Proficiency test for aflatoxin in pig feed

I.J.W. Elbers and W.C.M. de Nijs

RIKILT
WAGENINGEN UR

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P.O. Box 230, 6700 AA Wageningen, The Netherlands, T +31 (0)317 48 02 56, E info.RIKILT@wur.nl, www.wageningenUR.nl/en/rikilt. RIKILT is part of Wageningen UR (University & Research centre).

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Distribution list:
- 13 participating laboratories of which 12 European and 1 American participant.

Contents

Summary

In January 2015 a proficiency test (PT) for aflatoxin B1, B2, G1 and G2 in pig feed was organized by RIKILT Wageningen UR. This PT-test enabled laboratories to evaluate their competence for the analysis of aflatoxins in pig feed.

For this proficiency test, three test materials were prepared:
- Blank pig feed;
- Pig feed containing AFB1 aimed at 8 µg/kg;
- Pig feed containing AFB1 aimed at 12 µg/kg, AFB2 at 2 µg/kg, AFG1 at 8 µg/kg and AFG2 at 2 µg/kg;

The fortified materials were all prepared by spiking slurried pig feed, followed by extensive mixing and lyophylization. During homogeneity testing, all materials proved to be sufficiently homogenous for proficiency testing. The stability test demonstrated that no statistically significant losses of AFB1, AFB2, AFG1 and AFG2 occurred during the timescale of the proficiency test.

Thirteen laboratories subscribed for participation in this test. A variety of purification methods was applied but no specific method achieved optimal results.

A total number of six questionable/unsatisfactory z-scores was reported and three false positive results were reported. For AFB1 in material B and AFB1, AFB2 in material C the variation in the reported results was high but the results could still be statistically evaluated. For AFG1 and AFG2 in material C the variation in the reported results was too high, so the results could not be statistically evaluated. Six participants obtained satisfactory performance by obtaining satisfactory z-scores, reporting no false positive or false negative results and by correctly assigning the samples as being compliant/non-compliant.

From this proficiency test it can be concluded that the quantification of aflatoxins in animal feed needs additional attention to decrease the high variation in the results.

1 Introduction

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 test was to give laboratories the possibility to evaluate or demonstrate their competence for the analysis of aflatoxins in pig feed. The preparation of the materials, including the suitability testing of the materials and the evaluation of the quantitative results were carried out according to ISO 17043 [3], but this matrix-compound combinations is not part of the scope of RIKILTs accreditation.

2 Material and methods

This proficiency test focused on aflatoxin, a mycotoxin which is produced by several fungi. The maximum allowable content for AFB1 in pig feed with a moisture content of 12% in the Netherlands/EU is 20 µg/kg [13].

2.1 Sample preparation

The contaminated materials were prepared by adding the content of commercial available and certified ampoules containing aflatoxin(s) to a slurried blank pig feed aiming at the levels as presented in Table 1. For material A the blank material was used as such. Materials B and C were homogenized using a blender according to in-house standard operating procedures [5], freeze-dried and homogenized again.

Table 1
Target amount of AFB1, AFB2, AFG1 and AFG2 in the proficiency test materials.

Material code	Target amount (µg/kg)			
	AFB1	AFB2	AFG1	AFG2
A	0	0	0	0
B	8	0	0	0
C	12	2	8	2

2.2 Sample identification

After freeze-drying and homogenization, all materials were divided into sub-portions of at least 40 gram and stored in polypropylene containers. The samples for the participants were randomly selected and coded using a website application. For each laboratory a sample set was prepared consisting of one at random selected sample of each material A, B and C.
The codes of the samples belonging to each sample set are presented in Annex 1. The remaining samples were used for homogeneity and stability testing.

2.3 Participants

Thirteen laboratories subscribed for participation in the proficiency test of which twelve are situated within Europe and one in South-America. Each participant was asked to report the results and whether the samples were compliant or non-compliant using a web application designed for proficiency tests.

2.4 Homogeneity study

The homogeneity of the materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [7] and ISO 13528 [8], taking into account the insights discussed by Thompson [9] 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 standard deviation for proficiency assessment derived from the Horwitz equation (σ_H, §3.3). The method applied for homogeneity testing was considered suitable if $s_w < 0.5*\sigma_H$ and a material was considered adequately homogeneous if $s_s < 0.3* \sigma_H$.

Ten containers of materials B and C were analysed in duplicate for AFB1 to determine the homogeneity of the material. The results of the homogeneity study and their statistical evaluation are presented in Annex 2. Both materials demonstrated to be sufficiently homogeneous for use in the proficiency test.

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 three coded samples (Annex 1) were sent to the participating laboratories on January 26th, 2015. The sample sets were packed in a box and were dispatched to the participants immediately by courier. The samples were accompanied by a letter (Annex 3) describing the requested analyses, the reporting requirements and an acknowledgement of receipt form. By e-mail the laboratories received instructions on how to use the web application to report results. All samples arrived in good condition.

The laboratories were asked to store the samples until analysis according to their own laboratory procedure. A single analysis of each sample was requested. The deadline for submitting the quantitative results was March 13th, 2015, allowing at least six weeks for the analysis.

2.6 Stability

On January 26th, 2015, the day the materials were distributed to the participants, four randomly selected samples of each material were stored at <-70 °C. It is assumed that the compounds included in this proficiency test are stable at this storage condition. Four samples were stored at +4°C and four were stored at room temperature.

On April 3rd 2015, 67 days after distribution of the samples, four samples that had been stored at <-70°C, four at +4°C and four at room temperature were analysed. For each set of samples, the average of the results and the standard deviation were calculated.

First it was determined if a 'consequential instability' occurred [7,8]. A consequential instability occurs when the average value of the samples at +4°C or at room temperature is more than $0.3\sigma_H$ below the average value of the samples stored at <-70°C. If so, the instability has a significant influence on the calculated z-scores. Secondly, it was determined whether a statistically significant instability occurred using a Students t-test [8]. The results and statistical evaluation of the stability test are presented in Annex 4.

For AFB1, AFB2, AFG1 and AFG2 no consequential nor a statistical significant difference was observed among the samples stored at <-70°C, the samples stored at <+4°C and the samples stored at room temperature. The samples were considered sufficiently stable.

3 Statistical evaluation

The statistical evaluation of the quantitative part of the study was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [7], elaborated by ISO, IUPAC and AOAC and ISO 13528 [8] in combination with the insights published by the Analytical Methods Committee [11,12] regarding robust statistics.

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

3.1 Calculation of the consensus value (X)

The consensus value (X) was determined using robust statistics [8,11,12]. 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 do 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 consensus value [8,11].

3.2 Calculation of the uncertainty of the consensus value (u)

The uncertainty of the consensus value is calculated to determine the influence of this uncertainty on the evaluation of the laboratories. A high uncertainty of the consensus value will lead to a high uncertainty of the calculated participants z_a-scores. If the uncertainty of the consensus 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 consensus value is not taken into account.

The uncertainty of the consensus value (the robust mean) is calculated from the estimation of the standard deviation of the consensus value and the number of values used for the calculation of the consensus value [8]:

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

where:

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

According to ISO 13528 [8] the uncertainty of the consensus 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 consensus value;
σ_P = Standard deviation for proficiency assessment (§3.3).

In case the uncertainty of the consensus value does not comply with this criterion, the uncertainty of the consensus value should be taken into account when evaluating the performance of the participants regarding the accuracy (§3.4). In case the uncertainty is > $0.7\sigma_P$ the calculated z-scores should not be used for evaluation of laboratories' performance and are not presented.

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

According to Commission Decision 2002/657/EC [6], the coefficient of variation for the repeated analysis of a reference or fortified material under reproducibility conditions, shall not exceed the level calculated by the Horwitz equation. The Horwitz equation, $\sigma_H = 0.02c^{0.8495}$, presents a useful and widespread applied relation between the expected relative standard deviation of a singular analysis result under reproducibility conditions, and the concentration, c (g/g). It expresses inter-laboratory precision expected in inter-laboratory trials. Therefore, this relation is suitable for calculating the standard deviation for proficiency assessment (σ_P).

Thompson [7] demonstrated that the Horwitz equation is not applicable to the lower concentration range (<120 µg/kg). Therefore a complementary model is applied:

For analyte concentrations <120 µg/kg:
$\sigma_P = 0.22c$

where:
σ_P = standard deviation in proficiency assessment;
c = concentration of the analyte (µg/g).

3.4 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, ISO 13528 [8] is applied. According to these guidelines z_a-scores are classified as presented in Table 2.

Table 2
Classification of z_a-scores.

$\lvert z_a \rvert \leq 2$	Satisfactory
$2 < \lvert z_a \rvert < 3$	Questionable
$\lvert z_a \rvert \geq 3$	Unsatisfactory

If the calculated uncertainty of the consensus value complies with the criterion mentioned in §3.2, 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;
$\bar{x}$ = the result reported by the laboratory;
X = consensus value;
σ_P = standard deviation for proficiency assessment (Horwitz or Thompson).

However, if the uncertainty of the consensus value does not comply with the criterion mentioned in §3.2, it could influence the evaluation of the laboratories. Although, according to ISO 13528 in this case no z-scores can be calculated, 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 [8]:

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

where:
z'_a = accuracy z-score taking into account the uncertainty of the consensus value;
$\bar{x}$ = the result of the laboratory;
X = consensus value;
σ_P = standard deviation for proficiency assessment (Horwitz or Thompson);
u = uncertainty of the consensus value.

If a consequential instability of the proficiency test materials is observed, this can influence the evaluation of the laboratory performance. Then 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 case of a consequential instability, the accuracy z-score for the laboratories that reported a concentration below the consensus value is corrected for this instability by:

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

where:
z_{ai} = accuracy z-score taking into account the instability of the consensus value;
$\bar{x}$ = the result reported by the laboratory;
X = consensus value;
σ_P = standard deviation for proficiency assessment (Horwitz or Thompson);
Δ = difference between average concentration of compound stored at -70°C, stored at +4°C or at room temperature.

In some cases the uncertainty of the consensus value does not comply with the criterion in §3.2 and a consequential instability is observed. In this case the z'_a score for the laboratories that reported an amount below the consensus value is corrected for this instability by:

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

where:
z'_{ai} = accuracy z-score taking into account the uncertainty and instability of the consensus value;
$\bar{x}$ = the result reported by the laboratory;
X = consensus value;
σ_P = standard deviation for proficiency assessment (Horwitz or Thompson);
Δ = difference between average concentration of compound stored at -70 °C, stored at +4°C or at one day room temperature;
u = uncertainty of the consensus value.

4 Methods and results

4.1 Applied methods

Thirteen laboratories reported results for the proficiency test for aflatoxin in pig feed. An overview of the applied methods is presented in Annex 5. One participant (PT188) applied two methods. Of the thirteen participants, five reported the use of immuno affinity chromatography or filtration as clean-up. Others reported a clean-up based on SPE, extraction with water, organic solvent or a combination of these. Five labs used an internal standard. Thirteen labs applied instrumental detection techniques like LC-MS/MS and LC-FLD and one lab applied an immunorecepter assay. Reported LODs for AFB1 varied from 0.2 to 0.5 µg/kg and LOQs from 0.5 to 1.5 µg/kg.

4.2 Material A

No aflatoxins were added to material A. Every participant, except PT184, assigned this sample as compliant. PT184 did not report whether the sample was compliant or non-compliant. Lab PT188, which applied the immunoreceptor assay, reported a sum concentration of aflatoxins of 2 µg/kg, which is considered a false positive result. The performance of the individual labs is summarized in Annex 8.

4.3 Material B

All participants reported results for AFB1 in material B (Annex 6). Every participant, except PT184, assigned this sample as compliant according to Direction 2002/32/EC [13]. Two false positive results were reported in this proficiency test by lab PT183. This lab reported the presence of 1.7 µg/kg AFG1 and 0.4 µg/kg AFG2.
The lowest value AFB1 reported was 2.4 µg/kg and the highest value was 10.86 µg/kg. The consensus value was 7.2 µg/kg with a robust standard deviation of 2.8 µg/kg. This is almost two times higher than the value suggested by Thompson: 1.6 µg/kg. The uncertainty of the consensus value was 0.94 µg/kg which exceeded $0.3\sigma_P$ (§3.2), so the uncertainty was taken into account in the evaluation. With respect to the accuracy one result was questionable (PT187).

4.4 Material C

Lab PT188 reported a sum concentration of aflatoxins of 7 µg/kg based on the immunoreceptor assay. Every participant, except PT184, assigned this sample as compliant.

4.4.1 AFB1

All participants reported results for AFB1 in material C (Annex 7). The lowest value reported was 4 µg/kg and the highest value was 13.4 µg/kg. The consensus value was 9.3 µg/kg with a robust standard deviation of 3.9 µg/kg. This is almost two times higher than the value suggested by Thompson: 2.1 µg/kg. The uncertainty of the consensus value was 1.3 µg/kg which exceeds $0.3\sigma_P$ (§3.2), so the uncertainty was taken into account in the evaluation. With respect to the accuracy two results were questionable (PT184 and PT187).

4.4.2 AFB2

All participants reported results for AFB2 in material C (Annex 7). The lowest value reported was 1.3 µg/kg and the highest value was 8.2 µg/kg. The consensus value was 2.2 µg/kg with a robust standard deviation of 0.68 µg/kg. This was almost 1.5 times higher than the value suggested by Thompson: 0.48 µg/kg. The uncertainty of the consensus value was 0.24 µg/kg which exceeds $0.3\sigma_P$ (§3.2), so the uncertainty was taken into account in the evaluation. With respect to the accuracy one result was questionable (PT191) and two were unsatisfactory (PT182 and PT192).

4.4.3 AFG1

All participants reported results for AFG1 in material C (Annex 7). The lowest value reported was 2.7 µg/kg and the highest value was 19.69 µg/kg. The consensus value was 7.3 µg/kg with a robust standard deviation of 3.3 µg/kg. This was two times higher than the value suggested by Thompson: 1.6 µg/kg. The uncertainty of the consensus value was 1.1 µg/kg which exceeds $0.7\sigma_P$ (§3.2), so statistical evaluation was not appropriate (§3.2). However, Annex 7 shows z'_a-scores, but these are presented for information only.

4.4.4 AFG2

All participants reported results for AFG2 in material C (Annex 7). The lowest value reported was 0.6 µg/kg and the highest value was 4.09 µg/kg. The consensus value was 2.2 µg/kg with a robust standard deviation of 1.4 µg/kg. This was three times higher than the value suggested by Thompson: 0.48 µg/kg. The uncertainty of the consensus value was 0.50 µg/kg which exceeds $0.7\sigma_P$ (§3.2), so statistical evaluation was not appropriate (§3.2). However, Annex 7 shows z'_a-scores, but these are presented for information only.

5 Conclusions

Thirteen laboratories subscribed for the proficiency test of aflatoxin in pig feed and received. Six labs (46%) showed optimal performance by obtaining |z-scores| <2, reporting no false positive or false negative results and assigning the samples correctly as compliant. A variety of purification methods was applied but no specific method achieved optimal results.

A total number of six questionable/unsatisfactory z-scores was reported and three false positive results were reported. For AFB1 in material B and AFB1, AFB2 in material C the variation in the reported results was high but could still be statistically evaluated. For AFG1 and AFG2 in material C the variation in the reported results was too high to be statistically evaluated.
From this proficiency test it can be concluded that the quantification of aflatoxins in animal feed needs additional attention since a high variation in the reported results occurred. No causes for deviations could be identified.

References

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11 Analytical Methods Committee. 1989. Robust statistics – How not to reject outliers Part 1. Basic concepts. Analyst 114:1693-1697.

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Annex 1 Codification of the samples

Lab number	Material A*	Material B*	Material C*
PT096	782	850	451
PT181	556	167	546
PT182	209	997	458
PT183	440	854	498
PT184	416	120	237
PT185	362	513	814
PT186	253	221	464
PT187	606	381	762
PT188	577	478	255
PT189	951	343	244
PT190	214	564	450
PT191	299	834	126
PT192	740	851	519

* All sample codes start with AFLA/2014/FEED/.

Annex 2 Statistical evaluation of homogeneity data

AFB1 in material B (µg/kg)		
Sample number	Replicate 1	Replicate 2
Hom/B001	7.9	8.2
Hom/B002	8.2	7.9
Hom/B003	8.1	8.1
Hom/B004	8.1	8.2
Hom/B005	8.3	8.2
Hom/B006	8.1	8.1
Hom/B007	8.2	7.8
Hom/B008	7.9	7.8
Hom/B009	7.9	7.8
Hom/B010	8.0	8.0
Grand mean	8.0	
Cochran's test		
C	0.363	
C_{crit}	0.602	
$C<C_{crit}$?	NO OUTLIERS	
Target s = σ_H	Horwitz/Thompson: 1.77	
s_x	0.12	
s_w	0.15	
s_s	0.05	
Critial = $0.3\sigma_H$	0.53	
s_s<critical?	ACCEPTED	
$s_w<0.5\sigma_H$?	ACCEPTED	

AFB1 in material C (µg/kg)		
Sample number	Replicate 1	Replicate 2
Hom/C001	12.0	12.1
Hom/C002	12.3	12.2
Hom/C003	12.0	12.2
Hom/C004	12.0	12.0
Hom/C005	12.7	12.1
Hom/C006	12.3	12.0
Hom/C007	12.4	12.7
Hom/C008	12.1	12.6
Hom/C009	12.4	12.3
Hom/C010	12.6	12.2
Grand mean	12.3	
Cochran's test		
C	0.353	
C_{crit}	0.602	
$C<C_{crit}$?	NO OUTLIERS	
Target s = σ_H	Horwitz/Thompson: 2.70	
s_x	0.18	
s_w	0.23	
s_s	0.08	
Critial = $0.3\sigma_H$	0.81	
s_s<critical?	ACCEPTED	
$s_w<0.5\sigma_H$?	ACCEPTED	

Annex 3 Instruction letter

RIKILT
WAGENINGEN UR

For quality of life

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

DATE
January 15, 2015

SUBJECT
Proficiency test aflatoxin B1 in animal feed

OUR REFERENCE
15/RIK0067

POSTAL ADDRESS
P.O. Box 230
6700 AE WAGENINGEN
The Netherlands

VISITORS' ADDRESS
Wageningen Campus
Building 123
Akkermaalsbos 2
6708 WB WAGENINGEN

INTERNET
www.wageningenUR.nl/rikilt

COC NUMBER
09098104

HANDLED BY
Ir. I.J.W. Elbers

TELEPHONE
+31 (0)317 480 314

EMAIL
pt.rikilt@wur.nl

Dear participant,

Thank you very much for your interest in the proficiency test for the analysis of aflatoxin B1 in animal feed.
Hereby I send you a parcel containing three randomly coded samples.

Please fill out the accompanied 'acknowledgement of receipt form' and return it immediately upon receipt of the samples, preferably by e-mail.

Instructions:

- After arrival store the samples according to your laboratory's procedures.
- Homogenize them according to your laboratory's procedures.
- Please analyze the samples according to your routine method for aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2.
- Carry out a **single analysis** for each sample.
- Report the results in µg/kg, product based.
- Also report whether the samples are compliant or non-compliant.
- Please upload your results via the webapplication (https://crlwebshop.wur.nl/apex/f?p=307:1000) before **March 13 2015.**

- Your username is:
- Your password is:
- Your labcode to enter the proficiency test site is:

- A report of the test will be dispatched in July 2015.
- Results of the test will be presented anonymously.
- RIKILT is allowed to use the anonymous results of the test in presentations, seminars and publications.

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

With kind regards,

IJWElbers

Ingrid Elbers

Wageningen UR (Wageningen University and various research institutes) is specialised in the domain of healthy food and living environment.

RIKILT, part of Wageningen UR, carries out research into the safety, quality and health of food and feed and provides consultancy services to (inter)national governmental authorities. RIKILT is ISO 17025 and ISO 17043 accredited (the accredited tests are described on www.rva.nl (no. L014 and R013).

Annex 4 Statistical evaluation of stability data

Statistical evaluation for AFB1 in material B			
Storage temp	-70 °C	+4°C	room temperature
Time in freezer (days)	67	67	67
Calculated amounts (µg/kg)	8.3	8.3	7.9
	7.6	7.8	7.8
	7.5	8.3	8.2
	8.2	7.8	7.9
Average amount (µg/kg)	7.9	8.0	7.9
n	6	6	6
Standard deviation (µg/kg)	0.40	0.25	0.17
Difference		-0.1	0.0
$0.3\sigma_H$		0.52	0.52
Consequential difference? Diff < 0.3 σ_H		NO	NO
t		0.56	0.16
t_{crit}		2.45	2.45
Statistical difference? T < t_{crit}		NO	NO

Statistical evaluation for AFB1 in material C			
Storage temp	-70 °C	-20°C	room temperature
Time in freezer (days)	67	67	67
Calculated amounts (µg/kg)	8.9	9.3	9.6
	8.7	9.3	9.0
	9.1	9.4	9.6
	9.3	9.2	9.1
Average amount (µg/kg)	9.0	9.3	9.3
n	6	6	6
Standard deviation (µg/kg)	0.28	0.08	0.31
Difference		-0.3	-0.3
$0.3\sigma_H$		0.59	0.59
Consequential difference? Diff < 0.3 σ_H		NO	NO
t		2.13	1.39
t_{crit}		2.45	2.45
Statistical difference? T < t_{crit}		NO	NO

Statistical evaluation for AFB2 in material C			
Storage temp	-70 °C	-20°C	room temperature
Time in freezer (days)	67	67	67
Calculated amounts (µg/kg)	2.7	2.6	2.7
	2.4	2.6	2.6
	2.7	2.7	2.7
	2.7	2.7	2.6
Average amount (µg/kg)	2.6	2.6	2.6
n	6	6	6
Standard deviation (µg/kg)	0.14	0.06	0.09
Difference		0.0	0.0
$0.3\sigma_H$		0.17	0.17
Consequential difference? Diff < 0.3 σ_H		NO	NO
t		0.07	0.02
t_{crit}		2.45	2.45
Statistical difference? T < t_{crit}		NO	NO

Statistical evaluation for AFG1 in material C			
Storage temp	-70 °C	-20°C	room temperature
Time in freezer (days)	67	67	67
Calculated amounts (µg/kg)	7.7	7.8	7.5
	7.7	7.6	7.4
	7.7	7.8	7.4
	7.8	7.6	7.3
Average amount (µg/kg)	7.7	7.7	7.4
n	6	6	6
Standard deviation (µg/kg)	0.04	0.14	0.10
Difference		0.0	0.3
$0.3\sigma_H$		0.51	0.51
Consequential difference? Diff < 0.3 σ_H		NO	NO
t		0.38	6.73
t_{crit}		2.45	2.45
Statistical difference? T < t_{crit}		NO	YES

Statistical evaluation for AFB2 in material C			
Storage temp	-70 °C	-20°C	room temperature
Time in freezer (days)	67	67	67
Calculated amounts (µg/kg)	2.1	2.1	2.0
	2.0	2.0	2.0
	2.2	2.1	2.0
	2.1	2.0	2.1
Average amount (µg/kg)	2.1	2.1	2.0
n	6	6	6
Standard deviation (µg/kg)	0.05	0.03	0.03
Difference		0.0	0.1
$0.3\sigma_H$		0.14	0.14
Consequential difference? Diff < 0.3 σ_H		NO	NO
t		1.22	2.04
t_{crit}		2.45	2.45
Statistical difference? T < t_{crit}		NO	NO

Annex 5 Overview of the applied methods

Lab	Sample purificiation	Internal standard	Detection method
PT096	SPE	yes	MS/MS
PT181	Immuno affinity	None	HPLC Fluorescence
PT182	none	U-[13C17]-AFB1	LC-MS/MS
PT183	immunoafinity column		HPLC-FLD
PT184	extraction acetone 1-% aqueous NaCl (4:1), 15% aqueous lead acetate, filtration, defatting liquid-liquid extraction with hexane, aqueous phase was reextracted by chloroform-acetone	-	HPLC-FLD and LC-MS/MS
PT185	Immuno-affinity	-	HPLC-FLD
PT186	Extraction organic solvent	-	LC-MS/MS
PT187	Immuno-filtratie	-	HPLC-FLD
PT188	imunoaffinity column		Postcolumn derivatisation with FLD
PT188	extraction with water	-	Immunoreceptor assay utilizing lateral flow technology
PT189	none	C13 aflatoxin B1	LC-MS/MS
PT190	Een bepaalde hoeveelheid monster wordt in een mengsel van methanol en water geextraheerd. Het extract wordt na filtratie over een microfilter m.b.v. HPLC chromatografisch geanalyseerd.		LC-MS/MS
PT191		C-13 afla's	UPLC-MS/MS
PT192	LC	Aflatoxin B1 13C17	MS/MS

Annex 6 Results for AFB1 in material B

	AFB1 X: 7.2 u: 0.94 σ_P: 1.6 Robust sd: 2.8		Compliant?
Lab code	Result (µg/kg)	z_{ai}-score	
PT096	8.73	0.81	yes
PT181	8	0.41	yes
PT182	9.9	1.44	yes
PT183	7	-0.13	yes
PT184	3.7	-1.91	
PT185	7.2	-0.02	yes
PT186	7.4	0.09	yes
PT187	2.4	**-2.62**	yes
PT188 (LC-FLD)	5.3	-1.05	yes
PT188 (immuno)	5 (sum aflatoxin)	-1.21	yes
PT189	9.89	1.43	yes
PT190	5.4	-0.99	yes
PT191	9.8	1.38	yes
PT192	10.86	1.96	yes

X consensus value

u uncertainty

sd standard deviation

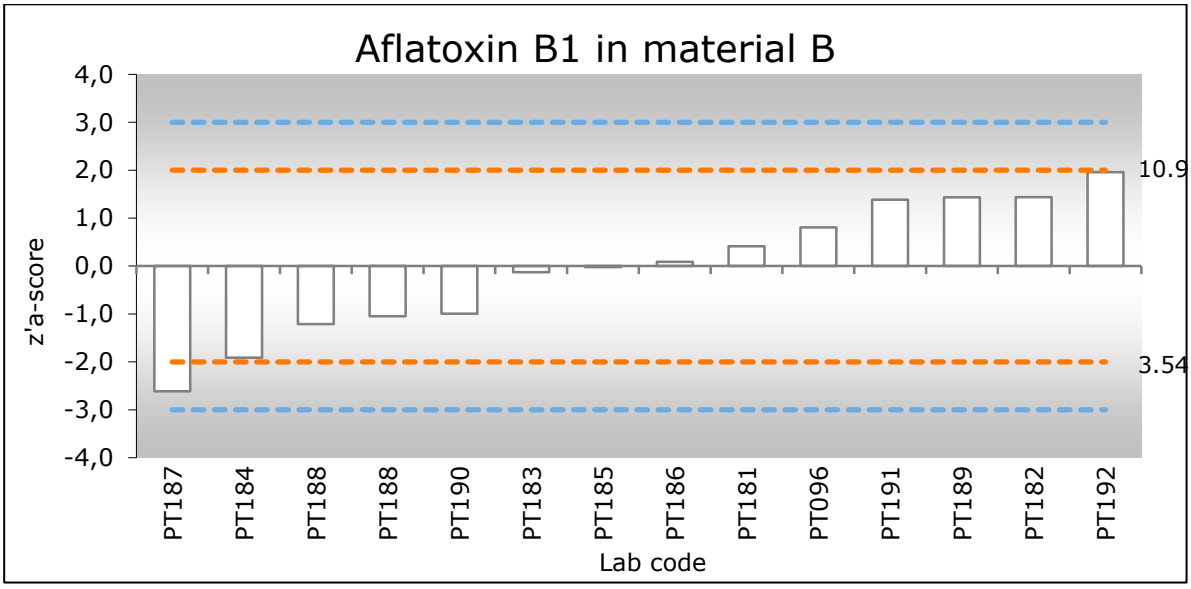

Figure a *Graphical representation of the reported results for AFB1 in material B. The X ± 2σ_p lines (dotted) are calculated according to equation II in §3.4.*

Annex 7 Results for material C

Lab code	AFB1 X: 9.3 u: 1.3 σ_p: 2.1 Robust sd: 3.9		AFB2 X: 2.2 u: 0.24 σ_p: 0.48 Robust sd: 0.68		AFG1 X: 7.3 u: 1.1 σ_p: 1.6 Robust sd: 3.3		AFG2 X: 2.2 u: 0.50 σ_p: 0.48 Robust sd: 1.4		Compliant?
	Result (µg/kg)	z'_a	Result (µg/kg)	z'_a	Result (µg/kg)	z'_a PRESENTED FOR INFORMATION ONLY	Result (µg/kg)	z'_a PRESENTED FOR INFORMATION ONLY	
PT096	11.7	0.97	3.01	1.50	9.27	1.02	2.75	0.82	yes
PT181	10.11	0.32	1.84	-0.67	10.33	1.56	1.23	-1.37	yes
PT182	13.4	1.67	8.2	**11.12**	9.6	1.18	9	**9.84**	yes
PT183	8.7	-0.25	2.1	-0.19	7.1	-0.09	1.9	-0.41	yes
PT184	3.8	**-2.25**	2.2	-0.01	2.7	**-2.33**	0.7	**-2.14**	
PT185	7.8	-0.62	2	-0.38	4.4	-1.47	1	-1.71	yes
PT186	8.8	-0.21	2	-0.38	6	-0.65	1.4	-1.13	yes
PT187	4	**-2.17**	1.4	-1.49	3.9	-1.72	1.3	-1.27	yes
PT188	7.9	-0.58	1.7	-0.93	6.6	-0.35	0.6	**-2.28**	yes
PT188	sum aflatoxin 7 µg/kg								yes
PT189	12.66	1.36	2.28	0.14	10.12	1.45	3.53	1.94	yes
PT190	6.3	-1.23	1.3	-1.67	5.1	-1.11	4.6	**3.49**	yes
PT191	12.2	1.18	3.5	**2.41**	7.5	0.11	2.5	0.46	yes
PT192	13.78	1.82	5.16	**5.48**	19.69	**6.33**	4.09	**2.75**	yes

X consensus value

sd standard deviation

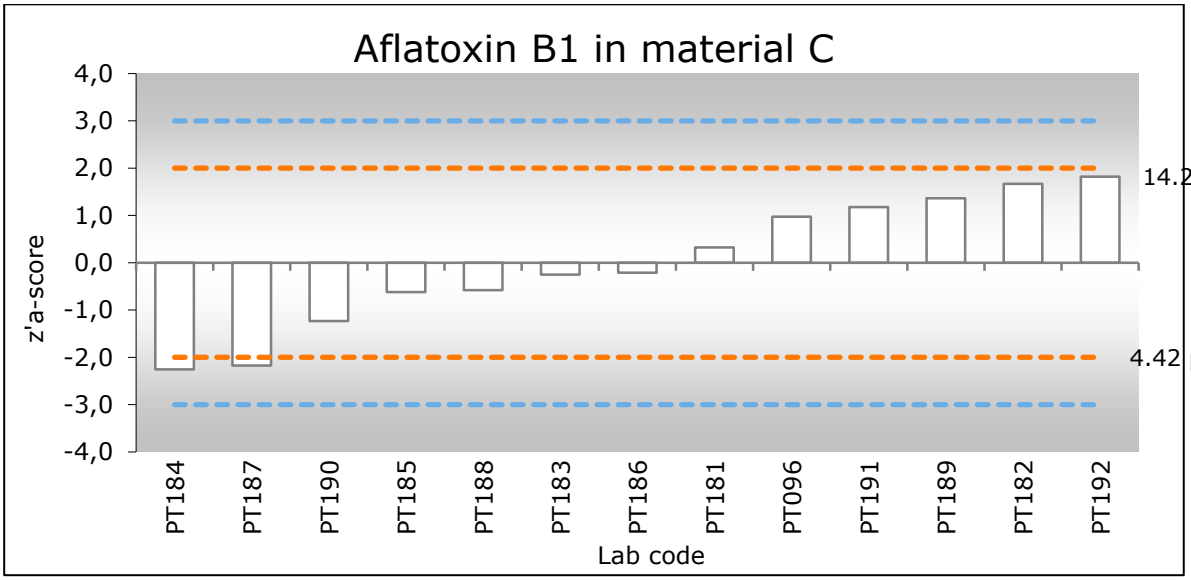

Figure a *Graphical representation of the reported results for AFB1 in material C. The X ± 2σp lines (dotted) are calculated according to equation II in §3.4.*

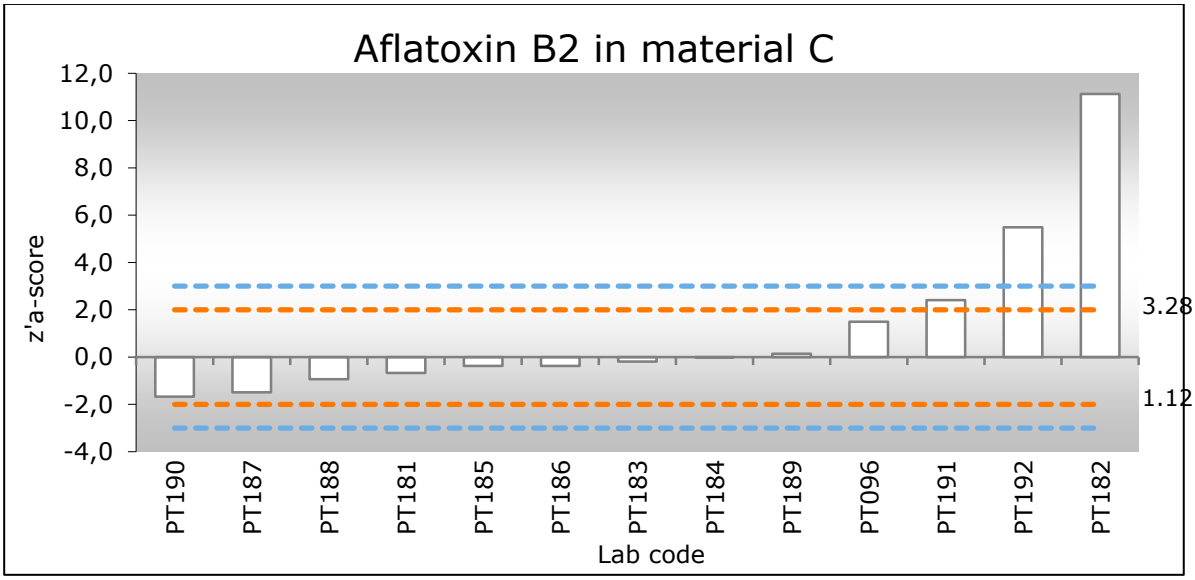

Figure a *Graphical representation of the reported results for AFB2 in material C. The X ± 2σp lines (dotted) are calculated according to equation II in §3.4.*

Annex 8 Overall performance

Lab	Performance
PT096	optimal performance
PT181	optimal performance
PT182	1 u
PT183	2 FP
PT184	1 q, did not report compliancy of samples
PT185	optimal performance
PT186	optimal performance
PT187	2 q
PT188	1 FP
PT189	optimal performance
PT190	optimal performance
PT191	1 q
PT192	1 u

FP	false positive
FN	false negative
q	questionable result
u	unsatisfactory result

RIKILT Wageningen UR
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
www.wageningenUR.nl/en/rikilt

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

RIKILT Wageningen UR
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
www.wageningenUR.nl/en/rikilt

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