WHO FOOD ADDITIVES SERIES: 71-S2

Prepared by the eightieth meeting of the
Joint FAO/WHO Expert Committee
on Food Additives (JECFA)

Safety evaluation of certain food additives and contaminants

Supplement 2:

Pyrrolizidine alkaloids

Food and Agriculture Organization of the United Nations

World Health Organization

WHO FOOD ADDITIVES SERIES: 71-S2

Prepared by the eightieth meeting of the
Joint FAO/WHO Expert Committee
on Food Additives (JECFA)

Safety evaluation of certain food additives and contaminants

Supplement 2:

Pyrrolizidine alkaloids

World Health Organization, Geneva, 2020

Food and Agriculture Organization of the United Nations

World Health Organization

Safety evaluation of certain food additives and contaminants: prepared by the eighteenth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Supplement 2: Pyrrolizidine alkaloids

(WHO Food Additives Series, No. 71-S2)

ISBN (WHO) 978-92-4-001267-7 (electronic version)

ISBN (WHO) 978-92-4-001268-4 (print version)

ISBN (FAO) 978-92-5-133522-2

ISSN 0300-0923

Suggested citation. Safety evaluation of certain food additives and contaminants: prepared by the eighteenth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Supplement 2: Pyrrolizidine alkaloids. Geneva: World Health Organization and Food and Agriculture Organization of the United Nations; 2020 (WHO Food Additives Series, No. 71-S2). Licence: CC BY-NC-SA 3.0 IGO.

Cataloguing-in-Publication (CIP) data. CIP data are available at http://apps.who.int/iris.

CONTENTS

PREFACE

The monograph contained in this volume was prepared following the eightieth meeting of the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), which met at FAO headquarters in Rome, Italy, from 16 to 25 June 2015. This monograph summarizes the data on one contaminant group reviewed by the Committee. Monographs on seven food additive groups and another contaminant group discussed at the meeting have been published in WHO Food Additives Series 71 and a separate supplement in WHO Food Additive Series 71, respectively.

The eightieth report of JECFA has been published by WHO as WHO Technical Report No. 995. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 2 of the present publication.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives, the Codex Committee on Contaminants in Food and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and dietary exposure and toxicological monographs, such as that contained in this volume, on substances that they have considered.

The monograph contained in this volume is based on a working paper that was prepared by JECFA experts. A special acknowledgement is given at the beginning of the monograph to those who prepared this working paper.

Special acknowledgement is due to the Dutch National Institute for Public Health and the Environment (RIVM) for the tremendous contribution in preparing this evaluation following a systematic review approach. Grateful thanks are also extended to Patrick Mulder, Wageningen Food Safety Research, Wageningen University & Research, the Netherlands, for all his work preparing the structural formulae of the chemical compounds mentioned in this monograph, to Coen Graven, Linda Razenberg, Wim Mennes and Jeanine Ridder of the RIVM, and Rob de Vries, Systematic Review Center for Laboratory Animal Experimentation (SYRCLE), Radboud University Medical Centre, the Netherlands, for their contribution to the preparation of this monograph. Editorial assistance was provided by Susan Kaplan.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the organizations participating in WHO concerning the legal status of any country, territory, city or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the organizations in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological or toxicological properties of or dietary exposure to the compounds evaluated in this publication should be addressed to: WHO Joint Secretary of the Joint FAO/WHO Expert Committee on Food Additives, Department of Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

Pyrrolizidine alkaloids

First draft prepared by
Astrid Bulder,[1] Antonio Agudo,[2] Diane Benford,[3] Scott Blechinger,[4] Peter John Cressey,[5] Lianne de Wit,[1] John Alexander Edgar,[6] Mark Feeley,[4] Tracy Hambridge,[7] Suzanne Jeurissen,[1] Jennifer Ratcliffe,[8] Leah Rosenfeld,[9] Klaus Schneider,[10] Gary Williams,[11] Gerrit Wolterink[1] and Monique de Nijs[12]

[1] National Institute for Public Health and the Environment (RIVM), the Netherlands
[2] Catalan Institute of Oncology (ICO), L'Hospitalet de Llobregat, Spain
[3] Food Standards Agency, London, England
[4] Bureau of Chemical Safety, Food Directorate, Health Canada, Ottawa, Canada
[5] Institute of Environmental Science and Research, Christchurch, New Zealand
[6] Commonwealth Scientific and Industrial Research Organization (CSIRO) Food and Nutritional Sciences, NSW, Australia
[7] Food Standards Australia New Zealand (FSANZ), Canberra, Australia
[8] ILS Inc., Durham, USA
[9] Center for Food Safety and Applied Nutrition (CFSAN), United States Food and Drug Administration, College Park, USA
[10] Forschungs- und Beratungsinstitut Gefahrstoffe GmbH (FoBiG), Freiburg, Germany
[11] Pathology and Clinical Public Health, New York Medical College, Valhalla, USA
[12] Wageningen Food Safety Research, Wageningen University & Research, the Netherlands

1. Explanation

1.1 Introduction

Pyrrolizidine alkaloids (PAs) are toxins produced by an estimated 6000 plant species. More than 600 different PAs, mainly 1,2-unsaturated PAs, including their associated nitrogen oxides (*N*-oxides) are known, and new PAs continue to

be identified in both new and previously studied plant species. The main plant sources are the families Boraginaceae (all genera), Asteraceae (tribes Senecioneae and Eupatorieae) and Fabaceae (genus *Crotalaria*). Different plant species in these families produce characteristic mixtures of 1,2-unsaturated PAs and their saturated analogues and varying amounts of their corresponding *N*-oxides. The PAs present in these plants are esters of pyrrolizidine diols. The pyrrolizidine moieties are referred to as necines, and the esterifying acids involved are necic acids. These PAs can be classified as open-chain monoesters, open-chain diesters and macrocyclic diesters.

The pyrrolizidine ring system consists of two fused, five-membered rings with a nitrogen atom at the bridgehead. Pyrrolizidines and PAs[1] are, by definition, fully saturated and have no double bonds. However, the term "saturated PA" is sometimes used to emphasize the fact that there are no double bonds present. The terms "1,2-unsaturated" or "1,2-dehydro" PAs indicate that the alkaloids being referred to are modified PAs having a double bond between carbons 1 and 2. The term "free base" means that the nitrogen lone pair electrons on the alkaloids are not protonated by acids or oxidized to *N*-oxides. The *N*-oxide forms of PAs occur naturally together with the PA parent molecules.

In this monograph, the term "PAs" used by itself refers to saturated and 1,2-unsaturated PAs and their associated *N*-oxides and the term "1,2-unsaturated PAs" refers to all 1,2-unsaturated PAs and their associated *N*-oxides. An overview of the structural formulae of all PAs mentioned in this monograph is provided in Fig. 1.

PAs have not been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), but they have been evaluated by a World Health Organization (WHO) Task Group on Environmental Health Criteria for Pyrrolizidine Alkaloids (coordinated by the International Programme on Chemical Safety (IPCS)) in 1988 and the International Agency for Research on Cancer (IARC) in 1976, 1983, 1987 and 2002. IPCS concluded that, based on animal data, a potential cancer risk for humans should be seriously considered; however, as no information was found on the long-term follow-up of humans exposed to PAs, it was not possible to make an evaluation of the cancer risk of PAs for humans. IARC evaluated several PAs and classified lasiocarpine, monocrotaline and riddelliine as Group 2B (possibly carcinogenic to humans) and hydroxysenkirkine, isatidine, jacobine, retrorsine, seneciphylline, senkirkine and symphytine as Group 3 (not classifiable as to their carcinogenicity to humans).

The Fifth Session of the Codex Committee on Contaminants in Foods (CCCF) discussed PAs and concluded that significant new information had become available since the evaluation by WHO-IPCS in 1988, warranting an

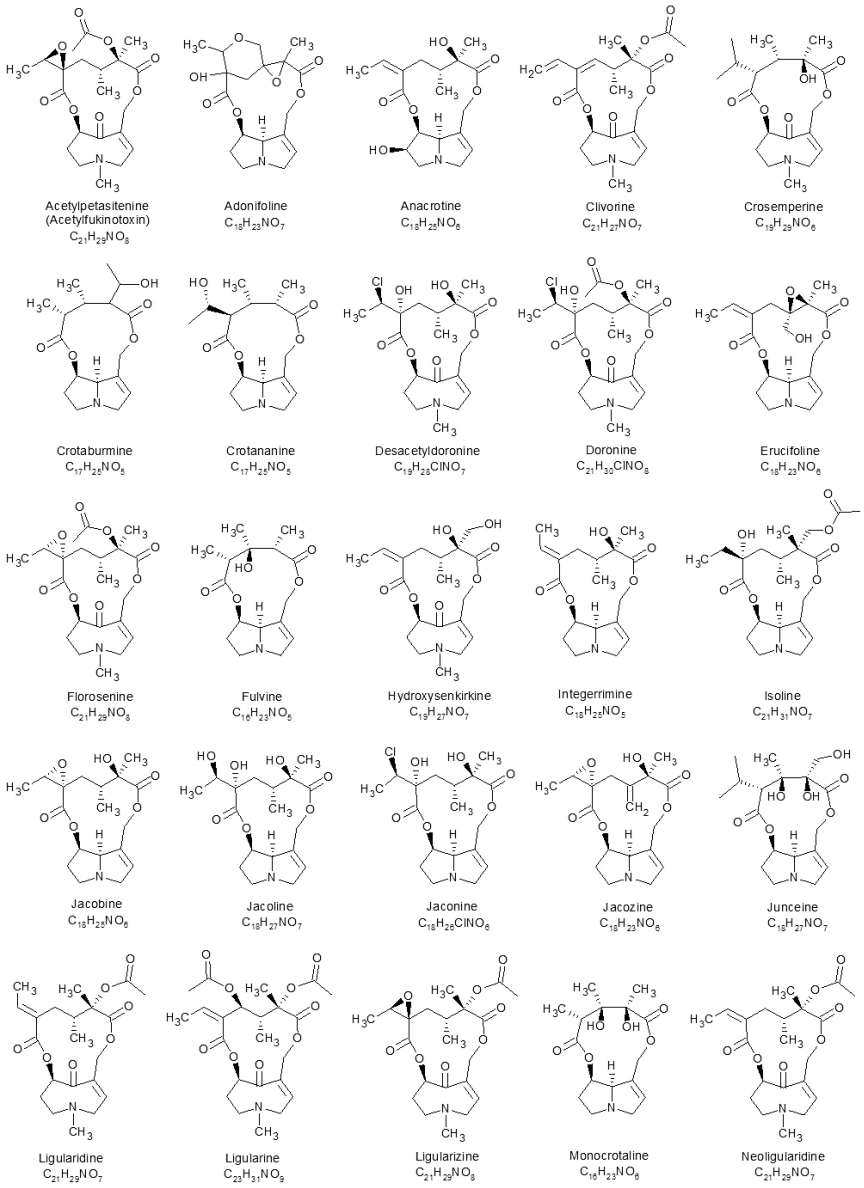
[1] The term "alkaloid" (alkali-like) refers to naturally occurring plant secondary chemicals with a basic nitrogen atom.

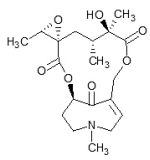
Fig. 1

The structural formulae of all PAs mentioned in this monograph

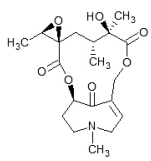
(1,2-dehydropyrrolizidine alkaloids)

A. Macrocyclic diesters

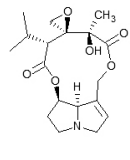




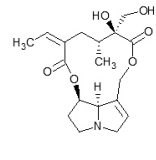
Otosenine
$C_{18}H_{27}NO_7$



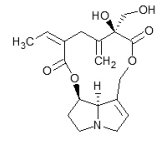
Petasitenine (Fukinotoxin)
$C_{18}H_{27}NO_7$



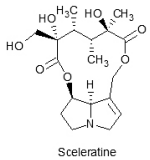
Pumiline A
$C_{18}H_{25}NO_6$



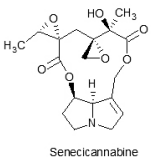
Retrorsine
$C_{18}H_{25}NO_6$



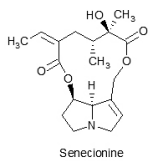
Riddelliine
$C_{18}H_{23}NO_6$



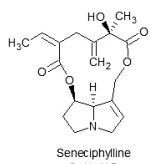
Sceleratine
$C_{18}H_{27}NO_7$



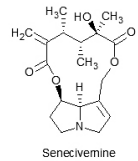
Senecicannabine
$C_{18}H_{23}NO_7$



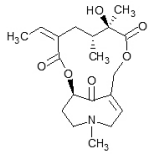
Senecionine
$C_{18}H_{25}NO_5$



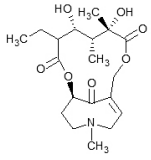
Seneciphylline
$C_{18}H_{23}NO_5$



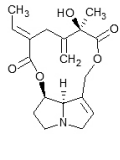
Senecivernine
$C_{18}H_{25}NO_5$



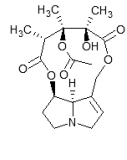
Senkirkine
$C_{19}H_{27}NO_8$



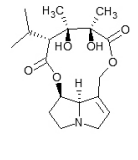
Synellesine
$C_{19}H_{25}NO_7$



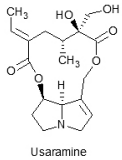
Spartioidine
$C_{18}H_{23}NO_5$



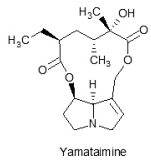
Spectabiline
$C_{18}H_{25}NO_7$



Trichodesmine
$C_{18}H_{27}NO_6$

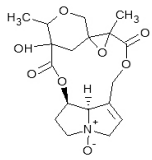


Usaramine
$C_{18}H_{25}NO_6$

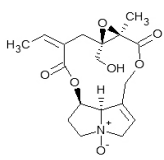


Yamataimine
$C_{18}H_{27}NO_5$

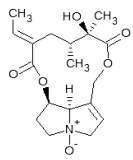
B. Macrocyclic diesters *N*-oxides



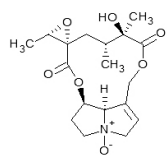
Adonifoline N-oxide
$C_{18}H_{23}NO_8$



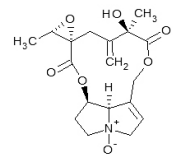
Erucifoline N-oxide
$C_{18}H_{23}NO_7$



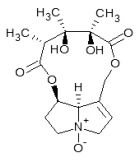
Integerrimine N-oxide
$C_{18}H_{25}NO_6$



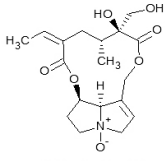
Jacobine N-oxide
$C_{18}H_{25}NO_7$



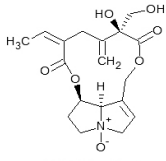
Jacozine N-oxide
$C_{18}H_{23}NO_7$



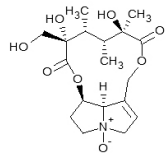
Monocrotaline N-oxide
$C_{18}H_{23}NO_7$



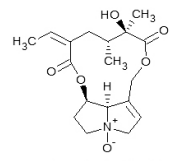
Retrorsine N-oxide (Isatidine)
$C_{18}H_{25}NO_7$



Riddelliine N-oxide
$C_{18}H_{23}NO_7$

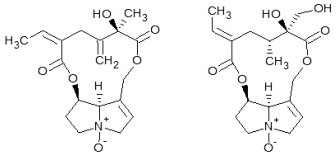


Sceleratine N-oxide
$C_{18}H_{27}NO_8$

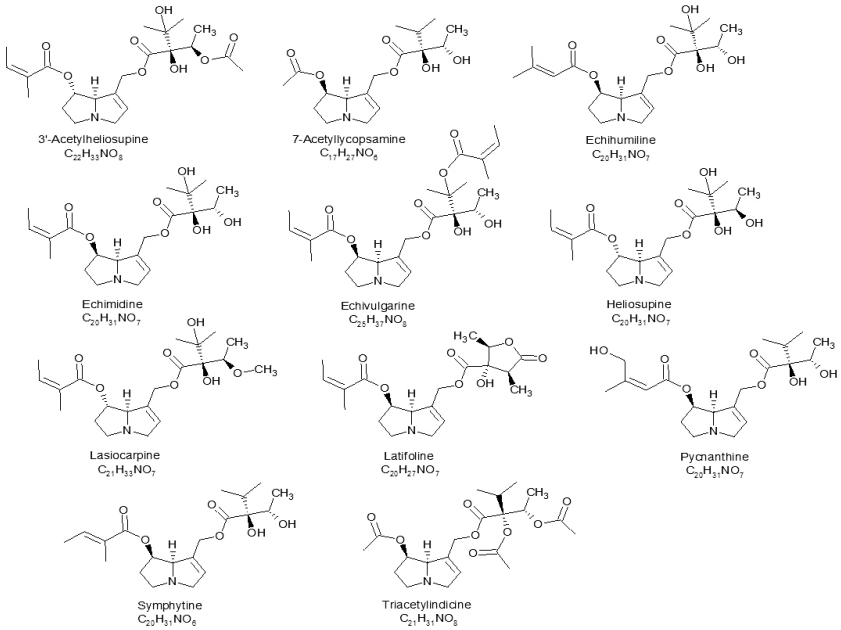


Senecionine N-oxide
$C_{18}H_{25}NO_6$

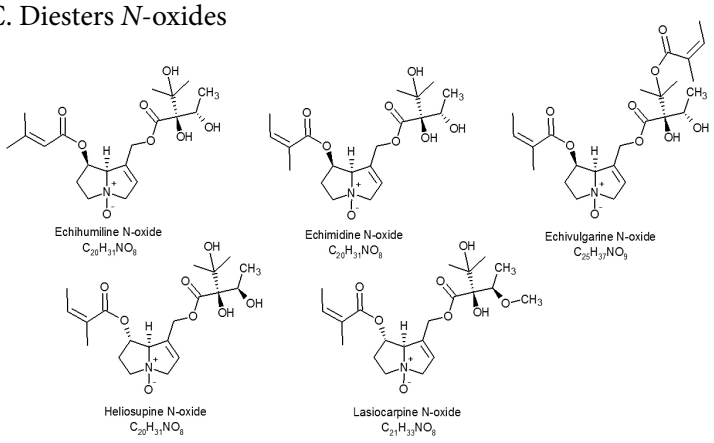
Fig. 1 (continued)



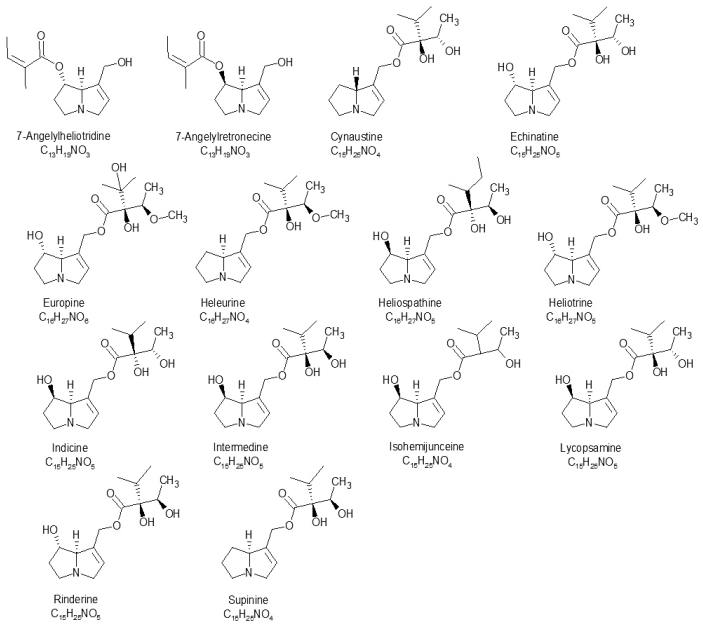
Diesters



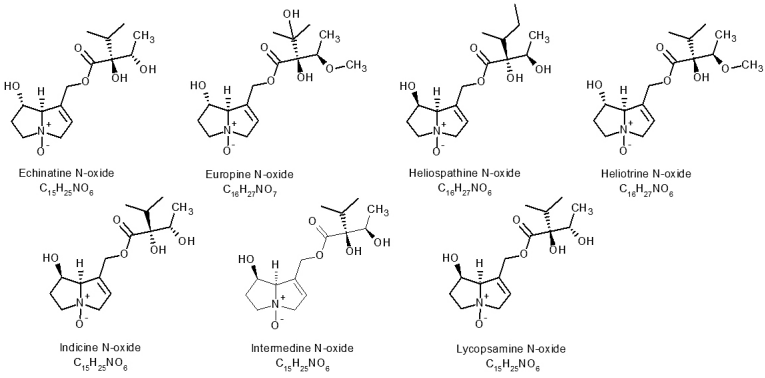
C. Diesters *N*-oxides



D. Monoesters



E. Monoesters *N*-oxides



(Saturated pyrrolizidine alkaloids)

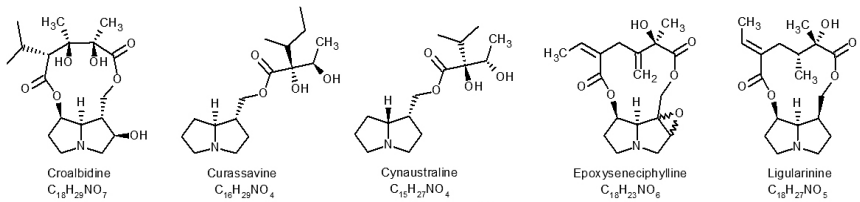
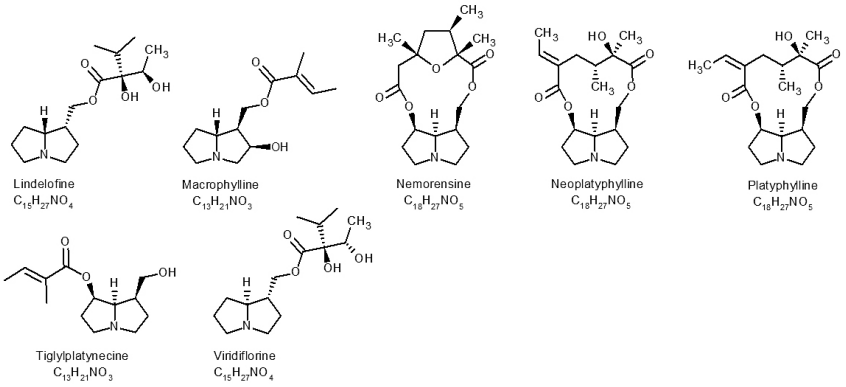
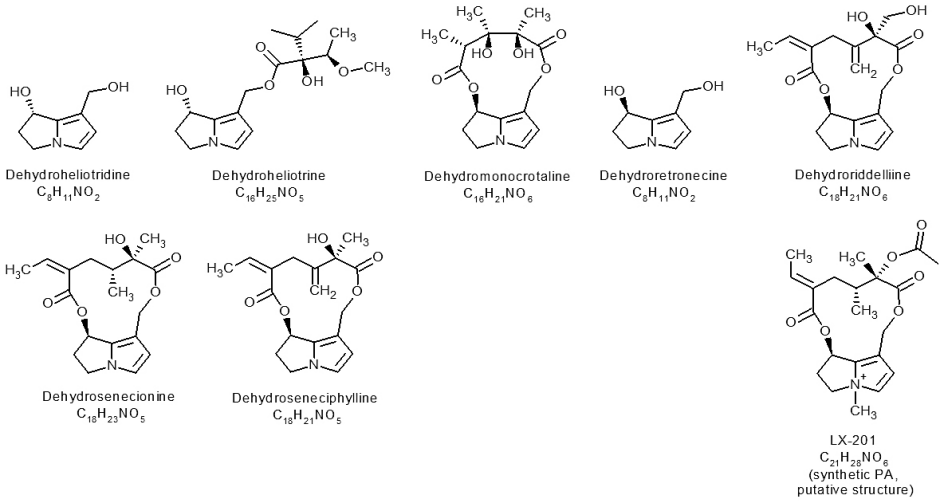


Fig. 1 (continued)

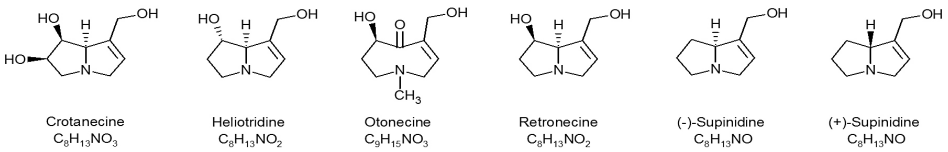


(Pyrrolic metabolites (6,7-didehydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine metabolites) and synthetic pyrrolic derivatives)

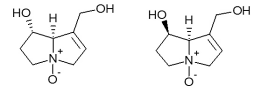


(Necines)

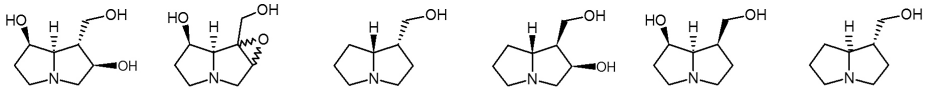
A. 1,2-dehydronecines



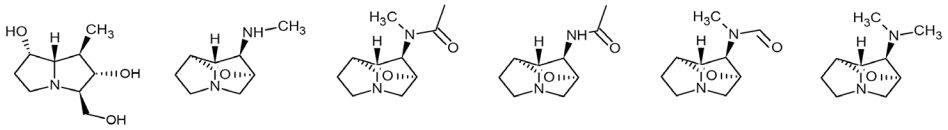
B. 1,2-dehydronecines *N*-oxides



C. Saturated necines



D. Lolines and other pyrrolizidine alkaloids



updated assessment by JECFA. CCCF therefore placed PAs on its priority list and requested JECFA to:

- perform a full risk assessment;
- identify the most relevant PAs (in terms of both occurrence and toxicity) for human health;
- identify data gaps; and
- consider PAs in feed, as PAs can transfer from feed to animal products.

1.2 Systematic review approach

A systematic review (SR) approach was used to gather data for the biochemical and toxicological aspects of the evaluation. An SR is a literature review aimed at identifying, selecting, evaluating, interpreting and synthesizing all available studies relevant to a particular research question by means of prespecified and standardized methods. The aim of an SR is to minimize bias and increase the transparency, objectivity and reproducibility of assessments. It is performed as sequential steps: literature search, selection based on title/abstract, full text

selection, data extraction from studies, quality assessment of studies, synthesizing included data, presenting data and results, and interpreting results and drawing conclusions. The process uses a predefined protocol with specified selection and quality criteria. The method has been used in human health research, mainly on narrow clinical and epidemiological questions, and is being implemented increasingly for questions related to animal toxicity. It was tested in this JECFA evaluation of PAs to determine its usefulness in a broad risk assessment.

The SR methodology was applied to find all relevant literature addressing the topics as described in 1.1 Explanation. The methodology used followed the guidance published by the European Food Safety Authority (EFSA, 2010) and the National Toxicology Program (NTP) of the United States of America (USA) (NTP, 2015). The steps taken in the current SR will be discussed briefly here and the protocol can be found in Annex 4.

1.2.1 Objective

The main objective of the SR was to identify all relevant literature necessary for performing a full human health risk assessment on PAs. In addition, literature on PAs occurring in feed was identified, since PAs can be transferred from feed to food via animals. Data gaps were also identified.

1.2.2 Research questions

To translate the objectives into research questions, several key elements were first identified, in accordance with the EFSA and NTP guidance (EFSA, 2010; NTP, 2015). For the WHO part of the evaluation, the key elements – population (P), exposure (E), comparator (C) and outcome (O) – were identified for the topics biochemical aspects and toxicology separately. In addition, the key element P was split into in vitro systems, animals and humans. This led to six research questions, the so-called PEO questions (since the comparator (C) could not be defined), and thereby six separate SRs, to obtain all relevant data on the biochemistry and toxicology of PAs (Table 1). These PEO questions formed the starting point for defining search terms to use in the SRs. The search terms were identified in close collaboration with an information specialist, and were found by consulting the Medical Subject Headings (MeSH) database of MEDLINE[*1] and (the keywords of) known publications about PAs, including the discussion paper on PAs (prepared for the CCCF5, 2011) and the IPCS report on PAs (1988). The search terms that were identified are presented in the protocol (Annex 4).

For the FAO part of the evaluation, the same approach was tried to cover all FAO topics. However, performing one or more SRs for this part was

[1] The search strategy was first developed for a MEDLINE® search and thereafter adjusted to be applicable for other databases. Hence, the MeSH database was used for finding search terms.

Table 1
Defined research questions for SRs for biochemical, toxicological and epidemiological parts

Biochemical aspects
1. What are the biochemical aspects of pyrrolizidine alkaloids in in vitro systems?
P = in vitro systems
E = pyrrolizidine alkaloids
C = *not defined*
O = biochemical aspects
2. What are the biochemical aspects of pyrrolizidine alkaloids in animals?
P = animals
E = pyrrolizidine alkaloids
C = *not defined*
O = biochemical aspects
3. What are the biochemical aspects of pyrrolizidine alkaloids in humans?
P = humans
E = pyrrolizidine alkaloids
C = *not defined*
O = biochemical aspects
Toxicology
4. What are the toxic effects of pyrrolizidine alkaloids in in vitro systems?
P = in vitro systems
E = pyrrolizidine alkaloids
C = *not defined*
O = toxic effects
5. What are the toxic effects of pyrrolizidine alkaloids in animals?
P = animals
E = pyrrolizidine alkaloids
C = *not defined*
O = toxic effects
6. What are the toxic effects of pyrrolizidine alkaloids in humans?
P = humans
E = pyrrolizidine alkaloids
C = *not defined*
O = toxic effects

not feasible as the search could not be made specific and sensitive enough. The search strategy used to obtain literature for the topics belonging to the FAO part is described further below.

1.2.3 Search strategy systematic review

The search terms identified for research questions 1–6 were subsequently used for the development of a search strategy. To this end, first the information sources

to be searched were identified. These sources can be divided into databases with (peer-)reviewed scientific published literature and sources of grey literature. The first group included MEDLINE (via OvidSP and via PubMed), SCOPUS, Scisearch (via Web of Science), EMBASE, Toxcenter, CABA and FSTA. Grey literature was found via several entries (see protocol in Annex 4), including specific grey literature search systems, websites of institutes and toxicological databases.

The search strategy for databases with (peer-)reviewed published scientific literature was first developed for MEDLINE (via OvidSP) and then adjusted for the other databases. The identified search terms for P, E and O were combined together with the Boolean operator AND, whereas the identified search terms within P, E and O were combined with OR. The search strategy for MEDLINE, which was adapted for the other databases, can be found in the protocol in Annex 4. All results of the search in databases with (peer-)reviewed published scientific literature were captured in separate EndNote™ databases per database consulted and per research question.[1]

The search strategy for sources of grey literature consisted of only searching on the term "pyrrolizidine alkaloids" (or synonyms/other languages), plant names and the individual PAs owing to practical limitations of the data sources. The grey literature search was therefore not based on a specific research question, and the results of the search were manually checked and assigned, if applicable, to the relevant research question. Due to limited resources during the process, the data sources were divided into databases and websites of high priority and low priority based on expectations as to which data sources were most likely to contain relevant literature. In the end, only the data sources with high priority were searched, because searching a selection of the low priority data sources with the term "pyrrolizidine alkaloids" (or synonyms/other languages) did not yield additional relevant information. The data sources of high priority were searched with the term "pyrrolizidine alkaloids" followed by nine PA names (representing the four PA bases: angelylpetasinecine, echimidine, heliotrine, intermedine, jacobine, monocrotaline, platynecine, retrorsine, senecionine) and three plant names (Comfrey, Gynura, Senecio) as a test. When there were no hits from any of these 12 names in a certain data source, this source was only searched with the general term "pyrrolizidine alkaloids". All search results obtained via the grey literature search strategy were gathered as pdfs, and recorded using a predefined outline.

[1] In addition, references were found that could not automatically be assigned to one of the three populations. Therefore, so-called rest-databases were created and these were manually checked for relevant studies.

1.2.4 Inclusion and exclusion of studies

Literature searches were conducted in early 2015. All references obtained from the different databases were combined into an EndNote™ library corresponding to each review question, from which the duplicates were removed. The resulting EndNote™ libraries were then cleared of references published before 1988, references in which monocrotaline was only used as a tool to induce a certain condition, and of references in which crotaline is related to crotaline snakes rather than to PAs. It was recognized that this is actually part of the study selection process (see below); however, due to time constraints this approach was chosen as the most effective way to remove these references. The chances of introducing bias by following this approach are thought to be minimal.

The references in the cleaned EndNote™ libraries were then independently assessed by two reviewers for relevance based on title/abstract screening (TIAB). To facilitate this process, the web-based Early Review Organizing Software (EROS v2.0) was used. When there was a persistent disagreement between the two reviewers, a third reviewer decided whether a reference was to be included or excluded. The inclusion/exclusion criteria for references are presented in Table 2. If there was doubt whether a reference was relevant according to these criteria, it was included for full text retrieval, to allow further assessment. Besides these criteria, labels were also added to enable classification of the studies assessed. For example, when a reference in the database concerning toxicity in animals contained information specifically on genotoxicity, a label "genotoxicity" was added to this reference to facilitate identification of all studies on genotoxicity in one database at a later stage. Also, when a reference captured in the database that covered biochemical aspects in in vitro systems, properly speaking (or also) belonged to the database on toxicity in animals, that reference was excluded for that review question but the labels "toxicity" and "animals" were added to indicate that the reference was to be included in the other database. When a reference was not relevant for the WHO part of the evaluation but was relevant for the FAO part, the label "FAO" was added.

After screening the titles/abstracts for their relevance, full texts were retrieved for all included references for each review question. As working through six research questions proved to be very labour-intensive and there was insufficient time available to complete this process before the JECFA meeting, stages of the SR subsequent to the title/abstract selection were not performed according to the protocol, and full text selection was done using the critical appraisal method regularly used in the preparation of JECFA monographs. Further, as a double-check, the list of excluded references was rechecked to make sure no relevant articles were excluded.

Table 2
Inclusion and exclusion criteria per review question

Biochemical aspects
1. What are the biochemical aspects of pyrrolizidine alkaloids (PAs) in in vitro systems?
Language Inclusion: all languages Exclusion: –
Publication date Inclusion: from 1988 onwards Exclusion: before 1988
Type of study Inclusion: original research manuscripts, and review papers/reports, also including expert opinions Exclusion: narrative reviews that only summarize current status of knowledge
Type of exposure Inclusion: PAs (all exposure characteristics, e.g. dose, exposure routes, duration, formulation) Exclusion: studies not dealing with PAs, or studies in which a PA is used to induce a certain condition without the intention to look at the effects or the mode of action of the PA itself, or a study on crotaline in combination with venom
Type of population Inclusion: studies in in vitro systems, including ex vivo Exclusion: animal studies and all studies in humans, including e.g. case reports, epidemiological studies, clinical experience and anecdotal observations
Outcome measures Inclusion: biochemical aspects describing the kinetics, dynamics and transfer of PAs in the system Exclusion: outcomes other than biochemical aspects
2. What are the biochemical aspects of PAs in animals?
Language Inclusion: all languages Exclusion: –
Publication date Inclusion: from 1988 onwards Exclusion: before 1988
Type of study Inclusion: original research manuscripts, and review papers/reports, also including expert opinions Exclusion: narrative reviews that only summarize current status of knowledge
Type of exposure Inclusion: PAs (all exposure characteristics, e.g. dose, exposure routes, duration, formulation) Exclusion: studies not dealing with PAs, or studies in which a PA is used to induce a certain condition without the intention to look at the effects or the mode of action of the PA itself, or a study on crotaline in combination with venom
Type of population Inclusion: animal studies Exclusion: studies in in vitro systems, including ex vivo, and all studies in humans, including e.g. case reports, epidemiological studies, clinical experience and anecdotal observations

Outcome measures
Inclusion: biochemical aspects describing the kinetics, dynamics and transfer of PAs in the system
Exclusion: outcomes other than biochemical aspects

3. What are the biochemical aspects of PAs in humans?

Language
Inclusion: all languages
Exclusion: –

Publication date
Inclusion: from 1988 onwards
Exclusion: before 1988

Type of study
Inclusion: original research manuscripts, and review papers/reports, also including expert opinions
Exclusion: narrative reviews that only summarize current status of knowledge

Type of exposure
Inclusion: PAs (all exposure characteristics, e.g. dose, exposure routes, duration, formulation)
Exclusion: studies not dealing with PAs, or studies in which a PA is used to induce a certain condition without the intention to look at the effects or the mode of action of the PA itself, or a study on crotaline in combination with venom

Type of population
Inclusion: all studies in humans, including e.g. case reports, epidemiological studies, clinical experience and anecdotal observations
Exclusion: studies in in vitro systems, including ex vivo and animal studies

Outcome measures
Inclusion: biochemical aspects describing the kinetics, dynamics and transfer of PAs in the system
Exclusion: outcomes other than biochemical aspects

Toxicology

4. What are the toxic effects of PAs in in vitro systems?

Language
Inclusion: all languages
Exclusion: –

Publication date
Inclusion: from 1988 onwards
Exclusion: before 1988

Type of study
Inclusion: original research manuscripts, and review papers/reports, also including expert opinions
Exclusion: narrative reviews that only summarize current status of knowledge

Type of exposure
Inclusion: PAs (all exposure characteristics, e.g. dose, exposure routes, duration, formulation)
Exclusion: studies not dealing with PAs, or studies in which a PA is used to induce a certain condition without the intention to look at the effects or the mode of action of the PA itself, or a study on crotaline in combination with venom

Type of population
Inclusion: studies in in vitro systems, including ex vivo
Exclusion: animal studies and all studies in humans, including e.g. case reports, epidemiological studies, clinical experience and anecdotal observations

Outcome measures
Inclusion: studies describing the toxic effects of PAs in in vitro systems, including ex vivo
Exclusion: outcomes other than toxicity; beneficial effects. Studies in cell systems based on Drosophila or plants

Table 2 (continued)

5. What are the toxic effects of PAs in animals?
Language Inclusion: all languages Exclusion: –
Publication date Inclusion: from 1988 onwards Exclusion: before 1988
Type of study Inclusion: original research manuscripts, and review papers/reports, also including expert opinions Exclusion: narrative reviews that only summarize current status of knowledge
Type of exposure Inclusion: PAs (all exposure characteristics, e.g. dose, exposure routes, duration, formulation) Exclusion: studies not dealing with PAs, or studies in which a PA is used to induce a certain condition without the intention to look at the effects or the mode of action of that PA itself, or a study on crotaline in combination with venom
Type of population Inclusion: animal studies Exclusion: studies in in vitro systems, including ex vivo, and all studies in humans, including e.g. case reports, epidemiological studies, clinical experience and anecdotal observations
Outcome measures Inclusion: studies describing the toxic effects of PAs in animals Exclusion: outcomes other than toxicity; beneficial effects. Studies in *Drosophila*
6. What are the toxic effects of PAs in humans?
Language Inclusion: all languages Exclusion: –
Publication date Inclusion: from 1988 onwards Exclusion: before 1988
Type of study Inclusion: original research manuscripts, and review papers/reports, also including expert opinions Exclusion: narrative reviews that only summarize current status of knowledge
Type of exposure Inclusion: PAs (all exposure characteristics, e.g. dose, exposure routes, duration, formulation) Exclusion: studies not dealing with PAs, or studies in which a PA is used to induce a certain condition without the intention to look at the effects or the mode of action of the PA itself, or a study on crotaline in combination with venom
Type of population Inclusion: all studies in humans, including e.g. case reports, epidemiological studies, clinical experience and anecdotal observations Exclusion: studies in in vitro systems, including ex vivo, and animal studies
Outcome measures Inclusion: studies describing the toxic effects of PAs in humans Exclusion: outcomes other than toxicity; beneficial effects

Table 3
Overview of the number of studies retrieved in each step of the systematic review

	Raw results	Cleaned up, in EROS	TIAB inclusion	TIAB exclusion
Biochemistry in vitro	2 592	1 087	112	975
Biochemistry animal	3 704	1 463	95	1 368
Biochemistry human	1 989	1 067	20	1 047
Biochemistry total	8 285	3 617	227	3 390
Toxicity in vitro	3 175	1 312	222	1 090
Toxicity animal	5 011	2 094	457	1 637
Toxicity human	2 619	1 467	98	1 369
Toxicity total	10 805	4 873	777	4 096
Toxcenter	4 408	1 678	604	1 075
				Manual
Biochemistry rest	1 186	–	43	1 143
Toxicity rest	1 453	–	58	1 395
Overall total	26 137	10 168	**1 709**	1 1099
Update of the literature search	1 427	–	**57** [a]	1 370

EROS: Early Review Organizing Software, v2.0; TIAB: title/abstract screening.
[a] The number of references that were eventually included for full text retrieval and critical appraisal is shown in bold type.

The grey literature results were manually checked for relevance by two independent reviewers. Few of the grey literature results were assessed to be relevant for the monograph.

Table 3 gives an overview of the number of studies handled in each step of the SR. More than 10 000 references were identified for title/abstract screening and 1709 references were included for full text retrieval and critical appraisal. Owing to the longer than anticipated time needed for finalization of the monograph, an update of the literature was conducted in October/November 2016 via a quick literature search and manual assignment of possible relevant references to the different sections of the monograph. This led to inclusion of an additional 57 references for critical appraisal. Additionally, EFSA published an update of the risk characterization for PAs in 2017, which is summarized in section 9.

The Committee also revisited the evaluations reported by IPCS and EFSA during the meeting to determine whether any of the studies included could provide more information on the relative potency of PAs. Other studies were also

identified that were relevant to the assessment of PAs, but had not been included in either of these evaluations.

1.2.5 Search strategy: exposure assessment and risk management strategies

The Committee reviewed studies relevant to exposure assessment and strategies for risk management of PAs obtained through a search of peer-reviewed literature in PubMed and Web of Science. The literature search on the occurrence of and dietary exposure to PAs was run using three databases (Scopus, PubMed and Ovid) and a cut-off date of 1988.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Absorption

The IPCS evaluation (WHO-IPCS, 1988) concluded that absorption of PAs occurs, although no specific absorption studies in humans were available. The conclusion on absorption is based on studies investigating the tissue distribution after oral exposure and the excreted amounts of PAs and their metabolites in urine, faeces and bile of animals. In one study in rats, which used a crude mixture of PAs (*N*-oxides) from comfrey (*Symphytum officinale*), it was shown that oral absorption was about 20–50 times higher than dermal absorption. Furthermore, in vitro studies showed that PAs can transfer across isolated ileum and jejunum, but not the stomach.

The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) reviewed PAs in 2011 (EFSA, 2011) and concluded that they are readily absorbed following oral ingestion. Depending on the PA, peak plasma concentrations were reached within 30–70 minutes.

(i) In vitro studies

Hessel et al. (2011) reported that active transport appears to play a role in the uptake of PAs. Senecionine, but not its *N*-oxide, was transported from the apical to the basolateral compartment in Caco-2 cells. In addition, oxidation of senecionine as well as reduction of senecionine *N*-oxide was observed, indicating metabolism by Caco-2 cells.

In an abstract, Yang M et al. (2013) reported that five retronecine-type PA *N*-oxides (not further specified) appear to be less well absorbed in the intestinal tract than their parent PAs. The transporters involved in the active uptake have not been identified. Hessel et al. (2014) conducted an in vitro study to assess the passage of the monoester heliotrine, the open-chained diester echimidine and the cyclic diesters senecionine and senkirkine across small intestinal epithelium using differentiated Caco-2 cells. As measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS), the P-glycoprotein (P-gp) (also called ABCB1) efflux transporter transports the noncyclic PAs to the lumen of the intestine, but does not transport the cyclic PAs. The transfer rates from the apical to the basolateral compartments (i.e. the absorption) for cells exposed to 0.25 µM of each PA, were senecionine > senkirkine > heliotrine > echimidine; and, for transfer from the basolateral to the apical compartment (i.e. the excretion), were echimidine >> heliotrine > senecionine > senkirkine. Following pretreatment

with cyclosporine A for one hour to inhibit the P-gp transporter, the efflux ratio comparing the apparent permeability in each direction returned to one for echimidine, heliotrine and senkirkine (senecionine was already at one and was unchanged), indicating equal rates of efflux in each direction. Compared to wild-type cells, Madin-Darby canine kidney II (MDCKII) cells stably transfected with the P-gp transporter showed that the basolateral to apical transport of echimidine was increased while the apical to basolateral passage was reduced. The basolateral efflux was also reduced for heliotrine, while no changes were observed for the cyclic PAs senecionine or senkirkine. The efflux ratios were substantially enhanced for the noncyclic PAs but not for the cyclic PAs. Inhibition experiments with cyclosporine A inhibited the increase in efflux ratio for the cyclic PAs. The authors concluded that the P-gp efflux transporter returns the noncyclic PAs to the lumen of the intestine, but does not affect the cyclic PAs.

(ii) In vivo studies

The studies mentioned below were included in the EFSA opinion and are described here in more detail. In a study designed to compare the in vivo toxicokinetics of the cyclic diesters senecionine and adonifoline in 8-week-old male Sprague-Dawley rats (eight per group), Wang C et al. (2011) compared the plasma concentrations of these PAs and their metabolites after intravenous and oral administration, as measured through collection of blood by a jugular catheter. Senecionine and adonifoline were intravenously administered at 1.5 and 4.0 mg/kg body weight (bw), respectively. Senecionine was administered by gavage at target doses of 6, 12 and 24 mg/kg bw (later in the article indicated as 5.7, 11.5 and 22.9 mg/kg bw) and adonifoline was administered by gavage at 16, 32 and 64 mg/kg bw. PAs and metabolites were measured using ultra-performance liquid chromatography/electrospray ionization-mass spectrometry. Following oral gavage, peak concentrations in plasma were observed at 25 minutes for senecionine and 70 minutes for adonifoline, and the area under the curve (AUC) increased in a dose-dependent manner. The absolute bioavailabilities of senecionine and adonifoline after oral administration were 8.2 ± 2.7% and 33.4 ± 8.4%, respectively. When calculations included the *N*-oxides, the absolute bioavailabilities were 67.7 ± 22.9% for senecionine and 25.4 ± 5.3% for adonifoline. The authors suggested that in addition to formation in the liver, senecionine *N*-oxide may also be formed in the intestine by the intestinal microflora. The authors also proposed that another reason for the increased availability may be enterohepatic circulation. These results indicate a large first-pass effect after oral administration for senecionine. The metabolic conversion rate is higher for senecionine than for adonifoline.

Williams et al. (2002) examined the toxicokinetic properties of orally gavaged 10 mg/kg bw riddelliine mixture (92% riddelliine, 5% retrorsine, 1.3%

seneciphylline) in 6–8-week-old Fisher rats (five per group for females and three per group for males) and male and female B6C3F1 mice (six per group). PAs and metabolites were measured using LC-MS. The concentration of PAs in the plasma peaked within 30 minutes, indicating that they are rapidly absorbed in both sexes and both species. AUCs were determined to be retronecine < riddelliine < riddelliine *N*-oxide, except for the female rat, where the internal exposure was in the order of retronecine < riddelliine *N*-oxide < riddelliine.

(b) Distribution

WHO-IPCS (1988) identified several studies investigating distribution of PAs, and concluded that only a relatively small amount of absorbed PAs remains in the body, much of this in the form of metabolites bound to tissue constituents. WHO-IPCS also identified one study indicating transfer of PAs from feed to milk.

EFSA (2011) identified new studies with radiolabelled PAs showing distribution to liver, red blood cells (RBCs), plasma and kidneys and also noted that studies have documented transfer to milk, meat and eggs. In addition, EFSA noted that binding of PAs to biological macromolecules was observed both in vitro and in vivo. EFSA described two studies on distribution of PAs in detail. One study had found that male rats showed a larger apparent volume of distribution of riddelliine and greater internal exposure to riddelliine *N*-oxide and dehydroretronecine metabolites (AUC) than females, which is consistent with the higher sensitivity to riddelliine of male rats compared to female rats. Another study in rats presented evidence for enterohepatic circulation of senecionine *N*-oxide after administration of the parent PA.

(i) In vitro

Tu et al. (2013) examined the role of organic cation transporter (OCT1) in uptake of monocrotaline and retrorsine. In MDCK cells transfected with human OCT1 (hOCT1), increasing retrorsine concentrations reduced uptake of 1-methyl-4-phenylpyridinium (MPP+), a known hOCT1 substrate. Monocrotaline uptake was also increased in hOCT1 transfected cells and blocked by the addition of tetraethylammonium bromide (TEA), 4-(4-(dimethylamino)stryyl)-*N*-meththylpyridinium iodide (ASP+), (+)-tetrahydropalmatine (THP), or quinidine, which were described as OCT inhibitors. Primary cultured rat hepatocytes also showed decreased retrorsine uptake when exposed to the same inhibitors.

In a similar study, Tu et al. (2014) examined the ability of retrorsine to be transported by OCT1 and OCT3. In hOCT1 transfected MDCK cells, increasing retrorsine concentrations reduced uptake of MPP+, but no effect was seen in hOCT3 transfected cells. Retrorsine uptake was also increased in hOCT1

transfected cells and blocked by the addition of TEA, ASP+, THP or quinidine. In primary cultured rat hepatocytes, retrorsine uptake was also decreased when exposed to the same inhibitors. Further, the interaction of retrorsine with the efflux transporters P-gp, breast cancer resistance protein (BCRP) and human multidrug and toxin compound extrusion-1 (hMATE1) was investigated in transfected MDCK or Lewis lung carcinoma (LLC) cells. The uptake of retrorsine in P-gp transfected cells was increased compared to MDCK-mock cells, and cyclosporine A inhibited the efflux of retrorsine. No significant uptake was observed in BCRP and hMATE1 transfected cells nor was this affected by inhibitors of these transporters. The authors concluded that retrorsine was both a substrate and an inhibitor of OCT1, which is involved in active uptake into hepatocytes. OCT1 is also involved in the active uptake of monocrotaline. In addition, retrorsine is a substrate of P-gp, although to a lesser extent, but not of OCT3, MATE1 and BCRP.

Yang et al. (2011) investigated monocrotaline and its metabolites using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). The authors incubated human blood or components of human blood (RBCs, plasma and/or human serum albumin (HSA)) with monocrotaline, dehydromonocrotaline, dehydroretronecine and retronecine. After incubation with plasma, monocrotaline was stable in plasma and weakly associated with plasma proteins; dehydromonocrotaline rapidly hydrolysed to monocrotalic acid and monocrotalic acid lactone; retronecine did not react with endogenous metabolites or proteins in plasma; and dehydroretronecine disappeared immediately. As the same results were obtained for dehydroretronecine after incubation with HSA, it was suggested that dehydroretronecine readily binds to plasma proteins. In experiments in which monocrotaline was incubated with RBCs for 0.5 hours, based on the concentration remaining in the supernatant, 46% of the monocrotaline was calculated to have been taken up into the RBCs. The percentage increased to 51.7% after 1.5 hours and remained at that level when measured at 2 hours. Dehydromonocrotaline was also shown to be taken up into RBCs. Although the pyrrole ring was not directly detectable, signals from the monocrotalic acid and monocrotalic acid lactone breakdown products were. The calculated uptake of dehydromonocrotaline was 48.9%. Retronecine was also able to enter the RBCs without binding to the RBC proteins, whereas dehydroretronecine was either not taken up in the RBCs or reacted rapidly with RBC proteins. The latter explanation was considered more likely by the authors. In contrast to these results, when monocrotaline or dehydromonocrotaline was incubated with whole blood, which was subsequently fractionated, the alkaloid fractionated with the plasma and not the RBCs.

(ii) Ex vivo/in situ

Studies involving the in situ perfusion of lung and liver with ^{14}C-labelled monocrotaline (99.3% pure) found that RBCs helped in the transport of the PA from the liver to the lungs (Pan et al., 1991). Male Sprague-Dawley rats had both lung and liver perfusions performed in tandem and separately with and without the addition of RBCs (n = 5). When RBCs were added to the tandem perfusions, the covalent binding of monocrotaline metabolites to the lung tissue was significantly increased.

(iii) In vivo

To look at the tissue distribution, Estep et al. (1991) administered ^{14}C-labelled monocrotaline (60 mg/kg, 200 μCi/kg, n = 3) subcutaneously to male Sprague-Dawley rats and collected tissues at 4 and 24 hours for determination of tissue distribution and covalent binding. At 4 hours, the levels of monocrotaline equivalents in tissue were 85, 74, 67, 36 and 8 nmol/g and the covalent binding levels were 125, 132, 39, 64 and 44 pmol/mg of protein in the RBCs, liver, kidney, lung and plasma, respectively. At 24 hours, levels of monocrotaline equivalents in tissue were 49, 25, 9, 10 and 2 nmol/g and the covalent binding levels were not determined, 74, 28, 55 and not determined pmol/mg of protein in the RBCs, liver, kidney, lung and plasma, respectively. Plasma levels of radioactivity decreased from 113 nmol/g of monocrotaline equivalents to 11 nmol/g while levels in the RBCs decreased from 144 nmol/g to 81 nmol/g between 4 and 24 hours. The authors concluded that the retention of monocrotaline or its metabolites in the RBCs suggests that they may function as a carrier in the transport to other organs.

In a study comparing metabolism of monocrotaline and trichodesmine, male Sprague-Dawley rats (4–6 rats per group; age and weight not reported) were dosed intraperitoneally with 25 or 90 mg/kg bw monocrotaline or 25 mg/kg bw trichodesmine (Huxtable et al., 1996). The authors considered the higher dose of monocrotaline and the dose for trichodesmine to be equitoxic or equilethal. Tissues were harvested 18 hours after intraperitoneal administration, and later tested for pyrrole-bound metabolites using Ehrlich's reagent for detection. At the higher dose of monocrotaline, the greatest level of bound pyrrolic metabolites was found in the liver, followed by the lung, but higher levels of pyrroles in all tissues, except the brain, were found compared to tissues of rats treated with trichodesmine. Pyrroles were found in the brains of rats (~3 nmol pyrrole/g tissue) following intraperitoneal injection of 25 mg/kg bw trichodesmine and significantly lower levels (0–2 nmol pyrrole/g tissue) were identified in rats injected intraperitoneally with 25 or 90 mg/kg bw monocrotaline. From these studies, based on differences in the PAs' ionization constants and partition coefficients, and the fact that trichodesmine underwent more extensive

metabolism than monocrotaline resulting in metabolites with a longer half-life, these authors concluded that trichodesmine had greater access to the brain than monocrotaline and hence has a greater neurotoxicity and lethality. Three reasons for this difference were suggested: a greater release of dehydrotrichodesmine from the liver, a longer half-life of dehydrotrichodesmine and a higher liposolubility of trichodesmine/dehydrotrichodesmine.

Lamé et al. (1997) administered 5 mg/kg bw ^{14}C-labelled dehydromonocrotaline intravenously to male Sprague-Dawley rats (n = 4) and the animals were killed at 4 and 24 hours following the injection. Tissue distribution of radioactivity was measured at 24 hours (n = 4). The measurements (^{14}C-labelled dehydromonocrotaline equivalents per gram tissue) in RBCs, lung, kidney, heart, liver, plasma and muscle were 95.1, 23.7, 11.6, 10.2, 4.1, 1.5 and 1.0, respectively. Additionally, two groups of five rats were administered 5 mg/kg bw [^{3}H]-labelled dehydromonocrotaline intravenously and killed at 4 and 24 hours after injection, respectively. RBCs were collected and globin chains were also recovered. An additional group of four rats was administered 10 mg/kg bw and sacrificed 2 hours later; blood was collected and heme and ghosts of RBCs were recovered from some samples. Washing the RBCs did not reduce the associated [^{3}H]-labelled dehydromonocrotaline equivalents. While the amount of tritium in the RBCs remained constant between 4 and 24 hours, the level in the plasma dropped 2.3-fold. In the fractionated parts, at 2 hours the detection of [^{3}H]-labelled dehydromonocrotaline equivalents for unwashed RBCs was 288 nmol/g, for washed RBCs it was 328 nmol/g, plasma, 18.9 nmol/g, globin chains 870 pmol/mg protein, ghosts 236 pmol/mg and heme 226 pmol/mg. The authors concluded that this study showed that, like monocrotaline, radiolabelled dehydromonocrotaline resulted in extensive radiolabelling of RBCs.

Dimande et al. (2007) administered a crude extract of *Senecio inaequidens*, containing retrorsine, senecionine and two unidentified compounds also likely to be PAs, at a dose of 0.049, 0.142, 0.196 or 0.245 mg/kg crude extract (equal to 0.012–0.06 mg/kg bw retrorsine) by gavage to one male Sprague-Dawley rat (8–9 weeks old; 115–140.5 g) per dose. PAs were found in the livers of all rats at 0.1–214.8 µg/g retrorsine or retrorsine equivalents, which was inversely proportional to the amount of extract administered. PAs were also detected in the kidneys (54.5 µg/g retrorsine or equivalents) of the rat dosed with 0.142 mg/kg and in the kidneys (31.2 µg/g retrorsine or equivalents) and the lungs (32.7 µg/g retrorsine *N*-oxide) of the rat dosed with 0.245 mg/kg. One male Dorper sheep (8 months old; 41 kg) was given escalating doses of crude *Senecio inaequidens* extract by stomach tube, starting with 49.5 mg/kg on the first day to 198 mg/kg on the fourth day. The sheep was killed one day after the last dose and PAs were detectable in urine (82 µg/g retrorsine or equivalents), the liver (53.10 µg/g retrorsine

or equivalents), kidneys (29.4 µg/g retrorsine or equivalents), bile (6 µg/g retrorsine *N*-oxide) and lungs (11.5 µg/g retrorsine) (Dimande et al., 2007).

Wang C et al. (2011) (design described in section 2.1.1 Absorption) administered senecionine and adonifoline to rats at 1.5 and 4.0 mg/kg bw, respectively. The volumes of distribution (Vd) for senecionine and adonifoline were 11 and 4 L/kg, respectively, indicating distribution to the organs. The Vd of the senecionine metabolites (except M5, which is a hydroxylation product of senecionine *N*-oxide and its isomerides) was smaller than that of senecionine. However, for adonifoline the Vd of the metabolites was larger than that of the parent compound.

Schoch, Gardner & Stegelmeier (2000) dosed sows (group size of 3) with 3, 10 or 15 mg/kg bw of riddelliine for around 40 days. Pyrrolic metabolites were detected in the liver and blood of the treated animals. The level of metabolite did not correlate with the dose administered and the authors described significant intra-sample variability in the same animal. In observational studies, PAs were detected in the blood of horses (Seawright et al., 1991a) and yaks (Winter et al., 1993) following suspected ingestions of PA-containing plants.

(c) Excretion

WHO-IPCS (1988) concluded that PAs are rapidly metabolized and that the parent compound and metabolites are almost completely excreted within 24 hours. EFSA (2011) concluded that more than 80% of the subcutaneously or intravenously administered radiolabelled PAs in mice and rats is excreted in the urine and faeces within 24 hours.

(i) Ex vivo

In excretion studies in perfused rat liver, monocrotaline and its metabolites were shown to be excreted into bile (Lamé et al., 1995; Yan & Huxtable, 1994, 1995a). Based on studies of four livers from male Sprague-Dawley rats perfused with 40 µM [^{14}C]monocrotaline for 90 minutes and where bile was collected at 15-minute intervals, Lamé et al. (1995) reported that 20% of the radioactivity was excreted into bile, peaking between 15 and 30 minutes. Metabolites recovered included glutathione (GSH)-monocrotaline, Cys-Gly-DHP, and GSH-1-formyl-7-hydroxy-6,7-dihydro-5H-pyrrolizidine (GSH-DHP), but the metabolites were not quantified.

Yan & Huxtable (1994) perfused rat livers with buffer containing 500 µM monocrotaline and collected bile every 10 minutes for 1 hour (n = 20). They found that monocrotaline was removed from the perfusate at a constant rate of 2.6 µmol/g liver per hour. Of the monocrotaline delivered, 45% remained in the perfusion buffer and 0.4% was found in the bile. Metabolites were detected at

the following percentages of the amount administered; dehydromonocrotaline at 1.6% and GS-DHP at 3.0% in bile, dehydroretronecine and GS-DHP at 2.0% in perfusate and 1.0% liver bound, and the remaining 47.6% was unaccounted for.

Yan & Huxtable (1995a) perfused rat livers with buffer containing 125, 250, 500, 1000 or 1500 μM monocrotaline and collected bile every 10 minutes for 1 hour ($n = 4$). Of the initially delivered monocrotaline, the amount remaining in the perfusate ranged from 41.9% to 25.6%. From the administered monocrotaline 0.4 to 0.6% was detectable in the bile, 2.4–4.6% was bound to the Sepharose 6B resin from the perfusate, 1.3–4.9% was GS-DHP in the bile, 1.0–5.8% DHP in the perfusate and 1.0–3.1% was bound to the liver. From 49.1 to 55.6% of the monocrotaline was not accounted for, and was considered by the authors to include monocrotaline *N*-oxide and metabolites with a broken pyrrolizidine ring. The absolute amount of dehydromonocrotaline released into the perfusate increased with increasing concentration, although the fraction converted decreased. Similarly, the percentage metabolized to biliary pyrroles, which were found to be almost exclusively GS-DHP, decreased with increasing concentration.

(ii) In vivo

Williams et al. (2002) administered 10 mg/kg bw riddelliine (92% riddelliine, 5% retrorsine, 1.3% seneciphylline) by gavage to 6–8-week-old Fisher rats (five per group for females and three per group for males) and B6C3F1 mice (six per group). Blood was collected for time points up to 24 hours. PAs and metabolites were measured using LC-MS. The elimination half-lives ($t_{½}$) were determined to be riddelliine < retronecine < *N*-oxide. The elimination half-life of riddelliine was lower in mice (3.0–3.2 hours) than in rats (4.2 hours), but there was no significant sex difference. The elimination half-life of riddelliine *N*-oxide was, however, lower in rats (7.0 hours for males and 11.9 hours for females) than in mice (15.4 hours for males and 28.9 hours for females), but with no significant sex differences. The elimination half-life of retronecine was comparable between rats (6.7 hours for females and 8.2 hours for males) and mice (6.9 hours for males and 8.1 hours for females) with no significant sex differences. The authors observed that there was a significant increase in the formation of *N*-oxides in male rats compared to female rats. Because *N*-oxides were eliminated more slowly than the parent compound, they suggested that non-excreted *N*-oxides could potentially be reduced back to the parent compound and further metabolized to a more reactive metabolite such as dehydroretronecine. This is consistent with their earlier report, which found that administration of *N*-oxides to rats showed the same toxicity as the parent compound or its dehydroretronecine metabolite (Fu et al., 2001). Thus the

authors concluded that the observed species/sex differences in riddelliine toxicity were not simply due to species/sex differences in toxicokinetics.

Wang C et al. (2011) published a study designed to compare the in vivo pharmacokinetics of senecionine and adonifoline in 8-week-old male Sprague-Dawley rats (eight per group). Following intravenous administration of doses of 1.5 and 4.0 mg/kg bw, respectively, both compounds showed a rapid decrease in concentration versus time, which slowed after around 230–300 minutes before dropping below the level of detection by 12 hours. Following oral gavage of 5.7, 11.5 and 22.9 mg/kg bw senecionine or 16, 32 and 64 mg/kg bw adonifoline, the $t_{½}$s for senecionine and adonifoline were short (≤1 hour). Also, the metabolites of these PAs had short half-lives (up to ~2 hours).

Estep et al. (1990) reported the results of a study on an unspecified number of male Sprague-Dawley rats to identify products excreted following subcutaneous injection with 60 mg/kg bw (10 µCi/kg bw) of ^{14}C-labelled monocrotaline or ^{14}C-labelled senecionine after they had undergone cannulation of both ureters. Sixty per cent of the radioactivity of monocrotaline was excreted in the urine within 5 hours, with unchanged monocrotaline accounting for 65% of this. The monographers suggest that this may indicate active secretion into the urine. Following administration of ^{14}C-labelled senecionine, 5% of the administered radioactivity was excreted unchanged in the urine with > 42% co-eluting with a senecionine *N*-oxide and 40% as Ehrlich-negative polar metabolites. The authors did not report what percentage of the radioactivity of senecionine was excreted in the urine. With both compounds, the Ehrlich-positive metabolite was identified as an *N*-acetylcysteine conjugate of (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (NAC-DHP).

Estep, Lamé & Segall (1990) treated rats (male, n = 4, 225–275 g) with 60 mg/kg bw (10 µCi/kg bw) ^{14}C-labelled senecionine administered intravenously and collected bile, urine and blood for 7 hours. Of the administered radioactivity, 43% was detected in the urine and 44% in the bile. The amount of radioactivity detected in the urine increased when bile was not collected, which the authors interpreted as suggesting enterohepatic circulation. Most of the excreted PAs were identified as senecionine *N*-oxide (52% in bile, 30% in urine). The parent compound, senecionine, accounted for 18% of the radioactivity in the urine and 5% in the bile.

After intravenous administration of ^{14}C-labelled monocrotaline (60 mg/kg, 10 µCi/kg), Estep et al. (1991) found rapid elimination of radioactivity with approximately 83% of the total radioactivity excreted in the urine and 12% in the bile at 7 hours. High-performance liquid chromatography (HPLC) analysis indicated that 62% of the excreted alkaloid was unchanged monocrotaline, and less than 4% was monocrotaline *N*-oxide.

A study comparing urinary excretion following intraperitoneal injection of 25 mg/kg bw retrorsine between male Sprague-Dawley rats (n = 7), Dunkin-Hartley guinea-pigs (n = 7), Balb/c mice (n = 4), and golden Syrian hamsters (n = 7) was done by Chu & Segall (1991). Urine was collected for 24 hours and assayed for various metabolites. In the guinea-pig, as a percentage of given dose, 2.5%, 4.6%, 4.8% and 21.7% of pyrrole, isatinecic acid, retrorsine and *N*-oxides were recovered, respectively, giving a total recovery of 24%. In the hamster, as a percentage of given dose, 9.8%, 29.0%, 16.9% and 4.0% of pyrrole, isatinecic acid, retrorsine and *N*-oxides were recovered, respectively, giving a total recovery of 60%. In the mice, as a percentage of given dose, 8.0%, 21.5%, 6.3% and 2.9% of pyrrole, isatinecic acid, retrorsine and *N*-oxides were recovered, respectively, giving a total recovery of 39%. In the rats as a percentage of given dose, 10.3%, 31.0%, 13.6% and 10.8% of pyrrole, isatinecic acid, retrorsine and *N*-oxides was recovered, respectively, giving a total recovery of 66%. The authors suggest that there may also be other unknown metabolites that account for the low recovery in mice and guinea-pigs. The Committee suggested that another explanation could be that part of the dose is excreted via bile.

Chu, Lamé & Segall (1993) investigated the excretion of the urinary metabolites of retrorsine and retrorsine *N*-oxide in male Sprague-Dawley rats (n = 4–7) following intraperitoneal or oral (via stomach tube) dosing at 25 mg/kg bw. Urine was collected for 24 hours and analysed for the presence of PAs and metabolites. After intraperitoneal administration of retrorsine, 31.0% was recovered as isatinecic acid, 10.3% as total pyrroles, 13.6% as unchanged retrorsine, 10.8% as total *N*-oxides and 0.39% as retronecine, given as percentage of dose. After intraperitoneal administration of retrorsine *N*-oxide, as percentage of dose, 6.2%, 1.8%, 1.1% and 47.5% of isatinecic acid, total pyrroles, retrorsine and total *N*-oxides was recovered, respectively. After oral dosing, recoveries were 43.9%, 11.9%, 4.5% and 11.2% for isatinecic acid, total pyrroles, retrorsine and total *N*-oxides, respectively. The authors suggested that the differences in excretion after intraperitoneal and oral dosing with retrorsine *N*-oxide are probably related to back-transformation of the *N*-oxide to the parent PA by gut flora.

In a study (described in section 2.1.1(b) Distribution) Lamé et al. (1997) intravenously administered 5 mg/kg bw ^{14}C-labelled dehydromonocrotaline to male Sprague-Dawley rats (n = 4) and the animals were sacrificed at 4 and 24 hours following the injection. Of the administered radioactivity, 82 ± 5% was excreted in the urine and 3 ± 1% in faeces in 24 hours. As described in more detail in section 2.1.5 (Transfer from feed to food), three dairy cows (4–5 years old; 600–700 kg) with fistulated rumens were treated with ragwort (*Senecio jacobeae*) through the fistula at doses of 0 for week 1, 50 g in week 2, 100 g in week 3, 200 g in week 4, and 0 g in week 5 (Hoogenboom et al., 2011). The ragwort contained mostly jacobine, jaconine, erucifoline, senecionine and seneciphylline and the

corresponding *N*-oxides. The total daily dose was split into two equal doses per day. Urine and faecal samples were taken twice weekly. Jacobine *N*-oxide and jacoline were identified in the urine, and jacoline was identified in the faecal samples.

A summary of the in vivo excretion data of (radiolabelled) PAs for which this information is available can be found in Table 4.

(iii) Humans

Twenty human patients (age 1 month – 7 years) were admitted to a South African hospital with veno-occlusive disease suspected to be due to PA toxicity from reported or suspected exposures to herbal medicines (Steenkamp, Stewart & Zuckerman, 2000). Using colorimetric methods, urine was analysed from the four patients for whom urine samples were available from within 72 hours of admission, and all were positive for PAs. None of the healthy controls or those with other types of hepatic damage had alkaloid in the urine samples. No information on the excretion via faeces is available.

2.1.2 Biotransformation

WHO-IPCS (1988) and EFSA (2011) summarized metabolism of 1,2-unsaturated PAs. Metabolism of PAs occurs predominantly in the liver and three Phase I metabolic pathways have been found:

- the formation of necine bases and necic acids through cleavage of the ester bonds by carboxylesterases on position C7 and C9,
- *N*-oxygenation, and
- oxidation leading to formation of reactive intermediates, 6,7-dihydropyrrolizine esters (pyrrolic esters).

Hepatic monooxygenases, cytochrome p450 enzymes (CYPs) and flavin-containing monooxygenases (FMOs) are involved in metabolism routes 2 and 3 yielding the *N*-oxide and pyrrolic ester metabolites. After the formation of the primary metabolites, they can be further conjugated by glutathione-S-transferase (GST).

(a) In vitro

The studies on in vitro metabolism of PAs are summarized below.

The details are given in a separate table, available as supplementary material online (Supplementary Table 1).

Table 4
Overview of in vivo excretion studies with (radiolabelled) senecionine, monocrotaline or retrorsine

Pyrrolizidine alkaloid	Species	Time	Route	Dose	Urine	Bile	Study
^{14}C-labelled monocrotaline	SD rats	5 hours	Subcutaneous	60 mg/kg bw (10 µCi/kg bw)	60%[a] (65% unchanged)	–	Estep et al. (1990)
^{14}C-labelled senecionine	SD rats	5 hours	Subcutaneous	60 mg/kg bw (10 µCi/kg bw)	Unknown total excreted in urine[a] (5% unchanged in urine, >42% senecionine *N*-oxide)	–	Estep et al. (1990)
^{14}C-labelled senecionine	SD rats	7 hours	Intravenous	60 mg/kg bw (10 µCi/kg bw)	43%[a, b] (18% unchanged, 30% *N*-oxide)	44%[a] (5% unchanged, 52% *N*-oxide)	Estep, Lamé & Segall (1990)
^{14}C-labelled monocrotaline	SD rats	7 hours	Intravenous	60 mg/kg (10 µCi/kg)	83%[a] (62% unchanged, <4% *N*-oxide)	12%[a] (<5% unchanged)	Estep et al. (1991)
Retrorsine	SD rats	24 hours	Intraperitoneal	25 mg/kg bw	66%[c] (10.3% pyrrole; 31.0% isatinecic acid; 13.6% retrorsine; 10.8% retrorsine *N*-oxide)	–	Chu & Segall (1991)[e]
Retrorsine	Dunkin-Hartley guinea-pigs	24 hours	Intraperitoneal	25 mg/kg bw	24%[c] (2.5% pyrrole; 4.6% isatinecic acid; 4.8% retrorsine; 21.7% retrorsine *N*-oxide)	–	Chu & Segall (1991)[e]
Retrorsine	Balb/c mice	24 hours	Intraperitoneal	25 mg/kg bw	39%[c] (8.0% pyrrole; 21.5% isatinecic acid; 46.3% retrorsine; 2.9% retrorsine *N*-oxide)	–	Chu & Segall (1991)[e]
Retrorsine	Golden Syrian hamsters	24 hours	Intraperitoneal	25 mg/kg bw	60%[c] (9.8% pyrrole; 29.0% isatinecic acid; 16.9% retrorsine; 4.0% retrorsine *N*-oxide)	–	Chu & Segall (1991)[e]
Retrorsine	SD rats	24 hours	Intraperitoneal	25 mg/kg bw	31.0% isatinecic acid; 10.3% total pyrroles; 13.6% retrorsine; 10.8% total *N*-oxides; 0.39% retronecine	–	Chu, Lamé & Segall (1993)
Retrorsine *N*-oxide	SD rats	24 hours	Intraperitoneal	25 mg/kg bw	6.2% isatinecic acid; 1.8% total pyrroles; 1.1% retrorsine; 47.5% total *N*-oxides	–	Chu, Lamé & Segall (1993)
Retrorsine *N*-oxide	SD rats	24 hours	Oral (stomach tube)	25 mg/kg bw	43.9% isatinecic acid; 11.9% total pyrroles; 4.5% retrorsine;	–	Chu, Lamé & Segall (1993)

Pyrrolizidine alkaloid	Species	Time	Route	Dose	Urine	Bile	Study
^{14}C-labelled dehydromonocrotaline	SD rats	24 hours	Intravenous	5 mg/kg bw	82 ± 5%[a]	3 ± 1%[d]	Lamé et al. (1997)

bw: body weight; SD: Sprague-Dawley
[a] Percentage of radioactivity.
[b] Increased to 59% when bile not collected.
[c] Percentage of dose.
[d] Measured in faeces.
[e] Recoveries in urine were very low, see also study description.

(i) Phase I metabolism

Metabolism has been studied using liver microsomes from rats, other rodents, sheep, cattle and other ruminants, and humans. In addition, purified metabolism enzymes and ex vivo perfused livers, sometimes in closed systems with isolated lungs, were used to investigate the in vitro metabolism of PAs.

Rat, guinea-pig and human liver microsomes, as well as recombinant CYPs have been shown to metabolize PAs to 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) (Buhler et al., 1991; Chung & Buhler, 2004; Duringer, Buhler & Craig, 2004; Fashe et al., 2015a,b; He YQ et al., 2010a; Huxtable et al., 1996; Lin et al., 2007; Reid et al., 1998a; Ruan et al., 2014a; Wang YP et al., 2005a,b; Wang YP, Fu & Chou, 2005; Xia et al., 2003, 2004; Xia et al., 2013; Yang et al., 2001a). Induction of CYPs in animals prior to microsome isolation has been shown to increase DHP formation following incubation of PAs with the induced microsomes (Buhler et al., 1991; Chung, Miranda & Buhler, 1995; Huan et al., 1998a; Miranda et al., 1992; Reid et al., 1998a; Wang YP et al., 2005a; Wang YP, Fu & Chou, 2005; Xia et al., 2004, 2006; Yang et al., 2001). Inhibition of CYPs, particularly the CYP3A-family enzymes, has been shown to decrease formation of pyrrolic adducts and DHP formation (Chung & Buhler, 2004; Duringer, Buhler & Craig, 2004; Fashe et al., 2015a; Huan et al., 1998b; Lin et al., 2002, 2007; Liu XQ et al., 2001, 2002; Reid et al., 1998a; Wang J et al., 2009; Wang YP et al., 2005a,b; Wang YP, Fu & Chou, 2005; Xia et al., 2003, 2004, 2006, 2013). CYPs have also been demonstrated to be involved in the formation of PA *N*-oxides (Dueker et al., 1992; Wang YP et al., 2005b; Wang YP, Fu & Chou, 2005; Williams et al., 1989a,b). In guinea-pigs, in vitro studies have indicated that the CYP2B isoform plays an important role in the activation of PAs, as was observed with senecionine (Chung, Miranda & Buhler, 1995).

Studies that looked at differences in metabolic activation between different types of PAs using human recombinant CYPs showed that CYP3A4 and 3A5 were involved in the case of otonecine-type PAs (clivorine and senkirkine), with the highest activity for 3A5. For retronecine-type PAs, CYP3A4 and 3A5 were also predominantly responsible for their metabolic activation except for monocrotaline, for which CYP2A6 was the major activating enzyme. In addition, CYP2A6 played a role in the metabolic activation of retrorsine, riddelliine, senecionine and seneciphylline. Other CYPs investigated, including CYP1A1, 1A2, 2B6, 2C9, 2C19, 2D6 and 2E1, were involved to a much lesser extent (Ruan et al., 2014a). Platyphylline also undergoes CYP3A4-mediated metabolism, and to a lesser extent metabolism mediated via CYP2C19, 1A1, 1A2, 2D6 and 2B6, in human liver microsomes to form the predominant unreactive metabolite dehydroplatyphylline carboxylic acid, which can be readily excreted (Ruan et al., 2014b).

Inhibition of FMOs reduced formation of *N*-oxides from monocrotaline when incubated with male rat or guinea-pig liver microsomes (Chung & Buhler, 2004; Wang J et al., 2009), or with senecionine incubated with sheep or cow liver microsomes (Duringer et al., 2004). With hamster or sheep microsomes, inhibition of FMOs reduced formation of DHP and senecionine *N*-oxide from senecionine (Huan et al., 1998a).

As demonstrated by incubation of riddelliine *N*-oxide, retrorsine *N*-oxide and monocrotaline *N*-oxide with rat or human liver microsomes, *N*-oxides can be transformed to DHP and parent PA. Inhibition of CYP3A reduced the formation of DHP significantly but the reduction to the corresponding parent was unaffected indicating that hepatic reductase activity is involved (Chou et al., 2003a; Wang YP et al., 2005b).

Carboxylesterases have been implicated as one pathway in the hydrolysis of the PAs clivorine, jacobine, senecionine and monocrotaline, as demonstrated in microsomes from guinea-pig (Chung & Buhler, 1995; Dueker et al., 1992; Lin et al., 2002, 2007), hamster and sheep (Huan et al., 1998a), and to a lesser extent in rat microsomes (Lin et al., 2002, 2007). However, this hydrolysis reaction was not observed with monocrotaline in rat microsomes (Dueker et al., 1992). The structure of the ester functionality of different PAs also has an impact, since steric hindrance impairs de-esterification of PAs with branched chain esters (EFSA, 2011; Mattocks, 1982).

Species differences in the rate of metabolic activation were observed with lasiocarpine (Fashe et al., 2015a). Extensive metabolism was observed in all species tested (mice, rats, pigs, sheep, humans) except for rabbits. Observed differences between human microsomes and those of the other species include a relatively high rate of reactive metabolite formation in human microsomes, as measured by the formation of GSH conjugate (see also below) and dehydrogenation metabolites, and a low rate of demethylation metabolite formation.

(ii) Phase II metabolism

Glucuronidation after incubation of senecionine, monocrotaline, isoline or adonifoline with liver microsomes was seen in some species but not others in the following order (from higher activity to lower): rabbits, humans, cattle, pigs and sheep >> guinea-pigs and dogs ≥ rats and mice (He YQ et al., 2010a). Using human liver microsomes and recombinant uridine 5′-diphospho-glucuronosyltransferases (UGTs), He YQ et al., 2010b showed activity from UGT1A4 and to a small degree from UGT1A3.

In vitro studies on the role of GSH in PA metabolism have generally found production of GSH-PA metabolites (Lin, Cui & Hawes, 1998). Reaction of dehydromonocrotaline with GSH yielded 7-GS-DHP adducts and the relatively

stable 7,9-diGS-DHP adducts, and a highly unstable mono-GS-DHP adduct, which was tentatively assigned as 9-GS-DHP (Ma et al., 2015). The existence of 9-GS-DHP was confirmed by Chen M, et al. (2016) who found it to be the predominant GSH conjugate formed for retronecine-type PAs. Other GSH conjugates include 7-GS-DHP, 7R,9-diGS-DHP and 7 S,9-diGS-DHP. In acidic conditions, 9-GS-DHP can be spontaneously converted to 7-GS-DHP and 7,9-diGS-DHP. The formation of GSH conjugates is suggested to occur spontaneously without being mediated by GSTs as incubation of dehydromonocrotaline with human microsomal GSTs or cytosolic GSTs in the presence of GSH did not show significant differences compared to vehicle incubations (Chen M, et al., 2016). The production of the GSH-PA mono- or diGSH metabolites can compete with production of other metabolites (Cui & Lin, 2000; Duringer et al., 2004; Fashe et al., 2014; Fashe et al., 2015a; Lin, Cui & Hawes, 2000). For example, Huan et al. (1998a) found a reduction in DHP formation but no effect on *N*-oxide formation when senecionine was incubated with hamster or sheep liver microsomes.

The rate of GSH-jacobine production remained unchanged following the addition of rat GST, while the addition of guinea-pig GST increased the rate of GSH-jacobine production over the non-enzymatic rate (Dueker et al., 1994).

The GSH contents of human and bovine artery endothelial cells were reduced following treatment with dehydromonocrotaline or dehydroretronecine (Reid et al., 1998b). Clivorine-exposed human liver L-02 cells had reduced cellular GSH and a reduced GSH/GSSG ratio (Ji, Chen & Wang, 2008; Ji et al., 2009). Increasing the GSH concentration led to increased DHP-GSH formation (Buhler et al., 1991; Reed et al., 1992).

Based on incubation of a number of PAs (monocrotaline, retrorsine, seneciphylline, senecionine, senkirkine, heliotrine, lycopsamine, lasiocarpine, clivorine, integerrimine, platyphylline and riddelliine) with human microsomes in the presence or absence of GSH, Ruan et al. (2014a) concluded that formation of pyrrole–GSH conjugates and pyrrole–protein adducts are competitive reaction pathways. The GSH conjugate and pyrrole–protein conjugates are formed simultaneously and competitively; the authors suggest that this means that the protein conjugate forms even in the presence of adequate amounts of GSH.

Dehydro-PAs, as found for riddelliine and monocrotaline in rat and human liver microsomes, were also found to be able to react with *N*-acetylcysteine (NAC) to generate 7-NAC-DHP or with cysteine to form 7-cysteine-DHP (7-Cys-DHP) (He X et al., 2016a,b). In addition, 7-Cys-DHP was also found to be generated via metabolism of 7-GS-DHP, not catalysed by CYPs. As both 7-GS-DHP and 7-Cys-DHP were formed in liver microsomes without addition of GSH or cysteine, these secondary pyrrolic metabolites may also be formed in vivo (He X et al., 2016b).

(b) **Ex vivo**

Male Sprague-Dawley rats underwent lung and liver perfusions, each in isolation and together in tandem, with ^{14}C-labelled monocrotaline (99.3% pure) (n = 5). When liver perfusions were performed alone, 65–85% of the radiolabelled monocrotaline was converted into polar metabolites that included monocrotalic acid and monocrotaline *N*-oxide; unchanged monocrotaline was also identified. In the lung perfusions, only 5% of the radiolabelled monocrotaline was converted into metabolites (Pan et al., 1991). Monocrotaline perfusion decreased the GSH concentration in isolated perfused rat liver (Nigra & Huxtable, 1992). Further, rat livers depleted of GSH (by in vivo administration of choloroethanol, diethyl maleate or buthionine sulfoximine), which were subsequently perfused with monocrotaline, showed a decrease in GSH bound metabolites released into bile and an increase in protein bound pyrroles (Yan & Huxtable, 1995b).

(c) **In vivo**

The in vivo studies investigating the metabolism of PAs in animals, which included rats, mice, pigs, sheep, horses and cows, are summarized below. The details are given in a separate table, available as supplementary material online (Supplementary Table 2).

The predominant metabolites identified following exposure via a variety of routes included DHP, PA *N*-oxides, GS-DHP, pyrroles (often identified without determining what they are conjugated to), GSH conjugates and NAC-conjugates. These metabolites have been found in many organs and in body fluids including liver, blood (whole blood, serum and RBCs), lung, heart, kidney, urine, bile and brain (Chen M et al., 2016; Chou et al., 2003a; Chu, Lamé & Segall et al., 1993; Dimande et al., 2007; Estep et al., 1990; Estep et al., 1991; Estep, Lamé & Segall, 1990; Huxtable et al., 1996; Lamé et al., 1990; Maia et al., 2014; Mattocks & Jukes, 1992a,b; Panariti et al., 1997; Schoch, Gardner & Stegelmeier, 2000; Wang C et al., 2011; Wang YP et al., 2005a,b; Williams et al., 2002; Xiong et al., 2009 a,b; Yan & Huxtable, 1995a; Yan & Huxtable, 1996a,b; Yang YC et al., 2001a).

In a rat study (design described in section 2.1.1 Absorption), Williams et al. (2002) found that riddelliine was rapidly converted to the *N*-oxide, although female rats had lower serum concentrations. In a later study in rats, Wang C et al. (2011) (design described in ection 2.1.1 Absorption) administered senecionine intravenously at 1.5 mg/kg bw or orally via gavage at 5.7, 11.5 or 22.9 mg/kg bw, or adonifoline intravenously at 4.0 mg/kg bw, or orally via gavage at 16, 32 or 64 mg/kg bw. The most common metabolite was the *N*-oxide for both senecionine and adonifoline, but the metabolic rate of transformation from the parent compound to the *N*-oxide was faster with senecionine than adonifoline by either route. The authors concluded from the data that the metabolic rate of

transforming senecionine to senecionine *N*-oxide was 35-fold higher than for adonifoline transformation to adonifoline *N*-oxide after intravenous dosing and 143-fold higher after oral dosing. Rapid conversions to the *N*-oxide were observed in all groups except in female rats, and the metabolite, retronecine, was identified in all groups.

(d) Overview biotransformation

A simplified overview of the biotransformation of four main characteristic pyrrolizidine moieties, namely retronecine-type, otonecine-type, heliotridine-type and platynecine-type, is presented in Fig. 2.

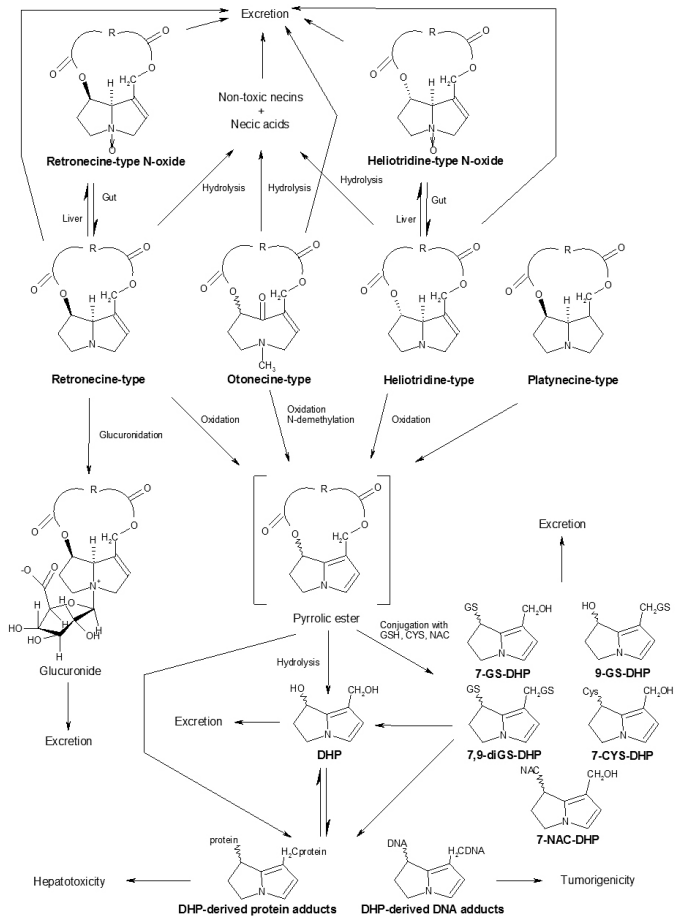
Metabolic activation of 1,2-unsaturated PAs starts with oxidation by CYP450 enzymes in the liver to 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine ester metabolites (DHP esters) and their hydrolysis products, (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine alcohols (DHPs, dehydronecines) (Mattocks, 1986; WHO-IPCS, 1988). CYP450 enzymes in the liver hydroxylate carbon atoms of 1,2-unsaturated PAs at positions 3 and 8 adjacent to the nitrogen to produce carbinolamines. Spontaneous dehydration of the carbinolamines then converts the ring system to an aromatic "pyrrole" ring. The DHP esters produced in this way readily release their esterifying acids and form stabilized, positively charged (carbonium) ions at C7 and C9, which rapidly react with electron-rich (nucleophilic) groups, such as amines, thiols, and also hydroxyl compounds they encounter, to produce DHP adducts on DNA, proteins and other nucleophilic molecules in the liver.

In the case of otonecine-type PAs, e.g. senkirkine, CYP450 hydroxylation of the *N*-methyl group leads to its removal as formaldehyde, and enables condensation of the 8-keto group with the NH group generated at position 4 to produce the corresponding DHP-ester metabolites leading to DHP adducts (Culvenor et al., 1971; Lin et al., 1998; Xia et al., 2004).

DHP esters are also readily hydrolysed, releasing DHP (dehydronecines) into the circulation. DHP is a less reactive alkylating agent than the precursor DHP esters but is still capable of forming DHP adducts with nucleophilic targets in the liver and also in many extrahepatic tissues (Mattocks, 1986). Some DHP adducted to weaker nucleophilic groups can release DHP or transfer it to stronger nucleophiles (Mattocks, 1986). Thus DHP linked to some circulating proteins and to other substances, for example GSH, that escape from the liver remain potentially active alkylating agents, prolonging toxicity and leading to extrahepatic effects (He X et al., 2016a,b; Huxtable et al., 1990; Mattocks, 1986; Prakash et al., 1999; Seawright, 1992, 1994; Xia et al., 2015; Zhao et al., 2014).

Fig. 2

Simplified overview of the biotransformation of 1,2-unsaturated PAs in humans leading to detoxification or activation



Notes: It is recognized that there are PA structures that do not contain a ring structure. For convenience of comparison these are not included in the scheme, but can be assumed to follow the routes of the PA-type to which they belong. Besides reaction with DNA and proteins, DHP may also react with other nucleophilic molecules.
Source: Adapted from He X et al. (2016a) and Zhu et al. (2016).

Ingested 1,2-unsaturated PA *N*-oxides, present as contaminants in food, are reduced to 1,2-unsaturated PAs in the digestive tract and to a lesser extent in the liver, and they too contribute to the hepatic metabolic activation and toxicity of the parent 1,2-unsaturated PAs (Mattocks, 1986). However, *N*-oxidation of 1,2-unsaturated PAs in the liver is a detoxification and results in excretion of the highly water soluble *N*-oxides produced (Mattocks, 1986). If 1,2-unsaturated PA *N*-oxides are injected intraperitoneally or intravenously they are largely excreted

and are not particularly toxic compared with dietary exposure where the *N*-oxides are reduced to, and display toxicity equal to, the corresponding 1,2-unsaturated PA free bases (Mattocks, 1986). Un-adducted DHP is also highly water soluble and, like the *N*-oxides, is also readily excreted.

Carboxylesterase hydrolysis is another detoxifying mechanism. It results in hydrolysis of 1,2-unsaturated PAs to non-toxic necines and monocrotalic acids (EFSA, 2011). The importance of this pathway is influenced by the level of hepatic esterase activity, which is lower in the rat than in other species such as the guinea-pig. The rat hepatic esterase activity is approximately equivalent to that of humans (Jewell et al., 2007). Further, glucuronidation by UGT1A4, and to a small degree by UGT1A3, has been identified as an additional detoxification route for the retronecine-type PAs senecionine, monocrotaline, isoline and adonifoline (He YQ et al., 2010a,b).

Saturated PAs are not converted to DHP esters or DHP and are therefore reportedly not genotoxic (EFSA, 2011; Mattocks, 1986; Ruan et al., 2014b).

(e) Ruminal microbial metabolism

Eight beef calves (135–185 kg) with rumen fistulas were exposed to PA-contaminated feed containing 5% tansy ragwort, 45% alfalfa hay, 47.5% grass hay and 2.5% molasses (Johnston et al., 1998). The ragwort was known to contain the PAs jacobine and seneciphylline. Prior to being fed, 1.5 L of sheep whole rumen fluid had been transferred into the calves via the fistula; the fluid was from sheep that had been fed 5% tansy ragwort for up to 3 weeks. The aim of the study was to determine if the transferred rumen fluid from the sheep would protect the calves, as sheep are able to ingest tansy ragwort with no adverse effects. All of the calves that received the transferred microbes exhibited degradation of the PA toxin at a much faster rate than controls and no increases in gamma-glutamyl transaminase (GGT) activity, except for one. The calf that showed no reaction to the transferred microbes was treated a second time and did react, but liver pathology showed damage. All control calves without protective sheep rumen fluid treatment had liver damage, as expected. This indicates that the microbial flora are able to metabolize the PAs to non-toxic metabolites.

Wachenheim, Blythe & Craig (1992) estimated the number of PA transforming bacteria in the guts of two Dorset ewes, an alpine goat and two shorthorn cows. The number of bacteria was estimated based on the maximum dilution at which microbial activity occurred. The authors incubated anaerobic cultures with 50 g of rumen from each animal and 0.3 ml of a 10 mg *Senecio jacobaea*/ml solution. *S. jacobaea* was noted to contain jacobine and seneciphylline. The authors found a biotransformation rate of the PAs from the *S. jacobaea*, based on the measurement of PA disappearance from culture,

to be 19.2 µg/ml per hour for the sheep, 25.6 µg/ml per hour for the goat, and 2.9 µg/ml per hour for the cows. The rates of PA disappearance were generally related to the estimated total numbers of PA-degrading bacteria.

Craig et al. (1992) also looked at the in vitro digestion of a *S. jacobaea* extract. Whole ruminal fluid (obtained from sheep pre-exposed to *S. jacobaea* plant) showed faster degradation of PAs than fractionated ruminal fluid, although the rate of PA depletion in the different supernatant fractions was not different. The fractionation was such that different fractions contained different combinations of microorganisms, and the authors concluded that some microorganisms in the pellet, such as larger protozoa, may contribute to the metabolism, although smaller protozoa or larger bacteria may not. In cultures with methanogenic medium or McDougalls medium (which simulates sheep saliva in composition and buffering capacity) up to 100 µg/ml of PAs was depleted in 24 hours, which was not the case in sterile controls or with denitrifying medium. The authors concluded that this indicates that fermentive organisms or methanogens are responsible for the PA metabolism.

Hovermale & Craig (2002) looked at metabolites from in vitro microbial digestions of a crude extract of *S. jacobaea*, identified as containing mostly jacobine and seneciphylline, but also senecionine, integerrimine, jacozine, jacoline and retrorsine (percentages of each PA were not determined). In addition the in vitro digestion of the individual PAs heliotrine, lasiocarpine and monocrotaline was investigated. Cultures used *Pseudostreptococcus heliotrinreducens*, which was isolated from a sheep rumen, as well as an enriched mixed culture, designated L4M2, also derived from a sheep rumen. *P. heliotrinreducens* completely metabolized heliotrine at a 100 µg/ml concentration in 16 hours, and L4M2 in 8 hours, and each produced the metabolite 7α-hydroxyl-1-methylene-8α-pyrrolizidine. *P. heliotrinreducens* completely metabolized lasiocarpine at a 100 µg/ml concentration in 16 hours, and L4M2 in 5 hours, and each produced the metabolites 7α-angelyl-1-methylene-8α-pyrrolizidine and 7α-tiglyl-1-methylene-8α-pyrrolizidine. A mixture of PAs derived from *S. jacobaea* or monocrotaline alone was not metabolized in 24 hours by *P. heliotrinreducens*. However, L4M2 metabolized the mixture of PAs in 12 hours, as did the culture with L4M2 and monocrotaline, and each produced 7β-hydroxyl-1-methylene-8α-pyrrolizidine. The authors concluded that the data are consistent with the prediction that *P. heliotrinreducens* is able to metabolize mono- and diester PAs but not macrocyclic ones.

Aguiar & Wink (2005) examined digestion of alkaloids in bovine rumen cultures. In a seven-day culture of 1 mM senecionine, using rumen inoculation from a cow not exposed to alkaloids in liquid media anaerobic cultures, no degradation was seen (monocrotaline was not tested). In a rumen simulation technique, which uses a semicontinuous rumen fermentation system consisting

of 4 to 6 vessels using 2 mM monocrotaline, 92.1% degradation was seen after 24 hours with no evidence of *N*-oxide formation (senecionine was not tested).

2.1.3 Effects on enzymes and other biochemical parameters

Several studies investigating the effects of PAs on enzymes involved in metabolizing PAs have been reported.

(a) In vitro CYP inhibition

Dai, Zhang & Zheng (2010) studied inhibition of individual human CYP isozymes in vitro by retrorsine or monocrotaline, based on the nicotinamide adenine dinucleotide phosphate (NADPH) dependence of the inhibition and the ability of substrates present during the incubation to block the inactivation of the enzyme. In their assay, the addition of 100 µM monocrotaline or retrorsine for 30 minutes showed that retrorsine, but not monocrotaline, mechanistically inhibited CYP3A4, but not the other CYPs tested, i.e. CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6 and 2E1.

(b) In vivo CYP induction

Gordon, Coleman & Grisham (2000) found that a single intraperitoneal dose of 30 mg/kg bw retrorsine in rats induced an increase in both the protein levels of CYP1A1/2, 2B1/2 and 2E1 and the mRNA levels in microsomes of CYP1A1, 2B1/2 and 2E1, which were obtained from the livers of these rats.

Luckert et al. (2016) found that of the four PAs senecionine, heliotrine, echimidine and senkirkine, only echimidine was able to activate the nuclear receptor – pregnane X receptor (PXR). Protein and mRNA level analysis in HEpG2 cells verified that echimidine can induce CYP3A4. To study whether only open-chain diesters are able to activate PXR, five additional PAs were studied (the PAs were not named, but were of the monoester, open-chain diester or cyclic diester type). Only the open-chain diesters echimidine and lasiocarpine were able to activate PXR.

(c) Effect on Phase II enzymes

One of the metabolic fates of PAs is conjugation to GSH. As described in section 2.1.2 Biotransformation, several studies have indicated effects on GSH levels in cell-based assays, perfused organs or in vivo studies following exposure to PAs.

Using cultured human pulmonary artery endothelial cells, Reid et al. (1998b) detected a 40% decrease in GSH in cells exposed to 100 µM dehydromonocrotaline and a 25% decrease in cells exposed to 100 µM dehydroretrorsine after 15 minutes of exposure.

Yan & Huxtable (1995c) found a significant increase of in vitro GSH synthesis rates in liver cytosol prepared from rats administered 65 mg/kg bw intraperitoneal monocrotaline or 15 mg/kg bw trichodesmine 24 hours before being sacrificed, compared to the rates in cytosols prepared from control rats.

Yan & Huxtable (1996a) also compared the activity of enzymes involved in GSH metabolism in several organs of rats administered 65 mg/kg bw monocrotaline by the intraperitoneal route for 24 hours. In the liver and lung, the activity of glutamate cysteine ligase (previously called gamma-glutamylcysteine synthetase), GSH synthase, gamma-glutamyl transpeptidase, dipeptidase and microsomal GSH-S transferase were significantly increased compared to controls. In the heart and kidney, gamma-glutamyl transpeptidase and cytosolic GSH-S transferase were significantly increased.

(d) Effect on other enzymes

Thirteen different PAs (3′-acetylheliosupine, echihumiline, echihumiline-*N*-oxide, echimidine, heliosupine, heliosupine-*N*-oxide, heliotrine, monocrotaline, pycnanthine, retronecine, riddelliine, senecionine and seneciphylline, purity >95%) were tested, using several concentrations for each PA, to determine if they affected acetylcholine (ACh)-related enzymes and various neuroreceptors. Brains from newly killed pigs were used to prepare receptor-rich membranes and in vitro binding assays were performed. Included in the analyses were ACh-converting enzymes, acetylcholinesterase, butyrylcholinesterase, choline acetyl transferase, and α1- and α2-adrenergic, nicotinergic, muscarinergic and serotonergic receptors.

Only two compounds, echihumiline and pycnanthine, inhibited butyrylcholinesterase activity (median inhibitory concentration (IC_{50}) values of 314.4 and 462.6 μM respectively); the activity of acetylcholine esterase and choline acetyl transferase were not affected by the PAs tested. All PAs except monocrotaline had an affinity for the muscarinic acetylcholine receptor, with echihumiline *N*-oxide being the most active (IC_{50} value = 8.7 μM) followed by senecionine (IC_{50} value = 43.0 μM), heliotrine (IC_{50} value = 52.2 μM) and seneciphylline (IC_{50} value = 52.5 μM). Only 3′-acetylheliosupine inhibited specific binding to the nicotinic ACh receptor (IC_{50} value of 159.7 μM). All PAs tested, except heliotrine *N*-oxide, retronecine and riddelliine, inhibited the specific binding to the serotonin receptor with IC_{50} values between 23.2 and 609 μM. 3′-Acetylheliosupine and heliosupine were the most active. 3′-Acetylheliosupine and heliosupine were also the only PAs to inhibit specific binding to the α1-adrenergic receptor with IC_{50} values of 39.1 and 148.1 μM, respectively. 3′-Acetylheliosupine, echihumiline, echihumiline-*N*-oxide, echimidine, heliosupine and seneciphylline inhibited the specific binding to the α2-adrenergic receptor, with 3′-acetylheliosupine being

the most active PA with an IC_{50} value of 2.9 µM followed by heliosupine with an IC_{50} of 15.0 µM (Schmeller, El-Shazly & Wink, 1997).

Australine was tested in in vitro assays for various metabolic enzyme activities using purified enzyme activity assays. Tropea et al. (1989) found that australine inhibits amyloglucosidase (an alpha 1-4 and alpha 1-6 exoglycosidase), but not beta glucosidase, alpha or beta mannosidases or alpha and beta galactosidases. It also inhibited glucosidase I but not glucosidase II. In a cell culture assay using influenza-infected MDCK cells, Tropea et al. (1989) reported that a 2-hour incubation with 500 µg/mL australine resulted in alterations in the oligosaccharide processing in isolated mature viruses.

(e) Unknown mechanism of action

In separate studies, Yan & Huxtable (1995d, 1996b) found changes in sulfur metabolism in bile.

Yan & Huxtable (1995d) administered 0.5 mM monocrotaline in the perfusion buffer of a rat liver perfusion study and found, in the first 60 minutes, cumulative increases (30-fold) in conjugated and free GSH release, and 2.6- and 2.3-fold increases in cysteine and cysteinylglycine, respectively, in the bile collected.

Yan & Huxtable (1996b) administered retrorsine, 20.4 mg/kg intraperitoneally, to male Sprague-Dawley rats and collected bile through a bile duct cannulation for 1 hour at 2, 4, 7, 12, 16, 24 and 48 hours. GS-DHP was present in the bile 2–3 hours after administration (0.24 mM) and then fell rapidly. Within 6 to 8 hours, biliary GSH increased significantly from time 0 (3 mM) and had risen by 12 hours to 24 mM, as did cysteine (from <1 mM at time 0 to 5 mM at 12 hours) and cysteinylglycine (1 mM at time 0 to 9 mM at 12 hours). Both results suggest a compensatory change in sulfur metabolism by the liver following exposure to PAs.

2.1.4 Physiologically based pharmacokinetic (PBPK) modelling

No information was available.

2.1.5 Transfer from feed to food

WHO-IPCS (1988) and EFSA (2011) concluded that PAs are able to transfer to milk, meat and eggs, but generally in low amounts.

(a) Milk

Studies in rodents have demonstrated transfer of 0.02 to 0.04% of 1,2-unsaturated PAs (retrorsine, senecionine, seneciphylline) into maternal milk (Eastman, Dimenna & Segall, 1982; Luthy, Heim & Schlatter, 1983).

Several studies examined the distribution of PAs and the transfer to milk in cattle and other animals utilized for milk production. Three dairy cows (4–5 years old; 600–700 kg) with fistulated rumens were treated with ragwort through the fistula at daily doses of 0 g for week 1, 50 g (2 × 25 mg) in week 2, 100 g (2 × 50 mg) in week 3, 200 g (2 × 100 mg) in week 4, and 0 g in week 5 (Hoogenboom et al., 2011). The PAs in the ragwort consisted mostly of jacobine, jaconine, erucifoline, senecionine and seneciphylline and the corresponding *N*-oxides. The total daily dose was divided into two equal doses per day. Milk was collected twice daily. Milk samples contained measurable levels of the following PAs: jacoline, jacobine, jaconine and senkirkine. No *N*-oxides were identified in the milk samples. Based on milk production and the total PA concentration, the transfer of the daily doses to milk was 0.1% of the overall daily dose of PAs. Although jacoline made up only 1% of the administered PAs, 4% of this PA and its *N*-oxide present in the plant material was transferred into the milk as a free base.

Similarly, Candrian et al. (1991) identified 0.16% of the total dose of radiolabelled PA in the milk of a cow given a single oral dose of 1 mg/kg bw of ^{3}H-labelled seneciphylline. At 18 hours after dosing, 100 ng/mL of seneciphylline was identified in the blood. When the cow was sacrificed 21 days post-dosing, radioactivity indicated that 0.06% of the original dose was in hepatic tissue.

Three merino sheep were treated with five capsules daily containing 6.5 mg of ^{14}C-labelled PAs (seneciphylline 91%, senecionine 6%, rest unidentified) for 5 days (Panariti, Xinxo & Leksani, 1997). The total dose was approximately 162.25 mg seneciphylline. At the end of the 5 days, levels in milk were 987 ng/mL seneciphylline and blood levels reached a maximum of 518.2 ng/mL during the last 2 days of treatment. Liver samples taken at the end of 10 days showed that 0.22% of the total dose administered was present in hepatic tissue.

Goats (Nubian, n = 3) received approximately 1% (500 g) of their body weight in tops of tansy ragwort per day, via cannula (Deinzer et al., 1982). 1,2-Unsaturated PAs and their *N*-oxides were determined in milk after conversion to retronecine. The authors estimated that this represented transfer of approximately 0.1% of the ingested PAs.

Milk was collected from a lactating goat that had been fed a diet supplemented with 0.5% of the seeds from *Crotalaria spectabilis*, a plant containing monocrotaline. The milk was collected twice daily for 8 weeks and was used to supplement the feed of rats (Medeiros, Górniak & Guerra, 1999). Twenty male Wistar rats (22 days old) received regular feed or the feed supplemented with the goat milk until they were killed on day 83. Histopathology revealed mild to moderate interstitial pneumonia with diffuse thickening of the alveolar septa in the lungs and apoptosis of hepatocytes and vacuolar degeneration in the liver suggesting that PAs were distributed to and excreted in the milk of the goat. The

concentration of PAs in the milk, and whether the PAs remained as the parent compound or were excreted as metabolites in the milk, was not determined. The amount of monocrotaline and the amount or type of metabolites found in the tissue and the blood of the rats were not reported.

(b) Avian eggs

Several studies looked at the distribution of PAs in avian eggs after administration of PAs to the mother animals. EFSA summarized two studies by Eröksüz et al. (2003b, 2008).

Diaz, Almeida & Gardner (2014) and Gardner et al. (2014) reported a study in which *Crotolaria pallida* seeds with 0, 0.54, 0.99 or 1.41 mg/kg of usaramine and its *N*-oxide were administered in the feed (0, 1%, 2% or 3% seeds containing 0.18% dry weight of usaramine and its *N*-oxide in the feed) for 5 weeks to 40 laying hens (10/group). By the end of the study, all treated hens had significant decreases in body weight compared to the controls, and egg mass production was reduced in the middle- and high-dose groups, but not the average egg weight. The average number of eggs laid per hen was reduced in the high-dose group. Usaramine was identified in egg; the maximum detected concentration was 885 ng/g for the eggs collected at day 14 in the high-dose group, the lowest concentration was 290 ng/g at day 7 in the low-dose group. Eggs collected 1 week after the end of the experiment (day 42) still contained detectable concentrations of usaramine (11.5, 9.5 and 43.5 ng/g for the low-, middle- and high-dose group, respectively).

In a study by Mulder et al. (2016), laying hens were fed diets containing 0.5% of dried common ragwort, common groundsel, narrow-leaved ragwort or viper's bugloss, or 0.1% of common heliotrope for 14 days. They found that the overall transfer rates from feed to eggs for the sum of PAs were between 0.02% and 0.23%, depending on the type of PAs in the feed. PAs were transferred mainly to the yolk of the eggs. Steady-state levels of 12, 21, 216, 2 and 36 µg/kg, respectively, were reached when the laying hens were exposed to concentrations of 5.5, 11.1, 53.1, 5.9 and 21.7 mg/kg in the feed, of common ragwort, common groundsel, narrow-leaved ragwort, viper's bugloss or common heliotrope, respectively. PAs measured in the eggs were primarily present in their free base form, and included mainly jacoline (38%), jacobine (17%), erucifoline (14%) and retrorsine (11%) when hens were given common ragwort. In eggs from hens that received common groundsel, the predominant PAs were retrorsine (58%), retrorsine *N*-oxide (7%), seneciphylline (6%), and an unknown retronecine macrocyclic ester (10%). When laying hens were fed narrow-leaved ragwort, the main PAs found in the eggs were retrorsine (67%), usaramine (8%) and otosenine (7%), and when fed viper's bugloss echimidine (75%), an unknown retronecine monoester

metabolite (9%) and an unknown retronecine diester (12%) were detected. After exposure to common heliotrope via the diet, the highest levels in eggs were found for europine (61%) and heliotrine (20%). When the PAs were withdrawn from the feed, the PA level in the eggs gradually decreased but could still be detected after 14 days in the group of laying hens exposed to the higher levels.

In another study, Eröksüz et al. (2003b) fed groups of 10 laying hens 0, 0.5, 2 or 4% ground aerial parts of *Senecio vernalis* for 210 days. Plant alkaloid content was 0.14%: 8.6% in the basic form and 91.4% in the *N*-oxide form. Specific alkaloids were senecionine (66.7%), senecivernine (10.4%), seneciphylline (8.5%), integerrimine (8.4%), retrorsine (3.0%), senkirkine (2.45%) and hydroxysenkirkine (0.7%). No PAs were found in the eggs sampled. Cumulative PA consumption for the groups was 0, 111, 291 and 468 mg/kg bw in the 0, 0.5, 2 and 4% groups, respectively.

Eröksüz et al. (2008) fed Japanese quail (40 per group) either untreated feed or feed treated with 390 mg/kg PA from *Heliotropium dolodum*, 450 mg/kg PA from *Heliotropium circinatum* or 420 mg/kg PA from *Senecio vernalis* from hatching to day 74. The major specific PAs in *H. dolodum* were: lasiocarpine (44.0%), europine (26.0%) and heliosupine (18.5%); in *H. circinatum*: europine (67.3%), heliotrine (16.3%) and lasiocarpine (8.1%); and in *S. vernalis*: senecionine (66.7%), senecivernine (10.4%) and seneciphylline (8.5%). Total alkaloid consumption for the animals fed *H. dolodum*, *H. circinatum* or *S. vernalis* was 1120, 1388 and 1274 mg/kg bw in the males and 1606, 1856 and 1702 mg/kg bw in the females, respectively. Alkaloids were present in the eggs, with europine (8.66 µg/g, 52 µg per egg) found in the eggs of animals treated with *H. dolodum*; europine (19.05 µg/g, 15.12 µg per egg) and heliotrine (1.46 µg/g, 9.29 µg per egg) in the eggs of animals treated with *H. circinatum*; and senecionine (3.21 µg/g, 22.27 µg per egg) in eggs of animals treated with *S. vernalis*. EFSA noted that not all PAs were transferred from the feed to the eggs and calculated from this study that transfer was 0.35% for the animals treated with *H. dolodum*, 1.08% for the animals treated with *H. circinatum*, and 0.22% for the animals treated with *S. vernalis*.

(c) Avian meat

In the study performed by Mulder et al. (2016), laying hens were slaughtered shortly after the last exposure to PA concentrations in the feed of 5.5, 11.1, 53.1, 5.9 and 21.7 mg/kg bw originating from various PA-containing plants (see above). The concentrations in the meat were slightly lower than the PA concentrations in the eggs, while the PA concentrations in the livers and kidneys were somewhat higher. After PA exposure was stopped, PA concentrations in meat and especially kidneys and livers decreased.

(d) Mammalian meat

In a radiotracer study, a dairy cow received a single oral dose of 1 mg/kg bw of ^{3}H-labelled seneciphylline (Candrian et al., 1991). At slaughter, 0.06% of the dose was found in the liver.

Crotalaria novae-hollandiae subsp. *novae-hollandiae*, *Heliotropium amplexicaule* and *Senecio brigalowensis* were fed at approximately 15% of the diet to weaned calves for 6 weeks to achieve a dose of 5.5, 15 and 2.5 mg/kg bw per day, respectively (Fletcher et al., 2011a). Alkaloids present in *C. novae-hollandiae* subsp. *novae-hollandiae* were identified as monocrotaline, pumiline A, trichodesmine and crosemperine; in *H. amplexicaule* as mainly indicine and as a minor component heliospathine (both mainly in *N*-oxide form); and in *S. brigalowensis* as sceleratine (mainly as *N*-oxide), senkirkine, otosenine, desacetyldorinine, florosenine and dorinine. After exposure to *C. novae-hollandiae* subsp. *novae-hollandiae*, PAs were detected in blood at levels up to 150 µg/kg, plateauing around days 7–28 and decreasing thereafter. PAs were also detected in muscle and liver tissue and followed the same trend tending to plateau at maximum levels of 250 µg/kg and 2500 µg/kg, respectively. PA adducts were detected in blood samples, with increasing levels up to days 14–21 after which they decreased. In tissues, the following order was found for PA-adduct levels: liver > kidney ≈ heart > muscle. After exposure to *H. amplexicaule*, PA levels in tissues were below or at the limit of quantification of 1 µg/kg. PA adducts were detected in blood, muscle, liver, kidney and heart samples, and tended to increase during the trial. In the tissues, PA-adduct levels were in the order liver > kidney ≈ heart ≈ muscle. After exposure to *S. brigalowensis*, free PAs, all of the otonecine type, were detected in blood and liver reaching a plateau after 2–3 weeks at levels up to 90 µg/kg and 400 µg/kg, respectively, and decreasing to 30 µg/kg and 40 µg/kg, respectively, at the end of the trial. PA adducts were detected in blood and tissue samples in increasing amounts up to 35 days, after which the levels declined. The PA adducts were detected in the order liver > kidney > heart ≈ muscle.

2.2 Toxicological studies

Many studies have been performed using extracts or material from PA-containing plants. Most of these studies did not specify the PA content, making it impossible to relate the toxicity to a dose of a specific PA. Therefore the Committee decided not to include studies on unspecified extracts or plant material in the evaluation.

As in vitro toxicity data would not be useful for deriving a point of departure for risk assessment, mainly in vivo toxicity data were included in the evaluation. Only in section 2.2.4 Genotoxicity, have in vitro studies been included.

For reasons given in the paragraph on "inclusion of studies" in section 1, studies in cell systems based on *Drosophila* or plants were excluded.

2.2.1 Acute toxicity

The acute toxicity of PAs was evaluated by IPCS in 1988 (IPCS, 1988) and EFSA in 2011 (EFSA, 2011). Acute toxicity studies for which median lethal dose (LD_{50}) values were described in these evaluations, as well as new publications are summarized in Tables 5–8. For the previous evaluations (EFSA, 2011; IPCS, 1988), mainly studies in which PAs were administered by intraperitoneal or intravenous injection were available. More recently, several acute toxicity studies using oral administration have been performed (Table 5). Since toxicity studies using intraperitoneal or intravenous administration are not very relevant for the risk assessment of PAs in food, the Committee focused on the available oral toxicity data.

EFSA (2011) noted, based on earlier studies with limited details, that chronic liver lesions are induced by single doses of PAs, including lasiocarpine, heliotropine, heliotrine, retrorsine, riddelliine, seneciphylline, senkirkine and hydroxysenkirkine. The liver and lung were the main organs affected, with the most common lesion being confluent haemorrhagic necrosis in the liver, followed by lesions in the central and sublobular veins of the liver. IPCS (1988) noted that the liver effects are similar whether caused by a single sublethal dose or multiple small doses.

Oral LD_{50} values are reported in mice for senecionine (57 mg/kg bw), isoline (123 mg/kg bw), adonifoline (163 mg/kg bw) and monocrotaline (154 mg/kg bw) and in rats for retrorsine (34–38 mg/kg bw), retrorsine *N*-oxide (48 mg/kg bw), riddelliine (80 mg/kg bw), lasiocarpine (110 mg/kg bw), monocrotaline (75, 151, 501, 510 mg/kg bw), echimidine (518 mg/kg bw) and heliotrine (510 mg/kg bw) (Table 5). These studies indicate that senecionine, retrorsine (and its *N*-oxide) and riddelliine are among the more acutely toxic PAs, whereas echimidine and heliotrine appear less toxic. IPCS (1988) and EFSA (2011) noted that the large macrocyclic diesters, such as retrorsine and senecionine, having a higher potency compared to the monoesters, such as heliotrine, correlated with the different toxicokinetic profiles. However, since only one oral LD_{50} value is available for a monoester (heliotrine), this cannot be confirmed based on the available oral acute toxicity data.

Heliotrine, monocrotaline, riddelliine and echimidine were tested in oral acute toxicity studies with the same study design, according to OECD Test Guideline 425 with some modifications (using male instead of female animals, determination of survival at 72 instead of 24 hours, and an observation period of 7 days instead of 14 days) (Dalefield, Gosse & Mueller, 2015; Dalefield et al., 2012 a,b). As noted by the authors, the nature of the liver lesions was generally

Table 5
Acute oral toxicity studies of pyrrolizidine alkaloids in rodents using single administration

Compound (purity)	Species	Dose (mg/kg bw)	Effect	LD_{50}[b] (confidence interval) [LOAEL] (mg/kg bw)	NOAEL (mg/kg bw)	Reference
Acetyl-lycopsamine	Mouse (m)	750	Biochemical and liver changes	[750]	NA	Robinson et al. (2012); Robinson et al. (2014)
Adonifoline	Mouse[a]	–	Lethality	163	NA	Wang C et al. (2011); Xiong et al. (2012)
Echimidine	Rat (m)	50, 160, 518, 1650	Lethality, weight loss, clinical signs, liver histopathology	518 (229–654)	160	Dalefield, Gosse & Mueller (2015)
Heliotrine (98%)	Rat (m)	50, 160, 510, 1600	Lethality, weight loss, clinical signs, liver histopathology	510 (405–142)	160	Dalefield et al. (2012a)
Isoline (≥85%)	Mouse (m)	0, 100	Biochemical changes	[100]	NA	Liu TY et al. (2010)
Isoline	Mouse[a]	–	Lethality	123 (106–142)	NA	Liu TY et al. (2010)
Isoline (≥90.5%)	Mouse (m)	0, 110	Biochemical and gene changes	[110]	NA	Wang Z et al. (2012)
Isoline (≥95%)	Mouse (m,f)	0, 110	Biochemical and gene changes	[110]	NA	Liang et al. (2011a)
Isoline (≥95%)	Mouse (m)	0, 105	Biochemical changes	[105]	NA	Liang et al. (2011b)
Lasiocarpine	Rat (m)	–	Lethality	110	NA	Newberne & Rogers (1970b)
Monocrotaline base equivalents (crude alkaloid from *Crotalaria assamica*)	Mouse[a]	128, 144, 161, 180	Lethality	154 (147–161)	NA	Chan MY, Zhao & Ogle (1989)
Monocrotaline	Rat (m)	–	Lethality	75	NA	Newberne & Rodgers (1970b)
Monocrotaline	Rat[a]	140, 145, 150, 155	Lethality	151	NA	Marar & Devi (1996)
Monocrotaline	Rat (f)	46.4, 100, 215, 464, 1000, 2150	Lethality	501	NA	Sharma, Prakash & Bhatnagar (2007)
Monocrotaline (99%)	Rat (m)	160, 510, 1600	Lethality, weight loss, clinical signs, liver histopathology	510 (308–814) [160]	NA	Dalefield et al. (2012b)
Retrorsine	Rat[a]	–	Lethality	34–38	NA	Mattocks (1971), cited by EFSA (2011)
Retrorsine equivalents (crude extract of *Senecio inaequidens*)	Rat (m)	0, 12, 35, 48, 60	Biochemical and liver changes	[12]	NA	Dimande et al. (2007)
Retrorsine *N*-oxide	Rat[a]	–	Lethality	48	NA	Mattocks (1971), cited by EFSA (2011)
Riddelliine (92%)	Rat (m)	25, 80, 255	Lethality, weight loss, clinical signs, liver histopathology	80 (47–152)	25	Dalefield et al. (2012b)

Compound (purity)	Species	Dose (mg/kg bw)	Effect	LD_{50} [b] (confidence interval) [LOAEL] (mg/kg bw)	NOAEL (mg/kg bw)	Reference
Senecionine	Mouse[a]	–	Lethality	57	NA	Wang C et al. (2011); Xiong et al. (2012)
Senecionine	Rat (m)	0, 35	Biochemical and liver changes	[35]	NA	Xiong et al. (2014)

f: female; m: male; –: not reported; NA: not applicable

[a] Sex unknown.

[b] The route of administration is not clear in Newberne & Rogers (1970); however, from a subsequent publication by these authors (Rogers & Newberne, 1971) it can be assumed that the PAs were administered orally in this study.

similar between the four PAs studied, except that the endothelial cells in the central veins appeared normal in the study with echimidine. Also, in contrast to heliotrine and monocrotaline, echimidine and riddelliine did not cause pulmonary changes consistent with acute pulmonary hypertension. The results observed also suggested that all four PAs inhibited gastric emptying. Based on the calculated LD_{50} values (and taking into account the skewed confidence limits), the relative acute toxicities would be riddelliine > echimidine > monocrotaline > heliotrine. However, when ranked based on histopathological no-observed-adverse-effect levels (NOAELs) the order would be riddelliine > monocrotaline > echimidine ≈ heliotrine. A firm conclusion on relative acute toxicity cannot be drawn because of the small number of animals studied (Dalefield, Gosse & Mueller, 2015).

The acute oral toxicity of monocrotaline has also been investigated in goats, sheep and donkeys (Table 6). The animals died or were killed moribund at the lowest-observed-adverse-effect levels (LOAELs) reported in Table 6. For 31 PAs, LD_{50} values were available after intraperitoneal administration. Based on the intraperitoneal LD_{50} values, heliosupine, lasiocarpine, retrorsine, senecionine, seneciphylline and spectabiline were most toxic, whereas europine, heliotridine, heliotrine *N*-oxide, indicine and intermedine were of lower toxicity (Table 7). Acute toxicity of riddelliine administered by the intraperitoneal route has not been reported. For PAs for which both oral and intraperitoneal LD_{50} values were available, the toxicity via the oral route tends to be lower, except for retrorsine for which the toxicity via the two routes was comparable. Based on the LD_{50} values after intraperitoneal administration, male rats seem more susceptible to the acute toxicity of basic PAs than females. EFSA (2011) indicated that the relative hepatotoxicity of 62 PAs has been investigated by administering single intraperitoneal doses (in the range of 0.025–3.2 mmol/kg bw) to male and female rats (Culvenor et al., 1976 as cited by EFSA, 2011). The livers of surviving rats were examined 4 weeks later. Most 1,2-unsaturated PAs caused death with liver necrosis within 1 week, or liver megalocytosis observed at necropsy after 4 weeks. A number of heliotridine esters did not cause liver toxicity under the conditions of the experiment. The 1,2-saturated PAs platyphylline (118 mg/kg bw) and cynaustraline (67 mg/kg bw) caused rapid death after intraperitoneal administration, without hepatotoxicity. LD_{50} values were not derived in this study (Culvenor et al., 1976, as cited by EFSA, 2011; Jago, 1970, as cited by EFSA, 2011). On a molar basis, diesters of heliotridine and retronecine were about four times more toxic than retronecine esters. Crotonecine esters were less toxic than retronecine esters (Culvenor et al., 1976, as cited by EFSA, 2011).

For 10 PAs, LD_{50} values after intravenous injection were available. The LD_{50} values ranged between approximately 40 and 100 mg/kg bw except for heliotrine (255–274 mg/kg bw) and retrorsine *N*-oxide (834 mg/kg bw). Oral

Table 6

Acute oral toxicity studies of pyrrolizidine alkaloids in non-rodents using single administration

Compound (purity)	Species	Dose (mg/kg bw)	Effect	LOAEL(mg/kg bw)	NOAEL (mg/kg bw)	Reference
Monocrotaline (*Crotalaria retusa* seeds, 4.96% monocrotaline content, constituting >99% of the total DHPA content)	Goat	0, 148.3, 248.0, 347.0	Biochemical and clinical changes, organ changes, lethality	248	148.3	Maia et al. (2013)
Monocrotaline (*Crotalaria retusa* seeds, 1.4% monocrotaline)	Sheep	0, 35, 70, 140, 280, 560	Biochemical and clinical changes, organ changes, lethality	70	35	Nobre et al. (2005)
Monocrotaline (*Crotalaria retusa* seeds, 6.84% monocrotaline)	Sheep	0, 205.2, 273.6	Biochemical and clinical signs, organ changes, lethality	205.2	–	Anjos et al. (2010)
Monocrotaline (*Crotalaria retusa* seeds, >99% of DHPA content)	Donkey	0, 149.7, 299.5	Biochemical and clinical changes, organ changes, lethality	299.5	149.7	Pessoa et al. (2013)

DHPA, dehydropyrrolizidine alkaloid.

Table 7
Acute intraperitoneal toxicity studies of pyrrolizidine alkaloids using single administration

Compound (purity)	Species (sex)	Dose (mg/kg bw)	Effect	LD_{50} (LOAEL) (mg/kg bw)	Reference
7-O-angeloylheliotridine	Rat (m)	–	Lethality	250	Downing & Peterson (1968), cited by EFSA (2011)
Clivorine	Rat (m,f)	–	Lethality	91 ± 3 (m) 114 ± 9 (f)	Lin, Cui & Liu (2003)
Cynaustine	Rat (m)	–	Lethality	260	Bull, Culvenor & Dick (1968), cited by EFSA (2011)
Echimidine	Rat (m)	–	Lethality	200	Culvenor et al. (1969), cited by EFSA (2011)
Echinatine	Rat (m)	–	Lethality	350	Culvenor et al. (1969), cited by EFSA (2011)
Europine	Rat (m)	–	Lethality	>1000	Culvenor et al. (1969), cited by EFSA (2011)
Heleurine	Rat (m)	–	Lethality	140	Culvenor et al. (1969), cited by EFSA (2011)
Heliosupine	Rat (m)	–	Lethality	60	Culvenor et al. (1969), cited by EFSA (2011)
Heliotridine	Rat (m)	–	Lethality	1500	Downing & Peterson (1968), cited by EFSA (2011)
Heliotrine	Rat (m,f)	–	Lethality	296 (m) 478 (f)	Bull, Dick & McKenzie (1958), cited by EFSA (2011)
Heliotrine *N*-oxide	Rat (m)	–	Lethality	>5000	Bull, Dick & McKenzie (1958), cited by EFSA (2011)
Indicine	Rat (m)	–	Lethality	>1000	Schoental (1968), cited by EFSA (2011)
Intermedine	Rat (m)	–	Lethality	1500	Cheeke & Shull (1985), cited by EFSA (2011)
Jacobine	Rat (f)	–	Lethality	138	Bull, Culvenor & Dick (1968), cited by EFSA (2011)
Jaconine	Rat (f)	–	Lethality	168	Bull, Culvenor & Dick (1968), cited by EFSA (2011)
Lasiocarpine	Rat (m, f)	–	Lethality	77 (m) 79 (f)	Bull, Culvenor & Dick (1968), cited by EFSA (2011)
Lasiocarpine	Rat (m)	80	Liver changes	[80]	Rao et al. (1991)
Lasiocarpine *N*-oxide	Rat (m, f)	–	Lethality	547 (m) 181 (f)	Bull, Dick & McKenzie (1958), cited by EFSA (2011)
Latifoline	Rat (m)	–	Lethality	125	Bull, Culvenor & Dick (1968), cited by EFSA (2011)
Lycopsamine	Rat (m)	–	Lethality	1500	Cheeke & Shull (1985), cited by EFSA (2011)
Monocrotaline (extracted from *Crotalaria retusa*)	Mice (m)	0, 5, 50, 100	Biochemical and brain changes	[5]	Honorio Jr. et al. (2012)
Monocrotaline	Rat (m)	0, 65	Biochemical changes	[65]	Yan & Huxtable (1995c)

Compound (purity)	Species (sex)	Dose (mg/kg bw)	Effect	LD_{50} (LOAEL) (mg/kg bw)	Reference
Monocrotaline	Rat (m)	0, 65	Biochemical changes	[65]	Sun et al. (1998)
Monocrotaline (extract from *C. spectabilis*)	Rat (m)	0, 65	Biochemical changes	[65]	Yan & Huxtable (1996a)
Monocrotaline	Rat (m)	0, 60	Biochemical and clinical changes	[65]	Coll et al. (2011)
Monocrotaline	Rat (m)		Lethality	175	Bull, Culvenor & Dick (1968), cited by IPCS (1988)
Monocrotaline	Rat (m)	–	Lethality	175	Culvenor et al. (1969), cited by EFSA (2011)
Monocrotaline	Rat (m, f)	–	Lethality	109 (m) 230 (f)	Mattocks (1972), cited by EFSA (2011)
Monocrotaline	Minipig[a]	0, 12.0	Lung changes	[12.0]	Zeng et al. (2013)
Platyphylline	Rat (m)	–	Lethality	252	Downing & Peterson (1968), cited by EFSA (2011)
Retrorsine	Mouse[a]	–	Lethality	65–69	White, Mattocks & Butler (1973), cited by EFSA (2011)
Retrorsine	Rat (m)	0, 20.4	Biochemical changes	[20.4]	Sun et al. (1998)
Retrorsine	Rat (m)	0, 20.4	Biochemical changes	[20.4]	Yan & Huxtable (1995e)
Retrorsine	Rat (m)	–	Lethality	34–38	Mattocks (1971), cited by EFSA (2011)
Retrorsine	Rat (f)	–	Lethality	153	Mattocks (1972), cited by EFSA (2011)
Retrorsine	Quail[a]	–	Lethality	279	White, Mattocks & Butler (1973), cited by EFSA (2011)
Retrorsine	Fowl[a]	–	Lethality	85	White, Mattocks & Butler (1973), cited by EFSA (2011)
Retrorsine	Hamster[a]	–	Lethality	81	White, Mattocks & Butler (1973), cited by EFSA (2011)
Retrorsine	Guinea-pig[a]	–	Lethality	>800	White, Mattocks & Butler (1973), cited by EFSA (2011)
Retrorsine *N*-oxide	Rat (m)	–	Lethality	250	Mattocks (1971), cited by EFSA (2011)
Rinderine	Rat (m)	–	Lethality	550	Bull, Culvenor & Dick (1968), cited by EFSA (2011)
Senecionine	Rat (m)	–	Lethality	85	Culvenor et al. (1969), cited by EFSA (2011)
Senecionine	Rat (m)	–	Lethality	50	Mattocks (1972), cited by EFSA (2011)
Seneciphylline	Rat (m, f)	–	Lethality	77 (m) 83 (f)	Bull, Culvenor & Dick (1968), cited by EFSA (2011)
Senkirkine	Rat (m)	–	Lethality	220	Hirono et al. (1979), cited by EFSA (2011)

Table 7 (continued)

Compound (purity)	Species (sex)	Dose (mg/kg bw)	Effect	LD_{50} (LOAEL) (mg/kg bw)	Reference
Spectabiline	Rat (m)	–	Lethality	50	Bull, Culvenor & Dick (1968), cited by EFSA (2011)
Supinine	Rat (m)	–	Lethality	450	Culvenor et al. (1969), cited by EFSA (2011)
Symphytine	Rat (m)	–	Lethality	130	Hirono et al. (1979), cited by EFSA (2011)
Symphytine	Rat (m)	–	Lethality	300	Cheeke & Shull (1985), cited by EFSA (2011)
Triacetylindicine	Rat (m)	–	Lethality	164	Mattocks (1972), cited by EFSA (2011)
Trichodesmine	Rat (m)	0, 15	Biochemical changes	[15]	Yan & Huxtable (1995c)

f: female; m: male; –, not reported
[a] Sex unknown.

LD_{50} values were quite comparable with intravenous LD_{50} values for PAs that were tested by both routes, except for retrorsine *N*-oxide, which was much less toxic when administered intravenously (Table 8). In rats and dogs, pulmonary lesions were observed after a single intravenous or subcutaneous injection of monocrotaline (Miller et al., 1978, as cited by EFSA, 2011; Valdivia et al., 1967a,b, as cited by EFSA, 2011).

In previous evaluations (EFSA, 2011; IPCS, 1988), it was noted that the route of administration did not have a great influence on the hepatotoxicity of basic PAs. The relative toxicity of retrorsine *N*-oxide compared with that of the basic alkaloid depended on the administration route. When given orally to rats, retrorsine *N*-oxide was reported to be as toxic as its basic alkaloid; however, when given intravenously or intraperitoneally, it was much less toxic. Heliotrine *N*-oxide and lasiocarpine *N*-oxide were also less toxic than their basic alkaloids when administered intraperitoneally (Table 7). This could be explained by the conversion of *N*-oxides to their basic alkaloids in the gut, which needs to take place before toxic pyrroles can be formed by hepatic microsomes.

2.2.2 Short-term studies of toxicity

The short-term toxicity of PAs in animals was evaluated by WHO-IPCS in 1988 and EFSA in 2011. Both WHO-IPCS and EFSA concluded that the most common toxic effect of repeated exposure to 1,2-unsaturated PAs is hepatotoxicity, characterized by megalocytosis[1] (enlarged hepatocytes containing hyperchromatic nuclei), and centrilobular necrosis, fibrosis and bile duct hyperplasia. EFSA reported in 2011 that in all animal species and for all ingested plant species investigated by them, lesions in the liver had been observed.

WHO-IPCS reported that almost simultaneously with or shortly after haemorrhagic necrosis of the liver cells, various levels of change appear in the central and sublobular veins of the liver lobules. These changes consist of subintimal oedema or even necrosis, deposits of fibrin, thrombosis and occlusion of the lumen, which later becomes organized. In humans, poisoning with 1,2-unsaturated PAs is characterized by acute hepatic veno-occlusive disease (HVOD), as reported by EFSA in 2011. The acute disease is associated with high mortality, and a subacute or chronic onset may lead to liver cirrhosis.

Both WHO-IPCS and EFSA reported that, besides hepatotoxicity, pulmonary toxicity has been observed; the structural requirements for toxicity in the lung are the same as those for toxicity in the liver and the toxicity is caused by metabolites produced in the liver. Pulmonary toxicity manifests as pulmonary

[1] The terms "cytomegalocytosis" and "(hepato-)cytomegaly" that are used in particular in older studies are considered to be synonymous with "hepatocellular hypertrophy", which is the term that is presently more commonly used in the literature.

Table 8
Acute intravenous toxicity studies of pyrrolizidine alkaloids

Compound[a]	Species[b]	LD_{50} (mg/kg bw)	Reference
Heliotrine	Mouse	255	Mattocks (1986), cited by EFSA (2011)
Heliotrine	Rat	274	Mattocks (1986), cited by EFSA (2011)
Integerrimine	Mouse	78	Mattocks (1986), cited by EFSA (2011)
Jacobine	Mouse	77	Mattocks (1986), cited by EFSA (2011)
Lasiocarpine	Mouse	85	Mattocks (1986), cited by EFSA (2011)
Lasiocarpine	Rat	88	Mattocks (1986), cited by EFSA (2011)
Lasiocarpine	Hamster	67.5	Mattocks (1986), cited by EFSA (2011)
Retrorsine	Mouse	59	Mattocks (1986), cited by EFSA (2011)
Retrorsine	Rat	38	Mattocks (1986), cited by EFSA (2011)
Retrorsine *N*-oxide	Mouse	834	Mattocks (1986), cited by EFSA (2011)
Riddelliine	Mouse	105	Mattocks (1986), cited by EFSA (2011)
Senecionine	Mouse	64	Mattocks (1986), cited by EFSA (2011)
Senecionine	Hamster	61	Mattocks (1986), cited by EFSA (2011)
Seneciphylline	Mouse	90	Mattocks (1986), cited by EFSA (2011)
Spartioidine	Mouse	80	Mattocks (1986), cited by EFSA (2011)

[a] Purity not specified.
[b] Sex not specified.

hypertension and can lead to cardiac right-ventricular hypertrophy. Acute lesions include alveolar oedema and effects on the alveolar wall. Chronic lesions can include extensive pleural effusion or necrotizing pulmonary arthritis.

Short-term toxicity studies in mouse, rat, chicken, quail, pig, sheep, cow and horse that were identified during the SR are described below. Some studies were appropriate for the derivation of a LOAEL or NOAEL. These studies are summarized in Table 9. Other studies provided information that indicated the hazardous properties of several PAs but were not found suitable for the derivation of a NOAEL due to limitations of the study design and description. Hence, these studies were considered to be supportive and are therefore described in less detail.

(a) Mouse

The toxicity of riddelliine was investigated in $B6C3F_1$ mice in a 2-week and a 13-week study (Chan et al., 1994; NTP, 1993) as part of a larger effort involving a chronic 2-year study conducted by the US NTP (see section 2.2.3 for a description of the 2-year study). In the 2-week study, groups of five male and five female mice (6 weeks of age) were dosed with 0 (vehicle control), 0.33, 1.0, 3.3, 10 or 25 mg/kg bw riddelliine (95% riddelliine, 5% retrorsine, 0.2% seneciphylline) in 0.1 M phosphate buffer by gavage for 5 days a week plus 2 consecutive doses (in total 12 administrations in 16 days). Averaged over the week, corrected doses amounted

Table 9

Overview of derived LOAELs and/or NOAELs from short-term toxicity studies with pyrrolizidine alkaloids

Species description	Compound (purity)	Length of study	No. per group	Dose (mg/kg diet)	Dose (mg/kg bw per day)[a]	Route	Effect	LOAEL (mg/kg bw)	NOAEL (mg/kg bw)	Reference
Mouse B6C3F1	Riddelliine (purity 92%)	13 weeks (plus a recovery period of 7 or 14 weeks for selected animals)	40 (20 per sex)	n/a	0, 0.33, 1, 3.3, 10 or 25 for 5× per week. Averaged over the week: 0, 0.24, 0.71, 2.4, 7.1 or 18	Gavage	Reduced body weight gain, affected organ weights, hepatocytomegaly	7.1	2.4	Chan et al. (1994); NTP working group (1993)
Mouse Swiss	*Heliotropium dolosum* (0.13% total PAs; lasiocarpine 78%, heliosupine 12%, echimidine 5.4%, heliotrine 3.8%)	24 weeks	12 (males only)	0, 13, 39, 65 or 130	0, 2.0, 5.9, 9.8 or 19.5 for total PAs	Feed	Mortality, gross liver lesions including periportal and midzonal hepatic megalocytosis, karyomegaly, necrosis and bile duct proliferation, acute or chronic hepatitis, kidney lesions, lung oedema	2.0	n/a	Eröksüz et al. (2001)
Rat Wistar	Echimidine (95.5%)	28 days	20 (10 per sex)	n/a	0, 0.60, 1.22 or 2.46 (m) 0, 0.61, 1.23 or 2.57 (f)	Feed	–	n/a	2.5	Dalefield, Gosse & Mueller (2016)
Rat Wistar	Lasiocarpine (94.1%)	28 days	20 (10 per sex)	n/a	0, 0.63, 1.23 or 2.78 (m) 0, 0.67, 1.29 or 2.76 (f)	Feed	Reduced body weight and body weight gain, increased serum ALT, decreased albumin, decreased absolute liver weights	2.8	1.2	Dalefield, Gosse & Mueller (2016)
Rat F334/N	Riddelliine (95%)	2 weeks	10 (5 per sex)	n/a	0, 0.33, 1.0, 3.3, 10 or 25 for 5× per week plus two consecutive doses. Averaged over the week: 0, 0.24, 0.71, 2.4, 7.1 or 18	Gavage	Histopathological changes in liver (including haemorrhagic centrilobular hepatic necrosis, karyomegaly) and lung (including focal haemorrhage, oedema), increased relative liver weight	0.71	0.24	Chan et al. (1994); NTP working group (1993)

Table 9 (continued)

Species description	Compound (purity)	Length of study	No. per group	Dose (mg/kg diet)	Dose (mg/kg bw per day)[a]	Route	Effect	LOAEL (mg/kg bw)	NOAEL (mg/kg bw)	Reference
Rat F334/N	Riddelliine (92%)	13 weeks (plus a recovery period of 7 or 14 weeks for selected animals)	40 (20 per sex)	n/a	0, 0.1, 0.33, 1.0, 3.3 or 10 for 5× per week; averaged over the week: 0, 0.071, 0.24, 0.71, 2.4 or 7.1	Gavage	Foci of hepatocellular alteration, karyomegaly, increased S-phase DNA synthesis in liver, haematological changes	0.71	0.24	Chan et al. (1994); NTP working group (1993)
Rat F334/N	Riddelliine (92%)	8 consecutive days, or 6 weeks (5× per week)	7 (males only)	n/a	0, 1.0 or 2.5. averaged over the week for 6-week study: 0, 0.71 or 1.4	Gavage	Hepatocyte vacuolization and hypertrophy, endothelial changes, reduction in hepatocyte mitosis, increased liver weights	1.0	n/a	Nyska et al. (2002)

ALT: alanine aminotransferase; n/a: not applicable.

to 0, 0.24, 0.71, 2.4, 7.1 and 18 mg/kg bw. Animals were observed twice daily and were weighed at the start of the experiment, weekly thereafter, and at necropsy. Clinical observations were recorded daily. At termination, complete necropsies were performed on the animals. Organs and tissues were weighed, examined for gross lesions, and subjected to histopathological and microscopical examinations.

No deaths occurred during the study, and mean body weights and mean body weight gains in the dose groups were similar to the controls. Slight emaciation (one male at day 5 and one female at day 4) and hunching (same female at day 3) were observed in the 10 mg/kg bw group but disappeared later. No clinical signs were noted in the other dose groups. Statistically significant and dose-related increases in absolute and relative mean liver weight were recorded in males in the 10 and 25 mg/kg bw dose group and in females in the 3.3, 10 and 25 mg/kg bw dose group. In addition, the absolute mean liver weight was significantly increased in females in the 0.33 and 1.0 mg/kg dose groups. Relative thymus weight in females in the 25 mg/kg bw dose group was statistically significantly increased compared to controls, and a positive trend was also observed in the other dose groups. No treatment-related gross lesions were noted. Microscopically, cytomegaly was reported in the livers of both males and females in the highest dose group. However, the actual organ weights and histopathology incidence data were not reported, as this investigation was intended as a dose-finding study for the 13-week study.

In the 13-week study, male and female mice (6 weeks old, 20 animals per sex per group) were administered the same dose levels of riddelliine (92% riddelliine, 5% retrorsine, 1.4% seneciphylline) as in the 2-week study by gavage 5 times a week. Averaged over the week, corrected doses amounted to 0, 0.24, 0.71, 2.4, 7.1 and 18 mg/kg bw. Animals were observed twice daily, and body weight and clinical observations were recorded weekly. Ten animals from each dose group were autopsied at the end of the treatment period, while groups of five animals were maintained without further treatment for a recovery period of 7 or 14 weeks. Postmortem examinations were the same as for the 2-week study, with the addition of some extra tissues, e.g. forestomach and reproductive system evaluations, and selected mice underwent clinical pathology evaluations.

Several deaths (3 males and 10 females) were reported, but none of them were considered to be treatment-related. Mean body weights and mean body weight gain were notably lower in both males and females in the 10 and 25 mg/kg bw groups compared to controls (body weights were 81 and 73% of those of controls in males and 82 and 74% in females, respectively) at the end of the base study, which did not recover in animals assigned to recovery periods. As feed consumption was not monitored in this study it is unclear whether these reductions were caused by reduced feed intake or represented toxicity of riddelliine. No clinical signs were observed during the dosing or recovery period.

Relative mean organ weights showed a dose-related increase compared to controls for the brain (17% (males), 18% (females)[1]), lung (45% (males), 27% (females)) and spleen (51% (males), 99% (females)) in male and female mice, the right testis (12%) in male mice, and the liver (7%) in female mice. At 10 and 25 mg/kg bw, statistically significant decreases were noted for absolute heart and liver weight in males (21% and 9%, respectively[1]) and females (36% and 13%, respectively[1]), absolute kidney weight in males (18%) and absolute brain and thymus weights in females (5% and 25%, respectively), while an increase in absolute weight was seen for lung in male mice (25%) and spleen in both male and female mice (31% and 63%, respectively). These differences were mainly observed in the two highest dose groups. At the end of the recovery periods, absolute and relative organ weights did not seem to have recovered towards normal.

Haematology evaluations revealed statistically significant increases in RBC count, haematocrit and haemoglobin concentration in male mice in the 0.33, 10 and 25 mg/kg bw groups, in reticulocyte counts in female mice in the 10 and 25 mg/kg bw groups, in mean cell volume in males in the 10 and 25 mg/kg bw groups and in females in the 25 mg/kg bw groups, and in mean platelet volume in males in the 0.33 and 25 mg/kg bw group and in females in the 25 mg/kg bw group. A statistically significant decrease in platelet counts was noted for males in the 10 and 25 mg/kg group and in females in all dose groups except 1.0 mg/kg bw.

No gross necropsy findings related to riddelliine administration were observed. Microscopically, centrilobular cytomegaly in the liver, characterized by increased amounts of pale-staining, finely granular cytoplasm in the hepatocytes, was found in high-dose males (10/10) and females (10/10) with a slightly greater severity in the females (mild to moderate) than in the males (minimal to mild), but was not observed at the next lowest dose of 10 mg/kg bw (0/10 males, 0/10 females). The centrilobular cytomegaly persisted throughout the recovery period in females (4/5) but not in males. Further, minimal bile duct hyperplasia was observed in high-dose females (3/5) at the end of the recovery periods. In addition, mild hyperplasia of the stratified squamous epithelium of the forestomach was noted in male and female mice from the 10 (8/10 and 2/10, respectively) and 25 (8/10 and 1/10) mg/kg bw groups, which regressed during the recovery periods. This was probably due to a direct irritant effect of riddelliine given by gavage. There were no biologically significant findings in males with respect to sperm morphology. Vaginal cytology evaluations, however, showed a prolonged estrus cycle length in females administered 25 mg/kg bw per day.

Based on histopathological changes, the study authors derived a NOAEL of 3.3 mg/kg bw, i.e. 2.4 mg/kg bw per day (Chan et al., 1994; NTP, 1993).

[1] Percentages given for 25 mg/kg bw dose group.

In a short-term toxicity study, male Swiss mice (12 animals per group, 11–15 weeks old) were given feed with 0, 1, 3, 5 or 10% *Heliotropium dolosum* seed (w/w) for 24 weeks (Eröksüz et al., 2001). Feed intake was recorded every 7 days, or more often in case of death in a cage. Surviving mice were euthanized after 24 weeks. From all animals, tissue samples of liver, lung, brain, kidney, pancreas, small intestine, large intestine and heart were taken for analysis.

The *H. dolosum* seeds were analysed by gas chromatography–mass spectrometry (GC-MS) and contained 0.13% total PAs; lasiocarpine (78.8%), heliosupine (12.0%), echimidine (5.4%) and heliotrine (3.8%). The total PA content in the final feed was therefore 0 ppm, 13 ppm, 39 ppm, 65 ppm and 130 ppm, respectively. This was equivalent to 0, 2.0, 5.9, 9.8 or 19.5 mg/kw bw per day using the JECFA conversion table for mouse (JECFA, 2016). A dose-dependent decrease in feed consumption was observed, which reached statistical significance in the 5% dose group. In addition, a dose-dependent decrease in survival times was noted, which reached statistical significance in the 5% and 10% groups. Mortality rates were 0% in the control group, 33.3% in the 1% group, 50% in the 3% group, 58.3% in the 5% group and 100% in the 10% group. In the 10% group, all animals died within 8 weeks.

Gross lesions, including dark red, congested and soft liver, atrophy and granuloma, were seen in the livers of animals given feed with 3%, 5% and 10% *H. dolosum* seeds. Histological changes in livers from all dose groups indicated acute hepatitis in seven animals and chronic hepatitis in 39 animals. In mice that survived less than 5 weeks on 10% or 5% seed levels, acute (sub)massive acidophilic coagulation necrosis or randomly scattered multifocal hepatocellular necrosis, moderate to severe sinusoidal dilatation, congestion and stasis, and mild to moderate karyomegaly was found. In animals that died after 5 weeks, hepatic megalocytosis, mainly located in periportal and midzonal regions, was detected; this increased in severity and distribution with increasing seed concentrations and survival time. Mice from the 10% group had necrotic megalocytes. Most animals in the 1%, 3% and 5% groups also displayed mild to moderate periportal mononuclear cell infiltration, hypertrophy, acidophilic degeneration in parenchymal cells, mild fibrous thickening of the liver capsule, mild to moderate bile duct hyperplasia and mild bile duct proliferation. In the kidneys of mice given 3%, 5% and 10% *H. dolosum* seeds, megalocytosis and karyomegaly were observed. Oedema in the lungs was seen in all dose groups. The authors reported that samples of brain, pancreas, small and large intestine and heart were taken, but no results for these organs were presented.

The authors concluded that dietary exposure to *H. dolosum* seeds caused specific lesions characteristic of PA intoxication in mice, and that the seeds of *H. dolosum* may pose a potential health hazard for domestic animals (Eröksüz et al., 2001). No NOAEL or LOAEL was reported by the authors. As adverse effects

were already seen in the lowest dose group, no NOAEL could be determined. The LOAEL was 2.0 mg/kg bw per day, the lowest dose tested, based on reduced feed consumption, increased mortality, various histopathological changes in the liver indicative of acute hepatitis and chronic hepatitis, and oedema in the lungs.

To investigate various model systems to study the hepatotoxic potential of PAs with expected antitumour activity, several experiments were conducted in mice and rats. Only those that used administration by the oral route are included here (Moore et al., 1989). Adult male CDF1 mice (21–25 g, age not specified, 7–10 animals per group) were administered 0, 1000, 2000 or 3000 mg/kg bw per day indicine, 0, 1200, 2000, 3000 or 4000 mg/kg bw per day indicine *N*-oxide or 0, 1000, 2000 or 4000 mg/kg bw per day retronecine *N*-oxide (purities not specified). Administration was via stomach tube for 5 consecutive days per week for 4 weeks; control animals received the vehicle only (0.3% hydroxypropyl-cellulose). Averaged over the week, adjusted doses amounted to 0, 714, 1429 or 2143 mg/kg bw per day for indicine, 0, 857, 1429, 2143 or 2857 mg/kg bw per day for indicine *N*-oxide, and 0, 857, 1429 or 2857 mg/kg bw per day for retronecine *N*-oxide. Animals were observed daily and deaths were recorded. Animals surviving on day 30 were killed and liver samples were taken for histology.

Mortality was observed in all but one dose group: for indicine mortality rates were 2/10, 9/10 and 10/10 for increasing doses, respectively; for indicine *N*-oxide 1/10, 0/10, 8/10 and 10/10 for increasing doses, respectively; and for retronecine *N*-oxide 1/10, 5/7 and 7/7 for increasing doses, respectively. Survival and incidence of histopathology findings were not reported for the vehicle control groups. Centrilobular haemorrhagic necrosis was observed in mice given indicine and indicine *N*-oxide at acutely lethal doses (nominal), and in one surviving mouse given indicine. Other histopathological changes included megalocytosis characterized by enlarged hepatocytes with varying degrees of nuclear enlargement primarily occurring in the centrilobular zone, observed in all dose groups, as well as eosinophilic cytoplasmic swelling. Retronecine *N*-oxide administration led mainly to megalocytosis, and to cytoplasmic swelling at the lowest dose, but not to centrilobular haemorrhagic necrosis (Moore et al., 1989).

(b) Rat

The toxicity of echimidine (purity 95.5%, impurities were other PAs, derived from *Echium vulgare*) and lasiocarpine (purity 94.1%, derived from *Heliotropium europaeum*) was studied in a 28-day feeding study in Wistar rats (strain not further specified) (Dalefield, Gosse & Mueller, 2016). The study adhered to good laboratory practice, but was not fully compliant with the corresponding OECD testing guideline 407 (only four tissues and gross lesions were analysed by histopathology, no determination of estrus cyclicity or neurofunctional

observations). Animals (12 weeks of age, 10 animals per sex per group) were exposed to target doses of 0, 0.6, 1.2 or 2.5 mg/kg bw per day echimidine or lasiocarpine. Echimidine and lasiocarpine were added to the feed suspended in food grade rice bran (RB) oil to a concentration no more than 5% of the weight of the feed. RB oil was also added to the control diet at 5% (w/w). Doses were selected based on the dose range-finding study for lasiocarpine in rats by the US NTP (NTP, 1978). Fresh diets were prepared weekly with the amount of PA adjusted based on predicted body weight and feed consumption. During the experiment, rats were observed for clinical signs and feed consumption daily, and measured for body weight and body weight gain twice weekly. The following parameters were measured: absolute and relative weights of adrenals, brain, epididymis, heart, kidneys, liver, prostate, spleen, testes, thymus, seminal vesicles, uterus and ovaries, urinalysis including bilirubin ketones and sediment analysis, histopathology (limited to heart, lungs, stomach, liver and gross lesions), haematology and clinical chemistry.

For echimidine, the animals were estimated to have received a mean daily exposure of 0, 0.60, 1.22 or 2.46 mg/kg bw (males) and 0, 0.61, 1.23 or 2.57 mg/kg bw (females) (reported by the authors as adjusted doses based on measured feed consumption). There were no effects on survival, body weight and body weight gain, feed consumption, absolute or relative (to body- or to brain weight) organ weights for brain, heart, spleen, liver, kidneys, adrenals, ovaries, epididymis or testes. Furthermore, no clinical signs were observed.

There were no statistically significant differences in haematology parameters between dose groups and controls, except for an increase in white blood cell counts in the high-dose group as compared to the controls for both sexes (males: high dose $17.4 \pm 2.05 \times 10^9$/ml; control $12.8 \pm 2.25 \times 10^9$/ml; females high dose $12.2 \pm 1.73 \times 10^9$/ml, control $9.1 \pm 2.11 \times 10^9$/ml), and a corresponding, statistically significant but clinically mild elevation in the absolute lymphocyte and monocyte counts in the high-dose groups, both males and females. According to the authors, the biological relevance of these variations in white blood cell counts is probably negligible as in biological terms the elevations are considered very mild. The values remained within normal ranges for the laboratory ($8.8–17.4 \times 10^9$/ml for males and $5.6–14.2 \times 10^9$/ml for females), and there were no other leucogram changes supportive of an active inflammatory process or treatment-associated source of inflammation identified within the organs evaluated in the histological report. The Committee concurs with this view.

Overall, there were no statistically significant changes in clinical chemistry parameters. In one male and one female from the high-dose group, however, high levels of blood urea and creatinine were measured. These findings were associated with increased urinary protein levels and histological changes in the kidneys, identified as marked, diffuse, bilateral chronic progressive nephropathy

(CPN), in these two animals. The authors considered that these changes reflect a background finding unrelated to administration of the test substance because CPN is a common spontaneous finding in rats, signs of early CPN like pale or mottled kidneys were also recorded in rats from other groups, and it occurred with a low incidence in the highest dose group. The Committee agrees with this opinion.

Based on the absence of treatment-related adverse effects, the authors derived a NOAEL of 2.5 mg/kg bw per day, the highest dose of echimidine tested. In the same study, rats were estimated to have received a mean daily exposure of 0, 0.63, 1.23 or 2.78 mg/kg bw lasiocarpine for males and 0, 0.67, 1.29 or 2.76 mg/kg bw for females (reported by the authors) (Dalefield, Gosse & Mueller, 2016). No clinical signs were reported nor were any effects observed on survival or feed consumption. Both males and females in the high-dose group showed a reduced body weight and body weight gain (final body weight: males control 520.6 ± 38.9, high dose 474.3 ± 40.2 (91% of control); females control 302.0 ± 27.3, high dose 283.9 ± 24.3 (94% of control)). Body weight gains in males in the mid-dose group were also slightly reduced (final body weights 95% of control). The decrease in body weight gain was consistent throughout the study. There was no such effect in females in the mid-dose and low-dose groups. Data on statistical significance were not available. Since group mean feed consumption was unaffected by ascending lasiocarpine dose, the decrease in body weight gain was not due to a confounding effect of declining palatability.

Absolute heart and liver weights of females in the high-dose group were statistically significantly reduced (by 11 and 12%, respectively). These organ weights were also reduced in females in the mid-dose group, but statistical significance was not reached. No differences were observed for absolute organ weights in any other tissues in males or in females. There were no statistically significant differences in relative organ weights.

No statistically significant differences in haematology parameters between dose groups and controls were noted, except for a statistically significant but clinically mild elevation in the mean total white blood cell counts relative to the control group ($12.7 \pm 1.94 \times 10^9$/ml versus $9.1 \pm 2.11 \times 10^9$/ml), and a corresponding, statistically significant but clinically mild, elevation in the neutrophil ($2.0 \pm 0.82 \times 10^9$/ml versus $1.2 \pm 0.51 \times 10^9$/ml) and lymphocyte ($10.3 \pm 1.57 \times 10^9$/ml versus $7.6 \pm 2.26 \times 10^9$/ml) counts in high-dose females. As for echimidine, these changes were not considered to be clinically significant. No biologically relevant effects on clinical chemistry parameters were observed. Only slight but statistically significant increases in serum ALT levels (up to 1.5- and 1.4-fold greater than control values in males and females, respectively) were observed in animals of the high-dose group, but there were no corresponding histopathological changes in the liver.

A very slight but statistically significant decrease in albumin was seen in females in the high-dose group (43 ± 2.6 g/L) relative to the mean in the controls (46 ± 2.1 g/L). Potassium levels were statistically significantly increased in males in the high-dose group (from 7.9 ± 0.94 to 9.4 ± 1.67). The study author reported that potassium levels overall were very high for any mammalian species, but there was no evidence of any increase with dose, or of a difference between treated animals and controls. Quality control was normal, and in most cases the high result initiated an automatic rerun by the analyser, producing a value the same as or within 0.2 mmol/L of the original result. In the absence of any other biochemical changes that could suggest a pathological cause for hyperkalaemia, or any report of pre-mortem clinical signs attributable to hyperkalaemia, this finding was considered to be artefactual by the authors.

The authors derived a NOAEL of 0.6 mg/kg bw per day, based on reduced body weights in male rats receiving the high and mid-dose (Dalefield, Gosse & Mueller, 2016[1]). The effects on body weight are, however, considered very minimal (5% decrease). Therefore, the NOAEL was set at 1.2 mg/kg bw per day.

The toxicity of riddelliine was investigated in F344/N rats in a 2-week and a 13-week study (Chan et al., 1994; NTP, 1993). These studies are part of a larger effort involving a chronic 2-year study conducted by the US NTP (see section 2.2.3 for a description of the 2-year study). In the 2-week study, groups of five male and five female rats (6 weeks of age) were dosed with 0 (vehicle control), 0.33, 1.0, 3.3, 10 or 25 mg/kg bw riddelliine (95% riddelliine, 5% retrorsine, 0.2% seneciphylline) in 0.1 M phosphate buffer by gavage for 5 days a week plus two consecutive doses (in total 12 administrations in 16 days). Corrected doses amounted to 0, 0.24, 0.71, 2.4, 7.1 and 18 mg/kg bw. Animals were observed twice daily and were weighed at the start of the experiment, weekly thereafter, and at necropsy. Clinical observations were recorded daily. At termination, complete necropsies were performed on the animals. Organs and tissues were weighed, examined for gross lesions, and subjected to histopathological and microscopical examinations.

In the 2-week study, four males from the 25 mg/kg bw dose group died or were killed for humane reasons due to poor condition before the end of the study (survival 20%) whereas survival was 100% for all other exposure and vehicle control groups. Dose-related decreases in mean body weight and mean body weight gains were observed in male rats from the 10 and 25 mg/kg bw groups (90% and 68% of vehicle controls respectively), whereas, in females, body weights up to the high dose of 25 mg/kg bw were similar to those of controls (96%

[1] Part of the information in this summary is based on the study reports (study director Cayzer) and is not published in the article by Dalefield, Gosse & Mueller. This information was made available to the monographers before the meeting.

of vehicle controls). In both males and females a dose-related saliva response was noted when riddelliine was administered at 3.3 mg/kg bw or higher. Clinical signs were observed in male rats from the 25 mg/kg bw group starting at day 10, and included hunching, hypoactivity, ruffled fur, slight ataxia, moderate dyspnoea and emaciation. In addition, one female from the highest dose group showed a hunched posture and nasal discharge in week 2. Dose-related increases were observed for absolute and relative lung and spleen weight in both male and female rats; for relative liver weight in male rats that received 1.0 mg/kg or higher doses and in females that received 1.0 or 3.3 mg/kg bw; for relative kidney weights in males given 10 or 25 mg/kg bw and in females given 25 mg/kg bw. Dose-related decreases were noted in absolute and relative ventricular weights in males given 3.3 mg/kg bw or higher doses and in females given 10 or 25 mg/kg bw, and in absolute heart weight in females.

Gross necropsy revealed hepatotoxicity, characterized by enlarged, firm, mottled or reddened liver lesions, in rats receiving doses of 1.0 mg/kg bw and higher in a dose-dependent manner with all rats affected at 10 and 25 mg/kg bw. In addition, icterus and darkened and/or enlarged lymph nodes were observed in many males and females from the highest dose group, a darkened thymus in both males and females receiving doses of 1.0 mg/kg bw and higher, and oedema of the pancreas in males in the highest dose group. Microscopically, at 1.0 mg/kg bw and above, increased incidence of lesions in the liver (haemorrhagic centrilobular hepatic necrosis, hepatocytic karyomegaly and cytological alterations) and lung (focal haemorrhage and oedema) were observed. Diffuse cytological changes of lesser severity, characterized primarily by cytoplasmic granularity and eosinophilia, were seen in all males administered 1.0 mg/kg or above. At doses of 3.3 mg/kg bw per day, increased incidences of lesions of the spleen (splenic extramedullary haematopoiesis), and pancreas (pancreatic oedema) were observed in male rats. Similar effects were observed in liver, lung and spleen, but with lower severity, in female rats administered riddelliine at 3.3 mg/kg bw or greater. The NOAEL was 0.33 mg/kg bw, i.e. 0.24 mg/kg bw per day, based on the histopathological changes described above as observed in males given 1.0 mg/kg bw, i.e. 0.71 mg/kg bw per day.

In the 13-week study, male and female rats (6 weeks old, 20 animals per sex per group) were administered 0 (vehicle control), 0.1, 0.33, 1.0, 3.3 or 10 mg/kg bw riddelliine (92% riddelliine, 5% retrorsine, 1.4% seneciphylline) by gavage 5 times a week. Averaged over the week, amortized doses amounted to 0, 0.071, 0.24, 0.71, 2.4 and 7.1 mg/kg bw. Animals were observed twice daily, and body weight and clinical observations were recorded weekly. Ten animals from each dose group were autopsied at the end of the treatment period, while groups of five animals were maintained without further treatment for a recovery period of 7 or 14 weeks. Postmortem examinations were the same as for the 2-week

study with the addition of some extra tissues, e.g. pancreas and salivary gland, as well as reproductive system evaluations, and selected rats had clinical pathology evaluations.

Mortality was observed in the highest dose group, from which 19 of the 20 males died before the end of the 13-week base study, and 5 of 10 females died during the recovery period. Dose-related decreases in mean body weight (up to 6% in males and 15% in females) and in mean body weight gain were noted, but, after the recovery periods, body weights were only slightly lower than in controls. Clinical signs were observed in both males and females in the highest dose group and included jaundice, abnormal posture, ruffled fur, discoloured urine, urine-stained fur, diarrhoea, emaciation and alopecia. Statistically significant increases were observed for mean absolute and relative weights of lung and spleen in males[1] (25% and 46%, respectively, for lung and 23% and 47%, respectively for spleen) and females[1] (46% and 59%, respectively, for lung and 162% and 185%, respectively for spleen), for relative weights of brain and right testis in males (14% and 9%, respectively), and for absolute and relative weights of brain (3% and 12%, respectively) and right kidney (37% and 50%, respectively) and relative weights of heart (11%) and liver (2%) in females. A statistically significant decrease in absolute and relative weight was noted for heart (20% and 8%, respectively) and liver (23% and 9%, respectively) in male rats, and for thymus (24% and 17%, respectively) and absolute left ventricle (6%) and relative right ventricle (22%) in females. During the recovery periods, partial recovery of the organ weights in females was observed.

Several haematology and clinical chemistry parameters were affected during the study. Significant increases were noted for RBC count and haematocrit (both males and females, up to 20%) and for haemoglobin concentration (females, up to 18%) in the 1.0 and 10 mg/kg bw groups on day 14; they were similar to or lower than control values on day 30, and were similar or elevated again compared to controls at week 13 in the groups given 10 mg/kg bw. Also in other dose groups, significant increases compared to control values were observed. Significantly increased reticulocyte counts were observed in male rats from the highest dose group at all three time points (up to 336%), in the males in the 3.3 mg/kg bw group at week 13 (up to 86%), and in females at week 13 in all dose groups (up to 444%). Mean RBC volume was increased in all treated males except in the 0.33 mg/kg bw group. These findings were reported by the authors to indicate a sequence of dehydration, anaemia and regenerative response. A significant reduction in platelet counts was seen in males from the 10 mg/kg bw group at all time points (up to 90%) and in females from the 10 mg/kg bw group at week 13 (up to 81%).

[1] Percentages given for the 3.3 mg/kg bw dose group for males due to high mortality at the highest dose, and for the 10 mg/kg bw dose group for females.

Leukocyte counts were significantly increased in males and females (up to 64%), which was generally accompanied by an increase in lymphocytes in females given high doses on day 14 and at week 13 (up to 62%), and in males from all dose groups, except the 0.33 mg/kg bw group, at week 13 (up to 27%). The changes in RBC parameters indicated regenerative anaemia. At 10 mg/kg bw the increased reticulocyte count (males only) and extramedullary haematopoiesis in the spleen (both sexes) had already occurred by day 14 and increased in magnitude through week 13. The observation of increased spleen weight, erythrophagocytosis in lymph nodes and intravascular macrophages further indicated possible RBC damage and increased turnover. However, the lack of associated haemosiderosis does not support a haemolytic process, and the cause of regenerative anaemia in riddelliine-treated rats is unclear. Serum alkaline phosphatase (ALP) activity was significantly increased at all time points in all high-dose males (up to 147%), and on day 14 in males from the 1.0 mg/kg bw dose group (up to 28%), and at week 13 in males from the 0.33 and 3.3 mg/kg bw groups (up to 52%). In females, this was observed in the 3.3 and 10 mg/kg bw dose groups at week 13 (up to 76%). Increased activity of alanine aminotransferase (ALT) occurred in males (up to 319%) and females (up to 196%) from the highest dose group on days 14 and 30, and in females in the 1.0 and 3.3 mg/kg bw groups on day 30 (up to 60%) and week 13 (up to 43%), respectively. Sorbitol dehydrogenase (SDH) activity was increased in high-dose males and females on day 14, day 30 (males only) and at week 13 (up to 247% in males, up to 260% in females), and in females from the lower dose groups at week 13 (up to 53%). The increases in ALT and SDH are consistent with the hepatotoxicity observed histopathologically, while the increased ALP activity is consistent with the histologically observed biliary hyperplasia.

A dose-related increase in unscheduled DNA synthesis (UDS) in hepatocytes was observed following 5 or 30 days of riddelliine treatment. An increase in S-phase DNA synthesis was observed at both time points in at least one dose group in male and female rats. Although the data were not dose-dependent and did not correspond well to the liver weight changes, the findings may not be inconsistent in view of the reported antimitotic effect of PAs. Thus riddelliine may be toxic to the hepatocytes, causing DNA damage and cell death, but at the same time inducing DNA synthesis. Megalocytosis development might be the result of the antimitotic effect of riddelliine.

Gross necropsy findings included ascites, icterus, firm and granular livers, dark/red and enlarged lymph nodes, pancreatic oedema, mottled hearts, darkened loops of small bowel, and firm, granular and enlarged spleens, and were most frequently reported for the animals in the highest dose group that died before the end of the study. Findings were in general similar in animals examined after week 13 and after the recovery period, but were less frequently observed in the

latter. Microscopically, the major findings were noted in the liver, in all animals in the highest dose group, and these included: primarily haemorrhagic centrilobular necrosis and karyocytomegaly and proliferative lesions of hepatocytes, and bile duct hyperplasia. Females that were included in the recovery groups showed the same effects, which persisted or progressed in severity, especially a marked increase in bile duct proliferation in females (Table 10). Three females from the highest dose group – one of which was assigned to the 14-day recovery group and died during the recovery period – had hepatocellular adenomas (3/20, 6%), and in two of the three cases, multiple adenomas.

Accumulations of macrophages were identified in the lung and kidney of most animals in the highest dose groups, and in the lung, kidney and liver of female rats after the recovery periods. As they were adherent to the underlying endothelium, they may be indicative of riddelliine-induced endothelial damage. Further lesions in the highest dose groups included mild to moderate haematopoietic cell proliferation and lymphoid depletion in the spleen, bone marrow hyperplasia, congestion, erythrophagocytosis and accumulation of macrophages in lymph nodes, haemorrhagic lesion of the heart, pancreatic oedema, kidney lesions, haemorrhage and oedema along the gastrointestinal tract, and atrophy of the testis, epididymis, seminal vesicle, prostate gland, preputial gland, ovary and thymus (Chan et al., 1994; NTP, 1993).

Although marginal effects on liver indicative of liver toxicity (increased incidence of karyomegaly, increased S-phase synthesis) were already observed at 0.33 mg/kg bw per day, the monographers considered the NOAEL was 0.33 mg/kg bw, i.e. 0.24 mg/kg bw per day, based on foci of hepatocellular alteration, karyomegaly and increased S-phase DNA synthesis in liver cells, and haematological changes observed at 1.0 mg/kg bw, i.e. 0.71 mg/kg bw per day.

In a repeated-dose liver micronucleus assay (see section 2.2.4 for details on the assay), groups of five adult male Sprague-Dawley rats were initially administered 0, 3.75, 7.5 or 15 mg monocrotaline (purity > 98%)/kg bw per day for 28 days by oral gavage (Takashima et al., 2015). One animal in each of the 7.5 and 15 mg/kg bw per day dose groups was found dead on day 27. The assay was therefore repeated with lower doses: 0, 0.5 and 1.5 mg/kg bw per day once daily for 14 days or 0, 0.15, 0.5 or 1.5 mg/kg bw per day for 28 days. In addition, groups of five adult rats and five juvenile rats (26 days old) were administered monocrotaline at doses of 15, 30 or 60 mg/kg bw per day for 2 days. Vehicle control groups were also included. Rats were weighed prior to administration on days 1, 4, 8, 11 and 15 for the 14-day experiment and on days 1, 4, 8, 11, 15, 18, 22, 25 and 29 for the 28-day experiment. Rats were weighed prior to administration on days 1 and 2, and on days 4 and 6 for the 2-day experiment. Livers of rats exposed for 14 or 28 days were examined histopathologically.

Table 10
Incidence of liver lesions in F344/N rats after riddelliine exposure via gavage for 13 weeks[a]

	Dose (mg/kg)					
	Vehicle control	**0.1**	**0.33**	**1.0**	**3.3**	**10**
Male						
Survival						
13 weeks	10/10	10/10	10/10	10/10	10/10	1/10
7-week recovery	5/5	5/5	5/5	5/5	5/5	0/5
14-week recovery	5/5	5/5	5/5	5/5	5/5	0/5
Body weight						
13 weeks	361	347	339	332	308	195
7-week recovery	415	410	385	376	375	–
14-week recovery	413	428	439	414	398	–
Karyomegaly						
13 weeks	0	0	10	10	10	19[b]
7-week recovery	0	0	5	5	5	–[c]
14-week recovery	0	0	5	5	5	–
Cytomegaly						
13 weeks	0	0	0	0	10	19[b]
7-week recovery	0	0	0	0	5	–[c]
14-week recovery	0	0	0	0	5	–
Necrosis, hepatocyte						
13 weeks	0	0	1	0	0	19[b]
7-week recovery	0	0	1	0	1	–[c]
14-week recovery	0	0	0	2	1	–
Bile duct hyperplasia						
13 weeks	0	0	0	0	1	19[b]
7-week recovery	0	0	0	0	1	–[c]
14-week recovery	0	0	0	0	1	–
Foci of cellular alteration						
13 weeks	0	0	1	0	1	0[b]
7-week recovery	0	0	0	3	2	–[c]
14-week recovery	0	0	0	1	3	–
Female						
Survival						
13 weeks	10/10	10/10	10/10	10/10	10/10	10/10
7-week recovery	5/5	5/5	5/5	5/5	5/5	4/5
14-week recovery	5/5	5/5	5/5	5/5	5/5	1/5
Body weight						
13 weeks	209	205	203	197	192	193
7-week recovery	232	235	232	230	208	225
14-week recovery	253	243	244	227	216	255
Karyomegaly						
13 weeks	0	0	7	10	10	10
7-week recovery	0	0	5	5	5	8[d]

	Dose (mg/kg)					
	Vehicle control	**0.1**	**0.33**	**1.0**	**3.3**	**10**
14-week recovery	0	0	5	5	5	2[e]
Cytomegaly						
13 weeks	0	0	0	0	1	10
7-week recovery	0	0	0	0	0	8[d]
14-week recovery	0	0	0	0	0	2[e]
Necrosis, hepatocyte						
13 weeks	0	0	0	0	0	10
7-week recovery	0	0	0	0	0	8[d]
14-week recovery	0	0	0	0	0	2[e]
Bile duct hyperplasia						
13 weeks	0	0	0	0	0	10
7-week recovery	0	0	0	0	1	8[d]
14-week recovery	0	0	0	0	0	2[e]
Foci of cellular alteration						
13 weeks	0	0	0	0	1	2
7-week recovery	0	0	0	0	0	1[d]
14-week recovery	0	0	0	0	0	0[e]
Nodular hyperplasia						
13 weeks	0	0	0	0	0	1
7-week recovery	0	0	0	0	0	2[d]
14-week recovery	0	0	0	0	0	2[e]
Adenoma						
13 weeks	0	0	0	0	0	2
7-week recovery	0	0	0	0	0	0[d]
14-week recovery	0	0	0	0	0	1[e]

[a] Incidence is the number of animals with lesions out of 10 animals (13-week study) or five animals (recovery period studies).
[b] $n = 20$, including 19 early deaths and one euthanized animal at termination.
[c] No male survivors for recovery studies in the 10 mg/kg bw group.
[d] $n = 8$, including four scheduled euthanization and four early deaths during 7-week recovery period.
[e] $n = 2$, including one scheduled euthanization and one early death during the 7-week to 14-week recovery period.
Source: NTP (1993).

Neither significant body weight changes nor mortality were observed in the lower dose groups (0.15–1.5 mg/kg bw per day). However, one animal in the 7.5 mg/kg bw per day and one in the 15 mg/kg bw per day dose group died and body weights of animals that received ≥ 3.75 mg/kg bw per day in the 28-day experiment were significantly lower than those of the vehicle control group (up to 23%). In rats exposed for 2 days, significantly lower body weights were observed in the juvenile rats from the 30 and 60 mg/kg bw dose groups on day 6 (up to 13%). In adults, the mean body weight of animals treated with monocrotaline at 60 mg/kg bw was significantly lower than that of the controls on days 4 and 6 (up to 9%). Hepatotoxic changes became evident at doses of 3.75 mg/kg bw

per day and above and included diffuse oedema in the connective tissue of the central vein (graded minimal), congestion in the acinus (graded minimal to mild), diffuse hepatocellular hypertrophy (graded minimal to moderate) and centrilobular single-cell necrosis (graded minimal). At higher doses, diffuse single-cell necrosis and diffuse fibrosis of Glisson's sheath (both graded minimal) were also observed. However, these effects did not always show dose-dependency (Takashima et al., 2015). A dose-dependent increase in micronucleus formation in hepatocytes was observed in the rats treated with monocrotaline for 14 or 28 days. This is further described in the genotoxicity section (2.2.4).

In a study to find biomarkers for drug-induced vascular injury, male Crl:(CD)SD rats (6 animals per group) were administered, among other non-PA compounds, monocrotaline (purity not specified) at doses of 0, 1, 10 or 30 mg/kg bw per day for 4 days (Dalmas et al., 2011). Monocrotaline was given orally via gavage in 0.9% sodium chloride (5 ml/kg bw). Rats were 12–14 weeks old upon arrival and were acclimatized to local housing conditions for a minimum of 2 weeks prior to the initiation of dosing. Exact age at the start of dosing and the exact acclimatizing period were not reported. Rats were observed at least twice daily for clinical signs. Approximately 24 hours after the last dose, rats were euthanized for necropsy. Histopathological analysis included the evaluation of mesenteric vascular damage (necrosis, haemorrhage, fibrosis, hypertrophy and perivascular inflammatory cell infiltrates). In addition, gene analysis was performed to identify toxicologically relevant genes (not further discussed). At 1 and 10 mg/kg per day, none of the rats showed histological evidence of mesenteric vascular damage. One of the rats that received 30 mg/kg per day showed mononuclear cell infiltrates (Dalmas et al., 2011).

In a study performed by Morris, O'Neill & Tanner (1994), the relationship between copper and PA hepatotoxicity was investigated in the rat. Male Wistar rats were exposed to retrorsine in two separate experiments.

In experiment 1, male Wistar rats (4 weeks old, 8 animals per group) were exposed to 0 or 25 mg/kg bw retrorsine (purity not specified) by gavage once a week for 15 weeks with or without the addition of copper (2 g/kg diet copper sulfate, final content 513 mg/kg), that was also given to the control group. Body weight and health of all animals were recorded twice weekly. Blood samples were taken every 2–3 weeks for liver function tests, and, at 3 and 8 weeks, liver biopsies were taken from half of the groups. Rats were euthanized after 15 weeks and liver samples were taken for histology and measurement of copper content. No deaths were reported in the control or copper-treated groups. Increased mortality was found in the retrorsine (37.5%) and in the retrorsine + copper (62.5%) group. No significant differences were noted for body weight.

In the copper group, plasma AST and ALT levels were significantly increased compared to controls at all time points. In the retrorsine group, after

12.5 weeks, plasma level of albumin was slightly but statistically significantly decreased (88% of control), and plasma levels of AST and ALT were statistically significantly increased (147% and 150% of control, respectively). Also, a marked but not statistically significant increase was seen in bilirubin levels after 12.5 weeks. In the retrorsine + copper group, plasma AST and ALT levels increased significantly up to 7.5 weeks (54% and 72%, respectively) where they plateaued, and remained at this level during the rest of the study. In addition, plasma albumin levels decreased over time down to 70% of control, and bilirubin level increased sharply up to individual levels of more than 50 μM (control 0.2–0.5 μM).

Liver histology revealed mild focal hepatitis in the copper group. In the retrorsine and retrorsine + copper groups, histological analysis showed signs of inflammation with nuclear dysplasia, megalocytosis, bile duct proliferation and haemosiderin.

In experiment 2, male Wistar rats (3 weeks old, 16 animals per group) were divided into the same four groups as in experiment 1. This time, however, the retrorsine was mixed with the diet, 25 mg/kg feed, for the first 4 weeks, and then reduced to 15 mg/kg feed for the rest of the exposure period due to high mortality in the retrorsine + copper group. According to the authors, this was equivalent to 5 mg per day, assuming a rat of 100 g and a feed intake of 20 g per day. Using the conversion factor of 0.1 for young rats from the JECFA conversion table, retrorsine intake would be equivalent to 2.5 mg/kg bw per day for the first 4 weeks and 1.5 mg/kg bw per day for the rest of the experiment. Body weight and health were checked twice a week. Blood samples were taken every 2–3 weeks. Rats were euthanized after 9 weeks (four rats per group for the control, copper and retrorsine groups), after 12 weeks (four rats per group for the control and copper groups), and after 15 weeks (remaining rats), and the liver was taken for histological analysis.

No mortality was noted among the rats in the control or copper group. Increased mortality was seen in the retrorsine (12.5%) and retrorsine + copper (81.3%) group. Body weights were significantly lower in the retrorsine and retrorsine + copper groups (325 ± 10 g and 313 ± 5 g, respectively at the end of the experiment) compared to controls (control: 421 ± 12 g; copper: 410 ± 12 g).

In the copper group, plasma levels of AST and ALT were increased (123% and 113%, respectively) at week 14. In the retrorsine group, plasma AST levels increased up to week 7 (312%) after which they decreased slightly but remained significantly higher (205% at week 14) than in the control group. Plasma ALT and bilirubin levels were also greatly increased (163% and 1789%, respectively, at week 14) and plasma levels of albumin were decreased (31% at week 14) compared to controls. In the retrorsine + copper group, plasma AST and ALT levels were significantly increased relative to the control group at all time points,

with the highest values at 7 weeks (385% and 272% of control, respectively). In addition, bilirubin levels rapidly increased, with individual levels of more than 50 μM. Plasma albumin levels were significantly decreased at all time points (62% of control at the end of the experiment).

Normal liver histology was observed in the control and copper group. Cytomegaly, hepatocellular necrosis, inflammatory infiltrates and biliary reduplication were observed in livers from retrorsine- and retrorsine + copper-treated rats. Severity differed widely and no relationship with copper content could be identified. Copper content in the liver remained unchanged in controls, and was significantly increased in the copper-treated rats compared to controls (by approximately a factor of 5–7). Also, in retrorsine-treated rats, the copper content was increased by approximately the same factor as in the copper-treated rats, and, in the retrorsine + copper-treated rats, large increases were noted of up to a factor of 400. The authors concluded that retrorsine caused copper accumulation in the liver and copper and retrorsine together led to more severe hepatic dysfunction and higher mortality than retrorsine alone (Morris, O'Neill & Tanner, 1994).

In a short-term toxicity study on pulmonary lesions caused by monocrotaline, male Wistar rats (age not reported, six animals per group) were administered 0 or 10 mg/kg bw per day monocrotaline (purity not specified) intragastrically for 15 days (Marar & Davi, 1995). Many details were not reported by the authors, including the statistical analysis performed and how the control group was treated (untreated or vehicle treated). After the experimental period, animals were euthanized and histopathology was performed on lung tissue. Bronchoalveolar lavage fluid was used to measure lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6DPH), ALP, acid phosphatase and protein.

Lung histopathology showed interstitial oedema and haemorrhage in the exposure group. Macrophage and mononuclear infiltration was seen in the alveolar wall, and around bronchi and the blood vessels. The exposure group showed significantly increased levels of LDH (119%), G6DPH (98%), ALP (65%), acid phosphatase (231%) and protein (200%) compared to the control group. The authors concluded that the results from the analysis of lung fluid correlated well with the morphological changes in the lungs due to monocrotaline exposure (Marar & Davi, 1995).

To develop a diagnostic test, male albino Wistar rats (Porton strain, two animals per group, weighing 200–250 g) were treated with 0 or 20 mg/L monocrotaline (purity not specified) in drinking water for 12 or 25 days and thereafter euthanized, or for 25 days and then kept for a recovery period of 17 days (Mattocks & Jukes, 1992a). Measured parameters were not reported, but liver and blood samples were taken.

Total alkaloid dose was estimated based on measured water intake: 27 mg/kg for rats exposed for 12 days and 55 mg/kg for rats exposed for 25 days. The authors estimated the administered dose to be equal to 0 or 2.25 mg/kg bw per day. Rats euthanized after 12 days of alkaloid treatment appeared normal, whereas rats treated for 25 days showed gross signs of lung damage (panting breath, pleural effusions) and some showed liver damage. Blood samples showed the presence of pyrrolic metabolites at all time points; they were also observed in fresh and fixed livers after 25 days of exposure. Pyrrolic metabolites were not observed in fresh rat liver after the recovery period of 17 days, but they were seen in livers after fixation. Lung tissue was not found to contain pyrrolic metabolites. The authors concluded that low-level exposure to monocrotaline resulted in detectable pyrrolic metabolites in blood and liver, but that a longer time was needed before they became detectable in liver tissue compared to blood (Mattocks & Jukes, 1992a,b).

In a study to investigate Na+/K+ adenosine triphosphatase-dependent activities of K+-return relaxation and 86Rb uptake in pulmonary arteries from rats with pulmonary hypertension induced by monocrotaline, three experiments were performed (Shubat, Bowers & Huxtable, 1990).

In the first experiment, male Sprague-Dawley rats (60–200 g, various ages) were exposed to 0 or 20 mg/L monocrotaline (extracted from *Crotalaria spectabilis*, purity not specified) via drinking water for 0, 4, 8, 12 or 20 days. This dosage is equivalent to approximately 2–3 mg/kg bw per day according to the JECFA conversion table. Rats of different ages received monocrotaline water such that all rats weighed approximately 200 g at termination of the study. After treatment, rats were euthanized and the pulmonary artery was removed. The force of the artery was measured in four ring segments as a parameter for contraction and relaxation. In addition, vessel diameters and wall thickness were determined.

The ratio of right ventricle weight to left ventricle plus septum was significantly greater in rats exposed to monocrotaline for 20 days than in control animals, indicating pulmonary hypertension. Monocrotaline treatment resulted in significantly decreased norepinephrine or 5-hydroxytryptamine-induced contraction and K+-return relaxation. Effects on the relaxation rate and force were more pronounced and appeared earlier (after 4 days) than effects on the contraction rate and force. The ratio of relaxation/contraction was unchanged. Wall thickness was increased in animals exposed to monocrotaline for 12 or 20 days.

In the second experiment, male Sprague-Dawley rats (100 g, age not specified) were given 0 or 20 mg/L monocrotaline (extracted from *C. spectabilis*, purity not specified) via drinking water for 4–5 days. Control animals received tap water. After treatment, rats were euthanized and the pulmonary artery of the third and fourth lobes of the right lung was removed. Radiolabelled rubidium

(Rb) uptake was measured in whole artery and in lumen. In the third experiment, Sprague-Dawley rats (sex not specified, 100 g, age not specified) were pretreated with 0 or 20 mg/L monocrotaline (extracted from *C. spectabilis*, purity not specified) via drinking water for 5 days, after which they were euthanized and the lungs were isolated. The transport of radiolabelled Rb was measured again, together with effects on Na^+K^+-ATPase-dependent relaxation.

No effect on uptake at any of the Rb incubation time points was found after 4 days of treatment with monocrotaline. However, after 5 days, the lungs of rats in the treatment group retained more radiolabelled Rb than controls. In addition, the uptake of radiolabelled Rb was significantly faster and reached equilibrium sooner, with the maximum uptake being the same as for controls.

Overall, the authors concluded that monocrotaline-induced changes are already occurring after 4–5 days, in both mechanical and biochemical properties of pulmonary arteries, and that the Na^+K^+-pump is involved (Shubat, Bowers & Huxtable, 1990).

In a study to determine the minimum monocrotaline concentrations in drinking water and the duration of exposure necessary to produce pneumotoxicity, four experiments were performed in rats (Shubat, Hubbard & Huxtable, 1989).

In one experiment, male Sprague-Dawley rats (100 g, age not specified, four animals per group) were dosed with monocrotaline (purity not specified) in drinking water at 0, 5, 10, 20, 40 or 60 mg/L for 0, 1, 2, 4, 6, 10 or 20 days. All animals were euthanized at day 20. Animals given monocrotaline for fewer than 20 days were given tap water until euthanasia. In a separate experiment, rats (three animals per group) were given 20 mg/L monocrotaline via drinking water for 0, 4 or 6 days and euthanized at 14, 21 or 28 days. In a third experiment, juvenile rats (50 g, 3–4 animals per group) were first pretreated with 65 mg/kg bw intraperitoneal phenobarbital and then maintained on 0.1% phenobarbital via drinking water for 7 days to induce CYP enzyme activity before they (now weighing 100 g) were exposed to 20 mg/L monocrotaline via drinking water for 0, 1, 2, 4, 6, 10 or 15 days. In all experiments, body weights were measured and heart, lungs, thymus, kidneys and liver were removed and wet weights recorded.

Mortality was observed in animals exposed to 40 mg/L for 4 days (25%) and for 6 days (50%), and in animals exposed to 60 mg/L for 4 days (25%; however, two additional animals died during transport for necropsy). No deaths were reported among animals exposed to these concentrations for a longer period. Decreased body weight gain was observed in several exposure groups, but this was not always statistically significant. Organ weights of kidney, liver and thymus were generally unchanged; only for animals exposed to 40 mg/L for 20 days was a significant increase in kidney weight observed. Right ventricle weight, expressed as a fraction of left ventricular + septal weight, was significantly increased at doses of 10 mg/L or higher and this was largely due to cellular hypertrophy.

This was observed for rats exposed to 10 mg/L after 10 days of exposure, for 20 mg/L and 40 mg/L after 4 days, and for 60 mg/L after 1 day. Relative lung weight was increased at doses of 10 mg/L or higher. For rats exposed to 10 mg/L monocrotaline, statistically significant changes were seen after 10 days, for 20 or 40 mg/L after 6 days or longer, and for 60 mg/L after 2 days of exposure to monocrotaline. In the 60 mg/L group, oedema was observed in the heart, lungs, liver and kidneys, and in some animals fluid was found in the thoracic cavity. Dose equivalents, expressed as total amount ingested, were calculated by the authors, based on 180 mL/kg per day water consumption, for those combinations of exposure and minimum time that produced right-ventricular hypertrophy: 10 mg/L for 10 days was calculated to be equivalent to 18.0 mg/kg bw (1.8 mg/kg bw per day), 20 mg/L for 4 days to 14.4 mg/kg bw (3.6 mg/kg bw per day), 40 mg/L for 4 days to 28.8 mg/kg bw (7.2 mg/kg bw per day), and 60 mg/L for 1 day to 10.8 mg/kg bw. Animals that received 20 mg/L for 4 or 6 days, and were euthanized at different time points, showed right-ventricular hypertrophy at all time points. Lung weights were significantly increased in both exposure groups when euthanized at 2 weeks, and in the 6-day exposure group when euthanized at 3 weeks, but not when euthanized at 4 weeks.

Significantly increased right ventricle weight and lung weight were observed in animals pretreated with phenobarbital and dosed with monocrotaline for 4 days or longer compared to controls, but differences were generally not statistically significant when compared to animals given monocrotaline alone. Only lung weight was significantly increased compared to the controls when rats were exposed to monocrotaline + phenobarbital for 4 days, while this was not the case for rats exposed to monocrotaline alone.

In another experiment by the same authors, male Sprague-Dawley rats (100–125 g, age not specified, 5–6 animals per group) were exposed to monocrotaline in drinking water at 20 mg/L for 0, 1, 2, 4 or 6 days. Animals were euthanized and cells were lavaged from the lungs. The protein concentration of the lavage fluid was significantly increased (~182%) in animals exposed to 20 mg/L monocrotaline in drinking water for 6 days. No significant differences were observed in percentage of lymphocytes, macrophages or polymorphonuclear leukocytes present in the lung fluid of treated animals (Shubat, Hubbard & Huxtable, 1989).

In an additional experiment, male Sprague-Dawley rats (age not specified, number of animals per group unknown) were exposed to monocrotaline in drinking water at 20 mg/L for 0, 1, 2, 4, 6, 10 or 20 days. One group of animals was euthanized on the last day of exposure and the other groups on day 20. The lungs were subjected to histological evaluation. Wall thickness of the pulmonary arteries increased with longer duration of exposure from 23 to 48 μm in animals

exposed for 0, 1 or 2 days and to 56 to 90 μm in animals exposed for 4 days or more and evaluated at 20 days.

Overall, the authors concluded that the oral threshold dose equivalent of monocrotaline to produce toxic effects is in the range 11–14 mg/kg bw. In addition, they concluded that this study provided evidence that it is the cumulative dose that determines the toxicity of monocrotaline as comparable toxic effects were noted after 1 day of exposure to 60 mg/L (11 mg/kg bw) and after 10 days of exposure to 10 mg/L (14 mg/kg bw) (Shubat, Hubbard & Huxtable, 1989).

In a short-term study with riddelliine, male F334/N rats (7 per group, 5–7 weeks old) were exposed to 0 (vehicle control), 1.0 or 2.5 mg/kg bw riddelliine (purity 92%, 5% retrorsine, 1.4% seneciphylline) dissolved in corn oil, by gavage, for eight consecutive daily doses or for 6 weeks on weekdays, i.e. five times a week (Nyska et al., 2002). In the latter case, the corrected daily doses amounted to 0, 0.71 or 1.4 mg/kg bw per day. Animals were observed twice daily for clinical signs, and body weights were recorded weekly. At termination, blood and liver samples were collected and animals were subjected to necropsy.

There were no dose-related effects on body weight. Liver weight was not affected after eight daily doses, but was significantly increased (~10%) after 6 weeks in rats from both dose groups. No effects were found on serum vascular endothelial growth factor (VEGF) concentration either after 8 days or 6 weeks.

Following eight doses of 1.0 or 2.5 mg/kg per day, an increase in number of cases and severity of apoptosis, cytomegaly and karyomegaly and S-phase nuclei (statistically significant at the highest dose) was noted in nonparenchymal (i.e. endothelial) and parenchymal cells. In addition, the number of hepatocellular mitoses was greatly reduced compared to controls. p53 Protein was minimally active in nonparenchymal cells. Mild to moderately increased expression of VEGF protein was observed in hepatocytes. Lipid in the space of Disse was observed in animals in the high-dose group.

Following 6 weeks of exposure to 1.0 and 2.5 mg/kg per weekday, decreased apoptosis (statistically significant at the highest dose), increased S-phase nuclei (statistically significant at the highest dose), and, sporadically, p53 nuclear positivity were noted in nonparenchymal cells. In parenchymal cells, apoptosis was also decreased (significantly at the highest dose), while S-phase nuclei were decreased (significantly at the highest dose) in contrast to in nonparenchymal cells. Reduced numbers of hepatocytes undergoing mitosis (none observed), minimal to mild hepatocellular hypertrophy, lipid in the space of Disse and centrilobular fatty vacuolization were also reported in nonparenchymal cells after 30 doses of 1.0 or 2.5 mg/kg bw per day, while karyomegaly, cytomegaly, endothelial cell swelling and hypertrophy were found in animals in the highest dose group only. The number of S-phase nuclei was higher in younger rats than

in older rats. Mild to moderately increased expression of VEGF protein was observed in hepatocytes.

The authors concluded that riddelliine interacts with DNA in endothelial cells. The LOAEL was 1 mg/kg bw per day based on hepatocyte vacuolization and hypertrophy, endothelial changes (cell degeneration, lipid in space of Disse, swelling, karyomegaly), reduction in hepatocyte mitosis and increased liver weights (Nyska et al., 2002).

In a study to investigate the pathological changes in rats after dietary exposure to *Heliotropium circinatum*, male rats (12–16 weeks old, strain not specified, 16 animals per group) were fed 0, 1, 3, 5 or 10% dried and ground parts of *H. circinatum* added to the feed for 20 weeks (Eröksüz et al., 2003a). PA content of *H. circinatum* was determined by GC-MS analysis. Body weight and feed consumption were recorded weekly. At the end of the 20-week exposure period, rats were euthanized and tissue samples of liver, lungs, kidneys, pancreas, small and large intestine and heart were taken for histological analysis.

The total PA content of *H. circinatum* was 0.15% and the free base content was 0.018%. Of the total PAs, 88% was present as *N*-oxide and 12% as free base. PAs found in *H. circinatum* were identified in fraction A (free base) and fraction B (free base + *N*-oxide), respectively, as europine (4.37% and 67.33%), heliotrine (4.88% and 16.34%), lasiocarpine (75.35% and 8.12%), heleurine (2.56% and 4.18%), echinaine (2.56% and 1.56%), 7-angelylheliotrine (2.05% and 1.19%) and an unknown PA (9.23% and 1.28%).

Feed intake and body weight were significantly and dose-relatedly reduced in all exposure groups when compared to the control group. Rats in the control group had an average cumulative feed intake over 20 weeks of 3189.0 g, which was equal to an average of 22.8 g per animal per day over 20 weeks. Rats receiving 1%, 3%, 5% and 10% *H. circinatum* had an average cumulative feed intake over 20 weeks of 2896.6, 2831.2, 2863.0 and 2547.1 g per animal over 20 weeks, which was equal to an average of 20.7, 20.2, 20.5 and 18.2 g feed per animal per day over 20 weeks, respectively. Total PA intakes calculated by the Committee, based on feed intake and body weights were 0, 0.95, 2.0, 4.9 and 11.1 mg/kg bw per day, respectively.

Animals in the highest dose group were generally inactive and anorectic, and exhibited gradual alopecia and hair loss. Animals in the 5% and 10% group showed mildly fibrotic livers. Several mild to moderate histological changes in the liver were seen in animals from all exposure groups (including hepatic megalocytosis, focal liver necrosis, periportal fibrosis and bile duct proliferation). Histological changes were also observed in the kidneys (including focal interstitial nephritis), lungs (including thickening of pulmonary artery and arterioles), heart (including oedema and fibrosis in the right ventricle) and spleen (including haemosiderin deposits). The frequency of most histological changes increased

with increasing dosage. The most common histological effects were hepatic megalocytosis and periportal fibrosis and infiltration.

The authors concluded that *H. circinatum* has limited toxic potential as it caused minimal to moderate histological changes and no mortality (Eröksüz et al., 2003a). The Committee, however, disagreed with the authors' conclusion because many organs were affected and the effects were already evident at total PA doses as low as 0.95 mg/kg bw per day.

In a study to investigate hepatotoxicity of comfrey, inbred dark Aguti rats (aged 3–3.5 months, sex not specified) were given a single dose of 200 mg/kg bw PAs, or 50 or 100 mg/kg PAs extracted from locally collected Russian comfrey by gavage 3 times a week for 3 weeks (Yeong et al., 1991). The presence of PAs was confirmed by thin-layer chromatography (TLC) and mass spectrometry (MS), but not further specified other than by retention times of the comfrey extract by TLC. Exposure groups consisted of four animals per group; a control group for each treatment consisting of two animals was also included. At the beginning of the fourth week, the animals were euthanized and liver samples were taken for histopathological analysis using light microscopy and electron microscopy. One rat that received a single dose of 200 mg/kg bw was euthanized at the end of week 1.

Livers of exposed rats were firm in consistency and plum-coloured with nodular surfaces. Severe sinusoidal congestion, extravasation of RBCs and necrosis were observed in exposed rats. Most terminal hepatic venules were narrowed. Electron microscopy revealed nuclear, mitochondrial and cytoplasmic swelling. In addition, extensive loss of sinusoidal lining cells and disruption of hepatocyte cellular margins was reported. Spillage of organelles and debris into sinusoidal tracts was seen. RBCs were found in extravascular compartments. The same changes were seen in all exposure groups, but necrosis, intimal narrowing of terminal hepatic venules and vascular congestion were less pronounced in the low-dose group than in the high-dose group.

The authors concluded that PAs from comfrey had been demonstrated to produce veno-occlusive lesions in rat livers. Effects were observed at all doses tested and showed a dose-related increase in severity (Yeong et al., 1991).

In a study to investigate low-dose hepatotoxicity, inbred dark Aguti rats (male and female, age not specified) were fed PAs extracted from comfrey at doses of 0 or 50 mg/kg bw (control group: two animals, exposure group: eight animals) by gavage once a week for 6 weeks. Qualitative and quantitative composition of the PAs extracted from comfrey were not further specified. The animals were euthanized 1 week after the last dose and immediately dissected. Liver samples were taken and prepared for examination using light microscopy and electron microscopy.

The liver abnormalities in all animals were similar, with no differences between sexes. There was a moderate to marked degree of sinusoidal dilatation

and congestion. The remaining liver tissues showed several signs of hepatocyte damage, including loss of definition of hepatocyte cellular and sinusoidal walls, loss of microvilli, breakdown of the cell membrane, nuclear swelling, and variable sized and shaped vacuoles. Bile canaliculi showed loss of microvilli with swelling and clubbing of those remaining. The most striking change in the hepatocytes was the formation of surface blebs.

The authors concluded that chronic low-dose comfrey-derived PA toxicity leads to sinusoidal fibrosis, sloughing of endothelial cells and hepatocyte membrane damage, most prominently seen in the sinusoidal borders (Yeong, Wakefield & Ford, 1993).

(c) Chicken

In a 35-day feeding trial, commercial ISA Babcock Brown laying hens (32 weeks old, 10 per group) were fed a commercial layer diet mixed with dried and ground *Crotalaria pallida* seeds at 0% (vehicle control), 1%, 2% or 3% (w/w) over the course of 5 weeks (Diaz, Almeida & Gardner, 2014). Feed intake and body weight were monitored at weekly intervals. Blood samples were collected on day 0, 14 and 28. AST, ALT, LDH, GGT and creatine kinase were determined in the blood samples. At the end of the experiment, six hens from each group were euthanized and examined for gross lesions. Liver, lungs, heart, spleen, kidneys, proventriculus, gizzard and pancreas were removed and weighed. Liver and lung were histologically examined.

C. pallida seeds were found to contain usaramine (0.16%) and its corresponding *N*-oxide (0.02%); the total PA content was about 0.18% (dry weight basis). The authors estimated the usaramine dose received by the hens to be 0, 0.54, 0.99 or 1.41 mg/kg per day, respectively, but did not report how this estimate was calculated.

No mortality was reported in any of the treatment groups. At the end of the 5-week period, body weight was significantly lower in all of the exposure groups compared to the control group; 8.7% lower in the 1% group, 14.8% lower in the 2% group and 21.1% lower in the 3% group. Feed intake and feed conversion were also decreased in all dose groups compared to the control group, while egg mass production was affected in the 2% and 3% groups (13% and 25% less egg mass, respectively) and the number of eggs was significantly lower in the 3% group only. Average egg weight was not affected in any of the dose groups. Statistically significant differences in relative organ weights compared to controls were noted for the livers of animals from the 3% group (23% decrease) and for lung weights in all dose groups (up to 33% increase).

No differences in creatine kinase or GGT activity were found between the groups at any of the sampling points (0, 14 and 28 days). AST and LDH activities

were up to 1.5-fold higher in the exposed groups compared to the control group at day 28. ALT activity was up to 1.9-fold higher in the exposed groups than in the control group at day 14.

No histological abnormalities were observed in hens from the control or the 1% dose group. Livers from animals in the 2% and 3% dose groups showed abnormal discoloration and were firmer than normal. In addition, distended gallbladders were observed. Histological abnormalities were seen, including hepatocyte necrosis, megalocytosis and bile duct proliferation, and in the 3% group, hepatocyte anisokaryosis was also observed. No histological alterations were noted in lung tissue.

The authors concluded that feeding laying hens ≥ 2% *C. pallida* seeds caused significant adverse effects on health, and dietary levels of 1% *C. pallida* led to mild adverse effects (Diaz, Almeida & Gardner, 2014). As effects were observed at all doses, the LOAEL in this study was 0.54 mg/kg bw.

In a dietary study with *Senecio vernalis*, Hyline W-77 laying hens (16 weeks old, 10 animals per group) were fed a diet containing 0, 0.5%, 2% or 4% dried and ground aerial parts of *S. vernalis* for 30 weeks (Eröksüz et al., 2003b). Feed consumption was recorded weekly and body weight every 2 weeks. At the end of the trial, animals were euthanized and blood was collected. Serum was used for biochemical evaluations. At study termination, tissues were examined grossly and major organs were weighed. Liver, spleen, heart, kidneys, pancreas, small and large intestine were subjected to histological evaluation.

The plant material contained 0.14% of total alkaloids (8.57% free base; 91.43% *N*-oxide), identified in fraction A (free base) and fraction B (free base + *N*-oxide), respectively, as senecionine (27.95% and 66.65%), senecivernine (10.15% and 10.37%), seneciphylline (5.19% and 8.51%), integerrimine (6.39% and 8.44%), retrorsine (0.59% and 3.03%), senkirkine (37.38% and 2.35%) and hydroxysenkirkine (12.41% and 0.65%). Based on these data, the diets had a total alkaloid content of 0, 7, 28 or 56 mg/kg diet, respectively. Cumulative feed intake and total egg production were significantly reduced in the 2% and 4% dose groups compared to the control group. Statistically significantly reduced body weight compared to control animals was observed in all dose groups. Relative organ weights were significantly lower for spleen, lung and kidney in the 2% and 4% groups, and for liver in the 4% group.

Increased serum values of total bilirubin (4% group), AST (all exposure groups) and gamma-GT (2% and 4% groups) were noted. In addition, decreased serum values of total protein, albumin and ALP in the 2% and 4% groups were reported.

Macroscopic and histological changes (including fibrosis, bile epithelial hyperplasia and bile duct proliferation) were seen in liver, and the changes were most pronounced in the 4% group, less at 2%, and were generally mild or absent in

the 0.5% group. In addition, in spleens of animals in the 4% dose group, lymphoid hyperplasia of white pulp and diffuse congestion of red pulp were observed. No data were presented on the histological evaluation of the heart, kidneys, pancreas and intestine.

The authors concluded that, at the 2% and 4% dietary levels, mild to moderate histological and biochemical changes were observed together with a significant reduction in feed intake and egg production (Eröksüz et al., 2003b).

The effect of oral administration of sunn hemp (*Crotalaria juncea*), which is closely related to showy crotalaria (*Crotalaria spectabilis*), on chicken was investigated (Hess & Mosjidis, 2008). Sunn hemp seeds have been reported to contain junceine, riddelliine, senecionine, seneciphylline and trichodesmine. In feeding trials, male and female broilers (strain not reported, 0 days old at start, 80 animals per group) were given starter feed with sunn hemp seeds for 21 days. In the first trial, animals were fed 0, 0.5 or 5% whole sunn hemp seeds. In the second trial, animals were fed 0, 0.5 or 2% ground sunn hemp seeds to prevent self-selection. Body weight, feed conversion efficiency and mortality were evaluated at 7 and 21 days. Results were only reported for 21 days.

In trial 1, the broilers showed significant seed refusal. Body weights and weight gains were significantly reduced (~6% and ~7%, respectively) in broilers fed 5% sunn hemp, but not in broilers fed 0.5% sunn hemp. Feed consumption was reduced (~5%) and feed conversion was poorer in the 5% group. No effects on mortality were noted. In trial 2, no effects were found for hens fed 0.5% sunn hemp. Body weights and weight gains were significantly reduced (~25% and ~27%, respectively) in broilers fed 2% sunn hemp. Feed consumption was reduced (~13%) and feed conversion was poorer in the 2% group. No effects on mortality were seen.

The authors concluded that the sunn hemp would not have contained enough of the toxins associated with showy crotalaria to be toxic. The Committee does not agree with this conclusion, as the presence of toxins associated with showy crotalaria was not investigated in sunn hemp seed. Further, in the low-level inclusion trial, body weight and body weight gain were severely affected in animals fed 2% sunn hemp. As no pathological investigations were performed, toxicity may have remained unnoticed in these trials and therefore it cannot be concluded that sunn hemp was not toxic to chicks through low-level inclusion in the diet (Hess & Mosjidis, 2008).

In a study to compare the toxicity of a reduced, crude comfrey alkaloid extract with that of the purified lycopsamine and intermedine, California white chicks (*Gallus gallus domesticus*, 1 week old, five chicks per group) were administered five doses of reduced crude comfrey extract, or the monoesters lycopsamine or intermedine, i.e. 0 (vehicle control), 0.04, 0.13, 0.26, 0.52 or 1.04 mmol/kg bw per day PA free base, for 10 days (Brown et al., 2016). For

lycopsamine and intermedine this amounted to doses of approximately 0, 12.0, 38.9, 77.8, 155.6 or 311.3 mg/kg bw per day. The daily dose was given in two halves via gavage, one half in the morning and the other half in the afternoon. Chicks were weighed three times per week and doses were recalculated before the next treatment. Further, they were monitored at least twice daily during treatment and for another seven days after treatment until termination and necropsy. Animals that became moribund were euthanized and necropsied. Gross abnormalities were reported and the entire brain, heart, spleen and right liver lobe, and samples of the lung, kidney, testicle, crop, proventriculus, ventriculus, small intestine, colon, caecum and bursa of Fabricius were collected for histopathological analysis. In addition, blood samples were taken and the left liver lobe was taken for pyrrole adduct analysis.

The crude comfrey extract contained mainly lycopsamine, intermedine and their acetylated derivatives. No differences in weight gain compared to control animals were noted for chicks in the lycopsamine and intermedine dose groups. Chicks that received the crude extract showed a dose-related decrease in body weight gain, which was significantly lower than in controls at 0.52 and 1.04 mmol/kg bw per day (~43% and ~78%, respectively). Of these, all chicks in the 1.04 mmol/kg bw group and one in the 0.52 mmol/kg bw group suffered from ascites, and two from the 1.04 mmol/kg bw group had to be euthanized after 7 and 10 days, respectively.

The serum activities of sorbitol dehydrogenase (SDH) and bile acids were significantly increased, compared to controls, in the two highest crude extract dose groups, ~422% and ~439%, respectively, for SDH, and ~167% and ~457%, respectively, for bile acids. Glucose levels were decreased in all the highest dose groups for each of the three treatments, but this was only statistically significant for the crude extract group. No differences were noted for total protein, triglycerides, cholesterol, GGT, aspartate aminotransferase (AST), LDH or creatine phosphokinase.

At necropsy, relative liver weight was significantly lower in the highest dose group that received crude extract of comfrey. Histologically, centrilobular necrosis was observed in the liver in the most severely affected animals, with small foci or individual hepatocytes surrounded by haemorrhage and with pyknotic nuclei in the less severely affected ones. The most severely affected chicks were those in the crude extract group and the severity increased with dose. Angiectasis was observed in animals from the lycopsamine group at the three highest doses, and from the crude extract group at all but the lowest dose. Regeneration, mainly present in portal areas, with bile duct proliferation was found in animals that received 0.26 and 1.04 mmol/kg bw crude extract. Mild to severe capsular serositis was observed in chicks from the two highest dose groups administered crude comfrey extract. Mild to moderate periportal inflammation was reported in all

exposed animals, including controls, with no differences between the groups. No information was given about observations made in other organs.

Pyrroles were detected in the livers of chicks that received 0.13, 0.26, 0.52 and 1.04 mmol/kg bw crude extract or lycopsamine. In the intermedine group, pyrroles were found in chicks dosed with 0.04, 0.52 and 1.04 mmol/kg bw, in lower concentrations than in the other treatment groups. The concentration of pyrroles tended to increase with increasing dose.

The authors concluded that, although all treatments resulted in evidence for toxicity, the crude extract of comfrey was more toxic than an approximately equivalent dose of the individual pure PAs (Brown et al., 2016).

(d) Quail

In a feeding study using three PA-containing plant species, male and female Japanese quail (20 per sex per group, age 74 days) were fed a diet containing 30% dried and ground plant material of *Heliotropium dolosum* (HD group), *Senecio vernalis* (SV group) or *Heliotropium circinatum* (HC group) for 6 weeks (Eröksüz et al., 2008). The control group (C group) received 0% plant material.

The alkaloid content of the feed was calculated to be 390 mg/kg for the HD group, 450 mg/kg for the SV group, and 420 mg/kg for the HC group. Major PAs included europine (67.3%), heliotrine (16.3%) and lasiocarpine (8.1%) for the HC plant material; senecionine (66.65%), senecivernine (10.37%) and seneciphylline (8.51%) for the SV plant material; and lasiocarpine (43.97%), europine (25.95%) and heliosupine (18.49%) for the HD plant material. Only for the HD group, were the PA content and composition redetermined; for the other groups it was assumed to be the same as in earlier studies (Eröksüz, 2003a,b). *H. dolosum* contained 0.13% of total alkaloids (53.85% *N*-oxide), identified in fraction A (free base) and fraction B (free base + *N*-oxide), respectively, as europine (8.37% and 25.95%), heliotrine (1.14% and 4.81%), lasiocarpine (76.31% and 43.97%), echimidine (3.76% and 6.78%) and heliosupine (10.42% and 18.49%).

Feed consumption was determined on a weekly basis; the mean ratio between males and females was calculated by individually feeding five quail of each sex and group for 2 weeks and applied on the feed consumption values to determine the feed consumption of the males and females separately. Biochemical parameters, including direct bilirubin, total bilirubin, albumin, total protein, AST, ALP and GGT, were determined at the end of the sixth week. All tissues were grossly examined, and body weight and absolute and relative major organ weights were determined. Liver, spleen, heart, kidneys, pancreas, and small and large intestines were taken for further analysis.

Mean cumulative feed consumption in both males and females from all test groups was significantly less than that of the controls (~27% for the HD

group; ~13% for the HC group; ~13% for the SV group). The authors reported that the cumulative alkaloid intake for the HD, HC and SV groups was 1120 mg/kg, 1388 mg/kg and 1274 mg/kg for males, respectively, and 1606 mg/kg, 1856 mg/kg and 1702 mg/kg for females, respectively. The monographer calculated that this would be equal to 26.7, 33.0 and 30.3 mg/kg per day for males and 38.2, 44.2 and 40.5 mg/kg per day for females.

No mortality or clinical signs were reported. Final body weight was significantly decreased in all the test groups (~29% HD, ~20% HC, ~22% SV for males; ~41% HD, ~22% HC, ~19% SV for females) compared to the control group. Total egg production (in g) was also significantly reduced in all test groups. With respect to organ weights, statistically significant decreases were noted for absolute liver, heart and testes weights in males from all test groups.

In the HD group, a statistically significant increase in serum AST (~5% and ~24%, respectively), GGT (~167% and ~139%, respectively) and total bilirubin (~60% and ~283%, respectively) levels was noted for both males and females, while a significant decrease was observed for ALP (~87%, females only), serum total protein (~53% and ~74%, respectively) and albumin (~59% and ~59%, respectively) levels. In the HC group, serum total bilirubin and GGT levels were significantly increased in males (~52% and ~220%, respectively), and serum total protein and albumin levels significantly decreased in both males (~68% and ~47%, respectively) and females (~65% and ~47%, respectively). In the SV group, a significant increase was observed for serum GGT levels in males (~100%) and for serum AST levels in females (~26%). A significant decrease was found for serum total protein levels in both males and females (~74% and ~78%, respectively) and for serum ALP levels in females (~52%). It should be noted that for both organ weights and biochemical parameters, data were presented for 10 males and females, respectively, instead of for all animals.

No remarkable gross changes were observed in quails from the test groups. The severity and incidence of microscopic lesions were somewhat more pronounced in females than in males and were most prominent in quails fed the HD diet. The main histological change in the liver and bile ducts was periportal or irregular oval cell proliferation (HD group: 9/20 (males), 12/20 (females); HC group: 8/20 (males), 8/20 (females); SV group: 6/20 (males), 9/20 (females)), and mild cytomegaly was seen. Further, in birds in the HD diet group, bile duct hyperplasia and fibrosis in the liver together with bile pigment deposits were reported. In addition, in the HD and HC diet groups, the proximal tubules in the kidneys also showed mild cytomegaly.

The results of this study showed that exposure via the diet to the PA-containing plants *H. dolosum, H. circinatum* and *S. vernalis* may lead to decreased feed intake, decreased body weight and histopathological changes in the liver of

Japanese quail with the most prominent effects caused by *H. dolosum* (Eröksüz et al., 2008).

(e) Pig

In a toxicity study on riddelliine, pigs (0, 3, 6, 12 or 52 weeks old, strain and sex not specified, 12 animals per group) were exposed by gavage to 0, 5, 10 or 20 mg/kg bw per day purified riddelliine (final purity not specified) for 14 days (Stegelmeier et al., 2004). All pigs were monitored daily for clinical signs and weighed weekly when blood samples were taken. After dosing for 14 days, the pigs were euthanized and tissues were collected for histological and chemical analysis.

In all age groups, pigs dosed with riddelliine showed a statistically significant reduction in weight gain or even lost weight. Weight changes were correlated to dose for all age groups. Body weight changes normalized for body size did not reveal age differences.

At the highest dose, animals suffered from severe hepatic disease characterized by icterus, anorexia, scruffy-dry skin and reluctance to move. Pigs that were 6 weeks old when exposure started also had extensive ascites and visceral oedema with swollen livers displaying red lobular patterns and subcapsular oedema. At doses of 5 and 10 mg/kg bw, similar effects were observed but were less severe. Pigs that were 3 and 6 weeks old at the beginning of the experiment showed the most severe effects and several of the animals in the high-dose groups had to be euthanized early.

Serum levels of ALP, aspartate amino transferase, gamma-glutamyl transferase and creatine kinase activities were elevated in all dosed animals. In addition, pigs from the high-dose group had increased serum bilirubin and bile acid concentrations. Animals that were 0, 3 or 6 weeks old when exposure started showed reduced serum albumin and total protein levels, which were significantly different from levels in animals that were older at the beginning of the study, dosed with 10 or 20 mg/kg bw per day. Serum enzyme activities, bilirubin levels and bile acid concentrations tended to be higher in pigs that were 6 weeks or older at the beginning of the study than in younger animals.

Histopathology showed significant hepatocellular swelling, vacuolization and necrosis with associated collapse of hepatic cords, haemorrhage and inflammation in all pigs from all dose groups. Lesions were most severe in 3- and 6-week-old pigs, while inflammation was most prominent in 12- and 52-week-old pigs. No extrahepatic lesions were found except in 6-week-old pigs from the high-dose group, which showed vascular media oedema and fibrosis with mild endothelial hyperplasia in small arteries. The livers from treated animals contained pyrroles, but the amount detected was not consistent or correlated with dose.

The authors concluded that riddelliine is toxic in pigs at doses comparable to toxic doses in other species. Poisoning resulted in hepatic lesions and animals that were 3 or 6 weeks old at the beginning of the study were more severely affected than other age groups, suggesting that weanling pigs near 3–6 weeks of age are most susceptible to PA poisoning (Stegelmeier et al., 2004). The monographer noted that the results were mainly qualitatively described in the text and not presented in tables and figures. Therefore, the results cannot be confirmed.

In an experiment to reproduce acute poisoning with *Crotalaria spectabilis*, 16 pigs (nine females and seven males, 5–12 kg) were fed seeds of *C. spectabilis* (Ubiali et al., 2011). Three pigs received 0.5 g/kg and three other pigs 1.25 g/kg daily for 10 days. Two pigs were fed a daily dose of 2.5 g/kg for 5 and 8 days, respectively. The other pigs received a single dose of 2.5 g/kg (n = 3), 5 g/kg (n = 4) or 9.5 g/kg (n = 1). Clinical examinations were performed and, at necropsy, fragments of the liver, lungs, central nervous system, kidneys, heart, lymph nodes, tonsils and gastrointestinal tract were preserved for histological analysis.

Clinical signs were noted at earlier time points with increasing dose and included anorexia, apathy, depression and tremors. Hepatocellular necrosis and haemorrhage was observed in seven pigs, of which two received 2.5 g/kg per day, and five single doses of 5 or 9.5 g/kg. No other alterations were reported for these single-dose groups. In some pigs receiving daily doses of 0.5 g/kg and 1.25 g/kg for 10 days, liver fibrosis, megalocytosis, bile duct hyperplasia and haemorrhage were observed. No histological observations were noted for the pigs that received a single dose of 2.5 g/kg seeds (Ubiali et al., 2011).

(f) Sheep

Ground seeds from *Crotalaria retusa* were used in two experiments in crossbred Santa Inês hair sheep, approximately 9 months old (Anjos et al., 2010). The ground seeds contained 6.84% of monocrotaline (5.62% as free base and 2.2% as *N*-oxide).

In the first experiment, crossbred Santa Inês hair sheep (three animals per group) were given 2 g/kg bw of ground seeds of *C. retusa*, mixed with concentrated feed, equivalent to 1% of the sheep body weight, for a 70-day period (daily dose of monocrotaline of 136.8 mg/kg bw), or 3 or 4 g/kg bw of ground seeds mixed with water and administered by stomach tube as a single dose (monocrotaline dose of 205.2 mg/kg bw and 273.6 mg/kg bw, respectively). In the second experiment, crossbred Santa Inês hair sheep (three animals per group) received increasing doses of ground *C. retusa* seeds mixed in the feed; first 2 g/kg bw per day for 20 days (monocrotaline exposure of 136.8 mg/kg bw per day), followed by 4 g/kg bw per day for 7 days (monocrotaline exposure of 273.6 mg/kg bw per day) and finally a single dose of 5 g/kg bw (monocrotaline exposure of 342 mg/kg bw). The

C. retusa seeds were given mixed with concentrated feed in an amount equivalent to 1% body weight, but the single dose was given via stomach tube. Three sheep served as controls.

Blood samples were collected before the experiments and on days 20, 30, 45 and 75 after the start of the experiment. Serum activities of AST, GGT, and total protein values were determined, but the authors did not report the results of these determinations. All sheep were observed for 270 days. Sheep that died were necropsied and the thoracic and abdominal organs and the central nervous system were subjected to further analysis.

The authors reported that the sheep that were fed 2 g/kg bw seeds for 70 days did not show clinical signs. The authors did not report whether all the monocrotaline-containing concentrated feed was consumed by each of the sheep. Also, sheep that were administered increasing doses of ground seeds did not show clinical signs. Sheep that received single doses of 3 g/kg bw seeds (except one sheep) or 4 g/kg bw seeds did show clinical signs. These signs generally appeared within the first week after administration, except for one sheep that received 4 g/kg bw seeds that developed clinical signs after 68 days. Clinical signs included restlessness, apathy, abdominal pain, anorexia, increased respiratory and cardiac rates, mild yellowish ocular or oral mucosa, marked depression and recumbence. All sheep died within a week after onset of the clinical signs.

Serum activities of AST and GGT and serum values of proteins were within normal ranges for sheep fed 2 g/kg bw seeds and control animals. In sheep that received 3 or 4 g/kg bw seeds, serum levels of AST and GGT were increased and serum levels of total proteins were decreased, and returned to normal in the surviving sheep administered 3 g/kg bw seeds. In two of the three sheep that received increasing doses of ground seeds, no changes in GGT and AST activities or in protein concentrations were seen. In the third animal, a transient increase of AST was noted, which later returned to normal, but GGT activity and total protein concentrations were unchanged.

At necropsy, two sheep that received 3 g/kg bw seeds and one sheep that received 4 g/kg bw seeds displayed mild jaundice, ascites and haemorrhages of the mucosa and/or serous membranes of abomasum, spleen, heart, small and large intestines, diaphragm and larynx, enlarged liver with rounded edges and increased lobular pattern. Microscopically, periacinar necrosis of the liver with degenerated hepatocytes was observed. In the other two sheep that received 4 g/kg bw seeds, the liver was firm with an increased lobular pattern and yellowish-white and red colouring. The gallbladder was enlarged and oedematous, and the abomasum showed oedema in the folds. In one animal, jaundice, a yellow/orange liver, dark kidneys and dark brown urine was noted. Microscopically, these animals had megalocytosis, degenerated and vacuolated hepatocytes, bile duct proliferation and fibrosis. In one animal, hepatocytes were severely degenerated

or necrotic, and degeneration or necrosis within the tubules of the kidneys was reported. The other animal also showed vacuoles in the cerebellar peduncles and medulla, and in the boundary between the white matter and grey matter of the brainstem.

Based on these two experiments, the authors concluded that sheep are susceptible to acute intoxication by monocrotaline, but can develop strong resistance when exposed daily to non-toxic doses. Further, chronic poisoning was observed after single toxic doses but not after repeated ingestion of non-acutely toxic doses. It was noted that the resistance to the adverse effects that is developed by sheep is not a general phenomenon in other species (Anjos et al., 2010).

In a dosing trial to reproduce an outbreak of PA toxicosis, one male Dorper sheep (8 months old) was exposed to incremental doses of the crude extract of *Senecio inaequidens* for 4 days via oral gavage (Dimande et al., 2007). The doses administered were 49.5 mg/kg bw on day 0 and day 1, 99.0 mg/kg bw on day 2 and 198 mg/kg bw on day 3. Clinical examination was performed daily and the sheep was observed twice a day for clinical signs. Blood and urine samples were collected every day during the dosing period. The sheep was euthanized on day 4 for necropsy and microscopic examination. In addition, liver, bile, kidney and lung samples were collected to determine PA concentrations.

LC-MS/MS and GC-MS analyses revealed the presence of four different PAs in *S. inaequidens*: retrorsine (major component, *N*-oxide:free base ratio of 4.12:1), senecionine (*N*-oxide:free base ratio of 3.8:1), and two unidentified compounds. The average total PA (free base plus *N*-oxide) concentration in plant parts of *S. inaequidens* was 0.18%.

On day 1, ruminal motility was decreased, and feed refusal was observed on day 4. The sheep was subsequently euthanized. Albumin concentrations and albumin/globulin ratios were below the normal reference ranges before and during the dosing trial. Total serum protein was below normal reference values before the start of dosing and within normal reference ranges during the dosing period. AST activity increased slightly on day 1 of the dosing trial and glutamate dehydrogenase activity was elevated on day 3 and day 4. The remaining analytes were within the reference ranges or did not differ much from the values determined during the pre-dosing period.

At necropsy, the liver was swollen and pale-coloured, and had rounded edges. Histopathology revealed swollen hepatocytes with vacuolization of the cytoplasm, mild oedema in the portal area, and necrosis of single cells with mild neutrophil infiltration. The spleen was congested with white pulp hyperplasia. A mild infiltration by mononuclear cells and neutrophilic leukostasis was reported in the lungs. In the small intestines, severe accumulation of mononuclear cells in the (sub)mucosa and a dispersing distribution of coccidian parasites were found.

Retrorsine and senecionine were detected in tissue and body fluids. No PAs were found in the serum. The highest concentration of retrorsine was found in urine on day 4, i.e. 82 µg/g, while the liver contained 53.10 µg/g, the kidneys 29.4 µg/g, the bile 6 µg/g and the lungs 11.5 µg/g retrorsine equivalents.

The authors concluded that sheep were not the optimal species to reproduce PA poisoning in cattle as they were less susceptible (Dimande et al., 2007).

(g) Cows

In a feeding trial in cattle, female Hereford calves (three animals per group) were either exposed to *Senecio riddellii* (total PA content 2.46% of dry weight; 45 mg/kg bw; free base to *N*-oxide ratio 1:9) by gavage, to 4.5 mg/kg bw pure riddelliine (purity not specified; isolated from *S. riddellii*), to 40.5 mg/kg bw riddelliine *N*-oxide (purity not specified), or to a mixture of riddelliine and riddelliine *N*-oxide by infusion via blind rumen fistula (Molyneux et al., 1991). Doses were given daily for 20 days. A control group was included. Calves were observed daily for clinical signs and blood samples were collected twice weekly up to 78 days in surviving calves. Calves that approached a moribund state or were determined to be in a state of irreversible wasting were euthanized and necropsied. Tissues from lung, liver, gallbladder, heart, kidney, pancreas and brain were taken and examined microscopically.

Signs of depression, reduced feed intake and ataxia of the hind limbs were noted in calves that received *S. riddellii*, riddelliine *N*-oxide or riddelliine + riddelliine *N*-oxide. In addition, signs of chronic seneciosis were found in *S. riddellii*-treated calves, while calves exposed to riddelliine *N*-oxide or riddelliine + riddelliine *N*-oxide stood with the head slightly lowered and had a dull, staring look. Calves exposed to 4.5 mg/kg bw riddelliine showed no clinical signs of toxicosis.

In calves fed with *S. riddellii*, moribund condition was reached after 20 to 34 days. Body weights were decreased by 13% at necropsy. Serum GGT and AST activity were increased 13- to 18-fold and 2- to 4-fold, respectively. Calves receiving riddelliine at 4.5 mg/kg bw showed no changes in body weight, body weight gain or serum enzyme activities. Calves exposed to 40.5 mg/kg bw riddelliine *N*-oxide were in a moribund condition 41 to 50 days after the beginning of the experiment. At termination, body weights were the same as on day 0, i.e. no net body weight gain, and reduced compared to the control group. Serum GGT and AST activities were increased 5- to 11-fold and 2- to 5-fold, respectively. Calves fed a combination of riddelliine and its *N*-oxide were euthanized 41 to 66 days after the start of the study. Mean body weights were slightly decreased compared to body weight at the start (2%) and were reduced compared to the

control group. Serum GGT and AST activities were increased 5- to 17-fold and 2- to 3-fold, respectively.

Ascites, oedema, petechial haemorrhages, enlarged gall bladder containing thick, dark bile, and enlarged liver, which was mottled and discoloured or pale and showing fibrotic and congested sections were found in animals in all groups except controls. The enlargement of the gallbladder was markedly less in the riddelliine + riddelliine *N*-oxide group than in the other treated groups. Animals in this group also had moderate oedema in the lungs, whereas this was not found in the other groups. No data were presented on the other organs.

Microscopically, livers from the affected treatment groups showed hepatocellular necrosis, collapse of lobules, portal oedema, anisokaryosis with some cytomegaly, spongiosus of white matter in the basal nuclei or periventricular region, and bile duct proliferation. Again, the riddelliine + riddelliine *N*-oxide group was less severely affected.

The authors concluded that the *N*-oxide form of the alkaloid alone is capable of inducing typical Senecio toxicosis in cattle and that the free base level of the plant cannot be considered to be the sole factor in assessing the toxicity of *S. riddellii* (Molyneux et al., 1991).

Skaanild and colleagues performed two limited feeding trials with *Senecio vernalis* in cattle (Skaanild, Friis & Brimer, 2001). In the first trial, two RDM heifers (aged 11–15 months) were fed 40 or 80 g dried plant material per day for 10 days. Daily doses were 0.34 mg/kg bw senecionine + 0.2 mg/kg bw senkirkine and 0.68 mg/kg bw senecionine + 0.4 mg/kg bw senkirkine, respectively. Blood samples were collected regularly until 14 days after the start of dosing. In the second trial, three RDM heifers (aged 11–15 months) were fed 200, 400 or 1000 g of fresh whole *Senecio vernalis* plants per day for 8 days. The fresh plant material contained 25 µg/g senecionine and 25 µg/g senkirkine. The administration of 1000 g whole plant was estimated to give an oral daily dose of 0.13 mg/kg bw senecionine + 0.13 mg/kg bw senkirkine. The cattle were observed for clinical signs. Blood samples were taken regularly from 3 days before until 15 days after the start of dosing. Enzyme activities of GGT, ALP and ALT were measured. Liver samples were taken and analysed for PA content and underwent a pathological examination.

In both studies, alkaloid concentrations in plasma were below the limit of detection (LOD) of 0.01 µg/mL in all samples. No changes in serum activity of GGT, ALT and ALP were noted in any of the animals. In addition, no clinical signs were noted in the cattle, nor morphological changes in the livers.

The authors concluded that limited short-term intake of *S. vernalis* does not seem to affect heifers and that this lack of effect may be due to fast liver metabolism of PAs or the short dosing period (Skaanild, Friis & Brimer, 2001).

Five calves (8 months old, 140 kg) were orally fed with 0.38 g/kg per day dry leaves of *Senecio brasiliensis* for 24 days (Torres & Coelho, 2008). Five liver biopsies were taken, before the experiment and then every 15 days up to day 60. Clinical examination was performed daily.

Clinical signs were noted at week 3 and consisted of anorexia, tenesmus, and dry and hard faeces. One calf died on day 45. Postmortem examination showed shrunken and fibrotic livers, ascites and oedema of the mesentery, gastric wall, gallbladder wall and subcutaneous tissue. Microscopic findings included progressive and irreversible liver damage as indicated by hepatocellular ballooning, necrosis, apoptosis and megalocytosis and centrilobular, pericellular and portal fibrosis was observed from day 30 onwards (Torres & Coelho, 2008).

(h) Horses and donkeys

In a feeding trial, donkeys (1–2 years old, sex not specified, one animal per group) were exposed via feed to *Crotalaria retusa* milled seeds added to the diet at levels of 0, 25, 50 or 100 mg/kg bw per day or to *Crotalaria juncea* milled seeds at dietary levels of 0, 300, 600 or 1000 mg/kg bw per day for 365 days (Pessoa et al., 2013). Animals were observed for clinical signs daily. Blood samples were collected weekly and serum activities of AST, GGT and serum concentration of total protein were measured. Donkeys that died naturally or were euthanized in extremis were necropsied.

In a separate experiment, repeated high daily doses of 5000 mg/kg bw for 48 days or 3000 mg/kg bw for 83 days of *C. juncea* milled seeds (n = 1) or 1000 mg/kg bw for 7 days of *C. retusa* milled seeds (n = 1), or single high doses of 2500 or 5000 mg/kg bw *C. retusa* milled seeds (n = 1) were administered via nasogastric tube. Serum activities of AST and GGT were determined at different time points. In addition, liver biopsies were taken of one donkey given a single dose of *C. retusa* milled seeds at a dose of 2500 mg/kg bw.

Analysis of the *C. retusa* seed showed that the 11-membered macrocyclic diester monocrotaline was the major PA (>99% of total content) present at 5.99% weight/dry weight (w/dw). *C. juncea* seeds had a total alkaloid content of 0.074% w/dw for the first experiment and of 0.078% w/dw for the second experiment. Major PAs present included the 11-membered macrocyclic diesters trichodesmine (0.016% and 0.022%, respectively) and junceine (0.008% and 0.01%, respectively), and the monoesters isohemijunceines (0.05% and 0.055%, respectively).

In the first experiment, no clinical signs or lung lesions were observed in any of the donkeys. Further, no changes in serum total protein and AST or GGT activities were noted. The only change observed was mild megalocytosis in the liver with occasional intranuclear vacuolization of the hepatocytes, which

was found in all animals receiving *C. retusa* and donkeys receiving *C. juncea* at dietary levels of 600 and 1000 mg/kg bw per day, but not at 300 mg/kg bw per day.

In the second experiment, with higher dietary levels of *C. juncea*, clinical signs started at day 40 at the dose level of 5000 mg/kg bw per day seeds, and at day 70 at the dose level of 3000 mg/kg bw per day seeds, and included reluctance to move, increased audibility of breathing sounds and heartbeats in the lung fields, anorexia and severe dyspnoea with dilated nostrils. The affected animals were euthanized at day 50 and day 90, respectively. No changes were observed in GGT or AST serum activities. At necropsy, inspection and microscopic investigation showed that the lungs were severely affected, presenting, among others, nodules corresponding to interstitial proliferation of Clara cells and smooth muscle cells surrounded by connective tissue, alveolar oedema and mild infiltration of neutrophils within the alveoli. No liver lesions or changes in other organs examined (not specified) were observed.

The donkey that received a single dose of 5000 mg/kg bw *C. retusa* milled seeds displayed clinical signs 4 days after administration, while the donkey that received 1000 mg/kg bw *C. retusa* seeds for 7 days showed clinical signs at the end of the administration period. Clinical signs in both animals included apathy, anorexia, jaundice, tachypnoea, tachycardia and positive jugular pulse. The donkeys died 9 and 10 days after the beginning of the experiment, respectively. The donkey that received a single dose of 2500 mg/kg bw seeds did not show any clinical signs.

Serum activity of AST was elevated in the donkey that received milled *C. juncea* seeds at a dose of 5000 mg/kg bw just before death, while in the donkey that received repeated doses of 1000 mg/kg bw *C. retusa* milled seeds, GGT activity was elevated before death and AST activity remained normal. In the donkey that was given a single dose of 2500 mg/kg bw seeds, serum activity of GGT was elevated on day 10 and returned to normal on day 20, and serum activity of AST remained within normal ranges.

Necropsy of the animals that died revealed petechial haemorrhages and suffusions in the gastrointestinal tract, urinary bladder mucosa, lungs, liver, diaphragm, mesentery, endocardium, epicardium and heart valves. In addition, bloody contents in the small intestine, and oedema of the mesentery, mucosa of the caecum and colon, trachea and lungs, were reported. The stomach showed multiple erosions on the mucosa. The liver showed increased lobular pattern, and histologically, centrilobular necrosis was the main lesion. The donkey that survived without clinical signs showed no significant lesions in the liver biopsies.

The authors concluded that the seeds of *C. juncea* were associated with respiratory disease, whereas seeds of *C. retusa* caused exclusively liver lesions (Pessoa et al., 2013).

In a gavage study, 1-year-old horses (sex not specified, strain not specified, 2–3 animals per group) were exposed to *Cynoglossum officinale* at a total PA dose of 0, 5 or 15 mg/kg bw per day via gavage for 15 days (Stegelmeier et al., 1996). Serum, plasma, whole blood and liver biopsies were collected weekly. Horses were euthanized following the onset of clinical illness. The remaining clinically normal animals were euthanized 252 days post-exposure.

In the low-dose group, transient effects were found, including depression and weight loss. These effects gradually resolved. In the high-dose group, depression, anorexia and icterus were noted, indicating severe liver disease. Their condition deteriorated until they were euthanized before week 5 of the study.

No statistically significant biochemical changes were noted in the low-dose group; however, sporadic increases in bile acids, GGT and sorbitol dehydrogenase were noted. Biochemical changes in the high-dose group included a statistically significant increase in serum sorbitol dehydrogenase, bile acids, ALP and bilirubin levels, and a significant decrease in blood urea nitrogen concentrations. Two of the horses that received high doses had neutrophil granulation, vacuolated monocytes and platelets, lymphopenia and eosinopenia. Low thrombocyte counts prior to euthanasia were reported but this was not statistically significantly different from controls.

Necropsy revealed caecal and colonic mucosal oedema, and soft red and wet livers with lobular patterns in the horses exposed to 15 mg/kg bw per day. In addition, two animals had caecal or ileal infarctions and one animal had severe necrotizing dermatitis with crust formation on the nose, face and thorax. No significant gross lesions were observed in the control animals or the animals exposed to 5 mg/kg bw per day.

Histological analysis of animals in the low-dose group showed mild hepatocellular necrosis, biliary hyperplasia and focal neutrophilic periportal inflammation at day 28, which resolved, and hepatocellular apoptosis, anisokaryosis and megalocytosis after week 30. Immunoreactive cells were significantly increased compared to control animals on day 28. In the high-dose group, extensive hepatocellular necrosis with mild periportal fibrosis and biliary hyperplasia were observed at day 28, which are typical of acute PA toxicosis. In addition, immunoreactive cells were increased compared to controls but the difference was not statistically significant.

Pyrroles were detected in the liver of all horses that received high doses, and in trace amounts in the liver from one horse treated with a low dose.

The authors concluded that acute PA toxicosis due to *C. offinicale* exposure caused marked biochemical changes and histopathological hepatocellular necrosis without megalocytosis. Chronic or low-dose intoxication caused minimal biochemical changes with dramatic hepatocyte megalocytosis (Stegelmeier et al., 1996).

After an outbreak of *Crotalaria retusa* poisoning in horses, this was experimentally reproduced in one adult horse and four adult donkeys (Nobre et al., 2004). The horse received 100 g (0.4 g/kg) of *C. retusa* seeds daily for 52 days and the donkeys received dried whole *C. retusa* mixed with grass at daily doses of 2.5, 5 or 10 g/kg for 120, 90 and 30 days, respectively. One donkey received no plant material and served as a control. The horse died after 52 days of dosing, and the donkey that received 5 g/kg per day died after 48 days. The other two donkeys were euthanized after 120 days. Biopsies were taken from liver, lung, heart, brain and kidneys for histological examination. The monocrotaline content of the plant material given to the donkeys was 0.5%.

After 15 days of dosing, the horse showed progressive anorexia, bilateral nasal discharge, depression and incoordination. After 51 days, the horse was agitated, walking in circles, restless and uncoordinated and eventually died within 24 hours. All donkeys presented with anorexia, depression, a low head and jaundice. The donkey that died 48 days after the start of the experiment showed nervous signs, including incoordination and muscle tremors 24 hours before death.

All animals suffered from loss of body condition and poor nutritional status. At necropsy, the horse had fluid in the abdominal cavity, a hard liver with dark red and white areas on the surface, dark red lungs, slightly congested kidneys and the intestinal content was more fluid than normal. Observations reported for the donkeys that were euthanized were similar to those in the horse.

Microscopically, the main alterations were in the liver, with severe disorganization of the parenchymal cells, megalocytosis, severe vacuolization of hepatocytes and periportal fibrosis. The lungs showed mild oedema and congestion, and in the kidneys discrete multifocal vacuolization of tubular cells and congestion was noted. The liver of the donkeys that received 2.5 and 10 g/kg showed periportal fibrosis, severe vacuolization in the hepatocytes – mainly of the centrilobular region – necrosis, bile-retaining hepatocytes and megalocytosis. Haemorrhages in the centrilobular region were also observed, mainly in the animal that received 10 g/kg *C. retusa*. The donkey that died during the experiment exhibited periportal fibrosis, megalocytosis, disorganization of the parenchymal cells, necrotic hepatocytes, diffuse congestion and centrilobular haemorrhages, as well as biliary retention and bile duct proliferation. Lesions were also observed in the lungs of the donkeys that received 5 and 10 g/kg and in the kidneys and brains of the one fed 5 g/kg. Clinical signs and pathology were similar to what was observed in the poisoning cases (see section 2.3), except that Alzheimer type II astrocytes were only found in the donkey that died (Nobre et al., 2004).

2.2.3 Long-term studies of toxicity and carcinogenicity

Several old studies on carcinogenicity, that were not performed according to OECD or similar guidelines, with various purified PAs such as lasiocarpine, monocrotaline, retrorsine, retronecine, heliotrine, senecionine, symphytine, clivorine, petasitenine and senkirkine and/or crude plant extracts containing PAs have been performed, using various durations, dosing regimes and routes of administration. Most of these studies were performed in rats. These studies were previously evaluated by IPCS (1988), NTP (2008), Committee on Toxicity of Chemicals in Food (2008), EFSA (2011) and Chen L et al. (2017). In summary, these studies indicate that tumours were most commonly observed in the liver, lung and blood vessels. In addition PA-related tumours were observed in various other organs such as breast, kidneys, pancreas, urinary bladder, pituitary, bone, peritoneal tissue and skin.

For the present evaluation, the Committee considered in detail the two long-term toxicity and carcinogenicity studies on lasiocarpine and riddelliine, which were evaluated by IPCS (IPCS, 1988) and/or EFSA (EFSA, 2011). These are the studies performed by the United States National Cancer Institute (NCI) on lasiocarpine (NCI, 1978) and by the United States NTP on riddelliine (NTP, 2003, also partly published in Chan PC et al., 2003). No new long-term studies published subsequent to those two evaluations were identified by the Committee.

(a) Mouse

(i) Riddelliine

The carcinogenic potential of riddelliine was studied in B6C3F1 mice in a long-term carcinogenicity bioassay (NTP, 2003). Groups of 50 male and 50 female animals, 5–6 weeks of age, were administered riddelliine (purity 92%; major impurities: retrorsine 5% and seneciphylline 1.4%) in sodium phosphate buffer by gavage at doses of 0 or 3 mg/kg bw per day, 5 days per week, for 105 weeks. Additional groups of 50 male mice received 0.1, 0.3 or 1 mg/kg bw per day, 5 days per week, for 105 weeks. A wide dose range was used in male mice to better characterize the dose–response curve. This decision was made as there was a limited amount of riddelliine available and females were chosen for investigating the low-dose effects of riddelliine in rats. Dose levels are equivalent to 0, 0.071, 0.21, 0.71 or 2.1 mg/kg bw per day on a 7 days per week basis (multiplied by 5/7, not corrected for purity). Animals were observed twice daily for clinical signs of toxicity and body weights were recorded on day 0 and every 4 weeks during the study. Animals found dead and surviving to the end of the study were necropsied and underwent extensive histopathological examination.

Survival of males and females administered 3 mg/kg bw was significantly less than that of the vehicle controls. Twenty out of 50 male mice (40%) and

17 out of 50 female mice (34%) survived until study termination compared to survival of 39/50 (78%) and 34/50 (68%) in males and females in the control group, respectively; survival in the other male dose groups was not different from controls. At 3 mg/kg bw per day, body weight gains were reduced in both females and males (at the end of the study, mean body weights were 19% and 33% lower, respectively) as compared to controls. In the 1 mg/kg bw per day dose group, on average the body weight at termination was 6% less than in the controls. There were no clinical findings attributable to riddelliine.

The results from the histopathological examination of the liver are presented in Table 11. The incidences of single haemangiosarcoma (14/50) and multiple haemangiosarcomas (17/50) of the liver in male mice in the 3 mg/kg bw dose group were significantly higher than those in the vehicle controls (2/50 and 0/50, respectively) and exceeded the historical ranges in controls (20/659). Liver haemangiosarcoma was the primary cause of death in 22/50 of the male mice in the 3 mg/kg bw group and occurred with an overall rate of 62% compared to an overall rate of 4% in the control groups (with poly-K correction for reduced survival: 66.7 and 4.4%, respectively). The incidence was not elevated in the other dose groups. Although most haemangiosarcomas occurred in the liver of male mice, sporadic haemangiosarcomas also occurred in other tissues, but the sporadic incidences of these haemangiosarcomas were similar to those in the vehicle controls, were within the historical range in controls, or occurred in only one animal in a dose group. Therefore, haemangiosarcomas in tissues other than the liver were not considered to be related to dosing. Metastases of the liver haemangiosarcomas were seen in the lung of five males from the 3 mg/kg bw dose group. One male each in the 1 and 3 mg/kg bw groups had endothelial cell hyperplasia, which is considered to be a preneoplastic change. Haemangiosarcoma in the liver was also noted in one female from the 3 mg/kg dose group. Sporadic incidences of this lesion appear also in historical control data (3/655), and therefore the single case in females was not considered to be related to dosing. One female treated with 3 mg/kg bw had a cholangioma.

The incidences of hepatocellular neoplasms occurred with a negative trend in males, and the incidence was significantly decreased in females in the 3 mg/kg bw group; the incidences in the 3 mg/kg bw male and female groups were less than the historical ranges in controls (Table 11). The reduced incidences of hepatocellular neoplasms were considered by the study authors to be possibly due to early mortality and lower body weight in males and females given the high dose, or the ability of PAs to inhibit division of hepatocytes.

Incidences of hepatocyte cytomegaly[1] and karyomegaly in males administered 0.3 mg/kg bw or greater and females given 3 mg/kg bw were

[1] Synonymous with hepatocellular hypertrophy.

Table 11

Incidences of neoplasms and non-neoplastic lesions of the *liver* in mice in the 2-year gavage study of riddelliine

	Vehicle control	0.1 mg/kg	0.3 mg/kg	1 mg/kg	3 mg/kg
Male					
Number examined microscopically	50	50	50	50	50
Survival to study termination	39/50 (78%)	41/50 (82%)	40/50 (80%)	38/50 (76%)	20/50 (40%)§
Relative body weight (% of controls)					
Week 1–13 (mean)	100%	101%	101%	100%	98%
Week 14–53 (mean)	100%	100%	99%	97%	91%
Week 54–101 (mean)	100%	98%	97%	96%	88%
Neoplastic lesions					
Haemangiosarcoma, multiple[a]	0	0	0	0	17**
Haemangiosarcoma (includes multiple)					
Overall rate[b]	2/50 (4%)	1/50 (2%)	0/50 (0%)	2/50 (4%)	31/50 (62%)
Adjusted rate[c]	4.4%	2.2%	0.0%	4.4%	66.7%
Terminal rate[d]	2/39 (5%)	1/41 (2%)	0/40 (0%)	2/38 (5%)	8/20 (40%)
First incidence (days)	729 (T)	729 (T)	–[e]	729 (T)	550
Poly-3 test[f]	$P<0.001$	$P=0.495$	$P=0.227$ N	$P=0.694$	$P<0.001$
Background incidence from HCD[g]	20/659 (3.1%)				
range 2–4%					
Hepatocellular adenoma, multiple	3	8	2	0	0
Hepatocellular adenoma (includes multiple)	16	18	14	5**	0**
Hepatocellular carcinoma, multiple	16	10	9	5**	0**
Hepatocellular carcinoma (includes multiple)	23	21	19	20	3**
Hepatocellular adenoma or carcinoma					
Overall rate[b]	36/50 (72%)	39/50 (78%)	33/50 (66%)	23/50 (46%)	3/50 (6%)
Adjusted rate[c]	73.4%	80.0%	66.0%	49.2%	7.5%
Terminal rate[d]	26/39 (67%)	32/41 (78%)	24/40 (60%)	19/38 (50%)	2/20 (10%)
First incidence (days)	475	542	567	566	590
Poly-3 test[e]	$P<0.001$ N	$P=0.299$	$P=0.281$ N	$P=0.011$ N	$P<0.001$ N
Background incidence from HCD[h]	304/659 (47.8%) range 28–72%				
Non-neoplastic lesions					
Endothelial cell, hyperplasia	0	0	0	1 (2.0)[i]	1 (2.0)
Hepatocyte, cytomegaly	4 (1.5)	4 (1.8)	16** (1.7)	33** (1.7)	43** (2.5)
Hepatocyte, karyomegaly	4 (1.5)	4 (1.8)	15** (1.7)	33** (1.8)	43** (2.5)
Bile duct, hyperplasia	2 (2.0)	0	1 (1.0)	3 (1.7)	6 (2.3)
Hepatocyte, centrilobular, necrosis	0	1 (3.0)	3 (3.3)	4 (3.3)	10** (3.0)
Hepatocyte, necrosis, focal	18 (1.3)	9* (1.3)	5** (1.8)	6** (2.2)	21 (2.6)
Haemorrhage, focal	0	2 (2.5)	1 (3.0)	6* (2.3)	21** (2.8)
Syncytial alteration, focal	38 (1.2)	30 (1.2)	31 (1.2)	27* (1.0)	0**

Table 11 (continued)

	Vehicle control	0.1 mg/kg	0.3 mg/kg	1 mg/kg	3 mg/kg
Female					
Number examined microscopically	49	–	–	–	50
Survival to study termination	34/50 (68%)	–	–	–	17/50 (34%)***
Relative body weight (% of controls)					
Week 1–13 (mean)	100%	–	–	–	98%
Week 14–53 (mean)	100%	–	–	–	82%
Week 54–101 (mean)	100%	–	–	–	74%
Neoplastic lesions					
Cholangioma	0	–	–	–	1/50 (2%)
Background incidence from HCD[j]	0/655 (0%)	–	–	–	–
Haemangiosarcoma	0/49 (0%)	–	–	–	1/50 (2%)
Background incidence from HCD[k]	3/655 (0.5%)				
range 0–4%	–	–	–	–	
Hepatocellular adenoma, multiple	2	–	–	–	0
Hepatocellular adenoma (includes multiple)	9	–	–	–	0**
Hepatocellular carcinoma, multiple	1	–	–	–	0
Hepatocellular carcinoma (includes multiple)	8	–	–	–	0**
Hepatocellular adenoma or carcinoma					
Overall rate[b]	16/49 (33%)	–	–	–	0/50 (0%)
Adjusted rate[c]	36.9%	–	–	–	0.0%
Terminal rate[d]	11/34 (32%)	–	–	–	0/17 (0%)
First incidence (days)	419	–	–	–	–
Poly-3 test[f]		–	–	–	$P<0.001$ N
Background incidence from HCD[l]	143/655 (21.8%) range 12–40%				
Non-neoplastic lesions					
Hepatocyte, cytomegaly	0	–	–	–	49** (2.3)
Hepatocyte, karyomegaly	0	–	–	–	49** (2.3)
Bile duct, hyperplasia	0	–	–	–	28** (1.9)
Infiltration cellular, mixed cell	29 (1.4)	–	–	–	41** (1.8)

(T) Terminal sacrifice

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Poly-3 test; ** $P \leq 0.01$;

*** Significantly decreased survival from the vehicle control group by life table pairwise comparison ($P<0.001$).

[a] Number of animals with lesions.

[b] Number of animals with neoplasms per number of animals with liver examined microscopically.

[c] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality.

[d] Observed incidence at terminal kill.

[e] Not applicable; no neoplasms in animal group.

[f] Beneath the vehicle control incidence (males only) the *P*-value associated with the trend test is presented. Beneath the dosed group incidence, the *P*-values corresponding to pairwise comparisons between the vehicle controls and that dosed group are presented. The Poly-3 test accounts for differential mortality in animals that did not survive until the end of the study. A negative trend or a lower incidence in a dosed group is indicated by N.

[g] Background incidence of haemangiosarcoma in male mice from historical control data (HCD) for 11 2-year studies with controls (3 gavage, 4 feeding, 1 drinking water, 2 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 20/659 (mean 3.1% ± 1.1% SD), range 2–4%. Note: these HCD data include tumour incidence from the riddelliine study (NTP, 2003).

[h] Background incidence of hepatocellular adenoma or carcinoma in male mice from HCD for 11 2-year studies with controls (3 gavage, 4 feeding, 1 drinking water, 2 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 304/659 (mean 47.8% ± 12.9% SD), range 28–72%. Note: these HCD data include tumour incidence from the riddelliine study (NTP, 2003).

[i] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

[j] Background incidence of cholangioma in female mice from HCD for 11 2-year studies with controls (3 gavage, 4 feeding, 1 drinking water, 2 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 0/655. Note: these HCD data include tumour incidence from the riddelliine study (NTP, 2003).

[k] Background incidence of haemangiosarcoma in female mice from HCD for 11 2-year studies with controls (3 gavage, 4 feeding, 1 drinking water, 2 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 3/655 (mean 0.5% ± 1.2% SD), range 0–4%. Note: these HCD data include tumour incidence from the riddelliine study (NTP, 2003).

[l] Background incidence of hepatocellular adenoma or carcinoma in female mice from HCD for 11 2-year studies with controls (3 gavage, 4 feeding, 1 drinking water, 2 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 143/655 (mean 22.8% ± 9.6% SD), range 12–40%. Note: these HCD data include tumour incidence from the riddelliine study (NTP 2003).

Source: NTP (2003).

significantly greater than those in the vehicle controls; the severities of these lesions were also increased in the 3 mg/kg bw groups. Incidences of minimal to mild bile duct hyperplasia were slightly increased in males that received 3 mg/kg bw and significantly increased in females given 3 mg/kg bw. In males in the 3 mg/kg bw group, the incidence of centrilobular necrosis of hepatocytes was significantly increased, and the incidence of focal hepatocyte coagulative necrosis was slightly increased. Although there was no dose-related change in the incidence of focal hepatocyte necrosis, the severity of the focal necrosis increased with increasing dose. The necrosis was mostly present in livers that exhibited other lesions, especially haemangiosarcomas. Incidences of focal haemorrhage, which was usually concomitant with haemangiosarcoma, were significantly increased in males administered 1 or 3 mg/kg bw. The incidence of mixed cell cellular infiltration was significantly increased in females that received 3 mg/kg bw. A dose-related decrease in the incidence of syncytial (multinucleated) cell alteration was noted in the males in the 3 mg/kg bw group.

The results from the histopathological examination of the lungs are presented in Table 12. Compared to those in the vehicle controls, the incidences of alveolar/bronchiolar adenoma and adenoma or carcinoma (combined) were significantly increased in females in the 3 mg/kg bw group, and the incidence of alveolar/bronchiolar carcinoma in this group was increased, also compared to historical control values. In contrast, in males, except in the 1 mg/kg bw group, which had significantly decreased incidences of alveolar/bronchiolar carcinoma and alveolar/bronchiolar adenoma or carcinoma (combined), the incidences of alveolar/bronchiolar neoplasms in dosed males were similar to those in the vehicle controls. In females in the 3 mg/kg bw group, the incidence and severity of alveolar epithelial hyperplasia showed a slight non-statistically significant increase (6/50 versus 1/50 in controls, $P = 0.056$), and a statistically significant increase in histiocytic hyperplasia (9/50 versus 2/50 in controls, $P = 0.0256$).

The results from the histopathological examination of the kidney are presented in Table 13. Common, age-related nephropathy was observed in all males in all groups, in the females in the 3 mg/kg bw group and in 18/49 of the animals in the female control group. The severity of this lesion was increased in the males and females in the 3 mg/kg bw groups. Nephropathy was considered

Table 12
Incidences of neoplasms and non-neoplastic lesions of the *lung* in mice in the 2-year gavage study of riddelliine

	Vehicle control	0.1 mg/kg	0.3 mg/kg	1 mg/kg	3 mg/kg
Male					
Number examined microscopically	50	50	50	50	50
Neoplastic lesions					
Alveolar/bronchiolar adenoma, multiple[a]	4	1	1	3	0
Alveolar/bronchiolar adenoma (includes multiple)	12	10	11	8	12
Alveolar/bronchiolar carcinoma, multiple	4	2	4	1	
Alveolar/bronchiolar carcinoma (includes multiple)	7	8	6	1*	5
Alveolar/bronchiolar adenoma or carcinoma					
Overall rate[b]	18/50 (36%)	16/50 (32%)	15/50 (30%)	9/50 (18%)	17/50 (34%)
Adjusted rate[c]	39.1 %	34.7%	31.1%	19.7%	39.7%
Terminal rate[d]	15/39 (39%)	16/41 (39%)	13/40 (33%)	7/38 (18%)	8/20 (40%)
First incidence (days)	599	729 (T)	567	689	559
Poly-3 test[e]	$P = 0.424$	$P = 0.413$ N	$P = 0.276$ N	$P = 0.033$ N	$P = 0.564$
Female					
Number examined microscopically	50	–	–	–	50
Neoplastic lesions					
Alveolar/bronchiolar adenoma[f]	1	–	–	–	9**
Alveolar/bronchiolar carcinoma, multiple	1	–	–	–	1
Alveolar/bronchiolar carcinoma (includes multiple)	1	–	–	–	4
Alveolar/bronchiolar adenoma or carcinoma[g]					
Overall rate	2/50 (4%)	–	–	–	13/50 (26%)
Adjusted rate	4.7%	–	–	–	30.5%
Terminal rate	1/34 (3%)	–	–	–	6/17 (35%)
First incidence	(days) 419	–	–	–	587
Poly-3 test		–	–	–	$P<0.001$
Background incidence from HCD	53/654 (8.1%) range 0–12%	–	–	–	–
Non-neoplastic lesions					
Alveolar epithelium, hyperplasia	1 (1.0)[h]	–	–	–	6 (1.5)
Respiratory system, lung, histiocytic hyperplasia	2 (4%)	–	–	–	9 (18%)*

(T) Terminal sacrifice
* Significantly different ($P \leq 0.05$) from the vehicle control group by the Poly-3 test; ** $P \leq 0.01$.
[a] Number of animals with lesions.
[b] Number of animals with neoplasms per number of animals with lung examined microscopically.
[c] Poly-3-estimated neoplasm incidence after adjustment for intercurrent mortality.
[d] Observed incidence at terminal kill.
[e] Beneath the vehicle control incidence (males only) is the *P*-value associated with the trend test. Beneath the dosed group incidence are the *P*-values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A lower incidence in a dosed group is indicated by N.
[f] Background incidence of haemangiosarcoma in female mice from historical control data (HCD) for 11 2-year studies with controls (3 gavage, 4 feeding, 1 drinking water, 2 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 37/654 (5.4% ± 4.0%), range 0–12%. Note: these HCD data include tumour incidence from

the riddelliine study (NTP, 2003).
[g] Background incidence of haemangiosarcoma in female mice from HCD for 11 2-year studies with controls (3 gavage, 4 feeding, 1 drinking water, 2 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 53/654 (7.6% ± 4.7%), range 0–12%. Note: these HCD data include tumour incidence from the riddelliine study (NTP, 2003).
[h] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
Source: NTP (2003).

Table 13
Incidences of non-neoplastic lesions of the *kidney* in mice in the 2-year gavage study of riddelliine

	Vehicle control	0.1 mg/kg	0.3 mg/kg	1 mg/kg	3 mg/kg
Male					
Number examined microscopically	49	49	50	50	50
Nephropathy[a]	46 (1.3)[b]	48 (1.5)	48 (1.8)	50 (2.1)	50 (2.8)
Glomerulus, glomerulosclerosis	0	1 (1.0)	0	42** (1.8)	41** (2.5)
Renal tubule, accumulation, hyaline droplet	0	2	1 (3.0)	1 (3.0)	3 (3.0)
Renal tubule, karyomegaly	0	0 (3.0)	0	0	12** (1.4)
Renal tubule, dilatation	16 (1.2)	17 (1.1)	24 (1.3)	29** (1.5)	22 (1.9)
Female					
Number examined microscopically	49	–	–	–	50
Nephropathy	18 (1.3)	–	–	–	47** (3.4)
Glomerulus, glomerulosclerosis	0	–	–	–	40** (2.7)
Renal tubule, accumulation, hyaline droplet	2 (2.5)	–	–	–	14** (2.6)
Renal tubule, pigmentation	2 (2.0)	–	–	–	27** (2.8)
Renal tubule, karyomegaly	0	–	–	–	1 (2.0)

** Significantly different ($P \leq 0.01$) from the vehicle control group by the Poly-3 test
[a] Number of animals with lesion.
[b] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
Source: NTP (2003).

the primary cause of death in one male and 19 females in the 3 mg/kg bw groups. Morphologically, the lesions were similar to background nephropathy.

The incidences and severity of glomerulosclerosis in males that received 1 mg/kg bw and males and females that received 3 mg/kg bw were significantly increased. Compared to those of the vehicle controls, the glomeruli from dosed mice had increased amounts of collagen and carbohydrate-containing substances. These findings are expected in sclerotic glomeruli. Immune complex-mediated glomerulosclerosis was not the primary mechanism for the accumulation of hyaline material within the glomeruli of the riddelliine-treated mice.

The incidences of renal tubule hyaline droplet accumulation and granular, brown pigment were significantly increased in females in the 3 mg/kg bw group.

Table 14
Incidences of chronic arterial inflammation in female mice in the 2-year gavage study of riddelliine

	Vehicle control	0.3 mg/kg
Small intestine (duodenum)[a]	47	46
Artery, inflammation, chronic[b]	0	13** (1.9)[c]
Large intestine (caecum)	48	47
Artery, inflammation, chronic, focal	0	18** (1.7)
Kidney	49	50
Artery, inflammation, chronic	1 (4.0)	16** (2.1)
Mesentery	23	29
Artery, inflammation, chronic, focal	1 (3.0)	19** (2.5)
Ovary	49	48
Artery, inflammation, chronic	0	26** (2.6)
Spleen	49	50
Artery, inflammation, chronic, focal	0	6* (2.0)
Uterus	49	50
Artery, inflammation, chronic, focal	0	21** (2.4)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Poly-3 test; ** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically.
[b] Number of animals with lesions.
[c] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
Source: NTP (2003).

These increased incidences appeared to parallel the increased incidences and/or severity of nephropathy. In contrast, the incidence of hyaline droplet accumulation was only slightly increased in males in the 3 mg/kg bw group. The pigment was Prussian blue-positive, indicating its haemosiderin nature.

The incidence of renal tubule karyomegaly was significantly increased in males in the 3 mg/kg bw dose group, and the incidence of renal tubule dilatation was significantly increased in males that received 1 mg/kg bw; mild karyomegaly was observed in one female in the 3 mg/kg bw group.

The results from the histopathological examination of the arteries are presented in Table 14. The incidence of chronic arteritis was significantly increased in females given 3 mg/kg bw. The organs most frequently affected were the small (duodenum) and large (caecum) intestines, kidney, mesentery, ovary, spleen and uterus. In these females, this lesion was also observed sporadically in the meninges of the brain, liver, lung (mediastinum), mesenteric lymph node, pancreas, salivary gland, thymus, urinary bladder, and the subcutis and blood vessels near the spinal ganglion. Sporadic cases of chronic arterial inflammation were also observed in the duodenum, heart, kidney and spleen of dosed males.

Table 15
Incidences of non-neoplastic lesions in mice in the 2-year gavage study of riddelliine

	Vehicle control	0.1 mg/kg	0.3 mg/kg	1 mg/kg	3 mg/kg
Male					
Spleen [a]	49	49	50	50	49
Haematopoietic cell proliferation[b]	18 (2.3)[c]	16 (2.6)	19 (2.6)	20 (2.7)	33** (2.8)
Skin (subcutaneous tissue)	50	50	50	50	49
Oedema	0	0	2 (2.0)	5* (2.0)	25** (2.0)
Female					
Adrenal cortex	50	–	–	–	50
Cytoplasmic alteration, focal	1 (1.0)	–	–	–	25** (2.0)
Large intestine (caecum)	48	–	–	–	47
Oedema	0	–	–	–	5*(3.0)
Haemorrhage, focal	0	–	–	–	6* (2.3)
Epithelium, erosion, focal	0	–	–	–	6* (1.8)
Small intestine (duodenum)	47	–	–	–	46
Haemorrhage, focal	0	–	–	–	2 (2.5)
Necrosis, focal	0	–	–	–	2 (3.0)
Spleen	49	–	–	–	50
Haematopoietic cell proliferation	32 (2.6)	–	–	–	43* (3.1)
Skin (subcutaneous tissue)	50	–	–	–	50
Oedema	0	–	–	–	1 (3.0)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Poly-3 test; ** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically.
[b] Number of animals with lesions.
[c] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
Source: NTP (2003).

The results from the histopathological examination of other organs are presented in Table 15. The incidence and severity of focal cytoplasmic alteration in the adrenal cortex was increased in females in the 3 mg/kg bw group.

Oedema, haemorrhage, mucosal erosion of the epithelium, and/or necrosis in the caecum and duodenum, possibly secondary to the primary submucosal vascular changes, were observed in females given 3 mg/kg bw. The incidences and severities of haematopoietic cell proliferation of the spleen in males increased with increasing dose and reached statistical significance in the males and females in the 3 mg/kg bw group. This change was considered to be secondary to the dose-related haemorrhage observed in the liver, caecum and duodenum.

Chemical-related, generalized subcutaneous oedema of the skin was noted in males that received doses of 0.3 mg/kg bw or higher, and the incidences in the 1 and 3 mg/kg bw groups were significantly higher than in the vehicle controls. Most mice that exhibited subcutaneous oedema also had malignant

neoplasms, usually haemangiosarcoma. One female in the 3 mg/kg bw group also had subcutaneous oedema.

In conclusion, there was a strongly increased incidence of liver haemangiosarcomas in the males that received the high dose, but not the lower doses. In females there was an increase of alveolar/bronchiolar neoplasms at 3 mg/kg bw (only one dose level studied). In both males and females the incidence of hepatocellular adenomas and carcinomas was reduced, in males this was also noted at 1 mg/kg bw. Based on the tumour findings in this study, the NTP concluded that "there was clear evidence of carcinogenic activity of riddelliine in male B6C3F1 mice based on increased incidences of hemangiosarcoma in the liver" and "clear evidence of carcinogenic activity in female B6C3F1 mice based on increased incidences of alveolar/bronchiolar neoplasms". The Committee concurred with this conclusion. The Committee noted that non-carcinogenic effects, including an increased incidence of hepatocyte karyomegaly and cytomegaly, and an increased incidence of subcutaneous tissue oedema were observed in males given doses of 0.3 mg/kg bw and higher. In females, enhanced nephropathy and increased incidences of bile duct hyperplasia and arteritis in various tissues were observed at 3 mg/kg bw (the only dose tested). For a benchmark dose (BMD) analysis of the carcinogenicity data see section 9.2.

(b) **Rat**

(i) Riddelliine

A chronic dietary study of the carcinogenic potential of riddelliine was conducted in F344/N rats (NTP, 2003). Groups of 50 male and female animals were administered riddelliine (purity 92%; major impurities: retrorsine 5% and seneciphylline 1.4%) in sodium phosphate buffer by gavage at doses of 0 or 1 mg/kg bw, 5 days per week, for 105 weeks. Additional groups of 50 female rats received 0.01, 0.033, 0.1 or 0.33 mg/kg bw for 105 weeks. Because there was a limited amount of riddelliine available, a wide dose range was used in female rats to better characterize the dose–response curve. Female rats were considered more suitable to investigate low-dose effects, as hepatocellular adenomas were found in the subchronic studies. Dose levels are equivalent to 0, 0.007, 0.024, 0.071, 0.236 or 0.71 mg/kg bw per day on a 7 days per week basis (multiplied by 5/7, not corrected for purity). Animals were observed twice daily for clinical signs of toxicity, and body weights were recorded on day 0 and every 4 weeks during the study. Animals found dead and those surviving to the end of the study were necropsied and underwent extensive histopathological examination.

All but three males in the 1 mg/kg bw group died before week 70. Due to the high mortality in this group, the male rat study was terminated at week 72. All females in the 1 mg/kg bw per day group died before week 97 of the study;

survival of all other dose groups of females was similar to that of the vehicle control group. Haemangiosarcoma was considered the cause of death of 37 males and 32 females administered 1 mg/kg bw that died early or were sacrificed moribund. The toxicity that led to regenerative hyperplasia was the primary cause of death in one male and four female rats treated with 1 mg/kg bw.

Mean body weights of males and females in the 1 mg/kg bw group were lower than those of the vehicle controls throughout most of the study. At the end of the study the mean body weights in these groups were 21% (male) or 18% (female) lower than in the controls. The body weights of the females in the other dose groups were not affected. The only clinical finding related to riddelliine administration was general debilitation, including thinness in males and females, prior to death.

The results from the histopathological examination of the liver are presented in Table 16. The incidences of haemangiosarcoma in males in the 1 mg/kg bw group (43/50) and females (38/50) were significantly greater than those in the vehicle controls (0/50); three females in the 0.33 mg/kg bw group also had this neoplasm. No haemangiosarcomas (0/609 and 0/659, respectively) have been noted in historical controls. The central portions of the large haemangiosarcomas apparently underwent necrosis and inflammation as the neoplasms grew, leaving a large, blood-filled cavity. The smaller neoplasms often consisted of irregular masses of neoplastic cells without a central cavity. Invasion and destruction of the hepatic parenchyma, frequently accompanied by prominent necrosis and haemorrhage, were common. In the group that received 1 mg/kg bw, approximately half of the hepatic haemangiosarcomas in males and one third of those in females metastasized to the lung (male: 26/50; female: 17/50); metastases also occurred in the mediastinal lymph node (male: 3/50; female: 2/50) and mesentery (male: 4/50; female: 2/50), and in the pancreas (2/50) and spleen (1/50) of males. Haemangiosarcoma in one female in the 0.33 mg/kg bw group metastasized to the lung. The metastatic lesions were morphologically similar to the primary neoplasms.

The incidences of hepatocellular adenoma in males in the 1 mg/kg bw group (4/50) and females (7/50), and of hepatocellular adenoma or carcinoma (combined) in females in the 1 mg/kg bw group (8/50) were significantly greater than those in the vehicle controls (0/50, 1/50 and 1/50, respectively) and exceeded the historical ranges in controls (5/659, 4/659 and 0/659, respectively).

The incidences of diffuse hepatocytic regenerative hyperplasia were significantly increased in males in the group given 1 mg/kg bw and in females given 0.33 and 1 mg/kg bw. The study authors considered that the toxicity that led to regenerative hyperplasia was the primary cause of death in one male and four female rats that received 1 mg/kg bw. These nodules are thought to represent a regenerative response, probably secondary to hepatic damage caused by

Table 16

Incidences of neoplasms and non-neoplastic lesions of the *liver* in rats in the 2-year gavage study of riddelliine

	Dose					
	Vehicle control	**0.01 mg/kg bw**	**0.033 mg/kg bw**	**0.1 mg/kg bw**	**0.33 mg/kg bw**	**1 mg/kg bw**
Male						
Number examined microscopically	50	–	–	–	–	50
Survival at study termination (early termination at 72 weeks of exposure)	49/50 (98%)	–	–	–	–	3/50 (6%)
Relative body weight (% of control)						
Weeks 1–13 (mean)	100%	–	–	–	–	98%
Weeks 14–53 (mean)	100%	–	–	–	–	93%
Weeks 54–69 (mean)	100%	–	–	–	–	84%
Neoplastic lesions						
Haemangiosarcoma, multiple[a]	0	–	–	–	–	24**
Haemangiosarcoma (includes multiple)[b]						
Overall rate[c]	0/50 (0%)	–	–	–	–	43/50 (86%)
Adjusted rate[d]	0.0%	–	–	–	–	92.5%
Terminal rate[e]	0/49 (0%)	–	–	–	–	1/3 (33%)
First incidence (days)	–[f]	–	–	–	–	307
Poly-3 test[g]	–	–	–	–	–	$P<0.00l$
Background incidence from HCD[h]	0/609 (0%)	–	–	–	–	–
Hepatocellular adenoma, multiple	0	–	–	–	–	1
Hepatocellular adenoma (includes multiple)						
Overall rate[c]	0/50 (0%)	–	–	–	–	4/50 (8%)
Adjusted rate[d]	0.0%	–	–	–	–	13.7%
Terminal rate[e]	0/49 (0%)	–	–	–	–	0/3 (0%)
First incidence (days)	–	–	–	–	–	398
Poly-3 test[g]	–	–	–	–	–	$P = 0.033$
Background incidence from HCD[i]	5/609 (0.8%) range 0–3%	–	–	–	–	–
Non-neoplastic lesions						
Hepatocyte, hyperplasia, regenerative	0	–	–	–	–	49** (3.6)[j]
Hepatocyte, cytomegaly	0	–	–	–	–	32** (2.3)
Necrosis, focal	0	–	–	–	–	23** (3.2)
Eosinophilic focus	3	–	–	–	–	15**
Mixed cell focus	3	–	–	–	–	7*
Basophilic focus	32	–	–	–	–	21
Haemorrhage	0	–	–	–	–	4* (3.5)
Female						
Number examined microscopically	50	50	50	50	50	50
Survival at study termination	33/50 (66%)	22/50 (44%)	28/50 (56%)	22/50 (44%)	29/50 (58%)	0/50 (0%)
Relative body weight (% of control)						

	Dose					
	Vehicle control	**0.01 mg/ kg bw**	**0.033 mg/ kg bw**	**0.1 mg/ kg bw**	**0.33 mg/ kg bw**	**1 mg/kg bw**
Weeks 1–13 (mean)	100%	100%	101%	99%	99%	98%
Weeks 14–53 (mean)	100%	100%	100%	99%	99%	94%
Weeks 54–69 (mean)	100%	100%	100%	98%	97%	88%
Neoplastic lesions						
Haemangiosarcoma, multiple	0	0	0	0	0	13**
Haemangiosarcoma (includes multiple)						
Overall rate[c]	0/50 (0%)	0/50 (0%)	0/50 (0%)	0/50 (0%)	3/50 (6%)	38/50 (76%)
Adjusted rate[d]	0.0%	0.0%	0.0%	0.0%	7.0%	89.7%
Terminal rate[e]	0/33 (0%)	0/22 (0%)	0/28 (0%)	0/22 (0%)	1/29 (3%)	0/0
First incidence (days)	–	–	–	–	524	350
Poly-3 test[g]	*P*<0.001	–[k]	–	–	*P*<0.118	*P*<0.001
Background incidence from HCD[l]	0/659 (0%)					
Hepatocellular adenoma, multiple	0	0	0	0	1	0
Hepatocellular adenoma (includes multiple)						
Overall rate[c]	1/50 (2%)	0/50 (0%)	0/50 (0%)	0/50 (0%)	1/50 (2%)	7/50 (14%)
Adjusted rate[d]	2.3%	0.0%	0.0%	0.0%	2.4%	32.3%
Terminal rate[e]	1/33 (3%)	0/22 (0%)	0/28 (0%)	0/22 (0%)	1/29 (3%)	0/0
First incidence (days)	729 (T)	–	–	–	729 (T)	426
Poly-3 test[g]	*P*<0.00l	*P* = 0.514N	*P* = 0.506N	*P* = 0.510N	*P* = 0.756	*P* = 0.002
Background incidence from HCD[m]	4/659 (0.6%) range 0–2%	–	–	–	–	–
Hepatocellular carcinoma	0	0	0	0	1	1
Hepatocellular adenoma or carcinoma						
Overall rate[c]	1/50 (2%)	0/50 (0%)	0/50 (0%)	0/50 (0%)	2/50(4%)	8/50 (16%)
Adjusted rate[d]	2.3%	0.0%	0.0%	0.0%	4.8%	36.1%
Terminal rate[e]	1/33 (3%)	0/22 (0%)	0/28 (0%)	0/22 (0%)	2/29 (7%)	0/0
First incidence (days)	729 (T)	–	–	–	729 (T)	426
Poly-3 test[g]	*P*<0.001	*P* = 0.514N	*P* = 0.506N	*P* = 0.510N	*P* = 0.493	*P*<0.001
Background incidence from HCD[n]	4/659 (0.6%) range 0–2%					
Non-neoplastic lesions						
Hepatocyte, hyperplasia regenerative	0	0	0	0	8** (2.8)	50** (3.6)
Hepatocyte, cytomegaly	0	0	7** (1.1)	23** (1.2)	32** (1.9)	29** (2.3)
Necrosis, focal	4 (1.8)	2 (1.5)	3 (2.3)	4 (1.5)	4 (1.8)	15** (2.7)
Eosinophilic focus	1	2	6	4	12**	13**
Mixed cell focus	8	10	10	11	23**	5
Clear cell focus	9	8	9	13	22	2
Basophilic focus	45	46	44	42	40	20*
Bile duct, hyperplasia	2 (1.0)	1 (1.0)	4 (1.8)	4 (1.5)	3 (2.0)	10** (1.7)

Table 16 (continued)

	Dose					
	Vehicle control	**0.01 mg/kg bw**	**0.033 mg/kg bw**	**0.1 mg/kg bw**	**0.33 mg/kg bw**	**1 mg/kg bw**
Haemorrhage	0	0	2 (3.5)	0	1 (4.0)	7** (3.0)

(T) Terminal sacrifice

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Poly-3 test; ** $P \leq 0.01$

[a] Number of animals with lesion.

[b] No comparable historical control incidence available because male rats were sacrificed at 72 weeks.

[c] Number of animals with neoplasm per number of animals with liver examined microscopically.

[d] Poly-3-estimated neoplasm incidence after adjustment for intercurrent mortality.

[e] Observed incidence at terminal kill.

[f] Not applicable; no neoplasms in animal group.

[g] Beneath the vehicle control group incidence (females only) is the *P*-value associated with the trend test. Beneath the dose group incidence are the *P*-values corresponding to pairwise comparisons between the vehicle controls and that dose group. The Poly-3 test accounts for the differential mortality in animals that did not reach terminal sacrifice. A lower incidence in a dose group is indicated by N.

[h] Background incidence of haemangiosarcoma in male rats from historical control data (HCD) for 10 2-year studies with controls (1 gavage, 4 feeding, 1 drinking water, 3 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 0/609. Note: these HCD data include tumour incidence from the riddelliine study (NTP, 2003).

[i] Background incidence of hepatocellular adenoma in male rats from HCD for 10 2-year studies with controls (1 gavage, 4 feeding, 1 drinking water, 3 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 5/609 (mean 0.8% ± 1.2% SD), range 0–3%. Note: these HCD data include tumour incidence from the riddelliine study (NTP, 2003).

[j] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

[k] Value of statistic cannot be computed.

[l] Background incidence of haemangiosarcoma in female rats from HCD for ten 2-year studies with controls (1 gavage, 4 feeding, 1 drinking water, 3 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 0/659. Note: these HCD data include tumour incidence from the riddelliine study (NTP, 2003).

[m] Background incidence of hepatocellular adenoma in female rats from HCD for ten 2-year studies with controls (1 gavage, 4 feeding, 1 drinking water, 3 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 4/659 (mean 0.7% ± 1.0% SD), range 0–2%. Note: these HCD data include tumour incidence from the riddelliine study (NTP, 2003).

[n] Background incidence of hepatocellular adenoma or carcinoma in female rats from HCD for 10 2-year studies with controls (1 gavage, 4 feeding, 1 drinking water, 3 inhalation, 1 whole body exposure) fed an NTP-2000 diet: total 4/659 (mean 0.7% ± 1.0% SD), range 0–2%. Note: these HCD data include tumour incidence from the riddelliine study (NTP, 2003).

Source: NTP (2003).

riddelliine exposure. In severely affected livers, most or all of the parenchyma was involved. Proliferation of the bile ducts and of small oval basophilic cells that resembled oval cells was occasionally seen, especially in the larger nodules.

Several other non-neoplastic lesions of the liver in males in the group given 1 mg/kg bw and in females that received 0.033 mg/kg bw or greater had incidences that were significantly greater than those in the vehicle controls.

Hepatocyte cytomegaly consisted of enlarged hepatocytes that had increased amounts of eosinophilic cytoplasm and variably sized, enlarged nuclei (karyomegaly); these cells were distributed randomly in the hepatic parenchyma or within the areas of hepatic parenchyma between the nodules of regeneration. Increased incidences and severities of focal necrosis were noted in both males and females given 1 mg/kg bw and consisted of few to many irregular focal areas of coagulative hepatocyte necrosis. The necrosis was mostly present in livers in which haemangiosarcoma was also observed, suggesting it was secondary to the presence of these neoplasms. However, a direct effect of riddelliine cannot be excluded.

The incidences of eosinophilic foci in males and females given 1 mg/kg bw and females given 0.33 mg/kg bw; mixed cell foci in males given 1 mg/kg bw and females given 0.33 mg/kg bw; and clear cell foci in females given 0.33 mg/kg bw were significantly increased. The incidences of basophilic foci of the liver in males and females that received 1 mg/kg bw, and of mixed cell foci and clear cell foci in females that received 1 mg/kg bw were decreased, and the decrease in the incidence of basophilic foci in females given 1 mg/kg bw was statistically significant. The low incidences of foci in the groups exposed to 1 mg/kg bw may have been related to the diffuse involvement of the liver with regenerative hyperplasia and neoplasms as well as to the decreased survival in those groups. The foci ranged from small to moderately large lesions composed of hepatocytes arranged in normal hepatic cords that merged imperceptibly with the surrounding normal hepatocytes. The foci usually caused little or no compression of the surrounding tissue.

The incidences of bile duct hyperplasia in females given 1 mg/kg bw and haemorrhage in males and females that received 1 mg/kg bw were also increased.

The incidences of mononuclear cell leukaemia are presented in Table 17. Mononuclear cell leukaemia is one of the most common neoplasms found in F344/N rats; there is a high variability in occurrence as indicated by the historical control data (range 16–42%). The incidences of mononuclear cell leukaemia in all dosed groups of rats, except the females in the 0.01 and 0.033 mg/kg bw dose groups, were increased; these increases were statistically significant in both males and females in the 1 mg/kg bw dose groups. The incidences of mononuclear cell leukaemia in the female dose groups were within or less than the historical ranges. Because the male rat study was terminated at week 72, the mononuclear cell leukaemia incidence in these animals cannot be compared directly to the 2-year historical control rate of 49.3% observed with the NTP-2000 diet. However, data from recent NTP studies show that the observed mononuclear cell leukaemia incidence in male vehicle control rats in the current study (4%) at 75 weeks of age (72 weeks in study = 16.5 months), is similar to the rates observed in comparably aged male control F344/N rats terminated at approximately 15 months (11/488; 2.2%). Moreover, although the overall rate of observed neoplasm incidence in females exposed to 1 mg/kg bw was similar to that in the vehicle controls, when survival differences are taken into account, the Poly-3 adjusted leukaemia incidences in females and males at this dose level are significantly higher than the respective incidences in controls. In addition, the overall trend in females is highly significant. Therefore, the increased incidences of mononuclear cell leukaemia in male and female rats were considered to be chemical-related. The Committee agreed with this view.

The histopathological changes in thyroid gland (C-cells) are presented in Table 18. The incidence of thyroid gland (C-cell) adenoma was significantly

Table 17
Incidences of mononuclear cell leukaemia in rats in the 2-year gavage study of riddelliine

	Dose					
	Vehicle control	0.01 mg/kg bw	0.033 mg/kg bw	0.1 mg/kg bw	0.33 mg/kg bw	1 mg/kg bw
Male						
Mononuclear cell leukaemia, all organs						
Overall rate[a]	2/50 (4%)	–	–	–	–	9/50 (18%)
Adjusted rate[b]	4.0%	–	–	–	–	28.5%
Terminal rate[c]	2/49 (4%)	–	–	–	–	0/3 (0%)
First incidence (days)	497 (T)	–	–	–	–	204
Poly-3 test[d]	–	–	–	–	–	$P = 0.004$
Background incidence from HCD[e]	11/488 (2.2%)	–	–	–	–	–
Female						
Mononuclear cell leukaemia, all organs[e]						
Overall rate	12/50 (24%)	8/50 (16%)	13/50 (26%)	18/50 (36%)	18/50 (36%)	14/50(28%)
Adjusted rate	27.0%	18.9%	9.9%	40.3%	39.0%	51.6%
Terminal rate	8/33 (24%)	1/22 (5%)	7/28 (25%)	6/22 (27%)	6/29 (21%)	0/0
First incidence (days)	393	513	532	451	463	352
Poly-3 test[d]	$P = 0.009$	$P = 0.262$N	$P = 0.475$	$P = 0.132$	$P = 0.158$	$P = 0.033$
Background incidence from HCD[f]	185/659 (28.1%)	–	–	–	–	–

(T) Terminal sacrifice
[a] Number of animals with neoplasm per number of animals necropsied.
[b] Poly-3-estimated neoplasm incidence after adjustment for intercurrent mortality.
[c] Observed incidence at terminal kill.
[d] Beneath the vehicle control group incidence (females only) the *P*-value associated with the trend test is presented. Beneath the dose group incidence, the *P*-values corresponding to pairwise comparisons between the vehicle controls and that dose group are presented. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A lower incidence in a dose group is indicated by N.
[e] Historical background incidence from studies with terminal kill after 15 months, as presented in the NTP (2003) report (mean ± standard deviation): mean 2.2% ± 6.3% SD range 0–33%.
[f] Historical incidence for 2-year studies with female controls given the NTP-2000 diet (mean ± standard deviation): 185/659 (29.1% ± 8.4%), range 16%–42%.
Source: NTP (2003).

increased in females in the 0.33 mg/kg bw dose group. However, the incidence in the vehicle controls (2%) was below the average incidence in historical controls (12%, range 4–21%), and the incidence of adenomas in females that received 0.33 mg/kg bw (22%) was just outside the historical control range. No adenomas were seen in females in the 1 mg/kg bw group; however, survival in this group was reduced, and many deaths occurred fairly early in the study. Hyperplasia, adenoma and carcinoma of the thyroid gland (C-cell) represent a morphological and biological continuum in the F344/N rat, and there was no increase in the incidence of carcinoma or hyperplasia in females in the 0.33 mg/kg bw group. The only carcinoma observed in female rats was in an animal in the vehicle control group, bringing the total incidence of C-cell neoplasms in that group to 3/49. In addition, the incidence of hyperplasia was not significantly increased in any dose

Table 18

Incidences of neoplasms and non-neoplastic lesions of the thyroid (C-cell) in rats in the 2-year gavage study of riddelliine

	Dose					
	Vehicle control	0.01 mg/kg bw	0.033 mg/kg bw	0.1 mg/kg bw	0.33 mg/kg bw	1 mg/kg bw
Male						
Number examined microscopically	50	–	–	–	–	50
Neoplastic lesions						
Thyroid gland						
C-cell, adenoma[a]	3 (6%)	–	–	–	–	3 (6%)
C-cell, carcinoma	1 (2%)	–	–	–	–	0
Thyroid gland (C-cell), adenoma[b]						
Overall rate[c]	3/50 (6%)	–	–	–	–	3/50 (6%)
Adjusted rate[d]	6.0%	–	–	–	–	10.3%
Terminal rate[e]	3/49 (6%)	–	–	–	–	1/3 (33%)
First incidence (days)	497 (T)	–	–	–	–	307
Poly-3 test[f]	–	–	–	–	–	$P = 0.479$
Thyroid gland (C-cell), adenoma or carcinoma[b]						
Overall rate	4/50 (8%)	–	–	–	–	3/50 (6%)
Adjusted rate	8.0%	–	–	–	–	10.3%
Terminal rate	4/49 (8%)	–	–	–	–	1/3 (33%)
First incidence (days)	497 (T)	–	–	–	–	307
Poly-3 test[f]	–	–	–	–	–	$P = 0.592$
Non-neoplastic lesions						
Thyroid gland[b]						
C-cell, hyperplasia	4 (8%)	–	–	–	–	1 (2%)
Female						
Number examined microscopically	49	50	49	49	50	[50]
Neoplastic lesions						
Thyroid gland						
Bilateral, C-cell, adenoma	1 (2%)	0	0	0	0	0
C-cell, adenoma	1 (2%)	4 (8%)	4 (8%)	6 (12%)	11 (22%)	0
C-cell, carcinoma	1 (2%)	0	0	0	0	0
Thyroid gland (C-cell), adenoma[g]						
Overall rate	2/49 (4%)	4/50 (8%)	4/49 (8%)	6/49 (12%)	11/50 (22%)	0/50 (0%)
Adjusted rate	4.8%	9.9%	9.6%	14.7%	25.1%	0.0%
Terminal rate	2/32 (6%)	1/22 (5%)	2/28 (7%)	5/22 (23%)	6/29 (21%)	0/0
First incidence (days)	729 (T)	613	647	682	570	—[h]
Poly-3 test[f]	$P = 0.169$	$P = 0.321$	$P = 0.337$	$P = 0.123$	$P = 0.008$	$P = 0.452$N
Thyroid gland (C-cell), adenoma or carcinoma[g]						
Overall rate	3/49 (6%)	4/50 (8%)	4/49 (8%)	6/49 (12%)	11/50 (22%)	0/50 (0%)
Adjusted rate	7.2%	9.9%	9.6%	14.7%	25.1%	0.0%

Table 18 (continued)

	Dose					
	Vehicle control	0.01 mg/kg bw	0.033 mg/kg bw	0.1 mg/kg bw	0.33 mg/kg bw	1 mg/kg bw
Terminal rate	3/32 (9%)	1/22 (5%)	2/28 (7%)	5/22 (23%)	6/29 (21%)	0/0
First incidence (days)	729 (T)	613	647	682	570	–[h]
Poly-3 test[f]	$P = 0.212$	$P = 0.482$	$P = 0.500$	$P = 0.228$	$P = 0.023$	$P = 0.336$N
Non-neoplastic lesions						
Thyroid gland						
C-cell, hyperplasia	22 (45%)	9 (18%)	24 (49%)	15 (31%)	20 (40%)	10 (20%)

(T) Terminal sacrifice
[a] Number of animals with lesion.
[b] No comparable historical control incidence available because male rats were sacrificed at 72 weeks.
[c] Number of animals with neoplasm per number of animals with thyroid examined microscopically.
[d] Poly-3-estimated neoplasm incidence after adjustment for intercurrent mortality.
[e] Observed incidence at terminal kill.
[f] Beneath the vehicle control group incidence (females only) is the *P*-value associated with the trend test. Beneath the dose group incidence are the *P*-values corresponding to pairwise comparisons between the vehicle controls and that dose group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A lower incidence in a dose group is indicated by N.
[g] Historical incidence for 2-year studies with controls given the NTP-2000 diet: adenomas: 12.3% (range: 4–21%); adenomas and carcinomas 15% (range: 6–26%).
[h] Not applicable; no neoplasms in animal group.
Source: NTP (2003).

group. Because there was no increase in the incidence of malignant neoplasms or hyperplasia, and because the significantly increased incidence of adenoma was due, in part, to the low incidence in the vehicle control group, the study authors did not consider the increase to be related to riddelliine administration.

The histopathological changes in other organs are presented in Table 19. The incidence of mammary gland fibroadenoma was significantly decreased in females given 1 mg/kg bw per day (vehicle control: 28/50; 0.01 mg/kg bw: 21/50; 0.033 mg/kg bw: 26/50; 0.1 mg/kg bw: 24/50; 0.33 mg/kg bw: 23/50; 1 mg/kg bw: 3/50). There is evidence that the incidence of mammary gland fibroadenoma in female F344/N rats is strongly correlated with body weight (Haseman et al., 1997), and females that received 1 mg/kg bw had reduced body weights. Thus the decreased incidence of fibroadenoma is probably due to a combination of reduced survival and reduced body weight, and possibly other factors.

The incidences of non-neoplastic lesions of the kidney, spleen, bone marrow, lung, glandular stomach, and mediastinal and mesenteric lymph nodes were increased in males and/or females in the 1 mg/kg bw groups. A primary haemangiosarcoma was observed in the lung of a single female that received 1 mg/kg bw (not presented in the table).

Minimal to moderate renal tubule necrosis of the kidney consisted of multiple scattered renal tubules lined by epithelial cells that had undergone coagulative necrosis and were sometimes filled with protein and cell debris.

Table 19
Incidences of non-neoplastic lesions in rats in the 2-year gavage study of riddelliine

	Dose					
	Vehicle control	0.01 mg/kg bw	0.033 mg/kg bw	0.1 mg/kg bw	0.33 mg/kg bw	1 mg/kg bw
Male						
Kidney	50[a]	–	–	–	–	50
Renal tubule, necrosis	0[b]	–	–	–	–	6** (2.2)[c]
Spleen	50	–	–	–	–	49
Congestion	0	–	–	–	–	24** (2.7)
Haematopoietic cell proliferation	1 (1.0)	–	–	–	–	23** (2.8)
Bone marrow	50	–	–	–	–	49
Hyperplasia	1 (3.0)	–	–	–	–	36** (3.0)
Lung	50	–	–	–	–	50
Haemorrhage	1 (1.0)	–	–	–	–	21** (3.1)
Oedema	0	–	–	–	–	5* (3.2)
Stomach, glandular	50	–	–	–	–	50
Erosion	0	–	–	–	–	10 (2.5)
Ulcer	0	–	–	–	–	6** (2.8)
Lymph node, mediastinal	50	–	–	–	–	50
Haemorrhage	3 (2.3)	–	–	–	–	20** (3.6)
Pigmentation	10 (1.4)	–	–	–	–	20** (2.8)
Lymph node, mesenteric	50	–	–	–	–	50
Haemorrhage	1 (1.0)	–	–	–	–	8** (2.3)
Female						
Kidney	50	50	50	50	50	50
Renal tubule, necrosis	0	0	0	1 (3.0)	1 (2.0)	6** (2.3)
Transitional epithelium hyperplasia	1 (1.0)	1 (3.0)	1 (2.0)	1 (1.0)	0	5* (1.4)
Spleen	50	50	50	50	50	50
Congestion	0	0	0	1 (2.0)	3 (2.3)	7** (2.6)
Haematopoietic cell proliferation	24 (1.8)	33* (1.5)	25 (2.0)	26 (2.0)	27 (1.9)	34** (2.9)
Bone marrow	50	50	50	50	50	50
Hyperplasia	6 (2.5)	3 (2.7)	8 (2.9)	7 (2.9)	10 (2.2)	32** (2.6)
Lung	50	50	50	50	50	50
Haemorrhage	4 (3.3)	7 (2.6)	1 (2.0)	3 (3.0)	5 (3.4)	19** (2.5)
Stomach, glandular	50	50	50	49	49	50
Erosion	0	0	0	2 (2.0)	1 (1.0)	9** (1.9)
Ulcer	0	0	0	2 (2.5)	0	7** (2.9)
Lymph node, mediastinal	50	50	50	50	50	50
Haemorrhage	5 (2.2)	8 (2.1)	9 (2.4)	5 (2.0)	7 (2.1)	25** (3.0)
Pigmentation	23 (1.9)	22 (2.2)	32* (2.1)	15 (2.0)	16 (2.0)	31** (2.1)
Lymph node, mesenteric	50	50	50	47	49	49
Haemorrhage	1 (1.0)	2 (1.5)	3 (2.7)	4 (2.3)	0	6** (1.8)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Poly-3 test; ** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically.
[b] Number of animals with lesion.

Table 19 (continued)

[c] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
Source: NTP (2003).

Hyperplasia of the transitional epithelium occurred in the kidney of females in the 1 mg/kg bw group.

Congestion of the spleen in the males and females in the 1 mg/kg bw dose groups was considered to be a terminal change related to early death. The incidences of haematopoietic cell proliferation of the spleen were increased in males and females that received 1 mg/kg bw. Bone marrow hyperplasia in males and females in the 1 mg/kg bw groups was characterized by an increase in the amount of haematopoietic tissue (both erythroid and myeloid). The changes in the bone marrow and spleen are suggestive of increased haematopoiesis secondary to chemical-related RBC and platelet sequestration as was seen in the 13-week NTP (1993) study of riddelliine.

Haemorrhage of the lung in males and females in the 1 mg/kg bw dose groups was characterized by alveolar spaces filled with free RBCs, and oedema in males given 1 mg/kg bw was determined by the presence of alveolar spaces filled with homogeneous, eosinophilic, proteinaceous fluid. This change was considered to be related to the presence of metastatic haemangiosarcomas in the lung. Males and females administered 1 mg/kg bw had increased incidences of focal or multifocal mucosal erosion and ulceration in the glandular stomach.

Haemorrhage in the mediastinal and mesenteric lymph nodes was considered to represent a terminal change in male and female rats in the 1 mg/kg bw dose groups that died early; mediastinal lymph node haemorrhage was secondary to the metastatic haemangiosarcomas in the lung. Pigmentation in the mediastinal lymph node was identified by the presence of brown granular material, apparently haemosiderin, within the macrophages and was considered secondary to the increased incidence of haemorrhage in this organ. However, an association between haemorrhage and pigmentation was not seen in dosed females or in control males and females.

The NTP concluded that "under the conditions of these studies, there was clear evidence of carcinogenic activity of riddelliine in male and female F344/N rats based on increased incidences of hemangiosarcoma in the liver". They also considered the increased incidences of hepatocellular adenoma and mononuclear cell leukaemia in male and female rats to be treatment-related.

The Committee endorsed these conclusions. It noted that non-carcinogenic effects including increased incidences of hepatocyte cytomegaly were observed in females at doses from 0.033 mg/kg bw per day upwards. Other toxic effects in liver (regenerative hepatocellular hyperplasia, focal hepatocyte

necrosis, eosinophilic, mixed cell and basophilic foci) were observed in females at higher dose levels and in males at 3 mg/kg bw per day (the only dose tested) (NTP, 2003). For a BMD analysis of the carcinogenicity data see section 9.2.

(ii) Lasiocarpine

Groups of 24 male and 24 female Fischer F344 rats were administered lasiocarpine (purity 97%) at 0, 7, 15 or 30 mg/kg diet, equivalent to 0, 350, 750 or 1500 µg/kg bw per day for 104 weeks (National Cancer Institute (NCI), 1978). Feed and water were available *ad libitum*. The animals were observed daily for signs of toxicity, and those that were moribund were killed and necropsied. Animals were weighed individually every other week for 12 weeks, and once every fourth week for the remainder of the study. Palpation for masses was carried out at each weighing. The pathological evaluation consisted of gross examination of major organs and tissues from killed animals and from animals found dead. Lungs and bronchi, spleen, liver, kidney, pituitary and testis, and gross lesions from all animals were examined microscopically. In addition, tissues of the stomach, urinary bladder, thyroid, uterus, ovary and brain were examined from the majority of the controls, and from treated animals only if a lesion was found at necropsy. A few of the tissues from animals that died early or from some animals in an advanced state of autolysis were not examined.

Mean body weights of the male and female rats administered the high dose were lower than those of the animals in the control groups throughout most of the study, whereas weights of the male and female rats in the mid-dose groups were lower only during the second year, and weights of animals in the low-dose groups were unaffected (data are only presented in figures). No data on feed consumption were recorded; hence it is unknown whether the reduction in body weight was connected to reduced feed consumption. There was a positive dose-related trend in mortality for both sexes. In male rats, 88% of the controls, 54% of the low-dose group, 17% of the mid-dose group and none of the high-dose group survived to termination of the study. In females, 92% of the controls, 42% of the low-dose group, 4% of the mid-dose group and none of the high-dose group survived to termination of the study.

In spite of the early deaths, all male rats (including controls), except one animal in the low-dose and one in the high-dose group, developed tumours. Among the females, 18 in the control, 23 in the low-dose, 22 in the mid-dose and 9 in the high-dose group developed tumours. Time-adjusted analysis of the incidence of tumours was only performed for the female rats, since for the females in the high-dose group substantial early mortality could have prevented development of late-appearing tumours;[1] while in virtually all males, tumours

[1] Data not presented in the tables.

were found at necropsy. As more than 50% of females administered the high dose died before week 52 of the study, the statistical analysis of female rats was performed using only those animals surviving more than 52 weeks: 24 in the control group, 24 in the low-dose group, 23 in the mid-dose group and nine in the high-dose group.

A variety of neoplastic lesions not related to dosing were observed in both the control and treated rats. The incidences of these neoplasms were comparable among the control and treated groups. The following neoplasms were randomly distributed throughout the control and treated groups: squamous-cell carcinoma of the skin, alveolar/bronchiolar adenomas of the lung and endometrial stromal polyps of the uterus.

Incidences and statistics on treatment-related primary tumours are presented in Table 20 (males) and Table 21 (females). In male rats, there was a dose-related increase in the incidence of haemangiosarcoma of the liver; furthermore, the incidences in the mid- and high-dose groups (11/24 and 13/24, respectively), but not in the low-dose group (5/24), were significantly higher than in the controls (0/24) and exceeded the historical ranges in controls (12/2320). In females, the incidences in both the low- and mid-dose groups (8/24 and 7/24, respectively), but not in the high-dose group (2/24), were significantly higher than in the controls (0/24) and exceeded the historical ranges in controls (3/2370). The study authors noted that the lower incidence in the females in the high-dose group, compared with those in the low- and mid-dose groups could be related to the increased mortality in the high-dose group. Metastatic angiosarcomas were present in the lungs from a few of the rats in all treatment groups (males: three in the low-dose, five in the mid-dose and seven in the high-dose groups; females: three in the low-dose, four in the mid-dose and one in the high-dose groups), but not in controls.

Females from the high-dose group showed a significant increase in the combined incidence of hepatocellular carcinoma and adenoma of the liver (7/24 versus 0/24 in controls, and exceeded the historical ranges in controls (74/2356)). A positive trend for these tumours was also observed in male rats, but incidences did not reach statistical significance. Hepatocellular carcinomas were found in only two males and one female in the high-dose group. Nodular hyperplasia was observed in animals in all treatment groups and of both sexes. Thus, lasiocarpine was associated with proliferative lesions of hepatocytes as well as with angiosarcomas arising from endothelial cells of the liver.

The combined incidence of lymphoma or leukaemia was significantly increased in females in both the low- and mid-dose groups (9/24 and 11/24, respectively) compared to controls (2/24) and exceeded the historical ranges in controls (448/2370), but not in the high-dose group (1/24), perhaps because of the early deaths of animals in this group. The combined incidences of these

Table 20
Analyses of the incidence of primary tumours in male rats fed lasiocarpine in the diet[a]

	Control	7 mg/kg feed (equivalent to 350 µg/kg bw)	15 mg/kg feed (equivalent to 750 µg/kg bw)	30 mg/kg feed (equivalent to 1500 µg/kg bw)
Haematopoietic system				
Lymphoma[b]	1/24 (4%)	2/24 (8%)	3/24 (13%)	1/24 (4%)
P-values[c]	NS	NS	NS	NS
Relative risk (control)[d]	–	2.000	3.000	1.000
Lower limit	–	0.111	0.265	0.013
Upper limit	–	112.815	150.246	75.218
Weeks to first observed tumour	103	88	80	59
Leukaemia[b]	3/24 (13%)	1/24 (4%)	8/24 (33%)	6/24 (25%)
P-values[c]	NS	NS	NS	NS
Relative risk (control)[d]	–	0.333	2.667	2.000
Lower limit	–	0.007	0.739	0.490
Upper limit	–	3.801	13.700	10.992
Weeks to first observed tumour	86	103	82	68
Lymphoma or leukaemia[b]	4/24 (17%)	3/24 (13%)	11/24 (46%)	7/24 (29%)
P-values[c]	NS	NS	$P = 0.030$	NS
Relative risk (control)[d]	–	0.750	2.750	1.750
Lower limit	–	0.121	0.968	0.515
Upper limit	–	3.951	9.911	7.075
Weeks to first observed tumour	86	88	80	59
Background incidence from HCD	699/2320 (30.1% ± 10.5 SD)	–	–	–
Liver				
Hepatocellular carcinoma[b]	0/24 (0%)	0/24 (0%)	0/24 (0%)	2/24 (8%)
P-values[c]	NS	NS	NS	NS
Relative risk (control)[d]	–	–	–	Infinite
Lower limit	–	–	–	0.305
Upper limit	–	–	–	Infinite
Weeks to first observed tumour	–	–	–	84
Hepatocellular carcinoma or adenoma, NOS[b]	0/24 (0%)	0/24 (0%)	3/24 (13%)	5/24 (21%)
P-values[c]	$P = 0.003$	NS	NS	$P = 0.025$
Relative risk (control)[d]	–	–	Infinite	Infinite
Lower limit	–	–	0.622	1.309
Upper limit	–	–	Infinite	Infinite
Weeks to first observed tumour	–	–	104	66
Background incidence from HCD	96/2320 (4.2% ± 3.9% SD)	–	–	–
Angiosarcoma[b]	0/24 (0%)	5/24 (21%)	11/24 (46%)	13/24 (54%)
P-values[c]	$P < 0.001$	$P = 0.025$	$P < 0.001$	$P < 0.001$
Relative risk (control)[d]	–	Infinite	Infinite	Infinite
Lower limit	–	1.309	3.478	4.223
Upper limit	–	Infinite	Infinite	Infinite
Weeks to first observed tumour	–	87	74	64

Table 20 (continued)

	Control	7 mg/kg feed (equivalent to 350 µg/kg bw)	15 mg/kg feed (equivalent to 750 µg/kg bw)	30 mg/kg feed (equivalent to 1500 µg/kg bw)
Background incidence from HCD	12/2320 (0.5% ± 1.1% SD)	–	–	–

HCD, historical control data; NS, not significant; NOS, not otherwise specified

[a] Treated groups received doses of 0 (control), 7 (low dose), 15 (mid-dose), or 30 (high dose) mg/kg feed.

[b] Number of tumour-bearing animals/number of animals necropsied (percentage).

[c] Beneath the incidence of tumours in the control group is the probability level for the Cochran–Armitage test when $P < 0.05$; otherwise, NS is indicated. Beneath the incidence of tumours in a treated group is the probability level for the Fisher exact test for the comparison of that treated group with the control group when $P < 0.05$; otherwise, NS is indicated.

[d] The 95% confidence interval of the relative risk between each treated group and the control group.

Table 21

Analyses of the incidence of primary tumours in female rats fed lasiocarpine in the diet[a]

	Control	7 mg/kg feed (equivalent to 350 µg/kg bw)	15 mg/kg feed (equivalent to 750 µg/kg bw)	30 mg/kg feed (equivalent to 1500 µg/kg bw)
Lung				
Angiosarcoma[b]	0/24 (0)	0/24 (0)	2/24 (8)	0/24 (0)
P-values[c]	NS	NS	NS	NS
Relative risk (control)[d]	–	–	Infinite	–
Lower limit	–	–	0.305	–
Upper limit	–	–	Infinite	–
Weeks to first observed tumour	–	–	68	–
Haematopoietic system				
Malignant lymphoma, NOS[b]	1/24 (4)	2/24 (8)	3/24 (13)	0/24 (0)
P-values[c]	NS	NS	NS	NS
Relative risk (control)[d]	–	2.000	3.000	0.000
Lower limit	–	0.111	0.265	0.000
Upper limit	–	112.815	150.246	18.289
Weeks to first observed tumour	104	70	39	–
Leukaemia[b]	1/24 (4)	7/24 (29)	8/24 (33)	1/24 (4)
P-values[c]	NS	$P = 0.024$	$P = 0.013$	NS
Relative risk (control)[d]	–	7.000	8.000	1.000
Lower limit	–	1.010	1.212	0.013
Upper limit	–	297.414	333.388	75.218
Weeks to first observed tumour	95	72	63	62
Lymphoma or leukaemia[b]	2/24 (8)	9/24 (38)	11/24 (46)	1/24 (4)
P-values[c]	NS	$P = 0.018$	$P = 0.004$	NS
Relative risk (control)[d]	–	4.500	5.500	0.500
Lower limit	–	1.072	1.395	0.009
Upper limit	–	38.367	45.074	8.943
Weeks to first observed tumour	95	70	39	62
Background incidence from HCD	448/2370 (18.9 ± 7.0 SD)	–	–	–

	Control	7 mg/kg feed (equivalent to 350 µg/kg bw)	15 mg/kg feed (equivalent to 750 µg/kg bw)	30 mg/kg feed (equivalent to 1500 µg/kg bw)
Liver				
Hepatocellular carcinoma[b]	0/24 (0)	0/24 (0)	0/24 (0)	1/24 (5)
P-values[c]	NS	NS	NS	NS
Relative risk (control)[d]	–	–	–	Infinite
Lower limit	–	–	–	0.055
Upper limit	–	–	–	Infinite
Weeks to first observed tumour	–	–	–	63
Hepatocellular carcinoma or adenoma, NOS[b]	0/24 (0)	5/24 (21)	1/24 (4)	7/24 (29)
P-values[c]	$P = 0.013$	$P = 0.025$	NS	$P = 0.005$
Relative risk (control)[d]	–	Infinite	Infinite	Infinite
Lower limit	–	1.309	0.055	2.021
Upper limit	–	Infinite	Infinite	Infinite
Weeks to first observed tumour	–	104	82	52
Background incidence from HCD	74/2356 (3.1% ± 3.2% SD)	–	–	–
Angiosarcoma[b]	0/24 (0)	8/24 (33)	7/24 (29)	2/24 (8)
P-values[c]	NS	$P = 0.002$	$P = 0.005$	NS
Relative risk (control)[d]	–	Infinite	Infinite	Infinite
Lower limit	–	2.382	2.021	0.305
Upper limit	–	Infinite	Infinite	Infinite
Weeks to first observed tumour	–	84	68	56
Background incidence from HCD	3/2370 (0.1% ± 0.5% SD)	–	–	–

HCD, historical control data; NS, not significant; NOS, not otherwise specified

[a] Treated groups received doses of 0 (matched control), 7 (low dose), 15 (mid-dose) or 30 (high dose) mg/kg feed.

[b] Number of tumour-bearing animals/number of animals necropsied (percentage).

[c] Beneath the incidence of tumours in the control group is the probability level for the Cochran–Armitage test when $P < 0.05$; otherwise, NS is indicated. Beneath the incidence of tumours in a treated group is the probability level for the Fisher exact test for the comparison of that treated group with the control group when $P < 0.05$; otherwise, NS is indicated.

[d] The 95% confidence interval of the relative risk between each treated group and the control group.

tumours in the males were significantly increased only in the males in the mid-dose group (11/24 versus 4/24 in controls, and exceeded the historical ranges in controls (699/2320)). This observation and the earlier appearance of leukaemias and lymphomas in the males in the high-dose group may indicate that also in males the development of these tumours might be enhanced by exposure to lasiocarpine.

The high and middle doses of lasiocarpine used in this bioassay were toxic, as shown by the decrease in body weights and in survival.

NCI (1978) concluded that "under the conditions of this bioassay, lasiocarpine was carcinogenic in Fischer 344 rats producing hepatocellular tumors and angiosarcomas of the liver in both sexes and hematopoietic tumours in female animals". The study authors did not derive either a NOAEL or a BMD (NCI, 1978).

The Committee noted that the animal groups were rather small (24/sex/dose). The high rate of intercurrent death, especially at the high dose, may have interfered with the development of relatively slow-growing tumours, resulting in an underestimation of development of such tumours. It was also noted that compared to current practice, the histological examination was rather limited (no full examination of all tissues, from all animals from all dose groups; only selected tissues). The Committee thus considered the study only as supportive.

2.2.4 Genotoxicity

IPCS (1988) and EFSA (2011) reviewed the genotoxicity of PAs. IPCS (1988) concluded that several PAs, PA-derivatives and related compounds have been shown to produce chromosome aberrations in plants and several cell culture systems. Heliotrine and lasiocarpine showed chromosome damaging properties in an in vivo micronucleus test. Genotoxicity of heliotrine, monocrotaline, seneciphylline and senkirkine was shown in the sister-chromatid exchange assay in V79 Chinese hamster cells, and several PAs showed induction of DNA repair in rodent hepatocytes. For dehydroretronecine, senkirkine, tussilagine and heliotrine it was shown that chromosomal aberrations occurred in human cell lines. Limited information was available on chromosome damage by PAs in humans. The most important study reviewed by IPCS described chromosome damage in the blood cells of children with veno-occlusive disease, probably caused by fulvine (IPCS, 1988).

EFSA (2011) concluded that the liver is the primary site for genotoxicity of 1,2-unsaturated PAs. The 1,2-unsaturated PAs from different structural classes (i.e. retronecine, heliotridine, and otonecine; diesters and monoesters) undergo metabolic activation to reactive pyrrolic intermediates and form a common set of DHP adducts at dG and dA sites in rat liver DNA. These findings suggest that a genotoxic carcinogenic mechanism is applicable for all 1,2-unsaturated-PA esters and their *N*-oxides, which can be metabolically converted into PAs. The concomitant induction of mutations compatible with DHP adduct formation in liver cells in transgenic rats, and the formation of haemangiosarcomas and hepatomas in riddelliine (retronecine-type PA)-treated male and female rats and mice, provide strong evidence for a genotoxic mechanism for hepatocarcinogenicity. In contrast to 1,2-unsaturated PAs, 1,2-saturated PAs do not undergo metabolic activation to reactive pyrrolic species responsible for hepatotoxicity and genotoxicity (EFSA, 2011).

The most relevant in vitro and in vivo studies summarized in IPCS (1988) and EFSA (2011; largely based on Chen, Mei & Fu, 2010) are included in Table 24 and Table 25. Studies in *Drosophila* were excluded (see also Table 2 in section

1.2 of the Explanation), since this less commonly used test method has limited validation.

(a) In vitro and in vivo genotoxicity studies

The genotoxicity of PAs and PA-containing preparations has been extensively studied in a variety of in vitro and in vivo assays. The individual results of in vitro and in vivo studies with PAs are shown in Table 22 and Table 23, respectively.

Detailed information about DNA adduct formation is described in the next section (Covalent binding to nucleic acids and/or proteins).

(b) Covalent binding to nucleic acids and/or proteins

WHO-IPCS (1988) noted several studies showing that a number of PAs are dose-dependent mutagens. These studies used *Drosophila melanogaster* and *Salmonella typhimurium* TA100 as model systems; however, no data describing covalent bonding of PAs to nucleic acids and/or proteins were discussed. In their opinion of 2011, EFSA described the formation of defined DHP adducts at dG and dA sites in the liver DNA in rats as part of the mode of action of PAs. Next to nucleoside adduct formation, binding to DNA of DHP or the pyrrolic esters may lead to DNA cross-linking and DNA-protein cross-linking. EFSA also mentioned that the same set of DNA adducts are formed in human and rat microsomes, with humans showing similar levels of adduct formation to the rat or two- to threefold higher levels. In addition, the measurement of pyrrolic species covalently bound to hepatic and serum proteins was mentioned as a potential biomarker for further investigation.

(i) In vitro

Binding to DNA was found when monocrotaline pyrrole was incubated with bovine pulmonary arterial endothelial cells that progressively increased with increasing incubation time (Thomas et al., 1996).

DHP–DNA adducts (DHP-3′-dGMP and DHP-derived dinucleotides) have been detected following incubation of monocrotaline with rat liver microsomes and calf thymus DNA (Wang YP et al., 2005a). The same eight DHP-derived DNA adducts were detected when rat liver microsomes and calf thymus DNA were incubated with riddelliine (Xia et al., 2003), riddelliine *N*-oxide, monocrotaline *N*-oxide and retrorsine *N*-oxide (Wang YP, Fu & Chu, 2005), clivorine (Xia et al., 2004), riddelliine (Yang et al., 2001a,b), heliotrine, riddelliine, riddelliine *N*-oxide, retrorsine, retrorsine *N*-oxide, monocrotaline (Xia et al., 2008), lasiocarpine (Xia et al., 2006) and riddelliine *N*-oxide (Chou et al., 2003a). Also incubations with human liver microsomes showed formation of the same eight DHP-derived DNA adducts (Wang YP, Fu & Chou, 2005; Xia et

Table 22
In vitro genotoxicity studies

Substance (purity)	Test system	End-point	Concentration	Results without metabolic activation	Results with metabolic activation	Reference
7-Angelyl retronecine (88%)	*Salmonella typhimurium* TA100	Reverse mutation	0, 0.25, 2.5, 25, 250 and 2000 µg/plate, +S9	NT	Negative[b]	Rubiolo et al. (1992)
Monocrotaline (99%)	*S. typhimurium* TA100	Reverse mutation	0, 250, 1000 or 2000 µg/plate, + S9	NT	Negative[b]	Rubiolo et al. (1992)
Retrorsine[a]	*S. typhimurium* TA98 and TA100	Reverse mutation	0, 0.4, 0.7, 1.3, 4 or 6.6 mg/plate, ± S9	Positive[b] (TA100)	Positive[b] (TA100)	Bovee et al. (2015)
Retrorsine (97% pure)	*S. typhimurium* TA97a, TA98, TA100, TA1535 and TA1538	Reverse mutation, + S9	0, 0.25, 2.5, 25, 250 and 2000 µg/plate	NT	Slightly positive[b]	Rubiolo et al. (1992)
Retrorsine *N*-oxide (97%)	*S. typhimurium* TA100	Reverse mutation	0, 250, 1000 and 2000 µg/plate	NT	Negative[b]	Rubiolo et al. (1992)
Retrorsine *N*-oxide	*S. typhimurium* TA98 and TA100	Reverse mutation	0, 0.4, 0.7, 1.3, 4 or 6.6 mg/plate, ± S9	Positive[b] (TA100)	Positive[b] (TA100)	Bovee et al. (2015)
Riddelliine (>92%)	*S. typhimurium* TA97, TA98, TA100 and TA1535	Reverse mutation	0–5000 µg/plate	Negative[b] (TA100 not tested)	Positive[b] (TA100)	Chan P (1993)
Senecionine[a]	*S. typhimurium* TA98	Reverse mutation	0, 0.05, 0.3, 0.5, 3 or 5 mg/plate, ± S9	Negative[b]	Negative[b]	Bovee et al. (2015)
Senecionine (90%, 6% seneciphylline, 1% integerrimine)	*S. typhimurium* TA100	Reverse mutation	0.25, 2.5, 25, 250 or 2000 µg/plate, +S9	NT	Negative[b]	Rubiolo et al. (1992)
Seneciphylline (83%)	*S. typhimurium* TA100	Reverse mutation	0, 0.25, 2.5, 25, 250 or 2000 µg/plate, + S9	NT	Negative[b]	Rubiolo et al. (1992)
Senecivernine (83%, 11% integerrimine, 3% senecionine)	*S. typhimurium* TA100	Reverse mutation	0–2000 µg/plate	NT	Negative[b]	Rubiolo et al. (1992)
Senkirkine[a]	*S. typhimurium* TA98 and TA100	Reverse mutation	0.05, 0.03, 0.3, 3 and 5 mg/plate, ± S9 (TA98), + S9 (TA100)	Negative[b]	Positive[b]	Bovee et al. (2015)
Monocrotaline (99%)	*Escherichia coli* PQ37	SOS chromotest[e]	0, 1.84, 3.68, 7.35, 14.7, 29.4, 58.8 and 117.6 µg, ± S9	Positive[c]	Positive[c]	Kevekordes et al. (1999)
Retrorsine (97%)	*E. coli* PQ37	SOS chromotest[e]	0, 2.38, 4.77, 9.55, 19.1, 38.2, 76.4, 152.8 µg, ± S9	Positive[c]	Positive[c]	Kevekordes et al. (1999)

Substance (purity)	Test system	End-point	Concentration	Results without metabolic activation	Results with metabolic activation	Reference
Retrorsine *N*-oxide (97%)	*E. coli* PQ37	SOS chromotest[e]	0, 0.03, 0.06, 0.12, 0.24, 0.48, 0.96, 1.91, 3.83, 7.65 and 15.3 µg/plate, ± S9	Negative[c]	Negative[c]	Kevekordes et al. (1999)
Senecionine (>98%)	*E. coli* PQ37	SOS chromotest[e]	0, 0.39, 0.78, 1.65, 3.13, 6.25, 12.5, 25, 50 µg	Negative[c]	Negative[c]	Kevekordes et al. (1999)
Senkirkine (98.5%)	*E. coli* PQ37	SOS chromotest	0, 0.62, 1.25, 2.5, 5.0, 10, 20, 40 and 80 µg, ± S9	Negative[c]	Negative[c]	Kevekordes et al. (1999)
Monocrotaline[a]	Mouse lymphoma L5178Y TK$^{+/-}$ cells	Forward mutation	NR, ± S9	Positive	Positive	Honma et al. (1999)
Monocrotaline[a]	TK6 human lymphoblastoid cells	Gene mutation (TK locus)	312.5–5000 µg/ml	Positive	NT	Honma & Hayashi (2011)
Monocrotaline[a]	WTK-1 human lymphoblastoid cells	Gene mutation (TK locus)	312.5–5000 µg/ml	Positive	NT	Honma & Hayashi (2011)
Monocrotaline[a]	Mouse lymphoma L5178Y TK$^{+/-}$ cells	Forward mutation	0–4000 µg/ml, ± S9	Positive	Positive	Sofuni et al. (1996)
Retronecine (>99%)	V79 cells	Forward mutation (HGPRT locus)	1.0, 10.0, 100 µM, 1.0, 10.0 mM (–S9), 1.0, 10.0 or 100 µM (+S9) 1.0, 10, 100 µM or 1 mM (+ hepatocytes)	Negative[d]	Negative[d]	Berry et al. (1996)
Retronecine *N*-oxide (>99%)	V79 cells	Forward mutation (HGPRT locus)	0.3, 1.0, 10.0 or 30.0 µM (+ hepatocytes)	NT	Negative[d]	Berry et al. (1996)
Riddelliine (>99%)	V79 cells	Forward mutation (HGPRT locus)	0.5, 5.0, 10.5 or 50 µM (+ hepatocytes)	NT	Positive[d]	Berry et al. (1996)
Riddelliine *N*-oxide (>99%)	V79 cells	Forward mutation (HGPRT locus)	1.0, 10.0, 100.0 µM, 1 mM (–S9) 5.0, 50.0, 100.0 or 500.0 µM (+S9) 0.3, 1.0, 3.0, 10.0, 50.0 µM (+ hepatocytes)	Negative[d]	Negative[d]	Berry et al. (1996)
Senecionine (>99%)	V79 cells	Forward mutation (HGPRT locus)	50 µM (–S9) 5.0, 50.0 or 500 µM (+S9) 0, 0.5, 5.0, 10.5 or 50 µM (+ hepatocytes)	Positive[d]	Positive[d]	Berry et al. (1996)
Seneciphylline (95%)	V79 cells	Forward mutation (HGPRT locus)	0.06, 0.2, 0.6, 2.0, 6.3 or 10.5 µM (+ hepatocytes)	NT	Positive[d]	Berry et al. (1996)
Monocrotaline[a]	V79 cells	Chromosomal aberration	3.16, 10, 31.6, 100, 316 and 1000 µM (± S9, ± rat hepatocytes)	Negative	Positive	Müller, Kasper & Kaufmann (1992)

Table 22 (continued)

Substance (purity)	Test system	End-point	Concentration	Results without metabolic activation	Results with metabolic activation	Reference
Retrorsine[a]	V79 cells	Chromosomal aberration	3.16, 10, 31.6, 100, 316 and 1000 µM (± S9, ± rat hepatocytes)	Positive	Positive	Müller, Kasper & Kaufmann (1992)
Retrorsine *N*-oxide[a]	V79 cells	Chromosomal aberration	3.16, 10, 31.6, 100, 316 and 1000 µM (± S9, ± hepatocytes)	Positive	Positive (+ hepatocytes only)	Müller, Kasper & Kaufmann (1992)
Riddelliine (>92%)	Chinese hamster ovary (CHO) cells	Chromosomal aberration	0–600 µg/mL	Negative	Positive	Chan P (1993)
Riddelliine (>92%)	CHO cells	Sister-chromatid exchange	0, 30, 100, 300 (−S9); 0, 3, 10, 30, 100 (+S9)	Positive	Positive	Chan P (1993)
Monocrotaline[a]	TK6 human lymphoblastoid cells	Micronucleus induction	312.5–5000 µg/ml	Positive	NT	Honma & Hayashi (2011)
Monocrotaline[a]	WTK-1 human lymphoblastoid cells	Micronucleus induction	312.5–5000 µg/ml	Positive	NT	Honma & Hayashi (2011)
Monocrotaline (99%)	Human lymphocytes	Micronucleus induction	0, 1, 10, 50, 150, 300 or 600 µM	Negative	Positive	Kevekordes et al. (2001)
Monocrotaline (99%)	HepG2 cells	Micronucleus induction	0, 1, 10, 50, 150, 300 or 600 µM	Positive	NT	Kevekordes et al. (2001)
Monocrotaline[a]	Rat hepatocytes	Micronucleus induction	0–10−5 mol/L (no exact concentrations reported)	Positive	NT	Müller-Tegethoff, Kasper & Müller (1995)
Retrorsine (97%)	Human lymphocytes	Micronucleus induction	0, 1, 5, 10, 50, 100 or 150 µM	Negative	Positive	Kevekordes et al. (2001)
Retrorsine (97%)	HepG2 cells	Micronucleus induction	0, 1, 5, 10, 50, 100 or 150 µM	Positive	NT	Kevekordes et al. (2001)
Retrorsine[a]	Rat hepatocytes	Micronucleus induction	0–10^{-5} mol/L (no exact concentrations reported)	Positive	NT	Müller, Kasper & Müller (1993)
Retrorsine *N*-oxide (97%)	Human lymphocytes	Micronucleus induction	0, 10, 50, 100, 250, 500 or 750 µM, ± S9	Negative	Negative	Kevekordes et al. (2001)

Substance (purity)	Test system	End-point	Concentration	Results without metabolic activation	Results with metabolic activation	Reference
Retrorsine *N*-oxide (97%)	HepG2 cells	Micronucleus induction	0, 10, 50, 100, 250, 500 or 750 µM	Negative	NT	Kevekordes et al. (2001)
Retrorsine *N*-oxide[a]	Rat hepatocytes	Micronucleus induction	0–10–3 M (no exact concentrations reported)	Positive	NT	Müller-Tegethoff et al. (1997)
Retrorsine *N*-oxide[a]	Hep G2 cells	DNA strand breaks (comet assay)	0, 500, 1000, 3000 or 9000 µM	Positive	NT	Uhl, Helma & Knasmüller (2000)
Monocrotaline (>99%)	Rat hepatocytes	Unscheduled DNA synthesis	1.0, 5.0 or 10.0 µM	Positive	NT	Berry et al. (1996)
Retronecine (>99%)	Rat hepatocytes	Unscheduled DNA synthesis	3.0 µM	Negative	NT	Berry et al. (1996)
Riddelliine (>99%)	Rat hepatocytes	Unscheduled DNA synthesis	0.2, 0.5, 1.0 or 5.0 µM	Positive	NT	Berry et al. (1996)
Riddelliine *N*-oxide (>99%)	Rat hepatocytes	Unscheduled DNA synthesis	10.0 mM	Positive	NT	Berry et al. (1996)
Senecionine (>99%)	Rat hepatocytes	Unscheduled DNA synthesis	0.2, 0.5, 1.0 or 5.0 µM	Positive	NT	Berry et al. (1996)
Seneciphylline (95%)	Rat hepatocytes	Unscheduled DNA synthesis	0.2, 0.5, 1.0 or 5.0 µM	Positive	NT	Berry et al. (1996)

NR, not reported; NT, not tested

[a] Purity not reported.

[b] Preincubation method (30 min, 37 °C) (in Chan P, 1993, 20 min, 37 °C).

[c] Results are expressed as an induction factor (IF). The study authors consider an IF <1.5 non-genotoxic, an IF between 1.5 and 2 marginally genotoxic and an IF >2 genotoxic. IFs >1.5 are marked as positive results in this table.

[d] Exposure times were 48 hours without activation, 23 hours with S9 activation and 45.4 hours with the hepatocyte-mediated assay.

[e] This test measures the increased expression of specific stress genes ("SOS genes", specifically sfiA, umuC, recA) as a consequence of DNA damage or an inhibition of DNA synthesis.

Table 23
In vivo genotoxicity studies

Substance (purity)	Test system	End-point	Dose	Results	Reference
Riddelliine (>97%)	Big Blue Fisher 344 transgenic rats, F	Gene mutation assay	0, 0.1, 0.3 or 1.0 mg/kg bw, 5 days/week for 12 weeks (gavage)	Positive	Mei et al. (2004a)
Riddelliine (>97%)	Big Blue Fisher 344 transgenic rats, F	Gene mutation assay	0 or 0.3 mg/kg bw per day, 5 days/week for 12 weeks (gavage)	Positive	Mei et al. (2004b)
Integerrimine[a]	C57B1/6 mice, M + F	Chromosome aberration	0, 18.75 and 37.50 mg/kg bw (single dose, intraperitoneal)	Positive[b]	Gimmler-Luz, Erdtmann & Balbueno (1990)
Heliotrine[a]	Swiss albino mice, F and fetuses	Micronucleus induction	225 and 300 mg/kg bw (single maternal dose, intraperitoneal)	Positive[c]	Sanderson & Clark (1993)
Heliotrine[a]	Fetuses of Swiss-Webster mice	Micronucleus induction	0, 0.3, 0.6 and 0.9 mmol/kg (single maternal dose, intraperitoneal)	Positive[d]	MacGregor et al. (1989)
Integerrimine[a]	BALB/c mice, M + F	Micronucleus induction	0, 18.75 or 37.5 mg/kg bw (single dose, intraperitoneal)	Positive[e]	Gimmler-Luz & Erdtmann (1997)
Lasiocarpine[a]	Fetuses of Swiss-Webster mice	Micronucleus induction	0, 0.03, 0.1 mmol/kg (single maternal dose, intraperitoneal)	Positive[f]	MacGregor et al. (1989)
Monocrotaline (>98%)	Sprague-Dawley rats, M	Micronucleus induction	2d: 0, 15, 30 or 60 mg/kg bw per day 14d: 0, 0.5 or 1.5 mg/kg bw per day (oral gavage) 28d: 0, 0.15, 0.5 or 1.5 mg/kg bw per day (oral gavage)	Positive[g] Positive[h] Positive[i]	Takashima et al. (2015)
Monocrotaline[a]	Sprague-Dawley rats, M	Micronucleus induction	0, 30, 60 or 120 mg/kg bw per day (single dose, oral gavage)	Positive[j]	Torous et al. (2003)
Monocrotaline (99%)	Sprague-Dawley rats, M + F	Micronucleus induction	0, 30, 60 or 120 mg/kg bw (single dose, gastric intubation)	Positive[k]	Proudlock, Statham & Howard (1997)
Monocrotaline[a]	Swiss albino mice, F and fetuses	Micronucleus induction	125 mg/kg bw (single maternal dose, intraperitoneal)	Positive[l]	Sanderson & Clark (1993)
Monocrotaline[a]	Swiss-Webster mice, M	Micronucleus induction	Diet containing 0, 1.5 or 3.0% ground seeds of *Crotalaria spectabilis* or equimolar amounts (0, 0.063 or 0.126%) monocrotaline (equal to 0, 135 and 270 mg/kg bw per day, respectively)	Positive[m]	MacGregor et al. (1990)
Monocrotaline[a]	Swiss albino mice, F and fetuses	Micronucleus induction	0, 0.3, 0.6 mmol/kg (single maternal dose, intraperitoneal)	Negative[n]	MacGregor et al. (1989)
Riddelliine[a]	B6C3F_1 mice, M + F	Micronucleus induction	0, 3.3, 10 or 25 mg/kg bw per day for 30 and 90 days (gavage)	Negative[o]	Witt et al. (2000)

Substance (purity)	Test system	End-point	Dose	Results	Reference
Riddelliine (92%, 5% retrorsine, 1.4% seneciphylline)	B6C3F$_1$ mice, M	Micronucleus induction	0, 75, 150 or 300 mg/kg (single dose, gavage)	Positive[p]	Chan PC et al. (1994)
Riddelliine[q] (92%, 5% retrorsine, 1.4% seneciphylline)	B6C3F$_1$ mice, M + F	Micronucleus induction	0, 3.3, 10.0 or 25.0 mg/kg bw per day for 5 days or for 29 days (gavage)	Negative[r]	Mirsalis et al. (1993) also reported in Chan PC et al. (1994)
Riddelliine[q] (92%, 5% retrorsine, 1.4% seneciphylline)	F344 rats, M + F	Micronucleus induction	0, 0.3, 1.0 and 3.3 mg/kg bw per day for 34 days (gavage)	Negative[r]	Mirsalis et al. (1993) also reported in Chan PC et al. (1994)
Riddelliine[q] (92%, 5% retrorsine, 1.4% seneciphylline)	B6C3F$_1$ mice, M + F	Unscheduled DNA synthesis and S-phase synthesis	0, 3.3, 10.0 or 25.0 mg/kg bw per day for 5 days or for 29 days (gavage)	Positive[s]	Mirsalis et al. (1993), also reported in Chan PC et al. (1994)
Riddelliine[q] (92%, 5% retrorsine, 1.4% seneciphylline)	F344 rats, M + F	Unscheduled DNA synthesis and S-phase synthesis	0, 0.3, 1.0 and 3.3 mg/kg bw per day for 5 days or for 34 days (gavage)	Negative[t]	Mirsalis et al. (1993), also reported in Chan PC et al. (1994)

M, male; F, female

[a] Purity not reported.

[b] Bone marrow cells were collected 6, 12 and 24 hours after treatment.

[c] Intraperitoneal injection between days 17 and 19 of gestation. Maternal bone marrow cells and fetal liver cells were collected 21 hours after treatment. Dose levels were reported to be equal to, respectively, 0.75 LD_{50} and LD_{50} of the test compound. Positive results were observed in both maternal and fetal tissues.

[d] Intraperitoneal injection between days 15 and 16 of gestation. Two fetuses per animal were collected 30 or 48 hours after dosing. Blood smears were prepared from each fetus. At the highest dose level, micronuclei were not scored in fetuses collected 48 hours after dosing due to suppression of the red blood cells.

[e] Mice were killed and bone marrow and peripheral blood samples were collected 24, 48 and 72 hours after treatment. Other mice were killed 2, 4, 7 and 21 days after treatment and peripheral blood samples were collected.

[f] Intraperitoneal injection between days 15 and 16 of gestation. Two fetuses per animal were collected 30 or 48 hours after dosing. Blood smears were prepared from each fetus. Small, statistically significant increases in micronucleated polychromatic and normochromatic red blood cells were observed; however, these were not consistently observed in males and females and at different time points.

[g] Both juvenile (26-day-old) and young adult (7-week-old) rats were used. Peripheral blood and liver were sampled at 48 hours and 4 days after the second dosing, respectively. Negative results were obtained in livers of 7-week-old rats and positive results were obtained in livers of juvenile rats and in peripheral blood of rats from both age groups.

[h] 7-Week-old rats were used. Hepatocytes and femur bone marrow cells were collected 24 hours after dosing. Positive results were obtained in hepatocytes, and negative results in bone marrow cells.

[i] 7-Week-old rats were used. Rats were initially treated with 3.75, 7.5 and 15 mg/kg bw per day, but some of the rats treated with 7.5 and 15 mg/kg bw per day died. Therefore the assay was repeated at lower dose levels. Hepatocytes and femur bone marrow cells were collected 24 hours after dosing. Positive results were obtained in hepatocytes and negative results in bone marrow cells.

[j] Peripheral blood was sampled 48 hours after treatment.

[k] Peripheral blood and bone marrow cells were collected 24 hours and 48 hours after treatment.

[l] Intraperitoneal injections between days 17 and 19 of gestation. Maternal bone marrow cells and fetal liver cells were collected 21 hours after treatment. The dose level was reported to be equal to 0.75 LD_{50} of monocrotaline. Positive results were observed in both maternal and fetal tissues.

[m] Peripheral blood was sampled on days 2, 3 and 6 after the beginning of the exposure.

[n] Intraperitoneal injection between days 15 and 16 of gestation. Two fetuses per animal were collected 30 or 48 hours after dosing. Blood smears were prepared from each fetus.

[o] Peripheral blood was collected from all animals.

[p] Bone marrow smears were prepared 24 hours after dosing and peripheral blood smears were prepared 48 hours after treatment.

[q] Not reported in Mirsalis et al. (1993) but specified in Chan PC et al. (1994).

[r] Femoral bone marrow cells were collected 24 hours after treatment. Animals were dosed daily, excluding weekends. During the final week of dosing, animals were dosed daily.

Table 23 (continued)

[s] Hepatocytes were collected 24 hours after treatment. Animals were dosed daily, excluding weekends. During the final week of dosing, animals were dosed daily. The unscheduled DNA synthesis (UDS) assay gave equivocal results in males in both the 5 and 30 hours treatment groups. The UDS assay gave a modest positive response in females after 30 hours of dosing. S-phase synthesis (SPS) was significantly elevated in males and females after 30 days of dosing, but not after 5 days. At higher doses, a depression in SPS was observed, which may have been a result of toxicity.

[t] Hepatocytes were collected 24 hours after treatment. Animals were dosed daily, excluding weekends. During the final week of dosing, animals were dosed daily. Results of the UDS assay were negative. SPS was significantly elevated in males and females at both 5 and 30 days of dosing. At higher doses, a depression in SPS was observed, which may have been a result of toxicity.

Table 24

Summary of results of the most frequently used in vitro genotoxicity tests with different PAs

Pyrrolizidine alkaloid	DNA adducts	Bacterial mutations	Mammalian cell mutations	Chromosomal aberrations	Micronucleus induction	Unscheduled DNA synthesis
Acetylpetasitenine (acetylfukinotoxin)						+[a,b]
7-Angelylretronecine		–[c]				
Clivorine	+[c,d]	+[a,d]				+[a,b]
Dihydroclivorine						+[a]
Dehydroretronecine		+[d]				
Epoxyseneciphylline						+[a]
Heliotrine	+[c,d]	+/–[d]		+[d]		
Indicine *N*-oxide	+[c]					
Jacobine						+[a]
Lasiocarpine	+[c,d]	+[b,d]		+[d]		+[a,b]
Ligularidine		+[a,d]				+[a]
Ligularinine[e]						–[a]
Ligularizine						+[a]
Lycopsamine		–[d]				
LX-201		+[d]				+[a,b]
Monocrotaline	+[c,d]	–[a,b,c,d]	+[c]	+[c,d]	+[c,d]	+[a,b,c]
Monocrotaline *N*-oxide	+[c]					
Neoligularidine						+[a]
Petasitenine (fukinotoxin)		+[a,b,d]		+[d]		+[a,b]
Platyphylline	–[c]					
Retronecine			–[c]			–[a,c]
Retronecine *N*-oxide		–	–[c]	+[c]		
Retrorsine	+[c,d]	+[c,d]		+[c,d]	+[c,d]	
Retrorsine *N*-oxide	+[c]	+/–[c,d]		+/–[d]	+/–[c,d]	
Riddelliine	+[c,d]	+/–[c,d]	+[c]	+[c,d]		+[a,c]
Riddelliine *N*-oxide	+[c]		–[c]			+[a,c]
Senecicannabine						+[a]
Senecionine		–[a,c,d]	+[c]			+[a,c]
Seneciphylline		+/–[a,c,d]	+[c]			+[a,c]
Senecivernine		+/–[c,d]				
Senkirkine		+/–[a,b,c,d]		+[d]		+[a,b]
Syneilesine						+[a]

[a] As reported in Mori et al. (1985).
[b] As reported in Williams & Mori (1980).
[c] As reported in Table 22 and the section on "Covalent binding to nucleic acids and/or proteins" in section 2.2.4 Genotoxicity.
[d] As reported in EFSA (2011).
[e] 1,2-saturated PA.

Table 25
Summary of results of in vivo genotoxicity testing with different PAs

Pyrrolizidine alkaloid	DNA adducts	Unscheduled DNA synthesis	Micronuclei	Chromosome aberration	Mutations in rodents
Clivorine	+[a]				
Fulvine				+[b]	
Heliotridine	+[a]				
Heliotrine	+[a]		+[a,b]		
Integerrimine			+[a,b]	+[a,b]	
Lasiocarpine	+[a]		+[a]		
Lycopsamine	+[a]				
Monocrotaline	+[a,b]		+[a,b]		
Platyphylline	–[a]				
Retrorsine	+[a,b]	+[b]			
Retrorsine *N*-oxide	+[a]				
Retronecine	–[a]				
Riddelliine	+[a,b]	+/–[b]	+/–[a,b]		+[a,b]
Riddelliine *N*-oxide	+[a]				
Senecionine	+[b]	+[b]			
Seneciphylline	+[b]	+[b]			
Senkirkine	+[a]				

[a] As reported in Table 23 and the section on "Covalent binding to nucleic acids and/or proteins" in section 2.2.4 Genotoxicity.
[b] As reported in EFSA (2011).
Source: IPCS (1988); EFSA (2011) and studies identified for this evaluation.

al., 2003). Indicine *N*-oxide was found to bind to tubulin and DNA in human cell lines (Appadurai & Rathinasamy, 2014).

In human liver microsomes, pyrrole–protein adducts were detected when incubated with retronecine-type and otonecine-type PAs but not with the platynecine-type PA platyphylline. The greatest amounts were found for the heliotridine-type open-ring lasiocarpine and the retronecine-type 12-membered macrocyclic diesters senecionine, seneciphylline, integerrimine, riddelliine and retrorsine, followed by the 11-membered macrocyclic diester monocrotaline and the otonecine-type 12-membered macrocyclic diesters clivorine and senkirkine. Only very small amounts were found for the heliotridine-type monoesters heliotrine and lycopsamine. Overall, the amounts of pyrrole–protein adducts were lower than the amounts of pyrrole–GSH conjugate but the tendency to form pyrrole–GSH conjugate and pyrrole–protein adducts was correlated between the different PAs (Ruan et al., 2014a).

Dehydromonocrotaline, dehydroriddelliine and dehydroheliotrine reacted with valine to form four DHP-derived valine (DHP–valine) adducts but with different yields and patterns. The same DHP–valine adducts were formed in

vitro from reaction of dehydromonocrotaline with rat haemoglobin. In contrast, dehydroretronecine did not react with valine or haemoglobin to form adducts. Stability experiments in aqueous medium with the DHP–valine adducts showed that they are highly unstable, easily decompose and interconvert between DHP–valine epimers. Further, the amount of some of the DHP–valine adducts decreased gradually, and DHP was formed increasingly in a time-dependent manner. The authors concluded that these findings suggest that DHP–protein adducts may also dissociate into protein and DHP in vivo. However, this was not confirmed as no DHP–haemoglobin adducts (measured as DHP–valine-phenylisothiocyanate (PITC) products due to high instability of DHP–valine adducts) were detected in blood samples of rats dosed with riddelliine or monocrotaline. The authors suggested that this was either caused by the method used to detect the DHP–haemoglobin adducts or because the reactive metabolite does not reach the circulation (Zhao et al., 2014). The chemical characteristics of the four DHP–valine-PITC showed differences in polarity and stability (Jiang et al., 2015). Xia et al. (2015) showed adduct formation following incubation of 7-GS-DHP with dG or dA or calf thymus DNA. In contrast, incubation of 7,9-diGS-DHP did not produce DHP-dG or DHP-dA adducts. 7-GS-DHP is more stable than the dehydro-PAs. Incubation of 7-NAC-DHP with the nucleotides dG or dA generated four DHP-dG or DHP-dA adducts, respectively (He X et al., 2016a). In addition, DHP was formed. Reaction with calf thymus DNA led to the formation of the same DHP-dG and DHP-dA adducts. These results indicate that 7-NAC-DHP is another potential reactive conjugate. He X et al. (2016b) showed that 7-Cys-DHP is capable of reacting with DNA to form DNA adducts, as observed when 7-Cys-DHP was incubated with calf thymus DNA. The same observations were made when 7-GS-DHP and 7-Cys-DHP were incubated with HepG2 cells leading to the formation of DHP–DNA adducts identical to those observed in the in vivo situation. Further, it was noted that 7-GS-DHP was degraded to 7-Cys-DHP (He, Xia & Fu, 2017). From the studies on secondary pyrrolic metabolites, He et al. noted that 7-NAC-DHP is less water soluble and relatively more stable than 7-valine-DHP, 7-GS-DHP, 7-cysteine-DHP and 7,9-diGS-DHP, thereby increasing the possibility of its translocation out of the liver (He X et al., 2016a).

(ii) In vivo

Riddelliine, retrorsine, monocrotaline and riddelliine *N*-oxide have all been shown to form DNA adducts in mice and rats following various methods of administration (Chou et al., 2003a,b; Chou et al., 2004; Fu et al., 2010; Wang YP et al., 2005a; Wang YP, Fu & Chu, 2005; Yan J et al., 2002; Yang YC et al., 2001a,b). Chou et al. (2003a) found that the level of DNA adducts from riddelliine *N*-oxide was 2.6-fold less than the level formed after the same dose of riddelliine in female

rats after oral gavage with 1.0 mg/kg bw for three consecutive days. Chou et al. (2004) found that the peak DNA adduct levels after oral administration of 1.0 mg/kg bw riddelliine to rats five times a week for 2 weeks were 1.5- to 1.9-fold higher in the endothelial cells than in the parenchymal cells and were 2.1- to 3.6-fold more persistent. The same was observed in mice given 3.0 mg/kg bw riddelliine via oral gavage for the same duration, but the values were lower. DNA adducts were similar across species and sex. The peak DNA adduct levels were found 3 days after the final dose and were higher in rats than in mice. Yan J et al. (2002) studied the presence of DNA adducts in blood after orally treating male and female rats (3 per group) with a single dose of 10.0 mg/kg bw riddelliine, or female rats with 0.1 or 1.0 mg/kg bw for three consecutive days. Maximum DNA adduct levels were measured 48 hours after a single dose, and were 4-fold higher in female than in male rats between 48 and 168 hours post-dose. A positive trend between dose and DNA adduct levels in blood was observed. Comparison with DNA adducts obtained from the livers showed similar DNA adducts in blood and in liver, with higher levels in the liver.

Investigators associated with the US Food and Drug Administration National Center for Toxicology Research found a similar set of eight DNA adducts formed from retronecine-type PAs (retrorsine, riddelliine and monocrotaline) (Wang YP, Fu & Chu, 2005; Wang YP et al., 2005a; Yang YC et al., 2001b), riddelliine *N*-oxide (Chou et al., 2003a), heliotridine-type PAs (lasiocarpine, heliotridine) (Xia et al., 2006, 2008), and an otonecine-type PA (clivorine) (Xia et al., 2004). Investigators from the same group identified four of the adducts that were formed in rats administered riddelliine: DHP-dA-3 and DHP-dA-4 (a pair of epimers of 7-hydroxy-9-(deoxyadenosin-N6-yl) dehydrosupinidine), and DHP-dG-3 and DHP-dG-4 (a pair of epimers of 7-hydroxy-9-(deoxyguanosin-N2-yl) dehydrosupinidine) (Zhao et al., 2012). A dose-related increase in the number of DNA adducts was observed in livers of female rats orally gavaged (5 days per week) with 0 (vehicle), 0.01, 0.033, 0.1, 0.33 or 1.0 mg/kg bw per day riddelliine for 3 or 6 months, as part of the NTP Program. Higher levels of DNA adducts were found in the livers from rats treated for 6 months than in those treated for 3 months at the same dose level (Yang YC et al., 2001b). A positive dose–response trend was also found by Fu et al. (2010) who treated female rats with oral daily doses of 0, 0.1, 1, 2 or 5 mg/kg bw riddelliine or 1 or 5 mg/kg bw monocrotaline for three consecutive days. Analysis of samples showed that DHP-dG-3 and DHP-dG-4 were formed to a greater extent than DHP-dA-3 and DHP-dA-4. DHP-dG-1, DHP-dG-2, DHP-dA-1 and DHP-dA-2 were generally not detected. To further study DNA adduct formation of different PAs, female F344 rats (n = 4 per group) were orally gavaged for three consecutive days with 4.5 or 24 μmol/kg bw per day of one of 11 PAs including a saturated macrocyclic

diester (platyphylline[1]: 1.5 or 8.1 mg/kg bw per day), cyclic and open-ring retronecine- and heliotridine-type diesters (riddelliine: 1.6 or 8.4 mg/kg bw per day, riddelliine *N*-oxide: 1.6 or 8.8 mg/kg bw per day, retrorsine: 8.5 mg/kg bw per day, monocrotaline: 1.5 or 7.8 mg/kg bw per day, lasiocarpine: 1.9 or 9.9 mg/kg bw per day), retronecine- and heliotridine-type monoesters (lycopsamine: 1.4 or 7.2 mg/kg bw per day, heliotrine: 1.4 or 7.5 mg/kg bw per day), cyclic otonecine-type diesters (clivorine: 1.8 or 9.7 mg/kg bw per day, senkirkine: 1.6 or 8.8 mg/kg bw per day), or a non-esterified unsaturated necine base (retronecine: 0.7 or 3.7 mg/kg bw per day). DNA isolated from the livers of rats 24 hours after injection was analysed for DNA adducts with DHP-dG-3, DHP-dG-4, DHP-dA-3 and DHP-dA-4 adducts identified in the livers of all exposure groups except animals treated with the retronecine-type monoester lycopsamine. The animals treated with lycopsamine formed only a small amount of DHP-dG-4 and DHP-dA-3, and the other two adducts were not detected. Also, no DNA adducts were detected in control animals or those treated with the non-esterified necine base retronecine, or the saturated cyclic diester PA platyphylline, which lacks a double bond in the pyrrolidine ring (Xia et al., 2013).

In male ICR mice, PA-derived DNA adducts, obtained from the liver, were dose-dependently increased after oral administration of 10, 20, 40 or 60 mg/kg retrorsine. DNA adduct persistence was determined following a dose of 40 mg/kg bw. Groups of mice were euthanized at 2, 4, 6, 8 and 12 hours, 1, 2 and 3 days, and 1, 2, 4 and 8 weeks. Elimination was biphasic with half-lives of 9 and 301 hours after single dosing. After multiple dosing for 8 weeks, DNA adducts accumulated in the liver without reaching a steady-state. Elimination again followed a biphasic pattern, but with much longer half-lives (approximately 7.3 and 72.3 days) (Zhu et al., 2017).

These studies of DNA binding and repair are consistent with the report of Mori et al. (1985) on induction of DNA repair in cultured hepatocytes of rat, mouse and hamster.

Ruan et al. (2014a) compared the relative levels of protein adducts in the serum and livers of male ICR mice (n = 3 per group) 24 hours after a single intraperitoneal injection of 20 μmol/kg bw[2] for one of 12 different PAs. These PAs included five retronecine-type 12-membered macrocyclic diesters (retrorsine, riddelliine, senecionine, seneciphylline and integerrimine), one 11-membered macrocyclic diester (monocrotaline), one heliotridine-type open-ring diester (lasiocarpine), two monoesters (heliotrine and lycopsamine), two otonecine-type 12-membered macrocyclic diesters (clivorine and senkirkine), and the platynecine-type platyphylline. A similar pattern of relative adduct levels was

[1] Mixture of 70% platyphylline and 30% neoplatyphylline.

[2] Equivalent to doses ranging from ~6.0 mg/kg bw lycopsamine to 8.2 mg/kg bw lasiocarpine, the PAs with, respectively, the lowest and highest molecular weight in this study.

found in the serum and liver for individual PAs; however, the highest levels of adducts detected were from lasiocarpine in the blood and from integerrimine in the liver. The highest adduct levels in both liver and blood were found for the diester retronecine- and heliotridine-type PAs, followed by relatively lower levels of the 11-membered macrocyclic diesters and the otonecine-type PAs, and negligible but detectable levels for the monoester retronecine- and heliotridine-type PAs. No adducts were seen in animals administered platyphylline.

Li et al. (2016) treated male Kunming mice with 60, 120 or 180 mg/kg bw monocrotaline, administered intraperitoneally, to explore the lysine modification of hepatic proteins 10 hours after dosing. A dose-dependent formation of the protein adduct 7-Lys-DHP was observed.

Xia et al. (2016) investigated the formation of protein adducts in blood of rats treated with 0.1, 1, 2 or 5 mg/kg bw riddelliine via oral gavage for 5 or 30 consecutive days. This study revealed a significant dose- and time-dependent increase in DHP–protein adduct formation with both plasma and haemoglobin. DHP–diprotein adducts (protein–protein cross-links) were formed only up to 10% with the DHP–protein adduct predominating. They also studied the protein adduct formation in rats treated with PAs of different necine bases and esterification (for further details see description of the study by Xia et al., 2013 above). The authors concluded that the most extensive DHP–protein adduct formation occurs with the cyclic and open-ring retronecine- and heliotridine-type diester PAs and their *N*-oxides, followed by otonecine-type PAs containing an n-methyl substituted necine ring, followed by monoester PAs, followed by PAs that lack a double bond in the necine ring. In the same study, a moderate but statistically significant correlation between DHP–protein adducts and DHP–DNA adducts (from Xia et al., 2013) was reported based on a Pearson product moment analysis ($r = 0.633$, $P = 0.04$) (Xia et al., 2016).

Based on the available data for covalent binding for PAs in in vitro studies, riddelliine, a retronecine-type 12-membered macrocyclic diester, appeared to create a comparable amount of DNA adduct to the heliotridine-type open-ring diester lasiocarpine (Xia et al., 2006), but more DNA adducts than clivorine, an otonecine type (Xia et al., 2004) or monocrotaline, a retronecine-type with an 11-membered ring (Wang YP et al., 2005a). Xia et al. (2008) found that the retronecine-type 12-membered macrocyclic diesters riddelliine and retrorsine created more DNA adducts than monocrotaline, a retronecine-type with an 11-membered ring, which were similar to the retronecine-type 12-membered macrocyclic diester *N*-oxides retrorsine *N*-oxide and riddelliine *N*-oxide. They all created more DNA adducts than the heliotridine-type monoester heliotrine. In studies in mice, Ruan et al. (2014a) found the highest levels of adducts in both liver and blood for the diester retronecine- and heliotridine-type PAs, followed by relatively lower levels of the 11-membered macrocyclic diesters and

the otonecine-type PAs, with negligible but detectable levels for the monoester retronecine- and heliotridine-type PAs.

In studies that directly compared more than one type of PA, retronecine-type 12-membered macrocyclic diesters and heliotridine- and retronecine-type open rings tended to form the most adducts with DNA or proteins, followed by retronecine-type 11-membered rings and otonecine type, and then heliotridine- and retronecine-type monoesters. The platynecine-type had the lowest level of adduct formation. However, there were some variations between individual studies.

(c) Results of additional assays with end-points related to genotoxicity

In a DNA laddering assay, HepG2 cells were treated with 25, 50, 100 and 200 µg/mL monocrotaline and incubated for 48 hours. At a dose level of 50 µg/mL, early stages of apoptosis with mild DNA damage were observed. Severe DNA damage was observed at 100 and 200 µg/mL (Kusuma et al., 2014.).

Several studies describe the formation of DNA cross-links by PAs. In a study by Hincks et al. (1991) MDBK epithelial cells were incubated for 2 hours with eight PAs in concentrations ranging from 50 to 500 µM in the presence of S9. All PAs tested induced DNA cross-links, which consisted primarily of proteinase-sensitive cross-links (DPC) and – to a smaller extent – DNA interstrand cross-links (ISC). None of the PAs induced detectable amounts of DNA single strand breaks. The PAs tested ranked from most potent to least potent were: seneciphylline (DPC > ISC), riddelliine (DPC > ISC), retrorsine (DPC > ISC), senecionine (DPC > ISC), heliosupine (DPC > ISC), monocrotaline (ISC = DPC), latifoline (DPC > ISC) and retronecine (ISC > DPC). In a comparable study in MDBK cells by Kim et al. (1999), the PAs tested ranked from most potent to least potent were: dehydrosenecionine, dehydromonocrotaline, dehydroriddelliine, dehydroretronecine and indicine *N*-oxide. In several other studies, DNA cross-link formation by dehydromonocrotaline (Coulombe, Drew & Stermitz, 1999; Coulombe, Kim & Stermitz, 1994; Kim, Stermitz & Coulombe, 1995; Pereira et al., 1998; Rieben & Coulombe, 2004; Tepe & Williams, 1999; Tepe, Kosogof & Williams, 2002; Wagner, Petry & Roth, 1993; Weidner, Sigurdsson & Hopkins, 1990), dehydroretronecine (Coulombe, Kim & Stermitz, 1994; Reed et al., 1988; Weidner, Sigurdsson & Hopkins, 1990), dehydrosenecionine (Coulombe, Drew & Stermitz, 1999; Coulombe, Kim & Stermitz, 1994; Kim, Stermitz & Coulombe, 1995), dehydroriddelliine and dehydroseneciphylline (Coulombe, Kim & Stermitz, 1994; Kim, Stermitz & Coulombe, 1995) and indicine *N*-oxide (Coulombe, Kim & Stermitz, 1994) was described.

(d) Summary and overall conclusion on genotoxicity

The genotoxicity of PAs has been extensively studied in a variety of in vitro and in vivo assays. Table 24 summarizes the results of the most frequently used bacterial and mammalian cell assays and Table 25 summarizes the results of in vivo genotoxicity studies. These tables summarize the studies identified for this monograph as well as the results reported in EFSA (2011) and additional studies identified during the meeting (Mori et al., 1985; Williams & Mori, 1980), as indicated by the footnotes to the tables.

In the reverse mutation assay in bacteria, some of the 1,2-unsaturated PAs tested gave negative results in the presence and/or absence of metabolic activation. For some PAs, positive results were obtained in mammalian cells in the presence but not in the absence of metabolic activation. Overall, the results of the assays in mammalian cell lines and the in vivo assays clearly demonstrate that all 1,2-unsaturated PAs that have been tested form DNA adducts and are mutagenic. An exception is the non-esterified necine base retronecine. In general, 1,2-saturated PAs have not been tested (except ligularinine, which gave negative results in the UDS assay and platyphylline, which did not form DNA adducts), but because they are not metabolized to pyrrolic esters (DHP esters), it is assumed that they are less likely to be genotoxic. Bioactivation of PAs to pyrrolic ester(s), and the subsequent formation of chemical-specific DNA adducts has been identified as the key pathway leading to genotoxic effects. Binding of PA to DNA leads to nucleoside adduct formation and DNA-protein cross-linking.

The Committee considered whether the genotoxicity data were adequate to assess potency for different 1,2-unsaturated PAs. The largest dataset comparing the genotoxicity of different PAs was for 17 PAs in the in vitro hepatocyte UDS assay (Mori et al., 1985). The lowest concentration resulting in a positive UDS response was in a similar range for 15 of these PAs. The exceptions were the non-ester retronecine, and the saturated PA ligularine. All 17 PAs showed cytotoxicity in similar concentration ranges (Table 26). There were insufficient in vivo genotoxicity studies using a comparable study design, species and end-point to allow a comparison.

2.2.5 Reproductive and developmental toxicity

WHO-IPCS reviewed the reproductive and developmental toxicity of PAs after intraperitoneal administration (WHO-IPCS, 1988). It was concluded that heliotrine at single doses of 50 mg/kg bw or more resulted in dose-related fetal abnormalities in rat after intraperitoneal injection during the second week of gestation. The abnormalities included retardation, musculoskeletal defects and other abnormalities. A single dose of 200 mg/kg bw resulted in fetal death or

Table 26

Overview of potency differences for pyrrolizidine alkaloids tested in the in vitro rat hepatocyte unscheduled DNA synthesis assay by Mori et al. (1985)

	Result of test	Toxic dose (M)	Lowest positive dose (M)	Net nuclear grains
Acetylpetasitenine	+	$2*10^{-4}$	$2*10^{-5}$	21
Epoxyseneciphylline	+	$2*10^{-4}$	$2*10^{-5}$	20
Clivorine	+	$2*10^{-4}$	$2*10^{-5}$	21
Dihydroclivorine	+	$2*10^{-4}$	$2*10^{-5}$	31
Jacobine	+	$2*10^{-4}$	$2*10^{-5}$	16
Ligularidine	+	$2*10^{-4}$	$2*10^{-5}$	44
Ligularinine	–	$>2*10^{-4}$	0	–
Ligularizine	+	$0.7*10^{-4}$	$0.7*10^{-5}$	18
Monocrotaline	+	$2*10^{-4}$	$2*10^{-5}$	15
Neoligularidine	+	$2*10^{-4}$	$2*10^{-5}$	27
Petasitenine	+	$2*10^{-4}$	$2*10^{-5}$	15
Retronecine	–	$>2*10^{-4}$	0	–
Senecicannabine	+	$2*10^{-4}$	$2*10^{-5}$	23
Senecionine	+	$0.7*10^{-4}$	$0.7*10^{-5}$	27
Seneciphylline	+	10^{-4}	10^{-5}	28
Senkirkine	+	$2*10^{-4}$	$2*10^{-5}$	24
Syneilesine	+	$2*10^{-4}$	$2*10^{-5}$	12

resorptions. The metabolic pyrrole derivative dehydroheliotridine was 2.5 times more potent on a molar basis than its parent PA.

The ability of PAs (and metabolites) to cross the placental barrier in the rat and to induce premature delivery or death of litters has been demonstrated. The embryo in utero appears to be more resistant to the toxic effects of PAs than the neonate. PAs (and metabolites) are known to have passed through the mother's milk to the weanlings (WHO-IPCS, 1988).

EFSA reviewed studies with lasiocarpine, heliotrine, integerrimine, monocrotaline, retrorsine, riddelliine and senecionine (2011). In most of these studies the PAs were administered during gestation where maternal and fetal or offspring toxicity was observed. The effects on the offspring included liver changes, cleft palate, micrognathios and deformation of the ribs and long bones, anophthalmia, microcephaly or anencephaly. EFSA concluded that developmental toxicity had mainly been observed following parenteral administration, and that it was not possible to determine if it is related to maternal toxicity. Therefore it was concluded that the effects could not be used in risk characterization (EFSA, 2011).

(a) Multigeneration reproductive toxicity

(i) Mouse

A preparation of riddelliine (92% riddelliine, 5% retrorsine, 1.3% seneciphylline) was administered by gavage to groups of 20 male and 40 female B6C3F1 mice at 0, 0.33, 3.3 or 25 mg/kg bw per day, on 5 days per week for 10 weeks before mating for both sexes, continuing through gestation and lactation for the females. Females were weighed on gestatation day (GD) 0 and 18, and postpartum on days 0, 5, 14 and 21 together with their pups. The number of live and dead pups was recorded and they were examined for gross malformations. On day 21 postpartum, dams and pups were killed without necropsy.

One male and one female died during the experiment due to dosing accidents, one female from the high-dose group was killed moribund after 8 weeks. Female mice in the high-dose group had prolonged estrus. No effects of riddelliine exposure were observed on fertility or gestation duration. Dam weights in the high-dose group were generally lower than controls during pregnancy and lactation (up to −18%), which was also the case for the dams in the mid-dose group at day 21 of lactation (−5%). In addition, there was a significant decrease in the percentage of pups born alive (96 versus 77%), pup weights (up to −23%) and pup survival (95% versus 85% at day 14) of mice treated with 25 mg/kg bw per day. In addition, litter size was reduced, although this decrease was not statistically significant (9.7 versus 5.1).

From the limited details available on the results of this study, it appears that the fetal NOAEL was 3.3 mg/kg bw per day in mice (2.4 mg/kg bw per day averaged over the week), and it is unclear whether or not the reported effects on the pups were secondary to maternal toxicity (Chan et al., 1994; NTP, 1993).

(ii) Rat

A preparation of riddelliine (92% riddelliine, 5% retrorsine, 1.3% seneciphylline) was administered by gavage to groups of 20 male and 40 female Fischer F344/N rats at doses of 0, 0.1, 1.0 or 10 mg/kg bw per day, on 5 days per week for 10 weeks before mating for both sexes, continuing through gestation and lactation for the females. Owing to excessive mortality in rats that received 10 mg/kg bw per day, surviving rats of this group were not mated. Females were weighed on GD0 and 19, and postpartum at days 0, 5, 14 and 21 together with their pups. The number of live and dead pups was recorded and they were examined for gross malformations. At day 21 postpartum, dams and pups were killed without necropsy.

Female rats in the high-dose group (10 mg/kg bw) had prolonged estrus. Dosing with riddelliine before and during mating and gestation had no effect on fertility or on length of gestation. Dam body weights in both dose groups

were generally lower than those of controls during pregnancy and lactation (up to −9%), although weight gains were similar during pregnancy. There were no significant differences with respect to the number of litters, litter size, percentage of pups born alive, pup survival or male pup weights. However, on days 14 and 21 of lactation, there was a significant decrease in female pup weight in the 1 mg/kg bw group (up to −9%). No gross malformations were noted.

From the limited details available about the results of this study, it appears that the fetal NOAEL was 1 mg/kg bw per day (0.7 mg/kg bw per day averaged over the week), the highest dose tested. It is unclear whether or not the decreased body weight in the female pups was secondary to maternal toxicity (Chan et al., 1994; NTP, 1993).

(iii) Chicken

Groups of 10 chicken hens (1377 ± 129 g) were fed a diet containing 0, 0.5, 2 or 4% *Senecio vernalis* plant for 210 days. Total alkaloid content in the extracts was determined by GC-MS. Total alkaloid content in the plant material was 0.14%, with 8.57% in the basic form and 91.43% in the *N*-oxide form. Based on feed consumption and average body weight of the hens, and the alkaloid content in plant material of 0.14%, the total PA intake was 0, 0.5, 1.3 and 1.9 mg/kg bw per day in the four dose groups. Specific alkaloids were senecionine (>65%), senecivernine, seneciphylline, integerrimine, retrorsine, senkirkine and hydroxysenkirkine.

Decreases in egg production, feed efficiency, feed intake and body weight occurred in animals in the two highest dose groups. Serum GGT and AST were slightly, but statistically significantly, elevated and serum albumin and protein were significantly decreased in hens fed the 2% and 4% diets. Total bilirubin was significantly higher in the group fed 4% *Senecio vernalis* plant. No free PAs were detected in eggs, indicating either that, at these levels of dietary exposure, they did not produce residues at the level of detection or that they were bound irreversibly to egg proteins. Hens fed 2% or 4% plant diets had mild to moderate chronic liver changes including periportal or septal fibrosis, megalocytosis, bile duct hyperplasia and early regenerative nodule formation. Based on the information available, the NOAEL was 0.5 mg/kg bw per day (Eröksüz et al., 2003b).

(b) **Developmental toxicity**

(i) Mouse

Mice (group size, strain, age and weight at the start of the study were not reported) were orally treated with retrorsine at 10 and 20 mg/kg bw per day or monocrotaline at 80 mg/kg bw per day (purities not reported) from gestational day 7 to 17. The control mice were treated with an equal volume of vehicle. Body

weight, body length and tail length were measured on GD 17 to assess the fetal physical development. The levels of adrenocorticotrophic hormone (ACTH) and corticosterone in fetal plasma were measured using the enzyme-linked immunosorbent assay (ELISA). Fetal liver and lung were histologically examined. Ehrlich reagent was applied to determine the bound pyrrole metabolite of PAs in fetal liver and lung, and the content of glutathione (GSH) in these tissues was measured simultaneously. In fetal liver, the activity of alanine transaminase/aspartate aminotransferase (ALT/AST) and of the GSH redox system including GST, GSH peroxidase (GPx) and GSH-reductase (GR) as well as other oxidative parameters, such as superoxide dismutase (SOD) activity and malonaldehyde (MDA) content, were measured.

When compared with control animals, treatment with 10 mg/kg bw retrorsine decreased the fetal body weight and length ($P<0.01$, $P<0.05$), whereas both 20 mg/kg bw retrorsine and 80 mg/kg bw of monocrotaline restricted the fetal physical development to some extent. The levels of fetal plasma corticosterone in both retrorsine groups were higher than those of controls. Retrorsine at 20 mg/kg bw per day also significantly elevated the level of fetal plasma ACTH. Local congestion was found in fetal liver and cellular necrosis was observed in fetal lung. A certain amount of bound pyrrole metabolites could be detected in fetal liver and lung from any PA-treated group. Retrorsine reduced the GSH content in fetal liver and lung compared to the controls but without any obvious dose–effect relationship, while monocrotaline had no significant effect on GSH content. The activities of ALT/AST, GST, GPx and GSH-reductase were all decreased. The content of MDA was augmented, especially in the 20 mg/kg bw retrorsine group ($P<0.05$), while the activity of SOD in all PA groups was lower than that of controls ($P<0.01$). No further details are available, since only an abstract was published (Kou et al., 2011).

Specific pathogen-free Kunming mice (9–10 weeks old at the start of the study, 25 ± 2 g) were divided into seven groups (10–14 animals per group): control group, retrorsine group, monocrotaline group, lipopolysaccharide (LPS) group, retrorsine + LPS group, and two monocrotaline + LPS groups. Pregnant mice treated with PAs were given 10 mg/kg bw retrorsine (purity: 95%) or 20 (only in combination with LPS) or 60 mg/kg bw monocrotaline (purity: 100%) intragastrically from GD 9 to GD 16, while animals from the control and LPS group were given equal volumes of vehicle. LPS was administered as a single intraperitoneal dose of 150 μg/kg dissolved in sterile saline at GD 15, and the control groups received an equal volume of vehicle at the same moment. The parturition of dams and newborns was checked every 2 hours after LPS injection. Delivery times and numbers of live and dead pups at delivery were recorded. Live pups were sacrificed at birth and their livers were taken for morphological examination and biochemical analysis, including measurement of ALT, AST,

GSH peroxidase (GSH-Px), GSH-reductase (GSH-Rd), GST and SOD activities, and thiobarbituric acid reactive substances (TBARS) and protein content. Dams were sacrificed on GD 21 and checked for retained dead fetuses.

There were no preterm births in control animals or in mice given monocrotaline alone. One preterm birth was noted in the retrorsine group, seven in the LPS group, three in the retrorsine + LPS group, eight in the 20 mg/kg bw monocrotaline + LPS group, and nine in the 60 mg/kg bw monocrotaline + LPS group. Duration of gestation was significantly shorter in animals in the retrorsine group and in the monocrotaline + LPS groups than in control animals. Stillbirths were noted in animals in all treatment groups, but not in vehicle controls. Mortality rates were 1/109 (1%) in the monocrotaline group, 9/108 (8%) in the retrorsine group, 69/129 (53%, $P<0.01$) in the LPS group, 57/91 (63%) in the retrorsine + LPS group, 95/113 (84%, $P<0.05$ versus monocrotaline group) in the 20 mg/kg bw monocrotaline + LPS group, and 75/95 (79%, $P<0.01$ versus monocrotaline group) in the 60 mg/kg bw monocrotaline + LPS group.

The livers of pups from dams treated with retrorsine or monocrotaline alone did not show a different histology from livers of pups from control dams. In the livers of pups from dams treated with LPS, marked haemorrhage was observed, which increased in severity and was accompanied by congestion when dams were concomitantly treated with retrorsine. Concomitant treatment with monocrotaline and LPS led to massive vacuolization in the hepatocyte nuclei, especially at the higher dose of monocrotaline.

ALT activity was significantly reduced compared to that of vehicle controls and to LPS alone in animals in the retrorsine + LPS group (−44% and −37%, respectively) and the 60 mg/kg bw monocrotaline + LPS group (−51% and −44%, respectively). AST activity was also reduced in these groups, but this decrease was not statistically significant. In addition, ALT and AST activities in the animals in the retrorsine and monocrotaline alone groups were lower than in controls, although this was also not statistically significant. There were no significant differences between hepatic TBARS levels in pups from dams treated with retrorsine, LPS or a combination. However, TBARS levels in livers of pups from dams exposed to 60 mg/kg bw monocrotaline + LPS were significantly higher than in the control group. No significant differences were observed in GSH-Rd levels in any treatment group. GSH-Px levels were significantly reduced, compared to controls, in all treated groups except for the LPS group. SOD levels were significantly lower in the monocrotaline, LPS, retrorsine + LPS, and 20 mg/kg bw monocrotaline + LPS group. GST levels showed a significant decrease in the retrorsine + LPS group and the 60 mg/kg bw monocrotaline + LPS group, when compared to retrorsine alone and monocrotaline alone, respectively.

The authors concluded that both retrorsine and monocrotaline augmented the effects of LPS in neonates from treated dams (Guo et al., 2013).

(ii) Rat

Four groups of 12 pregnant adult Wistar rats (260–280 g at the start of the study) were fed *ad libitum* from day 6 to day 21 of gestation with diets containing 0, 0.01, 0.015 or 0.02% monocrotaline (monocrotaline purity not reported), equivalent to 0, 6.7, 10 or 13.3 mg/kg bw per day. Monocrotaline was extracted from *C. spectabilis*. During the exposure period, no clinical signs of toxicity or mortality were observed in any dose group and food and water intake was not significantly different from that of controls. On day 21 of gestation, the females were anaesthetized with ether and the ovaries and uteri were removed by caesarean section. The number of corpora lutea in each ovary was recorded and the gravid uteri were weighed. The fetuses were removed from the uteri, dried of amniotic fluid, weighed, and examined for gross abnormalities. The placenta of the live fetuses was also weighed. The number of implantation sites and resorptions was recorded in both uterine horns. From each litter, one third of the fetuses were eviscerated, partly skinned, fixed and stained for skeletal examination, one third were fixed for visceral examination, and one third were used for weighing lung, liver and kidney, which were then prepared for histological analysis. Blood samples were taken from the dams for determination of serum alanine aminotransferase (ALT), AST, ALP, LDH, gamma glutamyltransferase (GGT), urea and creatinine. The dams were killed and lung, liver and kidneys were weighed and samples of these tissues were used for histopathological examination.

Liver weights of the dams from the three experimental groups were slightly (up to −11%), but statistically significantly, lower than those of animals from the control group. Serum levels of AST (+40%), GGT (+21%), LDH (+20%), urea (+19%) and creatinine (+13%) were slightly, but statistically significantly, higher in dams from the highest dose group; however, fatty acid levels were statistically significantly lower (−42%). No changes were noted in serum ALT levels. Mild to moderate interstitial pneumonia and liver lesions, consisting of mild megalocytosis characterized by enlarged hepatocytes with scanty and fragmented nuclear chromatin and hyaline droplet formation in the cytoplasm, vacuolar degeneration and karyolysis or apoptosis of periportal hepatocytes, were observed in dams of the highest dose group; however, incidence of these lesions was not reported. The weights of the placenta and fetuses of this group were slightly but statistically significantly lower than those of the control group. No significant differences were observed in the frequency of skeletal and visceral malformation or anomalies between the control and treated groups.

The LOAEL for maternal toxicity was 13.3 mg/kg bw per day based on lung and liver histopathology. The NOAEL for developmental toxicity was 10 mg/kg bw per day, as effects in fetuses were seen only at the highest dose level

(decreased body weight) (Medeiros, Górniak & Guerra, 2000). Data from the control and highest dose group were also reported by Soto-Blanco et al. (2001).

Groups of four pregnant female Wistar rats (age and weight at start of the study not reported) were fed a control diet or a diet with 0.012% monocrotaline (120 mg/kg diet, equivalent to 8 mg/kg bw per day) during the gestation and lactation (GLE) period or only during the lactation (LE) period. Monocrotaline was obtained from Sigma Chemical; however, purity was not reported. Soon after birth, four young female and male rats were randomly chosen to remain with their dams until weaning (day 21). The young rats of each litter were weaned and 10 days later the young rats were anaesthetized and blood samples were collected for ALT, AST, GGT, LDH, ALP, bilirubin, glucose, urea and creatinine analysis. After blood collection, the animals were euthanized and lungs, liver and kidney tissue were collected for histological examination.

The study authors provided a qualitative description of the effect of dosing. No indication of the size of the effects or actual data were presented. Pups in the GLE treatment group all died before blood sample collection at day 21. Pups in the LE treatment group showed increased levels of ALT and urea. The litters of both experimental groups showed alterations in the lungs and kidneys. The lungs showed interstitial pneumonia, thickened alveolar septa and mixed cell infiltration. There was an accumulation of exudate in the perivascular-peribronchiolar interstitium and thickening of the arterial wall. Alveolar sacs contained an abundance of foamy cells. Lesions in the kidneys were characterized as mild, toxic, tubular nephrosis; however, alterations in the liver were found only in the GLE litter group, which presented a partially preserved lobular hepatic architecture. Lesions predominated in focal areas of hepatocellular hydropic degeneration and nuclear changes (karyorrhexis, pyknosis and karyolysis). Periportal necrosis was observed, and there was mononuclear cell infiltration and haemorrhage. There was no fibrosis (Medeiros, Górniak & Guerra, 1998). The LOAEL was 8 mg/kg bw per day, the only dose tested.

Groups of 10 pregnant female rats (age and weight at start not reported) were given monocrotaline (purity not reported) by gavage during gestation (in total 18.84 mg monocrotaline per rat, in six divided doses on alternate days, starting on GD 10) or during lactation (in total 18.84 mg monocrotaline per rat, starting on postnatal day 2, in six divided doses once every 3 days) or both 18.84 mg monocrotaline per rat during gestation and 18.84 mg monocrotaline per rat during lactation (with similar dosing regimens), or were given saline (controls). The description of the study design and results is very concise. The authors indicated that all the pups were weighed at birth and later at monthly intervals. After birth the pups were nursed for 21 days. In each cage, five animals were kept and observed for up to 18 months. Periodic sacrifices and necropsies were made either monthly or once in 2 months and samples of a number of tissues

(not specified) were collected for histopathology. At times (not specified) only liver biopsies were taken. All the remaining animals were killed by 18 months.

All pups from all treated dose groups showed liver and lung lesions, indicating that monocrotaline or its metabolites reach the fetus through the placenta as well as through the milk, and cause effects. These effects were most severe during the first month after birth. Two dams treated during pregnancy, three dams treated during lactation and three dams treated during both periods died. Adverse effects were observed in their lungs, liver, kidneys and/or hearts (Sriraman, Gopal Naidu & Raman Rao, 1988).

Groups of 10 pregnant Wistar rats (180–200 g) were treated orally (gavage) from GD 6 to 20 with 0 (water vehicle), 3, 6 or 9 mg/kg bw of a butanolic residue (BR) of *Senecio brasiliensis* leaves. NMR analysis revealed the presence of integerrimine *N*-oxide only in the BR, and the purity of the BR was 69%; the remaining 31% of the BR was not identified. The integerrimine *N*-oxide doses were therefore 0, 2.1, 4.1 or 6.2 mg/kg bw per day. An additional pair-feeding group ($n = 9$) of dams were provided food for consumption matching that of the high-dose group. Maternal behaviour (weight gain, water and food consumption, retrieval, first nursing, fighting, dams with pups, intruder chases, full maternal behaviour), offspring development (weight, pinna detachment, hair growth, incisor eruption, eye opening, vaginal opening, testis descent) and offspring behaviour (palmar grasp reflex, surface righting reflex, negative geotaxis and auditory startle reflex) were tested.

By GD 14–21, maternal body weight gain and food consumption showed a statistically significant decrease at all doses of the integerrimine *N*-oxide groups and in the pair-feeding group. The decrease in body weight in the high-dose group ($P<0.0001$ compared to controls) was statistically significantly lower than in the corresponding pair-feeding group ($P<0.01$) suggesting that chemical toxicity was only partly responsible. Without corresponding pair-feeding in the low- and mid-dose groups, it cannot be ruled out that decreased food consumption was the principle cause of decreased body weight gain. No significant differences were found with respect to gestation duration, litter size, ratio of male/female pups and prenatal death. Cannibalism was observed in dams from the high-dose group.

Results showed that prenatal exposure to integerrimine *N*-oxide induced impairment of maternal behaviour and aggressive maternal behaviour, mainly in the highest dose group. Statistically significant differences were observed in the retrieval of the first, second, third and fourth pup, the number of pups present at the first nursing and in full maternal behaviour for the high-dose group when compared to controls. There were no significant differences in relation to retrieval of all pups or for the start of nursing for any of the groups. In addition, no differences were observed between any of the groups in relation to the open-field test, latency to first fight, and the time and number of pelvic and thoracic

attacks. The number and duration of fights were significantly reduced in the mid- ($P<0.05$) and high-dose ($P<0.001$) group and the high-dose group ($P<0.001$), compared to the control group. The frequency and time dams were with pups was also reduced in the treated groups compared to controls. The difference was significant for the mid- ($P<0.01$) and high-dose ($P<0.05$) group and the mid-dose group ($P<0.01$), respectively. In addition, the frequency ($P<0.05$) and time ($P<0.01$) for which the intruder chases the female and the time ($P<0.05$) for which the female chases the intruder were significantly induced in the high-dose group.

Between sexes comparison of pups showed significant differences in various parameters at the high dose, including lower body weights (−10.6–31% for males and −9.4–31.55% for females at different postnatal days (PNDs)), delays in pinna detachment ($P<0.05$), hair growth ($P<0.001$), eruption of incisor teeth ($P<0.05$ for males, $P<0.01$ for females), vaginal opening ($P<0.0001$), palmar grasp ($P<0.05$), surface righting reflex ($P<0.05$ for females) and negative geotaxis ($P<0.01$ for males and $P<0.05$ for females), while significant delays in eye opening and auditory startle reflex were seen at both the mid- and high-dose (at the mid-dose $P<0.001$ and $P<0.01$, respectively; at the high-dose $P<0.0001$ for both end-points). There were no significant differences between pups from different treatment groups in the open-field test, testes descent or adult gait.

The authors concluded that exposure during gestation to integerrimine *N*-oxide induced maternal toxicity and impairment of maternal care and that prenatal exposure induced delay in physical and behavioural development of the offspring (Sandini et al., 2014).

In a follow-up publication, Sandini et al. (2015) reported the effects of prenatal exposure to integerrimine *N*-oxide (see above) on behaviour and neurotransmitter levels in pups who reached adulthood. The pups were weaned at PND 21, separated according to sex and treatment group, and followed until adulthood (PND 75). For adult offspring studies, male and female offspring were randomly selected and individually evaluated on behaviour testing. However, in the open-field and elevated plus-maze test, the same animals were used (10 rats per sex and group). Animals were naive to the experimental situation for the catalepsy, forced swimming test and stereotype behaviour testing (8–9 rats per sex and group). Measurements of serum creatinine, urea, ALT, AST and GGT levels (10 rats per sex and group) and histological analysis (12 rats per group, males and females combined) were conducted in the same animals as were used for the open-field and plus-maze test. Monoamine levels were determined in 5–7 rats per group (males and females combined) that had not been used in any experimental setting.

In offspring from the high-dose group, locomotion frequency ($P<0.01$), open arms entries ($P<0.05$) and time spent in open-arm ($P<0.05$)

were significantly increased in males. Total time of swimming was significantly reduced in males ($P<0.05$), when compared to controls. In females, the time of climbing was significantly increased ($P<0.05$) in the high-dose group, and increased stereotyped behaviour was noted in the low- and high-dose group. No significant differences were reported for the rearing and grooming frequencies or the immobility time, defaecation and catalepsy.

Biochemical analysis showed significant differences, compared to controls, only in ALT levels in males in the high-dose group (+23%). Relative organ weights of liver, spleen, lungs and kidney did not differ between groups. Histopathology of liver and lungs revealed no oedema, necrosis, apoptosis or megalocytosis. However, in the liver from offspring of animals from the mid- ($P<0.01$) and high-dose ($P<0.05$) groups, an increased number of multinucleated cells was observed, when compared to the control group. In the brain monoamine studies, significant differences were only noted for the striatum in offspring from the mid-dose group. Dopamine (−74%), 3,4-dihydrophenylacetic acid (+123%), homovanillic acid (+253%), serotonin (+180%), and 5-hydroxyindolacetic acid (+112%) were different in the offspring of animals from the mid-dose group compared to controls.

The authors concluded that prenatal exposure to integerrimine *N*-oxide changed behaviour in adulthood and affected neurotransmitter levels in the striatum. The LOAEL for maternal toxicity was 2.1 mg/kg bw per day integerrimine *N*-oxide, since maternal body weight gain was decreased at the lowest dose tested. Based on the parameters tested in this study, the NOAEL for offspring toxicity was 2.1 mg/kg bw per day integerrimine *N*-oxide, since physical and developmental effects in the offspring were noted at higher doses. The critical effects were delayed opening of the eyes and delayed auditory startle reflex, as well as alterations in brain monoamine levels at 4.1 mg/kg bw per day (Sandini et al., 2015).

Pregnant Sprague-Dawley rats (9 weeks old, 4–11 animals per group) were dosed orally (by gavage) with 580 or 920 mg/kg bw per day of a methanol (MeOH) extract of *Senecio vulgaris* for 1–10 days postcoitum. Other groups were treated with doses of 10–30 mg/kg bw senecionine, senecionine *N*-oxide, seneciphylline, retrorsine, riddelliine or a mixture of senecionine plus seneciphylline on GD 3 or days 4–10. Purity or concentrations of PAs were not given for the substances or extracts. The control group was treated with the polyvinylpyrrolidone vehicle. Number of pregnant dams, implantation sites per pregnant animal and normal fetuses per pregnant animal at GD 16 were investigated.

The pregnancy rate and the number of implantation sites did not differ from those in controls, but the number of normal fetuses was decreased compared with the number of implantation sites in all dose groups treated with the MeOH extract. The same was observed in the groups treated with the PAs,

although the decrease was not always statistically significant. Livers from MeOH extract-treated rats revealed tissue damage and evidence of toxicity (e.g. jaundice) was seen in rats dosed with senecionine, senecionine *N*-oxide, and the mixture of senecionine and seneciphylline. Rats treated with 30 mg/kg bw senecionine all died before GD 16. The number of normal fetuses per pregnant dam was statistically decreased for animals in the MeOH extract groups and those treated with 20 mg/kg bw senecionine *N*-oxide (Tu ZB et al., 1988).

Groups of 12 pregnant rats (strain, age and body weight at the start of experiment not reported) were orally administered 0, 2, 4 or 6 mg/kg bw per day integerrimine *N*-oxide from GD 6 to 20. Purity of integerrimine *N*-oxide was not reported. Vehicle was not reported. Clinical signs, body weight gain and feed intake were measured daily. On GD 21, six females per group were killed to evaluate their reproductive parameters; the remaining dams were left to give birth. Pups were evaluated for gross alterations and weighed immediately after birth. On PND 70, 10 males per group were killed to evaluate lymphoid organs.

No differences in feed intake among the groups were noted; however, dams dosed with 6 mg/kg bw per day until GD 21 showed a reduction in body weight gain (no *P*-value reported). No statistical differences in the litter size and fetal/pup weight were observed. In relation to lymphoid organ analysis, no differences were noted in the parameters evaluated. No further details are available, since only an abstract was published (Hueza et al., 2011).

Pregnant Wistar rats (no information on group size, age or body weight at the start of the experiment was given) were treated by gavage with monocrotaline (purity not reported) at 0, 1, 3.5 or 7 mg/kg bw per day from GD 6 to GD 20. The control group received only water. Total body weight gain, water and food consumption were measured in mothers during the experimental period. After birth, the body weight of the offspring was measured and they were examined for physical characteristics (pinna detachment, eruption of incisor teeth, eye opening, testes descent, coat appearance, ear opening, vaginal opening, behavioural and reflex (palmar grasp, negative geotaxis and adult gait) development.

A dose-related reduction in number of pups born (no *P*-value reported) was observed. No significant differences between the experimental and control dams in their weight gain, food consumption and water intake during pregnancy were reported. Prenatal exposure to monocrotaline did not affect the offspring's body weight, motor activity (open-field behaviour), physical and reflex development. No further details are available, since only an abstract was published (Ricci et al., 2010).

Two-hundred and twenty pregnant Sprague-Dawley rats (age and weight at the start of the experiment not reported) were divided into 11 groups and treated by gavage with a water extract of *Senecio scandens* corresponding to doses of 0, 7.5, 15 or 30 g/kg bw of *S. scandens*, or with "Qianbai Biyanpian, a plant

preparation of *S. scandens* with total alkaloid at the same dose levels" from day 6 to day 15 of pregnancy. Body weight and the food consumption of pregnant rats, and fetal weight and length were measured. The numbers of absorbed and dead embryos were recorded. Fetuses were examined for visceral and skeletal abnormalities.

Weight and the food consumption of pregnant rats in the groups given high doses of Qianbai Byanpian and total alkaloids decreased. No significant changes were observed in the number of absorbed embryos, but stillbirths were significantly increased in the high-dose group of water extract and total alkaloids compared with the control group. Bone deformities such as fontanelle expansion, hypoplasia of parietal bone, occipital bone and cervical arch were observed. Rib abnormalities could also be seen in some rats. The information above is based on the English summary in a Chinese research paper (Zhao et al., 2010).

Groups of seven pregnant rats (no details on strain, age or weight at the start of the study were reported) were treated orally with monocrotaline (no purity reported) at 0, 3 or 5 mg/kg bw per day from GD 6 to 21. Clinical signs, body weight gain and feed intake were measured daily. Immediately after birth, pups were evaluated for gross alterations and weighed until PND 7. On PNDs 4 and 7, half of each litter was killed and lymphoid organs (thymus and spleen) were examined.

No clinical signs of monocrotaline toxicosis were observed in either dams or pups. Rats treated with monocrotaline did not show reduction in either body weight gain or feed intake (*P*-value not reported). No difference in body weight was noted in any group of pups immediately after birth; however, female pups from dams treated with 5 mg/kg monocrotaline showed a reduced body weight gain on PND 4. Only on PND 7, was a decrease in relative thymus weight observed in pups of both sexes from females in the mid-dose group, but not from the high-dose group. Male pups from dams in the mid-dose group showed splenomegaly on PND 7. No further details are available, since only an abstract was published (Benassi, Górniak & Hueza, 2008).

(iii) Hamster

Groups of pregnant golden Syrian hamsters (9 weeks old, group size and weight not reported) were administered 0 (vehicle control), 900, 1000, 1600 or 2000 mg/kg bw of a methanol extract of *Senecio vulgaris* (PA content not reported) in polyvinylpyrrolidone on days 1–8 of pregnancy. At autopsy (GD 14), the number of pregnant dams (defined as the presence of at least one implantation site) and the number of implantation sites, normal fetuses, dead-degenerating fetuses and corpora lutea were counted.

No adverse effects on pregnancy rates, number of implantation sites or fetal development were reported (Tu ZB et al., 1988).

2.2.6 Special studies

(a) Immunotoxicity

In the WHO-IPCS evaluation (1988), one study on immunosuppression was described, that "suggested that dehydroheliotridine selectively destroys or inactivates cells involved in the initial stages of antigen recognition and processing". No studies on immunotoxicity were described in the EFSA (2011) opinion.

For the current evaluation, several studies were identified in which anti-inflammatory effects of extracts from PA-containing plants were examined. As these were not considered immunotoxic effects, these studies were not assessed.

Five studies looked at aspects of possible PA-associated immunotoxic effects, three were in rats and two in mice; of those studies, four used monocrotaline and one (in rats) integerrimine *N*-oxide. In studies of monocrotaline in rats, at exposures below those that caused overt toxicity (7.0 mg/kg bw), Hueza et al. (2009) saw effects on macrophage function at lower doses (1.0 and 5.0 mg/kg bw) whereas Benassi et al. (2011) did not. Rats exposed to 2.07, 4.14 or 6.21 mg/kg bw integerrimine *N*-oxide (obtained by ethanolic extraction from *Senecio brasiliensis* leaves) by gavage for 28 days had reduced weight gain compared to controls, but did not show immunotoxicity (Elias et al., 2011). In mice exposed to monocrotaline, changes in body weight and immune organ weight were seen at doses higher than 75 mg/kg bw and, for some measures, above 50 mg/kg bw when also treated with an immune inducing agent. Both humeral and cell-mediated responses were diminished in some assays at or below the exposures causing general toxicity (Deyo & Kerkvleit, 1990, 1991).

(i) Mouse

In a study by Deyo & Kerkvliet (1990), female C57Bl/6 mice (7–8 weeks old, 6–8 animals per group) were dosed by gavage with 0 (vehicle), 10, 25, 50, 75 or 150 mg/kg bw per day monocrotaline for 14 consecutive days, and the immune response to sheep RBCs by intraperitoneal injection on day 9 was assessed. Liver, kidney, thymus, spleen and lungs were removed and weighed; in addition, part of the lungs was dried for determination of the wet to dry lung weight ratio. More than one experiment was conducted and (some of) the results were combined. It is, however, not clear how this was done and in what way the experiments differed from each other.

In two experiments with mice dosed with monocrotaline and sheep RBCs, final body weight (data not shown) and relative thymus weight (43% of control) were significantly decreased compared to controls in the highest exposure group. Relative spleen weight and spleen cell recovery were significantly decreased compared to controls in both the 75 and 150 mg/kg bw exposure groups (up to 42% decrease for relative spleen weight in mice exposed to the highest dose), and relative liver weight was increased, but not statistically significantly, in the 50, 75 and 150 mg/kg bw exposure groups. Generally, no changes were seen in relative lung weight ratios or wet to dry lung weight ratios. White blood cell counts were significantly decreased (−40%) in animals that received 75 mg/kg bw monocrotaline. No histopathological changes were noted in the kidneys. Nor were any histopathological changes seen in the lungs of animals given doses of 10 or 25 mg/kg bw, although some animals in the 50 mg/kg bw group were reported as having subtle type II pneumatocyte hypertrophy. The livers of animals at all dose levels showed various degrees of fatty changes.

In a separate experiment to examine alloimmunization, P815 mastocytoma cells were given by intraperitoneal injection on day 5 of treatment with monocrotaline at 25 or 75 mg/kg bw. Relative thymus weight was significantly decreased compared to that of controls following exposure to 25 or 75 mg/kg bw monocrotaline (−29% and −58%, respectively), and the expected increase in splenic size following P815 injection was not seen. In animals treated with 75 mg/kg bw monocrotaline, relative spleen weight was significantly decreased (−47%) compared to controls.

Two experiments examined antibody formation by looking at the plaque-forming cell assay or the haemolytic antibody isotope release assay, which are two different techniques for evaluating the ability of the harvested spleen cells from animals treated with sheep RBCs to lyse sheep RBCs. Both assays showed a reduced response in mice exposed to 25 mg/kg bw and little to no response in cells from mice given higher doses. Antibody titres in mice injected with P815 were decreased in the animals that received the 75 mg/kg bw dose. In an assay of the cytotoxic T-lymphocyte response of splenocytes to P815 cells from the mice injected with P815, the effector to target ratio and lytic unit 25 (LU_{25}) per spleen ratio, was significantly suppressed at a dose of 75 mg/kg bw monocrotaline.

The study authors concluded that both humoral and cell-based immunity can be suppressed in mice by monocrotaline, and immune suppression is evident at doses that do not otherwise cause gross toxicity in the organs examined (Deyo & Kerkvliet, 1990).

Female C57BL/6 mice (8–12 weeks old) were dosed by gavage with 0, 25, 50 or 100 mg/kg bw per day monocrotaline for 14 consecutive days. The gavage solution was obtained using a stock solution of dissolved monocrotaline in 1% phosphoric acid adjusted to pH 7 using sodium hydroxide (NaOH).

Some mice were sensitized by intraperitoneal injection to 25 μg trinitrophenyl-lipopolysaccharide (TNP-LPS) or 10 μg dinitrophenyl (DNP)-Ficoll in phosphate-buffered saline on day 10 or 9, respectively, and the antibody response was measured on day 15. In mice dosed only with monocrotaline (eight per group), significant changes in body weight (−7% compared to controls) and lymphoid organ weights (decrease of relative weight, −31% for spleen, −42% for thymus) were only observed in the 100 mg/kg bw dose group, which was also true for mice exposed to DNP-Ficoll, although the body weight change was also observed in mice exposed to 50 mg/kg bw monocrotaline. In mice that were also exposed to TNP-LPS, significant decreases in body weight (up to −25%), relative thymus weight (up to −58%), relative spleen weight (up to −43%) and total spleen cell recovery (up to −49%) were seen, beginning at 50 mg/kg bw monocrotaline.

In mice exposed only to monocrotaline, a decrease in peritoneal exudate cells (of −47%) was observed (four per group, each made of combined samples of two animals). Antibody responses were significantly decreased, in a dose-dependent manner up to almost 100% at the highest dose level, in all monocrotaline-treated mice, and were similar whether treated with TNP-LPS or DNP-Ficoll. The proliferation of splenic lymphocytes in response to conA or LPS, as assessed through measurements of incorporation of tritiated thymidine, was found to be significantly decreased in the lymphocytes isolated from monocrotaline-treated mice at all doses (up to around 90%). Exposure to monocrotaline at 50 and 100 mg/kg bw reduced the cytotoxicity of ex vivo natural killer cells (by −53 and −59%, respectively) in an assay that measures the lysis of YAC-1 tumour cell targets using lytic units.

The study authors concluded that monocrotaline is immunotoxic at doses lower than those that cause overt toxicity, and suggested that the B cell is a target for monocrotaline (Deyo & Kerkvliet, 1991).

(ii) Rat

Male Wistar rats (10 weeks old) were dosed with monocrotaline by gavage at the doses indicated below. The gavage solution was obtained by adjusting a stock solution of dissolved monocrotaline crystal in 1% phosphoric acid to pH 7 using NaOH, and dilution with distilled water to give gavage concentrations of 0, 0.3, 1.0, 3.0, 5.0 and 7.0 mg/ml. The authors did not report whether the stability of the solution was tested, nor whether new gavage solutions were made daily. The study was undertaken to evaluate the effects of low doses of monocrotaline on different branches of the immune responses in rats, a laboratory species known to be susceptible to the development of toxicosis in response to *Crotalaria* spp. The study comprised five experiments, 1A: monocrotaline toxicity, 1B: immunopathology,

2A: humoral immune response, 2B: cellular immune response, and 3: innate immunity – macrophage activity. Animals were killed on the 14th day.

For experiment 1A, rats were gavaged with 0, 1.0, 3.0 or 7.0 mg/kg bw per day monocrotaline (6 per group) for 14 days and the pulmonary LDH levels in the supernatants of lung lavage fluid, as well as histopathological examination of representative samples of the lung, heart, liver and kidney were assessed. Animals in the 3.0 and 7.0 mg/kg bw groups had reductions in body weight gain (up to −45%) but no significant reduction in food intake. Rats in the 7.0 mg/kg bw monocrotaline group had significant elevations in LDH enzymes (+467%) and histopathological changes: more macrophages in the alveolar lumen and alveolar epithelial type II cell hyperplasia in the lungs, megalocytosis in the liver and sporadic congestion foci in the kidneys. Therefore, for the rest of the assessment of immune toxicity the authors used doses lower than 7.0 mg/kg bw per day to avoid overt toxicity.

For experiment 1B, rats were gavaged with 0, 0.3, 1.0 or 3.0 mg/kg bw per day monocrotaline (10 per group) for 14 days. A significant reduction of 33% in bone marrow cellularity was seen in the 3.0 mg/kg bw monocrotaline group, but no significant changes were seen in splenic or thymic indices, splenic cellularity, or ratio of thymic cortical to medullar area among treatment groups.

For experiment 2A, rats were gavaged once per day with 0, 0.3, 1.0 or 3.0 mg/kg bw monocrotaline (10 per group) for 14 days. No significant change in titres of antibodies against sheep RBCs were found, nor was there a change in humoral immune response using a plaque-forming cells assay.

For experiment 2B, rats were gavaged once per day with 0, 1.0, 3.0 or 5.0 mg/kg bw monocrotaline (10 per group) for 14 days. No significant changes in foot pad swelling were observed following sensitization with keyhole limpet haemocyanin (KLH) on days 1 and 7 and challenge with heat-aggregated KLH injected into the foot pad.

For experiment 3, rats were gavaged with 0, 1.0, 3.0 or 5.0 mg/kg bw monocrotaline (10 per group). Macrophage activity was assessed by testing phagocytosis or zymosan particles (mean of four counts obtained from two slides from each rat), spontaneous hydrogen peroxide (H_2O_2) release, phorbol mistrate acetate-induced H_2O_2 release, and nitric oxide (NO) release of peritoneal macrophages (triplicate). The only significant effect seen on macrophage activity was a decrease in NO release in the animals given the 1.0 and 5.0 mg/kg bw doses (Hueza et al., 2009).

Male Wistar rats (10 weeks old, 10 per group) were given monocrotaline (purity 99.26%, extracted from *Crotalaria spectabilis* seeds) by gavage at doses of 0, 0.3, 1.0 or 3.0 mg/kg bw per day. The gavage solution was obtained by adjusting a stock solution of dissolved monocrotaline crystal in 1% phosphoric acid to pH 7 using NaOH, and diluting it with distilled water to yield gavage concentrations

of 0.0, 0.3, 1.0 and 3.0 mg/ml. The authors did not report whether the stability of the solution was tested, or whether new gavage solutions were made daily. The aim of the study was to determine whether monocrotaline administration to rats for 28 days induced the same modulatory effect on NO production by peritoneal macrophages as 14 days of administration.

Animals in each group were killed on the 29th day and peritoneal macrophages were collected to determine macrophage activity. Effects on phagocytosis of zymosan particles (mean of four counts obtained from two slides from each rat), spontaneous and phorbol myristate acetate (PMA)-induced H_2O_2 release (mean of four counts for each rat) and NO release of peritoneal macrophages (triplicate) were determined. No other examinations on the exposed animals were reported.

There were no significant differences between the various treatment groups in phagocytosis or in NO production by peritoneal macrophages. Rats treated with 3.0 mg/kg bw monocrotaline per day for 28 days had enhanced H_2O_2 production, both spontaneous and PMA-induced, when compared to untreated rats (respectively, 0.099 ± 0.007 versus 0.054 ± 0.004 and 0.16 ± 0.005 versus 0.074 ± 0.005, $P<0.01$). The study authors reported that these results were in contrast with a previous study in which monocrotaline was administered for 14 days using the same doses as above, where it was found that peritoneal macrophages from monocrotaline-treated animals were not able to produce NO. The study authors did not report mortality, but the numbers of animals in the results table were fewer than the original 10 animals per group (final group numbers were 6, 7, 8 and 7, respectively). Owing to the discrepancy in results from this 28-day study when compared with a previous 14-day study, the authors suggested that the experiments should be repeated (Benassi et al., 2011).

In a study looking at the haematological and immunological effects of integerrimine *N*-oxide isolated from *Senecio brasiliensis*, male Wistar Hannover rats (10 weeks old, 7 animals per group) were gavaged daily with 0, 3, 6 or 9 mg/kg bw of an extract of *Senecio brasiliensis*. The extract was determined to contain 69% integerrimine *N*-oxide. The authors calculated that the exposure to integerrimine *N*-oxide was 0, 2.07, 4.14 or 6.21 mg/kg bw per day, respectively. Rats were checked daily for clinical signs and mortality. Food consumption and body weight gain were measured every 3 days. On day 29, blood was collected and, after sacrifice, the thymus and spleen were harvested for lymphoid organ analysis. In addition, fragments of liver, spleen, thymus, lungs and kidneys were taken for histopathology.

No clinical signs or mortality were observed during the experiment. All of the treated rats were reported to have shown a reduction in body weight gain, although only the rats exposed to 6 mg/kg bw extract were reported to have a dose-related decrease in food consumption in all periods compared to controls.

No changes were seen in relative thymus weight, spleen cellularity or bone marrow cellularity. Increased relative spleen weight, mild to moderate splenic blood congestion, and mild megalocytosis in the liver, and hepatic and renal blood congestion were noted in the highest dose group. The other organs did not show any morphological changes.

Hepatic enzyme, AST and ALT, levels in the blood were significantly increased in animals given the 6 mg/kg bw dose (up to +158%) and 9 mg/kg bw dose (up to +199%), compared to controls. RBC counts (+12%), white blood cell counts (+62%), neutrophils (+136%) and lymphocytes (+48%) were increased in the highest dose group; the haematocrit, monocytes, and measurements of neutrophil phagocytosis and neutrophil oxidative burst were not significantly different between groups.

In additional experiments, rats (10 animals per group) were administered 0, 3, 6 or 9 mg/kg bw BR once daily by gavage for 28 days. In one experiment, on day 21, animals were immunized by intraperitoneal injection of sheep RBCs. On day 28, after euthanasia, blood samples were collected for humoral immune response analysis. In the second experiment, rats were sensitized with KLH, and on day 28 the animals were challenged with heat-aggregated KLH and swelling was measured. In a third experiment, after sacrifice on day 28, spleens were removed for lymphocyte analysis.

No significant differences were seen in antibody titres, in a plaque-forming cell assay or in delayed-type hypersensitivity as measured by changes in foot pad swelling after injection with KLH. At the 9 mg/kg bw dose, a decrease in splenocyte proliferation was observed in response to concanavalin A but not in response to lipopolysaccharide. In vitro treatment of splenocytes with integerrimine *N*-oxide at 0.8 mg/mL but not 0.08 mg/mL was seen to significantly increase the number of cells in G0/G1 and reduce the number in S-phase (group size of 8 in quadruplicate). The authors concluded that the treatments "did not promote a marked immunotoxic effect" (Elias et al., 2011).

(b) Neurotoxicity

WHO-IPCS (1988) noted that central nervous system poisoning can be an effect of pyrrolizidine poisoning in horses, cattle, sheep and pigs, and that Trichodesma alkaloids can cause intoxication of mice, rabbits and dogs. No additional information was presented by EFSA (2011).

Coll et al. (2011) injected eight male Wistar rats per group with a single intraperitoneal dose of 0 or 60 mg/kg bw monocrotaline and killed them 44 days later to examine the blood–brain barrier permeability. Prior to sacrifice, samples of cerebrospinal fluid were obtained for protein and glucose determination. In addition, blood samples were collected for determination of ALT, AST, ALP

activity and albumin concentration. Administration of trypan blue by intracardial perfusion and Evans blue by intravenous injection were used to determine brain permeability at necropsy. The protein and glucose content in the cerebrospinal fluid in treated rats was significantly increased (0.41 ± 0.07 g/L and 1.12 ± 0.08 g/L, respectively, $P<0.001$) compared to control rats (0.19 ± 0.04 g/L and 0.61 ± 0.14 g/L, respectively). Serum ALT (41 ± 4 UI/L), AST (149 ± 14 UI/L) and ALP (396 ± 16 UI/L) activities in treated rats were significantly decreased compared to control rats (69 ± 9 UI/L, 239 ± 25 UI/L, 698 ± 44 UI/L, respectively, $P<0.05$), whereas albumin concentration was significantly increased (4.13 ± 0.11 g/dL versus 3.22 ± 0.23 g/dL, $P<0.05$). Portal pressure was significantly increased in the treated rats (12.1 ± 1.0 mmHg, $P<0.01$) compared to controls (7.6 ± 0.2 mmHg). Trypan blue was identified in the hippocampi of the treated rats whereas no staining was observed in the controls. Evans blue dye was also identified in an increased concentration (8.34 ± 0.25 μg/g, $P<0.001$) within the brains of treated rats compared to those of the controls (6.12 ± 0.27 μg/g). The study suggests that altered blood–brain barrier permeability accompanied by alteration in the cerebrospinal fluid content is associated with monocrotaline toxicity (Coll et al., 2011).

(c) Photoisomerization

No information on photoisomerizing properties of PAs was included in previous opinions by WHO-IPCS (1988) and EFSA (2011).

Zhao et al. (2011) conducted in vitro studies to examine the potential for PAs and PA metabolites to initiate processes which may lead to skin cancer and/or PA-induced secondary photosensitization. Monocrotaline, riddelliine, heliotrine, retronecine, lasiocarpine, senkirkine, dehydromonocrotaline, dehydroheliotrine, dehydrolasiocarpine, dehydroretrorsine, dehydrosenecionine, dehydroseneciphylline, dehydroretronecine, monocrotaline *N*-oxide, riddelliine *N*-oxide, heliotrine *N*-oxide, retronecine *N*-oxide, 7-glutathionyl-6,7-dihydro-1-hydroxmethyl-5H-pyrrolizine (7-GSH-DHP) and 7-(deoxyguanosin-N2-yl) dehydrosupinidine (DHP-dG-1) at a concentration of 0.1 mM in methanol were added to a solution with 100 mM methyl linoleate and irradiated with 0, 14, 35 or 70 J/cm^2 of UVA light to determine if the mixture initiates lipid peroxidation compared to methyl linoleate irradiated alone. The results indicated that only the dehydro-PAs and 7-GSH-DHP induced a significantly increased production of methyl linoleate hydroperoxide, which increased in a UVA dose-dependent manner. Irradiation performed with the addition of either SOD or sodium azide decreased the lipid peroxidation with these two compounds. The formation of singlet oxygen and superoxide anion radicals was confirmed using electron spin resonance spin trapping (Zhao et al., 2011).

The same research group further tested for phototoxicity or photogenotoxicity of PAs using human HaCaT keratinocytes (transformed epidermal human cells) exposed to monocrotaline, riddelliine, lycopsamine, heliotrine, dehydromonocrotaline, dehydroriddelliine or dehydroretronecine. The cells were exposed to 250–500 μM of dehydromonocrotaline, dehydroretronecine, dehydroriddelliine, monocrotaline, riddelliine or control for 1 hour, followed by UVA treatment and incubation for 24 hours before being assayed. Cells were exposed to 1, 2 or 4 J/cm^2 of UVA and reduction of cell viability at 250 or 500 μM was measured using an MTS assay or induction of LDH release. Relative phototoxicity observed at 500 μM was dehydromonocrotaline ~ dehydroretronecine > dehydroriddelliine >> monocrotaline ~ riddelliine ~ control. Reduction in mitochondrial membrane potential was different in cells exposed to PAs with exposure to 4 J/cm^2 UVA compared to without for dehydroretronecine, but not for cells exposed to monocrotaline, dehydromonocrotaline, riddelliine, dehydroriddelliine, heliotrine or lycopsamine. DNA cleavage was observed only for dehydromonocrotaline and dehydroriddelliine in an assay for single strand breaks in supercoiled DNA, although the authors deduced the additional presence of DNA cross-linking based on the formation of low mobility products. HaCaT cells treated with 250 μM dehydromonocrotaline had increased levels of 8-OHdG DNA adducts, which were further increased with exposure to 4 J/cm^2 UVA. The authors additionally used an electron spin resonance spin trapping technique to show formation of reactive oxygen species, including hydroxyl radicals, singlet oxygen, superoxide and electron transfer reactions by UVA irradiation of dehydromonocrotaline and dehydoriddelliine (Wang CC et al., 2014).

2.3 Observations in domestic animals/veterinary toxicology

WHO-IPCS (1988) reported numerous cases of PA poisoning in several animal species, including cattle, sheep, horses and pigs starting from 1903 when the first cases were reported.

In 2011, EFSA concluded that all livestock species are susceptible to PA poisoning although susceptibility differs between species. In general, sheep, goats and rabbits are among the more resistant species, whereas horses, pigs and poultry are more sensitive. The intoxication can be acute or chronic, depending on the individual alkaloids present, the total amount of PAs ingested and the time span over which the ingestion took place. The onset of disease can be within 24–48 hours or after several days or months, and is characterized by lesions in the liver in all species. However, different overall patterns of lesions have been observed (EFSA, 2011).

This section provides an overview of data on livestock poisoning by PAs in the form of a short summary of the available literature. Consumption of PA-containing plants or feed contaminated with PA-containing plants has been reported to cause disease in pigs, horses, donkeys, goats, sheep, buffaloes, cattle, yaks and wombats. Under normal circumstances, PA-containing plants are not likely to be consumed by these species. However, in cases of severe drought, highly infested pastures and/or a marked increase in population, they may be consumed. Consumption also occurs when PA-containing plants are dried together with feed materials, such as in the production of hay.

(a) Pig

Pigs that were accidentally fed with *Crotalaria spectabilis* due to contamination of sorghum were reported to suffer from acute PA poisoning (Ubiali et al., 2011). Clinical signs started 24–48 hours after consumption and included depression, lethargy, apathy, loss of appetite, vomiting, pale or jaundiced mucous membranes, ascites, lateral recumbency, lateral position with paddling, and convulsions and death. The main lesions were increased liver size, reddish and yellowish coloured areas on the lobules, ascites, and hydrothorax with reddish-yellowish liquid containing filaments with aspect of fibrin, enlarged lymph nodes and pulmonary oedema.

(b) Horse

Numerous cases of PA toxicosis (acute and chronic) have been reported in horses after ingestion of PA-containing plants (Botha et al., 2012; Creeper et al., 1999; Crews & Anderson, 2009; de Lanux-Van Gorder, 2000; Fletcher et al., 2011b; Gherghiceanu et al., 2001; Lucena et al., 2010; Nobre et al., 2004; Passemard & Priymenko, 2007; Pearson, 1991; Pohlmann et al., 2005; Robinson & Gummow, 2015; Seawright et al., 1991a; Smith MRW et al., 2003; van Weeren et al., 1999; Wegge et al., 2009). The main reported symptoms include hepatic encephalopathy, liver parenchymal megalocytosis and fibrosis, head pressing, compulsive walking or circling, lameness and mortality. In most cases the presence of PAs in the plants consumed by the horses was confirmed by analysis. In addition, Seawright et al. (1991a) confirmed exposure to PAs by showing the presence of sulfur-bound pyrrolic metabolites on the haemoglobin.

In one study, poisoning was experimentally induced in one horse by administering 100 g of *Crotalaria retusa* seeds daily (see also section 2.2.2) resulting in the same clinical signs and pathology as described above, including death after 52 days (Nobre et al., 2004).

Liver biopsies that were taken from four horses suffering from PA toxicosis showed decreased expression of P-glycoprotein in bile canaliculi and intra-hepatic bile accumulation (cholestasis) (Wegge et al., 2009).

(c) Goat

Goats were affected by PA poisoning resulting from the consumption of seeds and leaves of *Crotalaria retusa* (Maia et al., 2013). They suffered from frothy salivation and lethargy, with death following in 13.3% of the animals within 24–48 hours. Histopathology of one goat showed accentuation of the lobular pattern of the liver with reddish/pale areas, centrilobular haemorrhagic necrosis of the liver, a focal area of haemorrhage on the liver surface with adhesions to peritoneum and diaphragm, multiple haemorrhages in subcutaneous tissue, abdominal external oblique muscle and greater omentum, reddish fluid present in the pericardial sac, and petechiae in the pericardium and endocardium. Analysis of the *C. retusa* seeds showed that monocrotaline was the predominant PA.

(d) Sheep

Although sheep are generally considered to be more resistant to PA poisoning than, for example, horses or cattle because of ruminal detoxification, a number of cases have been identified (Fletcher et al., 2011a). These were connected to severe shortage of forage and/or heavily infested land (Grecco et al., 2011; Ilha et al., 2001; Nobre et al., 2005). Both acute and chronic poisoning have been reported, characterized by clinical signs such as megalocytosis, proliferation of bile ducts and fibrosis or mortality (Giaretta et al., 2014; Grecco et al., 2011; Ilha et al., 2001; Lucena et al., 2010; Nobre et al., 2005; Riet-Correa et al., 2011).

(e) Buffaloes

One case was reported concerning 13 buffaloes that became ill after consuming *Senecio brasiliensis* in a time of severe drought, after foraging on a highly infested pasture. Eleven of the buffaloes died after showing clinical signs typical of PA poisoning, such as weakness, apathy, progressive weight loss, permanent decubitus and diarrhoea (Correa et al., 2008).

(f) Cows

PA poisoning in cattle has been reported by several authors (Bruckstein et al., 1996; Hill, Gaul & Noble, 1997; Karam & Motta, 2011; Karam et al., 2004; Lucena et al., 2010; Mendez & Riet-Correa, 1993; Moyano et al., 2006; Noble et al., 1994; Odriozola et al., 1994; Payne & Wight, 2013; Queiroz et al., 2013; Seawright et al., 1991b; Smith & Panariti, 1995; Shimshoni et al., 2015a; Stigger et al., 2014; Takeuti et al., 2011; Vos et al., 2002). The main clinical signs comprised progressive

emaciation, incoordination, tenesmus, diarrhoea, aggressiveness, depression and death. At necropsy, the main findings included a hard and enlarged liver with roughened capsular surfaces and slightly yellowish or reddish mottled cut surfaces, distended gallbladder, subcutaneous and mesenteric oedema, oedema of the abomasum, petechiae, ascites, and lesions in the digestive tract, kidneys and mesenteric lymph nodes. Histopathology showed fibrosis, megalocytosis and biliary duct proliferation of the liver, and spongy degeneration of the white matter in the brain. Besides the typical signs of PA poisoning, involvement of PAs was confirmed in several studies by the presence of PAs in the plants consumed or in contaminated feed, or by detection of pyrrolic metabolites in blood and liver samples (among others Karam et al., 2004; Noble et al., 1994; Seawright et al., 1991b; Shimshoni et al., 2015a; Stigger et al., 2014).

(g) Yaks

Two case reports of PA poisoning in yaks were available. PA poisoning started with characteristic skin lesions with hyaline parakeratosis, especially on the nose, the dorsal parts of the body and on the legs. At first, the hair became thinner and the skin roughened. Later, crusts were formed, which left a rough surface when removed. Similar lesions were also seen in the mucosa of the oral cavity. Several clinical symptoms may be observed, such as diarrhoea, abortion, premature or stillbirth, loss of milk production, etc., and several organs may be affected including lung, kidney and brain, but the main lesions were observed in the liver. This was mainly characterized by megalocytosis, fibrosis and proliferation of bile ducts. The PA poisoning was fatal in several animals (Mondal et al., 1999; Winter et al., 1990, 1992).

The involvement of PAs in these cases was confirmed by the detection of sulfur-bound pyrrolic metabolites in the blood and livers of affected animals and the presence of PA-containing plants in the pastures (Winter et al., 1990, 1992, 1993, 1994). Mondal et al. (1999) indicated that there is a lag period of about 25–30 days between consumption and appearance of symptoms. They suggest the accumulation of pyrrolic metabolites as an underlying cause.

(h) Wombats

Wombats were found to have extensive hair loss and dermatitis following PA ingestion. They were also jaundiced and thin or emaciated. A toxic hepatopathy, characterized by, among others, megalocytosis and multinucleation, cholestasis, bile duct proliferation and fibrosis, was identified accompanied by photosensitive dermatitis. Further investigations revealed the presence of PAs in faeces and gastrointestinal contents, consistent with the ingestion of *Heliotropium europaeum* (Woolford, Fletcher & Boardman, 2014).

2.4 Observations in humans

In 1988, WHO-IPCS concluded that exposure to PAs should be kept as low as practically achievable because of their known involvement in human poisoning and possible carcinogenicity. Human poisoning, with high mortality, usually manifested as acute veno-occlusive disease but could also manifest in a subacute way with vague symptoms and persistent hepatomegaly, and many cases progress to liver cirrhosis. Children were found to be particularly vulnerable.

In 2011, the EFSA issued a scientific opinion on PAs in food and feed (EFSA 2011). Effects on human health were mainly based upon large outbreaks of human poisonings including deaths associated with grain crops contaminated with PA-containing weeds, as well as case reports of poisonings due to PA-containing herbal medicines and teas. These reports have demonstrated the toxicity of PAs in humans, affecting predominantly the liver, characterized by acute hepatic veno-occlusive disease (HVOD). The acute disease is associated with high mortality, and a subacute or chronic onset may lead to liver cirrhosis. Taking into account the results from animal studies and the relevant mode of action, it is probable that PA exposure resulting in toxicity in humans could also lead to carcinogenicity. Exposure to PAs from herbal dietary supplements can potentially be much higher than dietary exposure and is known to have caused human illness.

2.4.1 Outbreaks

(a) Central India

An outbreak of HVOD probably caused by consumption of millet (*Panicum miliare*) contaminated with seeds of a local variety of the wild plant "jhunjhunia" (*Crotalaria* species) containing PAs was reported in the Sarguja district of Madhya Pradesh in central India in 1975; 28 (42%) of the 67 recorded cases died (Krishnamachari et al., 1977; Tandon et al., 1976). Histopathological studies of 11 patients (8 liver biopsies and 3 necropsies) revealed that the main findings were centrilobular hepatic necrosis and occlusion of the lobular and sublobular veins. Chemical analysis of the seeds of one dry plant of jhunjhunia indicated that they contained PAs closely similar (but not identical) to monocrotaline and fulvine (total amount of 5.3 g/kg of seeds expressed as monocrotaline). Assuming a maximum level of contamination with PA-containing seeds in the millet of up to 20 g/kg (106 mg PA/kg millet), and a daily average intake of 400 g millet/adult, it was estimated that the amount of alkaloid ingested was up to 40 mg per day, which corresponds to 0.66 mg PA/kg bw per day for a 60-kg adult. WHO-IPCS (1988) reported that crotananine and cronaburmine were the principal PAs.

(b) North-western Afghanistan

In an outbreak in north-western Afghanistan (Gulran district) many patients with massive ascites were identified during June to August 1975 (Mohabbat et al., 1976). Of the 7200 inhabitants of the affected villages, 22.6% showed evidence of liver disease, among whom 15% showed advanced disease; 21 patients were admitted to hospitals and three of them died. Pathological findings were based on the examination of 16 percutaneous liver biopsy specimens. The liver changes were regarded as characteristic of different stages of HVOD. Involvement of chemical fertilizers or pesticides was ruled out and aflatoxicosis seemed very unlikely. Examination of plants found to be growing extensively in the affected area, including the wheat fields, indicated that the outbreak was caused by consumption of bread made from wheat contaminated with the seeds of a weed, *Heliotropium popovi*, locally called charmac. Analyses carried out in two different laboratories found that the seeds contained PAs at concentrations reported to be 7.2 and 13.2–14.9 g/kg. Heliotrine and two other compounds similar to lasiocarpine were identified (75–100% as the *N*-oxides). The hepatotoxicity of crude extracts of seed was demonstrated in rats. The contaminated wheat contained an average of 300 mg of seeds per kg of wheat, and hence the PA concentration was 2.1–4.5 mg/kg wheat. The authors estimated that an adult consumed at least 700 g flour per day, containing approximately 2 mg of toxic alkaloids, which corresponded to 0.033 mg/kg bw per day PA for a 60-kg adult.

In the absence of any effective action in the interim, a second outbreak occurred from 1999 to 2001 with an estimated 400 cases and more than 100 deaths, and in February 2008, Afghanistan's outbreak and disease surveillance system was informed of a further outbreak of the so-called Gulran disease in the same district. To investigate this outbreak a case–control study was conducted among the inhabitants of the Gulran district, an area of approximately 150 km^2 with a population estimated at 110 000 people (Kakar et al., 2010). In total, 67 residents of the district were identified as having HVOD (onset of symptoms from October 2007 to February 2008) among 28 443 individuals and 3700 households surveyed using a standardized questionnaire. Three controls per case were selected: two matched by sex and age group from neighbouring households in the same village or from villages within the same district, neither of which reported cases, and another control from the same household as the case but not matched for age or sex; a total of 199 community controls were finally included. Wheat flour samples from affected households (n = 12) had median PA levels of 5.6 mg/kg wheat flour (0.16 mg/kg heliotrine, 5.4 mg/kg heliotrine-*N*-oxide and 0.045 mg/kg lasiocarpine) while PA levels in samples from control households (n = 20) were twofold lower (2.7 mg/kg wheat flour). However, since these data are based upon a relatively small sample, the difference was not statistically significant (P-value

0.1 for the Wilcoxon rank sum test). While "qurut" (whey) from the milk of goats was another minor source of PA exposure (median 0.09 mg PA/kg qurut), PAs were detected only at low levels or not detected at all in other foods and in drinking water. Although direct analysis of the bread consumed was not possible, contaminated wheat flour was the likely main source of PA causing the outbreak. This was supported by a positive association between odds of disease and the presence of PA-containing plants growing on the households' land (adjusted odds ratio (OR) = 8.5, 95% confidence interval (CI) 2.1–33.1). However, the most dramatic risk factor following multivariate analysis was frequent consumption of meals containing only bread, which was strongly associated with the disease (highest tertile adjusted OR = 35.8, 95% CI 7.6–168.2), while other nutritional factors were protective, including diets high in protein (adjusted OR = 0.1, 95% CI 0.02–0.9) and high in fruit (adjusted OR = 0.2, 95% CI 0.06–0.6). Following analysis of cases and controls from the same household (n = 19 of each) to reduce the impact of confounding at the household level, the positive association with frequent consumption of bread as the only food remained statistically significant (Mantel-Haenszel OR = 2.7, 95% CI 1.3–5.5). The daily intake of PAs was not estimated in this study. Overall, the authors concluded that prolonged, regular exposure to contaminated wheat in combination with a low-protein diet was chiefly responsible for clinical presentation. Those with a more varied diet were also less likely to be affected.

(c) Tajikistan

The Farkhar region of southern Tajikistan (approximately 98 000 inhabitants), was blockaded from May to November 1992. This led to a famine and a delay of 2 months in the wheat harvest. *Heliotropium lasocarpium* had time to grow in the fields and its seeds were therefore collected with the wheat. The contaminated wheat was distributed to the population, who milled it and made bread. The first case of liver toxicity occurred 6 weeks after the first consumption of the contaminated bread (Chauvin et al., 1994). Between October 1992 and March 1993, 3906 cases (2580 in those aged less than 15 years) had been recorded (attack rate 4%). Two of the 10 collective farms in the region were linked with 83.3% of the cases, with attack rates of 16.9% and 23.6%. The overall case fatality ratio was 1.3% (52 deaths) and increased with age. The liver injury was assumed to be HVOD, but a definitive diagnosis could not be established owing to the lack of liver biopsies. Therefore the case ascertainment was based upon clinical features. Four stages of illness were defined. Stage I was characterized by abdominal pain, nausea or vomiting and asthenia; all stage I patients (55.5%) recovered rapidly. Stage II was a combination of stage I and hepatomegaly (29.9%); stage III included ascites in addition to the symptoms of stages I and II (13.7%), and stage IV involved

alteration of consciousness (0.9%). It is not known how many patients went on to develop cirrhosis of the liver, for which HVOD is known to be an etiological factor. Wheat samples contained grains of *Heliotropium ellipticum*, of which *Heliotropium lasocarpium* is a variety. The average content was 510 mg *Heliotropium* per kg of flour, with 0.31% of PAs (PA concentration of 1.6 mg/kg wheat). The analytical method used, based upon Ehrlich's reagent, was able to identify unsaturated PAs and their *N*-oxides, and hence it is suitable for an untargeted PA-screening, but does not allow identification of the chemical structure of specific compounds. Accurate data on the consumption of contaminated flour by affected subjects were not available, but the authors estimated a daily consumption of 700 g for an adult, corresponding to approximately 360 mg of powdered grains of the plant, and 1.1 mg of PA per day (0.018 mg/kg bw for a 60-kg adult). Early in the outbreak, the population was informed of the danger of eating contaminated bread, but owing to the blockade and the consequent famine they continued to consume the contaminated flour. The local authorities started to exchange contaminated for uncontaminated flour in December 1992.

(d) Northern Iraq

From January to April 1994, 14 patients (eight males) were admitted to a hospital in Mosul with rapidly developing ascites and hepatomegaly; the 14 patients belonged to three Bedouin families (Altaee & Mahmood, 1998). A household survey among 45 families (556 members) in the area did not find any further cases. Seven of those hospitalized were children under the age of 15 years (age range 3–70 years). Liver biopsy from all patients confirmed that all of them had veno-occlusive liver disease. Two patients died of oesophageal bleeding. The outbreak was caused by the consumption of wheat accidentally contaminated with weeds. The plant isolated from the contaminated grain was identified as *Senecio vulgaris*. The presence of PAs in samples of the stored wheat and flour was confirmed by Erlich's reaction. Reliable estimates of the level of intake of PAs could not be obtained.

(e) Northern Ethiopia

Following the emergence of an unidentified fatal disease in Tahtay Koraro, rural farmers' village (Tsaeda Emba) in Tigray, northern Ethiopia since 2001, a team of experts investigated the problem in the field in November 2005. The study was a clinical-epidemiological survey using data collected from clinical histories, clinical evaluation of the affected cases in the area, relevant laboratory investigations and histopathological studies (Bane et al., 2012). Since the onset of the outbreak, up to 118 subjects were reported to have been affected by the illness, of whom 45 had died. At the time of the investigation, the study team evaluated

61 patients in the field (37 males and 24 females) with a mean age of 27 years (range 2–60 years). Duration of the illness ranged from 1 month to 3 years with a median of 6 months. The illness was reported to have started with abdominal cramp, febrile syndrome and abdominal distension. This was usually followed by jaundice, bleeding tendencies and oedematous states mainly in the form of rapidly filling ascites. Most patients had other family members affected by the same illness. Laboratory and sonography studies pointed to a disease spectrum from early hepatitis to chronic and terminal phases with complications, but also strengthened the clinical impression of noninfectious hepatitis. Postmortem liver biopsy of one patient (a 12-year-old male) revealed veno-occlusive disease of the liver with centrilobular haemorrhagic necrosis and congestion of the spleen (Schneider et al., 2012). A case–control study was undertaken to try to identify the probable etiological agent (Abebe et al., 2012), collecting data from the affected (case) area and a non-affected adjacent area (control). It was observed that almost all residents in the affected area relied on an unprotected well as a source of water for drinking and for other household purposes, while most people in the non-affected area obtained fresh water from a river or unprotected spring. Chemical and toxicological studies (Debella et al., 2012a,b) suggested that the intoxication stemmed from the consumption of the water from an unprotected well in which the PA-containing plant *Ageratum* grows abundantly. Accordingly, an immediate recommendation was made to ban the contaminated well water.

Although the disease was initially confined to Tseada Amba (also spelled Tsaeda Emba), cases have since been reported in adjacent villages within the same district of Kelakil and in the adjacent district of Kiburto in 2005. These villages are in the Hirmi Valley, and the disease is therefore referred to as Hirmi Valley liver disease; however, in 2008, cases were also reported in a village within 20 km west of the Hirmi Valley. Up to December 2009, the total number of reported cases was 591, including 228 deaths. From June 2008 to December 2009, a clinical and biomonitoring study was set up to characterize the clinical features of the disease, and to further explore the etiology of the outbreak (Robinson et al., 2012; Robinson et al., 2014). The full clinical histories of 32 patients were examined; nine patients underwent liver biopsy in hospitals; serum and urine samples were collected from cases and controls (healthy individuals from the affected villages). Samples of grains, millet and teff (a local gluten-free variety of millet) were collected from stores in six houses from the affected villages. Clinical presentation included epigastric pain, abdominal swelling, bloody diarrhoea, hepatomegaly, splenomegaly and ascites. Histology revealed acute injury characterized by centrilobular necrosis, or chronic injury with bile ductular reaction, cytomegaly and fibrosis but no hepatic vein occlusion. In phytochemical analysis, acetyl-lycopsamine, lycopsamine and intermedine were detected and quantified in three out of six grain samples; in the largest millet sample collected, 0.48 mg/kg of PA

was detected. Assuming a daily consumption of 700 g of millet for an adult, the estimated daily intake of PAs was 0.34 mg (0.006 mg/kg bw PA for a 60-kg adult). These PAs are known to be present in the invasive weed *Ageratum conyzoides*, which is abundant on the farmland of the affected villages.

Acetyl-lycopsamine was detected in urine samples taken from people living in the affected area. The concentrations were significantly greater (approximately 2-fold) in the 45 cases than in 43 controls. The relatively low toxicity of acetyl-lycopsamine and the unusual liver pathology of patients, atypical of the previously observed effects of PAs in humans, led the authors to hypothesize that an additional factor was involved in the pathogenesis of the disease. Residents of the affected areas reported adding dichlorodiphenyltrichloroethane (DDT) directly to stores of food grain to prevent storage pests. DDT and its metabolites are potent inducers of the hepatic enzymes CYP3A and CYP2B, which are responsible for the metabolic activation of PAs. Analysis of serum samples showed that 78% of them (31 out of 40) showed levels of total DDT above the upper detection limit (>125 ppb). Comparison of plate absorbance data indicated that the median concentration among 14 samples from cases was significantly greater ($P = 0.04$) than the concentration among 26 samples from controls. Furthermore, hepatotoxicity of DDT and acetyl-lycopsamine alone or in combination was explored in C57BL/6J mice. In mice, DDT (3 × 75 mg/kg) significantly increased the hepatotoxicity (measured by plasma ALT) of acetyl-lycopsamine (750 mg/kg), and in combination induced liver pathology similar to Hirmi Valley liver disease, including centrilobular necrosis and cytomegaly. The authors concluded that this form of liver disease appears to be caused by co-exposure to acetyl-lycopsamine and DDT. DDT availability has substantially declined since the Ethiopian authorities banned its use in 2009 and since 2010, only two further deaths have been attributed to Hirmi Valley liver disease.

2.4.2 Case reports

Two cases of poisoning were reported in infants from Arizona, USA, following ingestion of infusions of a herb called Gordolobo Yerba by the Mexican-American population and identified as *Senecio longilobus* (Fox et al., 1978; Huxtable, 1980; Stillman et al., 1977). The first case was a 6-month-old girl (body weight: 6 kg) who presented with portal hypertension and extensive hepatic fibrosis developed over 2 months, diagnosed with HVOD. PAs were found in the plant from which the infusion she had drunk was prepared and were largely identified as riddelliine and *N*-oxides of retrorsine, seneciphylline and senecionine. They were present in the herb at a concentration of 3 g/kg free alkaloid and 10 g/kg *N*-oxides. It was estimated that the girl received a total dose of between 70 and 147 mg of PAs during a period of 2 weeks, corresponding to an intake of 0.8–1.7 mg/kg

bw per day PA for the 6-kg girl, riddelliine and retrorsine being the principal alkaloids (WHO-IPCS, 1988). In another case, a 2-month-old boy (body weight unknown) was administered an infusion of the same herb for 4 days, after which he became progressively lethargic. At admission he was diagnosed with Reye's syndrome based upon clinical and laboratory findings, but subsequently he developed jaundice and possibly ascites. The infant's condition deteriorated and he died on the sixth day of hospitalization. At autopsy, extensive centrilobular haemorrhagic necrosis of the liver was seen; examination of the lungs revealed pulmonary oedema, atelectasis and necrotizing vasculitis. The sample of herb contained a PA concentration of 5 g/kg and a PA *N*-oxides concentration of 10 g/kg. It was calculated that the infant had probably consumed a total of 66 mg of mixed alkaloids over the 4-day period. Assuming the body weight of the boy to be 5.5 kg, this dose corresponds to an intake of 3 mg/kg bw per day PA, riddelliine and retrorsine being the principal alkaloids (WHO-IPCS, 1988).

From 1974–1977, three cases of HVOD were diagnosed in India. The affected patients had taken the same herb, *Heliotropium eichwaldii*, containing heliotrine in the form of the *N*-oxide at a concentration of 1–2% by weight. Approximate intake of heliotrine amounted to 200 mg per day (for a 60-kg adult corresponding to an intake of heliotrine of 3.3 mg/kg bw per day) in patients No. 1 and 2, and to 500 mg per day in patient No. 3 (for a 60-kg adult, corresponding to an intake of 8.3 mg/kg bw per day). The duration of treatment was 20 days in patient No. 1 and 50 days in patient No. 2; duration was not known for patient No. 3. Patients 1 and 2 presented with fulminant hepatic failure characterized by sudden onset of jaundice, abdominal pain, ascites, gastrointestinal bleeding and hepatic encephalopathy. Death occurred in the second week (patient No. 1) and within 12 weeks (patient No. 2). Cirrhosis of the liver was diagnosed in patient No. 3: the liver biopsy showed centrilobular haemorrhagic necrosis with reticulin collapse (Datta et al., 1978).

HVOD had been diagnosed as described by Kumana et al. (1983, 1985) in four young Chinese women with psoriasis who took herbal infusions, the toxic component of which has since been identified as *Heliotropium lasiocarpum* (Culvenor et al., 1986). The major alkaloids identified by MS and GC were heliotrine and lasiocarpine. The body weights of patients Nos 1, 2, 3 and 4 were 51, 61, 49 and 42 kg, respectively. Forty-five, 30 and 19 days after starting the herbal treatment, abdominal ascites and hepatomegaly developed in patients Nos 1, 2 and 3, respectively. Patients Nos 1 and 3 stopped drinking the tea when symptoms began and they experienced clinical and biochemical remission. The condition of patient No. 2, who continued taking the herbal remedy for 16 days after the onset of symptoms, deteriorated and she died of hepatic failure. Patient No. 4 stopped taking the herbal tea after 21 days. When assessed 77 days later, she had mild hepatomegaly only. The liver biopsies of all four patients revealed

histological features of HVOD. A detailed analysis of the alkaloid intake was carried out for each patient. The cumulative doses of alkaloid (base and *N*-oxide) ingested by patients Nos 1, 2, 3 and 4 were calculated to be 1350 mg over 45 days, 1380 mg over 46 days, 570 mg over 19 days, and 630 mg over 21 days, respectively (Culvenor et al., 1986; Kumana et al., 1983, 1985). These doses correspond to an intake of 0.59, 0.49, 0.60 and 0.71 mg/kg bw per day PA for patients Nos 1, 2, 3 and 4, respectively, heliotrine being the principal alkaloid (WHO-IPCS, 1988).

In a case report from the USA, HVOD was diagnosed in a 49-year-old woman. The patient had portal hypertension associated with obliteration of the smaller hepatic venules. A liver biopsy specimen showed centrilobular necrosis and congestion. Analysis of food supplements that the woman regularly consumed showed the presence of PAs. According to the authors, the major source was a powder purporting to contain ground comfrey (*Symphytum* spp.) root. For 4 months before admission, she had taken two capsules of "comfrey-pepsin" with each meal. Daily for 6 months before admission she had consumed a commercially available herbal tea. The herbal tea and the capsules were analysed for PAs using monocrotaline as a standard. PAs were found, but the compounds were not precisely identified. Based on the analysis of the PA content, the authors calculated that during the 6 months before she was hospitalized, the patient had consumed a total of at least 85 mg of PAs, corresponding to an intake of 0.015 mg/kg bw per day. The authors noted that the total PA consumption was relatively low and that it was possible that the patient had other sources of exposure and that she had probably been consuming PA-containing supplements for longer than established in the clinical history (Ridker et al., 1985).

A 13-year-old boy was admitted in July 1986 for investigation of hepatomegaly and ascites to a hospital in Bristol, England (Weston et al., 1987). Three years earlier, Crohn's disease had been diagnosed in the boy and treated with prednisolone and sulfasalazine with beneficial results. At his parents' request, these drugs were discontinued and he was treated with comfrey root prescribed by a naturopath. Up to 1986 he had been regularly given a herbal tea containing comfrey leaf. The exact quantities of leaves given and frequency of administration are unknown. On examination he had ascites and tender hepatomegaly but no dehydration, jaundice or heart failure and no stigmata of chronic liver disease. Percutaneous liver biopsy showed the thrombotic variant of HVOD. Common comfrey, *Symphytum officinale*, a native British plant, contains at least nine potentially hepatotoxic PAs in its leaves and roots.

A female neonate, aged 5 days, was admitted to intensive care with massive hepatomegaly, jaundice and ascites. An open liver biopsy was done when the infant was 27 days old, and she died 11 days later. Biopsy findings showed centrilobular fibrosis, neovascularization and iron deposition associated with widespread circumferential connective tissue occlusion of the small and

medium-sized hepatic veins suggesting a diagnosis of HVOD. The mother had daily consumed a herbal tea composed of 10 different plants, including *Tussilago farfara* (horsefoot, coltsfoot or coughwort). Chemical analysis by TLC showed a concentration of senecionine (measured with its *N*-oxide) of 0.60 mg/kg dry weight. Other PAs such as senkirkine could not be detected. No estimate of the dose to the mother or the fetus was reported. No abnormalities were seen during physical examination and blood tests on the mother (Roulet et al., 1988). In a comment to the previous report doubt was expressed as to whether the plant causing the illness was *Tussilago*, because no senkirkine, the major PA of that plant, was detected, but rather senecionine, which does not appear in European species (Sommer 1989). In an additional comment Spang (1989) reported that the herb containing PAs was not *Tussilago* but *Petasites officinalis*, whose roots were also present in the tea. Based on the data provided in the original report and some assumptions, it was estimated that if all the PA consumed by the mother throughout pregnancy was delivered to the fetus, by the time of its birth the baby had a cumulative exposure of 0.15 mg/kg of senecionine, a dose substantially lower than the estimates from other reports on toxic effects of PAs (Huxtable, 1989).

A 47-year-old woman, resident in New York (USA) in 1978, began to have vague complaints of abdominal pain and fatigue (Bach, Thung & Schaffner, 1989). Following the prescription of a homeopathic doctor she began consuming 10 cups of comfrey tea per day in addition to taking comfrey pills by the handful, which continued for more than 1 year. In 1986, she was hospitalized for massive ascites, hyponatraemia and confusion. Results from two liver biopsies (1987, 1988) revealed perivenular congestion with sinusoidal distension and dilatation, as well as non-thrombotic occlusion of small terminal hepatic venules by loose connective tissue; this pathological pattern suggests HVOD. An estimate of the amount of PAs in comfrey root tea yielded 26 mg per cup, while comfrey-pepsin capsules contain from 0.27 mg/g up to 2.9 mg/g total PAs. According to this estimate, the daily intake was 260 mg PA (4.3 mg/kg bw per day PA for a 60-kg adult) from tea alone. This intake is considerably higher than doses mentioned in previous reports on the harmful effects of comfrey (Ridker et al., 1985).

In Wellington, New Zealand, a 23-year-old male presented with a 3-month history of influenza-like symptoms followed by peripheral oedema and abdominal distension 3 weeks before admission to hospital (Yeong et al., 1990). On admission he had abdominal ascites, pleural effusions and hepatomegaly. The patient developed deep venous thrombosis in his left leg, with increasing ascites, deterioration of renal function and liver failure. A meso-atrial shunt was performed but the patient died 7 days post-intervention. Liver biopsy, liver angiography and autopsy showed histological features of veno-occlusive disease. In the 2 weeks before the onset of symptoms he ate young comfrey leaves steamed

and eaten as a vegetable (4–5 leaves every day). He took no alcohol or drugs apart from occasional marijuana before his illness.

In Southern Tyrol, Austria, veno-occlusive disease of the liver was diagnosed in an 18-month-old boy who had regularly consumed a herbal tea mixture since the third month of life (Sperl et al., 1995). The boy developed portal hypertension with severe ascites. He was given conservative treatment only and he recovered completely within 2 months. Histology of a thin-needle biopsy specimen showed centrilobular sinusoidal congestion with perivenular bleeding and parenchymal necrosis without cirrhosis. Despite the lack of a clear-cut vascular occlusion within the small biopsy, the diagnosis of acute veno-occlusive disease of the liver was established on the basis of the severe parenchymal changes. The tea contained peppermint and what the mother thought was coltsfoot (*Tussilago farfara*), but macroscopic and microscopic analysis of the leaf material indicated that *Adenostyles alliariae* had been erroneously gathered in place of coltsfoot. Seneciphylline and the corresponding *N*-oxide were identified as the major components by TLC, MS and NMR. It was calculated that the child had consumed at least 0.06 mg/kg bw per day of the PA mixture over 15 months.

A 73-year-old male was admitted to a hospital in Badajoz, Spain, with abdominal pain and distension and hepatomegaly. The liver biopsy revealed a histological pattern of HVOD. The patient was given conservative treatment and 1 month after discharge from hospital he was admitted to the emergency department with hepatic encephalopathy and died from massive gastric bleeding. During the 2 years prior to the onset of his illness, the patient had regularly taken two daily infusions of "hierba cana" (*Senecio vulgaris*) (Ortiz-Cansado et al., 1995).

In July 1994, a 25-month-old boy was admitted to a hospital in Rio Grande do Sul (Brazil), for investigation of ascites, hepatomegaly and restrictive respiratory failure (Magnabosco et al., 1997). The liver biopsy showed a histological pattern suggestive of HVOD. The child recovered completely after 6 months of conservative treatment. For about two years, the child had regularly been given the herbal tea "maria mole" (*Senecio brasiliensis*).

A 76-year-old female was admitted in May 1994 to a hospital in Tours, France, for investigation of potential heart failure; the examination revealed hepatomegaly without ascites (Bensaude et al., 1998). For 22 years, the patient had been regularly taking the herbal remedy Hérmoluol, containing *Senecio vulgaris*. This treatment was interrupted, and liver size was normal after 6 months. The patient had been taking about 1–2 blisters per month. Each blister contains 3 g of *Senecium* plus seven other compounds without known hepatotoxicity, as well as 3.3 g of alcohol. It was estimated that, over the 22 years, the patient had taken a cumulative dose between 396 and 792 mg of PA, or about 6.6–13.2 mg/kg.

A 23-year-old woman presented with abdominal distension and

ascites at a hospital in Corrientes, Argentina (Vilar, García & Cabrera, 2000). Histopathology of biopsied material revealed alterations characteristic of veno-occlusive disease and hepatic haemodynamic studies confirmed the diagnosis. The patient had been taking commercial preparations of herbal infusions that contained *Senecio vulgaris*. Outcome was favourable and periodic clinical check-ups were normal.

A 3.5-month-old female infant admitted to a hospital in Gauteng, South Africa, presented with diarrhoea, vomiting and ascites after having been administered a short course of treatment with a traditional herbal remedy (*muti*) (Zuckerman, Steenkamp & Stewart, 2002). The child developed peritonitis and septicaemia and died 3 months after admission. Postmortem liver histology confirmed the diagnosis of HVOD. Extracts of the traditional remedy were analysed by colorimetry and GC-MS. The colorimetric assay of an extract of *muti* indicated a yield of 1.0 mg/g, and of 1.2 mg/g by the method of Mattocks. In the analysis by GC-MS the PAs retrorsine, seneciphylline and platyphylline were identified. The results of chemical analyses of the *muti* were very similar to those obtained from the analysis of dried *Senecio latifolius*. Cell culture studies of human hepatoma cell lines indicated dose-related toxicity, with necrosis at high concentrations. The simple screening techniques used allowed rapid confirmation of the presence of toxic PAs in the remedy.

A pregnant 28-year-old woman was admitted to a hospital in Bonn, Germany, in the twenty-seventh week of gestation because of fetal ascites (Rasenack et al., 2003). During the thirty-second week, a male infant was delivered by emergency caesarean section (weight 2840 g) but died 12 hours later. At autopsy no internal or external malformations were detected. Liver histology showed HVOD. Teas used by the family were found to be free of PAs. However, a herbal mixture from Turkey, of which 2 g per day were used for daily cooking during the entire pregnancy, contained 6 mg/kg lycopsamine, 3.5 mg/kg interrimine and 3 mg/kg of their O7-acetyl derivatives. The patient had identified *Heliotropium* and *Symphytum* in the herbal mixture. The metabolites dehydrolycopsamine and dehydrointegerrimine were reportedly found in the fetal liver. Neither the maternal nor the fetal dose of total PAs resulting from the use of this mixture was estimated by the authors. However, assuming that the whole amount of the herbal mixture was consumed by the patient, her daily intake of PAs can be estimated as 0.025 mg; for a 70-kg weight (due to pregnancy), the estimated exposure was 0.00036 mg/kg bw per day PA. Since the herbal mixture was consumed during 27 weeks, the total amount of PAs ingested by the mother was 4.73 mg. Assuming that all the PA consumed by the mother throughout pregnancy was delivered to the fetus, by birth the baby had a cumulative exposure of 1.67 mg/kg. On the other hand, the fact that no gross malformations were noted suggests that it was PAs themselves that distributed to the fetus and formed reactive pyrroles in the

fetal liver, which was actually the site of toxicity. Owing to the estimated daily dose of 0.00036 mg/kg bw per day being only indicative in nature, the Committee did not use this dose for the evaluation.

The case histories of two sets of twins (age 1 month) admitted to a hospital in the region of Johannesburg, South Africa, who had been treated with traditional remedies are described by Conradie, Stewart & Steenkamp (2005). The herbal remedies were analysed using GC-MS. In one family, both siblings survived, albeit with hepatic damage. A diagnosis of HVOD was based upon clinical features and sonographic evaluation. In the other family, one twin died within 24 hours and the second 1 month after admission with a diagnosis of veno-occlusive disease of the liver associated with secondary necrosis diagnosed by autopsy. In both cases, the presence of the toxic PA retrorsine was identified in the traditional remedies. The level of the PA was much lower in the sample from the fatal case than from the survivors. These results, while seemingly contradictory, indicate that the children who died were being given higher or more frequent doses than those who survived. Unfortunately, the investigators were unable to obtain biological fluids or organs for examination. Of interest is the twin who died 24 hours after admission, who was reported to have had unspecified "respiratory problems". In rats, a PA (monocrotaline) has been shown to cause pneumotoxicity following transfer of active PA metabolites to the lung. Administration of monocrotaline or fulvine to experimental animals has been shown to induce pulmonary hypertension.

Three patients were admitted to a hospital in Zhejiang, China, in 2000 and 2002 (Dai HF et al., 2006), and in 2006 (Dai N et al., 2007). They were treated for HVOD after ingestion of *Gynura segetum*, a Chinese medicinal herb. The first patient was a 52-year-old man who presented with abdominal distension, ascites and hepatomegaly. Hepatic biopsy revealed histological features of veno-occlusive disease of the liver and necrosis of hepatocytes. He recovered after hepatic transplantation. He had been taking a decoction of *Gynura segetum* at a dose of 3 g per day. The second patient was a 39-year-old woman who presented with abdominal distension and hepatomegaly, but no ascites. Hepatic biopsy showed sinusoidal dilatation and necrosis of hepatocytes. She had taken a decoction of *Gynura segetum* at about 3 g per day for a period of 3 months. The third patient was a 62-year-old female with abdominal distension, hepatomegaly and ascites. Liver biopsies showed histological findings suggesting HVOD. She had ingested *Gynura* root (*tu san qi*) for 3 months before admission, at a dose of three slices of fresh *Gynura* root a day (about 2 g per day) soaked in rice wine. The authors indicated that six alkaloids have been isolated from *Gynura segetum*, two of which were identified as senecionine and seneciphylline, known to have hepatic toxicity.

A 66-year-old woman was admitted to the emergency department of

a hospital in Locarno, Switzerland, in November 2006 because of progressive dyspnoea (Györik & Stricker, 2009). Blood gas analysis showed severe partial respiratory insufficiency and laboratory analysis demonstrated a mild elevation of liver enzymes. A contrast-enhanced computed tomography (CT) scan of the chest excluded pulmonary emboli but showed mild polyserositis, and echocardiography revealed moderate pulmonary hypertension (PH). Two skilled pneumologists judged the CT images as not typical for pulmonary veno-occlusive disease. Despite extensive investigations, no explanation could be found and a final diagnosis of idiopathic PH was made. At follow-up the patient reported that she used a mixture of several herbs to make a tea; in the months prior to hospitalization she had drunk between one and one and a half litres per day of this tea. The tea contained a mixture of nine different herbal remedies, one of which was comfrey (*Symphytum officinale*). Although not proven, the authors suggested that the PH was possibly caused by the prolonged use of large quantities of boiled herbal remedies containing comfrey. One intriguing feature of this case was the mild polyserositis with ground glass opacities that disappeared after diuretics. One possible explanation is a capillary lesion produced by PAs, as has been reported in studies of monocrotaline-induced PH in rats. Another interesting aspect was the alteration of liver function without clear signs of decompensated right heart failure. Given the known hepatotoxicity, an association with the ingestion of comfrey cannot be ruled out, but no signs of HVOD could be seen either in the liver CT or in the magnetic resonance imaging (MRI) scan. Liver histology (biopsy) was not available.

A 63-year-old man presented to a hospital in Washington, DC, USA, with abdominal distension and ascites (Frist et al., 2010). Liver biopsy showed acute centrilobular necrosis, without inflammation or fibrosis, leading to a diagnosis of veno-occlusive disease of the liver. Despite diuretic therapy with initial clinical response, the patient's medical condition deteriorated and he developed fatal hepatic failure. He reported daily consumption of *Shen Chu Cha* tea, a supplement containing multiple herbs, often self-prescribed for general health. One of the ingredients is the root of Baikal skullcap (*Scutellaria baicalensis*). Although skullcap has not been associated with hepatic toxicity, skullcap harvests are often contaminated with germander, a plant containing the PA teucrin A. The authors did not report an estimate of the amount of tea consumed or the dose of PA, and there was no information on the actual composition of the herbal tea consumed by the patient.

A 54-year-old female complaining of abdominal distension and loss of appetite over the course of 4 months was admitted to a hospital in Shanghai, China. Physical examination revealed jaundice and ascites. Liver biopsy showed histological features indicating a diagnosis of hepatic sinusoidal obstruction syndrome (HSOS), previously called hepatic veno-occlusive disease (HVOD).

After treatment with diuretics, the patient had fully recovered 3 months after discharge from hospital (Lin et al., 2011). Before admission to hospital, she had consumed one to two cups of home-made herbal wine of *Tusanqi* daily for about 3 months to treat traumatic injuries. Cultivated herb from the patient's home was identified as *Gynura segetum*, an erroneous species substitute for *Sedum aizoon* used for the traditional Chinese medicinal herb *Tusanqi*. Two hepatotoxic PAs, senecionine and seneciphylline, were identified in *G. segetum* and its herbal wine by means of HPLC-UV–MS, but no PAs were found in *S. aizoon*. The concentrations of senecionine and seneciphylline in herbal wine made from *G. segetum* were 1.13 and 0.83 μg/ml, respectively. An LC-MS-based analytical approach was used to specifically determine serum PA–protein adducts. The same peak was observed in the chemically derivatized serum samples obtained from both the patient with HSOS and rats administered *G. segetum*, but no such peak was detected in either the serum samples obtained from human volunteers (blank control) or from rats treated with *S. aizoon* or vehicle. The animal study showed a good correlation of liver injury with the ingestion of *G. segetum*. The patient had continuously ingested home-made herbal wine containing *G. segetum* at approximately 50–100 ml per day for about 3 months. Based on the quantitative analysis of PA content in the wine, the estimated PA dose was approximately 98–196 μg per day (or 0.0016–0.0033 mg/kg bw per day). The protein adduct derived from reactive metabolites of PAs has been suggested to play a critical role in PA-induced liver toxicity. Hepatic tissue-bound PA metabolites, specifically the pyrrole–protein adduct, appear to be a reasonable biomarker of PA-induced HSOS.

A 63-year-old female was admitted to a hospital in Munich, Germany, with vomiting and abdominal pain, which started 3 hours after consumption of approximately 10 leaves (100 g) of self-collected *Petasites* and *Tussilago* (Schroff et al., 2013). Liver biopsy performed at day 14 found liver tissue with the characteristics of HVOD, showing intraluminal fibrin clots in the sinusoids. A liver biopsy 3 months later showed slight progression of the veno-occlusive disease of the liver.

A 10-month-old boy was admitted to a hospital in China, Hong Kong Special Administrative Region, in August 2009, with abdominal distension that had lasted for 1 week. Physical examination revealed hepatomegaly and a chest X-ray showed a right pleural effusion. His clinical (including imaging) features were compatible with Budd-Chiari syndrome. The patient responded well to treatment with diuretics and insertion of a chest drain. Repeat ultrasound of the abdomen 1 month later showed resolution of hepatic venous occlusion (Wu et al., 2013). Budd-Chiari syndrome is a rare disease in paediatric patients and an extensive workup performed to look for the underlying cause of Budd-Chiari syndrome was unrevealing, except for exposure to toxic PAs in herbal drinks. The mother had given the child herbal drinks for 3 months. The drinks were prepared

from plants grown in her garden that were boiled in water. She could not recall the exact frequency of administration or the amounts used. A pack of unused plants was examined and identified by a botanist to contain *Emilia sonchifolia* (tassel flower), *Elephantopus scaber*, *Centella asiatica* and *Hedyotis diffusa*. The toxic PA senkirkine was detected in the *E. sonchifolia* sample by LC-MS as well as by liquid chromatography time-of-flight mass spectrometry.

A 73-year-old man recently diagnosed with cryptogenic cirrhosis was admitted to a hospital in Los Angeles, USA, for progressively worsening ascites and altered mental status (Ng, Tran & Sundaram, 2014). MRI revealed hepatomegaly and heterogeneous signal intensity in the right hepatic lobe suggestive of an infiltrative malignancy. Liver biopsy demonstrated changes consistent with HSOS but no evidence of malignancy. The patient was placed on anticoagulants; however, he developed acute renal failure during his hospital stay and died shortly afterwards. The patient's family reported that he frequently ingested chipilin (*Crotalaria longirostrata*), a plant containing PAs.

A 43-year-old woman was admitted to a hospital in Geneva, Switzerland, 10 days after the onset of fatigue, vomiting and jaundice (Rollason, Spahr & Escher, 2016). She had no ascites or other signs or symptoms suggestive of chronic liver disease. She denied consuming any alcohol or illicit drugs. An infectious origin of the disease was ruled out. Abdominal ultrasonography showed that the liver size was normal. Serum bilirubin continued to rise to 138 μmol/L. A liver biopsy was performed; histological examination revealed extensive hepatocellular necrosis predominantly lobular, with portal inflammation composed of mononuclear cells and numerous polynuclear eosinophils, focal portal vein endotheliitis and signs of cholestasis. The patient reported having taken one tablespoon of home-made flower pollen daily for seasonal fatigue over a period of 3–4 weeks prior to the development of symptoms. The flower pollen was analysed. It contained a high concentration of echimidine (0.104 mg/kg), a PA with known hepatotoxicity. Assuming that a tablespoon contained about 15 g pollen, the patient had a daily intake of 0.0015 mg of PAs (or 26 ng/kg bw per day for a 60-kg adult). Phenotyping of CYP3A was performed and the patient was characterized as a rapid CYP3A metabolizer. Hepatotoxicity of the PA may be related to the formation of a reactive metabolite via CYP2B6 and CYP3A4. The authors concluded that the addition of the high echimidine concentration and the high metabolizing activity of CYP3A may have led to severe liver injury. It should be noted that the "severe liver injury" was never referred to by the authors as either veno-occlusive disease or sinusoidal obstructive syndrome. The histology showed some features found in these syndromes, such as the hepatocellular necrosis, predominantly lobular, but the overall morphological pattern did not correspond to HVOD or SOS.

2.4.3 Case series

In a study carried out in two hospitals in South Africa the authors identified 20 children suffering from HVOD thought to be caused by the administration of traditional remedies (*muti*) (Steenkamp, Stewart & Zuckerman, 2000). The female to male ratio was 1:1 and the mean age at admission was 2 years and 3 months (range, 1 month–7 years). The predominant clinical presentation was ascites of various degrees and hepatomegaly. Histological findings in all of the biopsy specimens were in keeping with veno-occlusive disease, the majority showing centrilobular congestion with sinusoidal distension and dilatation. There was a high morbidity and mortality in the young infants. Previous consumption of *muti* was confirmed or suspected in 15 out of the 20 patients. In four cases an on-admission urine specimen was available. In all of these, a simple colorimetric screening test (a modification of the method of Birecka et al. used for the detection of PAs in plants) confirmed the presence of PAs. Urine specimens from 40 healthy volunteers and also from 10 patients with liver disease of various etiologies were also analysed, but none of these specimens were positive for PAs, even in the presence of severe hyperbilirubinuria.

Eighteen cases diagnosed as *Gynura segetum*-induced HSOS from August 2009 to February 2011 in a hospital in Hangzhou, China, were retrieved through a search of medical records with the aim of describing the main CT and MRI features (Zhou H et al., 2014). None of the patients had liver disease before taking *Gynura segetum*, and the interval between consumption of *Gynura segetum* and initial CT or MRI examination ranged from 1 month to 6 months. The severity of the disease was evaluated radiologically, based on the abnormal hepatic patchy enhancement in post-contrast CT or MRI scans. Patients were also classified into three categories (mild, moderate or severe) based on their clinical presentations and outcomes. Ascites, patchy liver enhancement and narrowing of the main hepatic veins were the most frequent signs of herbal medicine induced HSOS.

2.4.4 Biomarkers

Measurement of pyrrolic species covalently bound to hepatic and serum proteins as a potential biomarker for PA metabolic activation associated with hepatotoxicity in experimental animals and humans has been reported (Lin et al., 2011) (details of this work are reported in section 2.4.2). Blood and liver tissue were collected from a 54-year-old female from Shanghai, China, diagnosed with HSOS attributed to previous consumption of *Gynura segetum*, a hepatotoxic herb containing senecionine and seneciphylline, erroneous species substitute for *Sedum aizoon,* which is used for the traditional Chinese medicinal herb *Tusanqi*. An LC-MS-based analytical approach was developed to specifically determine serum pyrrole–protein adduction. Individual serum samples were treated with silver nitrate ($AgNO_3$) in ethanol to release pyrroles

from the adducted proteins, followed by reaction with 3,5-diaminobenzoic acid to produce another compound, which was subjected to ultra-high-performance liquid chromatography (UHPLC)-MS analysis, with the mass spectrometer operated in positive ion mode with electrospray ionization (ESI) interface and mass range of 100–600 m/z. The same procedure was also followed for the chemical derivatization of pyrrole–GSH conjugate, a common metabolite of PAs. A peak with m/z 341 at a retention time of 2.2 minutes was observed in the chemically derivatized serum samples obtained from both the HSOS patient and from rats administered *G. segetum*. However, no such peak was detected in either the serum samples obtained from human volunteers (blank control) or from rats treated with *S. aizoon* or vehicle. These results verified the identity of pyrrole-derived serum protein adducts formed in the HSOS patient and the rats treated with *G. segetum*. Moreover, the observed pyrrole–protein adducts in the blood of rats treated with *G. segetum* appeared to gradually vanish from 100% (day 1) to 64.5% (week 1), 15.2% (week 2), 10.7% (week 3), to undetectable (week 4). The protein adduct derived from reactive metabolites of PAs has been suggested to play a critical role in PA-induced liver toxicity. Hepatic tissue-bound pyrrolic metabolites, specifically the pyrrole–protein adduct, appear to be a reasonable biomarker of PA-induced HSOS. Using this new analytical approach, pyrrole–protein adducts in the serum of an HSOS patient exposed to PA-containing *G. segetum* have been unequivocally identified. This is solid evidence for the involvement of metabolic activation of PAs in the development of HSOS induced by PAs. It provides direct support for the correlation between HSOS and PA intake. Based on the quantitative analysis of the PA content of the *G. segetum* wine, the estimated PA dose was about 98–196 μg of PA per day.

Using the technique developed in the previous work to detect pyrrole–protein adducts in blood, six consecutive patients taking *G. segetum* who were diagnosed with HSOS and admitted to a hospital in Shanghai, China, from January 2006 to August 2010 were studied (Gao et al., 2011; Gao et al., 2012). HSOS was diagnosed from its clinical features and using imaging techniques, although histological confirmation was also available from a liver biopsy in one patient. In addition to pyrrole–protein adducts in blood samples, PA in herbal preparations was detected by means of HPLC-UV. The patients (one male and five females) were 41–72 years old; five of them had ingested *G. segetum* for periods ranging from 3 weeks to 3 months, while the sixth had taken the herb for 2 years. Protein–pyrrole adducts were unequivocally identified in all six serum samples. Two PAs, senecionine and seneciphylline, were detected in herbal preparations provided by three of the patients. No estimation of the actual dose of PAs consumed by each patient was reported.

In another study, the presence of PAs was confirmed in the fetal liver

tissue from the autopsy of a male infant with HVOD delivered by caesarean section at the thirty-second week of gestation. The mother had consumed a herbal mixture containing *Heliotropium* and *Symphytum* during pregnancy (Rasenack et al., 2003) (details of this work are reported in section 2.4.2). The extract of liver tissue was evaporated to dryness and analysed by GC and GC-MS. The metabolites dehydrolycopsamine and dehydrointegerrimine were found in the fetal liver. However, although analysis and quantification of PAs was carried out in samples of the herbal mixture consumed by the mother, neither the maternal nor the fetal dose of total PAs resulting from the consumption of this mixture was estimated.

Identification of PAs in urine has also been used as a potential biomarker of PA exposure. For instance, an on-admission urine specimen was available from four of the 15 children with HVOD caused by ingestion of the traditional remedy *muti* in South Africa (Steenkamp, Stewart & Zuckerman, 2000) (details of this work are reported in section 2.4.3). A simple colorimetric screening test (a modification of the method of Birecka et al. used for the detection of PAs in plants) confirmed the presence of PAs. Urine specimens from 40 healthy volunteers and from 10 patients with liver disease of various etiologies were also analysed, but none of these specimens were positive for PAs, even in the presence of severe hyperbilirubinuria. The screening method is helpful for the detection of acute ingestion of PAs in large amounts, but is not sufficiently sensitive for the detection of PAs following chronic ingestion of smaller amounts.

Urine samples were also collected in the investigation of the outbreak of Hirmi Valley liver disease in northern Ethiopia (Robinson et al., 2014). Urine samples were analysed by UHPLC-MS. Data were available from samples obtained from 45 subjects with Hirmi Valley liver disease (cases), 25 household control samples, 18 village control samples and the acetyl-lycopsamine (AL) reference standard. AL eluted at approximately 3.9 minutes, with a dominant ion [M+H]+, at 342.19 m/z, which corresponds to the calculated monoisotopic mass of 341.18. AL was detectable in the majority of urine samples. The median relative AL concentration (RT-m/z ion intensity) was significantly greater among cases than in village controls ($P = 0.006$) or all controls combined ($P = 0.02$). No significant difference was found between cases and household controls ($P = 0.33$). A significant trend ($P = 0.007$) was observed with the greatest median intensity among cases, which was higher than among household controls, which in turn was higher than among village controls. Hirmi Valley liver disease has a histological pattern similar to HVOD, with acute injury characterized by centrilobular necrosis, or chronic injury with bile ductular reaction, cytomegaly and fibrosis but no hepatic vein occlusion. AL, as well as lycopsamine and intermedine, were detected in phytochemical analysis of the grain samples. Given the relatively low toxicity of AL and the unusual liver pathology, it has been suggested that this

form of liver disease appears to be caused by co-exposure to AL and DDT (see section 2.4.1).

2.5 Concluding remarks

The effects of PAs on human health have been mainly based upon the findings from outbreaks associated with grain crops contaminated with PA-containing weeds and on case reports of poisonings by herbal remedies or teas. In the vast majority of instances the toxicity primarily affected the liver, with a characteristic pattern of sinusoidal obstruction syndrome (SOS), usually termed hepatic veno-occlusive disease (HVOD). The causal link with PA-containing plants is supported in most instances by several criteria: clear identification of the outcome (in many instances based upon pathological findings); temporality (documented exposure to the suspected toxicant days to months prior to the onset of the disease); identification of a PA-containing plant (and in some instances identification of the PAs), and finally, other potential causes of the outcome were ruled out (infectious diseases, other hepatic toxicants such as alcohol, drugs and aflatoxins). A summary of the main features of the studies reviewed is presented in Table 27 (outbreaks) and Table 28 (case reports).

Six large outbreaks were reported, five in four Asian countries and one in Africa. The outbreak in central India in 1975 (Krishnamachari et al., 1977; Tandon et al., 1976) was caused by the consumption of millet contaminated by *Crotalaria*. The characterization of HVOD was based on pathological studies and the plant seeds were subjected to chemical analysis. The estimated dose of PA was 0.66 mg/kg bw per day; however, there was no clear indication of time from exposure to the identification of the first clinical cases or time from the onset of symptoms to liver biopsy. Furthermore, no details were provided on the analytical method used to identify PAs. The outbreak in 1973–1975 in Afghanistan (Mohabbat et al., 1976) was caused by the consumption of wheat flour contaminated with *Heliotropium popovii*. The estimated intake of PA was 0.033 mg/kg bw per day. The duration of exposure before the onset of the outbreak was about 2 years, the duration of the disease was from 1 to 6 months, and the time from onset of the disease to the biopsy was 1–9 months. The pathology findings therefore reflect both the early and advanced stages of HVOD. Details of the analytical methods used to identify PA were not provided but the hepatotoxicity of seeds was confirmed in rats. In a second outbreak in the same region in 2007 and 2008 (Kakar et al., 2010) cases were identified based only upon clinical findings, but the PAs heliotrine (and its *N*-oxide), and lasiocarpine were identified by means of LC-MS/MS. Wheat flour contaminated with another species of the same plant (*Heliotropium lasocarpium*) was the cause of the outbreak in 1992 and 1993 in Tajikistan (Chauvin, Dillon &

Table 27

Summary of observations in humans – outbreaks

Reference	Country, year	Population	Source	Plant	PA	Effect	Association by	Estimated dose	Remarks
Tandon et al. (1976); Krishnamachari et al. (1978)	India (Sarguja) 1975	67 cases, age 3–60+ years	Contaminated millet	Crotalaria	Crotananine, cronaburmine, monocrotaline	HVOD, 42% died; histopathological studies	Reported intake of millet and chemical analysis of seeds	0.66 mg/kg bw per day PA for a 60-kg adult	Dose estimated from assumed average intake
Mohabbat et al. (1976)	Afghanistan (Gulran) 1974	≈ 7000 inhabitants, age <14–80 years	Contaminated wheat flour	*Heliotropium popovii* (charmac)	Heliotrine	HVOD (in 23%; 14 biopsies, 8 autopsies)	Reported intake of bread and chemical analysis of seeds	0.033 mg/kg bw per day PA for a 60-kg adult	Dose estimated from assumed average intake
Kakar et al. (2010)	Afghanistan (Gulran) 2007–2008	67 cases and 199 controls, mean age 25 years, 60% male	Contaminated wheat flour	*Heliotropium popovii*	Heliotrine, lasiocarpine, heliotrine-*N*-oxide	HVOD (clinical: acute massive ascites)	Reported intake of bread and analysis of wheat flour samples	Not given	Intake; bread consumed 3+ times/day, odds ratio (OR) 35.8 (7.6–168)
Chauvin, Dillon & Moren (1994)	Tajikistan (Farkhat region) 1992–1993	3906 cases (2580 below 15 years)	Contaminated wheat flour	*Heliotropium lasocarpium*	No specific PAs identified	HVOD (based upon clinical features)	Reported intake of bread, analysis of contaminated wheat grains	1.1 mg/day PAs (0.018 mg/kg per day for a 60-kg adult)	Estimated average intake 360 mg/day of Heliotropium
Altaee & Mahmood (1998)	Iraq (Mosul) 1994	14 cases; 8 males, 7 aged <15 years	Contaminated wheat flour	*Senecio vulgaris*	No specific PAs identified	HVOD (liver biopsies)	Reported intake of bread and analysis of wheat	Not given	
Bane et al. (2012); Schneider et al. (2012); Abebe et al. (2012); Debella et al. (2012a,b)	Northern Ethiopia (Tigray, rural farmers) 2001–2005	118 cases reported; 61 cases studied (37 males), mean age 27 years	Drinking water from an unprotected well	*Ageratum conyzoides*	No specific PAs identified	HVOD, postmortem liver biopsy of one case	Reported drinking water consumption	Not given	
Robinson et al. (2012, 2014)	Northern Ethiopia (Hirmi Valley) (including Tigray) 2005–2009	591 cases; 228 deaths; clinical study of 32 cases (9 biopsies)	Grains, millet and teff (local gluten-free variety of millet)	*Ageratum conyzoides*	Acetyl-lycopsamine, lycopsamine, intermedine (+DDT)	Hirmi Valley liver disease	Reported intake of grains and teff, phytochemical analysis of the grain samples	Not given – 0.48 µg/g of PAs in the plant	Further analyses: PA in urine, DDT in serum, hepatotoxicity in mice

DDT: dichlorodiphenyltrichloroethane; HVOD: hepatic veno-occlusive disease.

Table 28
Summary of observations in humans – case reports

Reference	Country, (year)	Subjects	Source	Plant	PA	Effect	Association by	Estimated dose	Remarks
Stillman et al. (1977); Fox et al. (1978); Huxtable (1980)	Arizona, USA	Female infant aged 6 months; 6 kg weight	Infusion of herb	Gordolobo Yerba (*Senecio longilobus*)	Riddelliine, retrorsine, and possibly seneciphylline, senecionine	Hepatic veno-occlusive disease (HVOD) (biopsy)	Reported intake of infusion of herb, analysis of infusion	0.8–1.7 mg/kg bw per day PA during 2 weeks	Concentration in herb: 3 g/kg free PAs and 10 g/kg *N*-oxides
		2-month-old boy (weight unknown)				Reye's syndrome, HVOD (autopsy)		3 mg /kg bw per day PA, during 4 days (assuming 5.5 kg weight)	Concentration in herb: 5 g/kg free PAs and 10 g/kg *N*-oxides
Datta et al. (1978)	India	3 adults (assumed 60 kg body weight)	Infusion of herb	*Heliotropium eichwaldii*	Heliotrine (as *N*-oxide), 1–2% by weight	HVOD (biopsy)	Reported intake of infusion of herb	3.3 (2 patients) and 8.3 mg/kg bw per day PA (1 patient)	Not reported if analysis of infusion was performed
Kumana et al. (1983, 1985); Culvenor (1986)	China, Hong Kong Special Administrative Region	4 Chinese women (ages 23–28 years)	Herbal infusions (treatment of psoriasis)	*Heliotropium lasiocarpum*	Heliotrine and lasiocarpine	HVOD (biopsy)	Intake, plus biochemical analysis of the herbal tea	0.59, 0.49, 0.60 and 0.71 mg/kg bw per day PA	Biochemical analyses; mass spectrometry confirmed by gas chromatography
Ridker et al. (1985)	Arizona, USA	49-year-old woman	Capsules of "comfrey-pepsin" and commercial herbal tea	Ground comfrey root (*Symphytum* spp.)		HVOD (biopsy)	Intake, plus biochemical analysis of the capsules and herbal tea	15 µg/kg bw per day (over a 6-month period)	Biochemical analyses detected PA and *N*-oxides; no specific PAs
Weston et al. (1987)	Bristol, England (1986)	13-year-old boy	Herbal tea	Comfrey (*Symphytum officinalis*)		HVOD (liver biopsy)	Reported intake of herbal tea	Unknown	
Huxtable (1989); Roulet et al. (1988); Sommer (1989); Spang (1989)	Switzerland (1988)	5-day-old female infant	Herbal tea	*Petasites officinalis, Tussilago farfara*	Senecionine	HVOD (liver biopsy)	Reported intake by mother during pregnancy	0.125 mg/kg cumulative exposure at birth	Chemical analyses of the (unprepared) tea

Reference	Country, (year)	Subjects	Source	Plant	PA	Effect	Association by	Estimated dose	Remarks
Bach, Thung & Schaffner (1989)	New York, USA (1986)	47-year-old woman	Herbal tea and pills	Comfrey (*Symphytum officinalis*)		HVOD (liver biopsy)	Reported intake	26 mg PA per cup; pill: 2.9 mg/g PA	
Yeong et al. (1990)	Wellington, New Zealand, (1890?)	23-year-old man	Steamed leaves	Comfrey (*Symphytum officinalis*)		HVOD (liver biopsy and autopsy)	Reported intake of leaves eaten as a vegetable		4–5 leaves/day during 2 weeks
Sperl et al. (1995)	South Tyrol, Austria, (1993–1994)	18-month-old boy	Herbal tea	*Adenostyles alliariae* (Alpendost)	Seneciphylline (inc. *N*-oxide)	HVOD (liver biopsy)	Intake, plus biochemical analysis of the herbal tea	60 µg/kg bw per day PA over 15 months	Mistaken for *Tussilago farfara* (coltsfoot)
Ortiz-Cansado et al. (1995)	Badajoz, Spain (1993–1994)	73-year-old man	Herbal tea	*Senecio vulgaris*		HVOD (liver biopsy)	Reported intake of herbal tea		2 infusions daily of "hierba cana"
Magnabosco et al. (1997)	Rio Grande do Sul, Brazil (1994)	29-month-old boy	Herbal tea	*Senecio brasiliensis*		HVOD (liver biopsy)	Reported intake of herbal tea		Regular intake of herbal tea "maria mole"
Bensaude et al. (1998)	Tours, France (1994)	76-year-old woman	Hemoluol (herbal remedy)	*Senecio vulgaris*		HVOD (liver biopsy)	Reported intake of hemoluol	6.6–13.2 mg/kg bw over a 22-year period	Hemoluol, 1–2 blisters per month
Vilar, García & Cabrera (2000)	Corrientes, Argentina	23-year-old woman	Herbal tea	*Senecio vulgaris*		HVOD (liver biopsy)	Reported intake	Unknown	
Zuckerman, Steenkamp & Stewart (2002)	Gauteng, South Africa	3.5-month-old female infant	Traditional remedy (muti)	*Senecio latifolius* (hypothesized)	Retrorsine, seneciphylline, platyphylline	HVOD (autopsy)	Intake, plus biochemical analysis of the herbal tea	Not estimated	1–1.2 mg/kg in muti (herbal remedy)
Rasenack et al. (2003)	Bonn, Germany	Newborn (27th week of gestation)	Herbal mixture	*Heliotropium*, *Symphytum*	Lycopsamine, interrimine, and their O7-acetyl derivatives	HVOD (autopsy)	Intake, plus biochemical analysis of the herbal tea	Not estimated	3–6 mg/kg of PAs or O7-acetyl-PAs; PA metabolites in fetal liver as well
Conradie, Stewart & Steenkamp (2005)	Johannesburg, South Africa	Two pairs of 1-month-old twins (boy and girl)	Traditional remedies (herbal tea)	–	Retrorsine	HVOD (by sonography and autopsy)	Intake, plus biochemical analysis of the herbal tea	Not estimated	PA determined by GC-MS; quantification not provided

Table 28 (continued)

Reference	Country, (year)	Subjects	Source	Plant	PA	Effect	Association by	Estimated dose	Remarks
Dai HF et al. (2006); Dai N et al. (2007)	Zhejiang, China (2000, 2002, 2006)	52-year and 62-year-old females; 39-year-old male	Chinese medicinal herb	*Gynura segetum*	Senecionine, seneciphylline, other PA	HVOD (liver biopsy)	Reported intake of herbal tea	2–3 g/day of *Gynura* root over a 3-month period	No reported analyses of the medicinal herbs consumed
Györik & Stricker (2009)	Locarno, Switzerland (2008)	66-year-old woman	Herbal tea	Comfrey (*Symphytum officinalis*)		Idiopathic pulmonary hypertension	Reported intake of herbal tea	Not estimated	No reported analyses of the herbal tea
Frist et al. (2010)	Washington, DC, USA	63-year-old man	Shen Chu Cha tea	Germander (*Teucrium* spp.), herb contaminating skullcap)	Teucrin A (hypothesized)	HVOD (liver biopsy)	Reported intake of herbal tea	Not estimated	No reported analyses of the herbal tea
Lin et al. (2011)	Shanghai, China	54-year-old woman	Herbal wines (*Tusanqi?*)	*Gynura segetum* (erroneous substitution of *Sedum aizoon*)	Senecionine, seneciphylline	Hepatic sinusoidal obstruction syndrome (HSOS)	Intake, chemical analysis of herbal wine	98–196 µg/day (1.63–3.27 µg/kg bw, assuming body weight 60 kg)	Further analysis of PA adducts in serum proteins and hepatotoxicity studies in rats
Schroff et al. (2013)	Munich, Germany	63-year-old woman	Korean dish	*Tussilago and Petasites*		HVOD (liver biopsy)	Reported intake	100 g of leaves	No analyses of the leaves consumed
Wu et al. (2013)	China, Hong Kong Special Administrative Region, (2009)	10-month-old boy	Herbal drinks	*Emilia sonchifolia* (tassel flower)	Senkirkine	Budd-Chiari syndrome	Reported intake	Not reported	PA identified by LC-MS in plants consumed
Ng, Tran & Sundaram (2014)	Los Angeles, USA	73-year-old man	Herb (chiplin)	*Crotalaria longirostrata*		HSOS (liver biopsy)	Reported intake	Not reported	Preparation of herb not reported
Rollason, Spahr & Escher (2016)	Geneva, Switzerland	43-year-old woman	Home-made flower pollen	Not specified	Echimidine	Severe liver injury (liver biopsy)	Intake, chemical analysis of flower pollen	Not reported	Concentration in pollen: 104 mg/kg echimidine

GC-MS: gas chromatography–mass spectrometry; HVOD: hepatic veno-occlusive disease; LC-MS: liquid chromatography–mass spectrometry

Moren, 1994). The estimated dose of PAs was 0.018 mg/kg bw per day. The time from start of exposure to the first clinical case was about 6 weeks (the HVOD diagnosis was based upon clinical features). Wheat samples were analysed. The analytical method used was able to identify PAs and their *N*-oxides, but did not allow identification of the specific PA. Wheat flour contaminated with *Senecio vulgaris* was the cause of the outbreak of 1994 in Iraq (Altaee & Mahmood, 1998). Fourteen cases of HVOD were ascertained by liver biopsy; the average time from onset of clinical symptoms to biopsy/exploration was 2 weeks. Similarly to the previous report, the analytical method confirmed the presence of PAs in the flour but was unable to identify specific PAs. Finally, a huge outbreak was reported in 2001–2005 in northern Ethiopia (Bane et al., 2012). Clinical features of cases were compatible with HVOD, but pathological information was available only from the postmortem biopsy of one case. The duration of the illness ranged from 1 month to 3 years, with a median of 6 months. The most likely cause was found to be drinking water from an unprotected well contaminated with *Ageratum* plants. New cases of the disease were detected during the period 2005–2009, mostly in the Hirmi Valley region (Robinson et al., 2012; Robinson et al., 2014). Liver biopsies from some patients revealed centrilobular injuries compatible with SOS, but the histological pattern was slightly different from typical HVOD, and the illness was named Hirmi Valley liver disease. Those affected by the disease had consumed significantly greater amounts of millet contaminated with the invasive weed *Ageratum conyzoides*, and higher concentrations of the PAs acetyl-lycopsamine, lycopsamine and intermedine were found in urine. The disease was attributed to co-exposure to acetyl-lycopsamine and DDT. Neither the time pattern of the outbreak nor an estimate of the dose of PAs consumed was available.

A total of 24 case reports of poisoning by PA-containing teas or herbal remedies were identified; in all but one, the adverse health effect was hepatotoxicity. One case of Bud Chiari syndrome was reported and in another the outcome was described as "severe liver injury"; in the remaining 22 cases toxic effects on the liver were described as veno-occlusive disease or SOS, the vast majority of which were based upon histopathology findings. The species of PA-containing plants most often involved were *Symphytum* (7 cases) and *Senecio* (6 cases); other plant species reported were *Heliotropium*, *Tussilago*, *Petasites*, *Gynura*, *Adenostyles*, *Emilia* and *Teucrium*. Only in six case reports of histologically confirmed HVOD was the information detailed enough to verify involvement of PAs by chemical analysis of the product consumed by the patient, and to provide an estimate of the dose of PAs based on reported intake of the plant or herbal tea, and the duration of exposure.

In Arizona, USA, a female infant aged 6 months was exposed to an estimated dose of PAs of 0.8–1.7 mg/kg bw per day for a period of 2 weeks (Huxtable, 1980; Stillman et al., 1977), and a 2-month-old boy was exposed to

an estimated dose of 3 mg/kg bw per day over 4 days (Fox et al., 1978; Huxtable, 1980). In both cases the imputed PAs were riddelliine, retrorsine, and possibly seneciphylline and senecionine, contained in *Senecio longilobus*. Four Chinese women aged 23–28 years developed HVOD after consuming a herbal tea containing the plant *Heliotropium lasiocarpum* for 19, 21, 45 and 46 days (average 33 days); the estimated doses of PAs (Culvenor et al., 1986; Kumana et al., 1983, 1985) were, respectively 0.60, 0.71, 0.59 and 0.49 mg/kg bw per day (average 0.60 mg/kg bw per day). GC-MS identified heliotrine and lasiocarpine as the major alkaloids. During the 6 months prior to her hospitalization, a 49-year-old woman from Arizona, USA, had consumed a herbal tea made of the roots of *Symphytum* (Ridker et al., 1985). The estimated dose of PAs was 15 µg/kg bw per day. The analysis was based on a colorimetric procedure using monocrotaline as a standard, but the specific compounds present in the extract could not be precisely identified. An 18-month-old boy had been consuming a herbal mixture containing leaves of *Adenostyles alliariae* over 15 months, at an estimated dose of PAs of at least 60 µg/kg bw per day (Sperl et al., 1995). TLC, MS and NMR spectroscopy identified seneciphylline and the corresponding *N*-oxide as the major components of the tea. Finally, a 54-year-old female had consumed a home-made herbal wine containing *Gynura segetum* for about 3 months (Lin, 2011). Senecionine and seneciphylline were identified in the herbal wine by means of HPLC-UV–MS. The estimated dose of PAs was about 1.63–3.27 µg/kg bw per day. A LC-MS-based analytical approach showed the presence of serum PA–protein adducts in this patient.

Extrahepatic toxic effects in humans have seldom been reported. Only one article has described a case of pulmonary hypertension, in a 66-year-old woman (Györik & Stricker, 2009). This was possibly caused by prolonged consumption of large quantities of boiled herbal remedies containing comfrey (*Symphytum officinale*). No estimate of the amount consumed or any analysis of the tea were available. There was no evidence of liver toxicity. Some features of the pulmonary lesions found are similar to those reported in studies of monocrotaline-induced pulmonary hypertension in rats. In two case reports of hepatotoxicity, "respiratory problems" were also mentioned. In a 2-month-old boy with a diagnosis of Reye's syndrome and HVOD attributed to poisoning by riddelliine and retrorsine contained in *Senecio longilobus*, pulmonary oedema, atelectasis and necrotizing vasculitis were found at the autopsy (Fox et al., 1978). Later, a 1-month-old boy died within 24 hours after admission to hospital with a diagnosis of HVOD caused by a herbal remedy containing retrorsine (estimated dose not available); the child was reported to have had unspecified "respiratory problems", although no specific diagnosis was provided (Conradie, Stewart & Steenkamp, 2005). Although pulmonary hypertension caused by PAs has been consistently demonstrated in experimental animals, and they have provided a

useful model for the complex pathophysiology of pulmonary hypertension in humans, the data gathered in this review of observations in humans provide only weak support for the characterization of pulmonary hypertension as a toxic effect of consumption of PAs.

No studies were available that addressed the potential carcinogenic effects of PAs in humans. Animal experiments have demonstrated that certain PAs are genotoxic carcinogens; based on the present knowledge of the possible mechanism of carcinogenesis (metabolism, activation, DNA adduct formation), it may be anticipated that PAs may act as carcinogens in humans. The lack of epidemiological studies on cancer is a data gap; even though there are no findings confirming that such an effect exists, it cannot be concluded that PAs are not carcinogenic to humans.

To sum up the main features seen in the outbreaks of HVOD analysed, although there is usually a documented exposure to PA before the onset of the disease, there is variability regarding the timing, duration of the exposure, time to sampling and estimated levels of exposure. A clear description of the time aspects was available for the poisoning outbreak that occurred in Afghanistan: duration of exposure before onset of the outbreak was about 2 years, the duration of the disease from 1–6 months; the time from the onset of symptoms to biopsy was 1–9 months, and therefore pathology findings reflected both early and advanced stages of HVOD. In the outbreak in Tajikistan, the time that elapsed from the beginning of exposure to the first clinical cases was about 6 weeks. Data on the estimated dose of PAs (mg/kg bw per day) among cases were available in three outbreak reports, showing great variability: from 0.018 mg/kg bw per day in Tajikistan and 0.033 mg/kg bw per day in Afghanistan, up to 20 times higher in India, where the dose was estimated at 0.66 mg/kg bw per day.

Regarding the case reports of poisoning by PAs, a summary of the six with detailed data on methods and dose also showed a huge variability in the estimated dose of exposure: the lowest dose (in mg/kg bw per day) was 0.0025, the highest was 3 mg/kg bw per day, and the intermediate values in increasing order were 0.015, 0.06, 0.6 and 1.3 mg/kg bw per day. The duration of exposure ranged from short periods (4 days and 2 weeks) for those with the highest dose, up to chronic exposures lasting 15 months. Another source of variability is the analytical method used to identify the specific PA supposed to have caused the outcome. The two reports that identified riddelliine, retrorsine, and possibly seneciphylline and senecionine in *Senecio longilobus*, as well as the one that identified heliotrine and lasiocarpine in *Heliotropium lasiocarpium* used GC-MS. Seneciphylline and the corresponding *N*-oxide were identified as the major components in *Adenostyles alliariae* by means of TLC, MS and NMR spectroscopy, while senecionine and seneciphylline were identified in *Gynura segetum* and its herbal wine by means of HPLC-UV–MS. Finally, a spectrophotometric method

was used to analyse PA and PA *N*-oxides in *Symphytum* spp. using monocrotaline as a standard, but the compounds were not precisely identified. The methods in use are approximations to quantify PAs in complex mixtures. Currently, only MS provides the prerequisites to analyse PAs at trace levels; there are multiple variants of LC-MS and GC-MS but all of them require pre-concentration and sample clean-up prior to analysis. Further work is desirable using the best available analytical methods, certified authentic standards and inter-laboratory comparisons.

Overall, case reports of poisonings, including deaths, due to PA-containing herbal medicines and teas have clearly shown toxicity in humans, predominantly in the liver. However, the doses of PA resulting in disease in these reports are generally not well characterized. All the uncertainties discussed above must be taken into consideration in order to decide on the reliable information to be used as the basis for human health risk characterization and health-based guidance values.

3. Analytical methods

3.1 Chemistry and sources of PAs

The pyrrolizidine ring system consists of two fused, fully saturated five-membered rings with a nitrogen atom at the bridgehead position 4 (Fig. 3). All alkaloids with such a ring system are referred to as pyrrolizidine alkaloids (PAs).

Fig. 3

Molecular structures of pyrrolizidine molecules typically associated with pyrrolizidine alkaloids, showing the numbering system used

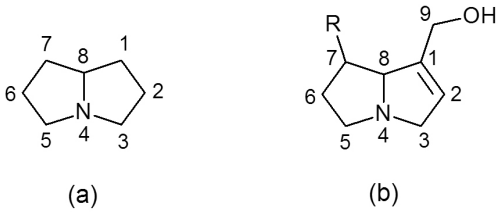
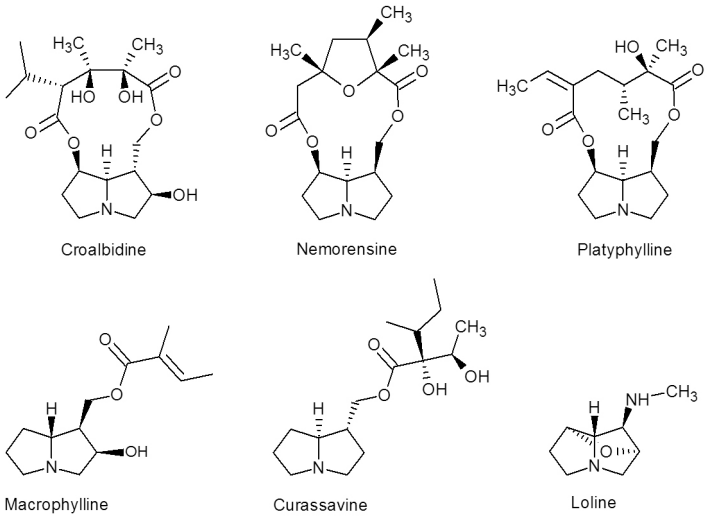


Fig. 4

The structures shown are typical of alkaloids that incorporate a saturated pyrrolizidine ring system



Typical PAs of the fully saturated type (Fig. 3a) are shown in Fig. 4. With the exception of the lolines, all saturated PAs of the type shown in Fig. 4 are found in plants belonging mainly to the families Boraginaceae, Asteraceae (Compositae) and Fabaceae (Leguminosae) (EFSA, 2011; Mattocks, 1986; Smith & Culvenor, 1981; WHO-IPCS, 1988). They are generally considered to be less toxic (Bull, Culvenor & Dick, 1968; Mattocks, 1986; WHO-IPCS, 1988) although platyphylline (Fig. 4) has been shown to be acutely toxic in rats by an unknown mechanism (Jago, 1970).

The loline alkaloids depicted in Fig. 4 occur in *Acremonium coenophialum*-infected tall fescue (*Festuca arundinacea* Schreb.). Lolines will not be considered further in this monograph.

A specific group of PAs, analogous to, and co-occurring with the saturated PAs depicted in Fig. 2 but with a 1,2 double bond (1,2-unsaturated PAs) (Fig. 3b), are also found in the families Boraginaceae, Asteraceae (Compositae) and Fabaceae (Leguminosae) as well as in in some genera of the Apocynaceae (EFSA, 2011; Mattocks, 1986; Smith & Culvenor, 1981; WHO-IPCS, 1988). As with the majority of the biosynthetically-related saturated PAs depicted in Fig. 4, they too have a hydroxymethyl grouping at C1 and, in many cases, a hydroxyl at C7 (and rarely an additional hydroxyl at C6). The hydroxyl groups at C9 and C7 (or C6) are esterified by the same characteristic, highly branched aliphatic acids that are present in the closely related saturated PAs shown in Fig. 4.

The structures of several typical 1,2-unsaturated PAs are shown in Fig. 3. Unlike their saturated analogues, for example, those shown in Fig. 4, the alkaloids in Fig. 5 are commonly found as contaminants in foods (EFSA, 2011; Mattocks, 1986; WHO-IPCS, 1988). Also included in this group of 1,2-unsaturated PAs are seco-alkaloids such as senkirkine (**6**) (Fig. 5) that strictly are only PAs when protonated. Senkirkine-like 1,2-unsaturated PAs have also been commonly found as food contaminants.

1,2-unsaturated PAs (e.g. those shown in Fig. 5) fall into four main categories: open-chain monoesters (e.g. heleurine (**1**) and intermedine (**2**)), open-chain diester (e.g. lasiocarpine (**3**)), macrocyclic diesters (e.g. senecionine (**4**) and anacrotine (**5**)) and seco-PAs (e.g. senkirkine (**6**)). There are five known 1,2-unsaturated pyrrolizidine moieties (Fig. 6). These are referred to as necines, as are their saturated analogues.

The characteristic aliphatic acids that esterify the necine hydroxyls of both saturated PAs (Fig. 4) and 1,2-unsaturated PAs (Fig. 5) are called necic acids (Mattocks, 1986). Typical necic acids are those esterifying the necine hydroxyls at C7 and C9 in Figs 4 and 5 (Bull, Culvenor & Dick, 1968; Mattocks, 1986). The necic acids display considerable branching, an important feature that sterically hinders esterase activity leading to detoxification of 1,2-unsaturated PAs in vivo (Mattocks, 1986). The highly branched necic acid moieties also provide good

Fig. 5
Typical 1,2-unsaturated PAs

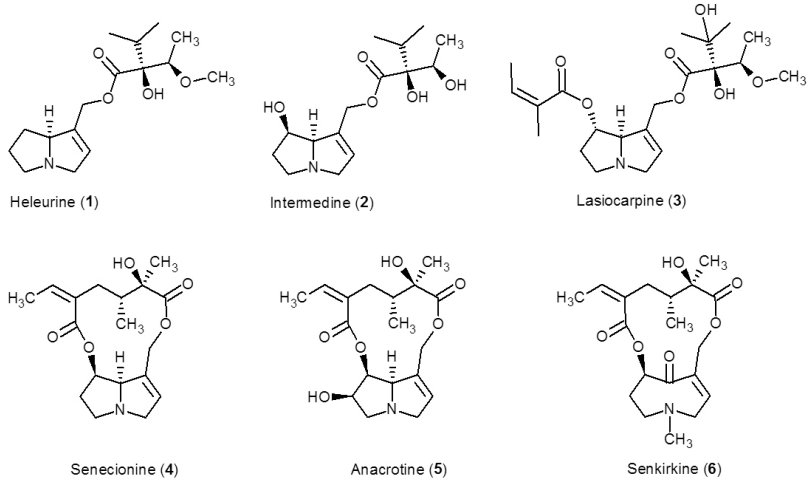
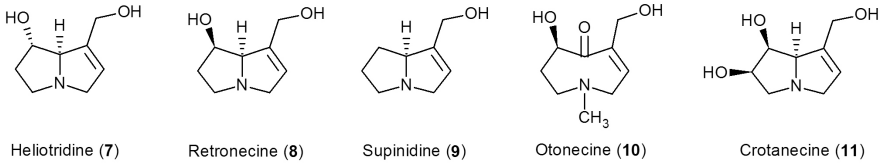


Fig. 6
The five necines characteristic of 1,2-unsaturated PAs



leaving groups in 1,2-unsaturated PAs' metabolites, facilitating the formation of carbonium ions at C7 and C9 of the metabolites involved in alkylation leading to the genotoxicity of 1,2-unsaturated PAs (see below) (Mattocks, 1986).

More than 600 different PAs, mainly 1,2-unsaturated PAs, and their associated nitrogen oxides (*N*-oxides) are produced by an estimated 6000 plant species and new PAs continue to be identified in both new and previously studied plant species (Mattocks, 1986; Smith & Culvenor, 1981; Stegelmeier et al., 1999; WHO-IPCS, 1988). Different plant species produce characteristic mixtures of 1,2-unsaturated PAs and varying amounts of their corresponding *N*-oxides (Bull, Culvenor & Dick, 1968; Mattocks, 1986; Smith & Culvenor, 1981; WHO-IPCS, 1988). As well as being characteristic of particular plant species, the relative composition of the constituent 1,2-unsaturated PAs and their *N*-oxides also

varies with: plant tissue (leaves, roots, flowers, seeds and pollen), stage of growth, climatic conditions, regional varieties, and hybrids (Adamczak, Gryszczyńska & Buchwald, 2013; Altamirano, Gratz & Wolnik, 2005; Betz et al., 1994; Castells, Mulder & Pérez-Trujillo, 2014; Eller & Chizzola, 2016; Mattocks, 1986; O'Dowd & Edgar, 1989; Witte et al., 1992).

3.2 Description of analytical methods

3.2.1 Introduction

(a) Summary of analytical issues

The analytical methods for screening and quantitation of PAs in food and feed reviewed and discussed in this section of the monograph include those that are currently available for all types of PAs but particularly the 1,2-unsaturated PAs and their *N*-oxides known to contaminate a wide variety of food samples. Also included are analytical methods for some saturated PAs, for example, the lolines and platyphylline (Fig. 2) that may at some stage become of interest as hazardous food contaminants. Currently, the only methods for quantitative analysis of trace levels in food and feed require MS to confirm identity and provide adequate limits of detection (LODs) (EFSA, 2011).

To be suitable for risk assessment purposes, it is important that sampling procedures (see section 4), sample preparation, LODs, limits of quantitation and inter-laboratory comparison studies conform to *Environmental Health Criteria 240: Principles and methods for the risk assessment of chemicals in food* (WHO-IPCS, 2009). Unequivocal chemical characterization is particularly important, especially in regard to complex, multicomponent analytes such as 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides.

Efficient extraction, isolation, separation and quantitation of individual 1,2-unsaturated PAs and their *N*-oxides that occur as contaminants in food, raise a number of challenges. The most obvious is the many hundreds of different 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides that occur in the plants that are the sources of food contamination (Smith & Culvenor, 1981; Mattocks, 1986; Stegelmeier et al., 1999; WHO-IPCS, 1988). This high variability is also likely to be reflected in similar variations in the PAs transferred into food.

A substantial number and a wide variety of 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides have been detected as contaminants in food products (see section 6), including: cereals, teas, salads, meat, herbs and spices, honey, milk, eggs, seed oils, and foods incorporating these, as well as in herbal medicines and food supplements (EFSA, 2011; WHO-IPCS, 1988). It is likely, however that, owing to differences in stability and extractability and a lack of authentic samples and analytical standards, some 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-

oxides may have gone undetected and unquantified in these and other foods. It is therefore important during analytical method validation to ensure that all 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides present in food samples are being efficiently isolated, detected and accurately quantified. However, this requires that all 1,2-unsaturated PAs and their *N*-oxides are available for validation studies and very many are currently not generally available (EFSA, 2011).

Several other issues can also hinder efficient analysis of 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides in items of food. As well as the current scarcity of a complete range of authentic 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides as analytical standards to ensure that all potential 1,2-unsaturated PA and 1,2-unsaturated PA-*N*-oxide food contaminants are accounted for and accurately quantified, other aspects also need to be considered. These include differences in the physical properties, for example, solubility, basicity and stability, between different 1,2-unsaturated PAs, and especially between 1,2-unsaturated PA free bases and their *N*-oxides, which can result in inefficiencies in the extraction and quantitative isolation of some 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides from food items (Bull, Culvenor & Dick, 1968; Mattocks, 1986; WHO-IPCS, 1988).

High concentrations of particular 1,2-unsaturated PAs and their *N*-oxides may occur in plant sources but, due to mixing and selective transfer, much lower and variable concentrations of these substances may be present in foods.

Contaminants from several different plant sources can also be present in some food samples, especially when blending has occurred during manufacture, for example in the case of honey, grains and milk.

The fact that 1,2-unsaturated PAs occur in plant sources and as contaminants in foods as both free bases and as *N*-oxides represents a particular challenge in efficiently extracting, isolating and quantifying all such substances of concern from a particular sample (Cao, Colegate & Edgar, 2008; Crews, 2013). Differences in basicity, solubility and relative stability of 1,2-unsaturated PAs and their *N*-oxides raise questions of how best to extract and isolate both forms of 1,2-unsaturated PAs from a wide range of food matrices and then how to quantify all 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides in subsequent analytical procedures (Bull, Culvenor & Dick, 1968; Crews, 2013; Crews, Berthiller & Krska, 2010; Mattocks, 1986). For example, analytical methods involving GC are not possible for 1,2-unsaturated PA-*N*-oxides as they are not volatile and decompose on injection (Bull, Culvenor & Dick, 1968; Crews, 2013; Mattocks, 1986; Roeder, 1999). The 1,2-unsaturated PA free bases are basic in character and usually lipophilic whereas their *N*-oxides are not basic and are hydrophilic. Some 1,2-unsaturated PA free bases, particularly those having necic acids incorporating vicinal diols, also tend to decompose on volatilization and they require appropriate derivatization prior to GC to enable them to be detected

and accurately quantified (Edgar, 1985; Mattocks, 1986). Decomposition of 1,2-unsaturated PAs, and particularly their *N*-oxides, can also occur during preparation of samples for analysis.

If the relative toxicities of all 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides detected in a particular food sample are to be taken into account in assessing food safety risk, each would theoretically need to be individually detected, identified, quantified and the level of hazard each represents factored into the equation to estimate overall risk to consumers. This would, however, require all naturally occurring 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides to be known and to be available for use as authentic analytical standards; for analytical method validation; and for standardized toxicity testing. While this would be the gold standard approach, other less challenging and more practical summation-based analytical approaches to assess dietary exposure, applicable to all 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides, are available (see below). For example, by assessing all 1,2-unsaturated PAs and their *N*-oxides as a cumulative assessment group, as proposed by EFSA (2008) for pesticides, and considering all 1,2-unsaturated PAs to be insufficiently different in genotoxic properties to require individual quantitation (*Bundesanzeiger*, 1992), summation methods that analyse all 1,2-unsaturated PAs and their *N*-oxides in a food sample as a group, described and reviewed later, provide a simpler and more effective approach to analysis of these substances for risk assessment and management purposes.

Several approaches have been employed in the past to simplify the quantification of 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides in food samples. However, not all of them have yet been adequately validated and some have also failed in regard to validation of the adequacy of 1,2-unsaturated PA and 1,2-unsaturated PA-*N*-oxide extraction and isolation. This is due in part to a lack of authentic standards. Approaches that have been used include: identifying the most common 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides detected in particular foods, for example honeys, or those known to be present in plants growing in the region in which particular food samples originated, ensuring that authentic samples of these 1,2-unsaturated PAs are available and only quantifying these substances as a measure of food safety. Analysts have sometimes also obtained as many authentic standards as they can and then sought to identify and quantify all of these in the food samples examined (Dübecke Beckh & Lüllmann, 2011; Griffin et al., 2013; Griffin et al., 2014; Griffin et al., 2015a,b; Kempf et al., 2011a).

While these approaches reduce the number of authentic analytical standards required, in some cases they will, as a consequence of the lack of a significant number of other authentic standards, lead to an underestimate of the PA content represented by a particular food sample. The results obtained

therefore need to be considered to be a minimum level rather than as representing the actual concentrations of all 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides in the foods analysed.

Methods for analysing individual PAs, 1,2-unsaturated PAs, 1,2-unsaturated PA-*N*-oxides and saturated PAs, can be significantly extended, in the case of MS analysis, by employing highly characteristic MS fragmentation patterns to identify and characterize all components that are saturated PAs or 1,2-unsaturated PAs, or saturated PA-*N*-oxides or 1,2 unsaturated PA-*N*-oxides (Bull, Culvenor & Dick, 1968; Mattocks, 1986). Saturated PAs and PA-*N*-oxides are recognizable from their characteristic fragmentation patterns, which differ from those characteristic of their 1,2-unsaturated analogues (Bull, Culvenor & Dick, 1968; Mattocks, 1986). Ionization techniques that produce strong molecular adduct peaks, monitoring of characteristic fragment ions, and exact mass measurements of molecular ions and fragment ions have been used to confirm the presence and tentative identity of possible 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides in plants and food samples, including some not available as authentic standards (Beales et al., 2004; Bull, Culvenor & Dick, 1968; Colegate & Gardner, 2008; Colegate et al., 2015; Edgar & Smith, 2000; Lin G et al., 1998; Mattocks, 1986). Currently this approach is best achieved using tandem mass spectrometry (MS/MS), collision-induced dissociation (CID) and multiple reaction monitoring (MRM) methods (Colegate et al., 2005; Crews, 2013; Dübecke, Beckh & Lüllmann, 2011; These et al., 2013; Xiong et al., 2009a; Zhou Y et al., 2010). Any additional, known or unknown 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides found in this way can be included in the risk assessment by using the cumulative assessment group summation approach to quantifying their contribution to sample toxicity. In such cases, however, quantitation would be based on a non-identical reference standard and not on an authentic analytical standard.

An additional simplification to the above approaches can be achieved by chemically reducing all of the 1,2-unsaturated PA-*N*-oxides present in food samples to 1,2-unsaturated PA free bases prior to total alkaloid extraction and quantitation (Bull Culvenor & Dick, 1968; Colegate et al., 2005; Mattocks, 1986). The need to isolate only 1,2-unsaturated PA free bases, rather than a mixture of 1,2-unsaturated PA free bases and 1,2-unsaturated PA-*N*-oxides, simplifies the extraction, reduces concerns relating to the instability of 1,2-unsaturated PA-*N*-oxides and simplifies the analysis. There are smaller numbers of analytes to be detected and quantified and the number of authentic standards required is reduced by 50%. MS/MS with in-source CID and MRM can also be more easily employed to extend the analysis to all the 1,2-unsaturated PA free bases present in samples (Colegate & Gardner, 2008). GC-MS methods also become a more viable option when all of the *N*-oxides present are converted to free bases.

Chemical reduction of the *N*-oxides present in a food sample is justified because the toxicity of 1,2-unsaturated PA-*N*-oxides requires their initial in vivo reduction to 1,2-unsaturated PA free bases before they can be metabolized to DHP esters and DHPs (Mattocks, 1986; WHO-IPCS, 1988). The chemical conversion of *N*-oxides in food sample extracts to free bases prior to analysis assumes that in vivo reduction of the *N*-oxides will be as efficiently achieved following ingestion of the food being analysed and the relative toxicities of the "total" 1,2-unsaturated PAs (the original 1,2-unsaturated PA free bases plus those produced by reduction of 1,2-unsaturated PA-*N*-oxides) then only needs to be considered to indicate the toxicity of a particular food sample.

As indicated above, rather than identifying and quantifying all individual 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides in a sample, a considerable analytical simplification and improved extraction efficiency is achieved by using summation methods for 1,2-unsaturated PA, 1,2-unsaturated PA-*N*-oxide and saturated PA analysis, which can more directly and easily indicate the sum of all 1,2-unsaturated PAs, saturated PAs and their *N*-oxides (BfR, 2011; Committee on Toxicity of Chemicals, 2008; EFSA, 2011).

One summation approach for 1,2-unsaturated PAs involves reduction of the 1,2-unsaturated PA-*N*-oxides to 1,2-unsaturated PA free bases followed by hydrolysis of the "total" 1,2-unsaturated PA free bases to necines (Fig. 6) and analysis of the necines to provide a measure of the total 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides in the sample. Such a method is likely to provide a more accurate measure of the 1,2-unsaturated PAs and their *N*-oxides because of the reduced number of analytes that need to be isolated, identified and individually quantified; a reduction in losses due to inefficiencies in extraction; reduced losses due to instability of 1,2-unsaturated PAs and especially 1,2-unsaturated PA-*N*-oxides during isolation, and; access to at most five 1,2-unsaturated necine authentic analytical standards is required (Fig. 6).

A comparison of the levels of 1,2-unsaturated PAs and their *N*-oxides present in samples of *Jacobaea vulgaris* honeys analysed by both a summation approach and liquid chromatography-ion electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis of individual 1,2-unsaturated PAs, reported that levels found by summation were generally much higher, by a factor of 2 to 124 (average 17) (Kempf et al., 2011a). This apparently reflected discrepancies due to a lack of authentic standards and differences in stability and extractability of individual 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides for LC-ESI-MS/MS analysis that were absent in the summation method used. The results appear to demonstrate the superiority of quantifying all 1,2-unsaturated PAs and their *N*-oxides present in the honeys by "summation" of necines over attempted analysis of individual 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides. However, the structures of the 1,2-unsaturated PAs and 1,2-unsaturated

PA-*N*-oxides present in samples are lost by summation and a complementary combination of summation and individual analysis methods can provide useful risk management information. Identification of individual 1,2-unsaturated PAs is particularly important in helping to ascertain the most important plants contributing to contamination of the food supply in particular regions and foods. Using both summation and individual analytical methods for food samples will also allow an assessment to be made of the relative reliability of the two approaches.

Another older and less precise "summation" screening method for 1,2-unsaturated PAs and their *N*-oxides involves conversion of all 1,2-unsaturated PA free bases in a sample to their *N*-oxides, subsequent conversion of these to DHP esters by treatment with acetic anhydride, followed by reaction of the DHP esters formed with Ehrlich reagent (*p*-dimethylaminobenzaldehyde) and spectrophotometric assessment of the characteristic colour produced (Mattocks, 1986). This "screening" summation method has been used, for example, to assess the level of 1,2-unsaturated PAs in samples of flour (Azadbakht & Talavaki, 2003) (see below). It has been made more suitable for quantitation purposes in the case of retronecine-based 1,2-unsaturated PAs by identification and quantitation of the coloured products generated using LC-MS, high-performance liquid chromatography–nuclear magnetic resonance spectroscopy (HPLC-NMR) and liquid chromatography with UV detection (HPLC-UV) analysis (Zhang et al., 2007).

Each of the issues and analytical approaches to detecting and quantifying 1,2-unsaturated PAs in food outlined above are considered and discussed individually and in more detail below.

(b) **Stability issues**

While most PAs are reasonably stable, the various ester linkages present are, to different degrees, quite readily hydrolysed, some under even mildly alkaline conditions (Bull, Culvenor & Dick, 1968; Crews, 2013; Crews, Berthiller & Krska, 2010; Mattocks, 1986; WHO-IPCS, 1988). This is especially true of simple ester linkages such as acetyl groups, which frequently esterify hydroxyls on both the necic acid and necine moieties (Bull, Culvenor & Dick, 1968; Mattocks, 1986). Exposure to alkaline conditions, for example, prior to solvent extraction from basified aqueous solutions during isolation and after dilute acid/zinc dust reduction of 1,2-unsaturated PA-*N*-oxides, should therefore be avoided or minimized. For example, the extraction solvent needs to be in place before basification of an aqueous acid solution by ammonium hydroxide and the pH should be raised no higher than pH 9–10 to reduce the possibility of ester hydrolysis although extraction at higher pH, while risking hydrolysis and losses,

could sometimes produce higher yields, particularly of more stable PAs (Bull, Culvenor & Dick, 1968; Mattocks, 1986).

Drying of plant samples is also known to cause significant decomposition of 1,2-unsaturated PAs and especially their *N*-oxides (Bull, Culvenor & Dick, 1968; Crews, 2013). Losses of 50–80% have been observed during drying of plant material (Culvenor & Smith, 1967; WHO-IPCS, 1988). 1,2-Unsaturated PA-*N*-oxides are considerably more vulnerable than the free bases to decomposition during heating, drying and storage of plant and food samples before and during analysis. Since the *N*-oxides can be the main form present in some plants and food samples (Boppré et al., 2008; Mattocks, 1986; O'Dowd & Edgar, 1989), this can lead to a significant underestimate of dietary exposure (Cao, Colegate & Edgar, 2008; Crews, 2013). For example, higher levels of *N*-oxides are reportedly isolated in sample extracts obtained by avoiding heating and extracting samples at room temperature, indicating that even mild heating can cause selective loss of *N*-oxides (Crews, 2013). Heating of pollen to 56 °C caused a 50% loss of senecionine-*N*-oxide, but no loss of senecionine (Boppré et al., 2008). One indication of the lability of 1,2-unsaturated PA-*N*-oxides during storage and heating is the observation that purified, crystalline samples of 1,2-unsaturated PA-*N*-oxides are "very largely converted into black tars in three or four years at room temperature" (Bull, Culvenor & Dick, 1968). This probably reflects the slow, spontaneous conversion of the 1,2-unsaturated PA-*N*-oxides to DHP esters that readily polymerize via self-alkylation to produce dark polymers (Mattocks, 1986).

Some necic acids are also susceptible to structural alteration under alkaline conditions, for example lasiocarpic acid, found in lasiocarpine and europine, can readily release acetone via a retro-aldol reaction (Bull, Culvenor & Dick, 1968). This is followed by more rapid hydrolysis of the resulting 2-hydroxy-3-methoxybutyrate and could lead to reduced detection of these two important 1,2-unsaturated PAs in food samples (Bull, Culvenor & Dick, 1968; Mattocks, 1986). Some macrocyclic 1,2-unsaturated PAs can also undergo similar retro-aldol reactions. Monocrotaline can release butane-2-one and junceine and trichodesmine can release 4-methylpentane-2-one (Mattocks, 1986). Epoxides on necic acids, for example, found in jacobine and jacozine, are prone to ring-opening following exposure to hydrochloric acid (Bull, Culvenor & Dick, 1968; Mattocks, 1986). Awareness of such degradations and losses is important to avoid underestimating levels of relatively labile 1,2-unsaturated PAs in food samples.

In summary, structural aspects of the necic acids and the particular lability of 1,2-unsaturated PA-*N*-oxides are the main concerns associated with instability and loss of toxins during sample preparation. Necines (Fig. 4) are relatively more stable elements than both 1,2-unsaturated PAs and, especially,

1,2-unsaturated PA-*N*-oxides and they are very suitable analytical targets for summation methods of analysis (discussed below).

(c) Solubility issues

Many 1,2-unsaturated PA free bases are relatively polar and water soluble, but some of the less hydroxylated 1,2-unsaturated PAs have limited solubility in water. When protonated by acid, however, they all become highly water soluble (WHO-IPCS, 1988). 1,2-Unsaturated PA-*N*-oxides and the necines of 1,2-unsaturated PAs shown in Fig. 4 are all highly soluble in water and can, as a result, be difficult to extract from aqueous solutions into non-polar solvent. Necines in particular are so soluble in water, even at high pH, that they can only be effectively extracted into non-polar solvents such as chloroform from basified aqueous solutions if, for example, the solution is saturated with potassium carbonate (Mattocks, 1986).

Colegate et al. (2013) have identified a retronecine-based, amphoteric 1,2-unsaturated PA, echiuplatine in *Cryptantha* spp., which has a free carboxylic acid group on its C9 necic acid and, as a result, was not extractable from aqueous solution and would not have been isolated by conventional alkaloid extraction methods. Such amphoteric 1,2-unsaturated PAs would probably be accounted for by summation analysis but are likely to be missed by conventional sample preparation for GC-MS and LC-MS methods of analysis.

(d) Extraction and isolation

The methods selected for efficiently isolating PAs and associated *N*-oxides from different food matrices, as well as needing to conform to best practice in regard to validation using authentic standards, also depend on the characteristics of the matrix, the purpose of isolation, the method of analysis to be used and a consideration of the time involved per sample.

The traditional, rather long, procedure for extraction and isolation of "total PAs" (free bases plus *N*-oxides) present in plants can be summarized as follows:

1) extraction of PAs and their *N*-oxides from samples with hot or cold methanol or ethanol;
2) filtration and evaporation of the alcohol extract;
3) dissolution of the residue in dilute aqueous acids, with zinc powder then added to reduce *N*-oxides;
4) filtration to remove excess zinc;
5) solvent extraction of the aqueous acid solution to remove non-basic components;

6) basification and extraction of PA free bases with, for example, chloroform;
7) drying of the chloroform with anhydrous sodium sulfate;
8) filtration and evaporation to yield the "total" PAs (free bases plus reduced *N*-oxides) isolate (Bull, Culvenor & Dick, 1968; EFSA, 2007; Koekemoer & Warren, 1951).

The isolate obtained using this procedure, but without the zinc reduction step, does not include the *N*-oxide component and has traditionally been used to isolate and separately measure the PA free base component in samples and, by difference, indicate the levels of PA-*N*-oxides.

The currently preferred method of isolation of 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides (and also necines) is by solid-phase extraction (SPE) using strong cation exchange resin (SCX) cartridges. The *N*-oxides are not retained by cation exchange, however, but by less specific binding to the resin (Crews, Berthiller & Krska, 2010; Mroczek, Glowniak & Wlaszcyk, 2002) and this could be a concern if some *N*-oxides are less effectively bound than others and the efficiency of binding and isolation has not been demonstrated using authentic standards.

Both 1,2-unsaturated PA free bases and *N*-oxides are reported to be simultaneously and apparently effectively isolated from plant and food extracts using SCX-SPE cartridges (Crew, Berthiller & Krska, 2010; Betteridge, Cao & Colegate, 2005; Colegate & Gardner, 2008; Kempf et al., 2008; Mroczek, Glowniak & Wlaszcyk, 2002). Dilute aqueous acid extracts or alcoholic extracts are applied to and adsorbed on prepared SCX-SPE cartridges, followed by washing of the cartridges with water and methanol to remove non-adsorbed impurities and then elution of the free base and *N*-oxide analytes using a small volume of ammoniated methanol. Subsequent evaporation and reconstitution of the residue in methanol, or another suitable solvent, produces samples ready for analysis of 1,2-unsaturated PAs, saturated PAs and their *N*-oxides, for example, by LC-MS/MS with CID and MRM assessment of the individual 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides present (Betteridge et al., 2005; Colegate et al., 2005; Colegate et al., 2015; Dübecke, Beckh & Lüllmann, 2011; These et al., 2013).

Other solid-phase columns have been used to concentrate PAs including: diatomaceous earth (Elut and Extrelut), non-polar octylsilane and octadecylsilane columns (Hösch et al., 1996; Mroczek, Glowniak & Wlaszcyk, 2002), but SCX-SPE columns are currently considered to be the most effective. Silica-based SCX resin cartridges are reportedly preferable to polystyrene cartridges (Colegate & Gardner, 2008), as the latter require much larger volumes of strong aqueous acids for effective elution of the PAs and PA-*N*-oxides and then further workup

is needed to prepare samples for analysis and quantitation (Betteridge, Cao & Colegate, 2005; Colegate & Gardner, 2008; Colegate et al., 2005).

An isolation method, referred to as QuEChERS (Quick Easy Cheap Effective Rugged Safe), developed for assessing pesticide in food matrices (Anastassiades et al., 2003) has also been applied to isolating 1,2-unsaturated PAs and their *N*-oxides in honey samples by Kempf et al. (2011a).

(e) Reduction of 1,2-unsaturated PA-*N*-oxides to free bases

Traditional methods for isolating and quantifying "total" PAs (PAs and their *N*-oxides) required the *N*-oxide component in a plant or food extract to be reduced to PA free bases prior to isolation (Bull, Culvenor & Dick, 1968; Mattocks, 1986). This method is very effective and continues to be used for sample preparation (Kempf et al., 2008). Dilute acid extracts, or initial alcohol extract residues taken up into dilute acid, are treated and stirred/agitated at a temperature below 45 °C for 3–4 hours with zinc dust to achieve reduction of the *N*-oxides (Bull, Culvenor & Dick, 1968; Crews, Berthiller & Krska, 2010). Zinc dust reduction is followed by removal of excess zinc by filtration, extraction of the acid solutions with ether or light petroleum to remove fats and non-alkaloidal components, followed by basification with ammonium hydroxide to pH 9–10 and immediate extraction of total PA free base into chloroform or methylene chloride. Some analysts recommend further basification with sodium hydroxide and further extraction to ensure complete isolation of all PAs, but at the risk of hydrolysis of ester linkages and retro-aldol reaction losses (Bull, Culvenor & Dick, 1968). After drying the combined extracts, filtration and evaporation produces a "total" 1,2-unsaturated PA sample ready for analysis. It has, however, been reported that the efficiency of zinc dust reduction can be variable depending on the batch of zinc dust and the length of the reduction time (Beales, Colegate & Edgar, 2004; Crews, 2013). Thus validation of the effectiveness of each zinc dust batch used is desirable. Overnight reduction has been reported to give lower yields than shorter reduction times (0.5–4 hours) (Beales, Colegate & Edgar, 2004). All of these aspects must be considered during method validation.

Reduction of 1,2-unsaturated PA-*N*-oxides is more conveniently achieved using reduced redox resin columns prepared by adsorption of indigocarmine onto an anion exchange resin (Beales et al., 2004; Colegate et al., 2005). The indigocarmine, adsorbed on the resin, is reduced with sodium dithionite to produce the 1,2-unsaturated PA-*N*-oxide-reducing agent which, when exposed to a mixed solution of 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides, converts the *N*-oxide component to 1,2-unsaturated PA free bases. A commercial product, called Serdoxit, was originally used for this purpose (Chizzola, 1994; Huizing & Malingré, 1979) but is no longer available. However, the redox resin

can be easily generated in the laboratory (Beales et al., 2004; Colegate et al., 2005). Sodium and potassium dithionite or metabisulfite also reduce *N*-oxides to free bases but the efficiency is apparently variable (Crews, 2013).

The relative levels of 1,2-unsaturated PA free bases and *N*-oxides can be determined by comparing the levels of 1,2-unsaturated PA free bases obtained with or without reduction of the *N*-oxide component (Bull, Culvenor & Dick, 1968) or, preferably by using a method of analysis such as LC-MS/MS that allows both 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides to be separated, identified and individually quantified.

(f) Introduction to methods of separation, detection and quantitation

Various forms of HPLC, GC, TLC and electrophoresis provide well-established methods for separating 1,2-unsaturated PAs and, in some cases, their *N*-oxides for identification and quantitative analysis (Crews, 2013; Crews, Berthiller & Krska, 2010; Mattocks, 1986; Roeder, 1999; WHO-IPCS, 1988). The 1,2-unsaturated PAs, their *N*-oxides and derivatives isolated from samples being analysed are best detected and identified, in the case of HPLC and GC, by their mass spectral properties, including determining their elemental composition by exact mass measurement of molecular ions and characteristic fragmentation ions. Nonspecific detection, by various detectors such as UV, flame ionization etc., requires the availability of authentic samples and analytical standards for comparison of retention times as the principle method of identification. In the case of TLC, the target analytes are located on TLC plates after chemical conversion to DHP derivatives (see below) and then the plates are sprayed with Ehrlich reagent to produce characteristic colour spots (Mattocks, 1986). Specific 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides can be tentatively identified by comparison of their colour response and Rf values with authentic samples. Some forms of electrophoresis have also used Ehrlich reagent or UV absorption to identify and locate 1,2-unsaturated PAs and their *N*-oxides. Only MS detection, however, meets current requirements for unequivocal chemical characterization and sensitive quantitation.

Quantitation by HPLC or GC methods is based on peak size relative to standard curves ideally generated using authentic standards. Many of these authentic standards, however, are not available and a representative substance is commonly selected for production of a standard quantitation curve for all 1,2-unsaturated PAs and, in the case of LC methods, their *N*-oxides in a sample. Summation methods, based on quantifying genotoxicity-indicating necines, are more amenable to production of standard curves based on the actual analytes being measured as there are only five 1,2-dehydronecines and all of these are

readily available or can be easily generated as authentic standards from available 1,2-unsaturated PAs.

Direct insertion fast atom bombardment–mass spectrometry with exact mass measurement has also been used to characterize the 1,2-unsaturated PAs in mixtures and to provide "fingerprints" of the 1,2-unsaturated PAs in food samples and in plant extracts, but has not been used for precise quantitation (Edgar & Smith, 2000; Edgar et al., 1992).

3.2.2 Screening methods for 1,2-unsaturated PAs

(a) Thin-layer chromatography (TLC)

TLC on both silica and aluminium oxide has been widely used to identify the presence of 1,2-unsaturated PAs and their *N*-oxides in combination with specific reagents for their detection (Crews, 2013; Crews, Berthiller & Krska, 2010; Mattocks, 1986). The original detection reagents used were nonspecific, for example, iodine vapour and also Dragendorff's reagent (Mattocks, 1986). However, with recognition and identification of the chemical character of the oxidation products and metabolites of 1,2-unsaturated PAs as DHPs, Ehrlich reagent has become a particularly effective and a relatively specific, colorimetric reagent for identifying 1,2-unsaturated PAs and their *N*-oxides on TLC plates (Mattocks, 1986). 1,2-Unsaturated PA spots on TLC plates can be oxidized to DHPs using iodine vapour and subsequent spraying with Ehrlich reagent produces characteristic blue or magenta spots (Culvenor et al., 1970). 1,2-Unsaturated PA detection on TLC plates is also achieved by converting 1,2-unsaturated PA free bases spots to 1,2-unsaturated PA-*N*-oxides by spraying with hydrogen peroxide or 3-chloroperbenzoic acid followed by acetic anhydride (Mattocks, 1986). After subsequent warming to produce DHPs, the plates are sprayed with Ehrlich reagent to generate coloured spots (Mattocks, 1986). An improved approach involves first spraying the plate with chloranil to oxidize the 1,2-unsaturated PAs to DHPs and then spraying with Ehrlich reagent (Molyneux & Roitman, 1980). Chloranil alone is also a less specific indicator of 1,2-unsaturated PAs on TLC plates (Huizing, de Boer & Malingré, 1980) and the additional spraying with Ehrlich reagent adds considerable specificity. Quantitation on TLC is achieved by visual comparison of spot intensity with a reference standard or by comparative densitometer scanning (Mroczek, Glowniak & Wlaszcyk, 2002).

(b) Electrophoresis

Paper electrophoresis has been extensively studied with a range of different electrolytes mainly to characterize 1,2-unsaturated PAs and their *N*-oxides (Frahn, 1969). Mobilities were related to pH of the electrolyte and to the pKas of the analytes. For example, at pH 4.6 the *N*-oxides are, unlike the free bases,

incompletely ionized and as a consequence they had much lower mobilities than their parent free bases.

Capillary electrophoresis (CE) has had limited use in 1,2-unsaturated PA analysis, but it reportedly has good ability to separate some 1,2-unsaturated PAs and provides quite high sensitivity for quantitation. Lebada et al. (2000) analysed senkirkine and senecionine free bases in *Tussilago farfara* using CE (Crews, 2013). Yu & Li (2005) applied the technique to separating and quantifying senkirkine, senecionine, retrorsine and seneciphylline free bases in Chinese herbal plants using UV detection at 220 nm and comparison with authentic standards (Feng et al., 2010). The corresponding *N*-oxides were not analysed. The LOD was reported to be 30 ppb. Pre-concentration techniques for CE reportedly improved the sensitivity (Yu et al., 2005). Electrophoresis has not been used for summation analysis of necines. With further development it may have potential as a rapid, high sensitivity method for 1,2-unsaturated PA analysis.

(c) Nuclear magnetic resonance spectroscopy (NMR spectroscopy)

Both proton (^{1}H) and carbon-13 (^{13}C) NMR have been employed for characterization and structural identification of pure, isolated 1,2-unsaturated PAs (Bull, Culvenor & Dick, 1968; Mattocks, 1986). Proton-NMR spectroscopy or ^{1}H-NMR spectroscopy for this purpose is comprehensively reviewed by Logie, Grue & Lidell (1994). Recent examples of the value of ^{1}H and ^{13}C NMR in the identification of previously unknown 1,2-unsaturated PAs are provided by Colegate et al. (2013) and Fletcher et al. (2011b).

In the past, however, ^{1}H-NMR and ^{13}H-NMR have also been used for quantitative analysis of 1,2-unsaturated PAs using the H2 proton signal at or near to δ 6 ppm, well away from other signals (Molyneux et al., 1979). Its exact position can be used to distinguish between macrocyclic 1,2-unsaturated PAs and open-chain esters. Pieters et al. (1989a) have used ^{1}H and ^{13}C NMR to determine total alkaloid levels in *Senecio jacobaea*. A comparison of ^{1}H and ^{13}C NMR with HPLC has also been made by Pieters & Vlietinck (1986) and NMR compared favourably to capillary GC for determining the total alkaloids in *S. vernalis* (Pieters et al., 1989a); however, the method probably lacks sufficient sensitivity for food safety risk assessment purposes.

(d) Immunological methods

ELISAs for 1,2-unsaturated PAs and their *N*-oxides have been developed and quite extensively studied (Bober et al., 1989, 1990; Cavallaro et al., 2004; Langer et al., 1996; Lee et al., 2001, 2004; Nivarlet et al., 2011; Oplatowska et al., 2014; Roeder & Pflueger, 1995; Roseman et al., 1992; Roseman, Wu & Kurth, 1996; Than et al., 2005; Zündorf et al., 1998). Specific antibodies against 1,2-unsaturated PAs

and 1,2-unsaturated PA-*N*-oxides could allow their selective isolation from and quantitative screening in food. However immunological detection, quantitation, isolation and screening of 1,2-unsaturated PAs and their *N*-oxides in food is currently limited by lack of antibodies that specifically bind all 1,2-unsaturated PAs and *N*-oxides with comparable affinity. Some of the antibodies produced have also been shown to bind PAs lacking the 1,2-double bond (Roeder, 1999; Zündorf et al., 1998). Antibodies binding both 1,2-unsaturated PA free bases and their *N*-oxides are also not available nor are they likely to be generated. For example, Zündorf et al. (1998) produced monoclonal antibodies against retrorsine and examined their affinity to 20 different PAs. While these monoclonal antibodies demonstrated considerable binding affinity with nine 1,2-unsaturated PAs, including retrorsine, they also bound to unsaturated PAs and failed to recognize heliotridine-based PAs, otonecine-based alkaloids, anacrotine or the necines heliotridine and retronecine. Thus the value of antibody preparations for the purpose of screening, quantifying and removing or isolating 1,2-unsaturated PAs and their *N*-oxides from food samples is currently limited (Cavallaro et al., 2004; Roeder, 1999; Roseman, Wu & Kurth, 1996; Than et al., 2005).

Retronecine-specific polyclonal antibody-based ELISAs have also been produced and studied (Bober et al., 1989, 1990; Roseman, Wu & Kurth, 1996). Development of sensitive ELISAs for quantifying necines could be useful in summation analysis methods for quantifying total 1,2-unsaturated PAs and *N*-oxides in food samples based on hydrolysis to, and quantitation of necines.

(e) Summation methods based on spectrophotometry

A spectrophotometric summation method was originally developed and used as a screening method for 1,2-unsaturated PAs and their *N*-oxides in plants with a LOD reportedly below 5 µg (Fig. 7) (Mattocks, 1986; Mattocks & Jukes, 1987). This method has also been applied to indicate levels of 1,2-unsaturated PAs and their *N*-oxides in flour (Azadbakht & Talavaki, 2003). The procedure involves conversion of 1,2-unsaturated PA free bases and 1,2-unsaturated PA-*N*-oxides in sample extracts to DHP esters in the presence of ascorbic acid, subsequent reaction with modified Ehrlich reagent containing boron trifluoride, and measurement of the colour produced (Mattocks, 1986). The ascorbic acid is present in order to provide a soluble alkylation target for the DHP esters that are formed. This reduces the likelihood of DHP-ester self-polymerization and precipitation leading to reduced colour intensity (Mattocks, 1986).

Both 1,2-unsaturated PAs and their *N*-oxides in sample extracts can be independently measured by selecting the appropriate reagents necessary to form DHP esters selectively from *N*-oxides or from free bases (Mattocks, 1986). This method, however, is not applicable to otonecine esters such as senkirkine.

Conversion of the 1,2-unsaturated PA free bases to DHPs is achieved directly by oxidation with *o*-chloranil or alternatively by conversion to *N*-oxides, which are then converted to DHPs with acetic anhydride (Mattocks, 1986). 1,2-Unsaturated PA-*N*-oxides in extracts are simply treated with acetic anhydride or iron (II) complexes and then Ehrlich reagent (Mattocks & Jukes, 1987).

While most saturated PAs present in extracts are not converted to DHP esters and therefore generally give little or no colour, one, rosmarinine, with a C2 hydroxyl group, can, to some extent, dehydrate to give a 1,2-unsaturated PA, and is reported to contribute slightly to the colour produced (Mattocks, 1986).

(f) 1,2-Unsaturated PA detection by insects

Certain butterfly and moth species have evolved a dependence on 1,2-unsaturated PAs (Edgar, 1975). The adult male butterflies seek out 1,2-unsaturated PA-producing plants based on their remarkable ability to detect volatile substances produced by wilting plants containing these toxins. The insects fly upwind to the plants and scratch the leaves to release 1,2-dehydro-PA-containing sap which they imbibe through their proboscis. To obtain the 1,2-unsaturated PAs from dried plants, the butterflies sometimes also regurgitate liquid and make an extract which they drink.

These butterflies, certain moths and other 1,2-unsaturated PA-dependent insects have evolved a capacity to store 1,2-unsaturated PAs in their tissues. This protects them from predators. In many if not all species of 1,2-unsaturated PA-dependent lepidoptera (e.g. butterflies of the subfamily Danainae and moths of the family Arctiidae) the males metabolize a proportion of the 1,2-unsaturated PAs they ingest to produce volatile pheromones related to DHP (Culvenor & Edgar, 1972). In producing these pheromones the hydroxyls of DHP are converted to aldehydes or ketones and/or the 7-hydroxymethyl group is, in some species, also converted to a methyl group. These modified, volatile DHP-related pheromones are no longer alkylating agents. They are applied to the head and antennae of the female by the males during courtship and they indicate to the female that the male has the capacity to store 1,2-unsaturated PAs in his tissues and that this capacity will be transferred to their offspring. Each species has a unique mixture of 1,2-unsaturated PA-based pheromones. They are sometimes also used to define territories by repelling other males. Only females find them attractive.

Species of 1,2-unsaturated PA-dependent Ithomiinae butterflies in central and South America produce a pheromone lactone based on a necic acid (Edgar, Culvenor & Pliske, 1976).

1,2-Unsaturated PA-dependent insects can identify the presence of 1,2-unsaturated PAs in plants and foods. Especially if deprived of PAs after emergence from the pupae, they will seek to obtain 1,2-unsaturated PAs. When

exposed to honeys or other foods that contain or do not contain 1,2-unsaturated PAs they are very effective in focusing their feeding on the former.

Examples of the use of such insects to find new 1,2-unsaturated PA-producing plants are reported by Colegate et al. (2015) and Boppré & Colegate (2015). Such insects have been used to screen food samples and commonly consumed herbal products for likely 1,2-unsaturated PA contamination (e.g. Colegate et al., 2015).

3.2.3 Quantitative methods

(a) Summation methods involving GC-MS or LC-MS

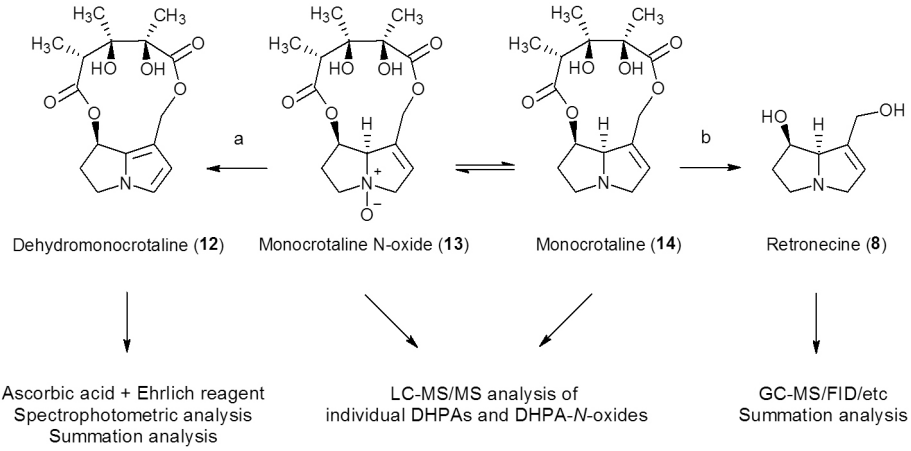
For the "summation" or "sum parameter" method for analysis of 1,2-unsaturated PAs and their *N*-oxides, they are converted to the necine moieties (Fig. 6), which are subsequently quantified using GC-MS or LC-MS.

This approach was used to quantify the retronecine-based 1,2-unsaturated PAs transferred into the milk of goats exposed to a plant, *Jacobaea vulgaris* (syn. *Senecio jacobaea)*, containing only retronecine-based 1,2-unsaturated PAs (Deinzer et al., 1982). In that case, the 1,2-unsaturated PAs present in the milk were hydrolysed with a solution of barium hydroxide ($Ba(OH)_2$) to retronecine (**8**) prior to analysis. $Ba(OH)_2$ was used so that when hydrolysis was complete, Ba^{++} could be precipitated as barium carbonate ($Ba(CO_3)_2$) using solid carbon dioxide (CO_2), the insoluble barium carbonate was removed by filtration and the retronecine then isolated by adsorption onto and subsequently eluted from an SCX column. The retronecine-containing fractions eluted with ammonium hydroxide/methanol were evaporated, derivatized to produce retronecine bis-heptafluorobutyrate and analysed by GC using an electron capture detector (Fig. 7). In this case the *N*-oxide component in the milk was apparently not measured. This would have required initial reduction of the *N*-oxides to 1,2-unsaturated PA free bases prior to hydrolysis.

An improved version of this approach has been used to measure retronecine-based 1,2-unsaturated PAs in honeys (Kempf, Reinhard & Beuerle, 2010; Kempf et al., 2008, 2010, 2011a,b). In these studies the "total" 1,2-unsaturated PA isolate (free bases plus reduced *N*-oxides) from each sample, produced by zinc dust reduction of the *N*-oxide components, was isolated using SCX-SPE cartridges or the QuEChERS method (Anastassiades et al., 2003) and then treated with a tetrahydrofuran solution of lithium aluminium hydride ($LiAlH_4$) to cleave the ester linkages and produce retronecine (**8**) (Kempf, Reinhard & Beuerle, 2010; Kempf et al., 2008, 2010, 2011a,b). Heliotridine-based alkaloids were shown not to be present in the honey samples and only retronecine (**8**) levels were measured in the honeys surveyed by Kempf, Reinhard & Beuerle (2010) and Kempf et al. (2008, 2010, 2011a,b). Heliotridine (**7**), produced by adding the precursor

Fig. 7

Several approaches to the analysis of 1,2-unsaturated PAs and their *N*-oxides in food sample extracts[a]



[a] Reagents used are: a, acetic anhydride (Ac2O) or $Fe(II)SO_4$/MeOH; b, lithium aluminium hydride ($LiAlH_4$) or barium hydroxide $Ba(OH)_2$.

1,2-unsaturated PA heliotrine to the samples prior to $LiAlH_4$ exposure, was used as an internal standard for quantitation (Kempf et al., 2008). The retronecine (**8**) produced and the heliotridine (**7**) internal standard were subsequently converted to their di-trimethylsilyl derivatives and analysed by GC-MS (Kempf, Reinhard & Beuerle, 2010; Kempf et al., 2008, 2010, 2011a,b).

Fig. 7 illustrates several approaches to the analysis of 1,2-unsaturated PAs and their *N*-oxides in food sample extracts, using, as an example, a mixture of monocrotaline (**14**) and monocrotaline-*N*-oxide (**13**). Approaches shown are:

1) Analysis of the individual 1,2-unsaturated PAs (e.g. (**14**)) and 1,2-unsaturated PA-*N*-oxide(s) (e.g. (**13**)), by LC-MS/MS (examples of this approach have been decribed by Griffin et al., 2015a,b; These et al., 2013);
2) Summation analysis by conversion of the *N*-oxides (e.g. (**13**)) to free bases (e.g. (**14**)) and hydrolysis of total free bases to necines (e.g. in the case of monocrotaline and its *N*-oxide, retronecine (**7**)) and quantitative analysis of the necines by, for example, GC-MS (examples of this approach have been described by Deinzer et al., 1982; Kempf et al., 2011a,b);
3) Summation analysis: by conversion of 1,2-unsaturated PA free bases (e.g. (**14**)) present to *N*-oxides (e.g. (**13**)) and subsequent

conversion of total *N*-oxides to the corresponding DHP esters (e.g. dehydromonocrotaline (**12**)), reaction with Ehrlich reagent in the presence of ascorbic acid and subsequent spectrophotometric analysis (described in Mattocks & Jukes, 1987; see also Azadbakht & Talavaki, 2003).

Methods 1 and 2, involving MS characterization and quantitation, are more suitable for quantification, whereas the summation method involving spectrophotometric analysis is probably best considered to be a semiquantitative screening assay.

Summation methods to date have been applied mainly to retronecine-based 1,2-unsaturated PAs and their *N*-oxides in food samples, but the other necines (heliotridine (**7**), supinidine (**9**) otonecine (**10**), and crotanecine (**11**)) also need to be considered. 1,2-Unsaturated PAs based on the necine otonecine (**10**) are problematic as otonecine (**10**) is not detectable by this particular summation approach (Crews, 2013; Mattocks, 1986). A photometric summation method of analysis of otonecine-based 1,2-unsaturated PAs has, however, been developed by Bartkowski, Wiedenfeld & Roeder (1997).

An alternative summation analysis based on necines involves trans-methylation of the 1,2-unsaturated PA esters using tetramethylammonium hydroxide to generate the corresponding necine methyl ethers. This has been used to determine 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides in Swiss honeys and the retronecine, heliotridine and supinidine methyl ethers produced in this way were directly analysed by GC-MS (Böhlen et al., 2011).

Zhang et al. (2007) have also developed a single pot summation method for retronecine-based 1,2-unsaturated PAs, which is possibly applicable to heliotridine (**7**) and supinidine (**9**) 1,2-unsaturated PAs. The method has been applied to the analysis of retronecine-based 1,2-unsaturated PA free bases in *Senecio scandens* spp. obtained from different regions of China. It involves isolation of the 1,2-unsaturated PA free bases from the plants, oxidation to their DHP esters with *o*-chloranil and then reaction with boron trifluoride diethyl etherate and Ehrlich reagent. The resulting purple-coloured derivative of 7-ethoxy-1-ethoxymethyl-dehydroretronecine was then identified and analysed by HPLC-UV, LC-MS and HPLC-NMR spectroscopy. The method was not applied to 1,2-unsaturated PA-*N*-oxides or to otonecine-based PAs.

Summation methods have several advantages over analytical methods involving isolation, separation, identification and quantitation of individual 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides in samples. These advantages include: greater certainty in quantifying all 1,2-unsaturated PAs and *N*-oxides present in the samples; no requirement for hundreds of authentic samples/analytical standards; and they provide a single measure of total

1,2-unsaturated PAs in a sample, with the current exception of otonecine-based 1,2-unsaturated PAs, which require separate quantitation.

(b) High-performance liquid chromatography (HPLC) and LC-MS

HPLC and UHPLC combined with MS/MS detection, CID and MRM methods have become the most widely used and accurate means of detecting, identifying and quantifying individual 1,2-unsaturated PAs and their *N*-oxides in plant and food extracts (Colegate & Gardner, 2008; Crews, 2013; Crews, Berthiller & Krska, 2010; Mulder et al., 2015). The simplest, most time-efficient, high-throughput sample preparation procedure applicable to most food samples involves aqueous acid extraction of the samples, direct application of extracts to SCX-SPE cartridges, adsorption of 1,2-unsaturated PA free bases and their *N*-oxides, washing, desorption of analytes with ammoniated methanol, evaporation, dissolution of the residue in methanol or other suitable solvent and then HPLC or UHPLC-MS/MS analysis with MRM (Colegate et al., 2005) to confirm known, and/or deduced identity of unknown 1,2-unsaturated PAs and their *N*-oxides, and the amount of each 1,2-unsaturated PA and 1,2-unsaturated PA-*N*-oxide detected (Colegate et al., 2005). Variations of this approach have been widely used in many recent surveys of 1,2-unsaturated PAs and their *N*-oxides in foods and herbal products (Adamczak, Gryszczyńska & Buchwald, 2013; Altamirano, Gratz & Wolnik, 2005; Avula et al., 2012; Beales et al., 2004, 2007; Beales, Colegate & Elgar, 2004; Betteridge, Cao & Colegate, 2005; Bodi et al., 2014; Bolechava et al., 2015; Boppré et al., 2008; Boppré, Colegate & Edgar, 2005; Bosi et al., 2013; Colegate et al., 2013; Cramer & Beuerle, 2012; Cramer et al., 2013; Crews & Anderson, 2009; Crews et al., 2009; Fragoso-Serrano et al., 2012; Griffin et al., 2013, 2014, 2015a,b; Hoogenboom et al., 2011; Kast et al., 2014; Kempf et al., 2011b; Liu F et al., 2009; Martinello et al., 2014; Mathon et al., 2014; Mulder et al., 2009; Mulder et al., 2015; Orantes-Bermejo et al., 2013; Qi et al., 2009; These et al., 2013).

MS/MS using atmospheric pressure ionization (API), electrospray ionization (ESI), and atmospheric pressure chemical ionization give strong molecular ion (MH^+) peaks that can provide exact mass molecular weight information and be subject to CID and MRM to produce structural identity information as well as accurate quantitation of all 1,2-unsaturated PAs present in samples (Colegate & Gardner, 2008).

Griffin et al. (2015b) have developed a fast, sensitive LC-MS/MS method, validated for a specific group of 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides, which is based on a very fast isocratic protocol. It involves rapid run times per sample of 6 minutes. This method allowed more than 200 honey samples to be effectively analysed for specific 1,2-unsaturated PAs and 1,2-unsaturated PA-

N-oxides in 24 hours. Rapid sample extraction and clean-up was achieved via SCX-SPE.

(c) Gas chromatography (GC) and GC-MS

GC with a variety of columns and detectors, including flame ionization, photodiode array and particularly – and most effectively – MS, with exact mass measurement and selective ion monitoring, has been used for many decades as a means of detecting and quantifying 1,2-unsaturated PA free bases in foods, animal feed and herbal products (Adamczak, Gryszczyńska & Buchwald, 2013; Culvenor, Edgar & Smith, 1981; Deinzer et al., 1982; Edgar, 1985; Edgar & Smith, 2000; Witte et al., 1993). While a number of 1,2-unsaturated PAs are amenable to GC without the need for derivatization, some are unstable or tend to decompose (Beales, Colegate & Edgar, 2004). GC performance can be greatly improved or, in some cases, made possible, with appropriate derivatization (Beales, Colegate & Edgar 2004; Edgar, 1985). Vicinal diol groups present on necic acids are, for example, thermally labile and 1,2-unsaturated PAs such as lasiocarpine and echimidine, both particularly toxic, do not readily volatilize under gas chromatographic conditions without derivatization (Beales, Colegate & Edgar, 2004). Cyclic alkylboronates and alkylboronate-trifluoroacetate derivatives also enable the difficult-to-separate diastereoisomers lycopsamine, indicine and rinderine to be resolved, but echinatine/intermedine co-chromatographed as a fourth peak and were not resolved on GC-MS (Edgar, 1985). The cyclic sulfite iso-propyl ester derivatives of the necic acid, (±)-trachelanthic acid can be resolved into + and – enantiomers on a chiral-phase column (Edgar, 1985). Trimethylsilyl, acetyl and trifluoroacetyl derivatives (Deinzer et al., 1978, 1982; Kempf et al., 2008) can also ensure that some labile 1,2-unsaturated PAs and necines chromatograph well, but TMS derivatization of 1,2-unsaturated PAs with vicinal tertiary hydroxyls (e.g. echimidine, europine and lasiocarpine) are reported not to chromatograph satisfactorily after trimethylsilylation (Edgar, 1985). Di-TMS-retronecine and di-TMS-heliotridine derivatives have been used in GC-MS summation analysis of retronecine-based 1,2-unsaturated PAs in honey samples (Kempf et al., 2008, 2010, 2011a,b).

GC-MS analysis of samples in which the *N*-oxides have been converted to free bases has been used frequently in the past (Betz et al., 1994; Bicchi, Rubiolo & Frattini, 1989; Bicchi et al., 1989; Hovermale & Craig, 1998; Huizing et al., 1986; Langer & Franz, 1997; Oberlies et al., 2004; Pawar et al., 2010; Stelljes et al., 1991; Witte et al., 1993; Wretensjö & Karlberg, 2003). Recently, however, HPLC-MS is increasingly favoured for analysing mixtures of 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides. This is partly because decomposition of some of the more labile 1,2-unsaturated PAs or their *N*-oxides is not considered to be

a concern and a time-consuming derivatization step is therefore not required. A comparison of performance of GC-MS and HPLC-MS for a small selection of 1,2-unsaturated PAs was made by Beales, Colegate & Edgar (2004).

3.2.4 Quality assurance considerations

When analysing for PAs, laboratory quality performance must be regularly monitored and actions must be taken when required. Since there are no harmonized methods currently available and no performance characteristics for PA analysis, the performance characteristics for mycotoxins in Regulation (EC) No 401/2006 amended with Regulation (EU) No 519/2014 might be followed. Ideally, ongoing monitoring of laboratory performance would include routine analysis of certified reference materials, preferably naturally contaminated materials. Furthermore, within-laboratory samples, containing naturally contaminated material, must be included in each batch of samples in order to monitor ongoing quality. The use of an internal standard may greatly facilitate quantification. A PA not expected to be present may be used as the internal standard (Kempf et al., 2008; Mulder et al., 2009) or preferably an isotopically-labelled PA. Isotopically-labelled 7-hydroxy-9-(deoxyguanosin-N2-yl)dehydrosupinidine adducts (DHP-dG) and 7-hydroxy-9-(deoxyadenosin-N6-yl)dehydrosupinidine adducts (DHP-dA) are used as internal standards for measurement of DNA adducts (Xia et al., 2013). Participation in proficiency testing programmes is also encouraged to allow comparison of the quality of analyses performed between laboratories.

3.2.5 Reference methods

There are currently no approved reference methods available for the analysis of PAs. However, several proficiency tests have been carried out in the European Union (EU) to compare the quality of the analyses performed by different laboratories.

De Nijs, Elbers & Mulder (2014) conducted an inter-laboratory comparison test in which 12 participating laboratories used their own method, with seven 1,2-unsaturated PAs and their *N*-oxides in animal feed samples, using spiked and incurred materials. *z*-Scores varied more for analysis of the free bases than for the *N*-oxides.

Tamošiūnas et al. (2013) carried out a proficiency test to investigate the measurement capacities of 28 laboratories for PAs in honey and hay and to provide a benchmark for their performance. Laboratories used their own method, which resulted in comparable results for the determination of the sum parameter covering all alkaloids containing a retronecine backbone compared to the determination of individual PAs in spiked honey. However, a significant difference was found for the analysis of naturally contaminated materials in

which the determination of individual PAs led to lower results than the sum parameter determination.

Ronczka et al. (2015) carried out a collaborative study on the determination of 17 PAs in honey and herbal tea by solid phase extraction (SPE)-LC-MS/MS. The method showed good performance for the detection of PAs in honey and tea.

4. Sampling protocols

Sampling protocols will play a crucial role in the precision with which the levels of PAs and DHP adducts are measured in the wide range of foods that are currently known to be subject to contamination. It seems likely that significantly more practical experience will be required before optimum sampling protocols emerge that are suitable for different foods and foods at different stages of manufacture. The situation with PAs and DHP adducts is, however, not unlike that associated with mycotoxin contamination and sampling protocols for PAs could initially be based on those specified for mycotoxins. Information on sampling for mycotoxins can easily be obtained from Codex Alimentarius standard CODEX STAN 193-1995 and its amendments, in which sampling protocols are compiled for several mycotoxins, but not specifically for PAs or other plant toxins (CXS, 2018). Furthermore, the guidelines in Commission Regulation (EC) No 401/2006 (EC, 2006) and its amendments for sampling of mycotoxins can be followed. Several different sampling protocols are likely to be required to ensure compliance with maximum limits established by food law. Aspects to be considered include:

- the types of foods to be accessed, for example,
  - particulate solids such as grains, teas, pollen granules, salads;
  - complex solids such as meats and eggs, or;
  - liquids such as honey and milk
- the size of the lot to be sampled
- the stage of processing and packaging that particular food products have reached
- the normal daily dietary intake of the various foods to be sampled
- the regional origins of the foods or food components to be monitored
- the likely plant sources of contamination in different regions, and
- the 1,2-unsaturated PAs, the saturated PAs and their *N*-oxides and the DHP adducts likely to be involved in contamination.

As with mycotoxins, cereals, for example wheat, harvested from individual farms are likely to be quite heterogeneously contaminated with plant parts containing PAs, and the level of contamination will be either diluted or increased when mixed with grain from other farms. The overall volume or weight of bulked grain will be one measure determining the number and size of the samples that need to be analysed to ensure compliance with regulations. Similarly, teas, salads and pollen granules are also likely to be quite heterogeneous in regard to contamination. Distribution of PAs in dry teas can be very inhomogeneous owing to variation in distribution of the plant particles with inherent PA through

the mix. Relatively more or larger-volume samples will probably be required for such foods than are needed for complex solids such as meat and especially liquid foods, such as milk, honey and mead, where more homogeneous contamination within a batch can be expected.

Lists of, and the density of, known PA-containing plant species, and any other suspected plant sources of PA, present in a particular region, are likely to be of considerable value to risk managers and analysts in considering the PAs and DHP adducts that could be present as contaminants. They would also be useful for identifying the specific authentic analytical standards that are likely to be required and perhaps also the sampling protocols to be used (van Raamsdonk et al., 2015).

Sampling protocols could also be selected based on the method of analysis. The use of analytical methods where the sum of PAs is determined, as described in section 3, are likely to significantly simplify sampling requirements.

Prior visual assessments of foods to be sampled may also, in some cases, be a useful indicator to suggest the most suitable sampling protocols. For example, counting the number of seeds of PA-containing plants present in grain samples upon receipt at commercial silos has traditionally been used in many countries to assess the safety of cereals for human consumption (IPCS, 1989) and such assessments could precede a decision on the sampling protocol to be used in particular cases. Visual classification of pollen sources in pollen granules for sale as food supplements could also be a useful preliminary assessment of likely contamination. Deep blue pollen granules from *Echium* spp., for example, are often very apparent in commercial bee pollen on retail sale. They are visible even to an untrained person (Edgar et al., 2011) and this could provide a preliminary visual indicator suggesting the presence of PA contamination. The presence of pollen from known PA sources in honey samples also provides an indication of PA contamination. In this case it is not normally an accurate indicator of the level of contamination in the honey (Kempf, Reinhart & Beuerle, 2010), but could assist in selecting an appropriate sampling protocol for risk assessment.

Ongoing assessment of the range of foods that could possibly be contaminated in particular regions, records of the individual PA contaminants and DHP adducts detected, particularly the necines present and their relative prevalence, will greatly assist in eventually establishing the best cross-regional sampling protocols as well as the most appropriate methods of analysis.

5. Effects of processing on levels of PA in food and feed

5.1 Food processing

PAs and DHP adducts are expected to be stable during processes applied for food and feed production, with the exception of processes that include feed fermentation. This is concluded from mainly indirect evidence of incidents in humans and animals and from molecular structures. The few studies that have been published on this subject concern the effects of processing on the concentrations of 1,2-unsaturated PAs and their *N*-oxides in both food and feed. No information was found on stability of saturated PAs and their *N*-oxides or DHP adducts during processing. The studies of processes in food production concerned the preparation of tea infusions, borage oil, honey and honey-derived products. Processing effects in feed production relate to compound feed production and ensiled roughage. It is evident that more information is required on the effects of processing on PAs and DHP adducts.

5.1.1 Fate of PAs during cleaning of cereals

Physical processing of grain by removal of the seeds from PA-containing plants will lower the overall concentration of PAs, but may not fully eliminate the 1,2-unsaturated PAs (Edgar, 1998, 2003; Molyneux et al., 2011).

5.1.2 Fate of PAs during preparation of black/herbal tea infusions

Dry teas obtained from retail stores in the EU in 2014 and 2015 were analysed for PAs and used for tea infusion making (Mulder et al., 2015). About 100% of the PAs were transferred to the tea infusions in which concentrations could be up to 7.99 µg/L PAs for rooibos tea infusion.

Tea infusions, from teas and herbal teas on the Swiss market, contained varying amounts of total 1,2-unsaturated PA, ranging from 0.105 to 4.77 µg/L (Mathon et al., 2014). The authors concluded that virtually all of the nine measured 1,2-unsaturated PAs from the dry teas were transferred to the infusion. Major PAs present in the 24 samples were: senecionine (92% of the samples positive), retrorsine (83%), seneciphylline (67%) and lycopsamine (50%).

Oberlies et al. (2004) found that the 1,2-unsaturated PAs symphytine and echimidine and their *N*-oxides are transferred from dry comfrey leaves in tea infusions. The ratio of transfer was not measured. They reported that the tea-making process that they used did not reduce the 1,2-unsaturated PA *N*-oxides to the free bases. Roitman (1981) estimated a transfer of 33% of the PA from comfrey root to the infusion (gelatinous substance removed).

The high transfer ratio of 1,2-unsaturated PAs from dry herbal tea to the corresponding tea infusion is underpinned by the indirect evidence of toxic effects in humans. The 1,2-unsaturated PAs are extracted with (hot) water and are fairly stable, at least for the short period of time between tea preparation and consumption (Roitman, 1981; Roulet et al., 1988).

5.1.3 Fate of PAs in processing of honey and honey products

1,2-Unsaturated PAs and their *N*-oxides are regularly detected in honey. Betteridge, Cao & Colegate (2005) detected both 1,2-unsaturated PAs and their *N*-oxides in *Echium vulgare* honeys. Their results also showed that the process of producing honey seems to have an intrinsic capacity to reduce the 1,2-unsaturated PA-*N*-oxides to their free bases (Betteridge, Cao & Colegate, 2005). Pollen present in honey quickly releases the PAs into the honey, making removal of pollen from honey to reduce the content of 1,2-unsaturated PAs and their *N*-oxides an impractical processing step to reduce exposure (Kempf et al., 2011b).

Kempf et al. (2011b) detected 1,2-unsaturated PAs in 13% of their samples of foods and beverages containing 5–27% honey, such as mead and candy. This study indicates that 1,2-unsaturated PAs can persist through the fermentation process for making mead and the candy-making process.

Echium-related PAs persisted in the process of making mead, with about 30–70% of the 1,2-unsaturated PA levels present in the honey found in the mead (Cao, Colegate & Edgar, 2013). The main 1,2-unsaturated PA detected in the mead was echimidine. *N*-oxides were not detected above the LOD in the honey for making the mead, and were, therefore, not detected in the mead.

5.1.4 Fate of PAs in pollen processing

Bee pollen sold as a commercial dry product (pollen granules, pollen nuggets, pollen pellets) can contain high levels of 1,2-unsaturated PAs and their *N*-oxides. Boppré et al. (2008) heated bee pollen at 56–60 °C for up to 100 hours. A 30% decrease in 1,2-unsaturated PA *N*-oxide was observed, but there was a 150–190% increase in 1,2-unsaturated PA. They concluded that the process of drying had little impact on total 1,2-unsaturated PA concentration (Boppré et al., 2008; Kempf, Reinhard & Beuerle, 2010).

5.1.5 Fate of PAs during borage oil processing

Wretensjö & Karlberg (2003) found a fourfold reduction of the spiked model compound crotaline in the first step of borage oil refining – the alkali refining. The subsequent step in the refining process, bleaching, resulted in an additional reduction of crotaline of 120-fold, whereas the final refining step, deodorizing,

added another 60-fold reduction of crotaline, with the overall result of a 30 000-fold reduction of the spiked model compound, crotaline, in the refining process.

Cramer et al. (2014) used sunflower seed oil spiked with senecionine and senecionine *N*-oxide as a model for 1,2-unsaturated PAs and their *N*-oxides in *Lappula squarrosa* oil to estimate the reduction of these PAs after washing with citric acid-phosphate buffer at various pHs, mimicking the first step of oil refining. At pH 2.2, the level of the spiked senecionine and senecionine *N*-oxide was reduced to 1.6% after one washing step and to 0.07% of the initial concentration after multiple washing steps.

Vacillotto et al. (2013) found that PA levels in a sample of *Boragio officinalis* seed oil were lower than 0.2 µg/kg. It is not possible to rule out the possibility that PAs are extracted with the oil but are lost during processing.

5.1.6 Indirect evidence for stability of PAs during processing

PAs were detected in 11 of 182 milk samples obtained from retail stores in the EU in 2014 and 2015 (Mulder et al., 2015). All samples were treated with heat, either pasteurization or ultra-high temperature processing (UHT).

Although PAs are detected in herbs and spices (section 6), no information was found on the identity of these products and the possible processes applied to make the end-products. However, the case described by Rasenack et al. (2003) clearly demonstrates that 1,2-unsaturated PAs are not reduced to any great extent by household use of kitchen herbs and spices in cooking. Mibei & Ojijo (2011) mention that alkaloids in general, not specifically the PAs, from edible African leafy vegetables (*Corchorus olitorius*, *Crotalaria ochraoleuca*, *Solanum scabrum* and *Cleome gynandra*) are reduced to between 40 and 70% of their initial concentrations during cooking.

Kakar et al. (2010) reported that the PAs in bread were strongly associated with HVOD symptoms in humans in Afghanistan, meaning that a good part of the PAs survive the bread making process, but no details on the bread making process were given. The PAs in the wheat flour used for bread making included heliotrine, heliotrine-*N*-oxide and lasiocarpine and came from charmac seeds (*Heliotropium popovii* H. Riedl subsp. *gillianum* H. Riedel), a weed co-harvested with the wheat used for bread making. A similar incident was reported in north-western Afghanistan where 22.6% of 7200 inhabitants showed liver disease due to consumption of *Heliotropium*-contaminated wheat (Mohabbat et al., 1976).

Qurut, a dried whey product made from goat's milk, contained heliotrine, heliotrine-*N*-oxide and trichodesmine, which survived the production process of that particular product (Kakar et al., 2010). The process of *qurut* making is not described in the paper. The PAs were transferred to the milk used for *qurut* making

when goats had been grazing on charmac-infested meadows (*Heliotropium popovii* H. Riedl subsp. *gillianum* H. Riedel).

5.1.7 Fate of PAs transferred to milk, eggs and meat

Occurrence of 1,2-unsaturated PAs in eggs and raw milk due to transfer is described (Hoogenboom et al., 2011; Mulder et al., 2015) but no information is yet available on transfer of saturated PAs and on co-occurring DHP adducts. The fate of the transferred PAs and the possibly co-occurring DHP adducts during processing has not been described so far.

1,2-Unsaturated PAs can be present in raw eggs due to transfer from feed (Diaz, Almeida & Gardner, 2014; Edgar & Smith, 2000; Mulder et al., 2016). However, no information is available on the stability of the 1,2-unsaturated PAs during the further processing of the contaminated eggs, for example, cooking, baking or drying for egg powder production. Mulder et al. (2016) suggest that PAs in eggs will be stable during processing of food and will add to the total exposure of humans to PAs.

Mulder et al. (2016) detected PAs in meat from laying hens, specifically in liver and kidney tissue. As for eggs, it is unknown how processing will affect the PAs present in meat. Again, Mulder et al. (2016) suggest that PAs in meat will be stable during processing of food and will add to the total exposure of humans to PAs.

5.2 Feed processing

Some information is available on the fate of 1,2-unsaturated PAs and their *N*-oxides during feed production. It is generally assumed that PA concentrations in feed do not decrease significantly when common feed preservation methods are used (EFSA, 2007). Processes for animal feed production on the farm involve drying and ensiling. This excludes pre-wilted forage. Commercially produced feed pellets may be subjected to a heating step. Pohlman et al. (2005) mention that drying of *Senecio jacobaea*, as occurs in hay making, makes the plant less bitter and thus more palatable to animals, which increases the risk for intoxication since PAs can still be present at high levels.

It has been reported that 1,2-unsaturated PAs and their *N*-oxides do occur in feeds but levels of PAs in the materials used for making the feed are unknown. Mulder et al. (2009) found 74% of 31 samples of alfalfa were positive for 1,2-unsaturated PAs and their *N*-oxides, at concentrations up to 5.4 mg/kg, as a result of contamination with *Senecio vulgaris*. This shows that 1,2-unsaturated PAs and their *N*-oxides can survive the process of drying alfalfa, making hay from alfalfa, pelleting and alfalfa crumb making. It was observed that in air-dried

material of *Senecio jacobeae*, one of the weeds contaminating roughage in the Netherlands, 80% of the 1,2-unsaturated PAs occurred as *N*-oxides. In samples in the survey, ratios between tertiary amines and *N*-oxides could vary from almost all tertiary amines to almost all *N*-oxides and included all ratios in between.

Hough et al. (2010) found that 1,2-unsaturated PAs and their *N*-oxides from ragwort were degraded to below the LOD during composting, at a cumulative temperature of 200 °C, in 4 weeks. The study of Crews et al. (2009) showed that the 1,2-unsaturated PAs and their *N*-oxides from *Senecio jacobaea* decreased to 11% of the initial concentration of 340 mg/kg in 4 weeks and to below the LOD after 10 weeks, when stored in plastic waste bags in the sun.

Becerra-Jiminez et al. (2013) showed that during ensiling, the concentrations of senecionine, seneciphylline, integerrimine and erucifoline were reduced quickly, while jacobine, jacoline, jaconine and jacozine showed slower degradation rates. The rate of reduction depended on temperature, storage conditions, air and amount of ensiled material. Since the reduction is a result of enzymatic processes, the degradation of 1,2-unsaturated PAs and their *N*-oxides stops at low pH owing to inhibition of the enzyme activity.

6. Levels and patterns of contamination in food commodities

PAs may be present in foods through three possible routes;

- as an inherent component of the food (e.g. herbal teas);
- through contamination of a food, particularly staple cereals, with PA-containing plant material (e.g. contamination of wheat with PA-containing weeds); and
- transfer of PAs from plant material consumed by animals into foods of animal origin.

Data on PA content of foods for evaluation in the present monograph were obtained from the scientific literature and from submissions to the Committee by FoodDrinkEurope, Brazil, Germany, Hungary and Luxembourg. It should be noted that all of the quantitative studies discussed in the following sections analysed only 1,2-unsaturated PAs and their *N*-oxides. No studies reported use of authentic standards of saturated PA.

6.1 Submitted data

6.1.1 Brazil

Brazil submitted data on the occurrence of 16 1,2-unsaturated PAs or their *N*-oxides in honey samples (n = 33), sampled in 2014. LODs were in the range 0.33 to 16.7 µg/kg, with the majority of LODs at the lower end of this range. The most frequently detected PAs were lycopsamine (100% of samples, mean = 21.0 µg/kg), senecionine (85%, mean = 34.1 µg/kg), retrorsine (64%, mean = 7.6 µg/kg) and lycopsamine *N*-oxide (33%, mean = 39.8 µg/kg). Ten of the 1,2-unsaturated PAs and *N*-oxides were not detected in any honey samples. Lower bound (values below the LOD set to zero, values between the LOD and limit of quantification (LOQ) set to the LOD) and upper bound (values below the LOD set to the LOD, values between the LOD and LOQ set to the LOQ) estimates were derived for the mean (69.5–73.9 µg/kg) and 95th percentile (165–178 µg/kg) concentration of 1,2-unsaturated PAs and their *N*-oxides.

6.1.2 FoodDrinkEurope

FoodDrinkEurope (formerly the Confederation of the Food and Drink Industries of the European Union) submitted data on the occurrence of 18 1,2-unsaturated PAs or their *N*-oxides in various foods and food ingredients (n = 34). LODs were in the range 0.5 to 5 µg/kg. PAs were detected in nine samples

(peppermint, basil, Bear's garlic, honey, honey powder, oregano, black tea and parsley (2 samples)). No 1,2-unsaturated PAs or their *N*-oxides were detected in more than four samples. Compounds detected in more than one sample were heliotrine *N*-oxide, lasiocarpine *N*-oxide, lycopsamine, retrorsine *N*-oxide, senecionine *N*-oxide, seneciphylline and seneciphylline *N*-oxide. Owing to the diversity of 1,2-unsaturated PAs and their *N*-oxides in different sample types it was assumed that analytical results less than the LOD represented true zero results. Concentrations of 1,2-unsaturated PAs and their *N*-oxides in the nine samples with detectable levels were in the range 4.3 to 4800 µg/kg, with the highest concentrations in Bear's garlic (4800 µg/kg), parsley leaves (335 µg/kg) and oregano leaves (330 µg/kg).

6.1.3 Germany

Germany submitted data on PAs (not further specified) in cereals and cereal products (n = 1137) for the period 2005 to 2010. PAs were not detected in 1120 (98.5%) samples. LOD ranged from 0.1 to 50 µg/kg. PAs were detected in 7 of 417 (1.7%) of cereal grains, including barley (3), maize (1), oats (2) and wheat (1). PAs were detected in 10 of 720 (1.4%) cereal products, including nonspecified cereals and cereal-based products (7) and bread and other cooked cereal products (3). Concentrations of PAs were generally low, ranging from 0.12 to 2.2 µg/kg, with concentrations in cereal products mostly less than 1 µg/kg.

6.1.4 Hungary

Hungary submitted data on PAs (not further specified) in cereals and cereal products (n = 165) for the period 2004 to 2010. The LOD was quite high (40 µg/kg) for all samples analysed and PAs were only detected in one sample of nonspecified cereals and cereal-based products, at a concentration of 55 µg/kg.

6.1.5 Luxembourg

Luxembourg submitted data on PAs (not further specified) in cereals and cereal products (n = 20) for 2009. PAs were not detected in any samples, but the LOD was very high for this data set (1000 µg/kg).

6.2 Literature data

Data on the occurrence and concentrations of 1,2-unsaturated PAs and their *N*-oxides in foods, from the scientific literature are summarized in the following sections.

6.2.1 Cereal and cereal products

During an outbreak of veno-occlusive disease of the liver in western Afghanistan, 32 samples of wheat flour were collected from 17 households and analysed for 10 1,2-unsaturated PAs and *N*-oxides by HPLC-MS/MS (Kakar et al., 2010). The median concentration of 1,2-unsaturated PAs and *N*-oxides in all samples was 4.0 mg/kg (4000 µg/kg), with median concentration from households with cases (n = 12) of 5.6 mg/kg, while the median concentration of samples from control households (n = 20) was 2.7 mg/kg. Heliotrine *N*-oxide accounted for greater than 95% of the PA content, with lesser amounts of heliotrine and lasiocarpine. PA contamination was believed to be due to the presence of charmac (*Heliotropium popovii* H. Riedl subsp. *gillianum* H. Riedl).

Wheat samples were collected from sites (n = 14) in the Mazandaran region of the Islamic Republic of Iran and analysed for PAs by a colorimetric method (Azadbakht & Talavaki, 2003). PAs were reported to be present in all wheat samples at concentrations in the range 33–98 mg/kg. Wheat samples were believed to be contaminated with common groundsel (*Senecio vulgaris*).

6.2.2 Teas and herbal teas

Table 29 summarizes the results of several recent studies on the content of 1,2-unsaturated PAs and their *N*-oxides in dried or infused teas and herbal teas.

There are substantial differences in the reported contents of 1,2-unsaturated PAs and their *N*-oxides in some common tea types. For example, Bodi et al. (2014) reported black tea from Germany to contain, on average, 256 µg/kg of 17 1,2-unsaturated PAs and *N*-oxides, while Griffin et al. (2014) found a maximum of 19 µg/kg in two black tea samples in Ireland. Bodi et al. (2014) reported intermedine to be the most commonly detected 1,2-unsaturated PA in dry tea and herbal tea samples in Germany (47% of samples), while this PA was not included in the analyses performed in the Irish and Swiss studies (Mathon et al., 2014).

Mathon et al. (2014) did not detect 1,2-unsaturated PAs or *N*-oxides in infusions of 10 black tea samples from Switzerland. While the different studies included differing ranges of 1,2-unsaturated PAs and their *N*-oxides, it is uncertain whether this is the reason for these differing results.

6.2.3 Milk and dairy products

Most studies of PAs in milk have analysed milk from animals intentionally fed known sources of PAs (Deinzer et al., 1982; Dickinson, 1980; Dickinson et al., 1976; Hoogenboom et al., 2011).

Dairy cows (n = 4) received 10 g/kg bw per day of dried tansy ragwort (*Senecio jacobaea L.*) for 2 weeks (Dickinson et al., 1976). The PA content

Table 29

Occurrence and concentrations of pyrrolizidine alkaloids (PAs) in teas and herbal teas

Study country[a]	Sample description	Sample form	Number of 1,2-unsaturated PAs and their *N*-oxides analysed	Number of samples	Percentage of samples > LOD	LOD/LOQ (µg/kg)	Concentration of 1,2-unsaturated PAs and their *N*-oxides (µg/kg or µg/L)[b]				Main 1,2-unsaturated PAs and *N*-oxides found
							Mean	Median	Minimum	Maximum	
Non-herbal teas											
Europe	Black	Dry Infusion	28	33	94	LOD:0.5–2.0 LOD: 0.007–0.027	556 7.6	93 1.6	<LOD <LOD	4060 54	Intermedine, intermedine *N*-oxide
Germany	Black	Dry	17	24	95	LOD: 0.5–2.0 LOQ:1.7–6.4	255.9	89.8	<LOD	1702.2	NS
Ireland	Black	Dry	14	2	50	LOD: 0.4–1.9 LOQ: 1.3–6.3	19	–	<LOD	19	Lycopsamine
Israel	Black	Dry	30	10	60	LOQ: 10–50	107	–	<LOQ	160	Intermedine *N*-oxide
Switzerland	Black	Infusion[c]	10	10	0	LOQ: 0.1–0.5 µg/L	–	–	<LOD	<LOD	NR
Europe	Green	Dry Infusion	28	26	85	LOD: 0.5–2.0 LOD: 0.007–0.027	448 5.7	33 0.3	<LOD <LOD	3920 52	Intermedine, retrorsine, senecionine and *N*-oxides
Germany	Green	Dry	17	23	87	LOD: 0.5–2.0 LOQ: 1.7–6.4	109	15	<LOD	697.5	NS
Ireland	Green	Dry	14	2	0	LOD: 0.4–1.9 LOQ: 1.3–6.3	–	–	<LOD	<LOD	NR
Israel	Green	Dry	30	10	60	LOQ: 10–50	81	–	<LOQ	116	Senecionine *N*-oxide
Switzerland	Green	Infusion[c]	10	6	0	LOQ: 0.1–0.5 µg/L	–	–	<LOD	<LOD	NR
Ireland	Oolong	Dry	14	2	50	LOD: 0.4–1.9 LOQ: 1.3–6.3	51	–	<LOD	51	Lycopsamine
Ireland	Yellow	Dry	14	1	0	LOD: 0.4–1.9 LOQ: 1.3–6.3	–	–	<LOD	<LOD	NR
Europe	Rooibos (red bush)	Dry Infusion	28	22	96	LOD: 0.5–2.0 LOD: 0.007–0.027	600 8.0	240 3.3	<LOD <LOD	4800 64	Retrorsine, senecionine and *N*-oxides
Germany	Rooibos (red bush)	Dry	17	24	100	LOD: 0.5–2.0 LOQ:1.7–6.4	1856.4	404.5	27.2	5647.2	NS

Study country[a]	Sample description	Sample form	Number of 1,2-unsaturated PAs and their *N*-oxides analysed	Number of samples	Percentage of samples > LOD	LOD/LOQ (µg/kg)	Concentration of 1,2-unsaturated PAs and their *N*-oxides (µg/kg or µg/L)[b]				Main 1,2-unsaturated PAs and *N*-oxides found
							Mean	Median	Minimum	Maximum	
Ireland	Rooibos (red bush)	Dry	14	1	100	LOD: 0.4–1.9 LOQ: 1.3–6.3	140	–	–	140	Senecionine *N*-oxide
Israel	Rooibos (red bush)	Dry	30	10	100	LOQ: 10–50	314	–	82	657	Senecionine *N*-oxide
Switzerland	Rooibos (red bush)	Infusion[c]	10	9	100	LOQ: 0.1–0.5 µg/L	1.47	0.625	0.235	3.86	Senecionine
Germany	Fruit tea	Dry	17	14	43	LOD: 0.5–2.0 LOQ: 1.7–6.4	<LOQ	–	<LOD	<LOQ	NS
Herbal teas											
Brazil	*Ageratum conyzoides*	Dry	20	3	100	NS	–	–	–	–	Lycopsamine, lycopsamine *N*-oxide
Europe	Chamomile	Dry Infusion	28	35	86	LOD: 0.5–2.0 LOD: 0.007–0.027	274 3.7	127 1.7	<LOD <LOD	1400 19	Various
Germany	Chamomile	Dry	17	39	90	LOD: 0.5–2.0 LOQ: 1.7–6.4	439.7	298.5	<LOD	3428.8	NS
Israel	Chamomile	Dry	30	10	100	LOQ: 10–50	564	–	74	1729	Lycopsamine *N*-oxide, intermedine, intermedine *N*-oxide, lycopsamine
Switzerland	Chamomile	Infusion[c]	10	10	70	LOQ: 0.1–0.5 µg/L	2.72	2.48	<LOD	3.94	Echimidine, senecionine, seneciphylline
USA	Comfrey	Infusion[d]	2	3	100	LOD: 0.1–0.4 µg/L	16.7	19.2	2.6	28.2	Echimidine, symphytine
Germany	Fennel	Dry	17	35	83	LOD: 0.5–2.0 LOQ: 1.7–6.4	51.7	1.6	<LOD	905.3	NS
Israel	Fennel	Dry	30	5	0	LOQ: 10–50	–	–	<LOD	<LOD	NR
Switzerland	Fennel	Infusion[c]	10	5	0	LOQ: 0.1–0.5 µg/L	–	–	<LOD	<LOD	NR
Switzerland	Linden	Infusion[c]	10	6	17	LOQ: 0.1–0.5 µg/L	0.27	–	<LOD	0.27	Senecionine, seneciphylline
Germany	Melissa (lemon balm)	Dry	17	16	100	LOD: 0.5–2.0 LOQ: 1.7–6.4	649.6	416.1	5	2579.2	NS
Israel	Melissa	Dry	30	5	0	LOQ: 10–50	–	–	<LOD	<LOD	NR

Table 29 (continued)

Study country[a]	Sample description	Sample form	Number of 1,2-unsaturated PAs and their *N*-oxides analysed	Number of samples	Percentage of samples > LOD	LOD/LOQ (µg/kg)	Concentration of 1,2-unsaturated PAs and their *N*-oxides (µg/kg or µg/L)[b]				Main 1,2-unsaturated PAs and *N*-oxides found
							Mean	Median	Minimum	Maximum	
Switzerland	Mint	Infusion[c]	10	8	50	LOQ: 0.1–0.5 µg/L	2.02	–	<LOD	4.77	Seneciphylline, senecionine, retrorsine
Mexico	Miracle (*Packera candidissima* and *P. bellidifolia*)	Dry tea bag	4	1	100	LOD: 0.5 µg/mL	8500	–	–	8500	Senecionine, senecionine *N*-oxide, retrorsine *N*-oxide
Europe	Mixed herbs	Dry Infusion	28	20	95	LOD: 0.5–2.0 LOD: 0.007–0.027	440 5.8	180 2.5	<LOD <LOD	1900 26	Various
Germany	Mixed herbs	Dry	17	43	91	LOD: 0.5–2.0 LOQ: 1.7–6.4	151.4	28.5	<LOD	1470	NS
Israel	Mixed herbs	Dry	30	10	50	LOQ: 10–50	315	–	<LOQ	723	Intermedine, senecionine *N*-oxide, lycopsamine
Switzerland	Mixed herbs	Infusion[c]	10	15	13	LOQ: 0.1–0.5 µg/L	0.13	–	<LOD	0.16	Heliotrine
Germany	Nettle	Dry	17	26	96	LOD: 0.5–2.0 LOQ: 1.7–6.4	238.8	150.5	<LOD	1143.7	NS
Europe	Peppermint	Dry Infusion	28	30	93	LOD: 0.5–2.0 LOD: 0.007–0.027	500 6.7	200 2.6	<LOD <LOD	4400 59	Various
Germany	Peppermint	Dry	17	29	86	LOD: 0.5–2.0 LOQ: 1.7–6.4	134.2	80	<LOD	766.1	NS
Israel	Peppermint	Dry	30	10	100	LOQ: 10–50	261	–	20	507	Heliotrine *N*-oxide, europine *N*-oxide
Ireland	Various[e]	Dry	14	10	60	LOD: 0.4–1.9 LOQ: 1.3–6.3	594	176	<LOD	1733	No consistent profile
Switzerland	Verbena	Infusion[c]	10	1	100	LOQ: 0.1–0.5 µg/L	0.53	–	–	0.53	Lycopsamine

NS; not stated: NR; not relevant: LOD; limit of detection: LOQ; limit of quantification

[a] Study details: Germany (Bodi et al., 2014), including data used in the German risk assessment (BfR, 2013a), Ireland (Griffin et al., 2014), Switzerland (Mathon et al., 2014), USA (Oberlies et al., 2004), Brazil (Bosi et al., 2013), Mexico (Fragoso-Serrano et al., 2012), Israel (Shimshoni et al., 2015b), Europe (Mulder et al., 2015).

[b] The summary statistics refer only to samples in which 1,2-unsaturated PAs and their *N*-oxides were detected, except for the study of Bodi et al. (2014) where summary statistics refer to all samples, including those in which 1,2-unsaturated PAs and their *N*-oxides were not detected.
[c] Samples were prepared at a standard infusion of 2 g of tea to 200 mL of hot water. The authors have carried out studies of extraction efficiency, suggesting that the figures given here can be multiplied by approximately 100 to give dry weight equivalents.
[d] Samples were prepared as a standard infusion of 10 g of tea to 1 L of hot water.
[e] Teas in this group were described by brand name or function, rather than herbal content.
[f] PAs were identified, but not quantified.

of the dried plant material was estimated to be 0.16% by weight. This equates to a PA exposure dose of 16 mg/kg bw per day. PAs were detected in milk at concentrations in the range 470–835 µg/L, after correction for recovery. Jacoline was the only 1,2-unsaturated PA detected in milk.

Dairy cows (Holstein Friesians, n = 3, weight 600–700 kg) were placed on a diet containing increasing amounts of ragwort (50, 100 or 200 g per day) for 3 weeks (Hoogenboom, et al., 2011). The concentration of 1,2-unsaturated PAs and their *N*-oxides in the plant material was 2.3 g/kg on a dry weight basis. Thus, exposure doses would have been in the range 0.16–0.77 mg/kg bw per day. 1,2-Unsaturated PAs and their *N*-oxides occurred in milk in a dose-dependent manner (concentrations in milk increased as quantity fed increased). During the three different feed periods, the mean concentrations of 1,2-unsaturated PAs and their *N*-oxides in milk were 2.1, 5.5 and 9.7 µg/L, respectively, predominantly jacoline.

In a radiotracer study, a dairy cow received a single oral dose of 1 mg/kg bw of [^{3}H] seneciphylline (Candrian et al., 1991). Concentrations of seneciphylline in milk reached a maximum of 102 µg/L after 14 hours and were still detectable after 64 hours. Goats (Nubian, n = 3) received approximately 1% (500 g) of their body weight in tops of tansy ragwort per day, via cannula (Deinzer et al., 1982). 1,2-Unsaturated PAs and their *N*-oxides were determined in milk after conversion to retronecine and found to be present at concentrations in the range 0.33–0.81 mg/L, expressed as jacobine (330–810 µg/L).

As part of an investigation of an outbreak of veno-occlusive disease in western Afghanistan, milk from goats believed to have grazed on a PA-containing plant (charmac, *Heliotropium popovii* H. Riedl subsp. *gillianum* H. Riedl) was analysed (Kakar et al., 2010). While the results of these analyses were not included in the original publication, they were provided to the Committee by WHO. PA concentrations (heliotrine, europine and their *N*-oxides) in three samples of goats' milk were in the range 1.5 to 3.1 µg/L. A whey product from the goats' milk (*qurut*) was also analysed, with three samples containing PA (heliotrine and europine and their *N*-oxides, lasiocarpine, trichodesmine) in the range 36.6–87.2 µg/kg.

A small number of studies determined PAs in commercial milk samples. Bulk milk samples (n = 21), from an area with a high reported incidence of ragwort poisoning in cattle, were analysed and no senecionine, seneciphylline or jacobine residues were detected (MAFF, 1994). However, samples were not analysed for jacoline, which the studies summarized above suggest is the main 1,2-unsaturated PA occurring in milk after ragwort ingestion by cows. LODs were 10 µg/kg for each PA.

Analysis of 63 commercial milk samples, including three goats' milk samples purchased in Belgium detected PAs (16 included in analysis) in eight

samples (12.7%) (Huybrecht & Callebaut, 2015). Total PA concentrations were very low (trace to 0.061 µg/kg), with retrorsine *N*-oxide being the main PA detected. Lycopsamine, heliotrine and senkirkine were also detected at lower concentrations.

As part of an EU-wide project, PAs (35 included in analysis) were determined in retail milk (n = 182), yoghurt (n = 27), cheese (n = 34) and infant formula milk powder (n = 25) (Mulder et al., 2015). PAs were detected in 11/182 milk samples (6.0%). No more than one PA or *N*-oxide was detected in any milk sample. Concentrations of PAs were in the range 0.05–0.16 µg/kg. Otosenine and senkirkine were the most commonly detected PAs, each detected in three samples. No PAs were detected in the other types of dairy product.

6.2.4 Eggs

Edgar & Smith (2000) reported on a case of PA poisoning among three flocks of layer chickens. The chickens had received feed wheat containing *Heliotropium europaeum* at an estimated concentration of 0.6%. The analytical technique used reduced *N*-oxides to the associated 1,2-unsaturated PA prior to analysis. The wheat was found to contain heliotrine (6.7 mg/kg), europine (9.5 mg/kg) and lasiocarpine (9.8 mg/kg), giving a total 1,2-unsaturated PA content of 26 mg/kg. Eggs from the layer birds were found to contain 19–168 µg/kg of 1,2-unsaturated PAs, with the higher concentrations relating to the period when birds were still receiving contaminated feed. Eggs were found to contain a wider range of 1,2-unsaturated PAs than observed in the feed available for analysis, indicating another unrecognized source of exposure.

Seeds of *Crotalaria pallida* were included in the diet of layer chickens at concentrations of 0, 1, 2 or 3% for 5 weeks (Diaz, Almeida & Gardner, 2014). Analysis of seeds detected usaramine and its *N*-oxide at a total alkaloid concentration of 0.18% dry weight. Usaramine was detected in eggs in a dose-dependent manner. The maximum usaramine concentration detected in eggs was 885 µg/kg in lyophilized egg, in the high-dose group after 14 days' feeding. The lowest measured concentration of usaramine was in eggs from the low-dose group after 7 days of feeding (290 µg/kg). Usaramine was still detectable in eggs laid 7 days after the cessation of feeding, at concentrations of 11.5, 9.5 and 43.5 µg/kg in eggs from the 1, 2 and 3% dose groups, respectively. All analyses were carried out on lyophilized eggs. The authors of the study estimated that the usaramine concentrations in fresh eggs would have been in the range 77–235 µg/kg.

In contrast to these findings are those of a Turkish study that included *Senecio vernalis* (PA content 0.14%) in the diet of layer chickens at concentrations of 0, 0.5, 2.0 and 4.0% for 210 days. The main PA in the plant material was

senecionine. No residues of PAs were detected in eggs at any dose level (Eröksüz et al., 2003b). In a more recent study by the same group, plant material from *Senecio vernalis* (SV), *Heliotropium dolosum* (HD) and *Heliotropium circinatum* (HC) was included in the feed of Japanese quail. The birds received feed that contained 70% normal feed and 30% PA-containing plant material for 6 weeks (Eröksüz et al., 2008). PA concentrations in the feeds were 420 mg/kg (SV), 390 mg/kg (HD) and 450 mg/kg (HC). Eggs were sampled and analysed at the end of week 6. The analytical technique included reduction of *N*-oxides to their associated 1,2-unsaturated PAs. In the SV group, only senecionine was detected, at a concentration of 3.21 mg/kg. In the HD group, only europine was detected, at a concentration of 8.66 mg/kg. In the HC group, europine (19.05 mg/kg) and heliotrine (1.46 mg/kg) were detected. It should be noted that the 1,2-unsaturated PA concentrations found in eggs in this study were at least an order of magnitude greater than those quantified in other studies (Diaz, Almeida & Gardner, 2014; Edgar & Smith, 2000).

Laying hens were fed for 14 days with diets containing 0.5% common ragwort (*Jacobaea vulgaris*), common groundsel (*Senecio vulgaris*), narrow-leaved ragwort (*Senecio inaequidens*), viper's bugloss (*Echium vulgare*) and common heliotrope (*Heliotropium europaeum*) (Mulder et al., 2016). Samples were analysed for more than 90 1,2-unsaturated PAs, *N*-oxides and associated metabolites. Total PA content of feeds was in the range 5.5–53.1 mg/kg. PAs were transferred to eggs, reaching "steady-state" concentrations in the range 2–216 µg/kg. While the feed materials contained higher concentrations of *N*-oxides than the associated PA, the reverse was the case in eggs.

As part of an EU-wide project, PAs (35 included in analysis) were determined in retail egg samples (n = 205) (Mulder et al., 2015). PAs were detected in 2/205 egg samples (1.0%). The PAs detected in the two samples were lycopsamine (0.12 µg/kg) and retrorsine (0.10 µg/kg).

6.2.5 Meat, including organ meat

While no information was found on PAs in retail meat, several studies have demonstrated the potential for PAs to be present in meat following feeding of PA-containing plant material to stock animals.

An Australian assessment reported that PAs may be present in the liver and kidneys of domestic animals at concentrations in the range <10 to 73 µg/kg (ANZFA, 2001).

In a radiotracer study, a dairy cow received a single oral dose of 1 mg/kg bw of [^{3}H] seneciphylline (Candrian et al., 1991). At slaughter, a concentration of 40 µg/kg was found in fresh liver.

An Australian study reported results of three experiments in which

PA-containing plant material was fed to weaned calves (Fletcher et al., 2011a). For animals receiving rattlepod (*Crotalaria novae-hollandiae* subspecies *novae-hollandiae*) at approximately 15% of a maintenance diet for 6 weeks (total PA intake 5.5 mg/kg bw per day), concentration of 1,2-unsaturated PAs and their *N*-oxides in muscle reached a maximum of 250 µg/kg, while in liver a maximum concentration of 2500 µg/kg was found. PAs present in the plant (monocrotaline, pumiline A, trichodesmine and crosemperine) were also detected in animal tissues, but in differing proportions. For animals receiving blue heliotrope (*Heliotropium amplexicaule*) at approximately 15% of a maintenance diet for 6 weeks (total PA intake 15 mg/kg bw per day) tissue concentrations of 1,2-unsaturated PAs and their *N*-oxides were at the LOQ (1 µg/kg) or below. PAs present in the plant material were indicine and heliospathine, predominantly present in the form of *N*-oxides. Analysis of tissue samples from 50 animals from 10 properties in an area where blue heliotrope was believed to be a pest detected trace PA concentrations (about 1% of concentrations found in feeding trials) in liver samples of animals from two properties. For animals given fireweed (*Senecio brigalowensis*) at approximately 15% of a maintenance diet for 6 weeks (total PA intake 2.5 mg/kg bw per day) concentrations of 1,2-unsaturated PAs and their *N*-oxides in liver reached a maximum of 400 µg/kg after 2–3 weeks, then decreased to 40 µg/kg. Muscle PA concentrations were not given, but were reported to follow a similar trend to those in the liver. An abattoir survey of cattle from a fireweed-affected area detected DHP adducts in liver samples from 80% of animals (n = 189), but the concentrations were about 1–10% of those found in calf livers in the feeding trial.

Transfer of PAs to poultry meat was demonstrated in a study in which laying hens were fed for 14 days with diets containing 0.5% common ragwort (*Jacobaea vulgaris*), common groundsel (*Senecio vulgaris*), narrow-leaved ragwort (*Senecio inaequidens*), viper's bugloss (*Echium vulgare*) and common heliotrope (*Heliotropium europaeum*) (Mulder et al., 2016). Animals were slaughtered just after the last exposure. Total PA concentrations (sum of more than 90 1,2-unsaturated PAs and their *N*-oxides and some metabolites) in muscle meats were in the range 0.3–67.1 µg/kg, while higher concentrations were found in organ meats (liver and kidney; 0.89–391 µg/kg). Patterns of PAs seen in meat were similar to those seen in eggs, with lower concentrations of *N*-oxides than parent PAs.

As part of an EU-wide project, PAs (35 included in analysis) were determined in retail meat (beef, pork and chicken) and liver (beef, pork and chicken) samples (n = 273) (Mulder et al., 2015). PAs were not detected in any meat or liver samples.

6.2.6 Honey

Honey is the food that has been investigated most frequently for the presence of 1,2-unsaturated PAs and their *N*-oxides. Summaries of relevant studies are given in Table 30.

It is difficult to draw conclusions regarding possible differences in the PA content of honey from different countries. The plants that bees collect pollen from will differ from region to region and it is plausible that the content of 1,2-unsaturated PAs and their *N*-oxides in honey could be dependent on geography. However, the studies summarized in Table 30 include a mixture of random retail surveys and surveys targeted at honeys with particular floral inputs.

Honey also appears to be a widely traded commodity and there is evidence that the retail surveys reported in Table 30 included products originating from a range of countries. For example, the BfR study (BfR, 2013b), data from which were used as the basis for the EFSA assessment (EFSA, 2011), included a country-level summary for the proportion of honey samples for which country of origin information was provided. The BfR report summarized concentration data for 1,2-unsaturated PAs and their *N*-oxides in honey from 36 countries, with mean concentrations of 1,2-unsaturated PAs and their *N*-oxides for positive samples ranging from 1 µg/kg (Czech Republic, Poland) to 163 µg/kg (Thailand).

6.2.7 Herbal supplements/medicines

Herbal products are an extremely diverse group of substances, which have the potential to contain very high concentrations of PAs.

Edgar et al. (1992) examined the PA composition of three Chinese medicinal herbs; "*pei lan*" (from *Eupatorium cannabinum*), "*cheng gan cao*" (from *Eupatorium japonicum*) and "*zi xiao rong*" (from *Crotalaria assamica*). *Zi xiao rong* was found to only contain monocrotaline. *Pei lan* contained predominantly 1,2-unsaturated PAs, such as viridiflorine and cynaustraline, whereas *cheng gan cao* contained similar proportions of saturated and 1,2-unsaturated PAs, including lycopsamine and intermedine. The total PA content of the herbal products was not determined.

Avula et al. (2015) analysed 1,2-unsaturated PAs and their *N*-oxides in dietary supplements ($n = 21$) claimed to contain material from *Petasites hybridus* (Avula et al., 2012; Avula et al., 2015). PAs (senecionine, senecionine *N*-oxide, integerrimine and senkirkine) were detected in 7 of the 21 dietary supplements. Results were expressed in terms of the recommended daily dose of the supplement, with 1,2-unsaturated PAs and their *N*-oxides in the range 0.1–8.4 mg per daily dose (Avula et al., 2012).

Mulder et al. (2015) analysed food supplements ($n = 30$) containing plant material based on PA-producing plants for 28 1,2-unsaturated PAs and

Table 30
Occurrence and concentrations of pyrrolizidine alkaloids (PAs) in honey

Study country	Sample description	Number of 1,2-unsaturated PAs and their *N*-oxides included	LOD/LOQ (µg/kg)	Number of samples	Percentage of samples > LOD	Concentration of 1,2-unsaturated PAs and their *N*-oxides (µg/kg)[a]				Main 1,2-unsaturated PAs or *N*-oxides found	Study reference
						Mean	Median	P95	Max		
Australia	Bulk "risk" honeys; retail and "non-risk" floral sources	15, results expressed as echimidine equivalents	LOD: 1.5	29 39	100 48.7	650 140	560 57	1640 470	2270 760	NS	Beales et al. (2004)
Australia	Honey for mead production, from area with large amounts of *Echium plantagineum*	7, expressed as heliotrine equivalents	LOD: 50	1	100	780	–	–	–	Echimidine	Cao, Colegate & Edgar (2013)
Belgium	Commercial	16	LOD: 0.004–0.05 LOQ: 0.03–0.17	16	93.4	12.3	7.0	–	65	Echimidine, lycopsamine, intermedine, retrorsine	Huybrechts & Callebaut (2015)
Brazil	Honey from federally-inspected establishments	8	LOD: 0.1–1.0 LOQ: 0.2–1.5	92	98.9	–	–	–	–	Senecionine, senecionine *N*-oxide	Valese et al. (2016)
Europe, various	Commercial	77	LOD: 0.5	13	76.9	24.3	15.5	76.3	87.4	Senecionine, lycopsamine, seneciphylline	Oplatowska et. al. (2014)
Germany	Retail Central/South American European Community	19	LOQ: 1–3	696 2 839 381	94 68 65	26 67 26	19 27 12		267 1 087 225	NS	Dübecke, Beckh & Lüllmann (2011)
Germany	New Zealand Echium honeys; imported raw honey; Netherlands ragwort honeys	16[b]	LOQ: 1, except monocrotaline and monocrotaline *N*-oxide (50)	8 8 31	100 75 93.5	920 261 179	920 57.5 69	 810	1 050 1 215 1 793	Echimidine, lycopsamine, senecionine, senecionine-*N*-oxide, seneciphylline, seneciphylline-*N*-oxide	Kempf et al. (2011c)
Germany	Retail (Germany, other European, Internet)	All retronecine-based PA, results expressed as retronecine equivalents[c]	NS	216	8.8	56	52	94	120	NS	Kempf et al. (2008)

Table 30 (continued)

Study country	Sample description	Number of 1,2-unsaturated PAs and their *N*-oxides included	LOD/LOQ (µg/kg)	Number of samples	Percentage of samples > LOD	Concentration of 1,2-unsaturated PAs and their *N*-oxides (µg/kg)[a]				Main 1,2-unsaturated PAs or *N*-oxides found	Study reference
						Mean	Median	P95	Max		
Germany	Medical honeys	All retronecine-based PA, results expressed as retronecine equiva-lents[c]	NS	19	94.7	83.6			495	NS	Cramer & Beuerle (2012)
Germany	German/Austrian beekeepers;	17	LOD: 0.06–0.23	15	46.7	6.1[d]	1.4[d]		28.2	NS	Bodi et al. (2014)
	brand products;			35	94.3	14.5	5.3		234.5		
	discount products;			17	94.1	13.3	11.1		52.8		
	other forms of sale			20	90.0	10.6	5.1		59.4		
Germany	Bulk	8[e]	LOD: 0.5–2.0	13 280	71	28–36[f]	6–15[f]	121–125[f]	2206–2212[f]	Echimidine, lycopsamine, echiumine	EFSA (2011)
		14	LOQ: 1.0–3.0	8 383	77	37–44	9–15	155–161	3298–3301		
		17		4 897	66	34–52	5–24	157–168	2334–2352		
	Retail	8		1 324	90	16–26	10–20	57–62	169–176		
		14		208	85	13–20	7–15	46–50	150–158		
		17		1 116	92.5	20–36	12–28	68–76	267–278		
Germany	Direct from beekeepers	28	LOD: 1	86	53.5	34	6.5		604	Senecionine *N*-oxide, seneciphylline *N*-oxide, seneciphylline	Neumann & Huckauf (2016)
Ireland	Retail	11	LOD: 13–31	50	16.0	1 891	838		5 614	Lycopsamine, echimidine	Griffin et al. (2013)
Ireland	Retail honey from Australian supermarkets	14	LOD: 0.4–2.8	59	69.5	155.8	117	341	932	Lycopsamine, echimidine	Griffin et al. (2015b)
Ireland	Retail	14	LOD: 0.5–3.9	150	22.7	87.1	30.4	420	546	Lycopsamine, echimidine	Griffin et al. (2015a)
Italy	Retail	9	LOD: 0.021–1.39	70	64	14.4	–	–	172	Echimidine, lycopsamine, senkirkine	Martinello et al. (2014)
Italy	Beekeepers	12	LOQ: 0.25	60	28.0	3.9	1.5	–	17.6	Retrorsine, seneciphyl-line, senecionine	Lucatello et al. (2016)

Study country	Sample description	Number of 1,2-unsaturated PAs and their *N*-oxides included	LOD/LOQ (µg/kg)	Number of samples	Percentage of samples > LOD	Concentration of 1,2-unsaturated PAs and their *N*-oxides (µg/kg)[a]				Main 1,2-unsaturated PAs or *N*-oxides found	Study reference
						Mean	Median	P95	Max		
the Netherlands	Retail	11	LOD: 1	171	28.7	23.6	6	59.4	365	Senecionine[g]	Nederlandse Voedsel en Warenautoriteit (NVWA) (2007)
New Zealand	Retail (specialist honeys)	15	LOD: 1	9	77.8	1 017	697		2 850	Echivulgarine, echimidine	Betteridge, Cao & Colegate (2005)
Spain	Commercial floral honeys, mainly of purple viper's bugloss	16	LOD: 1.0	103	94.2	48.1			237	Echimidine, lycopsamine	Orantes-Bermejo et al. (2013)
Switzerland	Retail	18	LOD: 1–3	71	53.5	6.7			55		Kast et al. (2014)
United Kingdom	Retail	5	LOD: 10	2	0.0	–			–	Senecionine, seneciphylline, jacobine	Crews, Startin & Clarke (1997)
	Honey from specific hives (near ragwort);			6	66.7	32.3			56		
	honeys with characteristics of ragwort contamination			2	100	950			1 480		
Honey-containing foods											
Germany	– mead	All retronecine-based PA, results expressed as retronecine equivalents[c]	LOQ: 10	19	15.8	192[h]			484	NS	Kempf et al. (2011b)
	– candy			10	20.0	25					
	– fennel honey			9	33.3	58					
	– soft drinks			5	0.0						
	– power bars and cereals			7	0.0						
	– jelly babies			3	0.0						
	– baby food			3	0.0						
	– supplements			3	0.0						
	– fruit sauce			1	0.0						

Table 30 (continued)

Study country	Sample description	Number of 1,2-unsaturated PAs and their *N*-oxides included	LOD/LOQ (µg/kg)	Number of samples	Percentage of samples > LOD	Concentration of 1,2-unsaturated PAs and their *N*-oxides (µg/kg)[a]				Main 1,2-unsaturated PAs or *N*-oxides found	Study reference
						Mean	Median	P95	Max		
Australia	Mead	7, expressed as heliotrine equivalents	LOD: 50	2	100	388			540	Echimidine	Cao, Colegate & Edgar (2013)

NS: not stated; P95: 95th percentile; Max: maximum; LOD: limit of detection; LOQ: limit of quantification

[a] Unless otherwise stated, summary statistics refer only to samples in which 1,2-unsaturated PAs and their *N*-oxides were detected.

[b] All samples were also analysed by the "sum parameter" method, which expresses results as retronecine base equivalents. However, only results for the sum of 16 1,2-unsaturated PAs and *N*-oxides are give here.

[c] Retronecine base has a molecular weight of 155. Associated 1,2-unsaturated PAs and PA-*N*-oxides have molecular weights in the range 299 to 413.

[d] Mean and median calculation included analytical results below the LOD.

[e] The data set included analyses from two sources covering different ranges of PAs. All analyses had 8 PAs in common (echimidine, echimidine-NO, heliotrine, lycopsamine, retrorsine, senecionine, seneciphylline and senkirkine). Analyses from one source included a further 6 PAs (heliotrine-NO, lasiocarpine, lycopsamine-NO, retrorsine-NO, senecionine-NO and seneciphylline-NO), giving a total of 14; the other source included 9 additional PAs (acetylechimidine, acetylechimidine-NO, acteylechiumine-NO, echiumine, echiumine-NO, echiuplatine, echiuplatine-NO, echivulgarine and echivulgarine-NO).

[f] Lower and upper bound estimates of summary statistics were derived by assigning results below the LOD/LOQ a value of zero (lower bound) or the respective LOD or LOQ (upper bound).

[g] The screen used in this survey did not include commonly detected 1,2-unsaturated PAs, such as lycopsamine and echimidine.

[h] Mean concentrations are only approximate, as the results were only presented in graphical format.

N-oxides. Concentrations of total PAs (on a dry plant material basis) were in the range ND–2.4 × 10^6 µg/kg, with the highest concentrations in products based on *Borrago officinalis* and *Eupatorium* spp. However, even in food supplements not containing plant material based on PA-producing plants (n = 140), total PA concentrations as high as 8500 µg/kg were found.

Chinese herbal medicines (n = 54) were obtained from retail outlets in Ireland and analysed for the presence of 14 1,2-unsaturated PAs and *N*-oxides (Griffin et al., 2014). PAs were detected in 42 samples (78%). The concentrations of 1,2-unsaturated PAs and their *N*-oxides were in the range 13–3668 µg/kg (mean 330 µg/kg, median 112 µg/kg, 95th percentile 1742 µg/kg). While a wide range of 1,2-unsaturated PAs and *N*-oxides were detected across the set of samples, senecionine (31 samples) and echimidine (30 samples) were the most commonly detected 1,2-unsaturated PAs or *N*-oxides.

Herbal medicines (n = 41), based on peppermint, chamomile, nettle, fennel, anise, caraway and melissa were analysed for the presence of 17 1,2-unsaturated PAs and *N*-oxides (Bodi, et al., 2014). 1,2-Unsaturated PAs and *N*-oxides were detected in 16 samples (39%), with 1,2-unsaturated PAs and *N*-oxides most frequently detected in products containing nettle (67%), melissa (60%) and anise (57%). Mean concentrations of 1,2-unsaturated PAs and their *N*-oxides ranged from 12.1 µg/kg (caraway) to 452.6 µg/kg (anise).

Products sold as "Miracle tea" are prepared from *Packera candidissima* and *Packera bellidifolia* and used in Mexico for a range of medicinal purposes (Fragoso-Serrano et al., 2012). Phytopharmaceutical products (n = 7) prepared from these plant sources were analysed for retrorsine and senecionine and their *N*-oxides. 1,2-Unsaturated PAs and *N*-oxides were detected in six samples, in concentrations ranging from 1 to 65 mg/kg (100 to 65 000 µg/kg, mean 15 500 µg/kg).

Extracts of comfrey (*Symphytum officinale* L.) root have been used for topical and internal medicinal purposes (Janes & Kreft, 2014). Comfrey root was analysed by a TLC method and estimated to contain 710 mg/kg of lycopsamine.

The dried extract of "*chuan zi wan*", a traditional Chinese herbal medicine prepared from *Ligularia* spp., was found to contain 7920 mg/kg of 1,2-unsaturated PAs (Tang, Cheng & Hattori, 2012). The predominant 1,2-unsaturated PAs were clivorine and ligularine. Low concentrations of saturated PAs (ligularinine and platyphylline) were also tentatively identified in this sample.

6.2.8 Culinary herbs

Culinary herb mixes available as convenience products (n = 21) were purchased from supermarkets and farmers' markets in Germany and analysed for PAs by the "sum parameter" method, in which 1,2-unsaturated PAs and and their *N*-oxides

are converted to the retronecine base (Cramer et al., 2013). PAs were detected in 15 samples (71%), with concentrations in the range 0.9–74 µg/kg retronecine equivalents fresh weight (mean of positive samples, 36 µg/kg). Fresh leaves of borage (*Borago officinalis*) were found to contain an average PA content of 133 µg/kg retronecine equivalents (253 µg/kg expressed as lycopsamine).

6.2.9 Other foods

(a) Pollen

Although pollen is not traditionally considered as a human food, it is used as a dietary supplement, particularly as products based on bee pollen (the form in which bees store pollen in the hive) (Kempf et al., 2010; Kennedy, 2005).

Pollen taken from the anthers of *Echium vulgare* in Germany ($n = 2$) was found to contain 8222 and 13 985 mg/kg 1,2-unsaturated PAs and their *N*-oxides, expressed as lasiocarpine-*N*-oxide equivalents (Boppré, Colegate & Edgar, 2005). The main 1,2-unsaturated PA or *N*-oxide detected was echivulgarine *N*-oxide (83 or 84% of 1,2-unsaturated PA and *N*-oxide content). Pollen was collected from the legs of bees (bee pollen) that had visited particular plant types (Boppré et al., 2008). For bees visiting *Echium vulgare*, the concentration of 1,2-unsaturated PAs and their *N*-oxides in bee pollen was 350 mg/kg lasiocarpine *N*-oxide equivalents. Echivulgarine *N*-oxide was the main 1,2-unsaturated PA or *N*-oxide detected, as would be expected from the composition of the pure plant pollen. 1,2-Unsaturated PAs and their *N*-oxides (in lasiocarpine *N*-oxide equivalents) were also determined in bee pollen from bees visiting *Eupatorium cannabinum* (120 mg/kg, major compound probably echinatine *N*-oxide), *Senecio jacobaea* (100 mg/kg, mainly erucifoline *N*-oxide, seneciphylline *N*-oxide and senecionine *N*-oxide), *Senecio ovatus* (70 mg/kg, mainly fully saturated PAs), *Echium plantagineum* (6–28 mg/kg, predominantly echimidine *N*-oxide). For pollen collected directly from the plant, higher PA concentrations were found, including up to 800 mg/kg from *Senecio jacobaea* and 155 mg/kg from *Senecio ovatus*.

1,2-Unsaturated PAs and their *N*-oxides were determined in pollen and flower samples from *Senecio vernalis* (4100 and 2300 mg/kg), *Senecio jacobaea* (3300 and 3400 mg/kg), *Eupatorium cannabinum* (600 and 4200 mg/kg), *Echium vulgare* (900 and 2000 mg/kg) and *Phalaenopsis* hybrids (600 and 4400 mg/kg) (Kempf et al., 2010). Except for *Phalaenopsis* hybrids, whose pollen predominantly contained 1,2-unsaturated PAs, PAs present in pollen samples were predominantly (69–98%) in the form of *N*-oxides.

Pollen products for sale as dietary supplements ($n = 55$) were purchased from supermarkets, mainly in Germany, France and Italy (Kempf et al., 2010). 1,2-Unsaturated PAs and their *N*-oxides were converted to retronecine base and reported as retronecine equivalents. PAs were detected in 17 (31%) of samples, with

concentrations in the range 1.08–16.35 mg/kg (1080–16 350 µg/kg) retronecine equivalents (mean 5168 µg/kg, median 2830 µg/kg). Of the PA-positive pollen samples, approximately half were labelled as coming from European countries, whereas for the remainder the country of origin was not specified.

Bee pollen samples (n = 119) were collected from a variety of retail sources and analysed for the presence of 19 1,2-unsaturated PAs and *N*-oxides (Dübecke, Beckh & Lüllmann, 2011). PAs were detected in 60% of bee pollen samples, with concentrations in the range 11 to 37 855 µg/kg (mean 1846 µg/kg, median 192 µg/kg).

(b) Plant oils

Some plant oils have gained attention as sources of ω-fatty acids (Cramer et al., 2014). Oils from the boraginaceous plant *Lappula squarrosa* were extracted and refined (Cramer et al., 2014). *L. squarrosa* seeds were found to contain 16 mg/kg retronecine equivalents, while the crude plant oil contained 29 µg/kg retronecine equivalents. Refining processes (washing, neutralization, bleaching and deodorization) were shown to decrease the PA content of the oil to 0.07% of the crude oil content. Analysis of commercial boraginaceous oils (n = 10) detected PA in three oils at concentrations in the range 0.08–0.6 µg/kg retronecine equivalents. Analysis of seed oil of *Borago officinalis* did not detect PAs above the LOD (0.2 µg/kg) (Vacillotto et al., 2013).

6.3 Animal feed

Feeding trials using materials known to contain high concentrations of 1,2-unsaturated PAs and their *N*-oxides have demonstrated that 1,2-unsaturated PAs and their *N*-oxides may be transferred to foods of animal origin (see sections 2.1.5, 6.2.3, 6.2.4 and 6.2.5). However, consideration should be given to whether such high concentrations of 1,2-unsaturated PAs and their *N*-oxides occur in actual animal feed.

Most of the information on 1,2-unsaturated PAs and their *N*-oxides in animal feed relates to forages and roughages (e.g. hay, silage, alfalfa/lucerne) rather than compounded animal feeds. Available information is summarized in Table 31. The Belgian study appears to have mainly examined feed for non-food-producing animals. High concentrations of PAs (138 and 411 µg/kg) were found in lucerne and lucerne-based feeds for horses. The main PAs detected in these feed samples were retrorsine and retrorsine *N*-oxide, seneciphylline and seneciphylline *N*-oxide, and senecionine and senecionine *N*-oxide.

While EFSA concluded that it was not possible to provide any reliable estimate of exposure of livestock to 1,2-unsaturated PAs and their *N*-oxides, a

Table 31
Pyrrolizidine alkaloid (PA) content of animal feeds

Study country	Feed type	Number of 1,2-unsaturated PAs and their *N*-oxides	Limit of detection (LOD) (µg/kg)	Number of samples	Percentage positive	Concentration of 1,2-unsaturated PAs and their *N*-oxides (µg/kg)[a]			Reference
						Mean	Min	Max	
Belgium	Horse	16	0.01–0.22	–	–	104	2.3	411	Huybrecht & Callebaut (2015)
	Rabbit			–	–	76.7	0.06	153	
	Rodent			–	–	10.5	–	10.5	
	Cat			–	–	1.6	0.3	3.6	
	Bird			–	–	0.35	0.07	1.9	
Czech Republic[b]	Hay	5	0.2–0.8	6	83	82.0	6.1	157	Bolechová et al. (2015)
	Silage			11	55	25.7	5.0	49.8	
	Alfalfa			4	50	7.9	5.4	10.3	
	Compound feed			20	40	54.1	5.3	137	
European Union[c,d]	Cereal grains	>50	4.5 [c,d]	6	17	4.9–23	–	30–48	EFSA (2011)
	Oil seeds and fruits			56	23	13–31	–	343–361	
	Legume seeds			1	0	0–18	–	–	
	Tubers and roots			1	0	0–18	–	–	
	Other seeds/fruits			3	33	12–30	–	36–54	
	Forages and roughage			252	49	272–290	–	22 753–22 771	
	Other plants and algae			32	62	316–334	–	3209–3306	
the Netherlands	Hay	40	10	37	3	–	–	549	Mulder et al. (2009)
	Silage			56	5	19.3	15	28	
	Grass			23	17	80.5	10	288	
	Alfalfa			31	74	613	10	5401	

[a] Summary statistics apply to positive samples only, unless otherwise stated.

[b] Concentrations of five 1,2-unsaturated PAs were not identifiable for individual samples. The minimum and maximum concentrations reported here are the minimum and maximum concentrations for any single 1,2-unsaturated PA. However, there was a low level of co-occurrence of individual 1,2-unsaturated PAs.

[c] The analytical data used by EFSA came from the Dutch monitoring programme for animal feed, between 2006 and 2010 and probably include the results of the study by Mulder et al. (2009), which were derived from the same programme for the period 2006 to 2008.

[d] To calculate lower and upper bound estimates of summary statistics, 1,2-unsaturated PAs were grouped into four groups of structurally related PAs (senecionine-like, lycopsamine-like, heliotrine-like and monocrotaline-like) and a group level LOD of 4.5 µg/kg was assigned. Lower bound estimates were based only on quantified results; upper bound estimates were calculated from individual estimates derived by adding 4.5 µg/kg to the quantified results for each group of PAs that were not detected in the sample. Mean estimates are for all samples, not just those in which PAs were detected.

"worst case" exposure was determined for farm livestock, based on the highest concentration of 1,2-unsaturated PAs and their *N*-oxides detected in alfalfa/lucerne (i.e. 6.2 mg/kg) (EFSA, 2011). If the lucerne was the sole forage for a high-yielding dairy cow then this would equate to an exposure level to 1,2-unsaturated PAs and their *N*-oxides of 0.14 mg/kg bw per day. Most of the feeding trial studies summarized in sections 6.2.3, 6.2.4 and 6.2.5 do not report the dose in terms of the animal body weight. The exception was the study of Fletcher et al. (2011a) where various plant sources of 1,2-unsaturated PAs and their *N*-oxides were fed to calves, giving doses of 1,2-unsaturated PAs and their *N*-oxides in the range 2.5–15 mg/kg bw per day. These dose levels are up to two orders of magnitude greater than the worst case exposure scenario proposed by EFSA.

The Committee calculated the dose range of 1,2-unsaturated PAs and their *N*-oxides used in the study of Hoogenboom et al. (2011) as 0.16–0.77 mg/kg bw per day for three dairy cows. At the lowest dose levels a mean concentration of 1,2-unsaturated PAs and their *N*-oxides of 2.1 µg/L was determined. The exposure dose of 1,2-unsaturated PAs and their *N*-oxides (16 mg/kg bw per day) was also estimated for the study of Dickinson et al. (1976) resulting in PA concentrations in milk in the range 470–835 µg/L.

7. Food consumption and dietary exposure assessment

Based on the toxicological evaluation by the Committee, both acute and chronic dietary exposures are relevant to 1,2-unsaturated PAs and their *N*-oxides; therefore, both were considered for this assessment. No specific at-risk population groups were identified; therefore, any dietary exposure estimates from the general population were considered.

No estimates of dietary exposure were submitted to the Committee for review and dietary exposure estimates summarized in the following section were obtained from a search of the literature. Owing to analytical differences between studies, the number of compounds that the dietary exposure estimates represent may vary.

7.1 National estimates of dietary exposure from the scientific literature

Few estimates of dietary exposure to 1,2-unsaturated PAs and their *N*-oxides were found in the scientific literature and those reported have often considered only some of the potential food routes of exposure.

7.1.1 Germany

The *Bundesinstitut für Risikobewertung* (BfR) conducted risk assessments for the presence of 1,2-unsaturated PAs and their *N*-oxides in honey and herbal teas and teas (BfR, 2013a,b). The risk assessments included both an acute and a chronic exposure assessment for children and adults. Food consumption data for adults (14–80 years) were taken from the NVS II National Food Consumption Study (n = 20 000, two independent 24-hour dietary recalls and a 4-week dietary history). Data on children's (age 6 months to less than 5 years) food consumption were taken from the VELS study (n = 816, two 3-day dietary diaries). For adult food consumption, the honey exposure assessment used the 4-week dietary history for chronic exposure estimates and the two independent 24-hour dietary recalls for acute exposure assessment. The two independent 24-hour dietary recalls were used for both chronic and acute exposure assessments for herbal teas and teas.

(a) Honey

Honey consumption included consumption of honey as a separate food and of honey as a component of recipes.

For the acute exposure assessment, children's honey consumption was based on the 97.5th percentile consumption level (consumers only) of 1.36 g/kg bw per day, while adult honey consumption was based on the 95th percentile

consumer-only consumption level of 0.88 g/kg bw per day. Chronic exposure estimates used overall respondent mean and 95th percentile consumption levels for children (mean 0.10 g/kg bw per day, 95th percentile 0.40 g/kg bw per day) and adults (mean 0.05 g/kg bw per day, 95th percentile 0.28 g/kg bw per day).

For data on 1,2-unsaturated PAs and their *N*-oxides in honey, results were taken from two laboratories, which had analysed different selections of individual 1,2-unsaturated PAs and *N*-oxides. Dietary exposure calculations were carried out based on the sum of analysed 1,2-unsaturated PAs and *N*-oxides as reported by each laboratory, the sum of only the eight 1,2-unsaturated PAs and *N*-oxides that the laboratories analysed in common, and the sum of 1,2-unsaturated PAs and *N*-oxides from the laboratory that provided most of the results and analysed honeys for 18 1,2-unsaturated PAs and *N*-oxides. Exposures were estimated based on lower, middle and upper bound estimates of 1,2-unsaturated PA and *N*-oxide concentrations.

Four chronic exposure scenarios were defined representing the four possible combinations of mean and 95th percentile consumption and concentration data from the two laboratories. Use of 95th percentile concentration data was taken to represent brand loyalty to products with high concentrations of 1,2-unsaturated PAs and their *N*-oxides. However, the Committee does not usually consider brand loyalty in relation to exposure to contaminants, and chronic dietary exposure estimates based on 95th percentile 1,2-unsaturated PA and *N*-oxide concentrations have not been summarized.

Adult acute exposures (18 1,2-unsaturated PAs and *N*-oxides), based on mean concentrations of 1,2-unsaturated PAs and their *N*-oxides, were in the range 0.018–0.037 µg/kg bw per day (95th percentile concentrations; 0.060–0.072 µg/kg bw per day). Acute child exposures, based on mean concentrations of 1,2-unsaturated PAs and their *N*-oxides, were in the range 0.028–0.057 µg/kg bw per day (95th percentile concentrations; 0.092–0.112 µg/kg bw per day). Mean chronic exposures for adults were in the range 0.001–0.002 µg/kg bw per day (95th percentile 0.005–0.012 µg/kg bw per day). Mean chronic child exposures were in the range 0.002–0.004 µg/kg bw per day (95th percentile 0.006–0.017 µg/kg bw per day).

(b) Herbal tea and tea

Assessments of exposure to 1,2-unsaturated PAs and their *N*-oxides from ingestion of herbal teas and teas used information from a survey of 1,2-unsaturated PAs and their *N*-oxides in dried tea material (Bodi et al., 2014). It was assumed that 200 mL of tea would be infused from 2 g of dried material and that 100% of 1,2-unsaturated PAs and their *N*-oxides would be extracted into the infusion. While exposure to PAs was derived separately for tea and herbal tea, the

Table 32
Consumption amounts (g/kg bw per day) of dried tea and dried herbal tea used for acute and chronic estimates of dietary exposure to 1,2-unsaturated PAs and their *N*-oxides in the BfR risk assessment

	Acute (95th percentile, consumers only)		Chronic (all respondents, mean (95th percentile))	
	Tea	Herbal tea	Tea	Herbal tea
Children	0.763	0.637	0.055 (0.231)	0.026 (0.137)
Adults	0.291	0.269	0.032 (0.153)	0.015 (0.093)

Source: Data from BfR (2013a).

consumption data for tea appear to include consumption of herbal tea. Exposure to 1,2-unsaturated PAs and their *N*-oxides from consumption of herbal tea should be considered to be a subset of exposure to 1,2-unsaturated PAs and their *N*-oxides from consumption of tea.

For the acute exposure assessment, tea consumption was based on the 95th percentile consumption level (consumers only), whereas, for the chronic exposure estimates, all respondent mean and 95th percentile consumption levels were used. Daily dried tea consumption used in the BfR risk assessment is summarized in Table 32.

Exposure estimates were calculated based on lower, middle and upper bound estimates of concentrations of 1,2-unsaturated PAs and their *N*-oxides. PA concentration data were taken as the aggregate of results for herbal tea varieties (fennel, chamomile, herbal, peppermint, melissa, nettle and baby fennel). These concentration estimates were applied to consumption data for tea and herbal tea. This approach will tend to overestimate exposure to 1,2-unsaturated PAs and their *N*-oxides from tea overall, as the non-herbal teas analysed generally contained lower concentrations of 1,2-unsaturated PAs and their *N*-oxides than the herbal teas. Four chronic exposure scenarios were defined representing the four possible combinations of mean and 95th percentile consumption and concentration data. Use of 95th percentile concentration data was taken to represent brand loyalty to products with high concentrations of 1,2-unsaturated PAs and their *N*-oxides. However, the Committee does not usually consider brand loyalty in relation to exposure to contaminants, and chronic dietary exposure estimates based on 95th percentile concentrations of 1,2-unsaturated PAs and their *N*-oxides have not been summarized. Acute exposure estimates were derived for mean and 95th percentile concentrations of 1,2-unsaturated PAs and their *N*-oxides.

Acute exposure to 1,2-unsaturated PAs and their *N*-oxides (lower bound-upper bound) for children, based on mean concentrations of 1,2-unsaturated PAs and their *N*-oxides, were 0.126–0.210 µg/kg bw per day (herbal tea) or 0.151–

Table 33
Chronic exposure to 1,2-unsaturated PAs and their *N*-oxides for German children and adults from consumption of tea and herbal tea

	Exposure to 1,2-unsaturated PAs and their *N*-oxides (µg/kg bw per day)[a]			
	Acute (95th percentile, consumers only)		Chronic (all respondents, mean (95th percentile))	
Scenario	Tea	Herbal tea	Tea	Herbal tea
Average consumer	0.011–0.014–0.018	0.005–0.007–0.009	0.006–0.008–0.010	0.002–0.003–0.003
95th percentile consumer	0.046–0.060–0.076	0.027–0.036–0.045	0.030–0.039–0.050	0.018–0.023–0.030

[a] The three figures in each cell represent exposure estimates using concentration data at the lower bound (values below LOD = 0, values below LOQ = LOD); middle bound (values below LOD = LOD/2, values below LOQ = LOQ/2); and upper bound (values below LOD = LOD, values below LOQ = LOQ).

0.252 µg/kg bw per day (tea overall) (95th percentile concentration; 0.574–0.655 µg/kg bw per day for herbal tea and 0.688–0.784 µg/kg bw per day for tea overall). For adults, acute exposure to 1,2-unsaturated PAs and their *N*-oxides, based on the mean concentration of 1,2-unsaturated PAs and their *N*-oxides, was 0.060–0.099 µg/kg bw per day for either herbal tea or tea overall (95th percentile concentration; 0.270–0.308 µg/kg bw per day for herbal tea or tea overall). Chronic exposure estimates are shown in Table 33.

(c) Culinary herbs

A scenario-based exercise estimated that the use of commercial culinary herb preparations in preparation of a traditional German dish could result in each consumer being exposed to 1.9 to 6.7 µg 1,2-unsaturated PAs and their *N*-oxides (0.032–0.112 µg/kg bw for a 60-kg adult) (Cramer et al., 2013).

7.1.2 Ireland

(a) Honey

Griffin et al. (2015b) estimated dietary exposure to 1,2-unsaturated PAs and their *N*-oxides from mean daily consumption of 20 g of honey or maximum daily consumption of 50 g honey by children (15 kg body weight) and adults (60 kg body weight). It should be noted that the the FAO/WHO Chronic Individual Food Consumption Database (CIFOCOss) gives a mean honey consumption for adult Irish for all respondents of 1.03 g per day, a consumers-only mean consumption of 10.3 g per day and a consumers-only 97.5th percentile of 42.9 g per day. This suggests that the scenarios reported by Griffin et al. (2015b) are best viewed as acute consumption scenarios, as they are unrealistically high for chronic consumption of honey. The scenarios considered consumption of honey containing a mean concentration of 1,2-unsaturated PAs and their *N*-oxides of 145 µg/kg (Australian and New Zealand honeys sourced from Irish

supermarkets) or 153 µg/kg (honeys sourced from Australian supermarkets). For children, this resulted in mean estimated dietary exposure to 1,2-unsaturated PAs and their *N*-oxides of 0.193 or 0.204 µg/kg bw per day (maximum exposure 0.483 or 0.510 µg/kg bw per day). Mean estimated dietary exposure for adults was 0.048 or 0.0512 µg/kg bw per day (maximum exposure 0.121 or 0.128 µg/kg bw per day). These exposure estimates will overestimate potential exposure to 1,2-unsaturated PAs and *N*-oxides for the Irish population as the average concentration of 1,2-unsaturated PAs and their *N*-oxides in non-Australian and New Zealand sourced honeys purchased in Ireland was reported to be 40 µg/kg.

(b) **Tea**

Concentrations of 1,2-unsaturated PAs and their *N*-oxides in herbal teas were equated to daily exposure on the basis of 1.5 g of dried tea per cup and three cups of tea per day (total 600 mL per day) (Griffin et al., 2014). The WHO CIFOCOss Food Consumption Database gives a mean adult population consumption for infused tea in Ireland of 605.8 g per day. Daily exposure estimates were in the range 0.09–7.8 µg per day (mean 1.89 µg per day) for herbal tea samples in which PAs were detected. PAs were detected in 9 of 18 tea samples. Assuming a body weight of 60 kg, this would give a range for potential exposures of 0.0015 to 0.130 µg/kg bw per day (mean 0.032 µg/kg bw per day). The upper end of this range is similar to German estimates of chronic exposure to 1,2-unsaturated PAs and their *N*-oxides from herbal tea for a 95th percentile consumer, exhibiting brand loyalty (always consuming tea with high 1,2-unsaturated PA and *N*-oxide concentrations).

(c) **Herbal medicines**

Although not strictly an exposure assessment based on actual consumption data, a survey of 1,2-unsaturated PAs and their *N*-oxides in Chinese herbal medicine products purchased in Ireland used PA concentration data and recommended dose information to calculate potential daily exposures to 1,2-unsaturated PAs and their *N*-oxides from use of Chinese herbal medicines (Griffin et al., 2014). Estimates of daily exposure were in the range 0.05–7.48 µg per day (mean 0.87 µg per day). Assuming a 60-kg adult body weight, this range equates to 0.0008–0.125 µg/kg bw per day (mean 0.015 µg/kg bw per day). However, the actual prevalence of use of Chinese herbal medicine in Ireland or elsewhere is unknown as is the frequency of consumption. These exposure estimates should be viewed as an additional potential source of exposure for users consuming the products at the recommended frequency.

7.1.3 Netherlands

A 24-hour duplicate-diet study (n = 63) was conducted measuring 32 1,2-unsaturated PAs and *N*-oxides in duplicate diets of adults (aged 25–65 years) in the Netherlands (de Wit et al., 2014). PAs (mainly integerrimine, senecionine, retrorsine and heliotrine) were detected in 23 of 63 diets (37%). The LOQ in this study was 0.1 µg/kg. In 16 of the 23 positive duplicate diets the respondents reported consuming honey or tea. PA concentrations in the duplicate diets ranged from 0.1 to 5.3 µg/kg (mean 0.29 µg/kg). The mean dietary exposure to 1,2-unsaturated PAs and their *N*-oxides was estimated by the study authors to be 0.00128 µg/kg bw per day (1.28 ng/kg bw per day). Women had higher mean exposure (2.26 ng/kg bw per day) than men (0.27 ng/kg bw per day). Given that the samples analysed represented a single 24-hour duplicate diet, the individual exposures should be viewed as acute estimates of dietary exposure. However, the mean, across all duplicate diets, can be used as an estimate of chronic population exposure.

(a) Herbal tea

A survey of 1,2-unsaturated PAs and their *N*-oxides in herbal teas purchased in the Netherlands detected 1,2-unsaturated PAs and their *N*-oxides in 11 herbal teas, with concentrations in the range 23–600 µg/kg (de Wit et al., 2014). Potential dietary exposure to 1,2-unsaturated PAs and their *N*-oxides was calculated based on mean (3 g tea per cup, 3 cups of 150 mL per day) and "worst case" (3 g tea per cup, 11 cups of 150 mL per day). This was based on 97.5th percentile consumer-only consumption from the Dutch National Food Consumption Survey (2007–2010) scenarios, except for two teas for which specific use recommendations were provided. Using a standard body weight of 70 kg, mean exposure estimates were in the range 0.003–0.13 µg/kg bw per day, while worst case exposures were in the range 0.01–0.26 µg/kg bw per day. These exposure estimates were treated as estimates of chronic exposure. This is consistent with information in the WHO CIFOCOss Food Consumption Database, which gives an all respondents mean intake for infused tea of 422 g per day.

(b) Herbal supplements

The survey mentioned in the previous section also detected 1,2-unsaturated PAs and their *N*-oxides in 20 herbal supplements purchased in the Netherlands (de Wit et al., 2014). Supplements were in the form of capsules or tablets. Mean exposure estimates were derived, based on recommended dose information. A worst case exposure was also calculated, based on consumption of 10 capsules/tablets per day. This figure was derived from the 97.5th percentile consumption level for supplements from the Dutch National Food Consumption Survey 2007–

2010 (10 g per day). Mean exposures to 1,2-unsaturated PAs and their *N*-oxides were in the range 0.0004–0.06 µg/kg bw per day, whereas worst case exposures were in the range 0.001–0.60 µg/kg bw per day.

7.1.4 USA

Avula et al. analysed PAs in dietary supplements (n = 21) claimed to contain material from *Petasites hybridus* (Avula et al., 2012; Avula et al., 2015). PAs (senecionine, senecionine-*N*-oxide, integerrimine and senkirkine) were detected in 7 of the 21 dietary supplements. Results were expressed in terms of exposures based on the recommended daily dose of the supplement, for 1,2-unsaturated PAs and their *N*-oxides – this would be in the range of 0.1–8.4 mg/daily dose. Assuming a 60 kg adult body weight, this range equates to 0.0017–0.14 µg/kg bw per day.

7.2 National estimates of dietary exposure derived by the Committee

Additional acute and/or chronic national estimates of dietary exposure were derived by the Committee for particular foods where:

- National food consumption information was available.
- Suitable concentration data had been submitted to the Committee or were available from the scientific literature.

Concentration data were considered unsuitable when:

- There was good evidence that sampling was not random or was not otherwise representative of food available for consumption.
- The data set contained non-quantified data with markedly different limits of quantification or excessively high limits of quantification (>10 µg/kg).

Finally, dietary exposure estimates for 1,2-unsaturated PAs and their *N*-oxides due to honey consumption in European countries were not derived when the published mean concentration data were within the range or below the range used for the EFSA risk assessment (EFSA, 2011). In such cases, it was assumed that the national estimate of dietary exposure to 1,2-unsaturated PAs and their *N*-oxides from honey consumption would be within or below the range of exposures determined in the EFSA study. This exclusion criterion was applicable to survey data from Germany (Bodi et al., 2014; Dübecke, Beckh & Lüllmann, 2011; Kempf et al., 2008), Italy (Martinello et al., 2014) and the Netherlands (Nederlandse Voedsel en Warenautoriteit (NVWA), 2007). Two retail surveys

have been reported for honey purchased in Ireland (Griffin et al., 2013; Griffin et al., 2015a). These surveys give widely varying estimates of the concentration of 1,2-unsaturated PAs and their *N*-oxides in honey purchased in Ireland, with the mean of positive samples in the earlier survey being greater than 1800 µg/kg, while the mean of positive samples in the later survey was 87.1 µg/kg. A lower-upper bound estimate of the mean concentration of 1,2-unsaturated PAs and their *N*-oxides for all samples in the recent survey was calculated as 19.7–33.7 µg/kg, which is within the range used for the EFSA risk assessment.

7.2.1 Australia

Information on 1,2-unsaturated PAs and their *N*-oxides (14 compounds) in honey purchased in Australia is available (Griffin et al., 2014). Samples of honey (n = 59) were purchased from retail outlets in and around Sydney, Australia during 2011 and 2012. The sampling plan was described as random and unbiased. Lower bound (values below the LOD set to zero, values between the LOD and LOQ set to the LOD) and upper bound (values below the LOD set to the LOD, values between the LOD and LOQ set to the LOQ) estimates were derived for the mean (108.2–129.3 µg/kg) and 95th percentile (331–351 µg/kg) concentration of 1,2-unsaturated PAs and their *N*-oxides. Information on honey consumption in Australia is available from the 1995 National Nutrition Survey (approximately 13 800 respondents) and the 2007 Australian National Children's Nutrition and Physical Activity Survey (4487 respondents) (Tracy Hambridge, Food Standards Australia New Zealand, personal communication). Honey consumption information and dietary exposure assessments (chronic and acute) for Australia are summarized in Table 34.

7.2.2 Brazil

Data were submitted to the Committee on the concentrations of 16 1,2-unsaturated PAs and *N*-oxides in honey samples (n = 33) from Brazil. Information on concentrations was provided for each of the individual 1,2-unsaturated PAs and *N*-oxides. Lower bound (values below the LOD set to zero, values between the LOD and LOQ set to the LOD) and upper bound (values below the LOD set to the LOD, values between the LOD and LOQ set to the LOQ) estimates were derived for the mean (69.5–73.9 µg/kg) concentration of 1,2-unsaturated PAs and their *N*-oxides. CIFOCOss contains information on the chronic consumption of honey by the general Brazilian population, with a population mean daily consumption of 0.0028 g/kg bw per day. These levels of consumption equate to a chronic exposure to 1,2-unsaturated PAs and their *N*-oxides for the Brazilian population of 0.0002–0.00021 µg/kg bw per day. No suitable food consumption information was available to carry out an assessment of acute exposure.

Table 34

Acute and chronic dietary exposure of Australian children (2–6 years) and adults (17+ years) to 1,2-unsaturated pyrrolizidine alkaloids (PAs) and their *N*-oxides from consumption of honey

Scenario	Children (2–6 years)	Adults (17+ years)
Mean honey consumption (high percentile), g/kg bw per day	0.104 (0.349)[a]	0.03 (0.2)[a]
Chronic dietary exposure, mean (high percentile), µg/kg bw per day	0.011–0.013 (0.038–0.045)	0.0032–0.0039 (0.022–0.026)
97.5th percentile single-day honey consumption, g/kg bw per day	1.84	0.92
Acute dietary exposure, based on mean concentration of 1,2-unsaturated PAs and their *N*-oxides in honey, µg/kg bw per day	0.199–0.238	0.100–0.119
Acute dietary exposure, based on 95th percentile concentration of 1,2-unsaturated PAs and their *N*-oxides in honey, µg/kg bw per day	0.609–0.646	0.305–0.323

[a] For children a 90th percentile value was available, whereas for adults a 95th percentile value was used.

7.3 Regional estimates of dietary exposure

7.3.1 Europe, 2011

In 2011, EFSA performed chronic exposure assessments for 16 European countries (Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, the Netherlands, Spain, Sweden and the United Kingdom) and acute exposure assessments for 21 countries (additionally Austria, Estonia, Poland, Slovakia and Slovenia) (EFSA, 2011). Consumption data came from country-specific nutrition surveys included in the EFSA Comprehensive European Food Consumption Database. Concentration data on 1,2-unsaturated PAs and their *N*-oxides in food were restricted to information on honey (bulk and retail) provided by Germany. Concentration data used in exposure estimates included lower bound (values below the LOD or LOQ set to zero) and upper bound (values below the LOD or LOQ set to the LOD or LOQ) estimates of the mean and 95th percentile, considering the sum of 8, 14 or 17 1,2-unsaturated PAs and *N*-oxides (see section 6.2.6).

Most honey would be blended before retail sale, but a proportion may be sold as specialty honey from one producer only (bulk honey). Bulk honey results used in this exposure assessment are reported to be mainly associated with honey imported from outside Europe. Retail and bulk honeys were considered separately.

For acute exposure estimates, mean and 95th percentile concentrations of 1,2-unsaturated PAs and their *N*-oxides were multiplied by the minimum and maximum mean and 95th percentile honey daily portion sizes, across the countries included. It is uncertain why this assessment included estimates of acute dietary exposure to 1,2-unsaturated PAs and their *N*-oxides based on mean daily portion sizes, as it is usual to base acute exposure estimates on a high-percentile

Table 35
Acute and chronic dietary 1,2-unsaturated pyrrolizidine alkaloid (PA) and *N*-oxide exposure assessments for European toddlers, other children and adults from consumption of retail or bulk honey

	1,2-unsaturated PA and *N*-oxide exposure (µg/kg bw per day, minimum LB-maximum UB)[a]					
	Consumption by toddlers		Consumption by other children		Consumption by adults	
Scenario	Mean	P95	Mean	P95	Mean	P95
Retail honey						
Acute – mean concentration		0.00332–0.114		0.00381–0.0735		0.00092–0.0493
Acute – P95 concentration		0.0117–0.254		0.0135–0.163		0.0032–0.110
Chronic – honey consumers only	0.00040–0.0374	0.00106–0.0778	0.00020–0.0180	0.00054–0.0470	0.00010–0.00903	0.00033–0.0260
Chronic – all respondents	0.00001–0.0051	0.0000–0.0567	0.00004–0.00276	0.0000–0.0179	0.00002–0.00122	0.0000–0.00941
Bulk honey						
Acute – mean concentration		0.00714–0.165		0.00821–0.106		0.00197–0.0712
Acute – P95 concentration		0.0309–0.546		0.0355–0.051		0.00850–0.236
Chronic – honey consumers only	0.00086–0.0541	0.00229–0.1123	0.00042–0.0260	0.00116–0.0680	0.00023–0.0130	0.00071–0.0375
Chronic – all respondents	0.00003–0.00737	0.0000–0.0819	0.00009–0.00398	0.0000–0.0259	0.00005–0.00177	0.0000–0.0136

P95: 95th percentile; LB: lower bound; UB: upper bound
[a] The range covers variation due to different honey consumption levels in different European countries and variation due to basing calculations on the sum of the concentrations of 8, 14 or 17 individual 1,2-unsaturated PAs and *N*-oxides.

portion size. The Committee summarized acute exposure estimates based on 95th percentile daily portion sizes, but not those based on mean daily portion sizes. For chronic exposure estimates, mean concentrations of 1,2-unsaturated PAs and their *N*-oxides were multiplied by minimum and maximum mean and 95th percentile honey consumption estimates, across all survey days, for honey consumers only and for all survey respondents, across the countries included. Three population groups were considered; toddlers, other children and adults. Exposure estimates are summarized in Table 35.

7.3.2 Europe, 2016

In a more recent dietary exposure assessment carried out by EFSA (2016), food consumption data for tea and herbal infusions were taken from the EFSA Comprehensive European Food Consumption Database containing information on chronic food consumption from 19 countries, while information from 7

additional countries was available for acute dietary exposure assessment. Where consumption data were expressed in terms of dry tea, they were converted to figures for infused tea by multiplying by a factor of 75 (2 g to 150 mL). Chronic consumption figures were based on the mean of at least two consumption days. Acute consumption figures were based on consumption days only.

Chronic dietary exposure to 1,2-unsaturated PAs and *N*-oxides from consumption of teas and herbal infusions was estimated by two approaches; using concentration data collected by EFSA and using concentration data submitted by Tea & Herbal Infusions Europe (THIE). Datasets were based on analyses including 16–28 1,2-unsaturated PAs and *N*-oxides. Further, estimates of dietary exposure to PAs were calculated for the whole population and for consumers only. Table 36 summarizes the dietary exposure estimates for the whole population.

Potential exposures to 1,2-unsaturated PAs and their *N*-oxides from consumption of plant extracts and pollen-based dietary supplements were also considered. For plant extracts intended to be consumed as infusions, potential exposures were estimated to be in the range 0.8–67 µg/kg bw per day, whereas for plant extracts intended to be consumed directly, maximum potential exposure was estimated at 1810 µg/kg bw per day. Based on a very limited number of records on pollen-based supplement consumption, chronic exposure was estimated to be in the range 0.001–0.018 µg/kg bw per day among consumers only. Acute exposure, using a 90th percentile concentration, was estimated to be in the range 0.003–0.044 µg/kg bw per day, for consumers only.

7.4 International estimates of dietary exposure

To obtain a global perspective and to permit comparisons across different dietary patterns of the potential exposure to a contaminant, the Committee usually estimates chronic dietary exposure for contaminants using the GEMS/Food consumption cluster diets (WHO, 2013) and concentrations of the contaminant in relevant foods as obtained for the assessment in question.

The available data on occurrence of 1,2-unsaturated PAs and their *N*-oxides were deemed to be inappropriate for use in determining international estimates of chronic dietary exposure in combination with the GEMS/Food consumption cluster diets. There were insufficient or inappropriate data for most relevant foods and/or not enough information about the samples to enable data to be combined from different sources. For example, data on 1,2-unsaturated PAs in cereals and cereal products were only available from three countries, all of which were European. Limits of detection for different countries varied across four orders of magnitude (0.1 to 1000 µg/kg) and no information was available on which individual PAs were covered by the analyses. Data on concentrations

Table 36
European acute and chronic dietary 1,2-unsaturated pyrrolizidine alkaloid (PA) and *N*-oxide exposure assessments for various age groups from consumption of tea and herbal tea

	1,2-unsaturated PA and *N*-oxide exposure (µg/kg bw per day, minimum LB-maximum UB)[a]					
	Acute (EFSA data)		Chronic (EFSA data)		Chronic (THIE data)	
Age group	Mean	P95	Mean	P95	Mean	P95
Infants	0.001–0.213	0.154–0.720	0.000–0.043	0.000–0.185	0.000–0.027	0.000–0.106
Toddlers	0.002–0.311	0.262–0.821	0.000–0.048	0.000–0.214	0.000–0.030	0.000–0.131
Other children	0.020–0.248	0.126–0.505	0.001–0.034	0.003–0.126	0.000–0.024	0.001–0.077
Adolescents	0.009–0.152	0.090–0.415	0.000–0.026	0.001–0.095	0.000–0.018	0.001–0.065
Adults	0.006–0.160	0.022–0.496	0.000–0.029	0.001–0.120	0.000–0.023	0.001–0.078
Elderly	0.029–0.162	0.082–0.389	0.003–0.040	0.015–0.123	0.001–0.032	0.003–0.079
Very elderly	0.033–0.168	0.110–0.383	0.004–0.042	0.016–0.127	0.001–0.033	0.004–0.077

THIE: Tea & Herbal Infusions Europe; P95: 95th percentile; LB: lower bound; UB: upper bound
[a] The range covers variation due to different tea and herbal infusion consumption levels in different European countries and, for chronic exposure, variation due to different treatments of left-censored data (lower-upper bound). For acute exposure, only estimates of dietary exposure based on upper bound treatment of left-censored data were given

of 1,2-unsaturated PAs and their *N*-oxides in animal products (milk, meat and eggs) were almost solely available from controlled feed trials, where animals were intentionally fed known amounts of material from known PA-containing sources. Data on 1,2-unsaturated PAs and their *N*-oxides in teas and herbal teas were available from several European countries, but in some cases gave very different results for the same beverage.

Therefore, no international estimates of chronic dietary exposure were prepared. The same issues with the occurrence data prevented estimation of acute dietary exposures using the WHO large portion (97.5th percentile) food consumption data.

7.5 Summary of dietary exposure estimates

Table 37 summarizes the various estimates of dietary exposure presented in the previous sections, to allow some comparisons between different foods and products in terms of their likely contributions to dietary exposure to 1,2-unsaturated PAs and their *N*-oxides.

The summary of dietary exposure estimates in Table 37 lacks information on a number of foods that are potential contributors to dietary exposure to 1,2-unsaturated PAs and their *N*-oxides.

While very high concentrations of PAs in cereals have been associated with veno-occlusive disease of the liver (Kakar et al., 2010), the concentrations of PAs measured in such instances are unlikely to be typical of background

Table 37

Summary of estimates of dietary exposure to 1,2-unsaturated pyrrolizidine alkaloids (PAs) and their *N*-oxides (lower bound-upper bound estimates)

	Dietary PA exposure, mean (95th percentile) (µg/kg bw per day)			
	Acute[a]		Chronic	
Study	**Children**	**Adults**	**Children**	**Adults**
Total (duplicate) diet				
Netherlands (de Wit et al., 2014)				0.00128
Honey				
Australia (current assessment)	0.199–0.238 (0.609–0.646)	0.100–0.119 (0.305–0.323)	0.011–0.013 (0.038–0.045)[b]	0.0032–0.0039 (0.022–0.026)
Brazil (current assessment)			0.00020–0.00021	
Europe (EFSA, 2011)[c]				
Retail honey	0.0033–0.114 (0.012–0.254)	0.0009–0.049 (0.0032–0.110)	0.00001–0.005 (0.0000–0.057)	0.00002–0.001 (0.0000–0.009)
Bulk honey	0.0071–0.165 (0.031–0.546)	0.0020–0.071 (0.0085–0.236)	0.00003–0.007 (0.0000–0.082)	0.00005–0.002 (0.0000–0.014)
Germany (BfR, 2013a)[d]	0.028–0.057 (0.092–0.112)	0.018–0.037 (0.060–0.072)	0.002–0.004 (0.006–0.012)	0.001–0.002 (0.005–0.017)
Ireland (Griffin et al., 2015b)[e]	0.193–0.204 (0.483–0.510)	0.048–0.051 (0.121–0.128)		
Tea				
Germany (BfR, 2013b)				
Tea	0.151–0.252 (0.688–0.784)	0.060–0.099 (0.270–0.308)	0.011–0.018 (0.046–0.076)	0.006–0.010 (0.030–0.050)
Herbal tea	0.126–0.210 (0.574–0.655)	0.060–0.099 (0.270–0.308)	0.005–0.009 (0.027–0.045)	0.002–0.003 (0.018–0.030)
Ireland (Griffin et al., 2014)[f]				0.0013–0.111
Netherlands (de Wit et al., 2014) – herbal tea[g]				0.003–0.13 (0.01–0.26)
Europe (EFSA, 2016)	0.001–0.311 (0.126–0.821)	0.006–0.168 (0.022–0.496)	0.000–0.048 (0.000–0.214)	0.000–0.042 (0.001–0.127)
Herbal medicines/supplements				
Ireland (Griffin et al., 2014)[h]				0.0008–0.125
Netherlands (de Wit et al., 2014)[i]				0.0004–0.06 (0.001–0.60)
USA (Avula et al., 2012)[j]				0.0017–0.14
Culinary herbs				
Germany (Cramer et al., 2013)[k]		0.032–0.112		

[a] Acute exposures summarized here are those based on a 95th or 97.5th percentile consumer, consuming food containing 1,2-unsaturated PAs and their *N*-oxides at either the mean concentration or the 95th percentile concentration, unless otherwise stated.

[b] In this case, the high percentile is a 90th percentile, rather than a 95th percentile.

[c] The results for children are those for toddlers (1–3 years), rather than "other children" (3–10 years). Chronic exposures are those estimated for all respondents, rather than consumers only.

[d] In the BfR assessment, chronic exposures were calculated with and without "brand loyalty". Brand-loyal consumers were considered to always consume honey at the 95th percentile of the concentration distribution. Results presented here are those based on the mean concentration.

[e] Based on mean consumption of 20 g honey per day or maximum consumption of 50 g honey per day.

[f] Based on daily consumption of three cups (600 mL) of herbal tea.

Table 37 (continued)

[g] Mean exposure estimates were based on consumption of three 150 mL cups of tea per day, each containing 3 g of tea. High percentile (97.5th percentile, consumer only) estimates of exposure were based on 11 × 150 mL cups, each containing 3 g of tea. For two of 11 teas, recommended dose information was used. Exposures were calculated assuming a 70 kg body weight.

[h] Exposure from use of herbal medicines at the recommended dose, assuming a 60 kg body weight.

[i] Exposure from use of herbal supplements at the recommended dose. Figures in parentheses are from use of supplements at a rate of 10 capsules/tablets per day. Exposures were calculated assuming a 70 kg body weight.

[j] The range represents 1,2-unsaturated PA and *N*-oxide exposure due to recommended use of dietary supplement based on *Petasites hybridus* containing 1,2-unsaturated PAs and their *N*-oxides, assuming a 60 kg body weight.

[k] Based on consumption of a traditional German meal in which surveyed commercial herb preparations may have been used, assuming a 60 kg body weight.

contamination levels in cereals and cereal products. Data on PAs in cereals submitted to the Committee from Germany, Hungary and Luxembourg, suggest that background concentrations of PAs in cereals are very low (<3 µg/kg). Unfortunately, the range of PAs represented by these data is not known and the datasets contained limits of detection/quantification that were so high that exposure estimates based on these concentration data were so uncertain (large range between lower and upper bound estimates), as to be uninformative. Lower bound exposure estimates, which are not affected by high limits of detection/quantification, suggest that dietary exposure to PAs from background levels in cereals may be 2–3 orders of magnitude lower than dietary exposure estimates due to consumption of honey or tea.

While the potential for foods of animal origin (meat, eggs and milk) to be contaminated with PAs, due to the animals feeding on PA-containing plant material, has been demonstrated, limited information is available on contamination in retail food samples. EFSA (2011) proposed a worst case intake of 1,2-unsaturated PAs and their *N*-oxides by "a high-yielding dairy cow" of 0.14 mg/kg bw per day. At PA doses just above this level (0.16 mg/kg bw per day) a mean concentration of 1,2-unsaturated PAs and their *N*-oxides in cows' milk of 2.1 µg/L was found. The highest daily consumption of milk reported in the WHO CIFOCOss Food Consumption Database is 42.6 g/kg bw per day for a 2–6-year-old Australian child. Combined with a concentration of 1,2-unsaturated PAs and their *N*-oxides in milk of 2.1 µg/L, this would equate to a daily exposure to 1,2-unsaturated PAs and their *N*-oxides from milk consumption of 0.089 µg/kg bw per day. However, this would require consistent and widespread feeding of dairy cows at the EFSA worst case level of PA exposure and a recent survey of 1,2-unsaturated PAs and their *N*-oxides in commercial milk samples found a maximum concentration of 0.16 µg/kg (Mulder et al., 2015), suggesting a maximum potential exposure to 1,2-unsaturated PAs and their *N*-oxides from this dietary source of 0.007 µg/kg bw per day.

Of the foods for which some dietary exposure estimates are available or are able to be derived, it appears that tea may make a greater contribution

to dietary exposure to 1,2-unsaturated PAs and their *N*-oxides than honey. However, it should be noted that the exposure estimates for tea are based on concentrations detected in herbal teas and it is uncertain what proportion of total tea consumption herbal teas represent. The available studies give very different information on the concentration of 1,2-unsaturated PAs and their *N*-oxides in conventional teas (those produced from *Camellia sinensis*) (Bodi et al., 2014; Griffin et al., 2014; Mathon et al., 2014).

It is worth noting that the two food types (honey and tea) for which dietary exposure estimates have been derived represent very different patterns of food consumption. According to country-level information in the WHO CIFOCOss Food Consumption Database, honey is consumed by at most 50% of the survey population in any age group in any country. The percentages of honey consumers for some country/population group combinations are as low as 1%. Mean daily intakes for any country/population group combination are no greater than 4.8 g per day. In comparison, tea as an infused beverage is consumed by up to 92% of survey respondents, with mean daily intakes up to 606 g per day.

Herbal medicines and herbal dietary supplements produced from PA-containing plants have the potential to be major contributors to total dietary exposure to 1,2-unsaturated PAs and their *N*-oxides. However, no information was available on the prevalence or frequency of use of such products.

7.6 Limitations of the dietary exposure assessment

Information on concentrations of 1,2-unsaturated PAs and their *N*-oxides in foods and estimates of dietary exposure are only available from a very small number of countries. While a number of studies have assessed the content of 1,2-unsaturated PAs and their *N*-oxides in honey, several of these are targeted to honey types for which there is an expectation of contamination with 1,2-unsaturated PAs and their *N*-oxides. Data from these studies are not suitable for dietary exposure assessment.

Very limited data are available on concentrations in foods other than honey, with the most information available for teas, including herbal teas. Tea is a product that is widely traded and there may be some expectation that the content of 1,2-unsaturated PAs and their *N*-oxides in tea would be similar in different countries. Unfortunately, disparities in the results from the available studies mean that data from these studies cannot be combined to provide a basis for international estimates of dietary exposure.

There were no estimates available of chronic dietary exposure to a number of foods that have the potential to contribute to overall dietary exposure to 1,2-unsaturated PAs and their *N*-oxides when it is likely that consumers may

eat a diet consisting of honey and tea and other foods containing 1,2-unsaturated PAs and their *N*-oxides. In some cases, concentration data are available, but are unusable owing to widely varying limits of detection and uncertainties about the range of 1,2-unsaturated PAs and their *N*-oxides analysed.

The overall scarcity and poor quality of data did not allow exposure estimates to be calculated using the GEMS/Food Cluster diets; therefore, exposures on a global scale could not be estimated or compared.

8. Prevention and control

The aim of prevention and control is to reduce the exposure of animals and humans to saturated PAs, 1,2-unsaturated PAs, their *N*-oxides and DHP adducts to levels that do not pose a risk to their health.

The main management strategy is to minimize the occurrence of plants with known adverse health effects in feed and food. This can be achieved by application of cutting strategies to reduce the unwanted plants (Bassler, Karrer & Kriechbaum, 2016; Winter et al., 2014). The spread of PA-containing plants to other regions and continents must be minimized and monitored (Eller & Chizzola, 2016; van Raamsdonk et al., 2015).

This strategy has resulted in management practices to minimize the occurrence of weeds currently known to contain 1,2-unsaturated PAs and their *N*-oxides in feed and food, and management practices to help prevent contamination and reduce the levels of 1,2-unsaturated PAs and their *N*-oxides in food and feed to alleviate possible consequences for human health from dietary exposure to these genotoxic carcinogens (CAC/RCP, 2014). Continuing improvements to management practices are, however, likely to be needed, subject to consideration of the effectiveness and evaluation of the Code of Practice as applied by landowners, occupiers, managers and food producers generally, and on hazard analysis and critical control points (HACCP)-based food safety systems applied by food business operators (in the food and feed supply chains).

When plants containing 1,2-unsaturated PAs and their *N*-oxides are permitted as feed or food, the levels of these PAs must be monitored.

8.1 Environmental management – weed control practices

Weed management practices provide the most effective first line of defence for reducing the level of contamination of food with 1,2-unsaturated PAs and their *N*-oxides (CAC/RCP, 2014; FAO, 2010; Long et al., 2016). These involve, for example: avoiding the spread of plants containing 1,2-unsaturated PAs and their *N*-oxides; eliminating weeds containing 1,2-unsaturated PAs and their *N*-oxides in and near crops and pastures prior to planting; and avoiding livestock grazing of rangelands and pastures with a high component of plants containing 1,2-unsaturated PAs and their *N*-oxides. Recently, additional recommendations have been made to avoid deliberately using plants containing 1,2-unsaturated PAs and their *N*-oxides for honey and pollen production (CAC/RCP, 2014; Dübecke, Beckh & Lüllmann, 2011; Edgar, Roeder & Molyneux, 2002). Also, the importance of monitoring and removing weeds containing 1,2-unsaturated PAs and their *N*-oxides in commercial salad production systems has been recognized

(BfR, 2007, 2011, 2013a,b). For many years, some herbal teas containing 1,2-unsaturated PAs and their *N*-oxides, for example, those made from comfrey, have been subject to strict regulations in many countries. Recently, a wide range of teas on sale in retail outlets have been shown to be contaminated by 1,2-unsaturated PAs and their *N*-oxides (Mulder et al., 2015) and, as a result, weed control during tea production is now also considered to be very important and strongly recommended (BfR, 2011, 2013a,b; Bodi et al., 2014; Griffin et al., 2014; Long, Van Wijk & Stander, 2016).

Weed control practices, such as those recommended in the aforementioned CCCF Code of Practice, have, for many decades, been used to prevent poisoning of livestock by 1,2-unsaturated PAs and their *N*-oxides. However, these measures need to be significantly improved and expanded to bring levels of 1,2-unsaturated PAs currently being detected in a wide range of foods of animal origin within tolerable levels. The need for control of the plant sources of 1,2-unsaturated PAs and their *N*-oxides in food production systems should be more widely publicized and encouraged by providing advice to all affected food producers and by introducing and rigorously enforcing national and international regulations, as has been recommended (CAC/RCP, 2014). Stronger regulations than those currently in force may be needed and this will become clearer once the effectiveness of various prevention and control practices has been evaluated. Ongoing evaluation of any changes to levels of 1,2-unsaturated PAs and their *N*-oxides in food will be required using the best available combination of analytical methods.

Total eradication of particular plants containing 1,2-unsaturated PAs and their *N*-oxides associated with food contamination is not considered feasible or ecologically desirable, except perhaps in countries where alien plants containing 1,2-unsaturated PAs and their *N*-oxides have been unintentionally or deliberately introduced and which, owing to a lack of indigenous phytophagous enemies, have become more widespread than in their home range (Eller & Chizzola, 2016). In this case biological control agents, sourced from the plant's normal range, are being considered for importation or have been imported and released. Introduction of 1,2-unsaturated PA-adapted and particularly 1,2-unsaturated PA-dependent insects is one approach to reducing the density of these undesirable and hazardous weeds (Boppré, 2011). However, in some cases, this could lead to control insects switching their host preference to indigenous 1,2-unsaturated PA-containing plants that currently do not present a food safety risk. This may have an undesirable ecological impact. Furthermore, insect damage may lead to increased levels of PA being present in plants.

8.2 Good manufacturing practices and HACCP

Pre- and post-harvest practices to reduce levels of 1,2-unsaturated PAs and their *N*-oxides mainly focus on: use of the correct cultivar of plants for use in teas or herbal treatments; establishing good tracking and tracing procedures to avoid mixing of plants after harvest, during transportation and storage; and carrying out correct cleaning procedures to eliminate co-harvested weeds. Application of HACCP-based food safety procedures is recommended to control co-occurring weeds and avoid high concentrations of 1,2-unsaturated PAs and their *N*-oxides when the use of plants containing 1,2-unsaturated PAs and their *N*-oxides is unavoidable (product specifications). These measures require education of personnel along the whole production chain.

There is a need to actively control concentrations of 1,2-unsaturated PAs and their *N*-oxides or provide instructions on labels of certain herbal teas or spices in cases where inclusion of PA-containing plants is unavoidable. The fetuses of pregnant women are at risk from 1,2-unsaturated PAs and their *N*-oxides that can pass through the placenta to the unborn child. Breastfed children are also at risk since 1,2-unsaturated PAs and their *N*-oxides can be transferred to mothers' milk (Rasenack et al., 2003), and, in many parts of the world, babies are given fennel tea that can contain toxic PA (Bodi et al., 2014).

8.3 Increased resistance of livestock to 1,2-unsaturated PAs

Some livestock species and individual animals are naturally more resistant to toxicity of 1,2-unsaturated PAs and their *N*-oxides than others owing to: lower levels of metabolic activation to DHP esters; and/or higher rates of detoxification, for example, by esterases, to produce highly water soluble, readily excreted non-toxic necines and necic acids (Fig. 5) and/or metabolic conversion to *N*-oxides that are also rapidly excreted (Bull, Culvenor & Dick, 1968; Mattocks, 1986; WHO-IPCS, 1988). These natural, in vivo 1,2-unsaturated PA-detoxifying processes could be amenable to enhancement leading to increased resistance of livestock to 1,2-unsaturated PAs and to a reduction in the transfer of 1,2-unsaturated PAs into food products, such as milk and meat. Chickens and other birds, pigs and horses show little natural resistance to 1,2-unsaturated PA toxicity. It seems likely that for these species the desired avoidance of 1,2-unsaturated PAs and, subsequently, DHP adducts in eggs, pork and horse meat, will only be achieved via monitoring and control of the feed provided. The same applies to bees involved in honey and pollen production.

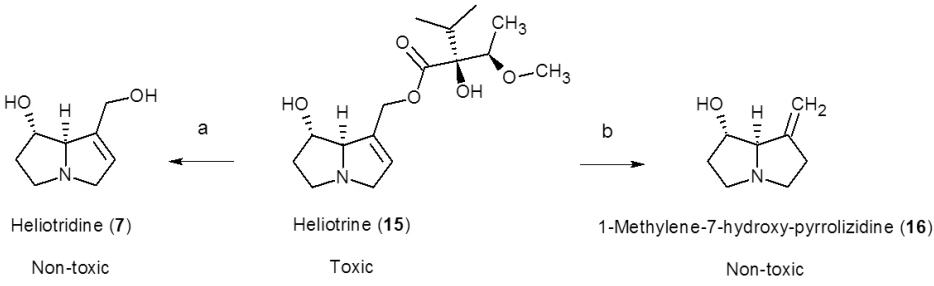
There is evidence that the use of certain feed additives with sulfhydryl groups can provide increased protection against 1,2-unsaturated PA toxicity. For example 2-mercaptoethylamine (cysteamine) (150–300 mg/kg bw) or 1%

cysteine in the diet of rats and mice significantly increased their resistance to monocrotaline, lasiocarpine and *Senecio jacobaea* alkaloids (Hayashi & Lalich, 1968; Miranda et al., 1981; Rogers & Newberne, 1971). Infusion of GSH into the hepatic portal vein has also been shown to prevent HSOS developing (DeLeve, Shulman & McDonald, 2002; Wang X, Kanel & DeLeve, 2000). Such protection is believed to be due to these sulfhydryl additives providing alkylation targets for the genotoxic DHP-ester metabolites of 1,2-unsaturated PAs and they act as scavengers for DHP esters in vivo. These observations, however, are not of practical use at present although they could suggest possible ways of increasing livestock resistance and detoxification in the future and thus means of reducing transfer of these compounds into some foods derived from animals.

Naive livestock are sometimes more susceptible to poisoning by 1,2-unsaturated PAs than animals that have had some previous exposure to plants containing 1,2-unsaturated PAs (Bull, Culvenor & Dick, 1968; Dick et al., 1963; Peterson et al., 1992). Ruminants in particular tend to become somewhat more resistant to 1,2-unsaturated PA toxicity over time (Lanigan, 1970, 1971, 1972; Lanigan & Smith, 1970; Mattocks, 1986; Peterson, Payne & Culvenor, 1992). This is believed to be due, in part, to an increase in the population of certain naturally occurring rumen bacteria, for example, *Peptostreptococcus heliotrinreducans*. *P. heliotrinreducans* has been shown to detoxify at least some 1,2-unsaturated PAs by hydrogenolysis to produce non-toxic 1-methylene and 1-methyl pyrrolizidine derivatives (Fig. 8) (Lanigan, 1970, 1971, 1972, 1976; Lanigan & Smith, 1970; Lanigan, Payne & Peterson, 1978; Mattocks, 1986; Peterson, Payne & Culvenor, 1992). This rumen detoxification is also likely to reduce the level of 1,2-unsaturated PA contaminants transferred into foods such as meat and milk. Thus, an inoculum of *P. heliotrinreducans* introduced into the rumen of naive livestock could help protect them against poisoning by 1,2-unsaturated PAs and reduce contamination of animal products such as milk and meat.

Methanogenic bacteria in the rumen are known to compete with *P. heliotrinreducans* for free hydrogen (Lanigan, Payne & Peterson, 1976; Peterson, Payne & Culvenor, 1992). They are also known to reduce animal productivity so that anti-methanogens introduced into the rumen of livestock could not only assist in reducing 1,2-unsaturated PA toxicity but also increase livestock production (May et al., 1995; McCrabb et al., 1997). One anti-methanogen product that has been shown to achieve this is a complex formed between cyclodextrin and bromochloromethane (May et al., 1995; McCrabb et al., 1997). This anti-methanogen has been incorporated into controlled release capsules that can be inserted into the rumen, or, alternatively, the bromochloromethane-cyclodextrin complex can also be incorporated as a component in livestock feed. Such anti-methanogens, as well as helping to increase the detoxification of 1,2-unsaturated PAs in the rumen and possibly increase livestock productivity

Fig. 8
1,2-unsaturated pyrrolizidine alkaloids such as heliotrine (15) are detoxified in vivo



Note: 1,2-unsaturated PAs such as heliotrine (**15**) are detoxified in vivo by: a) esterase hydrolysis to the component non-toxic necine heliotridine (**7**) and heliotric acid; and b) in ruminants by microbial hydrogenolysis in the rumen to non-toxic 1-methylene-7-hydroxy-pyrrolizidine (**16**) and 1-methyl-7-hydroxy-pyrrolizidine.

could also help to reduce global warming due to methane. Methane released by ruminants into the environment contributes significantly to global warming (Colombini et al., 2015). However, the active component of the anti-methanogen, bromochloromethane, is no longer manufactured or allowed in many countries because of the adverse effect it has on the ozone layer and therefore another more suitable anti-methanogen is required. The bromochloromethane-cyclodextrin complex was at one time patented but this patent has apparently now lapsed (May et al., 2001).

An attempt was made to vaccinate livestock against 1,2-unsaturated PAs, but vaccination was found to enhance rather than reduce toxicity (Culvenor, 1978). It is thought that this is due to the inability of the immune system to destroy the 1,2-unsaturated PAs. It was suggested that binding of 1,2-unsaturated PAs to anti-1,2-unsaturated PA antibodies may have protected them from other detoxification processes and thus they were retained for longer in in vivo circulation (Culvenor, 1978).

8.4 Management of livestock feed

While weed control to prevent livestock feed being contaminated by 1,2-unsaturated PAs and their *N*-oxides is desirable, it is not always achievable. Under certain conditions fodder, such as hay or silage, contaminated with 1,2-unsaturated PAs and 1,2-unsaturated PA-*N*-oxides can become less poisonous during storage. This is due to the decomposition of unstable 1,2-unsaturated PA-*N*-oxides and, to a lesser extent, 1,2-unsaturated PA free bases (e.g. Bull, Culvenor & Dick, 1968; Crews et al., 2009; Hough et al., 2010).

A novel, but as yet unexplored, means of facilitating the rate of destruction of 1,2-unsaturated PAs and their *N*-oxides in silage could be to inoculate the silage with microbes capable of destroying 1,2-unsaturated PAs. The anaerobe *P. heliotrinreducans* discussed above is one possible microbe that could be used to inoculate 1,2-unsaturated PA-contaminated silage and achieve 1,2-unsaturated PA detoxification. If effective it would also have the added potential advantage of inoculating the rumen of animals feeding on such silage.

9. Dose–response analysis and estimation of toxic/carcinogenic risk

9.1 Identification of key data for risk assessment

9.1.1 Pivotal data from biochemical and toxicological studies

Two studies were identified in section 2.2.3 providing dose–response information on the carcinogenicity of PAs, which is considered the most critical end-point after long-term exposure. These are the studies performed by the US National Cancer Institute on lasiocarpine (NCI, 1978) and by the US NTP on riddelliine (NTP, 2003). Lasiocarpine was administered to male and female Fischer 344 rats in feed for 105 weeks. Riddelliine was administered to female and male F344/N rats and B6C3F_1 mice via gavage for 105 weeks (5 days per week). Haemangiosarcoma of the liver was the most prominent and frequent tumour type in both studies on rats, and also in the riddelliine study on mice. Tables 38 and 39 provide an overview of the incidences of liver haemangiosarcoma observed in these studies.

9.1.2 Pivotal data from human clinical/epidemiological studies

No pivotal data were selected from the epidemiological studies for dose–response analysis.

9.1.3 Biomarker studies

No pivotal data were selected from the human biomarker studies for dose–response analysis.

9.2 General modelling considerations

9.2.1 Previous analyses of dose–response relationships

(a) United Kingdom Committee on Carcinogenicity, 2008

In 2008, the United Kingdom Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment presented a dose–response analysis of the data on lasiocarpine and riddelliine. Using the United States Environmental Protection Agency (US EPA) Benchmark Dose Software (BMDS), a benchmark dose–response level of 10%, and applying a limited set of models, a range of lower 95% confidence limits on the benchmark dose for a 10% response ($BMDL_{10}$) of 0.301–0.418 mg/kg bw per day was derived for the data on female rats, with the lowest $BMDL_{10}$ obtained with the gamma model. For male mice, a range of $BMDL_{10}$ of 1.04–1.175 mg/kg bw per day was derived from accepted models. For data on male rats that had received lasiocarpine in the diet, the range

Table 38
Dose–response relationship for haemangiosarcoma of the liver in F344N rats administered riddelliine via gavage for 2 years

Male rats			Female rats		
Gavaged dose (mg/kg, 5 days per week)	Dose converted to daily administration (mg/kg per day)	No. of animals with haemangio-sarcoma	Gavaged dose (mg/kg, 5 days per week)	Dose converted to daily administration (mg/kg bw per day)	No. of animals with haemangio-sarcoma
Vehicle controls	0	0/50	0	0.000	0/50
			0.01	0.007	0/50
			0.033	0.024	0/50
			0.1	0.071	0/50
			0.33	0.236	3/50
1	0.714	43/50	1	0.714	38/50

Source: NTP (2003).

Table 39
Dose–response relationship for haemangiosarcoma of the liver in Fischer 344 rats fed diets containing lasiocarpine for 2 years

Male rats			Female rats		
Concentration in feed (ppm)	Dose (mg/kg per day)	No. of animals with haemangio-sarcoma	Concentration in feed (ppm)	Dose	No. of animals with haemangio-sarcoma
Untreated controls	0	0/24	0	0	0/24
7	0.35	5/24	7	0.35	8/24
15	0.75	11/24	15	0.75	7/24
30	1.5	13/24	30	1.5	2/9

Source: NCI (1978).

of $BMDL_{10}$ values obtained was 0.078–0.20 mg/kg bw per day, the lowest value coming from the log-logistic model. No $BMDL_{10}$ was reported for the dataset on female rats as none of the models used provided an acceptable fit to the data (COC, 2008).

(b) EFSA, 2011 and 2017

In 2011, EFSA evaluated PAs in food. In this context dose–response modelling was performed for carcinogenicity data on riddelliine and lasiocarpine. For both substances a benchmark response level (BMR) of 10% extra risk was selected as recommended in the EFSA guidance document on benchmark dose modelling (EFSA, 2009). The US EPA software BMDS v2.1.2 was used for the analysis.

The daily doses of riddelliine administered by gavage to female rats were adjusted to obtain daily average doses by multiplying the doses by 5/7. Data on male rats were not considered as only a high-dose group and a control group of male rats were included in the NTP study. All models for quantal data available in BMDS were used for calculating values for $BMDL_{10}$ and EFSA's criteria for model selection were applied (EFSA, 2009). $BMDL_{10}$ values ranging from 0.180 to 0.299 mg/kg day were calculated. The lowest $BMDL_{10}$ of 0.18 mg/kg day was obtained with the multistage model and the multistage-cancer model.

For lasiocarpine data, again all models for quantal data available in BMDS were used for calculating $BMDL_{10}$ values. For the dataset on male rats, large variations in $BMDL_{10}$ between models were observed and differences between BMD_{10}s and $BMDL_{10}$ obtained with individual models were up to 1 or 2 orders of magnitude, indicating a high level of uncertainty. The lowest $BMDL_{10}$ was calculated with the Weibull model at 0.0018 mg/kg bw per day.

EFSA applied restrictions for some of the models (on slope, power or values for beta (all ≥1)) to reduce the variability, and obtained the lowest $BMDL_{10}$ from all restricted models of 0.08 mg/kg day (for the log-logistic model). EFSA further noted that a smaller difference between BMD and BMDL resulted from use of the unrestricted multistage model and chose the $BMDL_{10}$ obtained from this study (0.07 mg/kg bw per day) for risk characterization.

The dose–response data from female rats were modelled as well, but only one model yielded an acceptable fit. EFSA noted that the poor dose–response relationship was due to the high mortality observed in the middle and high-dose groups of females and that the confidence in the outcome of the dose–response analysis was poor. The data on female rats were therefore not considered for risk characterization. The $BMDL_{10}$ of 0.07 mg/kg bw per day calculated from data from the study of male rats exposed to lasiocarpine was used to assess the margin of exposure for the group of PAs in food.

In 2017, EFSA published a new statement on PAs including an updated dose–response analysis for the chronic effects of PAs in view of the updated guidance of the EFSA Scientific Committee on the use of benchmark modelling in risk assessment (ESFA, 2017a,b).

EFSA applied the BMD model averaging approach to the datasets on the incidence of liver haemangiosarcoma in male and female rats exposed to lasiocarpine (NTP, 1978) and riddelliine (NTP, 2003). For both substances, a BMR of 10% extra risk was selected as recommended in the EFSA guidance document on benchmark dose modelling (EFSA, 2009). Additional calculations were performed applying a BMR of 30% for comparing carcinogenic potencies of lasiocarpine and riddelliine. The R-package PROAST61.3 was used.

The BMD modelling of the incidence of liver haemangiosarcoma in male rats exposed to lasiocarpine and in female rats exposed to riddelliine led to BMD_{10}

confidence intervals (CIs) ($BMDL_{10}$–$BMDU_{10}$) of 8–343 and 237–548 μg/kg bw per day, respectively, based on model averaging. The BMD_{10} CI for lasiocarpine was affected by a high degree of uncertainty, with a $BMDU_{10}$ to $BMDL_{10}$ ratio of about 40-fold and $BMDL_{10}$–$BMDU_{10}$ intervals below the tested dose range for all the accepted individual models. On the other hand, the BMD modelling for riddelliine using model averaging resulted in a narrower $BMDL_{10}$ to $BMDU_{10}$ interval, fully included within the two higher tested doses (equivalent to 237–714 μg/kg bw per day), despite the relatively high uncertainty related to the poor information on the dose–response relationship.

BMD_{30} ($BMDL_{30}$–$BMDU_{30}$) of 491 (211–811) and 534 (373–622) μg/kg bw per day were calculated for lasiocarpine and riddelliine, respectively, using model averaging. This additional modelling supported the assumption that the two PAs can be considered to have similar carcinogenic potency. EFSA selected the $BMDL_{10}$ of 237 μg/kg bw per day, derived for the incidence of liver haemangiosarcoma in female rats exposed to riddelliine, as a reference point to assess the chronic risks of PAs (EFSA, 2017a).

(c) RIVM, 2014

RIVM (2014) performed a risk assessment of PAs in herbal preparations (report in Dutch, Annex on dose–response modelling in English). This report includes a dose–response analysis and benchmark dose calculation for liver haemangiosarcoma caused by riddelliine and lasiocarpine. Again, the analysis is based on the dose–response data from NCI (1978) for lasiocarpine and NTP (2003) for riddelliine. All analyses were performed using the dose–response software PROAST (version 36.9), following the EFSA guidance document on benchmark dose modelling (EFSA, 2009).

In the riddelliine study, mice proved to be less sensitive than rats; therefore, these data were not used for dose–response modelling. As no significant difference was detected between the tumour response data of male and female rats, for the modelling the two datasets were combined. No adjustment for gavage on only 5 days per week was made. For modelling the tumour incidence data, all models provided in PROAST were used and a BMR of 10% extra risk was selected. Seven models were identified showing an acceptable description of the dose–response data. Of those, the log-probit model provided the lowest $BMDL_{10}$ for the combined set of data from male and female rats, which was 0.307 mg/kg bw per day.

The authors of the report considered that the middle and high-dose group of treated females in the lasiocarpine dataset were affected by early deaths. Therefore, the data on female animals from the lasiocarpine study were excluded from further analysis. Further, they excluded the high-dose group of male rats,

due to high mortality and the presumed lower tumour incidence, which may have resulted from that. For male rats, RIVM adopted the following approach: data on lasiocarpine and riddelliine were combined (data on male and female rats from the riddelliine study, and data on male rats from the lasiocarpine study) and evaluated with PROAST, using "substance" as a covariate. Seven models provided an acceptable fit. Covariate "substance" did not result in different parameter estimates for the individual substance datasets. The Weibull model delivered the lowest $BMDL_{10}$ for the combined dataset of both substances (male and female rat data) of 0.274 mg/kg bw per day.

9.2.2 Dose–response modelling and BMD calculations

Haemangiosarcoma of the liver was the most prominent and frequent tumour type in the studies by the NCI on lasiocarpine (NCI, 1978) and the NTP on riddelliine (NTP, 2003). These tumours were observed in rats in both studies and also in the riddelliine study with mice, but mice proved to be less sensitive than rats (Fig. 9).

High lethality and reduced survival rates were observed in the riddelliine study at the highest dose and in the lasiocarpine study at the two highest doses in both sexes. When comparing doses, high mortality appeared to be related to doses of 0.7 mg/kg bw per day (and higher) for both substances in rats. Non-neoplastic lesions in the liver were observed in rats in the study with riddelliine at doses of 0.033 mg/kg per day (0.024 mg/kg per day after correction for continuous exposure) and with lasiocarpine starting at the lowest dose of 0.35 mg/kg per day.

Whereas, after riddelliine exposure, a clear dose–response relationship was observed and high incidences for liver haemangiosarcoma (86% in males, 76% in females) were noted in the highest dose group, in the lasiocarpine study, incidences in the highest dose group were lower (females) or only slightly higher (males) than in the lower dose groups. Therefore, it seems likely that tumour incidences in the highest dose groups of the lasiocarpine study (but not in the riddelliine study) were influenced by the low survival rates and the reduced time to develop tumours, resulting from the early deaths. Nevertheless, from the information obtained from the lower dose groups, it can be concluded that similar tumour numbers are induced at similar doses by lasiocarpine and riddelliine, although the different forms of administration in the two studies (administration by feed versus gavage) limit quantitative comparison (Fig. 10).

Modelling was carried out for the induction of haemangiosarcoma by riddelliine in female rats using the PROAST software (version 38.9). Eight models (including two families of nested models) were fitted to the data. Using a *P*-value of 0.1 as the criterion, all models provided acceptable fits and were used to derive BMD_{10} and $BMDL_{10}$ values for a BMR of 10% extra risk (Table 40). A

Fig. 9

Dose–response data on induction of haemangiosarcoma in male and female rats and information on survival rates in the studies of NCI (1978) with lasiocarpine and NTP (2003) with riddelliine

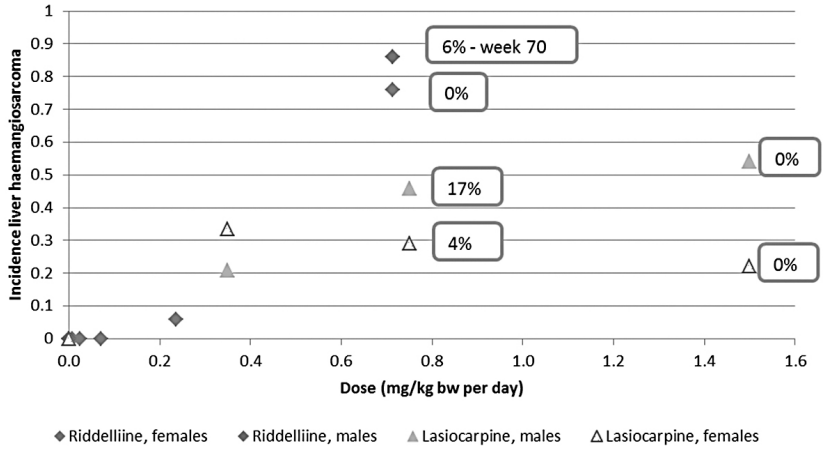


Table 40

BMD_{10} and $BMDL_{10}$ for liver haemangiosarcoma in female rats from riddelliine exposure based on NTP (2003)

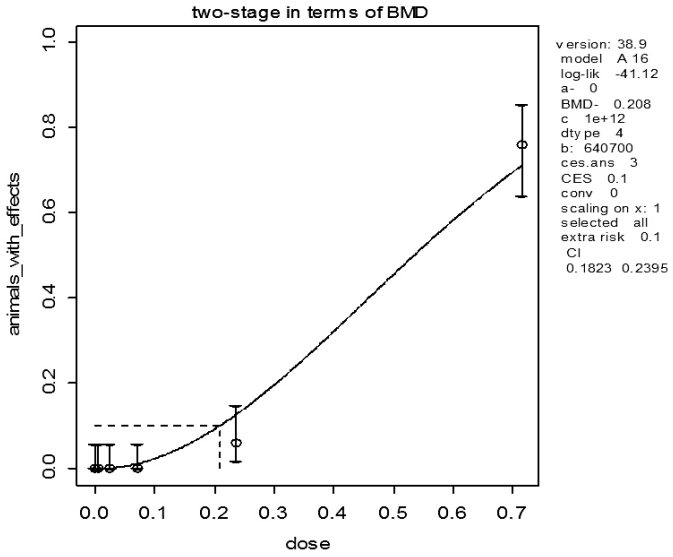
Model	npar	loglik	accept	BMD_{10}	$BMDL_{10}$
Null	1	−119.66	–	NA	NA
Full	6	−38.9	–	NA	NA
Two-stage	**3**	**−41.11**	**yes**	**0.208**	**0.182**
Log-logistic	3	−38.95	yes	0.278	0.216
Weibull	3	−39	yes	0.290	0.218
log−prob	3	−38.9	yes	0.270	0.215
gamma	3	−38.92	yes	0.277	0.215
logistic	2	−40.32	yes	0.363	0.299
LVM: E2	2	−39.64	yes	0.327	0.270
LVM: H5	4	−38.9	yes	0.247	0.215

Source: Based on NTP (2003).

range of BMD_{10} values of 0.208–0.363 mg/kg bw per day and a range of $BMDL_{10}$ values of 0.182–0.299 mg/kg bw per day was obtained from all accepted models. The lowest $BMDL_{10}$ value for riddelliine, derived from the NTP (2003) study with

Fig. 10

BMD_{10} and $BMDL_{10}$ for haemangiosarcoma induced by riddelliine using the two-stage model



Source: Produced using PROAST, with the experimental data from NTP (2003).

data for female rats with the two-stage model, was 0.18 mg/kg bw per day and was taken forward to risk assessment.

9.3 Relative potency factors

The Committee considered whether it was possible to identify relative potency factors for different 1,2-unsaturated PAs. In addition to the carcinogenicity studies used above, carcinogenicity studies on other PAs were conducted with non-standard protocols, and these do not allow comparison of carcinogenic potency. Based on short-term toxicity and genotoxicity, it appears that the potency is broadly in the order: macrocyclic esters > diesters > monoesters, although there may also be differences depending on the type of necine base and the stereochemistry (Merz & Schrenk, 2016). The two PAs that have been tested for carcinogenicity are among the more potent, and it is likely that many of the PAs present in food, such as lycopsamine, are less potent.

Ingested PA *N*-oxides are efficiently reduced to PA free bases in the digestive tract, and to a lesser extent in the liver. The Committee concluded that the data were not sufficient to make assumptions about the potency of the

N-oxides relative to the parent PA, and adopted the conservative approach of assuming equal potency.

10. Comments

Note: this section was updated after the report of the eightieth JECFA meeting had been published, taking new data into account.

10.1 Biochemical aspects

The vast majority of studies are on selected 1,2-unsaturated PAs, and there is little information on the saturated PAs. Only one systematic investigation of metabolic differences between different classes of PAs has been reported; investigations on other biochemical aspects are lacking. It is likely that the limited availability of individual purified PAs has impeded such studies.

Those 1,2-unsaturated PAs that have been investigated appear to be rapidly and extensively absorbed. In vitro studies indicate a role of active transport in the small intestine, favouring absorption of cyclic-ester PAs such as senecionine and senkirkine, and excretion of the open-chain diester PA echimidine, and of the monoester PA, heliotrine (Hessel et al., 2014). One in vitro study noted a role for P-glycoprotein in efflux of noncyclic PAs. Ingested PA *N*-oxides are efficiently reduced to PA free bases in the digestive tract, and to a lesser extent in the liver, and therefore contribute to PA toxicity (Mattocks, 1986).

Following absorption, PAs are distributed to the liver, RBCs, plasma, brain, lung and kidneys. In vitro studies indicate that the organic cation transporter (OCT1) could be involved in uptake of monocrotaline and retrorsine into hepatocytes (Tu et al., 2013, 2014). There is some evidence suggesting that uptake into the RBCs could facilitate transport of monocrotaline to other organs, such as the lung.

Elimination studies have been conducted with riddelliine, senecionine, adonifoline, monocrotaline, dehydromonocrotaline, retrorsine and retrorsine *N*-oxide (all of which are cyclic esters) in rats, mice, guinea-pigs and hamsters. Of the PAs tested, monocrotaline was mainly excreted unchanged, while other PAs were excreted mainly as metabolites. In general, the predominant route of excretion is in the urine and there is evidence for enterohepatic recirculation, possibly involving back-transformation of the *N*-oxides into the parent compounds.

Specific information on absorption, distribution and elimination of PAs in humans is not available; however, the reports of human poisoning demonstrate that PAs are absorbed and excreted in urine.

There are three main routes of metabolism of the 1,2-unsaturated PAs:

- cleavage of the ester bonds;

- *N*-oxygenation of the necine base of retronecine- and heliotridine-type PAs, leading to the more readily excreted *N*-oxides; and
- formation of reactive (+/−) 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) esters (see Fig. 2).

In vitro studies suggest that these metabolic routes are common to multiple species, including humans. In vitro studies have also shown that PAs can undergo glucuronidation at the nitrogen atom; however, the importance of this pathway in vivo has not been established.

Cleavage of the ester bonds is catalysed by carboxylesterases leading to formation of non-toxic necines and necic acids. The importance of this pathway is influenced by the level of hepatic esterase activity, which is lower in the rat than in other species such as the guinea-pig. The rat hepatic esterase activity is approximately equivalent to that of humans (Jewell et al., 2007). The structure of the ester functionality of different PAs also has an impact, since steric hindrance impairs de-esterification of PAs with branched chain esters (EFSA, 2011).

N-oxygenation is catalysed by CYPs and flavin-containing monooxygenases, and is generally considered a detoxification route since the *N*-oxides are more water soluble and hence more readily excreted (Dueker et al., 1992; Wang, Fu & Chou, 2005; Wang YP et al., 2005a,b; Williams et al., 1989a,b). However, the *N*-oxidation is reversible by the action of hepatic reductases and therefore it does not prevent subsequent formation of reactive metabolites (EFSA, 2011).

CYP3A4 and CYP3A5 are the major enzymes leading to formation of DHP esters from most 1,2-unsaturated PAs, with the exception of monocrotaline, for which CYP2A6 is the major activating enzyme. In vitro studies indicate a relatively high rate of reactive metabolite formation in humans compared to other species, as measured by the formation of GSH conjugate and dehydrogenation metabolites, and a low rate of demethylation metabolite formation. Hydroxylation at positions 3 and 8 adjacent to the nitrogen produces carbinolamines, which undergo spontaneous dehydration converting the ring system to an aromatic "pyrrole" ring. These DHP esters can form conjugates with GSH, cysteine or *N*-acetyl cysteine, but are also readily de-esterified to form stabilized, positively charged (carbonium) ions at C7 and C9. These ions rapidly react with electron-rich (nucleophilic) groups to produce DHP adducts in the liver (He X, Xia & Fu, 2017; Xia et al., 2015). DHP esters are also readily hydrolysed, releasing DHP (dehydronecines) into the circulation. DHP is a less reactive alkylating agent than the precursor DHP esters but is still capable of forming DHP adducts with nucleophilic targets in the liver and also in many extrahepatic tissues (Mattocks, 1986). Some DHP adducted to weaker nucleophilic groups can be released or transferred to stronger nucleophiles (Mattocks, 1986). Thus DHP linked to

some circulating proteins and to other substances, for example GSH, that escape from the liver, remain potentially active alkylating agents, prolonging toxicity and leading to extrahepatic effects (He X et al., 2016a,b; Huxtable et al., 1990; Mattocks, 1986; Prakash et al., 1999; Seawright, 1992, 1994; Xia et al., 2015; Zhao et al., 2014).

There is limited information on metabolism of saturated PAs; however, the absence of the 1,2-double bond means that they cannot be converted to DHP esters or to DHP.

In vitro studies demonstrate that ruminal microorganisms are able to metabolize PAs, but the metabolites have not been characterized.

PAs are able to transfer from animal feed into milk, eggs and meat at low levels (Diaz, Almeida & Gardner, 2014; EFSA, 2011; Gardner et al., 2014; Mulder et al., 2016).

10.2 Toxicological studies

10.2.1 Acute toxicity of PAs

Acute oral toxicity in rats and mice has been investigated only for ten 1,2-unsaturated PAs – adonifoline, echimidine, heliotrine, isoline, lasiocarpine, monocrotaline, retrorsine, retrorsine *N*-oxide, riddelliine and senecionine. These studies indicated that senecionine, retrorsine (and its *N*-oxide) and riddelliine are among the more acutely toxic PAs (LD_{50} values <100 mg/kg bw), whereas echimidine and heliotrine appear less toxic (LD_{50} values approximately 500 mg/kg bw). The liver and lungs are the main organs affected. Based on the LD_{50} values after intraperitoneal administration, male rats seem more susceptible to the acute toxicity of basic PAs than females. The relative toxicity of retrorsine *N*-oxide compared with that of the basic alkaloid depended on the administration route, being similar by the oral route, but less toxic following parenteral administration. This could be explained by the conversion of *N*-oxides to their basic alkaloids in the gut, which needs to take place before toxic pyrroles can be formed in the liver.

The 1,2-saturated PAs platyphylline (at 118 mg/kg bw) and cynaustraline (at 67 mg/kg bw) caused rapid death after intraperitoneal administration, without hepatotoxicity. In two other studies, LD_{50} values of 252 (platyphylline) and 260 mg/kg (cynaustraline) were obtained for these compounds.

10.2.2 Short-term toxicity

Short-term studies of toxicity have been conducted using a number of individual 1,2-unsaturated PAs (echimidine, indicine, indicine *N*-oxide, lasiocarpine, monocrotaline, retronecine *N*-oxide, retrorsine, riddelliine, riddelliine *N*-oxide) mainly in mice and rats. The most common toxic effect of repeated doses of

1,2-unsaturated PAs is hepatotoxicity, characterized by megalocytosis (enlarged hepatocytes containing hyperchromatic nuclei), and sometimes centrilobular necrosis, fibrosis and bile duct hyperplasia. The megalocytosis is considered to be due to an antimitotic effect, that is, the hepatocytes enter the cell cycle but do not divide (EFSA, 2011). Pulmonary toxicity is sometimes seen, particularly with monocrotaline; the structural requirements for toxicity in the lung are the same as those for toxicity in the liver and metabolites produced in the hepatocytes cause toxicity in the lung. The initial target appears to be the endothelial cells in the small blood vessels. Many of the studies were conducted at a single dose level, and therefore do not provide dose–response information. Riddelliine is the most studied PA, with rats being more sensitive than mice, and male rats more sensitive than females (Chan P, 1993; Chan PC et al., 1994). In a 13-week study in rats dosed with riddelliine by gavage, the NOAEL was 0.24 mg/kg bw per day, based on histopathological changes in the liver at a dose of 0.71 mg/kg bw per day (Chan P, 1993; Chan PC et al., 1994).

One 28-day study in rats tested both echimidine and lasiocarpine (Dalefield, Gosse & Mueller, 2016).[1] No effects were reported at the highest tested dose of echimidine (2.5 mg/kg bw per day), which was similar to the LOAEL for lasiocarpine (2.8 mg/kg bw per day for reduced body weight and the NOAEL was 1.2 mg/kg bw per day), indicating that echimidine might be less toxic than lasiocarpine.

10.2.3 Long-term toxicity

Megalocytosis in the liver is also seen in long-term studies of toxicity in experimental animals. Carcinogenicity is considered to be the critical end-point resulting from long-term exposure of laboratory animals to 1,2-unsaturated PAs. Several old studies on carcinogenicity that do not adhere to the current guidelines were conducted on various purified PAs, such as lasiocarpine, monocrotaline, retrorsine, retronecine, heliotrine, senecionine, symphytine, clivorine, petasitenine and senkirkine, and on crude plant extracts containing PAs. These studies were generally in rats, using various durations, dosing regimes and routes of administration (Chen L et al., 2017; EFSA, 2011). Tumours were observed in multiple tissues, most commonly the liver, lungs and blood vessels.

The Committee considered in detail the two long-term carcinogenicity studies on lasiocarpine and riddelliine, which were evaluated by IPCS and EFSA. No new long-term studies published since those two evaluations were identified by the Committee.

[1] Part of the information in this summary is based on the study reports (study director Cayzer) and is not published in the article by Dalefield, Gosse & Mueller. This information was made available to the monographers before the meeting.

(a) **Lasiocarpine (NCI, 1978)**

Groups of 24 rats of each sex were administered lasiocarpine (purity 97%) at doses of 7, 15 or 30 mg/kg feed (equivalent to 350, 750 and 1500 µg/kg bw per day, respectively), for 104 weeks. Matched controls consisted of groups of 24 untreated rats of each sex. Body weights at the end of the study were lower in animals in the mid- and high-dose groups than in controls. High mortality was noted in the mid- and high-dose groups of both sexes. None of the animals in the high-dose groups survived to the end of the study. Survival rate in male animals was 88% for controls, 54% in the low-dose group and 17% in the mid-dose group. In females, survival rates were 92% for controls, 42% in the low-dose group and 4% in the mid-dose group. As more than 50% of the females in the high-dose group died before week 52 of the study, the statistical analysis of female rats was performed using only those animals surviving more than 52 weeks: 24 in the control group, 24 in the low-dose group, 23 in the mid-dose group and 9 in the high-dose group.

Non-neoplastic lesions were not described in detail in the study report, but it was noted that nodular hyperplasia of the liver occurred in both male and female rats in all dose groups.

Male rats showed a dose-related increase in the incidence of haemangiosarcoma of the liver (controls 0/24, low dose 5/24, mid-dose 11/24, high dose 13/24). In females, the incidences in both the low- and mid-dose groups, but not that in the high-dose group, were significantly higher than that in the controls (controls 0/24, low-dose 8/24, mid-dose 7/24, high-dose 2/9). The study authors noted that the lower incidence in females that received the high dose, compared with the females in the low- and mid-dose groups, could be related to the increased mortality rate in the high-dose group. Furthermore, compared to controls, females in the high-dose group showed a significant increase in the combined incidence of hepatocellular carcinoma and adenoma of the liver. A positive trend for these tumours was also observed in male rats, but incidences did not reach statistical significance. The combined incidence of lymphoma or leukaemia was significant in females in both the low- and mid-dose groups, but not in the high-dose group, perhaps because of the early deaths of animals in this group. The combined incidences of these tumours in the males were not statistically significant.

NCI (1978) concluded that "*under the conditions of this bioassay, lasiocarpine was carcinogenic in Fischer 344 rats producing hepatocellular tumors and angiosarcomas of the liver in both sexes and hematopoietic tumors in female animals*".

(b) **Riddelliine (NTP, 2003)**

Groups of 50 male and female mice, 5–6 weeks of age, were administered riddelliine (purity 92%) by gavage at a dose of 0 or 3 mg/kg bw per day, 5 days/week, for 105 weeks (equivalent to 0 and 2.1 mg/kg bw per day on a 7 days/week basis). Additional groups of 50 male mice received 0.1, 0.3 or 1 mg/kg bw per day, 5 days/week, also for 105 weeks (equivalent to 0, 0.071, 0.21, 0.71 and 2.1 mg/kg bw per day on a 7 days/week basis).

Survival of males and females administered riddelliine at 3 mg/kg bw per day was significantly less than that of the vehicle controls. Twenty out of 50 male and 17 out of 50 female animals survived until study termination. Body weights were reduced in both females and males given 3 mg/kg bw per day (by 19 and 33%, respectively) as compared to controls. In the male animals administered 1 mg/kg bw per day, the body weight at termination was on average 6% lower than that of controls. There were no treatment-related clinical findings.

In males administered 0.3 mg/kg bw per day or more, and females given 3 mg/kg bw per day, incidences of hepatocyte cytomegaly, karyomegaly and necrosis were significantly increased compared to controls. Focal haemorrhage in the liver increased at doses from 1 mg/kg bw per day upwards. Increased incidences of various kidney effects were observed at higher doses in both males and females. These included glomerulosclerosis (in males in the two highest dose groups and in females in the high-dose group), nephropathy (females) and renal tubule karyomegaly (males). Further effects observed in females in the high-dose group were alveolar epithelial hyperplasia and chronic arterial inflammation in various organs.

Increased incidences of liver haemangiosarcomas were restricted to the males in the high-dose group (62% versus 4% in vehicle controls). Females in the high-dose group showed increased incidences of alveolar/bronchiolar adenoma or carcinoma (26% versus 4% in vehicle controls). Decreased incidences of liver adenoma and carcinoma were noted in both male and female mice dosed with riddelliine.

Groups of 50 male and 50 female rats were administered riddelliine (purity 92%) by gavage in sodium phosphate buffer at a dose of 0 or 1 mg/kg bw per day, 5 days/week for 105 weeks (equivalent to 0 and 0.71 mg/kg bw per day on a 7 days/week basis); additional groups of 50 female rats received 0.01, 0.033, 0.1 or 0.33 mg/kg bw per day, 5 days/week, also for 105 weeks (equivalent to 0, 0.007, 0.024, 0.071, 0.236 and 0.71 mg/kg bw per day on a 7 days/week basis).

Mean body weights were reduced in rats given high doses (by 21% and 18% in males and females, respectively) compared with vehicle controls. Survival days were reduced in both males and females in the high-dose groups, and only 3/50 male and 0/50 female animals survived until the end of the study. Because

of their high mortality, male rats were killed at week 72. The only clinical finding related to riddelliine administration was a general debilitation of the animals prior to death. Non-neoplastic effects observed in the liver of female rats included dose-related increases in the incidence of hepatocyte cytomegaly at doses starting from 0.033 mg/kg bw per day. Further histopathological observations (including regenerative hyperplasia, eosinophilic foci and clear cell foci) at the two highest doses, and necrosis and haemorrhage at the highest dose were also noted. All these effects were also evident at the only (high) dose investigated in male animals. Further, severe non-neoplastic lesions, such as renal tubule necrosis, were observed in males and females in the high-dose groups.

Haemangiosarcomas were present in the liver of 86% of males and 76% of females in the group that received riddelliine at a dose of 1 mg/kg bw per day and 6% of female animals in the group given 0.3 mg/kg bw per day. The incidences of hepatocellular adenoma and mononuclear cell leukaemia in males and females in the 1 mg/kg bw per day groups were also significantly increased.

NTP concluded that "*there was clear evidence of carcinogenic activity of riddelliine in male B6C3F*$_1$ *mice based on increased incidences of hemangiosarcoma in the liver*" and "*clear evidence of carcinogenic activity in female B6C3F*$_1$ *mice based on increased incidences of alveolar/bronchiolar neoplasms*".

NTP also concluded that *"under the conditions of these studies, there was clear evidence of carcinogenic activity of riddelliine in male and female F344/N rats based on increased incidences of hemangiosarcoma in the liver".* They also considered the increased incidences of hepatocellular adenoma and mononuclear cell leukaemia in male and female rats to be treatment-related.

The Committee agreed with the conclusions of the NTP.

10.2.4 Genotoxicity

The genotoxicity of PAs has been extensively studied in a variety of in vitro and in vivo assays. Overall these assays clearly demonstrate that the 1,2-unsaturated PAs that have been tested form DNA adducts and are mutagenic. Bioactivation of 1,2-unsaturated PAs to pyrrolic ester(s), and the subsequent formation of specific DNA adducts has been identified as the key pathway leading to genotoxic effects. Studies in rats have shown that levels of adducts and of mutations are higher in the endothelial cells of the liver than in the parenchymal cells (Chou et al., 2004). Binding to DNA leads to nucleoside adduct formation and protein cross-linking. From studies of DNA and/or protein-binding in vitro, including with human liver microsomes, and studies with rodents in vivo, it appears that capacity for adduct formation is in the following order: retronecine-type 12-membered macrocyclic diesters and open-ring heliotridine- and retronecine-type diesters > 11-membered macrocyclic diesters and otonecine-type macrocyclic diesters

> heliotridine- and retronecine-type monoesters. The unesterified necine base, retronecine, was not found to be mutagenic.

In general, the 1,2-saturated PAs have not been tested, but because they are not metabolized to pyrrolic esters, it is assumed that they are less likely to be genotoxic. This assumption is supported by studies showing that ligularine was negative in the UDS assay, and platyphylline did not form DNA adducts.

10.2.5 Reproductive and developmental toxicity

Multigeneration studies have been conducted in mice and rats administered a preparation of riddelliine by gavage (Chan et al., 1994; NTP, 1993). In the mice, which were treated with higher doses, effects included prolonged estrus and decreased body weights in the dams, and decreased pup weight and survival. The fetal NOAEL was 2.4 mg/kg bw per day in mice, and it is not clear whether fetal effects were secondary to maternal toxicity. Prolonged estrus and decreased body weights in the dams were also reported in the rat, and the fetal NOAEL was 0.7 mg/kg bw per day (the highest dose tested). Dietary administration of *Senecio vernalis* to chicken hens resulted in decreases in egg production, feed efficiency, feed intake and body weight. The NOAEL was 0.5 mg/kg bw per day total alkaloid (senecionine >65%, senecivernine, seneciphylline, integerrimine, retrorsine, senkirkine and hydroxysenkirkine).

Developmental studies in rats treated with various PAs have shown maternal toxicity (such as reduced body weight gain) at lower doses than those resulting in fetal toxicity (e.g. Medeiros, Górniak & Guerra, 2000; Sandini et al., 2014). Fetal effects included abnormalities in development, increased intrauterine death or resorption, and behavioural changes in adulthood. Because of the maternal toxicity it is not possible to determine whether effects on the fetus are secondary to maternal toxicity. One study indicated that exposure of pups to monocrotaline via lactation resulted in lung and kidney toxicity. Severe toxicity and mortality was observed in offspring exposed to the same dose of monocrotaline during gestation and lactation (Medeiros, Górniak & Guerra, 1998). These studies do not provide NOAELs, either because they were conducted at a single dose level, or because maternal toxicity was reported at all tested doses. Some studies indicated that, based on body weight, the fetus might actually be less sensitive to PA toxicity than the mother (Hueza et al., 2011; Sandini et al., 2015). This may be because the fetal liver is less able to metabolize PAs to the active pyrrole (Mattocks & White, 1973 in WHO-IPCS, 1988).

10.2.6 Special studies

Some, but not all, studies indicated that some PAs could have immunosuppressive effects. The Committee was unable to reach conclusions about this owing to the

inconsistency of the observations reported. Although one study showed that monocrotaline may alter the permeability of the blood–brain barrier and the content of the cerebrospinal fluid (Coll et al., 2011), the neurotoxicity of PAs has not been evaluated.

10.3 Observations in domestic animals/veterinary toxicology

Consumption of PA-containing plants or feed contaminated with PA-containing plants has been reported to cause disease in multiple species including pigs, horses, donkeys, goats, sheep, buffaloes, cattle, yaks and wombats. The observed symptoms are in line with the effects on the liver that have been observed in experimental animals. Other symptoms included spongy degeneration of brain tissue and skin lesions. EFSA (2011) reported that in general, sheep, goats, rabbits and guinea-pigs are among the more resistant species, whereas horses, pigs and poultry are more sensitive. These reports do not allow identification of the doses of PAs consumed.

10.4 Observations in humans

Reported effects of PAs on human health have been mainly based upon outbreaks associated with grain crops contaminated with PA-containing weeds and case reports of poisonings by herbal remedies or teas. In the vast majority of instances, the toxicity affected primarily the liver, with a characteristic pattern of SOS, termed hepatic veno-occlusive disease (HVOD) in most reports. The causal link between PA-containing plants and toxic effects is supported in most instances by several criteria: clear identification of the outcome (in many instances based upon pathological findings); temporality (documented exposure to the suspected toxicant days to months before the onset of the disease); identification of a PA-containing plant (and in some instances identification of the PAs), and, finally, other potential causes of the outcome were ruled out (infectious diseases, other hepatic toxicants such as alcohol, drugs and aflatoxins).

Six large outbreaks were reported, five in Asian countries and one in Africa. The outbreak in central India in 1975 was caused by the consumption of millet contaminated by Crotalaria. Consumption of wheat flour contaminated with *Heliotropium popovii* was the cause of the outbreak in 1973–1975 in Afghanistan, and was probably also the cause of a second outbreak in the same region in 2007–2008. Wheat flour contaminated by another species of the same plant (*Heliotropium lasocarpium*) was the cause of the outbreak in 1992–1993 in Tajikistan. The outbreak of 1994 in Iraq was caused by wheat flour contaminated with *Senecio vulgaris*. Finally, a huge outbreak was reported in 2001–2005 in

northern Ethiopia, whose most likely cause was found to be drinking water contaminated with Ageratum plant. New cases of the disease with liver injuries compatible with SOS were detected in 2005–2009, mostly in the Hirmi Valley region. These were attributed to consumption of large amounts of millet contaminated with the invasive weed *Ageratum conyzoides*.

To sum up, although in the outbreaks analysed there was usually a documented exposure to PA before the onset of the disease, there is variability regarding the timing, duration of the exposure, time to sampling and estimated levels of exposure. A clear description of the time aspects was available for the outbreak in Afghanistan: duration of exposure before the onset of the outbreak was about 2 years; the duration of the disease was from 1 to 6 months; the time from the onset of symptoms to the biopsy was 1–9 months. The estimated dose of PA among cases was 0.033 mg/kg bw per day. In Tajikistan, the time that elapsed from the beginning of exposure to the first clinical cases was about 6 weeks; the exposure was 0.018 mg/kg bw per day.

There are 24 case reports of poisoning by PA-containing teas or herbal remedies; in all but one of these, the health effect reported was HVOD or SOS and the vast majority of the diagnoses were based upon histopathological findings. The species of PA-containing plants most often involved were Symphytum (7 cases) and Senecio (6 cases); other plant species reported were Heliotropium, Tussilago, Petasites, Gynura, Adenostyles, Emilia and Teucrium. Only six case reports of histologically confirmed HVOD included enough details to provide information on the actual involvement of PAs by chemical analysis of the product consumed by the patient, estimated dose of PAs based on reported intake of the plant or herbal tea, and the duration of exposure. From these reports, the lower known doses of PAs associated with acute and/or short-term toxicity in humans are reported to be 3 mg/kg bw per day over 4 days in a 2-month-old boy (lethal outcome), 0.8–1.7 mg/kg bw per day for 2 weeks in a 6-month-old-girl, and 0.60 mg/kg bw per day over 19 days in a young woman. The lowest known dose of PA associated with longer-term toxicity is reported to be 15 µg/kg bw per day over a period of 6 months, and about 1.63–3.27 µg/kg bw per day for 3 months.

Overall, case reports of poisonings, including deaths, due to PA-containing herbal medicines and teas have clearly shown toxicity in humans, predominantly in the liver. These results could be used as indicative or supporting information for acute and non-cancer risk assessment. However, many uncertainties must be taken into consideration when deciding which information is reliable enough to be used as a basis for human health risk characterization. No studies were available on the potential carcinogenic effects of PAs in humans. Based on the present knowledge of the possible mechanism of carcinogenesis (metabolism, activation, DNA adduct formation) it is possible that PAs may act as carcinogens in humans. The lack of epidemiological studies on cancer is a data gap; even

though there are no findings confirming that such an effect exists, it cannot be concluded that PAs are not carcinogenic to humans.

10.5 Analytical methods

The Committee reviewed and identified the specific analytical issues associated with the screening and quantification of PAs – saturated and unsaturated PAs and their *N*-oxides – and DHP adducts in various foods and feeds.

The main issues related to analytical methods include:

- wide variations in PA concentrations in food and feed samples;
- variation in PA profiles between plants in various regions of the world;
- the stability of PAs during storage; and
- the issue of whether to quantify individual PAs or total necines.

PAs are extracted from plants and food samples with hot or cold methanol or ethanol, or dilute aqueous acid. The alcoholic or aqueous acid extracts are then applied to prepared strong cation exchange SPE cartridges, followed by washing of the cartridges with water and methanol to remove non-adsorbed impurities, and then elution of the PAs and *N*-oxide components using a small volume of ammoniated methanol. Subsequent evaporation and reconstitution of the residue in methanol or another suitable solvent produce samples ready for analysis of PAs.

Several screening methods are available, including TLC, electrophoresis, NMR and immunological methods. TLC with colorimetric detection of 1,2-unsaturated PAs is inexpensive, but the results are qualitative rather than quantitative. NMR has been used to determine the total alkaloid content but it probably lacks the sensitivity required for food safety risk assessment purposes. ELISA-based screening methods for 1,2-unsaturated PAs and their *N*-oxides have been developed, but are currently limited by a lack of antibodies that specifically bind all of the 1,2-unsaturated PAs and their *N*-oxides with comparable affinity. At the same time, antibodies developed for specific 1,2-unsaturated PAs or their *N*-oxides seem to lack specificity for other 1,2-unsaturated PAs or their *N*-oxides. The development of sensitive ELISAs for quantifying necines could be useful in summation analysis methods for quantifying total 1,2-unsaturated PAs and their *N*-oxides based on hydrolysis. However, results from ELISA should always be confirmed using quantitative reference methods, such as GC-MS and/or HPLC-MS/MS since immunological methods have limitations in selectivity and reproducibility.

Recent developments have shown the need for validated methods to analyse DHP adducts in liver tissue and foods of animal origin. Methods for sulfur-bound DHP adducts, DHP–DNA adducts and DHP–protein adducts are available, but not validated, for foods. The significance of DHP adducts in food is uncertain. Some DHP adducts are reversible and may be capable of transferring the DHP moiety to DNA in vivo; for example, DH-GSH has been shown to transfer DHP to DNA in vitro, and it and other DHP adducts in food could potentially cause mutations.

Quantitative analysis of PAs is based on the determination of individual PAs, using LC-MS/MS, or on analysis of common necine groups, using GC-MS detection. Some issues of concern are related to the instability of *N*-oxides during sample preparation and analysis.

Challenges common to all analytical methods are the lack of high-quality standards, internal standards and certified reference materials. Harmonized methods or performance criteria for PAs are currently not available, despite a number of proficiency tests carried out in the EU, which have shown promising results in tea, honey and several feedstuffs.

10.6 Sampling protocols

PA contamination can be non-homogeneous owing to the uneven distribution of plant parts in a batch of feed or food. Proper sampling will, therefore, be critical. Existing sampling protocols for other natural toxins such as mycotoxins should be followed in sampling protocols for PAs in bulk commodities and in consumer products. DHP adducts are expected to be more evenly distributed in foods of animal origin, and common sampling protocols for contaminants should be applied.

10.7 Effects of processing

Reports on the effects of food and feed processing on the concentration of PAs in the subsequently produced foods have been studied by the Committee.

The main focus of the reports in the literature is on 1,2-unsaturated PAs and their *N*-oxides. The 1,2-unsaturated PAs and their *N*-oxides are stable during tea infusion making. The removal of co-harvested seeds and weeds from the raw materials will reduce the content of 1,2-unsaturated PAs and their *N*-oxides significantly. Intoxications of humans related to 1,2-unsaturated PAs have involved consumption of tea, bread, yoghurt, kitchen herbs and spices. To a certain extent, this indicates the stability of PAs during various food preparation steps. The presence of PAs in foods and dietary supplements such as pollen and

honey is further confirmation of the stability of PAs during food processing. However, details on the rate of possible degradation during food processing are not available, with the exception of data on tea infusion.

The occurrence of PAs in animal feed shows that 1,2-unsaturated PAs and their *N*-oxides are fairly stable during feed production, although reliable data on the rate of degradation and the metabolites that are formed are lacking.

10.8 Prevention and control

Management strategies to prevent PA-containing plants from entering the food chain were evaluated by the Committee. Management practices currently focus on minimizing the occurrence of weeds containing 1,2-unsaturated PAs and their *N*-oxides in feed and food. Management practices to help prevent and reduce the levels of 1,2-unsaturated PAs and their *N*-oxides in food were indicated in a recently published Codex Alimentarius code of practice. Good agricultural practices, HACCP and good manufacturing practice strategies must be in place to prevent batches of food contaminated with PAs entering the food chain and mingling with uncontaminated products.

10.9 Levels and patterns of contamination in food commodities

Data on the content of 1,2-unsaturated PAs and their *N*-oxides in foods for evaluation by the Committee were obtained from the scientific literature and from submissions from Brazil, Germany, Hungary, Luxembourg and FoodDrinkEurope (formerly the Confederation of the Food and Drink Industries of the European Union). The total number of analytical results evaluated at the current meeting was 21 793. Table 41 summarizes the prevalence and concentration range of 1,2-unsaturated PAs and their *N*-oxides by food category. The concentration range (minimum to maximum) is based only on analytical results that were quantified. It should be noted that the number of 1,2-unsaturated PAs and their *N*-oxides included in individual studies varied substantially; from 2 to 30. In addition, some studies did not report the individual compounds analysed but reported only "total PAs", whereas other analytical methods converted 1,2-unsaturated PAs and their *N*-oxides to a common moiety for analysis.

While the specific 1,2-unsaturated PAs and their *N*-oxides detected vary with the plant species involved, frequently detected compounds included lycopsamine, heliotrine, echimidine, lasiocarpine, retrorsine, senecionine, seneciphylline, senkirkine and intermedine and *N*-oxides, particularly those of lycopsamine, retrorsine, senecionine, seneciphylline and intermedine.

Table 41
Summary of available data on 1,2-unsaturated PAs and their *N*-oxides in food products

Food category	*N*[a]	*n*<LOD[b]	Concentration range (µg/kg)
Cereals and cereal products	1 368	1 304	0.12–98 000[c]
Tea and herbal tea	377	60	0.021–8 500[d]
Culinary herbs	21	6	0.9–74
Herbal dietary supplements	21	14	0.1–8.4[e]
Miscellaneous food ingredients	34	25	4.3–4 800
Honey	19 698	5 672	0.3–5 600
Honey products	62	52	10–590
Bee pollen dietary supplements	174	86	11–38 000
Milk and milk products	27	21	1.5–87[c]
Oils	11	8	0.08–0.6

LOD: limit of detection.
[a] Number of samples for which analytical results are available.
[b] Number of samples for which the analytical result was left censored.
[c] Includes results from an investigation of an outbreak of veno-occlusive disease.
[d] Includes results for dry tea and for tea infusions.
[e] Concentration data were not available. Results are in units of µg/day.

Most of the data evaluated (99%) were from European countries. Patterns of contamination with 1,2-unsaturated PAs and their *N*-oxides are likely to differ between regions, and a better understanding of the worldwide situation would be aided by submission of data from other regions.

Transfer of 1,2-unsaturated PAs and their *N*-oxides from feed to foods, in particular eggs, milk and meat, is reported from studies using high doses. DHP adducts can also be expected in these products. The analytical results evaluated by the Committee lacked information on 1,2-unsaturated PAs and their *N*-oxides and DHP adducts in retail samples of foods of animal origin (milk, eggs and meat). The information on 1,2-unsaturated PAs and their *N*-oxides in cereals and cereal products included data from surveys of heavily contaminated cereals in Afghanistan and the Islamic Republic of Iran. Further information on background levels of 1,2-unsaturated PAs and their *N*-oxides in cereals would aid the evaluation. Data on 1,2-unsaturated PAs and their *N*-oxides in non-herbal teas are inconsistent, and further data on these beverages would be useful. Herbal medicines were found to contain high concentrations of 1,2-unsaturated PAs and their *N*-oxides (n = 103, n < LOD = 38, concentration range 1–7 900 000 µg/kg).

Data on the content of 1,2-unsaturated PAs and their *N*-oxides in animal feeds, for evaluation by the Committee, were obtained from the scientific literature. The total number of analytical results evaluated at the current meeting was 539. Table 42 summarizes the prevalence and concentration range of 1,2-unsaturated

Table 42
Summary of available data on 1,2-unsaturated PAs and their *N*-oxides in animal feed

Food category	Number of analytical results evaluated	*n*<LOD	Concentration range (µg/kg)
Forages and roughages	420	253	5.0–23 000
Compound feed	20	12	5.3–140
Other feed	99	64	4.9–3 300

LOD: limit of detection.

PAs and their *N*-oxides by feed category. The concentration range (minimum to maximum) is based only on analytical results that were quantified.

10.10 Food consumption and dietary exposure assessment

Based on the preliminary toxicological evaluation by the Committee, both acute and chronic dietary exposures are relevant to PAs; therefore, both were considered for this assessment. No specific at-risk population groups were identified; therefore, all dietary exposure estimates for the general population were considered.

No estimates of dietary exposure were submitted to the Committee for review, and the dietary exposure estimates summarized below were obtained from a search of the literature or from calculations made by the Committee. Owing to analytical differences between different studies, the number of compounds that the dietary exposure estimates represent may vary. All dietary exposure estimates performed to date relate to exposure to 1,2-unsaturated PAs and their *N*-oxides from a single food type, with the exception of a small (n = 63) duplicate-diet study carried out in the Netherlands. National and regional estimates of dietary exposure are summarized in Table 43.

No information was available on dietary exposure to 1,2-unsaturated PAs and their *N*-oxides from consumption of products of animal origin, excluding honey. Using EFSA estimates of "worst case" exposure of a "high-yielding dairy cow" to 1,2-unsaturated PAs and their *N*-oxides from consumption of contaminated feed, and transfer information from animal feeding trials, it was estimated that the maximum likely exposure to 1,2-unsaturated PAs from consumption of contaminated milk could be 0.089 µg/kg bw per day.

The available occurrence data for 1,2-unsaturated PAs and their *N*-oxides were deemed to be inappropriate for use in determining international estimates of chronic dietary exposure in combination with the GEMS/Food consumption cluster diets. There were insufficient or inappropriate data for most relevant

Table 43
Summary of estimates of dietary exposure to 1,2-unsaturated PAs and their *N*-oxides

	Mean (95th percentile) dietary exposure (µg/kg bw per day)[a]			
	Acute[b]		Chronic	
Study	**Children**	**Adults**	**Children**	**Adults**
Duplicate diet				
Netherlands (de Wit et al., 2014)	–	–	–	0.0013
Honey				
Australia (current assessment)	0.20–0.24 (0.61–0.65)	0.10–0.12 (0.31–0.32)	0.011–0.013 (0.038–0.045)[c]	0.0032–0.0039 (0.022–0.026)
Brazil (current assessment)			0.00020–0.00021	
Europe (EFSA, 2011)[d]				
Retail honey	0.0033–0.11 (0.012–0.25)	0.0009–0.049 (0.0032–0.11)	0.00001–0.005 (NS–0.057)	0.00002–0.001 (NS–0.009)
Bulk honey	0.0071–0.17 (0.031–0.55)	0.0020–0.071 (0.0085–0.24)	0.00003–0.007 (NS–0.082)	0.00005–0.002 (NS–0.014)
Germany (BfR, 2013a)[e]	0.028–0.057 (0.092–0.11)	0.018–0.037 (0.060–0.072)	0.002–0.004 (0.006–0.012)	0.001–0.002 (0.005–0.017)
Ireland (Griffin et al., 2015)[f]	0.193–0.20 (0.48–0.51)	0.048–0.051 (0.12–0.13)	–	–
Tea				
Germany (BfR, 2013b)				
Tea	0.151–0.25 (0.69–0.78)	0.060–0.099 (0.27–0.31)	0.011–0.018 (0.046–0.076)	0.006–0.010 (0.030–0.050)
Herbal tea	0.13–0.21 (0.5740.66)	0.060–0.099 (0.27–0.31)	0.005–0.009 (0.027–0.045)	0.002–0.003 (0.018–0.030)
Ireland (Griffin et al., 2014a)[g]	–	–	–	0.0013–0.111
Netherlands (de Wit et al., 2014)				
Herbal tea[h]	–	–	–	0.003–0.13 (0.01–0.26)
Herbal medicines/supplements				
Ireland (Griffin et al., 2014)[i]	–	–	–	0.0008–0.13
Netherlands (de Wit et al., 2014)[j]	–	–	–	0.0004–0.06 (0.001–0.60)
USA (Avula et al., 2012)[k]	–	–	–	0.0017–0.14
Culinary herbs				
Germany (Cramer et al., 2013)[l]	–	0.032–0.112	–	–

NS: not specified (some of the countries included in the European assessment did not provide 95th percentile estimates of honey consumption).

[a] All exposure estimates have been rounded to two significant figures and may appear different from the equivalent numbers in the original publications. Ranges show lower bound to upper bound estimates.

[b] Acute exposures summarized here are those based on a 95th or 97.5th percentile consumer, consuming food containing 1,2 unsaturated PAs and their *N*-oxides at either the mean concentration or the 95th percentile concentration, unless otherwise stated.

[c] In this case, the high percentile is a 90th percentile, rather than a 95th percentile.

[d] The results for children are those for a toddler (1–3 years), rather than "other children" (3–10 years). Chronic exposures are those estimated for all respondents, rather than consumers only.

[e] In the German assessment, chronic exposures were calculated with and without "brand loyalty". Brand-loyal consumers were considered to always consume honey at the 95th percentile of the concentration distribution. Results presented here are those based on the mean concentration.

[f] Based on mean consumption of 20 g honey per day or maximum consumption of 50 g honey per day.

[g] Based on daily consumption of three cups (600 mL) of herbal tea.

[h] Mean exposure estimates were based on consumption of three 150 mL cups of tea per day, each containing 3 g of tea. High percentile (97.5th percentile, consumer only) estimates of exposure were based on 11 × 150 mL cups, each containing 3 g of tea. For 2 of 11 teas, recommended dose information was used. Exposures were calculated using a 70 kg body weight.

[i] Exposure from use of herbal medicines at the recommended dose, assuming a 60 kg body weight.
[j] Exposure from use of herbal supplements at the recommended dose. Figures in parentheses are from use of supplements at a rate of 10 capsules or tablets per day. Exposures were calculated using a 70 kg body weight.
[k] The range represents exposure to 1,2 unsaturated PAs and their *N*-oxides from recommended use of PA-containing dietary supplements based on the common butterbur (*Petasites hybridus*), assuming a 60 kg body weight.
[l] Based on consumption of a traditional German meal in which surveyed commercial herb preparations may have been used, assuming a 60 kg body weight.

foods, and/or not enough information about the samples to enable data from different sources to be combined.

10.11 Dose–response analysis

Carcinogenicity is considered to be the most critical end-point following long-term exposure to certain PAs in experimental animals. The Committee reviewed the studies performed by the US NCI (NCI, 1978) on lasiocarpine and by the US NTP (NTP, 2003) on riddelliine, described in more detail above. Haemangiosarcoma of the liver was the most prominent and frequent tumour type in both studies on rats and also in the riddelliine study on mice, but mice proved to be less sensitive than rats. High lethality and reduced survival rates were observed in the riddelliine study at the highest dose and in the lasiocarpine study at the two highest doses in both sexes. When comparing doses, (similarly) high mortality appears to be related to doses of 0.7 mg/kg bw per day for both substances in these two studies on rats. Non-neoplastic lesions in the liver were observed in rats in the study with riddelliine at doses of 0.033 mg/kg bw per day (0.024 mg/kg bw per day after correction for continuous exposure) and with lasiocarpine starting at the lowest dose of 0.35 mg/kg bw per day.

Whereas a clear dose–response relationship was observed after riddelliine exposure and high incidences for liver haemangiosarcoma (86% in males, 76% in females) were noted in the highest dose group, incidences in the highest dose group in the lasiocarpine study were lower (females) or only slightly higher (males) than those in the lower dose groups. Therefore, it seems likely that tumour incidences in the highest dose groups of the lasiocarpine study were influenced by the low survival rates and consequently the shorter period of time during which tumours could develop. Nevertheless, from the information obtained from the lower dose groups, it can be concluded that similar tumour numbers are induced at similar doses by both lasiocarpine and riddelliine, although it must be noted that the different forms of administration in the two studies (administration in feed versus gavage) set limitations to a quantitative comparison.

Modelling was carried out for the induction of liver haemangiosarcoma by riddelliine in female rats using the software PROAST (version 38.9). Eight models (including two families of nested models) were fitted to the data. Using a *P*-value of 0.1 as the criterion, all models provided acceptable fits and were used

Table 44
Ranges of BMD_{10} and $BMDL_{10}$ values for liver haemangiosarcoma in rat after dietary exposure to riddelliine

End-point and study	BMD_{10} (μg/kg bw per day)	$BMDL_{10}$ (μg/kg bw per day)
Liver haemangiosarcoma in female rats (NTP, 2003)	208–363	182–299

to derive BMD and BMDL values for a BMR of 10% extra risk (see Table 44). The lowest $BMDL_{10}$ value for riddelliine, derived with the two-stage model, was 182 μg/kg bw per day or 0.182 mg/kg bw per day.

10.11.1 Relative potency

The Committee considered whether it was possible to determine the relative potency for different 1,2-unsaturated PAs. Based on short-term toxicity and genotoxicity, it appears that the potency is broadly in the order macrocyclic esters > diesters > monoesters, although there may also be differences depending on the type of necine base and the stereochemistry (Merz & Schrenk, 2016). The two PAs that have been tested for carcinogenicity are among the more potent, and it is likely that many of the PAs present in food, such as lycopsamine, are less potent.

Ingested PA *N*-oxides are efficiently reduced to PA free bases in the digestive tract, and to a lesser extent in the liver. The Committee concluded that the data were not sufficient to make assumptions about the potency of the *N*-oxides relative to the parent PA, and adopted the conservative approach of assuming equal potency.

10.11.2 Point of departure or health-based guidance value for acute or short-term toxicity

As there were many poisoning cases known, the Committee also considered deriving a point of departure or health-based guidance value for acute or short-term toxicity based on the observations in humans. However, the variability regarding the timing, duration of exposure, time to sampling and estimated levels of exposure, did not provide a sufficiently robust basis for a point of departure. For reference, the lowest reported exposure associated with human disease was 0.018 mg/kg bw per day following 6 weeks exposure in Tajikistan (Chauvin, Dillon & Moren, 1994).

11. Evaluation

The Committee noted that most studies of toxicity, and of occurrence of PAs in food, were focused on the 1,2-unsaturated PAs. The Committee concluded that while the saturated PAs could not elicit toxicity via the same mechanism as 1,2-unsaturated PAs, their toxicity in humans could not be excluded, but there were insufficient studies for evaluation. The Committee therefore decided to focus the evaluation on the 1,2-unsaturated PAs. Studies performed using extracts or material from PA-containing plants, which did not specify PA content did not allow the toxicity to be related to a dose of a specific PA, and were of limited relevance to the evaluation.

Exposure to 1,2-unsaturated PAs has been associated with a wide range of effects, with rats being the most sensitive species studied. In vitro studies on metabolic activation indicate that humans are also likely to be sensitive. Laboratory studies have identified the liver as the most sensitive organ in rats, following both short-term and long-term administration of a number of PAs. The 1,2-unsaturated PAs that have been tested form DNA adducts and are mutagenic. Based upon an understanding of their chemistry and metabolism, it is concluded that this property is common to all 1,2-unsaturated PAs, albeit with differing potencies, and that it is relevant to humans. PAs appear to be antimitotic in hepatocytes. A number of 1,2-unsaturated PAs have been shown to be carcinogenic in rodents, primarily causing haemangiosarcomas in the liver, i.e. originating in the endothelial cells rather than the hepatocytes. Carcinogenicity has not been investigated in case studies of human poisoning with PAs.

The Committee considered that derivation of a health-based guidance value for PAs was not appropriate in view of the genotoxic mode of action. From the carcinogenicity data in rats, a $BMDL_{10}$ of 182 μg/kg bw per day for liver haemangiosarcoma in female rats from the NTP study on riddelliine was calculated as the point of departure for use in a margin of exposure (MOE) approach.

The Committee considered whether it was possible to identify potency factors for different 1,2-unsaturated PAs, in order to evaluate the possible effects of combined exposure. Although the available data did not permit identification of relative potency factors, they did indicate that riddelliine was one of the more potent.

The Committee calculated MOEs between the BMDL of 182 μg/kg bw per day and mean and high-percentile (90th, 95th or 97.5th, depending on the study) chronic exposure estimates for children and adults from consumption of honey and tea, separately. As several national estimates of dietary exposure were available for each food, MOEs were calculated using a range from the lowest lower-bound mean or high-percentile dietary exposure to the highest upper-bound

mean or high-percentile dietary exposures. This range takes into account the uncertainty in measurements of 1,2,-unsaturated PAs and their *N*-oxides and the variability in their concentrations and national estimates of food consumption. MOEs are summarized in Table 45.

For adult consumption of honey, mean and high-percentile chronic dietary exposures to 1,2-unsaturated PAs are in the range 0.00002 to 0.0039 µg/kg bw per day and 0.005 to 0.026 µg/kg bw per day, respectively. These dietary exposures equate to MOEs in the range 46 000 to 9 million for mean exposures and 6900 to 36 000 for high-percentile exposures. For children consuming honey, the ranges of mean and high-percentile chronic dietary exposures to 1,2-unsaturated PAs are 0.00001 to 0.013 µg/kg bw per day and 0.006–0.082 µg/kg bw per day, equating to MOEs in the range 14 000 to 18 million for mean exposure and 2200 to 30 000 for high-percentile exposure.

For adult consumption of tea, mean and high-percentile chronic dietary exposures to 1,2-unsaturated PAs are in the range 0.0013 to 0.13 µg/kg bw per day and 0.01 to 0.26 µg/kg bw per day, respectively. These dietary exposures equate to MOEs in the range 1400 to 140 000 for mean exposure and 700 to 18 000 for high-percentile exposure. For children consuming tea, the range of mean and high-percentile chronic dietary exposures to 1,2-unsaturated PAs are 0.005 to 0.018 µg/kg bw per day and 0.027–0.076 µg/kg bw per day, respectively. These dietary exposures equate to MOEs in the range 10 000 to 36 000 for mean exposure and 2400 to 6700 for high-percentile exposure.

It should be noted that estimates of dietary exposure to 1,2-unsaturated PAs and their *N*-oxides from tea consumption are likely to be overestimates, as concentration data from herbal teas have been combined with information on total tea consumption.

There is currently insufficient information to determine MOEs for other food types or for the total diet. Although mean dietary exposure from a small duplicate-diet study in the Netherlands (0.00128 µg/kg bw per day) equates to a MOE of 140 000, no high-percentile exposure estimate from this study is available.

The Committee noted that a broad range of PAs has been reported in animal feed, but the data were not adequate to assess whether transfer to products of animal origin, such as milk, meat and eggs, could make a major contribution to dietary exposure.

The data were insufficient to identify a point of departure for use in calculating MOEs for acute exposure. However, the Committee noted that the estimates of mean and high-percentile acute exposure to 1,2-unsaturated PAs for children and adults were up to 0.784 µg/kg bw per day, which is 23-fold lower than the lowest reported exposure of 18 µg/kg bw per day associated with human disease following 6 weeks of exposure.

Table 45

Summary of margin of exposure calculations for 1,2-unsaturated PA exposure from consumption of honey, tea or duplicate diets

Population group	Consumption range (g/kg bw per day)	Dietary 1,2-unsaturated PA exposure (µg/kg bw per day)		Margin of exposure	
		LLB	HUB	LLB	HUB
Honey[a]					
Adults					
Mean	0.002–0.05	0.00002	0.0039	9 000 000	46 000
High percentile	0.20–0.28	0.005	0.026	36 000	6 900
Children					
Mean	0.001–0.14	0.00001	0.013	18 000 000	14 000
High percentile	0.35–1.58	0.006	0.082	30 000	2 200
Tea[b]					
Adults					
Mean	0.015–0.13	0.0013	0.13	140 000	1 400
High percentile	0.09–0.47[c]	0.010	0.26	18 000	700
Children					
Mean	0.026–0.055	0.005	0.018	36 000	10 000
High percentile	0.14–0.23	0.027	0.076	6 700	2 400
Duplicate diet[d]					
Adults					
Mean	NS	0.0013		140 000	

HUB: highest upper bound; LLB: lowest lower bound; NS: not stated

[a] From BfR (2013a), EFSA (2011) and the current Committee assessment.

[b] From BfR (2013b) and de Wit et al. (2014).

[c] Tea consumption is expressed in terms of dry tea weight. Typical conversion rates are 100 mL of tea infusion for 1 g of dry tea.

[d] From de Wit et al. (2014). Exposure estimates are mean estimates, rather than high percentile.

11.1 Conclusions[1]

Based on limited occurrence data, the Committee noted that the calculated MOEs for honey (high consumers) and tea (mean and high consumers) indicated a potential concern. It should be noted that PAs measured in these commodities might not be representative for all food groups and all regions. However, it provided a conservative risk estimate as it was compared to the $BMDL_{10}$ for the potent PA riddelliine, and most of the PAs commonly found in food are likely to be less potent than riddelliine.

The Committee considered it of concern that exposure to a single food product could result in such low MOEs. The Committee noted that exposure to PAs resulted from other food items as well, and animal products such as milk

[1] *Note*: This section has been updated since the publication of the report of the eightieth meeting of the Joint FAO/WHO Expert Committee on Food Additives as new data have become available.

might contribute to the total exposure as a result of the presence of PAs in feed. A first indication of total exposure could be obtained from a small duplicate-diet study, from which an MOE of 140 000 could be derived, but it was unclear how representative these data were.

The comparison of estimates of acute dietary exposure to PAs from honey and tea with the lowest reported dose causing human disease did not indicate a concern.

There was insufficient information to reach conclusions on food or beverages other than honey and tea.

11.2 Recommendations

The Committee noted that several gaps still exist in the overall PAs database, from toxicological and epidemiological aspects, to methods of analysis and occurrence levels in different food products, among others. As the missing information has precluded a more definitive assessment, in order to fill these data gaps, the Committee recommended the following:

- To establish internationally agreed high-quality standards, and certified reference materials, that would allow accurate analytical determination and quantification of the different PAs;
- To further study the effects of processing on the occurrence of PAs, taking into account possible metabolites formed during processing;
- To generate occurrence data from areas other than the EU and on food products other than honey, particularly foods of animal origin, in order to improve dietary exposure estimates for PAs across the range of potentially PA-containing foods and from different geographical regions;
- To conduct additional toxicological investigations in order to establish: 1) the relative potency of PAs, taking into account toxicokinetics and genotoxicity; and 2) a point of departure to be used in risk assessment of acute dietary exposure to PAs;
- To carry out epidemiological studies on long-term follow-up of incidents of PA contamination, with the aim to assess the carcinogenic potential of PAs in humans;
- To generate more information on:
  - toxicity and occurrence of saturated PAs, as most available data are on the 1,2-unsaturated PAs, and also because the saturated PAs elicit toxicity by a different mode of action;
  - transfer from feed to food, to estimate whether PA concentrations

in food resulting from PAs in feed could be of concern for human health.

12. References

Abebe D, Debella A, Tekabe F, Mekonnen Y, Degefa A, Mekonnen A et al. (2012). An outbreak of liver disease in Tahtay Koraro Woreda, Tigray region of Ethiopia: a case-control study for the identification of the etiologic agent. Ethiop Med J. 50(Suppl 2):17–25.

Adamczak AB, Gryszczyńska A, Buchwald W (2013). Content of pyrrolizidine alkaloids in the leaves of coltsfoot (*Tussilago farfara* L.) in Poland. Acta Soc Bot Pol. 82:289–93.

Aguiar R, Wink M (2005). Do naive ruminants degrade alkaloids in the rumen? J Chem Ecol. 31:761–87.

Altaee MY, Mahmood MH (1998). An outbreak of veno-occlusive disease of the liver in northern Iraq. East Mediterr Health J. 4:142–8.

Altamirano JC, Gratz SR, Wolnik KA (2005). Investigation of pyrrolizidine alkaloids and their N-oxides in commercial comfrey-containing products and botanical materials by liquid chromatography electrospray ionization mass spectrometry. J AOAC Int. 88:406–12.

Anastassiades M, Lehotay SJ, Stajnbaher D, Schenck FJ (2003). Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in products. J AOAC Int. 86:412–31.

Anjos BL, Nobre VMT, Dantas AF, Medeiros RM, Oliveira TS, Molyneux RJ et al. (2010). Poisoning of sheep by seeds of *Crotalaria retusa*: acquired resistance by continuous administration of low doses. Toxicon. 55:28–32.

ANZFA (2001). Pyrrolizidine alkaloids in food. A toxicological review and risk assessment. Technical Report Series No. 2. Canberra: Australia New Zealand Food Authority (http://www.foodstandards.gov.au/publications/documents/TR2.pdf, accessed 23 April 2015).

Appadurai P, Rathinasamy K (2014). Indicine N-oxide binds to tubulin at a distinct site and inhibits the assembly of microtubules: A mechanism for its cytotoxic activity. Toxicol Lett. 225:66–77.

Avula B, Wang Y, Mei Wang, Smillie TJ, Khan IA (2012). Simultaneous determination of sesquiterpenes and pyrrolizidine alkaloids from the rhizomes of *Petasites hybridus* (L.) G.M. et Sch. and dietary supplements using UPLCUV and HPLCTOFMS methods. J Pharm Biomed Anal. 70:53–63.

Avula B, Sagi S, Wang Y-H, Zweigenbaum J, Wang M, Khan IA (2015). Characterization and screening of pyrrolizidine alkaloids and N-oxides from botanicals and dietary supplements using UHPLC-high resolution mass spectrometry. Food Chem. 178:136–48.

Azadbakht M, Talavaki M (2003). Qualitative and quantitative determination of pyrrolizidine alkaloids of wheat and flour contaminated with *Senecio* in Mazandaran Province farms. Iran J Pharm Res. 2:179–83.

Bach N, Thung SN, Schaffner F (1989). Comfrey herb tea-induced hepatic veno-occlusive disease. Am J Med. 87:97–9.

Bane A, Seboxa T, Mesfin G, Ali A, Tsegaye Y, W/Tensae M et al. (2012). An outbreak of veno-occlusive liver disease in northern Ethiopia, clinical findings. Ethiop Med J. 50(Suppl 2):9–16.

Bassler G, Karrer G, Kriechbaum M (2016). The impact of different cutting regimes on population density of *Jacobaea aquatica* (Hill) G. Gaertn., B. Mey. & Scherb. and grassland vegetation. Agric Ecosys Environ. 226:18–24.

Bartkowski JPB, Wiedenfeld H, Roeder E. (1997). Quantitative photometric determination of senkirkine in *Farfarae folium*. Phytochem Anal. 8:1841–4.

Beales K, Colegate SM, Edgar JA (2004). Experiences with the quantitative trace analysis of pyrrolizidine alkaloids using GCMS and LCMS. In: Acamovic T, Stewart CS, Pennycott TW, editors. Poisonous plants and related toxins. Wallingford: CABI:453–8.

Beales KA, Betteridge K, Colegate SM, Edgar JA (2004). Solid-phase extraction and LC-MS analysis of pyrrolizidine alkaloids in honeys. J Agric Food Chem. 52:6664–72.

Beales K, Betteridge K, Boppré M, Cao Y, Colegate SM, Edgar JA (2007). Hepatotoxic pyrrolizidine alkaloids and their N-oxides in honey and pollen. In: Panter K, Wierenga T, Pfister J, editors. Poisonous plants: global research and solutions. Wallingford: CABI:94–100.

Becerra-Jiminez J, Kuschak M, Roeder E, Wiedenfeld H (2013). Toxic pyrrolizidinalkaloids as undesired contaminants in food and feed: degradation of the PAs from *Senecio jacobaea* in silage. Pharmazie. 68:636–9.

Benassi JC, Górniak SL, Hueza IM (2008). Evaluation of the possible immunotoxic effects of monocrotaline on the offspring of female rats treated during the gestation. Birth Defects Res. 82:382.

Benassi JC, Haraguchi M, Górniak SL, Hueza IM (2011). Comparative study of monocrotaline toxicity on peritoneal macrophage activity when dosed for 14 or 28 days. In: Riet-Correa F, Pfister J, Schild AL, Wierenga T, editors. Poisoning by plants, mycotoxins and related toxins. Wallingford: CABI:208–14.

Bensaude RJ, Monegier du Sorbier C, Jonville-Bera AP, Autret E, Ouyahya F, Metman EH (1998). Veno-occlusive disease after prolonged treatment with senecionine (Hemoluol). Gastroenterol Clin Biol. 22:363–4 [in French].

Berry DL, Schoofs GM, Schwass DE, Molyneux RJ (1996). Genotoxic activity of a series of pyrrolizidine alkaloids in primary hepatocyte-mediated V79 cell mutagenesis and DNA repair assay. J Natur Toxins. 5:7–24.

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 Agric Food Chem. 53:1894–902.

Betz JM, Eppley RM, Taylor WC, Andrzejewski D (1994). Determination of pyrrolizidine alkaloids in commercial comfrey products (*Symphytum* sp.). J Pharm Sci. 83:649–53.

BfR (2007). Salatmischung mit pyrrolizidinalkaloid-haltigem Greiskraut verunreinigt. Bundesamt für Risiokobewertung (BfR) Opinion No. 028/2007 (http://www.bfr.bund.de/cm/349/salad_mix_contaminated_with_groundsel_containing_pyrrolizidine_alkaloids.pdf, accessed 20 April 2015).

BfR (2011). Chemical analysis and toxicity of pyrrolizidine alkaloids and assessment of the health risk posed by their occurrence in honey. Bundesamt für Risiokobewertung (BfR) Opinion No. 038/2011 (English summary: http://www.bfr.bund.de/cm/349/chemical-analysis-and-toxicity-of-pyrrolizidine-alkaloids-and-assessment-of-the-health-risks-posed-by-their-occurence-in-honey.pdf. Full article in German: http://www.bfr.bund.de/cm/343/analytik-und-toxizitaet-von-pyrrolizidinalkaloiden.pdf, accessed 20 April 2015).

BfR (2013a). Analytik und Toxizität von Pyrrolizidinalkaloiden sowie eine Einschätzung des gesundheitlichen Risikos durch deren Vorkommen in Honig [Analysis and toxicity of pyrrolizidine alkaloids and an assessment of the health risk due to their occurrence in honey]. Bundesamt für

Risiokobewertung (BfR) Opinion 038/2011 ergänzt am 21. Januar 2013 (https://mobil.bfr.bund.de/cm/343/analytik-und-toxizitaet-von-pyrrolizidinalkaloiden.pdf, accessed, 20 April 2015).

BfR (2013b). Pyrrolizidine alkaloids in herbal teas and teas. Bundesamt für Risiokobewertung (BfR) Opinion 018/2013 (http://www.bfr.bund.de/cm/349/pyrrolizidine-alkaloids-in-herbal-teas-and-teas.pdf, accessed, 20 April 2015).

Bicchi C, Rubiolo P, Frattini C (1989). Capillary gas chromatography-Fourier transform infrared spectroscopy of pyrrolizidine alkaloids of *Senecio inaequidens* DC. J Chromatogr. 473:161–70.

Bicchi C, Caniato R, Tabacchi R, Tsoupras G (1989). Capillary gas-chromatography positive and negative-ion chemical ionization mass-spectrometry on pyrrolizidine alkaloids of *Senecio inaequidens* using ammonia and hydroxyl ions as the reagent species. J Nat Prod. 52:32–41.

Bober MA, Milco LA, Miller RB, Mount M, Wicks B, Kurth MJ (1989). A competitive enzyme-linked immunosorbent assay (ELISA) to detect retronecine and monocrotaline in vitro. Toxicon. 27: 1059–64.

Bober MA, Kurth MJ, Milco LA, Roseman DM, Miller RB, Segal HJ (1990). A pyrrolizidine alkaloid enzyme-linked immunosorbent assay detection strategy. In: Vanderlaan M, Stanker LH, Watkins BE, Roberts DW. Immunoassays or trace chemical analysis. ACS Symposium Series. Washington (DC): American Chemical Society:176–83.

Bodi D, Ronczka S, Gottschalk C, Behr N, Skibba A, Wagner M et al. (2014). Determination of pyrrolizidine alkaloids in tea, herbal drugs and honey. Food Addit Contam A. 31:1886–95.

Böhlen M, Kast C, Dübecke A, Zoller O (2011). Sum-analytical determination of pyrrolizidine alkaloids in Swiss honey by GC–MS. In: 5th International symposium on recent advances in food analysis, 1–4 November 2011, Prague (http://www.rafa2011.eu/pdf/boa2011.pdf, accessed 18 March 2015).

Bolechová M, Cáslavsky J, Pospíchalová M, Kosubová P (2015). UPLC–MS/MS method for determination of selected pyrrolizidine alkaloids in feed. Food Chem. 170:265–70.

Boppré M (2011). The ecological context of pyrrolizidine alkaloids in food, feed and forage: an overview. Food Addit Contam A. 28:260–81.

Boppré M, Colegate SM (2015). Recognition of pyrrolizidine alkaloid esters in the invasive aquatic plant *Gymnocoronis spilanthoides* (Asteraceae). Phytochem Anal. 26:215–25.

Boppré M, Colegate SM, Edgar JA (2005). Pyrrolizidine alkaloids of *Echium vulgare* honey found in pure pollen. J Agric Food Chem. 53:594–600.

Boppré M, Colegate SM, Edgar JA, Fischer OW (2008). Hepatotoxic pyrrolizidine alkaloids in pollen and drying-related implications for commercial processing of bee pollen. J Agric Food Chem. 56:5662–72.

Bosi CF, Rosa DW, Grougnet R, Lemonakis N, Halabalaki M, Skaltsounis AL et al. (2013). Pyrrolizidine alkaloids in medicinal tea of *Ageratum conyzoides*. Rev Bras Farmacogn. 23:425–32.

Botha CJ, Lewis A, du Plessis EC, Clift SJ, Williams MC (2012). Crotalariosis equorum ("jaagsiekte") in horses in southern Mozambique, a rare form of pyrrolizidine alkaloid poisoning. J Vet Diagn Investig. 24:1099–104.

Bovee TF, Helsdingen RJR, Hoogenboom LAP, De Nijs MWCM, Liu X, Vrieling K et al. (2015). Are effects of common ragwort in the Ames test caused by pyrrolizidine alkaloids? Mutat Res. 778:1–10.

Brown A, Stegelmeier BL, Colegate SM, Gardner DR, Panter KE, Knoppel EL et al. (2016). Comparative toxicity of a reduced, crude comfrey (*Symphytum officinale*) alkaloid extract and the pure, comfrey-

derived pyrrolizidine alkaloids, lycopsamine and intermedine in chicks (*Gallus gallus domesticus*). J Appl Toxicol. 36:716–25.

Bruckstein S, Tromp AM, Perl S (1996). Case report: Heliotropium poisoning. Isr J Vet Med. 51:75–7.

Buhler DR, Miranda CL, Kedzierski B, Reed RL (1991). Mechanisms for pyrrolizidine alkaloid activation and detoxification. Adv Exp Med Biol. 283:597–603.

Bull LB, Dick AT, McKenzie JS (1958). The acute toxic effects of heliotrine and lasiocarpine, and their N-oxides, on the rat. J Pathol Bacteriol. 75:17–25 (as cited by EFSA, 2011).

Bull LB, Culvenor CCJ, Dick AJ (1968). The pyrrolizidine alkaloids. Their chemistry, pathogenicity and other biological properties. Amsterdam: North Holland.

Bundesanzeiger (1992). 17 June, 4805; cited by Dtsch Apoth Ztg. 132:1406–8.

CAC/RCP 74-2014 (2014). Code of practice for weed control to prevent and reduce pyrrolizidine alkaloid contamination in food and feed (http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCAC%2BRCP%2B74-2014%252FCXP_074e.pdf).

Candrian U, Zweifel U, Lüthy J, Schlatter C (1991). Transfer of orally administered [3H]seneciphylline into cow's milk. J Agric Food Chem. 39:930–3.

Cao Y, Colegate SM, Edgar JA (2008). Safety assessment of food and herbal products containing hepatotoxic pyrrolizidine alkaloids: Interlaboratory consistency and the importance of N-oxide determination. Phytochem Anal. 19:526–33.

Cao Y, Colegate SM, Edgar JA (2013). Persistence of echimidine, a hepatotoxic pyrrolizidine alkaloid, from honey into mead. J Food Composit Anal. 29:106–9.

Castells E, Mulder PPJ, Pérez-Trujillo M (2014). Diversity of pyrrolizidine alkaloids in native and invasive *Senecio pterophorus* (Asteraceae): Implications for toxicity. Phytochemistry. 108:137–46.

Cavallaro V, Than KA, Colegate SM, Edgar JA (2004). An indirect competitive ELISA for pyrrolizidine alkaloids of *Heliotropium europaeum*. In: Acamovic T, Stewar CS, Pennycott TW, editors. Poisonous plants and related toxins. Wallingford: CABI:114–9.

CCCF (2011). Discussion paper on pyrrolizidine alkaloids (CX/CF 11/5/14). ftp://ftp.fao.org/codex/meetings/cccf/cccf5/cf05_14e.pdf.

Chan MY, Zhao ZL, Ogle CW (1989). A comparative study on the hepatic toxicity and metabolism of Crotalaria assamica and Eupatorium species. Am J Chi Med. XVII:165–70.

Chan P (1993). NTP technical report on the toxicity studies of riddelliine (CAS no. 23246–96–0) administered by gavage to F344 rats and B6C3F1 mice. Toxicity Rep Ser. 27:1-D9.

Chan PC, Mahler J, Bucher JR, Travlos GS, Reid JB (1994). Toxicity and carcinogenicity of riddelliine following 13 weeks of treatment to rats and mice. Toxicon. 32:891–908.

Chan PC, Haseman JK, Prejean JD, Nyska A (2003). Toxicity and carcinogenicity of riddelliine in rats and mice. Toxicol Lett. 144:295–311.

Chauvin P, Dillon JC, Moren A (1994). Épidémie d'intoxication alimentaire à l'héliotrope, Tadjikistan, novembre 1992 - mars 1993 [An outbreak of Heliotrope food poisoning, Tadjikistan, November 1992–March 1993]. Cahiers Sante. 4:263–8.

Cheeke PR, Shull LR (1985). Natural toxicants in feeds and livestock. Westport (CT): AVI Publishing Inc. (as cited by EFSA. 2011).

Chen L, Mulder PPJ, Louisse J, Peijnenburg A, Wesseling S, Rietjens IMCM (2017). Risk assessment for pyrrolizidine alkaloids detected in (herbal) teas and plant food supplements. Regul Toxicol Pharmacol. 86:292–302.

Chen M, Li L, Zhong D, Shen S, Zheng J, Chen X (2016). Glutathionyl-6,7-dihydro-1-hydroxymthyl-5H-pyrrolizine is the major pyrrolic glutathione conjugate of retronecine-type pyrrolizidine alkaloids in liver microsomes and in rats. Chem Res Toxicol. 29:180–9.

Chen T, Mei N, Fu PP (2010). Genotoxicity of pyrrolizidine alkaloids. J Appl Toxicol. 30:183–96.

Chizzola R (1994). Rapid sample preparation technique for the determination of pyrrolizidine alkaloids in plant extracts. J Chromatogr. 668:427–33.

Chou MW, Wang YP, Yan J, Yang YC, Beger RD, Williams LD et al. (2003a). Riddelliine N-oxide is a phytochemical and mammalian metabolite with genotoxic activity that is comparable to the parent pyrrolizidine alkaloid riddelliine. Toxicol Lett. 145:239–47.

Chou MW, Jian Y, Williams LD, Xia Q, Churchwell M, Doerge DR et al. (2003b). Identification of DNA adducts derived from riddelliine, a carcinogenic pyrrolizidine alkaloid. Chem Res Toxicol. 16:1130–7.

Chou MW, Yan J, Nichols J, Xia Q, Beland FA, Chan PC et al. (2004). Correlation of DNA adduct formation and riddelliine-induced liver tumorigenesis in F344 rats and B6C3F1 mice. Cancer Lett. 207:119–25.

Chu PS, Segall HJ (1991). Species difference in the urinary excretion of isatinecic acid from the pyrrolidizine alkaloid retrorsine. Comp Biochem Physiol C. 100:683–6.

Chu PS, Lamé MW, Segall HJ (1993). In vivo metabolism of retrorsine and retrorsine N-oxide. Arch Toxicol.67:39–43.

Chung WG, Buhler DR (1995). Major factors for the susceptibility of guinea pig to the pyrrolizidine alkaloid jacobine. Drug Metab Dispos. 23:1263–7.

Chung WG, Buhler DR (2004). Differential metabolism of the pyrrolizidine alkaloid, senecionine, in Fischer 344 and Sprague-Dawley rats. Arch Pharm Res. 27:547–53.

Chung WG, Miranda CL, Buhler DR (1995). A cytochrome P4502B form is the major bioactivation enzyme for the pyrrolizidine alkaloid senecionine in guinea pig. Xenobiotica. 25:929–39.

COC (2008). Pyrrolizidine alkaloids in food – initial assessment of carcinogenicity (CC/08/13 ANNEX C). Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment. (http://webarchive.nationalarchives.gov.uk/20090204144709/http://www.advisorybodies.doh.gov.uk/pdfs/cc0813c.pdf, accessed 10 December 2018).

Colegate SM, Gardner DR (2008). LC-MS of alkaloids: qualitative profiling, quantitative analysis, and structural identification. In: Fattorusso E, Taglialatela-Scafati O, editors. Modern alkaloids. Weinheim: Wiley-VCH Verlag:369–408.

Colegate SM, Edgar JA, Knill AM, Lee S (2005). Solid-phase extraction and HPLC-MS profiling of pyrrolizidine alkaloids and their N-oxides: a case study of *Echium plantagineum*. Phytochem Anal. 16:108–19.

Colegate SM, Gardner DR, Davis TZ, Betz JM, Panter KE (2013). Dehydropyrrolizidine alkaloids in two Cryptantha species: Including two new open chain diesters one of which is amphoteric. Phytochem Anal. 24:201–12.

Colegate SM, Boppré M, Monzón J, Betz JM (2015). Pro-toxic dehydropyrrolizidine alkaloids in the traditional Andean herbal medicine "asmachilca". J Ethnopharmacol. 172:179–94.

Coll C, Fernandez MA, Coll S, Coll T, Malliardi P, Perazzo JC et al. (2011). Effect of monocrotaline on blood-brain barrier permeability in rats. Lat Am J Pharm. 30:412–6.

Colombini S, Zucali M, Rapetti L, Crovetto GM, Sandrucci A, Sava L (2015). Substitution of corn silage with sorghum silages in lactating cow diets: In vivo methane emission and global warming potential of milk production. Agric Sys. 136:106–113.

Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (2008). COT statement on pyrrolizidine alkaloids in food. Food Standard Agency (https://cot.food.gov.uk/sites/default/files/cot/cotstatementpa200806.pdf).

Conradie J, Stewart MJ, Steenkamp V (2005). GC/MS identification of toxic pyrrolizidine alkaloids in traditional remedies given to two sets of twins. Ann Clin Biochem. 42:141–4.

Correa AMR, Bezerra PSJ, Pavarini SP, Santos AS, Sonne L, Zlotowski P et al. (2008). *Senecio brasiliensis* (Asteraceae) poisoning in Murrah buffaloes in Rio Grande do Sul. Pesqui Vet Bras. 28:187–9.

Coulombe RA, Drew GL, Stermitz FR (1999). Pyrrolizidine alkaloids cross-link DNA with actin. Toxicol Appl Pharmacol. 154:198–202.

Coulombe RA, Kim HY, Stermitz FR (1994). Structure-activity of pyrrolizidine alkaloid-induced DNA cross-linking. In: Colegate SM, Dorling PR, editors. Plant-associated toxins: agricultural, phytochemical and ecological aspects. Wallingford: CABI:125–30.

Craig AM, Latham CJ, Blythe LL, Schmotzer WB, O'Connor O (1992). Metabolism of toxic pyrrolizidine alkaloids from tansy ragwort (*Senecio jacobaea*) in ovine ruminal fluid under anaerobic conditions. Appl Environ Microbiol. 58:2730–6.

Cramer L, Beuerle T (2012). Detection and quantification of pyrrolizidine alkaloids in antibacterial medicinal honeys. Planta Medica. 78:1976–82.

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 Agric Food Chem. 61:11382–91.

Cramer L, Fleck G, Horn G, Beuerle T (2014). Process development of Lappula squarrosa oil refinement: Monitoring of pyrrolizidine alkaloids in Boraginaceae seed oils. J Am Oil Chem Soc. 91:721–31.

Creeper JH, Mitchell AA, Jubb TF, Colegate SM (1999). Pyrrolizidine alkaloid poisoning of horses grazing a native heliotrope (*Heliotropium ovalifolium*). Aust Vet J. 77:401–2.

Crews C (2013). Methods for analysis of pyrrolizidine alkaloids. In: Ramawat KG, Mérillon JM, editors, Natural products. Berlin: Springer-Verlag:1049–68.

Crews C, Anderson WAC (2009). Detection of ragwort alkaloids in toxic hay by liquid chromatography/time-of-flight mass spectrometry. Vet Rec. 165:568–9.

Crews C, Berthiller F, Krska R (2010). Update on analytical methods for toxic pyrrolizidine alkaloids. Anal Bioanal Chem. 396:327–38.

Crews C, Startin JR, Clarke PA (1997). Determination of pyrrolizidine alkaloids in honey from selected sites by solid phase extraction and HPLC-MS. Food Addit Contam A. 14:419–28.

Crews C, Driffield M, Berthiller F, Krska R (2009). Loss of pyrrolizidine alkaloids on decomposition of ragwort (*Senecio jacobaea*) as measured by LC-TOF-MS. J Agric Food Chem. 57:3669–73.

Cui Y, Lin G (2000). Simultaneous analysis of clivorine and its four microsomal metabolites by high-performance liquid chromatography. J Chrom A. 903:85–92.

Culvenor CCJ (1978). Prevention of pyrrolizidine alkaloid poisoning – animal adaptation or plant control? In: Keeler RF, van Kampen KR, James LF, editors. Effects of poisonous plants on livestock. New York: Academic Press:128–200.

Culvenor CCJ, Edgar JA (1972). Dihydropyrrolizine secretions associated with coremata of *Utetheisa moths* (family Arctiidae). Experientia. 28:627–8.

Culvenor CCJ, Smith LW (1967). The alkaloids of *Cynoglossum austral* R.Br. and C. amabile Stapf&Drummond. Aust J Chem. 20:2499–503.

Culvenor CCJ, Edgar JA, Smith LW (1981). Pyrrolizidine alkaloids in honey from *Echium plantagineum* L. J Agric Food Chem. 29:958–60.

Culvenor CC, Downing DT, Edgar JA, Jago MV (1969). Pyrrolizidine alkaloids as alkylating and antimitotic agents. Ann N Y Acad Sci. 163:837–47 (as cited by EFSA, 2011).

Culvenor CCJ, Edgar JA, Smith LW, Tweeddale HJ (1970). Dihydropyrrolizines III. Preparation and reaction of derivatives related to pyrrolizidine alkaloids. Aust J Chem. 23:1853–67.

Culvenor CCJ, Edgar JA, Smith LW, Jago MV, Peterson JE (1971). Active metabolites in the chronic hepatotoxicity of pyrrolizidine alkaloids, including otonecine esters. Nature. 229:255–6.

Culvenor CC, Edgar JA, Jago MV, Qutteridge A, Peterson JE, Smith LW (1976). Hepato- and pneumotoxicity of pyrrolizidine alkaloids and derivatives in relation to molecular structure. Chem Biol Interact. 12:299–324 (as cited by EFSA, 2011).

Culvenor CC, Edgar JA, Smith LW, Kumana CR, Lin HJ (1986). *Heliotropium lasiocarpum* Fisch and Mey identified as cause of veno-occlusive disease due to a herbal tea. Lancet. 26:1:978.

Dai J, Zhang F, Zheng J (2010). Retrorsine, but not monocrotaline, is a mechanism-based inactivator of P450 3A4. Chem Biol Interact. 183:49–56.

Dai HF, Gao Y, Yang M, Yu CH, Gu ZY, Chen WX (2006). Hepatic veno-occlusive disease induced by *Gymura segetum*: report of two cases. Hepatobiliary Pancreat Dis Int. 5:406–8.

Dai N, Yu YC, Ren TH, Wu JG, Jiang Y, Shen LG et al. (2007). Gynura root induces hepatic veno-occlusive disease: a case report and review of the literature. World J Gastroenterol. 13:1628–31.

Dalefield RR, Gosse MA, Mueller U (2015). Acute toxicity of echimidine in male Wistar rats. J Toxicol Res. 4:42–7.

Dalefield RR, Gosse MA, Mueller U (2016). A 28-day oral toxicity study of echimidine and lasiocarpine in Wistar rats. Reg Toxicol Pharmacol. 81:146–56.

Dalefield RR, Gosse MA, Bartholomaeus A, Schyvens CG, Mueller U (2012a). Acute toxicity of heliotrine in male Han Wistar rats. J Toxicol Res. 2:12–9.

Dalefield RR, Gosse MA, Bartholomaeus A, Schyvens CG, Hutchinson KJ, Mueller U (2012b). Determination of the single-dose 72-hour oral gavage LD50 values of monocrotaline and riddelliine in male Han Wistar rats using the up-and-down procedure. J Herb Med Toxicol. 6:153–65.

Dalmas DA, Scicchitano MS, Mullins D, Hughes-Earle A, Tatsuoka K, Magid-Slav M et al. (2011). Potential candidate genomic biomarkers of drug induced vascular injury in the rat. Toxicol Appl Pharmacol. 257:284–300.

Datta DV, Khuroo MS, Mattocks AR, Aikat BK, Chhuttani PN (1978). Veno-occlusive disease of liver due to heliotropium plant, used as medicinal herb (report of 6 cases with review of literature). J Assoc Physicians India. 26:383–93, vii–viii.

De Lanux-Van-Gorder V (2000). Tansy ragwort poisoning in a horse in southern Ontario. Can Vet J. 41:409–10.

De Nijs M, Elbers IJW, Mulder PPJ (2014). Inter-laboratory comparison study for pyrrolizidine alkaloids in animal feed using spiked and incurred material. Food Addit Contam A. 31:288–99.

de Wit L, Geraets L, Bokkers B, Jeurissen S (2014). Pyrrolizidine alkaloids in herbal preparations. Bilthoven: National Institute for Public Health and the Environment (RIVM Report 090437001).

Debella A, Abebe D, Tekabe F, Mamo H, Abebe A, Tsegaye B et al. (2012a). Toxicity study and evaluation of biochemical markers towards the identification of the causative agent for an outbreak of liver disease in Tahtay Koraro Woreda, Tigray. Ethiop Med J. 50(Suppl 2):27–35.

Debella A, Abebe D, Tekabe F, Degefa A, Desta A, Tefera A et al. (2012b). Physico-chemical investigation of consumables and environmental samples to determine the causative agent of liver disease outbreak in Tahitay Koraro Woreda, Tigray. Ethiop Med J. 50(Suppl 2):37–45.

Deinzer M, Thompson P, Griffin D, Dickinson E (1978). A sensitive analytical method for pyrrolizidine alkaloids. The mass spectra of retronecine derivatives. Biomed Mass Spectrom. 5:175–9.

Deinzer ML, Arbogast BL, Buhler DR, Cheeke PR (1982). Gas chromatographic determination of pyrrolizidine alkaloids in goat's milk. Anal Chem. 54:1811–14.

DeLeve LD, Shulman HM, McDonald GB (2002). Toxic injury to hepatic sinusoids: sinusoidal obstruction syndrome (veno-occlusive disease). Semin Liver Dis. 22:27–42.

Deyo JA, Kerkvliet NI (1990). Immunotoxicity of the pyrrolizidine alkaloid monocrotaline following subchronic administration to C57Bl/6 mice. Fundam Appl Toxicol. 14:842–9.

Deyo JA, Kerkvliet NI (1991). Tier-2 studies on monocrotaline immunotoxicity in C57BL/6 mice. Toxicology. 70:313–25.

Diaz GJ, Almeida LX, Gardner DR (2014). Effects of dietary *Crotalaria pallida* seeds on the health and performance of laying hens and evaluation of residues in eggs. Res Vet Sci. 97:297–303.

Dick AT, Dann AT, Bull LB, Culvenor CCJ (1963). Vitmain B12 and the detoxification of hepatotoxic pyrrolizidine alkaloids in rumen liquor. Nature. 197:207–8.

Dickinson JO (1980). Release of pyrrolizidine alkaloids into milk. Proc West Pharmacol Soc. 23:377–9.

Dickinson JO, Cooke MP, King RR, Mohamed PA (1976). Milk transfer of pyrrolizidine alkoloids in cattle. J Am Vet Med Assoc. 169:1192–6.

Dimande AFP, Botha CJ, Prozesky L, Bekker L, Rösemann GM, Labuschagne L et al. (2007). The toxicity of *Senecio inaequidens* DC. J South Afri Vet Assoc. 78:121–9.

Downing DT, Peterson JE (1968). Quantitative assessment of the persistent antimitotic effect of certain hepatotoxic pyrrolizidine alkaloids on rat liver. Aust J Exp Biol Med Sci. 46:493–502 (as cited by EFSA, 2011).

Dübecke A, Beckh G, Lüllmann C (2011). Pyrrolizidine alkaloids in honey and bee pollen. Food Addit Contam A. 28:348–58.

Dueker SR, Lamé MW, Morin D, Wilson DW, Segall HJ (1992). Guinea pig and rat hepatic microsomal metabolism of monocrotaline. Drug Metabol Disposition, 20: 275–80.

Dueker SR, Lamé MW, Jones AD, Morin D, Segall HJ (1994). Glutathione conjugation with the pyrrolizidine alkaloid, jacobine. Biochem Biophys Res Com. 198:516–22.

Duringer JM, Buhler DR, Craig AM (2004). Comparison of hepatic in vitro metabolism of the pyrrolizidine alkaloid senecionine in sheep and cattle. Am J Vet Res. 65:1563–72.

Eastman DF, Dimenna GP, Segall HJ (1982). Covalent binding of 2 pyrrolizidine alkaloids, senecionine and seneciphylline, to hepatic macromolecules and their distribution, excretion, and transfer into milk of lactating mice. Drug Metabol Disposit.10:236–40.

EC (2006). Commission Regulation (EC) No. 401/2006 of 23 February 2006 laying down the sampling methods and the methods of analysis for the official control of the levels of Fusarium toxins in foodstuffs. OJEU. L70:12–34 (http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32006R0401, accessed 20 December 2019).

Edgar JA (1975). Danainae (Lep.) and 1,2-dehydropyrrolizidine alkaloid-containing plants – with reference to observations made in the New Hebrides. Phil Trans Roy Soc of London, Series B. 272:467–76.

Edgar JA (1985). Gas chromatography of pyrrolizidine alkaloids. In: Seawright AA, Hegarty MP, James LF, Keeler RF, editors. Plant toxicology. Brisbane: Queensland Poisonous Plants Committee:191–9.

Edgar J (1998). Food safety & feed grain contamination: Pyrrolizidine alkaloids. Proceedings of the Grains Industry Meeting, 22 May 1998:1–16.

Edgar JA (2003). Pyrrolizidine alkaloids and food safety. Chem Aust. 70:4–7.

Edgar JA, Smith LW (2000). Transfer of pyrrolizidine alkaloids into eggs: Food safety implications. In: Tu AT, Gaffield W, editors, Natural and selected synthetic toxins: biological implications. ACS Symposium Series. 745:118–128.

Edgar JA, Culvenor CCJ, Pliske TE (1976). Isolation of a lactone, structurally related to the esterifying acids of pyrrolizidine alkaloids, from the costal fringes of male ithomiine. J Chem Ecol. 2:263–70.

Edgar JA, Roeder E, Molyneux RJ (2002). Honey from plants containing pyrrolizidine alkaloids: a potential threat to health. J Agric Food Chem. 50:2719–30.

Edgar JA, Lin HJ, Kumana CR, Ng MMT (1992). Pyrrolizidine alkaloid composition of 3 Chinese medicinal herbs, *Eupatorium cannabinum*, *E. japonicum* and *Crotalaria assamica*. Am J Chin Med. 20:281–88.

Edgar JA, Colegate SM, Boppré M, Molyneux RJ (2011). Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. Food Addit Contam A. 28:308–24.

EFSA (2007). Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the European Commission related to pyrrolizidine alkaloids as undesirable substances in animal feeds. EFSA J. 447:1–51 (https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2007.447, accessed 20 December 2018).

EFSA (2008). Opinion of the Scientific Panel on Plant Protection products and their residues to evaluate the suitability of existing methodologies and, if appropriate, the identification of new approaches to assess cumulative and synergistic risks from pesticides to human health with a view to set MRLs for

those pesticides in the frame of Regulation (EC) 396/2005. EFSA J. 704:1–84 (https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2008.705, accessed 20 December 2018).

EFSA (2009). European Food Safety Authority. Guidance of the Scientific Committee on Use of the benchmark dose approach in risk assessment. EFSA J. 1150:1–72.

EFSA (2010). European Food Safety Authority. Application of systematic review methodology to food and feed safety assessments to support decision making. EFSA J. 8:1637 (https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2010.1637, accessed 20 December 2018)

EFSA (2011). Scientific opinion on pyrrolizidine alkaloids in food and feed. EFSA J. 9:2406.

EFSA (2016). Dietary exposure assessment to pyrrolizidine alkaloids in the European population. EFSA J. 14:4572.

EFSA (2017a). EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain). Statement on the risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. EFSA J. 15:4908 (https://doi.org/10.2903/j.efsa.2017.4908).

EFSA (2017b). EFSA Scientific Committee. Update: use of the benchmark dose approach in risk assessment. EFSA J. 15:4658 (https://doi.org/10.2903/j.efsa.2017.4658).

Elias F, Latorre AO, Pípole F, Haraguchi M, Górniak SL, Hueza IM (2011). Haematological and immunological effects of repeated dose exposure of rats to integerrimine N-oxide from *Senecio brasiliensis*. Food Chem Toxicol. 49:2313–9.

Eller A, Chizzola R (2016). Seasonal variability in pyrrolizidine alkaloids in *Senecio inaequidens* from the Val Venosta (Northern Italy). Plant Biosystems. 150:1306–12.

Eröksüz Y, Eröksüz H, Özer H, Sener B, Tosun F, Akyüz Ç (2001). Toxicity of dietary *Heliotropium dolosum* seed to mice. Vet Human Toxicol. 43:152–5.

Eröksüz H, Eröksüz Y, Özer H, Çeribasi AO, Tosun F, Tamer U et al. (2003a). Toxicity of dietary *Heliotropium circinatum* to rats. Vet Human Toxicol. 45:198–201.

Eröksüz H, Eröksüz Y, Ozer H, Yaman I, Tosun F, Kizilay C et al. (2003b). Toxicity of *Senecio vernalis* to laying hens and evaluation of residues in eggs. Vet Hum Toxicol. 45:76–80.

Eröksüz Y, Çeribaşı AO, Çevik A, Eröksüz H, Tosun F, Tamer U (2008). Toxicity of *Heliotropium dolosum*, *Heliotropium circinatum*, and *Senecio vernalis* in parental quail and their progeny, with residue evaluation of eggs. Turk J Vet Anim Sci. 32:475–82.

Estep JE, Lamé MW, Segall HJ (1990). Excretion and blood radioactivity levels following [14C] senecionine administration in the rat. Toxicol. 64:179–89.

Estep JE, Lamé MW, Jones AD, Segall HJ (1990). N-acetylcysteine-conjugated pyrrole identified in rat urine following administration of two pyrrolizidine alkaloids, monocrotaline and senecionine. Toxicol Lett. 54:61–9.

Estep JE, Lamé MW, Morin D, Jones AD, Wilson DW, Segall HJ (1991). [14C]monocrotaline kinetics and metabolism in the rat. Drug Metabol Dis. 19:135–9.

FAO (2010). Pyrrolizidine alkaloids in foods and animal feeds. FAO Consumer Protection Fact Sheets No. 2. Rome: Food and Agriculture Organization of the United Nations:1–6.

Fashe MM, Juvonen RO, Petsalo A, Rahnasto-Rilla M, Auriola S, Soininen P et al. (2014). Identification of a new reactive metabolite of pyrrolizidine alkaloid retrorsine: (3H-pyrrolizin-7-yl) methanol. Chem Res Toxicol. 27:1950–7.

Fashe MM, Juvonen RO, Petsalo A, Vepsalainen J, Pasanen M, Rahnasto-Rilla M (2015a). In silico prediction of the site of oxidation by cytochrome P450 3A4 that leads to the formation of the toxic metabolites of pyrrolizidine alkaloids. Chem Res Toxicol. 28:702–10.

Fashe MM, Juvonen RO, Petsalo A, Räsänen J, Pasanen M (2015b). Species-species differences in the in vitro metabolism of lasiocarpine. Chem Res Toxicol. 28:2034–44.

Feng A, Tian B, Hu J, Zhou P (2010). Recent applications of capillary electrophoresis in the analysis of traditional Chinese medicine. Comb Chem High Throughput Screen. 13:954–65.

Fletcher MT, McKenzie RA, Reichmann KG, Blaney BJ (2011a). Risks from plants containing pyrrolizidine alkaloids for livestock and meat quality in northern Australia. In: Riet-Correa F, Pfister J, Schild AL, Wierenga T, editors. Poisonings by plants, mycotoxins and related toxins. Wallingford: CABI: 208–18.

Fletcher MT, Hayes PY, Somerville MJ, de Voss JJ (2011b). *Crotalaria medicaginea* associated with horse deaths in northern Australia: new pyrrolizidine alkaloids. J Agric Food Chem. 59:11888–92.

Fox DW, Hart MC, Bergeson PS, Jarrett PB, Stillman AE, Huxtable RJ (1978). Pyrrolizidine (Senecio) intoxication mimicking Reye syndrome. J Pediatr. 93:980–2.

Fragoso-Serrano M, Figueroa-González G, Castro-Carranza E, Hernández-Solis F, Linares E, Bye R et al. (2012). Profiling of alkaloids and eremophilanes in miracle tea (*Packera candidissima* and *P. bellidifolia*) products. J Natur Prod. 75:890–95.

Frahn JL (1969). Paper electrophoresis of pyrrolizidine alkaloids. Aust J Chem. 22:1655–67.

Frist M, Pandolfe F, Uradomo L, Borum M (2010). Death from herbal tea: Hepatic veno-occlusive disease and fulminant hepatic failure secondary to Chinese herbal tea ingestion. Abstracts of ACG 2010, American College of Gastroenterology Annual Scientific Meeting. October 15–20, 2010. San Antonio, Texas, USA. Am J Gastroenterol. 105(Suppl 1):S294.

Fu PP, Chou MW (2001) Metabolic activation of the tumorigenic pyrrolizidine alkaloid, riddelliine, leading to DNA adduct formation in vivo. Chemical Research in Toxicology, 14, 101-9.

Fu PP, Chou MW, Xia Q, Yang YC, Yan J, Doerge DR et al. (2001). Genotoxic pyrrolizidine alkaloids and pyrrolizidine alkaloid N-oxides – mechanisms leading to DNA adduct formation and tumorigenicity. Environ Carcino Ecotox Revs. C19:353–85.

Fu PP, Chou MW, Churchwell M, Wang Y, Zhao Y, Xia Q et al. (2010). High-performance liquid chromatography electrospray ionization tandem mass spectrometry for the detection and quantitation of pyrrolizidine alkaloid-derived DNA adducts in vitro and in vivo. Chem Res Toxicol. 23:637–52.

Gao H, Li N, Wang JY, Zhang SC, Lin G (2012). Definitive diagnosis of hepatic obstruction syndrome induced by pyrrolizidine alkaloids. J Dig Dis. 13:33–39.

Gao W, Wang JY, Lin G, Zhang SC (2011). A new diagnostic method to confirm the relationship between hepatic sinusoidal obstruction syndrome and *Gynura segetum*. The 21st Conference of the Asian Pacific Association for the Study of the Liver: oral presentations. Hepatol Int. 5:66.

Gardner D, Molyneux R, Downey D, Wall L, Almeida L, Diaz G (2014). Detection of dehydropyrrolizidine alkaloids in honey, pollen, eggs and the associated Senecio species. Planta Med 80 – PV5. Abstract only

Gherghiceanu DM, Giurgiu G, Mircean M, Ghergariu S (2001). A retrospective study concerning the hepatocerebral disease (H.C.D.) in horses in the "Clinic of internal diseases", Faculty of Veterinary Medicine Cluj-Napoca. Rev Rom Med Vet. 3:233–44.

Giaretta PR, Panziera W, Hammerschmitt ME, Bianchi RM, Galiza GJN, Wiethan IS (2014). Clinical and pathological aspects of chronic *Senecio* spp. poisoning in sheep. Pesq Vet Bras. 34:967–73.

Gimmler-Luz MC, Erdtmann B (1997). Clastogenic activity of integerrimine determined in mouse micronucleus assays. Braz J Genet. 20:405–9.

Gimmler-Luz MC, Erdtmann B, Balbueno RA (1990). The effect of the pyrrolizidine alkaloid integerrimine on the chromosomes of mouse bone marrow cells. Mutat Res. 241:297–304.

Gordon GJ, Coleman WB, Grisham JW (2000). Induction of cytochrome P450 enzymes in the livers of rats treated with the pyrrolizidine alkaloid retrorsine. Exp Mol Pathol. 69:17–26.

Gourlay H (2007a). Ragwort crown-boring moth (Information note). Lincoln: Manaaki Whenua New Zealand Landcare Research.

Gourlay H (2007b). Ragwort plume moth (Information note). Lincoln: Manaaki Whenua New Zealand Landcare Research.

Grecco FB, Estima-Silva P, Marcolongo-Pereira C, Soares MP, Collares G, Schild AL (2011). Chronic seneciosis in sheep in southern Rio Grande do Sul, Brazil. Pesq Vet Bras. 31:326–30.

Griffin CT, Danaher M, Elliott CT, Glenn Kennedy D, Furey A (2013). Detection of pyrrolizidine alkaloids in commercial honey using liquid chromatography-ion trap mass spectrometry. Food Chem. 136(3–4):1577–83.

Griffin CT, Gosetto F, Danaher M, Sabatini S, Furey A (2014). Investigation of targeted pyrrolizidine alkaloids in traditional Chinese medicines and selected herbal teas sourced in Ireland using LC-ESI-MS/MS. Food Addit Contam A. 31:940–61.

Griffin CT, O'Mahony J, Danaher M, Furey A (2015a). Liquid chromatography tandem mass spectrometry detection of targeted pyrrolizidine alkaloids in honeys purchased within Ireland. Food Anal Methods. 8:18–31.

Griffin CT, Mitrovic SM, Danaher M, Furey A (2015b). Development of a fast isocratic LC-MS/MS method for the high-throughput analysis of pyrrolizidine alkaloids in Australian honey. Food Addit Contam A. 32:214–28.

Guo Y, Ma Z, Kou H, Sun R, Yang H, Smith CV et al. (2013). Synergistic effects of pyrrolizidine alkaloids and lipopolysaccharide on preterm delivery and intrauterine fetal death in mice. Toxicol Lett. 221:212–8.

Györik S, Stricker H (2009). Severe pulmonary hypertension possibly due to pyrrolizidine alkaloids in polyphytotherapy. Swiss Med Wkly. 139:210–11.

Haseman JK, Young E, Eustis SL, Hailey JR (1997). Body weight-tumor incidence correlations in long-term rodent carcinogenicity studies. Toxicol Pathol. 25:256–63 (as cited in NTP, 2003).

Hayashi Y, Lalich, JJ (1968). Protective effect of mercaptoethylamine and cysteine against monocrotaline intoxication in rats. Toxicol Appl Pharmacol. 12:36–43, 223.

He X, Xia Q, Fu PP (2017). 7-Glutathione-pyrrole and 7-cysteine-pyrrole are potential carcinogenic metabolites of pyrrolizidine alkaloids. J Environ Sci Health C. 35:69–83.

He YQ, Yang L, Liu HX, Zhang JW, Liu Y, Fong A et al. (2010a). Glucuronidation, a new metabolic pathway for pyrrolizidine alkaloids. Chem Res Toxicol. 23:591–9.

He YQ, Liu Y, Zhang BF, Liu HX, Lu YL, Yang L et al. (2010b). Identification of the UDP-glucuronosyltransferase isozyme involved in senecionine glucuronidation in human liver microsomes. Drug Metabol Dis. 38:626–34.

He X, Ma L, Xia Q, Fu PP (2016a). 7-N-acetylcysteine –pyrrole conjugate – a potent DNA reactive metabolite of pyrrolizidine alkaloids. J Food Drug Anal. 24:682–94.

He X, Xia Q, Ma L, Fu PP (2016b). 7-cysteine-pyrrole conjugate: a new potential DNA reactive metabolite of pyrrolizidine alkaloids. J Environ Sci Health C. 34:57–76.

Hess JB, Mosjidis JA (2008). Effect of sunn hemp seed inclusion in broiler starter diets on live performance attributes. J Appl Anim Res. 33:105–8.

Hessel S, Gottschalk C, Klaffke H, Heinze L, Preiss-Weigert A, Lahrssen-Wiederholt M et al. (2011). Hepatotoxic pyrrolizidine alkaloids – Bioavailability and cellular effects on human HepG2 cells. Toxicol Lett. 205:S162.

Hessel S, Gottschalk C, Schumann D, These A, Preiss-Weigert A, Lampen A (2014). Structure-activity relationship in the passage of different pyrrolizidine alkaloids through the gastrointestinal barrier: ABCB1 excretes heliotrine and echimidine. Mol Nutri Food Res. 58:995–1004.

Hill BD, Gaul KI, Noble JW (1997). Poisoning of feedlot cattle by seeds of *Heliotropium europaeum*. Aust Vet J. 75:360–1.

Hincks JR, Kim HY, Segall HJ, Molyneux RJ, Stermitz FR, Coulombe RA (1991). DNA cross-linking in mammalian cells by pyrrolizidine alkaloids: structure-activity relationships. Toxicol Appl Pharmacol. 111:90–8.

Hirono I, Haga M, Fujii M, Matsuura S, Matsubara N, Nakayama M et al. (1979). Induction of hepatic tumors in rats by senkirkine and symphytine. J Nat Can Ins. 63:469–72.

Honma M, Hayashi M (2011). Comparison of in vitro micronucleus and gene mutation assay results for p53-competent versus p53-deficient human lymphoblastoid cells. Environ Mol Mutag. 52:373–84.

Honma M, Hayashi M, Shimada H, Tanaka N, Wakuri S, Awogi T et al. (1999). Evaluation of the mouse lymphoma tk assay (microwell method) as an alternative to the in vitro chromosomal aberration test. Mutagenesis.14:5–22.

Honorio Jr. JER, Vasconcelos GS, Rodrigues FTS, Filho JGS, Barbosa-Filho JM, Aguiar CCT et al. (2012). Monocrotaline: histological damage and oxidant activity in brain areas of mice. Oxid Med Cell Longev. doi:10.1155/2012/697541

Hoogenboom LAP, Mulder PPJ, Zeilmaker MJ, van den Top HJ, Remmelink GJ, Brandon EFA et al. (2011). Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows. Food Addit Contam A. 28:359–72.

Hösch G, Wiedenfeld H, Dingermann Th, Röder E (1996). A new high performance liquid chromatography method for the simultaneous quantitative analysis of pyrrolizidine alkaloids and their N-oxides in plant material. Phytochem Anal. 7:284–88.

Hough RL, Crews C, White D, Driffield M, Campbell CD, Maltin C (2010). Degradation of yew, ragwort and rhododendron toxins during composting. Sci Total Environ. 408:4128–37.

Hovermale JT, Craig AM (1998). A routine method for the determination of retronecine. Fresenius' J Anal Chem. 361:201–6.

Hovermale JT, Craig AM (2002). Metabolism of pyrrolizidine alkaloids by *Peptostreptococcus heliotrinreducens* and a mixed culture derived from ovine ruminal fluid. Biophy Chem.101–102:387–99.

Huan JY, Miranda CL, Buhler DR, Cheeke PR (1998a). Species differences in the hepatic microsomal enzyme metabolism of the pyrrolizidine alkaloids. Toxicol Let. 99:127–37.

Huan JY, Miranda CL, Buhler DR, Cheeke PR (1998b). The roles of CYP3A and CYP2B isoforms in hepatic bioactivation and detoxification of the pyrrolizidine alkaloid senecionine in sheep and hamsters. Toxicol Appl Pharmacol. 151:229–35.

Hueza IM, Benassi JC, Raspantini PCF, Raspantini LER, Sá LRM, Górniak SL et al. (2009). Low doses of monocrotaline in rats cause diminished bone marrow cellularity and compromised nitric oxide production by peritoneal macrophages. J Immunotoxicol. 6:11–18.

Hueza IM, Elias F, Haraguchi M, Górniak SL (2011). A postnatal evaluation of the immunotoxic effect of integerrimine n-oxide exposure during gestation to rats. Birth Defects Res A. 91:379.

Huizing HJ, Malingré ThM (1979). Reduction of pyrrolizidine-N-oxides by the use of a redox polymer. J Chromatogr. 173:187–189.

Huizing HJ, de Boer HJ, Malingré ThM (1980). Chloranil, a sensitive detection reagent for pyrrolizidine alkaloids on thin-layer chromatograms. J Chromatogr. 195:407–11.

Huizing HJ, Deboer F, Hendriks H, Balraadjsing W, Bruins AP (1986). Positive and negative-ion chemical ionization mass spectrometry of trimethylsilyl derivatives of pyrrolizidine alkaloids using (NH)H+ and (OH)H" as the reactant ions. Biomed Environ Mass Spectrom. 13:293–298.

Huxtable RJ (1980). Herbal teas and toxins: novel aspects of pyrrolizidine poisoning in the United States. Perspect Biol Med. 24:1–14.

Huxtable RJ (1989). Human embryotoxicity of pyrrolizidine-containing drugs. Hepatology. 9:510–11.

Huxtable RJ, Bowers R, Mattocks AR, Michnica M (1990). Sulfur conjugates as putative pneumotoxic metabolites of the pyrrolizidine alkaloid, monocrotaline. In: Witmer CM, Snyder RR, Jollow DJ, Kalf GF, Kocsis JJ. Biologically reactive intermediates IV – molecular and cellular effects and their impact on human health. New York: Plenum Press.

Huxtable RJ, Yan CC, Wild S, Maxwell S, Cooper R (1996). Physicochemical and metabolic basis for the differing neurotoxicity of the pyrrolizidine alkaloids, trichodesmine and monocrotaline. Neurochem Res. 21:141–6.

Huybrecht B, Callebaut A (2015). Pyrrolizidine alkaloids in food and feed on the Belgian market. Food Addit Contam A. 32:1939–51.

IARC (1976). Some naturally occurring substances. Lyon: International Agency for Research on Cancer (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 10).

IARC (1983). Some food additives, feed additives and naturally occurring substances. Lyon: International Agency for Research on Cancer; (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 31).

IARC (1987). Overall evaluations of carcinogenicity. An updating of IARC Monographs Volumes 1–42. Lyon: International Agency for Research on Cancer (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Supplement 7).

IARC (2002). Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Lyon: International Agency for Research on Cancer (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 82).

Ilha MRS, Loretti AP, Barros SS, Barros CSL (2001). Spontaneous poisoning in sheep by *Senecio brasiliensis* (Asteraceae) in southern Brazil. Pesq Vet Bras. 21:123–8.

IPCS (1988). Pyrrolizidine alkaloids. Geneva: World Health Organization, International Programme on Chemical Safety (Environmental Health Criteria 80).

IPCS (1989). IPCS International Programme on Chemical Safety. Health and Safety Guide No. 26 – pyrrolizidine alkaloids (http://www.inchem.org/documents/hsg/hsg/hsg026.htm, accessed 20 December 2018).

Jago MV (1970). A method for the assessment of the chronic hepatoxicity of pyrrolizidine alkaloids. Aust J Exp Biol Med Sci. 48:93–103.

Janes D, Kreft S (2014). TLC densitometric method for screening of lycopsamine in comfrey root (*Symphytum officinale* L.) extracts using retrorsine as a reference compound. Acta Pharmaceutica. 64:503–8.

JECFA (2016). Guidance document for WHO monographers and reviewers evaluating food additives. Table 1 (http://apps.who.int/iris/bitstream/10665/206184/1/9789241510394_eng.pdf).

Jewell C, Ackermann C, Payne NA, Fate G, Voorman R, Williams FM (2007). Specificity of procaine and ester hydrolysis by human, minipig, and rat skin and liver. Drug Metab Dispos. 35:2015–22.

Ji L, Chen Y, Wang Z (2008). Protection of S-adenosyl methionine against the toxicity of clivorine on hepatocytes. Environ Toxicol Pharmacol. 26:331–5.

Ji L, Liu T, Chen Y, Wang Z (2009). Protective mechanisms of N-acetyl-cysteine against pyrrolizidine alkaloid clivorine-induced hepatotoxicity. Journal of Cellular Biochemistry 108:424-32.

Jiang X, Wang S, Zhao Y, Xia Q, Cai L, Sun X et al. (2015). Absolute configuration, stability, and interconversion of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine valine adducts and their phenylthiohydantoin derivatives. J Food Drug Anal. 23:318–26.

Johnston WH, Craig AM, Blythe LL, Hovermale JT, Walker K (1998). Pyrrolizidine alkaloid detoxification by an ovine ruminal consortium and its use as a ruminal supplement in cattle. In: Garland T, Barr AC, editors. Toxic plants and other natural toxicants:185–190.

Kakar F, Akbarian Z, Leslie T, Mustafa ML, Watson J, van Egmond HP et al. (2010). An outbreak of hepatic veno-occlusive disease in Western Afghanistan associated with exposure to wheat flour contaminated with pyrrolizidine alkaloids. J Toxicol. 2010:1–7.

Karam FSC, Motta AC (2011). Pyrrolizidine alkaloid poisoning in cattle in the state of Rio Grande do Sul, Brazil. In: Riet-Correa F, Pfister J, Schild AL, Wierenga T, editors. Poisoning by plants, mycotoxins, and related toxins. Wallingford: CABI:175–6.

Karam FSC, Soares MP, Haraguchi M, Riet-Correa F, Méndez MC, Jarenkow JA (2004). Epidemiological aspects of seneciosis in southern Rio Grande do Sul, Brazil. Pesq Vet Bras. 24:191–8.

Kast C, Dübecke A, Kilchenmann V, Bieri K, Böhlen M, Zoller O et al. (2014). Analysis of Swiss honeys for pyrrolizidine alkaloids. J Apic Res. 53:75–83.

Kempf M, Reinhard A, Beuerle T (2010). Pyrrolizidine alkaloids (PAs) in honey and pollen-legal regulation of PA levels in food and animal feed required. Mol Nutr Food Res. 54:158–68.

Kempf M, Beuerle T, Bühringer M, Denner M, Trost D, von der Ohe K et al. (2008). Pyrrolizidine alkaloids in honey: risk analysis by gas chromatography-mass spectrometry. Mol Nutr Food Res. 52:1193–200.

Kempf M, Heil S, Hasslauer I, Schmidt L, von der Ohe K, Theuring C et al. (2010). Pyrrolizidine alkaloids in pollen and pollen products. Mol Nutr Food Res. 54:292–300.

Kempf M, Wittig M, Reinhard A, von der Ohe K, Blacquière T, Raezke K-P et al. (2011a). Pyrrolizidine alkaloids in honey: comparison of analytical methods. Food Addit Contam A. 28:332–47.

Kempf M, Wittig M, Schonfeld K, Cramer L, Schreier P, Beuerle T (2011b). Pyrrolizidine alkaloids in food: downstream contamination in the food chain caused by honey and pollen. Food Addit Contam Part A. 28:325–31.

Kempf M, Wittig M, Reinhard A, von der Ohe K, Blacquière T, Raezke K-P et al. (2011c). Pyrrolizidine alkaloids in honey: comparison of analytical methods. Food Addit Contam A. 28:332–47.

Kennedy J (2005). Herb and supplement use in the US adult population. Clin Ther. 27:1847–58.

Kevekordes S, Mersch-Sundermann V, Burghaus CM, Spielberger J, Schmeiser HH, Arlt VM et al. (1999). SOS induction of selected naturally occurring substances in *Escherichia coli* (SOS chromotest). Mutat Res. 445:81–91.

Kevekordes S, Spielberger J, Burghaus CM, Birkenkamp P, Zietz B, Paufler P et al. (2001). Micronucleus formation in human lymphocytes and in the metabolically competent human hepatoma cell line Hep-G2: results with 15 naturally occurring substances. Anticancer Res. 21:461–9.

Kim HY, Stermitz FR, Coulombe RA Jr. (1995). Pyrrolizidine alkaloid-induced DNA–protein cross-links. Carcinogenesis. 16:2691–7 (as cited by Chen, Mei & Fu, 2010).

Kim HY, Stermitz FR, Li KK, Coulombe RA (1999). Comparative DNA cross-linking by activated pyrrolizidine alkaloids. Food Chem Toxicol. 37:619–25.

Koekemoer MJ, Warren FL (1951). The Senecio alkaloids. Part VIII. The occurrence and preparation of the N-oxides. An improved method of extraction of the Senecio alkaloids. J Chem Soc. 66–8.

Kou H, Xiong J, Ma Z-g, Zheng J, Guo Y, Wang H (2011). Pyrrolizidine alkaloids induce fetal developmental toxicities in mice. Drug Metab Rev. 43:(suppl 2), 146–7.

Krishnamachari KA, Bhat RV, Krishnamurthi D, Krishnaswamy K, Nagarajan V (1977). Aetiopathogenesis of endemic ascites in Surguja district of Madhya Pradesh. Indian J Med Res. 65:672–8.

Kumana CR, Ng M, Lin HJ, Ko W, Wu PC, Todd D (1983). Hepatic veno-occlusive disease due to toxic alkaloid herbal tea. Lancet. 2:1360–1.

Kumana CR, Ng M, Lin HJ, Ko W, Wu PC, Todd D (1985). Herbal tea induced hepatic veno-occlusive disease: quantification of toxic alkaloid exposure in adults. Gut. 26:101–4.

Kusuma SS, Tanneeru K, Didla S, Devendra BN, Kiranmayi P (2014). Antineoplastic activity of monocrotaline against hepatocellular carcinoma. Anticancer Agents Med Chem. 14:1237–48.

Lamé MW, Morin D, Jones AD, Segall HJ, Wilson DW (1990). Isolation and identification of a pyrrolic glutathione conjugate metabolite of the pyrrolizidine alkaloid monocrotaline. Toxicol Lett. 51:321–9.

Lamé MW, Jones AD, Morin D, Segall HJ, Wilson DW (1995). Biliary excretion of pyrrolic metabolites of [14C]monocrotaline in the rat. Drug Metabol Dis. 23:422–9.

Lamé MW, Jones AD, Morin D, Wilson DW, Segall HJ (1997). Association of dehydromonocrotaline with rat red blood cells. Chem Res Toxicol. 10:694–701.

Langer T, Franz C (1997). Determination of pyrrolizidine alkaloids in commercial samples of borage seed oil products by GC-MS. Scientia Pharmaceut. 65:321–8.

Langer T, Möstl E, Chizzola R, Gutleb R (1996). A competitive enzyme immunoassay for the pyrrolizidine alkaloids of the senecionine type. Planta Medica. 62:267–71.

Lanigan GW (1970). Metabolism of pyrrolizidine alkaloids in the ovine rumen. II Some factors affecting rate of alkaloid breakdown by rumen fluid in vitro. Aust J Agric Res. 21:633–9.

Lanigan GW (1971). Metabolism of pyrrolizidine alkaloids in the ovine rumen. III. The competitive relationship between heliotrine metabolism and methanogenesis in rumen fluid in vitro. Aust J Agric Res. 22:123–30.

Lanigan GW (1972). Metabolism of pyrrolizidine alkaloids in ovine rumen. IV. Effects of chloral hydrate and halogenated methanes on rumen methanogenesis and alkaloid metabolism in fistulated sheep. Aust J Agric Res. 6:1085–91.

Lanigan GW (1976). *Peptococcus heliotrinreducans*, sp.nov., a cytochrome-producing anaerobe which metabolizes pyrrolizidine alkaloids. J Gen Microbiol. 94:1–10.

Lanigan GW, Smith LW (1970). Metabolism of pyrrolizidine alkaloids in the ovine rumen. I. Formation of 7α-hydroxy-1α-methyl-8α-pyrrolizidine from heliotrine and lasiocarpine. Aust J Agric Res. 21:493–500.

Lanigan GW, Payne AL, Peterson JE (1978). Antimethanogenic drugs and *Heliotropium europaeum* poisoning in penned sheep. Aust J Agric Res. 29:1281–92.

Lebada R, Schreier A, Scherz S, Resch C, Krenn L, Kopp B (2000). Quantitative analysis of the pyrrolizidine alkaloids senkirkine and senecionine in *Tussilago farfara* L. by capillary electrophoresis. Phytochem Anal. 11:366–9.

Lee ST, Schoch TK, Stegelmeier BL, Gardner DR, Than KA, Molyneux RJ (2001). Development of enzyme-linked immunosorbent assays for the hepatotoxic alkaloids riddelliine and riddelliine N-oxide. J Agric Food Chem. 49:4144–51.

Lee ST, Schoch TK, Stegelmeier BL Gardner DR, Than KA, Molyneux RJ (2004). The detection and estimation of pyrrolizidinine alkaloids in plants and feeds using an ELISA. In: Acamovic T, Stewart CS, Pennycott TW, editors. Poisonous plants and related toxins. Wallingford: CABI:387–93.

Leiss KA (2010). Management practices for control of ragwort species. Phytochem Rev. 10:153–63.

Li W, Wang K, Lin G, Peng Y, Zheng J (2016). Lysine adduction by reactive metabolite(s) of monocrotaline. Chem Res Toxicol. 29:333–41.

Liang Q, Sheng Y, Jiang P, Ji L, Xia Y, Min Y et al. (2011a). The gender-dependent difference of liver GSH antioxidant system in mice and its influence on isoline-induced liver injury. Toxicol. 280:61–9.

Liang QN, Sheng YC, Jiang P, Ji LL, Xia YY, Min Y et al. (2011b). The difference of glutathione antioxidant system in newly weaned and young mice liver and its involvement in isoline-induced hepatotoxicity. Arch Toxicol. 85:1267–79.

Lin G, Cui YY, Hawes EM (1998). Microsomal formation of a pyrrolic alcohol glutathione conjugate of clivorine firm evidence for the formation of a pyrrolic metabolite of an otonecine-type pyrrolizidine alkaloid. Drug Metabolism and Disposition 26:181-4.

Lin G, Cui YY, Hawes EM (2000). Characterization of rat liver microsomal metabolites of clivorine, an hepatotoxic otonecine-type pyrrolizidine alkaloid. Drug Metabol Dis. 28:1475–83.

Lin G, Cui YY, Liu XQ (2003). Gender differences in microsomal metabolic activation of hepatotoxic clivorine in rat. Chem Res Toxicol. 16:768–74.

Lin G, Zhou KY, Zhao XG, Wang ZT, But PPH (1998). Determination of hepatotoxic pyrrolizidine alkaloids by on-line high performance liquid chromatography mass spectrometry with an electrospray interface. Rapid Commun Mass Spectrom. 12:1445–56.

Lin G, Cui YY, Liu XQ, Wang ZT (2002). Species differences in the in vitro metabolic activation of the hepatotoxic pyrrolizidine alkaloid clivorine. Chem Res Toxicol. 15:1421–8.

Lin G, Tang J, Liu XQ, Jiang Y, Zheng J (2007). Deacetylclivorine: a gender-selective metabolite of clivorine formed in female Sprague-Dawley rat liver microsomes. Drug Metabol Dis. 35:607–13.

Lin G, Wang JY, Li N, Li M, Gao H, Ji Y et al. (2011). Hepatic sinusoidal obstruction syndrome associated with consumption of *Gynura segetum*. J Hepatol. 54:666–73.

Liu F, Wan SY, Jiang Z, Li SFY, Ong ES, Osorio JCC (2009). Determination of pyrrolizidine alkaloids in Comfrey by liquid chromatography-electrospray ionization mass spectrometry. Talanta. 80:916–23.

Liu TY, Chen Y, Wang ZY, Ji LL, Wang ZT (2010). Pyrrolizidine alkaloid isoline-induced oxidative injury in various mouse tissues. Exp Toxicol Path. 62:251–7.

Liu XQ, Lin G, Wang GJ, Qian ZY (2001). Metabolism of clivorine in female rat liver microsomes. Chi J Pharmacol Toxicol.15:413–7.

Liu XQ, Lin G, Wang GJ, Qian ZY (2002). Involvement of human CYP3A4 in the formation of hepatotoxic metabolites of clivorine. Chin J Pharmacol Toxicol. 16:15–20.

Logie CG, Grue MR, Liddell JR (1994). Proton NMR spectroscopy of pyrrolizidine alkaloids. Phytochemistry. 37:43–109.

Long HS, Van Wijk BE, Stander M (2016). *Senecio angustifoliais* the main pyrrolizidine alkaloid contaminant of rooibos tea. Sou Afri J Botany. 103:322.

Lucatello L, Merlanti R, Rossi A, Montesissa C, Capolongo F (2016). Evaluation of some pyrrolizidine alkaloids in honey samples from the Veneto Region (Italy) by LC-MS/MS. Food Anal Methods. 9:1825–36.

Lucena RB, Rissi DR, Maia LA, Dantas AFM, Flores MA, Nobre VMT et al. (2010). Poisoning by pyrrolizidine alkaloids in ruminants and horses in Brazil. Pesq Vet Bras. 30:447–52.

Luckert C, Braeuning A, Lampen A, Hessel S (2016). Structure-dependent activation of PXR and PXR-mediated induction of CYP3A4 expression by hepatotoxic pyrrolizidine alkaloids. Naunyn-Schmiedeberg's Arch Pharmacol. 389(S1):S48.

Luthy J, Heim T, Schlatter C (1983). Transfer of [3H]pyrrolizidine alkaloids from *Senecio vulgaris* L. and metabolites into rat milk and tissues. Toxicol Lett. 17:283–88.

Ma L, Zhao H, Xia Q, Cai L, Fu PP (2015). Synthesis and phototoxicity of isomeric 7,9-diglutathione pyrrole adducts: Formation of reactive oxygen species and induction of lipid peroxidation. J Food Drug Anal. 23:577–86.

MacGregor JT, Henika PR, Whitehand L, Wehr CM (1989). The fetal blood erythrocyte micronucleus assay: classification of RNA-positive erythrocytes into two age populations by RNA aggregation state. Mutagenesis. 4:190–9.

MacGregor JT, Wehr CM, Henika PR, Shelby MD (1990). The in vivo erythrocyte micronucleus test: measurement at steady state increases assay efficiency and permits integration with toxicity studies. Fundam Appl Toxicol. 14:513–22.

MAFF (1994). Naturally occurring toxicants in food. MAFF Food Surveillance Paper 42. London: Ministry of Agriculture, Fisheries and Food.

Magnabosco EM, Rivera ML, Prolla IR, de Verney YM, de Mello ES (1997). Hepatic veno-occlusive disease: report of a case. J Pediatr (Rio J). 73:115–8 [in Portuguese].

Maia LA, de Lucena RB, Nobre VM, Dantas AF, Colegate SM, Riet-Correa F (2013). Natural and experimental poisoning of goats with the pyrrolizidine alkaloid-producing plant *Crotalaria retusa* L. J Vet Diagn Investig. 25:592–5.

Maia LA, de Macedo Pessoa CR, Rodrigues AF, Colegate S, Medeiros Dantas AF, Medeiros RMT et al. (2014). Duration of an induced resistance of sheep to acute poisoning by *Crotalaria retusa* seeds. Ciencia Rural. 44:1054–9.

Marar T, Devi CSS (1995). Enzymic profile of lung lavage fluid after chronic monocrotaline ingestion. J Environ Biol. 16:343–6.

Marar T, Devi CSS (1996). Acute toxicity (oral LD50) of monocrotaline in rats. J Environ Biol. 17:125–6.

Martinello M, Cristofoli C, Gallina A, Mutinelli F (2014). Easy and rapid method for the quantitative determination of pyrrolizidine alkaloids in honey by ultra performance liquid chromatography-mass spectrometry: An evaluation in commercial honey. Food Control. 37:146–52.

Mathon C, Edder P, Bieri S, Christen P (2014). Survey of pyrrolizidine alkaloids in teas and herbal teas on the Swiss market using HPLC-MS/MS. Anal Bioanal Chem. 406:7345–54.

Mattocks AR (1971). Hepatotoxic effects due to pyrrolizidine alkaloid N-oxides. Xenobiotica 1:563–5 (as cited by EFSA, 2011).

Mattocks AR (1972). Toxicity and metabolism of Senecio alkaloids. In: Harborne JB, editor. Phytochemical ecology. London: Academic Press:179–200 (as cited by EFSA, 2011).

Mattocks AR (1982). Hydrolysis and hepatotoxicity of retronecine diesters. Toxicol Lett. 14:111-6.

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

Mattocks AR, Jukes R (1987). New improved field test for toxic pyrrolizidine alkaloids. J Nat Prod. 50:161–6.

Mattocks AR, Jukes R (1992a). Chemistry of sulphur-bound pyrrolic metabolites in the blood of rats given different types of pyrrolizidine alkaloid. Nat Toxins. 1:89–95.

Mattocks AR, Jukes R (1992b). Detection of sulphur-conjugated pyrrolic metabolites in blood and fresh or fixed liver tissue from rats given a variety of toxic pyrrolizidine alkaloids. Toxicol Lett. 63:47–55.

May C, Payne AL, Stewart PL, Edgar JA (1995). A delivery system for agents and composition. International Patent Application No. PCT/AU95/00733.

May C, Payne AL, Stewart PL, Edgar JA (2001). Delivery system for antimethanogenic agents. United States Patent US 6,251,879 B1 June. 26, 2001.

McCrabb G, Berger K, Magner T, May C, Hunter R (1997). Inhibiting methane production in Brahman cattle by dietary supplementation with a novel compound and the effects on growth. Aust J Agric Res. 48:323–9.

Medeiros RMT, Górniak SL, Guerra JL (1999). Comparative study of prenatal and postnatal monocrotaline effects in rats. In: Garland T, Barr AC, editors. Toxic plants and other natural toxicants, Wallingford: CABI:312–6.

Medeiros RMT, Górniak SL, Guerra JL (2000). Fetotoxicity and reproductive effects of monocrotaline in pregnant rats. J Ethnopharmacol. 69:181–8.

Mei N, Heflich RH, Chou MW, Chen T (2004a). Mutations induced by the carcinogenic pyrrolizidine alkaloid riddelliine in the liver cII gene of transgenic big blue rats. Chem Res Toxicol. 17:814–8.

Mei N, Chou MW, Fu PP, Heflich RH, Chen T (2004b). Differential mutagenicity of riddelliine in liver endothelial and parenchymal cells of transgenic big blue rats. Cancer Lett. 215:151–8.

Mendez MC, Riet-Correa F (1993). Intoxication by *Senecio tweediei* in cattle in southern Brazil. Vet Hum Toxicol. 35:55.

Merz KH, Schrenk D (2016). Interim relative potency factors for the toxicological risk assessment of pyrrolizidine alkaloids in food and herbal medicines. Toxicology Letters 263:44-57.

Mibei EK, Ojijo NKO (2011). Effects of processing on chemical composition of four African leafy vegetables. Electron J Environ Agric Food Chem. 10:3121–31.

Miller WC, Rice DL, Kreusel RG, Bedrossian CWM (1978). Monocrotaline model of noncardiogenic pulmonary edema in dogs. J Appl Physiol 45:962–5 (as cited by EFSA, 2011).

Miranda C L, Reed RL, Cheeke PR, Buhler D R (1981). Protective effects of butylated hydroxyanisole against the acute toxicity of monocrotaline in mice. Toxicol Appl Pharmacol. 59: 424–30.

Miranda CL, Rawson CL, Reed RL, Zhao X, Barnes DW, Buhler DR (1992). C3H/10T1/2 cells: a model to study the role of metabolism in the toxicity of the pyrrolizidine alkaloid retrorsine. In Vitro Toxicology. 5:21–32.

Mirsalis JC, Steinmetz KL, Blazak WF, Spalding JW (1993). Evaluation of the potential of riddelliine to induce unscheduled DNA synthesis, S-phase synthesis, or micronuclei following in vivo treatment with multiple doses. Environ Mol Mutagen. 21:265–71.

Mohabbat O, Younos MS, Merzad AA, Srivastava RN, Sediq GG, Aram GN (1976). An outbreak of hepatic veno-occlusive disease in north-western Afghanistan. Lancet. 2:269–71.

Molyneux RJ, Roitman JN (1980). Specific detection of pyrrolizidine alkaloids on thin-layer chromatograms. J Chromatogr. 195:412–15.

Molyneux RJ, Johnson AE, Stuart LD (1988). Delayed manifestation of Senecio-induced pyrrolizidine alkaloidosis in cattle: case reports. Vet Hum Toxicol. 30:201–5

Molyneux RJ, Johnson AE, Roitman JN, Benson ME (1979). Chemistry of toxic range plants. Determination of pyrrolizidine alkaloid content and composition in Senecio species by nuclear magnetic resonance spectroscopy. J Agric Food Chem. 27:494–9.

Molyneux RJ, Johnson AE, Olsen JD, Baker DC (1991). Toxicity of pyrrolizidine alkaloids from Riddell groundsel (*Senecio riddellii*) to cattle. Am J Vet Res. 52:146–51.

Molyneux RJ, Gardner DL, Colegate SM, Edgar JA (2011). Pyrrolizidine alkaloid toxicity in livestock: a paradigm for human poisoning? Food Addit Contam A. 28:293–307.

Mondal DB, Nandankar UA, Mohanty TK, Barari SK, Pal RN, Sarkar M (1999). Pyrrolizidine alkaloid poisoning in yak. Vet Rec. 144:508–9.

Moore DJ, Batts KP, Zalkow LL, Fortune Jr GT, Powis G (1989). Model systems for detecting the hepatic toxicity of pyrrolizidine alkaloids and pyrrolizidine alkaloid N-oxides. Toxicol Appl Pharmacol. 101:271–84.

Mori H, Sugie S, Yoshimi N, Asada Y, Furuya T, Williams GM (1985). Genotoxicity of a variety of pyrrolizidine alkaloids in the hepatocyte primary culture-DNA repair test using rat, mouse and hamster hepatocytes. Cancer Res. 4:3125–9.

Morris P, O'Neill D, Tanner S (1994). Synergistic liver toxicity of copper and retrorsine in the rat. J Hepatol. 21:735–42.

Moyano MR, García A, Rueda A, Molina AM, Mendez A, Infante F (2006). *Echium vulgare* and *Senecio vulgaris* poisoning in fighting bulls. J Vet Med, A. 53:24–5.

Mroczek T, Glowniak K, Wlaszcyk A (2002). Simultaneous determination of N-oxides and free bases of pyrrolizidine alkaloids by cation-exchange solid-phase extraction and ion-pair high-performance liquid chromatography. J Chromatogr. 949:249–62.

Mulder PPJ, Beumer B, Oosterink E, de Jong J (2009). Dutch survey pyrrolizidine alkaloids in animal forage. RIKILT report 2009.018 (http://edepot.wur.nl/135952, accessed 24 March 2015).

Mulder PPJ, Lopez P, These A, Preiss-Weigert A, Castellari M (2015). Occurrence of pyrrolizidine alkaloids in food. EFSA Supporting publication EN-859:114.

Mulder PPJ, de Witte SL, Stoopen GM, van der Meulen J, van Wikselaar PG, Gruys E et al. (2016).Transfer of pyrrolizidine alkaloids from various herbs to eggs and meat in laying hens. Food Add Contam A. 33:1826–39.

Müller L, Kasper P, Kaufmann G (1992). The clastogenic potential in vitro of pyrrolizidine alkaloids employing hepatocyte metabolism. Mutat Res. 282:169–76.

Müller K, Kasper P, Müller L (1993). An assessment of the in vitro hepatocyte micronucleus assay. Mutat Res. 292:213–24.

Müller-Tegethoff K, Kasper P, Müller L (1995). Evaluation studies on the in vitro rat hepatocyte micronucleus assay. Mutat Res. 335:293–307.

Müller-Tegethoff K, Kersten B, Kasper P, Müller L (1997). Application of the in vitro rat hepatocyte micronucleus assay in genetic toxicology testing. Mutat Res. 392:125–38.

NCI (1978). Bioassay of lasiocarpine for possible carcinogenicity. Bethesda (MD): United States Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, National Cancer Institute (Carcinogenesis Technical Report Series No. 39; DHEW Publication No. (NIH) 78–839).

Nederlandse Voedsel en Warenautoriteit (NVWA) (2007). Pyrrolizidine alkaloiden in honing (http://www.nvwa.nl/txmpub/files/?p_file_id=22703, accessed 8 April 2015).

Neumann H, Huckauf A (2016). Tansy ragwort (*Senecio jacobaea*): A source of pyrrolizidine alkaloids in summer honey? J Verbrauch Lebensm. 11:105–15.

Newberne PM, Rogers AE (1970). Effects of nutrition on response of liver toxins.Toxicol Appl Pharmacol. 17:290.

Ng V, Tran TT, Sundaram V (2014). An unexpected cause of an infiltrative liver mass. Gastroenterology. 147:12–13.

Nigra L, Huxtable RJ (1992). Hepatic glutathione concentrations and the release of pyrrolic metabolites of the pyrrolizidine alkaloid, monocrotaline, from the isolated perfused liver. Toxicon. 30:1195–202.

Nivarlet N, Andrianne D, Campbell K, Huet AC, Swinkels A, Crews C et al. (2011). Multiplex lateral flow immunoassays for the detection of pyrrolizidine, tropane and ergot alkaloids. Poster in: ASSET2011: food integrity and traceability conference, Belfast, 21–24 March 2011.

Noble JW, Crossley JB, Hill BD, Pierce RJ, McKenzie RA, Debritz M et al. (1994). Pyrrolizidine alkaloidosis of cattle associated with *Senecio lautus*. Aust Vet J. 71:196–200.

Nobre VMT, Riet-Correa F, Barbosa Filho JM, Dantas AFM, Tabosa IM, Vasconcelos JS (2004). Poisoning by *Crotalaria retusa* (Fabaceae) in equidae in the semiarid region of Paraíba. Pesq Vet Bras. 24:132–43.

Nobre VMT, Dantas AFM, Riet-Correa F, Barbosa Filho JM, Tabosa IM, Vasconcelos JS (2005). Acute intoxication by *Crotalaria retusa* in sheep. Toxicon 45:347–52.

NTP (1993). NTP Technical report on toxicity studies of riddelliine, administered by gavage to F344/N rats and B6C3F1 mice. Toxicity Report Series 27.

NTP (2003). Toxicology and carcinogenesis studies of riddelliine in F344/N rats and B6C3F1 mice (gavage studies). Research Triangle Park (NC): United States Department of Health and Human Services, Public Health Service, National Institutes of Health, National Toxicology Program (NTP Technical Report 508; NIH Publication No. 03-4442).

NTP (2008). Report on carcinogens background document for riddelliine. National Toxicology Program (https://ntp.niehs.nih.gov/ntp/roc/twelfth/2010/finalbds/riddelliine_final_508.pdf).

NTP (2015). Handbook for conducting a literature-based health assessment using OHAT approach for systematic review and evidence integration. Rockville (MD): Office of Health Assessment and Translation (OHAT). Division of the National Toxicology Program. National Institute of Environmental Health Sciences. (https://ntp.niehs.nih.gov/ntp/ohat/pubs/handbookjan2015_508.pdf).

Nyska A, Moomaw CR, Foley JF, Maronpot RR, Malarkey DE, Cummings CA et al. (2002). The hepatic endothelial carcinogen riddelliine induces endothelial apoptosis, mitosis, S phase, and p53 and hepatocytic vascular endothelial growth factor expression after short-term exposure. Toxicol Appl Pharmacol. 184:153–64.

Oberlies NH, Kim NC, Brine DR, Collins BJ, Handy RW, Sparacino CM et al. (2004). Analysis of herbal teas made from the leaves of comfrey (*Symphytum officinalis*): reduction of N-oxides results in order of magnitude increases in the measurable concentration of pyrrolizidine alkaloids. Pub Health Nutr. 7:919–24.

O'Dowd DJ, Edgar JA (1989). Seasonal dynamics in the pyrrolizidine alkaloids of *Heliotropium europaeum*. Aust J Ecol. 14:95–105.

Odriozola E, Campero C, Casaro A, Lopez T, Olivieri G, Melucci O (1994). Pyrrolizidine alkaloidosis in Argentinian cattle caused by Senecio selloi. Vet Hum Toxicol. 36:205–8.

Oplatowska M, Elliott CT, Huet AC, McCarthy M, Mulder PP, von Holst C et al. (2014). Development and validation of a rapid multiplex ELISA for pyrrolizidine alkaloids and their N-oxides in honey and feed. Anal Bioanal Chem. 406:757–70.

Orantes-Bermejo FJ, Serra Bonvehí J, Gómez-Pajuelo A, Megías M, Torres C (2013). Pyrrolizidine alkaloids: their occurrence in Spanish honey collected from purple viper's bugloss (Echium spp.), Food Addit Contam A. 30:1799–806.

Ortiz-Cansado A, Crespo Valadés E, Morales Blanco P, Sáenz de Santamaría J, González Campillejo JM, Ruiz Téllez T (1995). Veno-occlusive liver disease due to intake of *Senecio vulgaris* tea. Gastroenterol Hepatol. 18:413–6 [in Spanish].

Pan LC, Lamé MW, Morin D, Wilson DW, Segall HJ (1991). Red blood cells augment transport of reactive metabolites of monocrotaline from liver to lung in isolated and tandem liver and lung preparations. Toxicol Appl Pharmacol. 110:336–46.

Panariti E, Xinxo A, Leksani D (1997). Transfer of 14C-seneciphylline into sheep milk following multiple oral intakes. DTW – Dtsch Tierarztl Wochenschr.104:97–8.

Passemard B, Priymenko N (2007). L'intoxication des chevaux par les séneçons, une réalité en France. Revue Méd Vét. 185:425–30.

Pawar RS, Grundel E, Mazzola E, White KD, Krynitsky AJ, Rader JI (2010). Chiral stationary phases for separation of intermedine and lycopsamine enantiomers from *Symphytum uplandicum*. J Sep Sci. 33:200–5.

Payne J, Wight A (2013). Ragwort toxicity in cattle. Vet Rec. 173:77–8.

Pearson EG (1991). Liver failure attributable to pyrrolizidine alkaloid toxicosis and associated with inspiratory dyspnea in ponies: Three cases (1982–1988). JAVMA. 198:1651–4.

Pereira TN, Webb RI, Reilly PEB, Seawright AA, Prakash AS (1998). Dehydromonocrotaline generates sequence-selective N-7 guanine alkylation and heat and alkali stable multiple fragment DNA crosslinks. Nucl Acids Res. 26:5441–7.

Pessoa CRM, Pessoa AFA, Maia LA, Medeiros RMT, Colegate SM, Barros SS et al. (2013). Pulmonary and hepatic lesions caused by the dehydropyrrolizidine alkaloid-producing plants *Crotalaria juncea* and *Crotalaria retusa* in donkeys. Toxicon. 71:113–20.

Peterson JE, Payne AL, Culvenor CCJ (1992). *Heliotropium europaeum* poisoning of sheep with low liver copper concentrations and the preventive efficacy of cobalt and antimethanogen. Aust Vet J. 69:51–56.

Pieters LAC, Vlietinck AJ (1986). Comparison of high-performance liquid chromatography with 1H nuclear magnetic resonance spectrometry for the quantitative analysis of pyrrolizidine alkaloids from Senecio vulgaris. J Liq Chromatogr. 9:745–5.

Pieters LA, Hartmann T, Janssen J, Vlietinck AJ (1989a). Comparison of capillary gas chromatography with 1H and 13C nuclear magnetic resonance spectroscopy for quantitation of pyrrolizidine alkaloids from *Senecio vernalis*. J Chromatogr. 462:387–91.

Pieters LAC, van Zoelen AM, Vrieling K, Vlietinck AJ (1989b). Determination of the pyrrolizidine alkaloids from Senecio jacobaea by 1H and 13C NMR spectroscopy. Magn Reson Chem. 27:754–9.

Pohlmann J, van Loon G, Lefere L, Vanschandevijl K, Nollet H, De Clercq D et al. (2005). Hepatoencephalopathy caused by *Senecio jacobaea* intoxication in five horses. Vlaams Diergen Tijds. 74:440–5.

Porter JK (1995). Analysis of endophyte toxins: Fescue and other grasses toxic to livestock. J Anim Sci. 73:871–80.

Prakash AS, Pereira TN, Reilly PEB, Seawright AA (1999). Pyrrolizidine alkaloids in human diet. Mutat Res. 443:53–67.

Proudlock RJ, Statham J, Howard W (1997). Evaluation of the rat bone marrow and peripheral blood micronucleus test using monocrotaline. Mutat Res. 392:243–9.

Qi X, Wu B, Cheng Y, Qu H (2009). Simultaneous characterization of pyrrolizidine alkaloids and N-oxides in *Gynura segetum* by liquid chromatography/ion trap mass spectrometry. Rapid Commun Mass Spectr. 23:291–302.

Queiroz GR, Ribeiro RCL, Flaiban KKMC, Bracarense APFRL, Lisbôa JAN (2013). Spontaneous *Crotalaria incana* poisoning in cattle in the state of Paraná, Brazil. Semina:Ciencias Agrarias 34:823–32.

Rao MS, Subbarao V, Sato K, Reddy JK (1991). Alterations of pancreatic hepatocytes in rats exposed to carcinogens. Am J Pathol 139:1111–7.

Rasenack R, Muller C, Kleinschmidt M, Rasenack J, Wiedenfeld H (2003). Veno-occlusive disease in a fetus caused by pyrrolizidine alkaloids of food origin. Fetal Diagn Ther. 18:223–5.

Reed RL, Ahern KG, Pearson GD, Buhler DR (1988). Crosslinking of DNA by dehydroretronecine, a metabolite of pyrrolizidine alkaloids. Carcinogenesis. 9:1355–61.

Reed RL, Miranda CL, Kedzierski B, Henderson MC, Buhler DR (1992). Microsomal formation of a pyrrolic alcohol glutathione conjugate of the pyrrolizidine alkaloid senecionine. Xenobiotica. 22:1321–7.

Reid MJ, Lamé MW, Morin D, Wilson DW, Segall HJ (1998a). Involvement of cytochrome P450 3A in the metabolism and covalent binding of 14C-monocrotaline in rat liver microsomes. J Biochem Mol Toxicol. 12:157–66.

Reid MJ, Dunston SK, Lamé MW, Wilson DW, Morin D, Segall HJ (1998b). Effect of monocrotaline metabolites on glutathione levels in human and bovine pulmonary artery endothelial cells. Res Commun Mol Pathol Pharmacol. 99:53–68.

Ricci EL, Telloli CS, Dalmolin DP, Bernardi MM, Góniak SL, Spinosa HS (2010). Developmental toxicology of monocrotaline: Physical and neurobehavioral evaluation in rat offspring. Toxicol Lett. 196:183–4.

Ridker PM, Ohkuma S, McDermott WV, Trey C, Huxtable RJ (1985). Hepatic venocclusive disease associated with the consumption of pyrrolizidine-containing dietary supplements. Gastroenterology. 88:1050–4.

Rieben WK Jr., Coulombe RA Jr. (2004). DNA cross-linking by dehydromonocrotaline lacks apparent base sequence preference. Toxicol Sci. 82:497–503.

Riet-Correa F, Carvalho KS, Dantas AFM, Medeiros RMT (2011). Spontaneous acute poisoning by *Crotalaria retusa* in sheep and biological control of this plant with sheep. Toxicon. 58:606–9.

Roberts PD, Pullin AS (2007). The effectiveness of management interventions used to control ragwort species. Environ Manage. 39:691–706.

Robinson B, Gummow B (2015). A field investigation into a suspected outbreak of pyrrolizidine alkaloid toxicosis in horses in western Queensland. Prev Vet Med. 118:378–86.

Robinson O, Toledano MB, Goldin R, Want E, Hauser M, Fenwick A et al. (2012). Hirmi valley liver disease: A tale of two toxins. J Hepatol. 56:S33–S34.

Robinson O, Want E, Coen M, Kennedy R, van den Bosch C, Gebrehawaria Y et al. (2014). Hirmi Valley liver disease: a disease associated with exposure to pyrrolizidine alkaloids and DDT. J Hepatol. 60:96–102.

Roeder E (1999). Analysis of pyrrolizidine alkaloids. Curr Org Chem. 3:557–76.

Roeder E, Pflueger T (1995). Analysis of pyrrolizidine alkaloids: a competitive enzyme-linked immunoassay (ELISA) for the quantitative determination of some toxic pyrrolizidine alkaloids. Natural Toxins. 3:305–9.

Rogers AE, Newberne PM (1971). Lasiocarpine: Factors influencing its toxicity and effects on liver cell division. Toxicol Appl Pharmacol. 18:356–66.

Roitman JN (1981). Comfrey and liver damage. Lancet. 1:944.

Rollason V, Spahr L, Escher M (2016). Severe liver injury due to a homemade flower pollen preparation in a patient with high CYP3A enzyme activity: a case report. Eur J Clin Pharmacol. 72:507–8.

Ronczka S, These A, Bodi D, Preiß-Weigert A et al. (2105). International collaborative study for the determination of pyrrolizidine alkaloids in honey and herbal tea by SPE-LC-MS/MS. BfR ISBN 078-3-943963-26-7

Roseman DM, Wu X, Kurth MJ (1996). Enzyme-linked immunosorbent assay detection of pyrrolizidine alkaloids: immunogens based on quaternary pyrrolizidinium salts. Bioconjug Chem. 7:187–95.

Roseman DM, Wu X, Milco LA, Bober M, Miller RB, Kurth MJ (1992). Development of a class-specific competitive enzyme-linked immunosorbent assay for the detection of pyrrolizidine alkaloids in vitro. J Agric Food Chem. 40:1008–14.

Roulet M, Laurini R, Revier L, Calame A (1988). Hepatic veno-occlusive disease in newborn infant of a woman drinking herbal tea. J Pediatr. 112:433–6.

Ruan J, Liao C, Ye Y, Lin G (2014a). Lack of metabolic activation and predominant formation of an excreted metabolite of nontoxic platynecine-type pyrrolizidine alkaloids. Chem Res Toxicol. 27:7–16.

Ruan J, Yang M, Fu P, Ye Y, Lin G (2014b). Metabolic activation of pyrrolizidine alkaloids: insights into the structural and enzymatic basis. Chem Res Toxicol. 27:1030–9.

Rubiolo P, Pieters L, Calomme M, Bicchi C, Vlietinck A, VandenBerghe D (1992). Mutagenicity of pyrrolizidine alkaloids in the *Salmonella typhimurium*/mammalian microsome system. Mutat Res. 281:143–7.

Sanderson BJS, Clark AM (1993). Micronuclei in adult and foetal mice exposed in vivo to heliotrine, urethane, monocrotaline and benzidine. Mutat Res. 285:27–33.

Sandini TM, Udo MSB, Reis-Silva TM, Bernardi MM, Spinosa HDS (2014). Prenatal exposure to integerrimine N-oxide impaired the maternal care and the physical and behavioral development of offspring rats. Int J Dev Neurosci. 36:53–63.

Sandini TM, Udo MSB, Reis-Silva TM, Sanches D, Bernardi MM, Flório JC et al. (2015). Prenatal exposure to integerrimine N-oxide enriched butanolic residue from *Senecio brasiliensis* affects behavior and striatal neurotransmitter levels of rats in adulthood. Int J Dev Neurosci. 47:157–64.

Schmeller T, El-Shazly A, Wink M (1997). Allelochemical activities of pyrrolizidine alkaloids: interactions with neuroreceptors and acetylcholine related enzymes. J Chem Ecol 23:399–416.

Schneider J, Tsegaye Y, W/Tensae M, G/Selassie S, Haile T, Bane A et al. (2012). Veno-occlusive liver disease: a case report. Ethiop Med J. 50(Suppl 2):47–51.

Schoch TK, Gardner DR, Stegelmeier BL (2000). GC/MS/MS detection of pyrrolic metabolites in animals poisoned with the pyrrolizidine alkaloid riddelliine. J Nat Toxins. 9:197–206.

Schoental R (1968). Toxicology and carcinogenic action of pyrrolizidine alkaloids. Cancer Res. 28:2237–46 (as cited by EFSA, 2011).

Schroff F, Felgenhauer N, Pfab R, Stenzel J, Eyer F (2013). Acute liver failure after accidental intake of pyrrolizidine-containing plants. XXXIII International Congress of the European Association of Poisons Centres and Clinical Toxicologists (EAPCCT) 28–31 May 2013, Copenhagen, Denmark. Clin Toxicol. 51:264.

Seawright AA (1992). Potential toxicity problems with herbal medicines and food – new observations with pyrrolizidine alkaloids. Proc Nutr Soc Aust. 17:20–25.

Seawright AA (1994). Toxic plant residues in meat, in plant-associated toxins; agricultural, phytochemical and ecological aspects. In: Colegate SM, Dorling PR, editors. Plant-associated toxins: agricultural, phytochemical and ecological aspects. Wallingford: CABI:77–84

Seawright AA, Hrdlicka J, Wright JD, Kerr DR, Mattocks AR, Jukes R (1991a). The identification of hepatotoxic pyrrolizidine alkaloid exposure in horses by the demonstration of sulphur-bound pyrrolic metabolites on their haemoglobin. Vet Hum Toxicol. 33:286–7.

Seawright AA, Kelly WR, Hrdlicka J, McMahon P, Mattocks AR, Jukes R (1991b). Pyrrolizidine alkaloidosis in cattle due to Senecio species in Australia. Vet Rec. 129:198–9.

Sharma S, Prakash AO, Bhatnagar M (2007). Determination of LD50 of monocrotaline through oral route in adult female cyclic rats. J Herbal Med Toxicol. 1:75–6.

Shimshoni JA, Mulder PPJ, Bouznach A, Edery N, Pasval I, Barel S et al. (2015a). *Heliotropium europaeum* poisoning in cattle and analysis of its pyrrolizidine alkaloid profile. J Agric Food Chem. 63:1664–72.

Shimshoni JA, Duebecke A, Mulder PPJ, Cuneah O, Barel S (2015b). Pyrrolizidine and tropane alkaloids in teas and the herbal teas peppermint, rooibos and chamomile in the Israeli market. Food Addit Contam A. 32:2058–2067.

Shubat PJ, Hubbard AK, Huxtable RJ (1989). Dose-response relationship in intoxication by the pyrrolizidine alkaloid monocrotaline. J Toxicol Environ Health. 28:445–60.

Shubat PJ, Bowers RJ, Huxtable RJ (1990). Na+/K(+)-adenosine triphosphatase activity of pulmonary arteries after intoxication with the pyrrolizidine alkaloid monocrotaline. J Pharmacol Exp Ther. 252:70–6.

Skaanild MT, Friis C, Brimer L (2001). Interplant alkaloid variation and *Senecio vernalis* toxicity in cattle. Vet Human Toxicol. 43:147–51.

Smith LW, Culvenor CCJ (1981). Plant sources of hepatotoxic pyrrolizidine alkaloids. J Nat Prod. 44:129–52.

Smith MRW, Stevens KB, Durham AE, Marr CM (2003). Equine hepatic disease: the effect of patient- and case-specific variables on risk and prognosis. Equine Vet J. 35:549–52.

Smith RA, Panariti E (1995). Intoxication of Albanian cattle after ingestion of *Senecio subalpinus*. Vet Hum Toxicol. 37:478–9.

Sofuni T, Honma M, Hayashi M, Shimada H, Tanaka N, Wakuri S et al. (1996). Detection of in vitro clastogens and spindle poisons by the mouse lymphoma assay using the microwell method: interim report of an international collaborative study. Mutagenesis. 11:349–55.

Sommer M (1989). Hepatic veno-occlusive disease and drinking of herbal teas. J Pediatr. 115:659–60.

Soto-Blanco B, Medeiros RMT, Guerra JL, Górniak SL (2001). Lack of protective action of cysteine against the fetotoxic effect of monocrotaline. Food Chem Toxicol. 39:635–9.

Spang R (1989). Toxicity of tea containing pyrrolizidine alkaloids. J Pediatr. 115:1025.

Sperl W, Stuppner H, Gassner I, Judmaier W, Dietze O, Vogel W (1995). Reversible hepatic veno-occlusive disease in an infant after consumption of pyrrolizidine-containing herbal tea. Eur J Pediatr. 154:112–6.

Sriraman PK, Gopal Naidu NR, Raman Rao P (1988). Effect of monocrotaline, a pyrrolizidine alkaloid on the progeny of rats. Indian J Anim Sci. 58:1292–5.

Steenkamp V, Stewart MJ, Zuckerman M (2000). Clinical and analytical aspects of pyrrolizidine poisoning caused by South African traditional medicines. Ther Drug Monit. 22:302–6.

Stegelmeier BL, Gardner DR, James LF, Molyneux RJ (1996). Pyrrole detection and the pathologic progression of *Cynoglossum officinale* (houndstongue) poisoning in horses. J Vet Diagn Invest. 8:81–90.

Stegelmeier BL, Edgar JA, Colegate SM, Gardner DR, Schoch TK, Coulombe RA et al. (1999). Pyrrolizidine alkaloid plants, metabolism and toxicity. J Nat Toxins. 8:95–116.

Stegelmeier BL, James LF, Panter KE, Molyneux RJ, Gardner DR, Schoch TK et al. (2004). Toxicity of a pyrrolizidine alkaloid, riddelliine, in neonatal pigs. In: Acamovic T, Stewart CS, Pennycott TW, editors. Poisonous plants and related toxins. Wallingford: CABI:44–9.

Stelljes ME, Kelley RB, Molyneux RJ, Seiber JN (1991) GC–MS determination of pyrrolizidine alkaloids in four *Senecio* species. J Nat Prod. 54:759–73.

Stigger AL, Estima-Silva P, Fiss L, Coelho ACB, Santos BL, Gardner DR et al. (2014). *Senecio madagascariensis* Poir. (Asteraceae): a new cause of seneciosis in cattle in southern Brazil. Pesq Vet Bras. 34:851–5.

Stillman AE, Huxtable RJ, Consroe P, Kohnen P, Smith S (1977). Hepatic veno-occlusive disease due to pyrrolizidine poisoning in Arizona. Gastroenterology. 7:349–52.

Stroka J, Tamošiūnas V, Mulder PPJ, Mischke C (2013). Report on the 2012 Proficiency Test on pyrrolizidine alkaloids in honey and hay. European Comission, DG Joint Research Centre, Institute for Reference Materials and Measurements (JRC85378). Luxembourg: Publications Office of the European Union.

Sun Y, Hasal SJ, Yan CC, Brendel K, Huxtable RJ (1998). Pyrrolizidine alkaloid-induced depletion of taurine from rat liver in vivo and in vitro. Adv Exp Med Biol. 442:71–7.

Takashima R, Takasawa H, Wako Y, Yasunaga K, Hattori A, Kawabata M et al. (2015). Micronucleus induction in rat liver and bone marrow by acute vs. repeat doses of the genotoxic hepatocarcinogen monocrotaline. Mutat Res Genet Toxicol Environ Mutagen. 780–871:64–70.

Takeuti KL, Bandarra PM, Brum JS, Carvalho KS, Dalto AGC, Raymundo DL et al. (2011). Hepatic biopsy as a diagnostic tool for detecting Senecio spp. poisoning in live cattle. In: Riet-Correa F, Pfister J, Schild AL, Wierenga T, editors. Poisoning by plants, mycotoxins, and related toxins. Wallingford: CABI:194–7.

Tamošiūnas V, Mischke M, Mulder PPJ, Stroka J (2013). Report on the 2012 Proficiency test on pyrrolizidine alkaloids in honey and hay (https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/report-2012-proficiency-test-pyrrolizidine-alkaloids-honey-and-hay).

Tandon BN, Tandon HD, Tandon RK, Narndranathan M, Joshi YK (1976). An epidemic of veno-occlusive disease of liver in central India. Lancet. 7:271–2.

Tang J, Cheng M, Hattori M (2012). Pyrrolizidine alkaloid profile in a traditional Chinese herbal medicine Chuan Zi Wan (Ligulariae Radix et Rhizoma) by liquid chromatography/electrospray ionization ion trap mass spectrometry. Anal Methods. 4:2797–808.

Tepe JJ, Williams RM (1999). DNA cross-linking by a phototriggered dehydromonocrotaline progenitor. J Am Chem Soc. 121:2951–5.

Tepe JJ, Kosogof C, Williams RM (2002). DNA interstrand cross-link formation by reductive activation of dehydropyrrolizidine progenitors. Tetrahedron. 58:3553–9.

Than KA, Stevens V, Knill A, Gallagher PF, Gaul KL, Edgar JA et al. (2005). Plant-associated toxins in animal feed: Screening and confirmation assay development. Anim Feed Sci Technol. 121:5–21.

These A, Bodi D, Ronczka S, Lahrssen-Wiederholt M, Preiss-Weigert A (2013). 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. 405:9375–83.

Thomas HC, Lamé MW, Wilson DW, Segall HJ (1996). Cell cycle alterations associated with covalent binding of monocrotaline pyrrole to pulmonary artery endothelial cell DNA. Toxicol Appl Pharmacol. 141:319–29.

Torous DK, Hall NE, Murante FG, Gleason SE, Tometsko CR, Dertinger SD (2003). Comparative scoring of micronucleated reticulocytes in rat peripheral blood by flow cytometry and microscopy. Toxicol Sci. 74:309–14.

Torres MBAM, Coelho KIR (2008). Experimental poisoning by *Senecio brasiliensis* in calves: quantitative and semi-quantitative study on changes in the hepatic extracellular matrix and sinusoidal cells. Pesq Vet Bras. 28:43–50.

Tropea JE, Molyneux RJ, Kaushal GP, Pan YT, Mitchell M, Elbein AD (1989). Australine, a pyrrolizidine alkaloid that inhibits amyloglucosidase and glycoprotein processing. Biochem. 28:2027–34.

Tu M, Sun S, Wang K, Peng X, Wang R, Li L et al. (2013). Organic cation transporter 1 mediates the uptake of monocrotaline and plays an important role in its hepatotoxicity. Toxicology. 311:225–30.

Tu M, Li L, Lei H, Ma Z, Chen Z, Sun S, et al. (2014). Involvement of organic cation transporter 1 and CYP3A4 in retrorsine-induced toxicity. Toxicol. 322:34–42.

Tu ZB, Konno C, Doel Soejarto D, Waller DP, Bingel AS, Molyneux RJ et al. (1988). Identification of senecionine and senecionine N-oxide as antifertility constituents in Senecio vulgaris. J Pharm Sci. 77:461–3.

Ubiali DG, Boabaid FM, Borges NA, Caldeira FHB, Lodi LR, Pescador CA et al. (2011). Acute poisoning with *Crotalaria spectabilis* (Leg. Papilionoideae) seeds in pigs. Pesq Vet Bras. 31:313–8.

Uhl M, Helma C, Knasmüller S (2000). Evaluation of the single cell gel electrophoresis assay with human hepatoma (Hep G2) cells. Mutat Res. 468:213–25.

Vacillotto G, Favretto D, Seraglia R, Pagiotti R, Traldi P, Mattoli L (2013). A rapid and highly specific method to evaluate the presence of pyrrolizidine alkaloids in *Borago officinalis* seed oil. J Mass Spectrom. 48:1078–82.

Valdivia E, Lalich J, Hayashi Y, Sonnad J (1967a). Alterations in pulmonary alveoli after a single injection of monocrotaline. Arch Pathol. 84:64–76 (as cited by EFSA, 2011).

Valdivia E, Sonnad J, Hayashi Y, Lalich JJ (1967b). Experimental interstitial pulmonary oedema. Angiology. 18:378–83 (as cited by EFSA, 2011).

Valese AC, Molognoni L, de Sá Ploêncio LA, de Lima FG, Gonzaga LV, Górniak SL et al. (2016). A fast and simple LC-ESI-MS/MS method for detecting pyrrolizidine alkaloids in honey with full validation and measurement uncertainty. Food Control. 67:183–91

van Raamsdonk LWD, Ozinga WA, Hoogenboom LAP, Mulder PPJ, Mol JGJ, Groot MJ et al. (2015). Exposure assessment of cattle via roughages to plants producing compounds of concern. Food Chem. 189:27–37.

Van Weeren PR, Morales JA, Rodriguez LL, Cedeño H, Villalobos J, Poveda LJ (1999). Mortality supposedly due to intoxication by pyrrolizidine alkaloids from *Heliotropium indicum* in a horse population in Costa Rica: a case report. Vet Q. 21:59–62.

Vilar JH, García M, Cabrera P (2000). Veno-occlusive liver disease induced by *Senecio vulgaris* toxicity. Gastroenterol Hepatol. 23:285–6 [in Spanish].

Vos JH, Geerts AAJ, Borgers JW, Mars MH, Muskens JAM, van Wuijckhuise-Sjouke LA (2002). Jacobskruiskruid: bedrieglijke schoonheid. Vergiftiging met *Senecio jacobaea*. Tijdschr Diergeneeskd. 127:753–7.

Wachenheim DE, Blythe LL, Craig AM (1992). Characterization of rumen bacterial pyrrolizidine alkaloid biotransformation in ruminants of various species. Vet Human Toxicol. 34:513–7.

Wagner JG, Petry TW, Roth RA (1993). Characterization of monocrotaline pyrrole-induced DNA cross-linking in pulmonary artery endothelium. Am J Physiol. 264:L517–22 (as cited by Chen, Mei & Fu, 2010).

Wang C, Li Y, Gao J, He Y, Xiong A, Yang L et al. (2011). The comparative pharmacokinetics of two pyrrolizidine alkaloids, sencionine and adonifoline, and their main metabolites in rats after intravenous and oral administration by UPLC/ESIMS. Anal Bioanal Chem. 401:275–87.

Wang CC, Xia Q, Meng L, Wang S, Zhao Y, Tolleson WH et al. (2014). Metabolic activation of pyrrolizidine alkaloids leading to phototoxicity and photogenotoxicity in human HaCaT keratinocytes. J Environ Sci Health C. 32:362–84.

Wang J, Yang L, Wang C, Wang Z (2009). The action of cytochrome p450 enzymes and flavin-containing monooxygenases on the N-oxide of pyrrolizidine alkaloid monocrotaline. Asian J Tradi Med. 4(2):41–50.

Wang X, Kanel GC, DeLeve LD (2000). Support of sinusoidal endothelial cell glutathione prevents hepatic veno-occlusive disease in the rat. Hepatology. 31:428–34.

Wang YP, Fu PP, Chou MW (2005). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, retrorsine, leading to DNA adduct formation in vivo. Int J Environ Res Public Health. 2:74–9.

Wang YP, Yan J, Beger RD, Fu PP, Chou MW (2005a). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, monocrotaline, leading to DNA adduct formation in vivo. Cancer Lett. 226:27–35.

Wang YP, Yan J, Fu PP, Chou MW (2005b). Human liver microsomal reduction of pyrrolizidine alkaloid N-oxides to form the corresponding carcinogenic parent alkaloid. Toxicol Lett. 155(3):411–20.

Wang Z, Kang H, Ji L, Yang Y, Liu T, Cao Z et al. (2012). Proteomic characterization of the possible molecular targets of pyrrolizidine alkaloid isoline-induced hepatotoxicity. Env Toxicol Pharmacol. 34:608–17.

Wegge B, van der Heijden S, Vercauteren G, Koen C, Ducatelle R (2009). Decreased hepatic P-glycoprotein expression associated with Senecio intoxication in horses. ESVP/ECVP Proceedings. 141:311.

Weidner MF, Sigurdsson ST, Hopkins PB (1990). Sequence preferences of DNA interstrand cross-linking agents: dG-to-dG cross-linking at 5 '-CG by structurally simplified analogues of mitomycin C. Biochemistry. 29:9225–33.

Weston CF, Cooper BT, Davies JD, Levine DF (1987). Veno-occlusive disease of the liver secondary to ingestion of comfrey. Br Med J (Clin Res Ed). 295:183.

White INH, Mattocks AR, Butler WH (1973). The conversion of the pyrrolizidine alkaloid retrorsine to pyrrolic derivatives in vivo and in vitro and its acute toxicity to various animal species. Chem Biol Interact. 6:207–18 (as cited by EFSA, 2011).

WHO (2013). GEMS/Food Cluster Diets – 2012 (https://extranet.who.int/sree/Reports?op=vs&path=/WHO_HQ_Reports/G7/PROD/EXT/GEMS_cluster_diets_2012&userid=G7_ro&password=inetsoft123).

WHO-IPCS (1988). Pyrrolizidine alkaloids, Geneva: World Health Organization, International Programme on Chemical Safety (Environmental Health Criteria 80; http://www.inchem.org/documents/ehc/ehc/ehc080.htm, accessed 20 December 2018).

WHO-IPCS (2009). Principles and methods for the risk assessment of chemicals in food. Chapter 3: Chemical characterization, analytical methods and the development of specifications (Environmental health criteria 240; http://www.inchem.org/documents/ehc/ehc/ehc240_chapter3.pdf, accessed 20 December 2018).

Williams DE, Reed RL, Kedzierski B, Dannan GA, Guengerich FP, Buhler DR (1989a). Bioactivation and detoxification of the pyrrolizidine alkaloid senecionine by cytochrome P-450 enzymes in rat liver. Drug Metabol Dispo. 17:387–92.

Williams DE, Reed RL, Kedzierski B, Ziegler DM, Buhler DR (1989b). The role of flavin-containing monooxygenase in the N-oxidation of the pyrrolizidine alkaloid senecionine. Drug Metabol Dispo. 17:380–6.

Williams GM, Mori H (1980). Genotoxicity of pyrrolizidine alkaloids in the hepatocyte primary culture/DNA-repair test. Mutat Res. 79:1–5.

Williams L, Chou MW, Yan J, Young JF, Chan PC, Doerge DR (2002). Toxicokinetics of riddelliine, a carcinogenic pyrrolizidine alkaloid, and metabolites in rats and mice. Toxicol Appl Pharmacol. 182:98–104.

Winter H, Seawright AA, Mattocks AR, Jukes R, Tshewang U, Gurung BJ (1990). Pyrrolizidine alkaloid poisoning in yaks. First report and confirmation by identification of sulphur-bound pyrrolic metabolites of the alkaloids in preserved liver tissue. Aust Vet J. 67(11):411–2.

Winter H, Seawright AA, Hrdlicka J, Tshewang U, Gurung BJ (1992). Pyrrolizidine alkaloid poisoning of yaks (*Bos grunniens*) and confirmation by recovery of pyrrolic metabolites form formalin-fixed liver tissue. Res Vet Sci. 52:187–94.

Winter H, Seawright AA, Hrdlicka J, Mattocks AR, Jukes R, Wangdi K et al. (1993). Pyrrolizidine alkaloid poisoning of yaks: diagnosis of pyrrolizidine alkaloid exposure by demonstration of sulphur-conjugated pyrrolic metabolites of the alkaloid in circulating haemoglobin. Aust Vet J. 70:312–3.

Winter H, Seawright AA, Noltie HJ, Mattocks AR ,Jukes R, Wangdi K et al. (1994). Pyrrolizidine alkaloid poisoning of yaks: identification of the plants involved. Vet Rec. 134:135–9.

Winter S, Jung LS, Eckstein RL, Otte A., Donath TW, Kriechbaum M (2014). Control of the toxic plant *Colchicum autumnale* in semi-natural grasslands: effects of cutting treatments on demography and diversity. J Appl Ecol. 51:524–33.

Witt KL, Knapton A, Wehr CM, Hook GJ, Mirsalis J, Shelby MD et al. (2000). Micronucleated erythrocyte frequency in peripheral blood of B6C3F1 mice from short-term, prechronic, and chronic studies of the NTP carcinogenesis bioassay program. Environ Mole Mutag. 36:163–94.

Witte L, Ernst L, Adam H, Hartmann T (1992). Chemotypes of two pyrrolizidine alkaloid-containing Senecio spp. Phytochemistry. 31:559–66.

Witte L, Rubiolo P, Bicchi C, Hartmann T (1993). Comparative analysis of pyrrolizidine alkaloids from natural sources by gas chromatography-mass spectrometry. Phytochemistry. 32:187–96.

Woolford L, Fletcher MT, Boardman WSJ (2014). Suspected pyrrolizidine alkaloid hepatotoxicosis in wild southern hairy-nosed wombats (*Lasiorhinus latifrons*). J Agric Food Chem. 62:7413–8.

Wretensjö I, Karlberg B (2003) Pyrrolizidine alkaloid content in crude and processed borage oil from different processing stages. J Am Oil Chem' Soc. 80:963–70.

Wu JS, Poon WT, Ma CK, Chen ML, Pang KS, Mak TW et al. (2013). Budd-Chiari syndrome secondary to toxic pyrrolizidine alkaloid exposure. Hong Kong Med J. 19:553–5.

Xia Q, Chou MW, Kadlubar FF, Chan PC, Fu PP (2003). Human liver microsomal metabolism and DNA adduct formation of the tumorigenic pyrrolizidine alkaloid, riddelliine. Chem Res Toxicol. 16:66–73.

Xia Q, Chou MW, Lin G, Fu PF (2004). Metabolic formation of DHP-derived DNA adducts from a representative otonecine type pyrrolizidine alkaloid clivorine and the extract of *Ligularia hodgsonnii* Hook. Chem Res Toxicol. 17:702–8

Xia Q, Chou MW, Edgar JA, Doerge DR, Fu PP (2006). Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, lasiocarpine. Cancer Lett. 231:138–45.

Xia Q, Yan J, Chou MW, Fu PP (2008). Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, heliotrine. Toxicol Lett. 178:77–82.

Xia Q, Zhao Y, Von Tungeln LS, Doerge DR, Lin G, Cai L et al. (2013). Pyrrolizidine alkaloid-derived DNA adducts as a common biological biomarker of pyrrolizidine alkaloid-induced tumorigenicity. Chem Res Toxicol. 26:1384–96.

Xia Q, Ma L, He X, Cai L, Fu PP (2015). 7-Glutathione pyrrole adduct: a potential DNA reactive metabolite of pyrrolizidine alkaloids. Chem Res Toxicol. 28:615–20.

Xia Q, Zhao Y, Lin G, Beland FA, Cai L, Fu PP (2016). Pyrrolizidine alkaloid-protein adducts: Potential non-invasive biomarkers of pyrrolizidine alkaloid-induced liver toxicity and exposure. Chem Res Toxicol. 29:1282–92.

Xiong A, Yang L, He Y, Zhang F, Wang J, Han H et al. (2009a). Identification of metabolites of adonifoline, a hepatotoxic pyrrolizidine alkaloid, by liquid chromatography/tandem and high-resolution mass spectrometry. Rapid Comm Mass Spec. 23:3907–16.

Xiong A, Li Y, Yang L, Gao J, He Y, Wang C et al. (2009b). Simultaneous determination of senecionine, adonifoline and their metabolites in rat serum by UPLC-ESIMS and its application in pharmacokinetic studies. J Pharm Biomed Anal. 50:1070–4.

Xiong A, Yang L, Ji L, Wang Z, Yang X, Chen Y et al. (2012). UPLC-MS based metabolomics study on Senecio scandens and S. vulgaris: An approach for the differentiation of two Senecio herbs with similar morphology but different toxicity. Metabolomics. 8:614–23.

Xiong A, Yang F, Fang L, Yang L, He Y, Wan Y-J Y et al. (2014). Metabolomic and genomic evidence for compromised bile acid homeostasis by senecionine, a hepatotoxic pyrrolizidine alkaloid. Chem Res Toxicol. 27:775–86.

Yan CC, Huxtable RJ (1994). Quantitation of the hepatic release of metabolites of the pyrrolizidine alkaloid, monocrotaline. Toxicol Appl Pharmacol. 127:58–63.

Yan CC, Huxtable RJ (1995a). The relationship between the concentration of the pyrrolizidine alkaloid monocrotaline and the pattern of metabolites released from the isolated liver. Toxicol Appl Pharmacol. 130:1–8.

Yan CC, Huxtable RJ (1995b). Relationship between glutathione concentration and metabolism of the pyrrolizidine alkaloid, monocrotaline, in the isolated, perfused liver. Toxicol Appl Pharmacol. 130:132–9.

Yan CC, Huxtable RJ (1995c). The effect of the pyrrolizidine alkaloids, monocrotaline and trichodesmine, on tissue pyrrole binding and glutathione metabolism in the rat. Toxicon. 33:627–34.

Yan CC, Huxtable RJ (1995d) Effect of the pyrrolizidine alkaloid, monocrotaline, on bile composition of the isolated, perfused rat liver. Life Sci. 57:617–26.

Yan CC, Huxtable RJ (1995e). Effects of the pyrrolizidine alkaloid, retrorsine, on sulfur metabolism, in the liver. Proc West Pharmacol Soc. 38:37–40.

Yan CC, Huxtable RJ (1996a). Effects of monocrotaline, a pyrrolizidine alkaloid, on glutathione metabolism in the rat. Biochem Pharmacol. 51:375–9.

Yan CC, Huxtable RJ (1996b). The effect of the hepatotoxic pyrrolizidine alkaloid, retrorsine, on bile composition in the rat in vivo. Proc West Pharmacol Soc. 39:19–22.

Yan J, Nichols J, Yang YC, Fu PP, Chou MW (2002). Detection of riddelliine-derived DNA adducts in blood of rats fed riddelliine. Int J Mol Sci. 3:1019–26.

Yang YC, Yan J, Doerge DR, Chan PC, Fu PP, Chou MW (2001a). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, riddelliine, leading to DNA adduct formation in vivo. Chem Res Toxicol. 14:101–9.

Yang YC, Yan J, Churchwell M, Beger R, Chan P, Doerge DR et al. (2001b). Development of a ^{32}P-postlabeling/HPLC method for detection of dehydroretronecine-derived DNA adducts in vivo and in vitro. Chem Res Toxicol. 14:91–100.

Yang YC, Crowder J, Wardle NJ, Yang L, White KN, Wang ZT et al. (2011). 1H NMR study of monocrotaline and its metabolites in human blood. Food Chem Toxicol. 49:2793–9.

Yang M, Ruan J, Ye Y, Lin G (2013). Intestinal absorption of pyrrolizidine alkaloids and their n-oxides. Drug Metabol Rev. 45(S1):178.

Yeong ML, Wakefield SJ, Ford HC (1993). Hepatocyte membrane injury and bleb formation following low dose comfrey toxicity in rats. Int J Exp Pathol. 74:211–7.

Yeong ML, Swinburn B, Kennedy M, Nicholson G (1990). Hepatic veno-occlusive disease associated with comfrey ingestion. J Gastroenterol Hepatol. 5:211–4.

Yeong ML, Clark SP, Waring JM, Wilson RD, Wakefield SJ (1991). The effects of comfrey derived pyrrolizidine alkaloids on rat liver. Pathology. 23:35–8.

Yu LJ, Li SFY (2005). Dynamic pH junction-sweeping capillary electrophoresis for online preconcentration of toxic pyrrolizidine alkaloids in Chinese herbal medicine. Electrophoresis. 26:4360–7.

Yu LJ, Xu Y, Feng HT, Li SFY (2005). Separation and determination of toxic pyrrolizidine alkaloids in traditional Chinese herbal medicines by micellar electrokinetic chromatography with organic modifier. Electrophoresis. 26:3397–404

Zeng G, Liu R, Liao H, Zhang X, Qian Y, Liu B et al. (2013). Single intraperitoneal injection of monocrotaline as a novel large animal model of chronic pulmonary hypertension in Tibet minipigs. PLoS ONE 8e78965.

Zhang F, Wang CH, Xiong AZ, Wang W, Yang L, Branford-White CJ et al. (2007). Quantitative analysis of total retronecine esters-type pyrrolizidine alkaloids in plant by high performance liquid chromatography. Anal. Chim. Acta. 605:94–101.

Zhao Y, Liang A, Liu T, Li C, Wang X, Yi Y et al. (2010). Study on embryonic toxicity of *Senecio scandens*, Qianbai Biyanpian and total alkaloid from S. scandens in rats. China J Chinese Materia Medica. 35:373–7 [in Chinese].

Zhao Y, Xia Q, Yin JJ, Lin G, Fu PP (2011). Photoirradiation of dehydropyrrolizidine alkaloids – Formation of reactive oxygen species and induction of lipid peroxidation. Toxicol Lett. 205:302–9.

Zhao Y, Xia Q, da Costa GG, Yu H, Cai L, Fu PP (2012). Full structure assignments of pyrrolizidine alkaloid DNA adducts and mechanism of tumor initiation. Chem Res Toxicol. 25:1985–96.

Zhao Y, Wang S, Xia Q, Gamboa da Costa G, Doerge DR, Cai L et al. (2014). Reaction of dehydropyrrolizidine alkaloids with valine and hemaglobin. Chem Res Toxicol. 27:1720–31.

Zhou H, Wang YX, Lou HY, Xu XJ, Zhang MM (2014). Hepatic sinusoidal obstruction syndrome caused by herbal medicine: CT and MRI features. Korean J Radiol. 15:218–25.

Zhou Y, Li N, Choia FFK, Qiao CF, Songa JZ, Li SL et al. (2010). A new approach for simultaneous screening and quantification of toxic pyrrolizidine alkaloids in some potential pyrrolizidine alkaloid-containing plants by using ultra performance liquid chromatography-tandem quadrupole mass spectrometry. Anal Chim Acta. 681:33–40.

Zhu L, Xue J, Xia Q, Fu PP, Lin G (2017). The long persistence of pyrrolizidine alkaloid-derived DNA adducts in vivo: kinetic study following single and multiple exposures in male ICR mice. Arch Toxicol Arch Toxicol. 91:949–65.

Zuckerman M, Steenkamp V, Stewart MJ (2002). Hepatic veno-occlusive disease as a result of a traditional remedy: confirmation of toxic pyrrolizidine alkaloids as the cause, using an in vitro technique. J Clin Pathol. 55:676–9.

Zündorf I, Wiedenfeld H, Röder E, Dingermann T (1998). Generation and characterization of monoclonal antibodies against the pyrrolizidine alkaloid retrorsine. Planta Medica. 64:259–63.

Annex 1

Reports and other documents resulting from previous meetings of the Joint FAO/WHO Expert Committee on Food Additives

1. General principles governing the use of food additives (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).

2. Procedures for the testing of intentional food additives to establish their safety for use (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).

3. Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants) (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. I. Antimicrobial preservatives and antioxidants, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).

4. Specifications for identity and purity of food additives (food colours) (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. II. Food colours, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).

5. Evaluation of the carcinogenic hazards of food additives (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).

6. Evaluation of the toxicity of a number of antimicrobials and antioxidants (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).

7. Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).

8. Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).

9. Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).

10. Specifications for identity and purity and toxicological evaluation of food colours. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.

11. Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).

12. Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases. FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.

13. Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.

14. Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non-nutritive sweetening agents (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.

15. Toxicological evaluation of some flavouring substances and non-nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.

16. Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.

17. Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.

18. Specifications for the identity and purity of some antibiotics. FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.

19. Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.

20. Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances. FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.

21. Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives. FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.

22. Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.

23. Toxicological evaluation of some extraction solvents and certain other substances. FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.

24. Specifications for the identity and purity of some extraction solvents and certain other substances. FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.

25. A review of the technological efficacy of some antimicrobial agents. FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.

26. Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some

antioxidants (Fifteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.

27. Toxicological evaluation of some enzymes, modified starches, and certain other substances. FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.

28. Specifications for the identity and purity of some enzymes and certain other substances. FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.

29. A review of the technological efficacy of some antioxidants and synergists. FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.

30. Evaluation of certain food additives and the contaminants mercury, lead, and cadmium (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.

31. Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate. FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.

32. Toxicological evaluation of certain food additives with a review of general principles and of specifications (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).

33. Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents. FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.

34. Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers. FAO Food and Nutrition Paper, No. 4, 1978.

35. Evaluation of certain food additives (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.

36. Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives. FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.

37. Specifications for the identity and purity of some food colours, enhancers, thickening agents, and certain food additives. FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.

38. Evaluation of certain food additives: some food colours, thickening agents, smoke condensates, and certain other substances (Nineteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 55, 1975; WHO Technical Report Series, No. 576, 1975.

39. Toxicological evaluation of some food colours, thickening agents, and certain other substances. FAO Nutrition Meetings Report Series, No. 55A, 1975; WHO Food Additives Series, No. 8, 1975.

40. Specifications for the identity and purity of certain food additives. FAO Nutrition Meetings Report Series, No. 55B, 1976; WHO Food Additives Series, No. 9, 1976.

41. Evaluation of certain food additives (Twentieth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Food and Nutrition Meetings Series, No. 1, 1976; WHO Technical Report Series, No. 599, 1976.

42. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 10, 1976.

43. Specifications for the identity and purity of some food additives. FAO Food and Nutrition Series, No. 1B, 1977; WHO Food Additives Series, No. 11, 1977.

44. Evaluation of certain food additives (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.

45. Summary of toxicological data of certain food additives. WHO Food Additives Series, No. 12, 1977.

46. Specifications for identity and purity of some food additives, including antioxidants, food colours, thickeners, and others. FAO Nutrition Meetings Report Series, No. 57, 1977.

47. Evaluation of certain food additives and contaminants (Twenty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 631, 1978.

48. Summary of toxicological data of certain food additives and contaminants. WHO Food Additives Series, No. 13, 1978.

49. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 7, 1978.

50. Evaluation of certain food additives (Twenty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 648, 1980, and corrigenda.

51. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 14, 1980.

52. Specifications for identity and purity of food colours, flavouring agents, and other food additives. FAO Food and Nutrition Paper, No. 12, 1979.

53. Evaluation of certain food additives (Twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 653, 1980.

54. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 15, 1980.

55. Specifications for identity and purity of food additives (sweetening agents, emulsifying agents, and other food additives). FAO Food and Nutrition Paper, No. 17, 1980.

56. Evaluation of certain food additives (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.

57. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 16, 1981.

58. Specifications for identity and purity of food additives (carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents, and other food additives). FAO Food and Nutrition Paper, No. 19, 1981.

59. Evaluation of certain food additives and contaminants (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.

60. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 17, 1982.

61. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 25, 1982.

62. Evaluation of certain food additives and contaminants (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983, and corrigenda.

63. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 18, 1983.

64. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 28, 1983.

65. Guide to specifications – General notices, general methods, identification tests, test solutions, and other reference materials. FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.

66. Evaluation of certain food additives and contaminants (Twenty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 710, 1984, and corrigendum.

67. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 19, 1984.

68. Specifications for the identity and purity of food colours. FAO Food and Nutrition Paper, No. 31/1, 1984.

69. Specifications for the identity and purity of food additives. FAO Food and Nutrition Paper, No. 31/2, 1984.

70. Evaluation of certain food additives and contaminants (Twenty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 733, 1986, and corrigendum.

71. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 34, 1986.

72. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 20. Cambridge University Press, 1987.

73. Evaluation of certain food additives and contaminants (Thirtieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 751, 1987.

74. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 21. Cambridge University Press, 1987.

75. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 37, 1986.

76. Principles for the safety assessment of food additives and contaminants in food. WHO Environmental Health Criteria, No. 70. Geneva, World Health Organization, 1987 (out of print). The full text is available electronically at www.who.int/pcs.

77. Evaluation of certain food additives and contaminants (Thirty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 759, 1987, and corrigendum.

78. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 22. Cambridge University Press, 1988.

79. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 38, 1988.

80. Evaluation of certain veterinary drug residues in food (Thirty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 763, 1988.

81. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 23. Cambridge University Press, 1988.

82. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41, 1988.

83. Evaluation of certain food additives and contaminants (Thirty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 776, 1989.

84. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 24. Cambridge University Press, 1989.

85. Evaluation of certain veterinary drug residues in food (Thirty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 788, 1989.

86. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 25, 1990.

87. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/2, 1990.

88. Evaluation of certain food additives and contaminants (Thirty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 789, 1990, and corrigenda.

89. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 26, 1990.

90. Specifications for identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 49, 1990.

91. Evaluation of certain veterinary drug residues in food (Thirty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 799, 1990.

92. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 27, 1991.

93. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/3, 1991.

94. Evaluation of certain food additives and contaminants (Thirty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 806, 1991, and corrigenda.

95. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 28, 1991.

96. Compendium of food additive specifications (Joint FAO/WHO Expert Committee on Food Additives (JECFA)). Combined specifications from 1st through the 37th meetings, 1956–1990. Rome, Food and Agriculture Organization of the United Nations, 1992 (2 volumes).

97. Evaluation of certain veterinary drug residues in food (Thirty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 815, 1991.

98. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 29, 1991.

99. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/4, 1991.

100. Guide to specifications – General notices, general analytical techniques, identification tests, test solutions, and other reference materials. FAO Food and Nutrition Paper, No. 5, Rev. 2, 1991.

101. Evaluation of certain food additives and naturally occurring toxicants (Thirty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 828, 1992.

102. Toxicological evaluation of certain food additives and naturally occurring toxicants. WHO Food Additives Series, No. 30, 1993.

103. Compendium of food additive specifications: addendum 1. FAO Food and Nutrition Paper, No. 52, 1992.

104. Evaluation of certain veterinary drug residues in food (Fortieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 832, 1993.

105. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 31, 1993.

106. Residues of some veterinary drugs in animals and food. FAO Food and Nutrition Paper, No. 41/5, 1993.

107. Evaluation of certain food additives and contaminants (Forty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 837, 1993.

108. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 32, 1993.

109. Compendium of food additive specifications: addendum 2. FAO Food and Nutrition Paper, No. 52, Add. 2, 1993.

110. Evaluation of certain veterinary drug residues in food (Forty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 851, 1995.

111. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 33, 1994.

112. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/6, 1994.

113. Evaluation of certain veterinary drug residues in food (Forty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 855, 1995, and corrigendum.

114. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 34, 1995.

115. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/7, 1995.

116. Evaluation of certain food additives and contaminants (Forty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 859, 1995.

117. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 35, 1996.

118. Compendium of food additive specifications: addendum 3. FAO Food and Nutrition Paper, No. 52, Add. 3, 1995.

119. Evaluation of certain veterinary drug residues in food (Forty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 864, 1996.

120. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 36, 1996.

121. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/8, 1996.

122. Evaluation of certain food additives and contaminants (Forty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 868, 1997.

123. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 37, 1996.

124. Compendium of food additive specifications, addendum 4. FAO Food and Nutrition Paper, No. 52, Add. 4, 1996.

125. Evaluation of certain veterinary drug residues in food (Forty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 876, 1998.

126. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 38, 1996.

127. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/9, 1997.

128. Evaluation of certain veterinary drug residues in food (Forty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 879, 1998.

129. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 39, 1997.

130. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/10, 1998.

131. Evaluation of certain food additives and contaminants (Forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 884, 1999.

132. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 40, 1998.

133. Compendium of food additive specifications: addendum 5. FAO Food and Nutrition Paper, No. 52, Add. 5, 1997.

134. Evaluation of certain veterinary drug residues in food (Fiftieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 888, 1999.

135. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 41, 1998.

136. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/11, 1999.

137. Evaluation of certain food additives (Fifty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 891, 2000.

138. Safety evaluation of certain food additives. WHO Food Additives Series, No. 42, 1999.

139. Compendium of food additive specifications, addendum 6. FAO Food and Nutrition Paper, No. 52, Add. 6, 1998.

140. Evaluation of certain veterinary drug residues in food (Fifty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 893, 2000.

141. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 43, 2000.

142. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/12, 2000.

143. Evaluation of certain food additives and contaminants (Fifty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 896, 2000.

144. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 44, 2000.

145. Compendium of food additive specifications, addendum 7. FAO Food and Nutrition Paper, No. 52, Add. 7, 1999.

146. Evaluation of certain veterinary drug residues in food (Fifty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 900, 2001.

147. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 45, 2000.

148. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/13, 2000.

149. Evaluation of certain food additives and contaminants (Fifty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 901, 2001.

150. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 46, 2001.

151. Compendium of food additive specifications: addendum 8. FAO Food and Nutrition Paper, No. 52, Add. 8, 2000.

152. Evaluation of certain mycotoxins in food (Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 906, 2002.

153. Safety evaluation of certain mycotoxins in food. WHO Food Additives Series, No. 47/FAO Food and Nutrition Paper, No. 74, 2001.

154. Evaluation of certain food additives and contaminants (Fifty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 909, 2002.

155. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 48, 2002.

156. Compendium of food additive specifications: addendum 9. FAO Food and Nutrition Paper, No. 52, Add. 9, 2001.

157. Evaluation of certain veterinary drug residues in food (Fifty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 911, 2002.

158. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 49, 2002.

159. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/14, 2002.

160. Evaluation of certain food additives and contaminants (Fifty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 913, 2002.

161. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 50, 2003.

162. Compendium of food additive specifications: addendum 10. FAO Food and Nutrition Paper, No. 52, Add. 10, 2002.

163. Evaluation of certain veterinary drug residues in food (Sixtieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 918, 2003.

164. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 51, 2003.

165. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/15, 2003.

166. Evaluation of certain food additives and contaminants (Sixty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 922, 2004.

167. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 52, 2004.

168. Compendium of food additive specifications: addendum 11. FAO Food and Nutrition Paper, No. 52, Add. 11, 2003.

169. Evaluation of certain veterinary drug residues in food (Sixty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 925, 2004.

170. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/16, 2004.

171. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 53, 2005.

172. Compendium of food additive specifications: addendum 12. FAO Food and Nutrition Paper, No. 52, Add. 12, 2004.

173. Evaluation of certain food additives (Sixty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 928, 2005.

174. Safety evaluation of certain food additives. WHO Food Additives Series, No. 54, 2005.

175. Compendium of food additive specifications: addendum 13. FAO Food and Nutrition Paper, No. 52, Add. 13 (with Errata), 2005.

176. Evaluation of certain food contaminants (Sixty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 930, 2005.

177. Safety evaluation of certain contaminants in food. WHO Food Additives Series, No. 55/FAO Food and Nutrition Paper, No. 82, 2006.

178. Evaluation of certain food additives (Sixty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 934, 2006.

179. Safety evaluation of certain food additives. WHO Food Additives Series, No. 56, 2006.

180. Combined compendium of food additive specifications. FAO JECFA Monographs 1, Volumes 1–4, 2005, 2006.

181. Evaluation of certain veterinary drug residues in food (Sixty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 939, 2006.

182. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 2, 2006.

183. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 57, 2006.

184. Evaluation of certain food additives and contaminants (Sixty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 940, 2007.

185. Compendium of food additive specifications. FAO JECFA Monographs 3, 2006.

186. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 58, 2007.

187. Evaluation of certain food additives and contaminants (Sixty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 947, 2007.

188. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 59, 2008.

189. Compendium of food additive specifications. FAO JECFA Monographs 4, 2007.

190. Evaluation of certain food additives (Sixty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 952, 2009.

191. Safety evaluation of certain food additives. WHO Food Additives Series, No. 60, 2009.

192. Compendium of food additive specifications. FAO JECFA Monographs 5, 2009.

193. Evaluation of certain veterinary drug residues in food (Seventieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 954, 2009.

194. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 61, 2009.

195. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 6, 2009.

196. Evaluation of certain food additives (Seventy-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 956, 2010.

197. Safety evaluation of certain food additives. WHO Food Additives Series, No. 62, 2010.

198. Compendium of food additive specifications. FAO JECFA Monographs 7, 2009.

199. Evaluation of certain contaminants in food (Seventy-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 959, 2011.

200. Safety evaluation of certain contaminants in food. WHO Food Additives Series, No. 63/FAO JECFA Monographs 8, 2011.

201. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 9, 2010.

202. Evaluation of certain food additives and contaminants (Seventy-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 960, 2011.

203. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 64, 2011.

204. Compendium of food additive specifications. FAO JECFA Monographs 10, 2010.

205. Evaluation of certain food additives and contaminants (Seventy-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 966, 2011.

206. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 65, 2011.

207. Compendium of food additive specifications. FAO JECFA Monographs 11, 2011.

208. Evaluation of certain veterinary drug residues in food (Seventy-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 969, 2012.

209. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 66, 2012.

210. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 12, 2012.

211. Evaluation of certain food additives (Seventy-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 974, 2012.

212. Safety evaluation of certain food additives. WHO Food Additives Series, No. 67, 2012.

213. Compendium of food additive specifications. FAO JECFA Monographs 13, 2012.

214. Evaluation of certain food additives and contaminants (Seventy-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 983, 2013.

215. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 68, 2013.

216. Compendium of food additive specifications. FAO JECFA Monographs 14, 2013.

217. Evaluation of certain veterinary drug residues in food (Seventy-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 988, 2014.

218. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 69, 2014.

219. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 15, 2014.

220. Evaluation of certain food additives (Seventy-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 990, 2015.

221. Safety evaluation of certain food additives. WHO Food Additives Series, No. 70, 2015.

222. Compendium of food additive specifications. FAO JECFA Monographs 16, 2014.

223. Evaluation of certain food additives and contaminants (Eightieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 995, 2016.

224. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 71, 2015.

225. Compendium of food additive specifications. FAO JECFA Monographs 17, 2015.

226. Evaluation of certain veterinary drug residues in food (Eighty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 997, 2016.

227. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 72, 2016.

228. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 18, 2016.

229. Safety evaluation of certain food additives and contaminants. Supplement 1: Non-dioxin-like polychlorinated biphenyls. WHO Food Additives Series, No. 71-1, 2016.

230. Evaluation of certain food additives (Eighty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1000, 2016.

231. Compendium of food additive specifications. FAO JECFA Monographs 19, 2016.

232. Safety evaluation of certain food additives. WHO Food Additives Series, No. 73, 2017.

233. Evaluation of certain contaminants in food (Eighty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1002, 2017.

234. Evaluation of certain food additives (Eighty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1007, 2017.

235. Safety evaluation of certain contaminants in food. WHO Food Additives Series, No. 74, FAO JECFA Monographs 19 bis, 2018.

236. Compendium of food additive specifications. FAO JECFA Monographs 20, 2017.

237. Safety evaluation of certain food additives. WHO Food Additives Series, No. 75, 2019.

238. Evaluation of certain veterinary drug residues in food (Eighty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1008, 2018.

239. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 21, 2018.

240. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 76, 2019 (in press).

241. Evaluation of certain food additives (Eighty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1014, 2019.

242. Safety evaluation of certain food additives. WHO Food Additives Series, No. 77, 2020 (in press).

243. Compendium of food additive specifications. FAO JECFA Monographs 22, 2018.

Annex 2

Participants in the eightieth meeting of the Joint FAO/WHO Expert Committee on Food Additives[1]

Rome, 16–25 June 2015

Members

Dr D. Benford, Risk Assessment Unit, Science Evidence and Research Division, Food Standards Agency, London, England, United Kingdom

Dr M. DiNovi, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, MD, USA

Dr M. Feeley, Bureau of Chemical Safety, Food Directorate, Health Canada, Ottawa, Ontario, Canada

Dr D. Folmer, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, MD, USA

Dr Y. Kawamura, Division of Food Additives, National Institute of Health Sciences, Tokyo, Japan

Dr Madduri Veerabhadra Rao, Quality Control Department, Department of the President's Affairs, Al Ain, United Arab Emirates

Dr A. Mattia, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, MD, USA (*Vice-Chairperson*)

Mrs I. Meyland, Birkerød, Denmark (*Chairperson*)

Dr U. Mueller, Food Standards Australia New Zealand, Barton, ACT, Australia (*Joint Rapporteur*)

Dr J. Schlatter, Zurich, Switzerland

Mrs H. Wallin, Helsinki, Finland (*Joint Rapporteur*)

Professor G.M. Williams, Department of Pathology, New York Medical College, Valhalla, NY, USA

Secretariat

Dr A. Agudo, Unit of Nutrition and Cancer, Cancer Epidemiology Research Program, Institut Català d'Oncologia, L'Hospitalet de Llobregat, Spain (*WHO Expert*)

[1] Participants marked with an asterisk (*) did not attend the entire meeting.

Dr B. Amzal*, LASER, United Kingdom Headquarters, London, England, United Kingdom (*WHO Expert*)

Dr J.H. Andersen, National Food Institute, Technical University of Denmark, Søborg, Denmark (*WHO Expert*)

Dr S. Barlow, Brighton, East Sussex, England, United Kingdom (*WHO Expert*)

Ms A. Bruno, Joint FAO/WHO Food Standards Programme, Food and Agriculture Organization of the United Nations, Rome, Italy (*Codex Secretariat*)

Ms A.S. Bulder, Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands (*WHO Expert*)

Dr S. Cahill, Food Safety and Quality Unit, Agriculture and Consumer Protection Department, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Secretariat*)

Dr R. Cantrill, AOCS, Urbana, IL, USA (*FAO Expert*)

Mr P. Cressey, Risk and Response Group, ESR (Institute of Environmental Science and Research Ltd), Christchurch, New Zealand (*FAO Expert*)

Dr M. De Nijs, RIKILT Wageningen UR, Wageningen, the Netherlands (*FAO Expert*)

Dr E. Dessipri, General Chemical State Laboratory, Athens, Greece (*FAO Expert*)

Dr J.A. Edgar, CSIRO Food and Nutritional Sciences, North Ryde, NSW, Australia (*FAO Expert*)

Dr V. Fattori, Agriculture and Consumer Protection Department, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Joint Secretary*)

Professor H. Håkansson, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden (*WHO Expert*)

Ms T. Hambridge, Food Data Analysis Section, Food Standards Australia New Zealand, Barton, ACT, Australia (*WHO Expert*)

Dr S.M.F. Jeurissen, Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands (*WHO Expert*)

Professor F. Kayama, Department of Environmental & Preventive Medicine, School of Medicine, Jichi Medical University, Yakushiji, Shimotsuke-shi, Tochigi-ken, Japan (*WHO Expert*)

Mr J. Kim, Department of Food Safety and Zoonoses, World Health Organization, Geneva, Switzerland (*WHO Secretariat*)

Dr J.C. Leblanc, Food Safety and Quality Unit, Agriculture and Consumer Protection Department, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Secretariat*)

Dr T. Rawn, Food Research Division, Health Canada, Ottawa, Ontario, Canada (*FAO Expert*)

Dr K. Schneider, Forschungs- und Beratungsinstitut Gefahrstoffe GmbH (FoBiG), Freiburg, Germany (*WHO Expert*)

Ms M. Sheffer, Orleans, Ontario, Canada (*WHO Technical Editor*)

Dr J.R. Srinivasan, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, MD, USA (*FAO Expert*)

Professor I. Stankovic, Department of Bromatology, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia (*FAO Expert*)

Dr A. Tritscher, Department of Food Safety and Zoonoses, World Health Organization, Geneva, Switzerland (*WHO Joint Secretary*)

Dr T. Umemura, Division of Pathology, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan (*WHO Expert*)

Professor Dr M. Van den Berg*, Toxicology and Veterinary Pharmacology Division, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands (*WHO Expert*)

Dr Y. Wu, China National Center for Food Safety Risk Assessment, Beijing, China (*FAO Expert*)

Dr X. Yang, Guangdong Provincial Center for Disease Control and Prevention, Guangzhou, Guangdong Province, China (*WHO Expert*)

Dr H.J. Yoon, Food Contaminants Division, Food Safety Evaluation Department, Ministry of Food and Drug Safety, Cheongwon-gun, Chungcheongbuk-do, Republic of Korea (*FAO Expert*)

Dr Y. Zang, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, MD, USA (*WHO Expert*)

Annex 3

Abbreviations used in the monographww

ACTH	adrenocorticotropic hormone
AL	acetyl-lycopsamine
ALT	alanine aminotransferase
ALP	alkaline phosphatase
ALV	African leafy vegetables
APCI	atmospheric pressure chemical ionization
AST	aspartate transaminase or aspartate aminotransferase
AUC	area under the curve
BCRP	breast cancer resistance protein
BMD	benchmark dose
BMDS	United States Environmental Protection Agency (US EPA) Benchmark Dose Software
BMD_{10}	benchmark dose for a 10% response
BMDL	lower 95% confidence limit on the benchmark dose
$BMDL_{10}$	lower 95% confidence limit on the benchmark dose for a 10% response
BMR	benchmark response
BR	butanolic residue
CCCF	Codex Committee on Contaminants in Foods
CE	capillary electrophoresis
CFSAN	Center for Food Safety and Applied Nutrition
CID	collision-induced dissociation
CK	creatine kinase
CPN	chronic progressive nephropathy
CSIRO	Commonwealth Scientific and Industrial Research Organization
CT	computed tomography
CYP	cytochrome P450
DHP	6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine
DHPA	dehydropyrrolizidine alkaloid
DPC	proteinase-sensitive cross-links
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EROS	Early Review Organizing Software
ESI	electrospray ionization
FDA	(US) Food and Drug Administration

FSANZ	Food Standards Australia New Zealand
GC	gas chromatography
GC-MS	gas chromatography – mass spectrometry
GEMS/Food	Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme
GGT	gamma-glutamyl transaminase
GLE	gestation and lactation
GR	GSH reductase
GSH	glutathione
GST	glutathione-S-transferase
HC	*Heliotropium circinatum*
HCD	historical control data
HD	*Heliotropium dolosum*
HPLC	high-performance liquid chromatography
HPLC-MS/MS	high-performance liquid chromatography – tandem mass spectrometry
HSA	human serum albumin
HSOS	hepatic sinusoidal obstruction syndrome
HVOD	hepatic veno-occlusive disease
IARC	International Agency for Research on Cancer
IPCS	International Programme on Chemical Safety
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KLH	keyhole limpet haemocyanin
LB	lower bound
LC	liquid chromatography
LC-MS/MS	liquid chromatography – tandem mass spectrometry
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LLC	Lewis lung carcinoma
LOAEL	lowest-observed-adverse-effect level
LPS	lipopolysaccharides
LOD	limit of detection
LOQ	limit of quantification
MeSH	Medical Subject Headings
MOE	margin of exposure
MRI	magnetic resonance imaging
MRM	multiple reaction monitoring
MS	mass spectrometry
NA	not applicable
NaOH	sodium hydroxide
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)

NCI	National Cancer Institute (USA)
NMR	nuclear magnetic resonance
NOAEL	no-observed-adverse-effect level
NR	not reported
NS	not significant
NTP	National Toxicology Program (USA)
PA	pyrrolizidine alkaloid
PE	paper electrophoresis
PH	pulmonary hypertension
PMA	phorbol myristate acetate
PXR	pregnane X receptor
RB	rice bran
RBC	red blood cell
RIVM	Dutch National Institute for Public Health and the Environment
SD	standard deviation
SOD	superoxide dismutase
SOS	sinusoidal obstruction syndrome
SPE	solid-phase extraction
SPF	specific pathogen-free
SPS	S-phase synthesis
SR	systematic review
SV	*Senecio vernalis*
SYRCLE	Systematic Review Center for Laboratory Animal Experimentation
THIE	Tea & Herbal Infusions Europe
TLC	thin-layer chromatography
UDS	unscheduled DNA synthesis
UHPLC	ultra-high-performance liquid chromatography
UV	ultraviolet
UVA	ultraviolet A (radiation from about 320 to 400 nm in wavelength)
VEGF	vascular endothelial growth factor
VOD	veno-occlusive liver disease
WHO	World Health Organization

Annex 4

Systematic review protocol

Format by SYRCLE (www.syrcle.nl)
Version 2.0 (December 2014)

Item #	Section/Subsection/Item	Description	Check for approval
A. General			
1.	Title of the review	The toxic effects and biochemical aspects of pyrrolizidine alkaloids for use in JECFA risk assessment	
2.	Authors (names, affiliations, contributions)	Mw. Ir. A.S. Bulder (RIVM, project leader, reviewer) Mw. L. de Wit-Bos MSc. (RIVM, reviewer) Dr. R. de Vries (SYRCLE, Systematic Review specialist)	
3.	Other contributors (names, affiliations, contributions)	Dr. C. Graven (RIVM, reviewer) Ir. J.P. Ridder-Kools (RIVM, Information specialist) F. Geesink (SYRCLE, student assistant) A. Tosserams (SYRCLE, student assistant) T. Bolhuis (SYRCLE, student assistant)	
4.	Contact person + e-mail address	Astrid Bulder (astrid.bulder@rivm.nl)	
5.	Funding sources/sponsors	No sponsor (topic is contaminants), work is funded by the government of The Netherlands, ministry of Economic Affairs.	
6.	Conflicts of interest	None	
7.	Date and location of protocol registration		
8.	Registration number (if applicable)		
9.	Stage of review at time of registration	Searches + screening on title/abstract complete	
B. Objectives			
	Background		
10.	What is already known about this disease/ model/intervention? Why is it important to do this review?	Pyrrolizidine alkaloids (PAs) are natural occurring toxins. Their presence is confirmed in a wide variety of plants. PAs are probably the most widely distributed natural toxins that can affect wildlife, livestock and humans. The main target organ of PA toxicity is the liver, which may result in various degrees of progressive liver damage and veno-occlusive disease. Furthermore, riddelliine, lasiocarpine, and monocrotaline are classified as 'possibly carcinogenic to humans' (IARC Classification, Group 2B). Humans are exposed to PAs via the consumption of PA-contaminated food. Direct human poisoning via food is reported for 1) the direct and deliberate use of toxic plant species as herbal teas or traditional medicines, 2) the consumption of grain or grain products (i.e. flour or bread) contaminated with PA-containing seeds, 3) the intake of (drinking) water from wells surrounded with PA-containing plants, 4) the intake of foods prepared from agricultural crops (by contamination with plant parts that contain PAs), 5) the intake of honey, 6) intake of products from animal origin via transfer of PAs from feed to edible animal products. Direct animal poisoning is reported for animals consuming PA containing plants due to feed scarcity.	

Table (continued)

Item #	Section/Subsection/Item	Description	Check for approval
		Due to the serious threat PAs may pose to human (and animal) health all over the world, it is important to identify which PAs are most relevant with respect to toxicity and occurrence and to perform a full risk assessment. In addition, it is important to define the extent to which consumption of PA-contaminated feed by food producing animals contributes to human health risks, and identify critical data gaps. This systematic review will help to identify all relevant literature needed for addressing these topics. More specific, the systematic review will contribute to sections 2 and 9 of the JECFA Monograph.	
Research question			
11.	Specify the disease/health problem of interest	Toxic effects including progressive liver damage and acute veno-occlusive disease	
12.	Specify the population/species studied	1) In vitro systems 2) Animals 3) Humans	
13.	Specify the intervention/exposure	Pyrrolizidine alkaloids	
14.	Specify the control population	Not defined	
15.	Specify the outcome measures	1) Biochemical aspects 2) Toxic effects	
16.	State your research question (based on items 11-15)	1) What are the biochemical aspects of pyrrolizidine alkaloids in in vitro systems? 2) What are the biochemical aspects of pyrrolizidine alkaloids in animals? 3) What are the biochemical aspects of pyrrolizidine alkaloids in humans? 4) What are the toxic effects of pyrrolizidine alkaloids in in vitro systems? 5) What are the toxic effects of pyrrolizidine alkaloids in animals? 6) What are the toxic effects of pyrrolizidine alkaloids in humans?	
C. Methods			
Search and study identification			
17.	Identify literature databases to search	**X**MEDLINE via Ovid SP & PubMed **X**Scisearch via Web of Science **X**SCOPUS **X**EMBASE **X**Other, namely: Toxcenter, CAB abstracts, FSTA	
18.	Define electronic search strategies	Medline search strategy via OvidSP in Annex 1. Included in the search strategy are also two searches (nr 59 and 63 in Annex 1) using a combination of the Exposure and Outcome measure search terms (and not Population), resulting in two additional 'rest' Endnote databases consisting of references that may be relevant but were not indexed according to population. These additional references will be checked and manually assigned to one of the six research questions. The Medline search strategy for use via OvidSP is translated to strategies for searching SCOPUS, EMBASE, CAB abstracts and Toxcenter. The databases FSTA, Scisearch (via Web of Science), and	

Item #	Section/Subsection/Item	Description	Check for approval
		Medline via Pubmed (only last two years) are searched using the overall term 'pyrrolizidine alkaloids' in combination with the text search terms used in the Medline search strategy (thus without the MeSH terms).	
19.	Identify other sources for study identification	**X** Other, namely: Grey literature data sources (Annex 2)	
20.	Define search strategy for these other sources	The search strategy for finding grey literature will be simpler, due to practical limitations of the data sources. The grey literature search will consist of searching on the term pyrrolizidine alkaloids, individual PAs and plant names. The search strategy has been piloted for possible refinement. The first plan was to search all databases and websites identified as potential sources for grey literature using the general term 'pyrrolizidine alkaloids', all known PA names and plant names (see Annex 3). As this turned out to be too time consuming, it was later on decided to prioritize databases and websites (Annex 2). Furthermore, it was decided that the data sources of high priority will be first searched with the term pyrrolizidine alkaloids followed by 9 PA names (representing the four PA bases and known occurrence in foods) and 3 plant names (plant names expected to give hits) as a test. Thereafter, a selection of the data sources with lower priority will be screened for relevant information by searching only on the term pyrrolizidine alkaloids (or synonyms/other languages). It was decided not to search in the last group of data sources as these were expected not to generate any more relevant references in addition to the first two groups of data sources. The 9 PAs and 3 plant names selected are angeloylpetasinecine, echimidine, heliotrine, intermedine, jacobine, monocrotaline, platynecine, retrorsine, senecionine, Comfrey, Gynura and Senecio. The grey literature search will be used to identify relevant experimental studies that were not found in the other data sources. These studies will then be included in the systematic review by manually assigning them to the relevant research question. The other results of the grey literature search (e.g. other risk assessments) will be used as supporting information to the results of the systematic review and manually assigned to the relevant JECFA monograph section for reviewing. The results of the grey literature search will thus not be assessed according to the eligibility criteria defined below	
	Study selection		
21.	Define screening phases	1. Initial title/abstract screening of the Endnote databases to exclude publications published before 1988 (year in which last WHO opinion was published), and studies where PAs are only used to induce a specific effect in the test model (e.g. to test medicines against pulmonary hypertension or techniques for liver transplantation) or where crotaline is related to crotaline snakes instead to PAs, studies in other species than vertebrates, and studies that are not primary studies.	

Table (continued)

Item #	Section/Subsection/Item	Description	Check for approval
		2. Screening based on title/abstract in EROS. Excluded studies will be labelled for relevance for other research questions. These excluded references will be cross-checked for presence in the original Endnote databases for those research questions. Included studies will be labelled for the relevant parts of the JECFA monograph. 3. Retrieving the full texts of non-excluded studies. Full text screening in EROS was due to time constraints not possible.	
22.	Specify (a) the number of reviewers per screening phase and (b) how discrepancies will be resolved	One reviewer for the initial screening of the Endnote databases, with a second member of the team confirming the exclusion determination of the first reviewer. Two independent reviewers for title/abstract screening in EROS. Disagreements are solved by the two reviewers together. In case of persisting disagreement, a third independent reviewer will help to make a final decision whether to include the study or not.	
	Define all inclusion and exclusion criteria based on:		
23.	Type of study (design)	Inclusion criteria: Original research manuscripts Exclusion criteria: Narrative reviews	
24.	Type of animals/population (e.g. age, gender, disease model)	Inclusion criteria: • Questions 1 and 4: in vitro or ex vivo studies; • Questions 2 and 5: animal studies in vertebrates; • Questions 3 and 6: studies in humans, including case reports, epidemiological studies, clinical experience, and anecdotal observations. Exclusion criteria: • Questions 1 and 4: tests in cells of plants or non-vertebrates, animal studies, and all studies in humans, including case reports, epidemiological studies, clinical experience, and anecdotal observations; • Questions 2 and 5: studies in in vitro systems, including ex vivo, studies in non-vertebrates, and all studies in humans, including case reports, epidemiological studies, clinical experience, and anecdotal observations; • Questions 3 and 6: studies in in vitro systems, including ex vivo, and animal studies.	
25.	Type of intervention (e.g. dosage, timing, frequency)	Questions 1-6: Inclusion criteria: • PAs (All exposure characteristics, e.g. dose, exposure routes, duration, formulation) Exclusion criteria: • No exposure to PAs; • Studies in which PAs are only used to induce a certain condition in the model; • Studies in which crotaline is related to crotaline snake.	
26.	Outcome measures	Inclusion criteria: • Questions 1-3: biochemical aspects describing the kinetics, dynamics and transfer of PAs in the model; • Questions 4-6: studies describing the toxic effects of PAs.	

Item #	Section/Subsection/Item	Description	Check for approval
		Exclusion criteria: • Questions 1-3: other outcomes than biochemical aspects. • Questions 4-6: other outcomes than toxicity, beneficial effects.	
27.	Language restrictions	Inclusion criteria: All languages Exclusion criteria: -	
28.	Publication date restrictions	Inclusion criteria: Publication dates from 1988 onwards Exclusion criteria: Publication dates before 1988	
29.	Other	Inclusion criteria: - Exclusion criteria: -	
30.	Sort and prioritize your exclusion criteria per selection phase	Selection phase – Screening on title/abstract: 1. Reviews 2. Type of exposure 3. Type of population 4. Outcome measures Selection phase – Full text screening: 1. Reviews 2. Type of exposure 3. Type of population 4. Outcome measures	
Study characteristics to be extracted (for assessment of external validity, reporting quality) **Follows in principle Table 3 of OHAT NTP handbook, see Annex 4.**			
31.	Study ID (e.g. authors, year)	See Annex 4	
32.	Study design characteristics (e.g. experimental groups, number of animals)	See Annex 4	
33.	Animal model characteristics (e.g. species, gender, disease induction)	See Annex 4	
34.	Intervention characteristics (e.g. intervention, timing, duration)	See Annex 4	
35.	Outcome measures	See Annex 4	
36.	Other (e.g. drop-outs)	See Annex 4	
After the data extraction, the systematic review will end and the obtained data will be further handled according to the JECFA Guidelines for writing a JECFA Monograph.			
Final approval by (names, affiliations):		Date:	

MEDLINE search strategy (adapted for other databases)

Search strategy Medline (OvidSP) Pyrrolizidine alkaloids
20150312 RIVM

#	Searches	Results	Search Type
1	exp Pyrrolizidine Alkaloids/	2337	Advanced
2	((pyrrolizidine adj2 alkaloid*) or (senecio adj2 alkaloid*)).tw.	1192	Advanced
3	(Senecio/ or Eupatorium/ or exp Boraginaceae/ or Crotalaria/) and (*Alkaloids/ or *Heterocyclic Compounds/)	58	Advanced
4	1 or 2 or 3	2678	Advanced
5	(Adenostyles or Ageratum or Alkanna or Amsinckia or Anchusa or Asperugo or Borago or Brachyglottis or Cacalia or Caltha or Castilleja or Chromolaena or Comfrey or Conoclinium or Crassocephalum or Crotalaria or Cynoglossum or Doronicum or Echinacea or Echium or (Emilia and (sonchifolia or coccinea or asteraceae)) or Erechtites or Eupatorium or Farfugium or Fernaldia or Gynura or Hackelia or Heliotropium or Jacobaea or Lappula or Ligularia or Lindelofia or Lithospermum or Messerschmidia or Myosotis or Paracynoglossum or Parsonsia or Petasites or Prestonia or Pulmonaria or Rindera or Senecio or Solanecio or Solenanthus or Symphytum or Syneilesis or Tournefortia or Trichodesma or Tussilago or Ulugbeckia).mp.	4230	Advanced
6	Alkaloids/ or alkaloid*.tw.	41490	Advanced
7	5 and 6	617	Advanced
8	(acetoxysenkirkine or acetylanacrotine or acetylanonamine or acetylbulgarsenine or acetylcrotaverrine or acetylechimidine or acetylechinatine or acetylerucifoline or Acetylgynuramine or acetylhackelidine or acetylhadiensine or Acetylheliosupine or Acetylindicine or acetylintermedine or acetyllabumine or acetyllaburnine or Acetyllasiocarpine or acetyllithosenine or acetyllycopsamine or Acetylmadurensine or acetylneohadiensine or acetylotosenine or acetylpetasitenine or acetylplatynecine or acetylplatyphylline or acetylrinderine or acetylrosmarinine or Acetylscorpioidine or acetylsenecionine or Acetylseneciphylline or acetylsenkirkine or acetylspartioidine or Acetylsyneilesine or acetylvulgarine or acetylyamataimine or Adonifoline or Amabiline or Amadoline or Anacrotine or Anadoline or angeloyldihydroxyheliotridane or angeloylhastanecine or angeloylheliotridane or angeloylheliotridine or angeloylmacronecine or angeloylpetasinecine or angeloylplatynecine or angeloylretronecine or angeloylturneforcidine or Angelylechimidine or Angelylheliotrine or angularine or anhydroplatynecine or anonamine or Asperumine or aucherine or aureine or Axillaridine or Axillarine).mp.	62	Advanced
9	(Bisline or Brachyglottine or Bulgarsenine or butyrylretronecine or Callosine or Carategine or Chlorodeoxysceleratine or cholomethylbulgarsenine or cis-senaetnine or Clivorine or coromandaline or Crispatine or croaegyptine or croalbidine or Crobarbatine or Cromadurine or Cronaburmine or Crosemperine or Crotaflorine or Crotafoline or crotalaburnine or Crotalarine or Crotaleschenine or crotaline or Crotananine or Crotastriatine or Crotaverrine or Cruentine or Curassavinine or Cynaustine or cynaustraline or cynoglossine).mp.	182	Advanced
10	(deacetyldoronine or dehydrofuchsisenecionine or dehydroheliotridine or dehydroisosenaetnine or dehydrojaconine or dehydropyrrolizidine or dehydroretronecine or dehydrosenaetnine or dehydrosenkirkine or Deoxyaxillarine or desacetyldoronine or desacetyloxysenampeline or desacetylsenaetnine or Diacetyllycopsamine or diangeloylplatynecine or diangeloylretronecine or Dibenzoylretronecine or Dicrotaline or didehydroretronecine or dihydrolycopsamine or dihydroretorsine or dihydroretrorsine or dihydrosenkirkine or Dihydroxyerucifoline or Dihydroxytriangularine or Doriasenine or Doronenine or Doronine or Dororenine or Echimidine or Echimiplatine or Echinatine or Echiumine or echiuplatine or echivulgarine or Emiline or epineosarracine or episarracine or epoxintegerrimine or epoxisenecionine or Epoxyseneciphylline or Epoxyspartiodine or eruciflorine or Erucifoline or Europine).mp.	139	Advanced
11	(farfugine or Floricaline or Floridanine or Florosenine or fuchsisenecionine or fukinotoxin or Fulvine or Globiferine or Grahamine or Grantaline or Grantianine or Gynuramine or hackelidine or hadiensine or hastacine or hastanecine or hectorine or Heleurine or heliohoustine or heliospathine or Heliosupine or heliotridine or Heliotrine or Heliovinine or Heterophylline or heulerine or hydroxyrosmarinine or hydroxysarracine or hydroxyseneciphylline or hydroxysenkirkine or hydroxyspartioidine or hygrophylline).mp.	171	Advanced

#	Searches	Results	Search Type
12	(inaequidine or Incanine or (indicine not (taurine or bos or indicus or zebu or indicine cattle)) or Integerrimine or integrifoline or Intermedine or iodanthine or ipanguline or isatidine or Isocromadurine or isoechinatine or Isoline or isopterophorine or isoretrohoustine or isorosmarinine or isosenaetnine or isotussilagine or isotussilaginine or isovaleroylsenecicuadatine or Jacobine or Jacoline or Jaconine or Jacozine or Junceine).mp.	337	Advanced
13	(Labumine or Lasiocarpine or Latifoline or Leptanthine or Ligudentine or Ligularidine or Ligularine or Ligularinine or Ligularizine or lindelofoline or lithosenine or loline or longilobine or Lycopsamine or macronecine or macrophylline or Madurensine or merenscine or Merenskine or mikanoidine or Monocrotaline or Monocrotalinine or mucronatine or mucronatinine or mulgediifoline or Myoscorpine).mp.	1728	Advanced
14	(nemorensine or neoangularine or neo-isotussilagine or Neoligularidine or Neopetasitenine or neoplatyphylline or Neorosmarinine or Neosarracine or Neosarranicine or Neosenkirkine or neotriangularicine or Neotriangularine or neotussilagine or Nilgirine or Onetine or othonnine or Otosenine or oxynemorensine or oxyretroisosenine).mp.	24	Advanced
15	(Parsonsine or perloline or petasinecine or Petasinine or petasinoside or Petasitenine or petitianine or phalaenopsine or platiphylline or platynecine or platynecinium or platyphylline or procerine or pterophorine or pumiline or punctanecine or pyrrolizidine).mp.	1919	Advanced
16	(racemodine or racemonine or racemozine or renardine or retrohoustine or Retroisosenine or retronecine or Retrorsine or Retusamine or retusine or Riddelliine or Rinderine or rivularine or rosmarinecine or rosmarinine or ruwenine or ruzorine).mp.	338	Advanced
17	(sarracine or sarracineolyretronecine or sarracinoylretronecine or sarracinylplatynecine or sarranicine or Sceleratine or Scorpioidine or senaetnine or senampeline or Sencalenine or Senecicannabine or senecicaudatinal or semiacetal or senecifoline or Senecionine or senecioracenine or senecioylnorsenecicaudatine or senecioylplatynecine or Senecioylretronecine or senecioylsenecicaudatine or Seneciphylline or seneciphyllinine or Senecivernine or Senkirkine or Sincamidine or Spartioidine or Spectabiline or Spiracine or Spiraline or Spiranine or squalidine or strigosine or Supinine or Swazine or Symlandine or Symphytine or symviridine or Syneilesine).mp.	232	Advanced
18	(temulentiine or temuline or tetrahydrosenecionine or tigloylplatynecine or tigloylretronecine or tomentosine or trachelanthamidine or trachelanthylheliotridine or trachelanthyllaburnine or triangularicine or Triangularine or Trichodesmine or turneforcidine or tussilagine or tussilaginine or Uluganine or Uplandicine or Usaramine or Uspallatine or Vincamine or Viridiflorine or Yamataimine).mp.	436	Advanced
19	7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18	4101	Advanced
20	exp Toxicity Tests/ or exp Noxae/	793636	Advanced
21	exp Plants, Toxic/ or exp Plant Poisoning/ or exp Plants/ae, po, to [Adverse Effects, Poisoning, Toxicity]	32858	Advanced
22	exp Dose Response Relationship, Drug/ or exp Physiological Effects of Drugs/ or exp Structure-Activity Relationship/	4579497	Advanced
23	20 or 21 or 22	4957871	Advanced
24	(toxic* or intoxic* or toxin* or poison* or genotox* or neurotox* or hepatotox* or cytotox* or immunotox* or mutagen* or carcinogen* or oncogen* or clastogen*).tw.	1085409	Advanced
25	(lethal* or letal* or mortal* or fatal* or ld50 or ld 50 or acute or sub-acute or subacute or threshold or dose-response or dose response or dose-depend?nt or dose depend?nt or adverse effect*).tw.	1887110	Advanced
26	exp Drug-Induced Liver Injury/ or exp Liver Diseases/ or exp Hepatic Veno-Occlusive Disease/	442824	Advanced
27	exp Liver/ci, de, po, to [Chemically Induced, Drug Effects, Poisoning, Toxicity]	72773	Advanced
28	exp Lung Diseases/ or exp Lung/de [Drug Effects]	730665	Advanced
29	26 or 27 or 28	1211248	Advanced
30	((liver adj2 (disease* or damage* or injur* or lesion*)) or cirrhosis or sinusoidal or veno-occlusiv* or veno occlusiv* or ascites or hepatic* or hepato* or lung* or alveolar* or pulmonar* or neuromuscular*).tw.	1334211	Advanced
31	4 and (23 or 24 or 25 or 29 or 30)	2116	Advanced
32	exp Pyrrolizidine Alkaloids/ae, po, to	887	Advanced

Table (continued)

#	Searches	Results	Search Type
33	*Toxicology/ or toxicity.fs.	356566	Advanced
34	19 and (23 or 29 or 33)	2534	Advanced
35	31 or 32 or 34	2814	Advanced
36	Pyrrolizidine Alkaloids/bl, me, pk, ur [Blood, Metabolism, Pharmacokinetics, Urine]	274	Advanced
37	exp *Metabolic phenomena/ or exp Metabolism/ or exp Lung/me, pd or exp Liver/me, pd [Metabolism, Pharmacology]	1815749	Advanced
38	exp Molecular mechanisms of pharmacological action/ or exp Pharmacokinetics/	3206573	Advanced
39	37 or 38	4429727	Advanced
40	(metaboli* or kinetic* or pharmacokinetic* or absorption or excret* or biotransform* or milk or urine or faeces or feces or bile or blood or offal or egg or eggs or meat or transfer or carry-over).tw.	3278048	Advanced
41	39 or 40	6617571	Advanced
42	4 and 41	1435	Advanced
43	metabolism.fs.	4368499	Advanced
44	19 and (39 or 43)	2223	Advanced
45	36 or 42 or 44	2445	Advanced
46	Animals/	5406976	Advanced
47	(animal* or cattle or livestock or horse* or sheep* or cow* or fowl* or poultry or rat or rats or murine or mouse or mice).tw.	2809721	Advanced
48	46 or 47	5758399	Advanced
49	Humans/	13760465	Advanced
50	(human* or man or men or woman or women).tw.	3163734	Advanced
51	case reports.pt. or (case or epidemi*).tw.	2813950	Advanced
52	49 or 50 or 51	14680322	Advanced
53	exp In Vitro Techniques/ or exp Cells/	3829599	Advanced
54	(in vitro or tissue culture or hepatocyt* or microsom* or cell*).tw.	4355559	Advanced
55	53 or 54	5742250	Advanced
56	35 and 48	2381	Advanced
57	35 and 52	849	Advanced
58	35 and 55	1329	Advanced
59	35 not (56 or 57 or 58)	163	Advanced
60	45 and 48	1972	Advanced
61	45 and 52	685	Advanced
62	45 and 55	1241	Advanced
63	45 not (60 or 61 or 62)	210	Advanced

Grey literature data sources

Name	Website
Institutes (high priority)	
AGES	http://www.ages.at/
ANSES	https://www.anses.fr/en
ANZFA / FSANZ	http://www.foodstandards.gov.au/Pages/default.aspx
BfR	http://www.bfr.bund.de/de/start.html
CODEX	http://www.codexalimentarius.org/
COT/COC (UK)	http://cot.food.gov.uk/
AGRICOLA (NAL catalog)	http://agricola.nal.usda.gov/
ATSDR	http://www.atsdr.cdc.gov/
DAFF (South-Afrika)	http://www.daff.gov.za/
Danish EPA	http://eng.mst.dk/
DEFRA (UK)	https://www.gov.uk/government/organisations/department-for-environment-food-rural-affairs
DOHA (Australia)	http://www.health.gov.au/
DSEWPC (Australia)	http://www.environment.gov.au/
DTU	http://www.dtu.dk/
EFSA (incl compendium)	http://www.efsa.europa.eu/
EMA	http://www.ema-europa.eu/
EU	http://europa.eu/index_nl.htm
FAO	http://www.fao.org/home/en/
FDA	http://www.fda.gov/
Health Canada	http://www.hc-sc.gc.ca/index-eng.php
HSE	http://www.hse.gov.uk/
IARC	http://www.iarc.fr/
JECFA	http://www.who.int/foodsafety/databases/en/ http://apps.who.int/food-additives-contaminants-jecfa-database/search.aspx http://www.inchem.org/
Nordic Council	http://www.norden.org/en/nordic-council
NVWA	https://www.vwa.nl/
RIVM	http://www.rivm.nl/
USDA	http://www.usda.gov/wps/portal/usda/usdahome
US-EPA	http://www.epa.gov/
WHO	http://www.who.int/en/
Other sources (lower priority)	
CPDB - Cancer Potency DataBase	http://toxnet.nlm.nih.gov/cpdb/
DART/ETIC - Developmental and Reproductive Toxicology/Environmental Teratology Information Center	http://toxnet.nlm.nih.gov/newtoxnet/dart.htm
DART-Europe portal	http://www.dart-europe.eu/basic-search.php
GENE-TOX - Genetic Toxicology Data Bank	http://toxnet.nlm.nih.gov/newtoxnet/genetox.htm
GLIN – Grey Literature in the Netherlands / Picarta	http://picarta.pica.nl/
Grey literature report	http://www.greylit.org/

Table (continued)

Name	Website
Grey Net International	http://www.greynet.org/
NARCIS - National Academic Research and Collaborations Information System	http://www.narcis.nl/
NTP – National Toxicology Program	http://ntp.niehs.nih.gov/
OECD eChemPortal	http://www.echemportal.org/echemportal/index?pageID=0&request_locale=en
Open DOAR - Directory of Open Access Repositories	http://www.opendoar.org/
Open Grey	http://www.opengrey.eu/search/
RTECS - Registry of Toxic Effects of Chemical Substances	http://ccinfoweb.ccohs.ca/rtecs/search.html
TERIS - Teratogen Information System	http://depts.washington.edu/terisweb/teris/
World Cat	https://www.worldcat.org/
Other sources (not relevant)	
Abstracts	such as of World Mycotoxin Forum, World Plant Toxin Forum, SOT
AWIC	http://awic.nal.usda.gov/literature-searching-and-databases/databases/pharmacology-and-toxicology
BIBRA Information Services Ltd	http://www.bibra-information.co.uk/
British Library	http://explore.bl.uk/primo_library/libweb/action/search.do?vid=BLVU1
CCRIS - Chemical Carcinogenesis Research Information System	http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS
CHE (Collaborative on Health and the Environment) Toxicant and Disease Database	http://www.healthandenvironment.org/tddb
Chemical Hazard Information Library - EBSCO	http://www.ebscohost.com/academic/chemical-hazard-information-library
DOAJ – Directory of Open Access Journals	http://doaj.org/search
Experts	-
Google E-books	http://books.google.nl/
Google Scholar	http://scholar.google.nl/
Hagers Enzyklopädie	-
HSDB – Hazardous Substances Data Bank	http://toxnet.nlm.nih.gov/newtoxnet/hsdb.htm
IRIS (US EPA)	http://www.epa.gov/iris/
ISO	http://www.iso.org/iso/home.html
LIBER - Ligue de Bibliotheques Europeénes de Recherche (Association of European Research Libraries)	http://libereurope.eu/
Martindale	https://www.medicinescomplete.com/mc/martindale/current/
NDLTD - Networked Digital Library of Theses and Dissertation	http://www.ndltd.org/
NIH - National Institute of Health	http://www.nih.gov/
NLM Gateway - National Library of Medicine	http://gateway.nlm.nih.gov/
OAIster	http://www.oclc.org/oaister.en.html?urlm=168646

Name	Website
OCLC - Online Computer Library Center	http://www.oclc.org/nl-NL/home.html?redirect=true
Open Archives Initiative	http://www.openarchives.org/
Other governmental organizations from different countries	-
Lists of references (of key articles and relevant reviews)	-
Reprotox	http://www.reprotox.org/Default.aspx
Toxline	http://toxnet.nlm.nih.gov/newtoxnet/toxline.htm
ToxRefDB (US-EPA)	http://www.epa.gov/ncct/toxrefdb/

List of PA and plant names

List of PAs

acetoxysenkirkine
acetylanacrotine
acetylanonamine
acetylbulgarsenine
acetylcrotaverrine
acetylechimidine
acetylechinatine
acetylerucifoline
Acetylgynuramine
acetylhackelidine
acetylhadiensine
Acetylheliosupine
Acetylindicine
acetylintermedine
acetyllabumine
acetyllaburnine
Acetyllasiocarpine
acetyllithosenine
acetyllycopsamine
Acetylmadurensine
acetylneohadiensine
acetylotosenine
acetylpetasitenine
acetylplatynecine
acetylplatyphylline
acetylrinderine
acetylrosmarinine
Acetylscorpioidine
acetylsenecionine
Acetylseneciphylline
acetylsenkirkine
acetylspartioidine
acetylsyneilesine
acetylvulgarine
acetylyamataimine
Adonifoline
Amabiline
Amadoline
Anacrotine
Anadoline
angeloyldihydroxyheliotridane
angeloylhastanecine
angeloylheliotridane
angeloylheliotridine
angeloylmacronecine
angeloylpetasinecine
angeloylplatynecine
angeloylretronecine
angeloylturneforcidine
Angelylechimidine
Angelylheliotrine
angularine
anhydroplatynecine
anonamine

Asperumine
aucherine
aureine
Axillaridine
Axillarine
Bisline
Brachyglottine
bulgarsenine
butyrylretronecine
Callosine
Carategine
Chlorodeoxysceleratine
cholomethylbulgarsenine
Clivorine
coromandaline
Crispatine
croaegyptine
croalbidine
Crobarbatine
Cromadurine
Cronaburmine
Crosemperine
Crotaflorine
Crotafoline
crotalaburnine
Crotalarine
Crotaleschenine
crotaline
Crotananine
Crotastriatine
Crotaverrine
cruentine
Curassavinine
Cynaustine
cynaustraline
cynoglossine
deacetyldoronine
de-ethylretusamine
dehydrofuchsisenecionine
dehydroheliotridine
dehydroisosenaetnine
dehydrojaconine
dehydropyrrolizidine
dehydroretronecine
dehydrosenaetnine
dehydrosenkirkine
Deoxyaxillarine
desacetyldoronine
desacetyloxysenampeline
desacetylsenaetnine
Diacetyllycopsamine
diangeloylplatynecine
diangeloylretronecine
Dibenzoylretronecine
Dicrotaline
didehydroretronecine
dihydrolycopsamine
dihydroretorsine
dihydroretrorsine
dihydrosenkirkine
Dihydroxyerucifoline
Dihydroxytriangularine
Doriasenine
Doronenine
Doronine
Dororenine
Echimidine
Echimiplatine
Echinatine
Echiumine
echiuplatine
echivulgarine
Emiline
epineosarracine
episarracine
epoxintegerrimine
epoxisenecionine
Epoxyseneciphylline
Epoxyspartiodine
eruciflorine
Erucifoline
Europine

farfugine
Floricaline
Floridanine
Florosenine
fuchsisenecionine
fukinotoxin
Fulvine
Globiferine
Grahamine
Grantaline
Grantianine
Gynuramine
hackelidine
hadiensine
hastacine
hastanecine
hectorine
Heleurine
heliohoustine
heliospathine
Heliosupine
heliotridine
Heliotrine
Heliovinine
Heterophylline
heulerine
hydroxyintegerrimine
hydroxyjaconine
hydroxyneosenkirkine
hydroxyplatyphylline
hydroxyretroisosenine
hydroxyrosmarine
hydroxysarracine
hydroxyseneciphylline
hydroxysenkirkine
hydroxyspartioine
hygrophylline
inaequidine
Incanine
Indicine
Integerrimine
integrifoline
Intermedine
iodanthine
ipanguline
isatidine
Isocromadurine
isoechinatine
Isoline
isopterophorine
isoretrohoustine
isorosmarinine
isosenaetnine
isotussilagine
isotussilaginine
isovaleroylsenecicuadatine
Jacobine
Jacoline
Jaconine
Jacozine
Junceine
Labumine
Lasiocarpine
Latifoline
Leptanthine
Ligudentine
Ligularidine
Ligularine
Ligularinine
Ligularizine
lindelofoline
lithosenine
loline
longilobine
Lycopsamine
macronecine
macrophylline
Madurensine
merenscine
Merenskine
mikanoidine
Monocrotaline

Monocrotalinine
mucronatine
mucronatinine
mulgediifoline
Myoscorpine
nemorensine
neoangularine
neo-isotussilagine
Neoligularidine
Neopetasitenine
Neoplatyphylline
Neorosmarinine
Neosarracine
Neosarranicine
Neosenkirkine
neotriangularicine
Neotriangularine
neotussilagine
Nilgirine
Onetine
othonnine
Otosenine
oxynemorensine
oxyretroisosenine
Parsonsine
perloline
petasinecine
Petasinine
petasinoside
Petasitenine
petitianine
phalaenopsine
platiphylline
platynecine
platynecinium
platyphylline
procerine
pterophorine
pumiline
punctanecine
pyrrolizidine
racemodine
racemonine
racemozine
renardine
retrohoustine
Retroisosenine
retronecine
Retrorsine
Retusamine
retusine
Riddelliine
Rinderine
rivularine
rosmarinecine
rosmarinine
ruwenine
ruzorine
sarracine
sarracineolyretronecine
sarracinoylretronecine
sarracinylplatynecine
sarranicine
Sceleratine
scorpioidine
Scorpioidine
senaetnine
senampeline
Sencalenine
Senecicannabine
senecicaudatinal semiacetal
senecifoline
Senecionine
senecioracenine
senecioylnorsenecicaudatine
senecioylplatynecine
Senecioylretronecine
senecioylsenecicaudatine
Seneciphylline
seneciphyllinine
Senecivernine
Senkirkine

Sincamidine
Spartioidine
spartioine
Spectabiline
Spiracine
Spiraline
Spiranine
squalidine
strigosine
Supinine
Swazine
Symlandine
Symphytine
symviridine
Syneilesine
temulentiine
temuline
tetrahydrosenecionine
tigloylplatynecine
tigloylretronecine
tomentosine
trachelanthamidine
trachelanthylheliotridine
trachelanthyllaburnine
triangularicine
Triangularine
Trichodesmine
turneforcidine
tussilagine
tussilaginine
Uluganine
Uplandicine
Usaramine
Uspallatine
Viridiflorine
Yamataimine

List of plant genus
Adenostyles
Ageratum
Alkanna
Amsinckia
Anchusa
Asperugo
Borago
Brachyglottis
Cacalia
Caltha
Castilleja
Chromolaena
Comfrey
Conoclinium
Crassocephalum
Crotalaria
Cynoglossum
Doronicum
Echinacea
Echium
Emilia
Erechtites
Eupatorium
Farfugium
Fernaldia
Gynura
Hackelia
Heliotropium
Jacobaea
Lappula
Ligularia
Lindelofia
Lithospermum
Lollium
Messerschmidia
Myosotis
Paracynoglossum
Parsonsia
Petasites
Prestonia

Pulmonaria
Rindera
Senecio
Solanecio
Solenanthus
Symphytum
Syneilesis
Tournefortia
Trichodesma
Tussilago
Ulugbeckia

Data extraction elements.

Taken from: Handbook for Conducting a Literature-Based Health Assessment Using OHAT Approach for Systematic Review and Evidence Integration. Version 9 January 2015. Office of Health Assessment and Translation (OHAT), Division of the National Toxicology Program, National Institute of Environmental Health Sciences

Table 3. Key Data Extraction Elements to Summarize Study Design, Experimental Model, Methodology, and Results	
HUMAN	
Funding	Funding source(s)
	Reporting of conflict of interest (COI) by authors (*reporting bias)
Subjects	Study population name/description
	Dates of study and sampling time frame
	Geography (country, region, state, etc.)
	Demographics (sex, race/ethnicity, age or lifestage at exposure and at outcome assessment)
	Number of subjects (target, enrolled, n per group in analysis, and participation/follow-up rates) (*missing data bias)
	Inclusion/exclusion criteria/recruitment strategy (*selection bias)
	Description of reference group (*selection bias)
Methods	Study design (e.g., prospective or retrospective cohort, nested case-control study, cross-sectional, population-based case-control study, intervention, case report, etc.)
	Length of follow-up (*information bias)
	Health outcome category, e.g., cardiovascular
	Health outcome, e.g., blood pressure (*reporting bias)
	Diagnostic or methods used to measure health outcome (*information bias)
	Confounders or modifying factors and how considered in analysis (e.g., included in final model, considered for inclusion but determined not needed (*confounding bias)
	Substance name and CAS number
	Exposure assessment (e.g., blood, urine, hair, air, drinking water, job classification, residence, administered treatment in controlled study, etc.) (*information bias)
	Methodological details for exposure assessment (e.g., HPLC-MS/MS, limit of detection) (*information bias)
	Statistical methods (*information bias)
Results	Exposure levels (e.g., mean, median, measures of variance as presented in paper, such as SD, SEM, 75th/90th/95th percentile, minimum/maximum); range of exposure levels, number of exposed cases
	Statistical findings (e.g., adjusted β, standardized mean difference, adjusted odds ratio, standardized mortality ratio, relative risk, etc.) or description of qualitative results. When possible, OHAT will convert measures of effect to a common metric with associated 95% confidence intervals (CI). Most often, measures of effect for continuous data are expressed as mean difference, standardized mean difference, and percent control response. Categorical data are typically expressed as odds ratio, relative risk (RR, also called risk ratio), or β values, depending on what metric is most commonly reported in the included studies and on OHAT's ability to obtain information for effect conversions from the study or through author query.
	If not presented in the study, statistical power can be assessed during data extraction using an approach that can detect a 10% to 20% change from response by control or referent group for continuous data, or a relative risk or odds ratio of 1.5 to 2 for categorical data, using the prevalence of exposure or prevalence of outcome in the control or referent group to determine sample size. For categorical data where the sample sizes of exposed and control or referent groups differ, the sample size of the exposed group will be used to determine the relative power category. Recommended sample sizes to achieve 80% power for a given effect size, i.e., 10% or 20% change from control, will be compared to sample sizes used in the study to categorize statistical power. Studies will be considered adequately powered when sample size for 80% power is met.
	Observations on dose response (e.g., trend analysis, description of whether dose-response shape appears to be monotonic, non-monotonic)

Table 3. Key Data Extraction Elements to Summarize Study Design, Experimental Model, Methodology, and Results	
Other	Documentation of author queries, use of digital rulers to estimate data values from figures, exposure unit, and statistical result conversions, etc.
ANIMAL	
Funding	Funding source(s)
	Reporting of COI by authors (*reporting bias)
Animal Model	Sex
	Species
	Strain
	Source of animals
	Age or lifestage at start of dosing and at health outcome assessment
	Diet and husbandry information (e.g., diet name/source)
Treatment	Chemical name and CAS number
	Source of chemical
	Purity of chemical (*information bias)
	Dose levels or concentration (as presented and converted to mg/kg bw/d when possible)
	Other dose-related details, such as whether administered dose level was verified by measurement, information on internal dosimetry (*information bias)
	Vehicle used for exposed animals
	Route of administration (e.g., oral, inhalation, dermal, injection)
	Duration and frequency of dosing (e.g., hours, days, weeks when administration was ended, days per week)
Methods	Study design (e.g., single treatment, acute, subchronic (e.g., 90 days in a rodent), chronic, multigenerational, developmental, other)
	Guideline compliance (i.e., use of EPA, OECD, NTP or another guideline for study design, conducted under GLP guideline conditions, non-GLP but consistent with guideline study, non-guideline peer-reviewed publication)
	Number of animals per group (and dams per group in developmental studies) (*missing data bias)
	Randomization procedure, allocation concealment, blinding during outcome assessment (*selection bias)
	Method to control for litter effects in developmental studies (*information bias)
	Use of negative controls and whether controls were untreated, vehicle-treated, or both
	Report on data from positive controls – was expected response observed? (*information bias)
	Endpoint health category (e.g., reproductive)
	Endpoint (e.g., infertility)
	Diagnostic or method to measure endpoint (*information bias)
	Statistical methods (*information bias)
Results	Measures of effect at each dose or concentration level (e.g., mean, median, frequency, and measures of precision or variance) or description of qualitative results. When possible, OHAT will convert measures of effect to a common metric with associated 95% confidence intervals (CI). Most often, measures of effect for continuous data will be expressed as mean difference, standardized mean difference, and percent control response. Categorical data will be expressed as relative risk (RR, also called risk ratio).
	No Observed Effect Level (NOEL), Lowest Observed Effect Level (LOEL), benchmark dose (BMD) analysis, statistical significance of other dose levels, or other estimates of effect presented in paper. **Note:** The NOEL and LOEL are highly influenced by study design, do not give any quantitative information about the relationship between dose and response, and can be subject to author's interpretation (e.g., a statistically significant effect may not be considered biologically important). Also, a NOEL does not necessarily mean zero response. Ideally, the response rate at specific dose levels is used as the primary measure to characterize the response.
	If not presented in the study, statistical power can be assessed during data extraction using an approach that assesses the ability to detect a 10% to 20% change from control group's response for continuous data, or a relative risk or odds ratio of 1.5 to 2 for categorical data, using the outcome frequency in the control group to determine sample size. Recommended sample sizes to achieve 80% power for a given effect size, i.e., 10% or 20% change from

Table 3. Key Data Extraction Elements to Summarize Study Design, Experimental Model, Methodology, and Results	
	sample sizes to achieve 80% power for a given effect size, i.e., 10% or 20% change from control, will be compared to sample sizes used in the study to categorize statistical power. Studies will be considered adequately powered when sample size for 80% power is met.
	Observations on dose response (e.g., trend analysis, description of whether dose-response shape appears to be monotonic, non-monotonic)
	Data on internal concentration, toxicokinetics, or toxicodynamics (when reported)
Other	Documentation of author queries, use of digital rulers to estimate data values from figures, exposure unit, and statistical result conversions, etc.
IN VITRO	
Funding	Funding source(s)
	Reporting of COI by authors (*reporting bias)
Cell/Tissue Model	Cell line, cell type, or tissue
	Source of cells/tissue (and validation of identity)
	Sex of human/animal of origin
	Species
	Strain
Treatment	Chemical name and CAS number
	Concentration levels (as presented and converted to µM when possible)
	Source of chemical
	Purity of chemical (*information bias)
	Vehicle used for experimental/control conditions
	Duration and frequency of dosing (e.g., hours, days, weeks when administration was ended, times per day or week)
Methods	Guideline compliance (i.e., use of EPA, OECD, NTP or another guideline for study design, conducted under GLP guideline conditions, non-GLP but consistent with guideline study, non-guideline peer-reviewed publication)
	Randomization procedure, allocation concealment, blinding during outcome assessment (*selection bias)
	Number of replicates per group (*information bias)
	Percent serum/plasma in medium
	Use of negative controls and whether controls were untreated, vehicle-treated, or both
	Report on data from positive controls – was expected response observed? (*information bias)
	Endpoint health category (e.g., endocrine)
	Endpoint or assay target (e.g., estrogen receptor binding or activation)
	Name and source of assay kit
	Diagnostic or method to measure endpoint (e.g., reporter gene) (*information bias)
	Statistical methods (*information bias)
Results	No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC), statistical significance of other concentration levels, AC50, or other estimates of effect presented in paper. **Note:** The NOEC and LOEC are highly influenced by study design, do not give any quantitative information about the relationship between dose and response, and can be subject to author's interpretation (e.g., a statistically significant effect may not be considered biologically important). Also, a NOEC does not necessarily mean zero response.
	Observations on dose response (e.g., trend analysis, description of whether dose-response shape appears to be monotonic, non-monotonic)
Other	Documentation of author queries, use of digital rulers to estimate data values from figures, exposure unit, and statistical result conversions, etc.
Elements marked with an asterisk (*) are examples of items that can be used to assess internal validity/risk of bias in Step 4.	

Supplementary Table 1
Studies on the in vitro metabolism of pyrrolizidine alkaloids

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Lin G, Tang J, Liu XQ, Jiang Y, Zheng J (2007). Deacetylclivorine: a gender-selective metabolite of clivorine formed in female Sprague-Dawley rat liver microsomes. Drug Metab Dispos. 35:607–13.	Clivorine (>99%)	Female rat liver microsomes	250 µM	+	Clivoric acid	4.86±0.03 nmol/mg protein/h	+	Inhibition of CYP3A1/2 abolished formation of pyrrolic ester and thus of DHR, clivoric acid. Formation of deacetylclivorine not affected by inhibition of CYP2A1, CYP2C6/7/12, CYP2E1, CYP3A1/2	+	Deacetyl formation significantly inhibited by inhibition of carboxylesterases, particularly hydrolase A	-	-						
					Dehydroretronecine	5.73±0.80 nmol/mg protein/h												
					7-Glutathionyldehydroretronecine (racemic mixture)	No quantitative data												
					Deacetylclivorine	19.44±3.00 nmol/mg protein/h												
					Intact clivorine	178.41±20.70 nmol/mL												
					Tissue-bound pyrroles	No quantitative data												
		Female rat S9	250 µM	+	Formation of deacetylclivorine confirmed	No quantitative data							-	-	-	-	-	-
		Female rat cytosol	250 µM	-	Intact clivorine	250.40±15.70 nmol/mL												
Lin G, Cui YY, Liu XQ (2003). Gender differences in microsomal metabolic activation of hepatotoxic clivorine in rat. Chem Res Toxicol. 16:768–74.	Clivorine (>99%)	Male rat liver microsomes	250 µM	+	Clivoric acid	52.1±0.6 nmol/mg protein/h	+	CYP3A enzyme induction led to significantly increased formation rates of pyrrolic-related metabolites. P450 3A enzyme inhibition led to complete inhibition of formation metabolites generated from oxidative N-methylation pathway. Two novel metabolites found in female microsomes were not affected	-	-	-	-						
					Dehydroretronecine	8.9±0.6 nmol/mg protein/h												
					7-Glutathionyldehydroretronecine (racemic mixture)	7.4±0.2 nmol/mg protein/h												
					7, 9-Diglutathionyldehydroretronecine (racemic mixture)	1.8±0.1 nmol/mg protein/h												
					Tissue-bound pyrroles	1.3±0.2 nmol												
					Intact clivorine	129±0.9 nmol/mL												
		Female rat liver microsomes	250 µM		Clivoric acid	8.7±0.4 nmol/mg protein/h												
					Dehydroretronecine	8.8±0.8 nmol/mg protein/h												
					7-Glutathionyldehydroretronecine (racemic mixture)	2.4±0.1 nmol/mg protein/h												
					Tissue-bound pyrroles	0.4±0.1 nmol												
					Intact clivorine	129±2 nmol/mL												
					novel, non-pyrrolic metabolites formed	No quantitative data							-	-	-	-	-	-
		cDNA-expressed rat enzyme		+	Dehydroretronecine, 7-glutathionyldehydroretronecine , clivoric acid, and tissue-bound pyrroles were formed	-		Formation was mediated by CYP3A1 and CYP3A2					-	-	-	-	-	-
Xia Q, Chou MW, Lin G, Fu PP (2004). Metabolic formation of DHP-derived DNA adducts from a representative otonecine type pyrrolizidine alkaloid clivorine and the extract of Ligularia hodgsonnii hook. Chem Res Toxicol. 17:702–8.	Clivorine (>99%)	Male rat liver microsomes	0.2 mM/0.5 mM	+	(+/–)-6,7-Dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine	No quantitative data	+	Inhibition of CYP3A inhibited formation of DHP significantly	-	-	-	-	-	-	DNA adducts	5.6 ± 0.71 adducts/10^{-6} nucleotides (0.2 mM), 10.1 ± 1.48 adducts/10^{-6} nucleotides (0.5 mM). For comparison, riddelliine: 10.26 ± 1.36 adducts/10^{-6} nucleotides (0.2 mM), 13.6 ± 0.94 adducts/10^{-6} nucleotides (0.5 mM)	-	-
					Intact clivorine	No quantitative data												
	Extract *Ligularia hodgsonnii* (clivorine : ligularine 96:4)				(+/–)-6,7-Dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine	No quantitative data												
					Intact clivorine	No quantitative data	-	-					-	-	DNA adducts	5.4 ± 1.3 adducts/10^{-6} nucleotides	-	-
Lin G, Cui YY, Hawes EM (2000). Characterization of rat liver microsomal metabolites of clivorine, an hepatotoxic otonecine-type pyrrolizidine alkaloid. Drug Metab Dispos. 28:1475–83.	Clivorine (>99%)	Male rat liver microsomes	250 µM	+	Clivoric acid	147.39±12.20 nmol/mL (in absence of GSH); 225.81±6.55 nmol/mL (in presence of GSH)	-	-	-	-	-	-						
					dehydroretronecine	77.82±5.00 nmol/mL (in absence of GSH); 29.74±0.61 nmol/mL (in presence of GSH)												
					7-GSH-DHR	31.79±0.92 nmol/mL (in presence of GSH)												
					7,9-diGSH-DHR	7.04±0.18 nmol/mL (in presence of GSH)												
					Tissue-bound pyrroles	3.89±0.40 nmol (in absence of GSH); 2.15±0.44 nmol (in presence of GSH)												
					Intact clivorine	63.87±5.07 nmol/mL (in absence of GSH); 9.24±0.55 nmol/mL (in presence of GSH)							-	-	-	-	Without GSH, no pyrrolic alcohol GSH conjugates detected. An increase in GSH concentration leads to increase in formation of pyrrolic alcohol GSH conjugates and decrease in formation of DHR and bound pyrroles	-

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Lin G, Cui YY, Hawes EM (1998). Microsomal formation of a pyrrolic alcohol glutathione conjugate of clivorine firm evidence for the formation of a pyrrolic metabolite of an otonecine-type pyrrolizidine alkaloid. Drug Metab Dispos. 26:181–4.	Retrorsine (>99%)	Male rat liver microsomes	0.32 mM	+	7-GSH-DHP	No quantitative data	-	-	-	-	-	-	-	-	-	-	-	-
					7,9-diGSH-DHP	No quantitative data												
	Clivorine (>99%)	Male rat liver microsomes	0.25 mM	+	7-GSH-DHP	No quantitative data	-	-	-	-	-	-	-	-	-	-	-	-
Lin G, Cui YY, Liu XQ, Wang ZT (2002). Species differences in the in vitro metabolic activation of the hepatotoxic pyrrolizidine alkaloid clivorine. Chem Res Toxicol. 15:1421–8.	Clivorine (>99%)	Male rat liver microsomes	0.25 mM	+	Clivoric acid	21.9±0.2 nmol/mg protein/h	+	Inhibition of CYP3A inhibited metabolic rate of clivorine and formation of tissue-bound pyrroles significantly	-	-	-	-	-	-	-	-	-	-
					DHR	4.8±0.1 nmol/mg protein/h												
					7-GSH-DHR	3.8±0.2 nmol/mg protein/h												
					7,9-diGSH-DHR	2.3±0.1 nmol/mg protein/h												
					Tissue-bound pyrroles	0.7±0.1 nmol												
					Intact clivorine	152±5 nmol/mL												
		Male guinea-pig liver microsomes			Clivoric acid	50.5±1.4 nmol/mg protein/h	+	Inhibition of CYP3A abolished formation of DHR, 7-GSH-DHR, 7,9-diGSH-DHR and tissue-bound pyrroles, and significantly inhibited formation of clivoric acid. Formation of clivopic acid was significantly accelerated. Inhibition of CYP2B1 did not significantly affect the metabolic rate of clivorine, but inhibited the formation rates of clivopic acid, clivoric acid and 7-GSH-DHR markedly, while that of 7,9-diGSH-DHR increased significantly	-	-	-	-	-	-	-	-	-
					DHR	9.9±0.1 nmol/mg protein/h												
					7-GSH-DHR	8.4±0.2 nmol/mg protein/h												
					7,9-diGSH-DHR	5.8±0.2 nmol/mg protein/h												
					Tissue-bound pyrroles	0.6±0.2 nmol												
					Clivopic acid	70.2±1.7 nmol/mg protein/h							-	-	-	-	-	-
		Male guinea-pig whole liver homogenate			Clivopic acid	36.3±21.2 nmol/mL (14%)	-	-	+	Involvement suggested by results	-	-	-	-	-	-	-	-
		Male guinea-pig cytosolic fractions			Clivopic acid	220.9±21.8 nmol/mL (87%)	-	-	+	Involvement suggested by results	-	-	-	-	-	-	-	-
Liu XQ, Lin G, Wang GJ, Qian ZY (2002). Involvement of human CYP3A4 in the formation of hepatotoxic metabolites of clivorine. Chinese J Pharmacol Toxicol. 16:15–20.	Clivorine (purity unknown)	Human liver microsomes	250 mM	+	Clivoric acid	674±37 nmol/min/g	+	Inhibition of CYP3A4 significantly inhibited metabolism of clivorine, including formation of pyrrolic metabolites and bound pyrroles	-	-	-	-	-	-	-	-	-	-
					DHR	263±32 nmol/min/g												
					7-GSH-DHR	86±12 nmol/min/g												
					7,9-diGSH-DHR	45±2 nmol/min/g												
					Tissue-bound pyrroles	1.026 μmol/L												
					Intact clivorine	142±13 mmol/mL												
		cDNA expressed human CYPs			Clivoric acid	327±21 nmol/min/g		CYP3A4 was able to mediate formation metabolites										
					DHR	160±18 nmol/min/g												
					7-GSH-DHR	53±5 nmol/min/g												
					7,9-diGSH-DHR	32±3 nmol/min/g												
					Tissue-bound pyrroles	0.288 μmolL												
					Clivorine	203±4 mmol/L												
Liu XQ, Lin G, Wang GJ, Qian ZY (2001). Metabolism of clivorine in female rat liver microsomes. Chinese J Pharmacol Toxicol.15:413–7.	Clivorine (purity unknown)	Female rat liver microsomes	250 mM	+	Clivoric acid	8.8±0.3 μmol/L	+	Inhibition of CYP3A inhibited formation of pyrrolic metabolites significantly	+	Formation of novel metabolite M1 was independent of NADPH-generating system	+	Formation of novel metabolite M2 was significantly inhibited by FMO inhibitor	-	-	-	-	-	-
					DHR	9.2±1.1 μmol/L												
					7-GSH-DHR	1.6±0.6 μmol/L												
					Intact clivorine	138±16 mmol/mL												
					Two novel metabolites	no quantitative data												
		Recombinant CYPs			DHR	99±5 mmol/L (CYP3A1); 109±13 mmol/L (CYP3A2)												
					7-GSH-DHR	107±2 mmol/L (CYP3A1); 134±6 mmol/L (CYP3A2)												
					Clivoric acid	364±2 mmol/L (CYP3A1); 312±21 mmol/L (CYP3A2)		CYP3A1 and CYP3A2 were able to form metabolites	-	-	-	-	-	-	-	-	-	-
					Intact clivorine	135±10 mmol/L (CYP3A1); 147±3 mmol/L (CYP3A2)												

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Tamta H, Pawar RS, Wamer WG, Grundel E, Krynitsky AJ, Rader JI (2012). Comparison of metabolism-mediated effects of pyrrolizidine alkaloids in a Hep2G/C3A cell-S9 co-incubation system and quantification of their glutathione conjugates. Xenobiotica. 42:1038–48.	Echimidine (purity unknown)	Male rat S9 fraction	250 µg/mL (628.97µM)	+	7-GSH-DHR	16.50±1.20 µM	-	-	-	-	-	-	-	-	-	-	-
					7,9-diGSH-DHR	2.09±0.48 µM												
					Substrate turnover	2.95%												
	Heliotrine (purity unknown)	Male rat S9 fraction	250 µg/mL (798.31 µM)	+	7-GSH-DHR	2.14±0.28 µM												
					7,9-diGSH-DHR	0.33±0.05 µM												
					Substrate turnover	0.31%	-	-	-	-	-	-	-	-	-	-	-	-
	Lycopsamine (purity unknown)	Male rat S9 fraction	250 µg/mL (835.08 µM)	+	7-GSH-DHR	2.78±0.62 µM	-	-	-	-	-	-	-	-	-	-	-	-
					7,9-diGSH-DHR	0.39±0.03 µM												
					Substrate turnover	0.38%												
	Monocrotaline (purity unknown)	Male rat S9 fraction	250 µg/mL (768.37 µM)	+	7-GSH-DHR	6.79±0.82 µM												
					7,9-diGSH-DHR	0.79±0.20 µM												
					Substrate turnover	0.98%	-	-	-	-	-	-	-	-	-	-	-	-
	Retrorsine (purity unknown)	Male rat S9 fraction	250 µg/mL (711.46 µM)	+	7-GSH-DHR	42.27±5.11 µM	-	-	-	-	-	-	-	-	-	-	-	-
					7,9-diGHS-DHR	6.03±1.50 µM												
					Substrate turnover	6.79%												
	Senkirkine (purity unknown)	Male rat S9 fraction	250 µg/mL (684.12 µM)	+	7-GSH-DHR	15.65+H3092.22 µM												
					7,9-diGSH-DHR	2.25±0.64 µM												
					Substrate turnover	2.61%	-	-	-	-	-	-	-	-	-	-	-	-
Dueker SR, Lamé MW, Segall HJ (1995). Hydrolysis rates of pyrrolizidine alkaloids derived from *Senecio jacobaea*. Arch Toxicol. 69:725–8.	Jacobine (purity unknown)	Purified guinea-pig carboxylesterase GPH1	-	-	-		-	-	-	-	-	-	-	-	-	-	-	Not determined, enzymatic hydrolysis too low
	Jacozine (purity unknown)	Purified guinea-pig carboxylesterase GPH1	-	-	-		-	-	-	-	-	-	-	-	-	-	-	V_{max} = 1092±80, Km = 349.2±40.6
	Retrorsine (purity unknown)	Purified guinea-pig carboxylesterase GPH1	-	-	-		-	-	-	-	-	-	-	-	-	-	-	Not determined, enzymatic hydrolysis too low
	^{14}C riddelliine/ retrorsine (purity unknown)	Purified guinea-pig carboxylesterase GPH1	350 µg	+	Gradual disappearance of riddelliine over time. In addition, small decrease in retrorsine, suggesting it is enzymatically hydrolysed, albeit slowly		-	-	-	-	-	-	-	-	-	-	-	-
	Seneciphylline (purity unknown)	Purified guinea-pig carboxylesterase GPH1	-	-	-		-	-	-	-	-	-	-	-	-	-	-	V_{max} = 229±21, Km = 64.9±8.7
Chung WG, Buhler DR (1995). Major factors for the susceptibility of guinea pig to the pyrrolizidine alkaloid jacobine. Drug Metab Dispos. 23:1263–7.	[^{3}H]jacobine (purity unknown)	Male guinea-pig liver microsomes	0.5 mM	+	DHP	0.329 nmol/min/mg protein	-	-	-	-	-	-	-	-	-	-	-	-
					Jacobine *N*-oxide	0.104 nmol/min/mg protein												
					Ratio DHP/*N*-oxide	3.16												
		Purified guinea-pig carboxylesterases GPL1 and GPH1			Hydrolysis to necic acid and necine base	No quantitative data	-	-	+	GPH1 catalysed the esterolytic hydrolysis, but at a rate approximately one-third of that found for senecionine. No effect of GPL1	-	-	-	-	-	-	-	-
	[^{3}H]senecionine (purity unknown)	Male guinea-pig liver microsomes	0.5 mM		DHP	0.460 nmol/min/mg protein												
					Senecionine *N*-oxide	0.865 nmol/min/mg protein												
					Ratio DHP/*N*-oxide: 0.53	0.53	-	-	-	-							-	-
		Purified guinea-pig carboxylesterases GPL1 and GPH1			Hydrolysis to necic acid and necine base	No quantitative data	-	-	+	GPH1 hydrolysed at a low rate. No effect of GPL1							-	-
Winter CK, Segall HJ, Jones AD (1988). Determination of pyrrolizidine alkaloid metabolites from mouse liver microsomes using tandem mass spectrometry and gas chromatography/mass spectrometry. Biomed Environ Mass Spectrom. 15:265–73.	Monocrotaline (purity unknown)	Male mouse liver microsomes	1 mM	+	DHP	235 ng, 0.04% conversion	-	-	-	-	-	-	-	-	-	-	-	-
					Monocrotalic acid	260 µg, 34.6% conversion												
					Intact monocrotaline	No quantitative data												
					Monocrotaline *N*-oxide	Low formation, no quantitative data												
	Senecionine (purity unknown)	Male mouse liver microsomes	1 mM	+	DHP	182 ng, 0.03% conversion												
					Senecic acid	446 µg, 51.6% conversion												
					Intact senecionine	No quantitative data	-											
					Senecionine *N*-oxide	54 µg, 3.8% conversion		-	-	-	-	-	-	-	-	-	-	-
Nigra L, Huxtable RJ (1992). Hepatic glutathione concentrations and the release of pyrrolic metabolites of the pyrrolizidine alkaloid, monocrotaline, from the isolated perfused liver. Toxicon. 30:1195–202.	Monocrotaline (purity unknown)	Male rat liver perfusion	300 µM	+	Pyrrolic metabolites (bile)	0.81±0.47 mM (30 min); 1.14±0.16 mM (60 min)												
			900 µM		Pyrrolic metabolites (bile)	1.28±0.28 mM (30 min); 1.39±0.26 mM (60 min)						-					Monocrotaline perfusion lowered glutathione concentrations in the liver from 30 min onwards; correlates with the formation of glutathionyldehydroretronecine	

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Dueker SR, Lamé MW, Jones AD, Morin D, Segall HJ (1994). Glutathione conjugation with the pyrrolizidine alkaloid, jacobine. Biochem Biophys Res Comm. 198:516–22.	Jacobine (>98%)	Rat hepatic glutathione-S-transferase enzyme	2 mM	-	-		-	-	-	-	-	-	-	-	-	-	Amount of GSH-JAB produced over time: 0 min: 35.4±8.2 nmol/min, 25 min: 47.7±6.2 nmol/min, 120 min: 62.9±9.0 nmol/min. There was no significant difference between the rat GST mediated conjugation and the non-enzymatic rate of GSH-JAB formation	-
		Guinea-pig hepatic glutathione-S-transferase enzyme			-		-	-	-	-	-	-	-	-	-	-	Amount of GSH-JAB produced over time: 10 min: 57.4±5.0 nmol/min, 25 min: 124.7±9.8 nmol/min, 120 min: 254.8±44.8 nmol/min. The guinea-pig GST catalysed the formation of conjugate at approximately twice the non-enzymatic rate	-
Yang YC, Yan J, Doerge DR, Chan PC, Fu PP, Chou MW (2001). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, riddelliine, leading to DNA adduct formation in vivo. Chem Res Toxicol. 14:101–9.	Riddelliine (purity unknown)	Female rat liver microsomes	2 µmol in 50 µL	+	DHR	8.3±0.7 nmol/mg protein/30 min	+	Involvement of CYP2B6 and/or CYP3A4 suggested due to increased formation (3.8-fold for DHR, 3.4-fold for *N*-oxide) of metabolism in case of PB microsomes							DNA adducts	Eight DNA adducts were formed upon reaction of DHR with calf thymus DNA. Two of these adducts were identified as DHR-modified 7-deoxyguanosin-N2-yl epimers (DHR-3'-dGMP); the other six were DHR-derived DNA adducts, but their structures were not characterized. No adducts formed after incubation of riddelliine with calf thymus DNA		
					Riddelliine *N*-oxide	14.7±1.1 nmol/mg protein/30 min			-	-	-	-	-	-			-	-
Xia Q, Chou MW, Kadlubar FF, Chan PC, Fu PP (2003). Human liver microsomal metabolism and DNA adduct formation of the tumorigenic pyrrolizidine alkaloid, riddelliine. Chem Res Toxicol. 16:66–73.	Riddelliine (purity unknown)	Male rat liver microsomes	0.1 mM (metabolism), 0.1–1.6 mM (kinetics), 0.5 µmol (enzyme inhibition)	+	DHP	0.21±0.01 nmol/min/mg protein	-	-	-	-	-	-	-	-			-	Male: DHP: V_{max} = 1.12±0.04 nmol/min/mg protein, Km = 0.28±0.03 nmol/min/mg protein. Riddelliine *N*-oxide: V_{max} = 2.17±0.08 nmol/min/mg protein, Km =0.25±0.03 nmol/min/mg protein. Female: DHP: V_{max} = 0.48±0.03 nmol/min/mg protein, Km = 0.37±0.05 nmol/min/mg protein. Riddelliine *N*-oxide: V_{max} = 0.30±0.01 nmol/min/mg protein, Km =0.44±0.04 nmol/min/mg protein
		Female rat liver microsomes			Riddelliine *N*-oxide	0.48±0.02 nmol/min/mg protein									DNA adducts	Set of eight DHP-derived DNA adducts were formed. Two were identified as DHP-3'-dGMP adducts; the other six were characterized as DHP-derived dinucleotides. The same set of adducts formed in incubation with human microsomes as with rat microsomes. Quantities in humans either similar to or 2–3-fold higher than those in rat		
		Male human liver microsomes			DHP	0.14±0.02 nmol/min/mg protein												
					Riddelliine *N*-oxide	0.06±0.01 nmol/min/mg protein												
		Female human liver microsomes			Male: DHP (0.31–0.62 nmol/min/mg protein), riddelliine *N*-oxide (0.05–0.15 nmol/min/mg protein) Female: DHP (0.20–0.50 nmol/min/mg protein), riddelliine *N*-oxide (0.03–0.12 nmol/min/mg protein)		+	Inhibition of CYP3A4 significantly inhibited formation of DHP and riddelliine *N*-oxide	-	-	-	-	UGT1A4 and to a small degree UGT1A3 showed activity				-	Male: DHP: V_{max} = 0.95±0.02 nmol/min/mg protein, Km = 0.24±0.02 nmol/min/mg protein. Riddelliine *N*-oxide: V_{max} = 0.26±0.01 nmol/min/mg protein, Km =0.44±0.06 nmol/min/mg protein. Female: DHP: V_{max} = 1.70±0.09 nmol/min/mg protein, Km = 0.66±0.08 nmol/min/mg protein. Riddelliine *N*-oxide: V_{max} = 0.43±0.03 nmol/min/mg protein, Km =0.71±0.12 nmol/min/mg protein
Buhler DR, Miranda CL, Kedzierski B, Reed RL (1991). Mechanisms for pyrrolizidine alkaloid activation and detoxification. Adv Exp Med Biol. 283:597–603.	Senecionine (purity unknown) or [3H]-senecionine	Male rat liver microsomes	1 mM, 2 mM	+	DHP (in absence of added GSH)	3.3±0.19	+	Involvement suggested by higher formation of DHP (2.8-fold) and GS-DHP (2.7-fold) in case of PB-pretreated microsomes									When incubated with added GSH, GSH-DHP was formed, which was directly related to amount of GSH added. As GSH levels increased, concentration of free DHP decreased concomitant with increasing GSH-DHP conjugate formation. GSH-DHP was most likely predominantly formed by reaction between reactive intermediate and GSH, and not by reaction between DHP and GSH	
					GS-DHP (in presence of added GSH)	4.27±0.28												
					Senecionine	No quantitative data			-	-	-	-	-	-	-	-		-
Reed, RL, Miranda CL, Kedzierski B, Henderson MC, Buhler DR (1992). Microsomal formation of a pyrrolic alcohol glutathione conjugate of the pyrrolizidine alkaloid senecionine. Xenobiotica. 22:1321–7.	Senecionine (purified) or [3H]-senecionine	Male rat liver microsomes	1 mM	+	DHP	No quantitative data	-	-	-	-	-	-	-	-	-	-	When incubated with added GSH, GSH-DHP was formed. Formation of GSH-DHP increased with increasing GSH concentration. DHP-GSH predominantly formed by reaction between reactive intermediate and GSH, and not by reaction between DHP and GSH	-
					Senecionine *N*-oxide	No quantitative data												
						No quantitative data												

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Huxtable RJ, Wild SL (1994). Relationship between in vitro metabolism of pyrrolizidine alkaloids and extrahepatic toxicity in vivo. Proc West Pharmacol Soc. 37:109–11	Heliotrine (purified)	Male rat liver microsomes	0.5 mM	+	GSDHR/DHR	12.5±2.1 nmol pyrrole/mg protein	-	-	-	-	-	-	-	-	-	-	-	-
					Dehydroalkaloid	0.4±0.2 nmol pyrrole/mg protein (3.3%)												
	Monocrotaline (purified)		0.5 mM		GSDHR/DHR	12.0±1.4 nmol pyrrole/mg protein												
					Dehydroalkaloid	4.6±1.8 nmol pyrrole/mg protein (27.7%)	-	-	-	-	-	-	-	-	-	-	-	-
	Retrorsine (purified)		0.5 mM		GSDHR/DHR	32.7±2.9 nmol pyrrole/mg protein	-	-	-	-	-	-	-	-	-	-	-	-
					Dehydroalkaloid	2.4±0.4 nmol pyrrole/mg protein (6.8%)												
	Senecionine (purified)		0.5 mM		GSDHR/DHR	28.3±2.9 nmol pyrrole/mg protein												
					Dehydroalkaloid	9.5±3.2 nmol pyrrole/mg protein (25.5%)	-	-	-	-	-	-	-	-	-	-	-	-
	Trichodesmine (purified)		0.5 mM		GSDHR/DHR	43.9±1.3 nmol pyrrole/mg protein												
					Dehydroalkaloid	19.2±2.4 nmol pyrrole/mg protein (30.3%)	-	-	-	-	-	-	-	-	-	-	-	-
Wang YP, Yan J, Beger RD, Fu PP, Chou MW (2005). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, monocrotaline, leading to DNA adduct formation in vivo. Cancer Lett. 226:27–35.	Monocrotaline (purity unknown)	Female rat liver microsomes	2 µmol in 50 µL	+	DHP	3.6±0.7 nmol/mg /min	+	Induction liver microsomes with DX led to significantly increased formation of DHP (2.1-fold) and *N*-oxide (2.2-fold). Inhibition of CYP3A significantly inhibited DHP and *N*-oxide formation	-	-	-	-	-	-	DNA adducts	Set of eight DHP-derived DNA adducts were formed. Two were identified as DHP-3'-dGMP adducts, the other six were characterized as DHP-derived dinucleotides	-	-
					Monocrotaline *N*-oxide	3.3±0.6 nmol/mg /min												
		Female rat lung microsomes			DHP	ND		Metabolism detected when microsomes treated with DX	-	-	-	-	-	-			-	-
					Monocrotaline *N*-oxide	ND												
	Riddelliine (purity unknown)	Female rat liver microsomes	2 µmol in 50 µL		DHP	5.4±0.3 nmol/mg/min		Induction liver microsomes with DX led to significantly increased formation of DHP (2.1-fold) and *N*-oxide (1.3-fold). Inhibition of CYP3A significantly inhibited DHP and *N*-oxide formation	-	-	-	-	-	-	-	-	-	-
					Riddelliine *N*-oxide	17.7±2.3 nmol/mg/min												
		Female rat lung microsomes			DHP	ND		Metabolism detected when microsomes treated with DX	-	-	-	-	-	-	-	-	-	-
					Riddelliine *N*-oxid	ND												
Couet CE, Hopley J, Hanley AB (1996). Metabolic activation of pyrrolizidine alkaloids by human, rat and avocado microsomes. Toxicon. 34:1058–61.	Monocrotaline	Rat liver microsomes	-	+	DHR	40–80% of major, no quantitative data	-	-	-	-	-	-	-	-	-	-	-	-
					Unchanged monocrotaline	Major, no quantitative data												
		Human liver microsomes			DHR	<40–80% of major, no quantitative data												
					Monocrotaline *N*-oxide	<40–80% of major, no quantitative data												
					Unchanged monocrotaline	Major, no quantitative data	-	-	-	-	-	-	-	-	-	-	-	-
	Retrorsine	Rat liver microsomes	-	+	DHR	40–80% of major, no quantitative data	-	-	-	-	-	-	-	-	-	-	-	-
					Retrorsine *N*-oxide	<40–80% of major, no quantitative data												
					Unchanged retrorsine	Major, no quantitative data												
		Human liver microsomes			DHR	40–80% of major, no quantitative data												
					Retrorsine *N*-oxide	<40–80% of major, no quantitative data												
					Unchanged retrorsine	Major, no quantitative data	-	-	-	-	-	-	-	-	-	-	-	-
	Retrorsine *N*-oxide	Rat liver microsomes	-	+	DHR	<40–80% of major, no quantitative data	-	-	-	-	-	-	-	-	-	-	-	-
					Retrorsine	40–80% of major, no quantitative data												
					Unchanged retrorsine *N*-oxide	Major, no quantitative data												
		Human liver microsomes			DHR	<40–80% of major, no quantitative data												
					Retrorsine	40–80% of major, no quantitative data												
					Unchanged retrorsine *N*-oxide	Major, no quantitative data	-	-	-	-	-	-	-	-	-	-	-	-

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Huan JY, Miranda CL, Buhler DR, Cheeke PR (1998). Species differences in the hepatic microsomal enzyme metabolism of the pyrrolizidine alkaloids. Toxicol Lett. 99:127–37.	Senecionine (purified)	Rat liver microsomes	0.5 mM	+	DHP	0.85±0.22 nmol/min/mg	-	-	-	-	-	-	-	-	-	-	-	-
					Senecionine *N*-oxide	0.70±0.20 nmol/min/mg												
					Ratio DHP/*N*-oxide	1.25±0.08												
					Ratio senecionine after incubation: senecionine before incubation	69.7±9.3												
		Gerbil liver microsomes	0.5 mM		DHP	1.34±0.06 nmol/min/mg												
					Senecionine *N*-oxide	0.97±0.01 nmol/min/mg												
					Ratio DHP/*N*-oxide	1.39±0.08												
					Ratio senecionine after incubation: senecionine before incubation	42.2±3.0	-	-	-	-	-	-	-	-	-	-	-	-
		Hamster liver microsomes	0.5 mM		DHP	3.55±0.08 nmol/min/mg	+	SKF-525A (CYP inhibitor) inhibited almost completely the formation of DHP	+	TOCP and PMSF (esterase inhibitors) reduced DHP formation (23% and 10.4% respectively). No marked decreases in *N*-oxide formation	+	FMO inhibitors (methimazole and thiourea) reduced *N*-oxide formation 80% and 71%, respectively	-	-	-	-	When GSH was added, DHP formation was significantly reduced (63%), while *N*-oxide formation was unaffected	-
					Senecionine *N*-oxide	1.55±0.01 nmol/min/mg												
					Ratio DHP/*N*-oxide	2.29±0.07												
					Ratio senecionine after incubation: senecionine before incubation	23.1±1.2												
		Quail liver microsomes	0.5 mM		DHP	0.08±0.03 nmol/min/mg												
					Senecionine *N*-oxide	0.28±0.05 nmol/min/mg												
					Ratio DHP/*N*-oxide	0.30±0.05												
					Ratio senecionine after incubation: senecionine before incubation	91.5±2.3	-	-	-	-	-	-	-	-	-	-	-	-
		Cattle liver microsomes	0.5 mM		DHP	0.23±0.03 nmol/min/mg	-	-	-	-	-	-	-	-	-	-	-	-
					Senecionine *N*-oxide	0.59±0.04 nmol/min/mg												
					Ratio DHP/*N*-oxide	0.38±0.02												
					Ratio senecionine after incubation: senecionine before incubation	61.1±4.3												
		Rabbit liver microsomes	0.5 mM		DHP	1.71±0.14 nmol/min/mg												
					Senecionine *N*-oxide	0.81±0.05 nmol/min/mg												
					Ratio DHP/*N*-oxide	2.11±0.07												
					Ratio senecionine after incubation: senecionine before incubation	30.0±2.3	-	-	-	-	-	-	-	-	-	-	-	-
		Sheep liver microsomes	0.5 mM		DHP	0.45±0.07 nmol/min/mg	+	SKF-525A (CYP inhibitor) inhibited almost completely the formation of DHP	+	TOCP and PMSF (esterase inhibitors) reduced DHP formation (90.8% and 30.8% respectively). No marked decreases in *N*-oxide formation	+	FMO inhibitors (methimazole and thiourea) reduced *N*-oxide formation 37.7 and 55.8%, respectively	-	-	-	-	When GSH was added, DHP formation was significantly reduced (63%), while *N*-oxide formation was unaffected	-
					Senecionine *N*-oxide	1.76±0.08 nmol/min/mg												
					Ratio DHP/*N*-oxide	0.26±0.05												
					Ratio senecionine after incubation: senecionine before incubation	27.5±1.5												
		Chicken liver microsomes	0.5 mM		DHP	0.22±0.03 nmol/min/mg												
					Senecionine *N*-oxide	0.52±0.05 nmol/min/mg												
					Ratio DHP/*N*-oxide	0.44±0.06												
					Ratio senecionine after incubation: senecionine before incubation	84.5±1.2	-	-	-	-	-	-	-	-	-	-	-	-
Xia Q, Ma L, He X, Cai L, Fu PP (2015). 7-glutathione pyrrole adduct: a potential DNA reactive metabolite of pyrrolizidine alkaloids. Chem Res Toxicol. 28:615–20.	7-GS-DHP	-	0.5, 1 mM	-	-		-	-	-	-	-	-	-	-	DNA adducts	Incubation with DHP-dG or DHP-dA: Formation of DHP-dA and DHP-dG adducts. The reaction yields of DHP-dG-1 and DHP-dG-2 increased in a time- and concentration-dependent manner and were higher than that of DHP-dG-3 and DHP-dG-4. The yields of DHP-dA-1 and DHP-dA-2 were higher than that of DHP-dA-3 and DHP-dA-4. In addition, formation of DHP was seen, with a yield increasing in a time-dependent manner. Incubation with calf thymus DNA: Formation of all four DHP-dG and DHP-dA adducts. Yields generally increased in a time-dependent manner, with significantly higher levels of DHP-dG-3 and DHP-dG-4 adducts than of DHP-dG-2 and DHP-dG-1 adducts. Same for DHP-dA adducts	-	-
	7,9-diGS-DHP	-	1 mM	-	-		-	-	-	-	-	-	-	-	DNA adducts	No DHP-dG adducts detected. Only trace amounts of DHP-dA-3 and DHP-dA-4 adducts detected	-	-

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Miranda CL, Rawson CL, Reed RL, Zhao X, Barnes DW, Buhler DR (1992). C3H/10T1/2 cells: a model to study the role of metabolism in the toxicity of the pyrrolizidine alkaloid retrorsine. In Vitro Toxicol. 5:21–32.	Retrorsine	Rat liver microsomes	?	+	DHP	2.79 nmol/min/mg protein	+	Suggest involvement of CYP3A due to higher rate of metabolism in case of PB and DX microsomes	-	-	-	-	-	-	-	-	-	-
					Retrorsine *N*-oxide	0.93 nmol/min/mg protein												
					Unknown metabolites	No quantitative data												
		Guinea-pig liver microsomes			DHP	0.076 nmol/min/mg protein	+	Suggest involvement of CYP3A due to higher rate of metabolism in case of PB and DX microsomes										
					Retrorsine *N*-oxide	0.22 nmol/min/mg protein												
					Unknown metabolites	No quantitative data			-	-	-	-	-	-	-	-	-	-
Yang M, Ruan J, Lin G (2014). Biotransformation from pyrrolizidine alkaloid *N*-oxides to pyrrolizidine alkaloids in liver and gastrointestinal tract. Drug Metab Rev. 45:195.	Four PA *N*-oxides, including riddelliine *N*-oxide	Rat liver and intestine S9 preparations, rat intestinal microbiota	-	+	PAs (metabolic rate 17.9–33.1% (liver), 10.7–17.6% (intestine)), pyrrole–protein adducts (same as formed with PAs). Higher conversion rate found in hepatic microsome than in cytosol		+	Inhibition of CYPs significantly inhibted microsomal formations of both parent PAs and protein adducts	-	-	-	-	-	-	Protein adducts	The same pyrrole–protein adducts as generated by PAs were detected for all four PA *N*-oxides tested	-	-
Wang YP, Yan J, Fu PP, Chou MW (2005). Human liver microsomal reduction of pyrrolizidine alkaloid *N*-oxides to form the corresponding carcinogenic parent alkaloid. Toxicology Lett. 155:411–20.	Riddelliine *N*-oxide (purified)	Female rat liver microsomes	1.4 µmol	+	DHP	6.1±2.0 nmol/mg protein/60 min	+	Inhibition of CYP3A inhibited the formation of DHP significantly	-	-	-	-	-	-	DNA adducts	Eight DNA adducts were formed upon reaction of *N*-oxide with calf thymus DNA, including DHP-3'dGMP adducts and DHP-modified dinucleotide adducts. Similar peaks observed between *N*-oxides	-	-
					Riddelliine	435±25 nmol/mg protein/60 min												
		Human liver microsomes			DHP	4.2±0.5 nmol/mg protein/60 min	+	Inhibition of CYP3A inhibited the formation of DHP significantly							DNA adducts	Eight DNA adducts were formed upon reaction of *N*-oxide with calf thymus DNA, including DHP-3'dGMP adducts and DHP-modified dinucleotide adducts. Similar peaks observed between *N*-oxides		
					Riddelliine	851±216 nmol/mg protein/60 min			-	-	-	-	-	-			-	-
	Retrorsine *N*-oxide (purity unknown)	Female rat liver microsomes	1.4 µmol	+	DHP	4.0±0.3 nmol/mg protein/60 min	+	Inhibition of CYP3A inhibited the formation of DHP significantly	-	-	-	-	-	-	DNA adducts	Eight DNA adducts were formed upon reaction of *N*-oxide with calf thymus DNA, including DHP-3'dGMP adducts and DHP-modified dinucleotide adducts. Similar peaks observed between *N*-oxides	-	-
					Retrorsine	320±31 nmol/mg protein/60 min												
		Human liver microsomes			DHP	4.6±0.6 nmol/mg protein/60 min	+	Inhibition of CYP3A inhibited the formation of DHP significantly							DNA adducts	Eight DNA adducts were formed upon reaction of *N*-oxide with calf thymus DNA, including DHP-3'dGMP adducts and DHP-modified dinucleotide adducts. Similar peaks observed between *N*-oxides		
					Retrorsine	374±19 nmol/mg protein/60 min			-	-	-	-	-	-			-	-
	Monocrotaline *N*-oxide (purified)	Female rat liver microsomes	1.5 µmol	+	DHP	2.0±0.4 nmol/mg protein/60 min	+	Inhibition of CYP3A inhibited the formation of DHP significantly	-	-	-	-	-	-	DNA adducts	Eight DNA adducts were formed upon reaction of *N*-oxide with calf thymus DNA, including DHP-3'dGMP adducts and DHP-modified dinucleotide adducts. Similar peaks observed between *N*-oxides	-	-
					Monocrotaline	273±38 nmol/mg protein/60 min												
		Human liver microsomes			DHP	2.4±0.4 nmol/mg protein/60 min	+	Inhibition of CYP3A inhibited the formation of DHP significantly							DNA adducts	Eight DNA adducts were formed upon reaction of *N*-oxide with calf thymus DNA, including DHP-3'dGMP adducts and DHP-modified dinucleotide adducts. Similar peaks observed between *N*-oxides		
					Monocrotaline	154±28 nmol/mg protein/60 min			-	-	-	-	-	-			-	-
Fashe MM, Juvonen RO, Petsalo A, Rahnasto-Rilla M, Auriola S, Soininen P et al. (2014). Identification of a new reactive metabolite of pyrrolizidine alkaloid retrorsine: (3H-pyrrolizin-7-yl) methanol. Chem Res Toxicol. 27:1950–7.	Retrorsine (purity unknown)	Human liver microsomes	100 µM	+	7-GSH-DHP	No quantitative data	-	-	-	-	-	-	-	-	-	-	In presence of added GSH	-
					7,9-diGSH-DHP	No quantitative data												
					Retrorsine *N*-oxide	No quantitative data												
					Dehydroretrorsine	No quantitative data												
					Retrorsic acid	No quantitative data												
					New metabolite (3H-pyrrolozin-7-yl) methanol	No quantitative data												
Tang J, Zhang M, Wang ZT, Akao T, Nakamura N, Hattori M (2004). Simultaneous determination of isoline and its two major metabolites using high-performance liquid chromatography. J Anal Toxicol. 28:11–5.	Isoline (>99%)	Male mouse liver microsomes	1 mM	+	Bisline	0.567±0.026 mM	+	Higher metabolic conversion rate in PB pretreated microsomes										
					Bisline lactone	0.130±0.013 mM												
					Isoline	0.330±0.011 mM												
		Male rat liver microsomes			Bisline	0.801±0.014 mM												
					Bisline lactone	0.254±0.035 mM												
					Isoline	0.038±0.007 mM												
	Crotaline (purity unknown)	Male mouse liver microsomes			Clivorine	0.480±0.045 mM	-			3								
		Male rat liver microsomes			Clivorine	1.006±0.062 mM												
	Monocrotaline (purity unknown)	Male mouse liver microsomes	?		Monocrotaline	0.989±0.097 mM	-											
		Male rat liver microsomes			Monocrotaline	1.114±0.283 mM												

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
He YQ, Yang L, Liu HX, Zhang JW, Liu Y, Fong A et al. (2010). Glucuronidation, a new metabolic pathway for pyrrolizidine alkaloids. Chem Res Toxicol. 23:591–9.	Senecionine (>99%)	Mouse liver microsomes	100, 500, 1000 µM	-	-		+		-		-		+	Glucuronidation almost absent. Activity of glucuronidation observed in following order: rabbits, humans, cattle, pigs and sheep > guinea-pigs, dogs, rats, and mice	-		-	-
		Rat liver microsomes	100, 500, 1000 µM	-	-		+		-		-		+	Glucuronidation almost absent. Activity of glucuronidation observed in following order: rabbits, humans, cattle, pigs and sheep > guinea-pigs, dogs, rats, and mice	-		-	-
		Guinea-pig liver microsomes	100, 500, 1000 µM	-	-		+		-		-		+	No significant glucuronidation observed. Activity of glucuronidation observed in following order: rabbits, humans, cattle, pigs and sheep > guinea-pigs, dogs, rats, and mice	-		-	-
		Dog liver microsomes	100, 500, 1000 µM	-	-		+		-		-		+	No significant glucuronidation observed. Activity of glucuronidation observed in following order: rabbits, humans, cattle, pigs and sheep > guinea-pigs, dogs, rats, and mice	-		-	-
		Rabbit liver microsomes	Glucuronidation, metabolic activation: 100, 500, 1000 µM. Kinetics: 10–1000 µM	-	-		+	P450-mediated metabolic activation observed. Activities on metabolic activation in following order: rats, mice and guinea-pigs > dogs > humans, pigs, cattle, sheep and rabbits	-		-		+	Significant glucuronidation observed. Highest activity on glucuronidation compared with other species, nearly two times greater than cattle and sheep. Activity of glucuronidation observed in following order: rabbits, humans, cattle, pigs and sheep > guinea-pigs, dogs, rats, and mice	-		-	Glucuronidation: $V_{max} = 2.9 \pm 7.1 \times 10\text{-}2$ nmol/min/mg. Km = $1.3 \times 102 \pm 1.2 \times 10$ µM. Enzyme efficiency: 2.2×10-2 mL/min/mg
		Pig liver microsomes	Glucuronidation, metabolic activation: 100, 500, 1000 µM. Kinetics: 10–1000 µM	-	-		+		-		-		+	Significant glucuronidation observed. Activity of glucuronidation observed in following order: rabbits, humans, cattle, pigs and sheep > guinea-pigs, dogs, rats, and mice	-		-	Glucuronidation: $V_{max} = 9.9 \times 10\text{-}1 \pm 3.7 \times 10\text{-}2$ nmol/min/mg. Km = $1.0 \times 102 \pm 1.5 \times 10$ µM. Enzyme efficiency: 1.0×10-2 mL/min/mg
		Cattle liver microsomes	Glucuronidation, metabolic activation: 100, 500, 1000 µM. Kinetics: 10–1000 µM	-	-		+		-		-		+	Significant glucuronidation observed. Activity of glucuronidation observed in following order: rabbits, humans, cattle, pigs and sheep > guinea-pigs, dogs, rats, and mice	-		-	Glucuronidation: $V_{max}1 = 9.8 \times 10\text{-}1 \pm 3.0 \times 10\text{-}3$ nmol/min/mg. Km, 1 = $1.5 \times 10 \pm 2.0 \times 10\text{-}1$ µM. Enzyme efficiency, 1: 6.5×10-2 mL/min/mg. $V_{max}2 = 1.2 \pm 2.9 \times 10\text{-}2$ nmol/min/mg. Km, 2 = $8.3 \times 10 \pm 1.4 \times 10$ µM. Enzyme efficiency, 2: 1.5×10-2 mL/min/mg
		Sheep liver microsomes	Glucuronidation, metabolic activation: 100, 500, 1000 µM. Kinetics: 10–1000 µM	-	-		+		-		-		+	Significant glucuronidation observed. Activity of glucuronidation observed in following order: rabbits, humans, cattle, pigs and sheep > guinea-pigs, dogs, rats, and mice	-		-	Glucuronidation: $V_{max}1 = 3.1 \times 10\text{-}1 \pm 8.0 \times 10\text{-}3$ nmol/min/mg. Km, 1 = $9.6 \times 10 \pm 7.5$ µM. Enzyme efficiency, 1: 3.0×10-3 mL/min/mg. $V_{max}2 = 6.4 \times 10\text{-}1 \pm 3.1 \times 10\text{-}2$ nmol/min/mg. Km, 2 = $6.9 \times 102 \pm 7.2 \times 10$ µM. Enzyme efficiency, 2: 1.0×10-3 mL/min/mg
		Human liver microsomes	Glucuronidation, metabolic activation: 100, 500, 1000 µM	+	*N*-glucuronide metabolite detected	No quantitative data	+		-		-		+	Significant glucuronidation observed. Activity of glucuronidation observed in following order: rabbits, humans, cattle, pigs and sheep > guinea pigs, dogs, rats, and mice. In addition, inhibition of UGT 1A4 inhibited glucuronidation significantly	-		-	Glucuronidation: $V_{max} = 3.0 \pm 1.5 \times 10\text{-}1$ nmol/min/mg. Km = $7.1 \times 102 \pm 7.0 \times 10$ µM. Enzyme efficiency: 4.0×10-3 mL/min/mg
		Human intestinal microsomes	?	-	-		-	-	-	-	-	-	+	No glucuronidation observed	-	-	-	-

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
	Senecionine *N*-oxide (purity unknown)	Human liver microsomes	100, 500, 1000 µM										+	No glucuronidation observed				
		Mouse liver microsomes		-	-		-	-	-	-	-	-	+	No glucuronidation activity observed	-	-	-	-
		Rat liver microsomes		-	-		-	-	-	-	-	-	+	No glucuronidation activity observed	-	-	-	-
		Guinea-pig liver microsomes		-	-		-	-	-	-	-	-	+	No glucuronidation activity observed	-	-	-	-
		Dog liver microsomes		-	-		-	-	-	-	-	-	+	No glucuronidation activity observed	-	-	-	-
	Adonifoline (>99%)	Rabbit liver microsomes	100 µM	+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Pig liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Cattle liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Sheep liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Human liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Mouse liver microsomes		-	-	-	-	-	-	-	-	-	+	No glucuronidation activity observed	-	-	-	-
		Rat liver microsomes		-	-	-	-	-	-	-	-	-	+	No glucuronidation activity observed	-	-	-	-
		Guinea-pig liver microsomes		-	-	-	-	-	-	-	-	-	+	No glucuronidation activity observed	-	-	-	-
		Dog liver microsomes		-	-	-	-	-	-	-	-	-	+	No glucuronidation activity observed	-	-	-	-
	Monocrotaline (>99%)	Rabbit liver microsomes	100 µM	+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Pig liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Cattle liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Sheep liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Human liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Mouse liver microsomes		-	-	-	-	-	-	-	-	-	+	No glucuronidation activity observed	-	-	-	-
		Rat liver microsomes		-	-	-	-	-	-	-	-	-	+	No glucuronidation activity observed	-	-	-	-
		Guinea-pig liver microsomes		-	-	-	-	-	-	-	-	-	+	Weak glucuronidation activty observed	-	-	-	-
		Dog liver microsomes		-	-	-	-	-	-	-	-	-	+	Weak glucuronidation activty observed	-	-	-	-
	Isoline (>99%)	Rabbit liver microsomes	100 µM	+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Pig liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Cattle liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Sheep liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-
		Human liver microsomes		+	Glucuronide metabolite detected	No quantitative data	-	-	-	-	-	-	+	Significant glucuronidation activity observed	-	-	-	-

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Xia Q, Yan J, Chou MW, Fu PP (2008). Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, heliotrine. Toxicol Lett. 178:77–82.	Heliotrine (purity unknown)	Male rat liver microsomes	0.2 mM	+	Formation of dehydroheliotridine (DHH) (mixture of DHH and DHR, optical purity 61.4%)	No quantitative data	-	-	-	-	-	-	-	-	DNA adducts	Formation of eight DHP-derived DNA adducts. Two were identified as DHP-3'-dGMP adducts; the other six were characterized as DHP-derived dinucleotides: 23.1 ± 8.3 adducts/108 nucleotides	-	-
					Intact heliotrine	No quantitative data												
	Riddelliine (purity unknown)	Male rat liver microsomes	0.2 mM	-	-		-	-	-	-	-	-	-	-	DNA adducts	338.5 ± 23.2 adducts/108 nucleotides	-	-
	Riddelliine *N*-oxide (purity unknown)	Male rat liver microsomes	0.2 mM	-	-		-	-	-	-	-	-	-	-	DNA adducts	66 ± 10.8 adducts/108 nucleotides	-	-
	Retrorsine (purity unknown)	Male rat liver microsomes	0.2 mM	-	-		-	-	-	-	-	-	-	-	DNA adducts	196 ± 22.8 adducts/108 nucleotides	-	-
	Retrorsine *N*-oxide (purity unknown)	Male rat liver microsomes	0.2 mM	-	-		-	-	-	-	-	-	-	-	DNA adducts	76 ± 8.5 adducts/108 nucleotides	-	-
	Monocrotaline (purity unknown)	Male rat liver microsomes	0.2 mM	-	-		-	-	-	-	-	-	-	-	DNA adducts	88.7 ± 2.7 adducts/108 nucleotides	-	-
Xia Q, Chou MW, Edgar JA, Doerge DR, Fu PP (2006). Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, lasiocarpine. Cancer Lett. 231:138–45.	Lasiocarpine (>99%)	Male rat liver microsomes	0.2 mM	+	DHP	0.35±0.02 nmol/min/mg protein	+	Inhibition of CYP3A significantly inhibited microsomal formation of DHP. In addition, 1.6-fold higher formation of DHP in case of DX microsomes	-	-	-	-	-	-	DNA adducts	Formation of same eight DHP-derived DNA adducts as with riddelliine. Two were identified as DHP-3'-dGMP adducts; the other six were characterized as DHP-derived dinucleotides: 10.1 ± 3.3 adducts/106 nucleotides. For comparison, riddelliine: 10.2 ± 3.3 adducts/106 nucleotides	-	V_{max}=1.13 ± 0.07 nmol/min/mg protein. Km = 0.09 ± 0.016 mM
		Female rat liver microsomes			DHP	0.17±0.01 nmol/min/mg protein	+	Inhibition of CYP3A significantly inhibited microsomal formation of DHP. In addition, 3.5-fold higher formation of DHP in case of DX microsomes										V_{max}=0.31 ± 0.01 nmol/min/mg protein. Km = 0.05± 0.005 mM
Wang YP, Fu PP, Chou MW (2005). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, retrorsine, leading to DNA adduct formation in vivo. Int J Environ Res Publ Health. 2:74–9.	Retrorsine (purity unknown)	Female rat liver microsomes	2 µmol in 50 µL	+	DHP	4.8±0.1 nmol/mg/min	+	Inhibition of CYP3A significantly inhibited microsomal formations of DHP and *N*-oxide. In addition, higher rate of metabolism in case of DX microsomes	-	-	-	-	-	-	DNA adducts	Formation of same eight DHP-derived DNA adducts as with riddelliine. Two were identified as DHP-3'-dGMP adducts; the other six were characterized as DHP-derived dinucleotides	-	-
					Retrorsine *N*-oxide	17.6±0.5 nmol/mg/min												
		Female rat lung microsomes (pretreated with DX)			DHP	510±40 pmol/mg/min		Higher rate of metabolism in case of DX microsomes	-	-	-	-	-	-	-	-	-	-
					Retrorsine *N*-oxide	727±26 pmol/mg/min												
		Female rat kidney microsomes (pretreated with DX)			DHP	79±3 pmol/mg/min		Higher rate of metabolism in case of DX microsomes	-	-	-	-	-	-	-	-	-	-
					Retrorsine *N*-oxide	627±6 pmol/mg/min												
		Female rat spleen microsomes (pretreated with DX)			DHP	128±6 pmol/mg/min		Higher rate of metabolism in case of DX microsomes	-	-	-	-	-	-	-	-	-	-
					Retrorsine *N*-oxide	702±39 pmol/mg/min												
Huxtable RJ, Yan CC, Wild S, Maxwell S, Cooper R (1996). Physicochemical and metabolic basis for the differing neurotoxicity of the pyrrolizidine alkaloids, trichodesmine and monocrotaline. Neurochem Res. 21:141–6.	Monocrotaline (purified)	Male rat liver perfusion	0.5 mM	+	Dehydromonocrotaline	116±20 nmol/g liver	-	-	-	-	-	-	-	-	-	-	-	-
					GS-DHP (bile)	180±16 nmol/g liver												
					DHP (perfusate)	141±11 nmol/g liver												
					Tissue bound	57±8 nmol/g liver												
	Trichodesmine (purified)	Male rat liver perfusion	0.5 mM	+	Dehydrotrichodesmine	468±64 nmol/g liver												
					GS-DHP (bile)	80±7 nmol/g liver												
					DHP (perfusate)	220±19 nmol/g liver												
					Tissue bound	64±7 nmol/g liver	-	-	-	-	-	-	-	-	-	-	-	-
Cooper RA, Huxtable RJ (1996). A simple procedure for determining the aqueous half-lives of pyrrolic metabolites of pyrrolizidine alkaloids. Toxicon. 24:604–7.	Dehydro-7-acetyllycopsamine (purity unknown)	-	10 µmol in 100 µL	+	-	Hydrolysis half-life: 0.31±0.02 sec	-	-	-	-	-	-	-	-	-	-	-	-
	Dehydrolycopsamine (purity unknown)	-		+	-	Hydrolysis half-life: 1.02±0.22 sec	-	-	-	-	-	-	-	-	-	-	-	-
	Dehydroretrorsine (purity unknown)	-		+	-	Hydrolysis half-life: 1.06±0.20 sec	-	-	-	-	-	-	-	-	-	-	-	-
	Dehydroseneciphylline (purity unknown)	-		+	-	Hydrolysis half-life: 1.60±0.28 sec	-	-	-	-	-	-	-	-	-	-	-	-
	Dehydromonocrotaline (purity unknown)	-		+	-	Hydrolysis half-life: 3.39±0.99 sec	-	-	-	-	-	-	-	-	-	-	-	-
	Dehydrotrichodesmine (purity unknown)	-		+	-	Hydrolysis half-life: 5.36±0.92 sec	-	-	-	-	-	-	-	-	-	-	-	-
Yan CC, Huxtable RJ (1994). Quantitation of the hepatic release of metabolites of the pyrrolizidine alkaloid, monocrotaline. Toxicol Appl Pharmacol. 127:58–63.	Monocrotaline (purity unknown)	Male rat liver perfusion	500 µM	+	Dehydromonocrotaline	87.6±6.03 nmol/g liver (1.6%)												
					GS-DHP (bile)	165.1±33.4 nmol/g liver (3.0%)												
					DHP + GS-DHP (perfusate)	103.7±24.0 nmol/g liver (2.0%)												
					Unchanged monocrotaline (bile)	20.5±4.5 nmol/g liver (0.4%)												
					Tissue bound	54.1±9.5 nmol/g liver (1.0%)	-	-	-	-	-	-	-	-	-	-	-	-

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Chung WG, Buhler DR (2004). Differential metabolism of the pyrrolizidine alkaloid, senecionine, in Fischer 344 and Sprague-Dawley rats. Arch Pharm Res. 27:547–53.	Senecionine (purified)	Male rat liver microsomes	0.5 mM	+	DHP	2.13±0.3 nmol/min/mg protein (F344)	+	Inhibition of CYP inhibited mainly the formation of DHP significantly. Involvement supported by pH optimum study. Activity of CYP3A2, involved in DHP formation, higher in SD rats than in F344 rats. Activity of CYP2C11, involved in *N*-oxide formation, higher in F344 rats than in SD rats. May explain differences in strains	-	-	+	Inhibition of FMO inhibited mainly the formation of *N*-oxide significantly. Involvement supported by pH optimum study. Higher FMO activity in F344 rats compared to SD rats may explain higher *N*-oxide formation	-	-	-	-	-	-
					Senecionine *N*-oxide	5.91±0.8 nmol/min/mg protein (F344); significantly lower rates in SD rats (no quantitative data)												
		Female rat liver microsomes		+	DHP	0.50±0.1 nmol/min/mg protein (F344)	+	Inhibition of CYP inhibited mainly the formation of DHP significantly. Involvement supported by pH optimum study. Activity of CYP3A2 and CYP2C11 higher in F344 rats than in SD rats, while female specific activity of CYP2A1 is higher in SD than in F344 rats			+	Inhibition of FMO inhibited mainly the formation of *N*-oxide significantly. Involvement supported by pH optimum study. No differences in FMO activity between strains						
					Senecionine *N*-oxide	0.64±0.1 nmol/min/mg protein (F344); significantly higher rates in SD rats (no quantitative data)			-	-			-	-	-	-	-	-
Miranda CL, Reed RL, Guengerich FP, Buhler DR (1991a). Role of cytochrome P450IIIA4 in the metabolism of the pyrrolizidine alkaloid senecionine in human liver. Carcinogenesis. 12:515–9.	Senecionine (purified)	Human liver microsomes	0.5 mM	+	DHP	0.19-5.7 nmol/min/mg protein dependent on HLM donor	+	Inhibition of CYP3A4 reduced formation rates significantly	-	-	-	-	-	-	-	-	-	V_{max} = 3.6 nmol/min/mg protein. Km = 0.27 mM
					Senecionine *N*-oxide	0.09–2.24 nmol/min/mg protein dependent on HLM donor												V_{max} = 1.4 nmol/min/mg protein. Km = 0.28 mM
Dueker SR, Lamé MW, Morin D, Wilson DW, Segall HJ (1992). Guinea pig and rat hepatic microsomal metabolism of monocrotaline. Drug Metab Dispos. 20:275–80.	[14C]-monocrotaline (purified)	Male rat liver microsomes	1 mM	+	Monocrotalic acid	22.5±4.3 nmol/mg protein	+	Inhibition of CYP inhibited formation of all metabolites significantly	+	Inhibition of carboxylesterase had no effect on formation of metabolites	-	-	-	-	-	-	-	-
					Retronecine	Not detectable												
					DHP	5.3±2.5 nmol/mg protein												
					Monocrotaline *N*-oxide	5.0±0.8 nmol/mg protein												
		Male guinea-pig liver microsomes	1 mM	+	Monocrotalic acid	168.3±27.0 nmol/mg protein	+	Inhibition of CYP inhibited formation of DHP and *N*-oxide significantly. No effect on monocrotalic acid and retronecine	+	Inhibition of carboxylesterase inhibited formation of monocrotalic acid and retronecine significantly. No significant effect n DHP and *N*-oxide	-	-	-	-	-	-	-	-
					Retronecine	130.4±23.3 nmol/mg protein												
					DHP	6.8±1.5 nmol/mg protein												
					Monocrotaline *N*-oxide	6.6±3.1 nmol/mg protein												
Glowaz SL, Michnika M, Huxtable RJ (1992). Detection of a reactive pyrrole in the hepatic metabolism of the pyrrolizidine alkaloid, monocrotaline. Toxicol Appl Pharmacol. 115:168–73.	Monocrotaline (purity unknown)	Male rat liver microsomes	0.5 mM	+	In absence of resin: DHR (no quantitative data). Pyrrole production increased with monocrotaline concentration up to 1 mM and then plateaued. In presence of resin: bound and unbound pyrroles detected. With increasing resin concentrations, increasing concentrations of bound pyrrole. Bound pyrrole determined to be DHR (38%) and 07-ethyldehydroretronecine (62%), the latter also formed by reaction between DHM and resin		-	-	-	-	-	-	-	-	-	-	-	-
Mattocks AR, Jukes R (1992). Chemistry of sulphur-bound pyrrolic metabolites in the blood of rats given different types of pyrrolizidine alkaloid. Natural Toxins. 1:89–95.	Dehydroindicine (purity unknown)	Male rat blood	10 mg in 0.5 mL	+	Bound pyrrole to thiol group of haemoglobin (7β-hydroxy-9-ethoxy-)		-	-	-	-	-	-	-	-	-	-	-	-
	Dehydroanacrotine (purity unknown)	Male rat blood	10 mg in 0.5 mL	+	Bound pyrrole to thiol group of haemoglobin (7β-ethoxy-9-hydroxy-, 7α-ethoxy-9-hydroxy-)		-	-	-	-	-	-	-	-	-	-	-	-
	Dehydroheliotrine (purity unknown)	Male rat blood	10 mg in 0.5 mL	+	Bound pyrrole to thiol group of haemoglobin (7α-hydroxy-9-ethoxy-)		-	-	-	-	-	-	-	-	-	-	-	-

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Lamé MW, Jones AD, Morin D, Segall HJ (1991). Metabolism of [14C]-monocrotaline by isolated perfused rat liver. Drug Metab Dispos. 19:516–24.	[14C]-monocrotaline (purity unknown but checked)	Male isolated rat liver perfused	40 µmol in 120 mL	+	Unchanged monocrotaline (perfusate)	Major component	-	-	-	-	-	-	-	-	-	-	-	-
					Monocrotalic acid (perfusate)	Major metabolite												
					Monocrotaline *N*-oxide (perfusate)													
					1-formyl-7-hydroxy-6,7-dihydro-5H-pyrrolizine (perfusate)	Trace amounts												
					DHP (perfusate)	Trace amounts												
					1-hydroxymethyl-7-oxo-6,7-dihydro-5H-pyrrolizine (perfusate)	Trace amounts												
					GSH-DHP (bile)	Major metabolite												
					Unchanged monocrotaline (bile)													
					Monocrotalic acid (bile)													
Williams DE, Reed RL, Kedzierski B, Dannan GA, Guengerich FP, Buhler DR (1989). Bioactivation and detoxification of the pyrrolizidine alkaloid senecionine by cytochrome P-450 enzymes in rat liver. Drug Metab Dispos. 17:387–92.	Senecionine (purified)	Male rat liver microsomes	1 mM	+	DHP	2.0 nmol/min/nmol P-450	+	Involvement suggested by higher rate of metabolism in case of DX microsomes	-	-	-	-	-	-	-	-	-	-
					Senecionine *N*-oxide	5.7 nmol/min/nmol P-450												
		Rat liver enzymes	0.5 mM	+	Formation of DHP, *N*-oxide		+	Rat P450 enzymes displayed metabolizing activity towards senecionine, mainly in the formation of *N*-oxide. Inhibition of rat P-450 UT-A activity by IgG inhibited mainly the formation of *N*-oxide. Inhibition of rat P-450 PCN-E activity by IgG inhibited mainly the formation of DHP, followed by that of *N*-oxide									
		Female rat liver microsomes	1 mM	+	DHP	1.2 nmol/min/nmol P-450	+	Involvement suggested by higher rate of metabolism in case of DX microsomes	-	-	-	-	-	-	-	-	-	-
					Senecionine *N*-oxide	0.4 nmol/min/nmol P-450												
Chan MY, Zhao XL, Ogle CW (1989). A comparative study on the hepatic toxicity and metabolism of Crotalaria Assamica and Eupatorium species. Am J Chinese Med. 17:165–70.	*Crotalaria assamica*	Mouse liver microsomes	0.4125, 0.825, 1.65, 3.3 mM monocrotaline equivalents	+	Pyrrole	Increase in formation of both metabolites with increasing concentration. Levels of pyrrole formation higher than *N*-oxide formation	-	-	-	-	-	-	-	-	-	-	-	V_{max} = 1.89±0.27 nmol/mg protein/min, Km = 0.51±0.05 µM
					N-oxide													V_{max} = 0.86±0.18 nmol/mg protein/min, Km = 1.28±0.03 µM. Ratio pyrrole:*N*-oxide = 2.21
	Eupatorium japonicum	Mouse liver microsomes	0.4125, 0.825, 1.65, 3.3 mM monocrotaline equivalents	+	Pyrrole	Increase in formation of both metabolites with increasing concentration. Levels of *N*-oxide formation higher than pyrrole formation except at lowest concentration	-	-	-	-	-	-	-	-	-	-	-	V_{max} =1.59 nmol/mg protein/min, Km =0.28 µM.
					N-oxide													V_{max} = 9.62 nmol/mg protein/min, Km =3.56 µM. Ratio pyrrole:*N*-oxide = 0.17
	Monocrotaline (purity unknown)	Mouse liver microsomes	0.4125, 0.825, 1.65, 3.3 mM	+														V_{max} = 2.46±0.06 nmol/mg protein/min, Km = 1.27±0.05 µM
																		V_{max} = 0.92±0.16 nmol/mg protein/min, Km = 0.40±0.02 µM. Ratio pyrrole:*N*-oxide = 2.65
	Retrorsine (purity unknown)	Mouse liver microsomes	0.4125, 0.825, 1.65, 3.3 mM monocrotaline equivalents	+														V_{max} = 11.27±0.29 nmol/mg protein/min, Km = 0.51±0.04 µM
																		V_{max} = 4.00±0.87 nmol/mg protein/min, Km = 0.81±0.01 µM. Ratio pyrrole:*N*-oxide = 2.81
Chung WG, Buhler DR (1994). The effect of spironolactone treatment on the cytochrome P450-mediated metabolism of the pyrrolizidine alkaloid senecionine by hepatic microsomes from rats and guinea pigs. Toxicol Appl Pharmacol. 127:314–9.	Senecionine (purified)	Male rat liver microsomes	0.5 mM	+	DHP	Increased from control using microsomes from i.p. spironolactone treated rats	+	TAO had an inhibitory effect on the formation of both metabolites										
					Senecionine *N*-oxide	Decreased from controls using microsomes from i.p. spironolactone treated rats												
		Female rat liver microsomes		+	DHP	Increased from control using microsomes from i.p. spironolactone treated rats	+	TAO had an inhibitory effect on the formation of both metabolites										
					Senecionine *N*-oxide	Increased from controls using microsomes from spironolactone-treated rats												
		Male guinea-pig liver microsomes		+	DHP	Increased from control in i.p. and orally spironolactone treated guinea-pigs												
					Senecionine *N*-oxide	Increased using microsomes from orally sprironolactone treated guinea-pigs												
		Female guinea-pig liver microsomes		+	DHP	Increased from control in orally treated guinea-pigs												
					Senecionine *N*-oxide	Increased using microsomes from guinea-pigs orally treated with spironolactone												

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely	CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
He YQ, Liu Y, Zhang BF, Liu HX, Lu YL, Yang L et al. (2010). Identification of the UDP-glucuronosyltransferase isozyme involved in senecionine glucuronidation in human liver microsomes. Drug Metab Dispos. 38:626–34.	Senecionine	Human liver microsomes	50–1250 µM	+	Glucuronide metabolite detected							+	UGT1A4				Km 5.6 ± 0.46 x 102 µL, V_{max} 2.1 ± 0.080 nmol/min/mg
		Recombinant UGTs	100, 500, 1000 µM	+	Glucuronide metabolite detected							+	UGT1A4 and to a small degree UGT1A3 showed activity				
Ruan J, Yang M, Fu P, Ye Y, Lin G (2014). Metabolic activation of pyrrolizidine alkaloids: insights into the structural and enzymatic basis. Chem Res Toxicol. 27:1030–9.	Monocrotaline (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+	a.o. 7-GS-DHP	+	CYP 2A>>2E1>>3A4≈3A5 ≈2B6≈2D6> 1A1≈1A2≈2C9>2C19							Protein adducts	±250 nM		
	Retrorsine (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+	a.o. 7-GS-DHP	+	CYP 2A6> CYP 3A4> CYP 3A5>>all other CYPs							Protein adducts	±500 nM		
	Seneciphylline (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+	a.o. 7-GS-DHP	+	CYP 3A5>CYP 3A4>CYP 2A6>all other CYPs							Protein adducts	±750 nM		
	Senecionine (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+	a.o. 7-GS-DHP	+	CYP 2A6≈CYP 3A5≈CYP 3A4>2D6>all other CYPs							Protein adducts	±900 nM		
	Senkirkine (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+	a.o. 7-GS-DHP	+	CYP 3A5> CYP 3A4, other CYPS did not form adducts							Protein adducts	±150 nM		
	Heliotrine (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+		-	No or negligible pyrrole–protein adducts detected with microsomes, recombinant tests not done							Protein adducts	±20 nM		
	Lycopsamine (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+		-	No or negligible pyrrole–protein adducts detected with microsomes, recombinant tests not done										
	Lasiocarpine (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+	a.o. 7-GS-DHP	+	CYP 3A4> 3A5>1A1> all other CYPs							Protein adducts	±1000 nM		
	Clivorine (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+	a.o. 7-GS-DHP	+	CYP 3A5>>CYP 3A4, other CYPS did not form adducts							Protein adducts	±250 nM		
	Integerrimine (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+	a.o. 7-GS-DHP	+	CYP 3A5≈CYP 3A4>>all other CYPs							Protein adducts	±700 nM		
	Platyphylline (with 30% of its isomer neoplatyphylline) (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+		-	No or negligible pyrrole–protein adducts detected with microsomes, recombinant tests not done										
	Riddelliine (purity unknown)	Human liver microsomes Human recombinant CYP supersomes	200 µM	+	a.o. 7-GS-DHP	+	CYP 3A4≈3A5> all other CYPs							Protein adducts	±600 nM		
Wang J, Yang L, Wang C, Wang Z (2009). The action of cytochrome p450 enzymes and flavin-containing monooxygenases on the *N*-oxide of pyrrolizidine alkaloid monocrotaline. Asian J Tradit Med. 4:41–50.	Monocrotaline (>98%)	Male rat liver microsomes	0.05 mM	+	Monocrotaline *N*-oxide	+	CYP 450 inhibitor (SKF-525A) inhibited monocrotaline *N*-oxide formation by 92.66% at 20 µM and 93.91% at 100 µM			+	FMO inhibitor (methimazole) inhibited monocrotaline *N*-oxide by 44.52% at 20 µM and 59.46% at 100 µM						Km = 566.9 µM, V_{max} = 483.8 ± 14.04 nmol/min/mg protein for the formation of monocrotaline *N*-oxide. Km = 590.6 µM, V_{max} = 104.8 ± 4.125 nmol/min/mg protein for the metabolism of monocrotaline
			0.1 mM		Monocrotaline *N*-oxide		69.61 nmol/min/mg protein										
			0.5 mM		Monocrotaline *N*-oxide		236.43 nmol/min/mg protein										
			1.0 mM		Monocrotaline *N*-oxide		377.72 nmol/min/mg protein										

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Duringer JM, Buhler DR, Craig AM (2004). Comparison of hepatic in vitro metabolism of the pyrrolizidine alkaloid senecionine in sheep and cattle. Am J Vet Res. 65:1563–72.	Senecionine (purity unknown)	Male sheep liver microsomes	0.5 mM	+	DHP	0.50±0.10 nmol/min/mg protein	+	SKF-525A (P450 inhibitor) signficantly inhibited DHP and *N*-oxide formation. TAO (CYP3A inhibitor) inhibited DHP formation but less than in cattle	+	TOCP significantly inhibited DHP and *N*-oxide formation	+	Methimazole and thiourea reduced DHP and *N*-oxide formation					Addition of GSH decreased DHP formation. Addition of cytosol decreased *N*-oxide formation.	
					Senecionine *N*-oxide	1.08±0.06 nmol/min/mg protein												
		Female sheep liver microsomes		+	DHP	0.28±0.09 nmol/min/mg protein												
					Senecionine *N*-oxide	1.11±0.38 nmol/min/mg protein												
		Male cow liver microsomes		+	DHP	0.39±0.08 nmol/min/mg protein	+	SKF-525A (P450 inhibitor) signficantly inhibited DHP and *N*-oxide formation. TAO (CYP3A inhibitor) inhibited DHP formation but more than in sheep	+	TOCP significantly inhibited DHP and *N*-oxide formation	+	Methimazole and thiourea reduced DHP and *N*-oxide formation					Addition of GSH decreased DHP formation. Addition of cytosol decreased *N*-oxide formation.	
					Senecionine *N*-oxide	0.19±0.04 nmol/min/mg protein												
		Female cow liver microsomes		+	DHP	0.42±0.13 nmol/min/mg protein												
					Senecionine *N*-oxide	0.33±0.08 nmol/min/mg protein												
Reid MJ, Lamé MW, Morin D, Wilson DW, Segall HJ (1998). Involvement of cytochrome P450 3A in the metabolism and covalent binding of 14C-monocrotaline in rat liver microsomes. J Biochem Mol Toxicol. 12:157–66.	[14C]-monocrotaline (purity unknown)	Male rat liver microsomes	0.5 mM	+	Monocrotalic acid	5.85±0.16%	+	Troleandomycin (CYP 3A inhibitor) decreased metabolite percentages. Metabolite formation was increased using CYP3A inducers										
					Retronecine	0.23±0.14%												
					Monocrotaline *N*-oxide	1.76±0.07%												
					Dihydropyrrolizine	1.15±0.15%												
					Unchanged compound	90.10±0.87%												
Reid MJ, Dunston SK, Lamé MW, Wilson DW, Morin D, Segall HJ (1998). Effect of monocrotaline metabolites on glutathione levels in human and bovine pulmonary artery endothelial cells. Res Comm Mol Pathol Pharmacol. 99:53–68.	Dehydro monocrotaline (purity unknown)	Human pulmonary artery endothelial cells	15, 100 µM														Maximal GSH depletion at 15 min: 30% at 15 µM, 40% at 100 µM	
		Bovine pulmonary artery endothelial cells	15, 100 µM														Maximal GSH depletion at 15 min: 45% at 15 µM, 60% at 100 µM	
	Dehydroretronecine (purity unknown)	Human pulmonary artery endothelial cells	100 µM														Maximal GSH depletion at 15 min: 45% at 100 µM	
Chou MW, Wang YP, Yan J, Yang YC, Beger RD, Williams LD et al. (2003). Riddelliine *N*-oxide is a phytochemical and mammalian metabolite with genotoxic activity that is comparable to the parent pyrrolizidine alkaloid riddelliine. Toxicol Lett. 145:239–47.	Riddelliine *N*-oxide (synthesized from riddelliine, purified)	Female rat liver microsomes	0.5 mg/mL	+	DHP	12.9 ±0.01 nmol/60 min												
					Riddelliine	126.2 ±44 nmol/60 min												
	Riddelliine (purity unknown, but confirmed)	Calf thymus DNA	1.0 mg												DNA adducts	P4 and P6 were identified as a pair of diastereoisomers of 3 -monophosphate of 7-(deoxyguanosin-N2-yl)dehydrosupinidine (DHP-3 - dGMP), and the six DHP-derived adducts designated P1, P2, P3, P5, P7 and P8 were characterized as six DHP-modified dinucleotides. The quantity of DHP-DNA adducts generated in the in vitro metabolism of riddelliine (28.2±1.1 adducts/107 nucleotides) was 1.9-fold greater than that from riddelliine *N*-oxide (14.5±4.0 adducts/107 nucleotides)	
		Calf thymus DNA	1.0 mg															
Yan CC, Huxtable RJ (1995). Relationship between glutathione concentration and metabolism of the pyrrolizidine alkaloid, monocrotaline, in the isolated, perfused liver. Toxicol Applied Pharmacol. 130:132–9.	Monocrotaline (purity unknown)	Isolated perfused rat liver	0.5 mM	+	Dehydromonocrotaline (perfusate)	13.4±2.2 nmol/g liver/hr											Liver GSH concentrations correlated with amount of GS-DHP released into bile. Release of GS-DHP into bile was strongly reduced in GSH-depleted livers, and increased in livers where GSH levels were increased. Dehydromonocrotaline release in perfusate increased significantly in livers with increased GSH levels as well as with decreased GSH levels. Tissue-bound pyrrole levels increased when livers were exposed to strong GSH-depleting agents	
					GS-DHP (bile)	179.3±17.6 nmol/g liver/hr												
					DHP (perfusate)	192.5±18.5 nmol/g liver/hr												
					Tissue-bound	60.3±9.1 nmol/g liver/hr												
Yan CC, Huxtable RJ (1995). The relationship between the concentration of the pyrrolizidine alkaloid monocrotaline and the pattern of metabolites released from the isolated liver. Toxicol Appl Pharmacol. 130:1–8.	Monocrotaline (purity unknown)	Isolated perfused rat liver	125 µM	+	Dehydromonocrotaline	59.8±9.0 µM (4.6%)												
					GS-DHP (bile)	63.2±10.5 µM (4.9%)												
					DHP (perfusate)	75.3±12.8 µM (5.8%)												
					Tissue-bound	39.7±5.1 µM (3.1%)												
					Unchanged compound (perfusate)	330±100 µM (25.6%)												
					Unchanged compound (bile)	7.6±0.3 µM (0.6%)												
			250 µM	+	Dehydromonocrotaline	85.4±7.2 µM (3.3%)												
					GS-DHP (bile)	109.6±18.3 µM (4.2%)												
					DHP (perfusate)	91.4±17.1 µM (3.5%)												
					Tissue-bound	49.9±12.9 µM (1.9%)												
					Unchanged compound (perfusate)	600±200 µM (23.5%)												
					Unchanged compound (bile)	14.4±2.1 µM (0.6%)												

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
			500 µM	+	Dehydromonocrotaline	144.5±12.5 µM (2.7%)												
					GS-DHP (bile)	181.6±31.1 µM (3.4%)												
					DHP (perfusate)	142.3±11.3 µM (2.6%)												
					Tissue-bound	95.8±8.9 µM (1.8%)												
					Unchanged compound (perfusate)	2090±120 µM (38.9%)												
					Unchanged compound (bile)	23.5±0.3 µM (0.4%)												
			1000 µM	+	Dehydromonocrotaline	269.4±35.9 µM (2.6%)												
					GS-DHP (bile)	312.9±49.0 µM (3.0%)												
					DHP (perfusate)	210.2±10.4 µM (2.0%)												
					Tissue-bound	108.7±10.3 µM (1.0%)												
					Unchanged compound (perfusate)	4430±330 µM (41.9%)												
					Unchanged compound (bile)	49.4±6.7 µM (0.5%)												
			1500 µM	+	Dehydromonocrotaline	459.9±98.5 µM (2.4%)												
					GS-DHP (bile)	238.3±35.5 µM (1.3%)												
					DHP (perfusate)	423.7±136.6 µM (1.0%)												
					Tissue-bound	195.8±35.4 µM (1.1%)												
					Unchanged compound (perfusate)	7660±230 µM (41.0%)												
					Unchanged compound (bile)	64.4±4.1 µM (0.4%)												
Yan CC, Huxtable RJ (1995). Effect of the pyrrolizidine alkaloid, monocrotaline, on bile composition of the isolated, perfused rat liver. Life Sci. 57:617–26.	Monocrotaline (purity unknown)	Isolated perfused rat liver	0.5 mM	+	GS-DHP (bile)	No quantitative data											Significantly higher levels of GSH, cysteine and cysteinylglycine detected in bile in response to perfusion with monocrotaline	
Chung, Miranda & Buhler, 1995	Senecionine (purity unknown)	Male guinea-pig liver microsomes	0.5 mM	+	DHP	0.69±0.1 nmol/min/mg protein	-	-										
					Senecionine *N*-oxide	2.43±0.2 nmol/min/mg protein												
		Purified guinea-pig P450 enzymes HAP1 and HAP2	1 mM	+	DHP	1.98 nmol/min/nmol HAP1; 1.45 nmol/min/nmol HAP2	+	CYP2B (HAP1) main enzyme involved in mainly conversion to DHP, and to a lesser extent *N*-oxide.										
					Senecionine *N*-oxide	0.08 nmol/min/nmol HAP1; 13.30 nmol/min/nmol HAP2												
Fashe MM, Juvonen RO, Petsalo A, Vepsalainen J, Pasanen M, Rahnasto-Rilla M (2015). In silico prediction of the site of oxidation by cytochrome P450 3A4 that leads to the formation of the toxic metabolites of pyrrolizidine alkaloids. Chem Res Toxicol. 28:702–10.	Lasiocarpine (highest purity available) Retrorsine (highest purity available) Senkirkine (highest purity available)	In silico	N/A		C3 atom of lasiocarpine and retrorsine and C26 of senkirkine are the most favoured sites of hydroxylation that lead to the production of their toxic metabolites		+	CYP3A4									
	Lasiocarpine (highest purity available)	Human liver microsomes, recombinant CYP3A4	100 µM	+	Mono-GSH-DHP	No quantitative data, 2.5 and 19 times higher than that from retrorsine and senkirkine, respectively	+	CYP3A4 catalysed both DHP and *N*-oxide formation										
					Lasiocarpine *N*-oxide	No quantitative data, 10.5% of total metabolites												
					Another 10 metabolites	No quantitative data												
	Retrorsine (highest purity available)		100 µM	+	Mono-GSH-DHP	No quantitative data	+	CYP3A4 catalysed both DHP and *N*-oxide formation										
					Retrorsine *N*-oxide	No quantitative data, 50.3% of total metabolites												
					Another 2 metabolites	No quantitative data												
	Senkirkine (highest purity available)		100 µM	+	Mono-GSH-DHP	No quantitative data	+	CYP3A4 catalysed both DHP and *N*-oxide formation										
					Hydroxyl metabolite	No quantitative data												
Yan CC, Cooper RA, Huxtable RJ (1995). The comparative metabolism of the four pyrrolizidine alkaloids, seneciphylline, retrorsine, monocrotaline, and trichodesmine iin the isolated, perfused rat liver. Toxicol Appl Pharmacol. 133:277–84.	Seneciphylline (purity unknown but checked)	Isolated perfused rat liver	0.5 mM	+	Dehydroalkaloid	165±19 nmol/g liver												
					GS-DHP	404±33 nmol/g liver												
					DHP	232±7 nmol/g liver												
					Bound	91±5 nmol/g liver												
	Retrorsine (purity unknown but checked)	Isolated perfused rat liver	0.5 mM		Dehydroalkaloid	165±7 nmol/g liver												
					GS-DHP	881±45 nmol/g liver												
					DHP	374±35nmol/g liver												
					Bound	197±23 nmol/g liver												
	Monocrotaline (purity unknown but checked)	Isolated perfused rat liver	0.5 mM		Dehydroalkaloid	116±20 nmol/g liver												
					GS-DHP	180±16 nmol/g liver												
					DHP	141±11 nmol/g liver												
					Bound	57±8 nmol/g liver												

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
	Trichodesmine (purity unknown but checked)	Isolated perfused rat liver	0.5 mM		Dehydroalkaloid	468±64 nmol/g liver												
					GS-DHP	80±7 nmol/g liver												
					DHP	220±19 nmol/g liver												
					Bound	65±7 nmol/g liver												
Ruan J, Liao C, Ye Y, Lin G (2014). Lack of metabolic activation and predominant formation of an excreted metabolite of nontoxic platynecine-type pyrrolizidine alkaloids. Chem Res Toxicol. 27:7–16.	Retrorsine (purity unknown)	Rat liver S9 fraction	200 µM	+	Retrorsine *N*-oxide	No quantitative data												
					GSM	No quantitative data												
	Clivorine (purity unknown)	Rat liver S9 fraction	200 µM	+	Clivoric acid	No quantitative data												
					GSM	No quantitative data												
	Platyphylline (with 30% of its isomer neoplatyphylline) (purity unknown)	Rat liver S9 fraction	200 µM	+	Platyphylline epoxide	No quantitative data	+	For platyphylline: in rat liver microsomes 3A1/2 and 2D6 most important, in human supersomes 3A4 >> 2C19 ≥ 2B6 > 2D6 ≥ 2C9 > 1A1 ≥ 1A2			-	Platyphylline metabolism was not significantly inhibited by methimazole (non specific FMO inhibitor) in rat liver microsomes						
					Platyphylline *N*-oxide	No quantitative data												
					Dehydroplatyphylline carboxylic acid	No quantitative data, major metabolite												
					Dehydroneoplatyphylline carboxylic acid	No quantitative data												
		Rat liver microsomes		+	Platyphylline epoxide	No quantitative data, trace amounts	+	Inhibition of CYP enzymes significantly inhibited formation of platyphylline *N*-oxide and dehydroplatyphylline carboxylic acid. Formation of platyphylline *N*-oxide mainly catalysed by CYP1A1/2, CYP2B and CYP3A, formation of dehydroplatyphylline carboxylic acid mainly catalysed by CYP1A1/2, CYP2D, and CYP3A. Main contributions by CYP2D and CYP3A										
					Platyphylline *N*-oxide	No quantitative data												
					Dehydroplatyphylline carboxylic acid	No quantitative data												
		Human liver microsomes		+	Platyphylline epoxide	No quantitative data, metabolic rate lower than in rat	-											
					Platyphylline *N*-oxide	No quantitative data, metabolic rate lower than in rat												
					Dehydroplatyphylline carboxylic acid	No quantitative data, main metabolite, metabolic rate lower than in rat												
		Recombinant CYPs		+	Platyphylline epoxide	No quantitative data	+	CYP3A4 showed significantly high activity in mediating formation of platyphylline *N*-oxide and dehydroplatyphylline carboxylic acid. Formation of platyphylline *N*-oxide also mediated by CYP2C19, CYP1A1, CYP1A2. Formation of dehydroplatyphylline carboxylic acid also mediated by CYP2D6, CYP2B6 and CYP2C19										
					Platyphylline *N*-oxide	No quantitative data												
					Dehydroplatyphylline carboxylic acid	No quantitative data												
Dueker SR, Lamé MW, Segall HJ (1992). Hydrolysis of pyrrolizidine alkaloids by guinea pig hepatic carboxylesterases. Toxicol Appl Pharmacol. 117:116–21.	[14C]-seneciphylline (purified)	Purified GPH1 and GPL1 carboxylesterases	1 mM						+	Activity with GPH1								Km=29.5 ±5.9 µM, V_{max}=135 ±23.3 nmol/min/mg (GPH1)
		Male guinea-pig liver microsomes							+	Activity in microsomal incubation								
	[14C]-monocrotaline (purified)	Purified GPH1 and GPL1 carboxylesterases	1 mM						+	Activity with GPH1								Km=138.8 ±36.4 µM, V_{max}=63.6 ±19.6 nmol/min/mg (GPH1)
		Male guinea-pig liver microsomes							+	Activity in microsomal incubation								
	[14C]-senectionine 93% integerrimine 7% mixture	Purified GPH1 and GPL1 carboxylesterases	1 mM						+	Activity with GPH1								
		Male guinea-pig liver microsomes							+	Activity in microsomal incubation								
Miranda CL, Chung W, Reed RE, Zhao X, Henderson MC, Wang JL et al. (1991). Flavin-containing monooxygenase: a major detoxifying enzyme for the pyrrolizidine alkaloid senecionine in guinea pig tissues. Biochem Biophys Res Comm. 178:546–52.	Senecionine (purity unknown)	Male guinea-pig liver microsomes	0.5 mM	+	Senecionine *N*-oxide	Major metabolite, independent of pH (6.8–9.8): 9.34 nmol/min/mg protein at pH 8.7; 6.43 nmol/min/mg protein at pH 7.8		Anti-P450 reductase IgG inhibited DHP formation 100% and *N*-oxide formation 18% in liver microsomes. SKF-525A and 1-benzylimidazole inhibited DHP formation (almost) completely, *N*-oxide formation was inhibited by 22% to 35% dependent on pH			+	Methimazole inhibited *N*-oxide formation						
					DHP	0.074 nmol/min/mg protein at pH 8.7; 0.22 nmol/min/mg protein at pH 7.8												

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Huan JY, Miranda CL, Buhler DR, Cheeke PR (1998). The roles of CYP3A and CYP2B isoforms in hepatic bioactivation and detoxification of the pyrrolizidine alkaloid senecionine in sheep and hamsters. Toxicol Appl Pharmacol. 151:229–35.	Senecionine (purified)	Male guinea-pig kidney microsomes	0.5 mM	+	Senecionine *N*-oxide	No quantitative data, formation increased with increasing pH	-	Anti-P450 reductase IgG had a slight inhibitory effect on *N*-oxide formation (no greater than 20%) in lung and kidney microsomes. SKF-525A had a slight inhibitory effect on *N*-oxide formation in lung but not in kidney microsomes	-	-	+	Methimazole inhibited *N*-oxide formation	-	-	-	-	-	-
		Guinea-pig lung microsomes		+	Senecionine *N*-oxide	No quantitative data, formation increased with increasing pH	+		-	-	+	Methimazole inhibited *N*-oxide formation	-	-	-	-	-	-
		Male sheep liver microsomes		+	DHP Senecionine *N*-oxide Ratio DHP/*N*-oxide	0.45±0.07 nmol/min/mg 1.76±0.08 nmol/min/mg 0.26±0.05	+	Anti-sheep CYP3A IgG (20 mg/nmol P450) inhibited production of DHP (96.5%) and *N*-oxide (38.8%) and anti-sheep CYP2B IgG (20 mg/nmol P450) 47.7% and 24.6%, respectively CYP3A selective inhibitors inhibited production of DHP and were much less potent in inhibition of *N*-oxide formation	-	-	-	-	-	-	-	-	-	-
		Male hamster liver microsomes		+	DHP Senecionine *N*-oxide Ratio DHP/*N*-oxide	3.55±0.08 nmol/min/mg 1.55±0.01 nmol/min/mg 2.29±0.07	+	Anti-sheep CYP3A IgG (20 mg/nmol P450) inhibited production of DHP (69.5%) and *N*-oxide (41.3%) and anti-sheep CYP2B IgG (20 mg/nmol P450) 32.5.7% and 35.4%, respectively CYP3A selective inhibitors inhibited production of DHP and were much less potent in inhibition of *N*-oxide formation	-	-	-	-	-	-	-	-	-	-
Williams DE, Reed RL, Kedzierski B, Ziegler DM, Buhler DR (1989). The role of flavin-containing monooxygenase in the N-oxidation of the pyrrolizidine alkaloid senecionine. Drug Metab Dispos. 17:380–6.	Senecionine (purified)	Male rat liver microsomes	1 mM	+	Senecionine *N*-oxide DHP	No quantitative data No quantitative data	+	SKF-525A and metyrapone significantly reduced formation of both metabolites, being more effective inhibitors of DHP formation than *N*-oxidation. *N*-octylamine inhibited both DHP and *N*-oxide production in a comparable way. Rabbit anti-rat NADPH-cytochrome P-450 reductase IgG inhibited 75% of N-oxidation and over 90% of DHP production	-	-	+	FMO contributes at most about 20% of the senecionine *N*-oxidation	-	-	-	-	-	-
		Purified FMO from male pig microsomes	1 mM (0.05–1 mM for kinetic analysis)	+	Senecionine *N*-oxide													Km=0.3 mM, V_{max}=55 nmol/min/mg
		Purified FMO from rabbit lung	1 mM	+	No detectable activity													
Chen M, Li L, Zhong D, Shen S, Zheng J, Chen X (2016). Glutathionyl-6,7-dihydro-1-hydroxymthyl-5H-pyrrolizine is the major pyrrolic glutathione conjugate of retronecine-type pyrrolizidine alkaloids in liver microsomes and in rats. Chem Res Toxicol. 29:180–9.	Isoline (98%)	Rat liver microsomes	50 µM	+	7-GSH-DHP + diastereomer 9-GSH-DHP 7,9-diGSH-DHP + diastereomer		-	-	-	-	-	-	-	-	-	-	+	-
		Human liver microsomes		+	7-GSH-DHP + diastereomer 9-GSH-DHP 7,9-diGSH-DHP + diastereomer		- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ + +	- - -
	Retrorsine (>90%)	Rat liver microsomes	50 µM	+	7-GSH-DHP + diastereomer 9-GSH-DHP 7,9-diGSH-DHP + diastereomer		- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ + +	- - -
		Human liver microsomes		+	7-GSH-DHP + diastereomer 9-GSH-DHP 7,9-diGSH-DHP + diastereomer		- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ + +	- - -
	Monocrotaline (99%)	Rat liver microsomes	50 µM	+	7-GSH-DHP + diastereomer 9-GSH-DHP 7,9-diGSH-DHP + diastereomer		- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ + +	- - -
		Human liver microsomes		+	7-GSH-DHP + diastereomer 9-GSH-DHP		- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	+ +	- -
	Dehydromonocrotaline	Human liver microsomes	3, 10, 50 µM														No significant differences observed between microsomal, cytosolic and vehicle incubations suggesting conjugation with GSH took place spontaneously	
	Dehydromonocrotaline	Cytosol	3, 10, 50 µM															

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Fashe MM, Juvonen RO, Petsalo A, Räsänen J, Pasanen M (2015). Species-species differences in the in vitro metabolism of lasiocarpine. Chem Res Toxicol. 28:2034–44.	Lasiocarpine (>95%)	Human liver microsomes	10 µM	+	12 metabolites formed, main metabolites M9 (demethylation product), M7 (secondary product of unstable intermediate metabolite), M12 (hydroxylation product), M1 ((3H-pyrrolizin-7-yl)methanol). Presence of GSH reduced level of M1, M4 and M6 (secondary products of unstable intermediate metabolite), M10 (*N*-oxide)	Order of metabolic elimination (t1/2): HLM (16 min) > mouse (43 min) > pig (87 min) > rat (116 min) ≈ sheep (114 min) > human pooled (139 min) > rabbit (347 min). Highest rate of GSH conjugate formation observed in humans, pigs, rats and mice. Formation of M9 in following order: mouse > sheep > pig > rat > human > rabbit	+	Ketoconazole inhibited formation of all metabolites >50%									+	
		Pig liver microsomes	10 µM	+	11 metabolites formed, main metabolites M9, M8 (ester bond cleavage product), M7													
		Rat liver microsomes	10 µM	+	12 metabolites formed, main metabolite M9													
		Mouse liver microsomes	10 µM	+	11 metabolites formed, main metabolites M9, M1, M8, M7													
		Rabbit liver microsomes	10 µM	+	9 metabolites formed, main metabolite M9													
		Sheep liver microsomes	10 µM	+	11 metabolites formed, main metabolite M9													
		Sheep fetus liver microsomes	10 µM	-														
		Recombinant human CYP3A4	10 µM	+	10 metabolites formed (M3, M8 could not be formed)		+	Ketoconazole inhibited formation of all metabolites >60%										
Li W, Wang K, Lin G, Peng Y, Zheng J (2016). Lysine adduction by reactive metabolite(s) of monocrotaline. Chem Res Toxicol. 29:333–41.	Dehydromonocrotaline	-	-	-	-		-	-	-	-	-	-	-	-	Protein adducts	With NAL: formation of 7-NAL-DHP, 9-NAL-DHP, 7,9-di-NAL-DHP in a concentration-dependent manner. With Lys: formation of 7,9-di-Lys-DHP, 7-Lys-DHP, 9-Lys-DHP. With NAL-Ome: formation of 7,9-di-NAL-Ome-DHP, 7-NAL-Ome-DHP, 9-NAL-Ome-DHP with LPKPNQFR: lysine residue modified by DHM with BSA: formation of 7-Lys-DHP	-	-
Xia Q, Zhao Y, Von Tungeln LS, Doerge DR, Lin G, Cai L et al. (2013). Pyrrolizidine alkaloid-derived DNA adducts as a common biological biomarker of pyrrolizidine alkaloid-induced tumorigenicity. Chem Res Toxicol. 26:1384–96.	Senkirkine	Female rat liver microsomes	0.5 mM	+	DHP		+	TAO inhibited DHP formation with about 65%	-	-	-	-	-	-	-	-	-	-
He X, Ma L, Xia Q, Fu PP (2016). 7-*N*-acetylcysteine –pyrrole conjugate – a potent DNA reactive metabolite of pyrrolizidine alkaloids. J Food Drug Anal. 24:682–94.	Riddelliine (purity unknown)	Human liver microsomes	500 µM	+	DHP 7-NAC-DHP													
		Male rat liver microsomes	500 µM	+	DHP 7-NAC-DHP													
	Monocrotaline (purity unknown)	Human liver microsomes	500 µM	+	DHP 7-NAC-DHP													
		Male rat liver microsomes	500 µM	+	DHP 7-NAC-DHP													
	7-NAC-DHP	dG, dA	1 mM												DNA adducts	Formation of DHP-dG-1, DHP-dG-2, DHP-dG-3, DHP-dG-4 or DHP-dA-1, DHP-dA-2, DHP-dA-3, DHP-dA-4 adducts		
	7-NAC-DHP	Calf thymus DNA	500 µM												DNA adducts	Formation of DHP-dG-1, DHP-dG-2, DHP-dG-3, DHP-dG-4 or DHP-dA-1, DHP-dA-2, DHP-dA-3, DHP-dA-4 adducts		
Tang J, Akao T, Nakamura N, Wang ZT, Takagawa K, Sasahara M et al. (2007). In vitro metabolism of isoline, a pyrrolizidine alkaloid from Ligularia duciformis, by rodent liver microsomal esterase and enhanced hepatotoxicity by esterase inhibitors. Drug Metab Dispos. 35:1832–9.	Isoline (>98%)	Male mouse liver microsomes	1 mM	+	Isoline	0.407±0.025 µmol/h/mg protein	+	-	+	Formation of M1 and M2 significantly inhibited by carboxylesterase inhibitors								
					Bisline	No quantitative data												
					Bisline lactone	No quantitative data												
		Male rat liver microsomes			Isoline	0.272±0.019 µmol/h/mg protein		Metabolism enhanced when PB-pretreated microsomes were used	+	Formation of M1 and M2 almost completely inhibited by carboxylesterase inhibitors								
					Bisline	No quantitative data												
					Bisline lactone	No quantitative data												
	Clivorine (>98%)	Male mouse liver microsomes	?		Clivorine	0.270±0.019 µmol/h/mg protein		-										
		Male rat liver microsomes			Clivorine	0.028±0.026 µmol/h/mg protein	+	Metabolism enhanced when PB-pretreated microsomes were used										
	Monocrotaline (>98%)	Male mouse liver microsomes	?		Monocrotaline	0.030±0.040 µmol/h/mg protein		-										
		Male rat liver microsomes			Monocrotaline	No decrease	+	Metabolism enhanced when PB-pretreated microsomes were used										

Reference	PA (purity)	Incubation with (species)	Concentration	Metabolites	namely		CYP enzymes involved	namely	Carboxylesterases involved	namely	FMOs involved	namely	UGTs involved	namely	DNA adducts / protein adducts	namely	Glutathione involvement	Michaelis-Menten kinetics
Jiang X, Wang S, Zhao Y, Xia Q, Cai L, Sun X et al. (2015). Absolute configuration, stability, and interconversion of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine valine adducts and their phenylthiohydantoin derivatives. J Food Drug Anal. 23:318–26.	Dehydromonocrotaline (purity unknown)	Valine	10 mg in 1200 µL												Protein adducts	DHP-valine adducts-1, DHP-valine adducts-2 (both having a 7S absolute configuration), DHP-valine adducts-3, DHP-valine adducts-4 (both having a 7R absolute configuration). Stability suggested to be in the order: DHP-valine-3 > DHP-valine-1 > DHP-valine-4 > DHP-valine-2		
He X, Xia Q, Ma L, Fu PP (2016). 7-Cysteine-pyrrole conjugate: a new potential DNA reactive metabolite of pyrrolizidine alkaloids. J Environ Sci Health Part C. 34:57–76.	Riddelliine (purity unknown)	Male rat liver microsomes	500 µM	+	7-CYS-DHP DHP													
		Male human liver microsomes	500 µM	+	7-CYS-DHP DHP													
	Monocrotaline (purity unknown)	Male rat liver microsomes	500 µM	+	7-CYS-DHP DHP													
		Male human liver microsomes	500 µM	+	7-CYS-DHP DHP													
	7-GS-DHP	Male rat liver microsomes	100 µg	+	7-CYS-DHP DHP													
		Male human liver microsomes	100 µg	+	7-CYS-DHP DHP													
	7-CYS-DHP	Calf thymus DNA	500 µM												DNA adducts	DHP-dG and DHP-dA adducts were formed. DHP-dA-1/2 formed in lowest yield in incubations for 1, 3 and 5 days. DHP-dA-3/4 adducts were predominant and formation was in a time-dependent manner	Glutathione involved in formation 7-GS-DHP and 7-CYS-DHP	
Ma L, Zhao H, Xia Q, Cai L, Fu PP (2015). Synthesis and phototoxicity of isomeric 7,9-diglutathione pyrrole adducts: Formation of reactive oxygen species and induction of lipid peroxidation. J Food Drug Anal. 23:577–86	Dehydromonocrotaline	GSH	1:0.67 molar ratio	+	7-GS-DHP-1 + 7-GS-DHP-2	Major (12% yield)												
					7,9-diGS-DHP-1 + 7,9-diGS-DHP-2	Minor (0.5% yield)												
					9-GS-DHP	Minor (0.5% yield), highly unstable												
Ji L, Chen Y, Wang Z (2008). Protection of S-adenosyl methionine against the toxicity of clivorine on hepatocytes. Environ Toxicol Pharmacol. 26:331–5.	Clivorine (99.5%)	Human liver L-02 cells	0, 3, 10, 30, 50, 100 uM														Intracellular GSH was significantly reduced by clivorine at 30 and 50 µM and almost completely at 100 µM. GSSG were significantly reduced by clivorine only at 100 µM	
			50 uM														GSH, NAC, and SAM significantly protected cells against clivorine-induced toxicity. Intracellular GSH was also increased	
Ji L, Liu T, Chen Y, Wang Z (2009). Protective mechanisms of N-acetyl-cysteine against pyrrolizidine alkaloid clivorine-induced hepatoxicity. J Cell Biochem. 108:424–32.	Clivorine (>99.5%)	Human liver L-02 cells	50 µM														Intracellular GSH was significantly increased during early incubation time (24, 32h), while later was exhausted (after 40-48 h incubation)	
Pan LC, Lamé MW, Morin D, Wilson DW, Segall HJ (1991). Red blood cells augment transport of reactive metabolites of monocrotaline from liver to lung in isolated and tandem liver and lung preparations. Toxicol Applied Pharmacol. 110:336–46.	[14C]-monocrotaline (purity unknown)	Isolated perfused male rat lung	400 µM (4.5 µCi)	+	Unchanged compound	95%												
		Isolated perfused rat livers		+	Monocrotalic acid	No quantitative data												
					Monocrotaline *N*-oxide	No quantitative data												
					Unchanged compound	No quantitative data												

Supplementary Table 2
Studies on the in vivo metabolism of pyrrolizidine alkaloids

Reference	PA	Species	Number	Dose	Route	duration	Co-exposure	namely	Further exposure details	Results	Organ/cells	Metabolites	namely	CYP enzymes involved	namely	Carboxyl-ester-ases involved	namely	FMOs involved	namely	UGTs involved	namely	Protein adducts	namely	DNA adducts	namely	Glutathione involvement	Pharmaco-/toxicokinetics	Comments
Ruan J, Yang M, Fu P, Ye Y, Lin G (2014). Metabolic activation of pyrrolizidine alkaloids: insights into the structural and enzymatic basis. Chem Res Toxicol. 27:1030–9.	Monocrotaline	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+	Protein adducts in blood: lasiocarpine (175 nM) >integerrimine (140 nM) > senecionine ≈ retrorsine (110 nM) > riddelliine (100 nM) > seneciphylline (80 nM) ≫ clivorine (40 nM)> senkirkine≈ monocrotaline (25 nM)≫ lycopsamine (5 nM)≥ heliotrine (1 nM) Protein adducts in liver: integerrimine (1750 pmol/g) > lasiocarpine (1400 pmol/g) ≈ senecionine(1500 pmol/g) > retrorsine≈ riddelliine (1100 pmol/g) > seneciphylline (900 pmol/g)> senkirkine (750 pmol/g) ≫ clivorine (250 pmol/g) > monocrotaline (200 pmol/g) ≫ lycopsamine (20 pmol/g) ≥ heliotrine (10 pmol/g). No adducts seen in platyphylline Numbers estimated from bar graph					
											Liver (100 μg)											+					
	Retrorsine	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
	Seneciphylline	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
	Senecionine	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
	Senkirkine	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
	Heliotrine	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
	Lycopsamine	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
	Lasiocarpine	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
	Clivorine	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
	Integerrimine	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
	Platyphylline	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
	Riddelliine	Male ICR mice	3 per group	20 μmol/kg	Intraperitoneal injection	Single dose, 24 hours					Serum (100 μL)											+					
											Liver (100 μg)											+					
Chou MW, Wang YP, Yan J, Yang YC, Beger RD, Williams LD et al. (2003). Riddelliine N-oxide is a phytochemical and mammalian metabolite with genotoxic activity that is comparable to the parent pyrrolizidine alkaloid riddelliine. Toxicol Lett. 145:239–47.	Riddelliine	F344 rats	4 per sex per group	0 or 1.0 mg/kg per day	Gavage	3 days					Liver	+	6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP), DHP-DNA adducts											+	DHP-derived DNA adducts were also found in liver DNA of F344 rats fed riddelliine *N*-oxide or riddelliine. When rats received doses of 1.0 mg/kg riddelliine *N*-oxide for three consecutive days, the level of DNA adducts was 39.9±0.6 adducts/107 nucleotides, which was 2.6-fold less than that measured in rats treated with riddelliine at the same dose.			
	Riddelliine *N*-oxideRiddelliine	F344 rats	4 per sex per group	0 or 1.0 mg/kg per day	Gavage	3 days					Liver	+	6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP), DHP-DNA adducts											+					
Estep JE, Lamé MW, Morin D, Jones AD, Wilson DW, Segall HJ (1991). [14C]monocrotaline kinetics and metabolism in the rat. Drug Metabol Dis. 19:135–9.	[14C] Monocrotaline	Male Sprague Dawley rats	3, 5	60 mg/kg, 200 uCI/kg	Intravenous injection	24 hours					Urine, bile, plasma, RBC	+	MCT-NOX, PAs and their metabolites, *N*-acetylcystein-conjugated pyrrole													GSH conjugation discussed as possible mechanism for elimination		
Huxtable RJ, Yan CC, Wild S, Maxwell S, Cooper R (1996). Physicochemical and metabolic basis for the differing neurotoxicity of the pyrrolizidine alkaloids, trichodesmine and monocrotaline. Neurochem Res. 21:141–6.	Monocrotaline	Male Sprague Dawley rats	5, 6	25 mg/kg	Intraperitoneal injection	Single dose; 18 hours					Liver	+	Dehydroalkaloid, GSDHP (bile), DHP, pyrroles														Concentrations of metabolites were found in all tissues examined in nmol/g: liver (≈4), lung (≈6), heart (≈5), kidney (≈7), brain (≈3)	
	Trichodesmine	Male Sprague Dawley rats	5, 6	25 mg/kg	Intraperitoneal injection	Single dose; 18 hours					Liver	+	Dehydroalkaloid, GSDHP (bile), DHP, pyrroles														Concentrations of metabolites were found in all tissues examined in nmol/g except the brain: Liver (≈7), lung (≈4), heart (≈2), kidney (≈5)	
Lamé MW, Morin D, Jones AD, Segall HJ, Wilson DW (1990). Isolation and identification of a pyrrolic glutathione conjugate metabolite of the pyrrolizidine alkaloid monocrotaline. Toxicol Lett. 51:321–9.	[14C] Monocrotaline	Male Sprague Dawley rats	Unknown	60 mg/kg, 20 uCI/kg	Intravenous injection with cannulation to bile duct, ureters and carotid artery	3 hours					Bile	+	Pyrroles were detected in bile, also dehydrated form of GSH-DSR													Metabolite identified was a GSH-pyrrole conjugate		

Reference	PA	Species	Number	Dose	Route	duration	Co-exposure	namely	Further exposure details	Results	Organ/cells	Metabolites	namely	CYP enzymes involved	namely	Carboxyl-ester-ases involved	namely	FMOs involved	namely	UGTs involved	namely	Protein adducts	namely	DNA adducts	namely	Glutathione involvement	Pharmaco-/toxicokinetics	Comments
Maia LA, de Macedo Pessoa CR, Rodrigues AF, Colegate S, Medeiros Dantas AF, Trindade Medeiros RM et al. (2014). Duration of an induced resistance of sheep to acute poisoning by *Crotalaria retusa* seeds. Ciencia Rural. 44(6):1054–9.	Monocrotaline	2–3-year-old male Santa Ines sheep	3 per group with 1 control animal	Initial dosing: 117.2 mg/kg for 20 days followed by 234.4 mg/kg for 7 days; 293 mg/kg challenge dose	Gavage	20 days at low dose followed by 7 days at high dose; then challenge dose at 3, 7 or 15 days					Blood, abdominal and thoracic organs	+	Acetylmonocrotaline, monocrotaline-*N*-oxide, deoxymonocrotaline, dihydrodeoxymonocrotaline													
Mattocks AR, Jukes R (1992). Detection of sulphur-conjugated pyrrolic metabolites in blood and fresh or fixed liver tissue from rats given a variety of toxic pyrrolizidine alkaloids. Toxicol Lett. 63:47–55.	Heliotrine	Male albino Wistar rats	1 per dose	150 or 300 mg/kg	Intraperitoneal injection	Single dose, 30 hours					Liver, blood	+	Dehydroretronecine													
	Indicine	Male albino Wistar rats	1 per dose	150 or 500 mg/kg	Intraperitoneal injection	Single dose, 30 hours					Liver, blood	+	Not specified													
	Lasiocarpine	Male albino Wistar rats	1 per dose	40 or 80 mg/kg	Intraperitoneal injection	Single dose, 30 hours					Liver, blood	+	Not specified													
	Retrorsine	Male albino Wistar rats	1 per dose	9 or 17 mg/kg	Intraperitoneal injection	Single dose, 30 hours					Liver, blood	+	Dehydroretronecine													
	Senecionine	Male albino Wistar rats	1 per dose	17 or 33 mg/kg	Intraperitoneal injection	Single dose, 30 hours					Liver, blood	+	Not specified													
	Anacrotine	Male albino Wistar rats	1 per dose	45 or 90 mg/kg	Intraperitoneal injection	Single dose, 30 hours					Liver, blood	+	Racemic mixture of dehydrocrotanecine													
	Monocrotaline	Male albino Wistar rats	1 per dose	27 mg/kg	Intraperitoneal injection	Single dose, 30 hours					Liver, blood	+	S-bound pyrrolic metabolites													
	Monocrotaline	Male albino Wistar rats	2 or 4 per treatment	27 or 55 mg/kg (20 mg/L)	In water ad libitum	12 or 25 days of exposure to alkaloid, or 25 days exposure plus 17 subsequent days normal diet (only highest dose)					Liver, blood	+	S-bound pyrrolic metabolites													
Mattocks AR, Jukes R (1992). Chemistry of sulphur-bound pyrrolic metabolites in the blood of rats given different types of pyrrolizidine alkaloid. Nat Toxins. 1:89–95.	Heliotrine	Male albino Wistar rats	4	240 mg/kg	Intraperitoneal injection	Single dose; sacrificed at 20 hours					Liver, lungs, blood	+	9-ether S-bound pyrrolic metabolite, also small amount of di-ether													
	Indicine	Male albino Wistar rats	4	720 or 450 mg/kg	Intraperitoneal injection	Single dose; sacrificed at 20 hours					Liver, lungs, blood	+	9-ether S-bound pyrrolic metabolite													
	Anacrotine	Male albino Wistar rats	4	130 mg/kg	Intraperitoneal injection	Single dose; sacrificed at 20 hours					Liver, lungs, blood	+	Two dehydrocrotanecin 7-ethyl ethers													
Schoch TK, Gardner DR, Stegelmeier BL (2000). GC/MS/MS detection of pyrrolic metabolites in animals poisoned with the pyrrolizidine alkaloid riddelliine. J Nat Toxins. 9:197–206.	Riddelliine	Female pigs	3 per group	0, 3, 10 or 15 mg/kg bw	Oral, gelatin capsules	40 days					Liver, blood	+	Dehydro-riddelliine (DHR)													There appeared to be a lack of dose–response relationship in finding PA metabolites in the liver; possibly due to high variation in individuals' metabolic abilities
Wang YP, Fu PP, Chou MW (2005b). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, retrorsine, leading to DNA adduct formation in vivo. Int J Environ Res Public Health. 2:74–9.	Retrorsine	8-week-old F344 female rats	3 per group	0 or 1.0 mg/kg/day	Gavage	3 days					Liver	+	6,7-dihydro-7-hydroxyl-1-hydroxymethyl-5H-pyrrolizine (DHP) and retrorsine-*N*-oxide									+	DHP-derived DNA adducts: designated P1-P8. P4 and P6 are DHP-3'-dGMP, and the other 6 were DHP-derived dinucleotides		6,7-dihydro-7-hydroxyl-1-hydroxymethyl-5H-pyrrolizine (DHP) at a rate of 4.8 nmol/mg/min and retrorsine-*N*-oxide at a rate of 17.6 nmol/mg/min
Wang YP, Yan J, Beger RD, Fu PP, Chou MW (2005). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, monocrotaline, leading to DNA adduct formation in vivo. Cancer Lett. 226:27–35.	Monocrotaline	F344 female rats	4 per group	0, 10 mg/kg	Gavage	Single dose					Liver DNA	+	(+/-)6, 7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP)									+	DHP-derived DNA adducts		The DHP-derived DNA adducts were at a maximum 7 days after treatment. At this time point, the level of DHP-derived DNA adducts from riddelliine is about 8-fold higher than that from monocrotaline (about 990 adducts/10^8 nucleotides vs. about 130/10^8).
	Riddelliine	F344 female rats	4 per group	0, 10 mg/kg	Gavage	Single dose					Liver DNA	+	(+/-)6, 7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP)									+	DHP-derived DNA adducts		The DHP-derived DNA adducts were at a maximum 7 days after treatment. At this time point, the level of DHP-derived DNA adducts from riddelliine is about 8-fold higher than that from monocrotaline (about 990 adducts/10^8 nucleotides vs. about 130/10^8).

Reference	PA	Species	Number	Dose	Route	duration	Co-exposure	namely	Further exposure details	Results	Organ/cells	Metabolites	namely	CYP enzymes involved	namely	Carboxyl-ester-ases involved	namely	FMOs involved	namely	UGTs involved	namely	Protein adducts	namely	DNA adducts	namely	Glutathione involvement	Pharmaco-/toxicokinetics	Comments
Wang C , Li Y, Gao J, He Y, Xiong A, Yang L et al. (2011). The comparative pharmacokinetics of two pyrrolizidine alkaloids, sencionine and adonifoline, and their main metabolites in rats after intravenous and oral administration by UPLC/ESIMS. Anal Bioanal Chem. 401:275–87.	Senecionine	8-week-old male Sprague-Dawley rats	8 per group	1.5 mg/kg	Intravenous injection	Single dose					Plasma	+	Senecionine *N*-oxide, SEN-M2, SEN-M3, SEN-M4, SEN-M5												Cmax (μg/ min) 9.8 ± 6.8 Tmax (min) 2.0±0.0 AUC(0-t) (μg-min/ min) 69.2±24.6 CL (ml / min kg) 23.2±8.40	
	Adonifoline	8-week-old male Sprague-Dawley rats	8 per group	4.0 mg/kg	Intravenous injection	Single dose					Plasma	+	Adonifoline *N*-oxide, ADO-M2, ADO-M3												Cmax (μg/ min) 40.3±18.7 Tmax (min) 2.0±0.0 AUC(0-t) (μg-min/ min) 320.2±77.4 CL (ml / min kg) 12.7±3.5	
	Senecionine	8-week-old male Sprague-Dawley rats	8 per group	6, 12, 24 mg/kg	Gavage	Single dose					Plasma	+	Senecionine *N*-oxide, SEN-M2, SEN-M3, SEN-M4, SEN-M5												There was not a significant difference in metabolism between differing dose levels. Dose: 22.9 mg/kg Cmax (μg/ min) 1.27±1.58 Tmax (min) 21.11±15.16 AUC(0-t) (μg-min/ min) 76.30±77.40 CL (ml / min kg) 486.44±286.99	
	Adonifoline	8-week-old male Sprague-Dawley rats	8 per group	16, 32, and 64 mg/kg	Gavage	Single dose					Plasma	+	Adonifoline *N*-oxide, ADO-M2, ADO-M3												There was not a significant difference in metabolism between differing dose levels Dose: 64.0 mg/kg Cmax (μg/ min) 9.8±1.7 Tmax (min) 62.2±25.4 AUC(0-t) (μg-min/ min) 76.30±77.40 CL (ml / min kg) 486.44±286.99	
Williams L, Chou MW, Yan J, Young JF, Chan PC, Doerge DR (2002). Toxicokinetics of riddelliine, a carcinogenic pyrrolizidine alkaloid, and metabolites in rats and mice. Toxicol Appl Pharmacol. 182:98– 104.	Riddelliine	Fisher rats; B6C3F1 mice	Rats: 5 females, 3 males; mice: 6 males and females	10 mg/kg	Gavage	Single dose					Blood serum	+	Riddelliine *N*-oxide and retronecine												Riddelliine was completely absorbed within 30 min in all animals. There was a significant difference in exposure between sexes in rats. AUC rat Male: 516 ng h/ mL AUC rat Female: 1267 ng h/ mL	
Xiong A, Yang L, He Y, Zhang F, Wang J, Han H, et al (2009). Identification of metabolites of adonifoline, a hepatotoxic pyrrolizidine alkaloid, by liquid chromatography/tandem and high-resolution mass spectrometry. Rapid Comm Mass Spectrom. 23:3907–16.	Senecionine	Male Sprague Dawley rats	16	1.5 mg/kg	Intravenous injection	Single dose					Blood serum	+	Senecionine *N*-oxide												Cmax (μg/ml): 9.83 ± 6.85 Tmax (min): 2.00 ± 0.00 AUC(0–∞) (μg min/ml): 72.12 ± 24.37 MRT (min): 128.33 ± 99.61 Vd (ml/kg): 10,864.55 ± 8,736. CL (ml/min/kg): 23.23 ± 8.39	
	Adonifoline	Male Sprague-Dawley rats	16	4.0 mg/kg	Intravenous injection	Single dose					Blood serum	+	Adonifoline *N*-oxide												Cmax (μg/ml): 40.29 ± 18.74 Tmax (min): 2.00 ± 0.00 AUC(0–∞) (μg min/ml): 334.03 ± 85.36 MRT (min): 128.69 ± 46.20 Vd (ml/kg): 4,124.91 ± 1,270.22 CL (ml/min/kg): 12.74 ± 3.42	
Xiong A, Li Y, Yang L, Gao J, He Y, Wang C et al. (2009). Simultaneous determination of senecionine, adonifoline and their metabolites in rat serum by UPLC-ESIMS and its application in pharmacokinetic studies. J Pharm Biomed Anal. 50:1070–4.	Adonifoline	Male Sprague-Dawley rats	2 groups of 3 per group	78.5 mg/kg	Oral	Single dose			Animals were divided into groups based on sample type to be collected		Bile, urine, faeces	+	Adonifoline *N*-oxide, adonifoline hydroxylation (n = 4), M1 hydroxylation (n = 3), adonifoline hydrolysis (n = 2), M1 hydrolysis, retronecine, dehydroretronecine, adonifoline acid, 7-GSH-DHR, 7,9-diGSH-DHR, M16 oxidation, GSH-DHR conjugation (n = 2); 19 metabolites total									+		
Chu PS, Lamé MW, Segall HJ (1993). In vivo metabolism of retrorsine and retrorsine N-oxide. Arch Toxicol. 67:39–43.	Retrorsine Retrorsine-*N*-oxide	Male Sprague Dawley rats (150–200g) (*n*= 7, 4)	3, 7, or 8	25 mg/kg	Intraperitoneal injection Stomach tube	Single dose	+	TOCP (carboxylesterase inhibitor) given 24 hours prior to i.p. administration of PA or phenobarbital (cytochrome p450 inhibitor) given in drinking water for 14 days	Urine was collected for 24 hours		Urine	+	Isatinecic acid, pyrrolic metabolites (unidentified), retronecine, *N*-oxides and unmetabolized retrorsine	+	Phenobarbital treatment led to greater excretion of isatinecic acid (45 vs 31%) and pyrrolic metabolites (11.9 vs 10.3%) and level of retrorsine decreased (1.2 vs 13.6%)	+	TOCP pretreatment decreased *N*-oxide excretion (10.8 to 5.4%)							% of dose excreted in urine after 24 hours given by stomach intubation: % Isatinecic acid: 43.9 % pyrroles: 11.9 % retrorsine: 4.5 % *N*-oxides: 11.2	
Estep JE, Lamé MW, Jones AD, Segall HJ (1990). N-acetylcysteine-conjugated pyrrole identified in rat urine following administration of two pyrrolizidine alkaloids, monocrotaline and senecionine. Toxicol Lett. 54:61–9.	[14C] Monocrotaline	Male Sprague Dawley rats	unknown	60 mg/kg, 10 μCi/ kg subQ	Subcutaneous injection	Single dose					Urine	+	*N*-acetylcysteine conjugate of (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (NAC-DHP)													
	[14C] Senecionine	Male Sprague Dawley rats	unknown	60 mg/kg, 10 μCi/ kg subQ	Subcutaneous injection	Single dose					Urine	+	*N*-acetylcysteine conjugate of (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (NAC-DHP)													

Reference	PA	Species	Number	Dose	Route	duration	Co-exposure	namely	Further exposure details	Results	Organ/cells	Metabolites	namely	CYP enzymes involved	namely	Carboxyl-ester-ases involved	namely	FMOs involved	namely	UGTs involved	namely	Protein adducts	namely	DNA adducts	namely	Glutathione involvement	Pharmaco-/toxicokinetics	Comments
Estep JE, Lamé MW, Segall HJ (1990). Excretion and blood radioactivity levels following [14C] senecionine administration in the rat. Toxicol. 64:179–89.	[14C] Senecionine	Sprague Dawley rats	4 per group	60 mg/kg, 10 µCi/kg	Intravenous injection	Single dose					Urine, bile and blood	+	Senecionine *N*-oxide found as major metabolite in bile and urine													SEN appeared to be rapidly eliminated from plasma (≈90% in 7 hours), but was retained in the RBCs (≈66% decrease in 7 hours). Possible enterohepatic cycling.	
Dimande AFP, Botha CJ, Prozesky L, Bekker L, Rösemann GM, Labuschagne L et al. , Retief E (2007). The toxicity of *Senecio inaequidens* DC. J South Afri Vet Assoc. 78:121–9.	*Senecio inaequidens* crude extract (retrorsine/ senecionine and two unidentified PAs)	5 male Sprague Dawley rats (n=1/ group)	1 per group	0 (control), 0.049, 0.142, 0.196 or 0.245 mg/kg	Gavage	Single dose			Blood collected at sacrifice		Blood, liver, kidney and lung	+	Retrorsine or retrorsine equivalents found in livers (4 rats), kidneys (2 rats) and in lungs of one rat (retrorsine-*N*-oxide)													
		Male Dorper sheep (8 months old; 41 kg) (n=1)	1	49.5 mg/kg day 0 and 1; 99.0 mg/kg day 2 and 198 mg/kg day 3	Gavage	3 days			Blood and urine collected daily		Blood, urine, liver and lung	+	Retrorsine and senecionine found in liver; retrorsine found in kidneys, lungs, bile and urine													
Panariti E, Xinxo A, Leksani D (1997). Transfer of 14C-seneciphylline into sheep milk following multiple oral intakes. DTW – Dtsch Tierarztl Wochenschr. 104:97–8.	[14C] Seneciphylline	Lactating Merino sheep (62–69 kg)	4	0 or 32.4 mg	Oral, capsule	5 days	+	Senecionine 6% and other unidentified PAs 3%	Milk samples taken at 3 and 10 hours after first dose then daily afterwards for 5 days after dosing; blood samples taken at 1, 3 and 12 hours after first dose		Milk and blood	+	Radioactivity of seneciphylline determined												Alkaloid levels were 167 ng/ml 1 h after the first dose and 518 ng/mL after the 5th day	
Hoogenboom LAP, Mulder PPJ, Zeilmaker MJ, van den Top HJ, Remmelink GJ, Brandon EFA et al. (2011). Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows. Food Addit Contam A. 28:359–72.	Ragwort (jacobine and its *N*-oxide, jaconine and its *N*-oxide, erucifoline and its *N*-oxide, senecionine and its *N*-oxide, seneciphylline and its *N*-oxide)	Holstein Friesian dairy cows (4–5 yrs old; 600–700 kg)	3	Week 1: no dose; week 2: 50 g/day; week 3: 100 g/day; week 4: 200 g/day; week 5: no dose	Doses placed in rumen by fistula	3 weeks			Ragwort was dried and given in the rumen and contained all of the PAs listed; milk was tested twice daily and urine/ faeces twice weekly		Milk, faeces and urine	+	Jacoline (80.1%) found in milk; jacobine-*N*-oxide found in urine; jacoline in faeces													
Yan CC, Huxtable RJ (1996). Effects of monocrotaline, a pyrrolizidine alkaloid, on glutathione metabolism in the rat. Biochem Pharmacol 51:375–79.	Monocrotaline	Male Sprague Dawley rats	5 per group	65 mg/kg	Intraperitoneal injection	Single dose			Tissues collected at 24 hours for bound pyrrole content and glutathione status		Liver, kidney, lung, heart, brain	+	Tissue-bound pyrrole highest in liver followed by kidney and lung; lowest in heart and brain											Yes; significantly higher in liver and lung		
Yan CC, Huxtable RJ (1996). The effect of the hepatotoxic pyrrolizidine alkaloid, retrorsine, on bile composition in the rat in vivo. Proc West Pharmacol Soc. 39:19–22.	Retrorsine	Male Sprague Dawley rats	4–5 per data point (8 points)	20.4 mg/kg	Intraperitoneal injection	Single dose			Bile collected 2, 4, 8, 12, 16, 24, and 48 hours after injection to measure biliary sulfur metabolites		Liver/bile	+	GSH; GSH conjugated dehydroalkaloid of 7-glutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizine (GSDHP)											GSDPH in bile peaked at 2–3 hours after injection; GSH, cysteine and cysteinylglycine peaked in bile approximately 8 hours after injection		
Yan CC, Huxtable RJ. (1995). The effect of the pyrrolizidine alkaloids, monocrotaline and trichodesmine, on tissue pyrrole binding and glutathione metabolism in the rat. Toxicon. 33:627-34.	Monocrotaline	Male Sprague Dawley rats	6 per group	65 mg/kg	Intraperitoneal injection	Single dose			Livers collected 24 hours after injection			+	Pyrrolic metabolites (GSH conjugates) in liver, lung, heart, kidney higher after monocrotaline; level in brain higher after trichodesmine											Both alkaloids increased nonprotein sulfhydryl content of liver and increased GSH synthesis in liver		
	Trichodesmine		6 per group	15 mg/kg	Intraperitoneal injection																							
Chen M, Li L, Zhong D, Shen S, Zheng J, Chen X (2016). Glutathionyl-6,7-dihydro-1-hydroxymthyl-5H- pyrrolizine is the major pyrrolic glutathione conjugate of retronecine-type pyrrolizidine alkaloids in liver microsomes and in rats. Chem Res Toxicol. 29:180–9.	Isoline	Male Sprague Dawley rats	Unknown	30 mg/kg	Intraperitoneal injection	Single dose			Bile collected from predose to 6 h and from 6 h to 24 h preprocedure		Bile	+	Epimers of 7-GSH-DHP, 9-GSH-DHP, epimers of 7,9-GSH-DHP, with 9-GSH-DHP being major metabolite													
	Monocrotaline	Male Sprague Dawley rats	Unknown	60 mg/kg	Intraperitoneal injection	Single dose			Bile collected from predose to 6 h and from 6 h to 24 h preprocedure		Bile	+	Epimers of 7-GSH-DHP, 9-GSH-DHP, epimers of 7,9-GSH-DHP, with 9-GSH-DHP being major metabolite													
	Retrorsine	Male Sprague Dawley rats	Unknown	35 mg/kg	Intraperitoneal injection	Single dose			Bile collected from predose to 6 h and from 6 h to 24 h preprocedure		Bile	+	Epimers of 7-GSH-DHP, 9-GSH-DHP, epimers of 7,9-GSH-DHP, with 9-GSH-DHP being major metabolite													

References to Supplementary Table 1

Buhler DR, Miranda CL, Kedzierski B, Reed RL (1991). Mechanisms for pyrrolizidine alkaloid activation and detoxification. Adv Exp Med Biol. 283:597–603.

Chan MY, Zhao XL, Ogle CW (1989). A comparative study on the hepatic toxicity and metabolism of *Crotalaria Assamica* and *Eupatorium species*. Am J Chinese Med. 17:165–70.

Chen M, Li L, Zhong D, Shen S, Zheng J, Chen X (2016). Glutathionyl-6,7-dihydro-1-hydroxymthyl-5H-pyrrolizine is the major pyrrolic glutathione conjugate of retronecine-type pyrrolizidine alkaloids in liver microsomes and in rats. Chem Res Toxicol. 29:180–9.

Chou MW, Wang YP, Yan J, Yang YC, Beger RD, Williams LD et al. (2003). Riddelliine N-oxide is a phytochemical and mammalian metabolite with genotoxic activity that is comparable to the parent pyrrolizidine alkaloid riddelliine. Toxicol Lett. 145:239–47.

Chung WG, Buhler DR (1994). The effect of spironolactone treatment on the cytochrome P450-mediated metabolism of the pyrrolizidine alkaloid senecionine by hepatic microsomes from rats and guinea pigs. Toxicol Appl Pharmacol. 127:314–9.

Chung WG, Buhler DR (1995). Major factors for the susceptibility of guinea pig to the pyrrolizidine alkaloid jacobine. Drug Metab Dispos. 23:1263–7.

Chung WG, Buhler DR (2004). Differential metabolism of the pyrrolizidine alkaloid, senecionine, in Fischer 344 and Sprague-Dawley rats. Arch Pharm Res. 27:547–53.

Chung WG, Miranda CL, Buhler DR (1995). A cytochrome P4502B form is the major bioactivation enzyme for the pyrrolizidine alkaloid senecionine in guinea pig. Xenobiotica 25:929–39.

Cooper RA, Huxtable RJ (1996). A simple procedure for determining the aqueous half-lives of pyrrolic metabolites of pyrrolizidine alkaloids. Toxicon. 24:604–7.

Couet CE, Hopley J, Hanley AB (1996). Metabolic activation of pyrrolizidine alkaloids by human, rat and avocado microsomes. Toxicon. 34:1058–61.

Dueker SR, Lamé MW, Segall HJ (1992). Hydrolysis of pyrrolizidine alkaloids by guinea pig hepatic carboxylesterases. Toxicol Appl Pharmacol. 117:116–21.

Dueker SR, Lamé MW, Segall HJ (1995). Hydrolysis rates of pyrrolizidine alkaloids derived from *Senecio jacobaea*. Arch Toxicol. 69:725–8.

Dueker SR, Lamé MW, Morin D, Wilson DW, Segall HJ (1992). Guinea pig and rat hepatic microsomal metabolism of monocrotaline. Drug Metab Dispos. 20:275–80.

Dueker SR, Lamé MW, Jones AD, Morin D, Segall HJ (1994). Glutathione conjugation with the pyrrolizidine alkaloid, jacobine. Biochem Biophys Res Comm. 198:516–22.

Duringer JM, Buhler DR, Craig AM (2004). Comparison of hepatic in vitro metabolism of the pyrrolizidine alkaloid senecionine in sheep and cattle. Am J Vet Res. 65:1563–72.

Fashe MM, Juvonen RO, Petsalo A, Rahnasto-Rilla M, Auriola S, Soininen P et al. (2014). Identification of a new reactive metabolite of pyrrolizidine alkaloid retrorsine: (3H-pyrrolizin-7-yl) methanol. Chem Res Toxicol. 27:1950–7.

Fashe MM, Juvonen RO, Petsalo A, Vepsalainen J, Pasanen M, Rahnasto-Rilla M (2015a). In silico prediction of the site of oxidation by cytochrome P450 3A4 that leads to the formation of the toxic metabolites of pyrrolizidine alkaloids. Chem Res Toxicol. 28:702–10.

Fashe MM, Juvonen RO, Petsalo A, Räsänen J, Pasanen M (2015b). Species-species differences in the in vitro metabolism of lasiocarpine. Chem Res Toxicol. 28:2034–44.

Glowaz SL, Michnika M, Huxtable RJ (1992). Detection of a reactive pyrrole in the hepatic metabolism of the pyrrolizidine alkaloid, monocrotaline. Toxicol Appl Pharmacol. 115:168–73.

He X, Ma L, Xia Q, Fu PP (2016a). 7-N-acetylcysteine–pyrrole conjugate – a potent DNA reactive metabolite of pyrrolizidine alkaloids. J Food Drug Anal. 24:682–94.

He X, Xia Q, Ma L, Fu PP (2016b). 7-Cysteine-pyrrole conjugate: a new potential DNA reactive metabolite of pyrrolizidine alkaloids. J Environ Sci Health Part C. 34:57–76.

He YQ, Yang L, Liu HX, Zhang JW, Liu Y, Fong A et al. (2010a). Glucuronidation, a new metabolic pathway for pyrrolizidine alkaloids. Chem Res Toxicol. 23:591–9.

He YQ, Liu Y, Zhang BF, Liu HX, Lu YL, Yang L et al. (2010b). Identification of the UDP-glucuronosyltransferase isozyme involved in senecionine glucuronidation in human liver microsomes. Drug Metab Dispos. 38:626–34.

Huan JY, Miranda CL, Buhler DR, Cheeke PR (1998a). Species differences in the hepatic microsomal enzyme metabolism of the pyrrolizidine alkaloids. Toxicol Lett. 99:127–37.

Huan JY, Miranda CL, Buhler DR, Cheeke PR (1998b). The roles of CYP3A and CYP2B isoforms in hepatic bioactivation and detoxification of the pyrrolizidine alkaloid senecionine in sheep and hamsters. Toxicol Appl Pharmacol. 151:229–35.

Huxtable RJ, Wild SL (1994). Relationship between in vitro metabolism of pyrrolizidine alkaloids and extrahepatic toxicity in vivo. Proc West Pharmacol Soc. 37:109–11.

Huxtable RJ, Yan CC, Wild S, Maxwell S, Cooper R (1996). Physicochemical and metabolic basis for the differing neurotoxicity of the pyrrolizidine alkaloids, trichodesmine and monocrotaline. Neurochem Res. 21:141–6.

Ji L, Chen Y, Wang Z (2008). Protection of S-adenosyl methionine against the toxicity of clivorine on hepatocytes. Environ Toxicol Pharmacol. 26:331–5.

Ji L, Liu T, Chen Y, Wang Z (2009). Protective mechanisms of N-acetyl-cysteine against pyrrolizidine alkaloid clivorine-induced hepatoxicity. J Cell Biochem. 108:424–32.

Jiang X, Wang S, Zhao Y, Xia Q, Cai L, Sun X et al. (2015). Absolute configuration, stability, and interconversion of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine valine adducts and their phenylthiohydantoin derivatives. J Food Drug Anal. 23:318–26.

Lamé MW, Jones AD, Morin D, Segall HJ (1991). Metabolism of [^{14}C]-monocrotaline by isolated perfused rat liver. Drug Metab Dispos. 19:516–24.

Li W, Wang K, Lin G, Peng Y, Zheng J (2016). Lysine adduction by reactive metabolite(s) of monocrotaline. Chem Res Toxicol. 29:333–41.

Lin G, Cui YY, Hawes EM (1998). Microsomal formation of a pyrrolic alcohol glutathione conjugate of clivorine firm evidence for the formation of a pyrrolic metabolite of an otonecine-type pyrrolizidine alkaloid. Drug Metab Dispos. 26:181–4.

Lin G, Cui YY, Hawes EM (2000). Characterization of rat liver microsomal metabolites of clivorine, an hepatotoxic otonecine-type pyrrolizidine alkaloid. Drug Metab Dispos. 28:1475–83.

Lin G, Cui YY, Liu XQ (2003).Gender differences in microsomal metabolic activation of hepatotoxic clivorine in rat. Chem Res Toxicol. 16:768–74.

Lin G, Cui YY, Liu XQ, Wang ZT (2002). Species differences in the in vitro metabolic activation of the hepatotoxic pyrrolizidine alkaloid clivorine. Chem Res Toxicol. 15:1421–8.

Lin G, Tang J, Liu XQ, Jiang Y, Zheng J (2007). Deacetylclivorine: a gender-selective metabolite of clivorine formed in female Sprague-Dawley rat liver microsomes. Drug Metab Dispos. 35:607–13.

Liu XQ, Lin G, Wang GJ, Qian ZY (2001). Metabolism of clivorine in female rat liver microsomes. Chinese J Pharmacol Toxicol.15:413–7.

Liu XQ, Lin G, Wang GJ, Qian ZY (2002). Involvement of human CYP3A4 in the formation of hepatotoxic metabolites of clivorine. Chinese J Pharmacol Toxicol. 16:15–20.

Ma L, Zhao H, Xia Q, Cai L, Fu PP (2015). Synthesis and phototoxicity of isomeric 7,9-diglutathione pyrrole adducts: Formation of reactive oxygen species and induction of lipid peroxidation. J Food Drug Anal. 23:577–86.

Mattocks AR, Jukes R (1992). Chemistry of sulphur-bound pyrrolic metabolites in the blood of rats given different types of pyrrolizidine alkaloid. Natural Toxins. 1:89–95.

Miranda CL, Reed RL, Guengerich FP, Buhler DR (1991a). Role of cytochrome P450IIIA4 in the metabolism of the pyrrolizidine alkaloid senecionine in human liver. Carcinogenesis. 12:515–9.

Miranda CL, Chung W, Reed RE, Zhao X, Henderson MC, Wang JL et al. (1991b). Flavin-containing monooxygenase: a major detoxifying enzyme for the pyrrolizidine alkaloid senecionine in guinea pig tissues. Biochem Biophys Res Comm. 178:546–52.

Miranda CL, Rawson CL, Reed RL, Zhao X, Barnes DW, Buhler DR (1992). C3H/10T1/2 cells: a model to study the role of metabolism in the toxicity of the pyrrolizidine alkaloid retrorsine. In Vitro Toxicol. 5:21–32.

Nigra L, Huxtable RJ (1992). Hepatic glutathione concentrations and the release of pyrrolic metabolites of the pyrrolizidine alkaloid, monocrotaline, from the isolated perfused liver. Toxicon. 30:1195–202.

Pan LC, Lamé MW, Morin D, Wilson DW, Segall HJ (1991). Red blood cells augment transport of reactive metabolites of monocrotaline from liver to lung in isolated and tandem liver and lung preparations. Toxicol Applied Pharmacol 110:336–46.

Reed RL, Miranda CL, Kedzierski B, Henderson MC, Buhler DR (1992). Microsomal formation of a pyrrolic alcohol glutathione conjugate of the pyrrolizidine alkaloid senecionine. Xenobiotica. 22:1321–7.

Reid MJ, Lamé MW, Morin D, Wilson DW, Segall HJ (1998a). Involvement of cytochrome P450 3A in the metabolism and covalent binding of 14C-monocrotaline in rat liver microsomes. J Biochem Mol Toxicol. 12:157–66.

Reid MJ, Dunston SK, Lamé MW, Wilson DW, Morin D, Segall HJ (1998b). Effect of monocrotaline metabolites on glutathione levels in human and bovine pulmonary artery endothelial cells. Res Comm Mol Pathol Pharmacol. 99:53–68.

Ruan J, Yang M, Fu P, Ye Y, Lin G (2014a). Metabolic activation of pyrrolizidine alkaloids: insights into the structural and enzymatic basis. Chem Res Toxicol. 27:1030–9.

Ruan J, Liao C, Ye Y, Lin G (2014b). Lack of metabolic activation and predominant formation of an excreted metabolite of nontoxic platynecine-type pyrrolizidine alkaloids. Chem Res Toxicol. 27:7–16.

Tamta H, Pawar RS, Wamer WG, Grundel E, Krynitsky AJ, Rader JI (2012). Comparison of metabolism-mediated effects of pyrrolizidine alkaloids in a Hep2G/C3A cell-S9 co-incubation system and quantification of their glutathione conjugates. Xenobiotica. 42:1038–48.

Tang J, Zhang M, Wang ZT, Akao T, Nakamura N, Hattori M (2004). Simultaneous determination of isoline and its two major metabolites using high-performance liquid chromatography. J Anal Toxicol. 28:11–5.

Tang J, Akao T, Nakamura N, Wang ZT, Takagawa K, Sasahara M et al. (2007). In vitro metabolism of isoline, a pyrrolizidine alkaloid from Ligularia duciformis, by rodent liver microsomal esterase and enhanced hepatotoxicity by esterase inhibitors. Drug Metab Dispos. 35:1832–9.

Wang J, Yang L, Wang C, Wang Z (2009). The action of cytochrome p450 enzymes and flavin-containing monooxygenases on the N-oxide of pyrrolizidine alkaloid monocrotaline. Asian J Tradit Med. 4:41–50.

Wang YP, Fu PP, Chou MW (2005). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, retrorsine, leading to DNA adduct formation in vivo. Int J Environ Res Publ Health. 2:74–9.

Wang YP, Yan J, Beger RD, Fu PP, Chou MW (2005a). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, monocrotaline, leading to DNA adduct formation in vivo. Cancer Lett. 226:27–35.

Wang YP, Yan J, Fu PP, Chou MW (2005b). Human liver microsomal reduction of pyrrolizidine alkaloid N-oxides to form the corresponding carcinogenic parent alkaloid. Toxicology Lett. 155:411–20.

Winter CK, Segall HJ, Jones AD (1988). Determination of pyrrolizidine alkaloid metabolites from mouse liver microsomes using tandem mass spectrometry and gas chromatography/mass spectrometry. Biomed Environ Mass Spectrom. 15:265–73.

Williams DE, Reed RL, Kedzierski B, Dannan GA, Guengerich FP, Buhler DR (1989a). Bioactivation and detoxification of the pyrrolizidine alkaloid senecionine by cytochrome P-450 enzymes in rat liver. Drug Metab Dispos. 17:387–92.

Williams DE, Reed RL, Kedzierski B, Ziegler DM, Buhler DR (1989b). The role of flavin-containing monooxygenase in the N-oxidation of the pyrrolizidine alkaloid senecionine. Drug Metab Dispos. 17:380–6.

Xia Q, Chou MW, Kadlubar FF, Chan PC, Fu PP (2003). Human liver microsomal metabolism and DNA adduct formation of the tumorigenic pyrrolizidine alkaloid, riddelliine. Chem Res Toxicol. 16:66–73.

Xia Q, Chou MW, Lin G, Fu PP (2004). Metabolic formation of DHP-derived DNA adducts from a representative otonecine type pyrrolizidine alkaloid clivorine and the extract of *Ligularia hodgsonnii* hook. Chem Res Toxicol. 17:702–8.

Xia Q, Chou MW, Edgar JA, Doerge DR, Fu PP (2006). Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, lasiocarpine. Cancer Lett. 231:138–45.

Xia Q, Yan J, Chou MW, Fu PP (2008). Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, heliotrine. Toxicol Lett. 178:77–82.

Xia Q, Zhao Y, Von Tungeln LS, Doerge DR, Lin G, Cai L et al. (2013). Pyrrolizidine alkaloid-derived DNA adducts as a common biological biomarker of pyrrolizidine alkaloid-induced tumorigenicity. Chem Res Toxicol. 26:1384–96.

Xia Q, Ma L, He X, Cai L, Fu PP (2015). 7-glutathione pyrrole adduct: a potential DNA reactive metabolite of pyrrolizidine alkaloids. Chem Res Toxicol. 28:615–20.

Yan CC, Huxtable RJ (1994). Quantitation of the hepatic release of metabolites of the pyrrolizidine alkaloid, monocrotaline. Toxicol Appl Pharmacol. 127:58–63.

Yan CC, Huxtable RJ (1995a). Relationship between glutathione concentration and metabolism of the pyrrolizidine alkaloid, monocrotaline, in the isolated, perfused liver. Toxicol Applied Pharmacol. 130:132–9.

Yan CC, Huxtable RJ (1995c). The relationship between the concentration of the pyrrolizidine alkaloid monocrotaline and the pattern of metabolites released from the isolated liver. Toxicol Appl Pharmacol. 130:1–8.

Yan CC, Huxtable RJ (1995d). Effect of the pyrrolizidine alkaloid, monocrotaline, on bile composition of the isolated, perfused rat liver. Life Sci. 57:617–26.

Yan CC, Cooper RA, Huxtable RJ (1995). The comparative metabolism of the four pyrrolizidine alkaloids, seneciphylline, retrorsine, monocrotaline, and trichodesmine in the isolated, perfused rat liver. Toxicol. Appl Pharmacol. 133:277–84.

Yang M, Ruan J, Lin G (2014). Biotransformation from pyrrolizidine alkaloid N-oxides to pyrrolizidine alkaloids in liver and gastrointestinal tract. Drug Metabolism Reviews.

Yang YC, Yan J, Doerge DR, Chan PC, Fu PP, Chou MW (2001). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, riddelliine, leading to DNA adduct formation in vivo. Chem Res Toxicol. 14:101–9.

References to Supplementary Table 2

Chen M, Li L, Zhong D, Shen S, Zheng J, Chen X (2016). Glutathionyl-6,7-dihydro-1-hydroxymthyl-5H-pyrrolizine is the major pyrrolic glutathione conjugate of retronecine-type pyrrolizidine alkaloids in liver microsomes and in rats. Chem Res Toxicol. 29:180–9.

Chou MW, Wang YP, Yan J, Yang YC, Beger RD, Williams LD et al. (2003a). Riddelliine N-oxide is a phytochemical and mammalian metabolite with genotoxic activity that is comparable to the parent pyrrolizidine alkaloid riddelliine. Toxicol Lett. 145:239–47.

Chu PS, Lamé MW, Segall HJ (1993). In vivo metabolism of retrorsine and retrorsine N-oxide. Arch Toxicol. 67:39–43.

Dimande AFP, Botha CJ, Prozesky L, Bekker L, Rösemann GM, Labuschagne L et al. , Retief E (2007). The toxicity of *Senecio inaequidens* DC. J South Afri Vet Assoc. 78:121–9.

Estep JE, Lamé MW, Segall HJ (1990). Excretion and blood radioactivity levels following [14C] senecionine administration in the rat. Toxicol. 64:179–89.

Estep JE, Lamé MW, Jones AD, Segall HJ (1990). N-acetylcysteine-conjugated pyrrole identified in rat urine following administration of two pyrrolizidine alkaloids, monocrotaline and senecionine. Toxicol Lett. 54:61–9.

Estep JE, Lamé MW, Morin D, Jones AD, Wilson DW, Segall HJ (1991). [14C]monocrotaline kinetics and metabolism in the rat. Drug Metabol Dis. 19:135–9.

Hoogenboom LAP, Mulder PPJ, Zeilmaker MJ, van den Top HJ, Remmelink GJ, Brandon EFA et al. (2011). Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows. Food Addit Contam A. 28:359–72.

Huxtable RJ, Yan CC, Wild S, Maxwell S, Cooper R (1996). Physicochemical and metabolic basis for the differing neurotoxicity of the pyrrolizidine alkaloids, trichodesmine and monocrotaline. Neurochem Res. 21:141–6.

Lamé MW, Morin D, Jones AD, Segall HJ, Wilson DW (1990). Isolation and identification of a pyrrolic glutathione conjugate metabolite of the pyrrolizidine alkaloid monocrotaline. Toxicol Lett. 51:321–9.

Maia LA, de Macedo Pessoa CR, Rodrigues AF, Colegate S, Medeiros Dantas AF, Trindade Medeiros RM et al. (2014). Duration of an induced resistance of sheep to acute poisoning by *Crotalaria retusa* seeds. Ciencia Rural. 44(6):1054–9.

Mattocks AR, Jukes R (1992a). Detection of sulphur-conjugated pyrrolic metabolites in blood and fresh or fixed liver tissue from rats given a variety of toxic pyrrolizidine alkaloids. Toxicol Lett. 63:47–55.

Mattocks AR, Jukes R (1992b). Chemistry of sulphur-bound pyrrolic metabolites in the blood of rats given different types of pyrrolizidine alkaloid. Nat Toxins. 1:89–95.

Panariti E, Xinxo A, Leksani D (1997). Transfer of 14C-seneciphylline into sheep milk following multiple oral intakes. DTW – Dtsch Tierarztl Wochenschr. 104:97–8.

Ruan J, Yang M, Fu P, Ye Y, Lin G (2014a). Metabolic activation of pyrrolizidine alkaloids: insights into the structural and enzymatic basis. Chem Res Toxicol. 27:1030–9.

Schoch TK, Gardner DR, Stegelmeier BL (2000). GC/MS/MS detection of pyrrolic metabolites in animals poisoned with the pyrrolizidine alkaloid riddelliine. J Nat Toxins. 9:197–206.

Wang C , Li Y, Gao J, He Y, Xiong A, Yang L et al. (2011). The comparative pharmacokinetics of two pyrrolizidine alkaloids, sencionine and adonifoline, and their main metabolites in rats after intravenous and oral administration by UPLC/ESIMS. Anal Bioanal Chem. 401:275–87.

Wang J, Yang L, Wang C, Wang Z (2009) The action of cytochrome p450 enzymes and flavin-containing monooxygenases on the N-oxide of pyrrolizidine alkaloid monocrotaline.Asian J Tradit Med. 2009:4.

Wang YP, Yan J, Fu PP, Chou MW (2005) Human liver microsomal reduction of pyrrolizidine alkaloid N-oxides to form the corresponding carcinogenic parent alkaloid. Toxicol Lett. 155: 411–420.

Wang YP, Fu PP, Chou MW (2005b). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, retrorsine, leading to DNA adduct formation in vivo. Int J Environ Res Public Health. 2:74–9.

Wang YP, Yan J, Beger RD, Fu PP, Chou MW (2005a). Metabolic activation of the tumorigenic pyrrolizidine alkaloid, monocrotaline, leading to DNA adduct formation in vivo. Cancer Lett. 226:27–35 .

Williams L, Chou MW, Yan J, Young JF, Chan PC, Doerge DR (2002). Toxicokinetics of riddelliine, a carcinogenic pyrrolizidine alkaloid, and metabolites in rats and mice. Toxicol Appl Pharmacol. 182:98–104.

Xiong A, Yang L, He Y, Zhang F, Wang J, Han H, et al (2009a). Identification of metabolites of adonifoline, a hepatotoxic pyrrolizidine alkaloid, by liquid chromatography/tandem and high-resolution mass spectrometry. Rapid Comm Mass Spectrom. 23:3907–16.

Xiong A, Li Y, Yang L, Gao J, He Y, Wang C et al. (2009b). Simultaneous determination of senecionine, adonifoline and their metabolites in rat serum by UPLC-ESIMS and its application in pharmacokinetic studies. J Pharm Biomed Anal. 50:1070–4.

Yan CC, Huxtable RJ (1995). The effect of the pyrrolizidine alkaloids, monocrotaline and trichodesmine, on tissue pyrrole binding and glutathione metabolism in the rat. Toxicon. 33:627–34.

Yan CC, Huxtable RJ (1996a). Effects of monocrotaline, a pyrrolizidine alkaloid, on glutathione metabolism in the rat. Biochem Pharmacol. 51:375–9.

Yan CC, Huxtable RJ (1996b). The effect of the hepatotoxic pyrrolizidine alkaloid, retrorsine, on bile composition in the rat in vivo. Proc West Pharmacol Soc. 39:19–22.

This volume contains a monograph prepared at the eightieth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Rome, Italy, from 16 to 25 June 2015.

The toxicological and dietary exposure monograph in this volume summarizes the safety and dietary exposure data on a contaminant group (pyrrolizidine alkaloids) discussed at the eightieth meeting.

Monographs on seven food additives discussed at that meeting have been previously published in the WHO Food Additives series (FAS 71), and a monograph on a second contaminant group (non-dioxin-like polychlorinated biphenyls) has been published as a separate supplement in the WHO Food Additives series.

This volume and others in the WHO Food Additives series contain information that is useful to those who produce and use food additives and veterinary drugs and those involved with controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

9789240012677