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Investigations on Behaviour, Occurrence and Risk Assessment of Toxic Pyrrolizidine Alkaloids in Various Food and Feed Matrices

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Vorveröffentlichungen

Publikationen in wissenschaftlichen Fachjournalen

Kaltner F, Gottschalk C, Gareis M. Pflanzeninhaltsstoffe als Kontaminanten in der Lebensmittelkette. Nachrichten aus der Chemie 2018;66(2):109-113

Kaltner F, Rychlik M, Gareis M, Gottschalk C. Influence of Storage on the Stability of Toxic Pyrrolizidine Alkaloids and Their N-Oxides in Peppermint Tea, Hay and Honey. Journal of Agricultural and Food Chemistry 2018;66(20):5221-5228

Gottschalk C, Huckauf A, Dübecke A, Kaltner F, Zimmermann M, Rahaus I, Beuerle T. Uncertainties in the determination of pyrrolizidine alkaloid levels in naturally contaminated honeys and comparison of results obtained by different analytical approaches. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 2018;35(7):1366-1383

Kaltner F, Stiglbauer B, Rychlik M, Gareis M, Gottschalk C. Development of a Sensitive Analytical Method for Determining 44 Pyrrolizidine Alkaloids in Teas and Herbal Teas via LC-ESI-MS/MS. Analytical and Bioanalytical Chemistry 2019;411(27):7233-7249

Kaltner F, Rychlik M, Gareis M, Gottschalk C. Occurrence and Risk Assessment of Pyrrolizidine Alkaloids in Spices and Culinary Herbs from Various Geographical Origins. Toxins 2020;12(3):155

Kongressbeiträge

Kaltner F, Rychlik M, Gareis M, Gottschalk C. Lagerstabilität toxischer Pyrrolizidinalkaloide in Lebens- und Futtermitteln (Vortrag). 46. Deutscher Lebensmittelchemikertag, Würzburg 2017; ISBN 978-3-947197-03-3

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Kaltner F, Rychlik M, Gareis M, Gottschalk C. Sensitive determination of 44 toxic pyrrolizidine alkaloids in spices and culinary herbs using liquid chromatography tandem - mass spectrometry (Poster). XXII International Mass Spectrometry Conference, Florenz (Italien) 2018; ISBN 978-8-890738-85-2

Kaltner F, Rychlik M, Gareis M, Gottschalk C. Entwicklung einer Analysenmethode zur Bestimmung von 44 Pyrrolizidinalkaloiden in Kräutern und Gewürzen mittels LC-MS/MS (Poster). 47. Deutscher Lebensmittelchemikertag, Berlin 2018; ISBN 978-3-947197-08-8

Kaltner F, Rychlik M, Gareis M, Gottschalk C. Entwicklung einer LC-MS/MS-Methode zur Analyse von 44 Pyrrolizidinalkaloiden in Kräutern und Gewürzen (Poster). 59. Arbeits-tagung des Arbeitsgebietes Lebensmittelhygiene der Deutschen Veterinärmedizinischen Gesellschaft, Garmisch-Partenkirchen 2018

Kaltner F, Kukula V, Rychlik M, Gareis M, Gottschalk C. Screening von Nahrungs-ergänzungsmitteln auf toxische Pyrrolizidinalkaloide (Poster). 48. Deutscher Lebensmittel-chemikertag, Dresden 2019; ISBN 978-3-947197-15-6

Kaltner F, Kukula V, Rychlik M, Gareis M, Gottschalk C. Screening von Nahrungs-ergänzungsmitteln auf toxische Pyrrolizidinalkaloide (Poster). 60. Arbeitstagung des Arbeits-gebietes Lebensmittelsicherheit und Verbraucherschutz der Deutschen Veterinärmedizinischen Gesellschaft, Garmisch-Partenkirchen 2019

Kaltner F, Rychlik M, Gareis M, Gottschalk C. Vorkommen und Risikobewertung von Pyrrolizidinalkaloiden in Kräutern und Gewürzen (Vortrag). 71. Arbeitstagung des Regionalverbands Bayern der Lebensmittelchemischen Gesellschaft, Würzburg 2020

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Abbreviations

ALARA	As low as reasonably achievable
APCI	Atmospheric pressure chemical ionisation
BfR	Federal Institute for Risk Assessment (*Bundesinstitut für Risikobewertung*)
BGA	German Federal Health Agency (*Bundesgesundheitsamt*)
BMD	Benchmark Dose
$BMDL_{10}$	Benchmark Dose Lower Confidence Limit, 10 %
BMDU	Benchmark Dose Upper Confidence Limit
BMR	Benchmark Response
BW	Body weight
CoA	Coenzyme A
CV	Coefficient of variation
CYP450	Cytochrome P450 monooxygenase(s)
DAD	Diode array detector
DHP	Didehydro pyrrolizidine
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immune sorbent assay
ESI	Electrospray ionisation
EU	European Union
FLD	fluorescence detector
GC	Gas-chromatography
GSH	Glutathione
HBGV	Health Based Guidance Value
HPLC	High performance liquid-chromatography
HSOS	Hepatic sinusoidal obstruction syndrome
HSS	Homospermidine synthase
IS	Internal standard
LC	Liquid-chromatography
$LiAlH_4$	Lithium aluminium hydride
LOAEL	Lowest-Observed-Adverse-Effect-Level
LOD	Limit of detection
LOQ	Limit of quantification
MOE	Margin of Exposure

MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem-mass spectrometry
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
NCI	National Cancer Institute
NOAEL	No-Observed-Adverse-Effect-Level
NTP	National Toxicology Program
PA	Pyrrolizidine alkaloid(s)
PAH	Pulmonary arterial hypertension
PANO	Pyrrolizidine alkaloid *N*-oxide(s)
RE	Retronecine-equivalents
SPE	Solid phase extraction
TLC	Thin-layer chromatography
VOD	Veno-occlusive disease

Pyrrolizidine alkaloid abbreviations

AcIm	Acetylintermedine	AcImN	Acetylintermedine-*N*-oxide
AcLy	Acetyllycopsamine	AcLyN	Acetyllycopsamine-*N*-oxide
Em	Echimidine	EmN	Echimidine-*N*-oxide
En	Echinatine	EnN	Echinatine-*N*-oxide
Eu	Europine	EuN	Europine-*N*-oxide
Hd	Heliotridine	HdN	Heliotridine-*N*-oxide
Ht	Heliotrine	HtN	Heliotrine-*N*-oxide
Hs	Heliosupine	HsN	Heliosupine-*N*-oxide
Ic	Indicine	IcN	Indicine-*N*-oxide
Ig	Integerrimine	IgN	Integerrimine-*N*-oxide
Im	Intermedine	ImN	Intermedine-*N*-oxide
Jb	Jacobine	JbN	Jacobine-*N*-oxide
Jl	Jacoline	JlN	Jacoline-*N*-oxide
Jn	Jaconine	JnN	Jaconine-*N*-oxide
Lc	Lasiocarpine	LcN	Lasiocarpine-*N*-oxide
Ly	Lycopsamine	LyN	Lycopsamine-*N*-oxide
Mx	Merepoxine	MxN	Merepoxine-*N*-oxide
Mk	Merenskine	Mk	Merenskine-*N*-oxide
Mc	Monocrotaline	McN	Monocrotaline-*N*-oxide
Oc	Otonecine		
Rc	Retronecine	RcN	Retronecine-*N*-oxide
Rs	Retrorsine	RsN	Retrorsine-*N*-oxide
Rl	Riddelliine	RlN	Riddelliine-*N*-oxide
Rr	Rinderine	RrN	Rinderine-*N*-oxide
Sl	Sceleratine	SlN	Sceleratine-*N*-oxide
Sp	Seneciphylline	SpN	Seneciphylline-*N*-oxide
Sc	Senecionine	ScN	Senecionine-*N*-oxide
Sv	Senecivernine	SvN	Senecivernine-*N*-oxide
Sk	Senkirkine		
Sd	Spartioidine		
Td	Trichodesmine		
Us	Usaramine	UsN	Usaramine-*N*-oxide

1 Introduction

Alkaloids are known as a group of heterogeneous substances originating from the plant secondary metabolism. They are organic compounds consisting of carbon and hydrogen, which contain one or more basic nitrogen atoms in their heterocyclic ring systems. Further elements may occur within the molecules, like oxygen, sulphur, phosphorus or chlorine [1]. In the past, the term alkaloid was not strictly used for the above-named class of molecules, as it was also applicated for several natural substances isolated from a large variety of plants, fungi, animals and bacteria. Consequently, a satisfying and generally accepted definition does not exist.

Nowadays, more than 10,000 compounds belonging to the large group of alkaloids are known. Alkaloids are historically classified due to their common plant origin, chemical structure, similar biosynthesis or pharmaceutical effects. More recent classifications are based on similarities of the heterocyclic core structures [2]. Certain alkaloid compounds exhibit pharmacologic activities in human and animal organisms, and some are used for medical purpose in human and veterinary medicine (Table 1).

Table 1: Examples of naturally occurring alkaloids, their belonging alkaloid class and some pharmacological effects associate to them. Ergometrine is both a representative of toxic ergot alkaloids mainly produced by *Claviceps* fungi (effect-based classification) as well as of indole alkaloids (structural classification).

Alkaloid class	Exemplary compound	Pharmacological effect (examples)	Reference
Tropane alkaloids	Atropine	Increasing heart rate Decreasing saliva production Antidote for organophosphate poisoning	[3] [4] [5]
Purine alkaloid	Caffeine	Causing vasoconstriction	[6]
Ergot alkaloid, indole alkaloid	Ergometrine	Causing ergotism	[7]
Pyridine alkaloid	Nicotine	Disrupting blood-brain barrier	[8]
Steroidal alkaloid	Solanidine	Causing abortions in mice	[9]
Indolizidine alkaloid	Swainonine	Causing locoism disease	[10]
Pyrrolizidine alkaloid	Indicine *N*-oxide	Antitumor agent Decreasing number of leukocytes	[11] [12]

Several alkaloids can cause harmful or toxic effects in humans and animals after intake and their occurrence is undesirable in food and feed. These alkaloids occur unintendedly, thus regarded as food and feed contaminants. Indicine *N*-oxide is a representative of pyrrolizidine

alkaloids (PA), a group of toxic plant alkaloids contaminating food and feed and, thus, gaining increased attention in the field of food and feed safety during the last decade.

1.1 Structural characterisation and diversity of pyrrolizidine alkaloids

Alkaloids of the PA class share a similar pyrrolizidine (1-azabicyclo[3.3.0]octane) core structure, a bicyclic aliphatic hydrocarbon containing two five-headed rings and a nitrogen atom (Figure 1) [13].

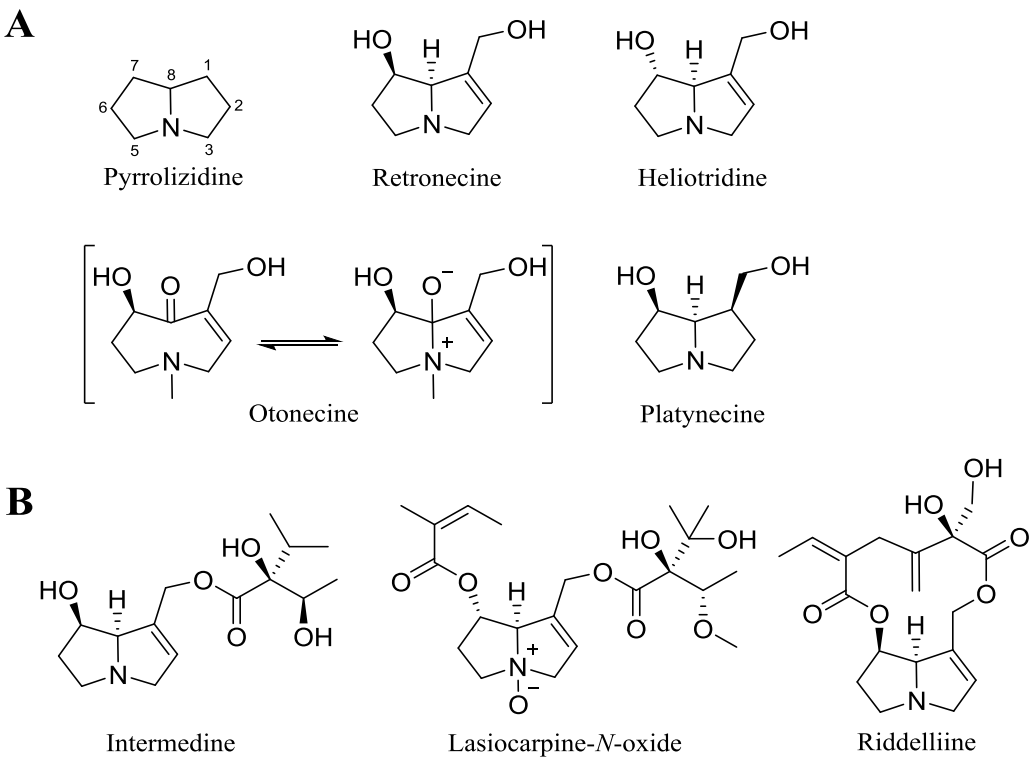

Figure 1: Overview of pyrrolizidine alkaloid (PA) structures. (**A**) Core structures (necine bases) of pyrrolizidine, the 1,2-unsaturated isomers retronecine and heliotridine, the tautomeric otonecine and the saturated PA platynecine. (**B**) Common examples of PA mono- (intermedine), di- (lasiocarpine-*N*-oxide) and cycloesters (riddelliine). Lasiocarpine-*N*-oxide additionally demonstrates the possibility of PA-*N*-oxide formation if the nitrogen atom is not methylated.

Typically, these alkaloids consist of a hydroxymethyl group at C1 and a hydroxy group at C7. Combined, this structure is called ‘necine base’. Normally, the necine base is esterified to one or more polyhydroxylated carboxylic acids, the so-called ‘necic acids’. PA can be further classified depending on their grade of esterification, stereochemistry or derivatisation of the necine base core structure. Naturally occurring derivatives of the PA core structure are unsaturated chemical bonds in position 1,2 (e. g. retronecine), *N*-methylation (e. g. otonecine) and *N*-oxidation (e. g. lasiocarpine-*N*-oxide) [14]. In general, PA without a methyl group bond

to the nitrogen atom may also occur in its corresponding PA-*N*-oxide (PANO) form: PANO presumably are the preferred storage compounds in vacuolated tissues of plants, while the more lipophilic PA are accumulated in the seeds [15]. As mentioned above, PA/PANO can be subdivided in further groups, according to their respective grade of esterification. An ester bond of a necic acid and the hydroxymethyl group at C1 is called PA/PANO monoester (e. g. intermedine). An additional ester bond at the C7 position forms an open-chained diester (e. g. lasiocarpine-*N*-oxide). In case the C1 hydroxymethyl group as well as the C7 hydroxy group are esterified to the same polycarboxylic acid in the final structure, the compound is called a cyclic diester (e. g. riddelliine).

Thus, PA/PANO molecules show an enormous diversity and a large potential variety of chemical structures mainly due to their branched necic acid side chains. Hence, more than 660 PA/PANO compounds were already reported and the number of so far unknown variants and compounds is constantly increasing [16-18]. While the necine bases share a common structure, in particular the necic acids show a broad structural variety (Figure 2) [19]. Normally, necic acids consist of two to ten carbon atoms and can strongly vary in their individual structure: Monocarboxylic acid side chains of PA/PANO range from C_2 to C_7 acids and include aliphatic and cyclic types of acids. Dicarboxylic acids consist of five to ten carbon atoms and form 11- or 12-membered rings with the necine base when incorporated in cyclic diester PA/PANO.

Acetic acid, the smallest necic acid, frequently occurs in very simple PA/PANO, e. g. 7-*O*-acetylretronecine [20]. Furthermore, it may be esterified to the hydroxy group at C7 position of the necine base, as it can be seen in acetylated PA/PANO (e. g. 7-*O*-acetylintermedine). Acetic acid can also be found esterified to hydroxy groups of other necic acids in more complex PA/PANO, like florosenine and floridanine [21]. Lactic acid represents an exotic necic acid and, so far, it is known to occur exclusively in the PA lactodine [22]. C_5 acid esters, like angelic acid, mainly appear associated to acetic acid or more complex, in particular C_7 acids, which can be observed in diesters, for instance echimidine, heliosupine or echivulgarine [16, 23]. Latifolic acid as an example of a cyclic C_7 acid is part of the PA latifoline [24]. Considering only the monoesters, aliphatic C_7 acids provide the majority of necic acids occurring in natural PA/PANO compounds. Commonly, they are linked to the hydroxymethyl group at C1 position of the respective necine bases. Consequently, several stereoisomers are known. For instance, due to the stereo configuration of the hydroxy groups within the branched C_7 necic acid, the PA compounds lycopsamine, intermedine and indicine differ only in the *S*-/*R*-configuration of the two hydroxy groups [25].

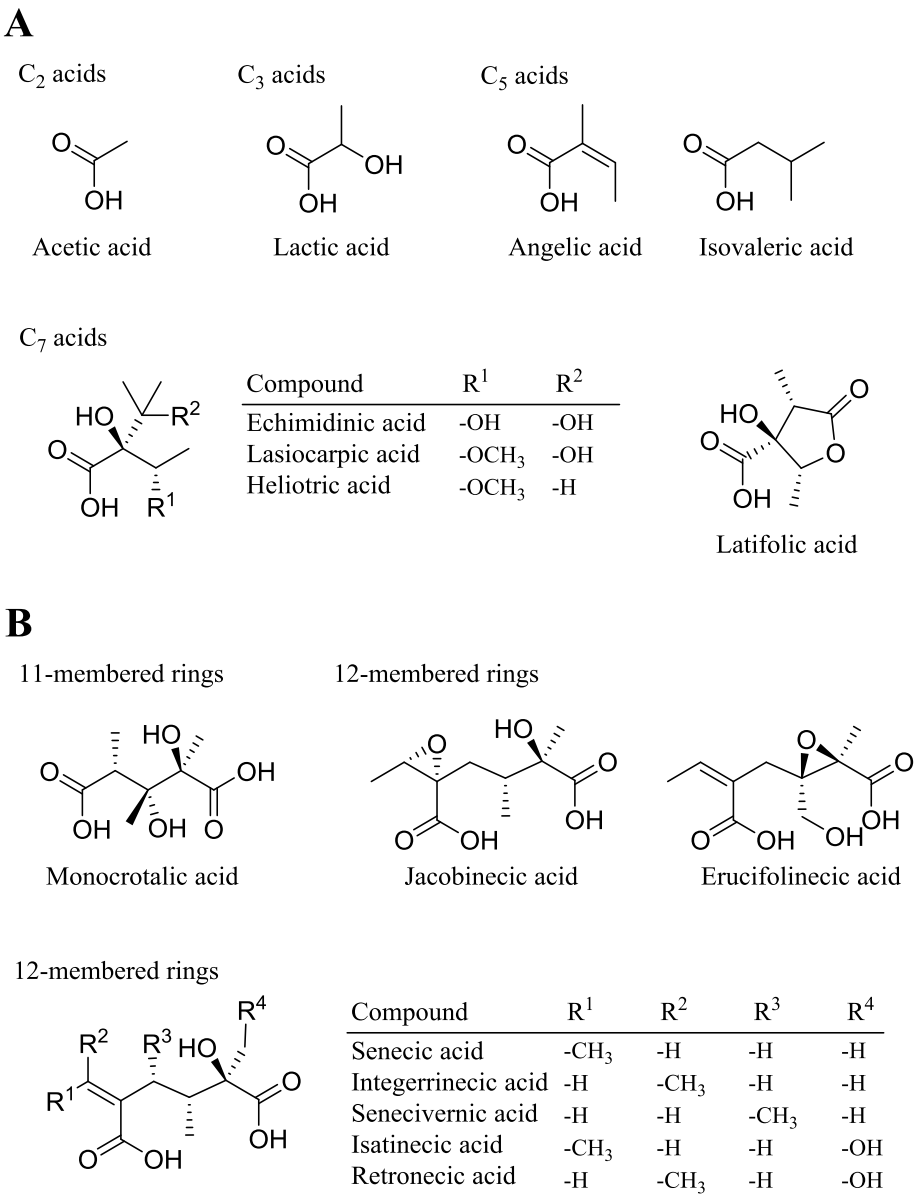

Figure 2: Examples of important necic acids. (**A**) Monocarboxylic aliphatic necic acids, grouped according to the number of their carbon backbones, (**B**) dicarboxylic acids occurring as necic acids in 11- or 12-membered macrocyclic diester pyrrolizidine alkaloids. Figure based on ROEDER et al. [26].

Dicarboxylic necic acids (Figure 2 B) are chemical structures typically found in macrocyclic PA/PANO. These necic acids consist of two eponymous carboxylic acid groups and a backbone of five or six carbon atoms. Linked to necine bases, 11- or 12- membered macrocyclic PA/PANO are formed. Usually, their branches show hydroxy and methyl groups that may occur unsaturated. Among the dicarboxylic acids, chlorine-containing structures are known, a component rarely observed in plant metabolites [19]. For instance, merenskine and jaconine as well as their corresponding *N*-oxides have chlorine included in their necic acids [27, 28].

1.2 Occurrence in plants

Plants containing PA/PANO are numerous and widespread and thus grow in most parts of the world. So far, more than 350 plant species are known to produce PA/PANO. Chemotaxonomic estimations predict these phytotoxins to occur in about 6,000 plant species, i. e. 3 % of all flowering plants worldwide [29]. Interestingly, the occurrence of PA/PANO is limited to only a few plant families.

1.2.1 Pyrrolizidine alkaloid-containing plant families

Main sources of PA/PANO are the plant families Asteraceae (tribes Senecioneae and Eupatorieae), Boraginaceae (all genera, mainly *Heliotropium* and *Symphytum*), Apocynaceae (tribe Echiteae) and Fabaceae (genus *Crotalaria*) [29, 30]. Herein, the different plant families and genera each produce only a few selected PA/PANO up to several dozens of structurally related compounds.

PA/PANO mainly occurring in the tribe Senecioneae of the Asteraceae family normally are derivatives of 12-membered cyclic diester alkaloids, consequently being named senecionine (Sc)-type PA/PANO (Figure 3). Lycopsamine (Ly)- and heliotrine (Ht)-type alkaloids are the second dominating group besides the Sc-type PA/PANO, including more than 100 structures and predominantly occurring in the Boraginaceae family.

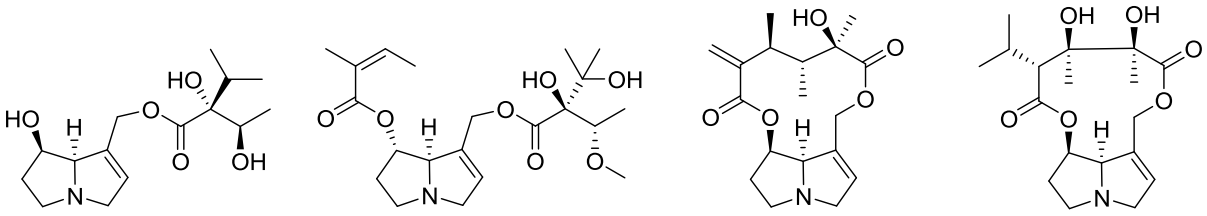

Figure 3: Exemplary structures of pyrrolizidine alkaloids (PA) of the four most important types occurring in plants: Lycopsamine (Ly) and heliotrine (Ht)-type: open-chained mono- and diesters; Senecionine (Sc)-type: 12-membered cyclic diesters; monocrotaline (Mc)-type: 11-membered cyclic diesters.

Ly-type PA/PANO are open-chained mono- and diesters based on the retronecine core structure. In contrast to the Sc-type, Ly-type PA/PANO seem to occur less in Senecioneae and Fabaceae tribes. The latter PA-type is dominantly formed in the tribe Eupatorieae of the Asteraceae family, in the Boraginaceae and in a few species of the Apocynaceae [31].

Like Ly-type PA/PANO, Ht-type alkaloids are also open-chained mono- and diesters, but are based on heliotridine. They occur nearly exclusively in species of the genera *Heliotropium*. Representative PA/PANO of the last group, the 11-membered cyclic diesters (monocrotaline (Mc)-type), are typically found in the genus *Crotalaria*, but were also reported to occur sporadically in some Boraginaceae species. Some species of the Orchidaceae family and of the subfamily Pooideae of the Poaceae family were also reported to produce PA/PANO, belonging to the less relevant phalaenopsine- and loline-type (Table 2) [32, 33].

Table 2: Distribution of pyrrolizidine alkaloid (PA) types within the taxa of PA-producing plant species. Senecionine (Sc)-type: 12-membered cyclic diesters; lycopsamine (Ly) and heliotrine (Ht)-type: open-chained mono- and diesters; monocrotaline (Mc)-type: 11-membered cyclic diesters. Some plant species can form PA of several types and thus are considered in more than one column. Table based on HARTMANN and WITTE [31].

Taxa	Total number of plant species containing PA	Number of species containing:			
		Sc-types	Ly-/Ht-types	Mc-types	Other types[1]
Apocynaceae	8	-	5	-	4
Asteraceae					
Eupatorieae	23	1	23	-	-
Senecioneae	231	204	31	1	7
Boraginaceae	145	2	144	5	11
Fabaceae					
Crotalaria	81	19	4	58	15
Other genera	37	22	-	-	16
Ochridaceae	35	-	-	-	35
Poaceae	4	1	-	-	3

[1] Miscellaneous PA-types, e. g. phalaenopsine- or loline-type

1.2.2 Biosynthesis

PA/PANO are alkaloids originating from the secondary metabolism of plants. The first investigations on the biosynthesis of PA/PANO were conducted in 1962, using radiolabelled acetate, propionate and ornithine [34]. On this basis, further studies utilizing stable isotope labelled precursors and nuclear magnetic resonance (NMR) experiments evidenced homospermidine, originating from putrescine and spermidine, as an intermediate in the PA/PANO necine base biosynthetic pathway [35]. Further investigations identified an enzyme from roots of *Eupatorium cannabinum* with homospermidine forming activity. The extracted enzyme named homospermidine synthase (HSS) was shown to be the key enzyme in formation of the necine base backbone. HSS catalyses the transfer of an amino butyl group of spermidine

to a putrescine to form homospermidine [36]. In contrast to spermidine and putrescine, homospermidine is resistant towards oxidative degradation. Via a copper-dependent diamine oxidation the 4,4'-iminodibutanal is formed, which cyclises to a pyrrolium cation and then to a pyrrolizidine-1-carbaldehyde in a Mannich-type reaction. Further reduction, desaturation and hydroxylation reactions lead to the retronecine and heliotridine necine bases (Figure 4) [19, 37].

Contrary to the common pathway of PA/PANO necine base formation, the different types of PA/PANO necic acids are distinctly formed. In general, the aliphatic mono- and dicarboxylic structures of the necic acids originate from branched amino acids like valine, leucine or isoleucine. Feeding experiments using radiolabelled substrates to alkaloid forming plants, for instance in *Datura meteloides* or *Cynoglossum officinale*, confirmed their C_5 necic acids to originate from isoleucine [38-40]. The sources of the synthesis of more complex necic acids were also revealed by feeding ^{14}C-labeled precursors to respective PA/PANO-forming plants: The C_7-acid of heliosupine and echimidine, echimidinic acid, is derived from valine and an additional, still unknown C_2 unit [41].

Dicarboxylic necic acids occurring in PA/PANO may be separated in two groups, forming 11- or 12-membered rings. The biosynthetic origin of monocrotalic acid, the main representative for PA/PANO with a 11-membered ring, was analysed in *Crotalaria spectabilis* and *Crotalaria retusa* by offering radiolabelled acetate and selected amino acids. The results showed that threonine and isoleucine were mainly incorporated into monocrotalic acid [42].

Senecic acid is the best characterised necic acid among the ones forming 12-membered rings in macrocyclic PA/PANO. Feeding experiments with ^{14}C-labeled L-isoleucine to *Senecio magnificus* identified this amino acid to be the biosynthetic source of senecic acid [43, 44]. Activation of the carboxylic groups of necic acids is revealed via addition of coenzyme A (CoA) and followed by a decarboxylation step. The esterification to hydroxyl groups of the necine base finally releases CoA. PANO are assumed to be the primary products of biosynthesis, and the corresponding tertiary amines are obtained via reduction [19].

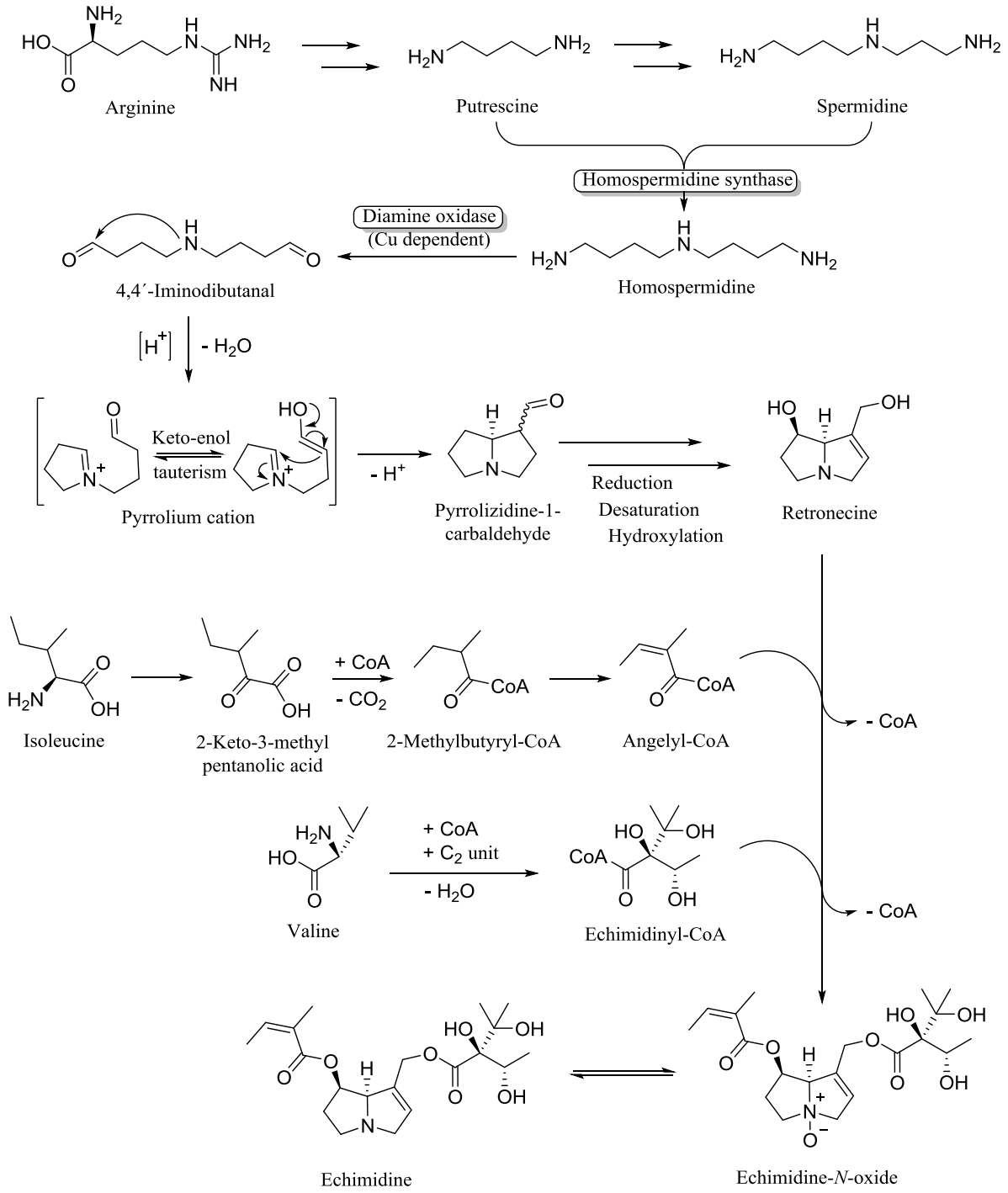

Figure 4: Scheme of the biosynthesis of retronecine-type pyrrolizidine alkaloids (PA) illustrated by the example of echimidine. The necine base core structure is formed by the amino acid arginine, via putrescine, spermidine and homospermidine as essential intermediates. After an enzymatic diamino oxidation, a Mannich-type reaction, followed by further reduction, desaturation and hydroxylation steps, leads to the heteroatomic bicyclic ring system of retronecine. Angelic acid originates from isoleucine, which is initially deaminated, decarboxylated and accompanied with coenzyme A (CoA) to the intermediate 2-methylbutyryl-CoA. Further hydroxylation and dehydration lead to angelyl-CoA. Its binding to retronecine releases CoA. Echimidinic acid is formed in a similar way out of valine and an unknown C_2 unit. PA *N*-oxides are assumed to be the primary products of biosynthesis, and the corresponding tertiary amines are obtained via reduction. Figure based on [19].

1.2.3 Role of pyrrolizidine alkaloids in ecology

The contemporaneous evolution of PA/PANO biosynthetic pathways in genetically unrelated plants indicated an evolutionary advantage or benefit. The PA/PANO formation in plants was suggested to pose a defence against herbivores, like it is assumed for the development of further plant secondary metabolites [45]. Grazing animals, in particular horses and cattle, are known to consume PA/PANO containing plants exclusively in times of very low feed supply due to the known bitterness of these plants [46]. In case of herbivorous insects, PA/PANO also show negative physiological effects, similar to humans and other mammals, that are not specialised in their plant diet (generalists), depending on the type and amounts of the alkaloids fed. MACEL et al. observed the influence of different PA/PANO under dietary conditions and derived a potential for selection of generalists on PA/PANO profiles and diversity in plants [47]. In addition, experiments using *Senecio* hybrids with varying PA/PANO contents instead of dietary feedings revealed correlations between feed-born damages in insects and PA/PANO amounts present in the used feeding plants [48].

The hypothesis of PA/PANO being defence compounds against animals is underlined by the existence of some insects specialised to plants containing PA/PANO, which can mainly be found in insects of the orders Lepidoptera or Coleoptera [49, 50]. The insects ingest PA/PANO to use them for their own purpose (sequestration). For instance, Arctiidae moths and some butterfly subfamilies were found to use PA/PANO as protective agents against the orb-weaving spider *Nephila clavipes*, which liberates adult insects unharmed from its web if their incorporated PA/PANO amounts are high [51]. Other moth and butterfly insects eat PA/PANO plants and incorporate the alkaloids to become poisonous to predators by storing of the phytotoxins or using them as precursors to synthesise own defence secretions [52].

Besides the use as defence chemicals, PA/PANO play an important role for pheromones of some insects: Male Danainae butterflies use PA/PANO as precursor for danaidone and its derivatives, which they need in order to gain attraction of the females [53, 54]. Interestingly, taste receptor neurons responding to PA/PANO have been identified in caterpillars of *Utetheisa ornatrix*, a phytophagous Lepidoptera using the alkaloids for defence and for pheromones, possibly explaining the hostplant selection of these insects [55].

In addition to their role in insect/plant interaction, PA/PANO are assumed to act as defence mechanism against phytopathogenic fungi. Extracts of *Jacobaea vulgaris* resulted in inhibitory effects on the mycelium growth of strains of the fungi *Fusarium* and *Trichoderma* [56].

1.3 Toxicity of pyrrolizidine alkaloids

Within the broad variety of PA/PANO, only singular compounds have been investigated regarding acute and chronic intoxications in mammals. Animal studies demonstrated 1,2-unsaturated PA to cause non-neoplastic effects in the liver and the kidney (e. g. hyperplasia and necrosis) and to act as genotoxic carcinogens. The corresponding PANO behave the same way, as they can be converted to the tertiary amines after ingestion [57]. The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain has issued a scientific opinion in 2011 which concluded that 1,2-unsaturated PA/PANO are toxic in humans [58].

1.3.1 Case reports on pyrrolizidine alkaloid intoxications

PA/PANO intoxications were already noted in literature about 120 years ago, but the origin was yet unknown. In nearly every case, the animals exhibited decreased body weight (BW), uncommon behaviour and died after a few weeks. The autopsy of the carcasses showed extremely enlarged and hardened livers. The so-called seneciose disease (German: *Schweinsberger Krankheit*) caused identical symptoms in horses like known diseases occurring in other parts of the world, for instance Winston's disease in New Zealand, Picton disease in Nova Scotia, Canada, or Zdár disease in Czechoslovakia [59, 60]. Further case studies of PA/PANO poisonings in animals were reported worldwide in the following century [61-65].

About 100 years ago, the symptoms of seneciosis were described in humans for the first time. WILLMOT and ROBERTSON investigated obscure sicknesses of whole families in the Cape province, South Africa, and identified *Senecio* plants as its origin. The patients had in common to belong to the poorer class of Europeans, typically eating bread as staple food. Seeds of *Senecio* plants were unintendedly incorporated in breads and identified to cause the observed symptoms that ranged from digestive derangements to death in humans [66].

Worldwide, more and more intoxications were identified to be caused by PA/PANO-containing plants in the last decades. Especially case studies reporting of hepatic sinusoidal obstruction syndrome (HSOS), previously termed veno-occlusive-disease (VOD), often were associated with a consumption of parts of these plants by the patients [67-69]. Besides the single case studies, also large outbreaks of HSOS occurred in the past, in particular in Afghanistan. In 1974, an epidemic in North-Western Afghanistan affecting 35,000 people in 98 villages was reported. More than 22 % of 7,200 examined inhabitants showed evidence of liver disease, and many patients died [70]. *Heliotropium* plants were found to grow extensively in the affected area and the wheat fields. Investigation of wheat samples from surrounding villages revealed high

amounts of *Heliotropium* seeds co-harvested with wheat kernels, which were not removed before milling. A further appreciable HSOS outbreak in Afghanistan due to PA/PANO-contaminated wheat flour was recorded in 2008 [71].

It seemed that, in particular, central Asia and the Indian subcontinent were haunted by PA/PANO-caused poisoning outbreaks: Further epidemic evidences of HSOS occurred in Tajikistan in 1992 and in the Sarguja district of India in 1975, which was caused by consumption of cereals mixed with seeds of *Crotalaria* plants. 28 of the 67 recorded patients (42 %) died, highlighting the toxicity of PA/PANO [72, 73].

1.3.2 Toxicokinetics

In general, only 1,2-unsaturated PA/PANO are verified to exhibit toxic properties; however, no quantitative data on the oral bioavailability in humans exist. Findings of animal studies proved that PA/PANO are quickly absorbed after ingestion from the gut and are primarily excreted via the kidney [74]. *In vitro* investigations on the bioavailability of PA/PANO have shown that these alkaloids cross the intestinal barrier differently, depending on their respective structure, and thus, the bioavailability of different PA/PANO may vary [75]. PA may be deemed as protoxins as they reveal their toxic potential in the liver, where they are metabolised to toxic agents by cytochrome P450 monooxygenases (CYP450) (Figure 5). PANO are assumed to be non-toxic in their original form, but they reveal toxic potential via reduction to their corresponding PA in the gut [76-79]. The transformation to pyrrole, namely didehydro pyrrolizidine (DHP) esters is exclusively possible for the 1,2-unsaturated PA/PANO, as the double bond at 1,2-position is prerequisite for formation of DHP. The DHP esters are further metabolised to DHP after hydrolysis [80-82]. Both the pyrrolic metabolites, the DHP as well as the DHP esters, are highly reactive alkylating agents, binding to nucleophilic groups of the DNA and proteins within the cell. DHP-derived adducts and crosslinks with DNA lead to organ dysfunctions and cancer in affected tissue [83].

The toxification of PA/PANO during the phase-I-metabolism may be regarded as unintended reactions within the detoxification pathways of the liver and the kidney. Normally, in these organs the water-solubility of xenobiotics is increased through unspecific enzymes via oxidation or addition of hydrophilic structures. For instance, PA can be *N*-oxygenised to be further excreted via the urine [76]. Also, the *N*-glucuronidation for the detoxication of PA was proven as a potential new metabolic pathway in humans and some animals [84]. Cleavage of the necic acids reveals non-toxic necine bases with free hydroxy groups, which can be esterified

within the phase-II-metabolism to glutathione (GSH) and form non-toxic and rapidly excreted conjugates [85, 86]. Recently, a study revealed differences in the metabolic degradation and GSH conjugate formation of PA due to the chemical structure: contrary to open-chained and cyclic diesters, monoesters did not form GSH conjugates using human liver microsomes, and the metabolic degradation in general seemed to be negatively correlated to the hydrophilicity of PA [87].

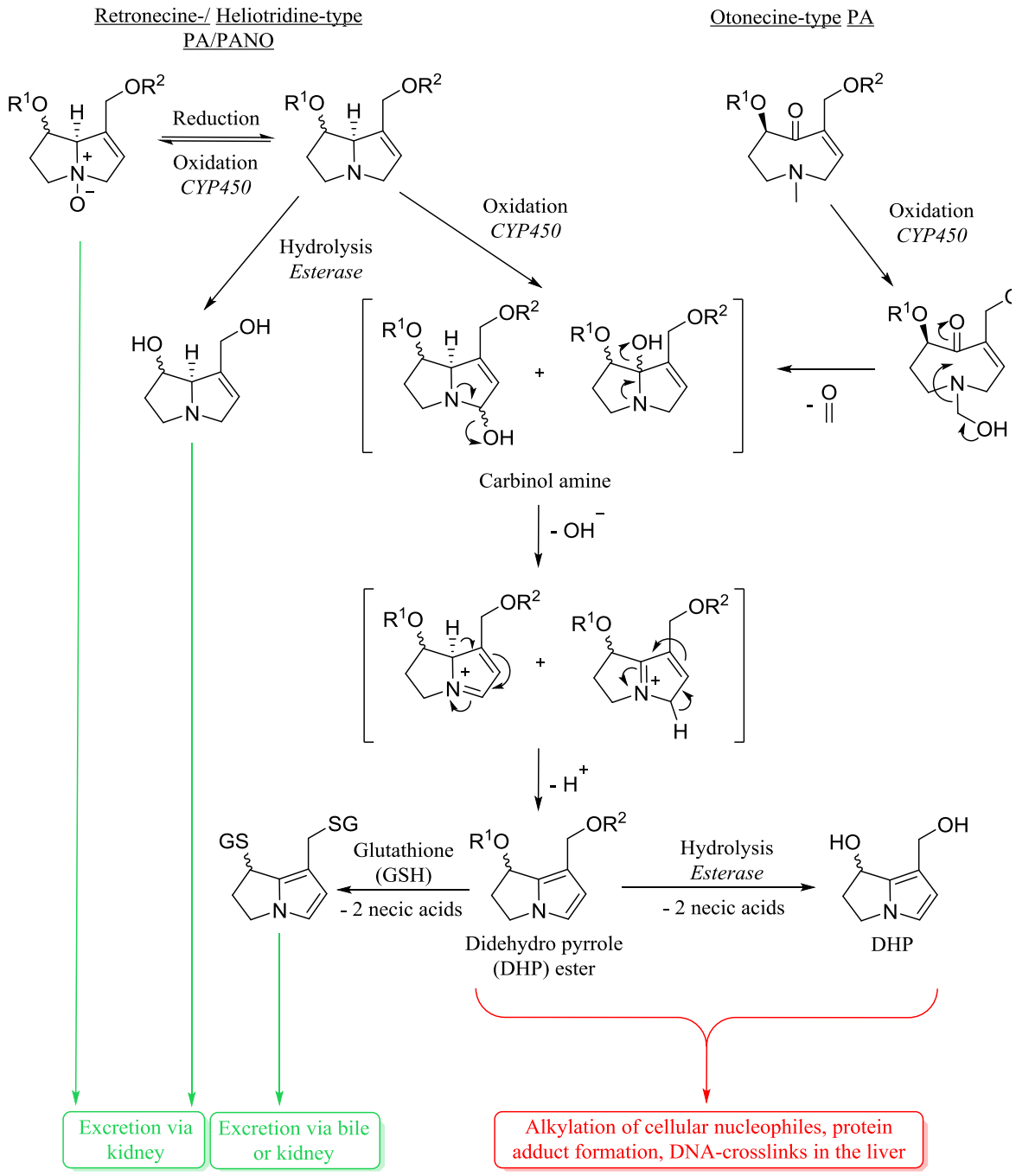

Figure 5: Phase-I- and -II-metabolism pathway of toxic 1,2-unsaturated pyrrolizidine alkaloids (PA) and *N*-oxides (PANO). After oral ingestion of PA/PANO, they are subject to *N*-oxidation or hydrolysis to increase the water-solubility before excretion via the kidney. Oxidation of the carbon atom in the bicyclic core structure or, in case of otonecine-type PA oxidative demethylation, forms carbinol amins. Elimination of water forms didehydro pyrrole (DHP) esters, which can be further conjugated with glutathione (GSH) and excreted in small amounts via the bile. Both DHP ester and, after its hydrolysis, DHP, are reactive agents, alkylating cellular nucleophiles to form DNA- or protein-adducts and DNA-crosslinks, which further induce geno- and hepatotoxicity as well as carcinogenicity and mutagenicity [76].

1.3.3 Acute and chronic toxicity

PA/PANO intoxications can be acute or chronic, mainly depending on the species, the total ingested amount and the period of time over which the toxins were ingested. The time between the intake and the first clinical findings can vary from one day to several days or months [76]. Several adverse effects are assumed to be caused by acute and chronic ingestion of 1,2-unsaturated PA/PANO, whereby the toxic potential may vary according to respective chemical structures. It is widely accepted that the toxicity increases from monoester via open chained diesters to macrocyclic diesters, which are assumed to be the most toxic PA/PANO compounds [14]. Recently, structure-dependent interim relative potency factors were proposed, respecting different toxicities of PA/PANO [88].

Depending on the ingested amounts, adverse acute toxic effects in the form of non-neoplastic damages mainly occur in the liver, which is the first target organ. Acute poisoning can cause non-carcinogenic, morphological changes of the tissue, and typically results in HSOS: harmful possible effects due to HSOS are fibrosis, necrosis of ambient liver tissue, hyperplasia, bile duct proliferation, eventually cirrhosis and liver failure. [68, 89-92]. In general, acute intoxications in humans due to ingestion of PA/PANO are a rare phenomenon occurring in certain regions (see 1.3.1), and chronic progressions of diseases seem to be more abundant.

Long-term exposure even to very low amounts of PA/PANO can also cause HSOS, pulmonary arterial hypertension (PAH) and carcinoma [26, 76, 89, 92]. PAH causes a remodelling of alveolar capillaries and pulmonary arteries, hyperplasia and hypertrophy of smooth muscles and expansion of the adventitial matrix. This can lead to increased pulmonary arterial pressure, the enlargement of the right ventricle and corpulmonale failure of the right heart [93, 94]. Furthermore, the carcinogenicity of PA/PANO was recently reviewed, summarising that to date more than 20 purified PA, one PANO and several dehydro-PA as well as plant extracts were demonstrated to induce carcinomas in rodents [81]. Several singular PA/PANO were also shown to induce damages, cross-links and strand breaks of DNA *in vitro* as well as *in vivo*, and thus to reveal genotoxic potential after being chronically ingested [82].

Furthermore, prenatal exposure to PA/PANO also seems to cause dysfunctions. Studies in rats prove negative effects in foetuses, such as hepatic and pulmonary impairments due to the formation of pyrrole metabolites and oxidative injury [95].

1.3.4 Data from long-term animal studies

Mammal species show different sensitivities towards PA/PANO exposition, depending on toxification and detoxification reactions during the phase-I- and phase-II- metabolism [76]. Cattle, horses and chicken are known to react more sensitive towards PA/PANO than goats, sheep and bunnies. The latter are more resistant due to the higher activity of GSH transferase and epoxide hydrolase [96]. *In vitro* experiments confirmed that rats are the optimum laboratory animals for extrapolation of results to humans in the field of toxicology: The liver microsome-induced metabolism of the cyclic diester PA riddelliine, retrorsine and monocrotaline to the corresponding dehydropyrroles happened in an extent similar to humans [97].

However, the number of long-term animal studies on carcinogenicity of PA/PANO is limited, with only two available references from cancer research institutes [74, 98]. In a study published in 1978 by the National Cancer Institute (NCI), groups of 24 each male and female F344 rats were fed for 104 weeks different doses of lasiocarpine (open-chained diester) in the feed (0, 7, 15, 30 µg/g). Compared to the control groups, losses in weight and high mortality with a positive dose-effect-relation were determined. In total, 40 % of male and 24 % of female rats developed cancer (angiosarcoma) during the study (Table 3).

In a study of the National Toxicity Program (NTP) from 2003, riddelliine (cyclic diester) was fed to each 50 male and female rats by gavage to investigate chronic toxicity. The given doses were much lower with a range of 0 to 1 mg/kg BW (0, 0.01, 0.033, 0.1, 0.33, 1 mg/kg BW). Here, only the higher dose of 1 mg/kg BW lead to a high evidence of angiosarcoma: 86 % in male and 76 % in female rats. In addition, the experiment was identically conducted using B6C3F1 mice.

Table 3: Development of angiosarcoma in male and female F344 rats fed with lasiocarpine or riddelliine for a maximum period of 104 weeks. Data according to studies performed by National Cancer Institute (NCI) and National Toxicity Program (NTP) [74, 98].

Sex	Dose[1] [mg/kg]	Number of hepatic angiosarcoma	Reference
Male	0 (control)	0 / 24	NCI 1978
	7	5 / 24	
	15	11 / 24	
	30	13 / 24	
	0 (control)	0 / 50	NTP 2003
	1	43 / 50	
Female	0 (control)	0 / 24	NCI 1978
	7	8 / 24	
	15	7 / 24	
	30	2 / 24	
	0 (control)	0 / 50	NTP 2003
	0.01	0 / 50	
	0.033	0 / 50	
	0.1	0 / 50	
	0.33	3 / 50	
	1	38 / 50	

[1] NCI study: lasiocarpine, doses calculated as mg/kg feed;
NTP study: riddelliine, doses calculated as mg/kg body weight

In case of the riddelliine study, the non-neoplastic and carcinogenic damages were equal in both groups. Riddelliine was shown to cause a higher evidence of angiosarcoma in rats as well as in mice. Lesions in livers and kidneys (hepatocyte cytomegaly, hyperplasia and necrosis; nephropathy) were the predominant non-neoplastic damages in both species. In case of neoplastic effects, hemangiosarcoma and cell leukaemia predominantly occurred in both rats and mice. The negative effects were mainly observed in the higher doses of fed riddelliine. Concludingly, the dose of 0.01 mg/kg BW was the highest dose without any observed adverse effects [74].

1.3.5 Toxicological reference points

Normally, data for the hazard characterisation of potential toxicological substances are obtained from *in vivo* animal studies, which are limited in extent due to high costs and animal welfare (see 1.3.4). The data are used to derive dose-response relationships, which are the basis for toxicological reference points for further risk assessment. One long-standing and common reference point is the highest dose tested in an animal study, where no significantly increased adverse effects between the test panel and the control panel are observed, the so-called

No-Observed-Adverse-Effect-Level (NOAEL, Figure 6 A). Consequently, the lowest dose tested showing adverse effects is called Lowest-Observed-Adverse-Effect-Level (LOAEL).

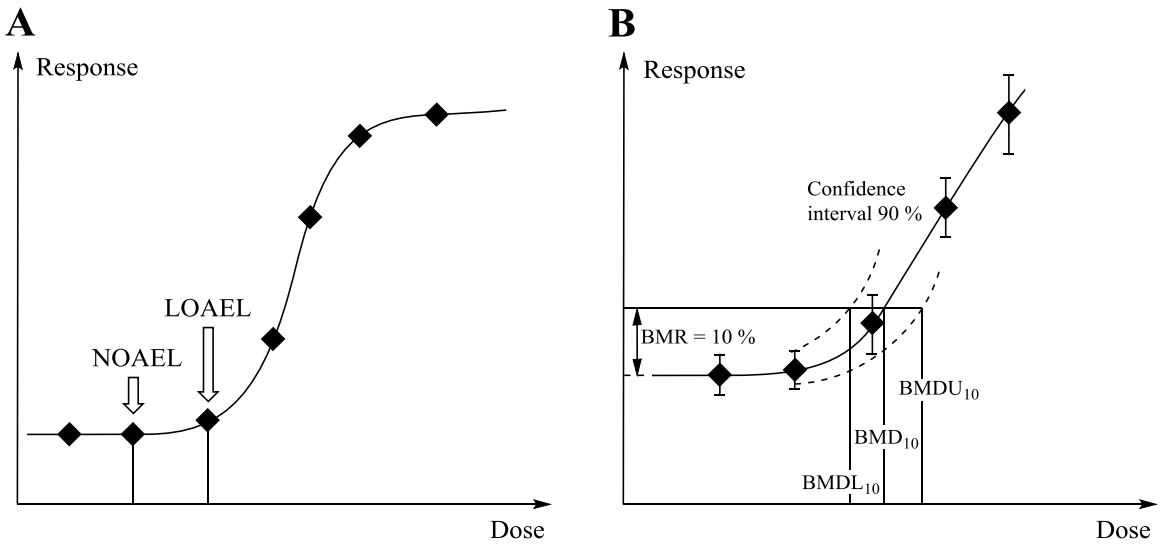

Figure 6: Exemplary comparison of dose-response models for deriving (**A**) the No-Observed-Adverse-Effect-Level (NOAEL) and the Lowest-Observed-Adverse-Effect-Level (LOAEL), and (**B**) the Benchmark Dose Lower Confidence Limit, 10 % ($BMDL_{10}$). The NOAEL represents the highest exposure without significantly increased adverse effects compared to the control. The $BMDL_{10}$ describes the lower 10 % confidence limit dose of a specific Benchmark Response (BMR) compared to a control group (here: Increased cancer risk of 10 %). Consequently, the Benchmark Dose Upper Confidence Limit, 10 % ($BMDU_{10}$) describes the upper 10 % confidence limit dose from a specific BMR and the Benchmark Dose (BMD) itself is the dose between $BMDL_{10}$ and $BMDU_{10}$.

In case of PA/PANO, the EFSA derived a NOAEL of 10 µg/kg BW/d on the basis of liver damages (hepatocytomegaly) of rats in a long-term study with riddelliine (see 1.3.4) [58]. The NOAEL is normally used for risk assessment of toxic substances with a specific dose-response relation, which is characteristic for the used animal species and way of administration. Additional safety factors ranging from ten to 1,000 are applied to the NOAEL to take differences between species and individuals into account.

To assess non-neoplastic, acute damages caused by PA/PANO, a so-called Health Based Guidance Value (HBGV) of 0.1 µg/kg BW/d was derived, using the NOAEL of 10 µg/kg BW/d and applying an uncertainty factor of 100. Concludingly, in case of an intake below the HBGV non-neoplastic damages due to PA/PANO are not expectable [99].

Main disadvantage is the use of only one dose in a series of tested doses to derive a NOAEL, without considering the slope of the dose-response curve. Further, it must be emphasised that

the genotoxic-carcinogenic effects are considered the most sensitive end point. In consequence, compliance with the HBGV does not offer any protection with regard to the genotoxic-carcinogenic effects of a chronic PA/PANO intake.

Recently, the Benchmark Dose (BMD) approach has acquired greater importance in assessing genotoxic or carcinogenic compounds. The BMD uses statistical methods to include and consider all values of the entire dose-response curve. The dose correlating with an increase or decrease of a specific Benchmark Response (BMR, e. g. an increase of cancer risk of 10 %) is called BMD. Intersections of the modelled upper or lower confidence interval of the dose-response curve and the BMD are called Benchmark Dose Lower/Upper Confidence Limit (BMDL or BMDU). For instance, the often-used $BMDL_{10}$ describes the lower 10 % confidence limit of a BMR of an increased cancer risk of 10 % compared to the control group (Figure 6 B). According to EFSA, the BMD approach should be used for future applications due to its advantages [100]. In case of PA/PANO, the EFSA derived a $BMDL_{10}$ of 70 µg/kg BW/d on the basis of the long-term study with lasiocarpine in rats [58]. In 2017, the EFSA updated this reference point: Due to the better quality of toxicological data, the long-term animal study with riddelliine was considered for the benchmark modelling, resulting in a $BMDL_{10}$ of 237 µg/kg BW/d [101].

Forming a dose-response relationship is not possible for genotoxic and carcinogenic substances as they have no effective response threshold. Consequently, the NOAEL approach is not appropriate for substances that are genotoxic and carcinogenic. In compliance with the EFSA, the Federal Institute for Risk Assessment (*Bundesinstitut für Risikobewertung*, BfR) recommends the additional consideration of the Margin of Exposure (MOE) concept [102, 103]. The MOE describes the margin between the relevant human exposure or estimated intake of all possible sources and the reference point derived from animal studies with the aid of the Benchmark Dose Approach (Equation 1).

Equation 1:

$$\text{Margin of Exposure (MOE)} = \frac{\text{Benchmark Dose lower Confidence Limit, 10 \% } (BMDL_{10})}{\text{Exposure}}$$

Uncertainties in the MOE concept, namely the inter- and intra-species variations as well as variabilities in the carcinogesis and the use of the low effect dose ($BMDL_{10}$), are covered by uncertainty factors of 10 each, resulting in an order of magnitude of 10,000. According to the

EFSA, a MOE of 10,000 or higher would be of low concern for public health and might reasonably be considered as a low priority for risk management actions [102]. However, the MOE of 10,000 may not be seen as a strict regulatory limit, but rather as a signal of action for risk management systems. Considering the MOE of 10,000 and the actual $BMDL_{10}$ of 237 µg/kg BW/d, an overall PA/PANO intake of more than 0.024 µg/kg BW/d can be seen as a threshold for risk management to take action [101].

1.4 Pyrrolizidine alkaloids as contaminants in the food chain

The ingestion of PA/PANO-contaminated food and feed can lead to massive intoxications of humans and animals (see 1.3.1). Also, the intended use of PA/PANO-containing plants for medical or culinary purposes was found to be responsible for poisonings in the past [69]. However, the unintended intake of these toxic alkaloids via contaminated plant-derived food and feed is still responsible for most of the reported intoxications [104]. Typical plant food products concerned by a contamination with PA/PANO are teas and herbal teas, spices and culinary herbs. Also, animals can directly be affected by impurities in feed like pelleted grass or hay. As a consequence, food of animal origin can also be contaminated with PA/PANO, namely milk and its products as well as most kind of animal tissue intended for human nutrition. Moreover, PA/PANO can also contaminate honey and honey based foodstuff due to honey bees collecting pollen and nectar from PA/PANO-containing plants [58, 105].

1.4.1 Plant-derived food and animal feed

A contamination of plant-derived food is commonly assumed to arise from a co-harvesting of PA/PANO-containing contaminant plants growing between the aimed culture plants: In a formerly reported case study, leaves of *Senecio vulgaris* accidently co-occurred with similar appearing leaves of rocket (*Eruca vesicaria* or *Diplotaxis tenuifolia*) in a salad mix in a German supermarket [106]. A yet disregarded source of plant food contamination may be a horizontal transfer of PA/PANO between living plants via the soil: SELMAR et al. demonstrated the ability of plants growing in the vicinity of PA/PANO-containing J*acobaea vulgaris* to accumulate the toxic alkaloids [107].

Concerning the occurrence of PA/PANO contamination, teas and herbal teas are the best investigated food matrices and many studies indicated the high prevalence of these toxins in the respective products (Table 4). BODI et al. investigated the occurrence of PA/PANO in overall 274 dry tea samples, including black tea, green tea, rooibos, peppermint, chamomile and mixed teas. The percentage of positive samples varied within each matrix, ranging from

86 % for peppermint teas to 100 % in case of rooibos teas, with a range from smaller than the limit of detection (LOD) to 5,650 µg/kg [108]. A more recent study investigated 168 samples from all over Europe and found 91 % of teas and herbal teas to contain at least one PA/PANO, with a median of 183 µg/kg and a maximum of 4,805 µg/kg, revealing that a contamination of these products is quite common [109]. At this point, it has to be emphasised that the total amounts of reported PA/PANO contents strongly depend on the considered set of compounds. Most of the studies back then measured 28 analytes [108, 109], and in total the number of PA/PANO analysed in the various studies ranged from 14 to 30 compounds [110, 111]. Furthermore, one study reported sum contents by using a sum-based analytical method [112].

Table 4: Selection of studies reporting contamination of plant-derived food and animal feed with pyrrolizidine alkaloids (PA) and PA *N*-oxides (PANO).

Matrix	N	Positive [%]	PA/PANO contents [µg/kg]			Reference
			Median	Mean	Maximum	
(Herbal) teas	274	83-100	1.6 - 416	52 - 1,856	97 – 5,647	[108][1]
	168	91	183	454	4,805	[109]
	169	14-57	---	5 - 253	21 – 5,668	[113][1]
	70	67	---	201	1,729	[114]
	44	86	---	50	391	[112][2]
	159	87	21	139	4,246	[111][3]
Spices/herbs	255	59	---	1,177	95,234	[115][3]
	40	60	4.9	265	4,995	[99]
	17	100	---	197	1,770	[111]
Food supplements	191	60	7.6	19,141	2,410,275	[109]
Liqueurs	38	24	---	0.5	9.5	[116]
Animal feed	115	18	2.9	4.8	30	[110]
	40	58	1	35	411	[117]
	147	21	---	121	5,401	[46]
	37	38	---	22	43	[118]

[1] PA/PANO median, mean and maximum contents were only reported for different types of teas
[2] Data reported as retronecine-equivalents (RE): µg RE/kg; [3] Matrices naturally containing PA/PANO were excluded

PA/PANO can also occur in spices and culinary herbs, as these products may be subject of the same way of contamination assumed for (herbal) tea plants [111]. Only a few reports on these contaminants in spices and herbs are available, presumably indicating a relevant contribution to consumers' exposure to PA/PANO. Data reported by the BfR revealed that many dried and freeze-dried spices and herbs are contaminated with high amounts of PA/PANO, up to a maximum of 95 mg/kg in oregano [115].

Data published by the EFSA indicate that also food supplements can be a relevant source of PA/PANO intake. Most of the food supplements investigated in the study showed only small amounts or traces of PA/PANO with a median of 7.6 µg/kg, but using parts of known PA/PANO-containing plants to produce food supplements can yield in contents up to multiple g/kg (Table 4) [109]. Therefore, the intake of PA/PANO via food supplements can be extremely high compared to other food and thus, they can greatly contribute to the overall exposure of consumers to these toxic plant alkaloids [99].

A recently published study investigated the occurrence of PA/PANO in herbal liqueurs and elixirs, revealing these toxins to be present in nine of 38 samples [116]. The detected small contents with a mean of 0.5 µg/kg and a maximum of 9.5 µg/kg indicated these matrices to be of little concern for the exposure of consumers to PA/PANO.

Several studies examining animal feed for the presence of PA/PANO were published in the past decade. The detected mean contents ranged from 4.8 µg/kg to 212 µg/kg with 18 % to 58 % positive samples [46, 110, 117, 118]. Herein, particularly alfalfa showed higher amounts and percentages of PA/PANO contamination compared to other animal feed. The results reveal a possible hazard to cattle and livestock, depending on the specific sensitivity of the species. Moreover, PANO seem to be metabolised in forage conservatives such as silage [110]. It remains unclear whether they degrade or they still present toxic potential. In heat-dried pelleted grass PA/PANO were proven to remain stable [119].

1.4.2 Food and food products of animal origin

It is known that grazing animals normally are able to discriminate PA/PANO-containing plants due to their bitterness [46]. Processing parts of these plants with further crop plants to animal feed, for instance silage, pelleted grass or hay, results in a masking of the bitter-tasting plants and thus, the animals cannot avoid or select the toxic plants any more [30]. As a consequence, PA/PANO ingested by livestock can get into food and food products of animal origin. This indirect contamination can concern meat and organs (e. g. liver or kidney), eggs, milk and dairy products [58].

Furthermore, the toxic alkaloids can be transferred into honey and other bee products by pollen and, in particular, nectar collected by honey bees [120, 121]. Contrary to most mammals, honey bees can tolerate high quantities of toxic PA/PANO up to 50 µg per individual adult bee without negative effects [122]. First reports of PA/PANO transfer from *Senecio* plants to honey were published 1977 [123]. However, honey is the matrix that most of occurrence studies on

PA/PANO in animal derived foods are dealing with. In the past, reported mean contents in honey ranged from 6 µg/kg to 283 µg/kg, with maximum concentrations of 64 µg/kg to 2,639 µg/kg (Table 5).

Table 5: Selection of studies reporting contamination of food of animal origin with pyrrolizidine alkaloids (PA) and PA *N*-oxides (PANO).

Matrix	N	Positive [%]	PA/PANO contents [µg/kg]			Reference
			Median	Mean	Maximum	
Honey	696[1]	94	19	26	267	[124]
	2,839[2]	68	27	46	1,087	[124]
	381[3]	65	12	17	225	[124]
	87	47 - 94	1.4 - 11	6.1 - 15	28 - 235	[108][4]
	54	74	---	7.7	64	[125]
	216	9	---	56	120	[126][5]
	48	85	---	283	2,639	[127]
Milk	182	6	---	---	0.2	[109]
Cheese	34	0	---	---	< LOD	[109]
Yoghurt	27	0	---	---	< LOD	[109]
Meat and liver	273	0	---	---	< LOD	[109]
Eggs	205	1	---	---	0.1	[109]

[1] Retail honey; [2] Bulk honey from European Union; [3] Bulk honey from Central and South America;
[4] PA/PANO median, mean and maximum contents were only reported for different types of honey
[5] Data reported as retronecine-equivalents (RE): µg RE/kg

Concerning milk, MULDER et al. reported in a survey across Europe PA/PANO to be present in 6 % of 182 retail milk samples from goats and dairy cows, but only in very small concentrations below 1 µg/kg [109]. First transfer studies revealed the toxic alkaloids to segue into milk with a transfer rate of 0.1 % when contaminated feed is ingested by dairy cows [128]. This finding was confirmed by more actual research, also showing compound-dependent differences and partly higher transfer rates (up to 4 %, depending on the analyte). An average transfer rate of 0.1 % was confirmed in the study [129]. Contrary to milk, no PA/PANO were detected in retail samples of the dairy products cheese and yoghurt investigated in the study of MULDER et al., raising the question of the fate of the alkaloids when contaminated milk is further processed. First results on the stability of PA/PANO during pasteurisation and cheese and yoghurt production indicated a decrease of the contaminants due to fermentative processes [130].

Concerning eggs, only very small total amounts of PA/PANO were detected in 205 samples, and no compounds were measurable in 273 meat samples (Table 5). However, a transfer of the

toxins into eggs and meat was already shown in former studies: A feeding study of laying hens with a diet of 0.5 % PA/PANO-containing plants in the feed confirmed the transfer of the toxic compounds into eggs with estimated transfer rates between 0.02 % and 0.23 % [131]. The determined maximum content in whole eggs was 216 µg/kg, whereas the alkaloids were more concentrated in the yolk than in the egg white. Furthermore, specific PA/PANO compounds seemed to enrich in the eggs, as the four major analytes contributed for 93 % of the total PA/PANO-level, and thus confirmed similar findings of earlier studies [132, 133]. Concerning a transfer of PA/PANO into tissue, the compounds were also found in muscle, liver and kidneys of the hens. Detected levels in liver and kidneys were in general much higher than in the breast muscle, with contents up to 392 µg/kg in kidney and 296 µg/kg in liver samples. Interestingly, the PA/PANO-levels were shown to significantly decrease in all four matrices after additionally feeding clean feed for 14 d [131].

1.4.3 National and international regulatory limits

Currently, no maximum levels of PA/PANO in food exist in the European Union (EU). General maximum levels for these alkaloids in food and feed have been discussed since years, but have not yet been implemented in the respective regulations. In the past, several national and international institutions recognised the potential risk of toxic PA/PANO to humans and livestock and established several regulatory limits, for instance in drugs or animal feed.

In 1988, the World Health Organization evaluated the acute and chronical toxicity originating from 1,2-unsaturated PA/PANO. For the first time, results of several toxicity studies were gathered and a toxic dose threshold of 10 µg heliotrine/kg BW/d was derived [134]. Due to the lack of reliable data, the carcinogenic risk potential in humans could not be assessed, and a recommendation to consequently minimise the exposure of consumers to PA/PANO was the only measure taken.

The former German Federal Health Agency (*Bundesgesundheitsamt*, BGA) established a step plan for maximum levels of 1,2-unsaturated PA/PANO in medicinal drugs in 1992 [135]. According to this plan, the maximum sum contents in drugs depend on the respective type and period of intended use: Based on a daily use of a drug, a maximum exposure of 100 µg PA/PANO (external use) or 1 µg (internal use) may not be exceeded. If the period of use of an internal drug is longer than six weeks, the maximum limit decreases to 0.1 µg/d. During pregnancy and breastfeeding period, the use of PA/PANO-containing drugs is prohibited. The

latter reference point was also used by the BfR in its first risk assessment on PA/PANO in mixed salad in 2007 [106].

Within the EU, the Netherlands established one of the strictest maximum levels for PA/PANO, with a maximum level of 1 µg/kg applying to herbal teas and other beverages and food products containing herbs or herbal extracts [136]. So far, the only maximum level within the entire EU is set for parts of *Crotalaria* spp. in animal feed (100 mg weed plants/kg feed; Directive 2002/32/EC) [137]. Today, the ongoing discussion on regulatory limits for PA/PANO in the EU may finally lead to their implementation in the Commission Regulation (EC) No. 1881/2006 within the next years [138]. However, PA/PANO are assumed to be genotoxic and carcinogenic, thus their occurrence in food and feed should generally be kept 'as low as reasonably achievable' (so-called ALARA principle) [99]. Furthermore, pursuant to Regulation (EEC) No. 315/93 generally no foodstuffs may be placed on the market that contain contaminants in amounts that are unacceptable to health [139].

1.5 Analysis of pyrrolizidine alkaloids

The toxicological concern of exposure to PA/PANO required the development of reliable, sensitive and accurate analytical methods as well as quick field tests for qualitative control of raw products.. Instrumental applications techniques should be able to accurately determine low contamination levels and to provide a profile of individual analytes.

Techniques used in the field of PA/PANO (trace) analysis cover robust thin-layer chromatography (TLC), enzyme-linked immune sorbent assay (ELISA) as well as gas- (GC) and liquid-chromatography (LC) techniques coupled to diode array detectors (DAD), fluorescence detectors (FLD) or (tandem-) mass spectrometry detectors (MS/MS). Prior to applying any detection methods, the analytes have to be extracted and potentially further concentrated. Due to the high polarity of PA/PANO compounds, aqueous acids or mixtures with polar alcohols or organic solvents are often used to extract the alkaloids from various matrices [140-142]. For detecting low levels of PA/PANO contamination further clean-up steps are often necessary to enrich the analytes in the extract. The used techniques evolved from repeated liquid-liquid extraction to the currently favoured solid phase extraction (SPE) techniques using cation exchange or C_{18} stationary phases [143, 144].

TLC as a robust, quick and cheap technique for the detection of PA/PANO in plant extracts was common at the beginning and the middle of the previous century. Both silica and aluminium oxide adsorbents have been used for separation or fractionation of PA/PANO, in particular prior

to identification of single compounds using other instrumental approaches. For detection of PA/PANO on TLC plates, Ehrlich's reagent is the most specific and common spray reagent used [145, 146]. Despite its mostly unsatisfying detection limits, analysis of PA/PANO via TLC can still be interesting as a rapid method for determination or confirmation of results [147].

ELISA tests seemed to enable a quick and easily performable detection of PA/PANO, especially for rapid field test. Single important PA or their common core-structure (e. g. retronecine; see Figure 1) were used for the production of antibodies. As PA are too small to cause an immune response, they were linked to larger antigenic carriers such as bovine serum albumin [148, 149]. The occurrence of a broad variety of PA/PANO analytes requires specific cross-reactivities, in particular to PANO, which were not satisfactory up to now [149]. This drawback can be avoided by a chemical reduction to the corresponding PA prior to analysis [150]. ELISAs thus may be useful for detecting PA/PANO contaminations higher than a certain cut-off, but result-confirming analyses should additionally be performed.

Contrary to ELISAs aiming to detect the overall PA/PANO content in a sample, a recently published study on the analysis of pyrrole-protein-adducts in blood as a marker for PA/PANO exposure revealed a well-performing application for ELISAs [151]. However, toxicological concern of PA/PANO requires a very high sensitivity. Thus, current analytical approaches use GC or LC coupled to MS/MS, enabling sensitive and accurate detection of target analytes.

1.5.1 Mass-spectrometry based sum analysis

Due to the lack of commercially available reference standards, analytical methods for determining the common core-structures of PA/PANO were developed, mostly based on sensitive and accurate MS detection hyphenated to chromatographic separation techniques. As a broad range of PA/PANO belong to retronecine- and heliotridine-type PA/PANO, a chemical reduction and analysis of the corresponding necine bases seems to be applicable for a reliable determination of PA/PANO occurrence. Since these compounds show unsatisfying chromatographical and MS behaviour, an additional derivatisation step of the resulting diols is necessary.

The first published sum-based approach was developed by KEMPF et al. in 2008, using GC-MS/MS [126]. After extraction of the PA/PANO, the PANO were reduced to the corresponding PA with zinc dust. Then, the raw extract was further purified using SPE cartridges. Afterwards, the necic acids were removed via a second reduction step using $LiAlH_4$ solution and further silylated. Thus, the originally non-volatile PA compounds were derived to

di-trimethylsilyl-retronecine derivatives and quantified using heliotrine as an internal standard (IS) (Figure 7 A). The total content of PA/PANO in a sample was expressed as retronecine-equivalents (RE).

An optimised method shortened the time-consuming sample preparation by quitting the first reduction step, implemented a stable isotope labelled IS and adapted the method for more practicable LC-MS/MS [152]. After a single reduction step for PA/PANO using $LiAlH_4$, the necine bases were esterified to retronecine- and heliotridine-diphthalates and quantified as RE referring to the IS ([9,9-$^2H_2$]-retronecine diphthalate, originating from 7-*O*-9-*O*-dibutyroyl [9,9-$^2H_2$]-retronecine) (Figure 7 B).

On the basis of this PA/PANO sum-based LC-MS/MS method, an approach using similar extraction, purification and reduction steps, but implementing *p*-toluenesulfonyl isocyanate as derivatisation reagent, was developed [112]. Once again, the stable isotope labelled IS 7-*O*-9-*O*-dibutyroyl[9,9-$^2H_2$]-retronecine was used and the toluenesulfonylcarbamic esters of the PA/PANO were detected via LC-MS/MS (Figure 7 C).

Sum-based analytical approaches can give sensitive results on the total content of PA/PANO in a sample, even if individual reference standards are unavailable. Since yet unknown compounds are also covered by a sum-based method, regularly higher levels are detected by this approach [142]. In consequence, sum-based approaches present excellent quantitative screening methods. A major disadvantage of these methods is the inability to detect otonecine-type PA. Moreover, mathematical conversion factors based on the molecular weight of the respective necine base used for the calculation of the total PA/PANO content are subject to uncertainties. Compared to assessing the singular compounds, the analysis of only one or two signals representing the sum of all derivated retronecine- or heliotridine-type PA/PANO leads to a lack of information on the contaminating botanical source in a sample. Furthermore, varying toxicity of the different compounds cannot be assessed any longer (see 1.3.3). Thus, the development and improvement of targeted trace analysis methods is still ongoing.

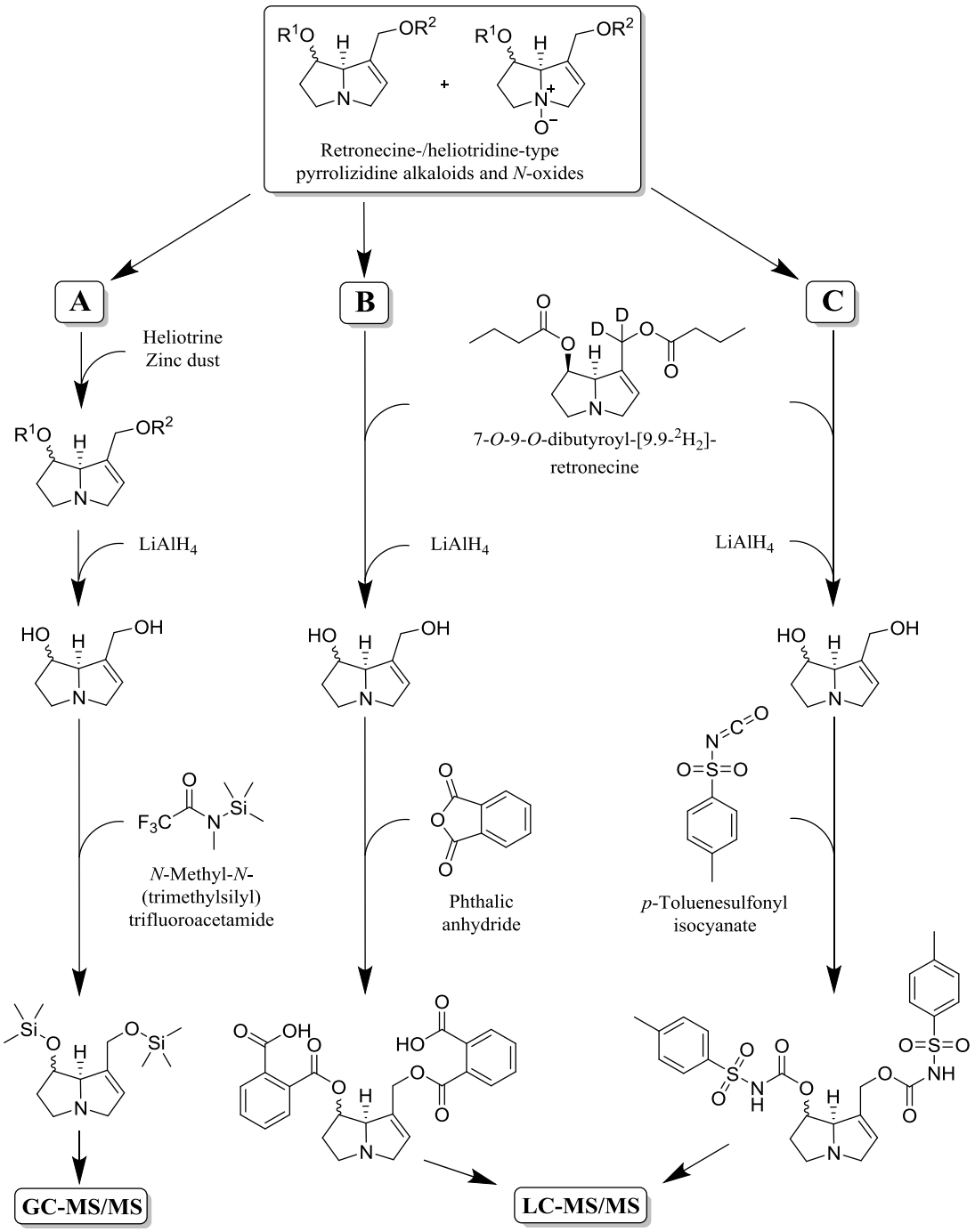

Figure 7: Schematic chemical reactions and derivatisations performed in three important sum-based methods for determining pyrrolizidine alkaloids (PA) and PA *N*-oxides (PANO). (**A)** The PA heliotrine, a heliotridine-type PA, was added as internal standard (IS) prior to a reduction using zinc and $LiAlH_4$. After silylation the analytes were detectable by gas chromatography tandem-mass spectrometry (GC-MS/MS). (**B**) The use of a stable isotope labelled retronecine derivative as IS also enabled the quantification of heliotridine-type PA and only one reduction step speeded up the method. Derivatisation of reduced PA using phthalic anhydride generated diphthalates, which were detectable by liquid chromatography (LC)- MS/MS. (**C**) Similar extraction and reduction steps according to method B were used, but derivatisation with *p*-toluenesulfonyl isocyanate yielded higher amounts of detectable derivatives. Presented reactions according to (A) Kempf 2008 [126], (B) Cramer 2013 [152] and (C) Maedge 2015 [112].

1.5.2 Mass-spectrometry based targeted analysis

In the field of PA/PANO trace analysis, atmospheric pressure chemical ionisation (APCI)-MS has been reported, but electrospray ionisation (ESI) was proven to be more suitable for polar compounds [153]. In case of the more polar PANO, ionisation was significantly higher in ESI than in APCI, thus being not applicable for the trace analysis of these compounds [153, 154]. On the other hand, ESI is known to be more prone to matrix-dependent ion suppression. In general, PA/PANO can ionise very differently, and thus their response factors are not equivalent. At this point, the matrix can still influence the response of the analytes and, thus, can lead to under- or overestimation of results. The lack of commercially available reference standards and isotope labelled reference standards remains a main factor of uncertainty in quantitative analysis of singular PA/PANO compounds [155].

Single-stage MS can only be of limited use as many compounds share identical molecular weight and chemical formulae [125]. In contrast, the application of MS/MS and, in particular, multiple reaction monitoring (MRM, Figure 8) enables very specific determination of precursor and fragment ions and thus generally results in lower LOD than single stage MS. Unsurprisingly, LOD and limits of quantification (LOQ) usually range below 1 µg/kg. The lowest LOQ yet reported for singular PA/PANO contamination in food ranged from 0.01 µg/kg to 0.76 µg/kg, using ultra-high performance LC-MS/MS techniques [156]. However, published LOD in modern analytical methods typically range from below 1 µg/kg up to 10 µg/kg [108, 157, 158].

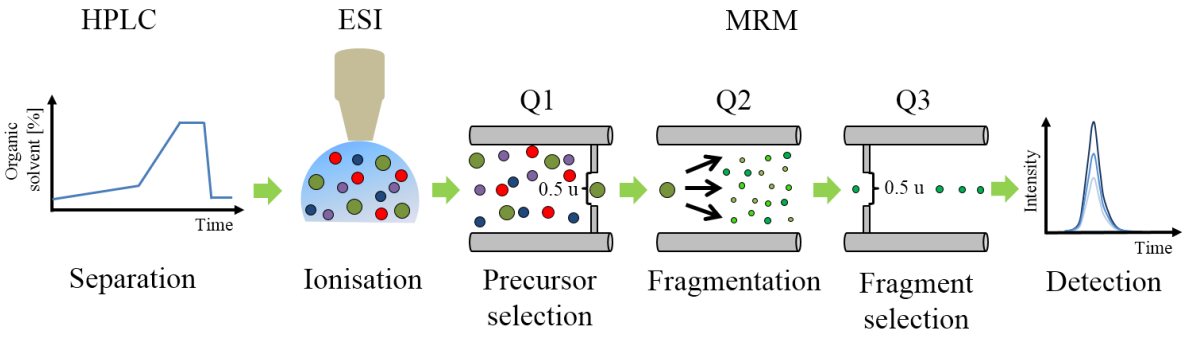

Figure 8: Principle of multiple reaction monitoring (MRM) using a triple quadrupole mass spectrometer. After a separation of analytes via high performance liquid chromatography (HPLC) the molecules are directed to an electrospray ionisation (ESI) source. In general, a single transition from the precursor ion to the product ion is recorded. In the first quadrupole (Q1), selection of precursor ions is performed using a mass-to-charge ratio (*m/z*) filter (0.5 u). After this, the precursor ions are fragmented in the collision cell (Q2). To specify the fragments, product ions are selected in a second *m/z* filter (0.5 u) in the Q3. Figure modified from [159].

Scans of precursor and product ions of PA/PANO enabled the development of targeted LC-MS/MS methods without the necessity of reference standards. Depending on the respective core-structure and type of esterification, PA/PANO were demonstrated to generate typical fragment ions. Triple quadrupole MS and high-resolution MS enabled the detection of patterns of fragments of the different PA-types, including the different esters and corresponding PANO. The observation of group-specific fragmentation patterns of PA/PANO enabled the development of LC-MS/MS methods for a screening and semi-quantification of samples by structural properties without requiring reference standards [160-162].

The existence of several hundred compounds also results in the presence of many known (and still unknown) isomers, complicating the MS-based detection due to the formation of identical fragment patterns and ions, especially in case of stereo isomers. A satisfying separation of all known isomers is hardly possible or can be exclusively achieved if uncommon techniques or instruments are used. For instance, stereo isomers of the lycopsamine-type (see Figure 3; varying stereo configuration of the hydroxy groups within the branched necic acid esterified to the C_7 hydroxymethyl group) were successfully separated by VAN DE SCHANS et al. in 2017, but only with an high instrumental effort. Online two-dimensional LC quadrupole time-of flight MS and quaternary gradient pumps were necessary as well as very long run times exceeding 60 min [163]. The problem of co-elution of important PA/PANO isomers is widely known: Interlaboratory comparison measurements and proficiency testings take it into account by reporting the contents of isomers in sum, e. g. the stereo isomers of the lycopsamine-type [164].

Due to increased sensitivity of modern MS detectors, so-called dilute-and-shoot methods can also be applied for PA/PANO determination. If medium or high levels of the analytes are expected and thus a maximum in sensitivity is not necessary, these techniques provide rapid results at the expense of the MS measurement, saving costs for sample pre-treatment [158].

1.6 References

1. Roberts MF. Alkaloids: biochemistry, ecology, and medicinal applications. 1998, Boston, MA: Springer Science & Business Media.
2. Fischer M, Glomb M. Moderne Lebensmittelchemie. 1. ed. 2015, Hamburg: Behr's.
3. Das G, Talmers FN, Weissler AM. New observations on the effects of atropine on the sinoatrial and atrioventricular nodes in man. The American Journal of Cardiology 1975;36(3):281-285.
4. Mirakhur RK. Comparative study of the effects of oral and i.m. atropine and hyoscie in volunteers. British Journal of Anaesthesia 1978;50(6):591-598.
5. Jokanović M. Medical treatment of acute poisoning with organophosphorus and carbamate pesticides. Toxicology Letters 2009;190(2):107-115.
6. Daniels JW, Molé PA, Shaffrath JD, Stebbins CL. Effects of caffeine on blood pressure, heart rate, and forearm blood flow during dynamic leg exercise. Journal of Applied Physiology 1998;85(1):154-159.
7. Moir JC. Ergot: From "St. Anthony's Fire" to the isolation of its active principle, ergometrine (ergonovine). American Journal of Obstetrics & Gynecology 1974;120(2):291-296.
8. Sajja RK, Rahman S, Cucullo L. Drugs of abuse and blood-brain barrier endothelial dysfunction: A focus on the role of oxidative stress. Journal of Cerebral Blood Flow & Metabolism 2015;36(3):539-554.
9. Friedman M, Henika PR, Mackey BE. Effect of feeding solanidine, solasodine and tomatidine to non-pregnant and pregnant mice. Food and Chemical Toxicology 2003;41(1):61-71.
10. Molyneux RJ, James LF. Loco intoxication: Indolizidine alkaloids of spotted locoweed (*Astragalus lentiginosus*). Science 1982;216(4542):190-191.
11. Poster DS, Bruno S, Penta J, Macdonald JS. Indicine-*N*-oxide: a new antitumor agent. Cancer Treatment Reports 1981;65(1-2):53-56.
12. Kovach JS, Ames MM, Powis G, Moertel CG, Hahn RG, Creagan ET. Toxicity and Pharmacokinetics of a Pyrrolizidine Alkaloid, Indicine *N*-Oxide, in Humans. Cancer Research 1979;39(11):4540-4544.
13. Molyneux RJ, Gardner DL, Colegate SM, Edgar JA. Pyrrolizidine alkaloid toxicity in livestock: a paradigm for human poisoning? Food Additives & Contaminants: Part A 2011;28(3):293-307.

14. Mattocks AR. Chemistry and Toxicology of Pyrrolizidine Alkaloids. 1986: Academic Press. 393 pp.
15. Toppel G, Witte L, Hartmann T. *N*-Oxidation and degradation of pyrrolizidine alkaloids during germination of *Crotalaria scassellatii*. Phytochemistry 1988;27(12):3757-3760.
16. Cairns E, Hashmi MA, Singh AJ, Eakins G, Lein M, Keyzers R. Structure of Echivulgarine, a Pyrrolizidine alkaloid isolated from the pollen of *Echium vulgare*. Journal of Agricultural and Food Chemistry 2015;63(33):7421-7427.
17. Kitajima M, Okabe K, Yoshida M, Nakabayashi R, Saito K, Kogure N, Takayama H. New otonecine-type pyrrolizidine alkaloid from *Petasites japonicus*. Journal of Natural Medicines 2019;73(3):602-607.
18. Robertson J, Stevens K. Pyrrolizidine alkaloids: occurrence, biology, and chemical synthesis. Natural Product Reports 2017;34(1):62-89.
19. Schramm S, Köhler N, Rozhon W. Pyrrolizidine alkaloids: Biosynthesis, biological activities and occurrence in crop plants. Molecules 2019;24(3):498.
20. El-Shazly A, Abdel-Ghani A, Wink M. Pyrrolizidine alkaloids from *Onosma arenaria* (Boraginaceae). Biochemical Systematics and Ecology 2003;31(5):477-485.
21. Roder E, Wiedenfeld H, Hoenig A. Pyrrolizidine alkaloids from *Senecio aureus*. Planta Medica 1983;49(1):57-59.
22. Ravi S, Ravikumar R, Lakshmanan AJ. Pyrrolizidine alkaloids from *Cynoglossum furcatum*. Journal of Asian Natural Products Research 2008;10(3-4):349-354.
23. Culvenor CC, Edgar JA, Smith LW. Pyrrolizidine alkaloids in honey from *Echium plantagineum* L. Journal of Agricultural and Food Chemistry 1981;29(5):958-960.
24. Crowley HC, Culvenor CCJ. Alkaloids of *Cynoglossum latifolium* r.br. Latifoline and 7-angelylretronecine. Australian Journal of Chemistry 1962;15(1):139-144.
25. Herz W, Kulanthaivel P, Subramanian PS, Culvenor CCJ, Edgar JA. Alkaloids of *Conoclinium coelestinum* (L.) DC., *Eupatorium compositifolium* walt., and *E. altissimum* L.: Isolation of crystalline intermedine from *C. coelestinum*. Experientia 1981;37(7):683.
26. Roeder E. Medicinal plants in Europe containing pyrrolizidine alkaloids. Pharmazie 1995;50(2):83-98.
27. Bradbury RB, Masamune S. The Alkaloids of *Senecio jacobaea L*. IV. The Structures of Jacobine, Jaconine, Jacoline and their Constituent Acids. Journal of the American Chemical Society 1959;81(19):5201-5209.

28. Bredenkamp MW, Wiechers A, van Rooyen PH. A new pyrrolizidine alkaloid from *Senecio latifolius* DC. Tetrahedron Letters 1985;26(7):929-932.
29. Smith LW, Culvenor CCJ. Plant sources of hepatotoxic pyrrolizidine alkaloids. Journal of Natural Products 1981;44(2):129-152.
30. Boppré M. The ecological context of pyrrolizidine alkaloids in food, feed and forage: an overview. Food Additives & Contaminants: Part A 2011;28(3):260-281.
31. Hartmann T, Witte L. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. Alkaloids: Chemical and Biological Perspectives, SW Pelletier, Editor. 1995, Elsevier. 155-233.
32. Lüning B, Tränkner H, Brandänge S. Studies on orchidaceae alkaloids V. A new alkaloid from *Phalaenopsis amabilis* Bl. Acta Chemica Scandinavica 1966;20(7):2011.
33. Wesseling AM, Demetrowitsch TJ, Schwarz K, Ober D. Variability of pyrrolizidine alkaloid occurrence in species of the grass subfamily pooideae (Poaceae). Frontiers in Plant Science 2017;8:2046.
34. Nowacki E, Byerrum RU. A study on the biosynthesis of the *Crotalaria* alkaloids. Life Sciences 1962;1(5):157-161.
35. Khan HA, Robins DJ. Pyrrolizidine alkaloids: Evidence for *N*-(4-aminobutyl)-1,4-diaminobutane (homospermidine) as an intermediate in retronecine biosynthesis. Journal of the Chemical Society, Chemical Communications 1981(11):554-556.
36. Böttcher F, Adolph RD, Hartmann T. Homospermidine synthase, the first pathway-specific enzyme in pyrrolizidine alkaloid biosynthesis. Phytochemistry 1993;32(3):679-689.
37. Robins DJ. Chapter 1: Biosynthesis of pyrrolizidine and quinolizidine alkaloids. Alkaloids: Chemistry and Pharmacology. 1995. 1-61.
38. Crout DHG. Pyrrolizidine alkaloids. Biosynthesis of the angelate component of heliosupine. Journal of the Chemical Society C: Organic Chemistry 1967:1233-1234.
39. Leete E, Murrill JB. Biosynthesis of the tiglic acid moiety of meteloidine in *Datura meteloides*. Tetrahedron Letters 1967;8(18):1727-1730.
40. McGaw BA, Woolley JG. The biosynthesis of angelic acid in *Cynoglossum officinale*. Phytochemistry 1979;18(10):1647-1649.
41. Crout DHG. Pyrrolizidine alkaloids. The biosynthesis of echimidinic acid. Journal of the Chemical Society C: Organic 1966:1968-1972.

42. Robins DJ, Bale NM, Crout DHG. Pyrrolizidine alkaloids. Biosynthesis of monocrotalic acid, the necic acid component of monocrotaline. Journal of the Chemical Society, Perkin Transactions 1 1974:2082-2086.
43. Crout DHG, Davies NM, Smith EH, Whitehouse D. Biosynthesis of the C10 necic acids of the pyrrolizidine alkaloids. Journal of the Chemical Society D: Chemical Communications 1970(11):635-636.
44. Davies NM, Crout DHG. Pyrrolizidine alkaloid biosynthesis. Relative rates of incorporation of the isomers of isoleucine into the necic acid component of senecionine. Journal of the Chemical Society, Perkin Transactions 1 1974:2079-2082.
45. Bennett RN, Wallsgrove RM. Secondary metabolites in plant defence mechanisms. New Phytologist 1994;127(4):617-633.
46. Mulder PPJ, Beumer B, Oosterink E, de Jong J. Dutch survey pyrrolizidine alkaloids in animal forage. RIKILT Report 2009.018, RIKILT Institute of Food Safety: Wageningen.
47. Macel M, Bruinsma M, Dijkstra SM, Ooijendijk T, Niemeyer HM, Klinkhamer PGL. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. Journal of Chemical Ecology 2005;31(7):1493-1508.
48. Wei X, Vrieling K, Mulder PPJ, Klinkhamer PGL. Testing the Generalist-Specialist Dilemma: The Role of Pyrrolizidine Alkaloids in Resistance to Invertebrate Herbivores in *Jacobaea* Species. Journal of Chemical Ecology 2015;41(2):159-167.
49. Hartmann T. Chemical ecology of pyrrolizidine alkaloids. Planta 1999;207(4):483-495.
50. Wink M. Plant secondary metabolites modulate insect behavior-steps toward addiction? Frontiers in Physiology 2018;9:364.
51. Silva KL, Trigo JR. Structure-activity relationships of pyrrolizidine alkaloids in insect chemical defense against the orb-weaving spider *Nephila clavipes*. Journal of Chemical Ecology 2002;28(4):657-668.
52. Hartmann T. Plant-derived secondary metabolites as defensive chemicals in herbivorous insects: A case study in chemical ecology. Planta 2004;219(1):1-4.
53. Boppré M. Insects pharmacophagously utilizing defensive plant chemicals (Pyrrolizidine alkaloids). Naturwissenschaften 1986;73(1):17-26.
54. Boppré M. Lepidoptera and pyrrolizidine alkaloids Exemplification of complexity in chemical ecology. Journal of Chemical Ecology 1990;16(1):165-185.

55. Bernays EA, Chapman RF, Lamunyon CW, Hartmann T. Taste receptors for pyrrolizidine alkaloids in a monophagous caterpillar. Journal of Chemical Ecology 2003;29(7):1709-1722.
56. Hol WHG, Van Veen JA. Pyrrolizidine alkaloids from *Senecio jacobaea* affect fungal growth. Journal of Chemical Ecology 2002;28(9):1763-1772.
57. Wang Y-P, Yan J, Fu PP, Chou MW. Human liver microsomal reduction of pyrrolizidine alkaloid *N*-oxides to form the corresponding carcinogenic parent alkaloid. Toxicology Letters 2005;155(3):411-420.
58. EFSA. Scientific opinion on pyrrolizidine alkaloids in food and feed. EFSA Journal 2011;9(11):2406.
59. Vanek J. Senecio poisoning (Senecio erraticus ssp. barbaraeifolius) as the cause of Zdár disease in horses; toxicity of isolated alkaloids. Review of Czechoslovak medicine 1957;3(4):299-314.
60. Petzinger E. Pyrrolizidine alkaloids and seneciosis in farm animals. Part 1: Occurrence, chemistry and toxicology. Tierarztliche Praxis Ausgabe G: Grosstiere - Nutztiere 2011;39(4):221-230.
61. Giles C. Outbreak of ragwort (*Senecio jacobea*) poisoning in horses. Equine Veterinary Journal 1983;15(3):248-250.
62. Hill BD, Gaul KL, Noble JW. Poisoning of feedlot cattle by seeds of *Heliotropium europaeum*. Australian Veterinary Journal 1997;75(5):360-361.
63. Fletcher MT, McKenzie RA, Blaney BJ, Reichmann KG. Pyrrolizidine alkaloids in *Crotalaria taxa* from Northern Australia: Risk to grazing livestock. Journal of Agricultural and Food Chemistry 2009;57(1):311-319.
64. Anjos BL, Nobre VMT, Dantas AFM, Medeiros RMT, Oliveira Neto TS, Molyneux RJ, Riet-Correa F. Poisoning of sheep by seeds of *Crotalaria retusa*: Acquired resistance by continuous administration of low doses. Toxicon 2010;55(1):28-32.
65. Anholt H, Britton A. Presumptive chronic pyrrolizidine alkaloid poisoning in 2 pygmy goats due to ingestion of tansy ragwort (*Jacobaea vulgaris*) in southwestern British Columbia. Canadian Veterinary Journal 2017;58(11):1171-1175.
66. Willmot F, Robertson G. *Senecio* disease, or cirrhosis of the liver due to *Senecio* poisoning. The Lancet 1920;196(5069):848-849.
67. Lyford CL, Vergara GG, Moeller DD. Hepatic Veno-Occlusive Disease Originating in Ecuador. Gastroenterology 1976;70(1):105-108.

68. Ridker PM, Ohkuma S, McDermott WV, Trey C, Huxtable RJ. Hepatic Venocclusive Disease Associated With the Consumption of Pyrrolizidine Containing Dietary Supplements. Gastroenterology 1985;88(4):1050-1054.
69. Bach N, Thung SN, Schaffner F. Comfrey herb tea-induced hepatic veno-occlusive disease. The American Journal of Medicine 1989;87(1):97-99.
70. Mohabbat O, Shafiq Younos M, Merzad AA, Srivastava RN, Ghaos Sediq G, Aram GN. An outbreak of hepatic veno-occlusive disease in north-western Afghanistan. The Lancet 1976;308(7980):269-271.
71. Kakar F, Akbarian Z, Leslie T, Mustafa ML, Watson J, Van Egmond HP, Omar MF, Mofleh J. An outbreak of hepatic veno-occlusive disease in Western Afghanistan associated with exposure to wheat flour contaminated with pyrrolizidine alkaloids. Journal of Toxicology 2010;2010.
72. Tandon BN, Tandon HD, Tandon RK, Narndranathan M, Joshi YK. An Epidemic of Veno-occlusive Disease of Liver in Central India. The Lancet 1976;308(7980):271-272.
73. Chauvin P, Dillon JC, Moren A. An outbreak of Heliotrope food poisoning, Tadjikistan, November 1992-March 1993. Sante 1994;4(4):263-268.
74. NTP. Toxicology and carcinogenesis studies of riddelliine. 2003, National Toxicology Program Technical Report 508.
75. Hessel S, Gottschalk C, Schumann D, These A, Preiss-Weigert A, Lampen A. Structure-activity relationship in the passage of different pyrrolizidine alkaloids through the gastrointestinal barrier: ABCB1 excretes heliotrine and echimidine. Molecular Nutrition and Food Research 2014;58(5):995-1004.
76. Fu PP, Xia Q, Lin G, Chou MW. Pyrrolizidine Alkaloids – Genotoxicity, Metabolism Enzymes, Metabolic Activation, and Mechanisms. Drug Metabolism Reviews 2004;36(1):1-55.
77. He X, Xia Q, Woodling K, Lin G, Fu PP. Pyrrolizidine alkaloid-derived DNA adducts are common toxicological biomarkers of pyrrolizidine alkaloid *N*-oxides. Journal of Food and Drug Analysis 2017;25(4):984-991.
78. Yang M, Ruan J, Gao H, Li N, Ma J, Xue J, Ye Y, Fu PP-C, Wang J, Lin G. First evidence of pyrrolizidine alkaloid N-oxide-induced hepatic sinusoidal obstruction syndrome in humans. Archives of Toxicology 2017;91(12):3913-3925.
79. Yang M, Ma J, Ruan J, Ye Y, Fu PPC, Lin G. Intestinal and hepatic biotransformation of pyrrolizidine alkaloid *N*-oxides to toxic pyrrolizidine alkaloids. Archives of Toxicology 2019;93(8):2197-2209.

80. Mattocks AR, Driver HE, Barbour RH, Robins DJ. Metabolism and toxicity of synthetic analogues of macrocyclic diester pyrrolizidine alkaloids. Chemico-Biological Interactions 1986;58:95-108.
81. Fu PP. Pyrrolizidine Alkaloids: Metabolic Activation Pathways Leading to Liver Tumor Initiation. Chemical Research in Toxicology 2017;30(1):81-93.
82. Chen T, Mei N, Fu PP. Genotoxicity of pyrrolizidine alkaloids. Journal of Applied Toxicology 2010;30(3):183-196.
83. Dusemund B, Nowak N, Sommerfeld C, Lindtner O, Schäfer B, Lampen A. Risk assessment of pyrrolizidine alkaloids in food of plant and animal origin. Food and Chemical Toxicology 2018;115:63-72.
84. He YQ, Yang L, Liu HX, Zhang JW, Liu Y, Fong A, Xiong AZ, Lu YL, Yang L, Wang CH, Wang ZT. Glucuronidation, a new metabolic pathway for pyrrolizidine alkaloids. Chemical Research in Toxicology 2010;23(3):591-599.
85. Xia Q, Ma L, He X, Cai L, Fu PP. 7-Glutathione pyrrole adduct: a potential DNA reactive metabolite of pyrrolizidine alkaloids. Chemical Research in Toxicology 2015;28(4):615-620.
86. Yan X, Kang H, Feng J, Yang Y, Tang K, Zhu R, Yang L, Wang Z, Cao Z. Identification of Toxic Pyrrolizidine Alkaloids and Their Common Hepatotoxicity Mechanism. International Journal of Molecular Science 2016;17(3):318.
87. Geburek I, Preiss-Weigert A, Lahrssen-Wiederholt M, Schrenk D, These A. In vitro metabolism of pyrrolizidine alkaloids – Metabolic degradation and GSH conjugate formation of different structure types. Food and Chemical Toxicology 2020;135:110868.
88. Merz KH, Schrenk D. Interim Relative Potency Factors for the Toxicological Risk Assessment of Pyrrolizidine Alkaloids in Food and Herbal Medicines. Toxicology Letters 2016;263:44-57.
89. Edgar JA, Colegate SM, Boppré M, Molyneux RJ. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. Food Additives & Contaminants: Part A 2011;28(3):308-324.
90. Schneider J, Tsegaye Y, Tensae MW, Selassie SG, Haue T, Bane A, Ali A, Mesfin G, Seboxa T. Veno-occlusive liver disease: A case report. Ethiopian Medical Journal 2012;50(SUPPL. 2):47-51.

91. Allgaier C, Franz S. Risk assessment on the use of herbal medicinal products containing pyrrolizidine alkaloids. Regulatory Toxicology and Pharmacology. 2015;73(2):494-500.
92. Edgar JA, Molyneux RJ, Colegate SM. Pyrrolizidine alkaloids: Potential role in the etiology of cancers, pulmonary hypertension, congenital anomalies, and liver disease. Chemical Research in Toxicology 2015;28(1):4-20.
93. Kay JM, Harris P, Heath D. Pulmonary hypertension produced in rats by ingestion of *Crotalaria spectabilis* seeds. Thorax 1967;22(2):176-9.
94. Burns J. The heart and pulmonary arteries in rats fed on *Senecio jacobaea*. The Journal of Pathology 1972;106(3):187-94.
95. Guo Y, Xiao D, Yang X, Zheng J, Hu S, Wu P, Li X, Kou H, Wang H. Prenatal exposure to pyrrolizidine alkaloids induced hepatotoxicity and pulmonary injury in fetal rats. Reproductive Toxicology 2019;85:34-41.
96. Teuscher E, Lindequist U. Biogene Gifte - Biologie-Chemie-Pharmakologie-Toxikologie. 3rd Edition ed. 2010, Stuttgart: Wissenschaftliche Verlagsgesellschaft.
97. Wang YP, Fu PP, Chou MW. Metabolic activation of the tumorigenic pyrrolizidine alkaloid, retrorsine, leading to DNA adduct formation in vivo. International Journal of Environmental Research and Public Health 2005;2(1):74-79.
98. NCI. Bioassay of lasiocarpine for possible carcinogenicity. 1978, National Cancer Insitute. 1-66.
99. BfR. Opinion No. 030/2016: Pyrrolizidine alkaloids: Levels in foods should continue to be kept as low as possible. 2016, BfR (Federal Institute for Risk Assessment): Berlin.
100. EFSA. Use of the benchmark dose approach in risk assessment. EFSA Journal 2009;1150:1-72.
101. EFSA. Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. EFSA Journal 2017;15(7).
102. EFSA. Opinion of the Scientific Committee on a request from EFSA related to A Harmonised Approach for Risk Assessment of Substances Which are both Genotoxic and Carcinogenic. EFSA Journal 2005;282:1-31.
103. BfR. Opinion No. 029/2005: Risk assessment of genotoxic and carcinogenic substances to be harmonised in the EU. 2005, BfR (Federal Institute for Risk Assessment): Berlin.
104. Ma C, Liu Y, Zhu L, Ji H, Song X, Guo H, Yi T. Determination and regulation of hepatotoxic pyrrolizidine alkaloids in food: A critical review of recent research. Food and Chemical Toxicology 2018;119:50-60.

105. Mulder PPJ, López Sánchez P, These A, Preiss-Weigert A, Castelari M. Occurrence of Pyrrolizidine Alkaloids in food. EFSA Supporting Publication 2015(EN-859):116 pp.
106. BfR. Opinion No. 028/2007: Salad mix contaminated with groundsel containing pyrrolizidine alkaloids. 2007, BfR (Federal Institute for Risk Assessment): Berlin.
107. Selmar D, Wittke C, Beck-von Wolffersdorff I, Klier B, Lewerenz L, Kleinwächter M, Nowak M. Transfer of pyrrolizidine alkaloids between living plants: A disregarded source of contaminations. Environmental Pollution 2019;248:456-461.
108. Bodi D, Ronczka S, Gottschalk C, Behr N, Skibba A, Wagner M, Lahrssen-Wiederholt M, Preiss-Weigert A, These A. Determination of pyrrolizidine alkaloids in tea, herbal drugs and honey. Food Additives & Contaminants: Part A 2014;31(11):1886-1895.
109. Mulder PPJ, López Sanchez P, Castelari M, Bodi D, Ronczka S, Preiß-Weigert A, These A. Occurrence of pyrrolizidine alkaloids in animal- and plant-derived food: Results of a survey across Europe. Food Additives & Contaminants: Part A 2018;35(1):118-133.
110. Gottschalk C, Ronczka S, Preiß-Weigert A, Ostertag J, Klaffke H, Schafft H, Lahrssen-Wiederholt M. Pyrrolizidine alkaloids in natural and experimental grass silages and implications for feed safety. Animal Feed Science and Technology 2015;207:253-261.
111. Picron JF, Herman M, van Hoeck E, Goscinny S. Analytical strategies for the determination of pyrrolizidine alkaloids in plant based food and examination of the transfer rate during the infusion process. Food Chemistry 2018;266:514-523.
112. Mädge I, Cramer L, Rahaus I, Jerz G, Winterhalter P, Beuerle T. Pyrrolizidine alkaloids in herbal teas for infants, pregnant or lactating women. Food Chemistry 2015;187:491-498.
113. Schulz M, Meins J, Diemert S, Zagermann-Muncke P, Goebel R, Schrenk D, Schubert-Zsilavecz M, Abdel-Tawab M. Detection of pyrrolizidine alkaloids in German licensed herbal medicinal teas. Phytomedicine 2015;22(6):648-656.
114. Shimshoni JA, Duebecke A, Mulder PPJ, Cuneah O, Barel S. Pyrrolizidine and tropane alkaloids in teas and the herbal teas peppermint, rooibos and chamomile in the Israeli market. Food Additives & Contaminants: Part A 2015;32(12):2058-2067.
115. BfR. Opinion No. 017/2019: Pyrrolizidine alkaloid levels in dried and deep-frozen spices and herbs too high. 2019, BfR (Federal Institute for Risk Assessment): Berlin.
116. Chmit MS, Wahrig B, Beuerle T. Quantitative and qualitative analysis of pyrrolizidine alkaloids in liqueurs, elixirs and herbal juices. Fitoterapia 2019;136.
117. Huybrechts B, Callebaut A. Pyrrolizidine alkaloids in food and feed on the Belgian market. Food Additives & Contaminants: Part A 2015;32(11):1939-1951.

118. Kowalczyk E, Kwiatek K. Determination of pyrrolizidine alkaloids in selected feed materials with gas chromatography-mass spectrometry. Food Additives & Contaminants: Part A 2017;34(5):853-863.
119. Gottschalk C, Ostertag J, Meyer K, Gehring K, Thyssen S, Gareis M. Influence of grass pellet production on pyrrolizidine alkaloids occurring in Senecio aquaticus-infested grassland. Food Additives & Contaminants: Part A 2018;35(4):750-759.
120. Edgar JA, Roeder E, Molyneux RJ. Honey from plants containing pyrrolizidine alkaloids: A potential threat to health. Journal of Agricultural and Food Chemistry 2002;50(10):2719-2730.
121. Lucchetti MA, Glauser G, Kilchenmann V, Dubecke A, Beckh G, Praz C, Kast C. Pyrrolizidine alkaloids from *Echium vulgare* in honey originate primarily from floral nectar. Journal of Agricultural and Food Chemistry 2016;64(25):5267-5273.
122. Reinhard A, Janke M, von der Ohe W, Kempf M, Theuring C, Hartmann T, Schreier P, Beuerle T. Feeding deterrence and detrimental effects of pyrrolizidine alkaloids fed to honey bees (*Apis mellifera*). Journal of Chemical Ecology 2009;35(9):1086-1095.
123. Deinzer M, Thomson P, Burgett D, Isaacson D. Pyrrolizidine alkaloids: their occurrence in honey from tansy ragwort (*Senecio jacobaea* L.). Science 1977;195(4277):497-499.
124. Dübecke A, Beckh G, Lüllmann C. Pyrrolizidine alkaloids in honey and bee pollen. Food Additives & Contaminants: Part A 2011;28(3):348-358.
125. Kowalczyk E, Sieradzki Z, Kwiatek K. Determination of Pyrrolizidine Alkaloids in Honey with Sensitive Gas Chromatography-Mass Spectrometry Method. Food Analytical Methods 2018;11:1345-1355.
126. Kempf M, Beuerle T, Bühringer M, Denner M, Trost D, Von Der Ohe K, Bhavanam VBR, Schreier P. Pyrrolizidine alkaloids in honey: Risk analysis by gas chromatography-mass spectrometry. Molecular Nutrition and Food Research 2008;52(10):1193-1200.
127. Letsyo E, Jerz G, Winterhalter P, Dübecke A, von der Ohe W, von der Ohe K, Beuerle T. Pyrrolizidine alkaloids in floral honeys of tropical Ghana: a health risk assessment. Food Additives &Contaminants: Part B 2017;10(4):300-310.
128. Dickinson JO, Cooke MP, King RR, Mohamed PA. Milk transfer of pyrrolizidine alkaloids in cattle. Journal of the American Veterinary Medical Association 1976;169(11):1192-1196.
129. Hoogenboom LAP, Mulder PPJ, Zeilmaker MJ, van den Top HJ, Remmelink GJ, Brandon EFA, Klijnstra M, Meijer GAL, Schothorst R, Van Egmond HP. Carry-over

of pyrrolizidine alkaloids from feed to milk in dairy cows. Food Additives & Contaminants: Part A 2011;28(3):359-372.

130. De Nijs M, Mulder PPJ, Klijnstra MD, Driehuis F, Hoogenboom RLAP. Fate of pyrrolizidine alkaloids during processing of milk of cows treated with ragwort. Food Additives & Contaminants: Part A 2017;34(12):2212-2219.
131. Mulder PPJ, de Witte SL, Stoopen GM, van der Meulen J, van Wikselaar PG, Gruys E, Groot MJ, Hoogenboom RLAP. Transfer of pyrrolizidine alkaloids from various herbs to eggs and meat in laying hens. Food Additives & Contaminants: Part A 2016;33(16):1826-1839.
132. Eröksüz Y, Çeribaşi AO, Çevik A, Eröksüz H, Tosun F, Tamer U. Toxicity of *Heliotropium dolosum*, *Heliotropium circinatum*, and *Senecio vernalis* in parental quail and their progeny, with residue evaluation of eggs. Turkish Journal of Veterinary and Animal Sciences 2008;32(6):475-482.
133. Diaz GJ, Almeida LX, Gardner DR. Effects of dietary *Crotalaria pallida* seeds on the health and performance of laying hens and evaluation of residues in eggs. Research in Veterinary Science 2014;97(2):297-303.
134. WHO. (World Health Organization) International Programme on Chemical Safety (IPCS): Pyrrolizidine Alkaloids. Environmental Health Criteria 80 1988.
135. Bundesgesundheitsamt. Pyrrolizidin-Alkaloide, Stufe II Abwehr von Arzneimittelrisiken. Bekanntmachung über die Zulassung und Registrierung von Arzneimitteln, Bescheid vom 5. Juni 1992. Bundesanzeiger 1992;111:4805.
136. De Wit L, Geraets L, Bokkers B, Jeurissen S. Pyrrolizidine alkaloids in herbal preparations. 2014, The Dutch National Institute for Public Health and the Environment (RIVM).
137. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. Official Journal of the European Communities L 2002;140:10-22.
138. June 2019 Stakeholder Update on Rapidly Developing Policy on Food Contaminants. Accessed 29.09.2019 Available from: https://www.food.gov.uk/news-alerts/consultations/june-2019-stakeholder-update-on-rapidly-developing-policy-on-food-contaminants.
139. Council Regulation (EEC) No. 315/93 of 8 February 1993 laying down Community procedures for contaminants in food. Official Journal of the European Communities L 1993;37:1-3.

140. Hoesch G, Wiedenfeld H, Dingermann T, Roeder E. A new high performance liquid chromatography method for the simultaneous quantitative analysis of pyrrolizidine alkaloids and their N-oxides in plant material. Phytochemical Analysis 1996;7(6):284-288.

141. Joosten L, Mulder PPJ, Vrieling K, Van Veen JA, Klinkhamer PGL. The analysis of pyrrolizidine alkaloids in *Jacobaea vulgaris*; a comparison of extraction and detection methods. Phytochemical Analysis 2010;21(2):197-204.

142. Kempf M, Wittig M, Reinhard A, von der Ohe K, Blacquière T, Raezke KP, Michel R, Schreier P, Beuerle T. Pyrrolizidine alkaloids in honey: comparison of analytical methods. Food Additives & Contaminants: Part A 2010;28(3):332-347.

143. Mroczek T, Glowniak K, Wlaszczyk A. Simultaneous determination of N-oxides and free bases of pyrrolizidine alkaloids by cation-exchange solid-phase extraction and ion-pair high-performance liquid chromatography. Journal of Chromatography A 2002;949(1-2):249-262.

144. Betteridge K, Cao Y, Colegate SM. Improved Method for Extraction and LC-MS Analysis of Pyrrolizidine Alkaloids and their *N*-Oxides in Honey: Application to *Echium vulgare* Honeys. Journal of Agricultural and Food Chemistry 2005;53(6):1894-1902.

145. Mattocks AR. Detection of pyrrolizidine alkaloids on thin-layer chromatograms. Journal of Chromatography A 1967;27:505-508.

146. Mattocks AR, Jukes R. Improved Field Test For Toxic Pyrrolizidine Alkaloids. Journal of Natural Products 1987;50(2):161-166.

147. Chizzola R. Rapid sample preparation technique for the determination of pyrrolizidine alkaloids in plant extracts. Journal of Chromatography A 1994;668(2):427-433.

148. Lee ST, Schoch TK, Stegelmeier BL, Gardner DR, Than KA, Molyneux RJ. Development of enzyme-linked immunosorbent assays for the hepatotoxic alkaloids riddelliine and riddelliine *N*-oxide. Journal of Agricultural and Food Chemistry 2001;49(8):4144-4151.

149. Sakamoto S, Nagamitsu R, Yusakul G, Miyamoto T, Tanaka H, Morimoto S. Ultrasensitive immunoassay for monocrotaline using monoclonal antibody produced by *N,N*'-carbonyldiimidazole mediated hapten-carrier protein conjugates. Talanta 2017;168:67-72.

150. Oplatowska M, Elliott CT, Huet AC, McCarthy M, Mulder PPJ, Von Holst C, Delahaut P, Van Egmond HP, Campbell K. Development and validation of a rapid multiplex

ELISA for pyrrolizidine alkaloids and their N-oxides in honey and feed Rapid Detection in Food and Feed. Analytical and Bioanalytical Chemistry 2014;406(3):757-770.

151. Li N, Zhang F, Lian W, Wang H, Zheng J, Lin G. Immunoassay approach for diagnosis of exposure to pyrrolizidine alkaloids. Journal of Environmental Science and Health, Part C 2017;35(3):127-139.
152. Cramer L, Schiebel HM, Ernst L, Beuerle T. Pyrrolizidine alkaloids in the food chain: Development, validation, and application of a new HPLC-ESI-MS/MS sum parameter method. Journal of Agricultural and Food Chemistry 2013;61(47):11382-11391.
153. Beales KA, Colegate SM, Edgar JA. Experiences with quantitative trace analysis of pyrrolizidine alkaloids using GC-MS and LC-MS. Poisonous Plants and Related Toxins, T Acamovic, CS Stewart and TW Pennycott, Editors. 2003, CABI Publishing: Wallingford, UK.
154. Crews C, Berthiller F, Krska R. Update on analytical methods for toxic pyrrolizidine alkaloids. Analytical and Bioanalytical Chemistry 2010;396(1):327-338.
155. Griffin CT, Sheehan A, Danaher M, Furey A. Pyrrolizidine Alkaloids in Food: Analytical, Toxicological and Health Considerations. Analysis of Food Toxins and Toxicants. 2017, John Wiley & Sons, Ltd. 267-318.
156. Chung SWC, Lam CH. Development of an Analytical Method for Analyzing Pyrrolizidine Alkaloids in Different Groups of Food by UPLC-MS/MS. Journal of Agricultural and Food Chemistry 2018;66(11):3009-3018.
157. Griffin CT, Mitrovic SM, Danaher M, Furey A. Development of a fast isocratic LC-MS/MS method for the high-throughput analysis of pyrrolizidine alkaloids in Australian honey. Food Additives & Contaminants: Part A 2015;32(2):214-228.
158. Valese AC, Molognoni L, de Sá Ploêncio LA, de Lima FG, Gonzaga LV, Górniak SL, Daguer H, Barreto F, Oliveira Costa AC. A fast and simple LC-ESI-MS/MS method for detecting pyrrolizidine alkaloids in honey with full validation and measurement uncertainty. Food Control 2016;67(Supplement C):183-191.
159. Picotti P, Aebersold R. Selected reaction monitoring – based proteomics: workflows, potential, pitfalls and future directions. Nature Methods 2012;9(6):555-566.
160. These A, Bodi D, Ronczka S, Lahrssen-Wiederholt M, Preiss-Weigert A. Structural screening by multiple reaction monitoring as a new approach for tandem mass spectrometry: presented for the determination of pyrrolizidine alkaloids in plants. Analytical and Bioanalytical Chemistry 2013;405(29):9375-83.

161. Zhu L, Ruan JQ, Li N, Fu PP, Ye Y, Lin G. A novel ultra-performance liquid chromatography hyphenated with quadrupole time of flight mass spectrometry method for rapid estimation of total toxic retronecine-type of pyrrolizidine alkaloids in herbs without requiring corresponding standards. Food Chemistry 2016;194:1320-1328.
162. Wang T, Frandsen HL, Christiansson NR, Rosendal SE, Pedersen M, Smedsgaard J. Pyrrolizidine alkaloids in honey: Quantification with and without standards. Food Control 2019;98:227-237.
163. van de Schans MGM, Blokland MH, Zoontjes PW, Mulder PPJ, Nielen MWF. Multiple heart-cutting two dimensional liquid chromatography quadrupole time-of-flight mass spectrometry of pyrrolizidine alkaloids. Journal of Chromatography A 2017;1503(Supplement C):38-48.
164. BfR. PT-PA01 final report: international proficiency testing on the determination of pyrrolizidine alkaloids in herbal teas. 2016, BfR (Federal Institute for Risk Assessment): Berlin.

2 Aim of the work

The toxic secondary plant metabolites PA and PANO gained increased importance in the field of food and feed safety, in particular during the last decade. Contrary to other naturally occurring toxins, e. g. myco- and phycotoxins, PA/PANO did not attract the same attention, although their hepatotoxicity has already been demonstrated long ago. These alkaloids can contaminate the food chain mainly via unintended co-harvesting of PA/PANO-containing plants or seeds and cause harmful intoxications of humans and livestock.

Despite the main PA/PANO-producing plant families as well as toxicological effects and mechanisms of these alkaloids are well-known or still investigated, little is known concerning their behaviour in unprocessed food and feed. Therefore, the first aim of this work was to investigate the stability of PA and PANO in relevant food and feed matrices. For this purpose, a storage experiment of these plant toxins in honey, peppermint tea, and hay for a storage period of six months should be conducted. Analysis of the PA/PANO contents using targeted LC-MS/MS should clarify possible differences in the individual stability of the compounds.

Findings published in the last years revealed partially inexplicable results on PA/PANO contents in samples, particularly in honey. Thus, the second aim was to compare the influence of different analytical approaches and methodologies on the reported contents of PA/PANO in a sample and to elucidate the impact of demixing effects or settling processes in stored honey on the PA/PANO distribution. To answer these questions, naturally PA/PANO contaminated bulk honeys obtained from bee keepers should be stored, allowed to settle and analysed by three participating laboratories each using a different analytical methodology.

Analytical methods for the determination of PA/PANO cover targeted methods detecting a specific set of analytes as well as sum-based approaches. A major disadvantage of the latter is the loss of information on the specific PA/PANO pattern in the analysed sample. In contrast, the analysis of a limited set of PA/PANO may lead to underestimations of the toxin levels due to unknown analytes. Selecting a set of PA/PANO compounds as a compromise requires comprehensive data on specific and typical PA/PANO profiles of contaminated samples. Thus, the third aim of the work was to develop and validate a sensitive LC-MS/MS method to quantify a large number of individual PA/PANO analytes in a broad range of economically important tea matrices. Further, the method should be applied on teas purchased from a local supermarket. Information about the botanical origin of potential contaminations due to typical PA/PANO patterns should also be gained.

Some studies indicated PA/PANO to presumably occur in spices and culinary herbs in high levels due to similar contamination ways already known for (herbal) tea plants. Moreover, contaminated spices and herbs were assumed to greatly contribute to the exposure of consumers to the toxins. Thus, the fourth aim of the current work was to expand knowledge on the occurrence of PA/PANO in several spices and culinary herbs to identify potential sources with relevance for food safety. Therefore, representative samples of 15 different spice and culinary herb varieties should be investigated for the presence of PA/PANO. The results should be used to perform a risk assessment in the investigated matrices using exemplary recipes and consumption data from nutritional studies.

3 Results

3.1 Influence of storage on the stability of toxic pyrrolizidine alkaloids and their *N*-oxides in peppermint tea, hay and honey

A reprint of the publication can be found at section 8.1 at pages 83-90.

Three relevant matrices free of PA/PANO were artificially contaminated with the toxic alkaloids. The plant-based food and feed matrices peppermint tea and hay were lyophilised, milled and fortified with identically treated PA/PANO-containing plants (*Jacobaea vulgaris* and *Echium vulgare*). In case of honey, the sample was divided in two subsamples and spiked with 25 PA and PANO compounds (No. I), or with twelve PANO and, as a control, the otonecine-type PA senkirkine (No. II), respectively. All samples, including the PA/PANO mix and calibration solutions, were stored up to six months (182 days). During the storage, their PA/PANO levels were determined in increasing intervals using a targeted LC-MS/MS method with matrix-matched external calibration.

Florian Kaltner concepted the whole study, and prepared all samples for the storage experiment. Next, Florian Kaltner extracted and analysed the samples as scheduled during the whole period of the experiment, evaluated all LC-MS/MS data and calculated the amounts of the respective PA/PANO compounds. Further, Florian Kaltner interpreted all data by means of tables and visualisation in figures. He wrote the manuscript draft, designed the figures and revised the manuscript according to the reviewers' comments.

PA/PANO were shown to be stable in plant-derived matrices during the whole time of storage. In case of the stored mix solutions, no significant impact of the storage temperature on the detected levels were observed within the storage period of six months. In contrast, the sum levels of PA/PANO in both honey subsamples quickly decreased within the first few weeks. This observation was exclusively due to the decrease of PANO, while a reduction to their corresponding PA was not observable. Moreover, the velocity of PANO decrease seemed to be dependent on the individual chemical structure, with heliotridine-type as the most stable PANO compounds. In total, this work enlarged the knowledge on the stability of toxic PA/PANO in relevant food and feed matrices and, for the first time, systematically investigated and confirmed the putative decrease of PANO in honey.

3.2 Uncertainties in the determination of pyrrolizidine alkaloid levels in naturally contaminated honeys and comparison of results obtained by different analytical approaches

A reprint of the publication can be found at section 8.2 at pages 93-110.

Naturally PA/PANO contaminated bulk honeys were provided by bee keepers from Schleswig-Holstein (Germany). 20 honeys were investigated by three participating laboratories and the results were compared. Two additional honeys were used for a time series analysis and for examining a potential demixing or settling effect. These honeys were stored in conical plastic bags up to a maximum of twelve weeks and sampled from the top and the bottom every four weeks. Honeys were quantified on their levels of PA/PANO by three different approaches typically used in the respective laboratories: Matrix-matched external calibration, standard addition and sum-based method.

In this collaborative study, Florian Kaltner contributed to the conceptualisation and investigated the time series analysis samples of his participating laboratory. Additionally, Florian Kaltner evaluated the obtained LC-MS/MS data and calculated the contents of the PA/PANO compounds. Florian Kaltner significantly contributed to the interpretation of the overall data obtained from the study and helped to write the methods, results and discussion part of the manuscript.

The study revealed a decrease of the sum of PA/PANO in both investigated naturally contaminated honeys over time, which was consistently observed by all three applied analytical approaches. The lower PA/PANO contents were due to decreased PANO levels, while the corresponding PA were quite stable. Concerning potential demixing effects, no significant differences in the PA/PANO levels from top or bottom sampled honeys were detected within the twelve weeks of storage, thus not affecting the distribution of the alkaloids in settled honeys. The interlaboratory comparison of analytical approaches showed that the results matched well in 14 of 20 samples. Observed differences between the three applied methods were mainly due to varying sets of considered analytes or higher yields of the sum-based method, compared to the targeted approaches. In summary, this study highlighted that PA/PANO levels in honey obtained by different analytical approaches can be subject to considerable uncertainties, regarding the amount of measured PA/PANO, the comparability of the method and consequently regarding the health risk assessment.

3.3 Development of a sensitive analytical method for determining 44 pyrrolizidine alkaloids in teas and herbal teas via LC-ESI-MS/MS

A reprint of the publication can be found at section 8.3 at pages 113-129.

In this study, a sensitive and accurate method for the determination of PA/PANO covering as many individual commercially available analytes as possible should be developed and validated. The final method should be applicable to economically important tea matrices. For this purpose, 44 available PA/PANO reference standards were purchased. A LC-MS/MS method was developed, whereby different LC solvents, gradients, chromatographic phases, and couplings were tested. The validation confirmed a good sensitivity and accuracy. Finally, the newly developed method was applied to 18 retail tea samples purchased in a local supermarket and covering the six tea matrices peppermint, fennel, chamomile, rooibos, green tea and black tea.

Florian Kaltner thoroughly prepared the stock solutions and implemented suitable MRM transitions for each of the 44 compounds. Florian Kaltner supervised the method development, which was performed by a master's student. Further, Florian Kaltner optimised the sample extraction methodology as well as the following clean-up of extracts by testing several SPE cartridges, and finally validated the new method. Florian Kaltner evaluated all the LC-MS/MS data from the small occurrence survey and calculated the contents of PA/PANO in the 18 tea samples. Moreover, Florian Kaltner interpreted all data by means of tables and visualisation in figures. He wrote the whole manuscript, designed the figures and tables and revised the manuscript according to the comments of the reviewers.

The validated method expanded the number of reliably detectable PA/PANO compounds. Moreover, the final method was used to validate a developed ELISA within the framework of a joint research project. Concerning the small applicability survey, PA/PANO were found in 17 of 18 retail tea samples at sum levels up to 48.3 µg/kg. The method was applicable to a broad variety of different sample matrices, ranging from teas from seeds, flowers as well as raw or even fermented teas. Covering the high number of 44 individual analytes, the method enabled the determination of the botanical origin of contaminating PA/PANO plants due to their alkaloid patterns. Confirming the good performance of the method, the study provided an analytical tool to reliably detect a large set of individual PA/PANO in (herbal) tea matrices.

3.4 Occurrence and risk assessment of pyrrolizidine alkaloids in spices and culinary herbs from various geographical origins

A reprint of the publication can be found at section 8.4 at pages 131-146.

Spices and herbs are suspected to contribute to the overall exposure of consumers to PA/PANO and to partially contain high amounts of these toxins. In this study, the newly developed analytical method for determining 44 PA/PANO was applied to broaden the knowledge on the occurrence of PA/PANO in spices and herbs. Therefore, 305 authentic and representative samples of spices and culinary herbs, covering 15 matrices from worldwide origin, were investigated on the presence of PA/PANO. The samples were provided by manufacturers and collected in collaboration with the German Spice Association and in compliance with requirements of Commission Regulation (EC) 401/2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

Florian Kaltner significantly contributed to the conceptionalisation of the study, performed the measurements of the sample extracts and the evaluation of the obtained data. He calculated the PA/PANO levels, interpreted the results and performed the risk assessment on the basis of the obtained occurrence data. Florian Kaltner designed all tables and figures for visualisation, wrote the manuscript, revised it according to the reviewers' comments and applied for open access funding.

The analysis of the sample set revealed particularly oregano, but also cumin and marjoram, to contain medium to high amounts of PA/PANO with a high incidence of more than 85 %. In particular samples originating from Turkey, Greece, Albany and also from Germany contained high amounts of PA/PANO. While Sc-type compounds (from Asteraceae plants) were predominant in samples from Central Europe, Ht-type PA/PANO (from *Heliotropium* plants) were more abundant in samples from Turkey, Syria and Greece. In total, 58 % of all 305 investigated samples contained PA/PANO, with an average sum content of 323 µg/kg, a median less than 1 µg/kg and a 95 % percentile of 665 µg/kg. The highest PA/PANO sum content was 24,600 µg/kg in an oregano sample from Turkey. The results were used to perform a risk assessment on the acute and chronic health risks due to PA/PANO intake in spices and herbs, revealing a potential health risk in particular in case of children in a worst case and high consumption scenario.

4 General discussion

PA/PANO are secondary plant metabolites that are known since decades to cause intoxications in humans and livestock after ingestion due to their acute hepatotoxic potential. In the last years, the compounds gained increased importance in the issue of food and feed safety. Due to their carcinogenic risk when ingested chronically, PA/PANO came more and more in focus of food control authorities. Thus, the current work aimed to enlarge the knowledge about PA/PANO, particularly in the fields of their behaviour in food and feed, the suitable and sensitive analysis of the compounds as well as estimating their risk to consumers' health due to possibly yet disregarded intake sources of PA/PANO with relevance for food safety. Therefore, the behaviour of 25 PA/PANO in artificially contaminated peppermint tea, hay and honey during long-term storage was investigated. Interlaboratory analysis of naturally contaminated honeys were conducted to compare the impact of varying analytical approaches on the results. Then, a sensitive LC-MS/MS method for the determination of 44 PA/PANO compounds was developed and validated for important (herbal) tea matrices. Finally, the method was applied on a large set of authentic spice and culinary herb samples and a risk assessment on the acute and chronic health risks was performed.

The current work was the first long-term experiment investigating the stability of twelve PA/PANO compounds in relevant plant-based matrices peppermint tea and hay, and demonstrated the toxins to remain preserved in freeze-dried plant matrices: The sum contents as well as levels of the twelve individual PA/PANO compounds neither decreased nor increased significantly during the observed period of storage. Thus, former findings of CANDRIAN et al. were confirmed, who verified in 1984 the stability of twelve partly unknown cyclic diester PA in two artificially contaminated greenfodder and dried hay samples [4.1]. In our study, *Jacobaea vulgaris* and *Echium vulgare* were used to fortify the samples. Hereby, the stability of overall twelve detectable PA/PANO naturally occurring in these plants was investigated, also covering open-chained mono- and diesters (e. g. retrorsine and echimidine). Studies on the behaviour of PA/PANO in dried fodder barely exist in literature, and thus the findings expanded the knowledge on the persistence of PA/PANO in dry plant-based food and feed matrices. Results confirm that a simple drying of plant matrices during production of feed seems to be an inappropriate way to reduce the PA/PANO levels. This was also shown in a study on hot air grass pellet production on the PA/PANO contents, whereby no reduction of their amounts was detected [4.2]. In consequence, heat dried plant products cannot be regarded as safe concerning a potential contamination of PA/PANO.

Contrary to unprocessed or heat-dried feed, the production of grass silage as a further processing of feed seems to strongly affect the PA/PANO contents remaining in the final products. GOTTSCHALK et al. demonstrated in 2015 a strong decrease of PANO in combination with a small increase of the corresponding PA during production of experimental grass silages, which may be due to activity of microbiota typical for fermentation processes [4.3]. In contrast, several studies reported also a compound-dependent decrease of the tertiary amines: RONZCKA et al. evaluated the degradation of senecionine, seneciphylline and their *N*-oxides as well as senkirkine originating from *Senecio vernalis* under laboratory conditions [4.4]. After a fermentation period of three days only traces of PA and no PANO were detectable. Interestingly, the otonecine-type PA senkirkine remained stable during the whole period of fermentation of 90 days. These findings were fully reconfirmed by a study KLEVENHUSEN et al. conducted in 2019, where the losses of senecionine and seneciphylline were negatively correlated with short-chain carboxylic acids [4.5]. An equal observation was made for honey, regarding the stability of senkirkine and PANO (see next paragraph). None of the studies examined the exact reason or fate of the decreased PA/PANO during ensiling. It is still unclear if the compounds are possibly detoxified due to a full hydrolysis, or whether the decrease has a bacterial or plant enzymatic background. Altogether, the question remains whether PA are also degraded during ensiling or not, and, more important, if the yet unknown metabolites of both PA and PANO still reveal toxicological potential.

Additionally to plant-based food and feed matrices, the behaviour of PA/PANO in honey as another important matrix concerning PA/PANO contamination was investigated in the current work. Hereby, the decrease of PANO in honey was systematically investigated for the first time, with examining the stability of overall 25 PA/PANO compounds during long-term storage of 182 days. Moreover, the behaviour of PA/PANO in honey was further investigated in naturally contaminated bulk honeys provided by beekeepers. Formerly observed diverging results of stored honey raised the question on possible demixing effects on PA/PANO distribution, e. g. due to pollen settling [4.6]. Thus, honey was examined with particular regard to settling effects during a twelve-week storage period and comparability of results obtained by different analytical approaches.

Contrary to the results obtained for the plant-based matrices peppermint tea and hay, the sum content of PA/PANO in the artificially fortified honey quickly decreased within the first days and weeks. The findings issued exclusively from reduced levels of PANO, however, without formation of the corresponding PA: Their contents remained stable over time. Again, the

otonecine-type PA senkirkine remained preserved and did not decrease. Thus, differences in the individual degradation kinetics of the analytes were observed, comparable to results published in studies on production of grass silage [4.3; 4.7]. Strikingly, a decrease of PANO without forming the corresponding PA was also observed in naturally contaminated honeys provided from beekeepers. Thus, in the current work the structure-dependent decrease of PANO in honey during storage was specifically investigated and confirmed for the first time.

The findings of decreased PA/PANO sum levels were consistently detected by the three laboratories from the interlaboratory comparison analysis of honey performed in the current work. Herein, also one sum-based analytical approach was used, detecting derivated necine base core structures. So, an assumed enzymatic cleavage of the necic acid side chains of PANO or a simple reduction to the corresponding tertiary amine would have led to stable total sum levels of PA/PANO using the sum-based approach. Instead, it also revealed a decrease of the overall PA/PANO amounts in the honeys stored for twelve weeks. This result further indicated a still unknown derivatisation or polymerysation of the necine base itself, possibly due to an increased reactivity of PANO compared to PA.

The decrease of PANO in honey was already noticed in a former study: BETTERIDGE et al. investigated in 2005 PA/PANO in *Echium vulgare* honeys and postulated a reduction of PANO to their tertiary amines in honey [4.8]. In fact, this finding could not be confirmed within the current work, as the contents of PA remained stable or only increased in traces. In case of using heated honey (80 °C, 10 min), the spiked amounts of PANO did not show any decrease during an observed period of seven days. Spiking of PANO to six other honeys confirmed their decrease, although the velocity of the overall loss was lower. In conclusion, the varying structure-dependent reaction kinetics of the PANO decrease, as well as their stability in thermally treated honey strongly indicate an enzymatic background. It is known that honey enzymes show less activity with increased age of the honey [4.9], thus the lower degradation rates of PANO in the six additionally spiked and stored honeys may be explained due to their advanced age and reduced enzymatic activity.

Consequently, the proven PANO decrease in fresh honey can have a strong impact on the risk assessment: The study indicated stable PA/PANO contents after a storage of 60 to 80 days due to almost fully degraded PANO. Thus, it remains questionable at which point a sampling of honey is conducted best in view of repeatable and reliable results. For instance, EFSA reported high contamination levels higher than 1 mg/kg of singular PANO in honey in the past [4.10].

Taking results from the current work on the PANO decrease into account, the originate contents of PANO in the respective honey sample of the EFSA survey must have been much higher. Assuming the average PANO decrease reaction kinetics elucidated in the current work (approx. 99 % decrease within a few weeks) and a content of 1 mg/kg in a honey sample, the originate level of singular PANO compounds could have been up to 100 fold higher, depending on the age of the honey. At this point, it has to be considered that the levels reported by EFSA were much higher compared to the total amounts in the spiked honeys, and thus the uncertainty of this presumption can be very high.

Moreover, the PANO modification in honey to still unknown metabolites raise the question of the behaviour of these compounds in further bee products. In honey based products, a decrease of PANO was not observed in every case. For instance, the PA echimidine was recently shown to persist intact from honey to mead [4.11]. Consequently, the described PA transfer into mead can be expected for further compounds, in particular if they are more stable than echimidine itself. Indeed, it has to be mentioned that the study only investigated the persistence of the tertiary amine and not of its *N*-oxide. Thus, investigations on the stability of PANO in mead remains part of future research. The stability of PA/PANO in another bee product, namely bee bread, was analysed by KAST et al. in 2019. Bee bread is a product consisting of pollen fermented by *Lactobacillus* bacteria in the honeycomb. Results revealed a stability dependence on the ambient temperature: At 15 °C, the PA/PANO contents remained relatively stable, while an increased temperature of 30 °C resulted in reduced PANO levels due to a yet unknown reason, and PA remained preserved [4.12], equally to silage [4.5].

Due to its role during fermentation of pollen to bee bread, *Lactobacillus* spp. are possibly involved in the PANO decrease, in particular in case of ambient temperatures occurring during fermentation processes. Thus, the findings may have an impact on certain products also relevant to PA/PANO and *Lactobacillus*, e. g. yoghurt made out of contaminated milk or silage; the latter was already discussed in detail [4.3; 4.5]. Concerning the fate of PA/PANO in yoghurt, DE NIJS et al. recently demonstrated the decrease of PA/PANO in contaminated milk during production of yoghurt [4.13]. Pre-heated milk was inoculated with a starter culture, consisting of two *Lactobacillus* and *Streptococcus* species, and fermented to yoghurt at 42 °C. A decrease of about 27 % after six hours was detected, compared to the levels in the yoghurt milk prior to fermentation, mainly due to reduced content of the most abundant PA present in the originate milk, jacoline. In consequence, the reduction of PA was assumed to result from microbiological action. Consequently, further research is needed to fully elucidate the source of PA/PANO

decrease in relevant products during storage or further processing. Therefore, an ongoing research project was recently started [4.14].

In summary, the results on the loss of PANO in fresh honey presented in the current work strongly indicate a derivatisation at the necine base, probably due to enzymes. In consequence, the results raise the question at which time a sampling of honeys should be performed best in light of possibly varying detected PA/PANO levels due to an ongoing decrease of PANO. At the moment, a maturation of three months for honeys seems to be suitable to minimise deviations in analytical results due to the observed PANO degradation. As a result, uncertainties due to unstable PANO in honey may strongly impact analytical results and, thus, affect a proper risk assessments of PA/PANO in honey. This phenomenon can have a great impact on food safety since it remains unclear if the degradations of PANO is equal to a putative detoxification, or if the analytes are only modified and can still reveal their toxic potential after ingestion.

Besides the stability of PA/PANO, their distribution in naturally contaminated bulk honeys provided by beekeepers was further investigated, with particular regard to possible settling effects during storage, e. g. of pollen, and comparability of results obtained by different analytical approaches. No influences of a potential settling, in particular concerning pollen, on the distribution of PA/PANO in honey after storage was detected in the current work. In consequence, a putative settling of PA/PANO containing pollen towards the bottom of glasses or containers with stored honey should be regarded as unproblematic, at least concerning a possible concentration of PA/PANO due to settled pollen.

In the past, studies comparing analytical results in honey often differed significantly, and revealed uncertainties concerning the reported levels of PA/PANO [4.15]. Thus, in the current work an interlaboratory comparison analysis of 20 honeys using three approaches was conducted. Although detected levels matched well in 14 of 20 samples, the PA/PANO levels detected by the sum-based approach were mostly higher compared to targeted analytical methods. In general, sum-based methods can additionally detect PA/PANO compounds, for which no reference standards are commercially available. Thus, these methods usually report analyte levels above average in interlaboratory comparison measurements or proficiency testings [4.16]. Results obtained by the sum approach yielded in up to four times higher levels in some samples, which where, moreover, also the samples with the highest contamination within the study. Since these PA/PANO sum levels consisted of only < 37 % PANO, presumably much higher PANO amounts would have been detected without storage of the

honeys. Consequently, in this case a possible conversion of PANO into metabolites with intact necine base core structures must be considered. Thus, they would have been still accessible via the sum-based method. In total, honeys with increased amounts of PA (compared to PANO) might have had much higher initial PA/PANO sum levels and could generally be subject to underestimations.

Although the sum-based approach detected higher amounts due to covered unavailable standards and, thus, consequently might report more 'accurate' values, the complete loss of information on the PA/PANO distribution is a major drawback of this approach. Varying toxic potentials of singular PA/PANO (see 1.3.3) could no longer be assessed, and, furthermore, information on the botanical origin of the contamination would be lost. Nevertheless, sum-based methods can be strong screening methods, in particular in case of reduced effort due to the use of stable isotope labelled internal standards [4.17].

The two laboratories using targeted methods only analysed 25 PA/PANO, which reflected the number of then available analytical standards. The PA/PANO patterns detected in the naturally contaminated bulk honeys were clearly dominated by analytes typically appearing in *Senecio* plants. Further compounds appearing in this plant genera and not covered in the current approach, were proven to contribute significantly to PA/PANO sum contents, particularly the *N*-oxide of integerrimine, but also *N*-oxides of spartioidine and riddelliine [4.18]. A future implementation of other important PA/PANO, if available, should be considered to exploit information on botanical sources of contaminating plants as well as data on contributions of individual PA/PANO. Honey is produced and traded worldwide, thus the botanical sources of a potential PA/PANO contamination may greatly vary. Former studies investigating honeys on the presence of a certain type of PA/PANO revealed patterns of these alkaloids in the honey that were identical to them in the nectar and pollen. For instance, LUCCHETTI et al. demonstrated PA/PANO originating from *Echium vulgare* to occur in honey, and identically distributed like in the pollen and nectar [4.19]. Furthermore, KAST et al. prove pollen from *Echium vulgare*, *Eupatorium cannabinum* and *Senecio* spp. to be distinguishable according to their PA/PANO fingerprinting, with contaminant patterns corresponding to them in the respective plants [4.20]. Thus, honeys containing these pollen could also be assignable to the contaminating plants. In consequence, if the pattern of PA/PANO naturally occurring in contaminating plants as well as their areas of growing are known, to a certain degree honey analysis could reveal the geographical origin of the sample. In consequence, the contaminant pattern may be seen as first

hint on the authenticity of samples, and could also be expanded on other matrices of concern for possible PA/PANO contamination.

To enlarge the knowledge on typical contamination patterns, more occurrence data on PA/PANO in regional honeys are necessary, as requested by BfR in 2011, in light of safety of regionally produced and marketed honey [4.21]. These data may also help defining a suitable, standardised set of compounds for analytical method developments, in particular in case of upcoming regulatory limits referring to the sum of PA/PANO. Therefore, targeted methods covering more PA/PANO compounds are definitely of high scientific value.

Thus, in the course of the current work, a further aim was to improve the sensitivity and reliability of PA/PANO analysis in relevant matrices. Comparative analysis of PA/PANO in bulk honeys revealed uncertainties in the reported levels due to the different applied analytical approaches and the respective set of analytes. Besides honey, (herbal) teas are still in focus of national and international food safety authorities and seen to be of concern regarding potential PA/PANO contaminations. A few years ago, a set of 21 PA/PANO analytes was suggested to be sufficient to be monitored in food and feed supply chains [4.22], despite the analysis of additional compounds was recommended by EFSA [4.23]. Thus, a sensitive analytical method for determining a large quantity of commercially available analytes in a broad variety of relevant (herbal) teas was developed and validated. The method consisted of an improved sample extraction, an optimised clean-up of sample extracts and a LC-MS/MS detection with ESI as well as a quantification of analytes using matrix-matched external calibration to correct for possible matrix effects as far as possible. Since stable isotope labelled standards of PA/PANO are hardly available and very expensive, at the moment they were not seen as suitable for daily routine multiple analyte methods, and, thus, not considered for the developed method.

The developed method covered 44 individual PA/PANO available back then, and thus the number of covered analytes was nearly doubled compared to former LC-MS/MS methods [4.24; 4.25]. A problem of the used kind of calibration is the availability of a sample material free from the aimed analytes: In particular, in case of complex or mixed matrices, providing a blank sample material seems to be a drawback of the method. Moreover, since PA/PANO gained more and more attraction in the field of food safety in the last years, the number of commercially available reference standards has also continuously increased to approximately 60 compounds nowadays (end of 2019). The enlarged number of analytes was accompanied

with several groups of isomers showing identical or interfering MRM transitions. Thus, the chromatographical separation became more important with increasing number of analytes included into the method. Approaches with a strong focus on chromatographic separation of isomeric PA/PANO were already reported, but are often impractical for daily applications due to very long run times or due to a high instrumental effort, e. g. two-dimensional chromatography systems with quaternary pumps [4.26]. The use of column phases with smaller particle sizes typically used in ultra-high performance did not allow satisfying separation of isomeric analytes, in particular of the isomeric lycopsamine-type compounds [4.27]. At least, core-shell particles enabled sharper peaks and thus an improved resolution compared to traditional porous reversed phase materials. The resulting total run-time of 23 min including the re-equilibration was proven practicable for daily routine analysis, for which the method was intended.

Several PA/PANO compounds showed similar transitions according to their respective structure. In particular, the fragments of mass-to-charge ratio (*m/z)* 120 and *m/z* 138 were proven to be typical for all additional PA, while fragments *m/z* 118 and *m/z* 136 were observed for many PANO, like it was already known for the set including 28 PA/PANO compounds [4.28]. Moreover, depending on the grade of esterification some fragments were observed more frequently, for instance a loss of a fragment with a molecular weight of 28 u was detected more often in cyclic diesters. The findings confirm published results on typical fragmentation patterns of the diverse types of esters within the PA/PANO compounds [4.29]. Thus, analytes newly implemented in the developed method may also be truthfully covered by untargeted analytical approaches based on structural screenings.

Validation proved the method to be sensitive and accurate, but also revealed discrepancies for the 7-*O*-acetylated *N*-oxides of lycopsamine and intermedine. Due to the methodology, the two compounds degraded in high yields about 95 % to their deacetylated forms, intermedine *N*-oxide and lycopsamine *N*-oxide. This observation appeared exclusively in these two compounds: Other open-chained diester *N*-oxides, e. g. echimidine *N*-oxide or lasiocarpine *N*-oxide, remained stable. In general, acetylintermedine *N*-oxide and acetyllycopsamine *N*-oxide were reported to naturally occur in some Borganiaceae plants in small amounts, e. g. *Echium plantagineum* [4.30]. Hence, when information on the PA/PANO pattern is focussed, the analytes may only be qualitatively determined. In case of sum levels of PA/PANO are of interest, the 7-*O*-acetylated *N*-oxides of lycopsamine and intermedine could be covered by detecting them via intermedine *N*-oxide and lycopsamine *N*-oxide in sum. This instability

phenomenon was described for the first time in the current work. However, for the moment the exact reason for this degradation remains unknown and must be part of future research.

The newly developed method was applied to a small number of 18 teas and herbal teas to examine its suitability in daily routine analysis. Results showed a high incidence of PA/PANO in economically relevant (herbal) tea matrices. The toxic alkaloids were identified in 17 of the 18 investigated samples, up to 48.3 µg/kg. The sum contents were comparably low. For instance, a comprehensive survey across Europe on the presence of 28 PA/PANO in food performed by EFSA revealed the toxic alkaloids to be widely present in teas, ranging from mean sum contents of 272 µg/kg in chamomile (n = 35) to 597 µg/kg in rooibos tea (n = 22) [4.31].

Although the method of our study included a higher number of compounds, there might occur underestimations due to still unknown or not covered analytes. A sample of herbal medicine investigated by CHEN et al. in 2019 contained relatively low PA/PANO sum contents of 131 µg/kg when respecting 28 compounds. In contrast, a total PA/PANO sum content of 7.883 µg/kg was detected when all PA/PANO from the comprehensive set of 89, mainly synthesised or derived, analytes covered by the used analytical method were considered. Herein, particularly the 1,2-unsaturated PA adonifoline occurred in high amounts in the sample [4.32].

Regulatory limits within the EU currently are discussed to range between 75 µg/kg and 400 µg/kg for herbal infusions, depending on matrix and consumer group [4.33]. Consequently, based on our data the investigated (herbal) tea samples may be regarded to be of no concern for consumers' health. Indeed, the low detected PA/PANO sum contents of up to 48.3 µg/kg may be a hint for successful weed management strategies applied in the meantime. Nevertheless, this may not be overrated, since only 18 (herbal) tea samples were investigated. To reliably prove successful and enduring weed management, further data on the presence of PA/PANO in (herbal) teas must be collected, at best for several years and from various origins.

The new method appeared to be suitable for flower-like matrices (chamomile), raw (green tea and peppermint tea) and fermented plant material (black tea and rooibos tea) as well as for seeds (fennel). Within this project, the method was also applied for analysing PA/PANO in spices and culinary herbs. Only few studies were available so far, but the authors suspected spices and culinary herbs to contribute to the overall exposure of consumers to toxic PA/PANO [4.34; 4.35]. Thus, a large set of 305 authentic spice and culinary herb samples was investigated on

the presence of PA/PANO to evaluate the status quo of the alkaloids' incidence and contents in these matrices and to identify particular spices or herbs of concern for food safety.

The set of samples consisted of 15 different spices and culinary herb matrices, primarily harvested in 2016 and 2017. To the best of our knowledge, the study was the first one investigating authentic and representative spice and herb samples, since they were directly provided by companies of the German spice and herb industry. Sampling was in compliance with Commission Regulation (EC) No. 401/2006, providing representative results for the sampled lots. Findings revealed 58 % of samples being contaminated with at least one PA/PANO above the LOD. The average sum content was 323 µg/kg, with a median of 0.9 µg/kg and a remarkable maximum sum content of 24.6 mg/kg in an oregano sample from Turkey.

As demonstrated in the current work, PA/PANO remain preserved in dry plant-based matrices. Consequently, the analytical result in the spice and herb samples are reliable concerning the detected amounts as well as in view of the analyte patterns, since they are not subject to a decrease as observed in honey. Moreover, due to the representative sampling in compliance with Commission Regulation (EC) No. 401/2006 samples can be assumed to be homogeneous. Thinking the other way round, high PA/PANO levels detected in homogeneous samples could mean extremely higher contents in retail samples due to the usually occurring spot contamination via parts of PA/PANO containing plants within a bigger spice or herb lot. In consequence, the homogeneous samples from the current work might contain lower PA/PANO amounts due to a 'dilution' with uncontaminated raw material, whereas individual retail samples could contain very high sum levels due to packaging of contaminated 'spots' from a large lot. To underline this hypothesis, more data must be collected in further occurrence studies on retail spice and herb sample. Up to now, retail samples of only one culinary herb matrix (oregano) were investigated, revealing high contents up to 32.4 mg/kg. In total, 29 of the 41 samples (71 %) were classified as harmful food in the study [4.36].

In the current work, highest incidence of PA/PANO was detected in cumin (100 %), curry (100 %), marjoram (97 %) and oregano (88 %). Particularly oregano was also identified to contain high PA/PANO sum contents, with a mean of 3,140 µg/kg and a median of 163 µg/kg. This was in accordance with the results from the survey on PA/PANO in oregano conducted from KAPP et al., showing average levels of 6,160 µg/kg and a median of 5,430 µg/kg [4.36]. Interestingly, the oregano samples were predominantly contaminated with Ht-type PA/PANO,

namely europine, lasiocarpine and their *N*-oxides. Our study revealed identical findings for oregano with europine *N*-oxide as the most abundant PA/PANO, presumably due to *Heliotropium* spp. that might typically grow in the cultivation region of our oregano samples (Turkey and South Eastern Europe).

The average sum content over all samples in our work was 323 µg/kg and the median was 0.9 µg/kg, and thus comparable to former studies: The BfR investigated 40 spice and herb samples in 2016 and reported a mean sum level of 265 µg/kg and a maximum of 4,990 µg/kg [4.33]. PICRON et al. showed an average sum content of 197 µg/kg and a maximum of 1,770 µg/kg in 17 spices and herbs. Strikingly, the latter study also reported Ht-type PA/PANO to mainly contribute to the total sum contents of singular samples [4.35]. In 2019, the BfR reported an average sum level of 2,680 µg/kg with a median of 10 µg/kg over all 263 spice and culinary herb samples [4.37]. Excluding the higher contaminated matrices oregano and lovage, as well as the originally PA/PANO containing borage, still showed an average of 186 µg/kg in the remaining 180 samples. In consequence, our results confirmed these findings and also strongly indicated oregano to be a main contributor to a PA/PANO exposure via spices and herbs.

An unintended co-harvesting of PA/PANO containing plants on the fields may explain high amounts of the contaminants in field spices like oregano, basil, parsley, or (herbal) tea plants. In contrast, even in spice matrices not being affected by co-harvesting on the fields, e. g. ginger roots (growing in the soil, 69 % positive samples) or pepper (growing on trees, 11 % positive samples), PA/PANO were found in small amounts. This observation indicates a possible cross-contamination with PA/PANO containing plants or parts of these plants during processing. This assumption may be the most probable in case of pepper. A further explanation for the PA/PANO contamination may be a horizontal transfer of the alkaloids in the soil [4.38]. Non-PA/PANO-producing parsley plants were grown in vicinity to *Senecio* plants, but thoroughly separated from each other and, thus, a direct contact of roots, stems or leaves could be ruled out. The only point of contact of the plants was the soil itself. Since this kind of contamination via the soil was shown for parsley, it may also be important in case of any other spice and herb growing on fields, where possible PA/PANO containing plants may grow in between.

However, the detected PA/PANO sum contents were too high in many spice and herbs samples. Applying the PA/PANO maximum levels which are discussed as being implemented in

Commission Regulation (EC) 1881/2006 in near future (dried herbs and cumin seeds: 400 µg/kg; dried oregano and dried marjoram: 1,000 µg/kg) [4.33], revealed 22 of 231 concerned samples (10 %) exceeding the maximum levels. In detail, in four of eleven cumin samples (36 %), in seven of 24 oregano samples (29 %), in one of 31 marjoram samples (3 %) and in ten of 165 other herb samples (6 %) the PA/PANO sum content would have been too high. Concludingly, in particular the high PA/PANO contents in oregano and cumin indicated the contamination originating from unsatisfying weed management on primary production stages. Ongoing trainings of farmers for an effective control and weed management may help to reduce the PA/PANO levels in near future. In total, even if our study is one of the most comprehensive available, there is still a lack of data on the PA/PANO contents in the addressed matrices, as data on singular matrices each ranged about 20 samples only.

Regarding health risk assessment of PA/PANO performed in this study for spices and herbs, two major uncertainties tackling the true intake of these foods must be taken into account: First, the reliability of ingestion data obtained from the used nutrition surveys [4.39; 4.40], in particular when singular spice and herb varieties are adressed, and second, if fresh or dried herbs are used for cooking. Therefore, the European Spice Association recommended the consideration of dehydration factors, [4.41]. They range from five to 13, depending on the matrix, compensate the loss of weight due to drying of spices and herbs, and thus relate to residual moisture contents of 8 % to 20 % in dry matter. While given contents of contaminants regularly relate to dried products, recipes mostly use fresh spices and culinary herbs. Thus, it could be unclear, if the used or ingested amounts of spices and herbs relate to dry or fresh matter.

To assess the acute health risk, exceedance factors of an HBGV of 0.1 µg/kg BW/d were calculated (see 1.3.5), with respect to the regarded scenario of PA/PANO sum content in the culinary herbs. Therefore, in the mean case scenario the average sum contents in herbs used in exemplary recipes were used (oregano: 3,140 µg/kg; thyme: 49.1 µg/kg, basil: 11.5 µg/kg), while for the worst case scenario the 95 %-percentiles of the sum contents were considered (oregano: 17,000 µg/kg; thyme: 191 µg/kg, basil: 68.3 µg/kg). To prevent overestimations of the intake when performing risk assessments on PA/PANO in spices and herbs, a dehydration factor of six (recommended for oregano, as the main contributor to the total PA/PANO levels in the exemplary recipes) was additionally considered in the current work.

Considering dried herbs for the recommended amounts of thyme, basil and oregano in both recipes, resulted for adults in exceedance factors of 0.3 and 1.7 in the mean case scenario and of 1.6 and 9.2 in the worst case scenario. In case of infants, the resulting HBGV exceedance factors were 1.3 and 1.2 in the mean case scenario and of 7.0 and 6.4 in the worst case scenario. In 2019, the BfR came to similar results, calculating a HBGV exceedance factor of 2.1 in case of tomato sauce with oregano and considering a worst case scenario of a PA/PANO sum level of 26,805 µg/kg [4.36]. Hereby, the BfR did not consider any dehydration factors.

When applying the dehydration factor of six, the acute intake resulted in HBGV exceedance factors of 0.0 and 0.3 for adults and 0.2 in case of children, when the mean case scenario was assumed. On the other hand, the worst case scenario resulted in exceedance factors of 0.3 and 1.5 for adults, as well as 1.2 and 1.1 for infants. The performed assessment on the acute health risk based on our collected data revealed an exceeding of the HBGV, even if dehydration factors were applied. In consequence, the acute ingestion of highly PA/PANO contaminated spices and culinary herbs may not be seen as safe regarding potential non-neoplastic effects.

To assess the chronic health risk, the MOE approach was used, based on a $BMDL_{10}$ of 237 µg/kg BW/d, whereby MOE values > 10,000 are seen as of little concern for public health (see 1.3.5). In case of adults, three consumption scenarios were included (median average: 0.77 g/d; mean average: 1.68 g/d; high: 6.09 g/d), based on [4.39]. For infants, an average consumption (0.70 g/d) and a high consumption scenario (1.40 g/d) was taken into account, based on [4.40]. In total, three risk groups concerning the PA/PANO sum contents were considered (low risk: 20 µg/kg; medium risk: 330 µg/kg, high risk: 5,500 µg/kg).

Once again, the influence of the dehydration factor was highlighted within the results. When the daily ingestion was assumed to be related to dry spices and herbs and thus no dehydration factor was applied, in case of high risk PA/PANO sum contents the MOE fell below 10,000 in every consumption scenario for both adults and infants. Additionally, this was even the case for the medium risk sum content of 330 µg/kg in both high consumption scenarios. Contrary to our results, the BfR already calculated MOE values < 10,000 in case of high consumption adults for medium PA/PANO sum contents of 1,000 µg/kg [4.37]. Again, dehydration factors were not considered and, additionally, the average/medium content was remarkably high, compared to 330 µg/kg used in the current work.

When a dehydration factor of six (average of the factors from the matrices concerned in the current study) was applied to the results, in case of adults the MOE fell below 10,000

exclusively in the high consumption scenario combined with the high risk sum content group. Remarkably, considering the latter content group resulted in MOE of 5,920 and 2,950 for average and high consumption scenarios of infants. Consequently, PA/PANO in spices and culinary herbs may be seen of concern for public health.

In summary, the differing results in acute health risk estimation highlighted the significance if fresh or dried herbs were considered for the ingested amounts. In general, risk assessments performed by the BfR are usually related to dry matter, and so, in consequence, a standardisation, or at least the additional consideration of dehydration factors should be aimed at in future assessments. At this point, it has to be mentioned, that generally all sources of PA/PANO have to be considered and included into the overall ingestion to perform a proper risk assessment. Apart from spices and herbs, (herbal) teas, food supplements as well as honey were shown to contribute to the overall exposure of consumers to PA/PANO [4.42].

In total, the risk assessment results revealed that further monitoring of PA/PANO in spices and culinary herbs must be of high priority, because spices and culinary herbs possess a not negligible impact on the overall exposure of consumers to toxic PA/PANO, in particular in cases of high consumption scenarios and children.

[4.1] Candrian U, Luethy J, Schmid P, Schlatter C, Gallasz E. Stability of pyrrolizidine alkaloids in hay and silage. Journal of Agricultural and Food Chemistry 1984;32(4):935-937

[4.2] Gottschalk C, Ostertag J, Meyer K, Gehring K, Thyssen S, Gareis M. Influence of grass pellet production on pyrrolizidine alkaloids occurring in *Senecio aquaticus*-infected grassland. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 2018;35(4):750-759

[4.3] Gottschalk C, Ronczka S, Preiß-Weigert A, Ostertag J, Klaffke H, Schafft H, Lahrssen-Wiederholt M. Pyrrolizidine alkaloids in natural and experimental grass silages and implications for food safety. Animal Feed Science and Technology 2015;207:253-261

[4.4] Ronczka S, Winter J, Pieper R, Korn U, Zentek J, Speer K, Pieper B. Investigation on the degradation of pyrrolizidine alkaloids in Eastern Groundsel (*Senecio vernalis*) during ensilage. Proceedings of the Society of Nutrition Physiology 2012;21:68

[4.5] Klevenhusen F, Pieper R, Winter J, Ronczka S, Speer K. Stability of pyrrolizidine alkaloids from *Senecio vernalis* in grass silage under different ensilage conditions. Journal of the Science of Food and Agriculture 2019;99(14):6649-6654

[4.6] Huckauf A. Projekt Nachweis von Pyrrolizidin-Alkaloiden in Honig -Endbericht- 2017. Stiftung Naturschutz Schleswig-Holstein, Molfsee 2017

[4.7] Becerra-Jimenez J, Kuschak M, Roeder H. Toxic pyrrolizidinalkaloids as undesired contaminants in food and feed: degradation of the PAs from *Senecio jacobaea* in silage. Pharmazie 2013;68:636-639

[4.8] Betteridge K, Cao Y, Colegate SM. Improved method for extraction and LC-MS analysis of pyrrolizidine alkaloids and their *N*-oxides in honey: Application to *Echium vulgare* honeys. Journal of Agricultural and Food Chemistry 2005;53(6):1894-1902

[4.9] Oddo LP, Piazza MG, Pulcini P. Invertase activity in honey. Apidologie 1999;30(1):57-65

[4.10] EFSA (European Food Safety Authority). Scientific opinion on pyrrolizidine alkaloids in food and feed. EFSA Journal 2011;9(11):134

[4.11] Cao Y, Colegate SM, Edgar JA. Persistence of echimidine, a hepatotoxic pyrrolizidine alkaloid, from honey into mead. Journal of Food Composition and Analysis 2013;29(2):106-109

[4.12] Kast C, Kilchenmann V, Reinhard H, Bieri K, Zoller O. Pyrrolizidine Alkaloids: The Botanical Origin of Pollen Collected during the Flowering Period of *Echium vulgare* and the Stability of Pyrrolizidine Alkaloids in Bee Bread. Molecules 2019;24(12):2214

[4.13] De Nijs M, Mulder PPJ, Klijnstra MD, Driehuis F, Hoogenboom RLAP. Fate of pyrrolizidine alkaloids during processing of milk of cows treated with ragwort. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 2017;34(12):2212-2219

[4.14] Gottschalk C, Kaltner F, Knappstein K, Lamp J, Gehling T, These A, Dänicke S. PA SAFE FEED - Studies on the carry-over of pyrrolizidine alkaloids in livestock 2019 [accessed 17th January 2020]. Available from: https://orgprints.org/36318/

[4.15] Breidbach A, Tamošiūnas V. Pyrrolizidine alkaloids in herbal tea and honey - report on the 2017 proficiency testing scheme. JRC Report (EUR 28960) 2017

[4.16] Tamošiūnas V, Mischke C, Mulder PPJ, Stroka J. Report on the 2012 Proficiency Test on pyrrolizidine alkaloids in honey and hay. JRC Report (EUR 26235) 2013

[4.17] Cramer L, Schiebel HM, Ernst L, Beuerle T. Pyrrolizidine alkaloids in the food chain: Development, validation, and application of a new HPLC-ESI-MS/MS sum parameter method. Journal of Agricultural and Food Chemistry 2013;61(47):11382-11391

[4.18] Cheng D, Nguyen VT, Ndihokubwayo N, Ge J, Mulder PPJ. Pyrrolizidine alkaloid variation in *Senecio vulgaris* population from native and invasive ranges. PeerJ 2017;5:e3686

[4.19] Lucchetti MA, Glauser G, Kilchenmann V, Dübecke A, Beckh G, Praz C, Kast C. Pyrrolizidine alkaloids from *Echium vulgare* in honey originate primarily from floral nectar. Journal of Agricultural and Food Chemistry 2016;64(25):5267-5273

[4.20] Kast C, Kilchenmann V, Reinhard H, Droz B, Lucchetti MA, Dübecke A, Beckh G, Zoller O. Chemical fingerprinting identifies *Echium vulgare*, *Eupatorium cannabinum* and *Senecio* spp. as plants species mainly responsible for pyrrolizidine alkaloids in bee-collected pollen. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 2018;35(2):316-327

[4.21] BfR (Federal Institute for Risk Assessment) Opinion No. 038/2011: Chemical analysis and toxicity of pyrrolizidine alkaloids and assessment of the health risks posed by their occurrence in honey. Berlin 2011

[4.22] BfR (Federal Institute for Risk Assessment) Opinion No. 002/2016: Provisional BfR recommendations on the analysis of pyrrolizidine alkaloids (PA) in herbal tea and tea (analyte spectrum and sampling method). Berlin 2016

[4.23] EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), Knutsen HK, Alexander J, Barregard L, Bignami M, Brüschweiler B, Ceccatelli S, Cottrill B, Dinovi M, Edler L, Grasl-Kraupp B, Hogstrand C, Hoogenboom LR, Nebbia CS, Oswald IP, Petersen A, Rose M, Roudot AC, Schwerdtle T, Vleminckx C, Vollmer G, Wallace H, Ruiz-Gomes JA, Binaglia M. Statement on the risk for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusion and food supplements. EFSA Journal 2017;15:4908-4942

[4.24] BfR (Federal Institute for Risk Assessment) Determination of pyrrolizidine alkaloids in honey with SPE-LC-MS/MS. Berlin 2013

[4.25] BfR (Federal Institute for Risk Assessment) Determination of pyrrolizidine alkaloids in plant material with SPE-LC-MS/MS. Berlin 2014

[4.26] Van de Schans MGM, Blokland MH, Zoontjes PW, Mulder PPJ, Nielen MWF. Multiple heart-cutting two dimensional liquid chromatography quadrupole time-of-flight mass spectrometry of pyrrolizidine alkaloids. Journal of Chromatography A 2017;1503(Supplement C):38-48

[4.27] Jeong WT, Lim HB. Determination and Chemical Profiling of Toxic Pyrrolizidine Alkaloids in Botanical Samples with UPLC-Q-TOFMS. Chromatographia 2019;82(11):1653-1664

[4.28] Bodi D, Ronczka S, Gottschalk C, Behr N, Skibba A, Wagner M, Lahrssen-Wiederholt M, Preiss-Weigert A, These A. Determination of pyrrolizidine alkaloids in tea, herbal drugs and honey. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 2014;31(11):1886-1895

[4.29] These A, Bodi D, Ronczka S, Lahrssen-Wiederholt M, Preiss-Weigert A. Structural screening by multiple reaction monitoring as a new approach for tandem mass spectrometry: presented for the determination of pyrrolizidine alkaloids in plants. Analytical and Bioanalytical Chemistry 2013;405(29):9375-9383

[4.30] Skoneczny D, Zhu X, Weston PA, Gurr GM, Callaway RM, Weston LA. Production of pyrrolizidine alkaloids and shikonins in *Echium plantagineum* L. in response to various plant stressors. Pest Management Science 2019;75(9):2530-2541

[4.31] Mulder PPJ, López Sánchez P, These A, Preiss-Weigert A, Castelari M. Occurrence of pyrrolizidine alkaloids in food. EFSA Supporting Publication 2015;8:18-31

[4.32] Chen L, Mulder PPJ, Peijnenburg A, Rietjens IMCM. Risk assessment of intake of pyrrolizidine alkaloids from herbal teas and medicines following realistic exposure scenarios. Food and Chemical Toxicology 2019;130:142-153

[4.33] June 2019 Stakeholder Update on Rapidly Developing Policy on Food Contaminants. Available from: (https://www.food.gov.uk/news-alerts/consultations/june-2019-stakeholder-update-on-rapidly-developing-policy-on-food-contaminants)

[4.34] BfR (Federal Institute for Risk Assessment) Opinion No. 030/2016: Pyrrolizidine alkaloids: Levels in foods should continue to be kept as low as possible. Berlin 2016

[4.35] Picron JF, Herman M, Van Hoeck E, Goscinny S. Analytical strategies for the determination of pyrrolizidine alkaloids in plant based food and examination of the transfer rate during the infusion process. Food Chemistry 2018;266:514-523

[4.36] Kapp T, Hägele F, Plate EM. Oregano - an aromatic, but rich in content culinary herb. Part I: Pyrrolizidine alkaloids. 2019. Available from: (http://www.ua-bw.de/pub/beitrag.asp?subid=1&Thema_ID=5&ID=3008&lang=DE&Pdf=No)

[4.37] BfR (Federal Institute for Risk Assessment) Opinion No. 017/2019: Pyrrolizidine alkaloid levels in dried and deep-frozen spices and herbs too high. Berlin 2019

[4.38] Selmar D, Wittke C, Beck-von Wolffersdorff I, Klier B, Lewerenz L, Kleinwächter M, Nowak M. Transfer of pyrrolizidine alkaloids between living plants: A disregarded source of contaminations. Environmental Pollution 2019;248:456-461

[4.39] MRI (Max Rubner Institute) Nationale Verzehrsstudie II: Lebensmittelverzehr und Nährstoffzufuhr auf Basis von 24h-Recalls (German National Nutrition Survey II: Food Consumption and Nutrient Intake on the Basis of 24h Recalls). Karlsruhe 2008

[4.40] Banasiak U, Heseker H, Sieke C, Sommerfeld C, Vohmann C. Estimation of the dietary intake of pesticide residues based on new consumption data for children. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 2005;48(1):84-98

[4.41] ESA (European Spice Association) European Spice Association recommends dehydration factors to assess pesticide residues on products of the spice industry. Journal of Consumer Protection and Food Safety 2008;3(4):474-478

[4.42] BfR (Federal Institute for Risk Assessment) Opinion No. 020/2018: Actual risk assessment concerning contents of 1,2-unsaturated pyrrolizidine alkaloids in food. Berlin 2018

5 Future research

PA/PANO are toxic secondary plant metabolites contaminating food and feed worldwide and causing harmful effects in humans and livestock. In the current work, the decrease of PANO in naturally contaminated bulk honeys as well as in artificially fortified fresh blank honey was systematically investigated. A structure-dependent decrease strongly indicated an enzymatic derivatisation. As an aim of future research, the potential PANO metabolites should be identified. Therefore, fresh honey should be spiked and stored, and the extracts should be investigated using untargeted metabolomics approaches combined with multivariate analysis. Identified metabolites can be further characterised using targeted high-resolution MS/MS, and possibly by NMR after isolation and purification of the compounds using (semi-) preparative LC. Moreover, the potential remaining toxicity of the derivatives should further be assessed for a proper risk characterisation. For this, a first estimation on the toxic behaviour can be obtained by comparing results of cytotoxicity tests of extracts of stored and freshly PANO-spiked honeys. Consequently, the background for the derivatisation of PANO in honey should be investigated via testing of enzymes typically occurring in honey, or recombination experiments with artificial honey may enlight the background of the PANO decrease.

Besides honey, the stability or behaviour of PA/PANO during processing of food and feed should generally be investigated in other matrices. In this context, more studies concerning the behaviour of PA/PANO after ingestion by livestock should be conducted to gain data on the transfer of the toxic alkaloids in animal-derived products. Therefore, feeding studies with dairy cows, goats and sheep can enlarge the knowledge on the transfer of the alkaloids into the milk of animals more or less prone to the known toxic effects of PA/PANO. Additionally, production of cheese and yoghurt on the basis of naturally and artificially contaminated milk can provide further insights into the behaviour of PA/PANO in processed milk products of animal origin.

In honey, variating analytical results were obtained, due to the decrease of PANO during honey maturation and, on the other hand, due to the used analytical approach and considered set of PA/PANO analytes. To cover as many analytes as possible, a targeted method for determining all back then commercially available 44 PA/PANO was developed. The newly developed method presented in the current work was shown to be capable of sensitively and accurately determining the PA/PANO profile in (herbal) tea matrices. Meanwhile, approximately 60 PA/PANO reference standards are available, and should be implemented in future methods. The developed method also provides the opportunity to be applied to further relevant matrices, e. g. dry animal feed, crop plants, flour, plant-based as well as honey-based food supplements.

In addition, an easy and fast screening method for the analysis of PA/PANO as a sum parameter should be developed, since the current sum-based methods targeting the necine backbone consist of many time-consuming steps for reduction and derivatisation. To ease such an analytical sum approach, a faster purification of extracts, for instance by using planar SPE, should be considered, as well as a possible 'downscaling' of the instrumental effort to common HPLC-FLD instead of MS detection, enabling a reliable control of raw materials up to certain cut-off level, e. g. 25 µg/kg, depending on the matrix. For this purpose, the derivatisation of the analytes would still be necessary. Increased sensitivity of MS should be used to develop more sensitive dilute-and-shoot approaches. A future generation of antibodies with a good cross-reactivity on the most important PA and maybe even PANO should be used to develop reliable and sensitive ELISA kits or dipstick tests for a quick assessment of raw materials directly on-site.

The analysis of a large set of spice and herb samples revealed the high incidence of toxic PA/PANO in these matrices, partially in very high contents. Moreover, some spices or herbs seemed to be more afflicted with a PA/PANO contamination than others were. A risk assessment based on the collected occurrence data in spices and culinary herbs revealed a not negligible impact on the total exposition of consumers to PA/PANO. Nevertheless, more data are necessary: Despite a quite high number of 305 samples has been investigated, the number of samples within each individual spice or herb matrix was around 20 samples or less. As an aim of future research, the quantity of reliable data obtained from analysing more authentic spice and culinary herb samples should be considerably increased to provide a better basis for overall exposure. Therefore, the intake of spices and culinary herbs should be included in future nutrition surveys. Furthermore, PA/PANO levels in spices and herbs of particular risk, for instance oregano and cumin, should be thoroughly monitored the next years to evaluate management actions aiming in reduction of PA/PANO contaminations.

Moreover, application of the newly developed targeted LC-MS/MS method revealed a broad variation of individual PA/PANO in the spice samples, strongly depending on the botanical source of the contaminating plant. Many spices and culinary herbs are nearly exclusively cultivated in relatively small regions of the world, e. g. pepper (Vietnam, Indonesia), nutmeg (Indonesia, Grenada) or cumin (India, China). Multivariate analysis based on additional reliable and authentic data on occurrence and distribution of PA/PANO in contaminated samples may be used in the future to generate some kind of a prediction model. Based on PA/PANO patterns

typically found in contaminating plants occurring in the cultivation region, the model could be applied to give a hint on potential food fraud in the respective spice or herb samples.

Besides spices and herbs, further food matrices were demonstrated to be potentially of concern regarding PA/PANO exposure. Thus, further plant- and animal-derived food matrices should be investigated on occurrence of PA/PANO, such as food supplements, wheats, flours, meat and eggs. The data should be used to enlarge the data basis and to evaluate the risk of PA/PANO exposure to consumers more properly.

Former studies indicated differences of the singular PA/PANO concerning their toxicity, and consequently structure-dependent interim relative potency factors were proposed. Consistent investigations on potency factors should be engaged, aiming in building up a database to reliably consider differences in the individual toxicity of the compounds. Such a database could have a strong impact on future risk assessment of PA/PANO.

6 Summary

Pyrrolizidine alkaloids (PA) and their corresponding *N*-oxides (PANO) are secondary metabolites produced by a wide variety of flowering plants against herbivorous insects. Currently, more than 660 compounds are known to presumably occur in more than 6,000 plant species worldwide. Though PA/PANO are assumed to be non-toxic in their originate form, the group of 1,2-unsaturated PA/PANO is known to act as protoxins. Ingestion reveals their toxic potential due to toxification processes in the liver by metabolic activation to highly reactive and thus, harmful pyrrolic derivatives via cytochrome P450 monooxygenases (CYP450). The activated PA/PANO metabolites can cause acute or chronic dysfunctions, damages of cells and may even induce carcinomas, particularly in the liver. PA/PANO can enter the food chain by unintended co-harvesting of the contaminating plants, by transfer of the toxins into culture plants via the ambient soil, or by transfer into honey due to honeybees collecting pollen and nectar from PA/PANO containing plants. Due to their harmful effects on humans and livestock, PA/PANO are unwanted to occur in food and feed.

Only few studies tackled the stability of the toxic alkaloids in food and feed so far. Thus, the first aim of the current work was to investigate the behaviour of PA/PANO in relevant food and feed matrices during long-term storage. For this purpose, the representative matrices honey, peppermint tea and hay were spiked with overall 25 PA/PANO reference standards, or, in case of the plant matrices, with freeze-dried and milled PA/PANO-containing plants to simulate a co-harvesting. The prepared samples were stored for six months in a dark and dry ambience and repeatedly analysed on their PA/PANO contents using targeted LC-MS/MS. To elucidate the impact of demixing effects or settling processes in stored honey on the PA/PANO distribution, a second study was conducted: Naturally PA/PANO contaminated bulk honeys were stored in conical plastic bags for a period of twelve weeks and allowed to settle. Honeys were sampled on the top and on the bottom, and PA/PANO contents were determined by three participating laboratories each using a different analytical methodology: One sum-based method and two targeted approaches using either standard addition or matrix-matched external calibration for the quantification of PA/PANO, respectively.

In case of plant-based matrices peppermint tea and hay, the PA/PANO were shown to be stable during the whole time of storage. Identical findings were obtained for stored mix solutions of PA/PANO and no impact of the storage temperature on the detected levels were observed within the storage period of six months. Contrary, the sum levels of PA/PANO in honey samples quickly decreased within the first few weeks. This observation was exclusively due to the

decrease of PANO, without a formation of the corresponding PA: While the contents of the tertiary amine PA remained stable during the observed period of 182 days, the loss of the twelve spiked PANO was 99 %. Depending on the analytes, differences in the individual velocity of the decrease were detected: For instance, after 14 d the loss of retronecine-type cyclic diesters was significantly higher (e. g. jacobine *N* oxide: -97 %), compared to heliotridine-type mono- or diesters (e. g. lasiocarpine *N* oxide: -69 %). The findings were confirmed in six additionally spiked and stored retail honeys of different floral and geographical origin.

Moreover, the results of decreased PANO in stored honey were also observed in the naturally contaminated bulk honeys stored for twelve weeks, which was consistently detected by the three participating laboratories. Due to reaction kinetics and structure-dependent degradation of PANO, an enzymatic background was assumed. Besides the uncertainties due to a PANO decrease, the three different analytical approaches applied by the laboratories (one sum-based and two targeted LC-MS/MS methods) revealed partially inexplicable results on PA/PANO contents in the honey samples. The ratios of PA/PANO sum levels detected in the respective bottom and top samples of the honeys ranged from 79 % to 118 %, depending on the honey sample and the laboratory. As a result, no potential effect of storage-related demixing on the PA/PANO distribution in honeys was observed. In addition, an interlaboratory comparison of analytical approaches by analysing 20 honeys showed that the results matched well in 14 samples. Differences between the three applied methods were mainly due to varying sets of considered analytes or higher yields of the sum-based method, compared to the targeted approaches. The study highlighted that PA/PANO levels in honey obtained by different analytical approaches can be subject to uncertainties due to instability of PANO or varying sets of covered analytes.

A major disadvantage of sum-based analytical methods (i. e. reduction of PA/PANO to their common necine base backbone and derivatisation) is the loss of information on the specific PA/PANO pattern in the analysed sample. Thus, a further aim of the work was to develop a sensitive LC-MS/MS method for quantifying all 44 back then available PA/PANO analytes and to validate it for important tea matrices. During method development, the sample extraction as well as the further clean-up of extracts was optimised. The validation revealed the method to be sensitive and accurate, with limits of detection (LOD) ranging from 0.1 µg/kg to 7.0 µg/kg and precisions were between 0.7 % and 16.1 %. Recoveries ranged from 60.7 % to 128.8 % for 40 of 44 analytes. The newly developed method was applied to 18 retail tea samples purchased from a local supermarket. PA/PANO were found in 17 of 18 retail tea samples at sum levels up

to 48.3 µg/kg. The method was shown to be applicable to a broad variety of different sample matrices, ranging from teas from seeds, flowers as well as raw or even fermented teas. The method also enabled conclusions on the botanical origin of contaminating plants by determining the respective alkaloid distribution due to the high number of 44 covered analytes.

Former studies indicated PA/PANO to presumably occur in spices and culinary herbs in high levels. Contaminated spices and herbs were assumed to greatly contribute to the exposure of consumers to the toxins. Thus, further aim of the work was to expand knowledge on the occurrence of PA/PANO in several spices and herbs to identify potential matrices with relevance for food safety. Therefore, a total of 305 authentic and representative samples of 15 different spice and culinary herb varieties of worldwide origin were examined. The results represent a first comprehensive data collection on the contamination of PA/PANO in spices and herbs, each considering the geographical origin and variety. Applying the developed method on the sample set revealed PA/PANO to be present in the majority of the spices and herbs. Particularly oregano, cumin and marjoram, contained medium to high amounts of PA/PANO in more than 85 % of samples. In detail, 58 % of all 305 investigated samples contained PA/PANO. The mean sum content was 323 µg/kg, with a median 0.9 µg/kg and a 95 % percentile of 665 µg/kg. The highest individual PA/PANO sum level was 24.6 mg/kg in a Turkish oregano sample. The collected occurrence data were used to perform a first risk assessment of PA/PANO in spices and herbs. Therefore, exemplary recipes and consumption data from nutritional studies were used. Considering a worst case scenario (17,000 µg/kg, i. e. 95 %-percentile of oregano samples), the Health Based Guidance Value (HBGV) for evaluating an acute health risk was exceeded by factors up to 9.3, depending on the use of fresh or dried herbs. The chronic risk assessment, using the Margin of Exposure (MOE) approach and considering high sum contents of PA/PANO (5,500 µg/kg; i. e. rounded median of spices and herbs within the group with sum contents > 1,000 µg/kg), revealed MOE values < 10,000 for children even in case of average consumption scenarios. Concludingly, the risk estimation revealed a not negligible impact of PA/PANO intake via spices and herbs on consumers' health, in particular in case of children or worst case scenarios concerning spice intake and PA/PANO levels.

7 Zusammenfassung

Pyrrolizidinalkaloide (PA) und deren korrespondierende *N*-Oxide (PANO) sind Sekundärmetabolite, die von einer Vielzahl an Pflanzen zum Schutz gegen Insekten gebildet werden. Momentan sind mehr als 660 Verbindungen bekannt, die vermutlich in mehr als 6.000 Pflanzenspezies vorkommen. PA/PANO sind in ihrer nativen Form nicht toxisch, 1,2-ungesättigte PA/PANO sind jedoch als Protoxine anzusehen. Nach der Aufnahme können sie ihr toxisches Potential durch Giftungsreaktionen in der Leber entfalten. Die metabolische Aktivierung durch Cytochrom-P450-Monooxygenasen (CYP450) führt hierbei zu hochreaktiven und somit schädlichen Pyrrolderivaten. Die auf diese Weise aktivierten PA/PANO Metaboliten können, insbesondere in der Leber, zu Dysfunktionen, Schäden bei Zellen und Organen oder zur Entstehung von Tumoren führen. PA/PANO gelangen meist über unbeabsichtigte Beiernte der alkaloidhaltigen Pflanzen in die Nahrungskette, können von diesen aber auch durch Transfer über den angrenzenden Boden an die Kulturpflanzen abgegeben werden. Ein weiterer Kontaminationsweg sind Honigbienen, die Pollen und Nektar PA/PANO-haltiger Pflanzen sammeln und die Alkaloide so in den Honig eintragen. Aufgrund ihrer schädlichen Wirkungen in Menschen und Tieren handelt es sich bei PA/PANO um in Lebens- und Futtermitteln unerwünschte Kontaminanten.

Bisher beschäftigten sich nur wenige Studien mit dem Verhalten der toxischen Alkaloide in Lebens- und Futtermitteln. Das erste Ziel der vorliegenden Arbeit war es somit, die Lagerstabilität von PA/PANO in relevanten Lebens- und Futtermittelmatrices zu untersuchen. Hierfür wurden die Matrices Honig, Pfefferminztee und Heu ausgewählt und mit insgesamt 25 PA/PANO-Standardsubstanzen dotiert oder, im Fall der pflanzlichen Matrices, mit gefriergetrockneten und gemahlenen PA/PANO-haltigen Pflanzen versetzt, um eine Beiernte dieser Pflanzen zu simulieren. Die präparierten Proben wurden für sechs Monate in einer dunklen und trockenen Umgebung gelagert und wiederholt mittels zielgerichteter Flüssigchromatographie-Tandemmassenspektrometrie (LC-MS/MS) Analytik auf ihre Gehalte an PA/PANO untersucht. In einer weiteren Studie wurde der Einfluss von Absetzeffekten von Pollen bei der lagerbedingten Entmischung von Honig auf die Verteilung der PA/PANO analysiert: Natürlich kontaminierte Honige wurden für zwölf Wochen in konischen Plastikbeuteln gelagert, um ein mögliches Absetzen der Pollen zu erreichen. Sowohl im oberen als auch im unteren Bereich wurden Proben der Honige entnommen und deren Gehalte an PA/PANO durch drei teilnehmende Labore untersucht, wobei drei unterschiedliche analytische Methodiken angewandt wurden: Eine summenbasierte und zwei unterschiedliche zielgerichtete

Methoden, die Analyten entweder mittels Standardaddition oder matrixangepasster externer Kalibrierung quantifizierten.

Bei den pflanzlichen Matrices Pfefferminztee und Heu zeigte sich, dass die PA/PANO Gehalte während des gesamten Lagerzeitraums der Proben stabil waren. Identische Ergebnisse wurden für die unter gleichen Bedingungen gelagerten PA/PANO Mischlösungen erhalten, wobei kein Einfluss der Lagertemperatur auf die Gehalte innerhalb des Lagerzeitraums von sechs Monaten festgesellt wurde. Im Gegensatz dazu sanken die PA/PANO Summengehalte in Honig innerhalb weniger Wochen schnell ab. Diese Beobachtung war ausschließlich auf die verringerten Gehalte an PANO zurückzuführen, wobei hierbei keine korrespondierenden PA gebildet wurden. Während die Gehalte der tertiären Amin PA über 182 Tage stabil blieben, zeigte sich in Summe ein Rückgang der zwölf dotierten PANO von 99 %. In Abhängigkeit der Analyten wurden individuell unterschiedliche Abbaugeschwindigkeiten verzeichnet: So war beispielsweise nach 14 d der Verlust an cyclischen Diestern des Retronecin-Typs (z. B. Jacobin-*N*-Oxid: -97 %) im Vergleich zu Mono- oder Diestern des Heliotridin-Typs (z. B. Lasiocarpin-*N*-Oxid: -69 %) signifikant höher. Die Resultate wurden in sechs weiteren dotierten und gelagerten Honigen unterschiedlicher geographischer Herkunft und Blütensorte bestätigt.

Die Ergebnisse zum Rückgang der PANO in gelagertem Honig wurden zudem auch in den natürlich kontaminierten und für zwölf Wochen gelagerten Honigen von allen drei an der Studie teilnehmenden Laboren beobachtet. Aufgrund der Reaktionskinetik und dem strukturabhängigen Abbau der PANO wurde ein enzymatischer Hintergrund vermutet. Das Verhältnis der PA/PANO Summengehalte in den im oberen und unteren Bereich entnommenen Proben der jeweiligen Honige lag je nach Honigprobe und Labor zwischen 79 % und 118 %. Folglich wurde keine potentielle Auswirkung einer lagerbedingten Entmischung oder Absetzeffekte auf die PA/PANO Verteilung in Honigen festgestellt. Zudem zeigten vergleichende Untersuchungen von 20 Honigen mittels der verschiedenen analytischen Methodiken eine gute Übereinstimmung der Ergebnisse für 14 Proben. Neben Schwankungen der Analysenergebnisse aufgrund des Rückgangs von PANO führten die drei unterschiedlichen Analysenmethoden der beteiligten Labore zu teilweise stark divergierenden Ergebnissen. Die beobachteten Unterschiede in den Ergebnissen der drei angewandten Methoden wurden auf die variable Anzahl an der zur Bildung des PA/PANO Summengehalts herangezogenen Analyten zurückgeführt. Die Unterschiede könnten jedoch auch durch die Erfassung bisher unbekannter Analyten durch die summenbasierte Methode bedingt sein. Insgesamt wurde in der Studie

hervorgehoben, dass Gehalte an PA/PANO in Honig je nach angewandter Analysenmethode Unsicherheiten unterliegen können.

Ein großer Nachteil summenbasierter analytischer Methoden ist der Verlust jeglicher Information des spezifischen PA/PANO Profils in der analysierten Probe. Somit war es ein weiteres Ziel dieser Arbeit, eine sensitive LC-MS/MS Methode zur Quantifizierung aller 44 zum Zeitpunkt der Methodenentwicklung kommerziell verfügbaren PA/PANO Analyten zu entwickeln und für wichtige Teematrices zu validieren. Im Rahmen der Methodenentwicklung wurden sowohl die Extraktion als auch die weitere Reinigung der Extrakte optimiert. Die Validierung der Methode belegte deren Sensitivität und Richtigkeit, mit Nachweisgrenzen zwischen 0,1 µg/kg und 7,0 µg/kg sowie Präzisionen zwischen 0,7 % und 16,1 %. Die Wiederfindungsraten lagen bei 40 der 44 Analyten bei 60,7 % bis 128,8 %. Die neu entwickelte Methode wurde auf 18 Teeproben aus einem lokalen Supermarkt angewandt. Diese umfassten Tees aus Samen und Blüten sowie rohe und fermentierte Tees. PA/PANO wurden dabei in 17 der 18 Einzelhandelsproben mit Summengehalten bis zu 48,3 µg/kg nachgewiesen. Somit wurde die Anwendbarkeit der Methode auf eine große Bandbreite verschiedener Matrices gezeigt. Die Analysenmethode erlaubte durch die hohe Anzahl an 44 Analyten auch Rückschlüsse auf den botanischen Ursprung kontaminierender Pflanzen anhand der jeweiligen Alkaloidverteilung.

Frühere Studien deuteten darauf hin, dass PA/PANO vermutlich auch in Gewürzen und Küchenkräutern in hohen Gehalten vorkommen und auf diese Weise beträchtlich zur Gesamtexposition der Verbraucher gegenüber diesen Toxinen beitragen können. Deshalb war es ferner Ziel dieser Arbeit, die Datenlage zum Vorkommen von PA/PANO in wichtigen Gewürzen und Kräutern zu erweitern und potentiell problematische Matrices mit Relevanz für die Lebensmittelsicherheit zu identifizieren. Hierfür wurden insgesamt 305 authentische und repräsentative Proben von 15 verschiedenen Gewürzen und Küchenkräutersorten weltweiten Ursprungs untersucht. Die Ergebnisse stellen die erste derart umfassende Datensammlung zur PA/PANO-Kontamination von Gewürzen und Kräutern dar, inklusive der Berücksichtigung deren geografischer Herkunft und Sorte. Die Anwendung der neu entwickelten Analysenmethode auf das Probenset ergab, dass PA/PANO in der Mehrheit der Kräuter und Gewürze enthalten waren. Insbesondere Oregano, Kreuzkümmel und Majoran waren in mehr als 85 % der jeweiligen Proben mit mittleren bis hohen Mengen an PA/PANO belastet. Insgesamt enthielten 58 % aller 305 untersuchten Proben PA/PANO, mit durchschnittlich 323 µg/kg, einem Median von 0,9 µg/kg sowie einem 95 %-Perzentil von 665 µg/kg. Der

höchste PA/PANO Summengehalt in einer Einzelprobe wurde mit 24,6 mg/kg in einer türkischen Oreganoprobe nachgewiesen. Die erhobenen Gehaltsdaten wurden zur Durchführung einer Risikobewertung von PA/PANO in Gewürzen und Kräutern verwendet. Hierfür wurde mit Beispielrezepten sowie Konsumdaten aus Ernährungsstudien gearbeitet. Bei Betrachtung eines *Worst Case* Szenarios (17.000 µg/kg, i. e. 95 %-Perzentil der Oreganoproben) wurde der zur Evaluierung des akuten Risikos herangezogene gesundheitsbezogene Richtwert (HBGV, *Health Based Guidance Value*) um einen Faktor von bis zu 9,3 überschritten, je nachdem, ob getrocknete oder frische Kräuter zur Berechnung herangezogen wurden. Die Bewertung des Risikos in Folge einer Langzeitexposition unter Verwendung des *Margin of Exposure* (MOE) Ansatzes und Annahme eines hohen PA/PANO Summengehalts (5.500 µg/kg; i. e. gerundeter Median aller Gewürze und Kräuter innerhalb der Gruppe mit Summengehalten > 1.000 µg/kg) ergab bei Kindern bereits bei durchschnittlicher Aufnahme von Kräutern und Gewürzen bedenkliche MOE-Werte < 10.000. Zusammenfassend ergab die Risikoschätzung einen nicht unerheblichen Einfluss von PA/PANO in Gewürzen und Kräutern auf das Gesundheitsrisiko von Verbrauchern, insbesondere von Kindern und im Falle von *Worst Case* Szenarien in Bezug auf Vielverzehrer und 95 %-Perzentil der PA/PANO Summengehalte.

8 Appendix

8.1 Influence of storage on the stability of toxic pyrrolizidine alkaloids and their *N*-oxides in peppermint tea, hay and honey

8.1.1 Bibliographic data

Title:	Influence of Storage on the Stability of Toxic Pyrrolizidine Alkaloids and Their *N*-Oxides in Peppermint Tea, Hay and Honey
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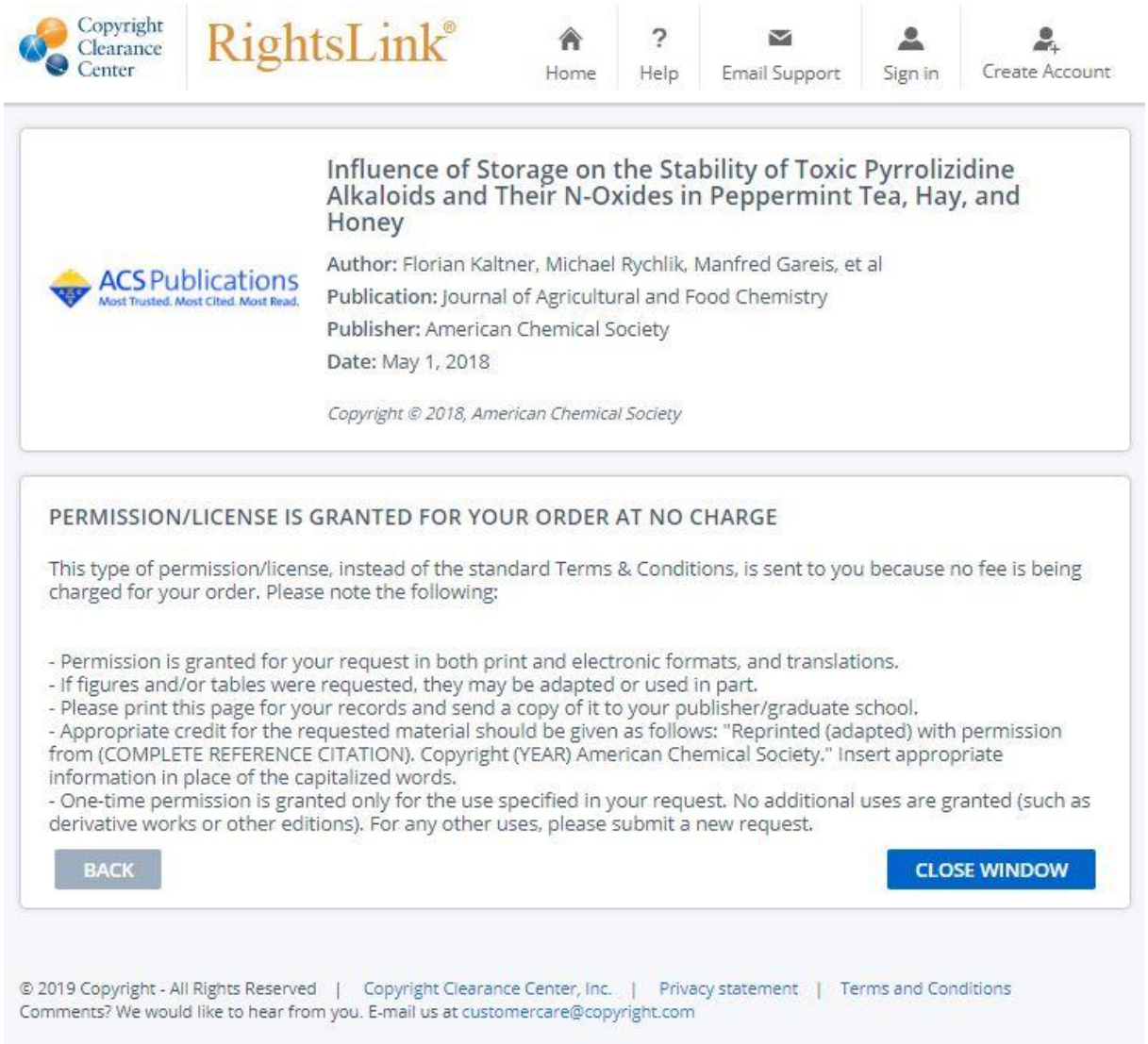

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Influence of Storage on the Stability of Toxic Pyrrolizidine Alkaloids and Their *N*-Oxides in Peppermint Tea, Hay, and Honey

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ABSTRACT: 1,2-Dehydropyrrolizidine alkaloids (PA) and PA-*N*-oxides (PANO) are phytotoxins, which presumably occur in more than 6,000 plant species worldwide. Plants containing PA/PANO are responsible for various food and feed poisonings recorded for decades. Main reasons of exposition of consumers and livestock are contaminations of food and animal feed with parts, seeds, pollen, or nectar of PA-containing plants. Concerning stability, effects of processing on PA were mainly investigated in the past. The current study examined the behavior of PA/PANO in unprocessed matrices peppermint tea, hay, and honey during storage. Blank samples were fortified with PA/PANO or contaminated with blueweed (*Echium vulgare*) and ragwort (*Senecio jacobaea*) and stored for 182 d. The time-series analysis using liquid chromatography−tandem mass spectrometry (LC−MS/MS) confirmed that all 25 analyzed PA/PANO compounds remained stable in herbal samples. However, the results showed a very fast decrease of PANO in honey samples within hours. These results were discussed with respect to potential consequences for health risk assessment.

KEYWORDS: *pyrrolizidine alkaloids, peppermint tea, hay, honey, stability, Mentha × piperita, ragwort, Senecio jacobaea, blueweed, Echium vulgare, liquid chromatography−tandem mass spectrometry, decrease, degradation*

INTRODUCTION

1,2-Dehydropyrrolizidine alkaloids (PA) and their corresponding *N*-oxides (PANO) are toxic plant secondary metabolites produced to prevent the plant against herbivorous insects (Figure 1).[1] It is assumed that more than 6,000 plant species, that is, 3% of all flowering plants, contain PA/PANO.[2] The majority of these plants belong to the families of Asteraceae (Senecioneae, Eupatorieae), Apocynaceae (Echiteae), Fabaceae (genus *Crotalaria*), and Boraginaceae.[3,4] PANO are more hydrophilic and show a higher water-solubility than PA. Therefore, in plants they are the preferred form of transport and are there generally found in higher amounts than the corresponding tertiary amines.[5]

PA and PANO show toxic activities in wildlife, cattle, and humans, and several cases of acute poisoning or chronic exposure of these substances were reported in the past years.[6−11] A long-term intake of low levels of PA/PANO in food can cause chronic diseases such as liver cirrhosis, pulmonary hypertension, cancer, and veno-occlusive disease.[8,12]

PA/PANO are biologically inactive and require metabolic activation by liver enzymes to develop their toxic potential. After hydroxylation in the course of phase-I-metabolism and elimination of H_2O highly reactive pyrrolic esters are formed, which can build adducts with DNA or proteins.[3,13−15] Regulatory limits for PA and PANO in food have not been established by the European Commission so far. Only in animal feed a maximum content of 100 mg/kg of plant parts of *Crotalaria* spp. was defined.[16] Considering a tolerable margin of exposure (MOE) of 10,000, for humans a maximum daily intake of 0.007 μg PA per kg bodyweight was calculated and recommended by the German Federal Institute for Risk Assessment (BfR),[17] based on a Benchmark Dose Lower Confidence Limit 10% ($BMDL_{10}$) of 73 μg per kg bodyweight from a toxicity study of lasiocarpine in male rats.[18]

The European Food Safety Authority (EFSA) recently adopted their opinion of 2011 and chose a new reference point for $BMDL_{10}$ calculation from a toxicity study based on riddelliine,[19] leading to a $BMDL_{10}$ of 237 μg per kg bodyweight. A MOE of 10,000 then corresponds to a maximum intake of 0.024 μg PA/PANO per kg bodyweight per day.[20]

Main sources of PA/PANO-exposure of humans are (herbal) teas, herbal food supplements, and honey.[21−23] Herbal teas can be contaminated with PA-containing plants via coharvesting of parts or whole plants of them. Another way of contamination of vegetable harvests is via mix-up with similar-looking weeds (e.g., rocket, *Diplotaxis tenuifolia*, and common ragwort, *Senecio vulgaris*), like it has already happened in the past.[24] Concerning contamination of honey, it is known that PA/PANO are transferred into bee products by nectar and pollen collected by honeybees.[25] Recently published work demonstrated floral nectar as main contributor of PA,[26] contrary to previous assumptions of pollen being their primary source.[27,28] Livestock can be exposed to PA by feed contaminated with PA-containing plants or seeds.[6,7,29] Preserved in feed mixtures, it seems that livestock is less able to detect the bitter-tasting PA-producing plants, which are normally unpalatable for grazing animals in their pure form.[4] Moreover, carry-over effects of PA from feed

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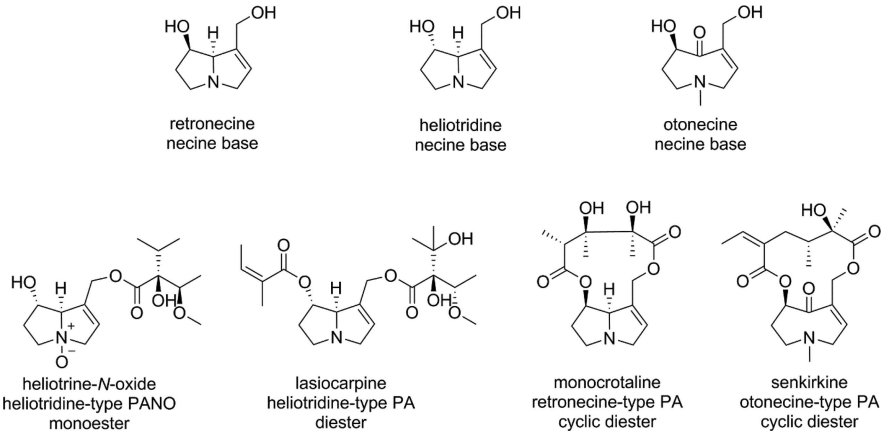

Figure 1. Overview of 1,2-dehydropyrrolizidine alkaloid (PA) and PA-*N*-oxide (PANO) basic structures and examples.

to milk, eggs and meat have been described in further studies.[30−33]

Regarding the behavior of PA and PANO during preservation of feed or composting of PA-plants, various studies have been conducted. Degrading effects on these compounds were described for ensiling of *Senecio alpinus*,[34] *Senecio vernalis*,[35] *Senecio jacobaea*,[36] and of composting on *Senecio jacobaea*.[37,38] Recently, Gottschalk et al. showed a compound-dependent loss especially of PANO from *Senecio vulgaris* during ensilage combined with stable or slightly increased amounts of PA.[39] In case of unprocessed feed, only Candrian et al. reported that 12 macrocyclic PA from *Senecio alpinus* remained preserved in artificial samples of hay and greenfodder during months.[34]

In conclusion, only few studies have been conducted about stability of PA in unprocessed food and feed matrices. Hence, the aim of the current study was to investigate the stability of PA and PANO in relevant food and feed matrices such as honey, peppermint tea, and hay during a period of 6 months (182 d).

MATERIALS AND METHODS

Chemical Reagents. Echimidine-*N*-oxide was obtained from Carl Roth (Karlsruhe, Germany), and other PA and PANO standards named echimidine, erucifoline, erucifoline-*N*-oxide, europine, europine-*N*-oxide, heliotrine, heliotrine-*N*-oxide, intermedine, intermedine-*N*-oxide, jacobine, jacobine-*N*-oxide, lasiocarpine, lasiocarpine-*N*-oxide, lycopsamine, lycopsamine-*N*-oxide, monocrotaline, monocrotaline-*N*-oxide, retrorsine, retrorsine-*N*-oxide, senecionine, senecionine-*N*-oxide, seneciphylline, seneciphylline-*N*-oxide, and senkirkine were purchased from Phytolab (Vestenbergsgreuth, Germany). Stock solutions (c = 1 mg/mL) of each PA and PANO were prepared with methanol. Two mixed spike solutions (c = 10 μg/mL of each analyte) were prepared by dilution with methanol: one solution containing 25 PA and PANO and another solution including 12 PANO and the otonecine-type PA senkirkine, which has no corresponding *N*-oxide. These two solutions were used to assess a putative reduction of PANO to their corresponding PA, while senkirkine served as control. For all experiments, LC−MS-grade water and methanol obtained from Th. Geyer (Renningen, Germany) were used. Sulfuric acid was purchased from Carl Roth (Karlsruhe, Germany), ammonia was acquired from Merck (Darmstadt, Germany), and LC−MS-grade ammonium formate and formic acid used as additive for LC−MS were obtained from Fluka (Steinheim, Germany), and Th, Geyer (Renningen, Germany), respectively.

Samples. In total, seven samples of honey harvested in 2016 were included. A floral honey sample (harvested in June 2016) originating from Murnau (Bavaria, Germany) was used for long-time storage experiments. Other honey samples used for confirmation experiments were commercially available forest honey from a local supermarket in Oberschleissheim (Bavaria, Germany), floral honey from Eutin (Schleswig-Holstein, Germany), floral honey and rape (*Brassica napus*) honey from Freising (Bavaria, Germany), floral honey from Murnau (harvested in July 2016), and floral honey from Cham (Bavaria, Germany). All samples were free of PA and PANO listed above.

Whole plants of blueweed (*Echium vulgare*) were collected near Munich (Bavaria, Germany) under dry weather conditions in July 2016, when the plant was in blossom. Plant samples of ragwort (*Senecio jacobaea*) were harvested in Eutin (Schleswig-Holstein, Germany) on August 2016. Leaves of peppermint plants (*Mentha* × *piperita*) were collected in Gross-Gerau (Hesse, Germany) on October 2016. Blank hay was obtained from the Bavarian State Research Centre for Agriculture.

Preparation of Storage Samples. Plant material samples were lyophilized using a freeze-dryer CTFD-10-PT (berrytec, Grünwald, Germany) and homogenized using a Grindomix GM200 mill (Retsch, Haan, Germany) to a particle size <0.5 mm. Stems and roots of blueweed and ragwort were discarded before lyophilization. Contaminated samples of peppermint and hay were prepared by mixing with different amounts of blueweed and ragwort as follows: peppermint/ragwort/blueweed (98:1:1, m/m/m dry-weight), hay/ragwort/blueweed (90:5:5, m/m/m dry-weight). Each contaminated plant sample was homogenized by overhead shaking (1 h, 30 rpm) and portioned in 36 units of 1.0 ± 0.1 g into centrifuge tubes.

The floral honey sample from Murnau used for long-term storage was split in two and spiked with mix solutions of 25 PA and PANO or 12 PANO and senkirkine in methanol (c = 10 μg/mL), respectively, to levels of 50 μg/kg of each analyte, homogenized by a stirring installation (1 h, 60 rpm, maximum water bath temperature 40 °C) and also each portioned in 36 units of 2.0 ± 0.1 g into centrifuge tubes.

For testing the homogeneity of the storage sample material, nine portions of each prepared sample type were chosen according to the "cross-riffling-schema",[40] indexed in three groups of three samples each, and analyzed. Measured amounts of single PA and PANO were tested via one-way analysis of variance (ANOVA, Tukey's Test) at a significance level of $p \leq 0.05$. The rest of portions was stored in the dark at 20 °C until analysis.

For confirmation of findings, each of the six additional honey samples from Holstein and Bavaria was portioned in 6 parts of 2.0 ± 0.1 g into centrifuge tubes, directly spiked with mix solutions of 25 PA and PANO to levels of 50 μg/kg of each analyte and stored at 20 °C in the dark until analysis.

Aliquots of both used spiking mix solutions (25 PA and PANO, 12 PANO and senkirkine) were diluted with methanol to a concentration of 500 ng/mL of each included analyte and stored at −19 °C, 6 °C and 20 °C to investigate the stability of standards under different storage temperature conditions typically used in laboratories. At each day of measurement aliquots of stored standard solutions were diluted with

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Table 1. Mass Spectrometric Conditions for the Analysis of Pyrrolizidine Alkaloids in Peppermint Tea, Hay, and Honey

compound	precursor [m/z]	quantifier [m/z]	qualifier [m/z]	declustering potential [V]	collision energy (quant/qual) [eV]	cell exit potential (quant/qual) [V]	retention time [min]
echimidine	398.3	120.1	220.0	96.0	35.0/25.0	20.0/12.0	17.9
-*N*-oxide	414.2	254.2	352.2	106.0	43.0/35.0	14.0/20.0	18.2
erucifoline	350.1	120.1	138.1	116.0	41.0/41.0	20.0/24.0	10.3
-*N*-oxide	366.2	118.0	136.0	111.0	49.0/45.0	20.0/24.0	12.4
europine	330.1	138.0	156.1	86.0	41.0/41.0	24.0/22.0	7.5
-*N*-oxide	346.1	172.1	270.1	106.0	45.0/35.0	30.0/16.0	9.2
heliotrine	314.2	138.2	94.0	86.0	29.0/47.0	24.0/16.0	12.3
-*N*-oxide	330.2	172.1	93.9	106.0	41.0/63.0	30.0/16.0	13.1
intermedine	300.2	138.0	120.1	96.0	29.0/37.0	24.0/20.0	5.6
-*N*-oxide	316.2	172.1	138.1	61.0	41.0/41.0	30.0/24.0	9.2
jacobine	352.1	280.2	155.2	111.0	33.0/41.0	16.0/26.0	10.7
-*N*-oxide	368.2	296.2	118.0	111.0	35.0/53.0	16.0/20.0	12.4
lasiocarpine	412.2	120.1	220.1	101.0	45.0/27.0	22.0/12.0	19.4
-*N*-oxide	428.2	254.1	352.3	91.0	41.0/35.0	14.0/20.0	20.0
lycopsamine	300.1	138.0	120.1	96.0	29.0/37.0	24.0/20.0	6.1
-*N*-oxide	316.2	172.1	138.0	106.0	41.0/41.0	30.0/24.0	9.7
monocrotaline	326.2	120.1	94.0	116.0	49.0/77.0	20.0/16.0	4.6
-*N*-oxide	342.1	137.1	118.0	116.0	53.0/69.0	22.0/20.0	9.0
retrorsine	352.2	138.1	324.3	121.0	43.0/39.0	24.0/18.0	12.6
-*N*-oxide	368.2	118.1	136.0	111.0	47.0/51.0	20.0/22.0	13.9
senecionine	336.2	120.0	308.3	116.0	41.0/37.0	20.0/16.0	16.2
-*N*-oxide	352.1	118.0	220.1	116.0	41.0/37.0	20.0/12.0	18.1
seneciphylline	334.1	120.1	94.1	111.0	41.0/51.0	20.0/16.0	13.8
-*N*-oxide	350.2	118.1	136.0	116.0	51.0/45.0	20.0/24.0	16.3
senkirkine	366.2	122.1	153.0	121.0	49.0/37.0	20.0/26.0	18.2

water (5:95, v/v) to a final concentration of 25 ng/mL of each PA/PANO and measured simultaneously with the other samples to proof the stability of standard solutions during the experiment.

Points of Measurement. A triple-determination of all long-term storage samples was conducted after 7, 14, 28, 42, 56, 84, 126, and 182 d of storage. Results from the homogeneity testing of plant material samples were used as t_0-values. The honey samples were additionally analyzed instantly, 3 h, and 1 d after spiking of mix solutions of PA/PANO or PANO and senkirkine, respectively. Obtained contents from honey analyzed instantly after spiking were considered as t_0-values for the honey samples. Confirmatory samples of honeys (n = 6) were analyzed in duplicate directly after spiking, 1 and 7 d after preparation.

Sample Extraction. Sample extracts were prepared according to a slightly modified method protocol of Bodi et al.[41] 25.0 mL of sulfuric acid (0.05 mol/L) was added to 2.0 ± 0.1 g of honey or 1.0 ± 0.1 g of plant material, respectively. The samples were shaken at room temperature for 30 min in a horizontal shaker. After centrifugation at 5000g for 10 min at 20 °C, plant material samples were filtered and 10.0 mL of extract were loaded onto SPE Bond Elut SCX 500 mg cartridges (Agilent, Waldbronn, Germany) preconditioned with 5 mL of methanol and 5 mL of sulfuric acid (0.05 mol/L). Centrifuged honey sample extracts were completely loaded onto preconditioned SPE cartridges. The loaded cartridges were washed with 6 mL of water and methanol, and analytes were eluted into a glass vial using 10 mL of ammoniated methanol (2.5%). The eluates were dried at 50 °C under a smooth stream of nitrogen. The residue was reconstituted in 1.0 mL of methanol/water (5:95, v/v), shaken with a laboratory shaker, and filtered into a glass vial using a 0.45 μm PVDF syringe filter (Berrytec, Grünwald, Germany). Extracts of peppermint tea and hay were diluted 100-fold with methanol/water (5:95, v/v) before measuring.

Quantification of PA/PANO by LC−MS/MS. For LC−MS/MS analysis, a Shimadzu Prominence LC20 System (Duisburg, Germany) hyphenated to an Applied Biosystems/AB Sciex API4000 triple quadrupole mass spectrometer (Darmstadt, Germany) was used. The separation of PA and PANO was performed using a 150 × 2.0 mm Synergi 4 μm Polar-RP 80 Å column (Phenomenex, Aschaffenburg, Germany) with a column oven temperature set to 30 °C. Solvents were water (A) and methanol (B), each containing 0.1% formic acid (100%) and 5 mmol/L ammonium formate. Gradient conditions were: 0−5 min 5% B, 10 min 25% B, 10−14 min 25% B, 22 min 95% B, 22−25 min 95% B. The column was equilibrated for 5 min before each run. The flow rate was 0.4 mL/min and the injection volume of samples was 20 μL. PA/PANO were analyzed in positive electrospray ionization (ESI+) mode as $[M + H]^+$ with two multiple reaction monitoring (MRM) transitions (Table 1) per analyte.

The concentration of 25 PA and PANO was determined by external matrix-matched calibration. Therefore, aliquots of the PA/PANO standard solutions were pipetted into glass vials, dried at 50 °C under nitrogen and reconstituted with extracts of the blank honey or plant material sample, respectively, to prepare calibration standards from 1.0−100 ng/mL (1.0, 5.0, 10, 25, 50, 100 ng/mL). Used blank extracts of peppermint tea and hay were 100-fold diluted with methanol/water (5:95, v/v) before using it for reconstitution. The matrix-matched calibration solutions were prepared freshly at each day of sample analysis. In case of plant materials, all results were related to dry matter. Mean recoveries (n = 9) of the method for plant material ranged between 62 and 114% for all analytes, except erucifoline and its *N*-oxide with recoveries of 53 or 49%, respectively. Relative standard deviations (RSD) of the recoveries were between 0.6 and 37.4%, depending on the analyte. The limits of detection (LOD) for plant material ranged between 0.20 and 1.60 μg/kg; the limits of quantitation (LOQ) were between 0.61 and 5.40 μg/kg. In case of honey, the mean recoveries (n = 9) ranged between 82 and 121% with RSD from 2.9 to 22%. The analytical limits for honey were between 0.01 and 0.19 μg/kg (LOD) and 0.03 and 0.59 μg/kg (LOQ). The precision of the method, expressed as coefficients of variation (CV), ranged from 0.4 to 5.3%.

RESULTS

Homogeneity of Long-Term Storage Samples. The artificially contaminated samples of plant material and honey were checked for homogeneity prior to storing. Obtained CV of

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single analyte amounts were less than 4% for honey storage samples within a measurement day. Also in storage samples of hay CV were <7%. Only individual analytes from the peppermint tea sample resulted in a CV > 10% (echimidine, intermedine-*N*-oxide, lycopsamine-*N*-oxide). Nevertheless, ANOVA analysis for testing the homogeneity of the prepared storage samples showed no significant difference ($p \leq 0.05$) within the amounts of each PA/PANO of the picked sample portions. Hereby, homogeneity of prepared food and feed long-term storage samples was successfully confirmed.

PA Stability in Solution. The stored standard mix solutions of PA and PANO showed no changes in contents of analytes during the whole experiment. The CV of the concentrations of the majority of single PA and PANO were less than 15% during the 182 d. Only lycopsamine and monocrotaline-*N*-oxide showed a CV > 20%, resulting from a single outlier in the middle of the time-series analysis, which was observed at every investigated storage temperature of −19 °C, 6 °C and 20 °C. Showing CV < 8%, sum concentrations of analytes of both stored standard solutions remained stable at the investigated temperatures during the complete period of storage. Therefore, neither the temperature nor the duration of storage showed a significant difference of analyte concentrations in the mix solutions (Figure 2). No increase or decrease of the analyte contents of solutions were observed (data not shown).

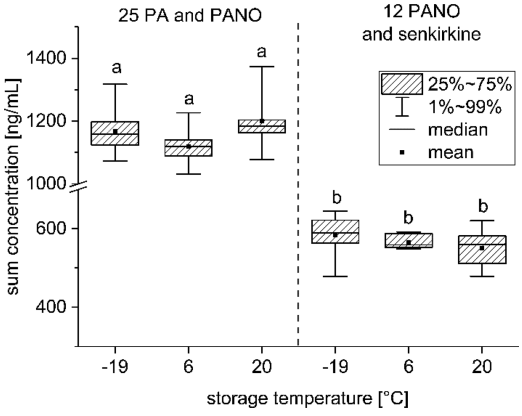

Figure 2. Average sum concentrations of 1,2-dehydropyrrolizidine alkaloid (PA) and PA-*N*-oxide (PANO)-standard mix solutions in methanol during 182 days of storage at different temperatures. Boxes topped by identical letters do not differ significantly ($p \leq 0.05$).

PA Stability in Samples of Peppermint Tea and Hay. Being prepared with both ragwort and blueweed, echimidine, erucifoline, jacobine, retrorsine, senecionine, seneciphylline, and their corresponding *N*-oxides were the prominent PA analytes in samples of artificially contaminated peppermint tea and hay. For both types of samples, smallest amounts were measured for retrorsine, while erucifoline-*N*-oxide was the most abundant compound. Mean contents of the single PA analytes ranged between 0.035 mg/kg and 9.9 mg/kg in peppermint tea and from 0.23 mg/kg to 39.8 mg/kg in hay. Within the triple determinations at each day of measurement, the CV ranged from 5% to 20% for the majority of analytes. Over the observed period of 182 d, in most cases the CV of means of individual PA/PANO-amounts were between 10% and 25%. Only analytes detected in relative small amounts, namely retrorsine, senecionine, seneciphylline, and erucifoline, showed a CV > 30%. Average sum contents of PA/PANO were 29.5 mg/kg in peppermint tea and 124.7 mg/kg in hay, both with a CV of 10%. As shown in Figure 3A and B, PA and PANO contents decreased neither for peppermint tea nor for hay in the observed period of 182 d and no trend could be identified.

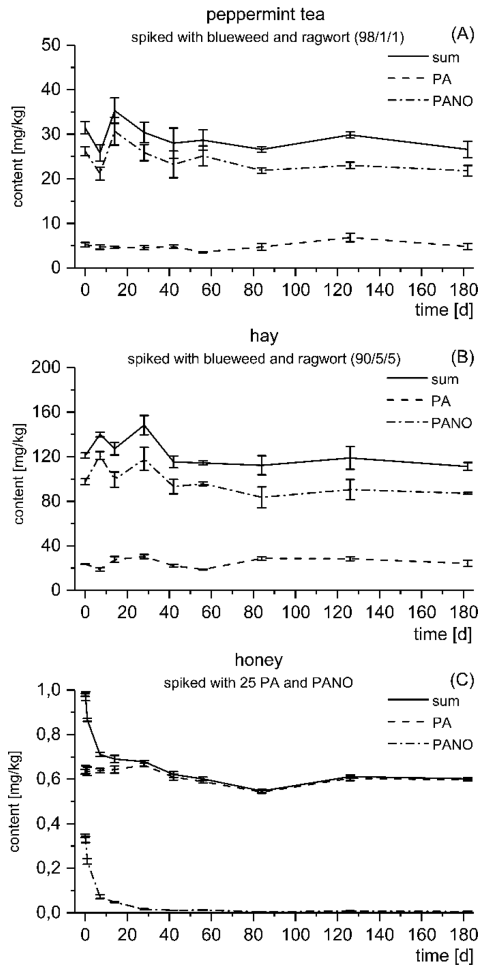

Figure 3. Sum contents of analytes, 1,2-dehydropyrrolizidine alkaloids (PA) and PA-*N*-oxides (PANO) in peppermint (A) tea and (B) hay artificially contaminated with tansy ragwort and blueweed and in (C) blank honey spiked with 25 PA/PANO during 182 days of storage at 20 °C.

PA Stability in Honey Samples. In contrast to the results of the time-series analysis in plant material, a decrease of analyte sum content was observed during long-term storage of honey samples (Figure 3C). The honey sample spiked with 25 PA and PANO showed a decrease of analyte sum content of 20% after storing of samples for 1 d and of 34% after 7 d. Targeted analysis revealed that the decline of sum of analytes in honey storage samples resulted exclusively from decreasing contents of PANO. While contents of tertiary amine PA remained stable during the period of 182 d (CV < 10%), amounts of PANO constantly decreased until only small amounts were detectable or even were smaller than the limit of detection.

The honey sample spiked with 12 PANO and senkirkine showed a decrease of analyte sum content of 14% from samples extracted 3 h after spiking and mixing compared to samples instantly extracted after spiking. The observation of the

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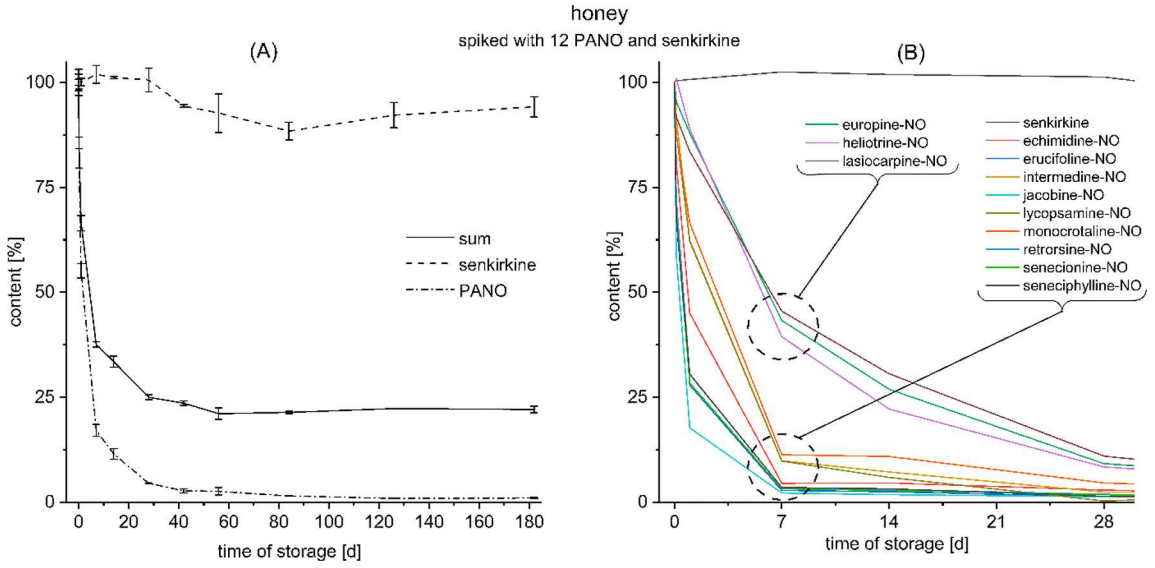

Figure 4. Relative amounts of (A) sum and (B) single contents of 1,2-dehydropyrrolizidine alkaloid-*N*-oxides (PANO) in blank honey spiked with 12 PANO and senkirkine during 182 days of storage at 20 °C. To improve the view, error bars were removed in panel B. NO = *N*-oxide.

decrease of PANO contents on storage of honey is illustrated in Figure 4A where the percentage of the original PANO and senkirkine present in honey samples instantly extracted after spiking is plotted against time of storing at 20 °C. Compared to the first measurement the sum content decreased to 66% on storing for 1 d and to 38% on storing for 7 d. During the observed period of 182 d, analyte sum content converged to a value about 20% of the sum content from the beginning of the study. Considering the stable amount of senkirkine during the whole storage period (CV < 5%), the observed loss of the 12 spiked PANO was 99%. Obtained data showed differences in the degradation rate of single PANO (Figure 4B). The decline of jacobine-*N*-oxide and erucifoline-*N*-oxide was particularly rapid, showing amounts less than 3% of the initial content already after 14 d. In contrast, the *N*-oxides of europine, heliotrine, and lasiocarpine were more persistent. The losses of these analytes were 73%, 78%, and 69%, respectively, after 14 d of storage.

The findings of the decrease of PANO amounts in honey samples on storing were further investigated by analyzing 6 additional honeys, which were spiked and stored equally to the honey sample of the long-term storage experiment. Since the highest changes of the contents occurred during the first week of sample storing, the honeys were only stored for 7 d after spiking. A decrease of PANO contents was observed in every honey sample after a storage of 7 d. The decreases of PANO sum amounts reached from 23% (sample Murnau, July 2016) to 55% (sample Cham) and thus were similar to the observations from the long-term storage honey sample. Contents of senkirkine remained stable in each of the six additional honey samples, like it was observed in the long term sample. The general decrease of PANO contents and the differences in the specific degradation rates of single PANO were also confirmed as europine-*N*-oxide was identified as the most stable PANO and *N*-oxides of erucifoline and jacobine again showed the highest rates of decrease (data not shown).

DISCUSSION

Stability of PA Standards in Methanol. For quality control of the results, a stability testing of standards was performed. The two mix solutions of 25 PA/PANO and 12 PANO and senkirkine were stored at different temperatures during the entire experiment to exclude a possible influence of storage conditions on the stability of PA/PANO. CV of concentrations of most analytes were <15% and thus within a typical range for interday precisions. In conclusion, sum concentrations showed no significant differences (Figure 2) and effects of the storing temperature on the measured concentrations were therefore excluded.

The results have demonstrated that the analytes remained stable in methanol for at least 182 d. Three temperatures were chosen to give also reliable new information on stability of standards in solution. Strikingly, also storage at room temperature did not influence the amounts of PA/PANO in methanol solution compared to 6 °C or −19 °C storage. However, it must be considered that only small concentrations of standards (c = 500 ng/mL) were investigated. A conclusion concerning stability of PA and PANO in higher concentrations of analytes or in different solvents cannot be derived from these results. Nevertheless, our findings of PA/PANO being stable during storage in methanol were in agreement with results from former studies, which reported the stability of indicine-*N*-oxide in water and under acidic conditions[42] or of retrorsine, senecionine, and seneciphylline in methanol for at least 12 weeks at −20 °C, respectively.[43] The same report also showed instabilities of seneciphylline at 4 °C and room temperature, which could not be confirmed in our study.

Depending on the analyte, vendors recommend the storage of solid reference standards between −20 °C or up to room temperature. According to the data sheets, a guarantee of the stability in solution is mostly not issued or limited to short terms of maximum a few weeks.

PA Stability in Lyophilized Peppermint Tea and Hay. As expected, the results showed high levels of contamination with PA and PANO typically occurring in tansy ragwort and blueweed, which were used for simulating a coharvesting. In the current experiment we found an average PANO to PA ratio of approximately 80:20. These results were similar to findings of Hartmann and Zimmer, who reported a PANO to PA ratio of 90:10 in various freshly harvested *Senecio* species.[44] PANO as

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the more water-soluble form are supposed to be the preferred storage compounds of PA in *Senecio* plants,[45] and so PANO were expectably detected in higher amounts than PA in our artificially contaminated peppermint tea and hay samples.

Our findings presented in Figure 3A and B showed that PA/PANO remain preserved in lyophilized peppermint tea and hay stored at 20 °C in the dark. The results confirmed a previous study of Candrian et al., who reported the stability of 12, partly unknown macrocyclic PA in 2 artificial hay and dried greenfodder samples prepared with various amounts of *Senecio alpinus*.[34] Although since that study it has been assumed that PA/PANO are stable in hay, a comparable work with a variety of overall 25 PA and PANO in two dry plant material samples over a period of 182 d was not available yet. Hence, the results of Candrian et al. were meaningfully expanded by our findings.

During the storage period, large errors of the means were only observed for individual PA/PANO and referable to small amounts of the respective analyte in the purified sample extracts (retrorsine, senecionine, seneciphylline, erucifoline). As a 100-fold dilution of plant sample extracts was applied, already small deviations in the analyzed absolute concentrations of individual PA/PANO resulted in higher differences of calculated analyte amounts and, therefore, in a higher CV. Furthermore, even smallest PA-plant fibers might contain high amounts of analytes and could have caused higher variations of PA/PANO sum amounts within the intra- and interday-repetitions. Grinding of samples after mixing with dry ice potentially would have led to more homogeneous dry plant samples.[41] Nevertheless, the homogeneity testing at the beginning of the experiment was successfully passed and we were able to confirm that the amounts of 25 PA and PANO in dry peppermint tea and hay samples neither increased nor decreased within the 182 d period of storage. In contrast to investigated dry sample material where PANO obviously remained preserved, especially in certain forage samples such as grass silages these compounds are known to decrease.[39]

Decrease of PANO in Honey. Contrary to plant material sample, a decrease of PA/PANO sum content caused by diminished PANO amounts was observed and systematically investigated in artificially contaminated honey samples. As the analyzed amounts of the otonecine-type PA senkirkine remained stable during the time-series analysis, it was guaranteed that these results were no errors of measurement. Our findings of the main experiment were confirmed by analyzing six additional spiked honeys. Although they differed in age, floral type, and local origin, a decrease of PANO contents was observed in every sample. Also a former study on settling effects of PA/PANO in naturally contaminated honey and comparability of results obtained by different analytical approaches reported a decrease of PANO during storage of honey.[46] A reduction of PANO to their corresponding PA, as it was assumed by Betteridge et al.,[47] could be excluded since the amounts of PA in analyzed honeys did not increase during storage. Even no reduction of smallest amounts of PANO to their corresponding PA was observed in the honey spiked with 12 PANO and senkirkine as no PA (excluding senkirkine) were detected during time-series analysis. Instead, the obtained results showed that contents of PANO in honey decreased continuously until the end of the time-series analysis and no corresponding PA were formed.

As a targeted LC−MS/MS approach was used in the current study, possible modifications of PANO prior to the detection would have led to decreased signals of the targeted analyte. Therefore, the observed decrease of PANO, on the one hand, could be explained by a simple chemical derivatization or a dimerization. On the other hand, the stability of single PANO seemed to depend on their respective chemical structure leading to differences in their degradation rates in honey (Figure 4), and such a behavior can usually be found in the substrate specificity of enzymes. Therefore, the observed PANO decrease could be a result of enzymatic activity in honey caused by bee digestive enzymes.

Concerning risk assessment of honey, an influence of the observed decrease of PANO on the trueness of analytical results has to be discussed. Consequently, our results raised the question at which time a honey sample is best taken to get reproducible results. The findings of the current study suggested that a PANO contamination is not to be expected anymore after a storage time of 60 to 80 days. Contrary to that, high contamination levels higher than 1 mg/kg of individual PANO were reported in the past, for example from the EFSA for some bulk and retail honeys.[29] Considering a structure dependent decrease of PANO in fresh honeys the respective samples must originally have contained very high PANO levels so that a potential enzymatic activity in the honey could have been limited to a certain extent. It must be considered that the sum amount of artificially added PANO in our study was only <1 mg/kg, and therefore, these levels maybe did not reflect the situation of honey in the EFSA assessment. Furthermore, it also must be checked whether the degradation of PANO involves a possible detoxification.

Additionally, it remains unclear at which time the decrease of PANO starts: already in the nectar, after being collected by the bee, or in the honeycomb. However, the exact fate of PANO in stored honey remained unclear and is part of future research. Further studies based on untargeted metabolomics approaches should clarify which possible metabolites are formed, whether they are still toxic, and which processes are responsible for the observed decrease. As herbal teas, teas and honey are known to be a main source of exposure to PA/PANO in Germany and the European Union,[48−50] the knowledge of the stability of the analytes during storage of such matrices reported in the current study should be useful for a proper risk characterization.

In conclusion, we found that PA and PANO showed a different behavior in several matrices concerning their stability during storage at 20 °C for 182 d. While the analytes remained stable in methanol solution and no changes of amounts could be observed in lyophilized peppermint tea and hay, contents of artificially added PANO readily decreased in honey while storing. At the same time, levels of PA did not increase and thus a simple reduction of PANO to their corresponding PA could be excluded. Differences in the degradation rates of single PANO suggested a compound-dependent derivatization.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ANOVA, analysis of variance; BfR, German Federal Institute for Risk Assessment; $BMDL_{10}$, benchmark dose lower confidence limit 10%; CV, coefficient of variation; EFSA, European Food Safety Authority; ESI+, positive electrospray ionization; LC−MS/MS, liquid chromatography−tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation; MOE, margin of exposure; MRM, multiple reaction monitoring; PA, pyrrolizidine alkaloid; PANO, pyrrolizidine alkaloid *N*-oxide

■ REFERENCES

(1) Hartmann, T.; Witte, L., Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. In *Alkaloids: Chemical and Biological Perspectives*; Elsevier: Oxford, UK, 1995; Vol. *9*, pp 155−233.

(2) Smith, L.; Culvenor, C. Plant sources of hepatotoxic pyrrolizidine alkaloids. *J. Nat. Prod.* **1981**, *44* (2), 129−152.

(3) Mattocks, A. R. *Chemistry and Toxicology of Pyrrolizidine Alkaloids*; Academic Press, 1986; p 393.

(4) Boppré, M. The ecological context of pyrrolizidine alkaloids in food, feed and forage: an overview. *Food Addit. Contam., Part A* **2011**, *28* (3), 260−281.

(5) Hartmann, T.; Toppel, G. Senecionine N-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of Senecio vulgaris. *Phytochemistry* **1987**, *26* (6), 1639−43.

(6) Hill, B. D.; Gaul, K. L.; Noble, J. W. Poisoning of feedlot cattle by seeds of Heliotropium europaeum. *Aust. Vet. J.* **1997**, *75* (5), 360−361.

(7) Anjos, B. L.; Nobre, V. M. T.; Dantas, A. F. M.; Medeiros, R. M. T.; Oliveira Neto, T. S.; Molyneux, R. J.; Riet-Correa, F. Poisoning of sheep by seeds of Crotalaria retusa: Acquired resistance by continuous administration of low doses. *Toxicon* **2010**, *55* (1), 28−32.

(8) Kakar, F.; Akbarian, Z.; Leslie, T.; Mustafa, M. L.; Watson, J.; Van Egmond, H. P.; Omar, M. F.; Mofleh, J. An outbreak of hepatic veno-occlusive disease in Western Afghanistan associated with exposure to wheat flour contaminated with pyrrolizidine alkaloids. *J. Toxicol.* **2010**, *2010*, 1.

(9) Stegelmeier, B. L.; Edgar, J. A.; Colegate, S. M.; Gardner, D. R.; Schoch, T. K.; Coulombe, R. A.; Molyneux, R. J. Pyrrolizidine alkaloid plants, metabolism and toxicity. *J. Nat. Toxins* **1999**, *8* (1), 95−116.

(10) Colegate, S. M.; Stegelmeier, B. L.; Edgar, J. A. Dietary exposure of livestock and humans to hepatotoxic natural products. In *Animal Feed Contamination: Effects on Livestock and Food Safety*, 2012; pp 352−382.

(11) Chauvin, P.; Dillon, J. C.; Moren, A. An outbreak of Heliotrope food poisoning, Tadjikistan, November 1992-March 1993. *Sante* **1994**, *4* (4), 263−268.

(12) Edgar, J. A.; Colegate, S. M.; Boppré, M.; Molyneux, R. J. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. *Food Addit. Contam., Part A* **2011**, *28* (3), 308−324.

(13) Fu, P. P.; Xia, Q.; Lin, G.; Chou, M. W. Pyrrolizidine Alkaloid - Genotoxicity, Metabolism Enzymes, Metabolic Activation, and Mechanisms. *Drug Metab. Rev.* **2004**, *36* (1), 1−55.

(14) Li, N.; Xia, Q.; Ruan, J.; Fu, P. P.; Lin, G. Hepatotoxicity and tumorigenicity induced by metabolic activation of pyrrolizidine alkaloids in herbs. *Curr. Drug Metab.* **2011**, *12* (9), 823−834.

(15) Ruan, J.; Yang, M.; Fu, P.; Ye, Y.; Lin, G. Metabolic activation of pyrrolizidine alkaloids: Insights into the structural and enzymatic basis. *Chem. Res. Toxicol.* **2014**, *27* (6), 1030−1039.

(16) EC. Directive 2002/32/EC of the European Parliament of 7 May 2002 on undesirable substances in animal feed. *Off J. Eur. Communities L*, 2002; Vol. *140*, p 10.

(17) BfR. *Opinion No. 038/2011: Chemical analysis and toxicity of pyrrolizidine alkaloids and assessment of the health risks posed by their occurrence in honey*; BfR (Federal Institute for Risk Assessment): Berlin, 2011.

(18) NTP. Bioassay of lasiocarpine for possible carcinogenicity. *NTP Technical Report 39*; NTP, 1978; pp 1−66.

(19) NTP. Toxicology and carcinogenesis studies of riddelliine. *NTP Technical Report 508*; NTP, 2003.

(20) EFSA. Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. *EFSA Journal* **2017**, *15* (7), e04908.

(21) EFSA. Dietary exposure assessment to pyrrolizidine alkaloids in the European population. *EFSA Journal* **2016**, *14* (8), 1.

(22) Schulz, M.; Meins, J.; Diemert, S.; Zagermann-Muncke, P.; Goebel, R.; Schrenk, D.; Schubert-Zsilavecz, M.; Abdel-Tawab, M. Detection of pyrrolizidine alkaloids in German licensed herbal medicinal teas. *Phytomedicine* **2015**, *22* (6), 648−656.

(23) Mulder, P. P. J.; López Sanchez, P.; Castelari, M.; Bodi, D.; Ronczka, S.; Preiß-Weigert, A.; These, A. Occurrence of pyrrolizidine alkaloids in animal- and plant-derived food: Results of a survey across Europe. *Food Addit. Contam., Part A* **2018**, *35* (1), 118−133.

(24) BfR. *Opinion No. 028/2007: Salad mix contaminated with groundsel containing pyrrolizidine alkaloids*; BfR (Federal Institute for Risk Assessment): Berlin, 2007.

(25) Edgar, J. A.; Roeder, E.; Molyneux, R. J. Honey from plants containing pyrrolizidine alkaloids: A potential threat to health. *J. Agric. Food Chem.* **2002**, *50* (10), 2719−2730.

(26) Lucchetti, M. A.; Glauser, G.; Kilchenmann, V.; Dubecke, A.; Beckh, G.; Praz, C.; Kast, C. Pyrrolizidine alkaloids from Echium vulgare in honey originate primarily from floral nectar. *J. Agric. Food Chem.* **2016**, *64* (25), 5267−5273.

(27) Boppre, M.; Colegate, S. M.; Edgar, J. A. Pyrrolizidine Alkaloids of Echium vulgare Honey Found in Pure Pollen. *J. Agric. Food Chem.* **2005**, *53* (3), 594−600.

(28) Kempf, M.; Heil, S.; Haßlauer, I.; Schmidt, L.; von der Ohe, K.; Theuring, C.; Reinhard, A.; Schreier, P.; Beuerle, T. Pyrrolizidine alkaloids in pollen and pollen products. *Mol. Nutr. Food Res.* **2010**, *54* (2), 292−300.

(29) EFSA. Scientific opinion on pyrrolizidine alkaloids in food and feed. *EFSA J.* **2011**, *9* (11), 134.

(30) Hoogenboom, L. A. P.; Mulder, P. P. J.; Zeilmaker, M. J.; van den Top, H. J.; Remmelink, G. J.; Brandon, E. F. A.; Klijnstra, M.; Meijer, G. A. L.; Schothorst, R.; Van Egmond, H. P. Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows. *Food Addit. Contam., Part A* **2011**, *28* (3), 359−372.

(31) Mulder, P. P. J.; de Witte, S. L.; Stoopen, G. M.; van der Meulen, J.; van Wikselaar, P. G.; Gruys, E.; Groot, M. J.; Hoogenboom, R. L. A. P. Transfer of pyrrolizidine alkaloids from various herbs to eggs and meat in laying hens. *Food Addit. Contam., Part A* **2016**, *33*, 1−14.

(32) Eröksüz, Y.; Çeribaşi, A. O.; Çevik, A.; Eröksüz, H.; Tosun, F.; Tamer, U. Toxicity of Heliotropium dolosum, Heliotropium circinatum, and Senecio vernalis in parental quail and their progeny, with residue evaluation of eggs. *Turk Vet Hayvanc Dergis* **2008**, *32* (6), 475−482.

(33) Dickinson, J. O.; Cooke, M. P.; King, R. R.; Mohamed, P. A. Milk transfer of pyrrolizidine alkaloids in cattle. *J. Am. Vet Med. Assoc.* **1976**, *169* (11), 1192−1196.

(34) Candrian, U.; Luethy, J.; Schmid, P.; Schlatter, C.; Gallasz, E. Stability of pyrrolizidine alkaloids in hay and silage. *J. Agric. Food Chem.* **1984**, *32* (4), 935−7.

(35) Ronczka, S.; Winter, J.; Pieper, R.; Korn, U.; Zentek, J.; Speer, K.; Pieper, B. Investigation on the degradation of pyrrolizidine alkaloids in Eastern Groundsel (Senecio vernalis) during ensilage. *Proc. Soc. Nutr Physiol* **2012**, *21*, 68.

DOI: 10.1021/acs.jafc.7b06036
J. Agric. Food Chem. 2018, 66, 5221−5228

(36) Becerra-Jiminez, J.; Kuschak, M.; Roeder, E.; Wiedenfeld, H. Toxic pyrrolizidinalkaloids as undesired contaminants in food and feed: Degradation of the PAs from Senecio jacobaea in silage. *Pharmazie* **2013**, *68* (7), 636−639.
(37) Crews, C.; Driffield, M.; Berthiller, F.; Krska, R. Loss of Pyrrolizidine Alkaloids on Decomposition of Ragwort (Senecio jacobaea) as Measured by LC-TOF-MS. *J. Agric. Food Chem.* **2009**, *57* (9), 3669−3673.
(38) Hough, R. L.; Crews, C.; White, D.; Driffield, M.; Campbell, C. D.; Maltin, C. Degradation of yew, ragwort and rhododendron toxins during composting. *Sci. Total Environ.* **2010**, *408* (19), 4128−4137.
(39) Gottschalk, C.; Ronczka, S.; Preiß-Weigert, A.; Ostertag, J.; Klaffke, H.; Schafft, H.; Lahrssen-Wiederholt, M. Pyrrolizidine alkaloids in natural and experimental grass silages and implications for feed safety. *Anim. Feed Sci. Technol.* **2015**, *207*, 253−261.
(40) van der Veen, A. M. H.; Nater, D. A. G. Sample preparation from bulk samples: an overview. *Fuel Process. Technol.* **1993**, *36* (1−3), 1−7.
(41) Bodi, D.; Ronczka, S.; Gottschalk, C.; Behr, N.; Skibba, A.; Wagner, M.; Lahrssen-Wiederholt, M.; Preiss-Weigert, A.; These, A. Determination of pyrrolizidine alkaloids in tea, herbal drugs and honey. *Food Addit. Contam., Part A* **2014**, *31* (11), 1886−1895.
(42) D'Silva, J. B.; Notari, R. E. Kinetics and mechanisms of aqueous degradation of the anticancer agent, indicine N-oxide. *Int. J. Pharm.* **1981**, *8* (1), 45−62.
(43) Tamosiunas, V.; Mischke, C.; Mulder, P. P. J.; Stroka, J. Report on the 2012 Proficiency Test on pyrrolizidine alkaloids in honey and hay; *JRC85378; JRC Report*; JRC, 2013.
(44) Hartmann, T.; Zimmer, M. Organ-specific Distribution and Accumulation of Pyrrolizidine Alkaloids during the Life History of two Annual Senecio Species. *J. Plant Physiol.* **1986**, *122* (1), 67−80.
(45) Toppel, G.; Witte, L.; Hartmann, T. N-oxidation and degradation of pyrrolizidine alkaloids during germination of Crotalaria scassellatii. *Phytochemistry* **1988**, *27* (12), 3757−3760.
(46) Gottschalk, C., Huckauf, A.; Dübecke, A.; Kaltner, F.; Zimmermann, M.; Rahaus, I.; Beuerle, T. Uncertainties in the determination of pyrrolizidine alkaloid levels in naturally contaminated honeys and comparison of results obtained by different analytical approaches. *Food Addit. Contam., Part A* **2018**, (in press), DOI: 10.1080/19440049.2018.1468929.
(47) Betteridge, K.; Cao, Y.; Colegate, S. M. Improved method for extraction and LC−MS analysis of pyrrolizidine alkaloids and their N-oxides in honey: Application to Echium vulgare honeys. *J. Agric. Food Chem.* **2005**, *53* (6), 1894−1902.
(48) BfR. *Opinion No. 018/2013: Pyrrolizidine Alkaloids in Herbal Teas And Teas*; BfR (Federal Institute for Risk Assessment): Berlin, 2013.
(49) Mulder, P. P. J.; Sanchez, P. L.; These, A.; Preiss-Weigert, A.; Castellari, M. Occurence of Pyrrolizidine Alkaloids in food. *EFSA Journal* **2015**, *12*, 1.
(50) BfR. *Opinion No. 030/2016: Pyrrolizidine alkaloids: Levels in foods should continue to be kept as low as possible*; BfR (Federal Institute for Risk Assessment): Berlin, 2016.

DOI: 10.1021/acs.jafc.7b06036
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8.2 Uncertainties in the determination of pyrrolizidine alkaloid levels in naturally contaminated honeys and comparison of results obtained by different analytical approaches

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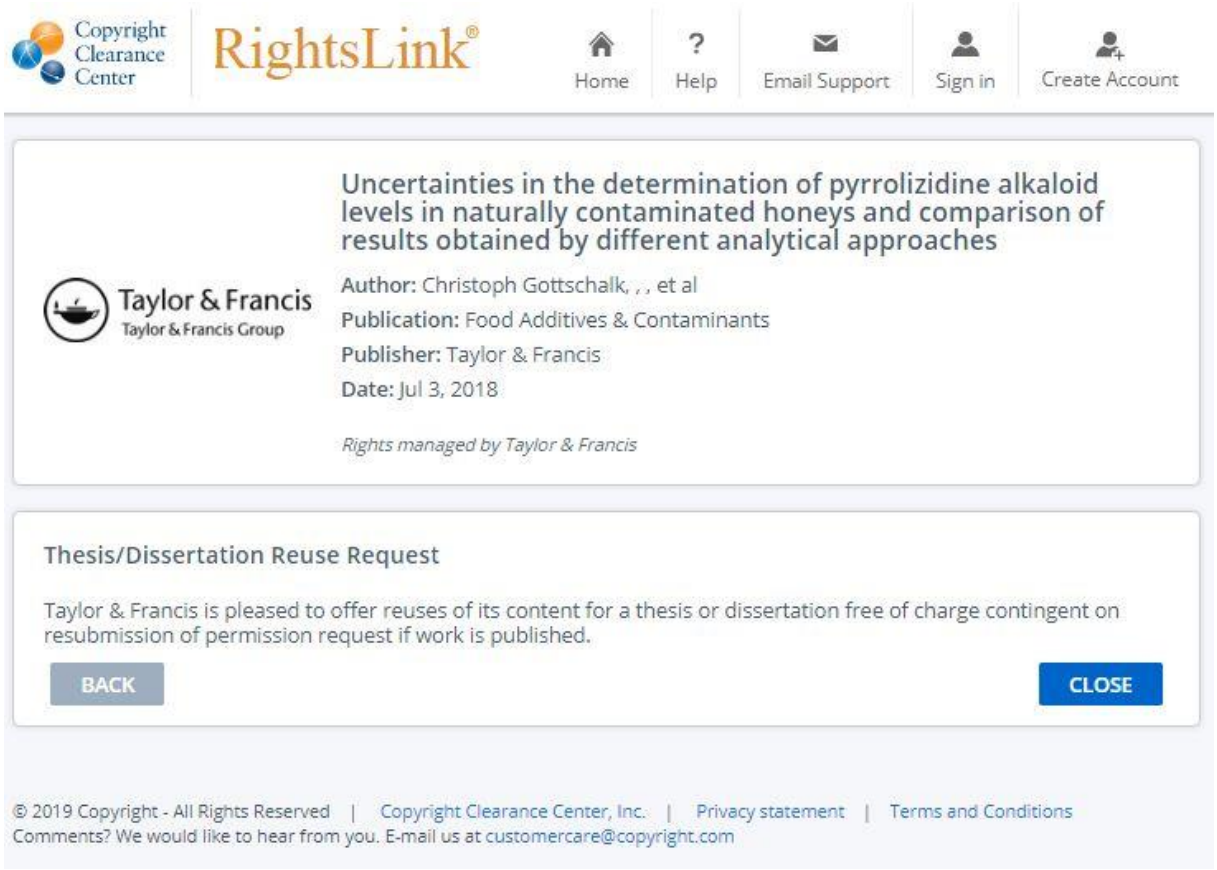

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Uncertainties in the determination of pyrrolizidine alkaloid levels in naturally contaminated honeys and comparison of results obtained by different analytical approaches

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ABSTRACT

The contamination of honey with hepatotoxic pyrrolizidine alkaloids (PAs) is a well-known hazard for food safety. While management strategies and controls of the honey industry aim to reduce the PA levels, uncertainties remain with regard to the safety of regionally produced and marketed honey. In addition, a previous study showed large differences of results obtained after various periods of storage and apparent differences between the analytical results of different laboratories. Therefore, this study aimed at examining these uncertainties by monitoring the impact of storage on the PA and PA *N*-oxide (PANO) content of two freshly harvested honeys and on possible demixing effects caused by pollen settling. Additionally, three analytical approaches – target analysis with matrix-matched calibration or standard addition and a sum parameter method – were applied for a comparative analysis of 20 honeys harvested in summer 2016. All samples originated from Schleswig-Holstein in Northern Germany where the PA plant *Jacobaea vulgaris* is currently observed on a massive scale. The results of the time series analyses showed that PANO levels markedly decreased within a few weeks and practically reached the LOD 16 weeks after harvest. Tertiary PAs, by contrast, remained stable and did not increase as a consequence of PANO decrease. The experiments on a putative demixing, which may result in a heterogeneous distribution of PAs/PANOs, revealed that there was no such effect during storage of up to 12 weeks. A comparison of the PA/PANO levels obtained by different analytical approaches showed that in some cases the sum parameter method yielded much higher levels than the target approaches, whereas in other cases, the target analysis with standard addition found higher levels than the other two methods. In summary, the results of this study highlight uncertainties regarding the validity and comparability of analytical results and consequently regarding health risk assessment.

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Introduction

Pyrrolizidine alkaloids (PAs) are secondary compounds produced by a wide variety of flowering plants as a chemical defence against herbivores. Currently, more than 660 different PAs have been identified in more than 350 plant species (BfR 2016a). Based on chemotaxonomic considerations, the potential number of PA-containing taxa has been estimated to be as high as 6,000 or 3% of the world's flowering plants (Smith and Culvenor 1981). Though the ability to form PAs is erratically scattered across angiosperm families that are only distantly related, more than 95% of the known PA-containing species belong to the four families *Asteraceae* (tribes *Senecioneae* and *Eupatoriae*), *Boraginaceae*, *Fabaceae* (genus *Crotalaria*) and *Orchidaceae* (Langel et al. 2011).

PAs are esters of a so-called 'necine base' (or simply necine), which consists of two fused five-membered rings with a nitrogen atom as a bridgehead in position 4 (pyrrolizidine), usually a hydroxymethyl group at C-1 and often a hydroxyl group at C-7. The hydroxyl groups can be esterified with one or two so-called 'necic acids', which usually are alkyl, or rarely aralkyl, mono- or dicarboxylic acids. PAs are usually classified according to the type of esterification (monoesters, open-chain diesters and cyclic diesters) or to the

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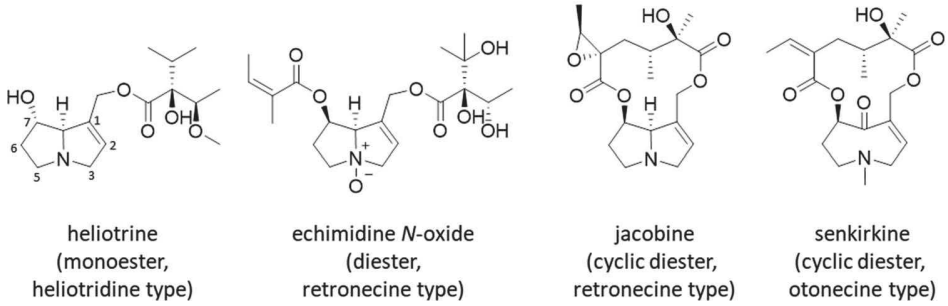

Figure 1. Examples of different types and structures of 1,2-unsaturated PAs and PANOs.

structure of the necine base (e.g. retronecine, heliotridine, otonecine and platynecine type, Figure 1).

Though PAs are considered not to be toxic per se (Hartmann and Witte 1995), many PAs are known to serve as protoxins, which can undergo a toxication process after ingestion leading to highly reactive and thus harmful pyrrolic intermediates (Mattocks 1968; Culvenor et al. 1969; Jago et al. 1970). As alkylating agents, the metabolites can bind to endogenous nucleophiles such as proteins and nucleic acids, which disturbs cell functions, results in cell damage or cell death and may even induce cancer formation (Mattocks 1986; Winter and Segall 1989). The metabolic activation is mainly caused by hepatic cytochrome P450 monooxygenases (Fu et al. 2004), and the liver is the primary target organ of the toxic action. Prerequisites for a PA to be protoxic are a double bond in 1,2-position, at least one esterified hydroxyl group and a branched carbon in at least one of the necic acid chains (Figure 1) (Schoental 1968; McLean 1970). Fully saturated PAs (e.g. of the platynecine type) and the necine bases and necic acids obtained by hydrolysis of the ester alkaloids seem not to be protoxic.

Most PAs except the otonecine-type compounds can occur in two forms: as tertiary amine and as PA *N*-oxide (PANO). In most – albeit not all – plants, PAs are translocated and stored predominantly in the polar, hydrophilic *N*-oxide form (Hartmann and Witte 1995). The *N*-oxides themselves cannot directly be metabolised into toxic pyrroles, but since they can at least partly be reduced to the corresponding tertiary amines in the gut, they are regarded as equally toxic (Mattocks 1971).

The hepatotoxic effect of PAs in humans is known from cases of acute, subacute or chronic poisonings caused by ingestion of herbal medicines or teas containing PAs or by accidental consumption of foodstuffs contaminated with PA-containing weeds (Prakash et al. 1999; Stewart and Steenkamp 2001; Brown 2017; De Boer and Sherker 2017). In animal studies, PAs have also been proven to be genotoxic and carcinogenic (Fu et al. 2004; Chen et al. 2010), but there are no long-term follow-up data or epidemiological studies available to judge whether continuous exposure to PAs might result in cancer in humans in the long term. Nevertheless, several European authorities such as the European Food Safety Authority (EFSA), the British Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) and the German Federal Institute for Risk Assessment (BfR) work on the assumption that 1,2-unsaturated PAs 'may act as carcinogens in humans as well' (EFSA 2011) .

Based on this assumption, the aforementioned authorities recommended that in the long term a daily intake of 0.007 µg 1,2-unsaturated PAs/kg body weight (bw) should not be exceeded (COT 2008; EFSA 2011; BfR 2013b). This figure was based on a BMDL10 of 0.073 mg kg^{-1} bw per day derived from a carcinogenicity study on lasiocarpine administered to rats (NCI 1978) allowing a margin of exposure (MOE) of at least 10,000 (EFSA 2012).

In July 2017, the EFSA Panel on Contaminants in the Food Chain (CONTAM) updated its former risk characterisation (EFSA 2017). As a result, the CONTAM Panel established a new reference point of 0.237 mg kg^{-1} bw per day to assess the carcinogenic risks of PAs, derived from a more recent carcinogenicity study on riddelliine (NTP 2003). Based on this new reference point and allowing the same MOE of 10,000, a daily exposure 'considered of low concern with respect to the carcinogenic effect' (EFSA 2012) of 0.0237 µg kg^{-1} bw per day can be deduced. This revised value has quite recently been adopted by the BfR (Dusemund et al. 2018).

In their latest statements on the risk for consumers resulting from the occurrence of PAs in foodstuffs, both the BfR (Dusemund et al. 2018) and the EFSA (2017) concluded that tea (including black, green, herbal and rooibos) and herbal infusions were by far the main average contributors to the total exposure to PAs. Yet, it was the possible contamination of honey with PAs that lately brought PAs into the focus of public attention, particularly in the northernmost German state Schleswig-Holstein. The reason for this sudden interest was that the abundance of a specific PA-producing flowering plant, tansy ragwort (*Jacobaea vulgaris*, syn. *Senecio jacobaea* L.), has significantly increased during the last 20 years: Formerly known as a sparsely growing species with a wide distribution, but usually confined to stands with single or only few plants, its population has largely increased in some areas and locally even formed extended mass occurrences. Though tansy ragwort is usually considered to be unattractive to honey bees, recent studies have demonstrated that under unfavourable conditions rather high PA levels can be detected in honeys from areas with *J. vulgaris* mass occurrences (Beuerle et al. 2011). The main flowering time of *J. vulgaris* falls into a period with a pronounced shortage of nectar sources in the landscape of Northern Germany, which is dominated by intensive agriculture. In some regions, especially in cool and rainy summers, when the few remaining alternatives like lime trees and white clover reduce their nectar production, honey bees seem to have little or no alternatives but to forage on tansy ragwort.

Against this background, the Ministry of Energy, Agriculture, the Environment and Rural Areas (MELUR) of the German federal state of Schleswig-Holstein initiated a pilot project 'Blüten für Bienen' ('Blossoms for Bees') in 2015 with the aim to screen summer honeys from ragwort-rich regions for contamination with PAs/PANOs. The project, which is managed by the Tansy Ragwort Competence Centre of the Nature Conservation Foundation Schleswig-Holstein (Jakobs-Kreuzkraut-Kompetenzzentrum, Stiftung Naturschutz Schleswig-Holstein), investigates possible correlations between the PA content of summer honeys from Schleswig-Holstein, the date of harvest, the occurrence of tansy ragwort and significant alternative bee pastures in the surroundings of the ragwort-affected apiaries. The participating apiarists have not been chosen randomly, but volunteered to take part in the study. Therefore, sampling is not representative for summer honeys from Schleswig-Holstein, but reflects a biased selection of honeys produced in high-risk areas. To enhance the sample size and compensate for annual variations, the project continues until 2018, allowing four harvests of summer honeys to be tested. Interim findings of the pilot phase and the project years 2015 and 2016 have already been published in German technical literature (Huckauf 2016, 2017; Neumann and Huckauf 2016). However, some findings remained inexplicable, suggesting that the determination of PAs in honey is – at least in case of some compounds – not without its problems. This observation is in agreement with the results of a recent proficiency testing scheme including 26 laboratories from nine EU-Member States and Singapore executed by the Joint Research Centre (JRC) of the European Commission (Breidbach and Tamošiūnas 2017).

Therefore, a series of systematic tests was conducted in the present study to answer the following questions:

- Do different testing methodologies or methods of quantification produce different results and if so, do certain PAs stand out to be particularly problematic?
- Can an observed decline of *N*-oxides be reproduced in different honeys and by different testing methodologies or methods of quantification? How fast is the progress of PA-decline, and are there differences between individual compounds?
- Do settling processes of pollen result in a demixing effect, i.e. in an uneven distribution of PAs/PANOs, when honey is allowed to stand?

Materials and methods

Samples

All investigated honeys (n = 22) originated from Schleswig-Holstein (Germany), from areas with a regionally high occurrence of *J. vulgaris* (tansy ragwort). Two samples (honeys A and B) were used for the time series analyses and for examining putative demixing effects caused by pollen settling. Twenty samples (honey 1–20) were applied for conducting

interlaboratory comparison measurements. A detailed sampling procedure for each of these experiments is described below.

Samples of honey for homogeneity testing and time series analyses

Samples of two freshly harvested, unblended summer honeys (A and B) from two different apiaries in Schleswig-Holstein were used for a three-month storage experiment. The two test samples were identified by Quality Services International (QSI) in the framework of the ongoing project 'Blossoms for Bees'. This first analysis of honey A (date of harvest 19 July 2016) and B (date of harvest 2 August 2016) was performed 2 and 8 days after harvest, respectively (time point $t = -2$). Aliquots of 10 kg of each honey were liquefied at 40°C in a heating incubator and homogenised for 10 min by means of a batch mixer. The samples were subdivided into plastic containers (2.5 kg) for shipment to each laboratory. Before shipment, aliquots of the samples were collected again for PA/PANO monitoring by QSI ($t = -1$). Upon arrival, the samples were homogenised again in each laboratory, and 500 g each were filled into three conical plastic bags for the demixing test (Figure 2) and into a honey jar for the time series analyses.

Analyses were performed in each lab immediately after arrival of the samples ($t = 0$) and repeated after approximately 4, 8 and 12 weeks ($t = 1$, 2 and 3). The age of the honeys A and B at the first time of the analyses done in all three labs was 4 and 6 weeks, respectively. The exact day of analysis for each measurement was recorded and used for the evaluation and presentation of the results. QSI provided results of six points of measurement; four measurements each were reported by the Chair of Food Safety, Ludwig-Maximilians-University Munich (LMU) and the Institute of Pharmaceutical Biology, Technische Universität Braunschweig (TUB). The two measurements by QSI of samples taken before the start of the collaborative analyses ($t = 0$) are labelled as $t = -1$ and $t = -2$ (Table 1). The honey samples for the time series analyses were homogenised prior to each analysis. For the demixing test, the conical bags were carefully opened, and samples were taken from the top and, after cutting off the tip, from the bottom (Figure 2).

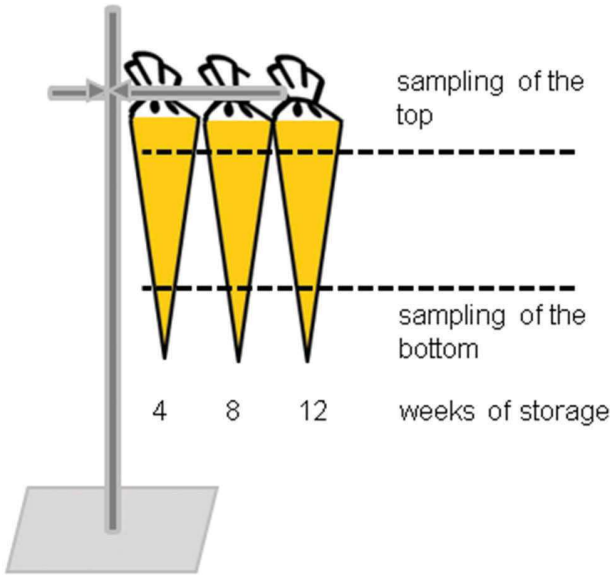

Figure 2. Scheme of the storage conditions and sampling plan of honey samples used in the test for demixing (two sampling positions (top and bottom), $n = 3$ conical plastic bags used for the analyses after 4, 8 and 12 weeks, respectively).

Sample set for interlaboratory comparison measurements

The sample set comprised honeys ($n = 20$) harvested in summer 2016 that had been tested for PAs/PANOs in the framework of the project 'Blossoms for Bees' and found to contain different amounts of PAs/PANOs. The floral origin of the PAs/PANOs could be attributed to plants of the genera *Senecio, Echium, Borago, Symphytum* and *Eupatorium* (Huckauf 2017). The honey samples (numbered 1–20, 500 g each) were thoroughly homogenised for 3 min by manual stirring and divided in three identical subsamples, which were sent to each of the three laboratories. The analyses were carried out in each lab immediately upon arrival as described below.

Chemicals

Reference standards for analyses at LMU (echimidine, echimidine *N*-oxide, europine, europine *N*-oxide, erucifoline, erucifoline *N*-oxide, heliotrine, heliotrine *N*-oxide, intermedine, intermedine *N*-oxide, jacobine, jacobine *N*-oxide, lasiocarpine, lasiocarpine *N*-oxide, lycopsamine, lycopsamine *N*-oxide, monocrotaline, monocrotaline *N*-oxide, retrorsine, retrorsine *N*-oxide, senecionine, senecionine *N*-oxide, seneciphylline and seneciphylline

Table 1. Results (individual PA and PANO compounds in µg kg^{-1}) of the time series analyses of honeys A and B.

					PAs/PANOs (µg kg^{-1}) detected in the two samples (time series analyses)[a]									
Laboratory	Honey sample	Point of sampling/analysis	Date of harvest	Date of analysis	Maturation time (days)	Echimidine	Echimidine *N*-oxide	Intermedine	Intermedine *N*-oxide	Lycopsamine	Lycopsamine *N*-oxide	Erucifoline	Erucifoline *N*-oxide	Jacobine	Jacobine *N*-oxide
QSI	A	$t = -2$	19.07.2016	21.07.2016	2	-	-	-	1.9	-	-	-	62	2.8	19
		$t = -1$	19.07.2016	02.09.2016	45	-	-	-	-	-	-	-	7.2	5.2	2.2
		$t = 0$	19.07.2016	07.09.2016	50	-	-	-	-	-	-	-	5.5	8.2	-
		$t = 1$	19.07.2016	10.10.2016	83	-	-	-	-	-	-	-	-	3.3	-
		$t = 2$	19.07.2016	20.10.2016	93	-	-	-	1.0	-	-	-	-	5.0	-
		$t = 3$	19.07.2016	08.12.2016	142	-	-	-	-	-	-	-	-	3.9	-
	B	$t = -2$	02.08.2016	10.08.2016	8	-	-	-	35	-	1.3	170	480	390	270
		$t = -1$	02.08.2016	01.09.2016	30	-	-	-	24	1.1	1.1	150	52	270	22
		$t = 0$	02.08.2016	08.09.2016	37	-	-	-	44	-	-	120	26	330	12
		$t = 1$	02.08.2016	10.10.2016	69	-	-	-	34	-	-	73	-	220	3.7
		$t = 2$	02.08.2016	20.10.2016	79	-	-	-	55	-	-	110	-	230	-
		$t = 3$	02.08.2016	14.12.2016	134	-	-	-	8.2	2.0	-	67	-	230	-
LMU	A	$t = 0$	19.07.2016	29.08.2016	41	0.1	-	-	-	-	-	0.2	0.2	1.6	0.4
		$t = 1$	19.07.2016	26.09.2016	69	0.1	-	-	-	-	-	0.1	0.1	1.4	-
		$t = 2$	19.07.2016	24.10.2016	97	0.1	-	-	-	-	-	0.1	-	1.7	-
		$t = 3$	19.07.2016	21.11.2016	125	0.1	-	-	-	-	-	0.1	-	1.7	-
	B	$t = 0$	02.08.2016	29.08.2016	27	0.3	-	0.4	-	-	-	18	7.8	189	4.4
		$t = 1$	02.08.2016	26.09.2016	55	0.2	-	0.2	-	-	-	15	0.5	119	1.8
		$t = 2$	02.08.2016	24.10.2016	83	0.2	-	0.2	-	-	-	21	0.2	130	0.9
		$t = 3$	02.08.2016	21.11.2016	111	0.2	-	0.1	-	-	-	20	0.2	132	1.2
TUB	A	$t = 0$	19.07.2016	29.08.2016	41	-	-	-	-	-	-	-	-	-	-
		$t = 1$	19.07.2016	26.09.2016	69	-	-	-	-	-	-	-	-	-	-
		$t = 2$	19.07.2016	26.10.2016	99	-	-	-	-	-	-	-	-	-	-
		$t = 3$	19.07.2016	23.11.2016	127	-	-	-	-	-	-	-	-	-	-
	B	$t = 0$	02.08.2016	29.08.2016	27	-	-	-	-	-	-	-	-	-	-
		$t = 1$	02.08.2016	26.09.2016	55	-	-	-	-	-	-	-	-	-	-
		$t = 2$	02.08.2016	26.10.2016	85	-	-	-	-	-	-	-	-	-	-
		$t = 3$	02.08.2016	23.11.2016	113	-	-	-	-	-	-	-	-	-	-

			PAs/PANOs (µg kg^{-1}) detected in the two samples (time series analyses)[a]														
Laboratory	Honey sample	Point of sampling/ analysis	Retrorsine	Retrorsine *N*-oxide	Riddelliine[b]	Riddelliine *N*-oxide[b]	Senecionine	Senecionine *N*-oxide	Seneciphylline	Seneciphylline *N*-oxide	Senecivernine[b]	Senecivernine *N*-oxide[b]	Senkirkine	Sum of PAs/ PANOs	Sum of PAs	Sum of PANOs	PANO percentage[c]
QSI	A	$t = -2$	-	4.9	-	5.2	1.6	63	2.0	63	-	2.0	-	220	6.4	214	97
		$t = -1$	-	-	-	-	3.5	14	3.6	9.9	-	-	-	46	12	33	73
		$t = 0$	-	-	-	-	4.5	8.8	3.9	7.9	-	-	-	39	17	22	57
		$t = 1$	-	-	-	-	3.7	1.4	3.7	-	-	-	-	12	11	1.4	12
		$t = 2$	-	-	-	-	3.8	-	3.9	-	-	-	-	14	13	1.0	7.3
		$t = 3$	-	-	-	-	4.5	-	4.5	-	-	-	-	13	13	0	0.0
	B	$t = -2$	55	99	46	120	120	690	350	1200	5.2	61	1.3	3862	1086	2775	72
		$t = -1$	50	15	45	21	100	76	340	160	14	8.1	-	1261	911	350	28
		$t = 0$	61	8.6	48	6.3	150	50	340	82	6.6	4	1.4	1225	1002	223	18
		$t = 1$	46	-	47	-	110	5.4	320	8.1	6.6	-	1.0	821	770	51	6.2
		$t = 2$	52	-	45	-	120	-	300	-	-	-	-	867	812	55	6.3
		$t = 3$	74	-	56	-	140	1.2	350	-	9.1	-	1.1	874	864	9.4	1.1

(*Continued*)

Table 1. (Continued).

			PAs/PANOs (µg kg^{-1}) detected in the two samples (time series analyses)[a]														
Laboratory	Honey sample	Point of sampling/ analysis	Retrorsine	Retrorsine *N*-oxide	Riddelliine[b]	Riddelliine *N*-oxide[b]	Senecionine	Senecionine *N*-oxide	Seneciphylline	Seneciphylline *N*-oxide	Senecivernine[b]	Senecivernine *N*-oxide[b]	Senkirkine	Sum of PAs/ PANOs	Sum of PAs	Sum of PANOs	PANO percentage[c]
LMU	A	***t* = 0**	0.5	0.3	-	-	5.7	3.8	3.5	2.6	-	-	-	19	12	7.4	39
		t = 1	0.3	0.1	-	-	4.9	1.0	3.2	0.4	-	-	-	12	10	1.7	14
		t = 2	0.4	0.1	-	-	4.8	0.1	2.7	0.1	-	-	-	10	9.7	0.3	2.7
		t = 3	0.4	-	-	-	5.0	-	3.0	-	-	-	-	10	10	0.1	0.5
	B	***t* = 0**	78	3.3	-	-	206	34.3	314	40.7	-	-	0.8	897	806	91	10
		t = 1	27	1.2	-	-	205	3.99	294	3.8	-	-	1.0	673	661	11	1.7
		t = 2	45	0.3	-	-	178	1.2	289	1.9	-	-	0.7	668	663	4.6	0.7
		t = 3	49	0.3	-	-	203	1.4	329	1.5	-	-	0.9	738	733	4.7	0.6
TUB	A	***t* = 0**	-	-	-	-	-	-	-	-	-	-	-	70	-	-	-
		t = 1	-	-	-	-	-	-	-	-	-	-	-	29	-	-	-
		t = 2	-	-	-	-	-	-	-	-	-	-	-	26	-	-	-
		t = 3	-	-	-	-	-	-	-	-	-	-	-	20	-	-	-
	B	***t* = 0**	-	-	-	-	-	-	-	-	-	-	-	1917	-	-	-
		t = 1	-	-	-	-	-	-	-	-	-	-	-	1688	-	-	-
		t = 2	-	-	-	-	-	-	-	-	-	-	-	1738	-	-	-
		t = 3	-	-	-	-	-	-	-	-	-	-	-	1464	-	-	-

'*t* = 0' marks the time of the first analysis performed by all three laboratories. Previous analyses performed only by QSI are presented as '*t* = −1' or '*t* = −2'.

[a]PAs/PANOs less than LOD were not listed (monocrotaline, lasiocarpine, europine, heliotrine and their *N*-oxides).

[b]Compounds measured exclusively by QSI, but results were not included into the PA/PANO sum to allow direct comparison to LMU.

[c]PANO% = sum of PANOs/sum of (PAs + PANOs) × 100.

LMU, honey B, time point t =1, senecionine-N-oxide: concentration must be 4.0 and not 3.99.

N-oxide and senkirkine) were purchased from PhytoLab (Vestenbergsgreuth, Germany). Deionised water for extraction and chromatography was prepared by means of a Millipore water purification system (Schwalbach, Germany). Methanol and acetonitrile were used in LC-MS grade (LiChrosolv®, Merck, Darmstadt, Germany). Formic acid and ammonium formate used as eluent additive (LC-MS/MS grade) were purchased from Sigma Aldrich (Deisenhofen, Germany).

Reference standards for analyses at QSI were obtained from Phytolab, Vestenbergsgreuth, Germany (echimidine, echimidine *N*-oxide, erucifoline, erucifoline *N*-oxide, jacobine, jacobine *N*-oxide, lasiocarpine, lycopsamine, lycopsamine *N*-oxide, monocrotaline, monocrotaline *N*-oxide, retrorsine, retrorsine *N*-oxide, senecionine *N*-oxide, seneciphylline *N*-oxide and trichodesmine), Carl Roth, Karlsruhe, Germany (europine, europine *N*-oxide, heliotrine, heliotrine *N*-oxide, intermedine, intermedine *N*-oxide, lasiocarpine *N*-oxide, seneciphylline, senecivernine, senecivernine *N*-oxide and senkirkine) and Cfm Oskar Tropitzsch, Marktredwitz, Germany (senecionine) and PhytoPlan, Heidelberg (echinatine *N*-oxide). Additionally, echinatine and rinderine were kindly provided by Patrick Mulder (RIKILT, The Netherlands). For each PA, stock solutions were prepared which were used for preparation of a PA standard mix. Concentrations in the standard mix used for the standard additions were different for each PA according to their responses in order to stay within the linear range for all PAs at all standard addition levels. Formic acid, sulphuric acid, ammonia and ammonium acetate were purchased from Merck Chemicals (Darmstadt, Germany). Methanol was obtained from VWR Chemicals (Darmstadt, Germany). Water (J.T. Baker) was also obtained via VWR. Both solvents were HPLC-grade.

Lithium aluminium hydride solution (1 M in tetrahydrofuran) and pyridine AcroSeal were purchased from Acros Organics (New Jersey, USA). Isotopically labelled internal standard, 7-*O*-9-*O*-dibutyroyl-[9,9-$^{2}H_{2}$]-retronecine, was synthesised in the laboratory of TUB (Cramer et al. 2013). All other chemicals used were purchased from Roth (Karlsruhe, Germany) and Sigma Aldrich (Seelze, Germany). Solvents were of HPLC-MS grade; water was double distilled before use.

Determination of PAs/PANOs

Laboratory 1 (LMU) applied LC-MS/MS target analysis using matrix-matched calibration. Laboratory 2 (QSI) also applied LC-MS/MS target analysis but quantified the PAs/PANOs by using standard addition. Laboratory 3 (TUB) determined the total PA/PANO amounts as a sum parameter (retronecine equivalents (REs)) applying LC-MS/MS as well. For comparability of the results of LMU and QSI, the sum of the 25 PAs and PANOs (echimidine, erucifoline, europine, heliotrine, intermedine, jacobine, lasiocarpine, lycopsamine, monocrotaline, retrorsine, senecionine, seneciphylline and their *N*-oxides and senkirkine) that were quantified by both laboratories was used (without any conversion factor). The REs determined by TUB were converted into senecionine equivalents (approximating the total PA content) using the conversion factor 2.16 (see below). All measurements were carried out in duplicate, and the arithmetic means were used for the evaluation. The different analytical approaches are described as follows.

PA quantification by target analysis and matrix-matched calibration (LMU)

Target analysis with matrix-matched calibration was performed according to a previously published method (BfR 2013a; Bodi et al. 2014). In brief, 10 g honey was diluted in 30 ml 2% H_2SO_4 using a horizontal shaker (30 min, 300 rpm). The entire solution was passed through an HF BondElut LRC SCX column (500 mg/6 ml cartridge) from Agilent (Waldbronn, Germany). Washing was performed by using 2 × 3 ml methanol (LC-MS grade), and retained analytes were eluted with 2.5% ammonia in methanol. Extracts were evaporated to dryness under a gentle stream of nitrogen at 50°C and reconstituted in 1 ml 10% methanol. Measurements were performed with an LC-MS/MS system (API4000, Applied Biosystems MDS Sciex, Darmstadt, Germany) and a LC20-ADvp HPLC (Shimadzu, Duisburg, Germany) with a Synergi Polar-RP column (150 × 2.0 mm, 4.0 µm, Phenomenex, Aschaffenburg, Germany) in ESI+ mode. Detailed method parameters (HPLC

conditions, source parameters) are summarised in Gottschalk et al. (2015); mass transitions and substance-specific parameters are available as supplemental material (Table S1). Data analysis and integration was achieved with Analyst 1.6.2 Software (Applied Biosystems MDS Sciex, Darmstadt, Germany).

Quantification of 25 PAs/PANOs was performed by matrix-matched calibration according to Bodi et al. (2014) with a blank rape honey harvested in the area of Eutin, Ostholstein, Germany, in 2015. LOD and LOQ were determined by linear regression/calibration curve method following German Standard DIN ISO 32645 (DIN 2008) for dilutions of standards in honey matrix. The analytical limits ranged between 0.01 and 0.19 μg kg^{-1} (LOD) and 0.03 and 0.59 μg kg^{-1} (LOQ), depending on the analyte. Mean recoveries (n = 9) of the method were between 82% and 121% with RSDs ranging from 2.9% to 22%. Results were not corrected for the analytical recovery rate.

PA quantification by target analysis and standard addition (QSI)

Three aliquots (10 g each) were prepared for analysis. No standard mix was added to the first, 100 μl to the second and 200 μl to the third aliquot. To all aliquots, 30 ml of 0.05 M sulphuric acid was added, followed by 20 min of vigorous shaking (modified after Betteridge et al. 2005 and Kempf et al. 2008). The samples were then filtered (2 μm mesh) overnight to remove particles, which would block the solid-phase extraction (SPE) cartridges during the clean-up step using Chromabond PS-H^+ (3 ml, 200 mg) SCX-cartridges (Macherey-Nagel, Düren, Germany) with a 30 ml reservoir attached. Prior to SPE, the cartridges were washed with 3 ml methanol and conditioned with 3 ml of 0.05 M sulphuric acid. The samples were applied onto the cartridges without the use of negative pressure and subsequently eluted into 8 ml glass vials using ammoniated methanol (Kempf et al. 2008) and dried at 40°C in a stream of ambient air. The dried samples were reconstituted in 1 ml deionised water, shaken vigorously and filtered into a 2 ml glass vial using a 0.45 mm syringe filter.

The LC system consisted of a Shimadzu degasser (DGU-20A3) and two Shimadzu LC-20AD pumps controlled by a Shimadzu CBM-20A controller unit. Injection of 10 μl of sample was done using an HTC PAL autosampler of CTC Analytics AG. Separation was achieved using a Thermo Hypersil Gold C18 column (50 × 2.1 mm, 1.9 mm particle size) and a column temperature of 25°C. The column was eluted using a gradient flow (300 μl min^{-1}) of two solvents (Dübecke et al. 2011). Solvent A consisted of 99.5% water plus 0.5% formic acid; solvent B of 94.5% methanol, 5% water and 0.5% formic acid. To both solvents, oxalic acid and ammonium acetate were added; concentrations in both solvents were set to 0.1 and 2.0 mM, respectively. The mobile phase was maintained at 100% A for the first minute and then changed with a linear gradient to 68% solvent B over 8.5 min. Subsequently, solvent B was increased to 100% over one minute before a re-equilibration phase over 2.5 min restored the initial mobile phase of 100% solvent A. The short column allowed for an analysis time of only 13 min per sample. An Applied Biosystems API 4000 triple quadrupole mass spectrometer was used to detect the PAs. The instrument was set to ESI+ MRM mode (source temperature 650°C, cone voltage 5500 V, collision gas on 'high', curtain gas 25 psi, ion source gas 1 and 2 at 35 and 45 psi, respectively), using one MRM transition as quantifier and another two as qualifiers for each PA.

The analytical limits of the method ranged between 0.3 and 1.3 μg kg^{-1} (LOD) and 1.0 and 5.0 μg kg^{-1} (LOQ), depending on the analyte. Recoveries of the method were between 51% and 97% with RSDs ranging from 2.0% to 14%. Quantification was done using standard addition (see above), thus compensating for lower recovery rates.

PA quantification by sum parameter method (TUB)

Total PA/PANO amounts of the honey samples were determined according to recently published HPLC-ESI-MS/MS sum parameter methods (Cramer et al. 2013; Letsyo et al. 2016), with slight modifications. Briefly, 200 mg of the honey was extracted with 2.0 ml of 0.05 M sulphuric acid after adding 40 μl of isotopically labelled 7-*O*-9-*O*-dibutyroyl-[9,9-$^2H_2$]-retronecine internal standard (IST) from a 2.0 μg ml^{-1} stock solution in methanol. The extract was cleaned on a preconditioned SCX-SPE column (500 mg/6 ml, Phenomenex, Aschaffenburg, Germany), derivatised with phthalic anhydride/pyridine and finally analysed with the HPLC-ESI-MS/MS sum parameter method according to Cramer et al. (2013) using a 1200 Series HPLC

(Agilent, Waldbronn, Germany) equipped with binary solvent delivery in combination with ESI-MS/MS triple quadrupole detection (3200 QTrap, Applied Biosystems MDS Sciex, Darmstadt, Germany). The chromatographic separation was achieved using a 150 mm × 2.1 mm, 4 µm, Synergy Max-RP 80 Å column (Phenomenex, Aschaffenburg, Germany) including a pre-column cartridge of the same material. The following gradient was applied at a flow rate of 300 µl min^{-1} (solvent A: 0.3% formic acid in water, solvent B: 0.3% formic acid in acetonitrile): 0–2 min (95% A), 2–14 min (95–40% A), 14–15 min (40–0% A), 15–18 min (0% A), 18–19 min (0–95% A) and 19–30 min (re-equilibration 95% A). Injection volume was 5 µl, and the temperature of the column oven was set to 40°C. Four transitions per compound (analyte and IST) were chosen. For the analyte retronecine-diphthalate: 452.0 → 94.0 m/z (cell entrance potential (CEP), 18.0; collision energy (CE), 50), 452.0 → 120.0 m/z (CEP, 18.0; CE, 36), 452.0 → 149.0 m/z (CEP, 18.0; CE, 55) and 452.0 → 304.0 m/z (CEP, 24.0; CE, 40). For the IST [9,9-$^2H_2$]-retronecine-diphthalate: 454.0 → 96.0 m/z (CEP, 18.0; CE, 50), 454.0 → 122.0 m/z (CEP, 18.0; CE, 36), 454.0 → 149.0 m/z (CEP, 24.0; CE, 55) and 454.0 → 306.0 m/z (CEP, 24.0; CE, 40). Data analysis and integration was achieved with Analyst 1.6.2 Software (Applied Biosystems MDS Sciex, Darmstadt, Germany). The LOD/LOQ for honey was 0.1 µg RE kg^{-1} and 0.3 µg RE kg^{-1}, respectively. Recoveries ranged between 69% and 104% (Cramer et al. 2013).

Identification and validation of the analyte/internal standard signals were achieved by monitoring retention time and co-occurrence of the ion transitions m/z 452.0 → 120.0/452.0 → 94.0 m/z (analyte) and m/z 454.0 → 122.0/454.0 → 96.0 m/z (IST). As additional control, the relative intensities of the transition in comparison to the IST were considered. The peak area of the analyte was corrected by subtracting 4% of the peak area of the internal standard, reflecting the amount of non-deuterated IST in the used IST preparation. The total PA/PANO content in REs was calculated using Equation (1), representing the sum of 1,2-unsaturated retronecine/heliotridine-type PAs in a sample. To determine the result in the form of RE, the known amount of IST (Mw 297.391 g mol^{-1}) was set in relation to the molecular weight of retronecine (Mw 155.197 g mol^{-1}), resulting in an RE factor of 0.52. To allow a comparison of sum parameter results and target analysis results, the following approximation approach was conducted. Since *J. vulgaris* was the main source for the PA/PANO contamination of summer honeys from Schleswig-Holstein (Huckauf 2016, 2017; Neumann and Huckauf 2016), its major PA constituent senecionine (Mw = 335.394 g mol^{-1}) was used to calculate the conversion factor of 2.16 to convert RE (Mw of retronecine = 155.197 g mol^{-1}) into a total amount of PA/PANO expressed as senecionine equivalents (Table 1).

$$c[\mu g\ kg^{-1}]RE = \frac{\text{Amount(IST)}[\mu g] \times 0.52(\text{RE factor}) \times \text{corrected area(analyte } m/z\,452.0 \rightarrow 120.0)}{\text{Area(IST}m/z\,454.0 \rightarrow 122.0) \times \text{amount(honey)}[kg]}. \quad (1)$$

Results

Relevant PAs and PANOs

The analytes found in the samples of the time series experiment (honeys A and B, Table 1) and of the interlaboratory comparison measurements (samples 1–20, Table 2) were mainly typical representatives of *Senecio* plants and, to a lesser extent, PAs/PANOs from *Echium vulgare, Borago officinalis, Symphytum* spp. or *Eupatorium cannabinum*. In both laboratories that performed target analyses, the main contribution to the PA/PANO sum levels consisted of erucifoline, jacobine, senecionine, seneciphylline, retrorsine and the corresponding *N*-oxides, typical *J. vulgaris* PAs, therefore reflecting the natural mass occurrences of this plant in the surroundings of the sampled bee hives.

As was to be expected with regard to the natural flora of Schleswig-Holstein as well as cultivated plants, neither trichodesmine nor europine, heliotrine, lasiocarpine and monocrotaline (or corresponding *N*-oxides) were found. Echimidine, a compound typical for *Echium* spp. and *Symphytum* spp. (Smith and Culvenor 1981), was detected in eight samples (honeys A and B, samples no. 5–8, 15, 16) by LMU in amounts ranging from the LOD to 10.7 µg kg^{-1} (Tables 1 and 2). Senecivernine and senecivernine *N*-oxide were analysed only by QSI in trace amounts (samples 15 and 19) up to 61 µg kg^{-1} (honeys A and B). Riddelliine and riddelliine *N*-oxide, which were also only

Table 2. Results of the interlaboratory comparison measurements of PA and PANO contamination (µg kg^{-1}) in 20 honey samples[a].

Sample no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Analyte																				
Echimidine (QSI)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Echimidine (LMU)	-	-	-	-	0.1	0.2	0.1	0.1	-	-	-	-	-	-	10.7	0.3	-	-	-	-
Intermedine (QSI)	-	-	-	-	-	-	-	-	-	-	1.9	-	-	-	-	-	-	-	-	-
Intermedine (LMU)	-	-	-	-	-	0.1	-	0.1	0.1	-	1.8	-	-	0.2	-	0.9	-	-	-	-
Intermedine *N*-oxide (QSI)[b]	-	2.1	-	22.0	46.0	5.9	-	1.0	1.2	-	140.0	-	5.0	13.0	1.9	61.0	36.0	6.6	-	-
Intermedine *N*-oxide (LMU)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lycopsamine (QSI)	-	-	-	-	5.8	-	-	-	-	-	8.8	-	-	1.3	-	1.6	3.2	-	-	-
Lycopsamine (LMU)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lycopsamine *N*-oxide (QSI)	-	-	-	-	-	-	-	-	-	-	2.1	-	-	-	-	1.2	-	-	-	-
Lycopsamine *N*-oxide (LMU)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erucifoline (QSI)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	18.0	-	-	-	12.0	20.0
Erucifoline (LMU)	-	-	-	-	-	-	0.1	-	0.1	-	-	-	15.9	-	18.5	-	0.9	2.0	-	0.3
Erucifoline *N*-oxide (QSI)	-	-	-	-	-	-	-	-	-	-	-	-	7.5	-	-	-	-	-	26.0	8.8
Erucifoline *N*-oxide (LMU)	-	-	-	-	-	-	-	-	-	-	-	-	12.1	-	11.8	-	-	0.5	12.1	0.8
Jacobine (QSI)	-	-	-	-	-	-	1.1	-	1.3	-	-	-	12.0	-	53.0	-	3.9	30.0	11.0	20.0
Jacobine (LMU)	-	0.1	0.2	-	-	-	1.0	0.2	0.8	-	1.2	-	20.0	0.2	49.7	-	4.4	20.4	19.8	16.2
Jacobine *N*-oxide (QSI)	-	-	-	-	-	-	-	-	-	-	-	-	3.0	-	-	-	-	2.2	8.0	-
Jacobine *N*-oxide (LMU)	-		-	-	-	-	-	-	-	-	-	-	15.1	-	15.0	-	0.1	2.4	18.5	1.7
Retrorsine (QSI)	-	-	-	-	-	-	-	-	-	-	-	-	2.6	-	9.1	-	-	4.0	4.4	16.0
Retrorsine (LMU)	-	-	-	-	-	-	0.3	-	0.2	-	-	-	11.0	-	15.3	-	0.7	3.2	13.6	9.7
Retrorsine *N*-oxide (QSI)	-	-	-	-	-	-	-	-	-	-	-	-	3.4	-	-	-	-	-	10.0	6.8
Retrorsine *N*-oxide (LMU)	-		-	-	-	-	-	-	-	-	-	-	1.8	-	0.1	-	-	2.5	8.6	5.1
Riddelliine (QSI)[c]	-	-	-	-	-	-	-	-	-	-	-	-	*3.2*	-	*9.2*	-	-	*3.4*	*1.4*	*6.0*
Riddelliine N-oxide (QSI)[c]	-	-	-	-	-	-	-	-	-	-	-	-	*5.4*	-	-	-	-	-	*14.0*	-
Senecionine (QSI)	-	-	-	-	-	-	1.7	-	2.4	-	-	-	14.0	-	32.0	-	4.2	26.0	35.0	83.0
Senecionine (LMU)	0.1	0.3	0.5		-	1.3	2.5	0.8	3.3	-	1.0	0.2	18.8	0.9	48.2	0.8	9.1	51.1	40.0	10.3
Senecionine *N*-oxide (QSI)	-	-	-	-	-	-	-	-	-	-	-	-	27.0	-	5.0	-	-	15.0	95.0	31.0
Senecionine *N*-oxide (LMU)	-	0.4	0.1	-	-				0.4		-	-	29.0	0.1	18.2	-		13.2	65.1	24.9
Seneciphylline (QSI)	-	-	-	-	-	1.1	3.3	1.0	3.8	-	-	-	29.0	-	68.0	-	7.2	48.0	22.0	56.0
Seneciphylline (LMU)	-	0.5	0.8	-	-	0.8	2.9	0.9	3.2		1.2	0.2	25.0	0.3	67.3	1.0	8.8	47.9	22.4	40.7
Seneciphylline *N*-oxide (QSI)	-	-	-	-	-	-	-	-	-	-	-	-	37.0	-	7.4	-	-	22.0	59.0	24.0
Seneciphylline *N*-oxide (LMU)	-	0.5	0.1	-	-	-		-	0.5	-	-	-	31.7		22.8	-	0.6	14.1	38.3	13.7
Senecivernine (QSI)[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*1.8*	-	-	-	*0.3*	-
Senecivernine N-oxide (QSI)[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*2.0*	-
Senkirkine (QSI)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Senkirkine (LMU)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.0	-	-	-	-	0.1
Sum of PA/PANO (QSI)	0.0	2.1	0.0	22.0	51.8	7.0	6.1	2.0	8.7	0.0	152.8	0.0	140.5	14.3	194.4	63.8	54.5	153.8	282.4	265.6
PA (QSI)	0.0	0.0	0.0	0.0	5.8	1.1	6.1	1.0	7.5	0.0	10.7	0.0	57.6	1.3	180.1	1.6	18.5	108.0	84.4	195.0
PANO (QSI)	0.0	2.1	0.0	22.0	46.0	5.9	0.0	1.0	1.2	0.0	142.1	0.0	82.9	13.0	14.3	62.2	36.0	45.8	198.0	70.6
Percentage of PANO (QSI)[d]		100%		100%	89%	84%	0%	50%	14%		93%		59%	91%	7%	97%	66%	30%	70%	27%
Sum of PA/PANO (LMU)	0.1	1.8	1.7	0.0	0.1	2.4	6.8	2.2	8.6	0.0	5.2	0.3	180.2	1.7	280.7	3.1	24.7	157.4	238.4	123.4
PA (LMU)	0.1	0.9	1.5	0.0	0.1	2.4	6.8	2.2	7.8	0.0	5.2	0.3	90.6	1.6	212.8	3.1	24.0	124.7	95.8	77.3
PANO (LMU)	0.0	0.9	0.2	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	89.5	0.1	67.9	0.0	0.7	32.7	142.7	46.1
Percentage of PANO (LMU)[d]	0%	51%	12%		0%	0%	0%	0%	10%		0%	0%	50%	7%	24%	0%	3%	21%	60%	37%
Senecionine equivalents (TUB)	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**15.2**	**0.6**	**8.8**	**0.0**	**8.4**	**0.0**	**146.5**	**0.0**	**483.7**	**2.1**	**45.4**	**208.1**	**290.3**	**507.3**

[a]PAs/PANOs less than LOD were not listed (monocrotaline, lasiocarpine, europine, heliotrine and their *N*-oxides).
[b]Including further closely related stereoisomers of lycopsamine-type PA *N*-oxides.
[c]Compounds measured exclusively by QSI, but results were not included into the PA/PANO sum to allow direct comparison to LMU.
[d]PANO% = sum of PANOs/sum of (Pas + PANOs) × 100.

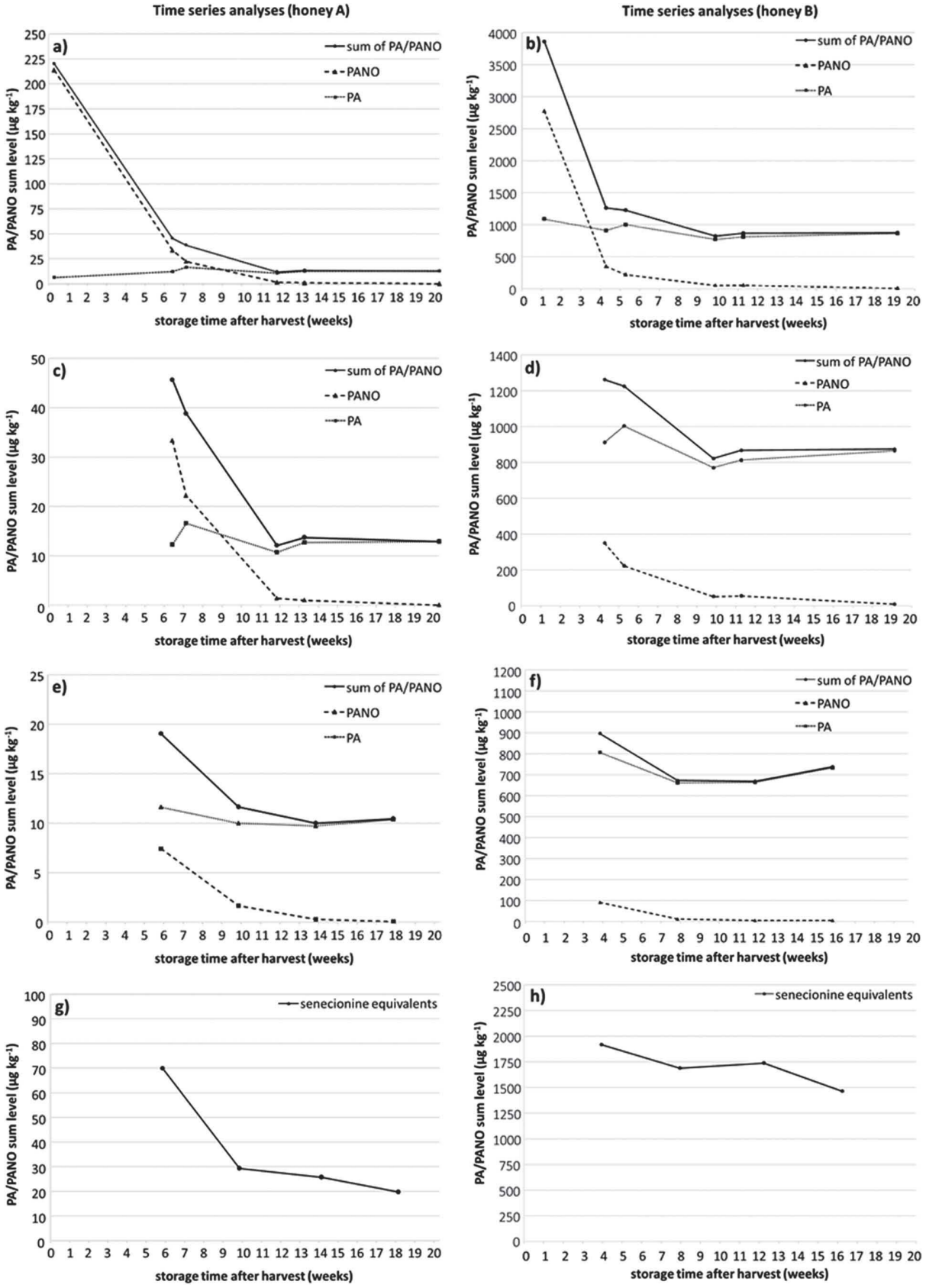

Figure 3. Results (PA, PANO and combined PA/PANO sum levels in µg kg^{-1}) of the time series analyses of honey A (left) and honey B (right) performed by three laboratories during a period of storage of up to 20 weeks after harvest. (a) and (b): Data of QSI, analyses from shortly after harvest until week 20 (six data points); (c) and (d): Data of QSI, for better comparability, time period adjusted to match the other two laboratories; (e) and (f): Data of LMU, analyses of weeks 4 or 6 to 16 or 18, respectively, after harvest; (g) and (h): Data of TUB, analyses of weeks 4 or 6 to 16 or 18, respectively, after harvest.

analysed by QSI, were detected in samples 14, 16 and 18–20 and in honeys A and B (up to 120 µg kg^{-1}). Both compounds occurred only in samples with high contents of senecionine or seneciphylline (Tables 1 and 2).

Time series analyses

A time series experiment was conducted to evaluate putative settling processes and the stability of PAs/PANOs in honey allowed to stand for defined time periods in order to investigate the reasons for the aforementioned differences of analytical results. For this purpose, two honeys with different levels of PA/PANO contamination (honeys A and B) were analysed shortly after harvest and again after approximately 4, 8 and 12 weeks of storage at room temperature. A decrease of the PA/PANO content was consistently observed by all applied analytical approaches (Figure 3). The target analyses clearly revealed that the decrease of the PA/PANO sum amount was exclusively due to a decrease of the PANOs, which were hardly detectable any more after 12 weeks, while the amount of tertiary PAs remained quite stable. While the percentage of PANOs was 97% or 71% of the PA/PANO sum amount in honey analysed 2 or 8 days after harvest (honeys A and B, respectively), it dropped to only 1% towards the end of the experiment (Table 1).

Strikingly, the PANO amounts of honeys A and B already decreased significantly between the first analysis (day 2 or 8 after harvest, respectively) and the second one (approximately 6 and 4 weeks after harvest). These initial measurements were only performed by QSI within the framework of the project 'Blossoms for Bees' (see above) (Figure 3(a,b), respectively). The results of QSI indicated a decrease of the PA/PANO sum amount of about 75% in honey A (initial PA/PANO sum level nearly exclusively attributable to PANOs, Figure 3(a)) and 66% in honey B (initial PA/PANO sum level attributable to PANOs to approximately 70%, Figure 3(b)) during the first weeks after harvest. Still, a further decrease was observable beginning from week 4 or 6 after harvest when LMU and TUB became involved in the analyses (t = 0, Table 1). Independent of the laboratory and the honey sample, the PA/PANO level was approximately 35–70% lower in honey A and 12–35% lower in honey B when comparing the first measurement of all laboratories and the second one (week 10 or 8 after harvest of honey A or B).

At the end of the experiment (weeks 18 or 16 after harvest related to analyses of honeys A and B by LMU and TUB; QSI provided their results 2–3 weeks later), both LMU and QSI only found PANO levels around the LOQ or below. Instead of the PA/PANO sum levels of 39, 19 and 70 µg kg^{-1} in honey A and 1225, 897 and 1917 µg kg^{-1} in honey B detected at the beginning of the collaborative experiment by QSI, LMU and TUB, respectively, only 13, 11 and 20 µg kg^{-1} were quantified in honey A and 874, 738 and 1464 µg kg^{-1} in honey B at the end of the experiment. While the PA/PANO sum results of LMU and QSI were more or less comparable after this 4-month period, the results of TUB were significantly higher. Comparing the initial amounts measured in honey A, TUB quantified about 3.7-fold more than LMU and

Table 3. Results (PA/PANO sum amounts in µg kg^{-1}) of the homogeneity measurements of two selected honey samples during storage of up to 12 weeks in conical plastic bags.

		Honey A			Honey B		
Laboratory	Weeks of storage	Top/µg kg^{-1}	Bottom/µg kg^{-1}	Bottom/top ratio (%)	Top/µg kg^{-1}	Bottom/µg kg^{-1}	Bottom/top ratio (%)
LMU	0	19.0	19.0	**100**	896.7	896.7	**100**
	4	11.7	12.0	**103**	652.2	650.1	**100**
	8	10.5	10.1	**96**	699.7	714.8	**102**
	12	10.2	10.2	**100**	699.8	739.0	**106**
QSI	0	45.6	45.6	**100**	1261.2	1261.2	**100**
	4	33.6	32.9	**98**	1224.0	1390.2	**114**
	8	13	12.3	**95**	770.0	829.0	**108**
	12	14.4	11.4	**79**	754.2	842.9	**112**
TUB	0	69.8	69.8	**100**	1916.9	1916.9	**100**
	4	30.5	26.7	**87**	1921.6	1792.3	**93**
	8	27.4	21.5	**79**	1700.0	1824.7	**107**
	12	20.3	24.0	**118**	1727.6	1699.5	**98**

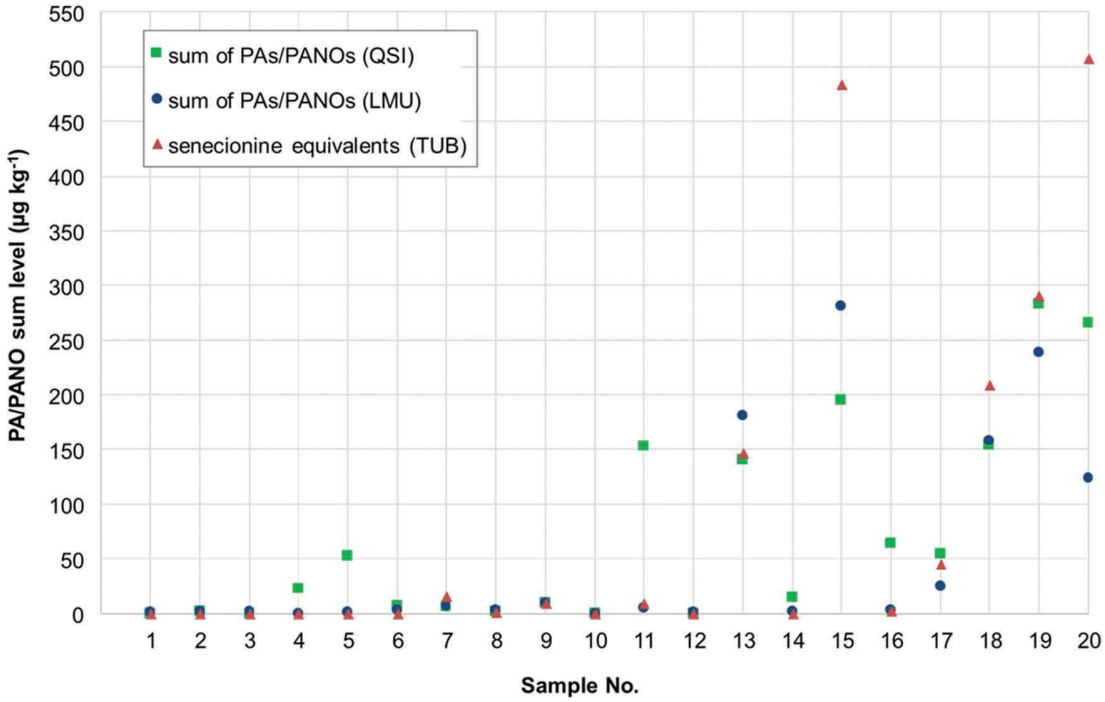

Figure 4. Results of the interlaboratory comparison measurements of LMU (target analysis, matrix-matched calibration), QSI (target analysis, standard addition) and TUB (sum parameter method).

1.5-fold more than QSI. In honey B, the corresponding factors were 2.1 and 1.5, respectively.

Test for demixing/settling effects

The results of the demixing test of two honey samples are shown in Table 3. The samples were stored over a defined period in conical plastic bags. After 4 weeks, the honey was tested by analysing one sample each from the top and the bottom of the first bag. After 8 weeks, the procedure was repeated with the second bag and after 12 weeks with the third. As shown in Table 3, the results of the samples taken from the top and from the bottom of the plastic bags did not indicate a demixing effect on PAs/PANOs over time (Table 3).

Interlaboratory comparison measurements

In 14 samples (1–3, 6–10, 12–14 and 17–19), the results of the three different approaches matched well (Figure 4). Especially the samples with a low or even unmeasurable PA content showed a high degree of consistency (Figure 4, Table 2). In two samples (15 and 20), the sum parameter approach yielded distinctly higher levels than the target analyses – as it was also observed for the samples of the time series experiment. For unknown reasons, these two samples also showed the highest observed variation between the values observed by the three applied analytical methods. This discrepancy occurred in samples with higher contaminations, e.g. TUB found 2.5- and 4.1-fold the amount registered by one of the target approaches (samples 15 and 20, respectively). However, in one lesser-contaminated sample (sample 7), TUB detected more than twice the amount of QSI and LMU as well. In addition, LMU found lower amounts than QSI and TUB in sample 20, but higher amounts in sample 15. QSI, on the other hand, found higher PA levels in samples 4–6, 11, 14, 16 and 17 (Table 2). In these seven samples, the results of QSI were higher than those of LMU and TUB (Figure 4). In all these cases, the surplus could be attributed to intermedine *N*-oxide which included further lycopsamine-type PA *N*-oxides. The parameter 'further lycopsamine *N*-oxides' refers to stereoisomers which are very closely related to intermedine *N*-oxide and lycopsamine *N*-oxide. These stereoisomers were detected by QSI but by neither LMU nor the sum parameter

method used by TUB (Table 2). The five honeys 13, 15 and 18–20 were identified as the samples with the highest PA/PANO contamination of this set of samples by all three analytical approaches with levels exceeding 140 μg kg^{-1}. The percentage of PANOs of the overall PA/PANO content in these honey samples ranged between 7% and 70%.

Discussion

Suitable honeys for the time series and storage experiments were chosen from the 'Blossom for Bees' sample of the summer 2016. Hence, for the initial PA/PANO content, only one value measured by QSI is available for each honey representing day 2 (honey A) or day 8 (honey B) after harvest, respectively. As indicated by the target analytical results, the PA patterns of both honeys were clearly dominated by *Senecio*-specific PAs/PANOs (Table 1 and Figure 3).

In the time series experiment, a decrease of the PA/PANO sum amount was observed by all laboratories (Figure 3). In the beginning, the PA pattern of both investigated honeys was dominated almost exclusively by the PANO forms (Figure 3(a,b); Table 1). However, the PANOs vanished rapidly, without resulting in an increase of tertiary PA levels, which remained almost constant according to the results of the target analyses. In this respect, our observations differ from those made by Betteridge et al. (2005), who discovered the capacity of *Echium* honey to reduce PANOs to their corresponding tertiary amines. A decrease exclusively of PANOs was also described after fermentation processes (silage preparation), where PAs remained stable as well (Gottschalk et al. 2015).

When comparing the PA/PANO content of honey A 4–6 weeks after the starting point $t = 0$ (first analysis carried out by all laboratories), TUB found a 3.7-fold higher PA/PANO sum amount (Figure 3(g)) than LMU (Figure 3(e)) and a 1.5-fold higher amount than QSI (Figure 3(c)). In honey B, the differences were 2.2- and 1.5-fold, respectively (Figure 3(d,f,h)). At the end of the storage experiment ($t = 3$, Table 1), when the PANO amounts were near or below the LOQ, those differences had become smaller: In honey A, TUB found a 1.8- and 1.5-fold higher PA/PANO content than LMU and QSI, respectively; in honey B, these factors were 2.0 and 1.6, respectively (Figure 3, Table 1).

One possible explanation for these findings might be that PANOs are less stable or more reactive towards other honey constituents, become structurally modified and hence vanish rapidly after harvest in target analysis approaches. Maybe a fraction of those former PANOs, which still contains the 1,2-unsaturated necine base part, is covered by the sum parameter approach, resulting in overall higher values after extended storage. Whether these postulated modified PANO structures would still be of toxicological relevance is unknown.

A second possible explanation might be that apart from the compounds targeted by the direct methods, additional PAs/PANOs occurred in the samples under investigation. This observation may be generalised, assuming that due to a lack of analytical reference standards, the results of target analytical investigations are prone to underestimate the actual contamination of honeys. LMU and QSI analysed only 25 and 28 compounds, respectively, which reflected the number of then commercially available reference standards. Meanwhile, this number has increased to approximately 50 compounds. Nevertheless, the dramatic drop in PA levels within 4–6 weeks after the harvest raises major concerns in terms of reproducibility and comparability of analytical data. In general, considering the now confirmed major impact the point of time of the analysis has in freshly harvested honeys, it would be strongly recommended to analyse only matured honeys to eliminate this obvious source of discrepancy.

The PA and PANO levels measured in the 20 honey samples of the interlaboratory comparison study showed a good consistency of all three approaches for 14 of the 20 samples (70%) (Table 2 and Figure 4; samples 1–3, 6–10, 12–14 and 17–19). For the other six samples (30%), either LMU and TUB or QSI and TUB showed a better consistency with each other than with the third laboratory, or both LMU and QSI quantified significantly less than than TUB. The latter was the case in three samples (15, 18 and 20) of the comparison analyses as well as for the honey sample B used for the storage experiments, where up to 4.1 times more PAs/PANOs were quantified by the sum parameter method (sample 20, Table 2). It is striking that those were the

samples with the highest PA/PANO contamination. Since the final values (Table 2) consisted of ≤37% PANOs, it can be assumed that much higher PANO concentrations would have been observed without any storage time. Hence, one possible explanation could be that some of the PANOs are converted into unknown PA metabolites that still bear the structural feature of the 1,2-unsaturated necine base and are still analytically accessible through a sum parameter approach. Matured honey samples with high amounts of tertiary PAs (representing an initially high load of PANOs) could therefore in general be subject to an underestimation by the target methods.

In sample 13, higher amounts of erucifoline and jacobine and their *N*-oxides and of retrorsine were determined by LMU, which accounted for a 40 µg kg^{-1} higher sum amount than determined by QSI. In sample 20, on the other hand, LMU quantified much lower amounts particularly of erucifoline, erucifoline *N*-oxide and senecionine than QSI (Table 2). Such differences between LMU and QSI may have been caused by the different ways of sample preparation and types of calibration. SCX columns of different manufacturers could influence recoveries and therefore the results of different laboratories. Additionally, probable matrix effects might have been better compensated by the standard addition approach of QSI, whose results were in some cases (samples 13, 17, 19 and 20) in better agreement with those of TUB. A better comparability of results obtained by this approach is known from evaluations of German proficiency tests (BfR 2015, 2016b). On the contrary, Breidbach and Tamošiūnas (2017) found no significant influence of the calibration method when evaluating results of the recent proficiency test organised by the JRC. However, this proficiency testing revealed a strong impact of individual compounds on the trueness of PA/PANO sum amounts. While echimidine, intermedine and senkirkine were determined with |z|-scores ≤2 by more than 85% of the participating laboratories, senecionine and seneciphylline showed |z|-scores ≥2 in 55% and 27% of the laboratories, respectively. Another factor influencing quantitative analytical results is the analytical standards. An international proficiency test organised by the German Federal Institute for Risk Assessment (BfR) revealed one or more compounds in laboratory-prepared standard solutions exceeding a |z|-score of 2 in 14 of 28 participating laboratories (BfR 2016b). Differences in samples with a low PA/PANO contamination can be explained by the lower LOD of the target methods: Seven samples were found to contain PAs/PANOs in amounts of only around 0.1 µg kg^{-1} by LMU, while REs were less than LOD (Table 2).

Differences caused by sample inhomogeneity could be excluded. As shown in the time series experiment with honey in conical bags, PAs and PANOs remained equally distributed in honey samples that had been allowed to stand for 3 months (Table 3). In the case of *Echium vulgare*, plant pollen showed much higher PA concentrations than nectar (factor of 500) (Lucchetti et al. 2016). However, a putative settling of PA/PANO containing pollen towards the bottom of a honey storage container – possibly interfering with analytical results – was not observed in this study. This can be explained either by an overall low amount of PA pollen or by a missing demixing/settling effect of pollen due to the high specific gravity of honey (>1.4 g ml^{-1}). Hence, storage time had no influence on the homogenous distribution of PAs/PANOs in stored honey (at least during the observed storage time of three months).

For seven samples (Table 2, samples 4–6, 11, 14, 16 and 17), the difference in the analytical results of all laboratories was mainly caused by the analyte intermedine *N*-oxide including the further lycopsamine-type PA *N*-oxides, which was exclusively detected by QSI. Intermedine is one out of five possible stereoisomers of the so-called lycopsamine-type PA group comprising lycopsamine, indicine, intermedine, rinderine and echinatine. These stereoisomers feature almost identical mass spectra and can usually not be completely separated chromatographically. The same holds for the corresponding *N*-oxides. These stereoisomers also turned out to exhibit problems in quantification within the recent proficiency testing organised by the JRC (Breidbach and Tamošiūnas 2017). It was striking that even at high levels (Table 2, Figure 4, sample 11), the appearance of intermedine *N*-oxide was not accompanied by the detection of the corresponding tertiary PA, as would have been expected from the general tendency of PA/PANO co-occurrence documented in Table 2. Furthermore, these putative PANOs were obviously not recorded by the sum parameter

method of TUB, even though they were confirmed by QSI as retronecine-type PANOs with a typical fragmentation behaviour by comparison with reference substances. If these peaks had not been taken into account by QSI, the results for all these samples apart from sample 17 would have satisfactorily matched across all analytical approaches. Given these circumstances, it remains questionable how to assess these analytical results. The occurrence of intermedine *N*-oxide and potential stereoisomers should be closely observed, and further studies are needed to investigate these phenomena.

Conclusions

In Germany, the target analysis of 17 compounds in honey and 21 compounds in tea is presently regarded as state of the art for assessing the PA/PANO contamination (BfR 2013a, 2014; Bodi et al. 2014). The latest statement of EFSA (2017) also recommended a set of 17 compounds for the monitoring of food and feed. However, our results showed that even for honeys produced in a relatively small region of Germany with a relatively narrow variety of PA/PANO-producing plants, assessments on the basis of 25 PA/PANO analytes are still prone to variations of analytical results, if different methods are applied. As we could demonstrate with the conducted time series experiment, a major factor for analytical uncertainty in the determination of PAs/PANOs in honey is time. To exclude major changes in PANO levels (affecting the final total amount of PAs/PANOs of a sample), it would make sense to compare analytical results obtained after a maturation period of three months. However, toxicological concerns regarding the 'disappearance' of PANOs are still a matter of debate.

Compounds like jacobine, jacobine *N*-oxide, jacoline, jaconine, riddelliine and riddelliine *N*-oxide, which are not part of the above-mentioned 'standard sets', have proven to contribute significantly to the PA/PANO sum amount of regionally produced honeys whenever they were 'unofficially' included in mass samplings carried out in the framework of the project 'Blossoms for Bees' (Huckauf, unpublished results). Hence, it seems imperative (a) to identify the relevant compounds and to complement the 'standard set' accordingly, (b) to improve the analytical methods in order to establish a reliable instrument for food and feed monitoring and (c) to elucidate the fate of PANOs during the maturation of honey. In addition, the inconsistent role of intermedine *N*-oxide and its stereoisomers (i.e. lycopsamine-type PAs and PANOs) in different analytical approaches remains a challenge for the future.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Betteridge K, Cao Y, Colegate SM. 2005. Improved method for extraction and LC-MS analysis of pyrrolizidine alkaloids and their N-oxides in honey: application to *Echium vulgare* honeys. J Agr Food Chem. 53:1894–1902.

Beuerle T, Blacquière T, von der Ohe W. 2011. Allgemeine Deutsche Imkerzeitung (ADIZ). 45:12–15.

[BfR] Federal Institute for Risk Assessment. 2013a. Determination of pyrrolizidine alkaloids in honey with SPE-LC-MS/MS. Method description of the BfR, Berlin (Germany).

[BfR] Federal Institute for Risk Assessment. 2013b. Pyrrolizidine alkaloids in herbal teas and teas. Scientific Opinion of the BfR No. 018/2013, Berlin (Germany).

[BfR] Federal Institute for Risk Assessment. 2014. Determination of pyrrolizidine alkaloids in plant material with SPE-LC-MS/MS. Method description of the BfR, Berlin (Germany).

[BfR] Federal Institute for Risk Assessment. 2015. International collaborative study for the determination of pyrrolizidine alkaloids in honey and herbal tea by SPE-LC-MS/MS. BfR Science Publication 013/2015, Berlin (Germany).

[BfR] Federal Institute for Risk Assessment. 2016a. Pyrrolizidine alkaloids: levels in foods should continue to

be kept as low as possible. Scientific Opinion of the BfR No. 030/2016, Berlin (Germany).

[BfR] Federal Institute for Risk Assessment. 2016b. International collaborative study on the determination of pyrrolizidine alkaloids in herbal tea and roiboos tea (BfR-PT-PA01). BfR Science Publication 03/2016, Berlin (Germany).

Bodi D, Ronczka S, Gottschalk C, Behr N, Skibba A, Wagner M, Lahrssen-Wiederholt M, Preiss-Weigert A, These A. 2014. Determination of pyrrolizidine alkaloids in tea, herbal drugs and honey. Food Add Contam A. 31:1886–1895.

Breidbach A, Tamošiūnas V 2017. Pyrrolizidine alkaloids in herbal tea and honey – report on the 2017 proficiency testing scheme. EUR 28960 EN, Publications Office of the European Union, Luxembourg, JRC 109959

Brown PA. 2017. Overview of toxins and toxicants in food and their regulatory limits. In: Wong Y-C, Lewis RJ, editors. Analysis of food toxins and toxicants. New Jersey: Wiley; p. 679–710.

Chen T, Mei N, Fu PP. 2010. Genotoxicity of pyrrolizidine alkaloids. J Appl Toxicol. 30:183–196.

[COT] Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment. 2008. Statement on pyrrolizidine alkaloids in food. https://cot.food.gov.uk/sites/default/files/cot/cotstatementpa200806.pdf accessed 2018 Feb 10

Cramer L, Schiebel HM, Ernst L, Beuerle T. 2013. Pyrrolizidine alkaloids in the food chain: development, validation, and application of a new HPLC-ESI-MS/MS sum parameter method. J Agr Food Chem. 61:11382–11391.

Culvenor C, Downing D, Edgar J, Jago MV. 1969. Pyrrolizidine alkaloids as alkylating and antimitotic agents. Ann N Y Acad Sci. 163:837–847.

De Boer YS, Sherker AH. 2017. Herbal and dietary supplement–induced liver injury. Clin Liver Dis. 21:135–149.

[DIN] German Institute for Standardisation. 2008. DIN 32654: 2008-11: chemical analysis – decision limit, detection limit and determination limit under repeatability conditions – terms, methods, evaluation. Berlin (Germany): Beuth German Standards.

Dübecke A, Beckh G, Lüllmann C. 2011. Pyrrolizidine alkaloids in honey and bee pollen. Food Add Contam A. 28:348–358.

Dusemund B, Nowak N, Sommerfeld C, Lindtner O, Schäfer B, Lampen A. 2018. Risk assessment of pyrrolizidine alkaloids in food of plant and animal origin. Food Chem Toxicol. 115:63–72.

[EFSA] European Food Safety Authority. 2011. Scientific opinion on pyrrolizidine alkaloids in food and feed. Efsa J. 9:2406.

[EFSA] European Food Safety Authority. 2012. MOE approach for the assessment of impurities which are both genotoxic and carcinogenic. Efsa J. 10:2578.

[EFSA] European Food Safety Authority. 2017. Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. Efsa J. 15:4908.

Fu PP, Xia Q, Lin G, Chou MW. 2004. Pyrrolizidine alkaloids – genotoxicity, metabolism enzymes, metabolic activation, and mechanisms. Drug Metab Rev. 36:1–55.

Gottschalk C, Ronczka S, Preiß-Weigert A, Ostertag J, Klaffke H, Schafft H, Lahrssen-Wiederholt M. 2015. Pyrrolizidine alkaloids in natural and experimental grass silages and implications for feed safety. Anim Feed Sci Technol. 207:253–261.

Hartmann T, Witte L. 1995. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. In: Pelletier SW, editor. Alkaloids: chemical and biological perspectives. Oxford (UK): Elsevier; p. 155–233.

Huckauf A. 2016. Das Pilotprojekt 'Blüten für Bienen'– ergebnisse des ersten Projektjahres 2015. Die neue Bienenzucht (DNB); p. 239–245.

Huckauf A. 2017. Das Pilotprojekt 'Blüten für Bienen' – ergebnisse des zweiten Projektjahres 2016. Die neue Bienenzucht (DNB); p. 190–196.

Jago MV, Edgar J, Smith L, Culvenor C. 1970. Metabolic conversion of heliotridine-based pyrrolizidine alkaloids to dehydroheliotridine. Molecular Pharmacol. 6:402–406.

Kempf M, Beuerle T, Bühringer M, Denner M, Trost D, von der Ohe K, Bhavanam VBR,Schreier P. 2008. Pyrrolizidine alkaloids in honey: risk analysis by gas chromatography-mass spectrometry. Mol Nutr Food Res. 52:1193–1200.

Langel D, Ober D, Pelser PB. 2011. The evolution of pyrrolizidine alkaloid biosynthesis and diversity in the Senecioneae. Phytochem Rev. 10:3–74.

Letsyo E, Jerz G, Winterhalter P, Horn G, Beuerle T. 2016. Survey of pyrrolizidine alkaloids in seven varieties of *Lappula squarrosa*: an alternative source of heart-healthy vegetable oil. Phytochem Anal. 27:133–139.

Lucchetti MA, Glauser G, Kilchenmann V, Dubecke A, Beckh G, Praz C, Kast C. 2016. Pyrrolizidine alkaloids from *Echium vulgare* in honey originate primarily from floral nectar. J Agr Food Chem. 64:5267–5273.

Mattocks A. 1968. Toxicity of pyrrolizidine alkaloids. Nature. 217:723–728.

Mattocks A. 1971. Hepatotoxic effects due to pyrrolizidine alkaloid N-oxides. Xenobiotica. 1:563–565.

Mattocks AR. 1986. Chemistry and toxicology of pyrrolizidine alkaloids. London (UK): Academic Press.

McLean EK. 1970. The toxic actions of pyrrolizidine (*Senecio*) alkaloids. Pharmacol Rev. 22:429–483.

[NCI] National Cancer Institute. 1978. Bioassay of lasiocarpine for possible carcinogenicity. Natl Cancer Inst Carcinog Tech Rep Ser. 39:1.

Neumann H, Huckauf A. 2016. Tansy ragwort (*Senecio jacobaea*): a source of pyrrolizidine alkaloids in summer honey? J Verbraucherschutz Lebensmittelsicherh. 11:105–115.

[NTP] National Toxicology Program. 2003. Toxicology and carcinogenesis studies of riddelliine (CAS No. 23246-96-0) in F344/N rats and B6C3F1 mice (gavage studies). Natl Toxicol Program Tech Rep Ser. 508:1.

Prakash AS, Pereira TN, Reilly PEB, Seawright AA. 1999. Pyrrolizidine alkaloids in human diet. Mutat Res. 443:53–67.
Schoental R. 1968. Toxicology and carcinogenic action of pyrrolizidine alkaloids. Cancer Res. 28:2237–2246.
Smith L, Culvenor C. 1981. Plant sources of hepatotoxic pyrrolizidine alkaloids. J Nat Prod. 44:129–152.
Stewart MJ, Steenkamp V. 2001. Pyrrolizidine poisoning: a neglected area in human toxicology. Ther Drug Monit. 23:698–708.
Winter C, Segall H. 1989. Metabolism of pyrrolizidine alkaloids. In: Cheeke PR, editor. Toxicants of plant origin. Boca Raton, (FL): CRC-Press; Vol. 1; p. 23–40.

8.3 Development of a sensitive analytical method for determining 44 pyrrolizidine alkaloids in teas and herbal teas via LC-ESI-MS/MS

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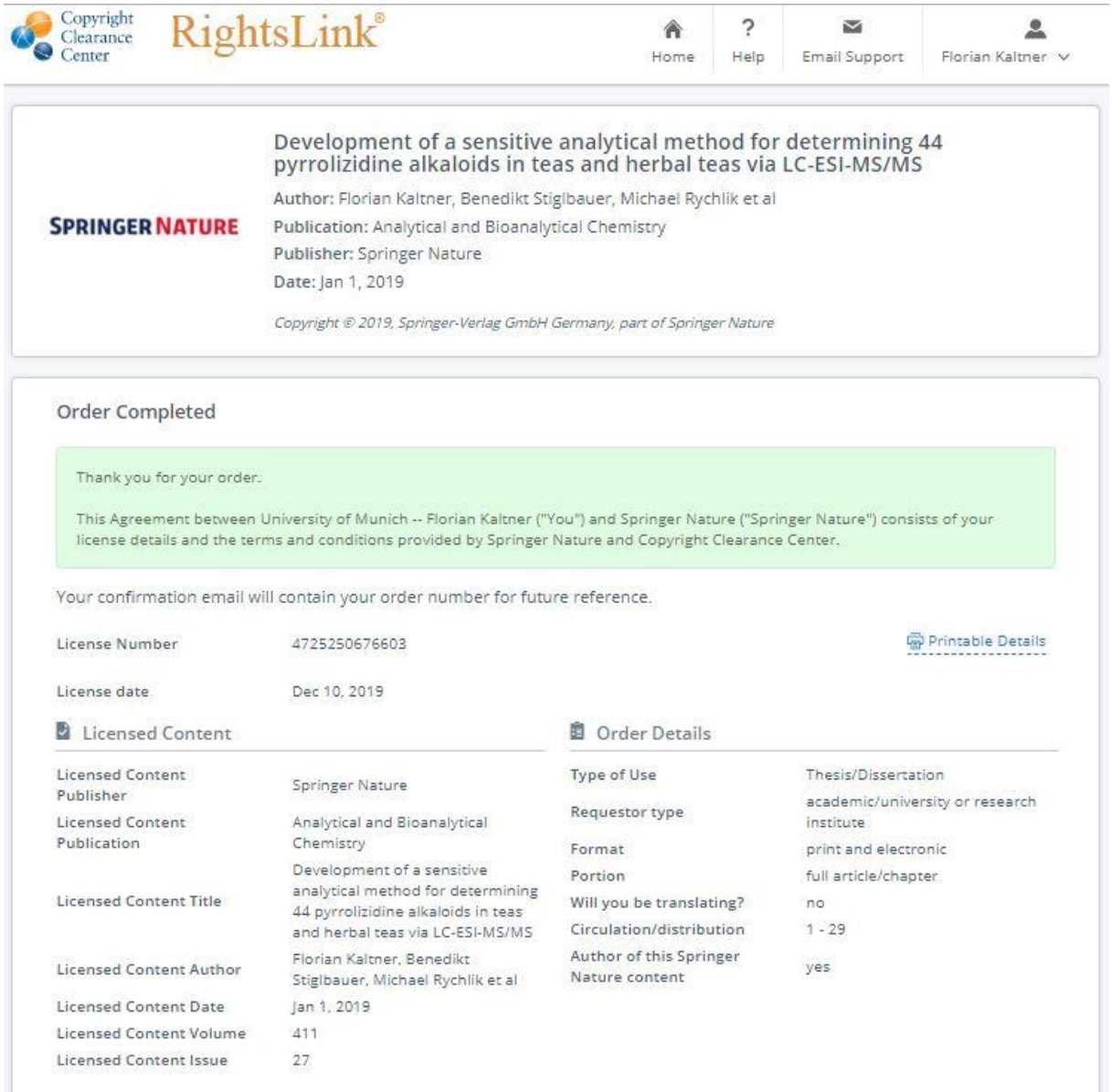

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RESEARCH PAPER

Development of a sensitive analytical method for determining 44 pyrrolizidine alkaloids in teas and herbal teas via LC-ESI-MS/MS

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Abstract

Pyrrolizidine alkaloids (PA) and PA-*N*-oxides (PANO) are a large group of secondary plant metabolites comprising more than 660 compounds. Exhibiting geno- and hepatotoxic properties, they are responsible for multiple cases of food and feed poisoning over the last 100 years. For food and feed safety reasons, relevant PA/PANO should be monitored extensively in the main sources of PA/PANO intake. In this study, a sensitive analytical method was developed for detecting a broad range of 44 commercially available PA/PANO compounds, and in-house validation procedures were performed for several (herbal) teas. Various extraction solvents and procedures, as well as solid phase extraction materials for sample clean-up and analyte concentration, were tested to establish the methods' efficiency and effectiveness. Chromatographic conditions were optimised to obtain the best possible separation of isomers for the 44 PA/PANO analytes. The final method was proven very sensitive and accurate, with detection limits ranging from 0.1 to 7.0 μg/kg and precisions between 0.7 and 16.1%. For 40 of the analytes, the recovery rates ranged from 60.7 to 128.8%. The applicability and trueness of the method were examined by analysing tea samples from a local supermarket and comparing them to a reference material. At least one PA/PANO analyte was detected in 17 of the 18 samples under investigation, and the sum contents of the samples ranged from 0.1 to 47.9 μg/kg. Knowledge of the PA/PANO composition in a sample can be used to indicate the botanical origin of the impurity and, thus, the geographical region of cultivation.

Keywords Pyrrolizidine alkaloids · Herbal tea · Method development · Liquid chromatography tandem-mass spectrometry

Abbreviations

AcIm	Acetylintermedine
AcImN	Acetylintermedine-*N*-oxide
AcLy	Acetylycopsamine
AcLyN	Acetylycopsamine-*N*-oxide
BfR	German Federal Institute for Risk Assessment
$BMDL_{10}$	Benchmark dose lower confidence limit 10%
Ec	Erucifoline
EcN	Erucifoline-*N*-oxide
Em	Echimidine
EmN	Echimidine-*N*-oxide
Eu	Europine
EuN	Europine-*N*-oxide
EFSA	European Food Safety Authority
ESI+	Positive electrospray ionisation
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
Ht	Heliotrine
HtN	Heliotrine-*N*-oxide
Ic	Indicine
IcN	Indicine-*N*-oxide
Ig	Integerrimine
IgN	Integerrimine-*N*-oxide
Im	Intermedine
ImN	Intermedine-*N*-oxide
Jb	Jacobine
Jb	Jacobine-*N*-oxide
Jl	Jacoline
JlN	Jacoline-*N*-oxide
Lc	Lasiocarpine

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LcN	Lasiocarpine-*N*-oxide
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
Ly	Lycopsamine
LyN	Lycopsamine-*N*-oxide
Mc	Monocrotaline
McN	Monocrotaline-*N*-oxide
Mk	Merenskine
MkN	Merenskine-*N*-oxide
MOE	Margin of exposure
MRM	Multiple reaction monitoring
Mx	Merepoxine
MxN	Merepoxine-*N*-oxide
MS	Mass spectrometry
MS3	Multiple tandem mass spectrometry
PA	Pyrrolizidine alkaloid
PANO	Pyrrolizidine alkaloid-*N*-oxide
RP	Reversed phase
Rs	Retrorsine
RsN	Retrorsine-*N*-oxide
RSD	Relative standard deviation
S/N	Signal-to-noise ratio
Sc	Senecionine
ScN	Senecionine-*N*-oxide
SCX	Strong cation exchange
Sl	Sceleratine
SlN	Sceleratine-*N*-oxide
Sp	Seneciphylline
SPE	Solid phase extraction
SpN	Seneciphylline-*N*-oxide
Sk	Senkirkine
Sv	Senecivernine
SvN	Senecivernine-*N*-oxide
Td	Trichodesmine

Introduction

Pyrrolizidine alkaloids (PA), and their corresponding *N*-oxides (PANO), are a large group of secondary metabolites plants produce to prevent damage caused by phytophagous insects [1]. So far, more than 660 PA/PANO structures are known and presumed to occur in more than 6,000 plant species worldwide, especially in plants of the families Asteraceae (Senecioneae, Eupatorieae), Apocynaceae (Echiteae), Boraginaceae and Fabaceae (genus *Crotalaria*) [2–4]. Chemically speaking, PA/PANO functionality typically consists of a pyrrolizidine core structure (necic base) esterified to one or more polyhydroxylated carboxylic acids (necic acids). Depending on the grade of esterification and stereochemistry, different groups of PA/PANO compounds have been identified (Fig. 1).

Only phytochemicals related to 1,2-dehydro-PA/PANO are of toxicological interest. In their original form, 1,2-dehydro-PA/PANO compounds are biologically inactive protoxins, which require metabolic activation by liver enzymes to achieve their toxic potential. 1,2-Dehydro-PA/PANO compounds are easily hydroxylated into highly reactive pyrrolic esters during phase I metabolism, which enables them to form the DNA or protein adducts responsible for the group's toxic effects [3, 5]. 1,2-Dehydro-PA/PANO compounds with more extensive esterification, i.e. transitioning from monoesters via diesters to cyclic diesters, exhibit greater toxicity levels [6]. Recently, relative potency factors for various PA/PANO compounds were proposed based on a comprehensive evaluation of the relevant literature on their toxic potential [7]. Several cases of acute poisoning or chronic exposure to 1,2-dehydro-PA/PANO compounds have been reported worldwide in the past decades [8–10]. Long-term intake of low doses of 1,2-dehydro-PA/PANO in food or feed is associated with chronic diseases, such as cancer, veno-occlusive disease, liver cirrhosis and pulmonary hypertension [10–14].

Concerning the occurrence of PA/PANO in the food chain, honey, food supplements, spices and (herbal) teas are important sources of PA/PANO exposure in humans [15]. Data published in the last few years by the European Food Safety Authority (EFSA) suggest that a set of 17 PA/PANO compounds are commonly encountered in food and feed supply chains. Nevertheless, to better understand the occurrence of PA/PANO in these matrices, EFSA also recommended monitoring the PA/PANO more frequently found in honey, tea and herbal tea products [15].

To date, regulatory limits have not been established by the European Commission, although human exposure to PA/PANO has reached critical levels for certain consumer groups. EFSA assessed the risk from PA/PANO exposure using the margin of exposure (MOE) approach based on a benchmark dose lower confidence limit 10% ($BMDL_{10}$) of 237 μg/kg body weight/day for the sum of the PA/PANO analytes under investigation [15]. So far, a maximum content of 100 mg/kg of PA/PANO-containing *Crotalaria* spp. was set in Directive 2002/32/EC for animal feed materials and compound feed [16]. However, due to PA/PANO derivatives' genotoxic and carcinogenic properties, their occurrence in food and feed supply chains should generally be kept as low as reasonably achievable (ALARA principle) [17].

Developing sensitive, reliable analytical methods to determine PA/PANO content in relevant food and feed matrices is very important since the toxicological concern raised by PA/PANO exposure has been brought increasingly into competent authorities' focus within the past decade. Typical modern methods are based on liquid chromatography tandem mass spectrometry (LC-MS/MS),

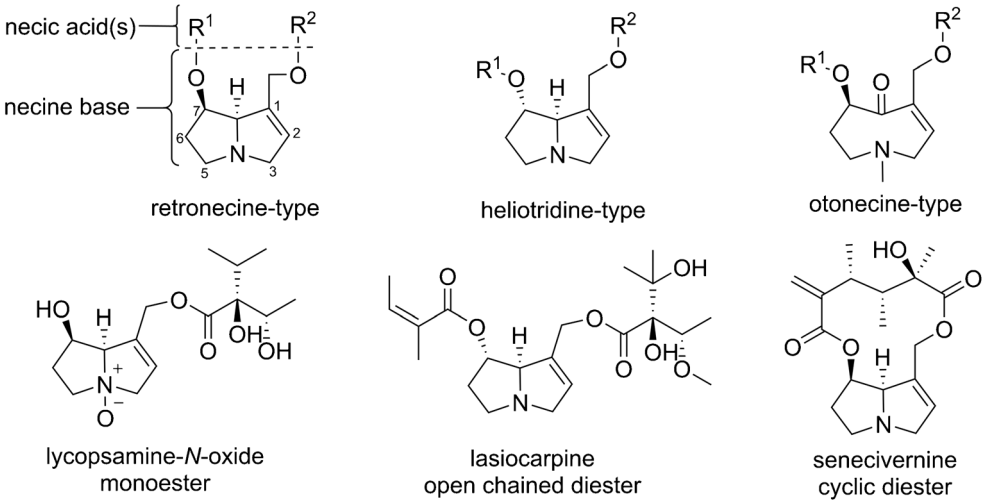

Fig. 1 Examples of different types of pyrrolizidine alkaloids and their *N*-oxides, depending on the core structure and grade of esterification

which is usually comprised of either solid phase extraction (SPE) clean-up of sample extracts, to obtain the highest possible sensitivity, or the use of simple dilute-and-shoot approaches [18, 19]. In general, two different analytical techniques are regularly applied to detect the PA/PANO content in samples: a sum parameter approach, which determines the sum of PA/PANO compounds in a sample after previous reduction and derivatisation procedures [20, 21], and the detection of single analytes using commercially available (or self-prepared) reference standards for a (semi-) quantification via standard addition or (matrix-matched) external calibration [22, 23]. The application of tandem mass spectrometry (MS/MS) results in low limits of detection (LOD), especially in cases where multiple-stage tandem mass spectrometers (MS3) are used [24]. Furthermore, PA/PANO compounds within the same esterification and/or stereochemistry group show similar or even identical mass transitions. This behaviour can be used to develop untargeted MS/MS approaches for analysing PA/PANO in samples without the need for analytical standards [25, 26].

A major disadvantage of the sum parameter approach is the loss of the specific PA/PANO profile present in the sample before analysis. In contrast, analysis of a limited set of PA/PANO can lead to underestimating the sum content due to the presence of unknown, yet related, analytes. Furthermore, selecting a set of PA/PANO compounds requires comprehensive data and knowledge of the occurrence and contribution of the individual PA/PANO to a sample's specific PA/PANO profile. Thus, the aim of the current study is to develop and validate a sensitive LC-MS/MS method to quantify a large number of specific PA/PANO analytes in a broad range of tea matrices. After development and in-house validation, the method was used to analyse the occurrence of PA/PANO in teas and herbal teas purchased from a local supermarket.

Materials and methods

Chemical reagents and standards The following PA/PANO standards were obtained from PhytoLab (Vestenbergsgreuth, Germany): 7-*O*-acetylintermedine (AcIm), 7-*O*-acetylintermedine-*N*-oxide (AcImN), 7-*O*-acetyllycopsamine (AcLy), echimidine (Em), echimidine-*N*-oxide (EmN), erucifoline (Ec), erucifoline-*N*-oxide (EcN), europine (Eu), europine-*N*-oxide (EuN), heliotrine (Ht), heliotrine-*N*-oxide (HtN), intermedine (Im), intermedine-*N*-oxide (ImN), jacobine (Jb), jacobine-*N*-oxide (JbN), lasiocarpine (Lc), lycopsamine (Ly), lycopsamine-*N*-oxide (LyN), monocrotaline (Mc), monocrotaline-*N*-oxide (McN), retrorsine (Rs), retrorsine-*N*-oxide (RsN), senecionine (Sc), seneciphylline (Sp), seneciphylline-*N*-oxide (SpN), senecivernine (Sv), senecivernine-*N*-oxide (SvN), senkirkine (Sk) and trichodesmine (Td). Further PA standards, namely 7-*O*-acetyllycopsamine-*N*-oxide (AcLyN), indicine (Ic), indicine-*N*-oxide (Ic), integerrimine (Ig), integerrimine-*N*-oxide (IgN), jacoline (Jl), jacoline-*N*-oxide (Jl), lasiocarpine-*N*-oxide (LcN), merenskine (Mk), merenskine-*N*-oxide (MkN), merepoxine (Mx), merepoxine-*N*-oxide (MxN), sceleratine (Sl), sceleratine-*N*-oxide (SlN) and senecionine-*N*-oxide (ScN), were purchased from CFM Oskar Tropitzsch (Marktredwitz, Germany).

With respect to solubility, stock solutions ($c = 1$ mg/mL) of each PA/PANO were prepared, either with acetonitrile (Em, Ec, EcN, Eu, Ht, Ic, IcN, Jb, Lc, LcN, Ly, Mc, SpN, Sk) or acetonitrile/water (50/50, v/v) (all other analytes), and stored at 6 °C in the dark. A PA/PANO mix solution ($c = 10$ µg/mL of each analyte) was prepared by combining stock solution aliquots and diluting the resulting mixture with acetonitrile/water (50/50, v/v). LC-MS-grade acetonitrile and methanol, purchased from Th. Geyer (Renningen, Germany), were used for all experiments. Ultrapure water was obtained by purifying water through an UltraClear™ TP UV UF TM from Evoqua

Water Technologies (Barsbüttel, Germany). Ammonia was acquired from Merck (Darmstadt, Germany). Sulphuric acid and formic acid were purchased from Th. Geyer (Renningen, Germany). Ammonium formate, used as an additive for LC-MS solvents, was obtained from Fluka (Steinheim, Germany).

Instrumentation Measurements were carried out on a Shimadzu high-performance liquid chromatography (HPLC) apparatus (LC-20AB, SIL-20AC HT, CTO-20AC, CBM-20A, Duisburg, Germany) coupled to an API4000 triple quadrupole MS (Sciex, Darmstadt, Germany). MS ion source parameters were set as follows: ionisation voltage, 2.500 V; nebuliser gas, 50 psi; heating gas, 50 psi; curtain gas, 30 psi; temperature, 600 °C; collision gas level, 7. Analyst (Version 1.6.2) and MultiQuant software (Version 3.0.1), both provided by Sciex, were used for data acquisition and processing. Statistical analyses of the processed data were performed using Microsoft Excel 2016 and OriginPro software (Version 2018G, OriginLab). Figure 1 was drawn with ChemBioDraw Ultra (Version 14.0.0.117, CambrideSoft), Fig. 2 was a chromatogram copied from Analyst (Version 1.6.2, Sciex) and Fig. 3 was drawn with OriginPro software (Version 2018G, OriginLab).

Samples A peppermint tea sample, which was free of any PA/PANO analyte and obtained from a previous study, was used for method development. For validation, the following additional tea and herbal tea matrices were chosen: green tea, black tea, fennel, chamomile and rooibos tea. These five samples were provided by Martin Bauer GmbH & Co. KG (Vestenbergsgreuth, Germany) and contained only traces of individual analytes of the 44 PA/PANO compounds listed above.

For a small survey, 18 tea samples ($n = 3$ for different products of the above-mentioned kinds of teas/herbal teas) were purchased from a local supermarket in Oberschleissheim (Germany). One sample of each investigated matrix originated from organic production. All samples were previously homogenised by grinding to a particle size of < 1.0 mm using an ultra-centrifugal mill ZM 200 from Retsch (Haan, Germany) and stored at room temperature in a dry, dark place until analysis.

Development of the LC-MS/MS method Multiple reaction monitoring (MRM) transitions were determined by direct injection of the individual PA/PANO standard solutions ($c = 100$ ng/mL) in methanol/water (50/50, v/v, containing 5 mmol/L ammonium formate) via a syringe pump (Harvard Apparatus, Holliston, USA) at a flow rate of 20 μL/min. MS instrument settings for each PA/PANO were optimised automatically with the compound optimisation tool included in the Analyst software. Injected PA/PANO solutions were tested in positive and negative electrospray ionisation (ESI+/−) mode. For the final method, two MRM transitions per analyte were chosen as $[M+H]^+$ in ESI+ mode.

After determining the MRM transitions for 44 analytes, various HPLC gradients were established and optimised. Depending on the LC solvents used, diluted mixes of standards ($c = 50$ ng/mL) in methanol/water (5/95, v/v) or acetonitrile/water (5/95, v/v) were utilised for these experiments. Water, methanol/water (10/90, 5/95, v/v) or acetonitrile/water (10/90, 5/95, v/v) were tested as solvent A candidates. Mixtures of methanol/water or acetonitrile/water were tested as solvent B candidates in ratios of 95/5 (v/v) or 90/10 (v/v). For 2D separations, methanol/water (90/10, v/v) or acetonitrile/water (90/10, v/v) were used as solvent C candidates. Ammonium formate was used as an additive in all LC solvents to obtain a final concentration of 5 mmol/L. If used, formic acid was added to the respective LC solvent at a final concentration of 26.5 mmol/L (= 0.1 vol%). Three different columns were tested, namely a 150 × 2.0 mm Synergi™ 4-μm Polar-RP C_{18}, a 150 × 2.1 mm Kinetex™ 5 μm EVO C_{18} column and, for additional 2D separation of highly polar compounds of the 44 PA, a 50 × 2.1 mm Kinetex™ 2.6 μm HILIC (all columns were from Phenomenex, Aschaffenburg, Germany). All LC columns were protected using suitable pre-columns (SecurityGuard™ Polar-RP 4 × 2.1 mm, SecurityGuard™ ULTRA EVO C_{18} 2.1 mm, SecurityGuard™ ULTRA HILIC 2.1 mm, all columns were from Phenomenex, Aschaffenburg, Germany). For 2D separation experiments, an additional Shimadzu LC pump LC-20AD (Duisburg, Germany) and a dynamic mixing chamber from Knauer (Berlin, Germany) were used to deliver a third solvent after C_{18} pre-separation. The oven temperature was kept at 30 °C during method development, and flow rates, ranging from 0.2 to 0.5 mL/min, were evaluated for suitability. The separation quality for neighbouring peaks of isomeric compounds was expressed as chromatographic resolution (Eq. 1).

Calculation of chromatographic resolution R_s of two peaks:

$$R_s = \frac{2(t_{R2} - t_{R1})}{w_{B1} + w_{B2}} \quad (1)$$

Where:

$t_{R1/2}$ retention time of peaks [min]
$w_{B1/2}$ baseline peak width of peaks [min]

Development of the sample extraction and clean-up After optimisation of PA/PANO separation, extraction and clean-up procedures were developed. Portions of blank peppermint tea (2.0 g) were artificially contaminated with the PA/PANO

mix solution to obtain a concentration of 20 μg/kg for each analyte, and the solvent was allowed to evaporate for 30 min. The samples were extracted and purified using 40 mL of various extraction solvents, SPE cartridges and SPE procedures.

The extraction solvents, aqueous sulphuric acid (0.05 mol/L), aqueous formic acid (0.53 mol/L), sulphuric acid (0.05 mol/L)/methanol (80/20, v/v) and formic acid (0.53 mol/L)/methanol (80/20, v/v), were partially used in previous studies [23, 27]. The extraction procedures under investigation were horizontal shaking (500 U/min, 30 min), sonication (20 min), homogenisation by an Ultra-Turrax™ disperser (1.0 min) (IKA, Staufen, Germany) and vortex shaking (0.5 min). To compare extraction efficiency, raw extracts of blank peppermint tea samples were prepared and spiked with the PA/PANO mix solution to a concentration of 1 ng/mL before SPE clean-up was conducted (i.e. the theoretical level of the analytes in the artificially contaminated sample extract after quantitative extraction was performed). The sample extracts were further purified using a non-optimised standard SPE protocol with strong cation exchange (SCX) Bond Elut SCX 6 mL 500 mg cartridges (Agilent, Waldbronn, Germany). SPE cartridges were activated, with 6 mL methanol and 6 mL of the respective extraction solvent, before they were loaded with 10 mL of the sample extract. The cartridges were washed with 12 mL of water and eluted with 5 mL of methanol and 5 mL of ammoniated methanol (2.5%). After evaporation under nitrogen at 50 °C, the residues were reconstituted with water/methanol (95/5, v/v). The area ratio of the peaks obtained for both samples (artificially contaminated peppermint tea/spiked raw extract of blank peppermint tea) expressed the extraction efficiency for the respective extraction solvents and procedures. Areas of PA/PANO measured in the spiked raw extract of blank peppermint tea were set as 100%.

For SPE purification of sample extracts in the final method, cartridges with SCX and reversed phase (RP) C_{18} material were tested, namely Bekolut SCX 6 mL 500 mg (Bekolut, Hauptstuhl, Germany), Discovery DSC-18 SPE 6 mL 500 mg (Supelco, Bellefonte, PA, USA), Chromabond SA 6 mL 500 mg (Macherey-Nagel, Dueren, Germany), Strata-X-C 6 mL 500 mg polymeric strong cation (Phenomenex, Aschaffenburg, Germany), Bond Elut SCX 6 mL 500 mg and Bond Elut Plexa PCX 6 mL 500 mg (both Agilent, Waldbronn, Germany). To identify the most suitable cartridge, blank peppermint tea spiked to a concentration of 80 μg/kg was extracted and purified with the SPE cartridges under investigation according to the manufacturer's instructions. The amounts of the respective PA/PANO compounds were compared after quantification, using an external matrix-matched standard calibration, as described in the next section.

Quantification of PA/PANO Quantification procedures were performed via external matrix-matched calibration. Aliquots of the PA/PANO mix standard solution were pipetted into glass vials, dried at 50 °C under nitrogen and reconstituted with extracts of a suitable blank tea or blank herbal tea sample, respectively, to prepare calibration standards at concentrations of 0, 1.0, 5.0, 10.0, 25.0 and 50.0 ng/mL. Calculation of the respective concentrations (linear regression) was performed using MultiQuant software and then back-calculated to the contents. In the presence of low endogenous contamination in the blank samples with certain PA/PANO compounds, the sample concentration was manually corrected. As such, the peak area in the blank sample was subtracted from the peak areas obtained for the spiked calibration standards. Matrix-matched calibration standards were freshly prepared for each day of sample analysis. If the content calculated for an individual compound was between the LOD and the LOQ, the amount indicated was 0.5 times that of the LOQ. Values below the LOD were considered as '0 μg/kg'. All results related to dry matter were not corrected via recovery rates.

Method validation The method was validated in-house by evaluating recovery rates, repeatability, trueness and both the LOD and limit of quantification (LOQ). Recovery experiments were performed using blank herbal tea and tea samples artificially contaminated at two concentrations (20 and 80 μg/kg of each PA/PANO compound) with five replicates each. The same data set was used to calculate the method's repeatability, which was expressed as the relative standard deviation (RSD) of the respective PA/PANO contents of the five replicates. Method performance parameters, such as linearity, LOD and LOQ, were determined following the calibration curve method for German standard DIN 32645 [28]. To this end, the 15 calibration standards were pipetted using the PA/PANO mix solution, dried under nitrogen at 50 °C and reconstituted with aliquots of blank sample extracts to final concentrations of 0.01, 0.03, 0.05, 0.07, 0.09, 0.1, 0.3, 0.5, 0.7, 0.9, 2.5, 5.0, 10.0, 25.0 and 50.0 ng/mL. After measurements were taken, a regression analysis of the calibration curve, with a 1% error probability and an uncertainty of 33.3%, was performed. The method's trueness was checked by analysing former inter-laboratory proficiency testing material (peppermint tea, chamomile tea, rooibos tea and balm tea) from an evaluation of the PA/PANO content in tea matrices provided by the German Federal Institute for Risk Assessment (BfR, Berlin) in 2015 [29]. Trueness was expressed as *z*-scores (Eq. 2) for the individual PA/PANO compounds considered in the proficiency testing report.

Calculation of participant's *z*-scores

$$z = \frac{(x - x_a)}{\sigma_p} \quad (2)$$

Where:

x participant's reported result
x_a appurtenant assigned value
σ_p standard deviation of proficiency

Quality control To ensure there was no analyte carry-over, a blank solvent was injected after the calibration curve standards and after every tenth run. The MS system's stability was tested by repeatedly injecting the 25ng/mL standard of the calibration curve after every eight to ten samples. The tolerated deviation of the calculated values from the theoretical concentration was set at ± 15%. PA/PANO compounds were confirmed present within a sample when they were eluted at the respective retention time (± 0.15 min) and with the correct area ratio (± 20%) of the two selected product ions.

Method applicability After the sample extraction procedure and the LC-MS/MS readings were developed and validated, the method's applicability was tested with a set of field samples. Eighteen samples were purchased from a local supermarket (three different samples for each validated matrix: peppermint tea, green tea, black tea, fennel tea, chamomile tea and rooibos tea) and were analysed in duplicate for the presence of the 44 PA/PANO compounds covered in the current method.

Results and discussion

Development of the LC-MS/MS method Solutions of each of the 44 PA/PANO compounds were directly injected to the MS via a syringe pump, and the MS parameters were optimised automatically for the ten fragment ions with the highest intensity. The behaviour of the transitions was further investigated under LC conditions, including matrix influences. Two transitions showing the best signal-to-noise (S/N) ratios were chosen as quantifier and qualifier transitions. Taking the concentration level of the PA/PANO mix solution used for optimisation into account, high signal intensities were achieved for all compounds. Only merenskine and its *N*-oxide had weak signal strength and poor S/N ratios for all MRM transitions; the weak signal intensities were assumed to be due to a chlorine atom within their chemical structure. There were no relevant negative ionisation signals associated with these analytes. Additionally, no optimal transitions could be identified for merenskine and its *N*-oxide. However, transitions for the other 42 compounds were intense and resulted in high S/N ratios. The optimised instrument settings for precursor and product ions of each compound are summarised in Table 1.

Due to similarities in their chemical structures, certain isomeric compound groups (namely, intermedine, indicine and lycopsamine, jacoline and sceleratine, acetylintermedine and acetyllycopsamine and lastly, senecivernine, integerrimine and senecionine) and their *N*-oxides were not distinguishable via MS. Many compounds also generated identical fragment ions, e.g. *m*/*z* 352/120 for jacobine, retrorsine, merepoxine and the *N*-oxides of integerrimine, senecivernints and senecionine. For successful chromatographic separation of these compounds, three different columns, as well as various solvents and gradients, were tested. The separation efficiency of neighbouring peaks was determined by evaluating chromatographic resolution (R_s, Eq. 1).

A Synergi™ C_{18} RP-polar column was initially applied, which had previously been used in separation methods covering up to 25 PA/PANO compounds [30–32]. This set only included two isomeric groups (intermedine/lycopsamine and their corresponding N-oxides). For the current application, adequate separation was not achievable with this system, neither via gradient optimisation nor through solvent variations (acetonitrile or methanol, with or without added formic acid). Adjacent peaks of critical isomeric groups were not separable to the baseline (= $R_s > 1.5$) and mostly co-eluted (= $R_s < 0.75$). Senecivernine, integerrimine and senecionine were completely co-eluted in every variation tested.

Alternatively, serial coupling between C_{18} and hydrophilic interaction liquid chromatography (HILIC) columns was used [33]. The Kinetex™ HILIC column was serially coupled after the Kinetex™ EVO C_{18} with a dynamic mixing chamber between the columns. Starting with a high concentration of the aqueous solvent A at the beginning led to retention of PA/PANO compounds on the RP C_{18} column. As water is the strong eluent in HILIC mode, mixtures with higher organic content (90%) were used as the solvent in a third pump (solvent C) to ensure additional retention of the PA/PANO on the HILIC column at the beginning of the gradient.

In contrast to the behaviour observed in RP chromatography, the *N*-oxides were generally eluted before their corresponding PA. Compared to the Synergi™ RP-polar C_{18} column, peak width and critical analyte separation were vastly improved. The isomers senecivernine, integerrimine and senecionine, as well as the corresponding *N*-oxides, were baseline separated. In contrast, co-elution was observed for acetylintermedine and acetyllycopsamine ($R_s = 0.8–1.1$) as well as for their *N*-oxides ($R_s < 0.75$). Additionally, isomers of intermedine, indicine and lycopsamine, including their *N*-oxides, co-eluted in all tested combinations of solvents and gradients. A major disadvantage of serial coupling was the long run time of more than 50 min. Although Van den Schans et al. [34] used a more complex type of multiple dimension liquid chromatography to separate the groups of isomers, special equipment, such as quaternary pumps and additional column selection valves, was needed, and run times of more than 60 min were typical. Since such an intensive effort would have made the current method impractical for daily routine analysis, it was decided to develop the LC-MS/MS method with a single Kinetex™ EVO C_{18} column since it

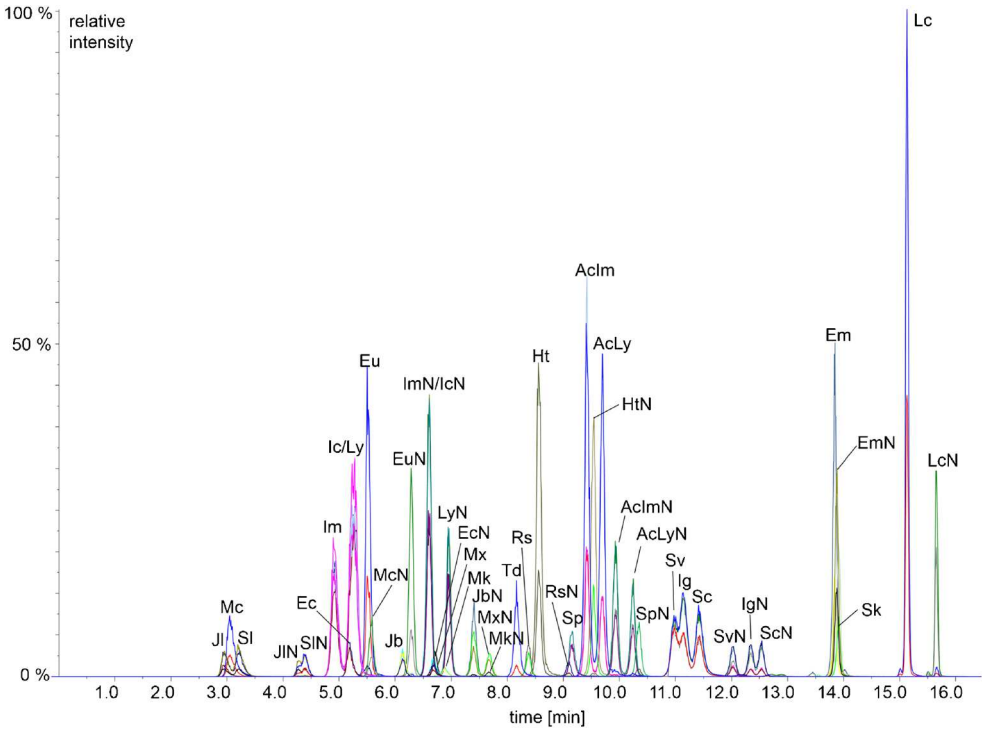

Fig. 2 LC-MS/MS chromatogram of a standard mix solution containing 44 pyrrolizidine alkaloids ($c = 25$ ng/mL), measured under optimised chromatographic and mass spectrometric conditions. For the abbreviations of the analytes, see Table 1

showed better resolution than the Synergi™ C_{18} RP-polar column and could be applied for the separation of some compounds that were poorly or not resolved via serial coupling.

Next, different types of solvents and gradients were tested. Fluctuations in the analytes' retention times were observed by exclusively using ammonium formate as a solvent additive, but this was remedied by using formic acid as an additive in both solvents. In general, better separation was obtained using acetonitrile, instead of methanol, as the organic component in solvent B. A flat gradient was used to achieve sufficient retention of the analytes on the column, starting with low concentrations of solvent B (≤ 5%). This resulted in better peak shapes and improved separation of the early eluting analytes. Resolution of the late-eluting PA/PANO compounds, however, did not improve. Thus, a steeper increase of the organic concentration was applied to the middle of the gradient to shorten the total run time. Finally, the best separation was achieved using water and acetonitrile/water (95/5, v/v) as solvents A and B, both containing formic acid and ammonium formate. This resulted in well-separated peaks within a run time of 16.5 min and an equilibration time of 6.5 min (Fig. 2).

Baseline separation was achieved for the isomers senecivernine-*N*-oxide and integerrimine-*N*-oxide ($R_s = 1.6$), and the latter could be effectively separated from senecionine-*N*-oxide ($R_s = 1.3$). Acetylintermedine and acetyllycopsamine were resolved ($R_s = 1.4$) as well as their *N*-oxides ($R_s = 1.4$). The isomers jacoline and sceleratine were well resolved ($R_s = 1.4$), whereas jacoline-*N*-oxide and sceleratine-*N*-oxide were sufficiently resolved ($R_s = 0.8$). Senecivernine and integerrimine were not completely separated ($R_s = 0.9$), but the latter could be baseline-separated to senecionine ($R_s = 1.3$). Only two isomer pairs, namely indicine and lycopsamine, as well as intermedine *N*-oxide and indicine-*N*-oxide, were co-eluted using the final method. In many published studies, the isomeric group consisting of intermedine, indicine, lycopsamine, rinderine and echinatine (also their *N*-oxides) were insufficiently separated and either reported as a combined group of lycopsamine isomers or were not included in the method at all [19, 35, 36]. Successful separation of these isomers was reported by Van den Schans et al. [34], but with intensive investment in instrumentation, laboratory resources and run time. In our study, however, we were able to achieve good separation for most of the PA/PANO compounds within a feasible run time using a single column and a flat gradient programme (Fig. 2).

Development of the sample extraction and clean-up

Artificially contaminated samples of blank peppermint tea were extracted using different solvents, extraction procedures and SPE cartridges to find the most suitable combination of high extraction efficiency and effective clean-up results. Figure 3 displays the average extraction efficiency for the 44 PA/PANO, obtained by analysing the spiked peppermint tea samples in duplicates, using four different solvents (sulphuric acid, formic acid, sulphuric acid/methanol and formic acid/methanol) and five extraction procedures (sonication + shaking, shaking, sonication, Ultra-Turrax and vortex shaking). The extraction efficiencies for the

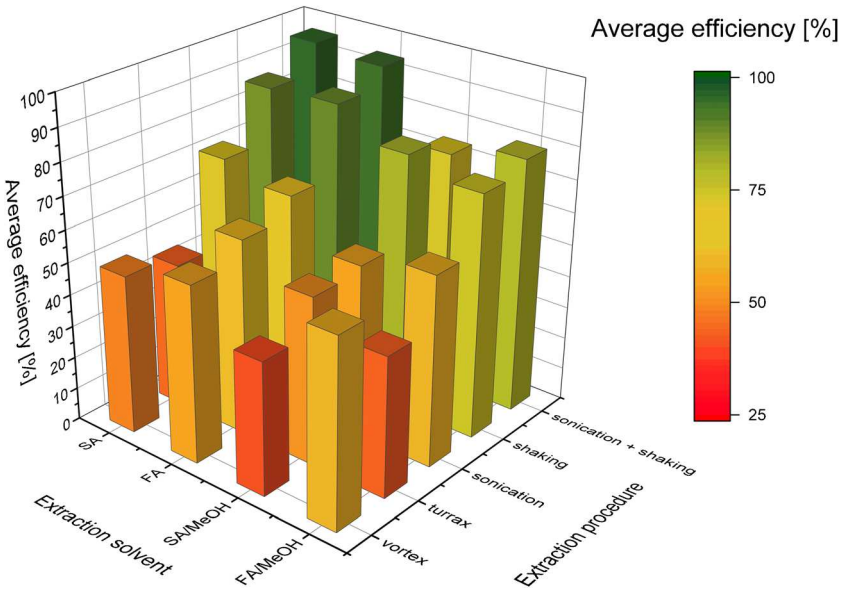

Fig. 3 Average extraction efficiencies for the 44 pyrrolizidine alkaloids and their *N*-oxides using different extraction solvents and procedures applied to artificially contaminated blank peppermint tea. SA sulphuric acid, FA formic acid, MeOH methanol

individual PA/PANO analytes were comparable across the various approaches.

In general, better results were obtained with either weak aqueous solutions of formic acid or sulphuric acid than with mixtures of the acids and methanol, which was commonly used in the past for pharmaceutical extracts [27]. Diluted sulphuric acid was previously used in several studies [20, 23, 24]. Joosten et al. compared weak formic acid and sulphuric acid to extract PA/PANO analytes. Even though similar results were obtained with both solvents, formic acid was more suitable for MS analysis [37]. As shown in Fig. 3, the various extraction procedures had a high impact on the respective extraction efficiency of the analytes. Sample treatment with a vortex laboratory shaker was quick and easy to handle but resulted in weak average efficiency rates between 41 and 58%. Ultra-Turrax homogenisation of the samples also resulted in low extraction efficiency rates (43 to 61%) and was subject to a time-consuming cleaning of the Turrax disperser after each sample. Ultra-sonication led to higher average extraction efficiencies (53 to 71%), but the results showed that there was poor precision, with standard deviations as high as 62% for individual analytes. The precisions noted for the other tested extraction procedures were lower than 25% for a single PA/PANO compound. This observation was independent of the type of extraction solvent used. Simple horizontal shaking treatment (500 U/min, 30 min) showed the best average efficiency rates of any single approach, with values between 75 and 88%. Average efficiency rates were then optimised using a combination of short vortex mixing for 10 s and sonication for 10 min prior to horizontal shaking for 20 min (74 to 96%). Although fast extraction is commonly the goal of any separation procedure, a minimum extraction time of 15 min was found to increase the efficiency of separation in our study when viewed in light of the shorter vortex or the Ultra-Turrax disperser treatments. Using formic acid and sulphuric acid resulted in average extraction efficiency rates of 96 or 94%, respectively. Due to the cleaner extracts acquired, sulphuric acid (0.05 mol/L) was chosen as the final extraction solvent for our method.

In total, the six different SPE cartridges, used to purify the raw extracts, were evaluated with regard to recovery rates and general handling during the extract purification procedure. Recovery of the SPE cartridges was tested with extracts of spiked blank peppermint tea, which were obtained using the newly developed extraction procedure (see above). Acidic extracts could be appropriately loaded directly onto the SCX cartridges or, after an additional neutralisation step had been performed, also onto the RP C_{18} cartridges. Both types of SPE cartridges are well established in the literature and commonly used for further purification of extracts and concentration of various PA/PANO analytes [23, 38].

The poorest recovery rates were seen using Bekolut SCX cartridges, with values ranging from 7% for sceleratine-*N*-oxide up to 71% for lasiocarpine. For all analytes, the average recovery was very low (25%), and only two analytes showed recovery rates within an acceptable range (60 and 120%) [39]. The RP cartridge Discovery DSC yielded an average recovery rate of 65%, ranging from 27 to 158%; for 24 of the analytes, this was within the range of 60 and 120%. However, the recovery rates achieved using the SCX cartridges were better than those obtained using the RP cartridges. Analyte recovery rates using Chromabond SA, Strata X and Bond Elut SCX were between 10 and 159%, and 26 or 27 analytes lay between 60 and 120%, with averages typically between 71 and 74%. Using Bond Elut PCX cartridges, the optimum range for recovery rates between 60 and 120% was achieved for 36

Table 1 Retention times and mass spectrometric conditions for determination of 44 pyrrolizidine alkaloids

Compound	Abbrev.	Retention time[1] [min]	Precursor ion [*m*/*z*]	Quantifier ion [*m*/*z*]	Qualifier ion [*m*/*z*]	DP [V]	CE [eV] (quant / qual)	CXP [V] (quant / qual)	Ion ratio[2] (qual / quant)
Jacoline	Jl	2.9	370.2	120.1	326.2	111	47 / 37	22 / 18	0.24
Monocrotaline	Mc	3.0	326.2	120.1	237.2	106	51 / 35	22 / 12	0.40
Sceleratine	Sl	3.2	370.2	138.1	342.2	116	43 / 39	24 / 18	0.20
Jacoline-*N*-oxide	JlN	4.3	386.3	120.1	172.1	111	59 / 51	22 / 30	0.38
Sceleratine-*N*-oxide	SlN	4.4	386.3	118.1	340.2	121	51 / 41	22 / 20	0.36
Intermedine	Im	4.9	300.1	138.0	94.0	91	29 / 39	24 / 16	1.09
Erucifoline	Ec	5.2	350.2	120.0	138.1	96	41 / 41	22 / 24	0.76
Indicine	Ic	5.3	300.2	138.0	156.1	91	29 / 41	24 / 28	0.80
Lycopsamine	Ly	5.3	300.2	94.0	138.1	101	39 / 29	16 / 24	0.85
Europine	Eu	5.5	330.2	138.1	254.1	86	33 / 27	24 / 14	0.40
Monocrotaline-*N*-oxide	McN	5.6	342.2	137.1	118.1	116	41 / 67	24 / 22	0.35
Jacobine	Jb	6.2	352.2	120.0	280.2	106	43 / 33	20 / 16	0.93
Europine-*N*-oxide	EuN	6.2	346.2	172.0	270.1	91	45 / 35	30 / 14	0.24
Indicine-*N*-oxide	IcN	6.6	316.2	172.1	94.1	101	39 / 61	30 / 16	0.58
Intermedine-*N*-oxide	ImN	6.6	316.2	172.0	94.1	96	39 / 59	32 / 16	0.63
Erucifoline-*N*-oxide	EcN	6.7	366.2	118.0	136.0	106	47 / 45	20 / 24	0.98
Merepoxine	Mx	6.8	352.1	120.1	324.2	141	45 / 39	22 / 32	0.45
Merenskine	Mk	6.9	388.2	120.0	138.0	121	51 / 45	22 / 24	0.81
Lycopsamine-*N*-oxide	LyN	6.9	316.2	172.1	138.1	86	39 / 39	30 / 24	0.69
Jacobine-*N*-oxide	JbN	7.4	368.2	120.1	296.2	101	53 / 35	22 / 16	0.68
Merepoxine-*N*-oxide	MxN	7.7	368.2	120.1	136.0	121	55 / 45	22 / 24	0.21
Merenskine-*N*-oxide	MkN	7.9	404.1	118.1	138.1	71	61 / 53	20 / 26	0.46
Trichodesmine	Td	8.1	354.2	222.2	308.2	86	41 / 30	12 / 15	0.13
Retrorsine	Rs	8.4	352.2	120.1	324.2	101	43 / 39	20 / 18	0.67
Heliotrine	Ht	8.5	314.2	138.0	156.0	76	29 / 39	24 / 28	0.32
Retrorsine-*N*-oxide	RsN	9.1	368.2	118.0	340.2	111	43 / 39	20 / 20	0.23
Seneciphylline	Sp	9.2	334.2	120.1	306.2	106	39 / 35	22 / 18	0.71
7-*O*-Acetylintermedine	AcIm	9.4	342.2	120.0	180.1	81	37 / 25	22 / 32	0.41
Heliotrine-*N*-oxide	HtN	9.5	330.2	172.2	111.0	96	39 / 59	30 / 20	0.34
7-*O*-Acetyllycopsamine	AcLy	9.6	342.2	120.0	180.1	81	35 / 25	22 / 32	0.29
7-*O*-Acetylintermedine-*N*-oxide	AcImN	9.9	358.2	214.1	180.1	91	39 / 41	38 / 32	0.49
7-*O*-Acetyllycopsamine-*N*-oxide	AcLyN	10.2	358.2	214.1	180.1	96	39 / 41	38 / 32	0.61
Seneciphylline-*N*-oxide	SpN	10.3	350.2	94.1	322.3	96	67 / 35	16 / 18	0.15
Senecivernine	Sv	10.9	336.2	120.1	308.2	96	43 / 39	22 / 18	0.69
Integerrimine	Ig	11.1	336.3	120.1	308.2	96	41 / 37	22 / 18	0.58
Senecionine	Sc	11.4	336.3	120.1	308.2	106	41 / 37	22 / 18	0.68
Senecivernine-*N*-oxide	SvN	12.0	352.2	118.1	324.3	96	45 / 37	22 / 18	0.74
Integerrimine-*N*-oxide	IgN	12.3	352.2	118.0	136.0	101	43 / 47	20 / 24	0.83
Senecionine-*N*-oxide	ScN	12.5	352.1	118.0	324.2	106	45 / 37	20 / 18	0.23
Senkirkine	Sk	13.8	366.3	168.2	150.1	96	43 / 39	8 / 26	0.45
Echimidine	Em	13.8	398.3	120.0	220.1	76	35 / 25	22 / 12	0.36
Echimidine-*N*-oxide	EmN	13.9	414.3	254.3	352.2	91	43 / 35	14 / 20	0.44
Lasiocarpine	Lc	15.1	412.3	120.0	220.2	86	39 / 27	22 / 12	0.53
Lasiocarpine-*N*-oxide	LcN	15.6	428.3	254.1	352.3	91	41 / 35	14 / 20	0.55

Entrance potential (EP) = 10 V for all analytes

Abbreviations: *DP* declustering potential, *CE* collision energy, *CXP* cell exit potential

[1] Determined using the final HPLC conditions

[2] Mean of three injections of a standard solution (c = 20 ng/mL)

analytes, with an average of 87% and a range from 52 to 152% considering all 44 analytes. Thus, these cartridges were applied in the final method.

Method validation Our final method was validated using blank samples of six common tea and herbal tea matrices. The samples were artificially contaminated with the individual analyte content of 20 and 80 μg/kg. LOD and LOQ were determined according the German standard DIN 32645. Results for the recovery rates and repeatability are displayed in Table 2, and the results for LOD and LOQ are shown in Table 3.

Overall, recovery rates between 60 and 120% were obtained in all validated matrices for at least 40 of the 44 analytes and thus fulfilled the requirements for trace analysis of the natural toxins occurring in food [39]. Merenskine had extremely high recovery rates in chamomile and rooibos teas, whereas its *N*-oxide had the highest recovery rate of all the

Table 2 Average recovery rates and repeatability (expressed as relative standard deviation) of the method for pyrrolizidine alkaloids in various tea and herbal tea matrices

Analyte[1]	Average recovery rate ± RSD [%] at 2 levels of fortification [μg/kg] (each $n = 5$ biological replicates)											
	Chamomile		Fennel		Green tea		Black tea		Rooibos		Peppermint	
	20	80	20	80	20	80	20	80	20	80	20	80
AcIm	104.6 ± 3.1	96.1 ± 4.2	121.5 ± 4.1	111.6 ± 2.0	99.7 ± 2.8	90.4 ± 2.5	112.0 ± 4.9	98.4 ± 2.7	78.1 ± 5.3	63.5 ± 13.9	81.7 ± 1.8	89.7 ± 2.9
AcImN	3.7 ± 16.2	3.8 ± 12.3	0.3 ± 15.6	0.2 ± 13.1	2.9 ± 14.4	2.4 ± 26.0	1.7 ± 4.2	2.0 ± 13.3	1.2 ± 15.4	0.9 ± 21.8	1.8 ± 9.4	1.8 ± 11.7
AcLy	86.6 ± 2.9	83.0 ± 6.1	116.9 ± 5.2	100.9 ± 1.6	87.3 ± 4.1	78.8 ± 4.5	106.3 ± 4.9	101.5 ± 3.1	78.1 ± 6.0	63.0 ± 14.8	78.6 ± 1.5	87.9 ± 2.0
AcLyN	4.8 ± 8.3	4.3 ± 18.5	0.3 ± 7.3	0.2 ± 12.4	3.0 ± 10.8	2.3 ± 23.0	1.6 ± 3.4	1.7 ± 13.2	1.6 ± 13.9	1.1 ± 21.8	2.0 ± 5.9	2.1 ± 12.2
Em	106.2 ± 2.0	97.8 ± 0.8	128.8 ± 4.4	114.2 ± 1.2	101.2 ± 2.0	94.7 ± 1.7	114.3 ± 2.7	100.0 ± 1.3	104.0 ± 2.1	83.1 ± 3.8	93.2 ± 2.0	96.4 ± 2.0
EmN	100.6 ± 3.2	93.8 ± 2.5	106.8 ± 4.3	89.1 ± 0.9	79.6 ± 3.7	74.0 ± 5.2	95.0 ± 6.2	81.0 ± 2.9	74.6 ± 5.7	63.6 ± 5.3	110.1 ± 4.9	101.5 ± 1.9
Ec	118.0 ± 3.5	90.2 ± 3.7	128.1 ± 4.1	112.0 ± 1.6	91.4 ± 5.9	86.1 ± 2.6	97.7 ± 10.7	103.3 ± 1.5	78.2 ± 3.1	69.7 ± 5.4	69.0 ± 2.4	90.5 ± 5.0
EcN	94.8 ± 5.1	87.1 ± 1.8	93.9 ± 4.1	84.5 ± 2.1	69.9 ± 6.0	68.9 ± 6.5	77.7 ± 6.6	72.8 ± 3.9	60.7 ± 8.5	55.3 ± 8.2	103.7 ± 3.5	98.1 ± 2.3
Eu	106.7 ± 3.9	102.4 ± 4.0	113.5 ± 5.2	96.0 ± 1.6	99.7 ± 2.2	97.5 ± 2.9	111.7 ± 6.1	102.9 ± 3.4	101.2 ± 2.6	89.7 ± 4.9	109.8 ± 2.9	105.9 ± 3.2
EuN	104.0 ± 9.4	97.1 ± 4.5	109.2 ± 3.9	87.7 ± 2.0	94.1 ± 3.5	89.9 ± 2.8	96.5 ± 5.4	90.9 ± 3.2	100.2 ± 2.9	92.4 ± 6.4	112.7 ± 3.2	108.1 ± 2.7
Ht	104.1 ± 3.9	99.1 ± 2.6	122.2 ± 4.4	104.8 ± 1.5	101.0 ± 4.5	92.4 ± 2.0	112.9 ± 4.1	103.8 ± 2.5	99.7 ± 1.4	88.6 ± 5.1	111.8 ± 3.5	99.5 ± 2.3
HtN	103.3 ± 1.7	96.5 ± 3.0	106.5 ± 3.1	89.5 ± 1.7	106.7 ± 2.3	90.8 ± 3.4	112.0 ± 7.1	90.4 ± 4.0	96.4 ± 1.7	87.6 ± 6.7	110.0 ± 3.5	105.0 ± 2.8
Ic	108.8 ± 3.3	102.1 ± 2.6	114.2 ± 4.5	105.5 ± 2.1	101.0 ± 2.1	97.1 ± 2.9	109.9 ± 9.1	97.9 ± 4.7	105.4 ± 4.5	97.8 ± 7.3	102.5 ± 2.5	105.7 ± 2.3
IcN	110.5 ± 2.3	104.1 ± 4.4	114.6 ± 4.1	101.0 ± 1.5	99.5 ± 4.9	83.3 ± 3.3	95.9 ± 3.9	83.9 ± 4.3	111.1 ± 3.3	114.1 ± 5.3	98.2 ± 5.2	103.0 ± 2.3
Ig	87.5 ± 2.4	87.1 ± 3.3	116.8 ± 3.5	113.3 ± 1.9	98.3 ± 3.5	94.3 ± 2.1	105.9 ± 6.1	102.8 ± 2.1	76.2 ± 4.3	69.8 ± 5.7	66.2 ± 1.2	82.9 ± 4.6
IgN	103.3 ± 4.3	93.4 ± 5.1	86.7 ± 7.0	78.6 ± 1.3	71.1 ± 4.0	71.6 ± 5.6	75.0 ± 5.7	68.3 ± 3.1	73.4 ± 6.6	63.4 ± 5.9	104.3 ± 2.8	98.5 ± 1.7
Im	106.4 ± 2.3	100.1 ± 1.3	124.8 ± 5.3	118.9 ± 2.4	103.2 ± 2.0	97.7 ± 2.0	115.6 ± 6.7	121.5 ± 2.6	115.9 ± 6.7	112.5 ± 12.7	102.3 ± 3.5	107.8 ± 1.4
ImN	105.9 ± 2.4	103.4 ± 2.1	103.8 ± 4.5	102.0 ± 1.4	102.2 ± 2.2	99.6 ± 3.1	71.7 ± 6.4	74.4 ± 14.0	102.0 ± 3.9	103.5 ± 4.4	103.7 ± 3.0	93.2 ± 3.5
Jb	94.6 ± 2.9	94.6 ± 3.0	117.2 ± 3.2	109.5 ± 2.7	115.2 ± 2.7	98.8 ± 2.8	115.8 ± 2.9	115.7 ± 1.9	78.7 ± 3.7	72.9 ± 8.0	83.0 ± 3.1	87.0 ± 5.4
JbN	90.3 ± 3.5	84.8 ± 2.8	80.9 ± 5.5	73.5 ± 3.2	80.2 ± 10.1	68.8 ± 8.1	75.5 ± 9.2	71.7 ± 4.8	50.8 ± 8.3	51.4 ± 9.6	104.9 ± 1.8	102.9 ± 1.3
Jl	99.2 ± 3.7	99.2 ± 1.3	123.5 ± 4.6	113.1 ± 1.9	101.8 ± 5.7	102.5 ± 2.0	115.0 ± 4.6	136.8 ± 3.2	99.6 ± 4.2	94.1 ± 5.8	64.5 ± 5.0	97.6 ± 6.5
JlN	105.7 ± 6.5	98.2 ± 3.1	88.4 ± 7.8	77.5 ± 2.9	71.7 ± 5.6	72.6 ± 7.8	89.3 ± 9.7	79.9 ± 6.0	69.8 ± 1.9	70.3 ± 8.1	97.3 ± 1.2	102.8 ± 2.2
Lc	107.6 ± 1.8	95.9 ± 1.7	125.0 ± 3.5	111.9 ± 1.0	89.3 ± 5.0	79.9 ± 2.5	99.9 ± 4.6	82.1 ± 2.3	94.9 ± 2.6	76.3 ± 4.4	103.6 ± 1.6	98.4 ± 1.0
LcN	101.9 ± 6.0	93.3 ± 4.7	97.9 ± 4.0	86.1 ± 2.5	84.7 ± 3.2	74.1 ± 4.1	85.9 ± 5.0	75.4 ± 1.4	78.7 ± 3.7	65.9 ± 6.1	101.3 ± 2.6	91.4 ± 1.2
Ly	109.9 ± 1.8	102.3 ± 2.9	103.9 ± 5.8	88.1 ± 2.4	105.1 ± 2.9	100.0 ± 2.0	105.8 ± 7.4	98.0 ± 3.6	105.6 ± 4.8	97.8 ± 7.9	101.8 ± 3.4	105.6 ± 2.7
LyN	106.7 ± 3.3	91.6 ± 3.3	108.5 ± 7.7	93.4 ± 3.8	107.7 ± 1.9	103.2 ± 3.2	104.1 ± 9.8	103.2 ± 3.6	91.9 ± 2.9	89.5 ± 4.7	99.4 ± 3.9	97.9 ± 2.4
Mk	162.0 ± 2.6	162.5 ± 4.8	121.3 ± 8.6	108.3 ± 3.4	123.1 ± 12.8	115.5 ± 7.0	116.1 ± 7.4	120.8 ± 5.1	170.0 ± 12.1	131.2 ± 11.4	92.6 ± 14.5	109.2 ± 4.9
MkN	100.6 ± 4.9	159.2 ± 13.2	98.9 ± 9.1	85.9 ± 4.2	44.0 ± 15.0	43.0 ± 15.6	109.7 ± 4.7	101.2 ± 6.2	265.4 ± 7.7	136.0 ± 8.4	140.4 ± 2.4	158.3 ± 2.3
Mx	72.1 ± 5.6	53.4 ± 5.5	97.5 ± 8.1	99.6 ± 1.6	98.1 ± 2.8	90.0 ± 2.4	111.7 ± 5.3	97.2 ± 3.2	76.5 ± 3.0	72.6 ± 6.6	54.7 ± 0.7	73.9 ± 4.0
MxN	76.6 ± 16.1	55.4 ± 4.3	81.5 ± 2.9	64.9 ± 2.1	62.2 ± 5.0	66.0 ± 7.3	67.9 ± 12.8	63.5 ± 5.8	54.8 ± 12.6	52.4 ± 11.2	87.5 ± 5.5	79.8 ± 3.2
Mc	91.7 ± 5.2	95.0 ± 3.3	118.7 ± 3.9	110.3 ± 2.3	97.8 ± 4.6	97.0 ± 2.5	128.2 ± 13.2	121.6 ± 4.3	86.7 ± 2.9	83.5 ± 4.5	76.2 ± 6.9	98.1 ± 4.9
McN	94.7 ± 2.7	90.5 ± 2.7	98.9 ± 4.3	87.9 ± 1.3	82.8 ± 4.7	82.5 ± 2.5	87.7 ± 7.5	80.9 ± 4.4	77.1 ± 6.6	67.9 ± 10.8	106.3 ± 6.4	101.1 ± 1.9
Rs	92.4 ± 5.3	90.7 ± 7.0	112.0 ± 6.3	105.5 ± 1.4	90.2 ± 4.3	82.7 ± 1.2	97.0 ± 6.2	95.9 ± 1.0	87.1 ± 2.7	78.2 ± 6.1	84.3 ± 6.6	88.9 ± 3.4
RsN	91.2 ± 4.1	92.4 ± 3.2	95.0 ± 4.0	83.2 ± 0.9	67.5 ± 2.2	67.7 ± 6.3	80.6 ± 5.3	76.7 ± 2.7	85.0 ± 7.7	68.9 ± 9.4	111.4 ± 4.1	100.4 ± 1.6
Sl	106.6 ± 9.2	99.3 ± 2.0	116.8 ± 3.5	106.2 ± 2.0	102.8 ± 3.8	98.8 ± 1.9	137.7 ± 4.4	137.2 ± 3.7	98.1 ± 5.2	92.7 ± 5.2	74.1 ± 2.0	98.1 ± 4.0
SlN	101.8 ± 4.7	101.3 ± 3.4	95.1 ± 9.2	83.6 ± 5.0	69.2 ± 4.1	69.9 ± 6.5	78.0 ± 4.0	76.9 ± 2.7	71.3 ± 9.3	65.9 ± 10.7	103.5 ± 1.0	104.9 ± 3.4
Sc	85.2 ± 4.2	90.5 ± 3.4	91.2 ± 5.1	84.7 ± 1.9	99.2 ± 4.4	94.9 ± 2.7	119.5 ± 5.0	110.4 ± 2.7	88.6 ± 4.1	74.2 ± 5.7	60.2 ± 4.0	85.9 ± 4.7
ScN	95.2 ± 4.8	88.9 ± 2.7	99.0 ± 4.1	88.6 ± 3.1	71.9 ± 2.1	71.5 ± 4.2	75.2 ± 5.0	68.7 ± 3.1	74.3 ± 8.7	63.1 ± 10.9	103.1 ± 3.6	98.0 ± 1.9
Sp	93.2 ± 5.1	86.7 ± 4.8	120.7 ± 4.5	112.4 ± 0.8	95.1 ± 1.9	86.8 ± 3.2	113.8 ± 6.3	107.9 ± 2.9	63.9 ± 12.9	64.2 ± 8.8	58.2 ± 5.1	82.1 ± 6.3
SpN	75.2 ± 4.9	65.4 ± 3.6	49.5 ± 6.2	43.5 ± 2.7	51.7 ± 2.3	51.7 ± 7.1	54.5 ± 6.7	50.3 ± 3.0	31.7 ± 12.1	29.4 ± 4.9	59.1 ± 1.4	57.3 ± 2.3
Sv	89.6 ± 6.2	84.6 ± 3.0	113.3 ± 7.2	96.7 ± 4.4	93.8 ± 2.7	91.6 ± 3.1	105.4 ± 4.7	103.5 ± 4.0	76.9 ± 3.1	69.7 ± 5.6	56.3 ± 1.9	84.5 ± 4.1
SvN	101.6 ± 6.7	94.9 ± 4.6	98.6 ± 3.4	90.4 ± 1.1	71.4 ± 5.1	69.2 ± 5.0	77.9 ± 6.9	71.0 ± 3.8	66.7 ± 5.7	60.9 ± 5.9	102.6 ± 3.4	95.8 ± 0.8
Sk	108.2 ± 1.1	98.1 ± 2.9	103.3 ± 4.8	89.0 ± 1.4	94.2 ± 2.9	86.6 ± 2.3	98.7 ± 5.0	84.2 ± 1.6	94.0 ± 2.0	79.6 ± 5.2	113.8 ± 3.1	101.3 ± 1.4

Table 2 (continued)

Analyte[1]	Average recovery rate ± RSD [%] at 2 levels of fortification [µg/kg] (each $n = 5$ biological replicates)											
	Chamomile		Fennel		Green tea		Black tea		Rooibos		Peppermint	
	20	80	20	80	20	80	20	80	20	80	20	80
Td	93.5 ± 5.7	92.3 ± 2.7	120.6 ± 4.9	113.4 ± 2.0	93.5 ± 3.0	89.2 ± 1.3	105.5 ± 9.3	102.7 ± 3.6	86.3 ± 2.2	80.9 ± 5.0	108.5 ± 10.0	98.4 ± 5.6

[1] For the abbreviations of the analytes, see Table 1

investigated PA/PANO in rooibos tea (up to 265%) and the lowest rate (below 45%) in green tea. However, merenskine and merenskine-*N*-oxide naturally occur in some *Senecio* species and may contribute to contamination of the food and feed supplies [40]. Thus, they were kept in the current method, but they should be additionally quantified using standard addition when qualitatively determined in a sample via this method.

The most conspicuous results were achieved for the *N*-oxides of acetylintermedine and acetyllycopsamine, which had extremely low, yet precise, recovery rates below 5% (Table 2). Further investigations with standard solutions and without a matrix showed that deacetylation transformed these compounds into the respective corresponding PANO intermedine-*N*-oxide and lycopsamine-*N*-oxide during the sample extraction and SPE clean-up procedures. In contrast, acetylintermedine and acetyllycopsamine did not degrade to intermedine and lycopsamine (data not shown). The reason for this phenomenon remains unclear and warrants further investigation. Given this, the recovery rates for the *N*-oxides of acetylintermedine and acetyllycopsamine were not taken into further consideration when evaluating the SPE cartridges' effectiveness. Considering the weak validation results for these two compounds, a reliable quantitation seems difficult. However, both compounds showed very low LODs (Table 3), resulting in detectable effective contents ranging from 3 µg/kg (green tea) to 10 µg/kg (rooibos tea) up to 67 µg/kg in fennel. Such amounts may easily occur in teas contaminated with lycopsamine-type PA/PANO, so acetylintermedine-*N*-oxide and acetyllycopsamine-*N*-oxide were kept as part of the analyte set. Consequently, these compounds may be determined only qualitatively using the final method.

The repeatability rates achieved for all analytes, expressed as RSD, were very satisfactory; values below 10% were obtained for most of the PA/PANO analytes under investigation. Only merenskine and merepoxine-*N*-oxide had RSD of up to 16.1% in more than one matrix. The low precision rates seen for the *N*-oxides of acetylintermedine and acetyllycopsamine were the result of their small total amounts after (undefined) chemical modification or degradation during sample pre-treatment. The good method precision would allow for corrections of results via the respective recovery rate, e.g. for analytes with lower recovery.

Thirty-one analytes had very low LODs < 1 µg/kg, depending on the respective tea or herbal tea matrix. With 7.0 µg/kg, the highest obtained LOD was for merenskine in the chamomile matrix; merenskine and its corresponding *N*-oxide had higher LODs in other matrices (1.2 to 6.8 µg/kg). As already mentioned, these PA/PANO analytes had weaker signal intensities for all MRM transitions, presumably due to their chlorine atom. However, their LODs were still acceptable. All other analytes within the framework of the method showed very good (< 0.1 µg/kg) to satisfactory LODs, which were comparable or better than those presented in other

Table 3 Limits of detection (LOD) and quantification (LOQ) of pyrrolizidine alkaloids in various tea and herbal tea matrices

Analyte[1]	Chamomile		Fennel		Green tea		Black tea		Rooibos		Peppermint	
	LOD [μg/kg]	LOQ [μg/kg]	LOD [μg/kg]	LOQ [μg/kg]	LOD [μg/kg]	LOQ [μg/kg]	LOD [μg/kg]	LOQ [μg/kg]	LOD [μg/kg]	LOQ [μg/kg]	LOD [μg/kg]	LOQ [μg/kg]
AcIm	0.7	2.9	0.1	0.6	0.1	0.2	0.1	0.4	0.1	0.2	0.2	0.8
AcImN	0.2	0.7	0.1	0.4	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1
AcLy	0.3	1.2	0.2	0.8	0.1	0.1	0.1	0.3	0.1	0.1	0.2	0.6
AcLyN	0.3	1.1	0.2	0.5	0.1	0.1	0.1	0.3	0.1	0.1	0.1	0.2
Em	0.1	0.4	0.1	0.4	0.1	0.1	0.1	0.3	0.1	0.1	0.1	0.4
EmN	0.3	0.9	0.2	0.5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2
Ec	2.0	7.1	0.1	0.2	0.1	0.3	0.1	0.4	0.1	0.2	0.1	0.5
EcN	1.4	4.9	0.1	0.1	0.1	0.3	0.1	0.3	0.1	0.1	0.1	0.2
Eu	1.4	4.3	0.5	1.5	0.2	0.6	0.9	2.8	0.2	0.7	2.2	9.3
EuN	0.7	2.4	0.4	1.4	0.1	0.2	0.5	1.6	0.1	0.3	0.9	4.4
Ht	0.9	3.7	0.1	0.1	0.1	0.2	0.1	0.5	0.1	0.1	0.2	0.7
HtN	0.2	0.6	0.1	0.2	0.1	0.2	0.1	0.3	0.1	0.1	0.1	0.3
Ic	0.2	0.6	0.8	3.4	0.1	0.4	1.0	3.0	0.1	0.2	0.4	1.2
IcN	0.1	0.3	0.1	0.4	0.1	0.1	0.5	1.6	0.1	0.2	0.1	0.3
Ig	0.5	1.6	0.2	0.9	0.1	0.2	0.4	1.3	0.1	0.3	0.9	3.2
IgN	0.3	1.2	0.1	0.4	0.2	0.5	0.2	0.6	0.2	0.5	0.2	0.9
Im	0.3	1.1	0.2	0.9	0.1	0.2	0.2	0.7	0.1	0.3	0.9	2.7
ImN	0.1	0.2	0.1	0.3	0.1	0.2	0.4	1.3	0.1	0.1	0.1	0.4
Jb	1.0	3.1	2.2	6.6	1.4	4.9	1.8	5.5	0.1	0.5	1.3	4.4
JbN	0.7	2.3	0.2	0.8	0.3	1.0	0.1	0.5	0.2	0.5	0.1	0.4
Jl	2.5	7.6	1.5	4.5	0.2	0.8	5.0	15.0	0.7	2.5	1.5	5.4
JlN	0.4	1.3	0.7	2.6	0.4	1.5	0.7	2.5	0.7	2.3	0.7	2.5
Lc	0.2	0.5	0.2	0.6	0.1	0.1	0.1	0.3	0.1	0.1	0.1	0.3
LcN	0.3	0.9	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2
Ly	0.3	1.2	1.0	5.2	0.1	0.2	0.4	1.2	0.1	0.3	0.9	2.8
LyN	0.1	0.2	1.5	5.2	0.1	0.2	0.4	1.3	0.1	0.2	0.1	0.1
Mk	7.0	25.4	2.6	9.3	5.5	19.2	2.2	6.7	1.2	4.0	3.0	11.4
MkN	3.0	16.1	1.8	6.6	1.8	9.2	4.4	16.8	6.8	27.9	3.2	10.1
Mx	2.2	8.1	1.4	5.9	0.5	1.8	1.8	7.2	0.2	0.5	0.2	0.7
MxN	1.0	3.6	0.2	0.6	0.7	2.3	0.5	1.6	0.7	2.3	0.5	1.9
Mc	1.8	5.4	1.9	6.1	0.4	1.4	1.3	5.2	0.2	0.7	0.5	1.6
McN	0.8	2.6	0.1	0.5	0.9	3.0	0.1	0.3	0.1	0.4	0.1	0.4
Rs	1.1	3.4	2.2	6.8	0.4	1.4	1.3	5.5	0.1	0.4	0.7	2.5
RsN	0.7	2.4	0.8	2.8	0.5	1.7	0.2	0.7	0.2	0.7	0.2	0.6
Sl	2.5	7.5	2.4	7.2	1.1	3.9	3.0	9.4	0.6	2.3	0.8	3.6
SlN	1.9	6.7	1.0	3.3	0.6	2.2	0.6	2.2	0.4	1.3	0.3	1.1
Sc	1.1	3.8	0.6	2.2	0.2	0.6	0.7	2.7	0.2	0.7	0.6	2.0
ScN	0.2	0.6	0.2	0.7	0.1	0.4	0.1	0.4	0.1	0.3	0.2	0.7
Sp	0.5	2.0	2.1	6.7	0.5	1.6	0.8	2.9	0.5	1.6	0.5	1.8
SpN	0.2	0.8	0.2	0.6	0.2	0.6	0.1	0.3	0.2	0.6	0.1	0.4
Sv	1.2	4.6	0.5	1.8	0.6	2.2	0.3	1.3	0.2	0.8	0.6	2.0
SvN	0.6	2.1	0.2	0.7	0.2	0.8	0.2	0.6	0.2	0.5	0.1	0.3
Sk	0.1	0.2	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Td	0.9	3.1	2.3	8.1	0.2	0.8	0.7	2.5	0.3	0.9	0.5	2.1

[1] For the abbreviations of the analytes, see Table 1

methods in the literature [19, 23, 41]. Only studies using special and relatively uncommon equipment in their analytical methods, like MS3, reported LODs that were up to ten times smaller [24].

For each compound, calibration curve linearity was proven within the range from the LOD to 50.0 ng/mL (Mandels *F*-test). Correlation coefficients R^2 were > 0.99 for all analytes. A comparison of the slope of the calibration curves, in pure solvent and in the sample matrix, showed matrix suppression effects of about 40 to 60%; for jacoline in chamomile, this value was as high as 84% (data not shown). These results highlighted the need for matrix-matched calibration.

A major problem of this approach is the reliance on a blank, PA/PANO-free material, which has to be as similar to the investigated sample as possible. However, such materials are hardly available in some matrices, especially those for tea [42]. Alternatively, a standard addition approach before sample extraction could be used for quantification [43]. This would lead to results that have been corrected for recovery, but also to a greater consumption of costly standards. Isotopically labelled internal standards can cover the individual losses sustained during sample treatment and extraction procedures, but generally, these are quite expensive. According to one research group, 13C standards for PA/PANO analytes are only commercially available for senecionine, lycopsamine and their *N*-oxides [24]; thus, they are not applicable for multi-toxin determination. For these reasons, we decided to incorporate matrix-matched external calibration in our study.

The trueness of the method was determined by analysing four different herbal tea matrices from a former inter-laboratory proficiency test and then calculating the *z*-scores obtained for the PA/PANO compounds [29]. *Z*-scores of the analysed PA were between the targeted scores of − 2 and + 2 for every analyte, except for seneciphylline-*N*-oxide (Table 4), which had *z*-scores between − 2.9 and − 2.6 in four samples. Taking recovery rates into account, seneciphylline-*N*-oxide would have met the necessary requirements. For retrorsine and its *N*-oxide, their *z*-scores increased up to 2.2 and 2.6 in the rooibos tea sample. For nearly all analytes, passing the required *z*-score benchmark was confirmation of the final method's trueness.

In conclusion, the final method was successfully validated. Problems with stability or the signal intensity of individual analytes could not be fully resolved, namely those associated with the *N*-oxides of acetylintermedine and acetyllycopsamine, as well as merenskine and merenskine-*N*-oxide. Nevertheless, the 44 PA/PANO analytes were detectable with a high level of sensitivity using the method developed in our study. To mitigate potential underestimation, a set of 44 PA/PANO

Table 4 *z*-scores of pyrrolizidine alkaloids in herbal teas without and with recovery correction. Samples were from a proficiency testing provided by the German Federal Institute for Risk Assessment in 2015 and analysed ($n = 2$) with the developed method

Analyte[1]	Peppermint		Melissa		Chamomile		Rooibos	
	z-score	*z*-score (corrected)	*z*-score	*z*-score (corrected)[2]	*z*-score	*z*-score (corrected)	*z*-score	*z*-score (corrected)
Em	− 0.7	− 0.6	–	–	− 1.4	− 1.4	–	–
EmN	–	–	–	–	− 1.6	− 1.4	–	–
Eu	–	–	− 1.1	− 1.3	–	–	–	–
EuN	–	–	− 1.4	− 1.7	–	–	–	–
Ht	− 0.1	− 0.1	− 0.1	− 0.1	–	–	–	–
HtN	− 1.5	− 1.6	− 1.1	− 1.3	–	–	–	–
Im/Ic/Ly[3]	− 1.0	− 1.0	− 0.9	− 0.9	− 0.1	− 0.1	–	–
ImN/IcN/LyN[3]	+ 0.3	+ 0.2	+ 0.6	+ 0.6	+ 0.1	0.0	–	–
Lc	–	–	+ 0.4	+ 0.2	–	–	–	–
LcN	–	–	− 1.2	− 0.9	–	–	–	–
Rs	− 1.4	− 1.3	− 1.0	− 0.7	− 1.3	− 1.1	+ 1.4	+ 2.2
RsN	− 1.5	− 1.5	− 1.1	− 1.1	− 0.9	− 0.6	+ 1.5	+ 2.6
Sc/Ig/Sv[3]	− 0.9	− 0.5	+ 0.3	+ 1.0	− 0.7	− 0.2	+ 1.0	+ 1.5
ScN/IgN/SvN[3]	− 0.5	− 0.4	+ 0.7	+ 0.7	− 1.2	− 0.9	− 0.5	+ 0.5
Sp	− 1.7	− 0.5	− 1.7	− 0.1	− 1.0	− 0.8	− 0.6	+ 0.9
SpN	− 2.5	− 2.0	− 2.9	− 1.9	− 2.6	− 2.0	− 2.7	0.0
Sk	− 0.3	− 0.3	–	–	–	–	+ 0.6	+ 0.9

[1] Only analytes addressed in the report of the proficiency test are listed. For the abbreviations of the analytes, see Table 1

[2] Corrected for recoveries of peppermint

[3] Equally to the report isomers are reported in sum

compounds was chosen to cover as many analytes as possible which may be present in the samples. The set encompassed all certified reference standards commercially available at the beginning of our study. Although reference standards were generally expensive, they were shown to be stable in solution for at least 6 months and, thus, could be used within this period [31].

Method applicability To check the applicability of our final method, a set of 18 tea samples covering all validated matrices was purchased from a local supermarket and analysed in duplicate for the presence of PA/PANO analytes. Results showed that 17 of the 18 samples contained at least one PA/PANO compound (Table 5). The analytes identified in positive samples varied over all known groups of PA/PANO types (Fig. 4). Sum contents in the investigated samples ranged from 0.3 up to 48.3 μg/kg, which was quite low compared to former reports on teas purchased in the European Union. So, in 2015, Mulder et al. reported means of PA/PANO sum contents of 272 μg/kg in chamomile tea ($n = 35$), 437 μg/kg in peppermint tea ($n = 30$), 571 μg/kg in black tea ($n = 33$) and 597 μg/kg in rooibos tea ($n = 22$) [42]. Schulz et al. also investigated a set of 169 medicinal herbal tea samples purchased in Germany on the presence of PA/PANO [44]. The group reported relatively low maximum sum contents in single-ingredient herbal teas, ranging from 21 μg/kg in peppermint to 66 μg/kg in nettle tea. Contrary to that, extremely high maximum amounts were found in melissa tea (1,596 μg/kg) and especially in tea mixtures (up to 5,668 μg/kg).

Interestingly, in our investigated set of herbal teas, the six organically cultivated samples contained comparably low amounts of PA/PANO analytes, and the only sample in which no analyte was detected was the organic peppermint tea. Nevertheless, due to the small number of samples, the results of our small survey could not be regarded as representative for the risk evaluation assessment of the tea samples.

The method itself was applicable to a broad variety of different tea samples, including teas from seeds (fennel), flowers (chamomile), raw (green tea and peppermint) and fermented plant material (black tea and rooibos). Thus, possibilities for using the developed analytical method in other plant-derived products or feed seemed quite obvious. As fennel is also a seed spice, the method's applicability for culinary herbs and spices is possible. Consequently, animal feed of dried (hay) or fermented origin (silage) will be part of further studies regarding the applicability of this method.

Summarised final sample preparation and LC-MS/MS conditions A sample (2.0 ± 0.01 g) was weighed into a 50 mL centrifuge tube, and 25.0 mL of aqueous sulphuric acid (0.05 mol/L) was added. Samples were vortexed (15 s), treated in an ultra-sonic bath (10 min) and then shaken at room temperature in a horizontal shaker (500 rpm, 20 min). After centrifugation (5000×*g*, 10 min, 20 °C), samples were filtered and 10.0 mL of extract was loaded onto SPE Bond Elut Plexa PCX 500 mg cartridges (Agilent, Waldbronn, Germany) preconditioned with 5 mL of methanol and 5 mL of aqueous sulphuric acid (0.05 mol/L). The loaded cartridges were washed with 5 mL of water and 5 mL of methanol; then the analytes were eluted into a glass vial using 10 mL of ammoniated methanol (5%). The eluates were dried at 50 °C under a smooth stream of nitrogen. The residue was reconstituted in 1.0 mL of solvent A (water containing 0.1% formic acid and 5 mmol/L ammonium formate), shaken with a vortex laboratory shaker and filtered into a glass vial using a 0.45 μm PVDF syringe filter (Berrytec, Grünwald, Germany).

For chromatographic separations of PA/PANO analytes, a 150 × 2.1 mm Kinetex™ 5 μm CoreShell EVO C_{18} 100 Å column protected by a SecurityGuard™ ULTRA EVO C_{18} 2.1x mm guard column (both Phenomenex, Aschaffenburg, Germany) was used. The column oven temperature was maintained at 30 °C, and the solvents used were water (A) and acetonitrile/water (95/5, v/v, B), each containing 0.1% formic acid and 5 mmol/L ammonium formate. The binary linear gradient conditions were as follows: 0.0 min 2% B, 10.0 min 9.5% B, 15.0 min 39% B, 15.1 min 100% B and 16.5 min 100% B. The column was re-equilibrated for 6.5 min prior to each run. The flow rate was 0.4 mL/min and the injection volume of the samples was set to 20 μL.

Table 5 Occurrence of pyrrolizidine alkaloids in retail tea and herbal tea samples ($n = 18$). Samples originated from three brands, each producing conventionally (con) or organically (org)

Brand	PA/PANO sum contents [μg/kg] (mean ± standard deviation of 3 biological replicates)					
	Matrix					
	Chamomile	Fennel	Green tea	Black tea	Rooibos	Peppermint
No. 1 (con)	5.9 ± 1.7	26.2 ± 1.9	9.4 ± 0.1	17.2 ± 1.0	48.3 ± 3.6	40.4 ± 2.3
No. 2 (con)	13.2 ± 0.2	0.3 ± 0.0	11.1 ± 1.0	6.8 ± 1.4	42.1 ± 7.6	5.1 ± 3.0
No. 3 (org)	4.1 ± 1.0	3.2 ± 0.4	0.7 ± 0.4	33.9 ± 22.4	12.7 ± 0.2	< LOD

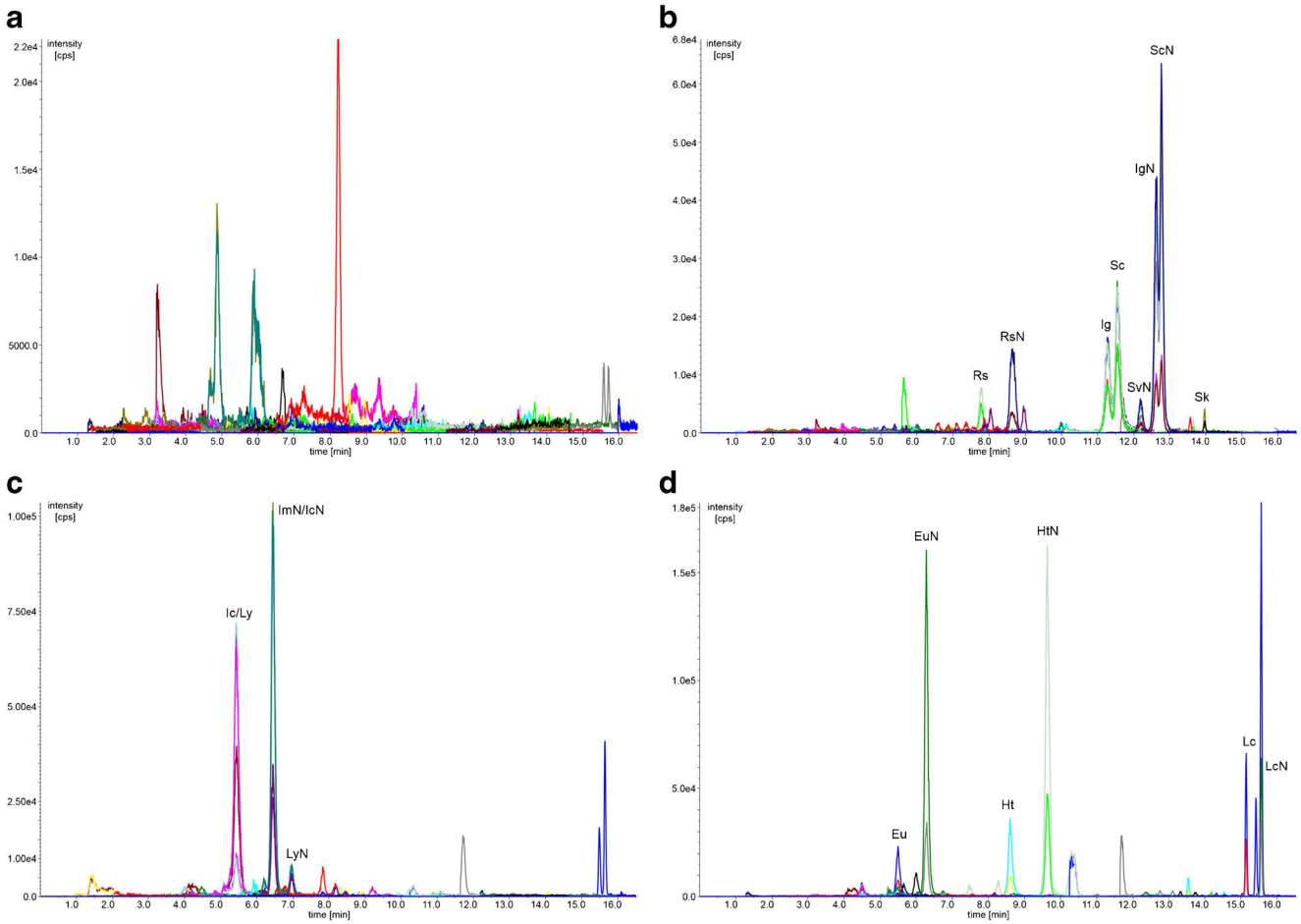

Fig. 4 LC-MS/MS chromatograms (**A**) of a peppermint tea sample free from pyrrolizidine alkaloids, (**B**) of a rooibos tea sample containing senecionine-type pyrrolizidine alkaloids, (**C**) of a black tea sample containing lycopsamine-type pyrrolizidine alkaloids and (**D**) of a fennel tea sample containing heliotrine-type pyrrolizidine alkaloids. Each chromatogram is normalised to its largest peak; thus, the *y*-axes differ from **A** to **D**. For the abbreviations of the analytes, see Table 1

Conclusion

A sensitive analytical method for determining the profile of 44 commercially available PA/PANO analytes was developed and validated for important tea and herbal tea matrices, namely peppermint tea, green tea, black tea, fennel tea, chamomile tea and rooibos tea. Separation of the analytes allowed for clear differentiation of most isomeric analytes, with a total run time of 23 min. Data presented on extraction efficiency of various procedures, as well as the tests undertaken with different types of SPE cartridges, gave valuable information for future applications and method development processes. Strong matrix effects, which depended on the respective type of tea sample under investigation, highlighted the need for matrix-matched calibration to achieve accurate quantification.

The problems arising from the analysis of acetylated PANO seemed to be related to their extensive deacetylation during preparation procedures. Nevertheless, the method's validation had good results for precision, sample recovery rates and LODs. Analysis of former proficiency testing materials showed that the method delivered accurate results and generally performed well. Applicability was confirmed by analysing tea samples from a local supermarket. Here 17 of the 18 samples investigated contained at least one PA/PANO analyte. Successful validation and application of the newly developed method over a broad range of different tea matrices suggest possible uses for other matrices prone to PA/PANO contamination, such as herbs, spices and plant-based animal feed.

Covering the high number of 44 analytes, the developed method was shown to be capable of determining the sum contents of PA/PANO in a contaminated sample to be more reliable, compared to targeted methods covering less analytes. Thus, our method can be used to effectively minimise health risks by reliably and accurately assessing the consumers' exposure to PA/PANO compounds in the global food and feed supply chain. Furthermore, reliable analysis of the 44 PA/PANO analytes allowed accurate determination of the PA/PANO composition of a contaminated sample, thus enabling us to establish the botanical origin of the contaminating plant

species, genus or family more precisely. This information is very valuable when it comes to identifying the region of cultivation for a particular herbal sample. Atypical PA/PANO compositions in samples of (herbal) tea, culinary herbs or spices, thus, may even indicate possible food fraud.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies involving human participants or animals performed by any of the authors.

References

1. Hartmann T, Witte L. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. In: Pelletier SW, editor. Alkaloids: chemical and biological perspectives: Elsevier; 1995. p. 155–233.
2. Smith LW, Culvenor CCJ. Plant sources of hepatotoxic pyrrolizidine alkaloids. J Nat Prod. 1981;44:129–52.
3. Mattocks AR. Chemistry and toxicology of pyrrolizidine alkaloids: Academic; 1986.
4. Boppré M. The ecological context of pyrrolizidine alkaloids in food, feed and forage: an overview. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2011;28:260–81.
5. Fu PP, Xia Q, Lin G, Chou MW. Pyrrolizidine alkaloids—genotoxicity, metabolism enzymes, metabolic activation, and mechanisms. Drug Metab Rev. 2004;36:1–55.
6. Roder E. Pyrrolizidine alkaloid containing medicinal plants. Dtsch Apoth Ztg. 1992;132:2427–35.
7. Merz KH, Schrenk D. Interim relative potency factors for the toxicological risk assessment of pyrrolizidine alkaloids in food and herbal medicines. Toxicol Lett. 2016;263:44–57.
8. Colegate SM, Stegelmeier BL, Edgar JA. Dietary exposure of livestock and humans to hepatotoxic natural products. Animal Feed Contamination: Effects on Livestock and Food Safety 2012:352–382.
9. Chauvin P, Dillon JC, Moren A. An outbreak of Heliotrope food poisoning, Tadjikistan, November 1992-March 1993. Sante. 1994;4:263–8.
10. Anjos BL, Nobre VMT, Dantas AFM, Medeiros RMT, Oliveira Neto TS, Molyneux RJ, et al. Poisoning of sheep by seeds of Crotalaria retusa: acquired resistance by continuous administration of low doses. Toxicon. 2010;55:28–32.
11. Kakar F, Akbarian Z, Leslie T, Mustafa ML, Watson J, Van Egmond HP, et al. An outbreak of hepatic veno-occlusive disease in western Afghanistan associated with exposure to wheat flour contaminated with pyrrolizidine alkaloids. J Toxicol. 2010;2010:313280.
12. Ruan J, Yang M, Fu P, Ye Y, Lin G. Metabolic activation of pyrrolizidine alkaloids: insights into the structural and enzymatic basis. Chem Res Toxicol. 2014;27:1030–9.
13. Edgar JA, Colegate SM, Boppré M, Molyneux RJ. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2011;28:308–24.
14. Schneider J, Tsegaye Y, Tensae MW, Selassie SG, Haue T, Bane A, et al. Veno-occlusive liver disease: a case report. Ethiop Med J. 2012;50:47–51.
15. EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), Knutsen HK, Alexander J, Barregard L, Bignami M, Brüschweiler B, et al. Statement on the risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. EFSA J. 2017;15:4908–42.
16. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. Off J Europ Communities L. 2002;140:10–22.
17. BfR. Opinion no. 030/2016: pyrrolizidine alkaloids: levels in foods should continue to be kept as low as possible. Berlin: BfR (Federal Institute for Risk Assessment); 2016.
18. Cao Y, Colegate SM, Edgar JA. Safety assessment of food and herbal products containing hepatotoxic pyrrolizidine alkaloids: interlaboratory consistency and the importance of N-oxide determination. Phytochem Anal. 2008;19:526–33.
19. Valese AC, Molognoni L, de Sá Ploêncio LA, de Lima FG, Gonzaga LV, Górniak SL, et al. A fast and simple LC-ESI-MS/MS method for detecting pyrrolizidine alkaloids in honey with full validation and measurement uncertainty. Food Control. 2016;67:183–91.
20. Cramer L, Schiebel HM, Ernst L, Beuerle T. Pyrrolizidine alkaloids in the food chain: development, validation, and application of a new HPLC-ESI-MS/MS sum parameter method. J Agric Food Chem. 2013;61:11382–91.
21. Mädge I, Cramer L, Rahaus I, Jerz G, Winterhalter P, Beuerle T. Pyrrolizidine alkaloids in herbal teas for infants, pregnant or lactating women. Food Chem. 2015;187:491–8.
22. Dübecke A, Beckh G, Lüllmann C. Pyrrolizidine alkaloids in honey and bee pollen. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2011;28:348–58.
23. Bodi D, Ronczka S, Gottschalk C, Behr N, Skibba A, Wagner M, et al. Determination of pyrrolizidine alkaloids in tea, herbal drugs and honey. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2014;31:1886–95.
24. Chung SWC, Lam CH. Development of an analytical method for analyzing pyrrolizidine alkaloids in different groups of food by UPLC-MS/MS. J Agric Food Chem. 2018;66:3009–18.
25. These A, Bodi D, Ronczka S, Lahrssen-Wiederholt M, Preiss-Weigert A. Structural screening by multiple reaction monitoring as a new approach for tandem mass spectrometry: presented for the determination of pyrrolizidine alkaloids in plants. Anal Bioanal Chem. 2013;405:9375–83.
26. Zhu L, Ruan JQ, Li N, Fu PP, Ye Y, Lin G. A novel ultra-performance liquid chromatography hyphenated with quadrupole time of flight mass spectrometry method for rapid estimation of total toxic retronecine-type of pyrrolizidine alkaloids in herbs without requiring corresponding standards. Food Chem. 2016;194:1320–8.
27. Roder E, Wiedenfeld H, Kersten R. The pyrrolizidine alkaloids of Senecio aquaticus huds. Sci Pharm. 1990;58:1–8.
28. DIN 32645:2008-11 Chemical analysis decision limit, detection limit and determination limit under repeatability conditions—terms, method, evaluation. Beuth Verlag GmbH: Berlin.
29. BfR. PT-PA01 final report: international proficiency testing on the determination of pyrrolizidine alkaloids in herbal teas. Berlin: BfR (Federal Institute for Risk Assessment); 2016.

30. Hessel S, Gottschalk C, Schumann D, These A, Preiss-Weigert A, Lampen A. Structure-activity relationship in the passage of different pyrrolizidine alkaloids through the gastrointestinal barrier: ABCB1 excretes heliotrine and echimidine. Mol Nutr Food Res. 2014;58: 995–1004.
31. Kaltner F, Rychlik M, Gareis M, Gottschalk C. Influence of storage on the stability of toxic pyrrolizidine alkaloids and their n-oxides in peppermint tea, hay, and honey. J Agric Food Chem. 2018;66: 5221–8.
32. Gottschalk C, Huckauf A, Dübecke A, Kaltner F, Zimmermann M, Rahaus I, et al. Uncertainties in the determination of pyrrolizidine alkaloid levels in naturally contaminated honeys and comparison of results obtained by different analytical approaches. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2018;35: 1366–83.
33. Greco G, Grosse S, Letzel T. Serial coupling of reversed-phase and zwitterionic hydrophilic interaction LC/MS for the analysis of polar and nonpolar phenols in wine. J Sep Sci. 2013;36:1379–88.
34. Van de Schans MGM, Blokland MH, Zoontjes PW, Mulder PPJ, Nielen MWF. Multiple heart-cutting two dimensional liquid chromatography quadrupole time-of-flight mass spectrometry of pyrrolizidine alkaloids. J Chrom A. 2017;1503:38–48.
35. Picron JF, Herman M, Van Hoeck E, Goscinny S. Analytical strategies for the determination of pyrrolizidine alkaloids in plant based food and examination of the transfer rate during the infusion process. Food Chem. 2018;266:514–23.
36. Mulder PPJ, López Sanchez P, Castelari M, Bodi D, Ronczka S, Preiß-Weigert A, et al. Occurrence of pyrrolizidine alkaloids in animal- and plant-derived food: results of a survey across Europe. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2018;35:118–33.
37. Joosten L, Mulder PPJ, Vrieling K, Van Veen JA, Klinkhamer PGL. The analysis of pyrrolizidine alkaloids in Jacobaea vulgaris; a comparison of extraction and detection methods. Phytochem Anal. 2010;21:197–204.
38. Colegate SM, Edgar JA, Knill AM, Lee ST. Solid-phase extraction and HPLC-MS profiling of pyrrolizidine alkaloids and their N-oxides: a case study of Echium plantagineum. Phytochem Anal. 2005;16:108–19.
39. EC. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (text with EEA relevance). Off J Europ Communities L. 2006;70:12–34.
40. Bredenkamp MW, Wiechers A, van Rooyen PH. A new pyrrolizidine alkaloid from Senecio latifolius DC. Tetrahedron Lett. 1985;26:929–32.
41. Griffin CT, O'Mahony J, Danaher M, Furey A. Liquid chromatography tandem mass spectrometry detection of targeted pyrrolizidine alkaloids in honeys purchased within Ireland. Food Anal Methods. 2015;8:18–31.
42. Mulder PPJ, López Sánchez P, These A, Preiss-Weigert A, Castelari M. Occurrence of pyrrolizidine alkaloids in food. EFSA Supporting Publication; 2015(EN-859): 116 pp.
43. Mathon C, Edder P, Bieri S, Christen P. Survey of pyrrolizidine alkaloids in teas and herbal teas on the Swiss market using HPLC-MS/MS. Anal Bioanal Chem. 2014;406:7345–54.
44. Schulz M, Meins J, Diemert S, Zagermann-Muncke P, Goebel R, Schrenk D, et al. Detection of pyrrolizidine alkaloids in German licensed herbal medicinal teas. Phytomedicine. 2015;22:648–56.

8.4 Occurrence and risk assessment of pyrrolizidine alkaloids in spices and culinary herbs from various geographical origins

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Occurrence and Risk Assessment of Pyrrolizidine Alkaloids in Spices and Culinary Herbs from Various Geographical Origins

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Abstract: Pyrrolizidine alkaloids (PA) and their *N*-oxides (PANO) are a group of toxic secondary plant metabolites occurring predominantly as contaminants in (herbal) teas, honeys and food supplements, as well as in spices and culinary herbs. Depending on the botanical origin of the contaminating plant, the pattern of PA/PANO can strongly vary within a sample. The current study aimed to broaden the existing data on the occurrence of PA/PANO in spices and culinary herbs. For this, 305 authentic samples covering 15 different matrices mainly harvested in 2016 or 2017 and originating from 36 countries were investigated for the presence of 44 PA/PANO. Fifty-eight percent of the samples contained at least one PA/PANO. The average sum content over all samples was 323 µg/kg (median of 0.9 µg/kg, 95% percentile of 665 µg/kg). The highest amount of 24.6 mg/kg was detected in an oregano sample. Additionally, conspicuous analyte patterns were discovered in samples from similar cultivation regions, indicating related botanical sources of PA/PANO contaminations. Particularly, oregano and cumin from Turkey often contained high amounts of PA/PANO. The results were used to assess the acute and chronic health risks related to PA/PANO intake via spices and culinary herbs, indicating a potential health risk in particular for adults and children with high consumption or when considering worst-case contamination scenarios of a sum content of 5500 µg/kg.

Keywords: pyrrolizidine alkaloids; spices; culinary herbs; food safety; contamination; LC-MS; risk assessment

Key Contribution: The findings presented in this study provide the first comprehensive data on PA/PANO contamination of spices and culinary herbs each considering variety and geographical origin. A high quality of results was assured by exclusively analyzing representative samples from large batches being provided by German spice and culinary herb companies. Exemplary recipes and consumption data from nutritional studies were used to perform a risk assessment for adults and children.

1. Introduction

Pyrrolizidine alkaloids (PA), including their corresponding *N*-oxides (PANO), are a group of toxic secondary metabolites presumably distributed in more than 6000 plant species worldwide, especially occurring in the families Asteraceae (tribus Senecioneae and Eupatorieae), Boraginaceae, Apocynaceae (tribus Echiteae) and Fabaceae (genus *Crotalaria*) [1,2]. More than 660 PA/PANO were

identified so far, occurring in presumably 3% of all flowering plants in varying compositions [3,4]. The 1,2-unsaturated types of PA/PANO, mainly of the retronecine-, heliotridine- and otonecine-type, are known to be toxic for humans and animals [5]. Cytochrome-P450 monooxygenase enzymes in the liver can transform these compounds into highly reactive pyrrole esters, which are able to bind to proteins and DNA and cause dysfunctions of affected proteins and exhibit genotoxic properties [6]. Adverse health effects of an acute PA/PANO intoxication are primarily characterised by the hepatic sinusoidal obstruction syndrome, formerly known as veno-occlusive disease. A long-term intake of small amounts of PA/PANO is associated with chronic diseases such as liver cirrhosis, cancer or pulmonary arterial hypertension [7–9].

PA/PANO can contaminate food and feed. Especially honey, (herbal) teas and herbal food supplements are well-known sources of PA/PANO exposure to humans [10]. A contamination of these products can occur in different ways: the contamination of honey is caused by bees collecting pollen and, in particular, nectar from PA/PANO producing plants [11]. Contrary to this, a contamination of herbal products with PA/PANO was assumed to be exclusively caused by impurities or unintended co-harvesting of the aimed culture plants and weed plants containing these toxins. Recently published data also report the horizontal transfer and exchange of PA/PANO between living plants via the soil as an unconventional and yet disregarded source of contamination [12].

Although the toxic effects of PA/PANO are known and thus contents in relevant food and feed matrices have been monitored for years, regulatory limits have not been established in the European Union so far. The risk of a PA/PANO exposure was assessed by the European Food Safety Authority (EFSA), using the margin of exposure (MOE) approach based on a Benchmark Dose Lower Confidence Limit 10% ($BMDL_{10}$) of 237 μg/kg body weight/day (BW/d) for the PA/PANO sum [10]. Until now, only a maximum content of 100 mg/kg of PA/PANO-containing *Crotalaria* spp. for animal feed materials and compound feed was set in Directive 2002/32/EC [13]. Regulatory limits for these toxic contaminants in food are still discussed and may be implemented in Commission Regulation (EC) No. 1881/2006 within the next year [14]. However, PA/PANO are known to be genotoxic and carcinogenic, thus their occurrence in food and feed supply chains should generally be kept as low as reasonably achievable (ALARA principle) [15].

PA/PANO can also occur in spices and culinary herbs, as these products are subject of the same way of contamination already known for (herbal) tea plants [16]. Considering the few available results, contaminated spices and herbs presumably may greatly contribute to the overall exposure of consumers to PA/PANO [17]. Thus, the aim of the present study was to expand knowledge on the occurrence of PA/PANO in different types of spices and culinary herbs to identify potential sources with relevance for food safety. Therefore, we analyzed 305 samples of 15 different varieties from numerous geographical origins from all over the world for the presence of 44 PA/PANO compounds. The results were evaluated with respect to their PA/PANO patterns, geographical and botanical origin. Additionally, data were used to perform a risk assessment for PA/PANO in spices and culinary herbs, using exemplary recipes and consumption data from nutritional studies for calculating exposure of adults and children.

2. Results

2.1. Occurrence of PA/PANO in Spices and Culinary Herbs

Samples were investigated for the presence of PA/PANO using an approach published earlier [18]. The analytes were extracted under acidic conditions and the raw extracts were further cleaned using polymeric cation exchange solid phase extraction (PCX-SPE) cartridges. The compounds were detected via liquid chromatography tandem mass spectrometry (LC-MS/MS). Quantification of PA/PANO was performed using matrix-matched external calibration.

Among 305 samples tested, 178 (i.e., 58.4%) were found to contain at least one PA/PANO above the limit of detection (LOD) (Figure 1). For calculation reasons, contents <LOD were further treated as 0.0 μg/kg and contents between LOD and the limit of quantification (LOQ) were calculated with

0.5 times the LOQ. High prevalence of PA/PANO was determined in marjoram, savory, oregano, cumin, curry and herbs of Provence, all showing contamination rates of more than 79%. Showing 11% positive samples, the smallest prevalence was obtained for pepper, and also basil, caraway, dill, rosemary and chive showed smaller contamination rates ranging from 30% to 36%. The remaining matrices, namely parsley, thyme and ginger showed a higher prevalence with 62% to 69% of the samples containing PA/PANO.

The sum contents in positive samples were markedly different and ranged from 0.1 µg/kg up to a maximum of 24,600 µg/kg (for exact contents, see Table S1). The median content over all samples tested was 0.9 µg/kg with an average content of 323 µg/kg and a 95% percentile of 665 µg/kg. The smallest average content was obtained for pepper (0.1 µg/kg). Further matrices with small average contents were thyme, dill, chive, marjoram and herbs of Provence, ranging from 49.1 to 83.4 µg/kg. High average contents above 100 µg/kg were determined in the matrices parsley (189 µg/kg), savory (150 µg/kg), cumin (641 µg/kg) and oregano (3140 µg/kg). In addition to their high amounts of PA/PANO, the two latter ones also showed comparably high median contents of 348 and 163 µg/kg, respectively.

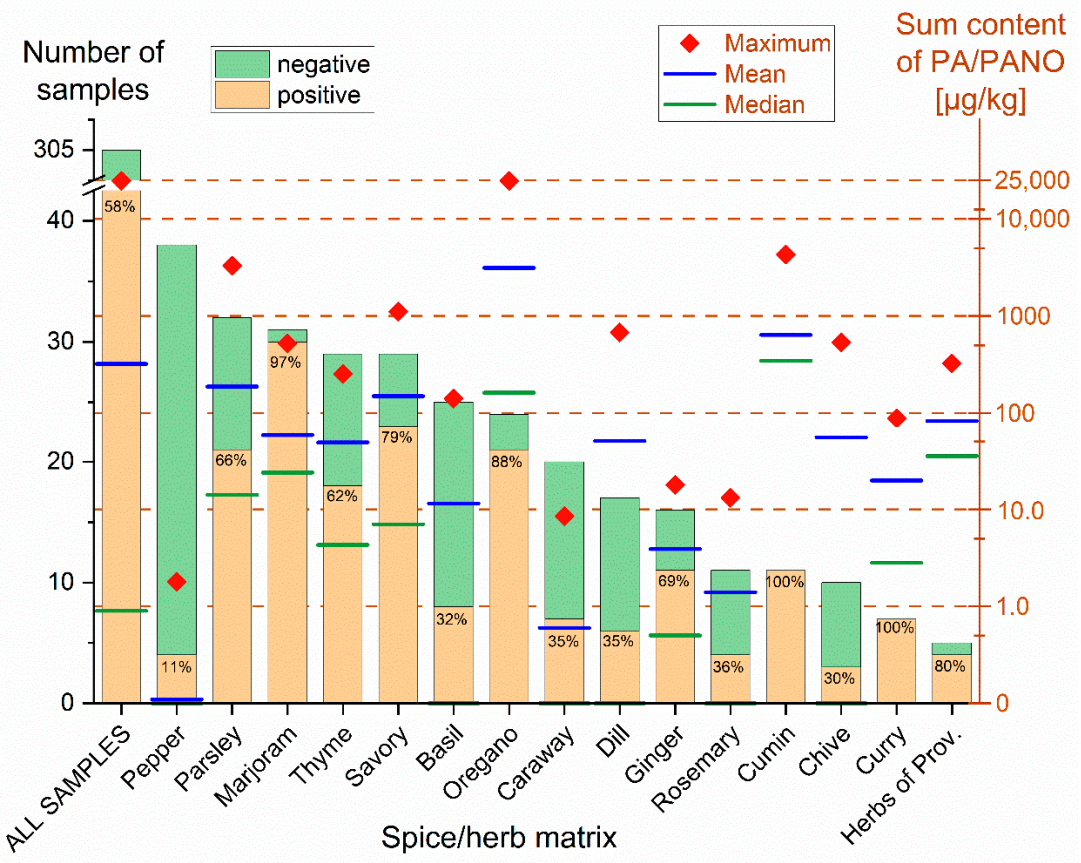

Figure 1. Prevalence and contents of pyrrolizidine alkaloids (PA) and PA *N*-oxides (PANO) in spice and culinary herb samples. The number of investigated samples of each matrix can be derived from the left Y-axis. The percentage on each bar represents the prevalence (positive samples/all tested samples) in the respective matrix (X-axis). Median, average and maximum PA/PANO sum contents can be taken from the right Y-axis. The median bar of spices and herbs with <50% positive samples is 0 µg/kg and thus not drawn. For the sake of clarity, the sum contents were displayed in a logarithmic manner.

2.2. Contents of PA/PANO in Samples from Different Countries

Notably, samples originating from South America were contaminated with levels near or below LOD (Table S1). Only three out of 12 samples (25.0%) were found to be positive, showing only small sum contents up to 41.6 µg/kg in one oregano sample from Chile. In total, 34 out of 62 samples (54.8%)

from Africa contained at least one PA/PANO, and the highest amount was determined in a dill sample from Tunisia, containing 681 μg/kg. The contamination rate of samples from Asia, including the Middle Eastern States Israel, Turkey and Syria, was comparable with the one of Africa (36 positive samples of 71, 50.7%). Contrary to this result, high amounts in individual samples were mostly detected in Asian samples, with 10 of 36 positive samples containing more than 400 μg/kg (1000 μg/kg for dried marjoram and dried oregano; these levels represent the currently discussed maximum levels for dried herbs for implementation in Commission Regulation (EC) No. 1881/2006 [14]). The maximum sum content in a sample from Asia was 24,600 μg/kg. Samples originating from Europe showed the highest prevalence, as PA/PANO were found in 76 of 118 spices and culinary herbs (64.4%). Here, the maximum content was 11,030 μg/kg in an oregano sample originating from Greece. With amounts of 11 of 76 positive samples exceeding 400 μg/kg, the detected sum contents in European samples were lower compared to those from other continents.

In Table 1, samples with contaminations of PA/PANO exceeding the discussed maximum level of 400 μg/kg are summarized. Remarkably, nine of the 24 samples were oregano, and ten were cultivated in Turkey. In twelve of 24 samples, a heliotrine (Ht)-type PA/PANO was the predominant compound (europine/-NO, heliotrine/-NO and lasiocarpine-*N*-oxide), and 22 of them were PANO. This evaluation revealed high contributions of single PA/PANO ranging from 26% to 86% of the total content in a sample.

Table 1. Sum contents, origins and matrices of the 24 samples with pyrrolizidine alkaloids (PA) and PA *N*-oxides (PANO) sum contents above the currently discussed maximum level of 400 μg/kg [14]. The predominant PA/PANO and their quantitative contribution to the sum contents are added.

PA/PANO Sum Content (μg/kg)	Place of Origin	Sample Type	Predominant PA/PANO	Contribution (% of Sum Content)
24,600	Turkey	Oregano	Europine-NO	82
18,100	Turkey	Oregano	Europine-NO	70
11,000	Greece	Oregano	Europine-NO	42
8300	Turkey	Oregano	Europine-NO	74
6800	Turkey	Oregano	Europine-NO	86
4310	Turkey	Cumin	Europine-NO	36
3300	Germany	Parsley	Senecionine-NO	31
1820	Turkey	Oregano	Europine	50
1780	Greece	Oregano	Europine-NO	55
1110	Albania	Savory	Lycopamine-NO	61
861	Turkey	Oregano	Lycopamine-NO	57
859	Albania	Savory	Lycopamine-NO	35
737	Germany	Parsley	Senecionine-NO	26
709	Albania	Savory	Lycopamine-NO	57
681	Tunisia	Dill	Lasiocarpine-NO	59
666	Unknown	Savory	Lycopamine-NO	63
661	Croatia	Savory	Lycopamine-NO	32
658	Germany	Parsley	Senecionine-NO	43
648	Poland	Oregano	Senecionine-NO	47
540	Germany	Chive	Erucifoline-NO	48
536	Turkey	Cumin	Europine-NO	40
524	Egypt	Marjoram	Senecionine-NO	34
493	Turkey	Cumin	Heliotrine	38
410	Turkey	Cumin	Heliotrine-NO	43

NO = *N*-oxide.

2.3. Patterns of PA/PANO in Samples from Various Geographical Origins

In total, 33 PA/PANO out of the tested set of 44 analytes were identified in the samples (Table S1). The most abundant PA/PANO were from senecionine (Sc)-type (49%), typically originating from species of the Asteraceae family. Within the covered analytes from the Sc-type, merenskine, merepoxine, sceleratine or their *N*-oxides were identified in no sample.

The next most frequently occurring group of analytes were from Ht-type (28%, from Boraginaceae) and lycopsamine (Ly)-type (23%, from Boraginaceae). In case of Ly-group, namely the *N*-oxides of acetylintermedine and acetyllycopsamine were not detected to occur in the samples.

Contrary to the Sc-, Ht- and Ly-type PA/PANO, no analytes belonging to the monocrotaline (Mc)-type (trichodesmine, monocrotaline and its *N*-oxide, from Fabaceae) were determined in the investigated set of 305 samples. Among positive samples, the five most frequently occurring individual PA/PANO were lasiocarpine (32.1%, Ht-type), senecionine-NO (31.8%, Sc-type), integerrimine-NO (29.2%, Sc-type), senecionine and senkirkine (both 25.9%, Sc-type). Contrary to that, the quantitatively predominant analytes were the *N*-oxides of europine, lasiocarpine and heliotrine, all belonging to Ht-type PA/PANO.

The distribution of individual analytes in positive samples originating from different countries and continents showed different predominant groups of PA/PANO (Figure 2). Sc-type compounds occurring in species of the Asteraceae family, covering retrorsine, senecionine, senecivernine, seneciphylline, integerrimine and their *N*-oxides as well as senkirkine, occurred more often in samples from Northern Africa and Central Europe, namely Germany and Poland. Contrary to that, Ly-type PA/PANO were predominantly found in samples originating from the Balkan region, with high percentages of indicine, lycopsamine and intermedine (+ *N*-oxides) in positive samples from Croatia and Albania. In spices and culinary herbs from Greece and, in particular, from Turkey, PA/PANO belonging to the Ht-type were predominant, and high amounts of europine, heliotrine and lasiocarpine as well as their *N*-oxides were detected.

2.4. Acute Health Risk Assessment of PA/PANO in Spices and Culinary Herbs

The determined levels of PA/PANO contamination in the investigated spices and culinary herbs were assessed for their risk regarding the health of consumers. The risk of an acute non-carcinogenic health damage was assessed on the basis of a health-based guidance value (HBGV) which has been derived from a No-Observed-Adverse-Effect-Level (NOAEL) of 10 µg/kg BW/d from a 2-year gavage study with riddelliine in rats [19]. The HBGV as a preliminary orientation value is quoted with 0.1 µg/kg BW/d (NOAEL/100) for the sum of PA/PANO and should not be exceeded [15].

To assess the acute health risk of adults (70 kg BW) resulting from a short-term exposure of PA/PANO, two exemplary recipes (No. 1: 'Mediterranean pesto'; No. 2: 'tomato sauce'; see 4.6) were assumed, including the culinary herbs basil, oregano and thyme. Median and mean contents as well as the 95% percentile (P95) of the sum contents of these matrices were calculated (Table 2).

Table 2. Number of samples and positive samples, median, mean and 95% percentiles (P95) of pyrrolizidine alkaloids (PA) and PA *N*-oxides (PANO) contents in culinary herbs selected for an acute health risk assessment.

Spice/Herb	*n*	Positive samples	Median (µg/kg)	Mean (µg/kg)	P95 (µg/kg)
Basil	25	8	0.0	11.5	68.3
Oregano	24	21	163	3140	17,000
Thyme	29	18	4.3	49.1	191
All samples	305	178	0.9	323	665

One portion of the pesto (50 g/adult) resulted in a consumption of 2.5 g of thyme and 3.75 g of oregano and basil each, and for the tomato sauce a consumption of 0.67 g of oregano was estimated (see Section 4.6). In case of two- to five-years aged children (16 kg BW), the 97.5% percentiles (P97.5) of intake of singular raw herbs retrieved from a study on dietary intake of pesticide residues were used [20].

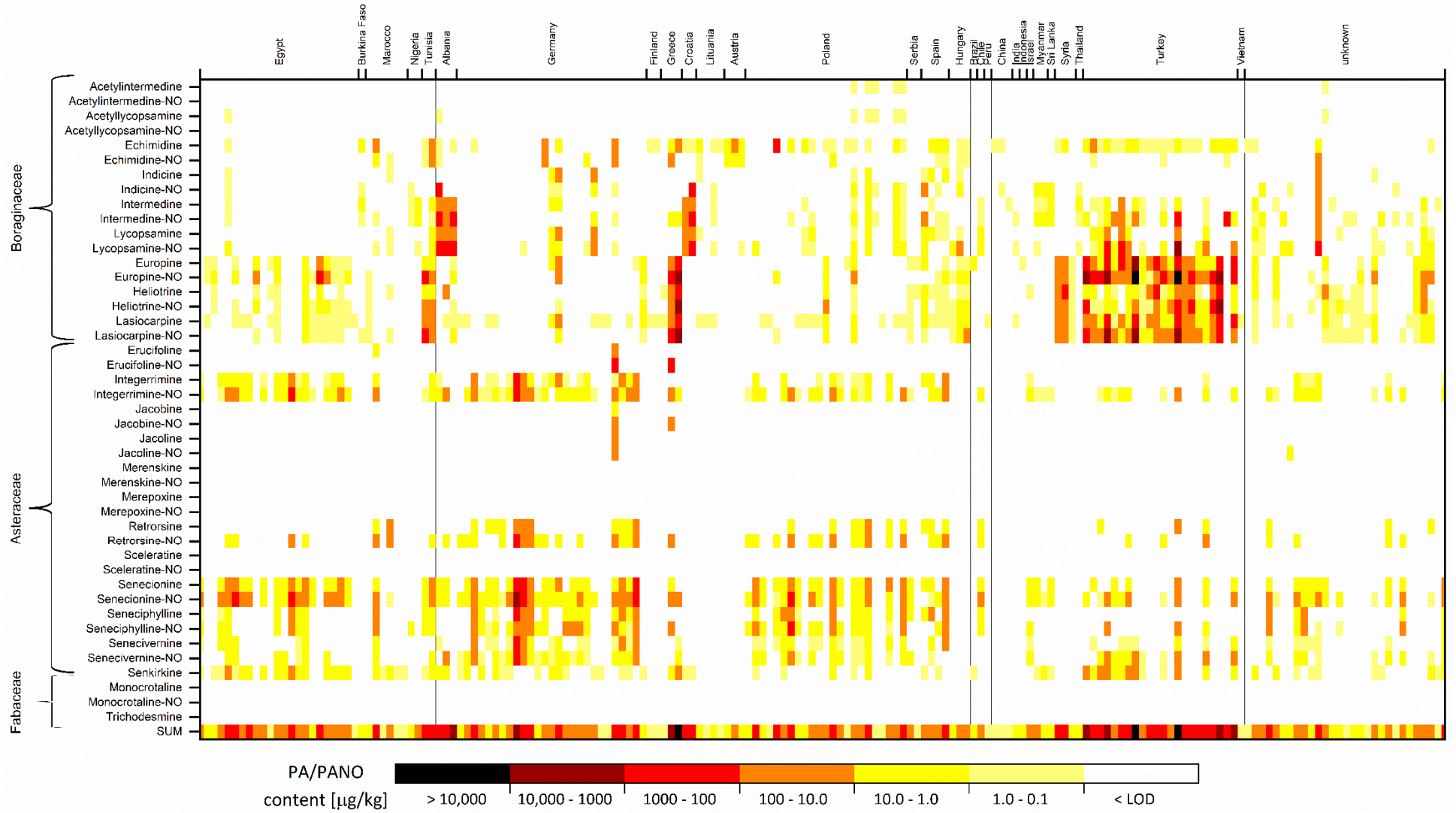

Figure 2. Heat map plot of amounts of pyrrolizidine alkaloids (PA) and PA *N*-oxides (PANO) in positive samples (n = 178). Analytes were grouped according to their botanical family of origin and countries of sample origins were arranged according their related continent (Africa, Europe, South America, Asia, unknown).

For PA/PANO exposure calculation, mean contents (mean-case scenario) or P95 contents (worst-case scenario) were considered to cover possibly occurring higher PA/PANO contaminations. In order to consider the impact of usage of fresh or dried produce, calculations were performed under application of drying or dehydration factors [21]. The acute health risk was expressed as an exceedance factor of the HBGV of 0.1 μg/kg BW/d (Table 3).

Table 3. Mean- and worst-case scenario for short-term (acute) exposure to pyrrolizidine alkaloids (PA) and PA *N*-oxides (PANO) due to consumption of two exemplary recipes containing culinary herbs. Intake by children were considered as given in [20]. With respect to consumption of fresh or dried herbs, exceedance factors were calculated with or without a dehydration factor (DF) of six, according to [21].

Recipe	Herb	BW[1] (kg)	Consumption (One Portion/d)		PA/PANO Content	PA/PANO-Intake	Exceedance Factor[4]	
			(g)	(g/kg BW)	(μg/kg)	(μg/kg BW)	no DF	DF = 6
					Mean case[5]			
Mediterranean pesto	Thyme	70[2]	2.50	0.036	49.1	0.002		
	Oregano		3.75	0.054	3140	0.170		
	Basil		3.75	0.054	11.5	0.001		
						0.173	1.7	0.3
	Thyme	16[3]	1.10	0.069	49.1	0.003		
	Oregano		0.60	0.038	3140	0.119		
	Basil		11.1	0.694	11.5	0.008		
						0.130	1.3	0.2
Tomato sauce	Oregano	70[2]	0.67	0.010	3140	0.031	0.3	0.1
	Oregano	16[3]	0.60	0.038	3140	0.119	1.2	0.2
					Worst case[6]			
Mediterranean pesto	Thyme	70[2]	2.50	0.036	191	0.007		
	Oregano		3.75	0.054	17,000	0.918		
	Basil		3.75	0.054	68.3	0.004		
						0.929	9.3	1.5
	Thyme	16[3]	1.10	0.069	191	0.013		
	Oregano		0.60	0.038	17,000	0.646		
	Basil		11.1	0.694	68.3	0.047		
						0.706	7.1	1.2
Tomato sauce	Oregano	70[2]	0.67	0.010	17,000	0.170	1.7	0.3
	Oregano	16[3]	0.60	0.038	17,000	0.646	6.5	1.1

[1] BW = body weight; [2] Adult, 70 kg BW; [3] child, 16 kg BW; [4] related to a health-based guidance value (HBGV) of 0.1 μg/kg BW/d; [5] mean levels of PA/PANO contamination; [6] 95% percentile levels of PA/PANO Contamination.

A consumption of one portion of the recipes prepared with mean contaminated, fresh culinary herbs (applying a dehydration factor of 6) resulted in HBGV exceedance factors of 0.1 and 0.3 for adults as well as each 0.2 in case of children. The exceedance factors in the worst-case scenario lay

between 0.3 and 1.5 for an adult and 1.1 and 1.2 for a child. When considering dried herbs for the recommended amounts of thyme, basil and oregano in both recipes, adults would exceed the HBGV by 0.3 and 1.7 in the mean-case and 1.7 and 9.3 in the worst-case scenario. In case of children, exceedance factors were 1.2 and 1.3 in the mean-case as well as 6.5 and 7.1 in the worst-case scenario. Due to the high levels within the oregano samples, this matrix was a main contributor for the acute intake of PA/PANO via the presented recipes.

2.5. Chronic Health Risk Assessment of PA/PANO in Spices and Culinary Herbs

The chronic, long-term exposure of consumers to PA/PANO was evaluated using the MOE approach based on a $BMDL_{10}$ of 237 μg/kg BW/d [10]. Therefore, the 305 investigated samples were arranged in four groups, with respect to their total PA/PANO contents (Table 4). Means and medians of each risk group were calculated and representative contents for a low, medium and high-risk scenario were derived. The mean contents were chosen for the low and medium-risk group. The rounded median was chosen as representative content for the high-risk group, being the more robust value with respect to the smaller quantity of samples.

Table 4. Number of samples classified in several risk groups depending on their sum contents of pyrrolizidine alkaloids (PA) and PA *N*-oxides (PANO). Median and mean contents were calculated for each risk group and representative contents were derived according to the approach applied in [17].

Group	*n*	%	Median (μg/kg)	Mean (μg/kg)	Representative Contents (μg/kg)
<Limit of detection (LOD)	127	41.6	n.c.	n.c.	n.c.
Low risk (LOD-100 μg/kg)	124	40.7	9.2	21.1	20
Medium risk (100–1000 μg/kg)	44	14.4	236	332	330
High risk (>1000 μg/kg)	10	3.3	5550	8120	5500

n.c., not calculable.

The derived representative contents for each risk group were used for calculating MOE values for different mean case and worst-case scenarios for adults and children (Table 5). As no specific intake data on individual spices and culinary herbs were available, the long-term consumption was estimated via the cumulated ingestion of culinary herbs from the two 24 h recalls of the German National Nutrition Survey II [17,22]. In this survey, only data from people of the age 14 to 80 were available, thus in case of children the overall daily intake of herbs reported in [20] was considered for the average consumption scenario. For a high consumption scenario of children, two times the average consumption was assumed. Equally to the acute health risk assessment approach, estimations were performed with respect to dried and fresh produce by applying a dehydration factor of six.

A MOE value > 10,000 is regarded as being of little concern for risk management and public health. When considering fresh herbs for the assumed daily intake (considering a dehydration factor of six), in case of adults a MOE of < 10,000 was only calculated in the high consumption scenario combined with PA/PANO sum levels of 5550 μg/kg (high risk group). Contrary to that, in the case of children, the MOE was below 10,000 in both consumption scenarios when a high contamination of the spices and herbs was assumed. Considering dried herbs for the daily spice and herb consumption and thus no dehydration factors, the results revealed for both adults and children MOE values < 10,000 in every assumed scenario with a high PA/PANO content of 5500 μg/kg. Notably, the high consumption scenario of dried spices and herbs already revealed MOE values < 10,000 for both children and adults when considering a sum content of 330 μg/kg (i.e., medium risk group).

Table 5. Margin of Exposure (MOE) values resulting for different average and high herb consumption scenarios for both adults and children. The considered sum contents of pyrrolizidine alkaloids (PA) and PA *N*-oxides (PANO) were derived from different risk groups derived from results of the 305 investigated spice and culinary herb samples (Table 4). With respect to consumption of fresh or dried herbs MOE values were calculated with or without a dehydration factor (DF) of six, according to [21].

Scenario	Consumption[1] (g/d)	Consumption[1] (g/kg BW/d)	PA/PANO Content[2] (µg/kg)	Intake (µg/kg BW/d)	Margin of Exposure[3] no DF	Margin of Exposure[3] DF = 6
Adult, 70 kg average consumption (median)	0.77	0.011	20	< 0.000	n.c.	n.c.
			330	0.004	59,300	356,000
			5500	0.061	3890	23,300
Adult, 70 kg average consumption (mean)	1.68	0.024	20	< 0.000	n.c.	n.c.
			330	0.008	29,600	178,000
			5500	0.132	1800	10,800
Adult, 70 kg high consumption (P95)	6.09	0.087	20	0.002	119,000	711,000
			330	0.029	8170	49,000
			5500	0.479	495	2970
Child, 16 kg average consumption (mean)	0.70	0.044	20	0.001	237,000	1,420,000
			330	0.015	15,800	94,800
			5500	0.242	979	5880
Child, 16 kg high consumption (2x average)	1.40	0.088	20	0.002	119,000	711,000
			330	0.029	8170	49,000
			5500	0.481	492	2950

[1] Based on [20,22]; [2] see Table 4; [3] Based on a Benchmark Dose Lower Confidence Limit 10% ($BMDL_{10}$) of 237 µg/kg BW/d for the sum of PA/PANO intake. n.c., not calculable.

3. Discussion

In total, in 178 of the 305 samples (58.4%) at least one PA/PANO was detected. The average content over all 305 samples was 323 µg/kg and the median content was 0.9 µg/kg. The results differed significantly, depending on the investigated spice or herb. Similar findings were recently published: In 2016, the German Federal Institute for Risk Assessment (BfR) investigated 40 spice and herb samples from various matrices and detected a mean content of 265 µg/kg, with amounts up to 4990 µg/kg in a cumin sample [15]. In addition, in 2019 the BfR reported an average sum level of 2680 µg/kg and a median level of 10 µg/kg obtained from a broader study with 263 spice and culinary herb retail samples, covering 17 varieties [17].

Due to the sampling in compliance with Commission Regulation (EC) No. 401/2006, the samples were assumed to be homogenous and representative for a large batch. The other way round, this indicated that PA/PANO levels in retail samples might be even higher, due to the usually occurring spot contamination via parts of PA/PANO containing plants within a bigger spice or herb lot. In consequence, the homogenised samples from the current study might represent lower PA/PANO amounts due to a 'dilution' with uncontaminated raw material, whereas individual retail samples could contain very high sum levels due to packaging of contamination 'spots' from a large lot. To confirm this hypothesis, more data must be collected in further occurrence studies on retail spice and culinary herb samples. Up to now, only retail samples of oregano were investigated in a prior study [23].

Our findings showed Ht-type PA/PANO as the quantitatively predominant contaminants, whereas Sc-type revealed a higher prevalence. The varying patterns of PA/PANO in the investigated samples indicated that spices and culinary herbs might be contaminated with diverse undesired weeds. PICRON et al. reported the presence of PA/PANO in 100% of the 17 investigated spice samples, with contents up to 1770 μg/kg and an average of 197 μg/kg [16]. Interestingly, this study also showed Ht-type PA/PANO to be the main contributors, being responsible for 85% among the total contamination of the study's samples. These results are in accordance to our findings of Ht-type alkaloids predominantly occurring in affected samples.

Remarkably, even 69% of ginger root samples were found to contain PA/PANO up to 17.8 μg/kg, although ginger is not an herb-based spice and, thus, should normally not be affected by a co-harvesting of contaminant plants. The ginger samples were obtained as dried and milled powder. Indeed, the detected contents seem to be unproblematic, but the findings may indicate a possible cross-contamination during processing in the companies. Another possible way of contamination may be the recently described horizontal transfer of PA/PANO between living plants via the soil [12], especially as ginger is a root spice. In summary, in all 15 spice or herb varieties investigated in the current study, traces of PA/PANO were detected in at least a few samples, indicating PA/PANO to be typical process contaminants.

Our findings showed that, in particular, oregano and cumin often were highly contaminated with PA/PANO. In previous studies, retail oregano was already identified to contain high amounts of PA/PANO. In a study from 2019, the BfR reported an average sum level of 4038 μg/kg and a median level of 942 μg/kg for 59 oregano samples from supermarkets [17]. KAPP et al. investigated 41 retail oregano samples and detected contents higher than 1000 μg/kg in 75% of them, with a mean of 6160 μg/kg, a median of 5430 μg/kg, and a maximum of 32.4 mg/kg [23]. In consequence, our results confirmed these findings, and, in addition, strongly indicated oregano to be one of the main contributors to the overall PA/PANO exposure via the intake of spices and herbs.

Recently, the PA/PANO sum contents in *Senecio vulgaris* L. were determined and shown to range from 0.16% to 0.49% related to dry matter, depending on the developmental stage and season [24]. Compared to the highest content of PA/PANO analytes detected in oregano in our current study (24.6 mg/kg), that indeed mainly originated from Ht-type compounds, this would correspond to a proportion of 1.54% to 0.50% of pure PA/PANO plants in this oregano sample. According to a specification of the European Spice Association (ESA), impurities of extraneous matter of up to 2% are quoted as tolerable for oregano to be marketed as pure oregano [25]. Considering the extremely high contamination levels detected in singular oregano samples in our study, the ESA specification was revealed to be inacceptable when the tolerated 2% extraneous matter were related to toxic plants, for instance PA/PANO containing plants. Thus, the ESA specification may not be seen as generally appropriate.

The maximum levels, which are discussed to be implemented in Regulation (EC) No. 1881/2006 are 400 μg/kg for cumin seeds and other dried herbs as well as 1000 μg/kg for dried marjoram and dried oregano [14]. Considering these limits, four of eleven cumin samples (36%), seven of 24 oregano samples (29%), one of 31 marjoram samples (3%) and ten of 165 other herb samples (6%) would have exceeded the maximum levels. In total, 22 of 231 samples (10%) concerned by the discussed maximum levels would have exceeded them. In conclusion, spices and culinary herbs should be further investigated on their PA/PANO contents. In particular, the results on PA/PANO sum levels of oregano and cumin seemed to be a more extensive problem, originating from the supplying farms itself. Thus, in the course of preventive consumer protection, weed management actions are necessary. In the meantime, trainings of the staff at early production stages were already initiated to tackle the problems of high PA/PANO contents, particularly in oregano.

The risk assessment results revealed the detected sum contents of PA/PANO to be of concern both in view of acute and chronic toxicity. Evaluation of the acute risk using spices and culinary herbs in typical recipes resulted in an exceedance of the HBGV in the worst-case scenario, even when dehydration factors for differences in weight of fresh and dried herbs were taken into account. In

consequence, a single ingestion of spices and culinary herbs highly contaminated with PA/PANO may not be seen as safe regarding potential non-neoplastic effects.

Concerning the risk assessment on the chronic intake of PA/PANO via spices and herbs, the presented ingestion scenarios for adults and children resulted in MOE values < 10,000 in some cases, particularly for scenarios considering a high spice and herb contamination of 5500 µg/kg. In conclusion, our findings entirely confirmed former risk assessment data on PA/PANO in freeze-dried herbs published by the BfR in 2019, where exceedance factors of the HBGV of up to 2.1 were calculated [17]. Hereby, the BfR did not consider any dehydration factors.

Indeed, our presented exposure estimations based on short-term and long-term intake of toxic PA/PANO may be afflicted by uncertainties. Two major problems have to be pointed out: the lack of reliable data concerning the consumption of individual spices and herbs, as well as in praxi strongly varying amounts of fresh or dried herbs used in cooking recipes. While reported contents of contaminants usually relate to the dried product, recipes mostly use fresh ingredients. Ignoring dehydration factors can cause a further uncertainty due to overestimations of the consumption of spices and herbs and, thus, of the PA/PANO intake. On the other hand, in case of children the intake was related on raw, unprocessed spices and herbs, and thus consequently lower compared to the data given for processed spices and herbs [20]. In conclusion, our results for risk assessment on the chronic intake of PA/PANO via spices and herbs represent a more conservative estimation, and the true intake might even be higher. In general, the current risk assessment procedure of summing-up the contents of all different types of PA/PANO is subject to uncertainties. This procedure does not respect different toxic potentials of the singular compounds. In order to respect these differences, MERZ and SCHRENK have proposed relative potency factors (RPF) that may improve the reliability of future risk assessments [26].

Additionally, the calculated content of 5500 µg/kg as representative for the high-risk group (see Table 4) may be regarded as biased, since seven of the ten samples within this group were oregano. On the other hand, the median of the PA/PANO sum content was used for these high risk calculations instead of the mean level. Moreover, more data on the occurrence of PA/PANO in spices and herbs and on their intake by different consumer groups are necessary to improve future risk assessment.

At this point it has to be considered that the intake of PA/PANO estimated in the current study exclusively originated from spices and culinary herbs. Actually, all sources of PA/PANO exposure have to be considered to properly assess the risk of adverse health consequences. In particular honey, (herbal) teas and food supplements are widely known as main contributors for PA/PANO exposure [10]. Formerly published studies on (herbal) teas, honeys and plant-based food supplements already reported MOE values < 10,000 without considering further sources of PA/PANO [27–29].

In conclusion, even if our study represents one of the most comprehensive available, there is still a lack of data on the PA/PANO contents in the addressed spice and herb matrices. Regarding single matrices, the number of samples was quite low; geographical and seasonal influences should be addressed in more detail. The presented data concerning the risk for consumers due to consumption of contaminated spices and culinary herbs may be seen as a first indication towards the problem of high contents of PA/PANO in these matrices. Although the total intake of spices and herbs is generally low, the current study undoubtedly revealed that these matrices may significantly contribute to the overall acute and chronic exposure of consumers to toxic PA/PANO.

4. Materials and Methods

4.1. Chemical Reagents and Standards

The following PA/PANO standards were purchased from PhytoLab (Vestenbergsgreuth, Germany): 7-O-acetylintermedine (AcIm), 7-O-acetylintermedine-N-oxide (AcImN), 7-O-acetyllycopsamine (AcLy), echimidine (Em), echimidine-N-oxide (EmN), erucifoline (Ec), erucifoline-N-oxide (EcN), europine (Eu), europine-N-oxide (EuN), heliotrine (Ht), heliotrine-N-oxide (HtN), intermedine (Im), intermedine-N-oxide (ImN), jacobine (Jb), jacobine-N-oxide (JbN), lasiocarpine (Lc), lycopsamine (Ly), lycopsamine-N-oxide (LyN), monocrotaline (Mc),

monocrotaline-N-oxide (McN), retrorsine (Rs), retrorsine-N-oxide (RsN), senecionine (Sc), seneciphylline (Sp), seneciphylline-N-oxide (SpN), senecivernine (Sv), senecivernine-N-oxide (SvN), senkirkine (Sk) and trichodesmine (Td). Other PA standards were obtained from CFM Oskar Tropitzsch (Marktredwitz, Germany), namely 7-O-acetyllycopsamine-N-oxide (AcLyN), indicine (Ic), indicine-N-oxide (Ic), integerrimine (Ig), integerrimine-N-oxide (IgN), jacoline (Jl), jacoline-N-oxide (Jl), lasiocarpine-N-oxide (LcN), merenskine (Mk), merenskine-N-oxide (MkN), merepoxine (Mx), merepoxine-N-oxide (MxN), sceleratine (Sl), sceleratine-N-oxide (SlN) and senecionine-N-oxide (ScN).

With respect to solubility, stock solutions (c = 1 mg/mL) of each PA/PANO were prepared, either with acetonitrile (Em, Ec, EcN, Eu, Ht, Ic, IcN, Jb, Lc, LcN, Ly, Mc, SpN, Sk) or acetonitrile/water (50/50, v/v) (all other analytes), and stored at 6 °C in the dark. A PA/PANO mix solution (c = 10 µg/mL of each analyte) was prepared by combining stock solution aliquots and diluting the resulting mixture with acetonitrile/water (50/50, v/v). Acetonitrile and methanol (both LC-MS grade) were used for all experiments and purchased from Th. Geyer (Renningen, Germany). Ultrapure water was obtained using an UltraClearTM TP UV UF TM system from Evoqua Water Technologies (Barsbüttel, Germany). Sulphuric acid and formic acid were purchased from Th. Geyer (Renningen, Germany). Ammonia was purchased from Merck (Darmstadt, Germany), and ammonium formate, used as an additive for LC-MS solvents, was obtained from Fluka (Steinheim, Germany).

4.2. Sampling

In total, 305 samples were provided by German spice and herb suppliers associated to the Association of the German Spice Industry (Table S1). The samples covered 15 spice and culinary herb matrices originating from 36 countries. They were harvested within the years 2014 to 2018, with a majority of samples originating from 2016 and 2017. Where possible, sampling was conducted according to European Commission Regulation No. 401/2006 concerning sampling and analysis of mycotoxins in foodstuff [30]. All samples were lyophilized and thus the weight of samples taken relied exclusively on dry matter.

4.3. Sample Pre-Treatment and Extraction

Each sample was homogenized to a particle size < 1 mm using a ZM 200 centrifugal mill from Retsch (Haan, Germany) and stored at 20 °C at a dry and dark place until analysis. Two independent biological replicates were generated from each sample according to an already described extraction procedure [18]: In brief, 40 mL of sulphuric acid (0.05 mol/L) were added to 2.0 g of dry sample material and vortexed, treated in an ultra-sonic bath (10 min) and horizontally shaken (500 U/min, 20 min). After centrifugation for 10 min at 5000× *g*, the raw sample extract was filtered through a folded filter and 10 mL were loaded on a solid phase extraction (SPE) cartridge (Agilent Bond Elut Plexa PCX, 500 mg/6 mL, Santa Clara, CA, USA), preconditioned with 5 mL each of methanol and sulphuric acid (0.05 mol/L). After washing with 5 mL of water and 5 mL of methanol, the analytes were eluted into a glass vial using 10 mL of ammoniated methanol (5%). The eluates were dried under nitrogen at 50 °C and reconstituted in 1.0 mL of LC solvent A (water containing 0.1% formic acid and 5 mmol/L ammonium formate), shaken with a vortex laboratory shaker, and filtered into a glass vial using a 0.45 µm PVDF syringe filter (Berrytec, Grünwald, Germany).

4.4. LC-MS/MS Instrumentation and Software

For all measurements, a Shimadzu high performance liquid chromatography (HPLC) apparatus, including binary pumps, degasser, autosampler, column oven and control unit (LC-20AB, SIL-20AC HT, CTO-20AC, CBM-20A, Duisburg, Germany) was used. The HPLC was coupled to an API4000 triple quadrupole mass spectrometer (MS) from Sciex (Darmstadt, Germany). MS ion source parameters were set as follows: ionization voltage, 2.500 V; nebulizer gas, 50 psi; heating gas, 50 psi, curtain gas, 30 psi, temperature, 600 °C; collision gas level, 7. For data acquisition and processing, Analyst (Version 1.6.2, Sciex, Framingham, MA, USA) and MultiQuant software (Version 3.0.1, Sciex,

Framingham, MA, USA) were used. Figures were drawn using OriginPro software (Version 2019, OriginLab, Northampton, MA, USA).

4.5. Measurements and Quantification

The measurements were conducted according to KALTNER et al. [18]: A 150 × 2.1 mm Kinetex™ 5 µm CoreShell EVO C18 100 Å column protected by a SecurityGuard™ ULTRA EVO C18 2.1 mm guard column (both Phenomenex, Aschaffenburg, Germany) was used for chromatographic separation of PA/PANO analytes. Used solvents were water (A) and acetonitrile/water (95/5, v/v, B), each containing 0.1% formic acid and 5 mmol/L ammonium formate. The column oven temperature was maintained at 30 °C and 20 µL of sample extract were injected. The binary linear gradient conditions at a flow rate of 0.4 mL/min were: 0.0 min 2% B, 10.0 min 9.5% B, 15.0 min 39% B, 15.1 min 100% B, 16.5 min 100% B and an additional re-equilibration of 6.5 min prior to each run.

The quantification was performed via external matrix matched calibration, or, in case of chive, curry and herbs of Provence samples, via standard addition, respectively. Aliquots of the PA/PANO mix standard solution were pipetted into glass vials, dried under nitrogen at 50 °C and reconstituted with extracts of a suitable blank sample to prepare calibration standards at concentrations of 0, 1.0, 5.0, 10.0, 25.0 and 50.0 ng/mL. MultiQuant software was used to calculate the respective concentrations in a sample vial applying linear regression on the calibration standards' peak areas. The matrix matched calibration standards were freshly prepared for each measurement day. LOD ranged from <0.1 µg/kg (LcN) to 2.6 µg/kg (Mk). If the content calculated for an individual compound was between the LOD and the limit of quantification (LOQ), it was calculated with 0.5 times the LOQ. Amounts smaller than the LOD were considered as '0.0 µg/kg' for calculation reasons. Overall, recoveries ranged from 50% (Sp) to 119% (Im) for 40 of 44 PA/PANO compounds. All quantitation results were related to dry matter and were not corrected for recovery rates.

4.6. Risk Assessment

For the evaluation of the risk of a short-term (acute) intake of PA/PANO from spices and culinary herbs, two exemplary recipes containing herbs were assumed. Recipe 1 'Mediterranean pesto' (https://www.kraeuter-buch.de/magazin/schmackhaftes-kraeuterpesto-zum-selber-machen-35.html, accessed on 15 January 2020) contained 10 g of thyme as well as 15 g of oregano and basil in 250 mL/200 g of the final pesto, resulting in 2.5 g of thyme and 3.75 g of oregano and basil per portion of 50 g pesto. Recipe 2 'tomato sauce' originated from a recipe book and was also used by the BfR in an earlier study [17]. Here, 2 g of oregano were assumed to be used for the whole recipe (= 3 portions), resulting in 0.67 g of oregano per portion. For comparison reasons, children were estimated to consume the same culinary herbs used in both recipes, but the ingested amounts of the singular herbs were taken form a special nutrition study for two- to five-years aged children [20]. Within this list, the consumption data of raw, unprocessed spices and herbs were used.

The risk of an acute non-carcinogenic health damage was assessed on the basis of a HBGV which has been derived from a NOAEL of 10 µg/kg BW/d from a 2-year gavage study with riddelliine in rats [19]. The HBGV as a preliminary orientation value was quoted with 0.1 µg/kg BW/d (NOAEL/100) for the sum of PA/PANO. The median and mean contents as well as the 95% percentile (P95) of the sum contents of the herbs basil, oregano and thyme were used (see Table 2).

For evaluating the risk of a long-term (chronic) PA/PANO intake, the MOE approach was used, based on a $BMDL_{10}$ of 237 µg/kg BW/d for the sum of PA/PANO intake. Therefore, all 305 investigated samples were grouped into four subgroups, according to their PA/PANO sum contents: <LOD, low content (LOD—100 µg/kg), medium content (100–1000 µg/kg) and high content (>1000 µg/kg). The means and medians of each group were calculated, rounded and used as representative contents for a low, medium and high-risk contamination group. In case of adults, data on the consumption of spices and culinary herbs (in g/kg BW/d) were taken from Table 3 from [17]; originally derived from the German National Nutrition Survey II [22]. For children, an average daily intake of herbs of 0.7 g was taken into account [20], and for the high consumption scenario of children, two times the average intake was suggested.

Consumption of spices and culinary herbs for the calculation of both the risk due to acute and the chronic exposure were related to an adult of 70 kg BW as proposed by EFSA [31]. In case of children, data were related to 16 kg BW as proposed by [20].

To consider the use of fresh or dried herbs for the exemplary recipes and the long-term intake of PA/PANO, the calculations were conducted with and without dehydration factors, respectively, as recommended by the ESA [21].

Supplementary Materials: The following what are available online at www.mdpi.com/2072-6651/12/3/155/s1, Table S1: Contents of pyrrolizidine alkaloids (PA) and PA-*N*-oxides in 305 investigated spice and culinary herb samples.

Author Contributions: Conceptualization, F.K. and C.G.; investigation, F.K.; data curation, F.K.; writing—original draft preparation, F.K.; writing—review and editing, M.R., M.G. and C.G.; visualization, F.K.; supervision, C.G.; project administration, C.G.; funding acquisition, C.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Boppré, M. The ecological context of pyrrolizidine alkaloids in food, feed and forage: An overview. *Food Addit. Contam. A Chem.* **2011**, *28*, 260–281.
2. Hartmann, T.; Witte, L. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. In *Alkaloids: Chemical and Biological Perspectives*; Pelletier, S.W., Ed.; Elsevier: Amsterdam, The Netherlands, 1995; Volume 9, pp. 155–233.
3. Smith, L.W.; Culvenor, C.C.J. Plant sources of hepatotoxic pyrrolizidine alkaloids. *J. Nat. Prod.* **1981**, *44*, 129–152.
4. Wiedenfeld, H. Plants containing pyrrolizidine alkaloids: Toxicity and problems. *Food Addit. Contam. A Chem.* **2011**, *28*, 282–292.
5. Ruan, J.; Yang, M.; Fu, P.; Ye, Y.; Lin, G. Metabolic activation of pyrrolizidine alkaloids: Insights into the structural and enzymatic basis. *Chem. Res. Toxicol.* **2014**, *27*, 1030–1039.
6. Robertson, J.; Stevens, K. Pyrrolizidine alkaloids: Occurrence, biology, and chemical synthesis. *Nat. Prod. Rep.* **2017**, *34*, 62–89.
7. Neuman, M.G.; Cohen, L.B.; Opris, M.; Nanau, R.; Jeong, H. Hepatotoxicity of pyrrolizidine alkaloids. *J. Pharm. Pharm. Sci.* **2015**, *18*, 825–843.
8. Edgar, J.A.; Colegate, S.M.; Boppré, M.; Molyneux, R.J. Pyrrolizidine alkaloids in food: A spectrum of potential health consequences. *Food Addit. Contam. A Chem.* **2011**, *28*, 308–324.
9. Kakar, F.; Akbarian, Z.; Leslie, T.; Mustafa, M.L.; Watson, J.; Van Egmond, H.P.; Omar, M.F.; Mofleh, J. An outbreak of hepatic veno-occlusive disease in Western Afghanistan associated with exposure to wheat flour contaminated with pyrrolizidine alkaloids. *J. Toxicol.* **2010**, *2010*, 313280.
10. EFSA (European Food Safety Authority). Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. *EFSA J.* **2017**, *15*, doi:10.2903/j.efsa.2017.4908.
11. Lucchetti, M.A.; Glauser, G.; Kilchenmann, V.; Dubecke, A.; Beckh, G.; Praz, C.; Kast, C. Pyrrolizidine alkaloids from Echium vulgare in honey originate primarily from floral nectar. *J. Agr. Food Chem.* **2016**, *64*, 5267–5273.

12. Selmar, D.; Wittke, C.; Beck-von Wolffersdorff, I.; Klier, B.; Lewerenz, L.; Kleinwächter, M.; Nowak, M. Transfer of pyrrolizidine alkaloids between living plants: A disregarded source of contaminations. *Environ. Pollut.* **2019**, *248*, 456–461.
13. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. *Off. J. Eur. Communities L.* **2002**, *140*, 10–22.
14. June 2019 Stakeholder Update on Rapidly Developing Policy on Food Contaminants. 2019 29.09.2019. Available online: https://www.food.gov.uk/news-alerts/consultations/june-2019-stakeholder-update-on-rapidly-developing-policy-on-food-contaminants (accessed 12 January 2020).
15. BfR (Federal Institute for Risk Assessment). *Opinion No. 030/2016: Pyrrolizidine Alkaloids: Levels in Foods should Continue to be Kept as Low as Possible;* BfR: Berlin, Germany, 2016. https://www.bfr.bund.de/cm/349/pyrrolizidine-alkaloids-levels-in-foods-should-continue-to-be-kept-as-low-as-possible.pdf (accessed on 01 February 2020).
16. Picron, J.F.; Herman, M.; Van Hoeck, E.; Goscinny, S. Analytical strategies for the determination of pyrrolizidine alkaloids in plant based food and examination of the transfer rate during the infusion process. *Food Chem.* **2018**, *266*, 514–523.
17. BfR (Federal Institute for Risk Assessment). *Opinion No. 017/2019: Pyrrolizidine Alkaloid Levels in Dried and Deep-Frozen Spices and Herbs too High*; BfR: Berlin, Germany, 2019. https://www.bfr.bund.de/cm/349/pyrrolizidine-alkaloid-levels-in-dried-and-deep-frozen-spices-and-herbs-too-high.pdf (accessed on 01 February 2020).
18. Kaltner, F.; Stiglbauer, B.; Rychlik, M.; Gareis, M.; Gottschalk, C. Development of a Sensitive Analytical Method for Determining 44 Pyrrolizidine Alkaloids in Teas and Herbal Teas via LC-ESI-MS/MS. *Anal. Bioanal. Chem.* **2019**, *411*, 7233–7249.
19. NTP (National Toxicology Program). Toxicology and Carcinogenesis Studies of Riddelliine. In *National Toxicology Program Technical Report 508*; NTP: Washington, DC, USA, 2003. https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr508.pdf?utm_source=direct&utm_medium=prod&utm_campaign=ntpgolinks&utm_term=tr508 (accessed on 01 February 2020).
20. Banasiak, U.; Heseker, H.; Sieke, C.; Sommerfeld, C.; Vohmann, C. Estimation of the dietary intake of pesticide residues based on new consumption data for children. *Bundesgesundheitsbla* **2005**, *48*, 84–98.
21. ESA (European Spice Association). Forum—European Spice Association recommends dehydration factors to assess pesticide residues on products of the spice industry. *J. Consum. Prot. Food S.* **2008**, *3*, 474–478.
22. MRI (Max Rubner Institut). *Nationale Verzehrsstudie II: Lebensmittelverzehr und Nährstoffzufuhr auf Basis von 24h-Recalls (German National Nutrition Survey II: Food Consumption and Nutrient Intake on the Basis of 24h Recalls);* MRI: Karlsruhe, Germany, 2008. https://www.mri.bund.de/de/institute/ernaehrungsverhalten/forschungsprojekte/nvsii/ (accessed on 01 February 2020).
23. Kapp, T.; Hägele, F.; Plate, E.-M. Oregano—An Aromatic, but Rich in Contente Culinary Herb. Part I: Pyrrolizidine Alkaloids. Available online: http://www.ua-bw.de/pub/beitrag.asp?subid=1&Thema_ID=5&ID=3008&lang=DE&Pdf=No (accessed 20 January 2020).
24. Flade, J.; Beschow, H.; Wensch-Dorendorf, M.; Plescher, A.; Wätjen, W. Occurrence of nine pyrrolizidine alkaloids in *Senecio vulgaris L.* depending on developmental stage and season. *Plants* **2019**, *8*, 54.
25. ESA (European Spice Association). *European Spice Association—Quality Minima Document*; ESA: Bonn, Germany; Brussels, Belgium, 2015, https://www.esa-spices.org/index-esa.html/publications-esa (accessed on 01 February 2020).
26. Merz, K.H.; Schrenk, D. Interim Relative Potency Factors for the Toxicological Risk Assessment of Pyrrolizidine Alkaloids in Food and Herbal Medicines. *Toxicol. Lett.* **2016**, *263*, 44–57.
27. Dusemund, B.; Nowak, N.; Sommerfeld, C.; Lindtner, O.; Schäfer, B.; Lampen, A. Risk assessment of pyrrolizidine alkaloids in food of plant and animal origin. *Food Chem. Toxicol.* **2018**, *115*, 63–72.
28. Chen, L.; Mulder, P.P.J.; Louisse, J.; Peijnenburg, A.; Wesseling, S.; Rietjens, I.M.C.M. Risk assessment for pyrrolizidine alkaloids detected in (herbal) teas and plant food supplements. *Regul. Toxicol. Pharm.* **2017**, *86*, 292–302.
29. Chen, L.; Mulder, P.P.J.; Peijnenburg, A.; Rietjens, I.M.C.M. Risk assessment of intake of pyrrolizidine alkaloids from herbal teas and medicines following realistic exposure scenarios. *Food Chem. Toxicol.* **2019**, *130*, 142–153.

30. Commission Regulation (EC). No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (Text with EEA relevance). *Off. J. Eur. Communities L.* **2006**, *70*, 12–34.
31. EFSA (European Food Safety Authority). Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. *EFSA J.* **2012**, *10*, 2579.