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REPORT ON THE COLLABORATIVE TRIAL
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Fluorimetric Detection

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Abstract

An inter-laboratory comparison was carried out to evaluate the effectiveness of a method based on immunoaffinity column clean-up followed by high performance liquid chromatography using fluorimetric detection (HPLC-FL). The method was tested for the determination of ochratoxin A (OTA) in animal feed at concentration levels relevant to those proposed according to Commission Recommendation 2006/576/EC (1). The test portion of the sample was extracted with methanol:water. The sample extract was filtered, diluted, passed over an immunoaffinity column for clean-up and then eluted with methanol. The separation and determination of the OTA was performed by reverse-phase high performance liquid chromatography (HPLC) with fluorescence detection with an excitation of 333 nm and emission of 467 nm.

The animal feed samples, both spiked and naturally contaminated with OTA, were sent to 35 laboratories in 15 EU Member States, Colombia, Canada and Japan. Each laboratory received 6 duplicate samples – 4 coded and 2 blanks for spiking with coded solutions. Blank marked test portions of the samples were spiked at levels of 76 µg/kg and 305 µg/kg OTA. The range of recovery values reported spanned from 47 % – 124 % with an average value of 82 % and 79 % for each level respectively. Based on results for spiked samples (blind duplicates at two levels), as well as naturally contaminated samples (blind duplicates at three levels), the relative standard deviation for repeatability (RSD_r) ranged from 3.1 % – 4.7 %. The relative standard deviation for reproducibility (RSD_R) ranged from 13.5 % – 14.6 %. After correction for recovery, the RSD_R values improved significantly and ranged from 5.6 % – 6.4 %for naturally contaminated test materials. This method therefore showed acceptable within-laboratory and between-laboratory precision for each matrix.

Introduction

Previous collaborative study projects with other mycotoxins (2, 3, 4, 5, 6) have shown that, with care and attention to detail during the organisation of a collaborative trial, it is possible to achieve impressive performance characteristics for a method suitable for low limits of detection. Due to the complexity of feed matrices, particular care was taken during preparation (blending of relevant matrix constituents and extensive homogenisation) and in demonstrating inter-unit homogeneity before undertaking the study. Furthermore the precise determination of the contamination levels in the matrices for which the legislative limits apply, require a robust and reliable analytical method.

Test Materials for the Collaborative Study

For this method validation study various animal feed constituents such as rye, rice, linseeds, soy, maize and sugar beet pellets were blended with a material highly contaminated with OTA, to obtain sufficiently high levels that were needed for this study.

Table 1: Levels of OTA obtained in the animal feed test materials after blending

Test Material	**Ingredients and Amount (%)**	**OTA in µg/kg**
blank	Rye 26%, Rice 19%, Linseed 8%, Soy 11%, Maize 25%, Sugar beet pellets 11%	<3
Level 1	Oat flakes 10 % Rye 1 % Sugar beet pellets 7 % Maize 54 % Linseed 4 % Rice 24 %	35
Level 2	Rye 12 % Sugar beet pellets 17 % Maize 23 % Soy 31 % Rice 17 %	178
Level 3	Rye 9 % Sugar beet pellets 14 % Maize 40 % Linseed 1 % Rice 14 % Cerealflakes 22 %	298

After blending and milling to < 0.3 mm, the materials were subsequently filled into 50 mL containers (approx. 30 g each container) and kept at –18° C until analysis for homogeneity or dispatch for collaborative trial testing.

Homogeneity of the Test Materials

According to generally accepted procedures for homogeneity testing, every 10th sample had been taken from the sequence during packing. The number of the first container from which the sampling started was randomly selected for each material. These selected test materials were analysed for homogeneity and results were evaluated by ANOVA. The method subjected to collaborative testing (Annex) was used to assess the homogeneity of the test materials. The study was done under repeatability conditions. The variation between sample units were statistically not different from within sample unit (F(calc)<F(crit)). Thus the homogeneity test (Table 2) showed that in all cases sufficiently homogeneous material was achieved based on ANOVA and a sufficient coefficient of variance (RSD_r) of the analysis of the materials[1].

Table 2: Homogeneity test results:

Material OTA [µg/kg]	Number of pairs	RSD_r	F_{crit}	F_{calc}
<3	-	-	-	-
35	10	4.2	3.02	2.27
178	10	9.7	3.02	0.93
298	10	6.7	3.02	1.49

[1] Only parts of the material lots were used for this collaborative study and the ANOVA results were calculated for these specific parts of the corresponding lots.

Organisation of Collaborative Study

The instructions for participants in the inter-laboratory comparison are given in the Annex of this report. A total of 33 collaborators, representing 15 EU Member States, Colombia, Canada and Japan as a cross-section of government, food control, academia and food industry affiliations registered in the collaborative trial. Thirty-one of them returned results: their names and addresses are given in Table 3.

Table 3: List of participants in the inter-laboratory comparison exercise that returned results for the determination of OTA in animal feed.

Participant	Institution
Henryka Wisniewska-Dmytrow	National Veterinary Research Institute
Jaroslava Petrová, Marie Mrkvilova	ÚKZÚZ
Pedro A. Burdaspal	Spanish Food Safety Agency
Jean-Claude Motte	VAR-CODA-CERVA
Stéphanie Aladenise	ROYAL CANIN Central Laboratory
Giuseppina Avantaggiato	CNR ISPA-BA
Ph. Bastijns	OLEOTEST n.v.
Theo de Rijk	RIKILT-Institute of Food Safety
Barbara de Santis	Istituto Superiore di Sanità
Gonzalo J. Diaz	National University of Colombia
József Dömsödi	Central Feed Investigation Laboratory
Eugene Gawalko	Canadian Grain Commission
Tetsuhisa Goto	Shinshu University
Jan Grajewski	Kazimierz Wielki University
Gudrun Hanschmann	Sächsische Landesanstalt für Landwirtschaft
Marie-Paul Herry	Laboratoire de RENNES
Yoshihito Ishihara	Food and Agricultural Materials Inspection Center (FAMIC)
John Keegan	Public Analyst's Laboratory
Renaud Le Bouquin	LAREAL
Marina Martins	Laboratório Nacional de Investigação Veterinária (LNIV)
Norma Perilla	Micotox Ltda
Elisabeth Pichler	Quantas Analytics GmbH
Mike Roscoe	Canadian Food Inspection Agency
Elisabeth Reiter	University of Veterinary Medicine
Ludovic Sarcher	Deltavit Laboratory
Fátima Silva	Universidade Católica Portuguesa
Alexey Solyakov	National Veterinary Institute,
Dionisis Theodosis	LGC Limited,
Hana Valenta	Friedrich-Loeffler-Institut
Klaas van Schalm	MasterLab BV
Jeroen Vancutsem	FAVV

For the collaborative trial each participant received:

1. Eight coded sample containers (blind duplicates at four concentration levels) plus four 'blank'-labelled ones for spiking.
2. One amber vial marked 'OTA Calibrant' containing OTA in a 99% acetonitrile-1% acetic acid solution, which was to be employed as the calibrant OTA solution, as described in the method.
3. Eight vials marked 'Spike solution A, B, C and D' to be used for spiking procedures.
4. Twelve immunoaffinity columns with antibodies against OTA.
5. A copy of the collaborative study method.
6. A copy of the spiking protocol.
7. A 'Collaborative Study Materials Receipt' form.
8. Report forms.
9. A results questionnaire.

Each participant was required to prepare one extract from each container and perform the analysis by HPLC. Additionally each participant was required to spike the four materials indicated as 'Blank' using the provided spike solutions.

Method of analysis

The method of analysis that was used in this study can be found in the Annex.

Results and Discussion

Collaborative trial results

All data submitted for the study is presented in Table 4. The data is given as individual pairs of results for each laboratory (identified with the lab ID codes that were used for reporting). Blank samples were spiked with 76 µg/kg and 301 µg/kg of OTA (identified as sample 'spL' and 'spH'). All other samples were blind duplicates of 'blank' (bl) and of naturally contaminated materials identified with the target level analogue to the spiked materials. The results for duplicate determinations of OTA are shown in chart form in the Annex as mean&range plots for spiked and naturally contaminated samples. The mean&range plot displays the individual duplicate results, shown as a bar for each laboratory, against the overall mean result. The results are also presented as Youden plots in the Annex. The Youden plots confirmed the validity of the identification of outlier laboratories shown in the mean&range plots and did not show any inconsistencies in the data used to generate the precision data from the collaborative trial results.

Table 4: Individual results of OTA determined using HPLC-FL

OTA concentration µg/kg (target values)												
Lab ID	**spL[1]**	**spL[1]**	**spH[1]**	**spH[1]**	**bl**	**bl**	**39[2]**	**39[2]**	**150[2]**	**150[2]**	**338[2]**	**338[2]**
101	55	52	209	216	<1	<1	36	34	131	137	291	299
102	62	61	248	240	<.25	<.25	40	38	153	147	339	336
103	62	61	**278**	**214**	<5	<5	40	38	149	127	311	265
105	45	47	177	184	<1	<1	31	30	114	117	265	254
106	54	54	210	211	<20	<20	36	33	135	140	315	313
107	70	72	278	262	<1	<1	44	44	178	171	428	388
108	77	84		302	3.9	6.7	**65**	**51**	197	183	435	412
109	67	68	259	261	0	0	43	42	167	164	354	383
110	64	65	264	263	1.3	<1	39	37	156	159	354	359
111	66	66	257	257	<1	<1	39	38	155	150	332	334
112	68	65	252	260	<20	<20	47	45	171	170	371	384
113	67	68	258	256	<10	<10	43	43	160	157	355	358
114	58	60	224	226	<20	<20	38	37	133	134	285	294
115	15	18	52	57	<1	<1	12	11	34	37	89	89
116	70	69	273	267	1.5	1.5	43	37	174	169	378	373
117	71	79	286	278	<2	<2	45	43	165	175	383	386
118	68	63	228	238	10.5	10.7	49	46	157	156	333	347
119	56	52	237	204	<0.1	<0.1	36	33	144	120	290	268
120	57	56	227	217	<5	<5	38	38	144	146	350	312
121	63	66	**240**	**144**	<5	<5	37	38	147	146	337	334
122	<4	<4	<4	<4	<4	<4	<4	<4	<4	6	<4	<4
123	49	44	184	200	<5	<5	26	32	97	119	251	265
124	62	62	243	250	<0.5	<0.5	39	39	159	150	353	352
126	78	77	327	310	<1	<1	52	51	198	205	490	455
127	54	57	197	200	4.9	4.8	34	37	126	121	315	271
130	52	53	182	196	4.4	4.4	34	34	119	121	264	274
131	67	65	233	256	<5	<5	43	43	155	156	345	328
132	58	62	242	241	<3	<3	37	38	145	148	323	338
133	48	50	207	247	0	0	33	32	132	128	313	306
134	70	73	280	292	<20	<20	43	47	155	160	409	353
135	62	60	251	237	<20	<20	36	35	147	158	338	333

	Invalid data that was removed prior statistical analysis[3]
	Outliers identified by statistical analysis

[1] Spiked Material (spL = 76 µg/kg and spH = 305 µg/kg)
[2] Naturally contaminated material
[3] For detailed information, see comments section

Comments of Participants

Generally the participants found that the method was easy to use and well documented. Participants (one exception), had previous experience in the analysis of OTA (6 months to 30 years) and in this study used various types of RP-HPLC columns at different flow rates (0.3 mL/min to 1.5 mL/min). The good method performance can be considered as an indicator for a robust HPLC part of this method. About one third of

the participants performed the analysis over two days, either analysing one part in one day and another the second, or performing the extraction and clean-up for all on one day and the HPLC part the next. No influence of over-night analysis breaks or splitted sample analysis was observed or resulted in outliers. Several participants deviated from the method description and used centrifugation either instead of filtration of extracts or instead of glass fibre filters, but no difference could be observed between these procedures. Glass fibre filtration was also performed without vacuum (gravity only) in several cases.

Several participants deviated in the preparation of the calibrant solutions or the solvent for injection. One commented that otherwise different peak size (shape) would be obtained. None of these deviations appeared to have a significant effect on the method performance.

One laboratory returned results below the LOQ or very low (LabID 122). From the questionnaire it could not be concluded what the reason for this was. The participants reported that one IAC was running very slow but this cannot explain these low results. The participant also had noted these unusual results. Results were excluded prior to statistical analysis. It has to be noted that such observations (one single participant returning results lower or very close to LOD/LOQ) have occurred in the past in other collaborative studies and the reason could so far never be elucidated, however that is a rare but known phenomenon, and whether shipping and storage conditions or other factors play a role is difficult to assess.

One participant (LabID 108) noted that IACs were running at different speed and had to slow down in some cases by restricting the flow rate. Also he found that one result could not be generated due to loss of sample during analysis. The two outliers that were identified during statistical analysis indicate that the application of extracts to IACs might be a step in the method that needs to be carefully controlled.

Another participant (LabID 114) reported that the extraction was carried out by ultra-sound rather than shaking, but this appears to have no influence on the method. This confirms the results of a previous proficiency test on OTA in which extraction by ultra-sound or shaking showed comparable results.

Statistical analysis of results

In some cases data was excluded from the statistical analysis. This was the case when statistical evaluation was impossible (values reported as "0" zero or below the detection limit) or when data-sets were identified as data intrinsically prone to error (only if pointed out in the Comments section). In three cases telephone contact was established with participants as a general bias by a certain factor for all their test results was observed. In two cases this could be solved, while in one case (LabID 115) no reason for miscalculation was identified and data could therefore not be corrected. As a conclusion of the above said, results from two participants (ID 115 and 122) were not considered for statistical analysis.

The collaborative trial results were also examined for evidence of individual systematic error ($p<0.025$) using Cochran's and Grubbs tests progressively (7). Pairs of results that were identified as outliers are indicated with shaded background in Table 4. For these results (excluding the data for blank materials) the maximum numbers of outliers identified was from two laboratories, giving acceptable data ranging from 26 to 29 laboratories.

Precision estimates were obtained using a one-way analysis of variance approach according to the IUPAC Harmonised Protocol (7, 8). Details of the average analyte concentration, the standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), the number of statistical outlier laboratories, the HORRAT ratio and the percentage recovery are presented in Table 5**.** In Table 6 the precision data for naturally contaminated materials is listed after recovery correction from the spiking experiments (mean recovery of all four spikes).

Table 5: Precision estimates[2]

Added ng/g	No. of Labs$^{m(n)}$	Mean ng/g	S_r ng/g	S_R ng/g	RSD_r (%)	RSD_R (%)	r ng/g	R ng/g	HORRAT value$^{H(Th)}$	Mean Recovery (%)
75.8	28(1)	62	2.07	8.90	3.33	14.3	5.80	24.9	0.6 (0.6)	82
305	26(2)	240	9.91	33.5	4.12	13.9	27.7	93.8	0.7(0.7)	79
nc (0)	29(0)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
nc(39)	28(1)	39.0	1.62	5.30	4.15	13.6	4.53	14.8	0.5(0.6)	n.a.
nc(150)	29(0)	150	6.60	21.9	4.40	14.6	18.5	61.2	0.7(0.7)	n.a.
nc(338)	29(0)	338	16.0	51.2	4.74	15.2	44.8	144	0.8(0.8)	n.a.

nc – naturally contaminated, n.a. – not applicable
number of laboratories, where m = number of labs retained after outliers removed and
(n) = number of outliers
R and r are calculated according to the IUPAC Harmonized Protocol.
HORRAT values, where H = the value calculated according to the classical Horwitz approach and
Th = value according to the modified approach by Thompson (9)

[2] The precision parameters in Tables 5 and 6 have been calculated using the Microsoft Excel spreadsheet 'CLSTD.XLT v3.6' provided with courtesy by Ken Mathieson (Central Science Laboratories, York, UK).

As EU legislation for food requires to consider analyte recovery for accepting or rejection of lots in official food control, the principle of recovery correction was applied in this study. As a result, the data sets of the analytical results from naturally contaminated material were corrected with the mean recovery value of the four recovery experiments (two duplicates). The result of this treatment on the calculated method performance is shown in Table 6. As a result, the performance increased significantly and reproducibility showed unexpectedly low values, indicating that for this type of analysis the correction for recovery results in a drastic improvement of the method performance under the conditions of this study (use of a common calibrant, common spiking procedure). The improved reproducibility after recovery correction is very close to the calculated repeatability. This can be seen in the according mean&range and Youdon plots in the Annex.

Table 6: Precision estimates for naturally contaminated materials after recovery correction of results

RCV ng/g	No. of Labs$^{m(n)}$	Mean ng/g	S_r ng/g	S_R ng/g	RSD_r (%)	RSD_R (%)	r ng/g	R ng/g	HORRAT Value$^{H(Th)}$
nc (0)	15(0)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
nc(39)	26(4)	48.7	1.76	2.73	3.61	5.60	4.92	7.63	0.2(0.3)
nc(150)	29(1)	186	8.90	10.1	4.79	5.46	24.9	28.4	0.3(0.3)
nc(338)	30(0)	420	19.1	26.8	4.55	6.38	53.5	75.0	0.4(0.4)

RCV – Recovery Corrected Value derived from the collaborative study after recovery correction
nc – naturally contaminated, n.a. – not applicable
number of laboratories, where m = number of labs retained after outliers removed and
(n) = number of outlier
R and r are calculated according to the IUPAC Harmonized Protocol.
HORRAT values, where H = the value calculated according to the classical Horwitz approach and
Th = value according to the modified approach by Thompson (9)

Precision characteristics of the method

Due to differences in reporting limits for not detectable the results for 'blank' materials were not analysed statistically. The results however clearly indicated that all participants could identify the blank pairs of samples for baby food as not containing detectable OTA.

Blank test portions of the samples were spiked at levels of 76 µg/kg and 305 µg/kg OTA. The range of recovery values reported spanned from 47 % – 124 % with an average value of 82 % and 79 % for each level respectively. Based on results for

spiked samples (blind duplicates at two levels), as well as naturally contaminated samples (blind duplicates at three levels), the relative standard deviation for repeatability (RSD_r) ranged from 3.1 % – 4.7 %. The relative standard deviation for reproducibility (RSD_R) ranged from 13.5 % – 14.6 %. After correction for recovery, the RSD_R values improved significantly and ranged for naturally contaminated test materials from 5.6 % – 6.4 %. This method therefore showed acceptable[5] within-laboratory and between-laboratory precision for each matrix.

Interpretation of results

The acceptability of the precision characteristics of the method was assessed on the basis of European legislation for methods of analysis for food (9), since no equivalent benchmark is available for feed. The fact that the benchmarking of methods for food is limited for a contamination range up to 10 µg/kg was not considered as critical and the method performance was benchmarked against the more stringent parameters in this regulation.

In order to benchmark the obtained performance characteristics, these values were compared with the performance requirements for food as given in Regulation (EC) 401/2006 (see Table 7) and are summarised in Table 8 with remarks on the qualification of each contamination level tested. Even though such qualifier benchmark (here for method in food analysis) is not available for feed analysis, this benchmark can be considered as a good basis for the evaluation of the fitness-for-purpose of the analytical methods for feed. As a result the method performance was satisfactory for all parameters and all levels tested.

Table 7: Acceptance criteria for analytical methods for OTA [9]

Level µg/kg	OTA		
	RSD_r %	RSD_R %	Recovery %
≤ 1	≤ 40	≤ 60	50 – 120
1 - 10	≤ 20	≤ 30	70 – 110

Table 8: Method performance parameters obtained in the collaborative trial[3]

Level[4] µg/kg	Obtained parameter			Qualified YES/NO[5]
	RSDr %	RSDR %	Recovery %	
blank	-	-	-	n.a.
76	3.1	13.5	82	YES
301	4.1	13.9	79	YES
nc(39)	4.2	13.6	-	YES
nc(150)	4.4	14.6	-	YES
nc(338)	4.7	15.2	-	YES

Materials for which no recovery data is given (marked with '–') were naturally contaminated.

Conclusions

The results of this inter-laboratory comparison regarding a method for determination of ochratoxin A in animal feed show precision characteristics which fulfil the criteria (RSD_r, RSD_R and recovery) at the levels of the guidance values that have been recommended by European Commission for mycotoxins in animal feed (1). A correction for recovery with the data generated by fortification experiments further improved the reproducibility performance of the method. In conclusion, the method qualified as a reliable method for monitoring ochratoxin A in animal feed. The JRC is currently transforming this method into CEN format and will submit it to CEN TC 327/WG 1 for adoption.

[3] Performance parameters prior correction for recovery.
[4] Mean level as reported in the collaborative trial.
[5] The qualification required that the performance parameters obtained in the collaborative trial fulfilled the requirements set in Regulation 401/2006/EC for methods for food.

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Annex I

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JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
Community reference laboratory for mycotoxins

Determination of Ochratoxin A in animal feed by immunoaffinity column clean-up and High Performance Liquid Chromatography with fluorescence detection.

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Foreword

THIS IS A STUDY FOR THE EVALUATION OF THE METHOD, NOT FOR ASSESSING THE PERFORMANCE OF THE LABORATORY. THE METHOD MUST BE FOLLOWED AS CLOSELY AS PRACTICABLE, AND ANY DEVIATIONS FROM THE METHOD AS DESCRIBED, NO MATTER HOW TRIVIAL THEY MAY SEEM, MUST BE NOTED ON THE REPORT FORM.

WARNING — the use of this protocol involves hazardous materials, operations and equipment. This protocol does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1. SCOPE

This protocol specifies a candidate method for the determination of Ochratoxin A (OTA) in cereal animal feed using liquid-chromatography with fluorescence detection. This candidate method will be validated for the determination of OTA via the analysis of naturally contaminated and spiked samples of cereal animal feed.

2. PRINCIPLE

OTA is extracted from the test material with a mixture of methanol – 3% aqueous sodium bicarbonate solution. The extract is filtered, diluted with PBS and purified using immunoaffinity columns (IAC). The purified OTA is eluted from the IAC using neat methanol and then water, brought to a defined volume with water and quantified by HPLC with fluorescence detection.

3. REAGENTS AND MATERIALS

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade. Solvents shall be of HPLC or better quality.

3.1. **Methanol,** technical grade.

3.2. **Methanol,** HPLC grade.

3.3. **Water,** ISO 3696 grade I (HPLC grade) and grade III water, or equivalent.

3.4. **Acetonitrile,** (CH3CN) HPLC grade.

3.5. **Acetic acid**, (CH3COOH) glacial 96% minimum.

3.6. **Sodium hydrogen carbonate, (NaHCO3) minimum 99% purity.**

3.7. **Phosphate buffered saline (PBS) concentrate**

Dissolve the following in 1800 ml of water (ISO grade I):
4 g KCl
160 g NaCl
72 g Na_2HPO_4*12 H_2O

3.8. **PBS Ready to use**

Dilute 100 ml of PBS concentrate (3.7) to 1000 ml with water (ISO grade I). Adjust to pH 7.4 with 1 M HCl and make up to 2000 mL with water (ISO grade I).

or

Phosphate buffered saline (PBS) tablet: f.i. Sigma P4417

One tablet dissolved in 200 mL of water (ISO grade I) yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C.

3.9. 3% aqueous sodium hydrogen carbonate solution.

Add 30 g of sodium hydrogen carbonate (3.6) to 1000 mL of water (ISO Grade III).

3.10. Extraction solvent, methanol – 3% aqueous sodium hydrogen carbonate solution 50:50 (v/v).

Add 500 mL of methanol (3.1) to 500 mL of 3% aqueous sodium hydrogen carbonate solution (3.9). Mix well.

3.11. Aqueous solution of glacial acetic acid.

Add 30 mL of glacial acetic acid (3.5) to 870 mL of water (ISO grade I) and filter.

3.12. HPLC mobile phase, acetonitrile - methanol – aqueous solution of glacial acetic acid 35:35:30 (v/v/v).

Mix 1050 mL of methanol (3.2) with 1050 mL of acetonitrile (3.4) and with 900 mL of aqueous solution of glacial acetic acid (3.11). Mix well and degas.

3.13. OTA stock solution.

A solution of OTA in acetonitrile:acetic acid (99:1); The concentration is 10 µg/mL OTA in case of this collaborative trial study.

3.14. OTA diluted stock solution for calibration.

Prepare 10 mL of working standard solution by diluting 100 times the stock standard solution (3.13) with mobile phase (3.12).

Prepare two separated diluted stock solutions!

3.15. Calibration solutions

From each of the two diluted stock solutions (3.14) prepare six levels of calibration solutions by adding the volumes of diluted stock solution listed below to a volumetric flask of the indicated volume and make up to the mark with mobile phase (3.12):

Calibrant	Diluted stock solution (3.14) [µL]	Volumetric flask (4.5) [mL]	Concentration OTA [ng/mL]
1	100	10.0	1
2	500	10.0	5
3	1000	10.0	10
4	1500	10.0	15
5	2000	10.0	20
6	2500	10.0	25

3.16. Immunoaffinity columns with antibodies specific to OTA

3.17. Test materials

Homogenized, ready-to-be-extracted, and coded; to be prepared once each.

3.18. Spiking materials

Homogenized, ready-to-be-extracted blank material for recovery determination; to be prepared once each.

3.19. Spiking solutions

OTA in a solution of acetonitrile/ acetic acid (99/1, v/v).

4. APPARATUS

Usual laboratory equipment and, in particular, the following:

4.1. Common laboratory glassware, such as graduated cylinders, beakers, volumetric pipettes…

4.2. Analytical balance, capable of weighing to 0.1 mg.

4.3. Horizontal or vertical shaker.

4.4. Automated SPE Vacuum System, Supelco Visiprep Extraction Manifold, or equivalent.

4.5. Volumetric flasks (class A, ISO 1042), 5 mL, 10 mL (tolerances: ±0.025 mL), 100 mL (tolerance: ±0.1 mL).

4.6. Filter paper pre-folded, Whatman No. 113 V, 18.5 cm or equivalent.

4.7. Screw-cap flasks, 250 mL and 500 mL.

4.8. Glass funnels, 9 cm ID.

4.9. Reservoirs, polypropylene, suitable for attachment to top of immunoaffinity column, 50 to 75 mL size.

4.10. Plastic syringes, 5 mL.

4.11. Calibrated displacement micropipette, 100, 500 and 1000 µL (variable volume).

4.12. Solvent vacuum filtration system, suitable for 47 mm filter.

4.13. Glass microfibre filter, Whatman GF/A 1.6 µm (47 mm), or equivalent.

4.14. Vortex mixer

4.15. HPLC syringe filter cartridges, Nylon with 0.45 µm pore size.

4.16. Ultrasonic bath.

4.17. Amber glass vials, ca 2 mL capacity and crimp caps or equivalent.

4.18. HPLC system consisting of:

4.18.1 Pump, pulse free, flow capacity 0.5 mL/min to 1.5 mL/min.

4.18.2 Injector system, manual or autosampler, with loop suitable for 100-300µL injections.

4.18.3 Fluorescence detector, suitable for measurements with excitation wavelengths 333 nm and emission at 467 nm.

4.18.4 Integrator, or PC workstation.

4.19. Analytical reversed phase HPLC column, C18 RP-column suitable to allow a sufficient separation of OTA from other interfering components; Fully End Capped with column dimensions preferably 250 mm X 4.6 mm I.D. stationary phase with particle size 5 µm.

4.20. Pre-column, with preferably the same stationary phase material as the analytical column and internal diameter of 4.0 mm, stationary phase with particle size 5 µm.

5. SPIKING PROCEDURE

Please follow the spiking instructions given in the additional SPIKING PROTOCOL.

6. SAMPLE PREPARATION

6.1. Extraction of OTA

- Weigh 25.0 g, to the nearest 0.1 g, of the test sample into a large enough container with lid, f.i. 500 mL flask (4.7).
- Add 200.0 mL of extraction solvent (3.10), cap, and shake vigorously by hand for a few seconds; so that the material disperses evenly (**check visually**).
- Put on a shaker (4.3) for 40 min. Choose speed such that the material is mixed well without collecting in the top of the flask. *A possible alternative is to ultrasonificate for 15 minutes; in this case shortly shake the extract by hand for a few seconds.* ***If you choose ultrasonification, please indicate this CLEARLY in question 6 of the questionnaire.***
- Allow the extracted sample to settle after extraction.
- Filter the extract through folded filter paper (4.6) and collect the filtered extract in a screw cap flask of 250 mL (4.7).
- Transfer approx. 10 mL of the filtered extract into the vacuum system and filter through glass fibre filter (4.13) by applying a slight vacuum (4.12). Discard this volume and filter again the remaining extract in another screw cap flask of 250 mL (4.7) to obtain a clear extract for further analysis. Proceed immediately with the immunoaffinity column clean-up procedure (6.2).

NOTE: do not apply a strong vacuum in the beginning of the filtration process, as this can lead to turbid filtered extract after filtration.

6.2. Clean up and test solution

- Take one immunoaffinity column (IAC, 3.16) per extract.
- Attach a reservoir (4.9), **do not** empty storage solution from column.
- Transfer 4.00 mL of double filtered extract into a 100 mL volumetric flask (4.5), fill to volume with PBS (3.8) and shake.
- Apply 50.0 mL of the diluted extract to the reservoir on the immunoaffinity column (see previous point). *This is equivalent to 2.00 mL of the double filtered and undiluted extract.*
- Open the immunoaffinity column outlet.
- Draw extract through the column by gravity at a steady flow rate until all extract has passed the column and the last solvent portion reaches the lower frit of the column.
- **Make note of columns with exceptionally fast or slow flow rates!**
- After the extract has passed completely, wash the IAC with 10 mL of water (ISO grade I). Make sure that the water is pH neutral.
- Pass air through the IAC (f.i., using a properly fitted large syringe) in order to expel excess water.

- Place a 5 mL volumetric flask (4.5) underneath the IAC and add 0.75 mL of methanol (3.2) to the IAC.
- Collect the eluate in the 5 mL volumentric flask.
- After the last drops of methanol have passed through the immunoaffinity column, allow the methanol to remain on the column for approx. 1 minute. Then add a further 0.75 mL of methanol (3.2) and continue to collect the eluate, followed by 0.50 mL of water (ISO grade I).
- Carefully pass air through the column in order to collect most of the applied methanol and water.
- Immediately add 1.5 mL of acetic acid solution (3.11) into the volumetric flask.
- Fill the volumetric flask up to the mark with water (ISO grade I) and shake using a vortex. In case of turbid samples, filter test solutions through a HPLC syringe filter (4.15) with a plastic syringe (4.10). This can be used directly as test solution.

NOTE: alternatively to a manual procedure the immunoaffinity clean-up and elution can be performed with an automatic sample preparation unit, provided that volumes and aliquots remain unchanged.

7. MEASUREMENTS:

7.1. HPLC operating conditions

Using the equipment outlined in 4.18, the following conditions have proven to produce satisfying results:

Mobile phase: as in 3.12

Flow rate: 0.7 – 1.5 mL/ min

Injection vol.: 100 µL

Column: as in 4.19

Guard column: C18

Fluorescence detector: Excitation λ: 333 nm

Emission λ: 467 nm

No temperature control

7.2. Determination of ochratoxin A in test solutions

Inject aliquots of the calibration solutions (3.15) and of the test solutions (6.2) into the chromatograph using the same operating conditions.

7.3. Batch (Sequence) composition

Each of the included test materials (3.17) and spiking materials (3.18) are to be prepared once. For each batch of runs all the calibration solutions (3.15) are to be run at the beginning and at the end of the batch. At the beginning of the batch run the six levels of calibrants prepared from the first diluted stock solution (3.14) from lowest to highest concentration followed by the six levels from the second diluted stock solution from highest to lowest. Then run the test solutions (6.2) once in random order. At the end of the batch repeat the calibration solutions in reversed order from the beginning. Therefore, a batch of runs would, for example, look like Table 1 below.

7.4. Calibration

Plot the signals (peak area or height) of the calibration solutions against the corresponding concentrations for OTA. With linear regression estimate the slope and the intercept of each of the two calibration functions. Use the values of the calibration curve Cal2 level 6 → level 1 (equivalent to injections 7 to 12, see section 7.5) to report the results for the test materials. For the collaborative study, all values (for all four calibration curves) must be reported.

7.5. Peak identification

Identify the Ochratoxin A peak in the test solution by comparing the retention time with those of the calibration solutions. The signal (peak area or height) of OTA in the test solution must fall within the calibration range. If the OTA signal in the test solution exceeds the signals of the highest calibration solution the test solution shall be diluted to bring it within calibration range, and be reanalysed twice (double injection). The dilution factor must be incorporated into all subsequent calculations.

Run	Identity	Run	Identity	Run	Identity
1	Cal1 level 1	12	Cal2 level 1	29	Cal2 level 5
2	Cal1 level 2	13	Material 1	30	Cal2 level 6
3	Cal1 level 3	14	Material 2	31	Cal1 level 6
4	Cal1 level 4	15	Material 3	32	Cal1 level 5
5	Cal1 level 5	16	Material 4	33	Cal1 level 4
6	Cal1 level 6	…	…	34	Cal1 level 3
7	Cal2 level 6	24	Material n	35	Cal1 level 2
8	Cal2 level 5	25	Cal2 level 1	36	Cal1 level 1
9	Cal2 level 4	26	Cal2 level 2		
10	Cal2 level 3	27	Cal2 level 3		
11	Cal2 level 2	28	Cal2 level 4		

Table 1: Example for the order of runs in a batch of runs

8. DETERMINATION OF CONCENTRATIONS

Using the estimated slopes and intercepts from linear regression (7.4) calculate the concentrations of OTA ($c_{T(OTA)}$) in the test solutions (6.2) as follows:

$$c_{T(OTA)} = \frac{\text{signal}_{OTA} - \text{intercept}_{OTA}}{\text{slope}_{OTA}} \text{ [ng/mL]} \quad \text{(I)}$$

If the test solution was diluted because of a signal above the calibration range (7.5) multiply the calculated concentrations of OTA ($c_{T(OTA)}$) with the dilution factor.

To calculate the mass fractions (c_{SMP}) of the analyte in the original materials use the following equation:

$$c_{SMP} = \frac{c_T \times Solvent \times Elution}{W \times Aliquot} \text{ [ng/g or µg/kg]} \quad \text{(II)}$$

with

c_T (ng/ml) = calculated concentration of OTA (I), corrected for dilution if needed.

W(g) = weight of the test material used for extraction (25.0 g).

Solvent = total volume of the extraction solvent (200.0 mL).

Aliquot = extract aliquot used for IAC clean-up (2.0 mL).

(4 mL / 100 mL X 50 mL = 2 ml)

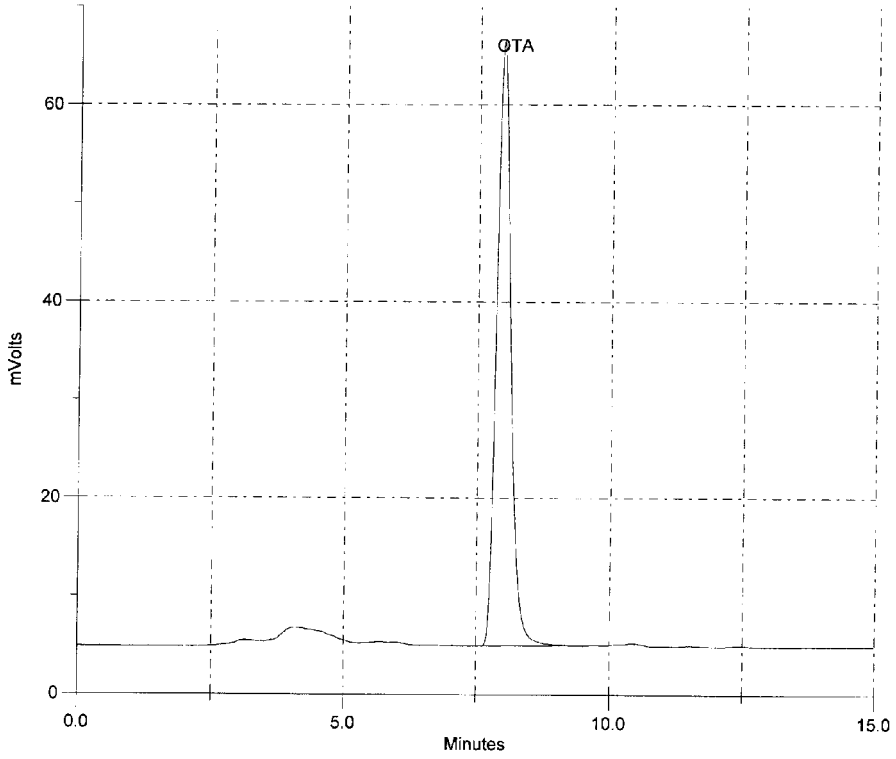
Elution = final volume achieved after elution from IAC (5 mL).

If the weight of the test material and the volumes described herein before are kept the above equation (II) can be simplified to:

$$c_{SMP} = c_T \text{ x } 20 \text{ [µg/kg]} \qquad \text{(III)}$$

9. Appendix A:

Example chromatogram (~200 µg/kg OTA in animal feed)



SPIKING PROTOCOL

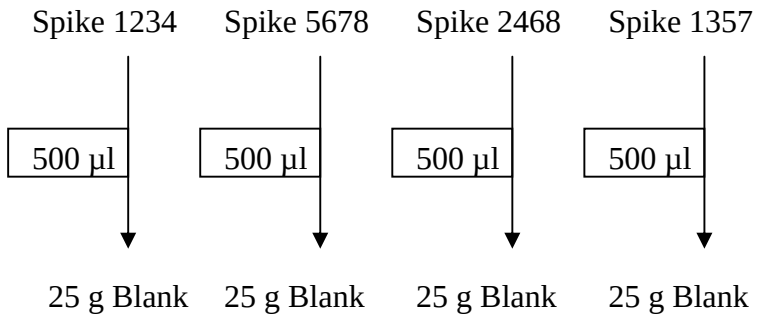
This box contains four sealed bags labelled "BLANK". These are blank materials to be used for recovery determinations.

There are also four ampoules labelled with a code. These are solutions of OTA in acetonitrile/acetic acid (99/1, v/v).

For the recovery determination proceed as follows (the references in parentheses refer to the method protocol):

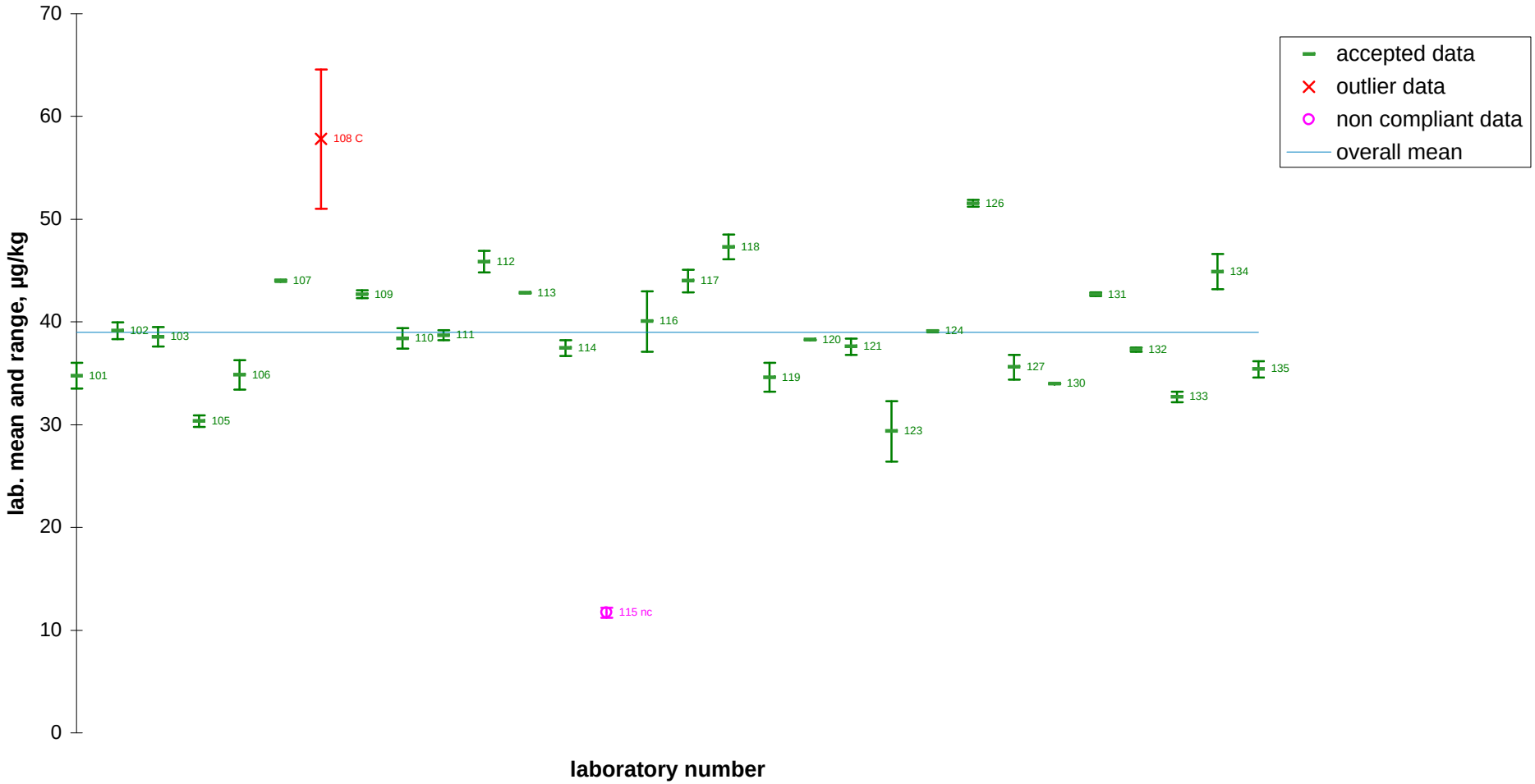
- Weigh 25.0 g, to the nearest 0.1 g, of the test sample into a large enough container with lid, f.i. 500 mL flask (4.7).
- Add **exactly** 500 µL of spiking solution to a blank marked test sample in the flask and repeat it for all the spiking solutions and blanks. You will end up with four spiked samples, each spiked with one portion of a spiking solution. For reporting, please use the code on the ampoules.
- Let stand for at least 30 min at room temperature to allow the acetonitrile/ acetic acid solution to evaporate and the OTA to migrate into the matrix.
- Analyse the content of OTA according to the method protocol. For this, proceed with the addition of 200.0 mL of extraction solvent (3.10) in section **6.1 Extraction of OTA** in the method protocol.

Example diagram:

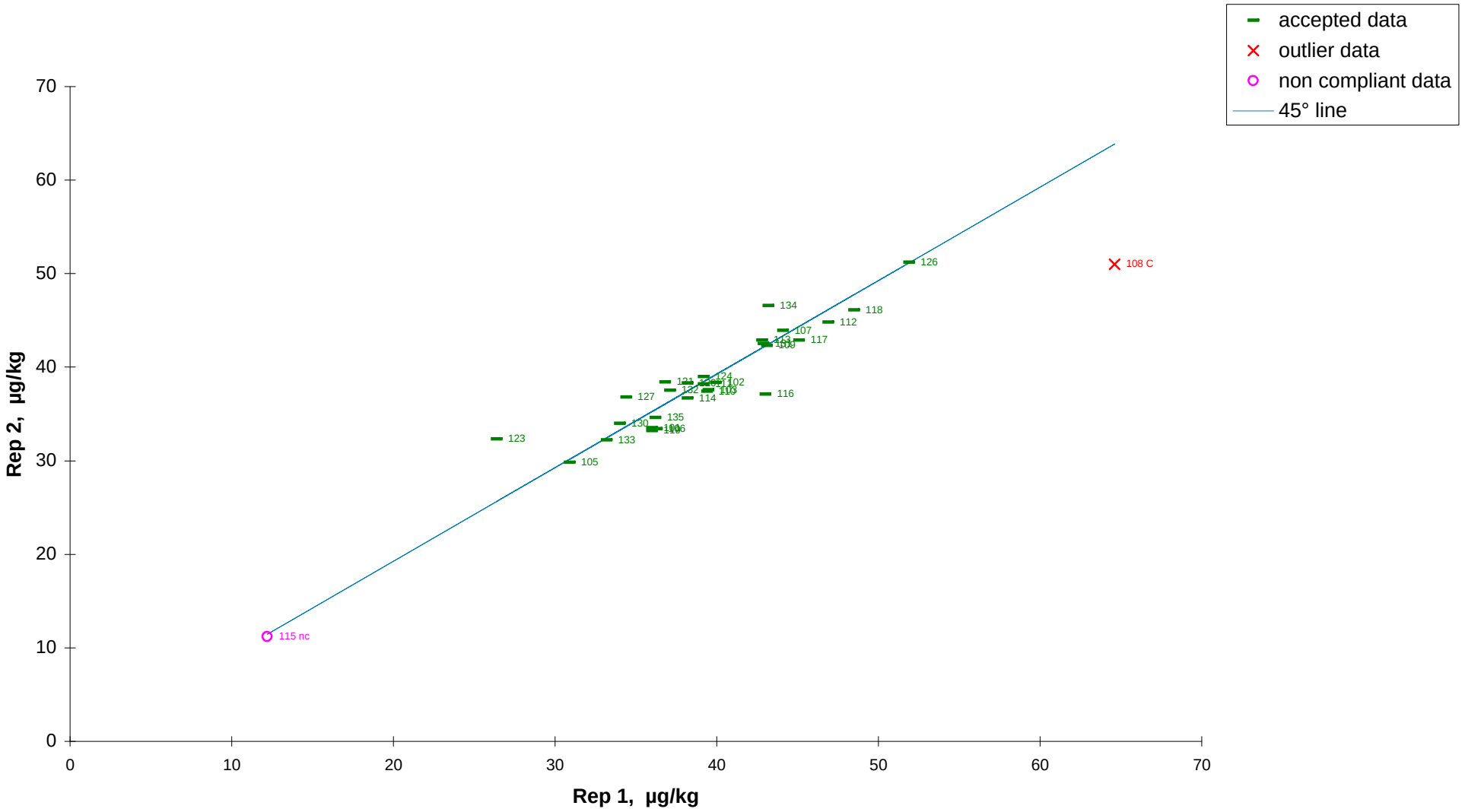


Figures

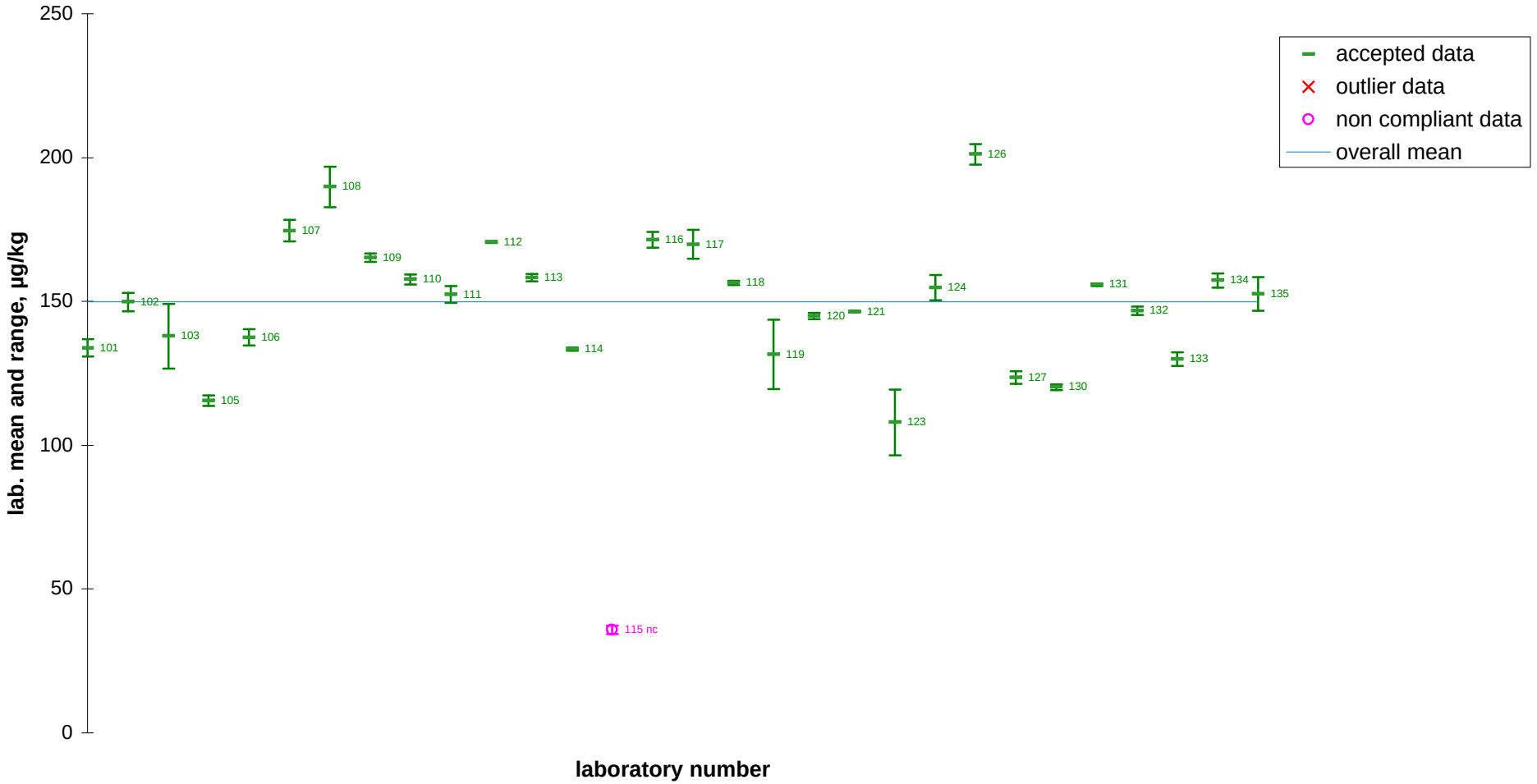
OTA in animal feed low level material - naturally contaminated - 39 µg/kg : blind replicates



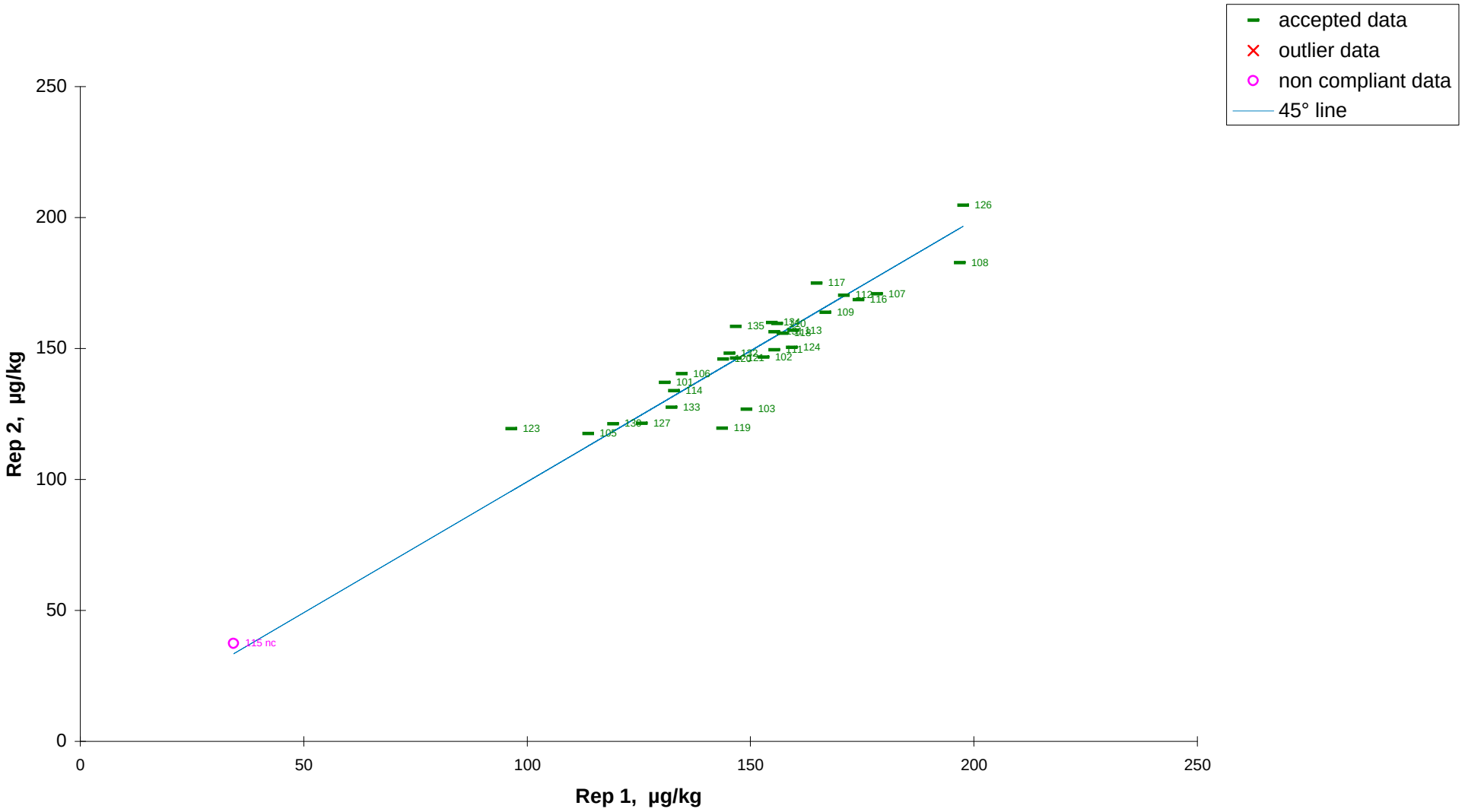
OTA in animal feed low level material - naturally contaminated - 39 µg/kg : blind replicates



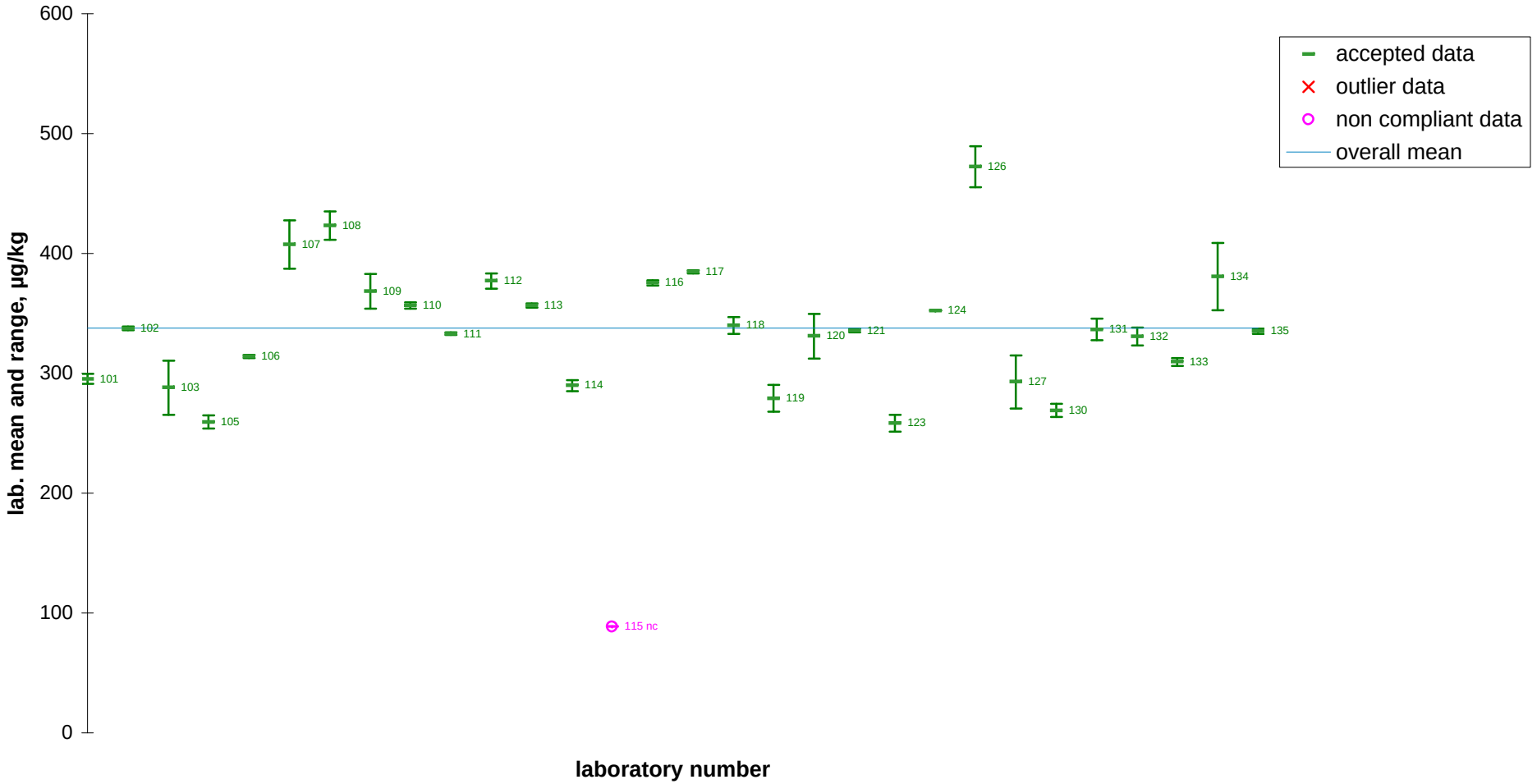
OTA in animal feed medium level material - naturally contaminated - 150 µg/kg : blind replicates



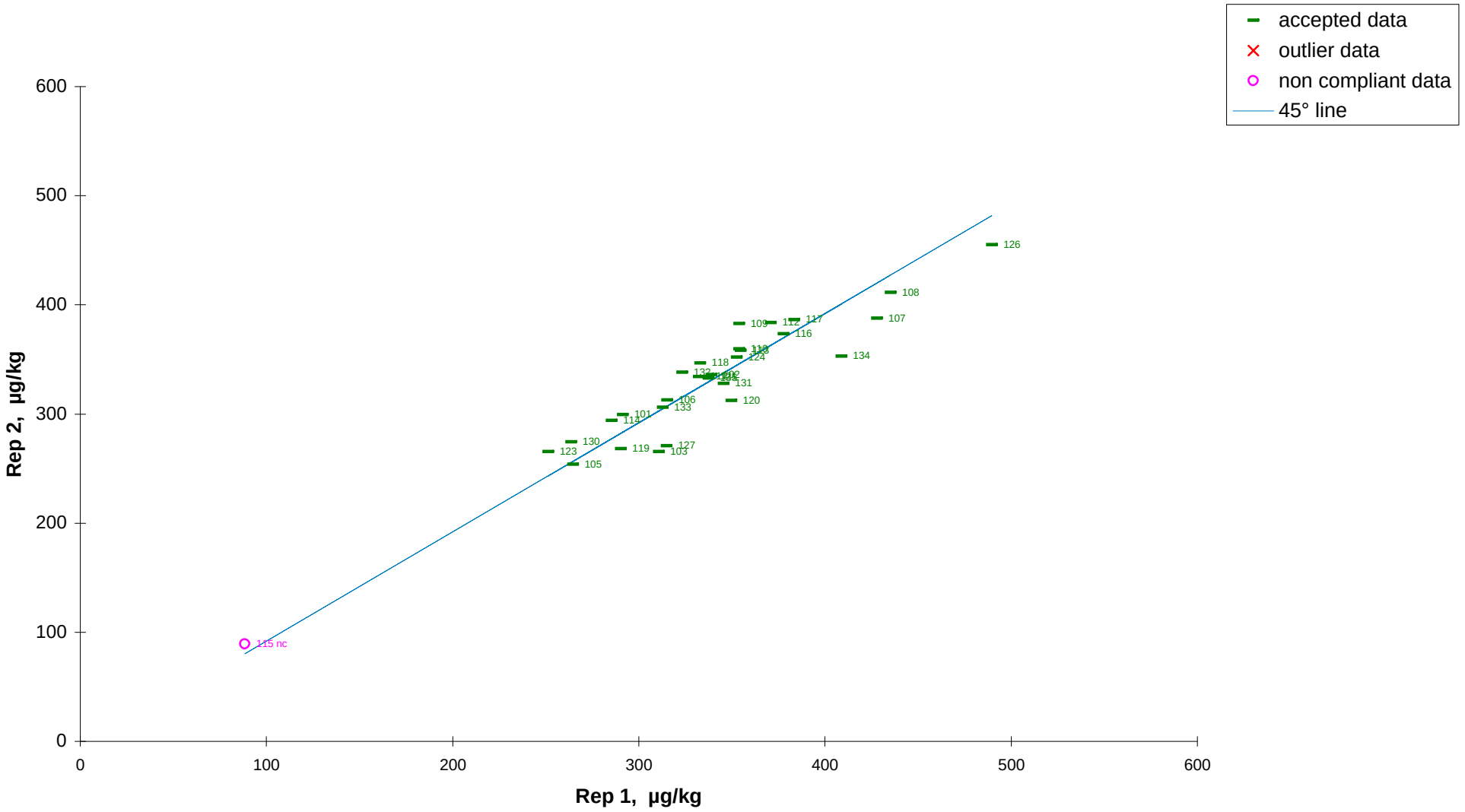
OTA in animal feed medium level material - naturally contaminated - 150 µg/kg : blind replicates

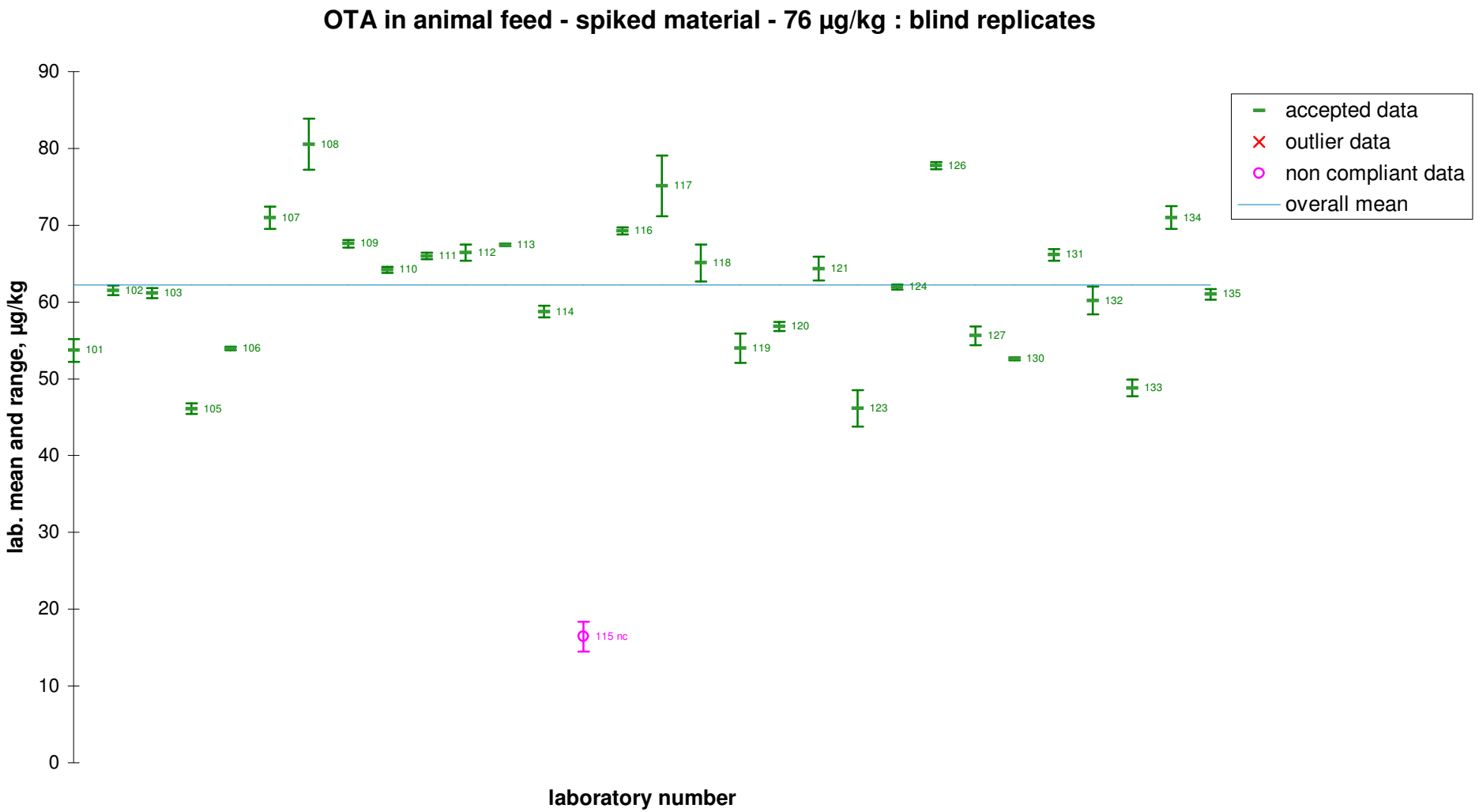


OTA in animal feed high level material - naturally contaminated - 338 µg/kg : blind replicates

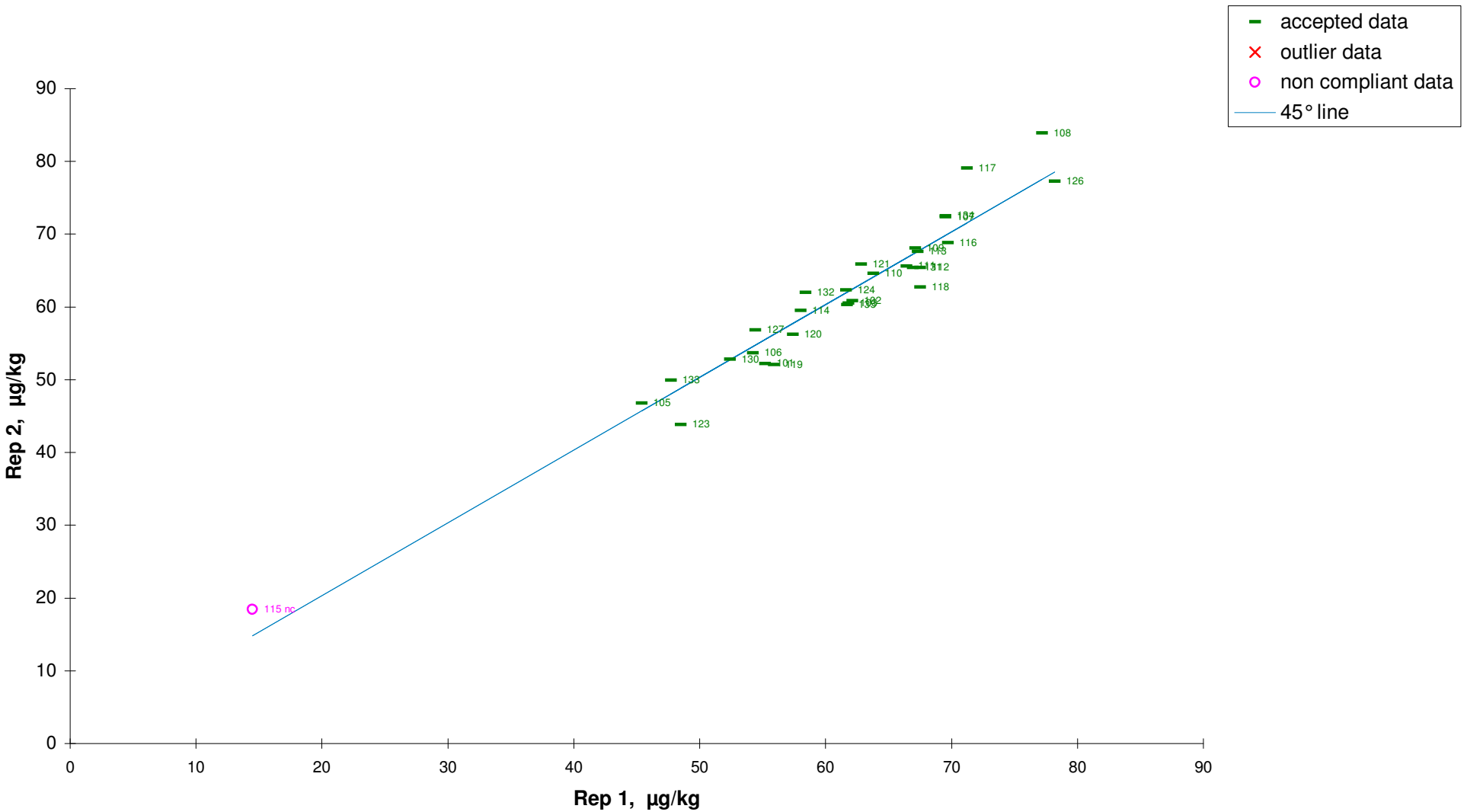


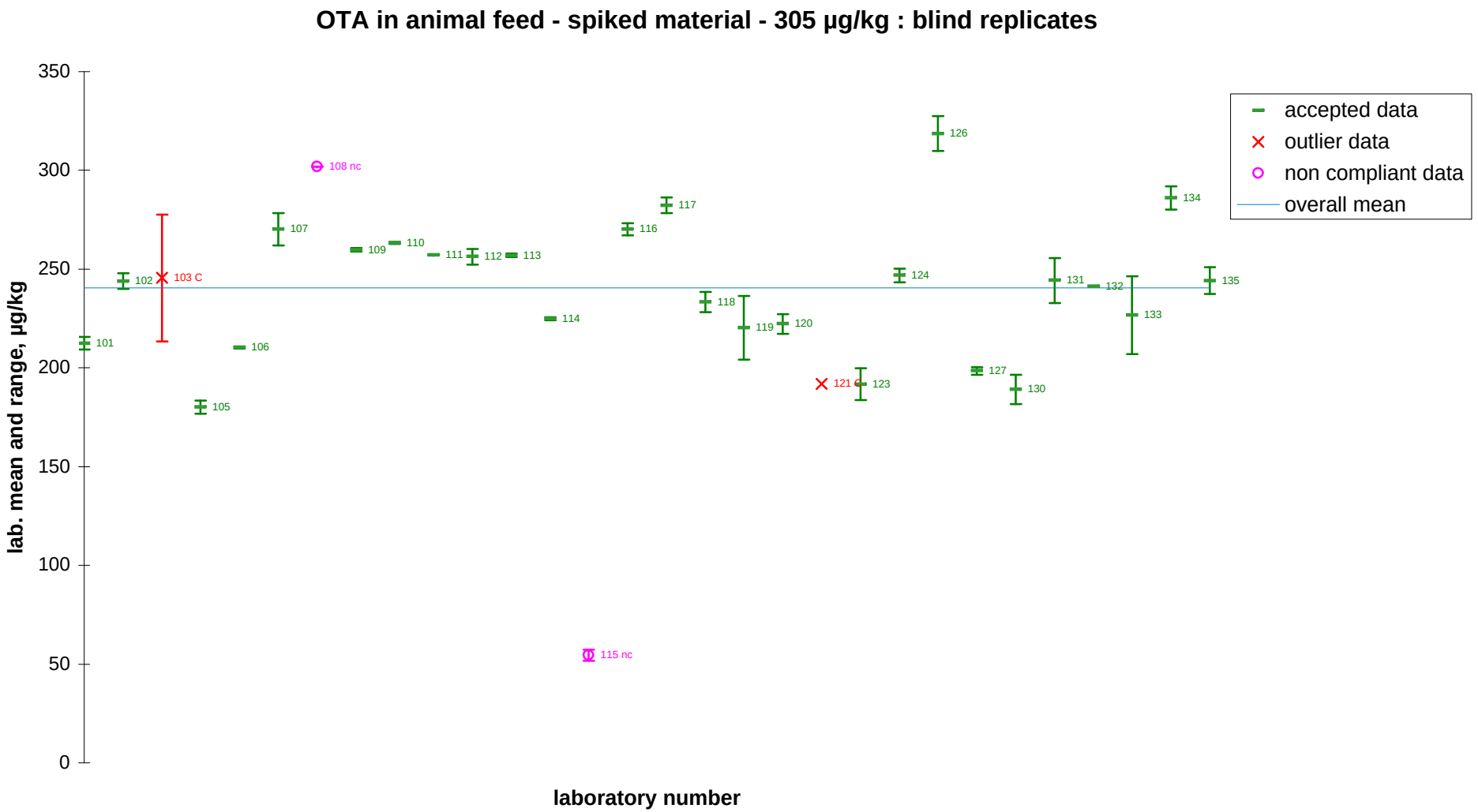
OTA in animal feed high level material - naturally contaminated - 338 µg/kg : blind replicates



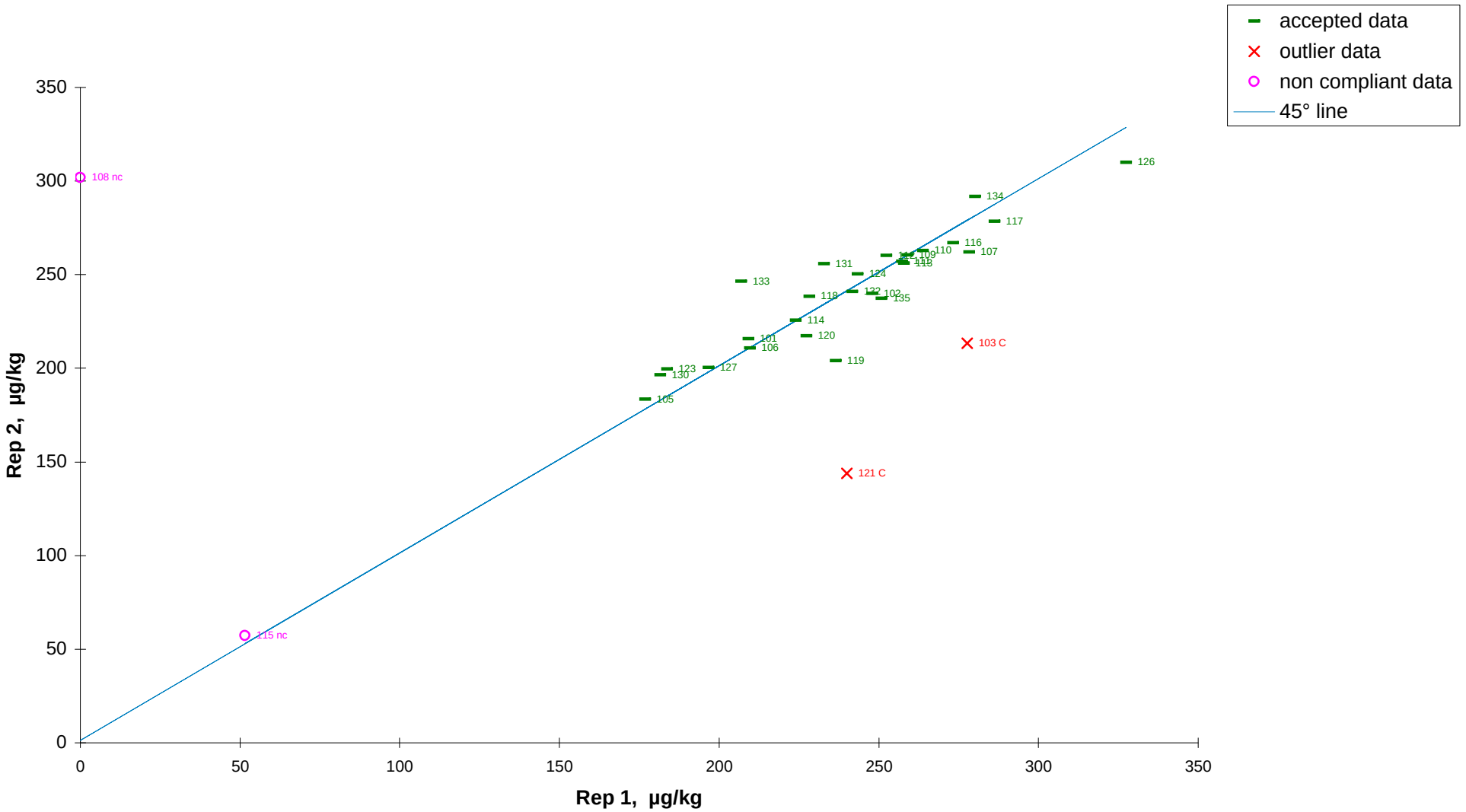

OTA in animal feed - spiked material - 76 µg/kg : blind replicates
lab. mean and range, µg/kg
laboratory number
accepted data
outlier data
non compliant data
overall mean
0
10
20
30
40
50
60
70
80
90
101
102
103
105
106
107
108
109
110
111
112
113
114
115 nc
116
117
118
119
120
121
123
124
126
127
130
131
132
133
134
135

OTA in animal feed - spiked material - 76 µg/kg : blind replicates

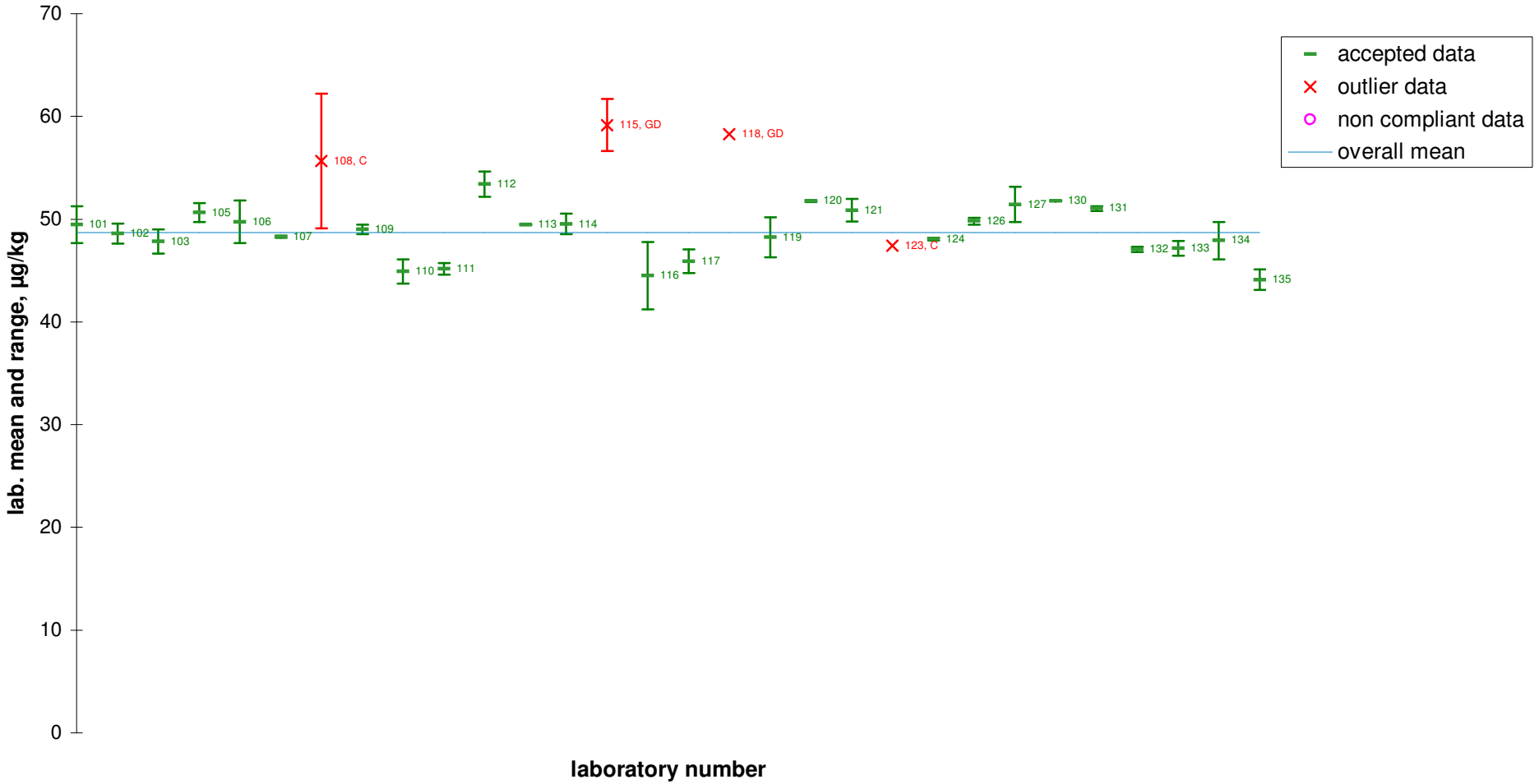



OTA in animal feed - spiked material - 305 µg/kg : blind replicates
lab. mean and range, µg/kg
laboratory number
accepted data
outlier data
non compliant data
overall mean
350
300
250
200
150
100
50
0
101
102
103 C
105
106
107
108 nc
109
110
111
112
113
114
115 nc
116
117
118
119
120
121 C
123
124
126
127
130
131
132
133
134
135

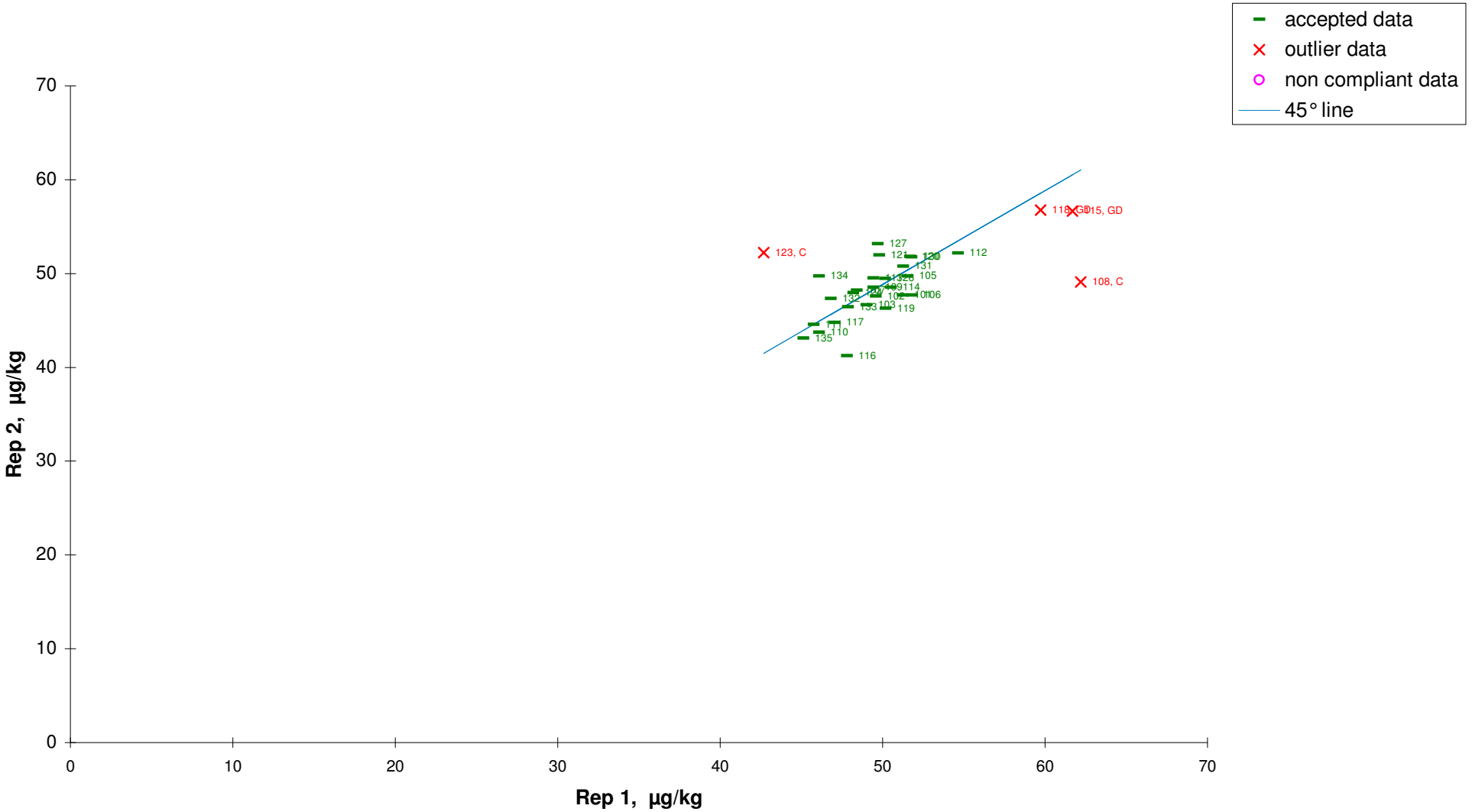
OTA in animal feed - spiked material - 305 µg/kg : blind replicates

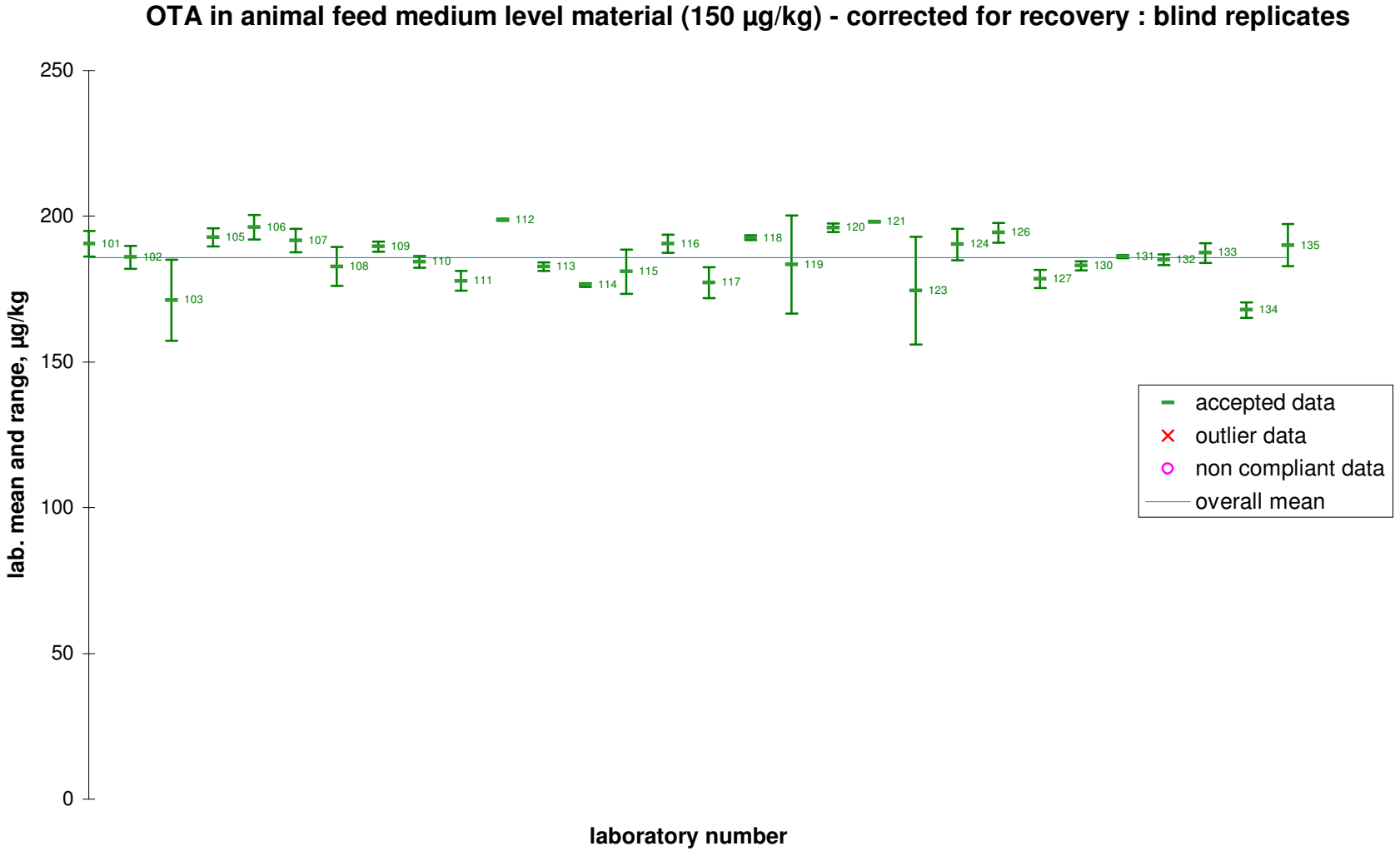


OTA in animal feed low level material (39 µg/kg) - corrected for recovery : blind replicates

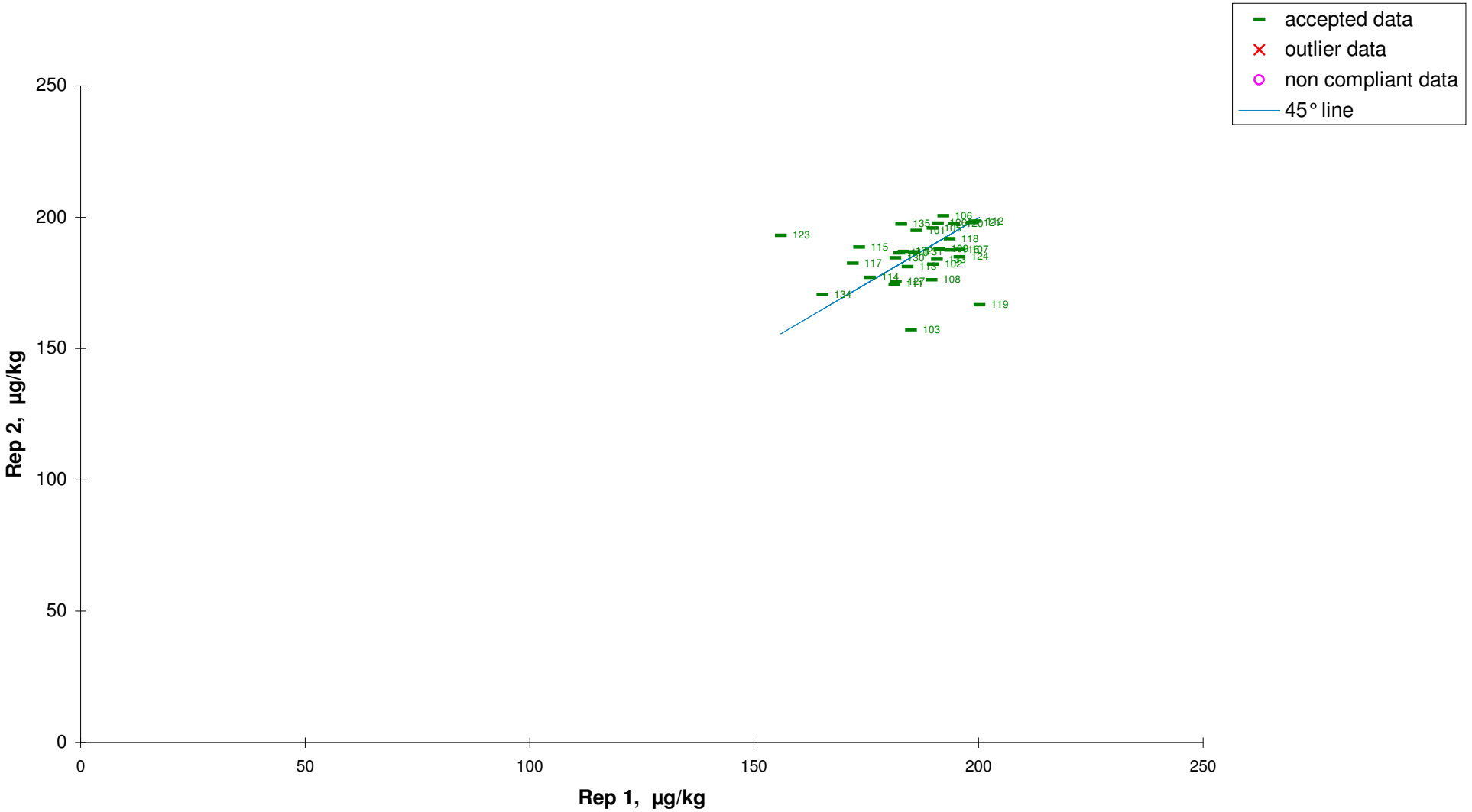


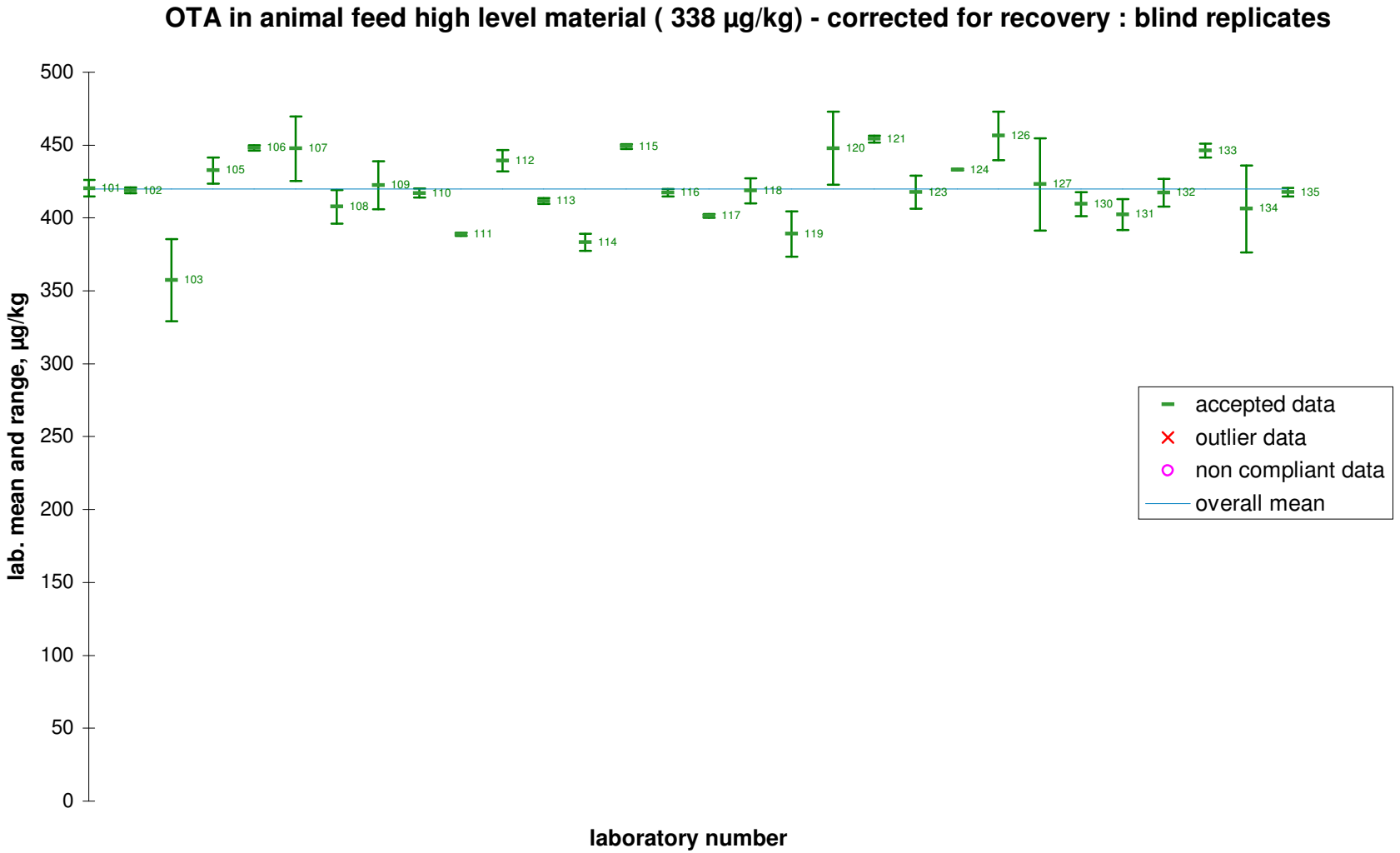
OTA in animal feed low level material (39 µg/kg) - corrected for recovery : blind replicates




OTA in animal feed medium level material (150 µg/kg) - corrected for recovery : blind replicates
lab. mean and range, µg/kg
250
200
150
100
50
0
101
102
103
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
123
124
126
127
130
131
132
133
134
135
accepted data
outlier data
non compliant data
overall mean
laboratory number

OTA in animal feed medium level material (150 µg/kg) - corrected for recovery : blind replicates




OTA in animal feed high level material (338 µg/kg) - corrected for recovery : blind replicates
lab. mean and range, µg/kg
500
450
400
350
300
250
200
150
100
50
0
101
102
103
105
106
107
108
109
110
111
112
113
114
115
116
117
118
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120
121
123
124
126
127
130
131
132
133
134
135
accepted data
outlier data
non compliant data
overall mean
laboratory number

European Commission

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Abstract

An inter-laboratory comparison was carried out to evaluate the effectiveness of a method based on immunoaffinity column clean-up followed by high performance liquid chromatography using fluorimetry (HPLC-FL). The method was tested for the determination of ochratoxin A (OTA) in animal feed at concentration levels relevant to those proposed according to Recommendation 576/2006/EC ([i]). The test portion of the sample was extracted with methanol:water. The sample extract was filtered, diluted, passed over an immunoaffinity column for clean-up and then eluted with methanol. The separation and determination of the OTA was performed by reverse-phase high performance liquid chromatography (HPLC) with fluorescence detection with an excitation of 333 nm and emission of 467 nm.

The animal feed samples, both spiked and naturally contaminated with OTA, were sent to 35 laboratories in 15 EU Member States, Colombia, Canada and Japan. Each laboratory received 6 duplicate samples – 4 coded and 2 blanks for spiking with coded solutions. Blank marked test portions of the samples were spiked at levels of 76 µg/kg and 305 µg/kg OTA. The range of single recovery values reported spanned from 47 % – 124 % with an average value of 82 % and 79 % for each level respectively. Based on results for spiked samples (blind duplicates at two levels), as well as naturally contaminated samples (blind duplicates at three levels), the relative standard deviation for repeatability (RSD_r) ranged from 3.1 % – 4.7 %. The relative standard deviation for reproducibility (RSD_R) ranged from 13.5 % – 14.6 %. After correction for recovery, the RSD_R values improved significantly and for naturally contaminated test materials ranged from 5.6 % – 6.4 %. This method therefore showed acceptable within-laboratory and between-laboratory precision for each matrix.

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