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Validation of an Analytical Method to Determine the Content of T-2 and HT-2 Toxins in Cereals and Compound Animal Feed by Immunoaffinity Column Clean-up and GC-MS

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VALIDATION OF AN ANALYTICAL METHOD

to

Determine the Content of T-2 and HT-2 toxins in Cereals and Compound Animal Feed

by

Immunoaffinity Column Clean-up and GC-MS

RESULTS OF THE COLLABORATIVE STUDY

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Contents

Abstract

A method for the determination of T-2 toxin and HT-2 toxin in cereals and compound animal feed was validated by collaborative study.

In short, the method is as follows: A test portion of a sample is extracted with a mixture of methanol/water (80/20, v/v). This raw extract is then diluted, filtered, and applied to an immunoaffinity column. After washing and elution with acetonitrile the eluate is evaporated to dryness. T-2 and HT-2 toxins in the dry residue are then derivatised with N-methyl-N-trimethylsilyl-trifluoroacetamid (MSTFA)/ trimethylchlorosilane (TMCS) (99/1, v/v), injected into a gas chromatograph, and detected and quantified by mass spectrometry.

Fourteen laboratories from ten different countries were selected to participate in the collaborative study. They received six different test materials as blind duplicates. The test materials consisted of a blank cereal mix, two cereal mixes naturally contaminated with T-2 and HT-2 toxins at different levels, a blank compound animal feed, and two compound animal feeds naturally contaminated with T-2 and HT-2 toxins at different levels. Furthermore, two blank cereal mixes and two blank compound animal feeds together with specific spiking solutions were provided for recovery determination. The sum of the mass fractions of T-2 & HT-2 toxins after spiking were 50 µg/kg in the cereal mix, and 75 µg/kg in the compound animal feed.

Reported apparent recoveries in the cereal mix ranged from 59 to 143% for the sum of T-2 & HT-2 toxins with a mean value of 105%. Reproducibility relative standard deviations (RSD_R) for the cereal matrix were 30% at a natural contamination level of ca. 25 µg/kg, 21% at ca. 87 µg/kg, and 27% for the spiked material at 50 µg/kg. For compound animal feed the recovery values ranged from 87 to 145% with 113% for the mean value. The RSD_R values were 25% at a natural contamination level of ca. 92 µg/kg, 19% at ca. 125 µg/kg, and 16% for the spiked material at 75 µg/kg. The Horwitz ratios (HorRat) ranged from 0.7 to 1.4.

European Commission Regulation 401/2006 [1] lays down method performance criteria for the control of foodstuffs only. Even though the validated method applies to cereals and compound feed it meets all of those criteria. Therefore it is suited for official feed control.

Introduction

The A-type trichothecenes T-2 toxin (4β,15-Diacetoxy-3α-hydroxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene, CAS No: 21259-20-1) and HT-2 toxin (15-Acetoxy-3α,4β-dihydroxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene, CAS No: 26934-87-2) present a non-negligible risk for humans and animals, because of their high toxicity and their prevalent occurrence in cereals. Therefore they have come into focus of competent food and feed authorities in the EU and they have been part of an exposure assessment study in 2003 [2]. In addition, T-2 and HT-2 toxins are planned to be regulated in the EU for human food, whereas only guidance values may be established for T-2 and HT-2 toxins in products intended for animal feeding [3]. The exposure assessment revealed that there was a lack of suitable methodology for the reliable determination of T-2 and HT-2 to make enforcement of regulations possible. Regulation 401/2006 lays down performance criteria for such methodology in foodstuffs [1].

Different methods for the determination of T-2/ HT-2 in cereals have been published. Biselli et al. [4] used LC/MS/MS with a MycoSep® clean-up to detect several mycotoxins including T-2/ HT-2. Only wheat flour was studied and recoveries were 71% for T-2 and 54% for HT-2 with a RSD of 3% for both. A HPLC-FLD method has been described by Visconti et al. [5] involving immunoaffinity clean-up. Recoveries were reported for pure wheat, maize or barley samples with a range of 70 to 100% and RSDs below 8%. Eskola et al. [6] and Jestoi et al. [7] used a MycoSep® clean-up with GC/MS to determine several mycotoxins including T-2 and HT-2. The method was validated with a mixture of wheat, rye and barley and the reported recoveries were around 100% with RSDs between 4 and 10%. But oat, which seems to be the cereal most affected with T-2/ HT-2 contamination, was not studied in any of the above mentioned studies.

We decided to develop a method based on immunoaffinity clean-up, because of its superior purification selectivity compared to other solid-phase clean-ups, and GC/EI-MS, because of its high chromatographic resolution, fast run times, robust ionisation characteristics and selectivity in single ion monitoring mode. The matrices studied were mixtures including all common cereals like oat, wheat, barley, rice, and maize, as well as soy, and other common ingredients found in compound animal feed. The method was subjected to an interlaboratory validation trial according to the *Guidelines for Collaborative Study Procedures To Validate Characteristics of a method of Analysis [10]*, so as to derive performance characteristics and to evaluate its suitability to be used for official purposes.

Materials and methods

Test materials for the collaborative study

For this study various types of cereals and brands of compound animal feed were purchased from local stores. After confirmation that these raw materials did not contain detectable amounts of T-2 or HT-2 toxins using an initial version of the described method they were milled and mixed as described below. The so obtained blank materials were again tested for the presence of T-2 and HT-2 toxins. Tables 1 and 2 describe the composition of those blank mixes.

To obtain naturally contaminated test materials, the blank materials were blended with different amounts of highly contaminated oat. (see Table 1 & 2).

Table 1: Composition of cereal mix test materials

Test Material	Ingredient	Amount (kg)
Blank	Wheat	5
	Rice	3
	Maize	5
	Soy	3
	Barley	2
	Oat	2
High	Blank mix	2
	Contam. oat	1
Medium	Blank mix	3
	Contam. oat	1

Full grains were first milled with a Romer RAS® mill prior to blending. All other materials were blended directly in a modified rotating-drum mixer for 30 minutes. After blending the whole lot was milled with a Retsch centrifugal mill (Model ZM 100) with a sieve of 3 mm. This milled material was again mixed in the rotating-drum mixer for 30 minutes and milled, for a second time, down to a particle size of < 1 mm in the centrifugal mill. Then the material was mixed again in the rotating-drum mixer for 2 – 3 hours and milled once more to a final particle size of < 0.5 mm. Subsequently the materials were filled into 50 mL polyethylene (PE) containers at approx. 30 g each. The containers were kept at –18° C until analysis for homogeneity or dispatch to the participating laboratories.

Table 2: Composition of compound animal feed test materials

Test Material	Ingredient	Amount (kg)	Composition
Blank	Pig feed	5	Peas, roasted soy, wheat, rye, manioc, cabbage, maize, animal adipose, salt, lime
	Chicken feed	3	Cereals, oil containing seeds, minerals, mussels, oils & fats
	Rabbit feed	5	Different cereals & seeds, herbal by-products, vegetables, minerals
	Maize	2	
	Oat	3	
	Soy	1	
	Barley	1	
High	Blank mix	3	(see: “blank”)
	Contam. oat	2	
	Alfalfa	1	
Low	Blank mix	2	(see: “blank”)
	Contam. oat	1	
	Horse feed	1	Oat, barley, flour pellets, maize, fibres, peas, molasse, oil
	Horse feed	1	Barley, oat, flour pellets, maize flakes, oil, alfalfa
	Rabbit feed	1	Different cereals & seeds, herbal by-products, vegetables, minerals

Homogeneity of the Test Materials and In-House Method Performance

For homogeneity testing, one tenth of all containers were selected from each batch during packing and subjected to the GC/MS method under study.

The selection was done such that out of the series of the first ten containers one was selected randomly and then every tenth container thereafter, e.g. container 3, 13, 23,…, and so on until 10% of all containers were selected. After thorough mixing the content of each selected container was split into two equal parts and analysed.

Analysis of Variance (ANOVA) was used to compute within-container and between-container mean sum-of-squares for the two analytes and their sum at each contamination level in the two materials. Homogeneity of the packaged material was accepted when a F-Test of those two mean sum-of-squares resulted in a probability p of larger than 0.1. This α error of falsely rejecting possible homogeneity was chosen with 10 % to keep the β error of falsely accepting homogeneity small.

Furthermore, the overall means for the two analytes and their sum at each contamination level and its associated relative standard deviation under repeatability conditions (RSD_r) were also computed. Table 3 lists the results for the two contamination levels of the two different matrices which were prepared.

Calibration data were acquired on three different days at 10 different levels from 0 – 50 ng/µL in the injection solution for T-2 and 0 – 100 ng/µL for HT-2. A calibration function, and the minimum detectable level (MDL) were then computed as outlined in section "Statistical evaluation". The calibration data for T-2 showed a slight negative curvature best explained by a 2^{nd} degree polynomial. The calibration data of HT-2, because of higher variability, could be explained well by a 1^{st} degree polynomial. The calculated MDLs [ng] were 0.4 and 1.0 for T-2 and HT-2, respectively. That means, if 0.4 ng of T-2, or 1.0 ng of HT-2, were eluted from the IAC column, assuming complete derivatization, one could be 95% confident that a detectable peak would result.

Apparent recoveries were determined by spiking a blank material with the two toxins and subjecting the spiked material to the GC/MS method under study. The determined mass fractions were then compared to the added mass fractions. Since preliminary experiments with a food matrix at three different mass fraction levels (three preparations, duplicate injections) showed no indication for a concentration dependency of the apparent recoveries for either T-2 (p=0.3) or HT-2 (p=0.6) it was decided to determine apparent recoveries only at one mass fraction for each of the two matrices. Those mass fractions were chosen such that they might reflect relevant contamination scenarios. Table 4 lists those results.

Acceptable performance criteria as set forth in European legislation [1] are RSD_r values smaller than 40% for HT-2 mass fractions of 100 – 200 µg/kg and T-2 mass fractions of 50 – 250 µg/kg, and recoveries in the range of 60 – 130 per cent. Whereas these criteria refer to collaborative study performance, within-laboratory performance characteristics should also fulfil these criteria, of course. The recovery percentages found were all within the range of acceptance except for compound animal feed spiked with HT-2 at a level of 50 µg/kg (141 %). Given the fact that the performance criteria for recovery are valid for much higher mass fractions of HT-2 (100-200 µg/kg), this slight exceedance was considered acceptable.

Table 3: Results of the homogeneity test and relative standard deviation under repeatability conditions (RSD_r)

Toxin	Material[1]	Level	MEAN	RSD_r	N	p
T-2	Cereal[1]	low	7.7	15	10	0.97
		high	18.7	7	10	0.25
	Animal feed	low	10.5	6	20	0.46
		high	38.4	11	10	0.23
HT-2	Cereal[1]	low	16.4	9	10	0.98
		high	34.5	11	10	0.39
	Animal feed	low	47.4	9	20	0.20
		high	84.3	11	10	0.38
Sum T-2/HT-2	Cereal[1]	low	24.0	10	10	1.00
		high	53.2	9	10	0.36
	Animal feed	low	57.9	8	20	0.20
		high	122.6	10	10	0.29

1- Homogeneity of the cereal materials was tested with an early version of the proposed method without internal standard, VICAM IAC columns , and Tri-SIL-TBT as derivatization reagent

Table 4: Results of the recovery experiments, showing the added and the determined mass fractions of the respective toxins in µg/kg, the recovery in per cent, and the number of measurements

Toxin	Material	Added	Determined	%	N
T-2	Cereal	17	17.7	104	4
	Animal feed	25	25.2	101	4
HT-2	Cereal	33	37.7	114	4
	Animal feed	50	70.5	141	4
Sum T-2/HT-2	Cereal	50	55.5	111	4
	Animal feed	75	95.7	128	4

Statistical evaluation

Calculations of the precision parameters were done according to Youden & Steiner [8] and are detailed below:

The square root of the within-laboratory mean sum-of-squares (MS_0) was taken as estimate of the repeatability standard deviation.

$$s_r = \sqrt{MS_0} \qquad (1)$$

The laboratory related variance was calculated as the difference of the between-laboratory mean sum-of-squares (MS_L) and MS_0 divided by the number of replications per laboratory (n=2).

$$s_L^2 = \frac{MS_L - MS_0}{n} \qquad (2)$$

Finally the reproducibility standard deviation was calculated from the residual and the laboratory variances.

$$s_R = \sqrt{s_L^2 + s_r^2} \qquad (3)$$

Relative standard deviations (RSD) were calculated as standard deviation times 100 divided by the mean value:

$$RSD = \frac{s * 100}{\bar{x}} \qquad (4)$$

Repeatability and reproducibility were calculated by multiplying the respective standard deviation with 2.8 which gives roughly a 95% confidence at two replications:

$$r = 2.8 * s_r \quad (5)$$

$$R = 2.8 * s_R \quad (6)$$

The precision parameters were calculated using the EXCEL macro CLSTD.XLT (V3.6) [9].

Based on ISO guide 11843 Part 2 calibration functions for data with non-constant variance were calculated with iteratively re-weighted least square. The formulas were extended to calibration functions including a 2nd degree term. Minimum detectable levels (MDL) were then calculated from the estimated calibration and variance functions.

Design of the collaborative study

The design was based on the "Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis" [10].

The participants were selected from a list of interested parties and a total of 14 laboratories from ten different countries were chosen to participate in this collaborative trial (Figure1).

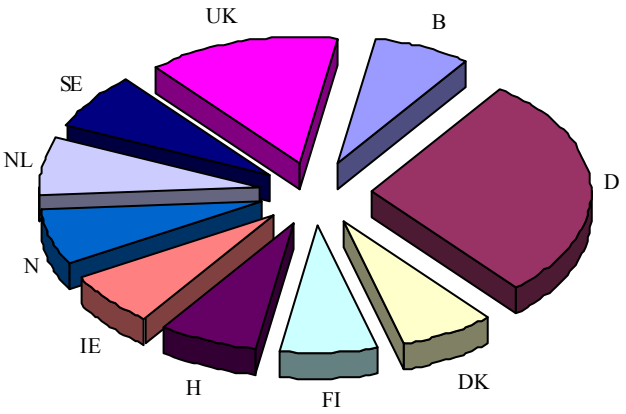

Figure 1: Distribution of the selected participants by country for the inter-laboratory comparison.

The participants represented governmental (64%), industrial (22%), and academic (14%) food control laboratories. Among these were seven National Reference Laboratories (NRL) for Mycotoxins appointed by European Union Member States.

All participants received cereal and animal feed samples. One selected laboratory returned the samples because of unavailability of a GC/MS system, and three selected laboratories received samples but were unable to conclude the analyses. Subsequently those laboratories were excluded from the evaluation. Table 5 lists the names and addresses of the laboratories which reported results and were included in the evaluation.

Table 5: List of included laboratories in alphabetical order

Participant	Institution	Address
Ingrid Bujara	SGS Germany GmbH, Laboratory Hamburg	Weidenbaumsweg 137, D-21035 Hamburg, Germany
Duncan Campbell	West Yorkshire Analytical Services	PO Box 11, Nepshaw Lane South, Morley, LS27 0UQ, Leeds, England
Per-Erik Clasen	National Veterinary Institute, Department of Feed and Food Hygiene- Toxicology, Chemistry and Microbiology	Ullevallsveien, 68, 0454 Oslo, Norway
Jozsef Dömsödi	National Institute for Agricultural Quality Control, Central Laboratory	Remeny u. 42, H-1144 Budapest, Hungary
Marika Jestoi	Finnish Food Safety Authority (Evira)	Mustialankatu 3, FIN-00790 Helsinki, Finland
John Keegan	Public Analyst's Laboratory	Sir Patrick Dun's Lower Grand Canal Street, Dublin 2, Ireland
Horst Klaffke	Bundesinstitut für Risikobewertung (Federal institute for risk assessment) - BfR	Thielallee 88-92, D-14195 Berlin, Germany
Peter Maynard	Kent Scientific Services	8 Abbey Wood Road, Kings Hill, ME19 6YT, West Malling, England
Alexey Solyakov	National Veterinary Institute, Department of Animal Feed	SE-75189 Uppsala, Sweden
Wim A. Traag	RIKILT-Instituut voor Voedselveiligheid	Bornsesteeg 45, 6700 AE Wageningen, The Netherlands

For the collaborative trial each participant received:

1. 12 containers of coded samples for determination of T-2/ HT-2 toxins mass fractions
2. 4 containers of samples for spiking identified as “Spike C” and “Spike A”
3. One ampoule identified as “T-2/HT-2 toxins standard in acetonitrile” (for calibration)
4. One ampoule identified as “Spike C solution in acetonitrile”
5. One ampoule identified as “Spike A solution in acetonitrile”
6. One ampoule identified as “ISTD solution in acetonitrile”
7. One ampoule identified as “TMS reagent”
8. 18 immunoaffinity columns for T-2/HT-2 toxins
9. One copy of the collaborative study method (see Annex III)
10. One copy of the spiking protocol (see Annex IV)
11. Report form (see Annex V)
12. Questionnaire (see annex VI)

The 12 sample containers contained blind duplicates of either blank, low, or highly contaminated cereal (see Table 1) or blank, low, or highly contaminated animal feed (see Table 2). Each of the 12 materials was to be prepared once and measured twice by each laboratory. For recovery determinations two blank materials, either cereal or animal feed, were provided to be spiked in duplicate with the respective spiking solution.

Results of the collaborative study and Discussion

As it is foreseen to regulate the maximum levels of the sum of the two type A trichothecenes T-2 and HT-2 detailed results are only reported for the summed-up mass fractions uncorrected for recovery. The results for the individual toxins can be found in Annex I.

Animal feed

Tables 6 lists the summed-up mass fractions of the reported values for T-2 and HT-2 toxins by laboratory, each row representing one laboratory identified by the codes used for reporting, and the columns representing the different materials. Cells show mass fractions as reported. Where no value was reported the cell is empty, n.d. indicates not detected (for computational purposes this was taken as zero). A gray shading indicates exclusion from the statistical evaluation, light gray for non-compliance, dark gray for being an outlying result (Grubbs and/or Cochran test). Non-compliance was established when a laboratory deviated from the protocol at points considered to be crucial, or when no or only one result per duplicate material was reported.

Table 6: Sum parameter (T-2 & HT-2 toxins) in animal feed

Lab ID	Blank [µg/kg]		Low [µg/kg]		High [µg/kg]		Spike [µg/kg]		App. Recovery [%]	
9	12.0	15.4	93.5	97.0	101.2	117.6	102.1	95.6	118	109
61	15.9	13.7	78.6	74.6	121.8	120.4	99.5	97.2	113	110
72	24.4	27.1	127.9	119.8	141.7	144.4	98.5	132.1	97	142
73	23.0	28.5	75.0	100.5			209.0	188.0	244	216
119	14.5	14.5	87.5	85.6	125.8	115.8	91.3	91.8	102	103
501	40.6	22.1	136.9	131.0	159.1	175.0				
502	n.d.	n.d.	88.0	95.0	127.0	112.0	109.0	104.0	145	139
503	0.0	0.0	105.4	95.6	144.6	122.3	0.0	196.5	0	262
504	17.9	15.5	67.6	78.0	90.9	83.3	89.9	81.6	98	87
505	20.4	20.2	57.8	55.2	115.0	133.3	104.6	101.7	112	109

Empty cell – no value reported; n.d. – not detected; Spike – nominal value 75 µg/kg; light gray – non-compliant; dark gray – outlying result

Table 7 lists the performance parameters of the tested method for animal feed. The mean of the reported results for the blank material indicates a contamination of 14.6 µg/kg for the sum of the two analytes. However, the associated relative reproducibility standard deviation of 65% indicates that this value cannot be quantified with sufficient

confidence. When looking at the results of the individual toxins in Annex I it can be seen that most of it is contributed by HT-2 toxin (mean HT-2 toxin mass fraction: 10.8 µg/kg, RSD_R HT-2 toxin: 73 %).

Table 7: Performance parameters for the sum of T-2 & HT-2 toxins in the animal feed

Level	Mean	N	nc	outl.	n	r	s_r	RSD_r	R	s_R	RSD_R	HoR_{mod}
Blank	14.6	10	0	1	9	5.09	1.82	12	26.7	9.52	65	3.0
Low	92.5	10	0	0	10	20.22	7.22	8	66.0	23.6	25	1.2
High	125.1	10	1	0	9	27.57	9.85	8	64.9	23.2	19	0.8
App. Recovery at 75 µg/kg	113	10	2	1	7	35.8	12.8	11	50.2	17.9	16	0.7

Legend: Mean –mean mass fraction [µg/kg] or mean percentage recovery; N – number of labs; nc – non-compliant laboratories; outl. – outlying laboratories; n – number of laboratories used for statistics; r – repeatability [µg/kg], s_r – repeatability standard deviation [µg/kg], RSD_r – relative standard deviation under repeatability conditions [%]; R, s_R, RSD_R– the respective values for reproducibility, HoR_{mod} – the HorRat value for reproducibility modified after Thompson [11]

Since for recovery determination the blank material was spiked the reported values for the spike have been corrected for the values reported in the blank material. The resulting mean apparent recovery is then 113 % for animal feed. Considering the repeatability of ca. 36 % it must be pointed out this is statistically not different from 100 %. The modified Horwitz ratios of 1.2 and 0.8 for the low and high contaminated materials, respectively, demonstrate acceptable performance. The apparent recovery and the values for the relative standard deviations of repeatability and reproducibility are well below limits for food control set forth in [1].

Cereals

All the results for the cereal mix are listed in Table 8 for which the same conventions apply as for Table 6.

Table 8: Sum parameter (T-2 & HT-2 toxins) in cereal mix

Lab ID	Blank [µg/kg]		Low [µg/kg]		High [µg/kg]		Spike [µg/kg]		App. Recovery [%]	
9	4.0		17.8	21.8	89.3	89.3	63.2	58.4	118	109
61			24.1	18.1	45.7	57.4		31.4		63
72	10.0	9.6	30.9	30.8	109.4	110.9	69.9	73.3	120	127
73	82.5	89.0	64.5	57.5	224.0	174.5	124.0	110.0	77	49
119	7.0	4.7	19.7	19.2	79.4	80.5	56.0	50.5	100	89
501	19.3	9.6	34.5	29.4	96.5	111.4	74.8	69.1	121	109
502	n.d.	n.d.	34.0	34.0	81.0	87.0	67.0	67.0	134	134
503	0.0	0.0	18.5	14.4	91.5	90.7	62.0	71.4	124	143
504	7.0	11.5	20.0	14.0	91.4	48.3	44.8	38.7	71	59
505	11.4	11.5	31.2	33.1	84.5	86.7	56.2	69.9	90	117

Empty cell – no value reported; n.d. – not detected; Spike – nominal value 50 µg/kg; light gray – non-compliant; dark gray – outlying result

Table 9: Performance parameters for the sum of T-2 & HT-2 toxins in the cereal mix

Level	Mean	N	nc	outl.	n	r	s_r	RSD_r	R	s_R	RSD_R	HoR_{mod}
Blank	7.3	10	2	1	7	8.18	2.92	40	16.7	5.95	82	3.7
Low	24.7	10	0	1	9	7.65	2.73	11	21.1	7.55	30	1.4
High	87.0	10	0	2	8	14.08	5.03	6	50.2	17.9	21	0.9
App. Recovery at 50 µg/kg	105	10	1	0	9	32.5	11.6	11	78.0	27.9	27	1.2

Legend: Mean –mean mass fraction [µg/kg] or mean percentage for recovery; N – number of labs; nc – non-compliant laboratories; outl. – outlying laboratories; n – number of laboratories used for statistics; r – repeatability [µg/kg], s_r – repeatability standard deviation [µg/kg], RSD_r – relative standard deviation under repeatability conditions [%]; R, s_R, RSD_R– the respective values for reproducibility, HoR_{mod} – the HorRat value for reproducibility modified after Thompson [11]

Table 9 lists the performance parameters of the tested method for the cereal mix. Again, as for the animal feed blank material, the mean of the reported results indicates a contamination of 7.3 µg/kg with an associated relative reproducibility standard deviation of 82 %. This means no reliable quantification is possible for this contamination. The mean apparent recovery after correction for the blank results is 105 % (no statistical difference to 100%). The performance for the low and high contaminated materials is acceptable (HoR_{mod} 1.4 and 0.9, respectively).

Graphical representations

When plotting the first replicate result of a laboratory versus the second so-called Youden plots are created. They display in one glance repeatability (closeness of points to the identity line) and reproducibility (tightness of the cloud of points). Figures 2 to 5 show the Youden plots for the sum of T-2 and HT-2 toxins for the blank, the two naturally contaminated, and the spiked animal feed material. For the blank, the two

naturally contaminated, and the spiked cereal mix figures 6 to 9 show the respective Youden plots. The Youden plots for the individual toxins are shown in Annex II.

Mean & range plots are a second way of displaying the data. Here the mean value for each laboratory and its range are plotted compared to the overall mean value. This kind of plot allows the quick identification of an individual laboratory's bias compared to the mean value. Figures 10 to 13 show the mean & range plots for the sum of T-2 and HT-2 toxins for the animal feed materials, and figures 14 to 17 for the cereal mix materials. The other mean & range plots can be found in Annex II.

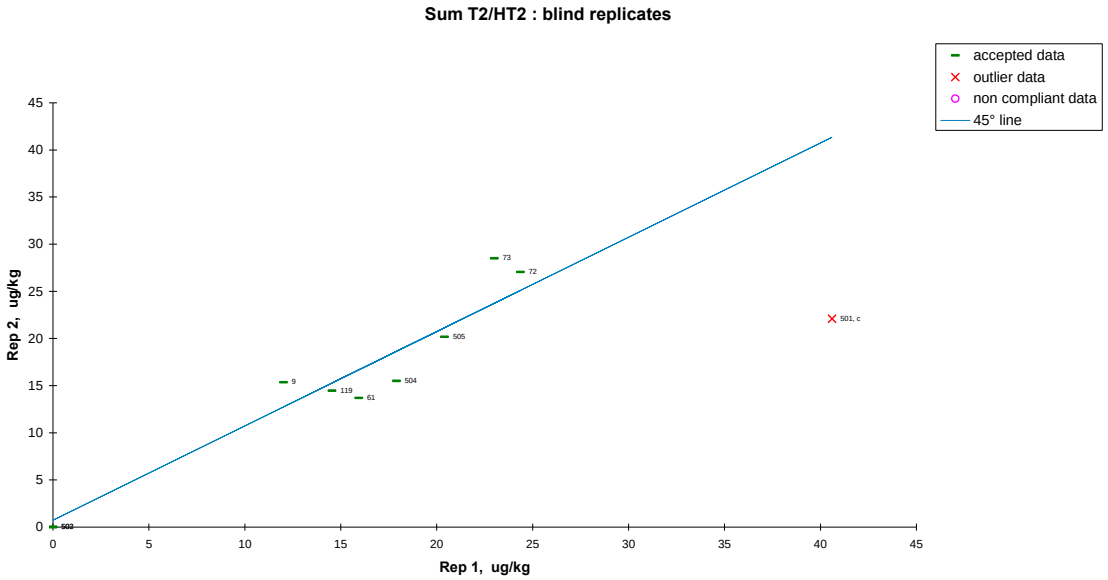

Figure 2: Youden plot of the sum of T-2 and HT-2 toxins for the blank animal feed material

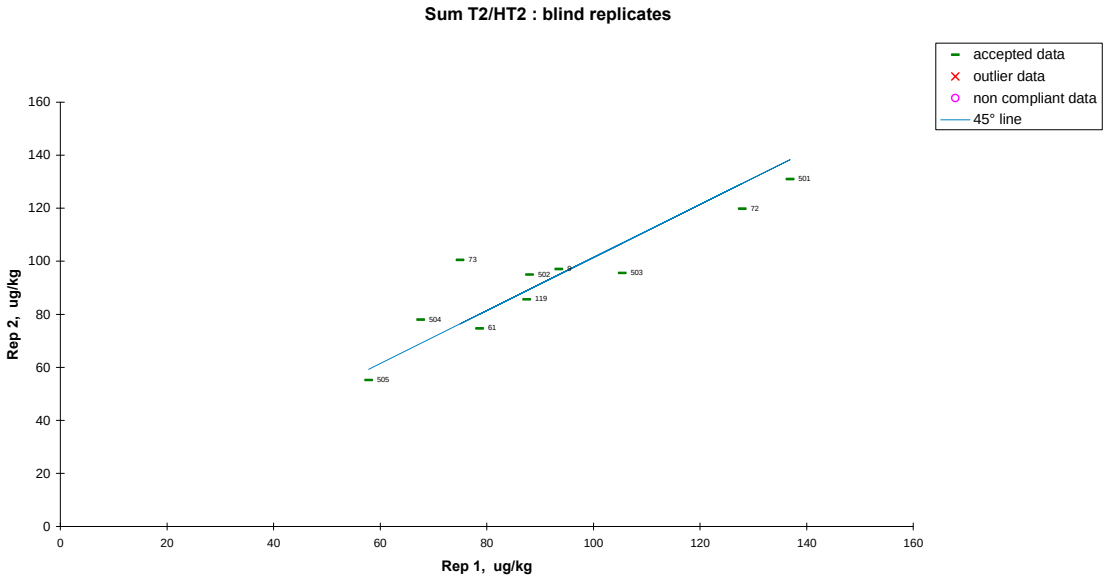

Figure 3: Youden plot of the sum of T-2 and HT-2 toxins for the low contaminated animal feed material

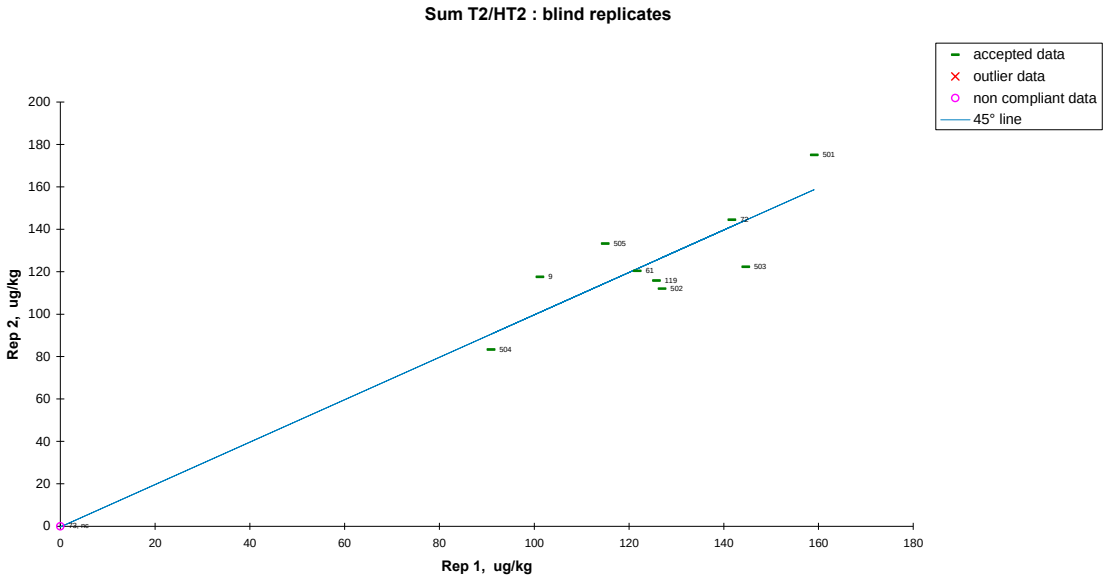

Figure 4: Youden plot of the sum of T-2 and HT-2 toxins for the highly contaminated animal feed material

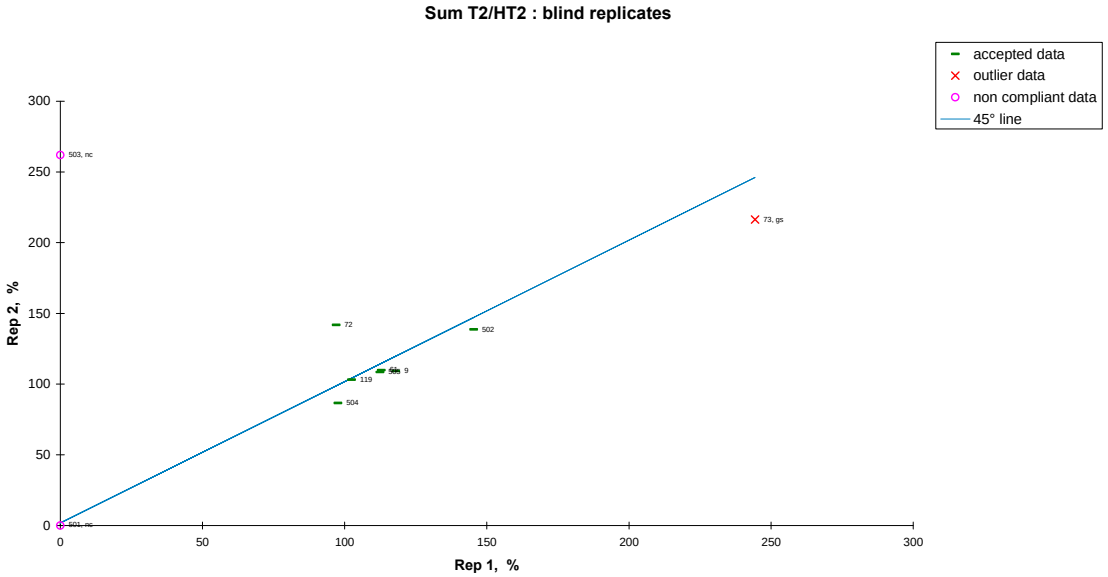

Figure 5: Youden plot of the sum of T-2 and HT-2 toxins for the recovery determination in animal feed material

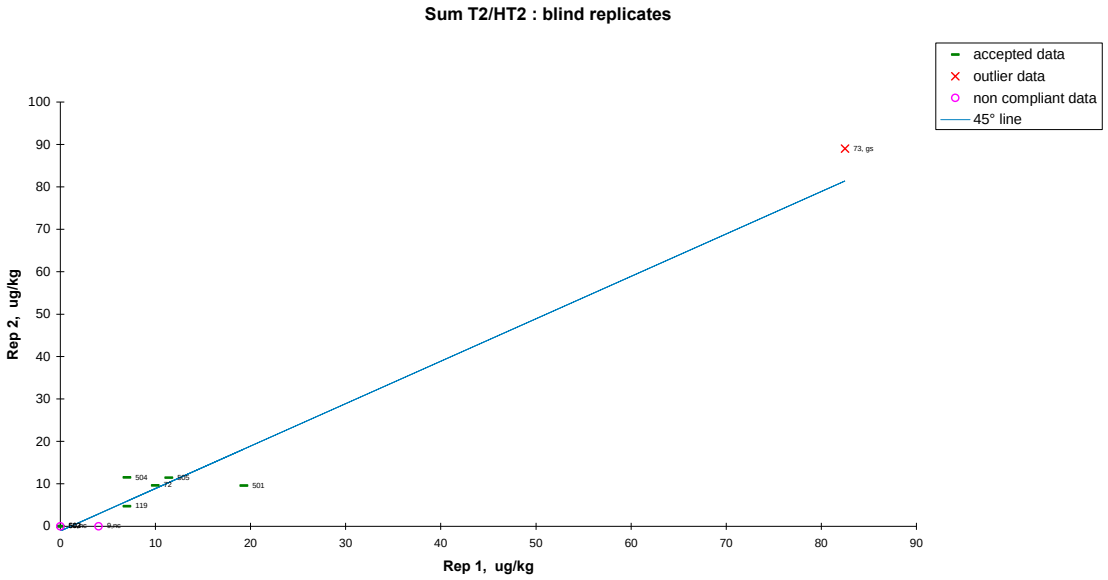

Figure 6: Youden plot of the sum of T-2 and HT-2 toxins for the blank cereal mix material

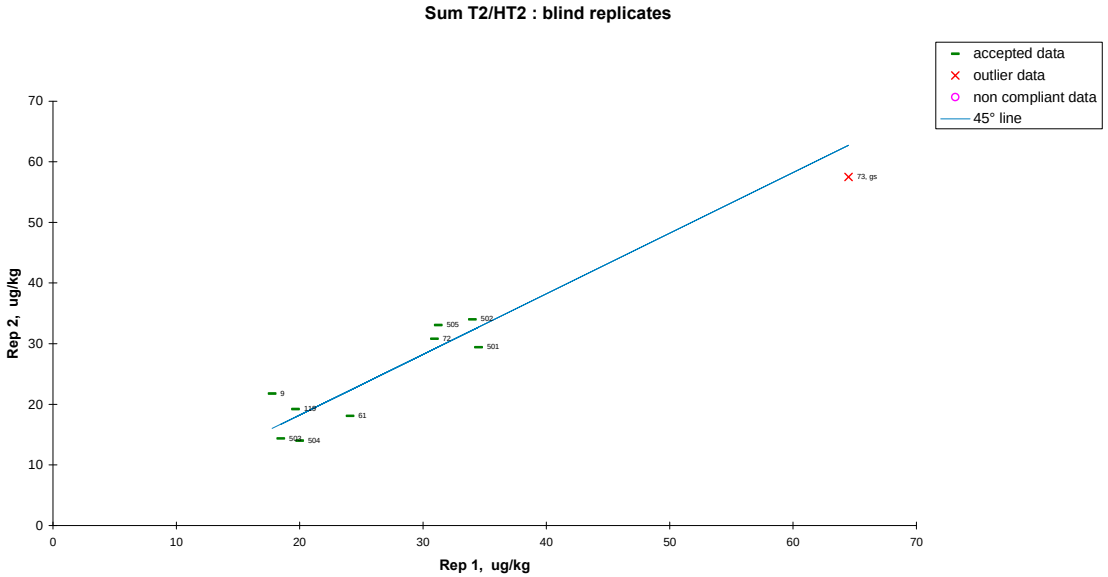

Figure 7: Youden plot of the sum of T-2 and HT-2 toxins for the low contaminated cereal mix material

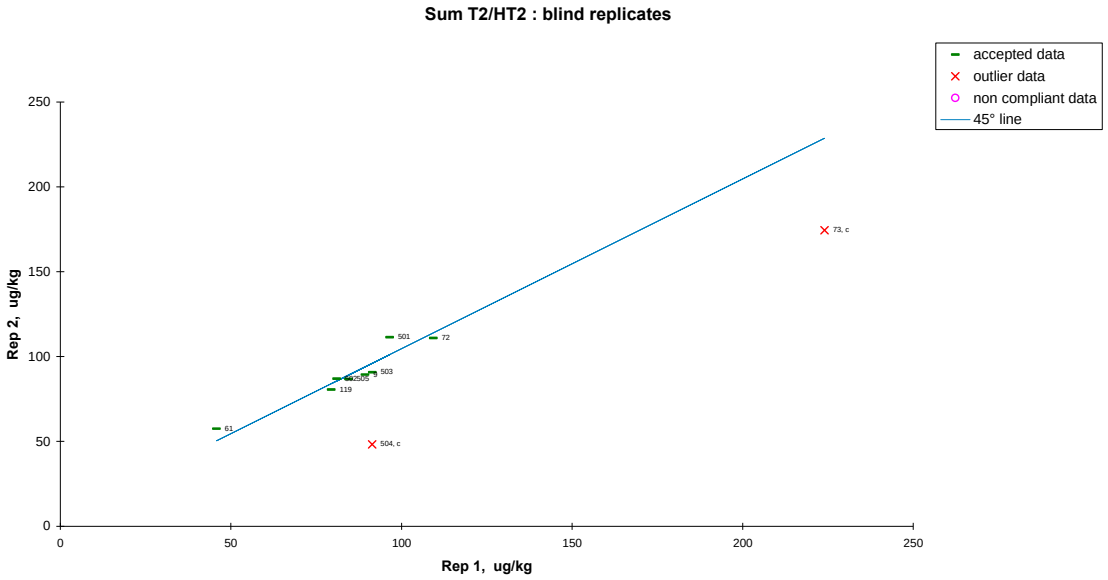

Figure 8: Youden plot of the sum of T-2 and HT-2 toxins for the highly contaminated cereal mix material

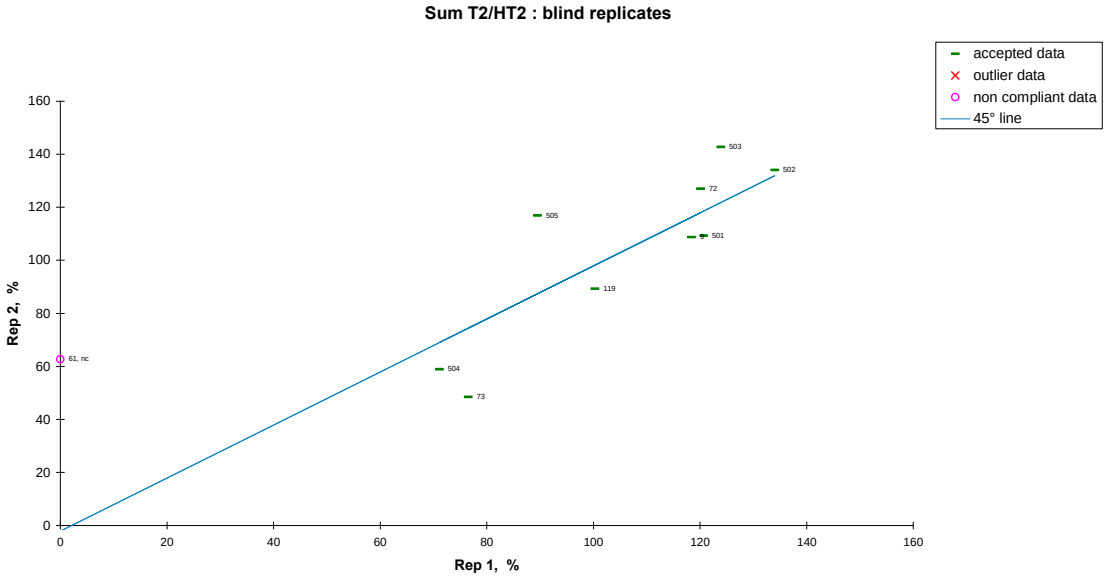

Figure 9: Youden plot of the sum of T-2 and HT-2 toxins for the recovery determination in the cereal mix material

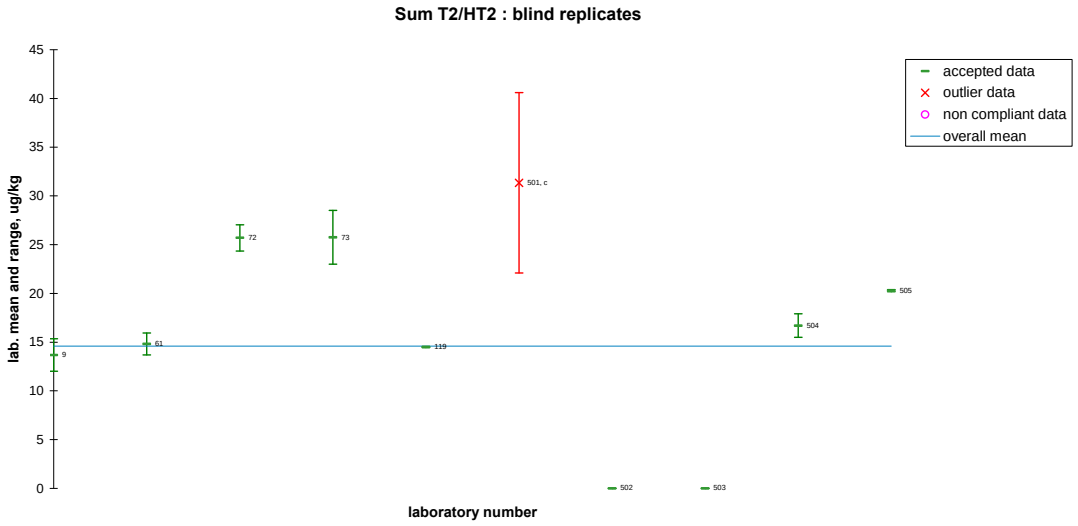

Figure 10: Mean & range plots of the sum of T-2 and HT-2 toxins for the blank animal feed material

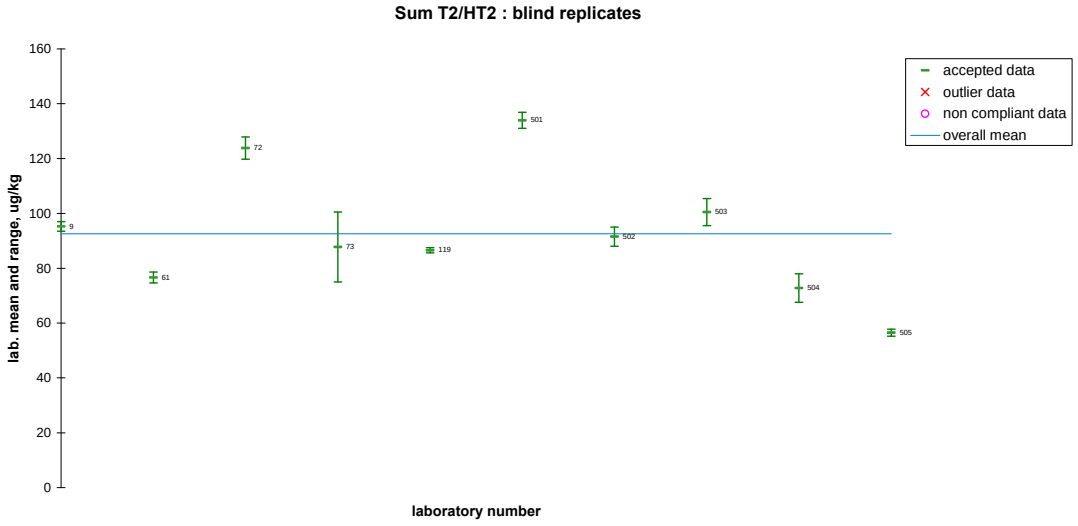

Figure 11: Mean & range plots of the sum of T-2 and HT-2 toxins for the low contaminated animal feed material

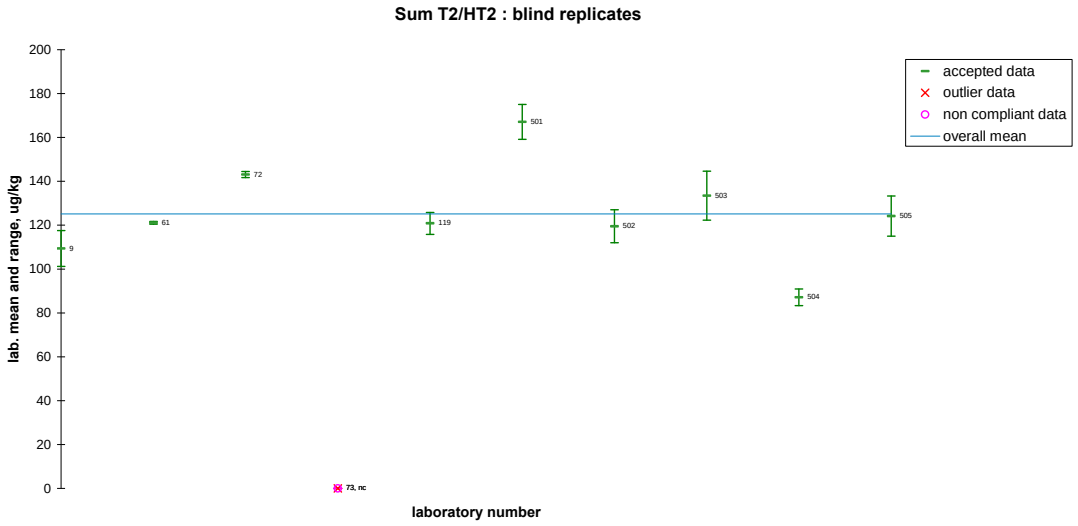

Figure 12: Mean & range plots of the sum of T-2 and HT-2 toxins for the highly contaminated animal feed material

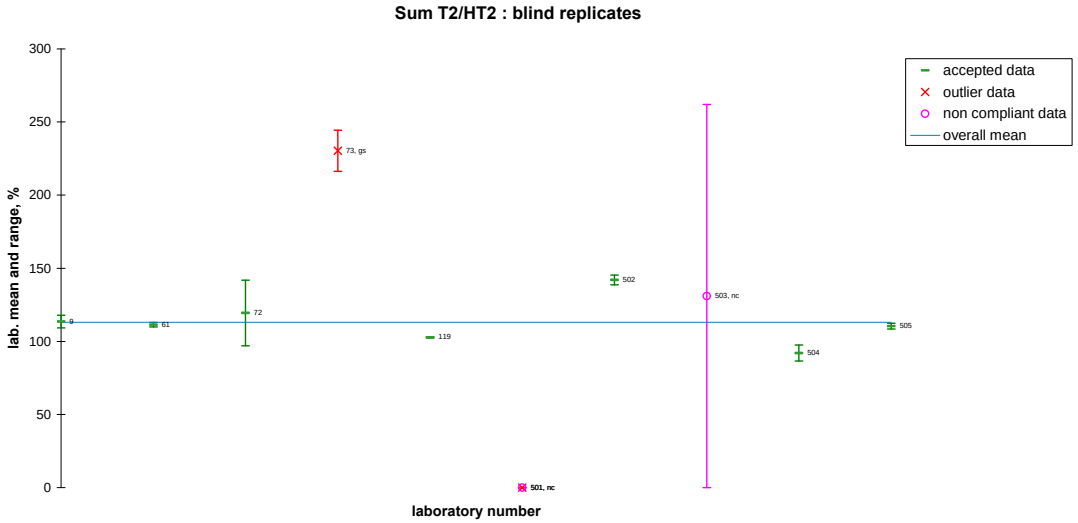

Figure 13: Mean & range plots of the sum of T-2 and HT-2 toxins for the recovery determination in animal feed material

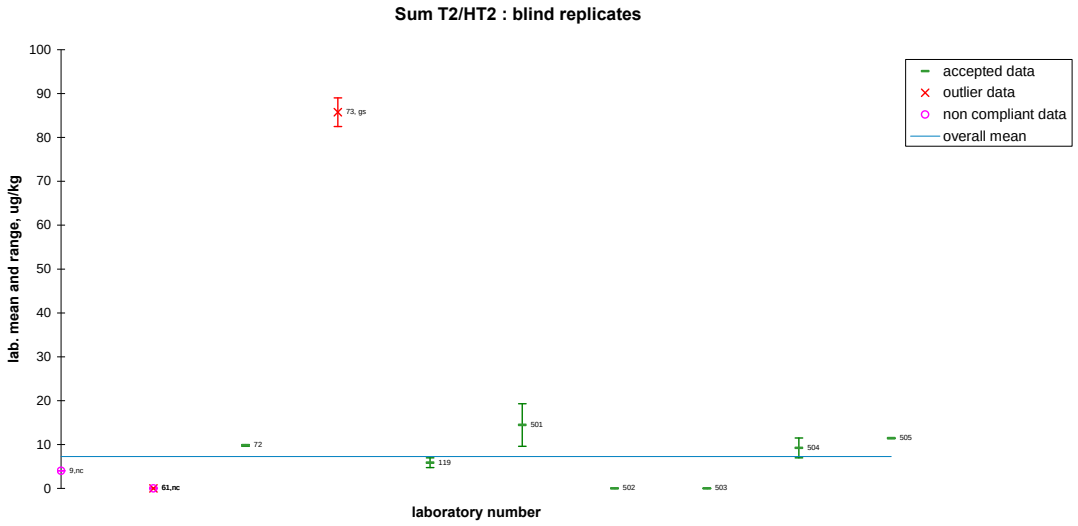

Figure 14: Mean & range plot of the sum of T-2 and HT-2 toxins for the blank cereal mix material

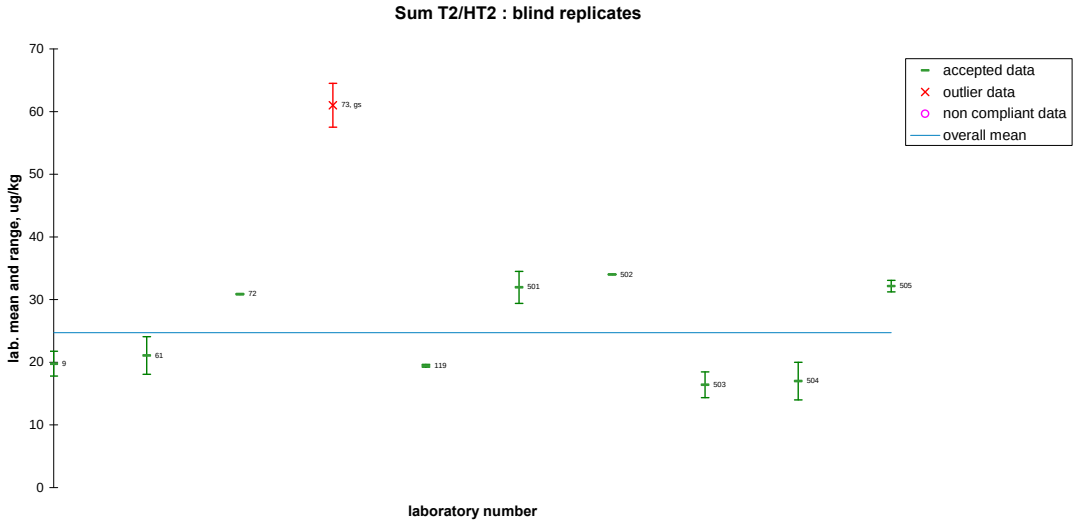

Figure 15: Mean & range plot of the sum of T-2 and HT-2 toxins for the low contaminated cereal mix material

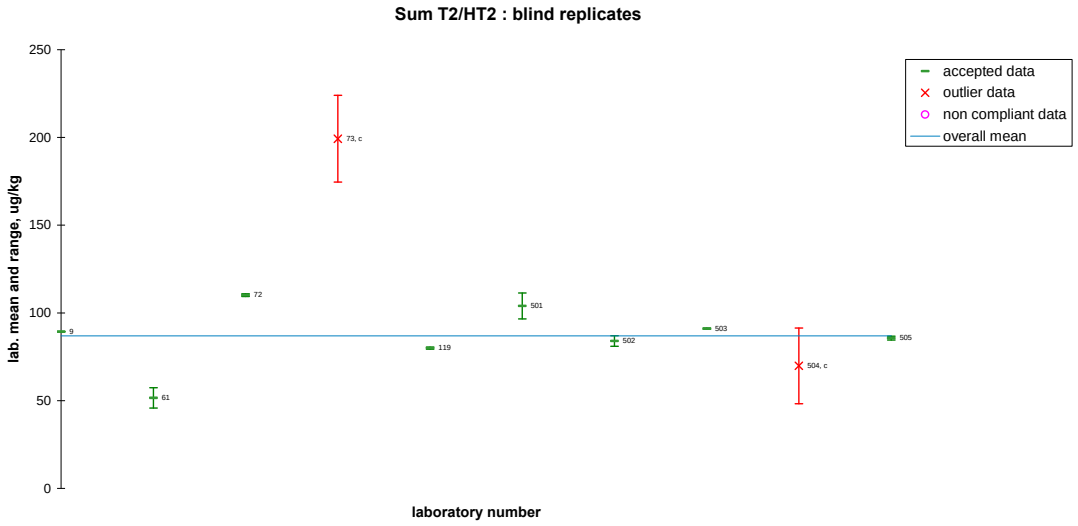

Figure 16: Mean & range plot of the sum of T-2 and HT-2 toxins for the highly contaminated cereal mix material

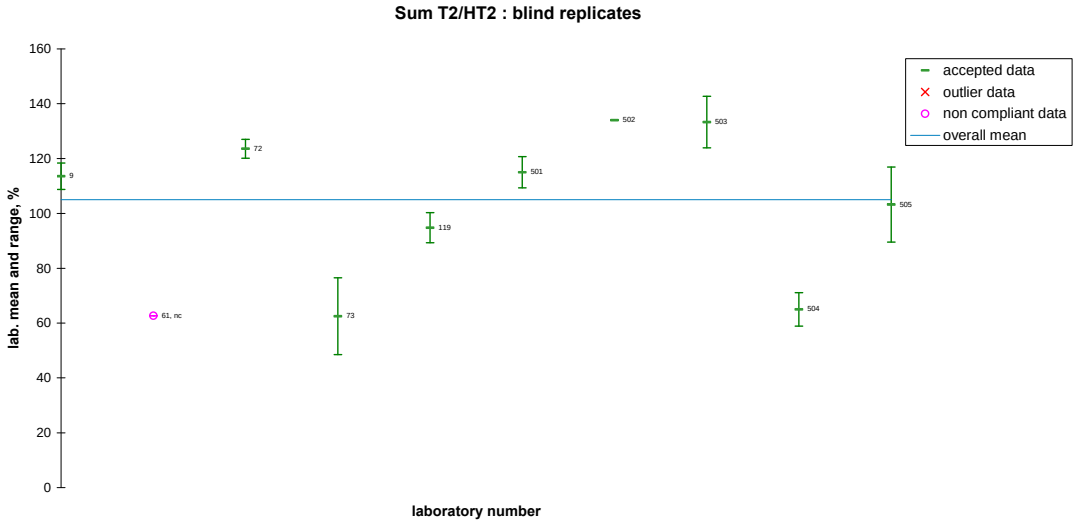

Figure 17: Mean & range plot of the sum of T-2 and HT-2 toxins for the recovery determination in the cereal mix material

Comments from collaborative trial participants

The questionnaire, which was sent along with materials and reagents, was filled in and returned by all 10 laboratories. Below are some of the answers given:

The question whether there was a deviation from the protocol was answered with "Yes" by seven labs, with "No" by one and two labs did not give an answer. Two labs which answered "Yes" also reported outlying results. One of those is Laboratory 73 which deviated by using a more polar column (DB1701), a temperature program starting at 80 ºC, and centrifugation instead of filtration to remove particulate matter from the raw extract. The other is Laboratory 501 which deviated by storing the IAC cleaned-up extracts overnight before proceeding with the method.

A third lab which reported outlying results did not answer whether it deviated from the protocol.

The question whether a similar method was being used before was answered with "Yes" by only three labs and with "No" by the other seven. A second question whether there was familiarity with the procedures used in the protocol was answered with "Yes" by seven labs and with "No" by the other three. Laboratories 61 and 501 were neither using similar methodology nor familiar with the procedures.

The question whether there were criticism or suggestions was answered with "Yes" by nine labs and the tenth lab did not answer. One criticism was that a method just for T-2 & HT-2 toxins was impractical. Another point that was raised was the lack of a familiarization opportunity like extra materials and IAC columns. One lab criticized the small injection solution volume. All other comments were related to the method protocol and its lack of details in some areas.

Concerning the low volume of the injection solution: We realize that the handling of such small volumes needs practice but we also see the small volume, which leads to a higher concentration of the analytes in the injection solution, as the strength of the novel derivatisation procedure.

Conclusions

The results of this collaborative trial show that immunoaffinity clean-up in combination with GC/MS is a suitable procedure to determine the two type A trichothecenes T-2 and HT-2 toxins in cereal and compound animal feed based matrices.

The novel trimethylsilylation reagent introduced in this trial works well if some precautions are considered: the efficient handling of the small injection volumes need some experience, the autosampler settings need to be adjusted such that no sample washes are performed, and initial oven temperatures below 120 ºC might be deleterious to successful separation and detection. We believe that the small injection solution volume has helped considerably in achieving the reported precision parameters.

Since almost all of the outlying results were reported by two laboratories which deviated from the protocol by either using centrifugation instead of filtration or storage of the cleaned-up extracts overnight it must be pointed out that particulate matter need to be removed from the raw extract by filtration and that derivatization should follow clean-up immediately.

Recently a fully ^{13}C labelled HT-2 toxin , analogue to the fully ^{13}C labelled T-2 toxin used in this study, has become available. Its inclusion is strongly recommended.

The blank materials of compound animal feed and cereal mix proved to be not blank but contaminated at a very low level. But the levels reported by the laboratories varied widely so that it must be said that determination at such a low concentration is not possible with an acceptable level of reliability.

The results of this collaborative study show precision characteristics for the sum of the concentrations of T-2 and HT-2 toxins which fulfil the criteria (RSD_r, RSD_R and recovery) as set forth by European legislation [1] for all tested levels in both cereal mix and animal feed.

The JRC is currently transforming this method into CEN format and will submit it to CEN TC 275/WG 5 for adoption.

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1. *Regulation (EC) No 401/2006*, E. Commission, Editor. 2006.
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7. Jestoi, M., A. Ritieni, and A. Rizzo, *Analysis of the Fusarium Mycotoxins Fusaproliferin and Trichothecenes in Grains Using Gas Chromatography-Mass Spectrometry.* J Agric Food Chem, 2004. **52**(6): p. 1464-1469.
8. Youden, W.J. and E.H. Steiner, *Statistical manual of the AOAC*. 1975: AOAC International.
9. Mathieson, K., *CLSTD.XLT*. 1998. p. Microsoft EXCEL template with macro collection.
10. *Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a method of Analysis*, in *J AOAC Int*. 2002.
11. Thompson, M., *Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing.* Analyst, 2000. **125**: p. 385-386.

Annex I:

Table 1: Reported results for T-2 toxin in animal feed

Lab ID	Blank [µg/kg]		Low [µg/kg]		High [µg/kg]		Spike [µg/kg]		App. Recovery [%]	
9	2.0	2.0	23.9	25.7	29.7	32.4	28.8	26.2	107	97
61	3.0	2.2	21.6	20.2	32.3	34.4	29.2	29.5	106	107
72	1.8	2.9	24.2	26.2	29.0	29.6	23.4	27.8	84	102
73	8.0		23.0	27.5			52.5	42.5	178	138
119	2.8	2.6	25.6	26.1	35.8	32.9	27.0	26.5	97	95
501	16.7	5.5	46.1	32.5	69.8	34.7				
502	n.d.	n.d.	35.0	30.0	44.0	40.0	37.0	34.0	148	136
503	0.0	0.0	26.7	21.0	36.8	31.3	0.0	53.7	0	215
504	5.8	5.8	18.4	22.4	23.3	21.5	25.5	25.0	79	77
505	3.2	3.8	10.0	10.0	26.0	27.6	24.9	24.2	86	83

Empty cell – no value reported; n.d. – not detected; Spike – nominal value 25 µg/kg; light gray – non-compliant; dark gray – outlying result

Table 2: Reported results for HT-2 toxin in animal feed

Lab ID	Blank [µg/kg]		Low [µg/kg]		High [µg/kg]		Spike [µg/kg]		App. Recovery [%]	
9	10.0	13.3	69.6	71.3	71.4	85.1	73.4	69.3	123	115
61	12.9	11.5	57.0	54.5	89.5	86.0	70.3	67.7	116	111
72	22.6	24.1	103.7	93.6	112.7	114.8	75.1	104.4	104	162
73	15.0	28.5	52.0	73.0			156.5	145.5	270	248
119	11.7	11.9	61.9	59.5	90.0	82.9	64.3	65.3	105	107
501	23.9	16.6	90.8	98.5	89.3	140.3				
502	n.d.	n.d.	53.0	65.0	83.0	72.0	72.0	70.0	144	140
503	0.0	0.0	78.7	74.6	107.8	90.9	0.0	142.8	0	286
504	12.1	9.7	49.2	55.6	67.6	61.8	64.4	56.6	107	91
505	17.2	16.3	47.8	45.2	89.1	105.7	79.7	77.5	126	121

Empty cell – no value reported; n.d. – not detected; Spike – nominal value 50 µg/kg; light gray – non-compliant; dark gray – outlying result

Table 3: Performance parameters for the two toxins in animal feed

		Mean	N	nc	outl.	n	r	s_r	RSD_r	R	s_R	RSD_R	HoR_{mod}
T-2	Blank	2.4	10	1	1	8	1.08	0.39	16	5.30	1.89	80	3.6
	Low	24.8	10	0	0	10	10.64	3.80	15	22.7	8.10	33	1.5
	High	31.7	10	1	1	8	5.96	2.13	7	16.9	6.03	19	0.9
	App. Recovery at 25 µg/kg	100	10	2	1	7	17.89	6.39	6	59.9	21.4	21	1.0
HT-2	Blank	10.8	10	0	2	8	3.28	1.17	11	22.1	7.88	73	3.3
	Low	67.7	10	0	0	10	17.98	6.42	9	50.6	18.1	27	1.2
	High	88.1	10	1	1	8	21.80	7.79	9	45.4	16.2	18	0.8
	App. Recovery at 50 µg/kg	120	10	2	1	7	46.1	16.5	14	52.9	18.9	16	0.7

Legend: Mean –mean mass fraction [µg/kg] or mean percentage; N – number of labs; nc – non-compliant laboratories; outl. – outlying laboratories; n – number of laboratories used for statistics; r – repeatability [µg/kg], s_r – repeatability standard deviation [µg/kg], RSD_r – relative standard deviation under repeatability conditions [%]; R, s_R, RSD_R– the respective values for reproducibility, HoR_{mod} – the HorRat value for reproducibility modified after Thompson [11]

Table 4: Reported results for T-2 toxin in cereal mix

Lab ID	Blank [µg/kg]		Low [µg/kg]		High [µg/kg]		Spike [µg/kg]		App. Recovery [%]	
9	1.2		4.5	4.9	24.9	28.1	18.0	19.1	99	105
61			11.0	8.6	23.0	20.0		21.9		129
72	1.4	1.4	4.9	5.5	24.8	26.1	18.3	18.0	99	97
73	1.0		4.0	4.5	29.5	31.5	19.0	17.5	106	97
119	2.8	2.1	5.7	5.8	25.2	24.3	18.7	18.9	96	97
501	15.2	5.8	16.0	9.5	24.4	32.0	19.6	20.6	54	59
502	n.d.	n.d.	14.0	13.0	33.0	32.0	26.0	27.0	153	159
503	0.0	0.0	0.0	0.0	22.5	22.7	14.3	18.4	84	108
504	2.4	4.8	10.8	4.4	21.6	22.0	13.0	12.3	55	51
505	2.9	3.2	5.9	6.5	22.0	22.0	16.7	18.5	80	91

Empty cell – no value reported; n.d. – not detected; Spike – nominal value 17 µg/kg; light gray – non-compliant; dark gray – outlying result

Table 5: Reported results for HT-2 toxin in cereal mix

Lab ID	Blank [µg/kg]		Low [µg/kg]		High [µg/kg]		Spike [µg/kg]		App. Recovery [%]	
9	2.9	2.7	13.3	16.9	64.4	61.3	45.2	39.3	129	111
61			13.1	9.5	22.8	37.5		9.5		29
72	8.6	8.2	26.0	25.4	84.6	84.8	51.5	55.4	131	142
73	81.5	89.0	60.5	53.0	194.5	143.0	105.0	92.5	60	22
119	4.2	2.6	14.0	13.4	54.2	56.2	37.3	31.6	103	85
501	4.1	3.8	18.5	19.9	72.1	79.4	55.2	48.5	155	135
502	n.d.	n.d.	20.0	21.0	48.0	55.0	41.0	40.0	124	121
503	0.0	0.0	18.5	14.4	68.9	68.0	47.7	52.9	145	160
504	4.6	6.7	9.2	9.6	69.8	26.3	31.8	26.4	79	63
505	8.5	8.3	25.3	26.5	62.5	64.7	39.5	51.4	94	130

Empty cell – no value reported; n.d. – not detected; Spike – nominal value 33 µg/kg; light gray – non-compliant; dark gray – outlying result

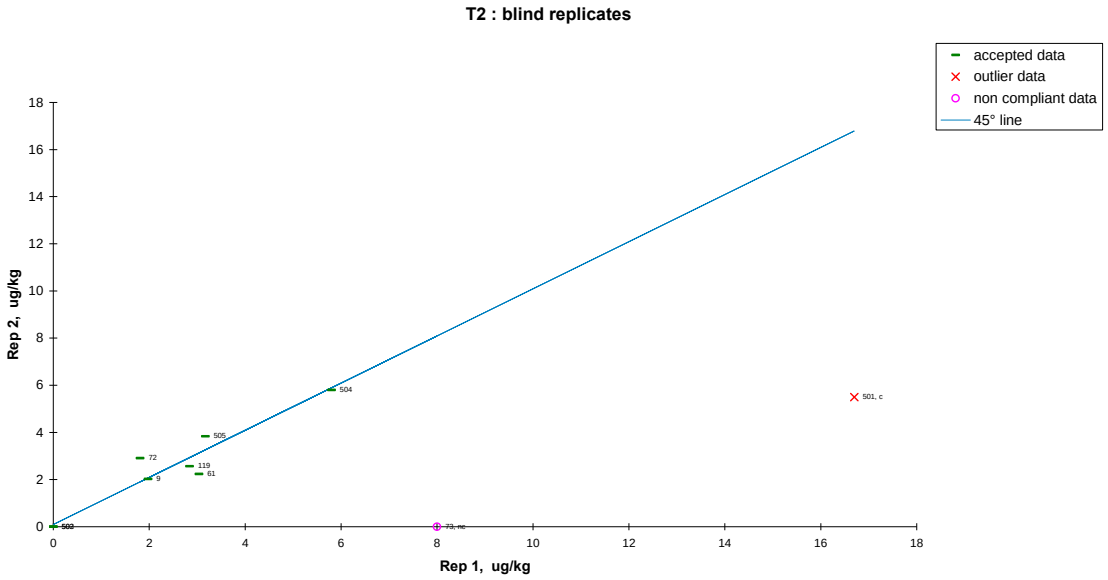
Table 6: Performance parameters for the two toxins in cereal mix

		Mean	N	nc	outl.	n	r	s_r	RSD_r	R	s_R	RSD_R	HoR_{mod}
T-2	Blank	1.7	10	3	1	6	2.02	0.72	41	4.54	1.62	93	4.2
	Low	7.0	10	0	0	10	5.98	2.13	31	12.2	4.36	63	2.8
	High	25.3	10	0	1	9	3.43	1.22	5	11.3	4.05	16	0.7
	App. Recovery at 50 µg/kg	94	10	1	0	9	20	7.16	8	84.7	30.3	32	1.5
HT-2	Blank	4.1	10	1	1	8	1.90	0.68	17	9.26	3.31	81	3.7
	Low	17.5	10	0	1	9	4.57	1.63	9	16.7	5.98	34	1.6
	High	61.5	10	0	2	8	12.89	4.60	7	47.3	16.9	27	1.2
	App. Recovery at 50 µg/kg	111	10	1	0	9	43.9	15.7	14	106	38.0	34	1.6

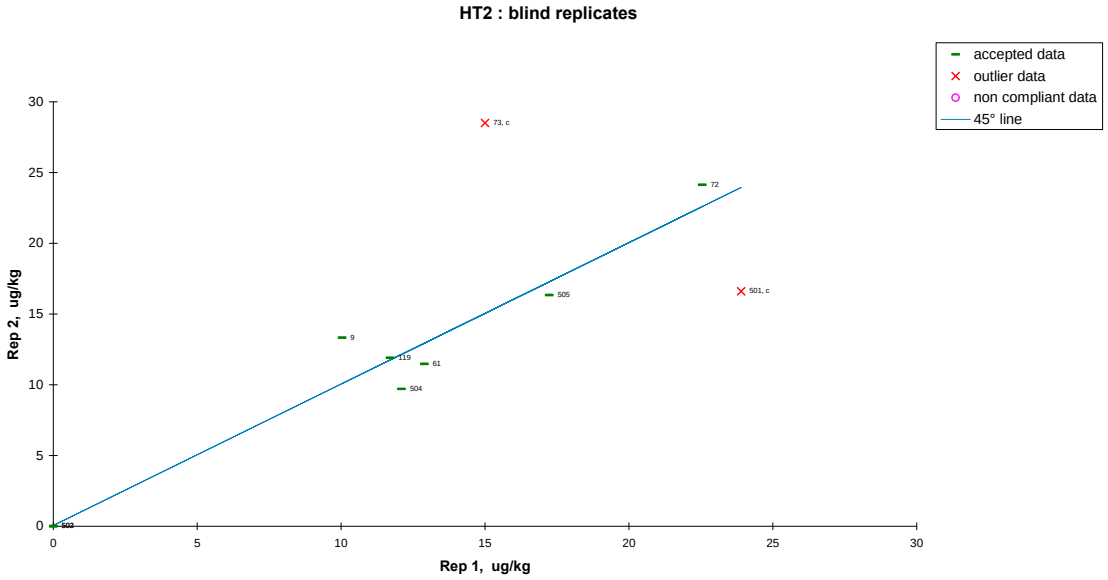
Legend: Mean –mean mass fraction [µg/kg] or mean percentage for recovery; N – number of labs; nc – non-compliant laboratories; outl. – outlying laboratories; n – number of laboratories used for statistics; r – repeatability [µg/kg], s_r – repeatability standard deviation [µg/kg], RSD_r – relative standard deviation under repeatability conditions [%]; R, s_R, RSD_R– the respective values for reproducibility, HoR_{mod} – the HorRat value for reproducibility modified after Thompson [11]

Annex II:

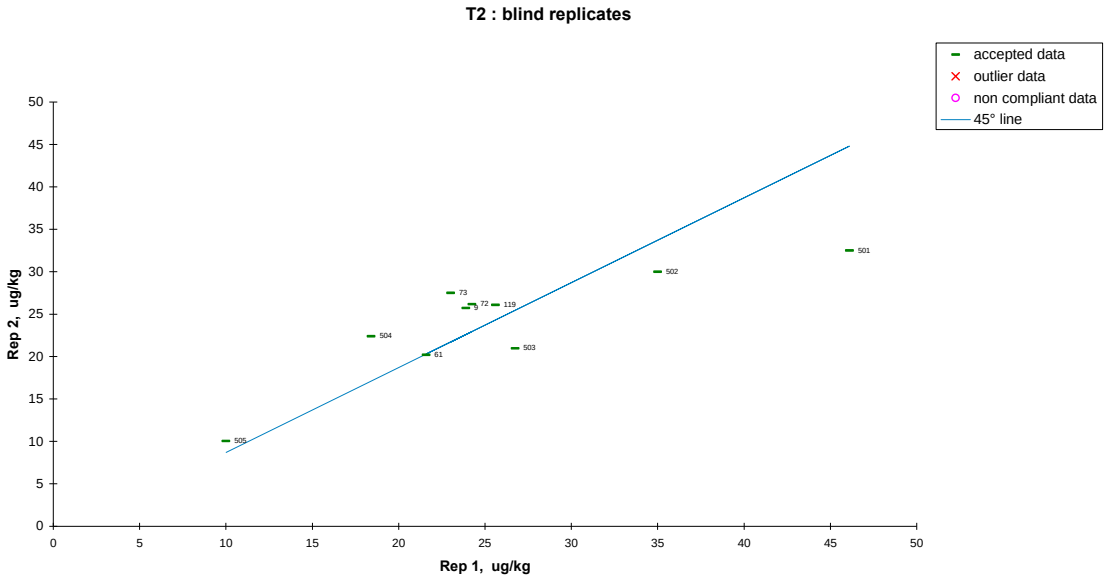
Youden plots:



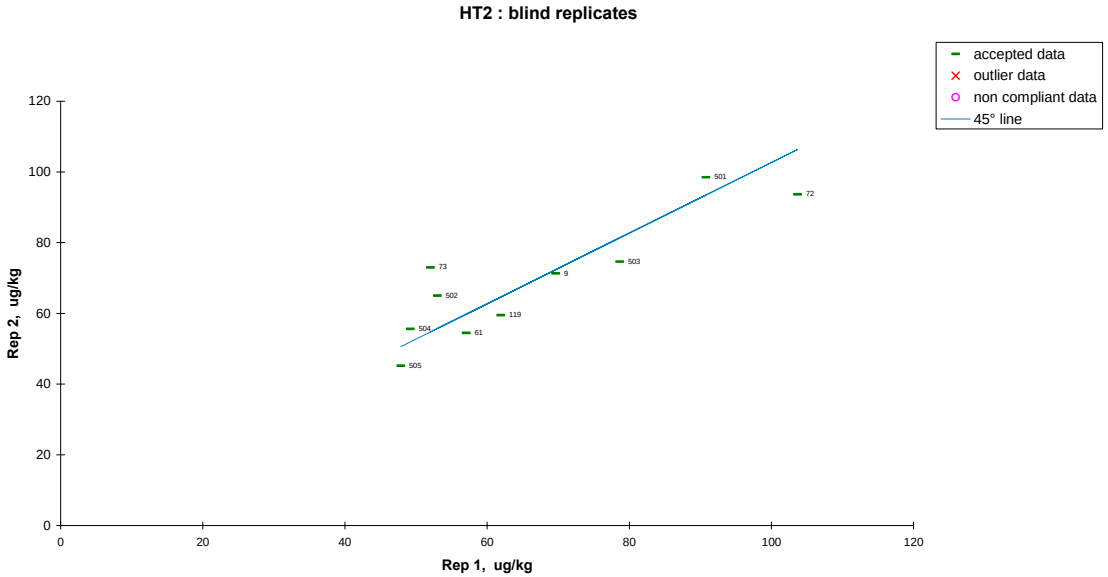
T2 toxin in blank compound animal feed



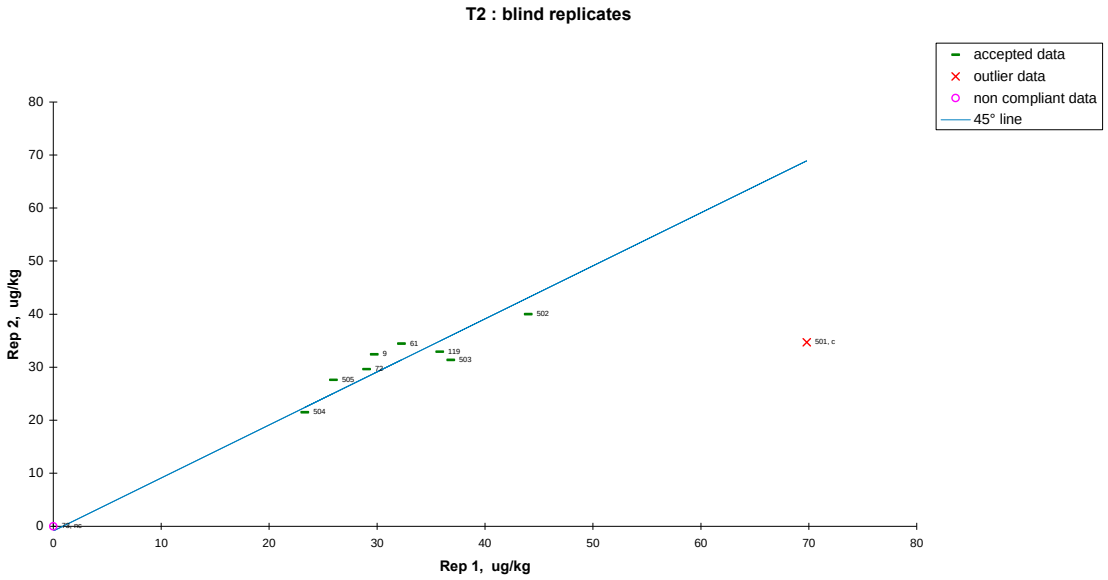
HT2 toxin in blank compound animal feed



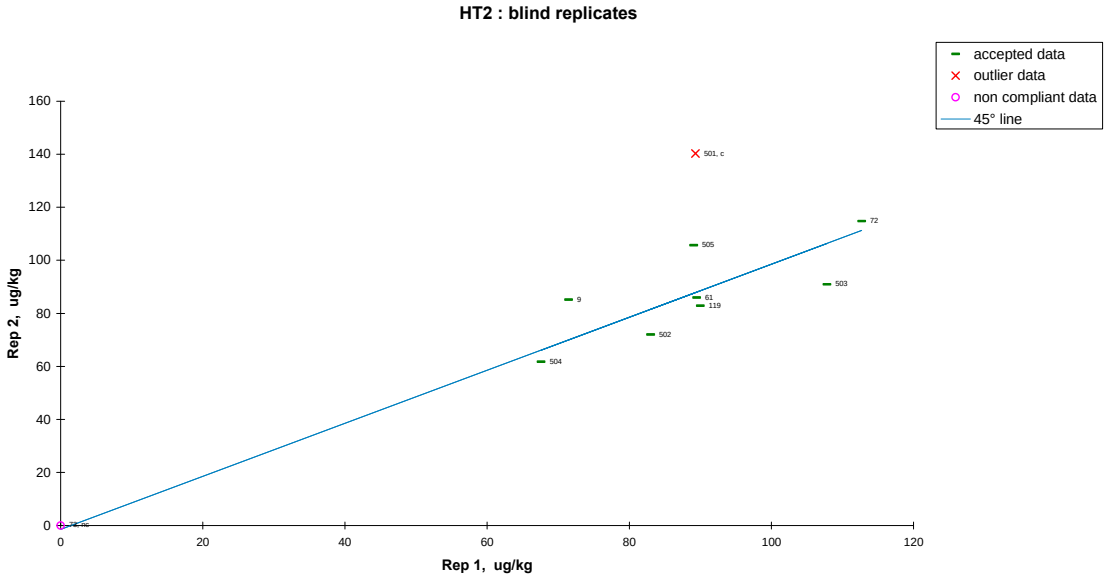
T2 toxin in low contaminated compound animal feed



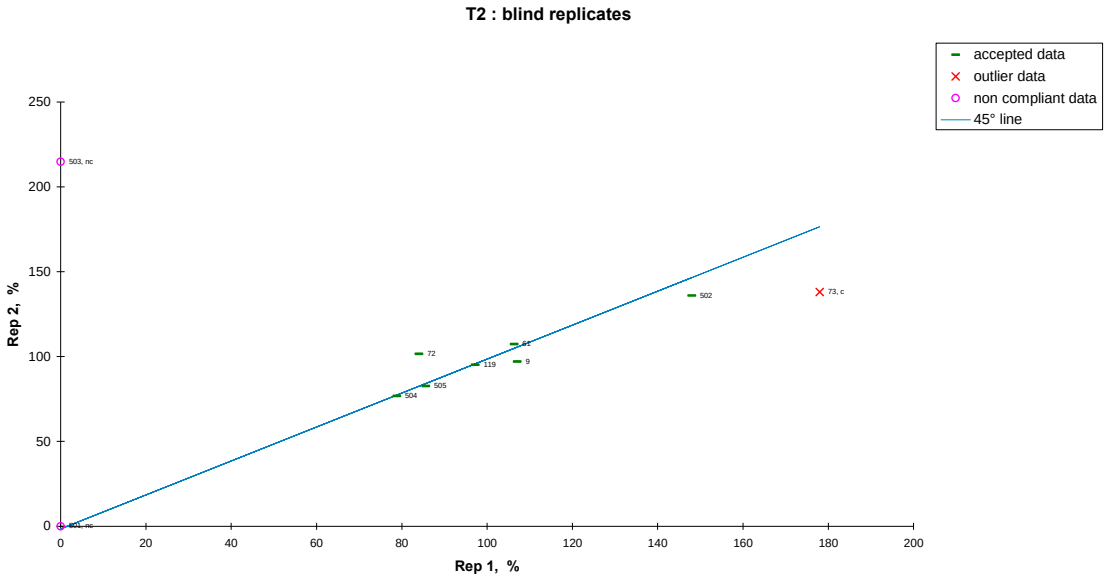
HT2 toxin in low contaminated compound animal feed



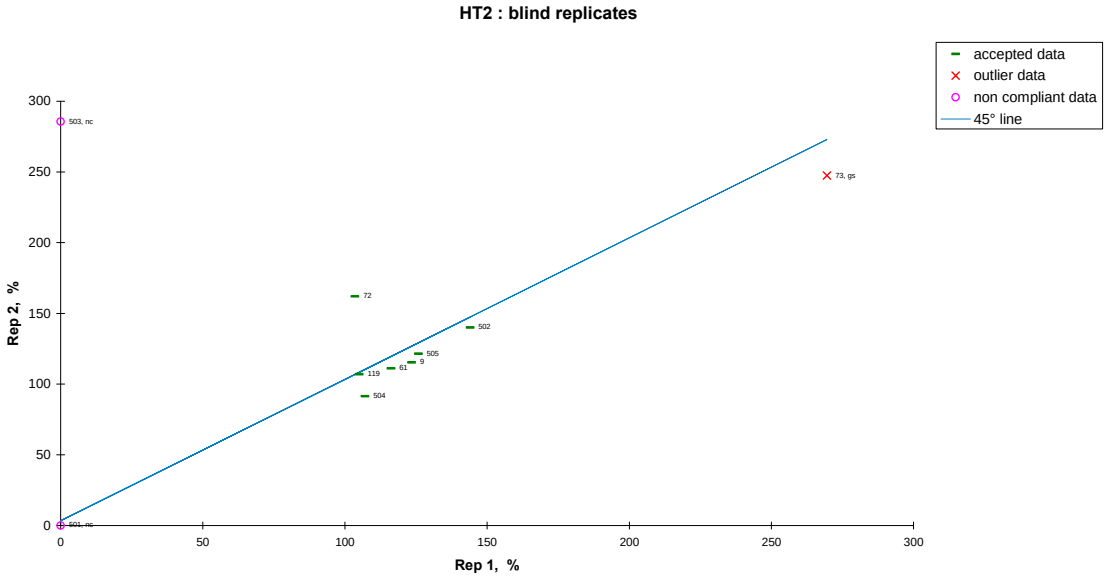
T2 toxin in highly contaminated compound animal feed



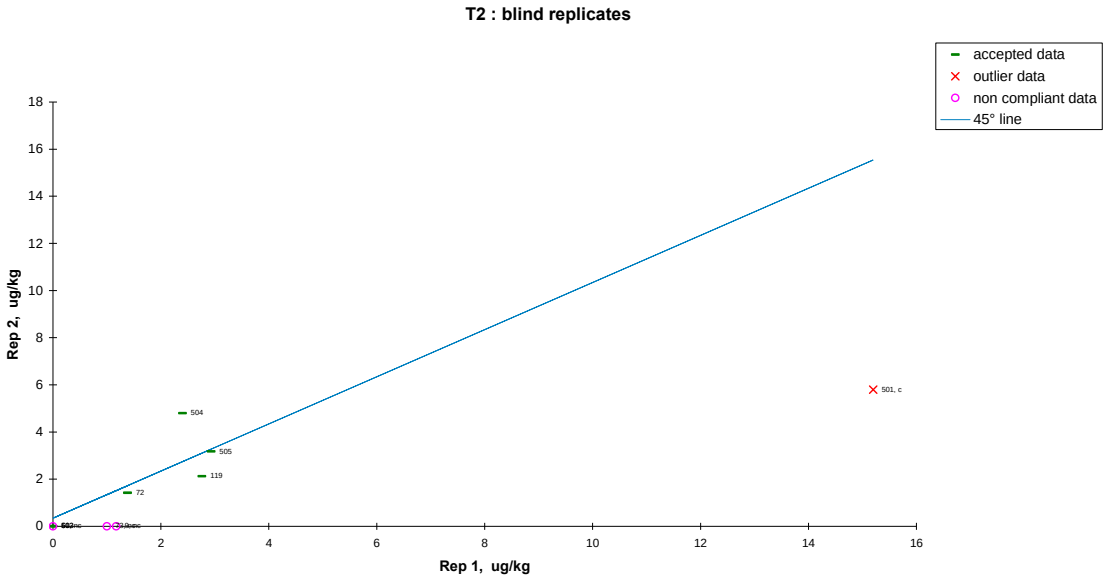
HT2 toxin in highly contaminated compound animal feed



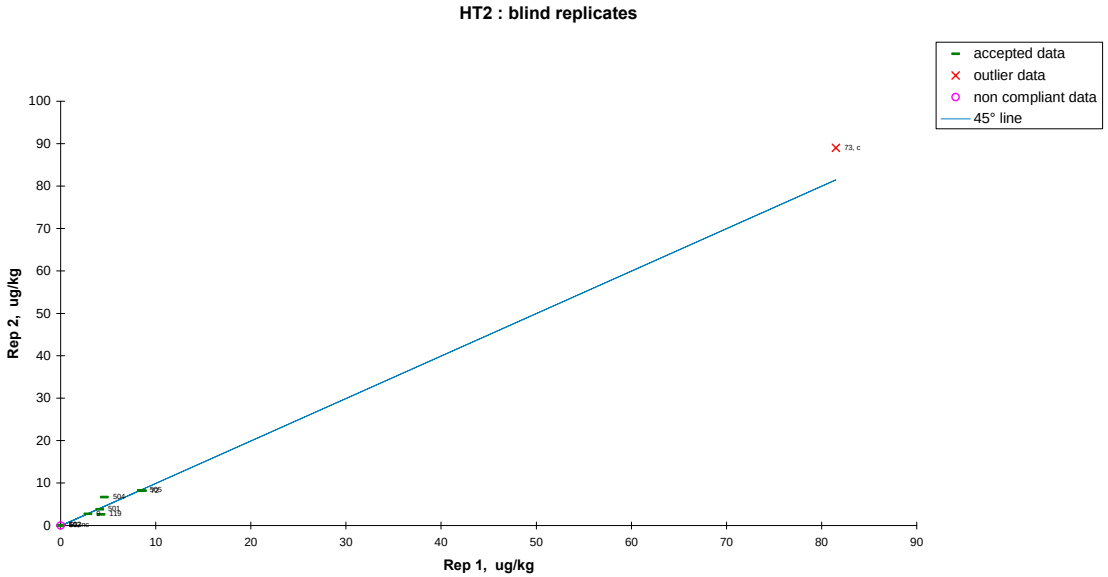
T2 toxin in spiked blank compound animal feed



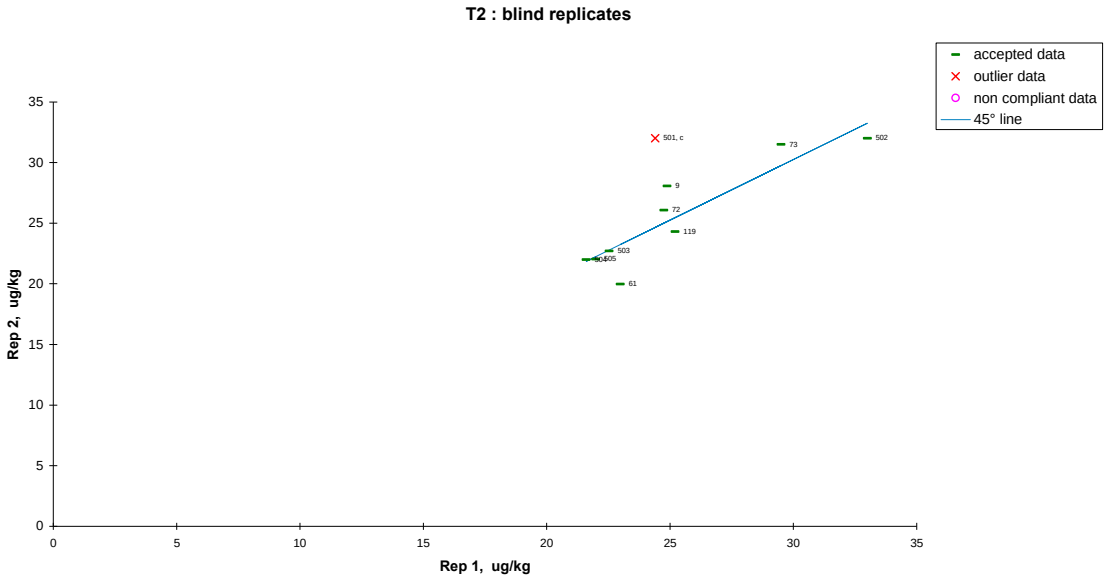
HT2 toxin in spiked blank compound animal feed



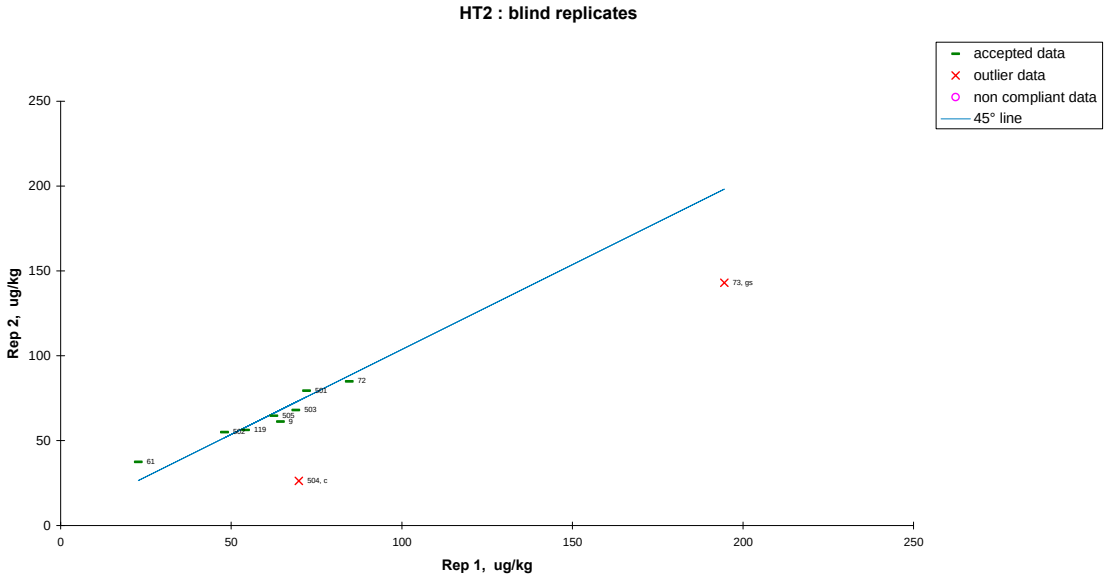
T2 toxin in blank cereal mix



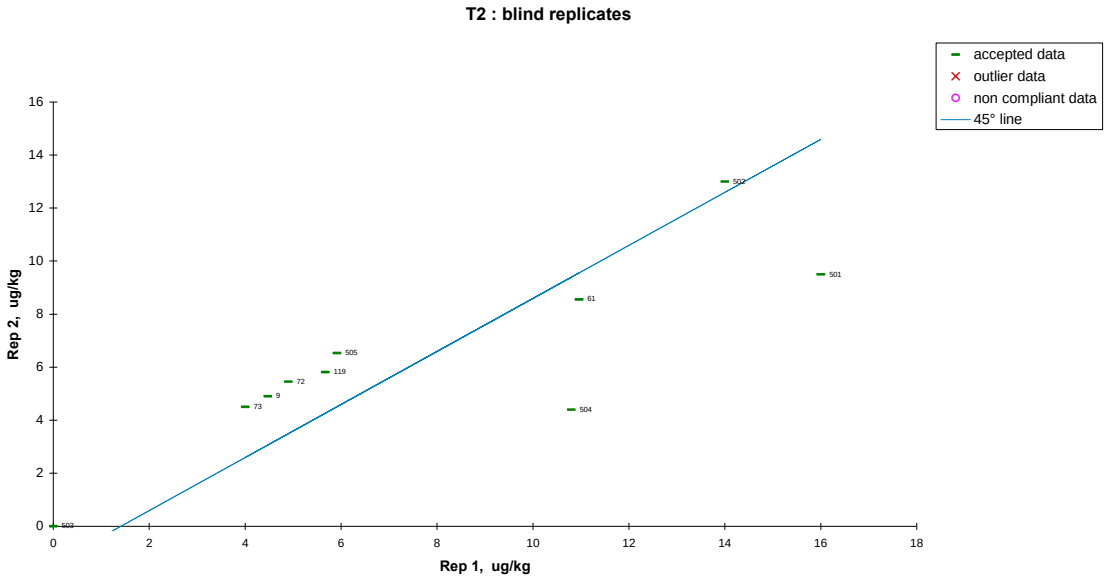
HT2 toxin in blank cereal mix



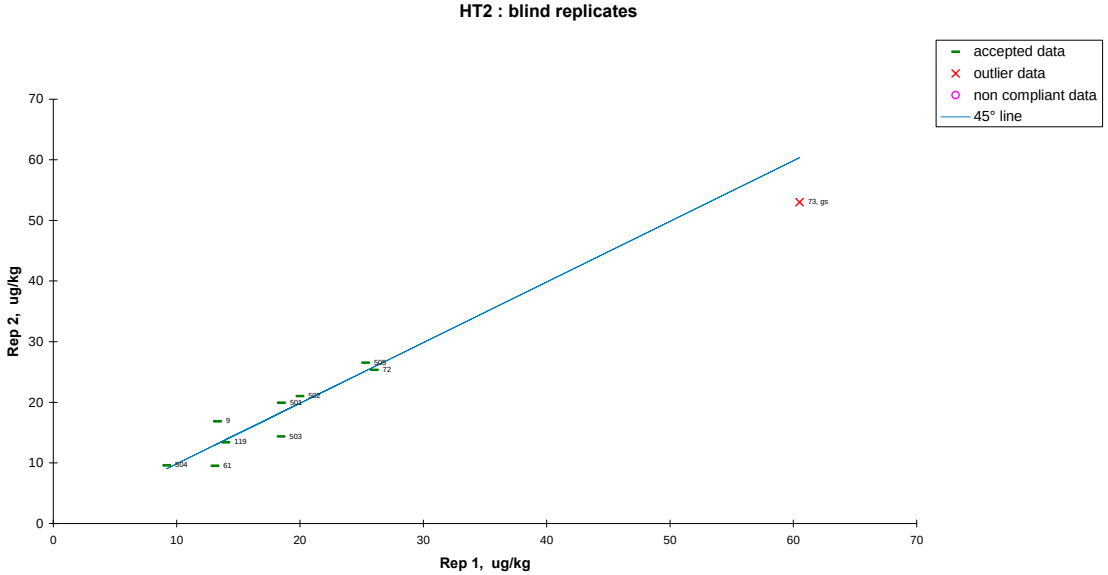
T2 toxin in highly contaminated cereal mix



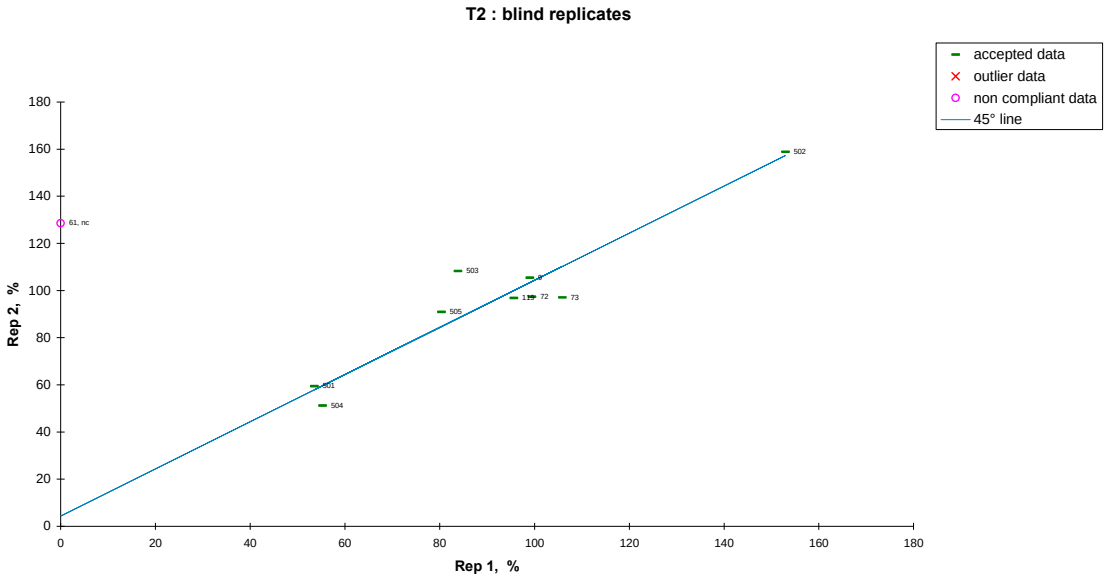
HT2 toxin in highly contaminated cereal mix



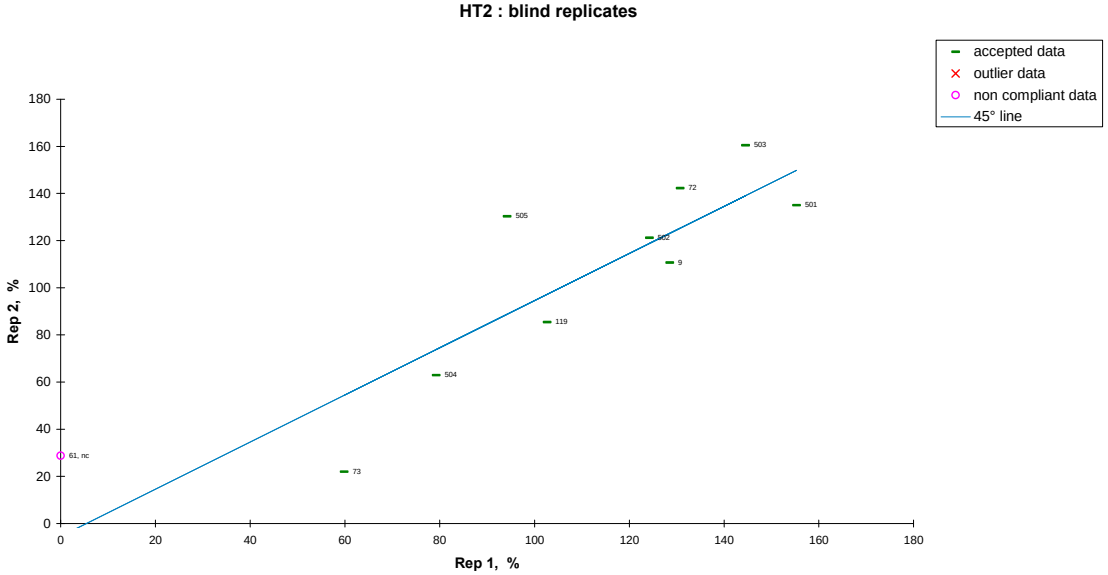
T2 toxin in low contaminated cereal mix



HT2 toxin in low contaminated cereal mix



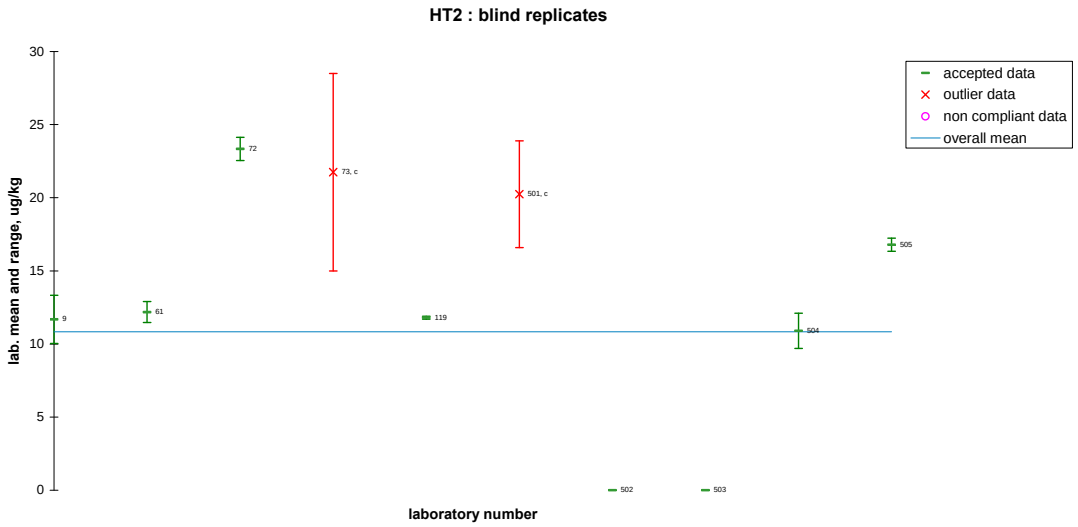
T2 toxin in spiked blank cereal mix



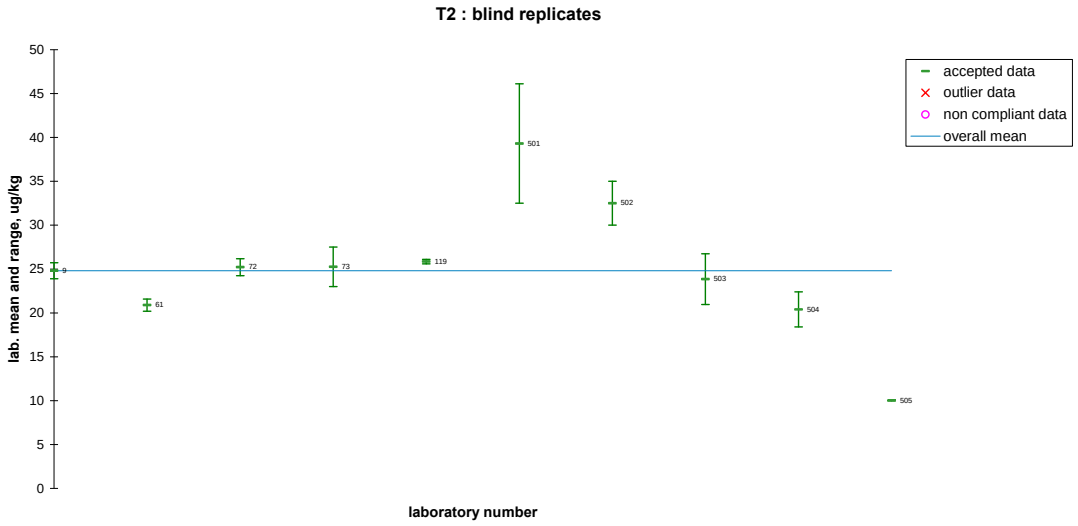
HT2 toxin in spiked blank cereal mix

Mean & Range plots:

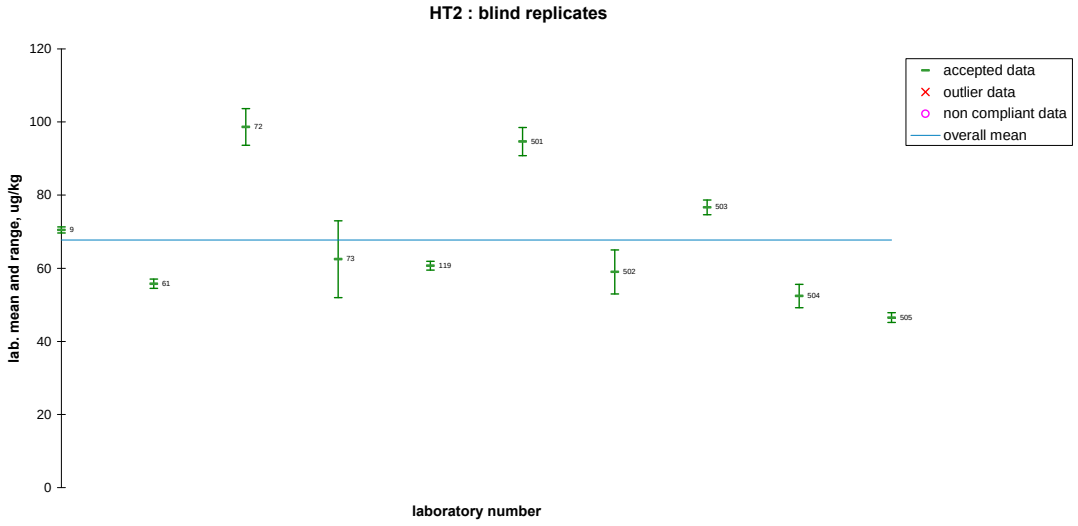
T2 toxin in blank compound animal feed



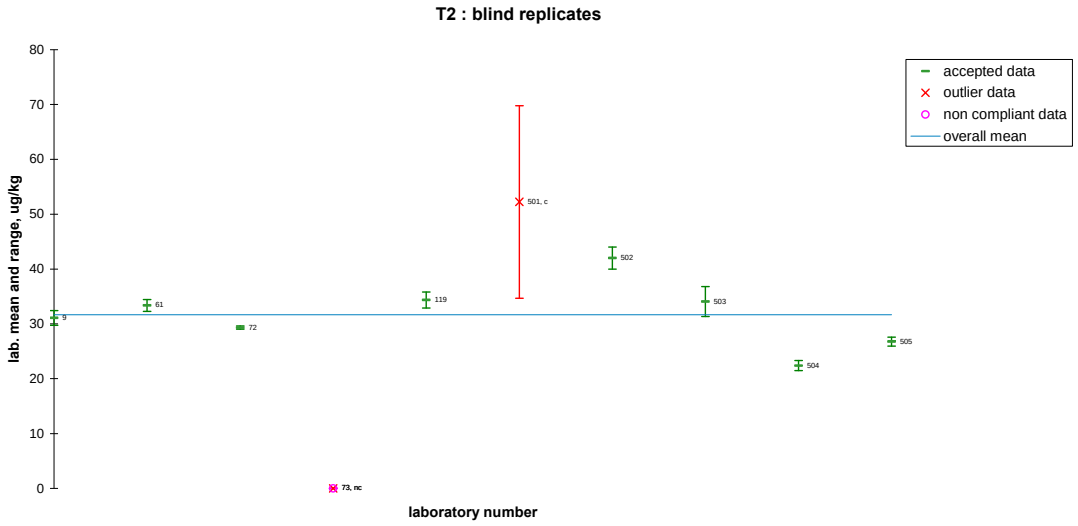
HT2 toxin in blank compound animal feed



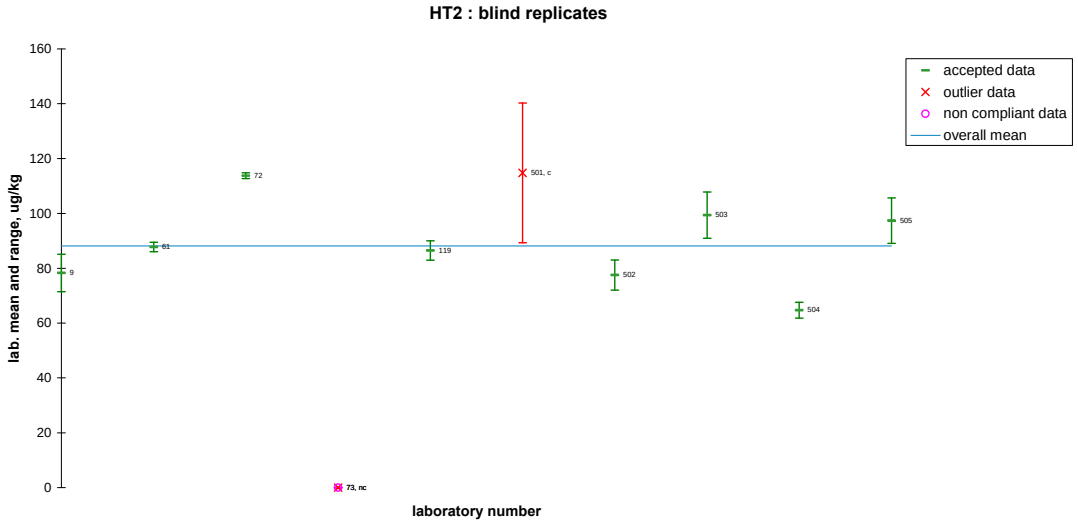
T2 toxin in low contaminated compound animal feed



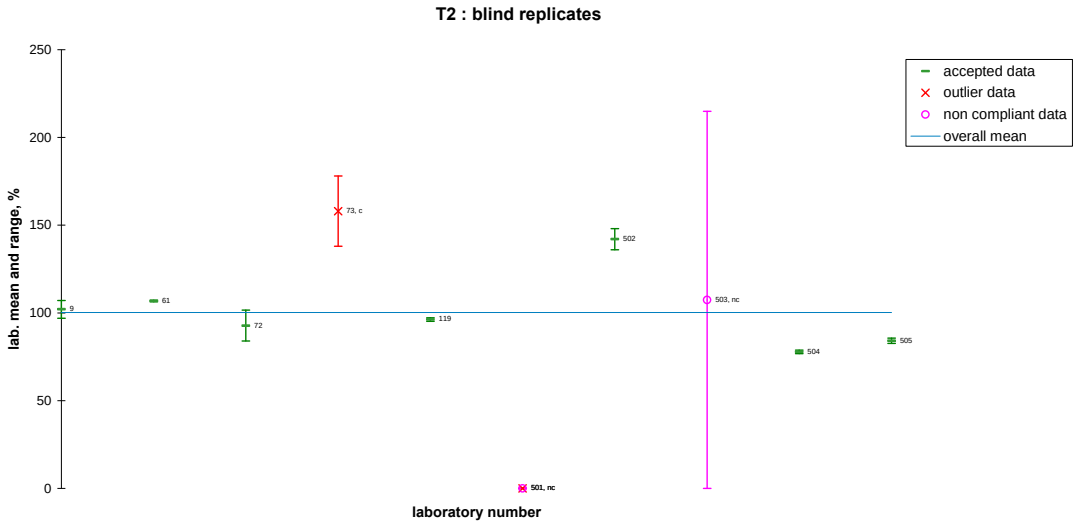
HT2 toxin in low contaminated compound animal feed



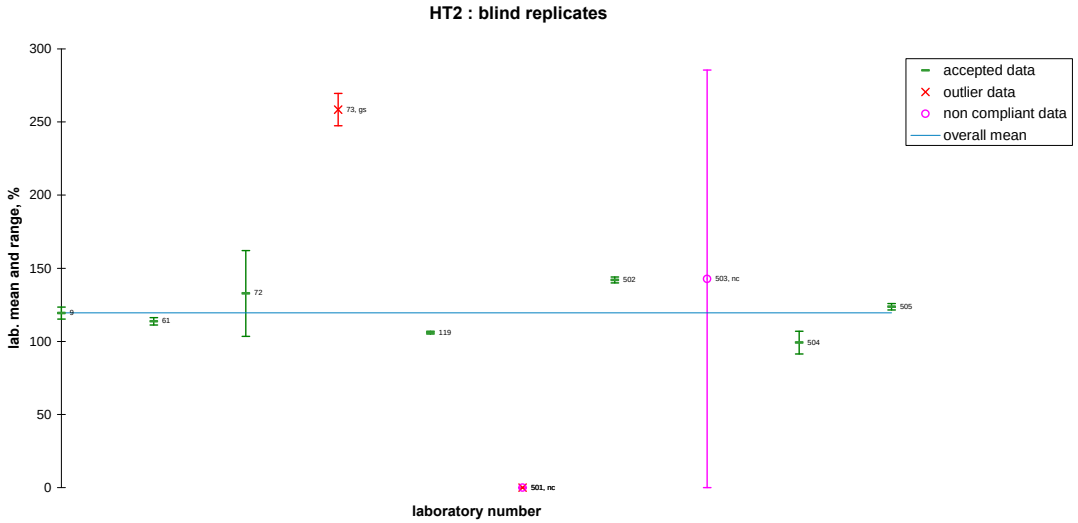
T2 toxin in highly contaminated compound animal feed



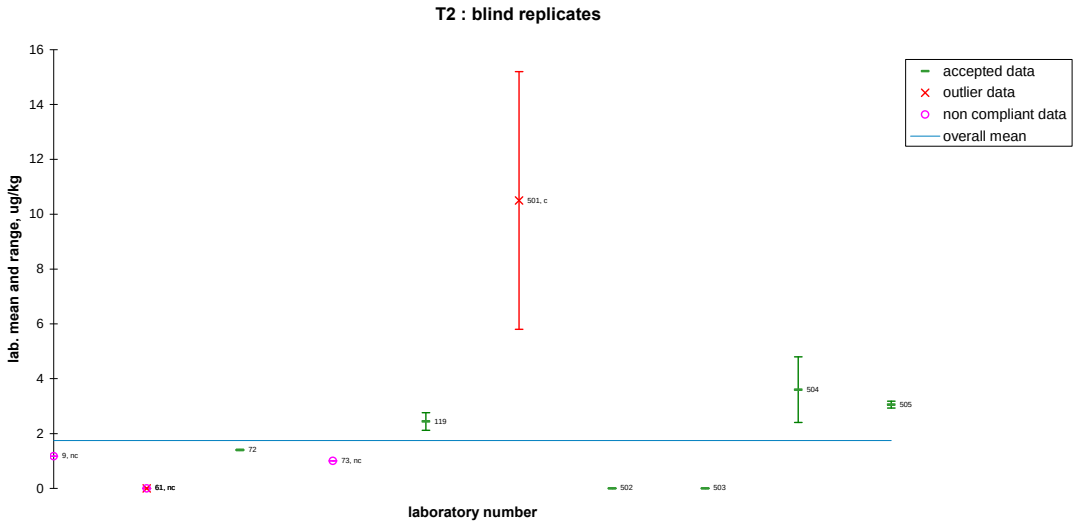
HT2 toxin in highly contaminated compound animal feed



T2 toxin in spiked blank compound animal feed



HT2 toxin in spiked blank compound animal feed



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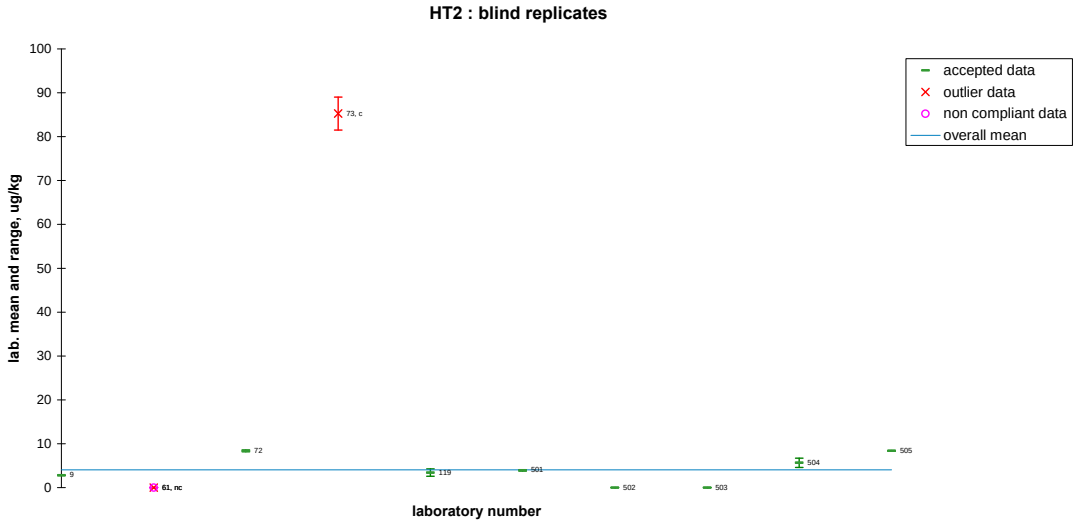
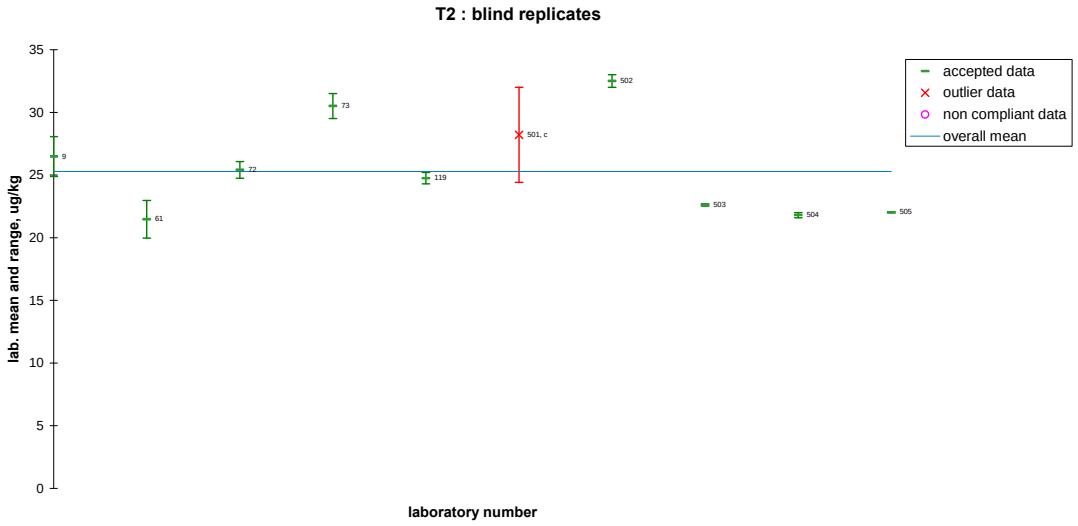
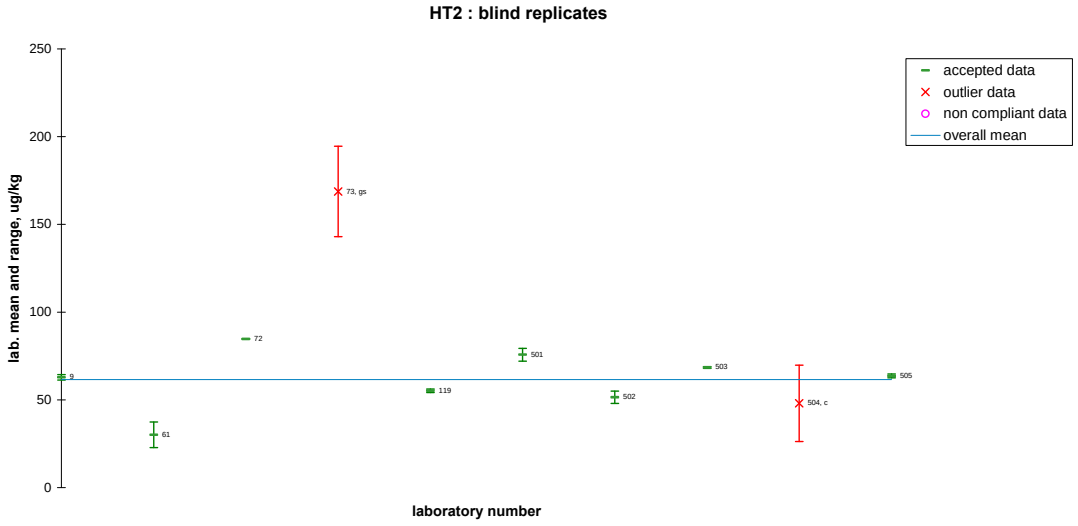


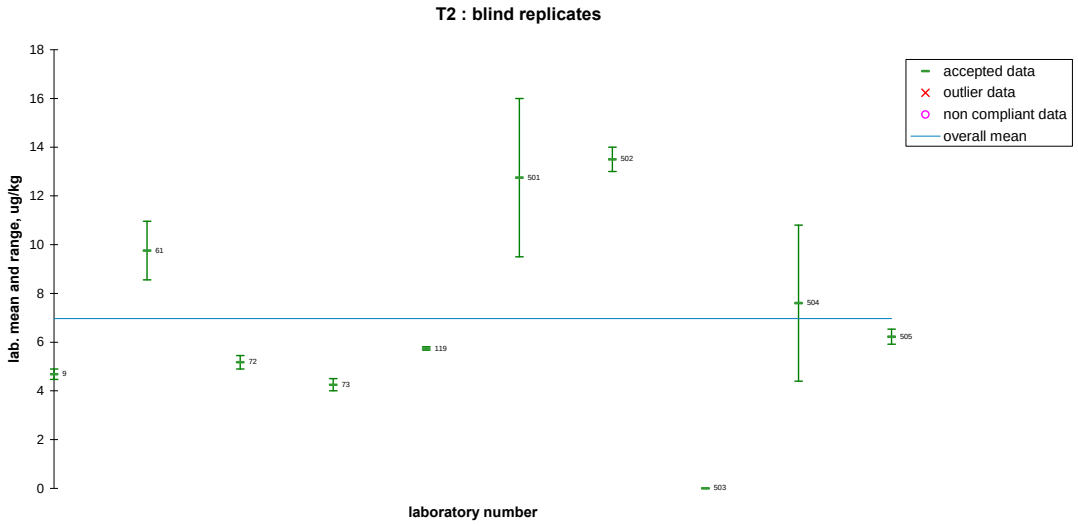
HT2 toxin in blank cereal mix



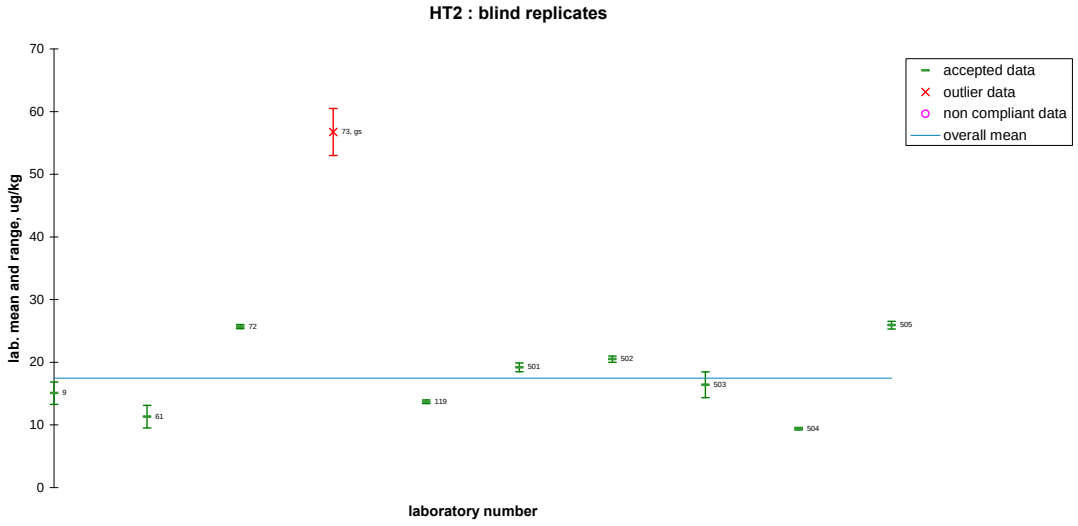
T2 toxin in highly contaminated cereal mix



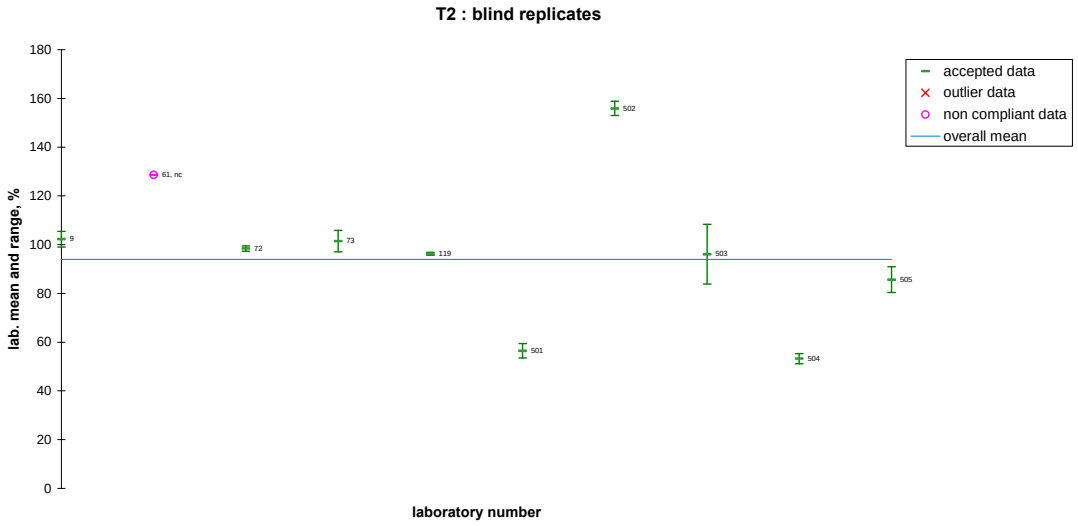
HT2 toxin in highly contaminated cereal mix



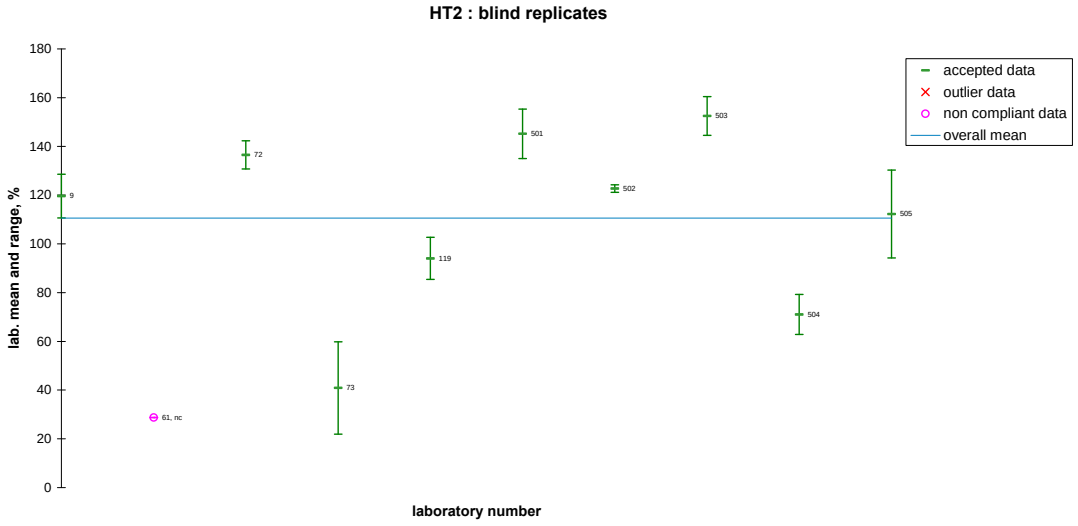
T2 toxin in low contaminated cereal mix



HT2 toxin in low contaminated cereal mix



T2 toxin in spiked blank cereal mix



HT2 toxin in spiked blank cereal mix

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Annex III

Foodstuffs – Determination of T-2 and HT-2 toxins in cereals, baby food and animal feed – GC/MS method with immunoaffinity clean-up AA

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Contents

Foreword

THIS IS A STUDY OF THE METHOD, NOT 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 can involve 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.

WARNING – trichothecenes are highly toxic. Gloves and safety glasses should be worn at all time and all standard and sample preparation stages should be carried out in a fume cupboard.

NOTE — Care should be taken to avoid contact of organic solvents (standard solutions, reagents, extracts) with plastics, like pipet tips, containers, etc., as much as possible. Constituents of the plastics, f.i. plasticizers, might dissolve in the organic solvent and cause increased background and additional peaks during the GC/MS run.

1. SCOPE

This protocol specifies a candidate method for the determination of T-2 and HT-2 in cereals, baby food and animal feed using gas-chromatography with mass spectrometric detection. This candidate method will be validated for the determination of T-2 and HT-2 via the analysis of naturally contaminated and spiked samples of cereals, baby food, and/or of animal feed at levels starting from 2 ng/g for the individual toxins or 10 ng/g for the sum of both toxins.

2. PRINCIPLE

T-2 and HT-2 are extracted from cereal, baby food, and animal feed with a methanol/water solution. The extract is cleaned up using immunoaffinity columns (IAC). T-2 and HT-2 are eluted from IAC using acetonitrile, which is evaporated to dryness. The dry residue is derivatized using a trimethylsilyl reagent. T-2 and HT-2 are quantitatively assessed by gas chromatography (GC)/ mass spectrometry (MS).

3. APPARATUS

Usual laboratory equipment and, in particular, the following:

3.1. Conical flasks

250 mL capacity with screw caps

3.2. Flask shaker

3.3. Bulb pipettes

2 mL (Class AS, ± 0.01 mL)

10 mL (Class AS, ± 0.02 mL)

20 mL (Class AS, ± 0.02 mL)

100 mL (Class AS, ± 0.08 mL)

3.4. Heating block with nitrogen gas supply or centrifugal vacuum evaporator

3.5. Vortex mixer

3.6. Analytical balance (d= 0.01g)

3.7. Folded filter paper (f.i. Whatman 113V 18.5 cm)

3.8. Glass micro fibre filter (f.i. Whatman GF/A 15 cm)

3.9. GC/MS instrumentation, comprising the following:

3.9.1. GC: capable of splitless injections onto capillary columns, f.i. Agilent GC 6890N

3.9.2. Autosampler: capable of injecting sufficient volumes of injection solution with sufficient repeatability

3.9.3. MS: with electron impact ionization, capable of measuring the ions described in 7.2 with sufficient repeatability, f.i. Agilent MSD 5973N with EI

3.9.4. Chromatographic column: capable of baseline separation of T-2 and HT-2 – The following column has shown to be suitable for this separation: DB-5MS, 30 m x 0.25 mm I.D. x 0.25 µm film thickness

3.10. Glass filter funnel, f.i. 11 cm O.D.

3.11. Autosampler vials of 2.0-2.5 mL with crimp caps

3.12. Conical inserts for autosampler vials (3.11) for small volumes

3.13. Reservoirs for immunoaffinity columns

20-50 mL capacity with adapter for connection to top of immunoaffinity columns

3.14. Volumetric flasks

20 mL (Class A, ± 0.04mL)

3.15. Gastight Hamilton syringes

100, 250, 500 and 1000 µL capacity

3.16. Support rack for immunoaffinity columns (12 mm O.D.)

4. REAGENTS AND MATERIALS

During the analysis, unless otherwise stated, use only solvents and reagents of recognized analytical grade and only distilled water or water of grade 1 as defined in EN ISO 3696. Solvents shall be of HPLC or better quality.

4.1. Double distilled or deionized water

4.2. Methanol

4.3. Extraction solvent

Mix 80 parts methanol (4.2) with 20 parts of water (4.1, v/v).

4.4. Methanol solution (16 %)

Mix 10 parts of extraction solvent (4.3) with 40 parts of water (4.1, v/v).

4.5. Acetonitrile

4.6. Dichloromethane

4.7. TMS reagent

5 mL N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA)/ Trimethylchlorosilane (TMCS) (99/1, v/v) are provided labelled as "TMS reagent". After opening transfer and store reagent in glass container with gas-tight, Teflon-lined cap.

4.8. ISTD solution

5 mL of a solution of 250 ng/mL $^{13}C_{24}$-T-2 toxin (Biopure, Tulln, Austria) in acetonitrile (4.5) is provided labelled as "ISTD solution in acetonitrile". After opening transfer and store solution in glass container with gas-tight, Teflon-lined cap.

4.9. T-2/ HT-2 stock solution

10 mL of a mixed standard solution of T-2 and HT-2 toxin in acetonitrile (4.5) at 100 and 200 ng/mL, respectively, are provided, labelled with "T-2/HT-2 standard in acetonitrile". After opening transfer and store solution in glass container with gas-tight, Teflon-lined cap.

4.10. T-2/ HT-2 diluted solution for calibration

To a 20 mL volumetric flask (3.14) add 2.0 mL of the T-2/ HT-2 stock solution (4.9) using a 2 mL bulb pipette (3.3) and make up to 20.0 mL with acetonitrile (4.5). This will result in a diluted solution with 10 and 20 ng/mL of T-2 and HT-2, respectively.

4.11. Calibration solutions

Using Hamilton syringes (3.15), to 6 autosampler vials (3.11) add:

Vial	T-2/ HT-2 stock solution (4.9) [µL]	T-2/ HT-2 diluted solution (4.10) [µL]	ISTD solution (4.8) [µL]	Amount (T-2/ HT-2) [ng]
1		100	100	1/ 2
2		500	100	5/ 10
3	200		100	20/ 40
4	350		100	35/ 70
5	500		100	50/ 100
6			100	Reagent blank

This will create 5 levels of calibration solutions containing 1, 5, 20, 35, and 50 ng T-2, and 2, 10, 40, 70, and 100 ng HT-2 plus a reagent blank.

4.12. Spiking solution

You are provided with two vials containing 3 mL each of spiking solutions of unknown T-2 and HT-2 concentrations in acetonitrile (4.5) labelled as "Spike C solution in acetonitrile" and one of either "Spike A solution in acetonitrile" or " Spike B solution in acetonitrile"**.**

4.13. Test samples

Eight naturally contaminated and four blind blank samples in coded plastic containers and four blank samples, labelled "Spike C" and one of either "Spike A" or "Spike B", are provided.

4.14. Immunoaffinity columns with antibodies specific to T-2 and HT-2

18 EASI-EXTRACT T-2 & HT2 columns are provided (R-BIOPHARM RHÔNE LTD, Glasgow, Scotland G20 0SP)

5. PROCEDURE

5.1. Extraction of T-2 and HT-2

Weigh, to the nearest 0.1 g, 25.0 g of the test sample and 1.0 g of sodium chloride into a 250 mL conical flask (3.1), add 100.0 mL of extraction solvent (4.3), cap and shake vigorously by hand, so that the material disperses evenly. Then put on a flask shaker (3.2) for 30 min. The material should be mixed well without collecting in the top of the flask.

Allow the sample to settle after shaking. Prepare a filter funnel (3.10) and filter paper (3.7). Filter the extracted sample into a 250 mL conical flask (3.1). To 10.0 mL of filtrate add 40.0 ml of water (4.1) and mix. Filter the diluted extract through a glass microfibre filter (3.8) and collect the filtrate.

5.2. Clean up

Take an immunoaffinity column (IAC, 4.14) and attach a reservoir (3.13), **do not** empty storage solution from column. To the reservoir add 5 ml of the 16 % methanol

solution (4.4), 100 µL of the ISTD solution (4.8, using a 100 µL gastight Hamilton syringe (3.15), and 10.0 mL of the diluted, filtered extract (5.1, equivalent to 0.5 g sample). Mix carefully and allow everything to pass slowly through the column. Preferably this is driven by gravity without application of any positive or negative pressure (vacuum). The result will be a flow rate of about one drop per second.

After the extract has passed completely through the IAC, wash with 10 ml of 16 % methanol solution (4.4) in order to avoid precipitation of extract constituents in the IAC sepharose gel. Then wash with 20 mL of water (4.1).

Using a large syringe, or something similar, with an appropriate adaptor to fit the IAC, pass air through the IAC in order to expel excess water. Then place an autosampler vial (3.11) under the IAC and elute with 0.75 mL of acetonitrile (4.5), collecting the eluate with the purified toxins. After all of the acetonitrile (4.5) has passed through the column, wait for approximately one minute. Then add another 0.75 mL of acetonitrile (4.5) and continue to collect the eluate in the same vial. Carefully pass air through the column in order to collect most of the applied acetonitrile (4.5).

5.3. Derivatization for gas chromatography:

Evaporate the eluted extract (5.2) or the calibration solutions (4.11) to dryness. This can be done either with a centrifugal vaccum evaporator or under a gentle stream of nitrogen at 50 °C in a heating block (3.4). To ensure that the evaporated samples are absolutely free of water for derivatisation add some dichloromethane (4.6) to the visually dry vials and dry again.

To the dry residue add 50 µL of MSTFA/TMCS (99/1, v/v), cap vial and shake on vortex mixer for 30 seconds, making sure the lower part of the vial is thoroughly washed with the reagent. **The reagent is moisture sensitive so the capping should be done immediately**. Incubate at room temperature for 30 minutes and transfer solution with a glass Pasteur pipet to an autosampler vial (3.11) with conical insert (3.12) for GC/MS analysis. **Again, because of the moisture sensitivity this should be done as quickly as possible to minimize contact with ambient air.**

6. SPIKING PROCEDURE

To 25.0 g of spiking material add 1.0 mL of the respective spiking solution (see also attached "Spiking Protocol"). Let stand for one hour before proceeding with the sample extraction (5.1).

7. MEASUREMENTS:

7.1. GC operating conditions

Using the equipment outlined in 3.9.1, the following conditions have proven to produce adequate separation:

— Injection volume: 1 µL with injection port at 250 °C;

— Injection mode: pulsed splitless with a 160 kPa pulse for 1 min (regular splitless will work with appropriate adjustment of the initial temperature hold time);

— GC oven conditions: initial temperature 140 °C for 1 min, 60 °C/ min to 250 °C, 10 °C/ min to 300 °C and hold for 3 min;

— Carrier gas: helium at a constant flow of 1.2 mL/min;

— **Attention: There is a substance co-eluting with HT-2 that might generate a significant m/z 185 signal. Care should be taken to optimize the separation to achieve baseline separation of this substance from HT-2. With the stated conditions this was possible (see attached chromatograms).**

7.2. MS operating conditions

— GC/MS: SIM mode with electron impact ionization using the following ions: **185, 350, 436** *m/z* for T-2, **185, 347, 466** *m/z* for HT-2, and **365**, **455** *m/z* for $^{13}C_{24}$-T-2 (ISTD).

7.3. Batch (Sequence) composition

Each of the 12 coded test samples and the four spike samples are to be prepared once and to be injected in duplicate. For each batch (sequence) of samples that is run on the GC prepare two reagent blanks and two sets of 5 levels of the calibration solutions (4.11). Inject each preparation of the reagent blank and the calibration

solutions once at the beginning of the batch of samples and again at the end. This results in four injections, two injections per preparation, of the reagent blank and each level of the calibration solutions.

7.4. OPTIONAL: In-house standards

If there are in-house standards for T-2 and HT-2 toxins dilute these standards such that a mixed stock solution of 100 ng/mL T-2 and 200 ng/mL HT-2 in acetonitrile (4.5) is created, according to 4.9. Then add three times 250 µL of this in-house stock solution to three autosampler vials (3.11). To each of these three vials also add 100 µL ISTD solution (4.8). Then dry and derivatize as described in 5.3. Run these three preparations in duplicate with a sample batch.

7.5. Identification criteria

For the reagent blank runs display the extracted ion chromatograms for the ions 365, and 455 m/z. There should be only one location where peaks line up for both traces. These peaks represent the ISTD (4.8). Note the retention time.

Next repeat the above with ions 185, 350, and 436 m/z for each of the calibration runs. At the retention time of the ISTD peaks in all three traces should line up. These peaks represent T-2. Calculate the on ratios of the peak heights of ion 350 divided by ion 185, and of ion 436 divided by ion 185.

Next repeat the above with ions 185, 347, and 466 m/z. Close to the already identified peak of T-2 peaks in all three traces should line up. These peaks represent HT-2. Note the retention time and calculate the ion ratios of the peak heights of ion 347 divided by ion 185, and ion 466 divided by ion 185.

The retention times and ion ratios of T-2 and HT-2 will be used to identify the respective substance in the sample chromatograms.

For this display the appropriate extracted ion chromatograms and near the retention time of the respective substance look for three peaks lining up, f.i. ions 185, 350, and 436 m/z of T-2 and the retention time of T-2. Only if peaks in all three traces line up and have similar ion ratios as established from the calibration runs of the respective substance (f.i., 350/185 and 436/185 for T-2) is the peak positively identified. Only positively identified peaks shall be used for the determination of the concentration.

7.6. Calibration

Using the extracted ion currents for ions (m/z) 436 (T-2), 466 (HT-2), and 455 (ISTD) integrate the peaks representing the respective substances. Divide the peak areas of T-2 (A_{436}) or HT-2 (A_{466}) by the peak area of the ISTD (A_{455}).

$$R_{T-2} = A_{436}/A_{455} \text{ or } R_{HT-2} = A_{466}/A_{455} \quad \text{(I)}$$

The resulting area ratios (R_{T-2}, R_{HT-2}) of the four injections of the reagent blank and the 5 levels of calibration solutions are then plotted versus the corresponding amounts (4.11). The expected calibration plot has a slight curvature to it best described by a second degree polynomial. But since the quadratic term is small and for simplicity reasons a simple linear regression of the form

$$y = b_0 + b_1 x \quad \text{(II)}$$

is to be performed, with y being the area ratio for either T-2 or HT-2, x the respective amount of T-2 or HT-2, b_0 the intercept, and b_1 the slope. This is the calibration function.

8. DETERMINATION OF CONCENTRATIONS

Inverting the calibration function (7.5, II) yields the analysis functions:

$$x = \frac{y - b_0}{b_1} \quad \text{(III)}$$

Inserting the intercept (b_0) and slope (b_1) of the calibration function and the area ratios (y) for either T-2 or HT-2 (7.5, I) of the unknown samples yields the estimated amounts x for T-2 or HT-2, respectively.

Since the cleaned-up extract represents 0.5 g of the original sample multiplying by two yields the final concentration estimate $C_{T-2/HT-2}$ in µg/kg:

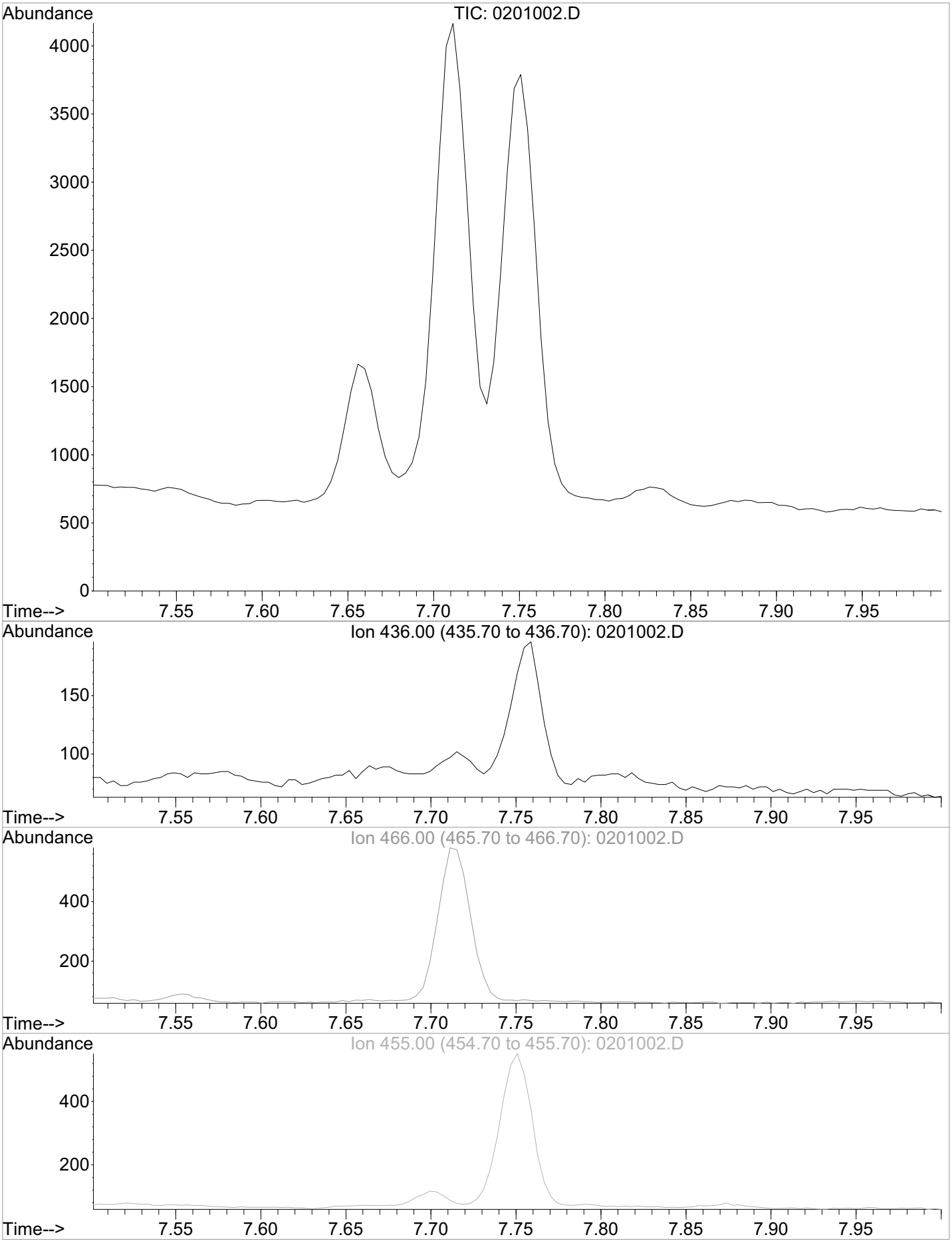
$$C_{T-2} = 2x_{T-2} \quad \text{(IV)}$$

$$C_{HT-2} = 2x_{HT-2} \quad \text{(V)}$$

9. Appendix A:

Example chromatograms

File :C:\MSDChem\1\DATA\2006\11 November\20061128\0201002.D
Operator : abr
Acquired : 28 Nov 2006 9:36 using AcqMethod T2HT2_10KMIN.M
Instrument : Instrument #1
Sample Name: Animal Feed
Misc Info :
Vial Number: 2



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Operator : abr
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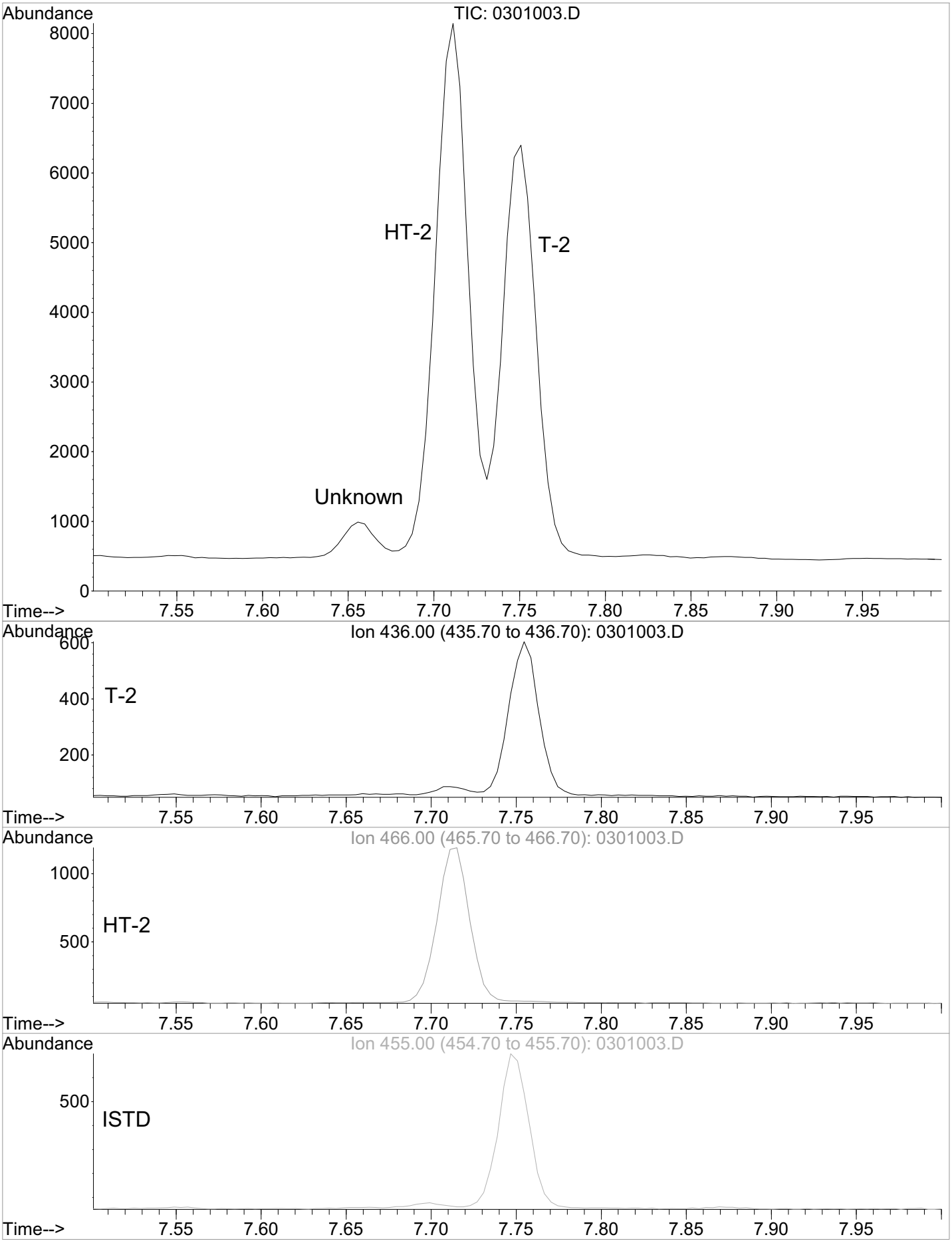
Instrument : Instrument #1
Sample Name: Calibration solution
Misc Info :
Vial Number: 3



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Annex IV:

Geel, 04. Dec, 2006
AB D(2006)

Spiking Protocol:

In order to calculate the recovery of the method you are supplied with 2 different vials containing 3 mL each of a mixed T-2/ HT-2 standard in acetonitrile

The vial marked with "Spike A solution" has to be used to spike the two samples marked "Spike A".

The vial marked with "Spike C solution" has to be used to spike the two samples marked "Spike C".

Prior to analysis shake all containers vigorously (e.g. with a vortex shaker).

*These containers are exclusively reserved for the spiking experiments and **MUST NOT** be used for direct analysis.*

After shaking the spike sample containers weigh, to the nearest 0.1 g, 25.0 g of the blank material into a clean flask and add exactly 1.0 mL of the respective spiking solution (4.12, e.i. "Spike A solution" for "Spike A" sample) using a 1000 µL Hamilton syringe (3.16). Dispense right onto the sample making sure that the spiking solution is evenly distributed across the sample. Do not dispense onto the glass surface of the flask. Gently shake the flask by hand to achieve some mixing. Leave the spiked test sample for at least 1 h before proceeding with the extraction as described in the protocol, section 5.1, paragraph 2.

Retieseweg 111, B-2440 Geel - Belgium. Telephone: (32-14) 571 211. http://irmm.jrc.ec.europa.eu
Telephone: direct line (32-14) 57 12 83. Fax: (32-14) 57 13 43.

E-mail: jrc-irmm-crl-mycotox@ec.europa.eu

Annex V:

T-2/ HT-2 collaborative trial GC/MS

Instructions :

Please carefully fill in all the requested information. If for a certain reason data is not available leave field empty and make a note in the "Remarks" column.

In the column "Sample Code" below enter the codes of the samples you have received.

In the columns "Mean Concentration" enter the average of the calculated concentration of the duplicate runs for either T-2 or HT-2 for each of the samples.

The column "Mean Concentration SUM" will then show the sum of both concentrations.

In the following four columns for the peak of T-2 enter the retention time (RT, 2 decimals), peak area (integer only) of ion 436 (Area 436), and ion ratios (3 decimals) of ions 350/185 and of ions 436/185, for the **first** injection of each injection solution .

In the next four columns for the peak of T-2 enter the respective data for the **second** injection of each injection solution.

In the next eight columns for the peak of HT-2 enter the respective data for both injections of each injection solution.

Finally, in the last six columns for the ISTD peak enter the respective information for both injections of each injection solution.

Fields for one full calibration are provided in this table. Also at the end fields for the optional in-house standard measurement are provided.

If more calibrations have been performed enter them in the "Additional calibrations" work sheet.

Laboratory:

Name of the participant

00X (code of the participant)

Results table:

				T-2								HT-2								ISTD						
				Run 1				Run 2				Run 1				Run 2				Run 1			Run 2			
Sample Code	Mean Concentration T-2	Mean Concentration HT-2	Mean Concentration SUM	RT	Area 436	Ratio 350/185	Ratio 436/185	RT	Area 436	Ratio 350/185	Ratio 436/185	RT	Area 466	Ratio 347/185	Ratio 466/185	RT	Area 466	Ratio 347/185	Ratio 466/185	RT	Area 455	Ratio 455/365	RT	Area 455	Ratio 455/365	Remarks
			0																							
			0																							
			0																							
			0																							
			0																							
			0																							
			0																							
			0																							
			0																							
			0																							
			0																							
			0																							
Spike A1			0																							
Spike A2			0																							
Spike B1			0																							
Spike B2			0																							
Spike C1			0																							
Spike C2			0																							
Reagent Blank/Prep 1			0																							
Reagent Blank/Prep 2			0																							
Cal 1/Prep 1	1	2	3																							
Cal 1/Prep 2	1	2	3																							
Cal 2/Prep 1	5	10	15																							
Cal 2/Prep 2	5	10	15																							
Cal 3/Prep 1	20	40	60																							
Cal 3/Prep 2	20	40	60																							
Cal 4/Prep 1	35	70	105																							
Cal 4/Prep 2	35	70	105																							
Cal 5/Prep 1	50	100	150																							
Cal 5/Prep 2	50	100	150																							
In-House Standard/Prep 1	25	50	75																							
In-House Standard/Prep 2	25	50	75																							
In-House Standard/Prep 3	25	50	75																							

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Mycotoxins

Annex VI:

Geel, 04. Dec, 2006
AB D(2006)

Results Questionnaire for the T-2/HT-2 Collaborative Study

Name of the participant **00X (code of the participant)**

PLEASE ANSWER THESE QUESTIONS AND USING THE SUBJECT LINE "T-2/ HT-2 trial" SEND THEM TO:

CRL on Mycotoxins

European Commission

DG Joint Research Centre

Institute for Reference Materials and Measurements

Retieseweg 111, B-2440 Geel, Belgium

Fax No: **+32-14-571343**

Email: JRC-IRMM-CRL-MYCOTOX@ec.europa.eu

AFTER YOU HAVE SUBMITTED YOUR RESULTS.

General comments on the method:

1. Did you already use a similar method for the determination of T-2/ HT-2 including immunoaffinity clean-up or solid phase extraction and GC/MS detection?

   ☐ ***Yes*** ☐ ***No***

   If yes, please state source:

2. Have you been familiar with the procedures used in this method?

   ☐ ***Yes*** ☐ ***No***

   If no, please specify briefly which procedures were unfamiliar to you:

3. Was the method description adequate?

   ☐ ***Yes*** ☐ ***No***

4. What criticisms and/or suggestions could you make concerning the method and/or its performance? (Please attach additional sheets if necessary).

Specific remarks about execution of the method:

5. What type of shaker was used for the initial extraction of materials?

   ☐ ***Side-to-side*** ☐ ***Wrist action*** ☐ ***Rotational***

   ☐ ***Other please specify:***

   Type/Manufacturer:

6. Did you encounter any problems during filtration (e.g. low flow rate)?

   ☐ ***Yes*** ☐ ***No***

   If yes, please state the exact problem and for which sample(s) this occurred (include codes):

7. What procedure was used for sample application to the IAC?

   ☐ ***Gravity*** ☐ ***Vacuum***

   ☐ ***Other please specify:***

8. Did you encounter problems during application of the extract to the IAC (e.g. slow flow rate, discolouration of immunoaffinity gel)?

   ☐ ***Yes*** ☐ ***No***

   If yes, please state the exact problem and for which sample(s) this occurred (include codes).

9. What was the average run time for sample application to the IAC

   _____ minutes

10. Did you encounter any problems during evaporation?

    ☐ ***Yes*** ☐ ***No***

    If yes, please state the exact problem and for which sample(s) this occurred (include codes):

11. What evaporation system was used?

    ☐ ***Vacuum centrifuge*** ☐ ***Heating block with N_2***

    ☐ ***Other please specify:***

12. How long were the samples dried for?

    _____ minutes

13. Specify manufacturer and model of the gas chromatography system used:

14. Specify manufacturer and model of the mass spectrometer used:

15. Specify specifications (Manufacturer, Name, Dimensions) of the GC column used:

16. Specify GC parameters:

    Injection volume in μL:

    Injector temperature [ºC]:

    Injection mode (split, splitless, or pulsed modes thereof):

    Temperature program [ºC]:

    Transfer line temperature [ºC]:

    Head pressure (if constant pressure mode) or flow rate (if constant flow):

    Carrier gas:

17. Specify MS parameters (if applicable):

    Ion source temperature [ºC]:

    Ionization energy [eV]:

    Scan speed [scans/s]:

18. Specify whether peaks were integrated valley-to-valley or using a horizontal baseline:

    ☐ ***Valley-to-Valley*** ☐ ***Horizontal baseline***

19. If manual integration was used, specify the samples and peaks affected and the reasons why:

20. Was any data smoothing applied before integration?

☐ ***Yes*** ☐ ***No***

If yes, please specify name of the algorithm:

21. Describe any deviation from the protocol other than what has been mentioned above:

Please supply copies of all chromatograms obtained (similar layout as the chromatograms in the appendix of the method protocol) and detailed calculations.

Thank you for completing this questionnaire.

European Commission

EUR 23560 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Validation of an Analytical Method to Determine the Content of T-2 and HT-2 toxinx in Cereals and Compound Animal Feed by Immunoaffinity Column Clean-up and GC-MS
Author(s): A. Breidbach, V. Povilaityte, C. Mischke, I. Doncheva, H. van Egmond, J. Stroka
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Abstract
A method for the determination of T-2 toxin and HT-2 toxin in cereals and compound animal feed was validated by collaborative study.

In short, the method is as follows: A test portion of a sample is extracted with a mixture of methanol/water (80/20, v/v). This raw extract is then diluted, filtered, and applied to an immunoaffinity column. After washing and elution with acetonitrile the eluate is evaporated to dryness. T-2 and HT-2 toxins in the dry residue are then derivatised with N-methyl-N-trimethylsilyl-trifluoroacetamid (MSTFA)/ trimethylchlorosilane (TMCS) (99/1, v/v), injected into a gas chromatograph, and detected and quantified by mass spectrometry.

Fourteen laboratories from ten different countries were selected to participate in the collaborative study. They received six different test materials as blind duplicates. The test materials consisted of a blank cereal mix, two cereal mixes naturally contaminated with T-2 and HT-2 toxins at different levels, a blank compound animal feed, and two compound animal feeds naturally contaminated with T-2 and HT-2 toxins at different levels. Furthermore, two blank cereal mixes and two blank compound animal feeds together with specific spiking solutions were provided for recovery determination. The sum of the mass fractions of T-2 & HT-2 toxins after spiking were 50 µg/kg in the cereal mix, and 75 µg/kg in the compound animal feed.

Reported apparent recoveries in the cereal mix ranged from 59 to 143% for the sum of T-2 & HT-2 toxins with a mean value of 105%. Reproducibility relative standard deviations (RSD_R) for the cereal matrix were 30% at a natural contamination level of ca. 25 µg/kg, 21% at ca. 87 µg/kg, and 27% for the spiked material at 50 µg/kg. For compound animal feed the recovery values ranged from 87 to 145% with 113% for the mean value. The RSD_R values were 25% at a natural contamination level of ca. 92 µg/kg, 19% at ca. 125 µg/kg, and 16% for the spiked material at 75 µg/kg. The Horwitz ratios (HorRat) ranged from 0.7 to 1.4.

European Commission Regulation 401/2006 [1] lays down method performance criteria for the control of foodstuffs only. Even though the validated method applies to cereals and compound feed it meets all of those criteria. Therefore it is suited for official feed control.

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