European Commission

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Pyrrolizidine alkaloids in herbal tea and honey

Report on the 2017 Proficiency testing scheme

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2017

EUR 28960 EN

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JRC Science Hub
https://ec.europa.eu/jrc

JRC109959

EUR 28960 EN

PDF ISBN 978-92-79-77167-5 ISSN 1831-9424 doi: 10.2760/219405

Luxembourg: Publications Office of the European Union, 2017

How to cite this report: Breidbach A, Tamošiūnas V, *Pyrrolizidine alkaloids in herbal tea and honey—Report on the 2017 Proficiency testing scheme*, EUR 28960 EN, Publications Office of the European Union, Luxembourg, 2017, ISBN 978-92-79-77167-5, doi 10.2760/219405, JRC109959.

European Union Reference Laboratory

Mycotoxins

268-PT Accredited by the
Belgian Accreditation Body (BELAC)

Executive Summary

Pyrrolizidine alkaloids (PAs) and their N-oxides (PANOs) are plant toxins which can enter the food chain through different paths. Two affected foods are herbal infusions and honey. This proficiency testing scheme was executed to assess the capabilities of laboratories to determine PAs. 29 laboratories from nine EU Member States plus Singapore registered. On 04. and 06.09.2017 test items and documentation were dispatched to all of those laboratories.

By the dead line of 24.10.2017 26 laboratories had reported back results and filled in a questionnaire. Test item HO (acacia honey) was fortified with six PAs/PANOs (Echimidine, Integerrimine, Intermedine, Senecionine, Seneciphylline-NO, and Senkirkine) and 23 laboratories reported results for this item. The same number of laboratories reported for test item HT (herbal infusion) which was naturally contaminated with four PAs after extraction under reductive conditions (Integerrimine, Retrorsine, Senecionine, and Senecivernine). Laboratories had to report the sums of PA and its respective PANO.

Satisfying outcomes could only be registered for Senecionine in test item HT and for Echimidine, Intermedine, and Senkirkine in test item HO with 74 %, 85 %, 85 %, and 91 %, respectively, of reported results having a z'-score smaller or equal to |2|. Only four laboratories reported for Integerrimine in both test items. Contrary to test item HT, Senecionine analysis in test item HO showed very unsatisfactory results. Of the 22 z'-scores calculated for Senecionine nine (41 %) were larger than 3. Senecivernine measurements in test item HT showed a similarly unsatisfying outcome with 47 % of reported results having *z'*-scores larger than 3.

Only three laboratories out of the 26 were able to test for all 10 measurands and only one reported all 10 values with z'-scores smaller or equal to |2|. Overall only five laboratories obtained satisfactory z'-scores (≤ |2|) for all their reported results. There are two groups of three isomeric PAs/PANOs each which apparently caused, for a number of laboratories, problems with quantification. This is an issue which deserves heightened attention.

The questionnaire contained queries regarding accreditation and experience, preparation conditions for the two test items, chromatographic separation conditions, detection conditions, calibration approach, and a comments section. The answers were evaluated and for selected questions their correlation to the z'-score of Senecionine in test item HO or Senecivernine in test item HT was analysed. For none of the tested questions a significant influence could be shown.

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1. Introduction

Pyrrolizidine alkaloids (PAs) are secondary metabolites of plants used as defence against plant eating animals. It is estimated that over 6000 plant species worldwide, mainly from the botanical families Boraginaceae (e.g. Heliotropium spp.), Asteraceae (e.g. Senecio spp.) and Fabaceae (e.g. Crotalaria spp.), produce at least 600 different PAs. Those with an unsaturated bond in position 1,2 of the pyrrolizidine ring system (Figure 1) are hepatotoxic. Additional diversity is created due to oxidation of the pyrrolizidine nitrogen to the N-oxide (PANO) which doubles the possible number of PAs.

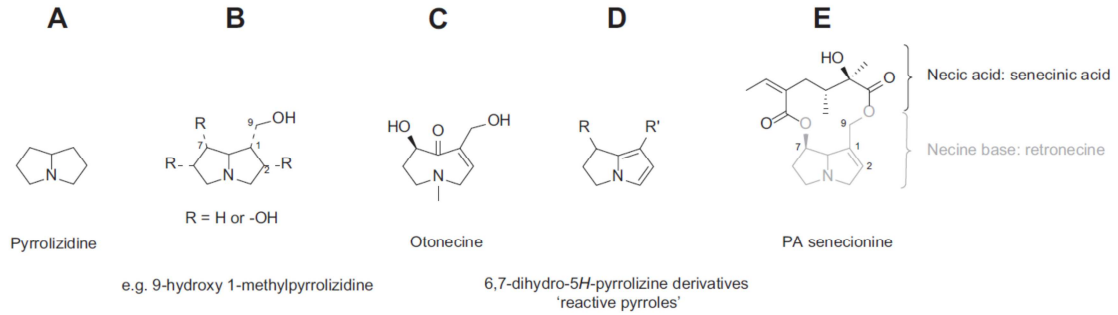


Figure 1: Structural features of PAs. (A) core structural motif pyrrolizidine (1,2,3,6,7,8-hexahydro-5Hpyrrolizine);(B) general description of the main necine base parts of naturally occurring PAs including the common necine base numbering; (C) necine base otonecine; a core structural motif of otonecine-type PAs; (D) general pyrrolizine structure motif and (E) structural example of 1,2-unsaturated ester PA senecionine (Figure taken from [1])

PAs may enter the food chain via different routes. Two possibly affected end products are teas / herbal infusions, if PA containing weeds had contaminated the leafs / herbs, and honey, if PA containing pollen was collected by the bees. In 2016 the European Food Safety Authority (EFSA) published a new scientific report about dietary exposure to PAs [1]. In that report teas / herbal infusions were identified as the largest contributors to total PA exposure. It has been recommended that for relevant food commodities, as honey and teas / herbal infusions, the effort to collect PAs occurrence data should be continued.

In this PT scheme a naturally contaminated rooibos herbal blend for infusion and a spiked acacia honey were sent out to 29 laboratories for PAs analysis. The two test materials were prepared, tested for homogeneity, stability, and characterized with respect to contamination level in-house. This report presents the results of these tests and characterizations as well as the reported results from the participating laboratories.

2. Scope

This PT scheme was executed to assess the capabilities of the participating laboratories to determine one or more of the following PAs and/or their corresponding PANOs:
Echimidine, Erucifoline, Europine, Indicine, Integerrimine, Intermedine, Jacobine, Lasiocarpine, Lycopsamine, Monocrotaline, Retrorsine, Senecionine, Seneciphylline, Senecivernine, Senkirkine, Trichodesmine,
in an herbal blend for infusion and acacia honey.

3. Set-up of PT scheme

3.1. Time frame

The PT registration was open from 19.05.2017 to 15.08.2017. Registration could be done through a dedicated web site. The test materials were dispatched to the participants on 04.09. and 06.09.2017. All shipments reached their respective recipients within 2 days. The initial deadline for reporting the results was 20.10.2017. This deadline was eventually extended to 24.10.2017 because two laboratories reported problems with entering the results.

3.2. Confidentiality

The procedures used for the organisation of PTs are accredited according to ISO 17043:2010 [2] and guarantee that the identity of the participants and the information provided by them is treated as confidential.

3.3. Distribution

In all, 29 laboratories from nine EU Member States plus Singapore registered for participation. The following was sent to each of them:

- one container with 40 – 50 g of a herbal blend for infusion naturally contaminated with PAs;
- one container with 50 – 70 g of acacia honey spiked with PAs
- the "Instructions for PT scheme" letter with the individual reporting password (Annex 1: Instructions for PT scheme);
- the "Proficiency testing materials receipt form" (Annex 2: Proficiency testing materials receipt form);
- a "Submission of results" document explaining the use of the reporting interface.

3.4. Instructions to participants

The "Instructions for PT scheme" letter included with the test item shipment gave detailed instructions to the participants. In brief, the participants were informed that one or more of the following PAs:
Echimidine, Erucifoline, Europine, Indicine, Integerrimine, Intermedine, Jacobine, Lasiocarpine, Lycopsamine, Monocrotaline, Retrorsine, Senecionine, Seneciphylline, Senecivernine, Senkirkine, Trichodesmine,
could be present in either one of the two test items. The participants had to check the test items for integrity and store them upon receipt at temperatures between -15 °C and -25 °C. Instructions on how to access the reporting web site and how to enter results were also included.

3.5. Standard deviation for proficiency assessment

Scoring of the reported results was to be done acc. to ISO 13528:2015 and was to be based on an independently determined reference value (x_{PT}) for each PA and a target standard deviation σ_{PT} of 0.22 x_{PT} acc. to the following equation:

$$z = \frac{(x_i - x_{PT})}{\sigma_{PT}}$$

with x_i the reported result of an individual PA from a laboratory. No scoring was to be provided for reported sums of total PAs.

For those analyte contents for which the associated uncertainty of x_{PT} (u(x_{PT})) was larger than 0.3 σ_{PT} the following equation was used:

$$z' = \frac{(x_i - x_{PT})}{\sigma'_{PT}}$$

with the adjusted target standard deviation $\sigma'_{PT} = \sqrt{\sigma^2_{PT} + u^2(x_{PT})}$.

4. Test items

4.1. Preparation

The herbal infusion blend (test material HT, mostly rooibos) was purchased from a provider within the EU. It was milled with a ZM200 centrifugal mill with a 0.5 mm sieve (Retsch, Hahn, Germany). After thorough homogenization the material was filled into 96 plastic screw-capped containers.

The acacia honey (test material HO) was courtesy of Breitsamer + Ulrich GmbH & Co KG (München, Germany). The honey was practically free of PAs (none detected above 3 µg/kg) and spiked with Echimidine, Integerrimine, Intermedine, Senecionine, Seneciphylline-NO, and Senkirkine. It was then thoroughly mixed with a ploughshare mixer (Lödige, Paderborn, Germany) and filled into 73 plastic screw-capped containers.

4.2. Homogeneity

For testing the homogeneity ten containers of HT and eight containers of HO were selected. Test portions were extracted/cleaned-up with SPE acc. to an internal protocol and measured in randomized order with a LC-HRMS. The obtained signals, without conversion to concentration units, were evaluated for sufficient homogeneity acc. to ISO 13528 Annex B [3]. More details can be found in Annex 3: Homogeneity testing.

For all analytes in both test materials the results indicated sufficient homogeneity.

4.3. Stability

The stability of the analytes in the two test materials was determined through an isochronous study. For this purpose randomly selected test units were stored frozen (-15 to –25 °C) and at 4 °C, and 40 °C for up to eight weeks. At the day of measurement all test units were prepared and measured as described for the homogeneity testing. More details can be found in Annex 4: Stability testing.

All analytes in both test materials were sufficiently stable during the PT period.

5. Assigned values and their uncertainties

For the scoring of this PT scheme individual mass fractions for all the PAs in the two test materials were determined. This was done with gravimetric standard addition using an internal standard

(ISTD) acc. to Hauswaldt et al [4]. These measurements were executed in early October for test item HT and early November for test item HO. This was due to time constraints and happened without prior knowledge of reported results. More details can be found in Annex 5: Determination of the assigned values and their uncertainties.

The assigned values x_{PT} and their associated standard uncertainties $u(x_{PT})$ were determined acc. to ISO 13528 clause 7 [3] and are listed in Table 1 for test item HT and Table 2 for test item HO.

6. Evaluation of the results

6.1. Test item HT

Test item HT was naturally contaminated with four PAs/PANOs at detectable levels: Integerrimine, Retrorsine, Senecionine, and Senecivernine. Table 1 lists relevant parameters. The reference values (x_{PT}) are for the sum of PA/PANO after reductive extraction. Since the associated uncertainties of the reference values were larger than 0.3 σ_{PT} in all cases the adjusted target standard deviation σ'_{PT} was used for scoring. The robust mean ($\overline{x}_{rob}$) was computed from the reported results using Algorithm A [3, Annex C] following the Recommendation 1 in [5]. A robust mean could not be calculated for the reported results of Integerrimine because of too few results and for Senecivernine because of too high a contribution of minor modes to the multi-modal distribution of reported results. For the other two robust means, the one of Senecionine falls within the expanded uncertainty range (k=2, ~ 95 % confidence) around x_{PT}, the one of Retrorsine does not.

For the four PAs present in test item HT a satisfying outcome can only be attested for the analysis of Senecionine. It was present at the highest level and 17 out of 23 (74 %) reported results showed z'-scores ≤ |2|. No reported result had a z'-score > |3|. At the opposite end is the outcome for the measurements of Senecivernine with 47 % of reported results with z'-scores larger than 3. For Integerrimine only four laboratories reported a result, two of them as "smaller than", even though it was present at the second highest level in this naturally contaminated material. Table 3 is listing all reported results and the corresponding z'-scores. The figures in Annex 6: Graphs for Test item HT visualise these results.

Integerrimine, Senecionine, and Senecivernine are three isomeric PAs with the elemental formula ($C_{18}H_{25}NO_5$). This makes these three compounds practically indistinguishable for mass spectrometry which was the detection mode used by all participating laboratories. Only proper chromatographic separation, which is challenging, can provide reliable results for the individual compounds. Insufficient separation between Integerrimine and Senecivernine, and false identification may have led to the large number of too high results for Senecivernine. A compounding factor may be that the majority of participating laboratories were apparently not testing for Integerrimine and, therefore, might not have been aware of any coelution issues. Integerrimine is not included in the list of PAs provisionally selected by the European Commission [1].

Laboratory 14 reported to have also detected Senkirkine at 10 µg/kg. No other laboratory reported any other PA above its respective LOQ. Laboratory 3 reported the sum of all PAs as "retronecine equivalents" at 78 µg/kg.

Table 1 Parameters of the four PAs and summary of the outcome in test item HT

	Integerrimine	Retrorsine	Senecionine	Senecivernine
x_{PT} [µg/kg]	30.6	19.8	80.4	5.7
$u(x_{PT})$ [µg/kg]	2.8	1.5	7.8	1.2
$\bar{x}_{rob}$ [µg/kg]		15.4	65.4	
σ_{PT} [µg/kg]	6.7	4.3	17.7	1.2
$u(x_{PT})$>0.3 σ_{PT}	YES	YES	YES	YES
σ'_{PT} [µg/kg]	7.3	4.6	19.3	1.7
Number reported	4	20	23	15
"<" or ">"	2	7	1	2
$z' \leq \|2\|$	2	9	17	6
$\|2\| < z' \leq \|3\|$	0	2	5	0
$z' > \|3\|$	0	2	0	7

6.2. Test item HO

Test item HO was an acacia honey free of PAs. It was spiked with Echimidine, Integerrimine, Intermedine, Senecionine, Seneciphylline-NO, and Senkirkine. Table 2 lists relevant parameters. These parameters were determined as described for test item HT above. Again, for Integerrimine too few results were reported to calculate a robust mean. Of the other robust means, the ones for the content of Echimidine and Senkirkine lay within the expanded uncertainty ranges (k=2, ~ 95 % confidence) around the respective x_{PT}, the ones for Intermedine, Senecionine, and Seneciphylline were below the lower limit of the expanded uncertainty ranges. Also, as for test item HT, the associated uncertainties of the reference values were larger than 0.3 σ_{PT} in all cases but for Senecionine. Yet for reasons of consistency the adjusted target standard deviation σ'_{PT} was used for scoring of all PAs including Senecionine.

Table 2 Parameters of the six PAs and summary of the outcome in test item HO

	Echimidine	Integer-rimine	Intermedine	Senecionine	Seneci-phylline	Senkirkine
x_{PT} [µg/kg]	70.8	54.4	43.9	29.1	12.9	62.1
$u(x_{PT})$ [µg/kg]	6.3	3.8	4.4	1.8	1.0	9.6
$\bar{x}_{rob}$ [µg/kg]	68.4		30.9	23.4	9.7	52.3
σ_{PT} [µg/kg]	15.6	12.0	9.7	6.4	2.8	13.7
$u(x_{PT})$>0.3 σ_{PT}	YES	YES	YES	NO	YES	YES
σ'_{PT} [µg/kg]	16.8	12.6	10.6	6.6	3.0	16.7
Number reported	20	4	20	22	22	23
"<" or ">"	1	2	1	1	3	1
$z' \leq \|2\|$	17	2	17	9	13	21
$\|2\| < z' \leq \|3\|$	1	0	2	3	3	0
$z' > \|3\|$	1	0	0	9	3	1

Table 3 Reported values and calculated z'-scores for the PAs content in test item HT

Lab Code	Integerrimine		Retrorsine		Senecionine		Senecivernine	
	reported [µg/kg]	z'-score	reported [µg/kg]	z'-score	reported [µg/kg]	z'-score	reported [µg/kg]	z'-score
1								
2	< 10		< 10		75	-0.3	< 5	
3	retronecine equivalents: 78 µg/kg							
4			17.2	-0.6	59.4	-1.1	8.16	1.5
5			15.7	-0.9	47.7	-1.7	12	3.7
6	< 2		16.58	-0.7	78.08	-0.1	7.84	1.3
7			3.7	-3.5	47.9	-1.7		
8			11	-1.9	58.14	-1.2	24.4	11.0
9			16.92	-0.6	68.21	-0.6	6.86	0.7
10			< 10		130	2.6	14.3	5.1
11			7.06	-2.8	47.54	-1.7	47.54	24.6
12			10.11	-2.1	62.62	-0.9		
13	21.5	-1.3	11.5	-1.8	38.5	-2.2	8.47	1.6
14			< 2.5		64	-0.9		
15			20.3	0.1	66.9	-0.7	< 5	
16			< 2.5		26.6	-2.8		
17			< 3		59.2	-1.1		
18					120	2.1	5.9	0.1
19								
20			19.7	0.0	111	1.6	36	17.8
21					30	-2.6		
22			< 10		93	0.7	39	19.6
23			< 20		< 30			
24			35.9	3.5	86.4	0.3	21.2	9.1
25					92.91	0.7		
26	36.4	0.8	14.8	-1.1	68.3	-0.6	4.9	-0.5

For this test item the measurements of the PAs Echimidine, Intermedine, and Senkirkine showed very satisfactory results with 85 % (17 of 20), 85 % (17 of 20), and 91 % (21 of 23), respectively, of the z'-scores below or equal to |2|. Analogous to item HT, only four laboratories reported for Integerrimine and, contrary to test item HT, the results for Senecionine in this item were very unsatisfactory.

Of the 22 z'-scores calculated for Senecionine nine (41 %) were larger than 3. This is all the more surprising since five of those nine laboratories (z'-scores 3.9 to 14.2) scored well for Senecionine in item HT (z'-scores -1.7 to 0.7). Additionally, seven laboratories (1, 5, 8, 10, 11, 20, 22) reported to have found Senecivernine in item HO and, of these, four scored well in this item for Senecionine with z'-scores between -1.0 and 0.9. The results are listed in Table 4 and visualised in Annex 6: Graphs for Test item HO. The results for Seneciphylline are also not convincing with only 13 out of 22 (59 %) z'-scores below or equal to |2|.

Laboratories 14 (83 µg/kg) and 19 (30 µg/kg) have reported to have found Lycopsamine and did not report results for Intermedine. Intermedine / Lycopsamine / Indicine form another group of

isomeric PAs ($C_{15}H_{25}NO_5$) which are difficult to separate. If laboratory 19 which indicated in the questionnaire that it reported Intermedine / Lycopsamine / Indicine as sum misidentified Intermedine as Lycopsamine then the reported value would have earned a z'-score of -1.3. Trichodesmine was reported to have been found by laboratory 12 (49 µg/kg). No other laboratory reported any other PA above its respective LOQ. Laboratory 3 reported the sum of all PAs as "retronecine equivalents" at 45 µg/kg.

Correct determination of the individual members of the isomeric groups "Integerrimine / Senecionine / Senecivernine" and "Intermedine / Lycopsamine / Indicine" seems to be an issue which needs attention.

Table 4 Reported values and calculated z'-scores for the PAs content in test item HO

Lab Code	Echimidine		Integerrimine		Intermedine		Senecionine		Seneciphylline		Senkirkine	
	reported [µg/kg]	z'-score	reported [µg/kg]	z'-score	reported [µg/kg]	z'-score	reported [µg/kg]	z'-score	reported [µg/kg]	z'-score	reported [µg/kg]	z'-score
1	57.6	-0.8			24.7	-1.8	74.7	6.9	9.1	-1.3	74	0.7
2												
3	retronecine equivalents: 45 µg/kg											
4	61.8	-0.5			34.4	-0.9	22.7	-1.0	10	-1.0	47.9	-0.9
5	69.3	-0.1			27.6	-1.5	22.8	-1.0	10.4	-0.8	54.4	-0.5
6	67.87	-0.2	< 0.5		39.75	-0.4	21.99	-1.1	12.37	-0.2	57.89	-0.3
7	34	-2.2			32.3	-1.1	83	8.1	25.3	4.1	37.2	-1.5
8	81.9	0.7			21.4	-2.1	27.6	-0.2	31.7	6.3	60.4	-0.1
9	72.15	0.1			38.24	-0.5	27.13	-0.3	7.61	-1.8	37.82	-1.5
10	77	0.4			29.4	-1.4	50	3.1	21.1	2.7	58.2	-0.2
11	45.92	-1.5			16.84	-2.6	11.07	-2.7	19.71	2.3	42.05	-1.2
12	69.45	-0.1			29.18	-1.4	58.84	4.5	0.34	-4.2	9.17	-3.2
13	82.5	0.7	37.4	-1.4	24.8	-1.8	13.6	-2.3	5.78	-2.4	48.9	-0.8
14							120	13.7	18	1.7	69	0.4
15	74.6	0.2			30.2	-1.3	19.5	-1.4	10.8	-0.7	49.9	-0.7
16	62.8	-0.5			33	-1.0	14.5	-2.2	9.9	-1.0	40.4	-1.3
17					48	0.4	116.33	13.1	< 3		54.73	-0.4
18	57	-0.8					52	3.4	7.9	-1.7	55	-0.4
19	122	3.1	< 1				52	3.4	< 1		53	-0.5
20	96.4	1.5			36.2	-0.7	28.5	-0.1	13.4	0.2	54.7	-0.4
21	62	-0.5			31	-1.2					44	-1.1
22	56	-0.9			31	-1.2	35	0.9	9	-1.3	49	-0.8
23	> 10				> 10		> 10		> 10		> 10	
24												
25					44.49	0.1	54.31	3.8	16.37	1.2	71.75	0.6
26	74	0.2	39.4	-1.2	57	1.2	16.2	-1.9	10.2	-0.9	49.2	-0.8

6.3. "Smaller than" / "Larger than" values and uncertainties

For the PAs listed in Table 1 and Table 2 16 results were reported as "smaller than". This declaration was only correct for three of the 16. For all others the actual mass fraction was

above the "smaller than" value. Five results were reported as "larger than" and all those declarations were correct.

Only for a minority (72 out of 173, i.e. 42 %) of results an associated measurement uncertainty was reported. Of those 72 expanded uncertainties 37 were under-estimated, meaning the uncertainty ranges around the reported value did not include the reference value. Another five reported uncertainty ranges included the value zero meaning the reported value within its uncertainty is not different from zero at the selected confidence range. In seven cases the estimate of the measurement uncertainty was overly conservative.

All affected laboratories are advised to check their LOD/LOQ and uncertainty estimations.

6.4. The questionnaire

All participating laboratories were asked to fill in a questionnaire addressing their accreditation and experience, preparation conditions for the two test items, chromatographic separation conditions, detection conditions, calibration approach, and any comments they felt like making. Each laboratory reporting results also filled in the questionnaire. For selected questions the correlation of answers to the z'-score of Senecionine results in test item HO or Senecivernine results in test item HT was analysed by single-factor analysis of variance (ANOVA) after variance stabilizing transformation of the response variable.

The vast majority of the laboratories (85 %) had more than 12 months of experience with PA analysis. Two laboratories reported to have less than 3 months and another two between 3 and 12 months of experience. Accreditation to ISO 17025 for PA analysis was held by 15 laboratories while 11 were not accredited. A question begging to be asked is whether holding accreditation has a positive effect on the reported results. Looking at the z'-scores of Senecionine results no difference in performance between accredited and non-accredited laboratories could be detected with ANOVA. The same is true for Senecivernine results. The data is depicted in Figure 6- 11 in Annex 6: Graphs.

The information provided about test item preparation showed that the laboratories used a median test portion size of 5 g for HO and 2 g for HT. This was then extracted with a median volume of 20 mL and 40 mL, for HO and HT, respectively. For both test items the median extraction time was 30 min. The composition of the extraction solvents for test item HO was either acidic aqueous (16), acidic aqueous/organic (2), neutral aqueous/organic (3), pure organic (1), or in one other case basic aqueous/organic. Three laboratories chose to not make a statement. For test item HT either acidic aqueous (19), acidic aqueous/organic (2), or neutral aqueous/organic (2) conditions were employed. Also here, three laboratories did not make statements. Neither for Senecionine results in HO nor for Senecivernine results in HT a significant effect of the extraction solvent composition on the z'-scores was detected by ANOVA.

In the next group of questions about chromatographic separation conditions the 26 laboratories all stated to have used liquid chromatography for separation of the PAs/PANOs. Methanol was used as organic mobile phase modifier by 19 and acetonitrile by five laboratories. Two laboratories did not provide information. Only in two cases the mobile phase did not contain any additives. Formate (formic acid with or w/o ammonium formate) was used by 17 participants, acetate (acetic acid with ammonium acetate) by 4, and one laboratory employed a basic mobile

phase with ammonium carbonate. Again, two laboratories did not provide information. Neither the organic modifier nor the additive in the mobile phase had a significant effect on the results of Senecionine in HO or Senecivernine in HT.

A hypothesis was raised above that the unsatisfactory result for Senecionine in HO was caused by insufficient chromatographic separation within the isomeric group "Integerrimine / Senecionine / Senecivernine". Ten laboratories reported to have used analytical columns with fully porous particles of larger than 2 µm size. The same number of laboratories used sub-2 µm fully porous particle columns. Since the sub-2 µm columns are capable of higher separation efficiencies it was tested whether that had a significant effect on the scores. But neither for Senecionine results in HO nor for the ones of Senecivernine in HT such an effect could be shown.

All laboratories have used mass spectrometry (MS) for detection with 20 laboratories using MS/MS in selected/multiple reaction monitoring (SRM/MRM), one using high resolution MS/MS, and three using selected ion monitoring (SIM). Two made no statement. The question how the PAs/PANOs were determined was answered by 24 laboratories with individual PAs and PANOs as such. One laboratory reduced all PAs/PANOs to a common analyte (retronecine) and derivatised with phthalic acid before measurement. And only one laboratory (Lab 19) stated that the sums of isomers were reported.

Questions about the calibration approach were not included in the initial questionnaire. Therefore, all 26 reporting laboratories received an email with these questions on 09.11.2017. All except one laboratory replied. For the test item HO 24 laboratories reported results and, of those, 12 used matrix-matched external standard calibration, six standard addition, three neat solvent external standard calibration, one isotope dilution, one "neat solvent external standard/standard addition", and one "prespiked matrix matched calibration". The same number (24) of laboratories reported at least one result for test item HT. Of those, nine used standard addition, six matrix-matched external standard calibration, five neat solvent external standard calibration, one isotope dilution, one "neat solvent external standard/standard addition", and one "prespiked matrix matched calibration". One of these laboratories did not reply to the inquiry. The influence of the calibration approach on the z'-scores for Senecionine in HO and Senecivernine in HT was tested with ANOVA and no difference could be found.

Altogether, 21 laboratories reported results for both test items and only five of them reported all their results with satisfactory z'-scores. Laboratory 26 using standard addition reported ten values with z'-scores between -1.1 and 2.0. Laboratory 6 reported eight values with scores between -1.1 and 1.3 and two "smaller than" results. Laboratory 4 reported eight values with scores between -1.1 and 1.5. Both laboratories used matrix-matched external standard calibration for test item HO and standard addition for HT. Laboratory 9 reported eight values between -1.8 and 0.7 using neat solvent external standard calibration. Finally, laboratory 15 reported seven values with scores between -1.5 and 0.2 using standard addition and one "smaller than" result.

In the comments section of the questionnaire laboratory 11 declared that it reported the sum of Senecionine and Senecivernine. It reported the same value for both in HT. That led to a satisfactory z'-score for Senecionine and an unsatisfactory one for Senecivernine. That Indicine and Intermedine could not be separated on a C18 column was stated by laboratory 8. Laboratory

14 commented that co-occurrence of the isomeric PAs might occur and affect their results. Co-elution of Lycopsamine and Indicine was remarked by laboratory 16.

7. Conclusions

This proficiency testing scheme was executed to assess the capabilities of laboratories to determine PAs/PANOs in herbal infusions and honey. 29 laboratories from nine EU Member States plus Singapore registered for this PT scheme and were sent two test items for analysis. By the dead line of 24.10.2017 26 laboratories had reported back results and filled in a questionnaire.

Only three laboratories out of the 26 were able to test for all 10 measurands and only one reported all 10 values with z'-scores smaller or equal to |2|. Overall only five laboratories obtained satisfactory z'-scores (≤ |2|) for all their reported results. The outcome of this scheme points to a possible problem by many laboratories to properly determine the individual members of the two isomeric groups "Integerrimine / Senecionine / Senecivernine" and "Intermedine / Lycopsamine / Indicine".

For test item HO, spiked with six PAs/PANOs, the measurements of the PAs Echimidine, Intermedine, and Senkirkine showed very satisfactory results with 85 % (17 of 20), 85 % (17 of 20), and 91 % (21 of 23), respectively, of the z'-scores below or equal to |2|. For the four PAs present in test item HT a satisfying outcome can only be attested for the analysis of Senecionine. It was present at the highest level and 17 out of 23 (74 %) reported results showed *z'*-scores ≤ |2|.

8. Acknowledgements

We want to thank the participating laboratories (see Annex 8: Participating laboratories, p.44) for their efforts in reporting the results on time and filling in the questionnaire. Our thanks also go to C. Mischke of the EURL for Mycotoxins for his support in test material production, and Vasiliki Exarchou for the qNMR measurements.

References:

1 EFSA (European Food Safety Authority), 2016. Dietary exposure assessment to pyrrolizidine alkaloids in the European population. EFSA Journal 2016;14:4572, 50 pp. doi:10.2903/j.efsa.2016.4572

2 ISO/IEC 17043 "Conformity assessment – General requirements for proficiency testing, International Organisation for Standardization, Geneva, Switzerland, 2010.

3 ISO 13528:2015 - Statistical methods for use in proficiency testing by interlaboratory comparison, International Organisation for Standardization, Geneva, Switzerland, 2015.

4 Hauswaldt, A.-L.; Rienitz, O.; Jährling, R.; Fischer, N.; Schiel, D.; Labarraque, G.; Magnusson, B. Uncertainty of standard addition experiments: a novel approach to include the uncertainty associated with the standard in the model equation. Accreditation and Quality Assurance 2012, 17, 129-138, 10.1007/s00769-011-0827-5.

5 Thompson, M.; Ellison, S.L.R.; Wood, R. The International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories (IUPAC Technical Report). *Pure Appl. Chem.* **2006**, *78*, 145-196.

Annex 1: Instructions for PT scheme

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers & Reference Materials (Geel/Ispra)
Food & Feed Compliance

Instructions for PT scheme "Pyrrolizidine alkaloids contamination in herbal tea and honey"

«AddressBlock»

«GreetingLine»

please read the following instructions carefully.

This PT scheme is to assess your laboratory's ability to determine pyrrolizidine alkaloids (PAs) in herbal tea and/or honey. The following PAs may or may not be present in the two test materials: Echimidine, Erucifoline, Europine, Indicine, Integerrimine, Intermedine, Jacobine, Lasiocarpine, Lycopsamine, Monocrotaline, Retrorsine, Senecionine, Seneciphylline, Senecivernine, Senkirkine, Trichodesmine.

It is your choice whether you measure just one or both of the two test materials and which of the 16 PAs you determine. You may report a sum parameter combining all PAs or results for the individual PAs. If results for individual PAs are reported you must report the sum of the amine and the N-oxide of the respective PA. This sum can either be calculated from individual values for the amine and N-oxide or it may be the measured value of the total amine after reduction of the N-oxide.

Please check the integrity of the test material containers and use the included material receipt form to report back to us your findings as soon as possible. Until commencement of the analysis the test materials should be stored at deep freeze temperatures (-15 - -25 °C). Before opening the containers they should be given sufficient time to equilibrate to room temperature. It is the responsibility of the laboratory to withdraw a representative test portion from the test material containers. Therefore, to minimize sub sampling uncertainties, the content of the container should be mixed well before withdrawal and the test portion be withdrawn in multiple smaller increments.

Scoring of the reported results will be done acc. to ISO 13528:2015 and be based on an independently determined reference value (x_{PT}) for each PA and a target standard deviation σ_{PT} of 0.22* x_{PT} acc. to the following equation:

$$z = \frac{(x_i - x_{PT})}{\sigma_{PT}}$$

with x_i the reported result of an individual PA from a laboratory. No scoring will be provided for reported sums of total PAs.

The reporting deadline is **20. Oct 2017**. After this date the reporting interface will be closed.

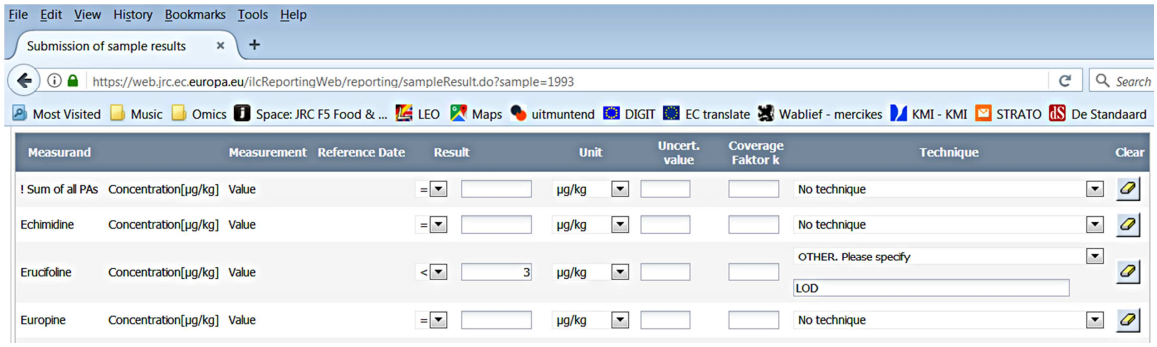
Open the following link (https://web.jrc.ec.europa.eu/ilcReportingWeb/) in a web browser (preferably use latest version Firefox or Internet Explorer) to enter your results.

Your personal password key is: «Part_key»

Enter this into the "Password key" field.

When entering your results use a "." (dot) as decimal divider. Do not use a "," (comma). To enter a result below LOD into the results table select the "<" (smaller as) sign from the drop down menu and enter the numerical value of the LOD into the results field. Then choose "OTHER, Please specify" in the Technique column and enter "LOD".

For example, a measurement of Erucifoline with a LOD of 3 µg/kg would be entered as is depicted below:



For entering a value below LOQ proceed accordingly. Leave the fields for PAs for which no measurements were performed empty. If you determined the sum of all PAs enter your result into the top row and leave all others empty. Results entry does not need to happen in one session. Entry can always be interrupted. Save what you have entered and come back later to continue. Only once you click the "Submit my results" button on the reporting home page the entries will be submitted.

If possible also provide an estimate of the measurement uncertainty and the coverage factor used for any reported results larger than LOQ. The annex provides further information on how to use the reporting interface.

There is also a questionnaire about your analysis that we ask you to fill in at the time of reporting.

To prevent falsification of results you must keep the password key confidential. Any attempt of collusion is contrary to professional scientific conduct and serves only to nullify the benefits of proficiency tests to costumers, accreditation bodies and analysts alike.

Good luck with your analysis.

Andreas Breidbach
PT coordinator

Annex 2: Proficiency testing materials receipt form

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
Food & Feed Compliance

Geel, 12 December 2017

PROFICIENCY TESTING MATERIALS RECEIPT FORM

Name:	**«Title» «Firstname» «Surname»**
Institute:	**«Organisation»**

NOTE: STORE MATERIAL IN A FREEZER AT -15 - -25 °C!

Please ensure that the items listed below have been received undamaged, and then check the relevant statement:

Date of receipt	
All items have been received undamaged	YES / NO
If NO, please list damaged items:	

Contents of the parcel:

a) 1 container with artificially contaminated honey (HO)
b) 1 container with naturally contaminated herbal tea (HT)
c) Instructions with information about storage and reporting
d) this material receipt form

Please scan in the completed form and e-mail to:

JRC-EURL-MYCOTOX@ec.europa.eu

Your Signature / Stamp here:

Annex 3: Homogeneity testing

For homogeneity testing of the HT test material 10 test units were randomly selected out of the 96 test units that were created. Of each of the 10 test units two 1 g test portions were weighed out for extraction and clean-up. Care was taken to ascertain that each test portion was representative of its respective test unit.

For homogeneity testing of the HO test material eight test units were selected from the 73 test units that were created. Since the test units were created from a more or less continuous material stream a random unit was selected from the first 10 units. Then every 10th following unit was selected until the end. In this particular case the units 3, 13, 23, 33, 43, 53, 63, and 73 were selected. Of each of the selected units 5 g were weighed into a new tube. Then 2.5 g of water were added to reduce viscosity of the honey. After thorough mixing two 3 g test portions, representing 2 g test material, were weighed into new tubes for clean-up.

The test portions were extracted/cleaned-up with SPE acc. to an internal protocol and measured in randomized order with a LC-HRMS. The obtained signals, without conversion to concentration units, were evaluated for sufficient homogeneity acc. to ISO 13528 Annex B [3]. Table 1- 1 shows the results for test material HT, Table 1- 2 the results for material HO.

Table 1- 1: Results of the homogeneity test for test material HT

Analyte	c	s^2_s	s^2_w
Integerrimine	0.178	0.0133	0.0905
Retrorsine	0.0142	0	0.00742
Senecionine	0.93	0.059	0.55
Senecivernine	0.00927	0.00234	0.00391

c- -critical value, s^2_s – between-sample variance, s^2_w – within-sample variance

Table 1- 2: Results of the homogeneity test for test material HO

Analyte	c	s^2_s	s^2_w
Echimidine	2.74	0	0.310
Integerrimine	1.08	0	0.0255
Intermedine	0.558	0.000472	0.0207
Senecionine	0.272	0.00101	0.00148
Seneciphylline	0.0493	0.000464	0.000899
Senkirkine	2.20	0.0221	0.0909

c- -critical value, s^2_s – between-sample variance, s^2_w – within-sample variance

For all analytes in both test materials the between-sample variance was smaller than the critical value indicating sufficient homogeneity.

Annex 4: Stability testing

The stability of the analytes in the two test materials was determined through an isochronous study. For this purpose randomly selected test units were stored frozen (-15 to –25 °C). Eight weeks before the measurement a set of test units was transferred to and stored at 40 and 4 °C, respectively. Four weeks before the measurement another set of test units was transferred to and stored at the same temperatures. At the day of measurement a set of test units stored frozen, the test units stored for 4 and 8 weeks at 40 °C, and the test units stored for 4 and 8 weeks at 4 °C were prepared and measured as described for the homogeneity testing. The test units stored frozen represented time point zero.

Based on the statistical model with y the measured mass fraction at time point x (in weeks)

$$y = \beta_0 + \beta_1 x$$

the coefficients β_0 and β_1 were computed via least square regression. The slope β_1 represents the analyte change per time unit. If the 95 % confidence range around β_1 includes zero there is no statistically significant instability. Table 2- 1 shows that this is the case for all analytes in test material HT but Retrorsine stored at 40 °C. The temperature level "40 °C" was included to simulate the worst case scenario for transportation. All shipments arrived in the respective laboratory within 2 days. After reception laboratories were required to store the test materials at deep-freeze temperatures. For Retrorsine in HT a 2 day (0.29 weeks) exposure to transportation temperature would result in a analyte change of -0.23 ng/g/week * 0.29 weeks = -0.066 ng/g. This is smaller than 0.3 σ_p (1.2 ng/g) and has, therefore, no impact on the calculated z-scores. This is indicated in the right-most column of Table 2- 1.

Table 2- 1: Results of the stability study for test material HT

Analyte	Temperature [°C]	β_1 [ng/g week]	*95 % CR* [ng/g week]	$\beta_1 \times max(t_T) > 0.3\ \sigma_p$
Integerrimine	4	0.029	-0.75 to 0.81	
	40	-0.22	-0.68 to 0.23	
Retrorsine	4	-0.16	-0.62 to 0.31	
	40	-0.23	-0.46 to -0.0032	NO
Senecionine	4	0.41	-1.2 to 2.0	
	40	-0.77	-1.9 to 0.35	
Senecivernine	4	-0.092	-0.29 to 0.11	
	40	-0.068	-0.23 to 0.10	

β_1: contamination change per week, *95 % CR:* 95 % confidence range of β_1, $\beta_1 \times max(t_T) > 0.3\ \sigma_p$: $\beta_1 \times$ maximum possible exposure time to respective temperature is larger than *0.3* × target standard deviation

Table 2- 2: Results of the stability study for test material HO

Analyte	Temperature [°C]	β_1 [ng/g week]	*95 % CR* [ng/g week]	$\beta_1 \times max(t_T) > 0.3\ \sigma_p$
Echimidine	4	-0.67	-1.2 to -0.16	NO
	40	-0.47	-1.0 to 0.11	
Integerrimine	4	-0.27	-0.64 to 0.086	
	40	0.052	-0.34 to 0.45	
Intermedine	4	-0.36	-0.69 to -0.028	NO
	40	0.015	-0.29 to 0.32	
Senecionine	4	-0.11	-0.29 to 0.064	
	40	-0.10	-0.35 to 0.15	
Seneciphylline	4	-0.010	-0.14 to -0.060	NO
	40	-1.2	-1.6 to -0.80	NO
Senkirkine	4	-0.026	-0.33 to 0.27	
	40	0.073	-0.38 to 0.53	

β_1: contamination change per week, *95 % CR:* 95 % confidence range of β_1, $\beta_1 \times max(t_T) > 0.3\ \sigma_p$: $\beta_1 \times$ maximum possible exposure time to respective temperature is larger than *0.3* × target standard deviation

Table 2- 2 lists the result for the test material HO. Here for Echimidine and Intermedine a statistically significant instability was detected at 4 °C storage but not at 40 °C. But even incorrect storage at 4 °C over the 7 week study period would not lead to a contamination change which would affect the z-score. For Seneciphylline instabilities at both tested temperatures were detected. Assuming a 2-day exposure to 40 °C during transportation and 7 weeks of storage at 4 °C an analyte change of 0.41 ng/g would result. The critical value 0.3* σ_p is 0.89 ng/g. All of this is indicated in the right-most column.

Annex 5: Determination of the assigned values and their uncertainties

This was done with gravimetric standard addition using an internal standard (ISTD) acc. to Hauswaldt et al. [4]. In brief, an amount of a mixed PAs solution was gravimetrically added to an aliquot of the test material. The mixed PAs solution was prepared from individual PA reference materials of which the purity was verified using HPLC, TGA-GC-MS, and qNMR. For the HT test material approx. 15 or 77 mg were added in duplicate to four aliquots. For the HO test material approx. 27, 56, or 80 mg were added to six aliquots in duplicate. Since the HO test material contained Seneciphylline-NO approx. 37 or 74 mg of a Seneciphylline-NO reference solution were added in duplicate to another four aliquots. For each test material two aliquots were prepared without addition of PAs. To all HT aliquots a constant amount of approx. 157 mg of an ISTD solution (Heliotrine) was added. To all HO aliquots approx. 192 mg ISTD were added. All additions were performed with a calibrated analytical balance with a readability of d = 0.01 mg.

Extraction and clean-up were performed the day after the additions to give the added reference materials sufficient time to equilibrate. To this end, H_2O/formic acid (95/5, v/v) and zinc were added to the aliquots. This created a reductive environment to reduce the N-oxides to the respective amines. After 60 min the zinc and other particulate matter were pelleted through centrifugation. The supernatant was adjusted to approx. pH 10 with conc. ammonia and an aliquot was cleaned-up with SPE. The dried eluate was reconstituted with H_2O/formic acid (999/1, v/v) and injected into a LC-HRMS. Almost baseline separation was achieved for the critical groups: intermedine/lycopsamine and senecivernine/integerrimine/Senecionine (see Figure 5- 1). The reductive extraction was chosen to reduce the complexity of the separation and to limit the number of reference materials necessary for this task.

Iteratively weighted least square regression was used to determine the intercept β_0 and the slope β_1 of the linear model:

$$R' \frac{m_y}{m_x} = \beta_0 + \beta_1 \frac{m_z}{m_x}$$

with
R' – ratio of peak area of analyte divided by peak area of ISTD;
m_y – mass of the ISTD solution;
m_x – mass of the test material aliquot;
m_z – mass of the mixed PAs solution.

With β_0 and β_1 known the mass fraction of the analyte w_x can be calculated:

$$w_x = \frac{\beta_0}{\beta_1} w_z$$

with w_z the mass fraction of the analyte in the mixed PAs solution.

Following the law of error propagation the uncertainty of w_x is

$$u(w_x) = \sqrt{\left(\frac{w_x}{w_z}\right)^2 u^2(w_z) - 2\frac{w_z w_x}{\beta_1^2} u(\beta_0, \beta_1) - \left(\frac{w_x}{\beta_1}\right)^2 u^2(\beta_1) + \left(\frac{w_z}{\beta_1}\right)^2 u^2(\beta_0)}$$

with

$u^2(w_z)$ – squared standard uncertainty of the analyte mass fraction in the mixed PAs solution;

$u(\beta_0, \beta_1)$ – co-variance of the intercept β_0 and the slope β_1 from the regression;

$u^2(\beta_1)$ – squared standard error of the slope β_1 from the regression;

$u^2(\beta_0)$ - squared standard error of the intercept β_0 from the regression.

Acc. to ISO 13528:2015 [3] the assigned value for an analyte in a test material is calculated as

$$x_{PT} = w_x + \delta_{homo} + \delta_{stab} + \delta_{trans}$$

with

x_{PT} – the assigned value;

w_x – the reference value determined through standard addition;

δ_{homo} – error due to possible inhomogeneity (assumed as zero);

δ_{stab} – error due to possible instability during the proficiency test period (assumed as zero);

δ_{trans} – error due to possible instability under transport conditions.

Since those errors were negligible they were included into the respective standard uncertainties. The associated combined uncertainty is calculated as

$$u(x_{PT}) = \sqrt{u^2(w_x) + u^2(homo) + u^2(stab) + u^2(trans)}$$

with

$u(x_{PT})$ – combined uncertainty of the assigned value;

$u^2(w_x)$ – squared uncertainty of the mass fraction of the analyte;

$u^2(homo)$ – squared uncertainty of the homogeneity determination incl. possible error;

$u^2(stab)$ - squared uncertainty of the stability determination incl. possible error;

$u^2(trans)$ - squared uncertainty due to transport conditions incl. possible error.

For all analytes $u(stab)$ was calculated as

$$u(stab) = \frac{RSD_y}{\sum(x_i - \overline{x})} \times 7 \times w_x$$

with

RSD_y – relative standard deviation of all mass fractions determined during the stability study for a given analyte;

x_i – individual time points;

$\overline{x}$ - average of all time points;

w_x – the reference value determined through standard addition;

the factor 7 represents the duration of the PT scheme in weeks.

For analytes for which a significant instability was detected the values for δ_{trans} and $u^2(trans)$ were calculated from the slope β_1 and its uncertainty u(β_1), respectively, by multiplying with 2/7 (transportation time of 2 days expressed in weeks). The main contributors to the uncertainty budgets of the assigned values were the uncertainties due to the determination of β_0 and β_1. Table 3- 1 lists the respective values for test material HT, and Table 3- 2 does the same for test material HO.

Table 3- 1: The assigned values and their uncertainties for the test material HT

Analyte	x_{PT} **[ng/g]**	***u*(w_x) [ng/g]**	***u(homo)* [ng/g]**	***u(stab)* [ng/g]**	***u(trans)* [ng/g]**	***u*(x_{PT}) [ng/g]**
Integerrimine	30.6	2.8	0.11	0.14	0	2.8
Retrorsine	19.9	1.5	0	0.099	0.11	1.5
Senecionine	80.4	7.8	0.24	0.36	0	7.8
Senecivernine	5.7	1.2	0.048	0.037	0	1.2

Table 3- 2: The assigned values and their uncertainties for the test material HO

Analyte	x_{PT} **[ng/g]**	***u*(w_x) [ng/g]**	***u(homo)* [ng/g]**	***u(stab)* [ng/g]**	***u(trans)* [ng/g]**	***u*(x_{PT}) [ng/g]**
Echimidine	71.1	6.3	0	0.099	0.31	6.3
Integerrimine	54.4	3.8	0	0.076	0	3.8
Intermedine	44.1	4.4	0.022	0.088	0.16	4.4
Senecionine	29.1	1.8	0.032	0.036	0	1.8
Seneciphylline	13.4	1.0	0.021	0.29	0.51	1.2
Senkirkine	62.1	9.6	0.15	0.086	0	9.6

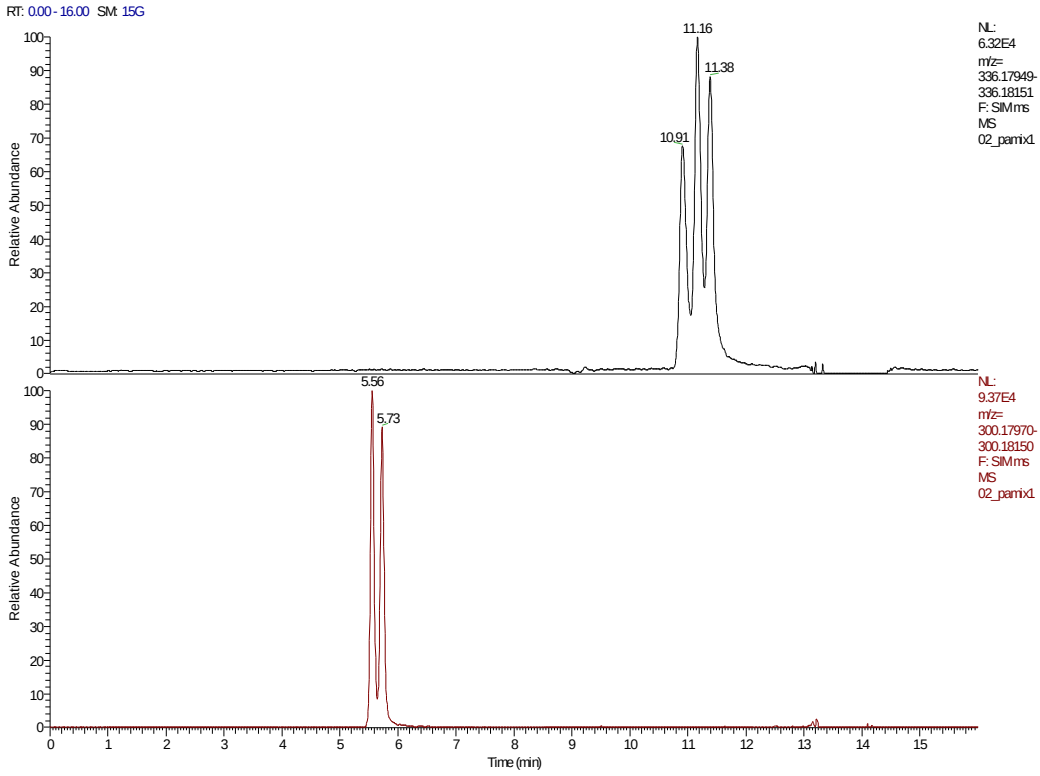


Figure 5- 1 Extracted ion chromatograms of a PA mixed reference solution; the top panel shows the separation of Senecivernine / Integerrimine / Senecionine (m/z 336.1805±3 ppm); the bottom panel shows the separation of Intermedine / Lycopsamine (m/z 300.1805±3 ppm); Indicine coelutes with Lycopsamine under these conditions; separation was afforded by a Phenomenex Kinetex C_{18} 100x2.1mm, 1.7 µm particle size.

Annex 6: Graphs

Test item HT

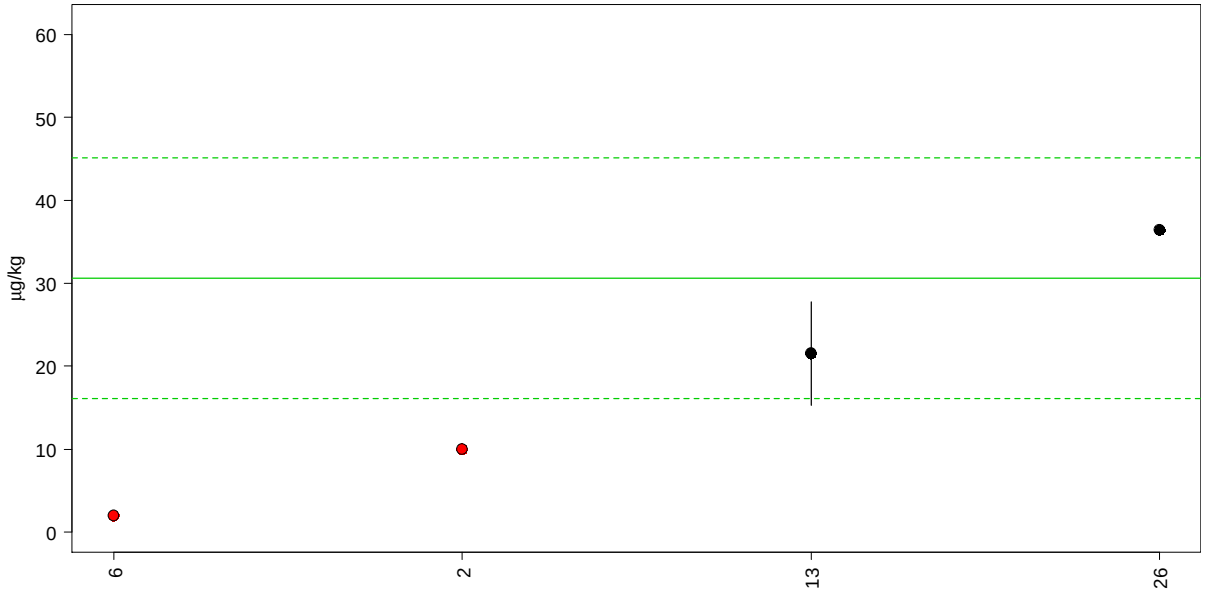


Figure 6- 1 Plot of results for Integerrimine from smallest to largest; black circles – reported results, red circles – reported "smaller than", black vertical line – reported measurement uncertainty, green solid line – assigned value, green broken line – two times adjusted target standard deviation σ'_{PT}

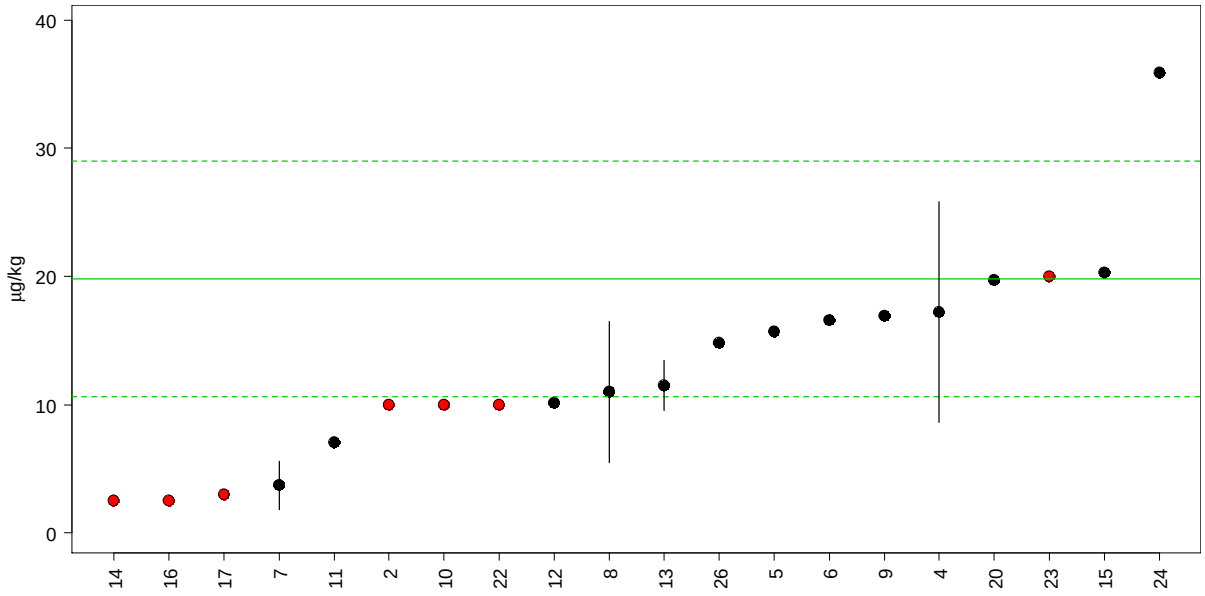


Figure 6- 2 Plot of results for Retrorsine from smallest to largest; black circles – reported results, red circles – reported "smaller than", black vertical line – reported measurement uncertainty, green solid line – assigned value, green broken line – two times adjusted target standard deviation σ'_{PT}

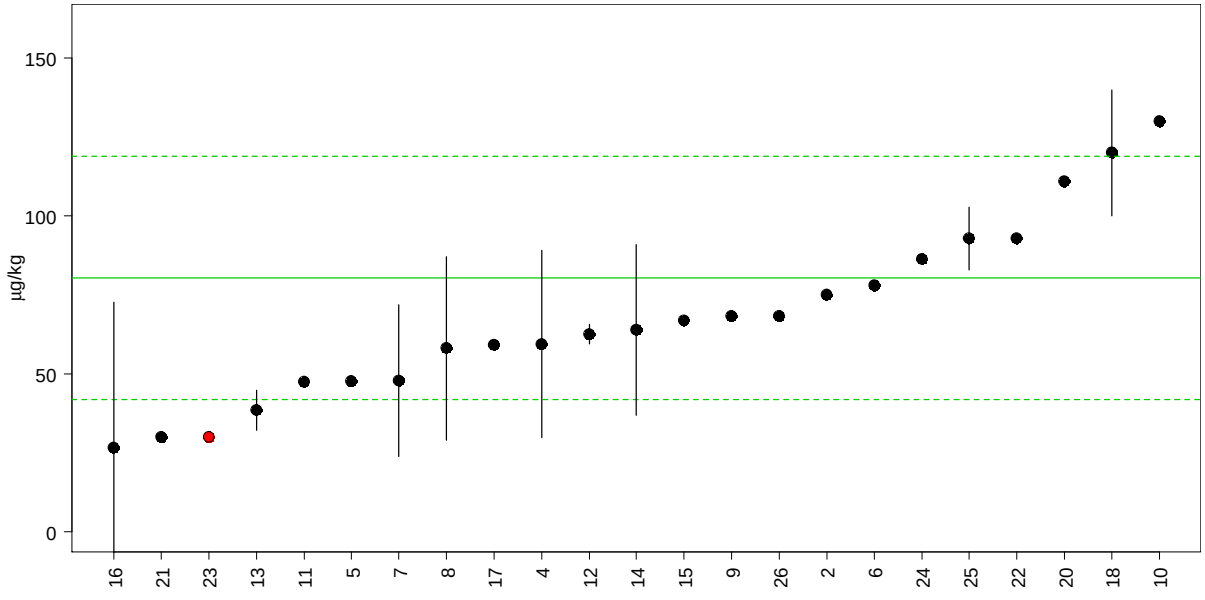


Figure 6- 3 Plot of results for Senecionine from smallest to largest; black circles – reported results, red circles – reported "smaller than", black vertical line – reported measurement uncertainty, green solid line – assigned value, green broken line – two times adjusted target standard deviation σ'_{PT}

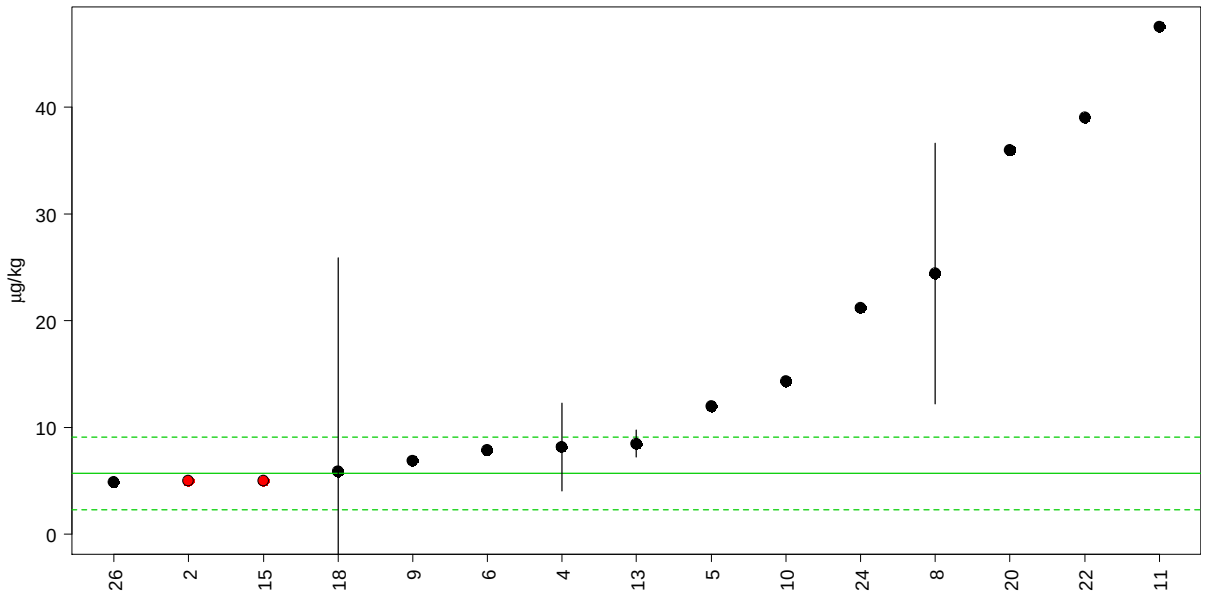


Figure 6- 4 Plot of results for Senecivernine from smallest to largest; black circles – reported results, red circles – reported "smaller than", black vertical line – reported measurement uncertainty, green solid line – assigned value, green broken line – two times adjusted target standard deviation σ'_{PT}

Test item HO

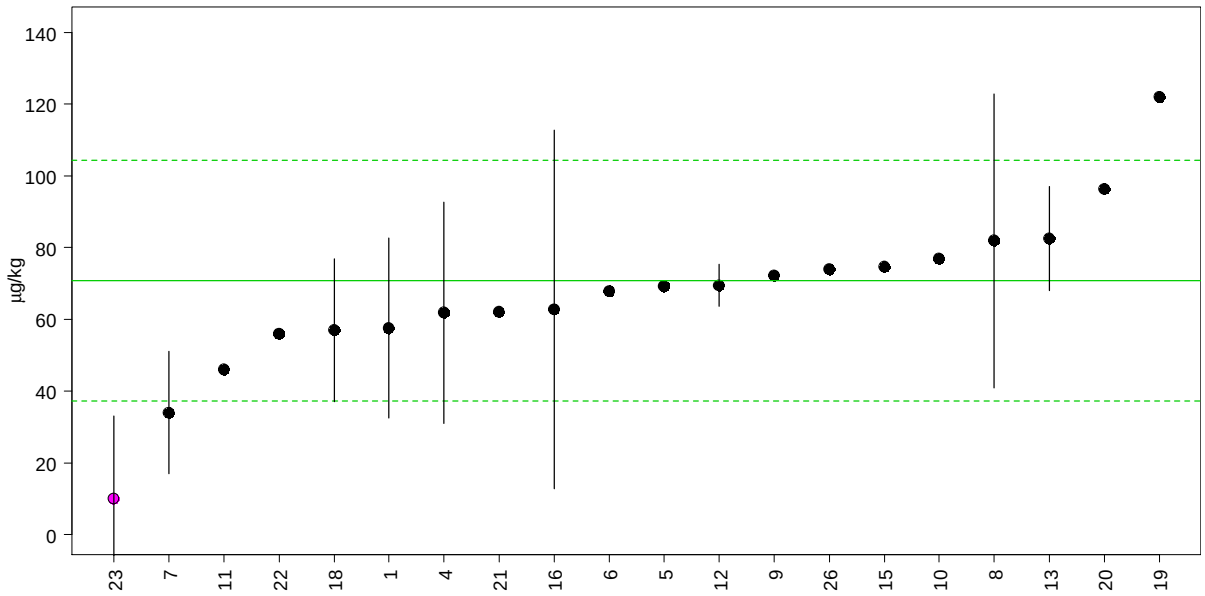


Figure 6- 5 Plot of results for Echimidine from smallest to largest; black circles – reported results, magenta colored circles – reported "larger than", black vertical line – reported measurement uncertainty, green solid line – assigned value, green broken line – two times adjusted target standard deviation σ'_{pT}

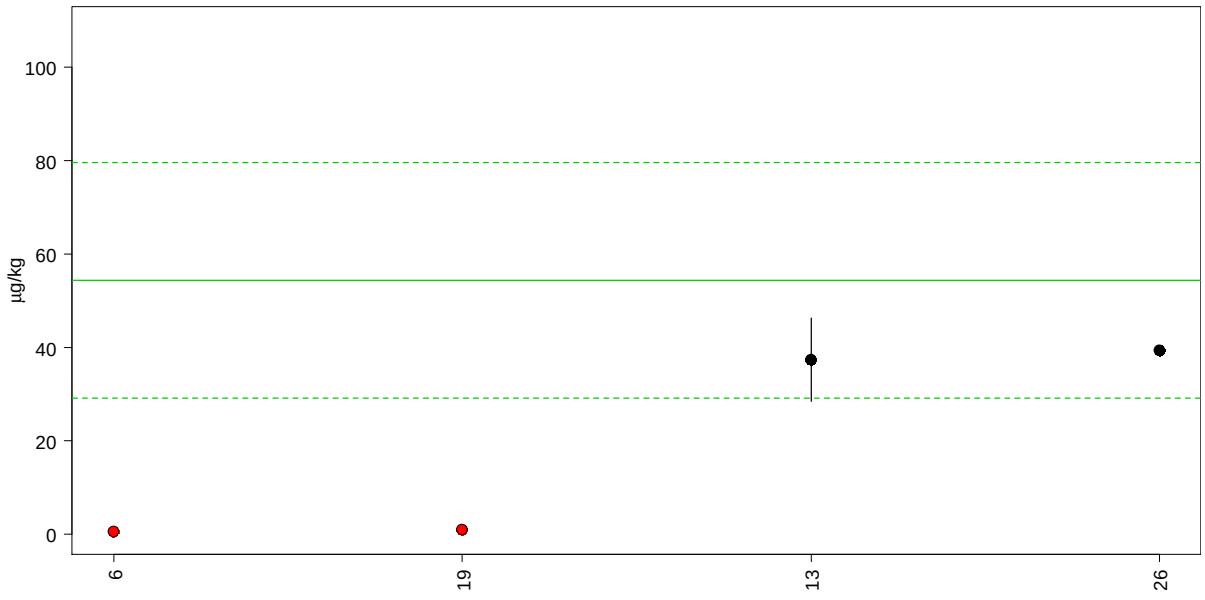


Figure 6- 6 Plot of results for Integerrimine from smallest to largest; black circles – reported results, red circles – reported "smaller than", black vertical line – reported measurement uncertainty, green solid line – assigned value, green broken line – two times adjusted target standard deviation σ'_{pT}

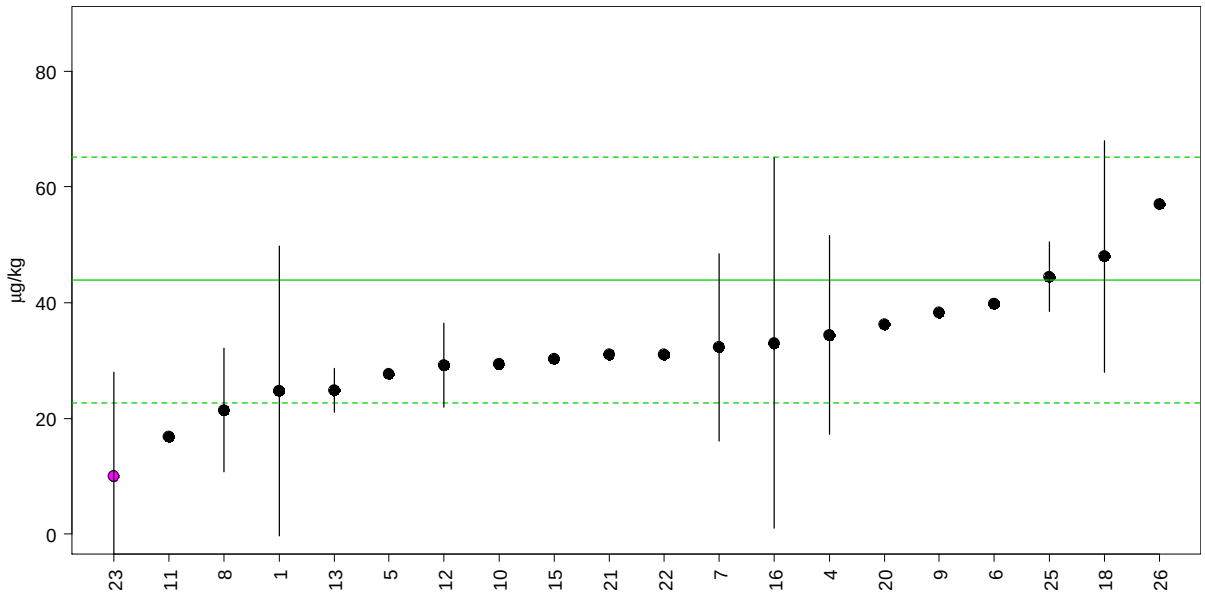


Figure 6- 7 Plot of results for Intermedine from smallest to largest; black circles – reported results, magenta colored circles – reported "larger than", black vertical line – reported measurement uncertainty, green solid line – assigned value, green broken line – two times adjusted target standard deviation σ'_{pT}

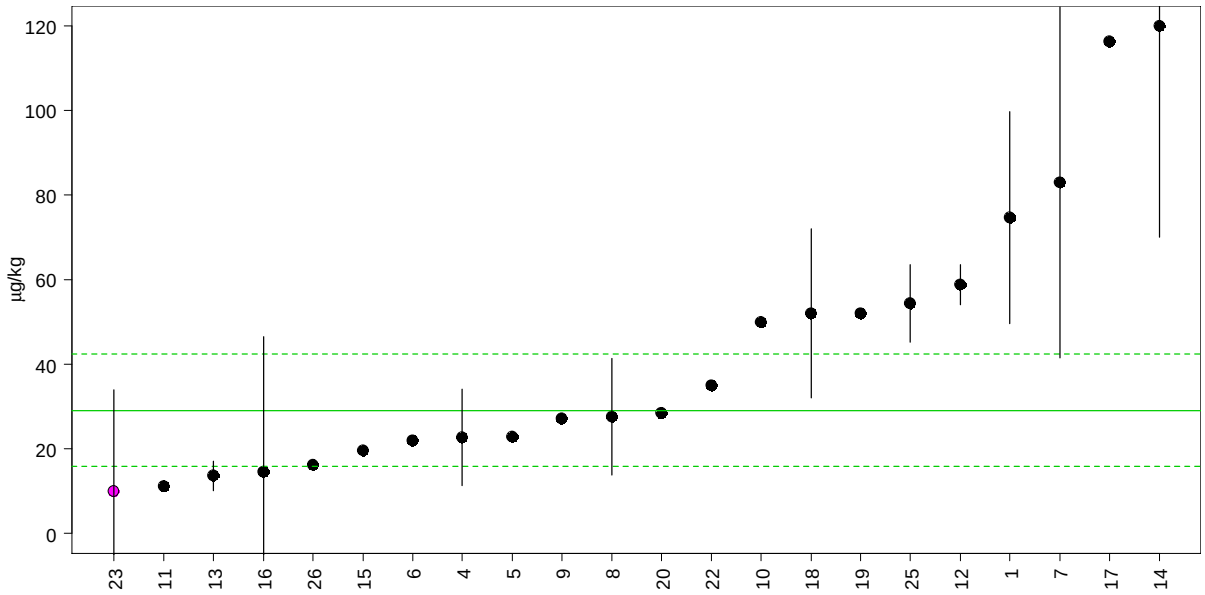


Figure 6- 8 Plot of results for Senecionine from smallest to largest; black circles – reported results, magenta colored circles – reported "larger than", black vertical line – reported measurement uncertainty, green solid line – assigned value, green broken line – two times adjusted target standard deviation σ'_{pT}

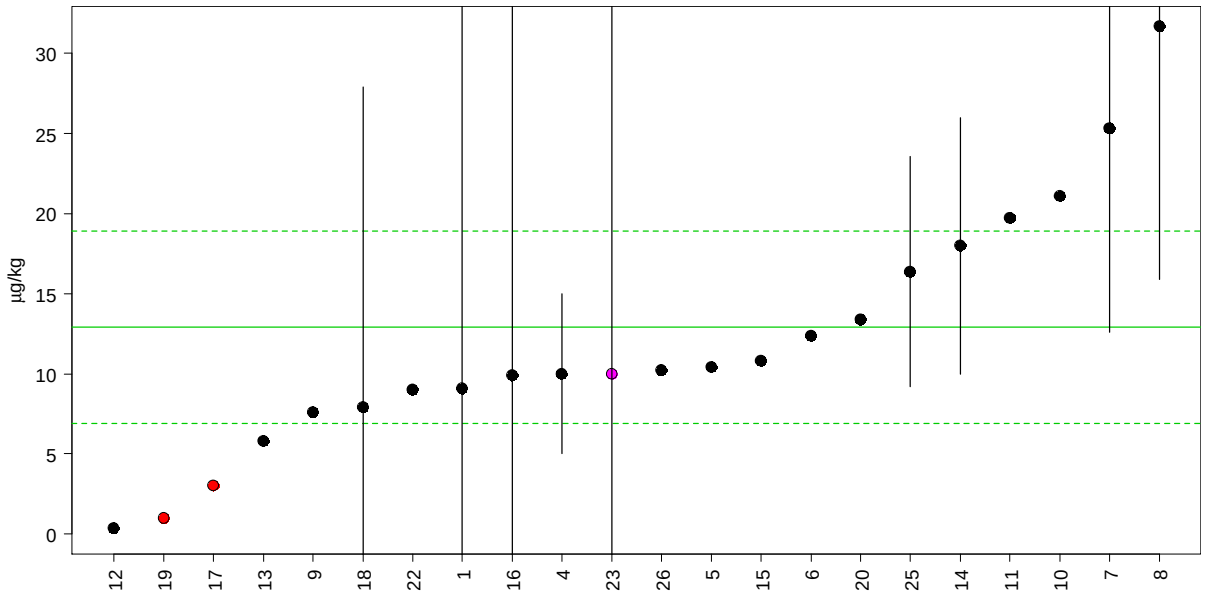


Figure 6- 9 Plot of results for Seneciphylline from smallest to largest; black circles – reported results, magenta colored circles – reported "larger than", red circles – reported "smaller than", black vertical line – reported measurement uncertainty, green solid line – assigned value, green broken line – two times adjusted target standard deviation σ'_{pT}

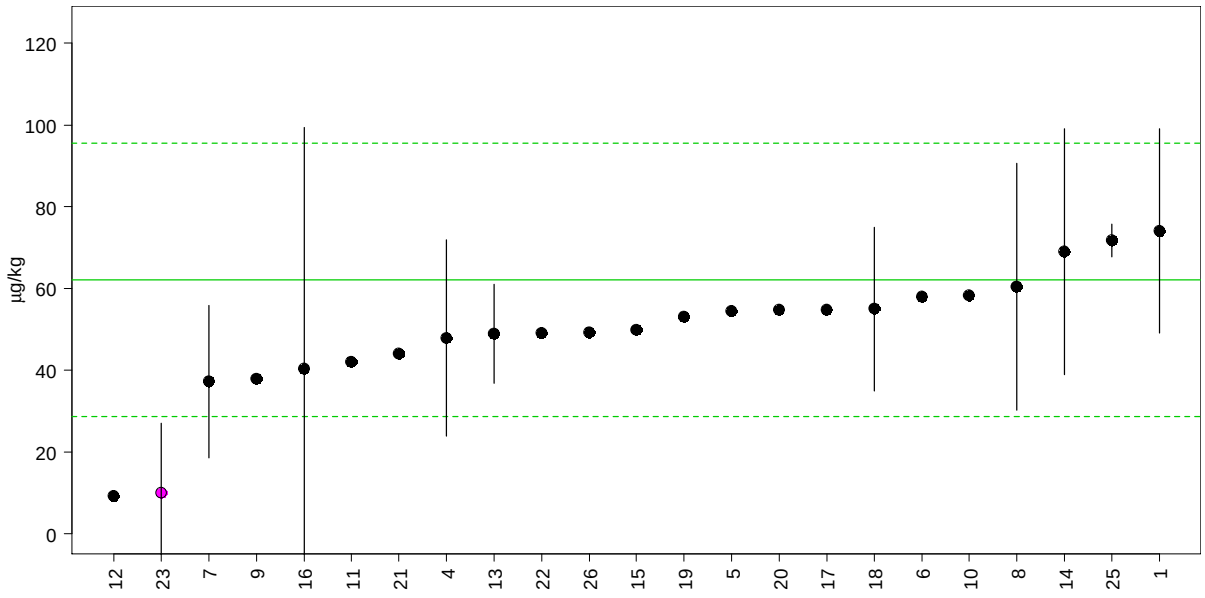


Figure 6- 10 Plot of results for Senkirkine from smallest to largest; black circles – reported results, magenta colored circles – reported "larger than", black vertical line – reported measurement uncertainty, green solid line – assigned value, green broken line – two times adjusted target standard deviation σ'_{pT}

Questionnaire data

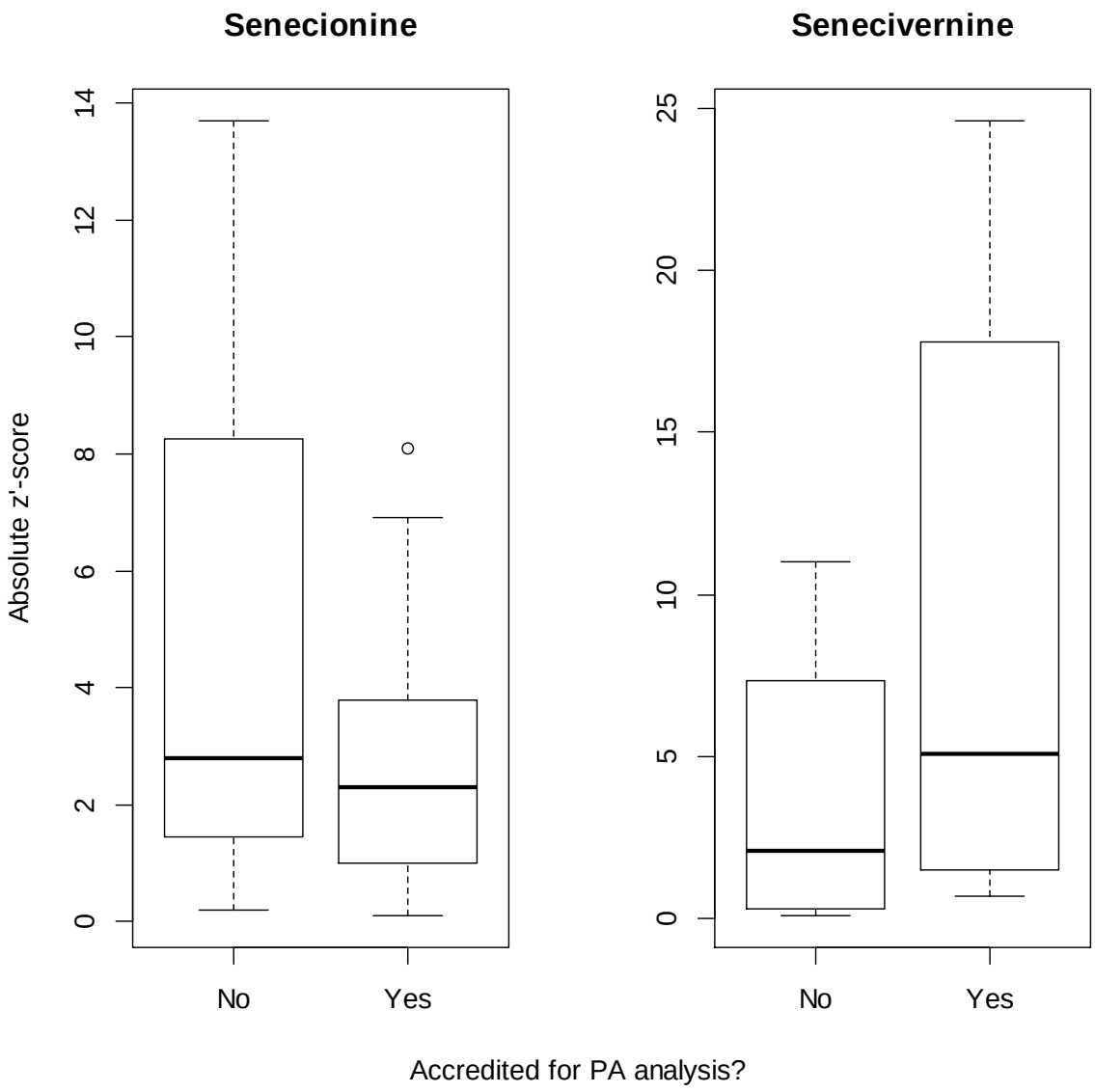


Figure 6- 11 Box & whisker plots of absolute z'-scores of Senecionine results in HO or Senecivernine results in HT grouped by whether an accreditation for PA analysis was held by the laboratories; bold horizontal line: median value, boxes extend from the first to the third quantile of the data.

Annex 7: Questionnaire data

Table 7- 1 Answers to questionnaire about experience and accreditation

Lab Code	Previous experience	ISO 17025 accredited	Scope of accreditation	Lab Code	Previous experience	ISO 17025 accredited	Scope of accreditation
1	>12 months	Yes	DAkkS	**14**	>12 months	No	
2	>12 months	No		**15**	>12 months	Yes	Honey, herbs and tea, grains
3	>12 months	No		**16**	>12 months	No	
4	>12 months	Yes	tea, honey	**17**	3-12 months	No	
5	0-3 months	No		**18**	0-3 months	No	
6	>12 months	Yes	flexible scope, determination of contaminants, plant toxins and mycotoxins by LC-MS/MS	**19**	>12 months	No	
7	>12 months	Yes	Food	**20**	>12 months	Yes	DAkkS Number D-PL-XXXXX-01-00, flexible
8	>12 months	No		**21**	>12 months	Yes	See on the DAKKS website under our accreditation number: D-PL-XXXXX-01-00
9	>12 months	Yes	HPLC-MS/MS	**22**	>12 months	Yes	Bee Products, Honey, Plant materials, tea and herbal tea, spices, feed
10	>12 months	Yes	food (tea, herbal tea, spices)	**23**	>12 months	No	
11	>12 months	Yes		**24**	3-12 months	Yes	D-PL-XXXXX-01-00
12	>12 months	Yes	Tea, herbal Tea, Honey	**25**	>12 months	Yes	HONEY
13	>12 months	Yes	flexible accreditation	**26**	>12 months	No	

Table 7- 2 Answers to questionnaire about treatment of test item HO (Labs 1-13)

Lab Code	HO test portion size [g]	HO extractant	HO extractant simplified	HO extractant vol. [mL]	HO extraction time [min]	HO cleaned up?	HO Clean-up type	HO calibration mode
1	5	water/acetonitrile	aqu/org	20	30	Yes	QuEChERs MIX I: MgSO4, NaCl, Na2H Citrate, Na3 Citrate; QuEChERS MIX III: Diamino, MgSO4	
2	0	N/A				No		N/A
3	0.2	0.05 mol/l H2SO4	aqu/acidic	5	720	Yes	SCX-SPE	isotope dilution
4	2	0.05 mol/l H2SO4	aqu/acidic	10	15	Yes	SPE	matrix-matched external STD
5	1.5	H2SO4 0,1M and ACN	aqu/org/acidic	10	30	Yes	QUECHERS	neat solvent external STD
6	5	0.05 mol/l H2SO4	aqu/acidic	30	10	Yes	HR-X (Macherey Nagel, C18)	matrix-matched external STD
7	10	0.05 mol/l H2SO4	aqu/acidic	30	30	Yes	SPE with HF Bond Elut LRC-SCX	matrix-matched external STD
8	10	0.05 mol/l H2SO4	aqu/acidic	30	30	Yes	SPE cartridge SCX 500 mg	matrix-matched external STD
9	2	Water, H2SO4	aqu/acidic	40	20	Yes	filter	neat solvent external STD
10	5	0.05 mol/l H2SO4	aqu/acidic	50	15	Yes	SPE with Chromabond HR-X	standard addition
11	2	2 % formic acid	aqu/acidic	40	30	Yes	centrifugated, filtered, SPE clean-up	matrix-matched external STD
12	10	0.05 mol/l H2SO4	aqu/acidic	30	30	Yes	Cation exchange (HF Bond Elut LRC-SCX, 500 mg)	matrix-matched external STD
13	5	acetonitrile (adjusted to alkaline pH w/ ammonia solution)	aqu/org/basic	10	10	No		matrix-matched external STD

Table 7- 3 Answers to questionnaire about treatment of test item HO (Labs 14-26)

Lab Code	HO test portion size [g]	HO extractant	HO extractant simplified	HO extractant vol. [mL]	HO extraction time [min]	HO cleaned up?	HO Clean-up type	HO calibration mode
14	5	acetonitrile:water, 1:1 (QuEChERS-like method)	aqu/org	20	2	No		matrix-matched external STD
15	0.5	Acetonitrile : Methanol : Water 1:1:1 (v:v:v)	aqu/org	10	15	No		neat solvent external STD / standard addition
16	5	20 g NaCl in 800 ml H2O, making up to 1 L with HCl 37%	aqu/acidic	20	60	No		neat solvent external STD
17	10	0.05 mol/l H2SO4	aqu/acidic	30	30	Yes	MCX SPE_Waters	Prespiked matrix matched calibration
18	1	0.1 n HCl in Water	aqu/acidic	20	60	Yes	NH2	matrix-matched external STD
19	4	Hydrochloricacid and Acetonitrile	aqu/org/acidic	8	10	Yes	MgSO4 and PSA	matrix-matched external STD
20	1	aqueous citric buffer solution	aqu/acidic	25	15	Yes	Solid phase extraction	matrix-matched external STD
21	10	no further information		0	0	Yes	no further information	standard addition
22	1	Acetonitrile	org	4	6	Yes	liquid liquid extraction	standard addition
23	10	0.05 mol/l H2SO4	aqu/acidic	30	30	Yes	SPE Bond Elut LRC SCX 500mg	matrix-matched external STD
24	0	not		0	0	No		
25	20	0.05 mol/l H2SO4	aqu/acidic	200	30	Yes	SCX SPE CARTRIDGE	standard addition
26	2	0.2% formic acid in water	aqu/acidic	20	30	Yes	Solid Phase Extraction on Phenomenex Strata X 200 mg/6 ml	standard addition

Table 7- 4 Answers to questionnaire about treatment of test item HT (Labs 1-13)

Lab Code	HT test portion size [g]	HT extractant	HT extractant simplified	HT extractant vol. [mL]	HT extraction time [min]	HT cleaned up?	HT Clean-up type	HT calibration mode
1	0	not analyzed		0	0	No		N/A
2	2	acetic acid and methanol	aqu/org/acidic	20	23	No		standard addition
3	0.1	0.05 mol/l H2SO4	aqu/acidic	5	720	Yes	SCX-SPE	isotope dilution
4	10	0.05 mol/l H2SO4	aqu/acidic	200	15	Yes	SPE	standard addition
5	1	0.05 mol/l H2SO4	aqu/acidic	25	30	Yes	SPE MCX	neat solvent external STD
6	2	0.05 mol/l H2SO4	aqu/acidic	40	30	Yes	HR-X (Macherey Nagel, C18)	standard addition
7	2	0.05 mol/l H2SO4	aqu/acidic	40	30	Yes	SPE with DSC-C18 SPE (Supelco)	neat solvent external STD
8	2	0.05 mol/l H2SO4	aqu/acidic	40	30	Yes	SPE DSC C18	standard addition
9	2	Water, H2SO4	aqu/acidic	40	20	Yes	filter	neat solvent external STD
10	2.5	0.05 mol/l H2SO4	aqu/acidic	50	30	Yes	SPE with Chromabond HR-X	standard addition
11	2	2 % formic acid	aqu/acidic	40	30	Yes	centrifugated, filtered, SPE clean-up	matrix-matched external STD
12	2	0.05 mol/l H2SO4	aqu/acidic	20	15	Yes	C18-SPE (Discovery DSC18, 6 ml, 500 mg)	neat solvent external STD
13	2	0.05 mol/l H2SO4	aqu/acidic	40	15	Yes	SPE (C18-ec)	matrix-matched external STD

Table 7- 5 Answers to questionnaire about treatment of test item HT (Labs 14-26)

Lab Code	HT test portion size [g]	HT extractant	HT extractant simplified	HT extractant vol. [mL]	HT extraction time [min]	HT cleaned up?	HT Clean-up type	HT calibration mode
14	2	acetonitrile:water, 1:1 (QuEChERS-like method)	aqu/org	20	30	No		matrix-matched external STD
15	0.5	Acetonitrile : Methanol : Water 1:1:1 (v:v:v)	aqu/org	10	15	No		neat solvent external STD / standard addition
16	2.5	20 g NaCl in 800 ml H2O, making up to 1 L with HCl 37%	aqu/acidic	25	60	No		neat solvent external STD
17	2	0.05 mol/l H2SO4	aqu/acidic	50	30	Yes	MCX SPE waters	Prespiked matrix matched calibration
18	0.5	0.1 n HCl in Water	aqu/acidic	20	60	Yes	GCB	matrix-matched external STD
19	4	Hydrochloricacid and Acetonitrile	aqu/org/acidic	8	10	No		NA
20	1	aqueous citric buffer solution	aqu/acidic	25	15	Yes	Solid phase extraction	matrix-matched external STD
21	1	no further information		0	0	Yes	no further information	standard addition
22	2	no further information		0	0	Yes	no further information	standard addition
23	2	0.05 mol/l H2SO4	aqu/acidic	40	30	Yes	SPE DSC-C18 (Supelco) 500mg	matrix-matched external STD
24	2	Water+sulfuric acid	aqu/acidic	20	15	Yes	SPE	
25	1	0.2% formic acid in water	aqu/acidic	40	30	Yes	Solid Phase Extraction on Phenomenex Strata X 200 mg/6 ml	standard addition
26	2	0.2% formic acid in water	aqu/acidic	40	30	Yes	Solid Phase Extraction on Phenomenex Strata X 200 mg/6 ml	standard addition

Table 7- 6 Answers to questionnaire about separation conditions (Labs 1-13)

Lab Code	Separation method	Sep. meth. other	Analytical column	Mobile phase	Flow rate [ml/min]
1	Liquid chromatography		C18, 50 mm, 2,0 mm, 2µm (YMC Ultra Hydrosphere 50*2,0mm; (Partikel 2µm) (Poren 12mm)	eluent A: distilled water (1 %CH3COOH, 0,05 % 10 M NH4(HCOO)) ; eluent B: methanol (1 %CH3COOH, 0,05 % 10 M NH4(HCOO))	0.2
2	Liquid chromatography		Thermo Hypersil Gold C18; 150x2,1mm	water and methanol	0.3
3	Liquid chromatography		Synergi Max-RP 4um, 150 x 2 mm	H2O/ACN Gradient, each phase containing 0.3 % formic acid	0.3
4	Liquid chromatography		C18, 150 x 2.0 mm	water/acetonitrile with ammonium formiate and formic acid	0.3
5	Liquid chromatography		Luna C8 150 x 2 mm 3 ?m	ammonio formiate in H2O and ammonio formiate in MeOH	0.25
6	Liquid chromatography		C18, 100mm, 2,1mm, 1,8 ?m (Eclipse Plus)	A: Methanol+0,1% FA B: Wasser+0,1% FA	0.3
7	Liquid chromatography		Kinetex C18, 100 mm, 2.1 mm, 2,6 µm	5 mM NH4COO and 0.1% COOH in water/ 5 mM NH4COO and 0.1% COOH in methanol	0.2
8	Liquid chromatography		C18, 200 mm, 2.1 mm, 1.9 µm, Hypersil Gold	water / Methanol with HCOOH 0.1% and ammonium formiate 315 mg/l	0.25
9	Liquid chromatography		N/A	N/A	0.4
10	Liquid chromatography		Acquity UPLC BEH C18 1,7 µm, 2,1 x 100 mm	ammonium formiate + formic acid in water / methanol	0.2
11	Liquid chromatography		Waters, ACQUITY UPLC BEH C18 150 x 2,1 mm 1,7 µm	0,1 % formic acid in water; 0,1 % formic acid in acetonitrile	0.2
12	Liquid chromatography		RP18 (Kinetex 2.6 µ EVO C18 100A, 150 * 2.1 mm)	Gradient H2O and MeOH (both 5 mM NH4HCO2 and 0.1% CH2O2)	0.35
13	Liquid chromatography		Waters BEH C18 (1.7 µm, 100 mm, 2.1 mm ID)	A: 0.005 M ammonium formate / formic acid in water (pH 3); B: A: 0.005 M ammonium formate / formic acid in methanol (pH 3)	0.35

Table 7- 7 Answers to questionnaire about separation conditions (Labs 14-26)

Lab Code	Separation method	Sep. meth. other	Analytical column	Mobile phase	Flow rate [ml/min]
14	Liquid chromatography		silica-based reversed-phase (C18) analytical column Acquity UPLC HSS T3 (lenght 100 mm, 2.1 mm inner diameter, 1.8 um particle size)	5mM ammonium formate and 0.2% formic acid both in methanol (A) and milli-Q water (B)	0.3
15	Liquid chromatography		Waters Acquity UPLC BEH C18 1.7µm 2.1 x 150 mm	water : methanol + ammoniumformiat and formic acid	0.3
16	Liquid chromatography		Phenomenex Kinetex 2.6 ?m XB-C18 column (100 × 4.6 mm)	MPA: H2O + 5 mM ammonium acetate + 0.05% acetic acid; MPB: acetonitrile	1.5
17	Other	LCMS-MS	C18,100mm,2.1um,3um	5mM ammonium acetate ,0.1% formic acid in MeOH/water	0.4
18	Liquid chromatography		Agilent InfinityLab Poroshell 120 EC-C18,2.1 × 100 mm, 2.7 µm (p/n 695775-902) with HPLC, Guard (p/n 821725-911) at 25 °C	0.025 % formic acid in 5 mM ammonium formate in Water (A), 0.025 % formic acid 5 mM ammonium formate in methanol (B),	0.3
19	Liquid chromatography		150mm*2mm, C18	Methanol/ammonium acetate	0.15
20	Liquid chromatography		Thermo Scientific TM Hypersil Gold C18 150 x 3 mm, 3 µm particle size	A: methanol + 5 mmol ammonium formate + 1% formic acid; B:water + 5 mmol ammonium formate + 1% formic acid	0.4
21	Liquid chromatography	no further informa-tion	no further information	no further information	0
22	Liquid chromatography		C18, 150mm, 2.1mm, 5µm	formic acid in water / formic acid in methanol	0.3
23	Liquid chromatography		Acclaim Vanquish PA2, 150 x 2.1 mm, 2.2 um particle size	A: Ammonium formate (315mg/L): formic acid (1ml/L) in water; B: Ammonium formate (315mg/L): formic acid (1ml/L) in methanol	0.3
24	Other	LC MS/MS	Hypersil gold 150 x 2,1mm; 175A, 3µm	Eluent A: Water Eluent B: Methanol	0.3
25	Liquid chromatography		C18, 150MM, 2.1MM,1.9UM	A:5mM AMMONIUM FORMATE, 0.1% FORMIC ACID IN METHANOL; B: 5mM AMMONIUM FORMATE, 0.1% FORMIC ACID IN ULTRAURE WATER	0.3

26	Liquid chromatography		Waters BEH C18, 150 x 2.1 mm, 1.7 um	A: 10 mM ammonium carbonate in water (pH 9.0), B: acetonitrile	0.4

Table 7- 8 Answers to questionnaire about detection conditions (Labs 1-13)

Lab Code	Detector	Ioniza-tion mode	Ionization mode other	MS mode	PAs determined as	PANOs determined as	Other	Deriva-tization?	Which deriv.
1	Mass spectrometry	ESI		MRM	Individual PAs	measured as such		No	
2	Mass spectrometry	ESI		SRM	Individual PAs	measured as such		No	
3	Mass spectrometry	ESI		MRM	Sum after conversion of PAs to common analyte	reduced to the amine before separation		Yes	Phthalic acid anhydrid
4	Mass spectrometry	ESI		SRM	Individual PAs	measured as such		No	
5	Mass spectrometry	ESI		SRM	Individual PAs	measured as such		No	
6	Mass spectrometry	ESI		MRM	Individual PAs	measured as such		No	
7	Mass spectrometry	ESI		sMRM	Individual PAs	measured as such		No	
8	Mass spectrometry	ESI		MRM	Individual PAs	measured as such		No	
9	Mass spectrometry	ESI			Individual PAs	measured as such		No	
10	Mass spectrometry	ESI		MRM	Individual PAs	measured as such		No	
11	Mass spectrometry	ESI		MRM	Individual PAs	measured as such		No	
12	Mass spectrometry	ESI		MRM	Individual PAs	measured as such		No	
13	Mass spectrometry	ESI		dynamic MRM	Individual PAs	measured as such		No	

Table 7- 9 Answers to questionnaire about detection conditions (Labs 14-26)

Lab Code	Detector	Ioniza-tion mode	Ionization mode other	MS mode	PAs determined as	PANOs determined as	Other	Deriva-tization?	Which deriv.
14	Mass spectrometry	ESI	n	HRMS/MS	Individual PAs	measured as such		No	
15	Mass spectrometry	ESI	n	MRM	Individual PAs	measured as such		No	
16	Mass spectrometry	ESI	n	SRM	Individual PAs	measured as such		No	
17	Mass spectrometry	ESI	n	MRM	Individual PAs	measured as such		No	
18	Mass spectrometry	ESI	n	SRM	Individual PAs	measured as such		No	
19	Mass spectrometry	Other	EI	SIM	Other	measured as such	some as individual, some as sum of several PA (eg. Lycopsamin/Indicin/Intermedin)	No	
20	Mass spectrometry	ESI	n	SIM	Individual PAs	measured as such		No	
21	Mass spectrometry	Other	no further informatio n	no further informatio n	Individual PAs	measured as such		TRUE	
22	Mass spectrometry	ESI	n	SRM	Individual PAs	measured as such		No	
23	Mass spectrometry	ESI	ESI positive mode	SIM	Individual PAs	measured as such		No	
24	Mass spectrometry	ESI	n	SRN	Individual PAs	measured as such		No	
25	Mass spectrometry	ESI	n	MRM	Individual PAs	measured as such		No	
26	Mass spectrometry	ESI	n	MRM	Individual PAs	measured as such		No	

Table 7- 10 Comments from Labs 1-13

Lab Code	Comments
1	
2	
3	
4	
5	I haven't the Indicine and Integerrimine standards, so I could't resolve these PAs from its isomers (Intermedine/Lycopsamine and Senecionine/Senecivernine). The sum of all PAs is the sum of the only PAs with a concentration >LOQ (the other PAs are considered as 0 ppb)
6	Indicine and Integerrimine are not part of our method
7	The Concentration and composition of the honey sample did not match our common samples (Concentration too high and too much N-Oxides)
8	indicine or intermedine are not separated on a C18 column
9	N/A
10	
11	On mass transiotion of Senecionone-NO we recognized a Peak with shifted retention time to Senecionine NO Peak. It also has differing ion rations. The Peak is poparbly a different PA for which we do not ahve any standard. The transiotions are typical for PA. Results of Senecivernine adn Senecionine are reported as the sum of both.
12	N/A
13	Wouldn't it have been wiser to report individual PA results and summarize them subsequently?

Table 7- 11 Comments from Labs 14-26

Lab Code	Comments
14	Under conditions described within the PT, several PAs were difficult to resolve chromatographically and spectrometrically (lycopsamine x indicine x intermedine; senecionine x integerrimine x senecivernine; retrorsine x jacobine); the reported results might be therefore affected by the co-occurrence of these PAs.
15	Low amounts of Senecivernin and the respectiv N-oxide, just below the respectiv LOQs where found in the herbal tea. Reporting as the sum of amine and the respective N-oxide is disadvantageous to accuracy and comparability.
16	The results are reported as sum of PA and its corresponding N-oxide, however, echimidine-N-oxide, intermedine-N-oxide, lycopsamine-N-oxide were not measured. Under our LC conditions there is co-elution of lycopsamine and indicine. HT material: a peak interfering with senecionine-N-oxide prevented proper peak integration, therefore result for this PANO is not reported.
17	
18	LOQs: very strong dependence on matrix components
19	
20	Meaning of "coverage factor" is unclear.
21	Section 3.3., 3.4., 4.3., 4.4., 5.4., 5.5.2 no further information
22	
23	Methods used were the BfR methods for honey and plant material. Tea sample was extracted as 2 x 20ml extrcation solvent for 15 min each extraction.
24	only tea, sum of amine and N-oxide
25	ION RATIO DISTORTION OBSERVED FOR RETRORSINE-N-OXIDE IN TEA SO VALUE FOR RETRORSINE NOT REPORTED. ION RATION DISTORTION WAS ALSO OBSERVED FOR ECHIMIDINE IN HONEY.
26	Quantification was performed by means of standard addition to the sample and using internal standard correction

Annex 8: Participating laboratories

Organisation	Part. key	Firstname	Surname	Country
Agri-Food and Veterinary Authority of Singapore	PLA11131621	Lim	Poh Choo	SINGAPORE
Austrian Agency for Health and Food Safety	CCAK1172028	Christoph	Czerwenka	AUSTRIA
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	GFBS1348628	Franziska	Gaßmann	GERMANY
Chemical and Veterinary Analytical Institute Muensterland-Emscher-Lippe	KOC41110468	Oliver	Keuth	GERMANY
Chemisches und Veterinäruntersuchungsamt Ostwestfalen-Lippe	AEC41082382	Elisabeth	Apel	GERMANY
CVUA Stuttgart	KTC10323649	Thomas	Kapp	GERMANY
Eurofins Dr. Specht Laboratorien GmbH	SEEQ1345165	Edda	Sassen	GERMANY
Fera Science Ltd.	MSFF1153945	Susan	MacDonald	UNITED KINGDOM
FoodQS GmbH	RNF12432073	Nadine	Raum	GERMANY
Gesellschaft für Bioanalytik mbH	JSG11185486	Stefan	Jäger	GERMANY
HEALTH SCIENCES AUTHORITY	LAHF1128167	Angela	Li	SINGAPORE
Intertek Food Services GmbH Bremen	ZHI12913009	Hauke	Zinow	GERMANY
IZSLER	CEIC1078150	ELISABETTA	CAPRAI	ITALY
Landesbetrieb Hessisches Landeslabor (LHL)	KJL13694049	Johannes	Kemme	GERMANY
Landesuntersuchungsamt Rheinland-Pfalz	KSLI1049678	Simone	Kasper	GERMANY
Lower Saxony State Office for Consumer Protection and Food Safety	DKL12243546	Kay	Dietrichkeit	GERMANY
PiCA GmbH	RAPF1342087	Anna	Romanotto	GERMANY
PMA Sindelfingen GmbH	GSP10165902	Stefan	Glöckler	GERMANY
QSI - Quality Services International GmbH & Co.KG	WTQQ1279373	Tobias	Wiezorek	GERMANY
RIKILT Wageningen University & Research	MHRN1018129	Hans	Mol	NETHERLANDS
SCL laboratoire de Strasbourg	GLSV1151252	Ledoux	Gérald	FRANCE
SGS Germany GmbH	MNS12889924	Nicolaus	Mouillard	GERMANY
Teekanne GmbH & Co.KG	BATL1197037	Andreas	Bollert	GERMANY
TU Braunschweig	BTTI1231664	Till	Beuerle	GERMANY
University of Chemistry and Technology Prague	KVUF1247054	Vladimir	Kocourek	CZECH REPUBLIC
Veterinary and Agrochemical Research Centre (CODA-CERVA)	MSVT1247439	Svetlana	Malysheva	BELGIUM

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KJ-NA-28960-EN-N

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Publications Office

doi: 10.2760/219405

ISBN 978-92-79-77167-5