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Report on the 2007 Proficiency Test of the Community Reference Laboratory Network

Determination of Aflatoxins in a Peanut Product and a Test Solution

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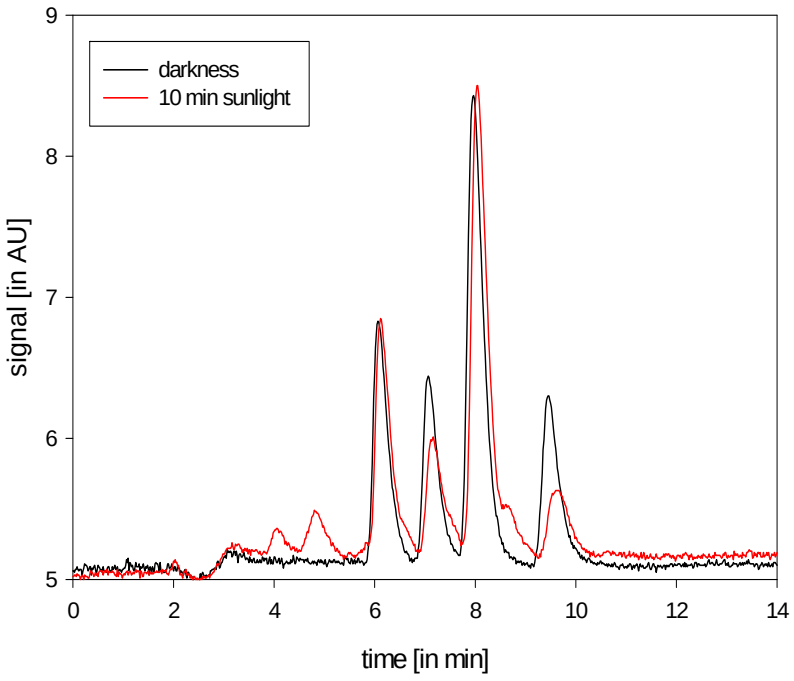

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The mission of the IRMM is to promote a common and reliable European measurement system in support of EU policies.

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Summary

A proficiency test was conducted with 31 European National Reference Laboratories (NRLs) for mycotoxins and one Laboratory from a candidate country. Test materials were a mixed aflatoxin (Af) solution in acetonitrile and two candidate Certified Reference Materials (CRM) - one "aflatoxin positive" and one "blank" material - that have not yet been released. Laboratories determined the aflatoxin content by reverse-phase high-performance liquid-chromatography (RP-HPLC) with either fluorescence or mass-selective detection against their own standard solutions as reference.

Applying the modified Horwitz equation according to Thompson[1] as a basis for the target standard deviation (22% in the case of this proficiency test), 26 out of 32 laboratories achieved z-scores of less than 2 and 17 laboratories reported values within the uncertainty range for both aflatoxin B1 and total aflatoxins in the candidate CRM after correction for recovery in both cases.

[1] M. Thompson (2000) *Analyst*, **125**, 385-386

Introduction

In 2006 the Institute for Reference Materials and Measurements (IRMM) in Geel was nominated as Community Reference Laboratory (CRL) for mycotoxins by the Directorate General for Health and Consumer Protection (DG SANCO). One of the main responsibilities of the CRL is to organise comparative testing to benchmark and harmonise the measurement capabilities of national reference laboratories (NRLs) working in the same field. Therefore, the CRL for mycotoxins together with the network of NRLs agreed to conduct the proficiency test in 2007 (PT2007) as follow up action to the PT 2006, this time on an aflatoxin solution in acetonitrile and a peanut material. This approach was chosen as it evaluates the first step, namely the calibration, as well as the analysis of the test material.

Methodology

Aflatoxin (Af) solutions were produced from the CRM calibrants that were used in the PT2006 and resulted in a mixed Af solution for which the assigned values are given in Table 1. In Table 2 the assigned values for the candidate CRM peanut material are given. The Af concentration values in the Af test solution was not known to the participants.

Table 1: Assigned values of the mixed aflatoxin test solution (the coverage factor k=2 corresponding to a level of confidence of about 95 %)

Mixed Af solution	µg/L	Uncertainty (k=2)
AfB1	20.85	0.61
AfB2	5.95	0.13
AfG1	5.90	0.21
AfG2	5.95	0.12

Table 2: Assigned values of aflatoxins in peanut (the coverage factor k=2 corresponding to a level of confidence of about 95 %)

Peanut CRM	µg/kg	Uncertainty (k=2)
AfB1	1.77	0.29
AfB2	0.48	0.07
AfG1	0.92	0.32
AfG2	0.31	0.12

A full report on the production and certification of the CRM calibrants that were used to prepare the mixed aflatoxin calibrant solution is available from the IRMM. The report concerning the peanut CRMs will be available upon the final certification of these materials by the RM-Unit of the IRMM.

Each participant received an ampoule containing the Af test solution and two peanut materials ("Af positive" and "blank"). Participants were asked to measure the Af positive peanut material and the test solution for four aflatoxins, and to spike the blank peanut material with their own calibrant, reporting the spiking level and amount found to obtain recovery information. The instructions as sent to the participants are included in the annex.

Results and Discussion:

For each tested material the individual aflatoxin results are listed in **Tables 3 – 7** in the annex. As the repeatability for the three required measurements for each material and aflatoxin was in the lower %-range with an average of 5 %, all further calculations for the performance were made on the mean values calculated from the three measurements for each material/aflatoxin. In addition, no correlation was observed between the calculated repeatability values and the obtained z-scores. z-scores were calculated on the basis of the modified Horwitz equation according to Thompson. As a result, in all cases a target standard deviation of 22% was taken for z = |1|.

Deviation from the reference values

Figure 1 depicts the ranking of the results of the participating laboratories for aflatoxin B_1 in peanut prior recovery correction and prior calibrant correction. The order is by increasing laboratory mean value. The reference value and its uncertainty are depicted by a black and a blue line, respectively. The limit for a z-score of z =|2| is indicated by red lines. For twelve of the laboratories the calculated laboratory mean values fell within that range of the assigned value. For twenty-six of the laboratories the calculated laboratory mean values fell within a z-score limit of 2. Seven laboratories reported values outside this limit.

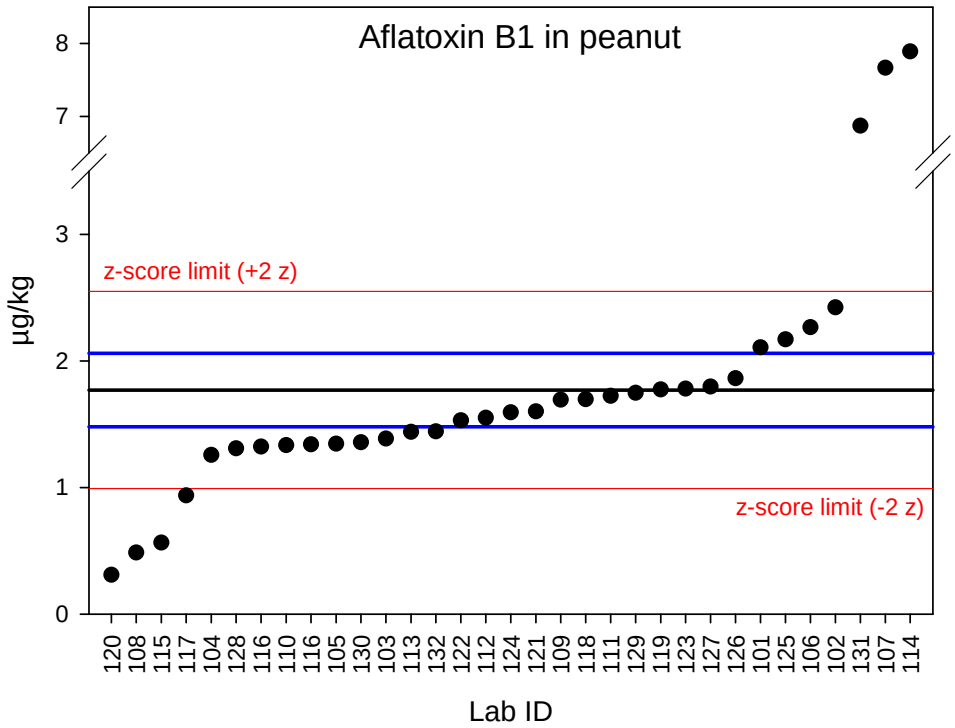
Figure 2 depicts the ranking of the results of the participating laboratories for aflatoxin B_2 in peanut prior recovery correction and prior calibrant correction. The order is by increasing laboratory mean value. The reference value and its uncertainty are depicted by a black and a blue line, respectively. The limit for a z-score of z = |2| is indicated by red lines for Figures 1 - 9. For thirteen of the laboratories the calculated laboratory mean values fell within that range of the assigned value. For twenty-four of the laboratories the calculated laboratory mean values fell within a z-score limit of 2.

Figure 3 depicts the ranking of the results of the participating laboratories for aflatoxin G_1 in peanut prior recovery correction and prior calibrant correction. The order is by increasing laboratory mean value. For twenty of the laboratories the calculated laboratory mean values fell within that range of the assigned value. For twenty-three of the laboratories the calculated laboratory mean values fell within a z-score limit of 2.

Figure 4 depicts the ranking of the results of the participating laboratories for aflatoxin G_2 in peanut prior recovery correction and prior calibrant correction. The order is by increasing laboratory mean value. For fourteen of the laboratories the calculated laboratory mean values fell within that range of the assigned value. For seventeen of the laboratories the calculated laboratory mean values fell within a z-score limit of 2.

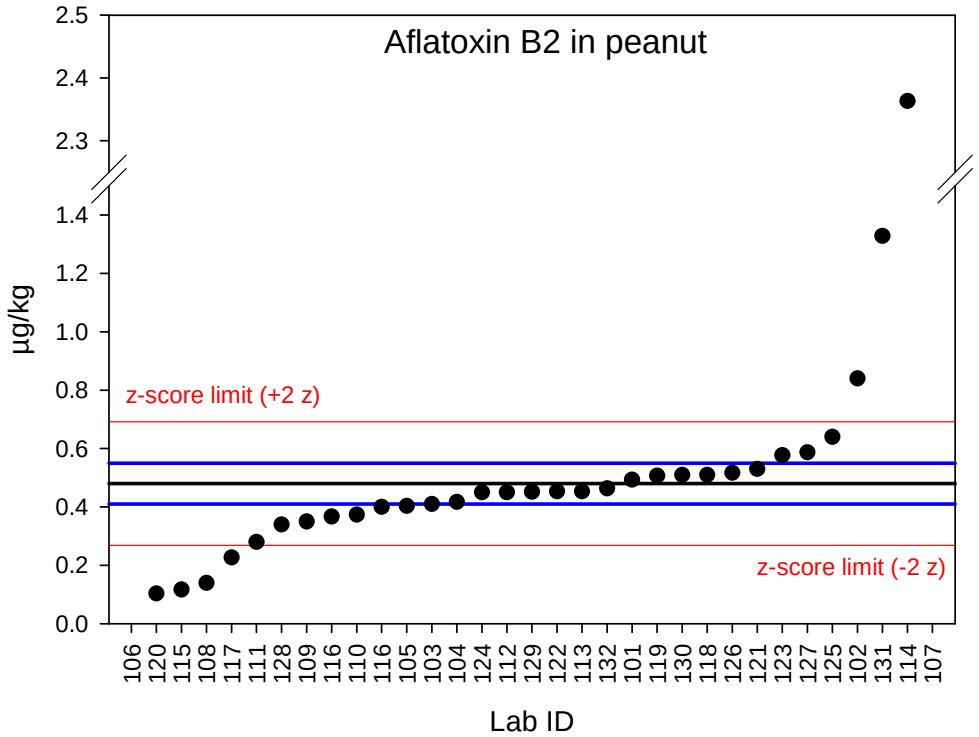
Figure 5 depicts the ranking of the results of the participating laboratories for the sum of aflatoxins (total Af) in peanut prior recovery correction and prior calibrant correction. The order is by increasing laboratory mean value. For eleven of the laboratories the calculated laboratory mean values fell within that range of the assigned value. For twenty-six of the laboratories the calculated laboratory mean values fell within a z-score limit of 2.

Figure 1: Plot of Aflatoxin B1 in peanut prior recovery correction.



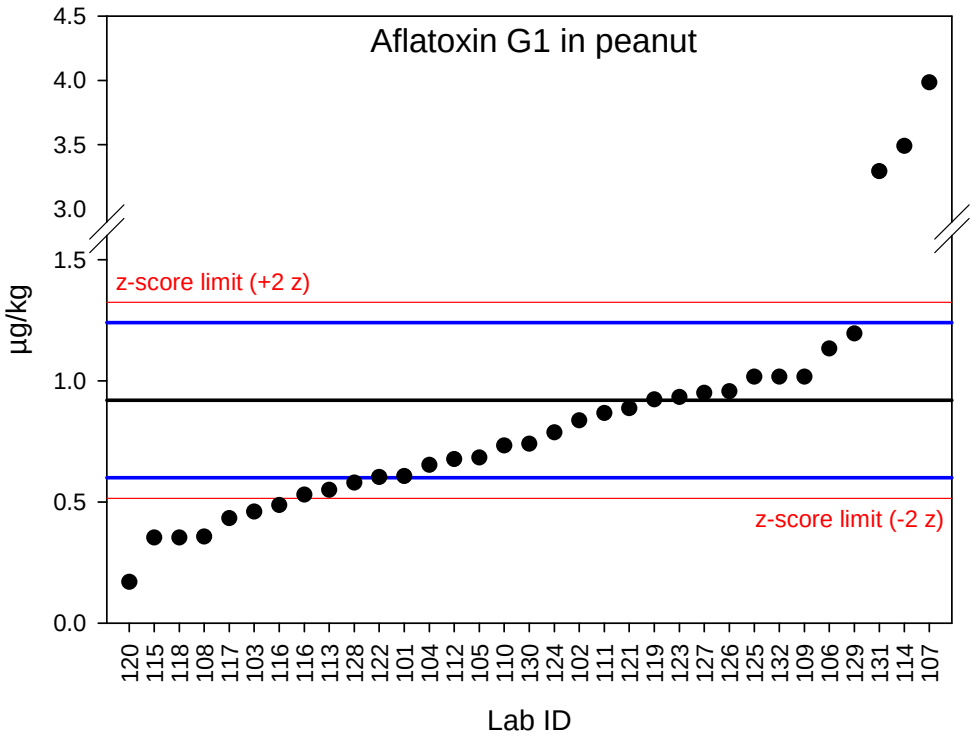
Plot of mean values from replicate measurements (n=3) for the determination of Aflatoxin B1 in a naturally contaminated peanut product. Blue lines reflect the uncertainty range of the reference value (black line), red lines the z-score limit of z=|2|.

Figure 2: Plot of Aflatoxin B2 in peanut prior recovery correction.



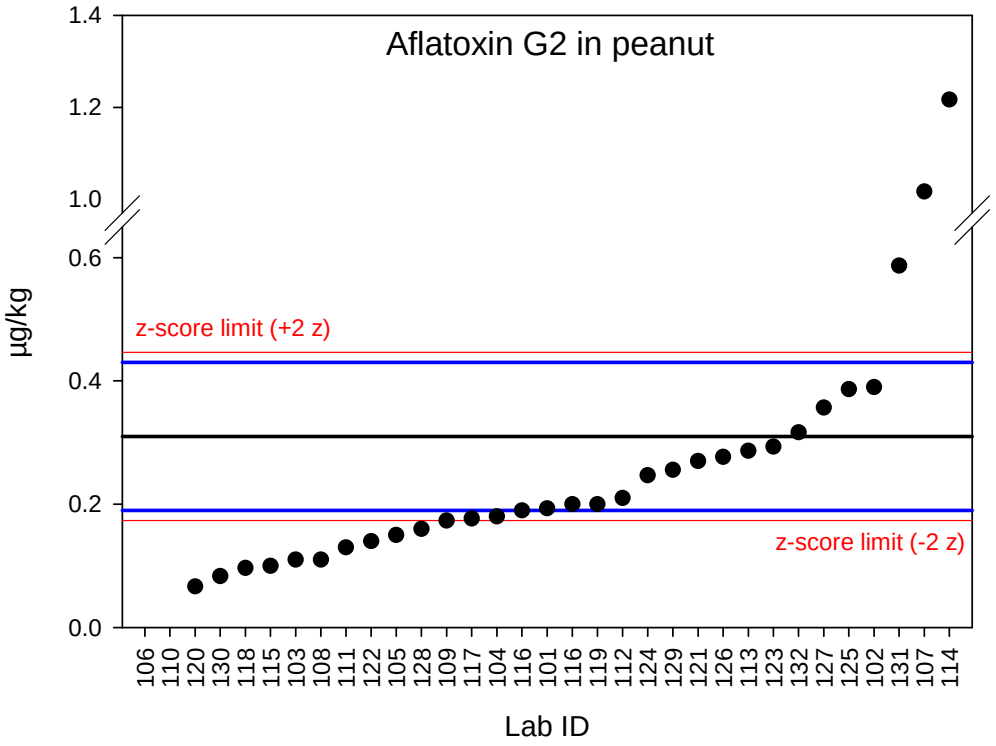
Plot of mean values from replicate measurements (n=3) for the determination of Aflatoxin B2 in a naturally contaminated peanut product. Blue lines reflect the uncertainty range of the reference value (black line), red lines the z-score limit of z=|2|.

Figure 3: Plot of Aflatoxin G1 in peanut prior recovery correction.



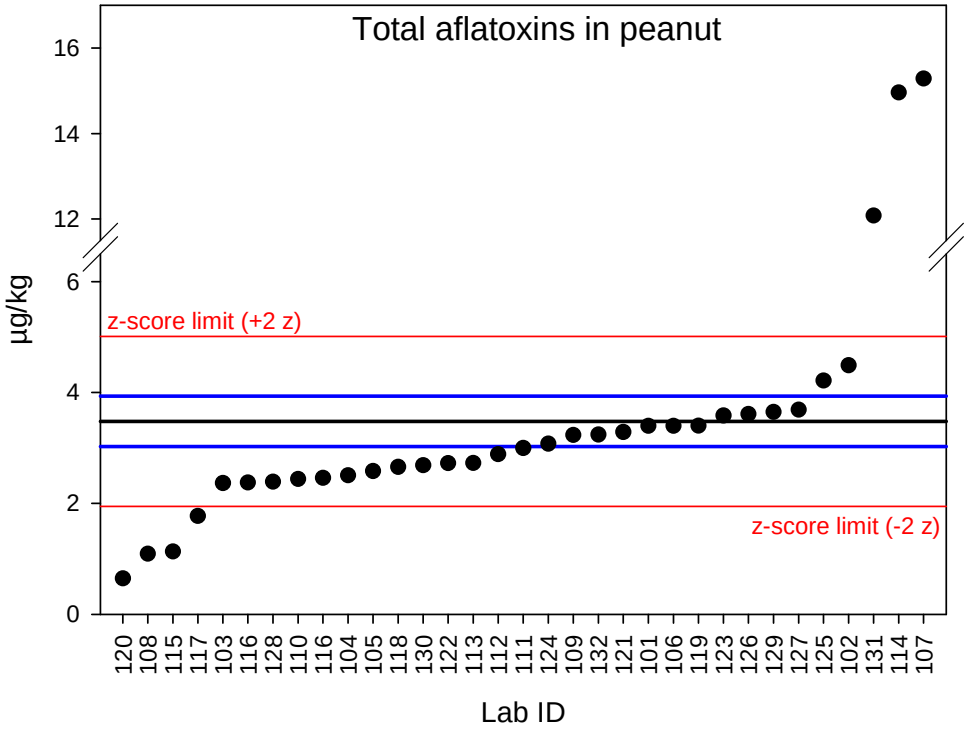
Plot of mean values from replicate measurements (n=3) for the determination of Aflatoxin G1 in a naturally contaminated peanut product. Blue lines reflect the uncertainty range of the reference value (black line), red lines the z-score limit of z=|2|.

Figure 4: Plot of Aflatoxin G2 in peanut prior recovery correction.



Plot of mean values from replicate measurements (n=3) for the determination of Aflatoxin G2 in a naturally contaminated peanut product. Blue lines reflect the uncertainty range of the reference value (black line), red lines the z-score limit of z=|2|.

Figure 5: Plot of total aflatoxins in peanut prior recovery correction.

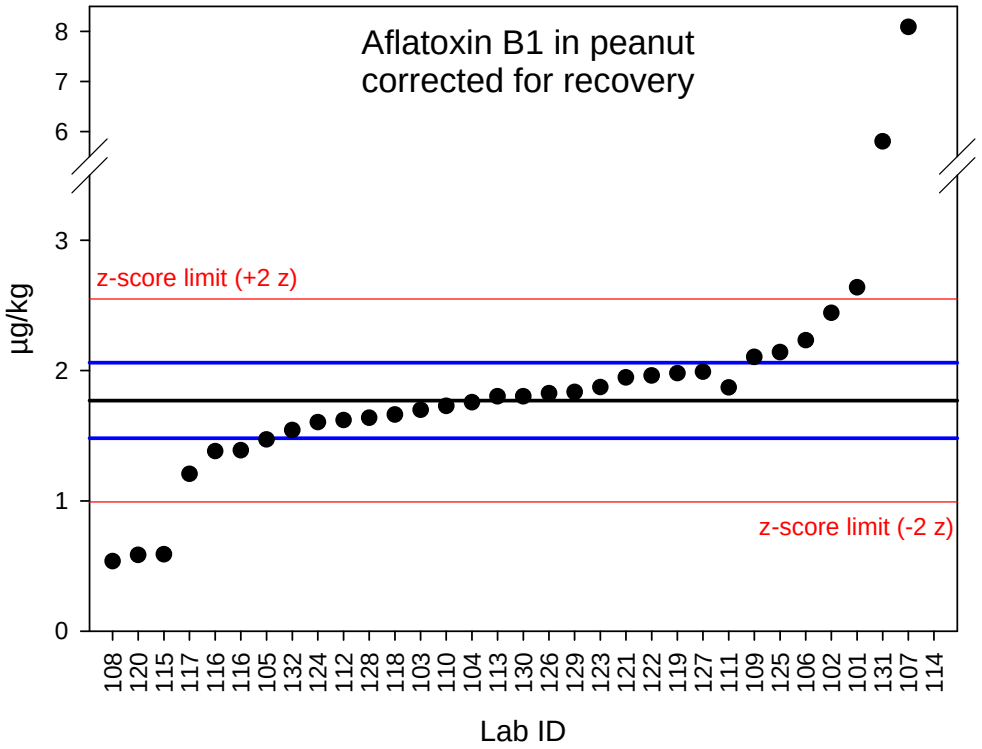


Plot of total aflatoxin values calculated from the sum of AfB1, AfB2, AfG1 and AfG2 in a naturally contaminated peanut product. Blue lines reflect the uncertainty range of the reference value (black line), red lines the z-score limit of z=|2|.

In addition to the analysis of a naturally contaminated sample ("aflatoxin positive") a blank peanut material was supplied. Participants were requested to indicate the level of aflatoxins spiked and analytically found, in order to calculate the recovery rate. Reported recoveries ranged from 53-118 % for AfB1, 56-142 % for AfB2, 46-106 % for AfG1 and 23-121 % for AfG2. The individually reported recovery figures were used to correct the results from the analysis of the "aflatoxin positive" material. For the determination of AfB1 and total Af results are shown in Figures 6 and 7. Table 3 summarises the effect by comparing the number of laboratories that reported values within the z-score limit and the uncertainty of the reference value.

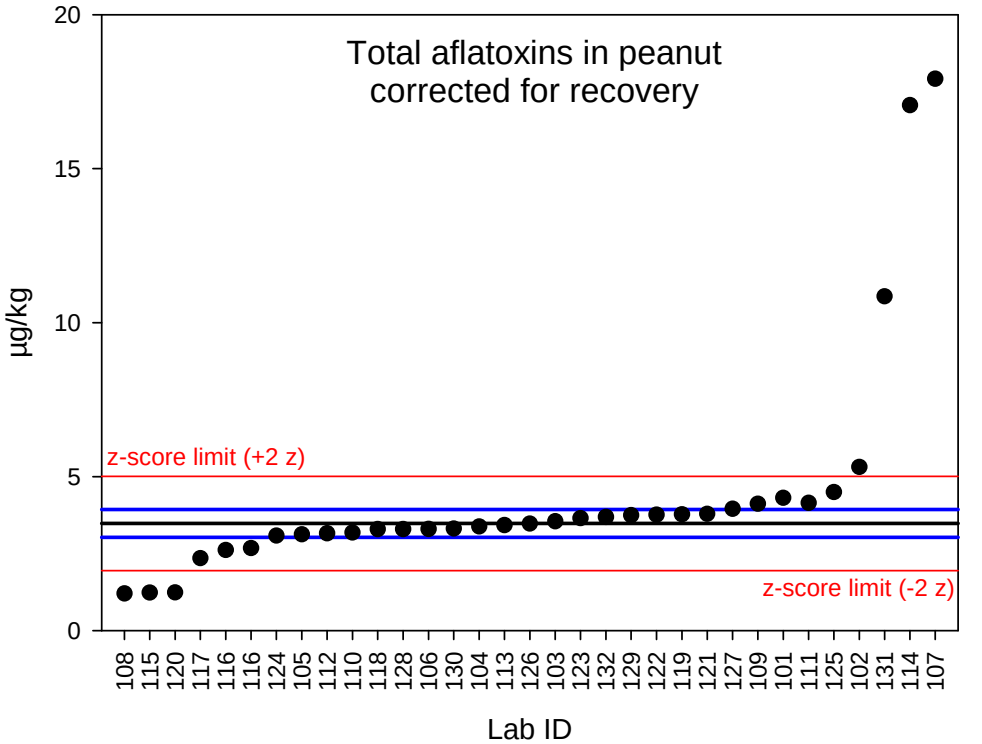
Furthermore the results for AfB1 and total Af were corrected by a factor that was calculated from the measurement of the Af test solution. This factor was obtained by multiplying the result with the assigned value of the Af test solution, divided by the reported value (mean of 3 determinations). By this procedure values are normalised for calibrant effects. The results are shown in Figures 8 and 9.

Figure 6: Plot of Aflatoxin B1 results in peanut after recovery correction.



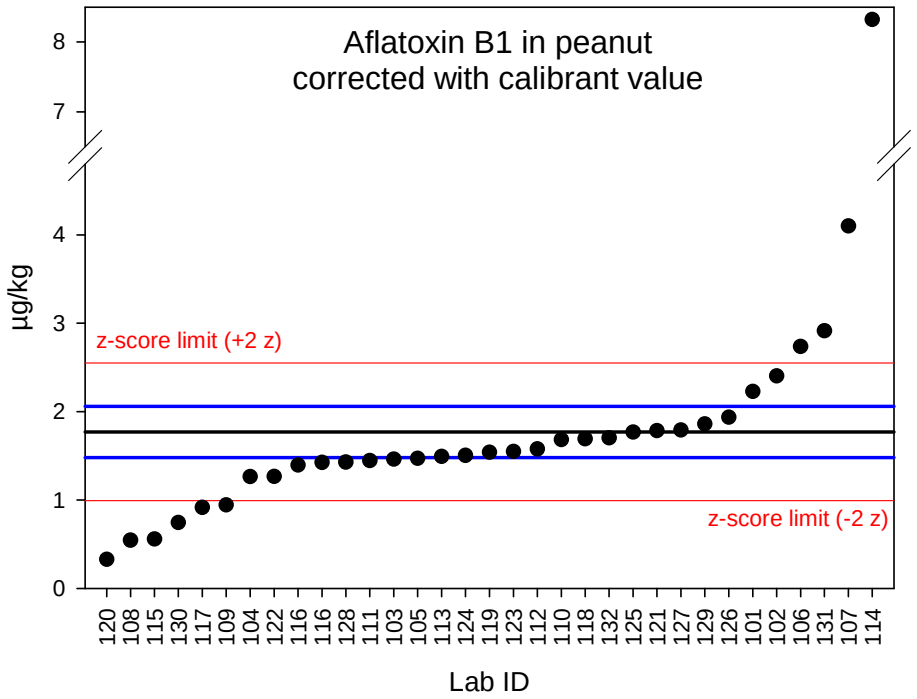
Plot of mean values from replicate measurements (n=3) for the determination of Aflatoxin B1 in a naturally contaminated peanut product after correction by recovery (mean of three replicate measurements). Blue lines reflect the uncertainty range of the reference value (black line), red lines the z-score limit of z=|2|.

Figure 7: Plot of total aflatoxins in peanut after recovery correction.



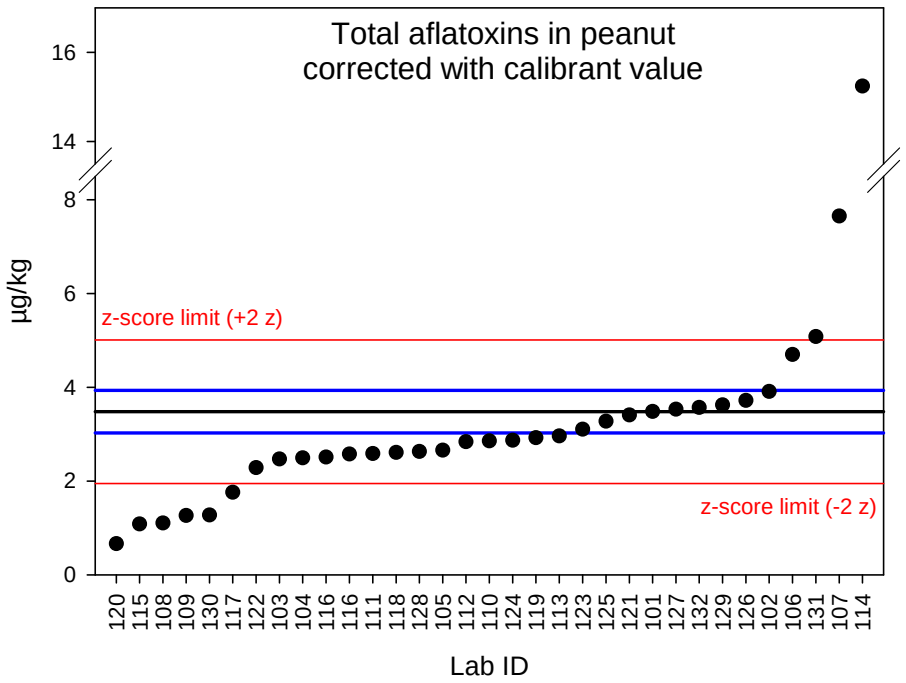
Plot of mean values from replicate measurements (n=3) for the sum of AfB1, AfB2, AfG1 and AfG2 in a naturally contaminated peanut product after correction by recovery (mean of three replicate measurements). Blue lines reflect the uncertainty range of the reference value (black line), red lines the z-score limit of z=|2|.

Figure 8: Plot of Aflatoxin B1 results in peanut after calibration bias correction.



Plot of mean values from replicate measurements (n=3) for the determination of Aflatoxin B1 in a naturally contaminated peanut product after correction for the calibrant (mean of three replicate measurements). Blue lines reflect the uncertainty range of the reference value (black line), red lines the z-score limit of z=|2|.

Figure 9: Plot of total aflatoxins results in peanut after calibration bias correction.



Plot of mean values from replicate measurements (n=3) for the sum of AfB1, AfB2, AfG1 and AfG2 in a naturally contaminated peanut product after correction for the calibrant (mean of three replicate measurements). Blue lines reflect the uncertainty range of the reference value (black line), red lines the z-score limit of z=|2|.

Table 3: Summary for values within z-score and certification limits

n=33	Number of results within z-score limit (z=|2|)					Number of results within certification limit			
	Af pos	Cal	Cal-cor	Rec-cor	Cal-rec	Af pos	Cal-cor	Rec-cor	Cal-rec
AfB1	26	29	23	26	25	12	13	17	17
AfB2	24	29	23	25	25	13	15	17	15
AfG1	23	29	21	26	25	20	17	21	23
AfG2	17	28	17	20	22	14	14	20	21
AfSUM	26	-	24	26	26	11	9	18	16

Af pos = Af positive peanut prior any correction. Cal = mixed Af test solution. Cal-cor = Af pos after correction with the values obtained from the determination of the mixed Af test solution. Rec-cor = Af pos after recovery correction. Cal-rec = Af pos after calibrant and recovery correction.

Correction for recovery

As can be seen from **Figures 1, 5 - 7** and **Table 3** the correction for recovery has a clear effect on analytical results for the analysis of peanut material. This procedure aims to normalise the effects that occur during the analysis (extraction, clean-up, etc.). Thus, recovery correction improved slightly the number of acceptable z-scores and to a considerable extent the number of results within the uncertainty ranges of the reference values. This supports the finding that the correction for recovery improves the comparability of analytical results between laboratories[2]. It should however be noted that the reported values which have been used for the correction were not obtained in a blinded experiment. Participants were asked to use their own calibrant, spike at a known level and report the found amount. From this the recovery was calculated.

Correction for calibrant

The intention to improve analytical results by normalising the effect that might be due to the quality of the calibrant was less effective than in the case of recovery or not effective at all. No improvement could be observed (see **Table 3**) as it was observed for recovery correction. An interesting observation is however that on an overall scale (all participants) the center-part of the ranked results in **Figure 8** is apparently more effected by the test solution correction than by recovery correction (**Figure 6**) for initial results of AfB1 (**Figure 1**). This is stressed by the fact that the "slope" of the plotted results is less steep in the middle region. This effect indicates that for a certain fraction of laboratories the test solution correction has a normalisation effect, too. Any further conclusion and evaluation would require a degree of data unscrambling that is beyond the scope of this proficiency test at this time.

Comparison of other observed trends within laboratories

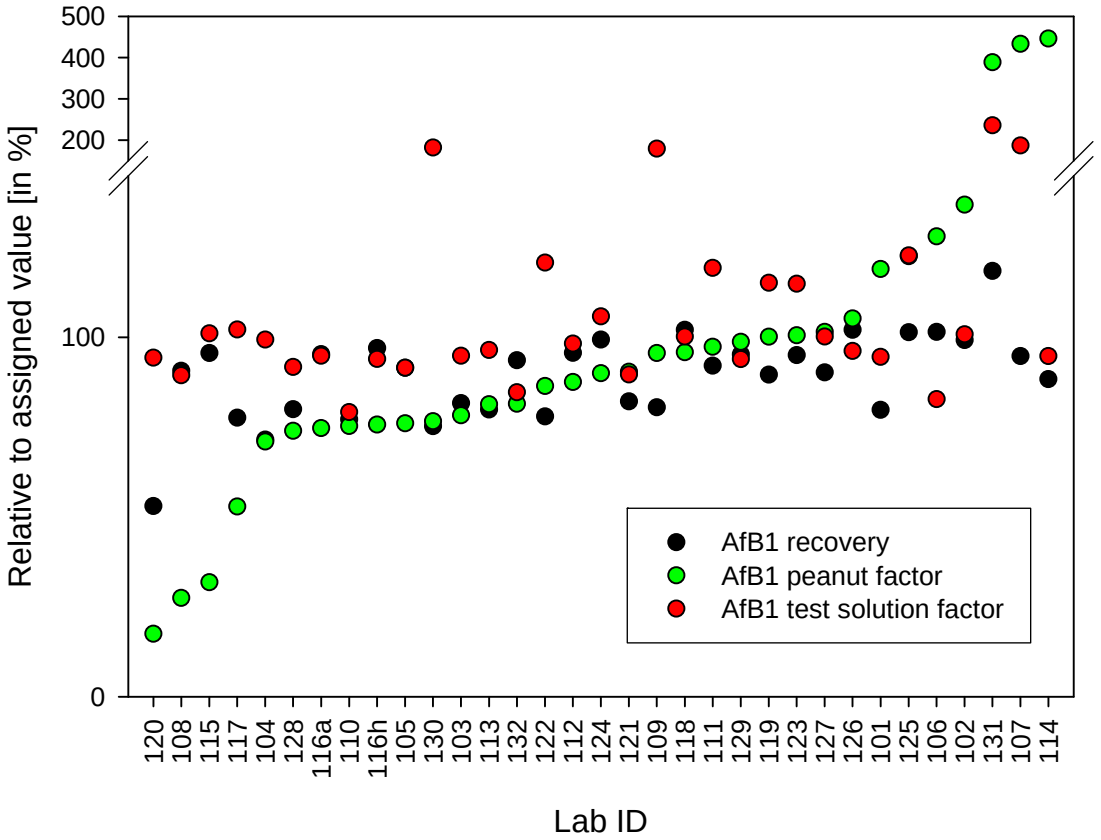
Certain effects could be observed by plotting the reported AfB1 concentration values (as the major constituent in all test materials) found in relation to the assigned values of the three test materials (aflatoxin positive peanuts, peanut spike and Af test solution).

Ideally, all values reported by a laboratory should be located near the 100 % mark. Spike recovery for AfB1 as well as recovery of the Af test solution varied, with some exceptions, in a range between 70-110 %, while the AfB1 content reported in the aflatoxin positive material by the laboratories showed the sigmoidal distribution, which is typical for this type of PT exercise (**Figure 10**). Laboratories 120, 108, 115 and 117 had difficulty recovering the AfB1 from naturally incurred materials, although spike recovery was satisfactory; likewise laboratories 107 and 114 grossly overestimated the AfB1 content in the peanut material, while having satisfactory spike recovery. These findings suggest that for some experiments spiked or naturally incurred materials behaved differently during analysis, giving rise to an under or over estimation of the true content that can not be corrected using the results of the

[2] C. von Holst, J. Stroka, E. Anklam (2002), *Food Additives Contaminants*, **19**, 701-708

recovery experiment. Laboratories 130, 122 and 109 overestimated the AfB1 content in the AfB test solution, while reporting acceptable values for the natural and the spiked peanut materials. Incorrect dilution or handling of the AfB test solution might have caused these deviations.

Figure 10: Plot of AfB1 values in dependency of the type of analysis



Values in the plot are ranked by the AfB1 value reported for the aflatoxin positive peanut material. Values were normalised to %-values of the respective assigned or fortified values.

Figure 11 shows that there is a general tendency to overestimate results for AfG2 in the Af test solution; this was not evident for the other aflatoxins. **Figure 12** illustrates that sample treatment (extraction, clean-up, etc.) increased the spread of Af values. However, the highest dispersion was found for AfG2 in this case with a tendency to lower values, which is in contrast to the findings for the Af test solution where generally higher values were found for AfG2. **Figure 13** shows that several laboratories reported considerable AfG2 losses in the recovery experiment, while for AfB1 the reported recoveries ranged from 80- 100 %. Nevertheless the low recovery values for AfG2 (and to a lesser extent also for AfG1) indicate a need for further investigation and corrective action, as such losses not necessarily occur at predictable rates and can thus lead to non-comparability and / or a misinterpretation of testing results. This is especially of relevance when AfG2 (and AfG1 – which is more likely) appear in higher concentrations in a material.

Figures 14 and **15** show the effectiveness of bias correction by using the Af testing solution. Based on the assumption that the recovery rate was properly established such a plot visualises effects related to the *in-house* calibrant (of *known* concentration) used by the laboratories. In cases were the values for the test solution results differ to a larger extent from the recovery corrected results of the Af, an *in-house* calibrant effect (=bias) is very likely. In those cases where the recovery corrected values for the peanut material were different from values of the Af test solution (values for the test solution being near 100%), a doubtful spike recovery estimate or problems in the course of the analytical procedure are likely.

These scenarios must be judged on the basis that the levels of aflatoxins G1 and G2 in the peanut material were rather low compared to levels for AfB1 and AfB2. This fact makes an exact evaluation/unscrambling of effects difficult for the G aflatoxins. Furthermore legislative limits exist currently only for AfB1 and total aflatoxins and thus these should be the final parameters focussed on. This however shall not mean that analytical performance for the less abundant aflatoxins can be neglected. An evaluation of effects from analytical procedures based on the questionnaire has not been performed, but all answers have been added to the annex to allow further unscrambling of data by the participants if necessary in particular cases.

Figure 11: Plot of the normalised aflatoxin values reported for the calibrant

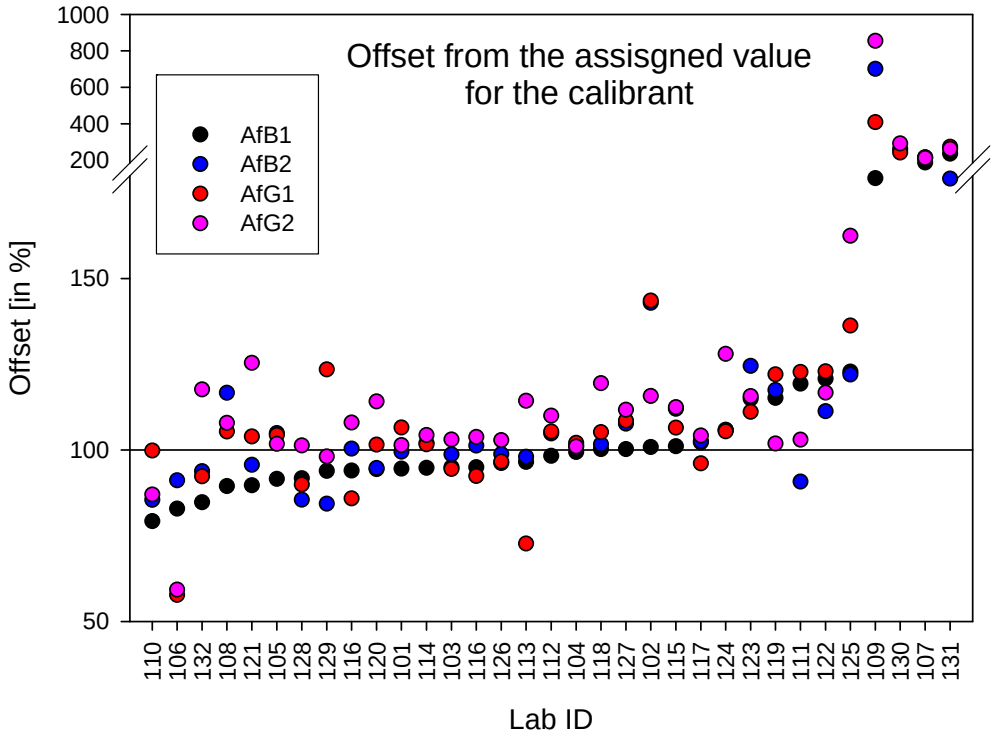


Figure 12: Plot of the normalised aflatoxin values reported for the peanut material

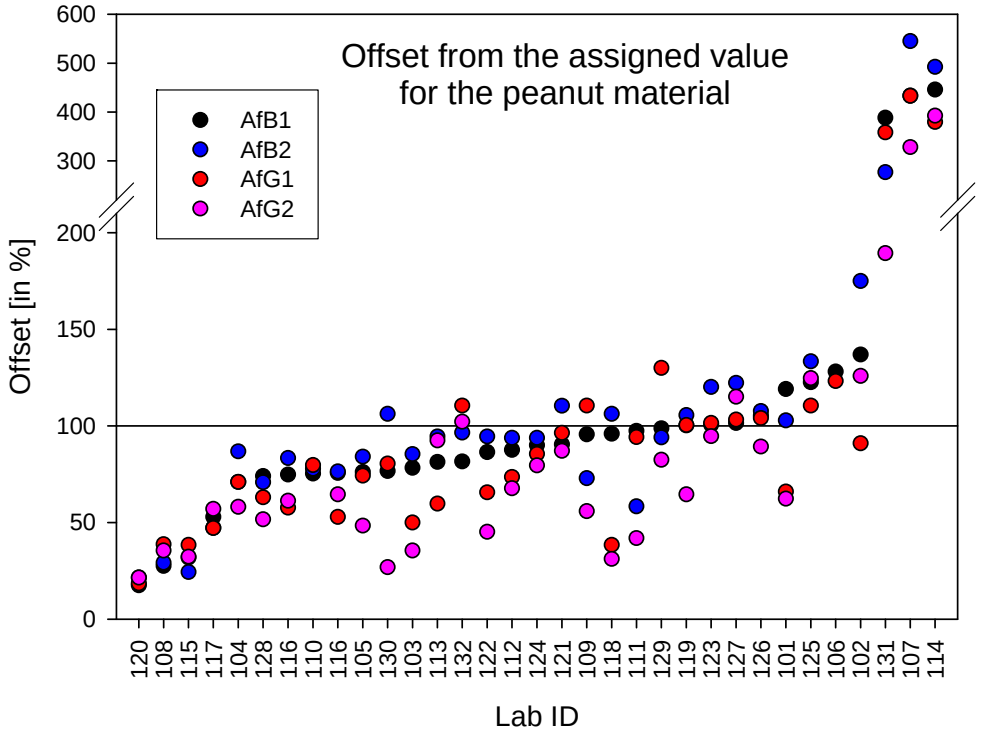


Figure 13: Plot of the aflatoxin recovery values reported for the spike

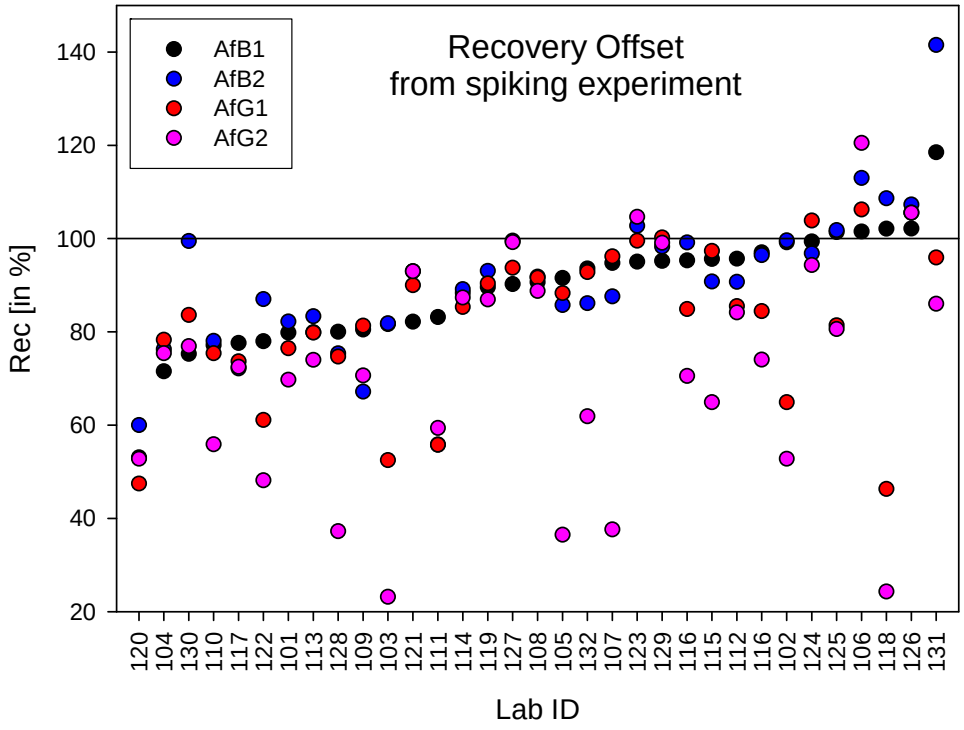


Figure 14: Plot of the normalised calibrant values for AfB1 and AfB2 against the recovery corrected result for the peanut positive result

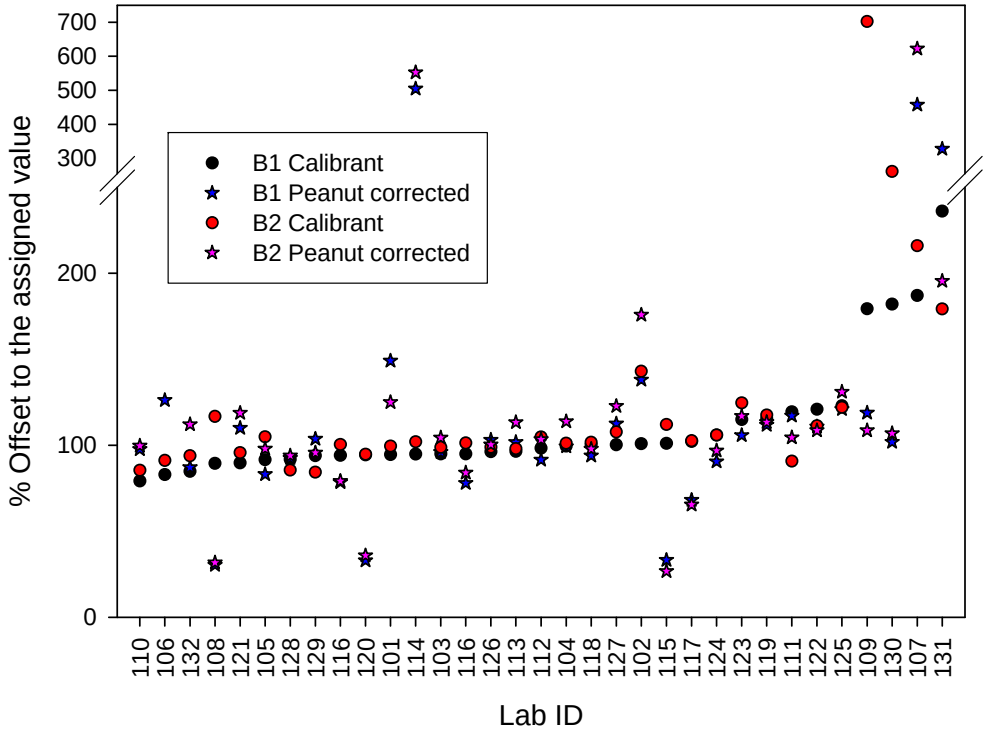
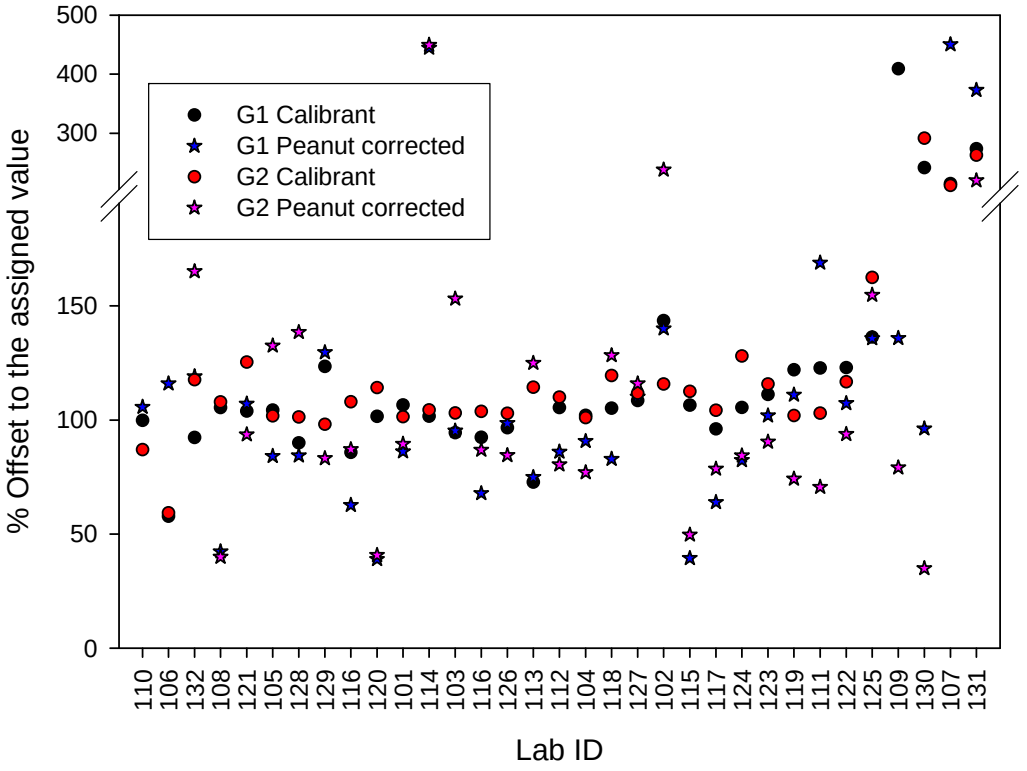


Figure 15: Plot of the normalised calibrant values for AfG1 and AfG2 against the recovery corrected result for the peanut positive result



Conclusion

With respect to the NRL proficiency test 2006 the situation has improved. The majority of laboratories reported values within z-scores of |2| taking the modified Horwitz equation as basis for the target standard deviation. For measurements of Af in a test solution 78% of the labs were able to report acceptable values for aflatoxin B1, while for aflatoxin G2 this was only the case for 59% of the laboratories. In general, the agreement of results among laboratories as well as in relation to the assigned values - improved after recovery correction. A further correction of results for AfB1 and total aflatoxins with a factor to take into account the calibration bias had no significant effect on the overall comparability of such corrected results, while a slight improvement for generally good performing labs could be observed. This indicates that a calibration bias correction can have a positive effect, however not in all cases.

Annex

Table 3: Individual results [in µg/kg] for aflatoxin B1 and B2 in peanut:

Laboratory code	AfB1	AfB1	AfB1	AfB2	AfB2	AfB2
101	2.07	2.11	2.14	0.49	0.49	0.5
102	2.65	2.21	2.41	0.71	0.86	0.95
103	1.39	1.39	1.38	0.41	0.41	0.41
104	1.28	1.28	1.21	0.38	0.37	0.5
105	1.39	1.28	1.37	0.43	0.39	0.39
106	2.1	2.4	2.3	<1	<1	<1
107	8.15	7.15	7.7	2.35	2.55	2.95
108	0.5	0.48	0.48	0.14	0.14	0.14
109	1.94	1.8	1.34	0.39	0.39	0.27
110	1.33	1.34	1.33	0.38	0.37	0.37
111	1.74	1.67	1.76	0.29	0.27	0.28
112	1.56	1.53	1.56	0.45	0.45	0.45
113	1.44	1.43	1.45	0.45	0.45	0.46
114	7.98	7.67	8.02	2.33	2.29	2.47
115	0.58	0.56	0.55	0.12	0.12	0.11
116	1.33	1.33	1.36	0.4	0.4	0.4
116	1.31	1.33	1.33	0.36	0.36	0.38
117	0.93	0.93	0.95	0.23	0.22	0.23
118	1.83	1.64	1.62	0.56	0.49	0.48
119	1.7	1.83	1.79	0.49	0.52	0.51
120	0.28	0.31	0.34	0.08	0.12	0.11
121	1.6	1.6	1.6	0.53	0.53	0.53
122	1.49	1.59	1.51	0.45	0.45	0.46
123	1.67	1.8	1.87	0.53	0.59	0.61
124	1.56	1.55	1.67	0.46	0.45	0.44
125	2.27	2.08	2.16	0.64	0.63	0.65
126	1.82	1.86	1.91	0.49	0.53	0.53
127	1.79	1.8	1.8	0.59	0.58	0.59
128	1.3	1.28	1.35	0.34	0.34	0.34
129	1.722	1.774	1.746	0.452	0.456	0.446
130	1.46	1.3	1.31	0.57	0.48	0.48
131	6.921	6.893	6.806	1.331	1.337	1.315
132	1.41	1.47	1.45	0.46	0.47	0.46

Table 4: Individual results [in µg/kg] for aflatoxin G1 and G2 in peanut:

Laboratory code	AfG1	AfG1	AfG1	AfG2	AfG2	AfG2
101	0.63	0.61	0.58	0.19	0.2	0.19
102	0.8	0.82	0.89	0.39	0.39	0.39
103	0.46	0.46	0.46	0.11	0.11	0.11
104	0.74	0.6	0.62	0.19	0.18	0.17
105	0.7	0.66	0.69	0.16	0.16	0.13
106	1.2	1	1.2	<1	<1	<1
107	4.1	3.7	4.15	1	1.25	0.8
108	0.35	0.35	0.37	0.11	0.11	0.11
109	1.11	1.01	0.93	0.19	0.18	0.15
110	0.75	0.74	0.71	<0.21	<0.21	<0.21
111	0.85	0.87	0.88	0.14	0.12	0.13
112	0.68	0.68	0.67	0.2	0.21	0.22
113	0.55	0.55	0.55	0.29	0.28	0.29
114	3.71	3.42	3.34	1.39	1.08	1.18
115	0.35	0.35	0.36	0.1	0.1	0.1
116	0.53	0.53	0.53	0.19	0.19	0.19
116	0.49	0.48	0.49	0.2	0.19	0.21
117	0.43	0.43	0.44	0.18	0.17	0.18
118	0.31	0.39	0.36	0.09	0.09	0.11
119	0.88	0.94	0.95	0.2	0.22	0.18
120	0.17	0.18	0.16	0.06	0.08	0.06
121	0.93	0.93	0.8	0.27	0.27	0.27
122	0.6	0.58	0.63	0.15	0.13	0.14
123	0.86	0.96	0.98	0.27	0.3	0.31
124	0.79	0.79	0.78	0.24	0.24	0.26
125	1.03	0.96	1.06	0.41	0.35	0.4
126	0.87	1.02	0.98	0.23	0.31	0.29
127	0.95	0.95	0.95	0.36	0.36	0.35
128	0.59	0.57	0.58	0.16	0.16	0.16
129	1.216	1.181	1.19	0.234	0.259	0.274
130	0.88	0.67	0.67	0.08	0.1	0.07
131	3.294	3.306	3.28	0.628	0.621	0.512
132	1.03	0.99	1.03	0.39	0.24	0.32

Table 5: Individual results [in µg/kg] for aflatoxin B1 and B2 in the aflatoxin test solution:

Laboratory code	AfB1	AfB1	AfB1	AfB2	AfB2	AfB2
101	19.4	19.78	19.8	5.86	5.89	5.95
102	21.48	21.07	20.33	8.87	8.78	7.77
103	20.06	19.72	19.42	6.04	5.82	5.7
104	21	21	20	6	6	6
105	18.98	19.01	19.1	6.28	6.28	6.1
106	16.43	17.188	18.044	5.425	5.727	5.059
107	39.5	38.54	38.59	12.99	12.62	12.8
108	18.514	18.631	18.627	6.893	6.928	6.939
109	39.85	35.18	36.79	34.94	40.07	49.87
110	16.3	16.9	16.2	5	5.1	5.1
111	24.89	24.85	24.69	5.43	5.384	5.331
112	20.381	20.413	20.503	6.255	6.198	6.187
113	19.94	19.98	20.25	5.81	5.78	5.84
114	19.919	19.786	19.415	6.066	6.125	5.955
115	21.11	20.86	21.08	6.64	6.63	6.65
116	19.23	19.69	19.71	5.86	6.12	6.03
116	20.12	19.64	19.44	6.04	5.97	5.85
117	21.689	21.303	20.732	6.167	6.058	6.023
118	20.82	20.97	20.73	6	6.12	5.96
119	24.21	23.97	23.67	7.08	6.85	6.97
120	19.799	19.235	19.823	5.667	5.481	5.703
121	18.73	18.6	18.6	5.67	5.67	5.67
122	25.32	25.02	25	6.58	6.52	6.7
123	23.685	23.999	24.002	7.388	7.394	7.37
124	22.001	22.003	22.016	6.276	6.277	6.278
125	24.86	25.18	26.55	6.95	7.1	7.65
126	19.286	19.951	20.77	5.708	5.859	6.011
127	20.835	20.665	21.021	6.363	6.31	6.475
128	18.27	18.68	20.26	4.98	5	5.23
129	19.41	19.64	19.52	5	5.06	4.94
130	37.93	37.95	37.7	15.71	15.48	15.28
131	48.262	49.117	49.82	10.09	10.807	10.972
132	17.669	17.56	17.628	5.633	5.503	5.551

Table 6: Individual results [in µg/kg] for aflatoxin G1 and G2 in the aflatoxin test solution:

Laboratory code	AfG1	AfG1	AfG1	AfG2	AfG2	AfG2
101	6.14	6.41	6.24	5.95	6.07	6.05
102	9.03	8.09	8.2	7.63	7.08	5.91
103	5.72	5.54	5.4	6.16	6.12	6.08
104	6	6	6	6	6	6
105	6.18	6.17	6.05	6.16	6.14	5.84
106	3.131	3.063	3.989	3.48	3.711	3.373
107	12.75	12.65	12.42	12.77	12.64	12.27
108	6.171	6.211	6.211	6.383	6.418	6.425
109	29.64	22.62	19.9	48.27	51.06	52.98
110	5.8	6	5.8	5.6	4.8	5.1
111	7.288	7.137	7.225	6.148	6.11	6.093
112	6.433	5.919	6.24	6.832	6.301	6.465
113	4.2	4.2	4.43	6.79	6.7	6.88
114	6.015	6.07	5.84	6.241	6.302	6.049
115	6.26	6.3	6.22	6.71	6.74	6.59
116	5.35	5.62	5.33	6.13	6.28	6.08
116	5.11	5.13	4.9	6.51	6.47	6.26
117	5.647	5.626	5.673	6.213	6.171	6.182
118	6.19	6.29	6.07	7.15	7.13	7.01
119	7.36	7.15	7.01	6.25	5.83	6.08
120	6.041	5.889	5.978	6.812	6.545	6.981
121	6.13	6.13	6.07	7.47	7.47	7.4
122	7.16	7.22	7.3	6.84	6.9	7.06
123	6.562	6.544	6.497	6.87	6.903	6.843
124	6.203	6.193	6.203	7.603	7.602	7.604
125	8.03	7.86	8.15	9.54	9.46	9.95
126	5.256	5.779	6.007	5.851	6.246	6.23
127	6.56	6.355	6.228	6.608	6.697	6.601
128	5.16	5.25	5.45	5.98	6.01	6.06
129	7.07	7.51	7.2	5.82	5.86	5.8
130	14.39	14.54	13.71	17.57	17.24	17.14
131	16.09	16.242	15.894	15.672	15.575	15.58
132	5.496	5.362	5.42	6.907	6.978	7.074

Table 7: Mean spike recoveries calculated for aflatoxin B1, B2, G1 and G2 [in %]:

Laboratory code	AfB1	AfB1	AfG1	AfG2
101	80	82	77	70
102	99	100	65	53
103	82	82	53	23
104	72	76	78	75
105	92	86	88	37
106	102	113	106	121
107	95	88	96	38
108	91	92	92	89
109	81	67	81	71
110	77	78	75	56
111	92	56	56	59
112	96	91	86	84
113	80	83	80	74
114	88	89	85	87
115	96	91	97	65
116	97	99	85	71
116	95	96	84	74
117	78	72	74	72
118	102	109	46	24
119	90	93	90	87
120	53	60	47	53
121	82	93	90	93
122	78	87	61	48
123	95	103	100	105
124	99	97	104	94
125	101	102	81	81
126	102	107	106	106
127	90	100	94	99
128	80	75	75	37
129	95	98	100	99
130	75	99	84	77
131	118	142	96	86
132	94	86	93	62

Table 8a: Evaluation of the Questionnaire:

Lab ID\ Question	Give a reference to your method	Extraction solvent used	Extraction solvent to sample ratio used during extraction (in mL/g)?
101	SOP	MeOH/H2O=4:1	4:1
102	Aflaprep	MeOH/H2O=6:4	5:1
103	EN 14123	MeOH/H2O=4:1	4:1
104	EN 14123:2003	MeOH/H2O=4:1	0.167g/mL
105	J AOAC vol.83/2 N°2, 2000	MeOH/H2O=4:1	4:1
106	In house method, LC-MS/MS	AcCN/H2O=4:1	4:1
107	SR EN 14123/2003	MeOH/H2O=7:3	5:1
108	SOP	MeOH	100mL/28.6g
109	VICAM Afla Test WB Instruction Manual	MeOH/H2O=7:3	5:1
110	AOAC 991.31	MeOH/H2O=7:3	5:1
111	ISO 16050	MeOH/H2O=7:3	5:1
112	Modified EN 14123	MeOH/H2O=4:1	4:1
113	PN-EN-14123:2004	MeOH/H2O=4:1	4:1
114	in-house developed	AcCN/MeOH=1:1 AcCN/MeOH/H2O=1:1:1	25:3
115	EN14123 (2003)	100 ml MeOH/H2O(4/1)+50 ml Hexan	6:1
116	EN 14123	MeOH/H2O=4:1	4:1
117	in-house (according EN 14123	MeOH/H2O=4:1	5:1
118	EN ISO 17375:2006	Acetone/H2O=85/15	5:1
119	Modified AOAC Official Method 991.31	MeOH/H2O 62.5%	4:1
120	EN 14123	MeOH/H2O	4:1
121	CEN/TC 275 EN 14123:2003	MeOH/H2O=4:1	4:1
122	LVS EN 14123:2003	MeOH/H2O	0,25 mL/g
123	J.Chromatogr, 1991, 543, 220-225	AcCN/H2O=6:4	5:1
124	Internal SOP	CHCl3	5:1
125	§64-LFGB L 48.00-1	Acetone/H2O=85:15	6:1
126	J. AOAC Int. 1994, 77 (1), 46-53	AcCN/H2O=4:1	5:1
127	Instruction manual for the columns MultiSep 226 AflaZon + (Romer lab).	Acetonitrile 84 % in water	4:1
128	CEN standard: prEN 14123	MeOH/H2O=4:1	4:1
129	JAOAC Int., 88, 2005, 526 – 535	MeOH/H2O=6:4	5:1
130	Method for determination of Aflatoxin B1 and total Aflatoxins in peanut butter, raw peanuts and corn	MeOH/H2O=7:3	5:1
131	Project SMT-CT96-2045	MeOH/H2O(4/1)+ Hexan	6:1
132	Senyuva H.Z., Gilbert J., J AOAC Int Vol88, No2, 2005	H2Oand MeOH	5:1

Table 8b: Evaluation of the Questionnaire:

Lab ID\ Question	extraction aids added	extraction mode and time	type of clean-up
101	0.5g NaCl	shaking 16 hours	IAC
102	4g NaCl	Shaking 30 minutes	IAC
103	5g NaCl	Blending 3 min	IAC
104	2.5g NaCl	Blending 3 min	IAC
105	25gNaCl/L	3min blending + 30min shaking	IAC
106	No	Shaking for 2 hours	none
107	5g NaCl	Blending 3min	IAC
108	5g NaCl	Shaking	IAC
109	5g NaCl	Blending 5 min	IAC
110	5g NaCl to 25g	Blend 2 min	IAC
111	5 g of NaCl	Shaking 10 min	IAC
112	0,1g NaCl/g	Blending 3 min	IAC
113	2,5 g NaCl/25 g	Blending 3 min	IAC
114	No	Blending:3 min solvent1, 2 min solvent2	IAC
115	2,5 g NaCl to 25 g sample	Shaking 30 min	IAC
116	5.0 g NaCl	Blending 2 min	IAC
117	1g NaCl/10g sample	blending 3 min	IAC
118	No	Shaking 1hr	IAC
119	10%w/w NaCl to dry sample	Blending 2.5 min	IAC
120	0,1g NaCl/g sanple	Shaking 30 min	IAC
121	5g NaCl and 100 ml n-hexane	Blending 3 min	IAC
122	No	Shaking 30 min	Romer Labs AflaStar
123	No	Blending 4 min	IAC
124	10g acid washed Celite 545/20g sample/100ml chloroform	Shaking 30 min	IAC
125	5g NaCl	blending 3 min	IAC
126	No	Shaking 1 hr	IAC
127	No	Shaking 30 min	MultiSep-column
128	5g NaCl	Blending 2 min	IAC
129	2g NaCl	Blending 1min after addition of H2O and 2min after addition of MeOH	IAC
130	5g NaCl	Blending 2 min	IAC
131	5g NaCl	Blending 3min	IAC
132	2g NaCl	Blending	IAC

Table 8c: Evaluation of the Questionnaire:

Lab ID\ Question	Extract evaporated prior injection	derivatisation method applied? If yes, please state the kind of method	any "over-night" stops in the analysis? If yes, please state at what point.
101	No	PBPB	No
102	Yes to 1mL	Iodine derivatisation	YES, there were a lot of measurements and the system with the external pump for pumping iodine can not be left alone during night.
103	Yes	Kobra cell	Yes before injections
104	No	Kobra cell	Yes, problem with Kobra cell
105	No	Kobra cell	Yes, After IA-clean-up
106	No	No	No
107	No	Kobra cell	No
108	No	Kobra cell	Yes, after extraction of the samples (28.6g with 100mL Methanol) and filtration of extracts, the extracts were stored in a freezer overnight.
109	Yes	TFA	No
110	No	Iodine derivatisation	No
111	No	Kobra cell	No
112	No	PBPB	No
113	No	PBPB	No
114	Yes	Kobra cell	No
115	No	PBPB	No
116	Yes	PBPB	No
117	No	Kobra cell	Yes, before clean-up
118	No	Kobra cell	No
119	No	Iodine derivatisation	No
120	Yes	TFA	No
121	No	PBPB	No
122	Yes	Kobra cell	Yes, Evaporation
123	No	Kobra cell	No
124	Yes, under N2	Kobra cell	No
125	Yes, under N2	Kobra cell	No
126	No	Kobra cell	No
127	Yes, 2 ml of extract was evaporated, dried and dissolved in 300 µl.	Kobra cell	Yes, after clean-up on MultiSep-column, the 2 ml extract for evaporation was frozen overnight
128	Yes	PBPB	Stop after elution from IAC
129	No	PBPB	No
130	Yes	PBPB	No
131	No	PBPB	No
132	No	Kobra cell	No

Table 8d: Evaluation of the Questionnaire:

Lab ID\ Question	use acid washed glass ware	protection against daylight	unusual observations
101	Yes	Yes	
102	Yes	Only at measurement by HPLC	No
103	Yes	Yes	
104	No	Yes, problem with Kobra cell	No
105	No	No	Blank material analysed unspiked was shown to contain very low concentrations of Aflatoxin
106	Yes	Yes	The signal for Aflatoxin B1 of the standard solution (10 times diluted) is out of the calibration range.
107	Yes	Yes	No
108	Yes, after extraction of the samples (28.6g with 100mL Methanol) and filtration of extracts, the extracts were stored in a freezer overnight.	Yes	No
109	Yes	Yes	
110	No	Yes	No
111	Yes	Yes	trace of aflatoxin B1 detected in the blank sample Though the requested store t was 4°, the t written on containers was –20°, so the samples were stored for some time in the freezer
112	Yes	Yes	No
113	Yes	Yes	No
114	No	Yes	The ‘positive’ sample was a different colour from the ‘blank’. Also it had a different (less ‘fresh’) odour
115	Yes	Yes	No
116	Yes	Yes	No
117	No	Yes	No
118	Yes	Yes	Yes, Low recovery for G1 and G2
119	Yes	Yes	No
120	No	No	No
121	No	Yes-Al foil	No
122	No	No	problem during extraction, sample material sticks to container
123	Yes	Yes	AFB1 was outside the calibration range prepared, therefore, further dilutions were made of the standard (x50) and AFB1 concentration was calculated from this
124	No	Yes	The samples contained a lot of fat, after chloroform extraction it is evaporated and re-dissolved in methanol a striking colour difference between the extract of the blank and the positive sample.
125	No	Yes	Yes, it was the first time we observed a second fluid layer after extraction and centrifugation. We didn´t analysed that solution for aflatoxins!
126	Yes	Yes	Some sample amount was always left on the bottom of the extraction glassware.
127	No	Yes	The blank sample for spiking gave peaks at the retention times of toxins. Blank subtraction was made.
128	No, but 0.1% acetic acid is added to the mobile phase, which is used for solution of standards and samples	Yes	No
129	No	Yes	No
130	Yes	Yes	No
131	Hypochlorite and Acetone/H2O	Yes	The ‘positive’ sample was a different colour from the ‘blank’.
132	No	Yes	for two blank samples G2 recovery were below 85%

Table 9a: z-scores for Aflatoxin B1

LAB ID	Af positive	Test Solution	Af positive TSC	Af positive REC	Af positive CTSREC
101	0.9	-0.3	1.2	2.2	2.6
102	1.7	0.0	1.6	1.7	1.7
103	-1.0	-0.2	-0.8	-0.2	0.0
104	-1.3	0.0	-1.3	0.0	0.0
105	-1.1	-0.4	-0.8	-0.8	-0.4
106	1.3	-0.8	2.5	1.2	2.4
107	15.1	3.9	6.0	16.2	6.6
108	-3.3	-0.5	-3.1	-3.2	-3.0
109	-0.2	3.6	-2.1	0.9	-1.5
110	-1.1	-1.0	-0.2	-0.1	1.1
111	-0.1	0.9	-0.8	0.3	-0.5
112	-0.6	-0.1	-0.5	-0.4	-0.3
113	-0.8	-0.2	-0.7	0.1	0.3
114	15.7	-0.2	16.8	18.4	19.6
115	-3.1	0.0	-3.1	-3.0	-3.0
116.1	-1.1	-0.3	-0.9	-1.0	-0.8
116.2	-1.1	-0.2	-1.0	-1.0	-0.8
117	-2.1	0.1	-2.2	-1.4	-1.5
118	-0.2	0.0	-0.2	-0.3	-0.3
119	0.0	0.7	-0.6	0.5	-0.1
120	-3.7	-0.3	-3.7	-3.0	-3.0
121	-0.4	-0.5	0.0	0.5	1.0
122	-0.6	0.9	-1.3	0.5	-0.4
123	0.0	0.7	-0.6	0.3	-0.4
124	-0.5	0.3	-0.7	-0.4	-0.7
125	1.0	1.0	0.0	1.0	-0.1
126	0.2	-0.2	0.4	0.1	0.3
127	0.1	0.0	0.1	0.6	0.6
128	-1.2	-0.4	-0.9	-0.3	0.0
129	-0.1	-0.3	0.2	0.2	0.5
130	-1.1	3.7	-2.6	0.1	-2.0
131	13.1	6.2	2.9	10.4	1.8
132	-0.8	-0.7	-0.2	-0.6	0.1

Af positive = Score rating on the neat result for the Af positive sample, prior any correction; Test Solution = Score rating for the result for the test solution; Af positive TSC = Score rating on the result of the Af positive sample, after correction with the test solution result; Af positive REC = Score rating on the result of the Af positive sample, after correction for recovery; Af positive CRC = Score rating on the result of the Af positive sample, after correction with the test solution result and recovery.

Table 9b: z-scores for Aflatoxin B2

LAB ID	Af positive	Test Solution	Af positive TSC	Af positive REC	Af positive CTSREC
101	0.1	0.0	0.2	1.1	1.2
102	3.4	1.9	1.0	3.4	1.0
103	-0.7	-0.1	-0.6	0.2	0.3
104	-0.6	0.0	-0.6	0.6	0.6
105	-0.7	0.2	-0.9	-0.1	-0.3
106	-4.5	-0.4	-4.5	-4.5	-4.5
107	20.2	5.2	6.9	23.7	8.6
108	-3.2	0.7	-3.4	-3.1	-3.3
109	-1.2	27.3	-4.1	0.4	-3.8
110	-1.0	-0.7	-0.4	0.0	0.8
111	-1.9	-0.4	-1.6	0.2	0.7
112	-0.3	0.2	-0.5	0.2	-0.1
113	-0.3	-0.1	-0.2	0.6	0.7
114	17.8	0.1	17.4	20.6	20.1
115	-3.4	0.5	-3.6	-3.3	-3.5
116.1	-0.8	0.0	-0.8	-0.7	-0.8
116.2	-1.1	0.0	-1.1	-0.9	-1.0
117	-2.4	0.1	-2.5	-1.6	-1.6
118	0.3	0.1	0.2	-0.1	-0.2
119	0.3	0.8	-0.5	0.6	-0.2
120	-3.6	-0.3	-3.5	-2.9	-2.8
121	0.5	-0.2	0.7	0.9	1.1
122	-0.3	0.5	-0.7	0.4	-0.1
123	0.9	1.1	-0.2	0.8	-0.3
124	-0.3	0.3	-0.5	-0.1	-0.4
125	1.5	1.0	0.4	1.4	0.3
126	0.3	-0.1	0.4	0.0	0.1
127	1.0	0.3	0.6	1.0	0.6
128	-1.3	-0.7	-0.8	-0.3	0.4
129	-0.3	-0.7	0.5	-0.2	0.6
130	0.3	7.3	-2.7	0.3	-2.7
131	8.0	3.6	2.5	4.3	0.4
132	-0.2	-0.3	0.1	0.5	0.9

Af positive = Score rating on the neat result for the Af positive sample, prior any correction; Test Solution = Score rating for the result for the test solution; Af positive TSC = Score rating on the result of the Af positive sample, after correction with the test solution result; Af positive REC = Score rating on the result of the Af positive sample, after correction for recovery; Af positive CRC = Score rating on the result of the Af positive sample, after correction with the test solution result and recovery.

Table 9c: z-scores for Aflatoxin G1

LAB ID	Af positive	Test Solution	Af positive TSC	Af positive REC	Af positive CTSREC
101	-1.5	0.3	-1.7	-0.6	-0.9
102	-0.4	2.0	-1.7	1.8	-0.1
103	-2.3	-0.3	-2.1	-0.2	0.0
104	-1.3	0.1	-1.4	-0.4	-0.5
105	-1.2	0.2	-1.3	-0.7	-0.9
106	1.1	-1.9	5.2	0.7	4.6
107	15.1	5.2	4.6	15.9	5.0
108	-2.8	0.2	-2.9	-2.6	-2.7
109	0.5	14.0	-3.3	1.6	-3.0
110	-0.9	0.0	-0.9	0.3	0.3
111	-0.3	1.0	-1.1	3.1	1.7
112	-1.2	0.2	-1.4	-0.6	-0.8
113	-1.8	-1.3	-0.8	-1.1	0.1
114	12.7	0.1	12.4	15.7	15.3
115	-2.8	0.3	-2.9	-2.8	-2.9
116.1	-1.9	-0.4	-1.7	-1.5	-1.2
116.2	-2.1	-0.7	-1.7	-1.7	-1.2
117	-2.4	-0.2	-2.3	-1.6	-1.5
118	-2.8	0.2	-2.9	-0.8	-1.0
119	0.0	1.0	-0.8	0.5	-0.4
120	-3.7	0.1	-3.7	-2.8	-2.8
121	-0.2	0.2	-0.3	0.3	0.1
122	-1.6	1.0	-2.1	0.3	-0.6
123	0.1	0.5	-0.4	0.1	-0.4
124	-0.7	0.2	-0.9	-0.8	-1.0
125	0.5	1.6	-0.9	1.6	0.0
126	0.2	-0.2	0.3	-0.1	0.1
127	0.1	0.4	-0.2	0.5	0.1
128	-1.7	-0.5	-1.4	-0.7	-0.3
129	1.4	1.0	0.2	1.3	0.2
130	-0.9	6.4	-3.0	-0.2	-2.7
131	11.7	7.8	1.4	12.4	1.7
132	0.5	-0.4	0.9	0.9	1.3

Af positive = Score rating on the neat result for the Af positive sample, prior any correction; Test Solution = Score rating for the result for the test solution; Af positive TSC = Score rating on the result of the Af positive sample, after correction with the test solution result; Af positive REC = Score rating on the result of the Af positive sample, after correction for recovery; Af positive CRC = Score rating on the result of the Af positive sample, after correction with the test solution result and recovery.

Table 9d: z-scores for Aflatoxin G2

LAB ID	Af positive	Test Solution	Af positive TSC	Af positive REC	Af positive CTSREC
101	-1.7	0.1	-1.7	-0.5	-0.5
102	1.2	0.7	0.4	6.3	4.8
103	-2.9	0.1	-3.0	2.4	2.2
104	-1.9	0.0	-1.9	-1.0	-1.1
105	-2.3	0.1	-2.4	1.5	1.4
106	-4.5	-1.9	-4.5	-4.5	-4.5
107	10.4	5.1	2.5	35.1	14.2
108	-2.9	0.4	-3.1	-2.7	-2.9
109	-2.0	34.3	-4.2	-0.9	-4.1
110	-4.5	-0.6	-4.5	-4.5	-4.5
111	-2.6	0.1	-2.7	-1.3	-1.4
112	-1.5	0.4	-1.7	-0.9	-1.2
113	-0.3	0.6	-0.9	1.1	0.4
114	13.3	0.2	12.6	15.9	15.0
115	-3.1	0.6	-3.2	-2.3	-2.5
116.1	-1.8	0.2	-1.9	-0.6	-0.7
116.2	-1.6	0.4	-1.8	-0.6	-0.9
117	-2.0	0.2	-2.1	-1.0	-1.1
118	-3.1	0.9	-3.4	1.3	0.3
119	-1.6	0.1	-1.7	-1.2	-1.2
120	-3.6	0.6	-3.7	-2.7	-2.9
121	-0.6	1.1	-1.4	-0.3	-1.1
122	-2.5	0.8	-2.8	-0.3	-0.9
123	-0.2	0.7	-0.8	-0.4	-1.0
124	-0.9	1.3	-1.7	-0.7	-1.6
125	1.1	2.8	-1.1	2.5	-0.2
126	-0.5	0.1	-0.6	-0.7	-0.8
127	0.7	0.5	0.1	0.7	0.2
128	-2.2	0.1	-2.2	1.8	1.7
129	-0.8	-0.1	-0.7	-0.8	-0.7
130	-3.3	8.7	-4.1	-3.0	-4.0
131	4.1	7.4	-1.3	5.5	-0.7
132	0.1	0.8	-0.6	3.0	1.8

Af positive = Score rating on the neat result for the Af positive sample, prior any correction; Test Solution = Score rating for the result for the test solution; Af positive TSC = Score rating on the result of the Af positive sample, after correction with the test solution result; Af positive REC = Score rating on the result of the Af positive sample, after correction for recovery; Af positive CRC = Score rating on the result of the Af positive sample, after correction with the test solution result and recovery.

Table 9e: z-scores for Total Aflatoxin

LAB ID	Af positive	Test Solution	Af positive TSC	Af positive REC	Af positive CTSREC
101	-0.1	-	0.0	1.1	1.2
102	1.3	-	0.6	2.4	1.4
103	-1.5	-	-1.3	0.1	0.3
104	-1.3	-	-1.3	-0.1	-0.2
105	-1.2	-	-1.1	-0.5	-0.4
106	-0.1	-	1.6	-0.2	1.4
107	15.4	-	5.4	18.9	7.1
108	-3.1	-	-3.1	-3.0	-3.0
109	-0.3	-	-2.9	0.8	-2.5
110	-1.4	-	-0.8	-0.4	0.3
111	-0.6	-	-1.2	0.9	0.2
112	-0.8	-	-0.8	-0.4	-0.5
113	-1.0	-	-0.7	-0.1	0.3
114	15.0	-	15.4	17.7	18.2
115	-3.1	-	-3.1	-2.9	-3.0
116.1	-1.4	-	-1.2	-1.0	-0.9
116.2	-1.3	-	-1.3	-1.1	-0.9
117	-2.2	-	-2.2	-1.5	-1.5
118	-1.1	-	-1.1	-0.2	-0.4
119	-0.1	-	-0.7	0.4	-0.3
120	-3.7	-	-3.7	-2.9	-2.9
121	-0.3	-	-0.1	0.4	0.6
122	-1.0	-	-1.6	0.4	-0.4
123	0.1	-	-0.5	0.2	-0.4
124	-0.5	-	-0.8	-0.5	-0.8
125	1.0	-	-0.3	1.3	0.0
126	0.2	-	0.3	0.0	0.1
127	0.3	-	0.1	0.6	0.4
128	-1.4	-	-1.1	-0.2	0.2
129	0.2	-	0.2	0.3	0.3
130	-1.0	-	-2.9	-0.2	-2.5
131	11.2	-	2.1	9.6	1.3
132	-0.3	-	0.1	0.3	0.7

Af positive = Score rating on the neat result for the Af positive sample, prior any correction; Test Solution = Score rating for the result for the test solution; Af positive TSC = Score rating on the result of the Af positive sample, after correction with the test solution result; Af positive REC = Score rating on the result of the Af positive sample, after correction for recovery; Af positive CRC = Score rating on the result of the Af positive sample, after correction with the test solution result and recovery.

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Abstract
A proficiency test was conducted with 31 European National Reference Laboratories (NRLs) for mycotoxins and one Laboratory from Turkey. Test materials were a mixed aflatoxin (Af) solution in acetonitrile and two candidate Certified Reference Materials ("aflatoxin positive" and "blank") that have not yet been released. Laboratories determined the aflatoxin content by reverse-phase high-performance liquid-chromatography (RP-HPLC) with either fluorescence or mass-selective detection against their own standard solutions as reference.

Applying the modified Horwitz equation according to Thompson as a basis for the target standard deviation (22% in the case of this proficiency test), 26 out of 32 NRLs achieved z-scores of less than 2 and 17 NRLs reported values within the uncertainty range for both aflatoxin B1 and total aflatoxins in the candidate CRM after correction for recovery in both cases.

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