Proficiency test for tropane alkaloids in food and feed

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Distribution list:
- 22 participating laboratories

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Summary

The proficiency test for tropane alkaloids was organized by RIKILT, Wageningen UR in accordance with ISO 17043. The primary goal of this study was to provide laboratories with the opportunity to implement a method in their laboratory and to evaluate or demonstrate their performance regarding quantitative analysis of tropane alkaloids in cereals intended for human consumption and in animal feed.

For this proficiency test, four test materials were prepared:
- Blank animal feed (A);
- Blank cereal for human consumption (buckwheat flour) (D);
- Animal feed containing atropine aimed at 1000 µg/kg and scopolamine at 250 µg/kg (C);
- Cereal for human consumption containing atropine aimed at 100 µg/kg and scopolamine at 50 µg/kg (B).

The fortified animal feed was prepared by mixing blank animal feed with homogenized ground *Datura stramonium* seeds. The buckwheat flour was prepared by spiking blank material with a solution of atropine and scopolamine to the required target concentrations followed by homogenization.

Homogeneity assessment showed that all materials were sufficiently homogenous for proficiency testing. The stability test for the buckwheat flour demonstrated that no statistically significant loss of atropine and scopolamine occurred during the timescale of the proficiency test. The stability test for the animal feed demonstrated a small consequential loss for atropine and scopolamine during the storage at room temperature as well as at 4 °C. This was accounted for in the calculations of the z-scores.

For calculating of the accuracy z-score for material B and C, the z'_a was used and a standard deviation of 25% was taken as an acceptable deviation for reproducibility conditions in this study. This means that there was a large variation present in the results of the participants for these materials.

Twenty-two laboratories submitted results for the proficiency study of tropane alkaloids in food and feed. Eleven laboratories (PT564, 565, 566, 568, 571, 577, 579, 580, 581, 583 and 588) showed optimal performance within the scope of their own method and of these laboratories nine (PT564, 565, 568, 571, 577, 579, 581, 583 and 588) did fully comply with the requirements of this proficiency test. They showed optimal performance by detecting all compounds with sufficient sensitivity, the absence of false positives and false negatives and a correct quantification of the tropane alkaloids atropine and scopolamine in materials B and C.

Based on the results of this proficiency study it is concluded that:
- There is a large variation in the results of the participants for both material B and C (high uncertainties). For material B 68% of the results for atropine were satisfactory and 64% for scopolamine. For material C this was respectively 62 and 71% (see Table 3).
- There is a need for improvement and harmonisation of quantitative methods for atropine and scopolamine. For the analysis of cereals intended for human consumption, methods preferably should be capable of detecting these compounds at low µg/kg concentrations.

1 Introduction

1.1 Proficiency testing

Proficiency testing is conducted to provide laboratories with a powerful tool to evaluate and demonstrate the reliability of the data that are produced. Next to validation and accreditation, proficiency testing is an important requirement of the EU Additional Measures Directive 93/99/EEC [1] and is required by ISO 17025:2005 [2].

The aim of this proficiency study was to provide laboratories with the opportunity to implement the method in their laboratory or to evaluate or demonstrate their competence for the analysis of the two most relevant tropane alkaloids; atropine and scopolamine, in cereal for human consumption and in animal feed.

The preparation of the materials, including the suitability testing of the materials and the evaluation of the quantitative results were carried out in accordance with guidelines ISO/IEC 17043 [3].

1.2 Tropane alkaloids

Tropane alkaloids are secondary metabolites produced by several plant families such as Erythroxylaceae (including coca), Solanaceae (including mandrake, henbane, deadly nightshade, datura), Proteaceae, Euphorbiaceae, Rhizophoraceae, Convolvulaceae and Cruciferae [11].

The class contains over 200 compounds, but the most common tropane alkaloids are atropine, hyoscyamine and scopolamine. Atropine is a racemic mixture of the D- and L-enantiomer of hyoscyamine. L-hyoscyamine is the pharmacologically active and toxicologically relevant form. Since most methods of analysis cannot separate the enantiomers, atropine is often used as a general indicator for L- (and D-)-hyoscyamine, to express the toxic content of plants or preparations, without stating the enantiomeric purity [12]. Tropane alkaloids are known to prevent binding of acetylcholine to its receptor and as a result have effects on heart rate, respiration and functions in the central nervous system [11].

Tropane alkaloids are found in all parts of the plant, but often the highest concentrations of atropine and scopolamine are found in the seed. The concentration of tropane alkaloids may range from 1-9 mg/g seed [13]. Most important species are *Datura stramonium* (thorn apple) and *Atropa belladonna* (deadly nightshade). Through accidental mixing of these plants with normal food and feed ingredients the consumers of these products may be exposed to the toxins [11,12].

Tropane alkaloids are not regulated in the European Union. However, European Directive 2002/32/EC [14] stipulates that the maximum allowed amount of *Datura* seeds in unground cereals intended for animal feed is 1000 mg/kg. From European Food Safety Authority (EFSA), there is considerable interest to generate occurrence data of tropane alkaloids in relevant food and feed commodities that could be used for exposure and risk assessment [11,13].

2 Materials en methods

2.1 Sample preparation

Two batches of complete feed for porcine (7 kg each) were prepared for the production of blank animal feed material A and animal feed material C. The latter was contaminated with ground *Datura* seeds. Two batches of buckwheat flour (7 kg each) were prepared for the production of blank buckwheat flour D and buckwheat flour B which was fortified with a spike solution of atropine and scopolamine, see Table 1. Material B was prepared by adding an acetone solution of atropine and scopolamine to blank buckwheat flour aiming at the levels as presented in Table 1. For material C *Datura* seeds were ground under cryogenic conditions and mixed with blank animal feed to obtain concentrations of approximately 1000 µg atropine and 250 µg scopolamine /kg. Each material was homogenized by extensive mixing.

Table 1
Target concentrations of tropane alkaloids in the proficiency test materials.

Material		Target concentration (µg/kg)	
		Atropine	Scopolamine
A	Blank animal feed	-	-
B	Spiked cereal	100	50
C	Contaminated animal feed	1000	250
D	Blank cereal	-	-

2.2 Sample identification

After homogenization, the materials were divided into sub-portions of 50 gram and stored in polypropylene airtight closed containers at room temperature. The samples for the participants were randomly selected and coded through a website application (crlwebshop). For each laboratory a sample set was prepared consisting of one randomly selected sample of each material A, B, C and D. The codes of the samples belonging to each sample set are presented in Annex 1.

2.3 Participants

Twenty-six laboratories registered for the participation in the proficiency test and twenty-two laboratories reported their results. Of the laboratories nineteen are situated within Europe, one in Asia, one in Central America and one in South America.

2.4 Homogeneity study

The homogeneity of the materials was assessed according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [5] and ISO 13528 [6], taking into account the insights discussed by Thompson [7] regarding the Horwitz equation. With this procedure the between-sample standard deviation (s_s) and the within-sample standard deviation (s_w) are compared with the target deviation σ_H. The method applied for homogeneity testing is considered suitable if $s_w < 0.5 * \sigma_H$ and a material is considered adequately homogeneous if $s_s < 0.3 * \sigma_H$.

Ten containers of material B and C were analysed in duplicate for atropine and scopolamine to determine the homogeneity of the materials. The results of the homogeneity study and their statistical

evaluation are presented in annexes 2-5. Materials B and C demonstrated to be sufficiently homogeneous for use in the proficiency test. Three containers of material A and D were analysed in duplicate for the presence of atropine and scopolamine. Blank material A contained traces of atropine and scopolamine (< 15 µg/kg). Based on these findings it was decided to apply a cut-off level of 25 µg/kg for this material. In material D no atropine and scopolamine were detected (< 5 µg/kg). Based on these findings it was decided to apply a cut-off level of 10 µg/kg for this material.

2.5 Sample distribution and instructions

Each of the participating laboratories received a randomly assigned laboratory code, generated by the website application. The sample sets with the corresponding number, consisting of four coded samples (Annex 1) were sent to the participating laboratories on October 7th, 2013. The sample sets were packed in an insulating box and were dispatched to the participants immediately by courier. All laboratories confirmed that the samples were received in good condition.

The samples were accompanied by a letter (Annex 6) describing the requested analyses and acknowledgement of receipt form. By e-mail the laboratories received instructions on how to use the web application to report the results.

The laboratories were asked to store the samples according to their own laboratory procedure and to analyse the samples according to their routine method. A single analysis result for atropine and scopolamine in each sample was requested. The deadline for submitting the quantitative results was December 13nd 2013, allowing ten weeks for the analysis.

2.6 Stability

On October 7th, the day the materials were distributed to the participants, six randomly selected samples of each material were stored at <-20 °C. It is assumed that the tropane alkaloids are stable at these storage conditions. Also, six samples of each material were stored at 4 °C and six at room temperature.

On December 16th, 70 days after the dispatch of the samples, the six samples that had been stored at -20 °C, at 4 °C and at room temperature were analysed for atropine and scopolamine. For each set of samples, the average of the results and the standard deviation were calculated.

First it was determined if storage at 4 °C or at room temperature could have led to a 'consequential instability' of the analytes [5,6]. A consequential instability is observed when the average concentration of an analyte in the samples stored at 4 °C or stored at room temperature is more than $0.3_{\sigma H}$ below the average concentration in the samples stored at <-20 °C. If so, the instability has a significant influence on the calculated z-scores. Second, it was determined whether a statistically significant instability occurred using a Students t-test [6]. The results and statistical evaluation of the stability test are presented in Annex 7 and 8.

For atropine and scopolamine in material B no consequential nor a statistical significant difference was observed among the samples stored at -20 °C, the samples stored at 4 °C and the samples stored at room temperature. These samples are considered sufficiently stable for the duration of the study.

For atropine and scopolamine in material C there was a consequential difference between the samples stored at -20 °C, at 4 °C and at room temperature. The average concentration at 4 °C and at room temperature was lower than the average of the samples that were stored at -20 °C. The concentration of atropine showed a decrease of 9.6% (from 868 to 785 µg/kg) during storage at 4 °C and 9.6% (from 868 µg/kg to 785 µg/kg) during storage at room temperature. The concentration of scopolamine showed a decrease of 6.9% (from 197 to 184 µg/kg) during storage at 4 °C and 8.7% (from 197 µg/kg to 180 µg/kg) during storage at room temperature (§ 4.4). The consequential instability for material C was taken into account for the calculation of the z_a scores (§ 4.4) for the participating laboratories.

3 Applied methods of analysis

Twenty laboratories carried out quantitative analyses for atropine and scopolamine. One laboratory reported only quantitative results of atropine and one laboratory reported screening results for atropine and scopolamine. An overview of the information provided by the participants regarding the quantitative confirmatory methods applied in this proficiency test is presented in Annex 9.

Seventeen laboratories applied (UP)LC-MS/MS for the determination of tropane alkaloids while five laboratories did not report their detection technique.

Nine laboratories used a mixture of formic acid in methanol/water as extraction solvent, among which six participants used 0.4% formic acid in methanol/water (60:40) and one participant used 1% formic acid in methanol/water (7:92). Three laboratories applied low pH extraction solvents using other acids: one participant used 0.1% acetic acid in methanol, one participant used heptafluorobutyric acid in an acetonitrile/water mixture, and one used 0.1 M hydrochloric acid in 75% ethanol (1:1). One laboratory applied alkaline extraction conditions using a mixture of dichloromethane, methanol and ammonia.

For sample purification one laboratory applied dispersive SPE with C_{18}, PSA and magnesium sulphate, one applied SPE based on C_{18} chemistry and one applied filtration on Chem Elut 1020, followed by purification on a Bond Elut Certify column. Other methods applied for purification of the extracts were filtration and liquid-liquid extraction. Several labs purified the extract through a 30 kD ultrafilter and one lab used a 0.2 µm filter. Three participants reported that they had not applied a clean-up step.

Seven laboratories reported their chromatographic conditions, of which four participants applied acidic chromatography and three participants applied alkaline chromatography.

Three laboratories used atropine-d_3 as an internal standard, one laboratory used nalorphine (a morphine derivative), one used sulfadimethoxine (a sulphonamide) and one used cocaine-d_3 (an opiate). No information was provided by the laboratories whether the internal standard was used for internal calibration or quantification or only for analytical quality control purposes.

4 Statistical evaluation

The statistical evaluation was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [5], elaborated by ISO, IUPAC and AOAC and ISO 13528 [6] in combination with the insights published by the Analytical Methods Committee [9,10] regarding robust statistics.

For the evaluation of the quantitative results, the assigned value, the uncertainty of the assigned value, a standard deviation for proficiency assessment and z-scores were calculated.

4.1 Calculation of the assigned value (X)

The assigned value (X) was determined using robust statistics [6,9,10]. The advantage of robust statistics is that all values are taken into account: outlying observations are retained, but given less weight. Furthermore, it is not expected to receive normally distributed data in a proficiency test. When using robust statistics, the data does not have to be normally distributed in contrast to conventional outlier elimination methods.

The robust mean of the reported results of all participants, calculated from an iterative process that starts at the median of the reported results using a cut-off value depending on the number of results, was used as the assigned value [6,9]. The assigned value is therefore a consensus value.

4.2 Calculation of the uncertainty of the assigned value (u)

The uncertainty of the assigned value is calculated to determine the influence of this uncertainty on the evaluation of the laboratories. A high uncertainty of the assigned value will lead to a high uncertainty of the calculated participants z_a-scores. If the uncertainty of the assigned value and thus the uncertainty of the z_a-score is high, the evaluation could indicate unsatisfactory method performance without any cause within the laboratory. In other words, illegitimate conclusions could be drawn regarding the performance of the participating laboratories from the calculated z_a-scores if the uncertainty of the assigned value is not taken into account.

The uncertainty of the assigned value (the robust mean) is calculated from the estimation of the standard deviation of the assigned value and the number of values used for the calculation of the assigned value [6]:

$$u = 1.25 * \frac{\hat{\sigma}}{\sqrt{n}}$$

where:

u = Uncertainty of the assigned value;
n = Number of values used to calculate the assigned value;
$\hat{\sigma}$ = The estimate of the standard deviation of the assigned value resulting from robust statistics.

According to ISO 13528 [6] the uncertainty of the assigned value (u) is negligible and therefore does not have to be included in the statistical evaluation if:

$u \leq 0.3\sigma_P$

where:

u = The uncertainty of the assigned value;

σ_P = Standard deviation for proficiency assessment (§ 4.3).

In case the uncertainty of the assigned value does not comply with this criterion, the uncertainty of the assigned value should be taken into account when evaluating the performance of the participants regarding the accuracy (§ 4.4). In case the uncertainty is > 0.7 σ_p the calculated z-scores should not be used for evaluation of laboratories performance and are presented for information only.

4.3 Calculation of the standard deviation for proficiency assessment (σ_P)

Based on RIKILTs extensive experience with the analysis of substances in animal feed, a fixed standard deviation of 25% was considered as an acceptable standard deviation for reproducibility conditions in this study for both materials:

$\sigma_P = 0.25c$

where:

σ_P = Standard deviation in proficiency assessment;

c = Concentration of the analyte (µg/kg).

4.4 Performance characteristics with regard to the accuracy

For illustrating the performance of the participating laboratories with regard to the accuracy a z_a-score is calculated. For the evaluation of the performance of the laboratories, the guidelines of ISO 13528 [6] are applied. According to these guidelines z_a-scores are classified as presented in Table 2.

Table 2

Classification of z_a-scores.

$\|za\| \leq 2$	Satisfactory
$2 < \|za\| < 3$	Questionable
$\|za\| \geq 3$	Unsatisfactory

If the calculated uncertainty of the assigned value complies with the criterion mentioned in § 4.2, the uncertainty is negligible. In this case the accuracy z-score is calculated from:

$$z_a = \frac{\overline{x} - X}{\sigma_P}$$ Equation I

where:

z_a = Accuracy z-score;

$\overline{x}$ = The average result of the laboratory;

X = Assigned value;

σ_P = Standard deviation for proficiency assessment.

However, if the uncertainty of the assigned value does not comply with the criterion mentioned in § 4.2, it could influence the evaluation of the laboratories. Although, according to ISO 13528 no z-scores can be calculated if a consensus value is used as the assigned value, we feel that evaluation of the participating laboratories is of main importance justifying the participating laboratories' effort. Therefore in this case, the uncertainty is taken into account by calculating the accuracy z-score [6]:

$$z'_a = \frac{\overline{x} - X}{\sqrt{\sigma_P^2 + u^2}}$$ Equation II

where:

z'_a = Accuracy z-score taking into account the uncertainty of the assigned value;
$\overline{x}$ = The average result of the laboratory;
X = Assigned value;
σ_P = Standard deviation for proficiency assessment;
u = Uncertainty of the assigned value.

If a consequential instability of the proficiency test materials is observed, this can influence the evaluation of the laboratory performance. Therefore, in that case the consequential instability is taken into account when calculating z-scores. Because instability only regards one side of the confidence interval (a decrease of the concentration) this correction only applies to the lower 2s limit and results in an asymmetrical confidence interval.

In the case of a consequential instability the accuracy z-score for the laboratories that reported an amount below the assigned value is corrected for this instability by:

$$z_{ai} = \frac{\overline{x} - X}{\sqrt{\sigma_P^2 + \Delta^2}}$$ Equation III

where:

z_{ai} = Accuracy z-score taking into account the consequential instability of the assigned value;
$\overline{x}$ = The average result of the laboratory;
X = Assigned value;
σ_P = Standard deviation for proficiency assessment;
Δ = Difference between average concentration of compound stored at -20 °C, 4 °C or room temperature.

In some cases the uncertainty of the assigned value does not comply with the criterion in § 4.2 and also a consequential instability is observed. In this case the z'_a score for the laboratories that reported an amount below the assigned value is corrected for this instability by:

$$z'_{ai} = \frac{\overline{x} - X}{\sqrt{\sigma_P^2 + \Delta^2 + u^2}}$$ Equation IV

where:

z'_{ai} = Accuracy z-score taking into account the uncertainty and consequential instability of the assigned value;
$\overline{x}$ = The average result of the laboratory;
X = Assigned value;
σ_P = Standard deviation for proficiency assessment;
Δ = Difference between average concentration of compound stored at -20 °C, 4 °C and room temperature;
u = Uncertainty of the assigned value.

5 Results

Twenty-six laboratories registered for participation in the proficiency test for tropane alkaloids in food and feed and twenty-two submitted results. Lab PT580 registered only for atropine and lab PT584 screened for the presence of atropine and scopolamine. The performance of individual laboratories is summarized in Annex 13.

Because it was observed during homogeneity assessment that the blank material A contained traces of atropine and scopolamine, it was decided to use in this study a cut-off level of 25 µg/kg for both compounds. Several laboratories reported the presence of atropine or scopolamine below the cut-off level in material A (see Annex 11) and these were not listed as false positive results. The homogeneity analysis of blank material D had shown the absence of any traces of atropine and scopolamine and in this study for both compounds a cut-off level was used of 10 µg/kg. None of the participants reported the presence of atropine or scopolamine below the cut-off level in material D.

An overview of the compounds reported in the samples is presented in Annex 10. Annex 11 gives an overview of false positive and false negative results. Five false positive results were reported for material D, seven false negative results were reported for material B and four false negative results were reported for material C. Laboratories PT573 and 576 reported false negative results for material B and false positive results for material D. Participant PT586 reported false negative results for materials B and C.

5.1 Material A (blank animal feed)

No laboratories reported results above the cut-off level of 25 µg/kg, so no false positive results were recorded.

5.2 Material B (spiked cereal)

Labs PT573, 576 and 586 reported false negative results for both atropine and scopolamine in material B. Lab PT566 could not detect atropine in material B and lab PT584 failed to detect scopolamine. Lab PT580 did not report results for scopolamine.

5.2.1 Atropine

As presented in Annex 12 the lowest value reported for atropine was 60.2 µg/kg and the highest was 4400 µg/kg. The assigned value of atropine is 102 µg/kg with a robust standard deviation of 33.6 µg/kg. This is 1.3 times higher than the value of the standard for proficiency assessment of 25.4 µg/kg. The uncertainty of the assigned value is 10.2 µg/kg which does exceed 0.3 σ_p (7.61 µg/kg, § 4.2). Therefore the uncertainty is taken into account in the evaluation of the laboratories. No consequential instability during storage of 70 days was observed, therefore z'_a-scores (§ 4.4, equation II) were calculated. With respect to atropine in material B, two participants (PT569 and 585) reported results with unsatisfactory accuracy.

It should be remarked that laboratory PT566 did not detect atropine in material B because it was below the LOD of their method (100 µg/kg).

5.2.2 Scopolamine

As presented in Annex 12 the lowest value reported for scopolamine was 23.9 µg/kg and the highest was 2500 µg/kg. The assigned value of scopolamine is 56.1 µg/kg with a robust standard deviation of 26.8 µg/kg. This is almost two times higher than the value suggested by the standard for proficiency assessment of 14.0 µg/kg. The uncertainty of the assigned value is 8.12 µg/kg which does exceed 0.3 σ_p (4.21 µg/kg, § 4.2). Therefore the uncertainty is taken into account in the evaluation of the laboratories. No consequential instability during storage of 70 days was observed, so z'_a-scores (§ 4.4, equation II) were calculated. With respect to the results for scopolamine in material B four laboratories (PT569, 572, 585 and 589) submitted unsatisfactory results.

5.3 Material C (contaminated animal feed)

Laboratory PT586 reported false negative results for both atropine and scopolamine, while labs PT576 and 584 missed the presence of scopolamine. Lab PT580 did not report results for scopolamine.

5.3.1 Atropine

As presented in Annex 13 the lowest value reported for atropine was 7.21 µg/kg and the highest was 3010 µg/kg. The assigned value of atropine is 597 µg/kg with a robust standard deviation of 354 µg/kg. This is more than two times higher than the value of the standard for proficiency assessment of 149 µg/kg. The uncertainty of the assigned value is 98.8 µg/kg which does exceed 0.3 σ_p (44.7 µg/kg, § 4.2). Therefore the uncertainty is taken into account in the evaluation of the laboratories. Also, a consequential instability during storage of 70 days at room temperature was observed (decrease of 9.6%) and this instability was taken into account by calculating the z'_{ai}-scores (§ 4.4, equation IV) for laboratories that reported a value below the assigned value. With respect to the results for atropine in material C three results were questionable (labs PT572, 573 and 576) and three were unsatisfactory (labs PT569, 575 and 582). When no instability was assumed and equation II was used instead, the only change was that the result for lab PT580 would change from satisfactory into a questionable result.

5.3.2 Scopolamine

As presented in Annex 13 the lowest value reported for scopolamine was 30.4 µg/kg and the highest was 750 µg/kg. The assigned value of scopolamine is 186 µg/kg with a robust standard deviation of 55.7 µg/kg. This is comparable to the standard for proficiency assessment of 46.6 µg/kg. The uncertainty of the assigned value is 16.4 µg/kg which does exceed 0.3 σ_p (14.0 µg/kg, § 4.2). Therefore the uncertainty is taken into account in the evaluation of the laboratories. Also, a consequential instability during storage of 70 days at room temperature was observed (decrease of 8.7%) and this instability was taken into account by calculating the z'_{ai}-scores (§ 4.4, equation IV) for labs that reported a value below the assigned value. With respect to the accuracy for scopolamine in material C one result was questionable (lab PT587) and two were unsatisfactory (labs PT569 and 572). When no instability was assumed and equation II was used instead, this did not change the number of questionable and unsatisfactory results.

5.4 Material D (blank cereal)

Three laboratories reported results above the cut-off level of 10 µg/kg, which are considered false positive results. Laboratory PT572 reported the presence of 17.6 µg/kg scopolamine in the blank material and laboratories PT573 and PT576 reported the presence of atropine and scopolamine with respectively concentration of 62.1 and 37 µg/kg for atropine and 116.6 and 30 µg/kg for scopolamine.

6 Discussion and conclusions

Twenty-two laboratories reported results for the proficiency test of tropane alkaloids in food and feed. The aim of this study was to offer laboratories the opportunity to implement the method in their laboratory and to evaluate or demonstrate their performance regarding the quantitative analysis of tropane alkaloids.

An overview of each participant's performance is shown in Annex 14. Eleven laboratories (PT564, 565, 566, 568, 571, 577, 579, 580, 581, 583 and 588) showed optimal performance within the scope of their own method and of these laboratories nine (PT564, 565, 568, 571, 577, 579, 581, 583 and 588) have fully complied with the requirements of this proficiency test. They showed optimal performance by detecting all compounds with sufficient sensitivity, the absence of false positives and false negatives and a correct quantification of the tropane alkaloids atropine and scopolamine in materials B and C.

Based on the analysis of the submitted data some speculations can be made regarding reporting errors or interchanging of samples. Labs PT573 and 576 reported false negative results for material B and false positive results for material D. In both cases the reported false positive values for material D are in line with the consensus values obtained for material B. This may indicate that these laboratories have exchanged the results of materials B and D.

Laboratory PT582 reported an unsatisfactory result for atropine in material C, but acceptable results for atropine in material B and acceptable results for scopolamine in material B and C. Possibly a reporting error was made for atropine in material B (reporting 7.21 µg/kg instead of 721 µg/kg).

Laboratory PT585 reported unsatisfactory results for atropine and scopolamine in material B, but acceptable results for both compounds in material C. The values reported for both compounds in the two materials are very similar, which may be an indication that lab PT585 accidentally analysed material C twice.

The results show that the variation in results for material C is larger than the variation in the results of material B. This was somewhat unexpected because the levels for atropine and scopolamine are higher in material C (assigned value of atropine 596 and scopolamine 186 µg/kg) than in material B (assigned value of atropine 102 and scopolamine 56 µg/kg). The larger variation may be related to the nature of the material; material B consists of plain buckwheat flour, while material C is an animal feed, that is composed of many ingredients. Furthermore, the analytes have been spiked to material B, while in material C they are present as constituents of the (ground) *Datura* seeds, mixed into the animal feed. Extraction of the analytes from seed may be more difficult than from spiked material.

Evaluation of the methods used by the participants is only possible to some extent. Although most participants gave information about the clean-up and detection technique only a few participants mentioned the chromatographic conditions of their analytical method. However, regarding reporting limits, based on the information provided by the participants it can be concluded that there is a rather large range, varying from 0.5 µg/kg up to 50 µg/kg and even 100 µg/kg in one case for atropine (see Annex 9). Nine laboratories reported with limits of 5 µg/kg or less, four reported with limits between 10 and 20 µg/kg, while three labs had reporting limits of 25 µg/kg or higher. For the analysis of tropane alkaloids in animal feed a higher reporting limit can still be acceptable in view of the relatively high maximum allowed content of *Datura* seeds in feed [14]. However for the adequate analysis of tropane alkaloids in products for human consumption a low detection limit will be required. EFSA in their 2013 Scientific Opinion has derived an acute reference dose (ARfD) for the group of tropane alkaloids of 0.016 µg/kg body weight [11]. For a person of 60 kg this corresponds to an daily intake of only 1 µg. Cereals constitute a substantial part of the Western diet, this implies that consumption of cereal products containing low µg/kg levels of tropane alkaloids can already result in exceedance of

the ARfD. Reliable quantitative analytical methods will thus be required that can determine individual tropane alkaloids at a level of 5 µg/kg and (preferably) lower.

Table 3

Overview of performance of laboratories in the proficiency test on tropane alkaloids.

Compound		Material B	Material C	Overall
	# of participants / results	Correct results (%)	Correct results (%)	Correct results (%)
Atropine	22	68	62	66
Scopolamine	21	64	71	67
Overall	43	65	67	

Based on the results of this proficiency study it is concluded that:

- There is a large variation in the results of the participants for both material B and C (high uncertainties). For material B 68% of the results for atropine were satisfactory and 64% for scopolamine. For material C this was respectively 62 and 71% (see Table 3).
- There is a need for improvement and harmonisation of quantitative methods for atropine and scopolamine. For the analysis of cereals intended for human consumption, methods preferably should be capable of detecting these compounds at low µg/kg concentrations.

References

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Annex 1 Codification of the samples

Lab number	Material A*	Material B*	Material C*	Material D
PT564	715	819	311	261
PT565	405	695	885	315
PT566	625	249	759	810
PT567	185	292	684	407
PT568	336	928	329	399
PT569	119	231	603	570
PT570	428	196	740	922
PT571	199	295	306	254
PT572	189	159	834	818
PT573	259	683	379	617
PT574	621	690	875	133
PT575	125	468	374	109
PT576	181	688	717	643
PT577	925	624	556	411
PT578	673	878	600	882
PT579	862	249	775	551
PT580	590	871	736	344
PT581	558	476	632	135
PT582	382	954	485	457
PT583	620	428	587	985
PT584	933	749	744	250
PT585	957	483	382	363
PT586	593	326	951	498
PT587	112	816	125	183
PT588	524	534	760	106
PT589	513	614	501	144

* All sample codes start with TA/2013/feedfood/.

Annex 2 Statistical evaluation of homogeneity data of material B for atropine

	Atropine (µg/kg)	
Sample number	Replicate 1	Replicate 2
Hom/B001	88.8	85.6
Hom/B002	86.8	85.7
Hom/B003	91.2	89.0
Hom/B004	99.1	102
Hom/B005	86.7	87.0
Hom/B006	85.3	82.3
Hom/B007	88.5	87.0
Hom/B008	90.9	88.7
Hom/B009	89.2	86.9
Hom/B010	91.2	91.9
Grand mean	89.2	
Cochran's test		
C	0.231	
C_{crit}	0.602	
$C<C_{crit}$?	NO OUTLIERS	
Target s = σ_H	Horwitz: 19.6	
S_x	4.47	
S_w	1.50	
S_s	4.35	
Critical = $0.3\sigma_H$	5.88	
S_s<critical?	ACCEPTED	
$S_w<0.5\sigma_H$?	ACCEPTED	

S_x = standard deviation of the sample averages.

S_w = within-sample standard deviation.

S_s = between-sample standard deviation.

Annex 3 Statistical evaluation of homogeneity data of material B for scopolamine

	scopolamine (µg/kg)	
Sample number	Replicate 1	Replicate 2
Hom/B001	43.2	43.3
Hom/B002	41.9	42.3
Hom/B003	45.4	42.7
Hom/B004	48.0	52.4
Hom/B005	41.7	41.4
Hom/B006	43.2	40.8
Hom/B007	43.6	43.7
Hom/B008	44.3	43.2
Hom/B009	43.2	40.4
Hom/B010	43.9	44.5
Grand mean	43.7	
Cochran's test		
C	0.462	
C_{crit}	0.602	
$C<C_{crit}$?	NO OUTLIERS	
Target s = σ_H	Horwitz: 9.6	
S_x	2.51	
S_w	1.44	
S_s	2.30	
Critical = $0.3\sigma_H$	2.88	
S_s<critical?	ACCEPTED	
$S_w<0.5\sigma_H$?	ACCEPTED	

S_x = standard deviation of the sample averages.

S_w = within-sample standard deviation.

S_s = between-sample standard deviation.

Annex 4 Statistical evaluation of homogeneity data of material C for atropine

	atropine (µg/kg)	
Sample number	Replicate 1	Replicate 2
Hom/C001	749	836
Hom/C002	703	684
Hom/C003	796	794
Hom/C004	694	751
Hom/C005	744	702
Hom/C006	706	795
Hom/C007	*	*
Hom/C008	776	706
Hom/C009	896	705
Hom/C010	691	724
Grand mean	747	
Cochran's test		
C	0.578	
C_{crit}	0.638	
$C<C_{crit}$?	NO OUTLIERS	
Target s = σ_H	Horwitz: 125	
S_x	40.2	
S_w	59.2	
S_s	0	
Critical = $0.3\sigma_H$	37.5	
S_s<critical?	ACCEPTED	
$S_w<0.5\sigma_H$?	ACCEPTED	

* Outlier according Cochran's test

S_x = standard deviation of the sample averages.

S_w = within-sample standard deviation.

S_s = between-sample standard deviation.

Annex 5 Statistical evaluation of homogeneity data of material C for scopolamine

	scopolamine (µg/kg)	
Sample number	Replicate 1	Replicate 2
Hom/C001	222	237
Hom/C002	214	202
Hom/C003	226	237
Hom/C004	207	220
Hom/C005	216	207
Hom/C006	211	234
Hom/C007	*	*
Hom/C008	232	210
Hom/C009	251	208
Hom/C010	211	209
Grand mean	219	
Cochran's test		
C	0.524	
C_{crit}	0.638	
$C<C_{crit}$?	NO OUTLIERS	
Target s = σ_H	Horwitz: 44.1	
S_x	9.11	
S_w	14.1	
S_s	0	
Critical = $0.3\sigma_H$	13.2	
S_s<critical?	ACCEPTED	
$S_w<0.5\sigma_H$?	ACCEPTED	

* Outlier according Cochran's test

S_x = standard deviation of the sample averages.

S_w = within-sample standard deviation.

S_s = between-sample standard deviation.

Annex 6 Instruction letter

RIKILT
WAGENINGEN UR

For quality of life

P.O. Box 230 | 6700 AE WAGENINGEN | The Netherlands

Contaminants & Toxins

DATE
September 30, 2013

SUBJECT
Proficiency study

OUR REFERENCE
13/RIK0853

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HANDLED BY
D.P.K.H. Pereboom - de Fouw

TELEPHONE
+31 (0)317 480 355

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pt.rikilt@wur.nl

Dear participant,

Thank you very much for your interest in the proficiency test for the analysis of tropane alkaloids in food and feed.
Hereby I send you a parcel containing four randomly coded samples. The samples contain 50 g of cereals for human consumption or animal feed. The samples may contain atropine (the racemic mixture of D - and L- hyoscyamine) and scopolamine.

Please fill out the accompanying acknowledgement of receipt form and return it immediately upon receipt of the samples, preferably by e-mail (pt.rikilt@wur.nl).

Instructions:

- After arrival store the samples according to your laboratory procedure.
- Homogenize the samples before analysis according to your laboratory procedure.
- Please analyze the samples according to your routine method and make use of your own reference standards. If you don't have a routine method available in your laboratory, you can receive a short description of the method used by RIKILT upon request.
- Carry out a **single analysis** for each sample. Report one result and not an average of multiple measurements.
- The deadline for this test is **December 13th 2013.**
- Please use the web application for entering your results (https://crlwebshop.wur.nl/apex/f?p=307:1000).
- Extra information about the use of this application can be found in the document 'webapplication'.

- Your username is:
- Your password is:
- Your lab code to enter this proficiency test is:

For reporting the results please use the table below:

Result	Decimals
<1 µg/kg	3
1< result < 10 µg/kg	2
10 < result < 100 µg/kg	1
result >100 µg/kg	0

Please contact me if you have any questions or need any assistance.

Good luck with the study and kind regards,

D.P.K.H. Pereboom - de Fauw

Annex 7 Statistical evaluation of stability data for material B

Statistical evaluation for atropine in material B			
Storage temp	-20 °C	4 °C	Room temperature
Time in freezer (days)	0	70	70
Calculated amounts (µg/kg)	104	101	106
	120	111	115
	108	111	106
	97.9	104	105
	108	109	108
	119	100	104
Average amount (µg/kg)	110	106	107
n	6	6	6
st. dev (µg/kg)	8.43	4.77	4.29
Difference		3.51	2.31
$0.3\sigma_H$	7.23		
Consequential difference? Diff < 0.3 σ_H		NO	NO
t		0.89	0.6
t_{crit}		2.23	2.23
Statistical difference? T < t_{crit}		NO	NO

Statistical evaluation for scopolamine in material B			
Storage temp	-20 °C	4 °C	Room temperature
Time in freezer (days)	0	70	70
Calculated amounts (µg/kg)	65.2	63.4	69.0
	77.9	72.1	71.2
	68.3	74.6	69.8
	65.3	68.7	67.7
	68.0	68.4	69.2
	76.7	61.1	69.4
Average amount (µg/kg)	70.2	68.0	69.4
n	6	6	6
st. dev (µg/kg)	5.63	5.08	1.13
Difference		2.20	0.88
$0.3\sigma_H$	4.64		
Consequential difference? Diff < 0.3 σ_H		NO	NO
t		0.71	0.37
t_{crit}		2.23	2.23
Statistical difference? T < t_{crit}		NO	NO

Annex 8 Statistical evaluation of stability data for material C

Statistical evaluation for atropine in material C			
Storage temp	-20 °C	4 °C	Room temperature
Time in freezer (days)	0	70	70
Calculated amounts (µg/kg)	891	781	782
	954	817	780
	852	712	844
	781	776	811
	714	781	768
	1014	841	725
Average amount (µg/kg)	868	785	785
n	6	6	6
st. dev (µg/kg)	110	43.7	40.0
Difference		83.1	82.9
$0.3\sigma_H$	42.6		
Consequential difference? Diff < 0.3 σ_H		YES	YES
t		1.72	1.73
t_{crit}		2.23	2.23
Statistical difference? T < t_{crit}		NO	NO

Statistical evaluation for scopolamine in material C			
Storage temp	-20 °C	4 °C	Room temperature
Time in freezer (days)	0	70	70
Calculated amounts (µg/kg)	201	189	179
	223	193	169
	197	173	187
	181	174	189
	163	179	176
	218	195	180
Average amount (µg/kg)	197	184	180
n	6	6	6
st. dev (µg/kg)	22.4	9.75	7.36
Difference		13.6	17.2
$0.3\sigma_H$	12.1		
Consequential difference? Diff < 0.3 σ_H		YES	YES
t		1.38	1.78
t_{crit}		2.23	2.23
Statistical difference? T < t_{crit}		NO	NO

Annex 9 Overview of the applied methods for tropane alkaloids

Lab	Clean-up	Internal standard	Reporting limit		Detection method
			Atropine (µg/kg)	Scopolamine (µg/kg)	
PT564			<12	<12	
PT565	no	no	<25	<25	LC-MS/MS
PT566	14 minutes; methanol - acetic acid 0.1%		<100	<20	
PT568	Filtration on column Chemelut 1020; purification on column Bondelut Certify	nalorphine	<4	<2	LC-MS-MS
PT569			<10	<10	
PT571	Extraction: acetonitril/water/heptafluorobutyric acid; SPE cleanup Oasis MCX	atropine-d3	<5	<5	LC-MS/MS
PT572	Filtration	Atropine D3	<0.5	<0.5	LC-MS/MS
PT573	methanol/water (60/40) 0.4% formic acid				
PT575	Excerpt from RIKILT SOP A 1070	Cocaine-D3			LC-MS/MS
PT576	Extraction 5 g sample in 50 mL HCl 0.1 M/EtOH 75% (1:1); Bath heating/mix at 60 C (30 min) 5 mL SPE C18 - dry under nitrogen at 50 C; Elute with 1 mL (MeOH/water/formic acid)	sulfadimethoxine	<12	<8	UPLC-MS-MS
PT577	Extraction with MeOH 60% with formic acid,	none	<1	<1	ULC-MS/MS
PT579	Same as RIKILT SOP A1070	none	<50	<50	LC-MS/MS
PT580	d-SPE with C18, PSA and Magnesium Sulfate	none			LC/MS/MS
PT581	Extraction with CH2Cl2/CH3OH/NH3. Shaking, centrifugation, adding of H2SO4, shaking. Liquid/liquid extraction with CH2Cl2, evaporation of extract to dryness, dilution with H2O/CH3OH.	none	<1	<1	LC/MS/MS
PT582	From 2 g sample extraction with 10 ml solvent (7% Methanol, 92% Water, 1% Formic Acid) 10 min ultrasonic bath, 2 ml filtered with 0.2Âµm.	no			LC-MS/MS
PT583	Extraction (1:20, w/v) with methanol/water/formic acid 60/40/0.4. Ultrafiltration using 30 kD Amicon ultrafilter.	no	<5	<5	LC-MS/MS
PT584					screening
PT585	Extract with 0.4% formic Acid in Methanol:Water (60:40), centrifuge and filter	no	<2.51	<1.76	UPLC-MS/MS
PT586	Extraction with mixture of water/ methanol/ formic acid; no additional clean-up	no			LC-MS/MS
PT587	no clean-up	no internal standard	<20	<20	LC-MS/MS
PT588	Extraction with CH3OH + 0.4% FA:water (60:40)	Atropine-d3	<1	<1	LC-MS/MS
PT589	none	no	<5.0	<6.4	LC-MS/MS

Reporting limits were taken from the submitted concentrations for atropine and scopolamine.

Annex 10 Overview of results

Lab	Material A	Material B	Material C	Material D
PT564		atropine scopolamine	atropine scopolamine	
PT565		atropine scopolamine	atropine scopolamine	
PT566		atropine <100 scopolamine	atropine scopolamine	
PT568		atropine scopolamine	atropine scopolamine	
PT569		atropine scopolamine	atropine scopolamine	
PT571		atropine scopolamine	atropine scopolamine	
PT572		atropine scopolamine	atropine scopolamine	**scopolamine (FP)**
PT573		**atropine (FN)** **scopolamine (FN)**	atropine scopolamine	**atropine (FP)** **scopolamine (FP)**
PT575		atropine scopolamine	atropine scopolamine	
PT576		**atropine (FN)** **scopolamine (FN)**	atropine **scopolamine (FN)**	**atropine (FP)** **scopolamine (FP)**
PT577		atropine scopolamine	atropine scopolamine	
PT579		atropine scopolamine	atropine scopolamine	
PT580		atropine **scopolamine (NR)**	atropine **scopolamine (NR)**	
PT581		atropine scopolamine	atropine scopolamine	
PT582		atropine scopolamine	atropine scopolamine	
PT583		atropine scopolamine	atropine scopolamine	
PT584		atropine **scopolamine (FN)**	atropine **scopolamine (FN)**	
PT585		atropine scopolamine	atropine scopolamine	
PT586		**atropine (FN)** **scopolamine (FN)**	**atropine (FN)** **scopolamine (FN)**	
PT587		atropine scopolamine	atropine scopolamine	
PT588		atropine scopolamine	atropine scopolamine	
PT589		atropine scopolamine	atropine scopolamine	

FN: false negative result.

FP: false positive result.

NR: no result reported.

Annex 11 False positives, false negatives and reported results for material A and D

False positive results reported for D above the cut-off level.

Lab code	Sample code	Material	Compound detected	Concentration (µg/kg)
572	818	D	scopolamine	17.6
573	617	D	atropine	62.1
573	617	D	scopolamine	116.6
576	643	D	atropine	37
576	643	D	scopolamine	30

The cut-off level applied is 10 µg/kg for atropine and scopolamine in material D.

False negative results reported for material B and C.

Lab code	Sample code	Material	Compound missed
573	683	B	atropine
573	683	B	scopolamine
576	688	B	atropine
576	688	B	scopolamine
576	717	C	scopolamine
584	749	B	scopolamine
584	744	C	scopolamine
586	326	B	atropine
586	326	B	scopolamine
586	951	C	atropine
586	951	C	scopolamine

Results reported for material A below the cut-off level.

Lab code	Sample code	Material	Compound detected	Concentration (µg/kg)
568	336	A	atropine	4
568	336	A	scopolamine	2
571	199	A	atropine	2.5
572	189	A	atropine	1.05
572	189	A	scopolamine	2.02
577	925	A	atropine	22.8
588	524	A	atropine	6.5

The cut-off level applied is 25 µg/kg for atropine and scopolamine in material A.

Annex 12 Results for material B

	Atropine AV: 102 µg/kg Uncertainty of AV: 10.2 µg/kg σ_P (25% of AV): 25.4 µg/kg Robust sd: 33.6 µg/kg		Scopolamine AV: 56.1 µg/kg Uncertainty of AV: 8.12 µg/kg σ_P (25% of AV): 14.0 µg/kg Robust sd: 26.8 µg/kg	
Lab.code	**Result (µg/kg)**	**z'_a -score**	**Result (µg/kg)**	**z'_a-score**
PT564	92	-0.35	49	-0.44
PT565	91.5	-0.37	49.6	-0.40
PT566			37	-1.18
PT568	73	-1.04	34.0	-1.37
PT569	4400	**157.18**	2500	**150.75**
PT571	109.3	0.28	46	-0.62
PT572	85.5	-0.59	192.7	**8.42**
PT573				
PT575	131	1.08	65	0.55
PT576				
PT577	96.9	-0.17	23.9	-1.99
PT579	115	0.49	77	1.29
PT580	122	0.75		
PT581	64.8	-1.34	31.2	-1.54
PT582	60.20	-1.51	77.4	1.31
PT583	77	-0.90	36.5	-1.21
PT584				
PT585	349.6	**9.07**	206.1	**9.25**
PT586				
PT587	141	1.44	48	-0.50
PT588	66	-1.30	30	-1.61
PT589	104	0.09	121.1	**4.01**

AV = assigned value.

sd = standard deviation.

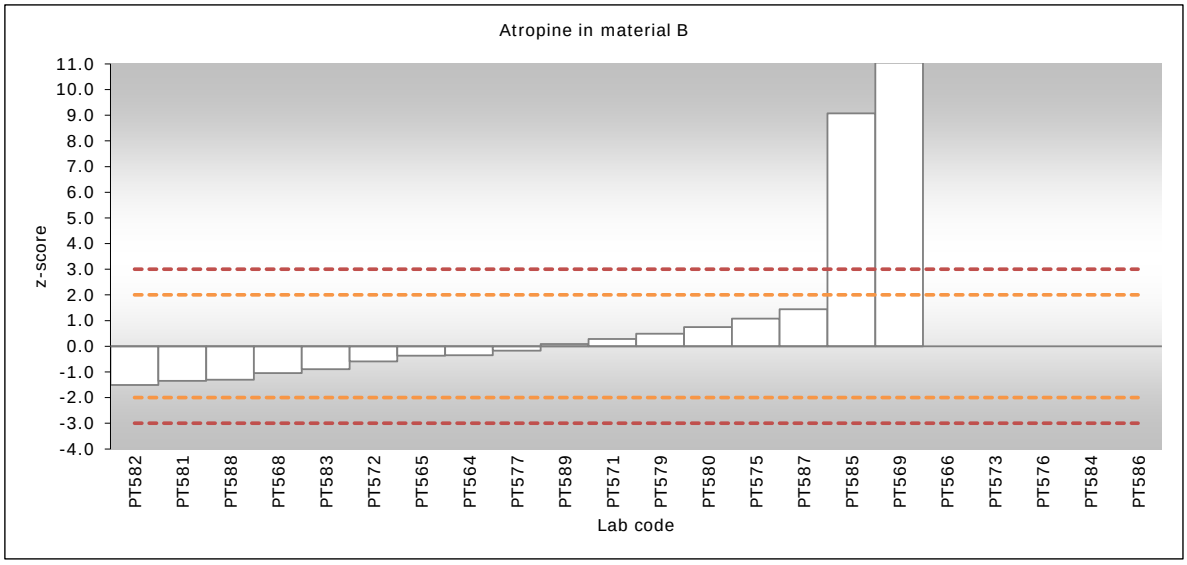

Figure A *Graphical representation of the reported results for atropine in material B. The X ± 2σ_P lines (dotted) are calculated according to equation II in § 4.4.*

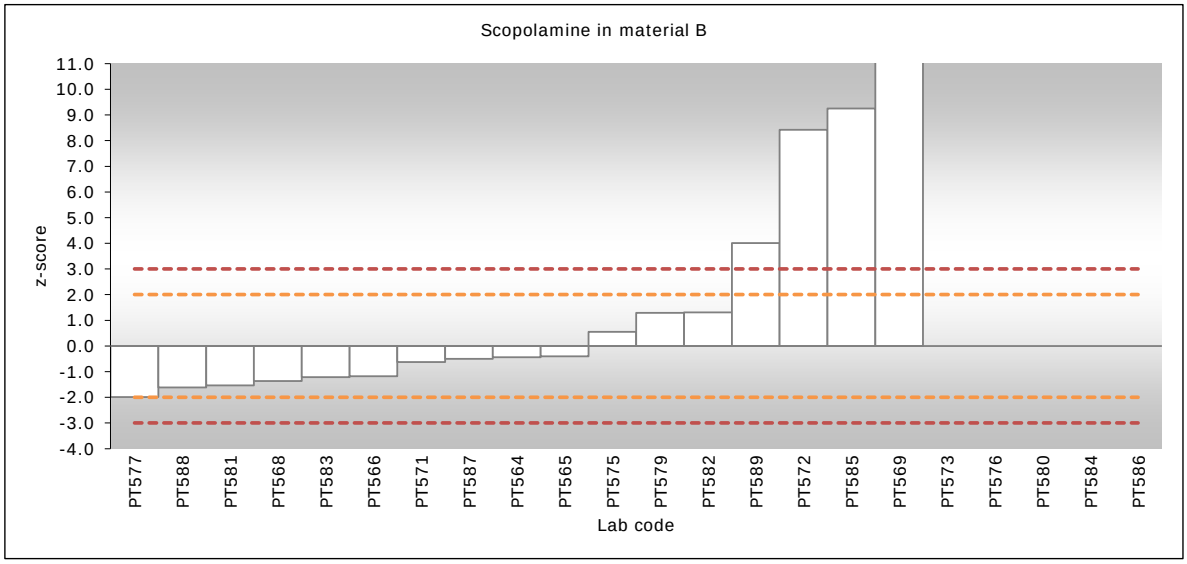

Figure B *Graphical representation of the reported results for scopolamine in material B. The X ± 2 σ_P lines (dotted) are calculated according to equation II in § 4.4.*

Annex 13 Results for material C

	Atropine AV: 597 µg/kg Uncertainty of AV: 98.8 µg/kg σ_P (25% of AV): 149 µg/kg Robust sd: 354 µg/kg		Scopolamine AV: 186 µg/kg Uncertainty of AV: 16.4 µg/kg σ_P (25% of AV): 46.6 µg/kg Robust sd: 55.7 µg/kg	
Lab.code	**Result (µg/kg)**	**z'$_{ai}$ -score**	**Result (µg/kg)**	**z'$_{ai}$-score**
PT564	520	-0,41	155	-0,60
PT565	483	-0,60	221	0,70
PT566	763	0,93	139	-0,91
PT568	489	-0,57	150	-0,70
PT569	3010	**13,49**	750	**11,41**
PT571	865.7	1,51	207.6	0,43
PT572	1005	**2,28**	30.4	**-3,00**
PT573	214.6	**-2,03**	187.4	0,02
PT575	1212	**3,44**	155	-0,60
PT576	163	**-2,31**		
PT577	494	-0,55	198	0,24
PT579	640	0,24	244	1,17
PT580	231	-1,95		
PT581	402	-1,04	134	-1,01
PT582	7.21	**-3,14**	208	0,44
PT583	828	1,29	152	-0,66
PT584				
PT585	383.6	-1,13	202.9	0,34
PT586				
PT587	922	1,82	295	**2,20**
PT588	550	-0,25	127	-1,14
PT589	697	0,56	229	0,86

AV = assigned value.

Sd = standard deviation.

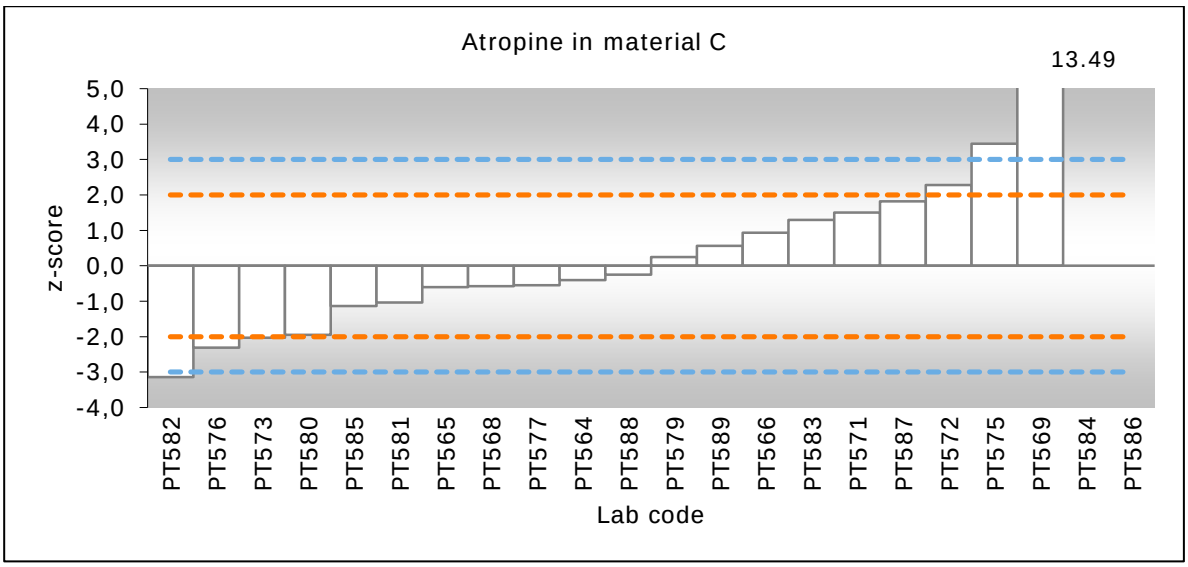

Figure C *Graphical representation of the reported results for atropine in material C. The X ± 2 σ_P lines (dotted) are calculated according to equation IV in § 4.4.*

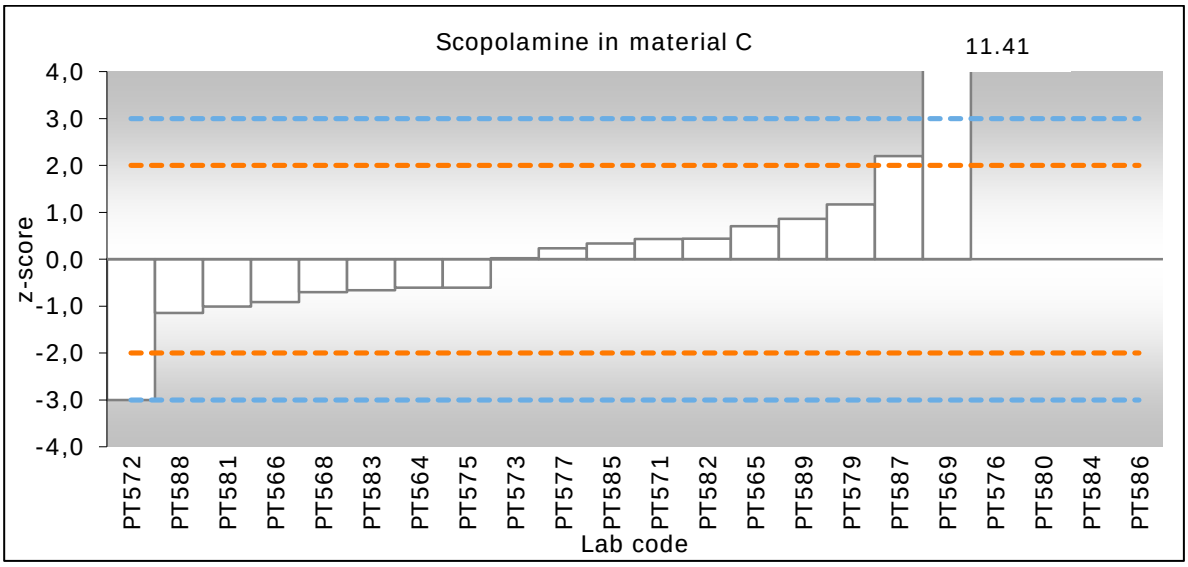

Figure D *Graphical representation of the reported results for scopolamine in material C. The X ± 2 σ_P lines (dotted) are calculated according to equation IV in § 4.4.*

Annex 14 Overall score participants

Lab	Correct z-scores	Questionable Za-scores	Unsatisfatory Za-scores	False positive result	False negative result	Incomplete or non-quantitative result
PT564	4					
PT565	4					
PT566	3				(1)	
PT568	4					
PT569			4			
PT571	4					
PT572	1	1	2			
PT573	1	1		2	2	
PT575	3		1			
PT576		1		2	3	
PT577	4					
PT579	4					
PT580	2					2
PT581	4					
PT582	3		1			
PT583	4					
PT584					2	4
PT585	2		2			
PT586					4	
PT587	3	1				
PT588	4					
PT589	3		1			

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RIKILT report 2014.008

RIKILT Wageningen UR is part of the international knowledge organisation Wageningen University & Research centre. RIKILT conducts independent research into the safety and reliability of food. The institute is specialised in detecting and identifying substances in food and animal feed and determining the functionality and effect of those substances.

The mission of Wageningen UR (University & Research centre) is 'To explore the potential of nature to improve the quality of life'. Within Wageningen UR, nine specialised research institutes of the DLO Foundation have joined forces with Wageningen University to help answer the most important questions in the domain of healthy food and living environment. With approximately 30 locations, 6,000 members of staff and 9,000 students, Wageningen UR is one of the leading organisations in its domain worldwide. The integral approach to problems and the cooperation between the various disciplines are at the heart of the unique Wageningen Approach.

To explore the potential of nature to improve the quality of life

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