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PhytoLab

Safety

in Laboratory and Regulatory Services for Herbal Products

Dr. Bernhard Klier, PhytoLab

SANA BOLGNA 2018: „Pyrrolizidine Alkaloid (PA) control“

Bologna, 10 September 2018

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Pyrrolizidine alkaloid (PA) control –

sampling, methods and analysis

Content

Introduction

(chronology, sources and strucure of PA)

Sampling

Analytical methods

(BfR method, challenges, validation)

Reference standards

Conclusion

Introduction

Some Pyrrolizidine Alkaloid (PA) - containing plants are used in pharmaceutical products, e.g. Symphytum or Farfara; besides these there are a few plants used in homoeopathic preparations in high potencies, for instance Eupatorium, Petasites or Senecio.

Codex Alimentarius Commission reported that PA - containing plants could be found worldwide in herbal teas, in diverse herbal remedies (e.g. TCM), in food supplements or in foodstuffs (Comfrey).

There has been a press release of BfR at 5th **July 2013** that high values of PAs have been found in certain herbal teas.

Introduction

Bundesinstitut
für Arzneimittel
und Medizinprodukte

1,0 µg per day final product

Bundesinstitut für Arzneimittel und Medizinprodukte
Federal Institute for Drugs and Medical Devices

(Informal translation for information in the European network only)

Announcement on testing of content of pyrrolyzidine alkaloids to ensure quality and safety of medicinal products containing herbal substances or herbal preparations or homeopathic preparations from starting material of plant origin

1st March 2016

Introduction

31 May 2016
EMA/HMPC/328782/2016
Committee on Herbal Medicinal Products (HMPC)

1,0 µg per day final product
reduction to 0,35 µg per day

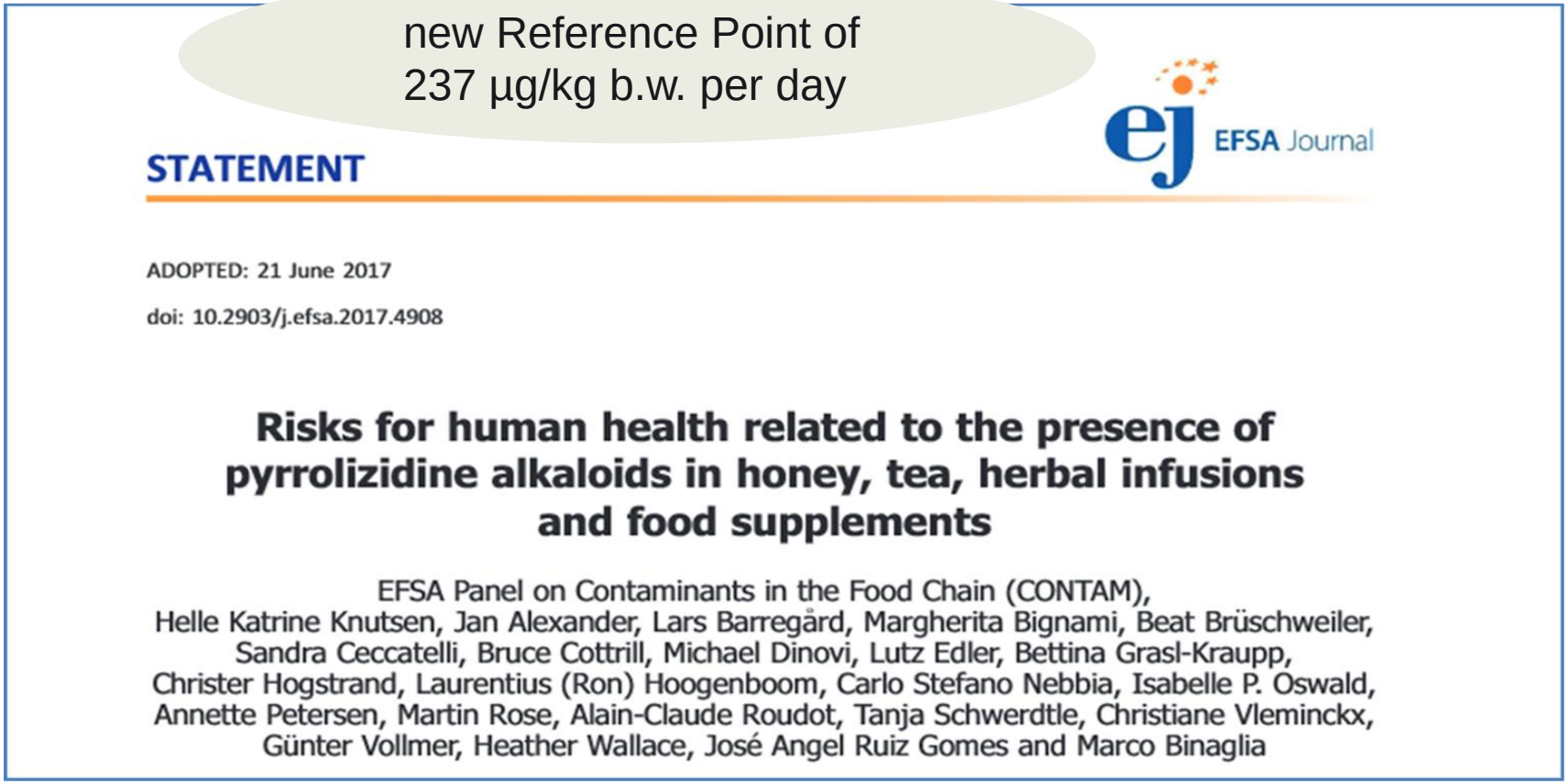
Public statement on contamination of herbal medicinal products/traditional herbal medicinal products[1] with pyrrolizidine alkaloids

Transitional recommendations for risk management and quality control

Recently, it has been shown that PA-containing weeds contaminate botanical raw materials used for the production of food and herbal medicinal products (HMPs)[2]. The botanical raw materials generally appear to be contaminated by (very) low levels of PAs, but due to newly developed analytical methods (LC-MS/MS) even trace amounts of PAs can now be detected and quantified.

Introduction

new Reference Point of
237 µg/kg b.w. per day



EFSA Journal

STATEMENT

ADOPTED: 21 June 2017

doi: 10.2903/j.efsa.2017.4908

Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements

EFSA Panel on Contaminants in the Food Chain (CONTAM),
Helle Katrine Knutsen, Jan Alexander, Lars Barregård, Margherita Bignami, Beat Brüschweiler, Sandra Ceccatelli, Bruce Cottrill, Michael Dinovi, Lutz Edler, Bettina Grasl-Kraupp, Christer Hogstrand, Laurentius (Ron) Hoogenboom, Carlo Stefano Nebbia, Isabelle P. Oswald, Annette Petersen, Martin Rose, Alain-Claude Roudot, Tanja Schwerdtle, Christiane Vleminckx, Günter Vollmer, Heather Wallace, José Angel Ruiz Gomes and Marco Binaglia

Source: https://www.efsa.europa.eu/de/efsajournal/pub/4908

Natural sources and structures of Pyrrolizidine alkaloids

The minimal structural requirements for toxicity of PAs are:

- a double bond in 1,2 position
- a hydroxymethyl substituent (C1 position) and hydroxyl group (C7 position)
- esterification of the primary hydroxymethyl group

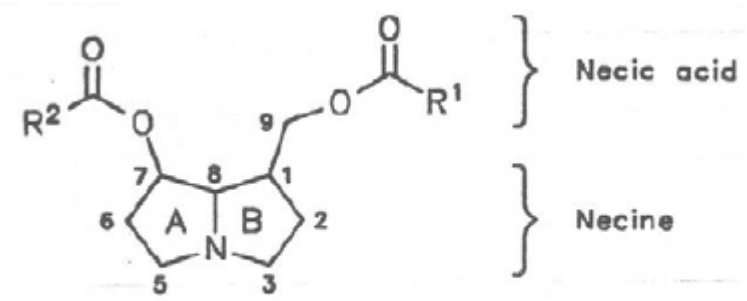
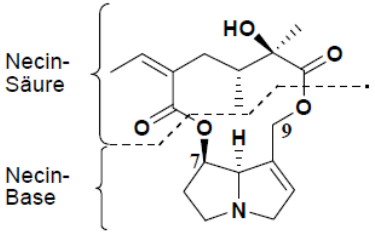


Fig. 1: general structure of PAs [ROEDER 2000]

(**EMA/HMPC/893108/2011**)

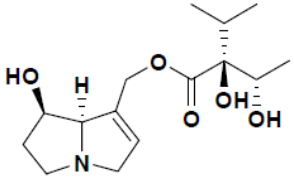
Most of all PA are sorted to following types (Hartmann und Witte 1995):

1. Retronecin Typ:



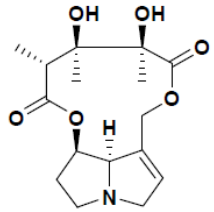
Senecionin (**7**) -Typ
(> 100 Strukturen)

Tribe
Senecioneae



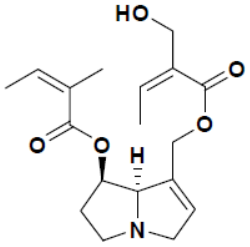
Lycopsamin (**10**) -Typ
(> 100 Strukturen)

Tribe
Eupatoriae,
Boraginaceae



Monocrotalin (**9**) -Typ
(> 30 Strukturen)

Genus
Crotalaria,
Boraginaceae



Triangularin (**8**) -Typ
(> 50 Strukturen)

Senecio ssp.,
Boraginaceae

2. Otonecin-Typ:

Asteraceen, Boraginaceen, Fabaceen (about 20 chem. structures)

Pyrrolizidine alkaloids

Pyrrolizidine Alkaloids (PAs) have been isolated from about 350 plant species and are estimated as compounds of more than 6.000 flowering plants worldwide (about 3% of all flowering plants).

13 families have been reported to produce PAs, toxic compounds have been found especially in following families:

Asteraceae (Senecio, Eupatorium, Tussilago, Petasites),
Boraginaceae (Heliotropium, Myosotis, Symphytum, Lithospermum, Echium),
Fabaceae (Crotalaria),

(Apocynaceae, Ranunculaceae, Scrophulariaceae, Orchidaceae, Convolvulaceae)

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Spotcontamination – theoretical calculation

Contamination with **senecio plant**, which has an content of **1.310 mg/kg PA**.

Using the same plant weight for weed (senecio) and crop:

	%	PA-content in mg/kg	Number of plants (basis 60.000 plants / ha)
Contamination	100	1.310	60.000
Contamination	10	131,0	6.000
Contamination	1	13,10	600
Contamination	0,1	1,310	60
Contamination	0,01	0,1310	6

Spotcontamination – theoretical calculation

Theoretical calculation shows that in the case of senecio very low contamination (0,01%) of PA containing weed in the field can contaminate crops with high levels of PA (Ph.Eur. foreign matter: max 2%).

Only 6 senecio plants per hectare are theoretically sufficient to get non compliant material.

It is very difficult to get representative values of inhomogenous raw material (high number of samples).

dried raw material → cut, homogenised → powdered → extracts

Preliminary recommendation of BfR (002/2016) about analysis of PA in herbal teas and tea
(scope of substances and sampling)

Recommendation on sampling for PA analysis in herbal tea and tea

- refering to sampling procedure in EFSA monitoring plan PA in foodstuff
- sampling according to sampling procedure described for aflatoxins in spices

  → **Regulation (EG) No. 401/2006, Annex I, E.4**
- spot contamination similar to mycotoxins

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Requirements to analytical method

- there are roughly 400 plants and parts of plants on the herbal market, as well as many different active substances (extracts, intermediate products) and final products

- analytical method must be valid for many **different matrices**
  →**Matrix effects** have to be adjusted (difficult matrix)
  →need of a **„multi method“** (multi matrices, multi substances)

- method must be **very sensitive** (sum of PA < 35µg/kg)

- method must be **suitable for routine testing** (multi samples)

- for correct quantification **reference standards** are needed

  →there are more than 500 PA alkaloids known (including diastereomers, isobaric compounds)
  →additionally pyrrolizidine N-Oxides existing

31 May 2016
EMA/HMPC/328782/2016
Committee on Herbal Medicinal Products (HMPC)

Public statement on contamination of herbal medicinal products/traditional herbal medicinal products[1] with pyrrolizidine alkaloids

Transitional recommendations for risk management and quality control

6.1. Analytical methods

Highly sensitive analytical methods are required to provide the level of quantification needed to control PAs due to contamination in HMPs. There are no official test methods currently available for PAs in HMPs.

The HMPC has therefore requested that the European Pharmacopoeia consider development of an appropriate analytical method for PAs in HMPs as a matter of priority.

Until such time as an official analytical method is available Marketing Authorisation Holders (MAHs) are advised to use the SPE-LC-MS/MS method as published by BfR (Federal Institute for Risk Assessment: BfR-PA-Tea-2.0/2014). Other suitable validated methods may be acceptable.

8.4 Flow chart of the sample preparation procedure

FEDERAL INSTITUTE FOR RISK ASSESSMENT

BfR-PA-Tea-2.0/2014

2 (± 0.1) g plant material

extraction

- add 20 mL 0.05 M H_2SO_4
- 1st extraction (ultrasonic bath, 15 min, RT)
  → centrifugation (10 min, 3800 x g), supernatant in a 50 mL vessel
- 2nd extraction (ultrasonic bath, 15 min, RT)
  → centrifugation (10 min, 3800 x g)
  combine both supernatants, adjust pH value to 6-7, filtration
  10 ml of the solution is used for the SPE

plant extract for the SPE

SPE - program

- conditioning step 1: 5 mL of methanol
- conditioning step 2: 5 mL of water
- sample load: 10 mL of sample
- wash step: 2 x 5 mL of water

dry the cartridge for 5 – 10 min under vacuum

- elution: 2 x 5 mL of methanol or
  in case of black/green tea: 2 x 5 mL of 2.5% ammonia in methanol

SPE - eluate

solvent exchange

- evaporate the sample to dryness (nitrogen stream)

residue

reconstitution

- add 1 mL methanol/wasser (5/95, v/v) to the residue
  dissolve the sample
- filtration

LC-MS/MS

Bundesinstitut für Risikobewertung

apparatus
ultrasonic bath; centrifuge; 50 ml vessel, 10ml pipette, filter

solvent
0.05 M H_2SO_4 – solution, NH_3-solution

apparatus
SPE – vacuum chamber

materials
DSC-C18 SPE cartridge, 500 mg

solvent
methanol; water; 2.5% ammonia in methanol

apparatus
evaporator

apparatus
HPLC-vials; membrane filter (0.2 µm)

solvent
methanol/water (5/95, v/v)

http://www.bfr.bund.de/cm/349/determination-of-pyrrolizidine-alkaloids-pa-in-plant-material.pdf

Method BfR-PA-Tea-2.0/2014

- Comminution of sample in minimum 0,5 mm (better **0,200 mm**, if possible)

- weight 2,0 g
- double extraction (0,05 M sulfuric acid)
- cleanup with SPE

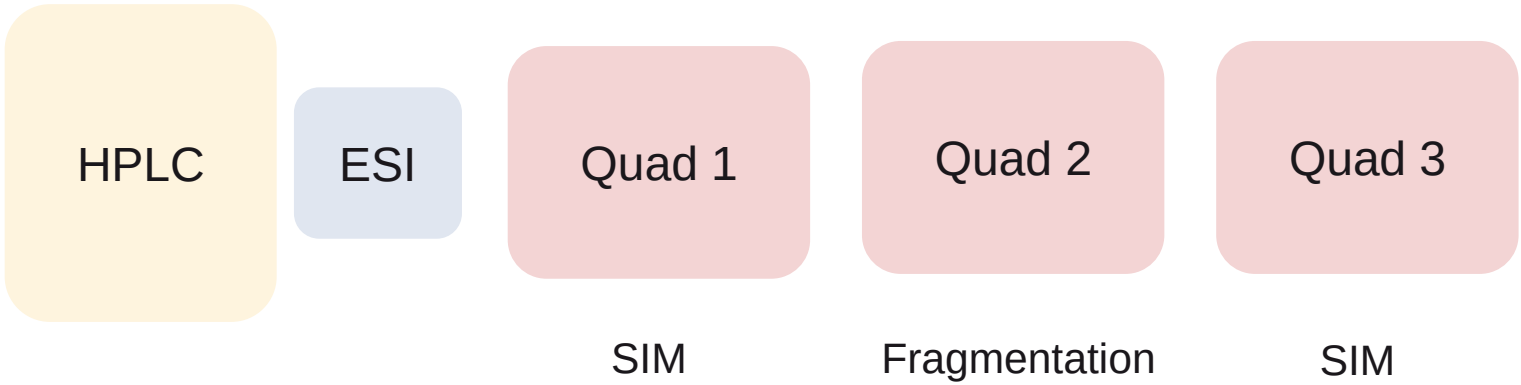
- chromatographic separation with RP18-HPLC-column

  → chromatographic conditions (equipment, column, gradients) are variable

- Detection with Triple Stage Quadrupole Mass Spectrometry **(HPLC-ESI-MS/MS** with **scheduled Multi-Reaction-Monitoring = sMRM)**

  → LC-MS/MS-equipment of different suppliers can be used

Triple-Quadrupole
Multiple Reaction Monitoring (MRM)



- ElectroSpray Ionisation
- only ions with selected m/z pass Quad 1
- fragmentation of all ions in Quad 2
- only selected daughter ions pass and are detected (Quad 3)

→ **very high selectivity and sensivity**

Method validation and acceptance criteria

Validation of „multi-methods“ (multi analytes and multi matrices) is not easy to perform.

An example how to handle this special issue is given in the case of pesticide residue analysis:

Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed

SANTE 11813/2017

implemented by 01/01/2018

Method validation and acceptance criteria

principle of matrix groups related to SANTE 11945/2015

- Representative matrices may be used to validate multi-residue methods with an so-called **„Initial full validation“** (as a minimum, one representative matrix from each relevant matrix group must be validated)

- Method validation must be supported and extended by **method performance verification** (on-going validation, extended validation) during routine analysis (wider variety of matrices).

Suitability of method, proficiency test

Suitability of analytical method and elegibility of laboratories (fit for purpose) could be reviewed in proficiency tests, for example BfR-PT-PA-01 from 2015 (started 01 June 2015, preliminery evaluation in February 2016, final evaluation 27.12.2016).

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BfR-PT-PA-01

- samples: mix of reference standards, peppermint (standard added), chamomile (nat.), melissa (nat.) and rooibos (nat.)
- participants from 27 laboratories: 25 LC-MS/MS, 1 inhouse method (sum method with „retronecin equivalent), 1 both
- 41% (11) of laboratories satisfied (z-scores |z| ≤ 2) with 5 samples evaluated;
  59% (16) satisfied with 4 samples evaluated (without rooibos)
- high standard deviation was observed by **quantifying pairs of isomers** and **analytes with low concentration**
- following parameters are influencing results:

  reference standards, extraction procedere, calibration/ standard addition, clean up, chromatographic separation

Challenges - analytical methods

- analysis must be done with many different matrices (> 1000)

  →starting material (herbal drug)
  →API (extracts)
  →finished product (containing excipients)

- checking and compensation of matrix effects (costs)

- many verifications are necessary (costs)

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Preliminary recommendation of BfR (002/2016) about analysis of PA in herbal teas and tea
(scope of substances)

Recommended scope of testing

- **Method BfR-PA-Tee-2.0/2014 → 28 PA**
- all reference standards available
- toxicological potential of substances has not been considered
- relevant PA-marker substances have been considered („Scientific Opinion on Pyrrolizidine alkaloids in food and feed“ der EFSA, 2011)
- **selection of substances based on data evaluation** of BfR study (466 herbal teas and teas): → **in minimum 21 PA**
- **further relevant PA should be added**

Recommendation of CONTAM Panel for monitoring programm (EFSA Journal 2017;15(7):4908)

Recommended scope of testing

- **selection of substances based on data evaluation of 2307 herbal teas and tea** (Dietary exposure assessment to pyrrolizidine alkaloids in the European population, EFSA Journal 2016;14(8):4572)
  → **in minimum 17 PAs**

- **further relevant PA should be added**

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Substances	Method BfR 17 (2013	Method BfR 28 (2014	Recommendation BfR 5. Januar 2016	Recommendation EFSA 21. Juni 2017
Echimidin	x	x	x	x
Echimidin-N-oxid		x	x	x
Erucifolin (Z)		x		
Erucifolin-N-oxid (Z)		x		
Europin		x	x	
Europin-N-oxid		x	x	
Heliotrin	x	x	x	
Heliotrin-N-oxid	x	x	x	
Intermedin	x	x	x	x
Intermedin-N-oxid		x	x	x
Jacobin		x		
Jacobin-N-oxid		x		
Lasiocarpin	x	x	x	x
Lasiocarpin-N-oxid	x	x	x	x
Lycopsamin	x	x	x	x
Lycopsamin-N-oxid		x	x	x
Monocrotalin	x	x		
Monocrotalin-N-oxid	x	x		
Retrorsin	x	x	x	x
Retrorsin-N-oxid	x	x	x	x
Senecionin	x	x	x	x
Senecionin-N-oxid	x	x	x	x
Seneciphyllin	x	x	x	x
Seneciphyllin-N-oxid	x	x	x	x
Senecivernin		x	x	x
Senecivernin-N-oxid		x	x	x
Senkirkin	x	x	x	x
Trichodesmin	x	x		

Different scopes of testing:

EFSA = 17
BfR = 21

24 (28) recommended

Scope of testing in view of relevant weed

Genus	Main alkaloides and N-Oxides
Crotalaria	Monocrotalin, Trichodesmin
Echium	Echimidin
Heliotropium	Europin, Heliotrin, Lasiocarpin
Myosotis	Intermedin, Lycopsamin
Senecio	Retrorsin, Senecionin, Seneciphyllin, Senecivernin, Senkirkin

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- Sampling is difficult and should be established depending on homogeneity of material.
- LC-MS/MS is a **target method** used for samples where typical pyrrolizidine alkaloids are expected. N-oxides can be determined simultaneously. The alkaloids were identified and quantified with suitable reference substances.
- LC-MS/MS method has been proposed from BfR using MRM-technic .This technique is often used analysing trace concentrations and shows a high specifity in spite of high sensitivity (10 µg/kg to 3 mg/kg).
- Other suitable methods than the proposed BfR method can be used if validation criteria are met.

Conclusion – analytical methods

- Proficiency tests showed that laboratories have comparable results although using different instruments and modified methods. Improvement could achieved in view of analysing pairs of isomers and low concentrations of analytes
- Scope of testing is in discussion. Reduction to 17, 21 or 24 substances?
- Implementation of method description, validation requirements and acceptance criteria to Ph.Eur. will improve situation

Thank you


Dr. Bernhard Klier
PhytoLab GmbH & Co. KG
Dutendorfer Straße 5-7
91487 Vestenbergsgreuth
Germany
Tel.: +49 9163 88-342
Fax.: +49 9163 88-349
Bernhard.Klier@phytolab

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