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LC-MS Based Method of Analysis for the Simultaneous Determination of four Mycotoxins in Cereals and Feed

Results of a Collaborative Study

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LC-MS/MS based method of analysis for the simultaneous determination of deoxynivalenol, HT-2 toxin, T-2 toxin, and zearalenone in unprocessed cereals and cereal-based compound animal feeds

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A. Breidbach, K. Bouten, K. Kröger-Negiota, J. Stroka, F. Ulberth

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EXECUTIVE SUMMARY

An LC-MS/MS based method of analysis to determine the four Fusarium toxins deoxynivalenol, HT-2 toxin, T-2 toxin, and zearalenone in cereals and cereal-based compound animal feed has been validated through a collaborative study. After extraction of the mycotoxins with ethyl acetate / water, and addition of sodium sulphate an aliquot of the organic phase was spiked with stable-isotope labelled isotopologues of the targeted analytes and dried down. The dry extract was then reconstituted with mobile phase and injected into a LC-MS. The described use of the isotopologues keeps costs down while still offering many of their benefits. This is evidenced by relative repeatability standard deviations (RSD_r) between 5 and 15 %. Exceptions were T-2 toxin at 7 µg/kg with 27%, and at 3.5 µg/kg with 35%, and zearalenone at 3.4 µg/kg with 32% RSD_r.

The tested contamination ranges were 88 to 559 µg/kg for deoxynivalenol, 22 to 178 µg/kg for HT-2 toxin, 3.5 to 50 µg/kg for T-2 toxin, and 3.4 to 430 µg/kg for zearalenone. For 10 of the 20 analyte / matrix combinations (four analytes in five matrices) Horwitz ratios between 0.6 and 0.9 were computed, for another six the ratios were below 1.5. The remaining four test samples were associated with Horwitz ratios between 2.0 and 4.4. They were the samples described above, two containing T-2 toxin and one zearalenone, plus one complex matrix sample containing zearalenone at a low contamination level. For this complex matrix sample we were able to show the importance of proper separation in LC-MS.

Because of the use of test materials having assigned reference values in this study trueness could be assessed. The observed biases were small and only significant for deoxynivalenol (-8%) and HT-2 toxin (-11%). For T-2 toxin and zearalenone they were insignificant. To facilitate the checking of compliance of a test result produced with this method with legislation a description on how to estimate measurement uncertainty based on these results is provided.

All of the above shows that the studied method is fit for the purpose of enforcing existing and anticipated legislative limits of the four Fusarium toxins deoxynivalenol, HT-2 toxin, T-2 toxin, and zearalenone in unprocessed cereals and cereal-based compound animal feed.

1. INTRODUCTION:

The accurate determination of mycotoxins in food and feed matrices for which EU legislative limits apply requires robust and reliable analytical techniques. Robustness and reliability are best shown through validation by a collaborative study. An area for which results of collaborative studies are lacking are methods of analysis for mycotoxins in food/ feed involving LC-MS techniques. While there are a large number of published LC-MS methods available those methods are, if anything, single-laboratory validated. The proof that LC-MS is actually capable of delivering fit-for-purpose results in the mycotoxin arena still needs to be shown.

The rationale behind this method of analysis was to have an easy-to-apply protocol for enforcement of legislative limits for unprocessed cereals and recommended limits for compound animal feed for most of the regulated or soon-to-be regulated Fusarium toxins. Therefore, compromises were made with regards to limit of detection and quantification. Lower limits of detection and of quantification are achievable but are not necessary for the purpose of this method

During the time the method was developed there was a shortage in production of acetonitrile which affected availability and prizes. For this and other reasons, other extraction solvent systems were tested. A binary system with ethyl acetate / water led to good extraction yields and lesser matrix effects than other more commonly used systems. A large solvent-to-sample ratio without subsequent concentration was sufficient for an adequate working range because of the sensitivity of selected reaction monitoring (SRM) with triple quadrupole mass spectrometers and of the latest generation of high mass-accuracy single MSs.

We forwent a clean-up of the extract as a possible source of error and instead focused on proper LC separation and control of possible matrix effects through the use of stable-isotope labelled isotopologues of the analytes. For the recommended mobile phase methanol was chosen as organic modifier and formic acid at 0.1% as additive to keep the mobile phase as generic as possible.

Test portion sizes of only 2 g are believed to be not large enough to avoid erroneous results due to sample inhomogeneities. With proper physical test material preparation (milling and mixing) this is not the case. The additional effort needed to mill the material to particle sizes < 500 µm and mixing it to homogeneity is, from our point-of-view, small against the benefits of saving large volumes of organic solvents.

We also realize that reconstituting dried down extracts containing T-2 toxin and/or zearalenone necessitates a high organic solvent content and injection solutions with high organic content might lead to peak broadening for early eluting analytes. No peak broadening of deoxynivalenol was observed in our set-up because the aforementioned sensitivity of the MSs allows, and the use of small particle-size analytical columns requires, small injection volumes.

2. METHOD DESCRIPTION

The full method protocol can be found in Annex A. Following is a brief description: Two gram of finely ground and homogeneous test material is suspended in 8 mL water. After addition of 16.0 mL ethyl acetate the sample is agitated for 30 min. Then sodium sulphate is added to facilitate phase separation and after 10 to 20 min the sample is centrifuged to pellet particulate matter at the bottom of the extraction tube. The organic phase is transferred to a clean vial for possible storage. 500 µL of the organic phase, an equivalent of one-sixteenth of a gram of the test portion, are mixed with stable isotopologues of the analytes and evaporated to dryness in deactivated glass vials. Adding the isotopologues to an aliquot of the extract is a compromise between accuracy requirements and acceptable costs. After reconstitution of the dry extract with 250 µL of organic mobile phase modifier, addition of 250 µL of water, and thorough mixing the analytes are quantified with a LC-MS system.

3. LAYOUT OF THE COLLABORATIVE STUDY

This collaborative study was planned according to guidelines of the AOAC Official Methods Program [1]. In particular, this means that five different materials had to be measured as blind duplicates representing a mix of different cereals with or without addition of soy, rape, and other components found in feed. Contrary to traditional study designs, where spiking of a material with known amounts of analytes is applied for recovery determination, assessment of trueness was done by assigning reference values to two of the materials by isotope dilution mass spectrometry.

Twenty-three laboratories of 13 Member States of the EU and USA and Canada were invited to participate (see Annex C for detail). Each laboratory received a box containing:

- Ten containers with ready-to-be-extracted test materials identified by a four digit code (blind duplicates of five materials)
- A vial with 1 mL of a multi-mycotoxin reference standard stock solution
- A vial with 1 mL of an isotopically labeled multi-mycotoxin internal standard (ISTD) stock solution
- 20 deactivated glass vials
- Method protocol (see Annex A)

These boxes were dispatched on 06.10.2011 with a courier service. No provisions for cooling were made.

Next to this box each invited laboratory received as email attachment the following documents in PDF format:

- Invitation letter with instructions
- Results reporting form

- Questionnaire regarding laboratory experience, employed equipment, and execution of analysis

- Materials receipt form

The reporting dead line was fixed to 02.12. 2011.

4. PREPARATION OF TEST MATERIALS

Three of the five test materials used in this study were prepared by an external provider (EFL1, EFL2, EFL3). The materials were provided milled to a particle size < 500 µm, homogenized, and packaged in clear polypropylene containers with screw caps. The other two materials were prepared at IRMM (IRMMFEED, IRMMCER). Particle size was also < 500 µm with additionally longer fibers in the cereal material (IRMMCER) because of the oat husks. Table 1 details the composition of the five materials.

Test Material	Constituents (%)
EFL1	Oat (6), Rye (12), Feed mix (10), Maize (23), Soya (20), Rice (29)
EFL2	Rye (25), Wheat (17), Maize (17), Oat (8), Rice (33)
EFL3	Soya (16), Sugar beet (8), Maize gluten (18), Bean (8), Rice (24), Oat (26)
IRMMFEED	Feed mix Horse (50; oat, barley, wheat), Feed mix Rabbit (25; wheat, alfalfa, sunflower seeds), Feed mix Chicken (25)
IRMMCER	Oat with husk (40), Maize (50), Wheat (10)

Table 1: Composition of the five test materials, in parentheses the percent content and, for mixes, the declared constituents (if known).

5. STATISTICAL ANALYSIS

To verify consistency of the reported data Mandel's h statistic [2], describing between-laboratory consistency, was computed and plotted per analyte for all materials and reporting laboratories. Laboratories with a consistent bias were excluded from further evaluation.

Robust statistical methods, as described in ISO 5725 Part 5 [3], were used to avoid the need to exclude individual "outlying" results for the estimation of repeatability and reproducibility. In particular, "Algorithm S" ([3], p. 36) was used to obtain a robust estimate of the standard deviation s^* of the differences between the blind duplicates per material and "Algorithm A" ([3], p. 35) to obtain a robust estimate of the standard deviation s_d of the averages of the blind duplicates per material.

The repeatability standard deviation s_r for duplicate measurements can then be calculated as:

$$s_r = s^* / \sqrt{2} \qquad (1)$$

The between-laboratory standard deviation s_L is derived from s_r and s_d:

$$s_L = \sqrt{s_d^2 - (s_r^2 / 2)} \qquad (2)$$

If the expression under the square root is negative s_L will be assigned a value of zero. Knowing s_L and s_r the reproducibility standard deviation s_R is calculated as:

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

Relative standard deviations (RSD) were calculated as:

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

Repeatability and reproducibility limits, which describe the maximum difference between two results obtained under the specified test conditions that can be attributed to method precision with a probability of 95%, were calculated by multiplying the respective standard deviation with 2.8:

$$r = 2 * \sqrt{2} * s_r = 2.8 s_r \qquad (5)$$

$$R = 2 * \sqrt{2} * s_R = 2.8 s_R \qquad (6)$$

The repeatability and reproducibility standard deviations can be expressed as functions of the mass fraction *w* acc. to ISO 5725 Part 2 [2]. For the data from this study a first order model with fixed term showed to be sufficiently accurate:

$$\hat{s}_{r,i} = a_{r,i} + b_{r,i} w_i \qquad (7)$$

$$\hat{s}_{R,i} = a_{R,i} + b_{R,i} w_i \qquad (8)$$

with $a_{r,i}$, $a_{R,i}$ being fixed contributions to the repeatability and reproducibility standard deviation, respectively, and $b_{r,i}$, $b_{R,i}$ being coefficients, representing relative repeatability and reproducibility standard deviation respectively, for the different analytes *i*.

For comparison, traditional statistics with outlier removal was also performed as described in ISO 5725 Part 2 [2]. Student's t-test was used to determine significances of differences between means.

To assess the trueness of the method the overall mean obtained from the participants results for the RMs was compared with their assigned values according to ISO 5725 Part 4 [5]. To that end the bias $\hat{\delta}$ was estimated as:

$$\hat{\delta} = \overset{=}{y} - \mu \qquad (9)$$

where $\bar{\bar{y}}$ is the overall mean of the material reported by the participants and μ is the assigned value. The standard deviation of the bias is then calculated as:

$$s_{\hat{\delta}} = \sqrt{\frac{s_R^2 - (1 - 1/n)s_r^2}{p}} \qquad (10)$$

where p is the number of laboratories and n the number of replicates per laboratory.

An approximate 95% confidence interval for the bias is calculated as As_R and if this interval covers the value zero the bias of the method is insignificant. The factor A is calculated as:

$$A = 1.96\sqrt{\frac{n(\gamma^2 - 1) + 1}{\gamma^2 pn}} \qquad (11)$$

with

$$\gamma = \frac{s_R}{s_r} \qquad (12)$$

All calculations were performed with “R” [4], a language and environment for statistical computing.

6. IN-HOUSE METHOD PERFORMANCE

The method was developed with ease of execution and low cost of operation in mind. Its intended purpose was to be applicable for the determination of deoxynivalenol (DON) in the range from 200 µg/kg to 2560 µg/kg, HT-2 toxin (HT2) in the range from 25 µg/kg to 400 µg/kg, T-2 toxin (T2) in the range from 15 µg/kg to 240 µg/kg, and zearalenone (ZON) in the range from 50 µg/kg to 240 µg/kg in unprocessed cereals and compound animal feed.

Validation of the method was done as follows: Unprocessed, finely-ground rice, wheat, maize, and oat and in addition unprocessed, finely-ground soy and a mix of all the before were used as test materials. All these materials were essentially free of the four analytes of interest except for a very low contamination of the oat material with HT2 and T2. Furthermore, three materials (EFL1, EFL2, EFL3) were tested which were naturally contaminated with the four analytes.

Each test material was prepared as is and after spiking with 25, 75, 490, and 800 µL of the multitoxin stock solution per g of material according to the spiking procedure (Sec. 6.4. Annex A). To determine possible matrix effects the calibration solutions were also prepared with blank raw extracts of the different materials. Repeatability was determined by preparing the three naturally contaminated materials 20 times each according to the method protocol. Two of the naturally contaminated materials, EFL1 and EFL3, were prepared by three different operators to assess intermediate precision. Operator 1 prepared the two materials on days 1, 2, and 6, operator 2 on days 3, 8, 9, and 21, and operator 3 on day 17. On each day new calibration solutions were prepared.

Robustness of the method was determined through a 11 factor, 12 run Plackett-Burman factorial design with the following factors: Sample weight, Volume Water, Volume Ethyl acetate, Mode of agitation, Duration of agitation, Amount of salt, Wait time after salt addition, Centrifuge time, Glass vials (deactivated/ non-deactivated), Reconstitution volume of methanol, Reconstitution volume of water. Each factor was varied at two levels of about 10% above and below the initial values. Next to the robustness test the stability of the raw extracts and the injection solutions were tested. Raw extracts of the two QC levels and a spiked material were stored in the dark at 2 – 10 ºC for several days and measured repeatedly. The same was done for injection solutions.

Within the stated ranges the method showed a linear correlation between signal and tested concentration, it proved to be selective, and due to the use of isotopologues as ISTDs matrix effects were negligible. Relative repeatability standard deviations within the working range were between 4 and 10 %, relative intermediate precision between 11 and 25 %. Recovery was only significantly different from 1 for DON with 0.83.

The robustness test showed only significant effects for sample weight and volume of ethyl acetate. As long as these two factors are well controlled the method is not sensitive to small changes in all other tested parameters except for ZON. There an effect is seen and care should be taken to follow the method protocol closely. Raw extract and injection solutions are stable at 2-10 °C for up to 7 days.

7. VERIFICATION OF SUFFICIENT TEST MATERIAL HOMOGENEITY

Sufficient homogeneity of the test materials was verified according to Thompson [6]. Even though that procedure is aimed at Proficiency Tests it is appropriate for method validation studies in the area of mycotoxins in food and feed as well. Reason is that method performance will be judged based on prescribed criteria thus there is a "target standard deviation" as in a PT.

To verify homogeneity of the test materials 10 units per material EFL1, EFL2, and EFL3 were selected at random For the other two materials IRMMCER and IRMMFEED only 6 and 7 units, respectively, were selected which represented approx. 10% of the total number of units because of a limited number of total units available. Two independent determinations were performed per unit with the method under investigation. The measurement batch order was randomized. Sufficient homogeneity was assumed if the between-unit variance (s^2_{sam}) was smaller than a critical factor c ([6], Sec 3.11.2, P 171).

The between-unit variance (s^2_{sam}) and the within-unit variance (s^2_{an}) were obtained from one-way analysis of variance (ANOVA). The allowable variance (σ^2_{all}) was calculated as $(0.3*\sigma_p)^2$ from the Horwitz equation modified by Thompson [7]. Table 2 lists the details of the homogeneity testing results of the five materials. For all materials the between-unit variance (s^2_{sam}) was smaller than the critical factor c and, therefore, sufficient homogeneity was assumed.

Material	Analyte	s^2_{sam}	s^2_{an}	σ^2_{all}	N	c
EFL1	**DON**	36.8	44.1	23.3	10	88.4
	HT-2	0	11.7	3.2	10	17.9
	T-2	0.262	1.52	0.213	10	1.93
	ZON	0.262	1.10	0.135	10	1.36
EFL2	**DON**	11.8	31.8	45.2	10	117
	HT-2	0.964	1.34	3.14	10	7.26
	T-2	0	1.48	0.188	10	1.85
	ZON	0	1.36	1.02	10	3.29
EFL3	**DON**	805	421	543	10	1450
	HT-2	21.2	131	117	10	354
	T-2	0.282	10.6	8.84	10	27.3
	ZON	626	336	669	10	1600
IRMMCER	**DON**	120	30.9	76.4	6	221
	HT-2	0	112	11.8	6	215
	T-2	1.90	3.23	0.257	6	6.02
	ZON	0.151	0.226	0.0258	6	0.439
IRMMFEED	**DON**	0	1005	239	7	1940
	HT-2	12.8	8.38	2.25	7	16.7
	T-2	0	0.611	0.0797	7	1.04
	ZON	0.245	6.41	1.36	7	12

Table 2: Results of the homogeneity test of the five test materials; s^2_{sam} – between-unit variance, s^2_{an} – analytical or within-unit variance, σ^2_{all} – allowable variance, N – number of units tested, c – critical value

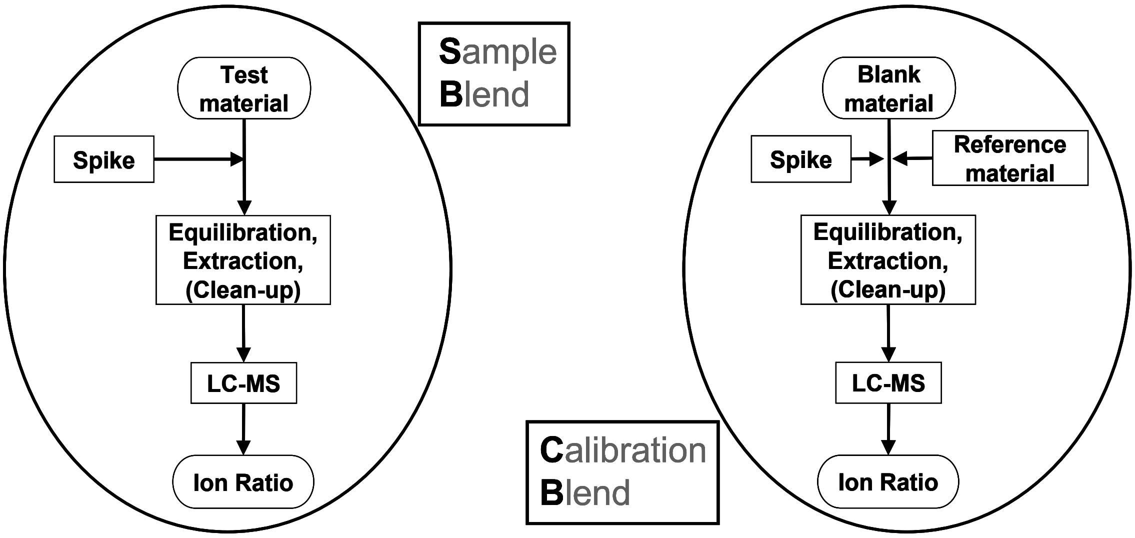

Figure 1: Depiction of the process of exact-matching, double isotope dilution mass spectrometry

8. ASSIGNED VALUES FOR SELECTED MATERIALS

Contrary to traditional collaborative study design bias was not estimated via spike recovery but reference materials with an assigned value were used instead.

Therefore, this study is able to provide a direct measure of the trueness of the studied method.

To this end the two materials EFL2 and EFL3 were characterized in our laboratory using Exact-Matching Double Isotope Dilution Mass Spectrometry (EMD-IDMS). Figure 1 depicts the flow scheme of this process. The "spike" is an isotopologue of the analyte. Two blends, sample blend (SB) and calibration blend (CB), are prepared and measured in sequence multiple times. The overall ratio of the isotope ratios in SB and CB is calculated from these measurements. If it is not close to unity, the process is repeated with new spike amounts until exact matching is achieved. Once the spike amounts for exact-matching have been determined several test portions are prepared with these amounts.

Calculation of the assigned values and their uncertainties

Since there were no significant signals of the isotopologues in the reference standards of the targeted analytes or test materials, and likewise no significant signals of the analytes in the spike solutions, which were completely ^{13}C labelled isotopologues, the following simplified model equation was used:

$$w_{s,i} = w_{c,i} \times \overline{R} \times \frac{m_{c,i}}{m_{ISTD,CB}} \times \frac{m_{ISTD,SB}}{m_{smp,i}} \qquad (13)$$

with

$w_{s,i}$ = mass fraction of analyte in test portion

$w_{c,i}$ = mass fraction of analyte in reference solution

$m_{c,i}$ = mass of reference solution added to calibration blend (CB)

$m_{ISTD,CB}$ = mass of the spike added to CB

$m_{ISTD,SB}$ = mass of the spike added to sample blend (SB)

$m_{smp,i}$ = mass of test portion

$\overline{R}$ = Mean ion ratio SB over CB

The combined uncertainty of $w_{s,i}$ is then given by:

$$\left(\frac{u(w_{s,i})}{w_{s,i}}\right)^2 = \left(\frac{u(w_{c,i})}{w_{c,i}}\right)^2 + \left(\frac{u(m_{c,i})}{m_{c,i}}\right)^2 + \left(\frac{u(m_{ISTD,SB})}{m_{ISTD,SB}}\right)^2 + \left(\frac{u(m_{ISTD,CB})}{m_{ISTD,CB}}\right)^2 + \left(\frac{u(m_{smp,i})}{m_{smp,i}}\right)^2 + \left(\frac{u(\overline{R})}{\overline{R}}\right)^2 \qquad (14)$$

The assigned value x_a was calculated as the average of all $w_{s,i}$ of the six preparations per test material:

$$x_a = \overline{w}_{s,i} \times F_u \tag{15}$$

with F_u being a factor of 1 representing the uncertainties of the individual $\overline{w}_{s,i}$. The combined uncertainty of x_a is then expressed by Eq. 16:

$$u_c(x_a) = x_a \sqrt{\left(\frac{u(\overline{w}_{s,i})}{\overline{w}_{s,i}}\right)^2 + \left(\frac{u(F_u)}{F_u}\right)^2} \tag{16}$$

where $u(\overline{w}_{s,i})$ = the standard error of the mean of $\overline{w}_{s,i}$ and $u(F_u)$ = the mean of all $u_{c,i}(w_{s,i})/ w_{s,i}$ per test material.

The assigned values of the two test materials are summarized in Table 3:

Analyte	**Assigned value x_a**	**Expanded uncertainty U (k=2)**
EFL2		
DON	282	26
HT-2	51	5
T-2	18	2
ZON	28	4
EFL3		
DON	605	49
HT-2	201	13
T-2	52	3
ZON	445	16

Table 3: Assigned values of the four analytes in two materials EFL2 and EFL3

For the full uncertainty budget see Annex B.

9. PILOT STUDY

To test the suitability of the method protocol a small scale pilot study was executed before the actual study. To that end material EFL1 was send to five laboratories and the laboratories were asked to measure five independent preparations of this material. Table 4 lists the results. All five laboratories (see Table C7 for details) reported that the method protocol was adequate. Based on the outcome of this pilot the execution of a full scale study was seen as feasible.

LABID	w_{DON}	s_{DON}	w_{HT2}	s_{HT2}	w_{T2}	s_{T2}	w_{ZON}	s_{ZON}	COMPLIANT
P1	98.1	3.33	48.6	10.71	19.2	5.54			NO
P2	89.6	5.75	41.4	4.06	20.8	1.96	14.1	2.04	YES
P3	69.4	6.32	26.0	3.80	11.2	2.73	4.8	1.14	YES
P4	80.2	7.35	39.3	1.74	16.6	1.84	13.0	0.80	YES
P5	80.3	4.68	47.8	2.93	18.7	2.19	17.5	1.63	NO
Overall	**83.5**	**5.49**	**40.6**	**4.65**	**17.3**	**2.85**	**12.4**	**1.40**	

Table 4: Results of the pilot study; w_{DON} – mean mass fraction of DON of five determinations, s_{DON} – standard deviation of mass fractions of DON (the results of he other analytes are indicated by the indices), COMPLIANT – were requirements of chromatographic resolution met?

10. RESULTS & DISCUSSION

Questionnaire and Compliance

All 23 invited laboratories received a questionnaire about their experience and how the analysis was performed. The filled-in questionnaire was returned by 21 of the 23 laboratories. Evaluation of the answers shows that of the 21 laboratories reporting three did not “perform mycotoxin analysis by LC-MS prior to this study” and of the ones with prior experience the vast majority (16) did so for more than 12 months. The question “Was the description of the method adequate?” was answered with yes by 19 laboratories (90%). Other questions dealt with the equipment used prior to and during the study, and with details of the LC and MS settings.

Important for us was question 6: “Did you at any step deviate from the method protocol sections 4.20, 5.12, or 6?”. Section 4.20 of the method protocol relates to the calibration, 5.12 to the instrument requirements, and 6 to the procedures for sample extraction and test solution preparation. Sections 6.1. “Sample preparation” and 6.4. “Spiking procedure” did have no bearing for this study and were in the protocol for future reference. Question 6 was answered with “Yes” by seven laboratories: Laboratory 2 reported to not have used deactivated vials, Laboratory 7 reported to have reconstituted the dried down extract with 100 µL organic and 400 µL aqueous solvent, Laboratory 9 reported it had added three more calibration points (4.20), Laboratory 11 reported to have not met the resolution requirement (5.12.3), Laboratory 17 reported to have reconstituted the dried down extract with 50 µL organic and 450 µL aqueous solvent and to have not met the resolution requirement (5.12.3), and Laboratories 18 + 20 reported to have not met the resolution requirement (5.12.3).

The deviation of Laboratory 9 was seen as acceptable since a note to clause 4.20. states that the number of levels can be adjusted to one’s needs. Four laboratories (11, 17, 18, 20) pointed out that the resolution of their separation was smaller than the prescribed value. Evaluation of chromatograms of calibration level 6 from all laboratories showed that far more laboratories did not meet this requirement, namely laboratories 1, 4, 6, 11, 12, 13, 18, and 20. Three more laboratories (9: plate number; 17, 21: minimum retention) did not meet clause 5.12.3. This did not lead to exclusion from the evaluation phase. The deviations of Laboratory 2 (deactivated vials) and Laboratories 7 + 17 (reconstitution) were at crucial steps in the method and seen as significant enough to justify exclusion from the evaluation phase for non-compliance.

Data Consistency

Of the 23 invited laboratories 21 reported results for the test materials sent to them. One of the two remaining laboratories did not report because of instrument problems. The other laboratory never submitted a report and did not reply to emails anymore. In Annex B the reported results of the four analytes per material of the 21 laboratories are listed. Before commencement of the evaluation the submitted data (measurements and questionnaire) were checked for consistency.

Plots of Mandel's h statistic were used to check for inconsistencies in the reported data (Annex D, Figures D 1 to D 4). Three laboratories are sticking out: Laboratory 3 reported consistently low for all analytes and all materials; Laboratory 13 did not report results for DON in any of the five materials which raised doubts about their detection capability; Laboratory 18 had significant high scores for three of the four analytes in material IRMMCER. On request (Email 21.12.2011) Laboratory 3 recalculated and confirmed its reported results. Figure 2 depicts the significant consistent bias with 11 out of 20 reported values falling beyond the 1% error limit. Figure 3 shows the situation for Lab 13 which on request was not able to provide data for DON (Email 22.12.2011). The high scores for IRMMCER for Lab 18 (Figure 4) appeared to be a problem with addition of the ISTD to the sample 1123 (Email 08.03.2012). Therefore, as a whole, Laboratories 3 and 13, and the IRMMCER results of Laboratory 18 were excluded from the evaluation phase (see also Table 5).

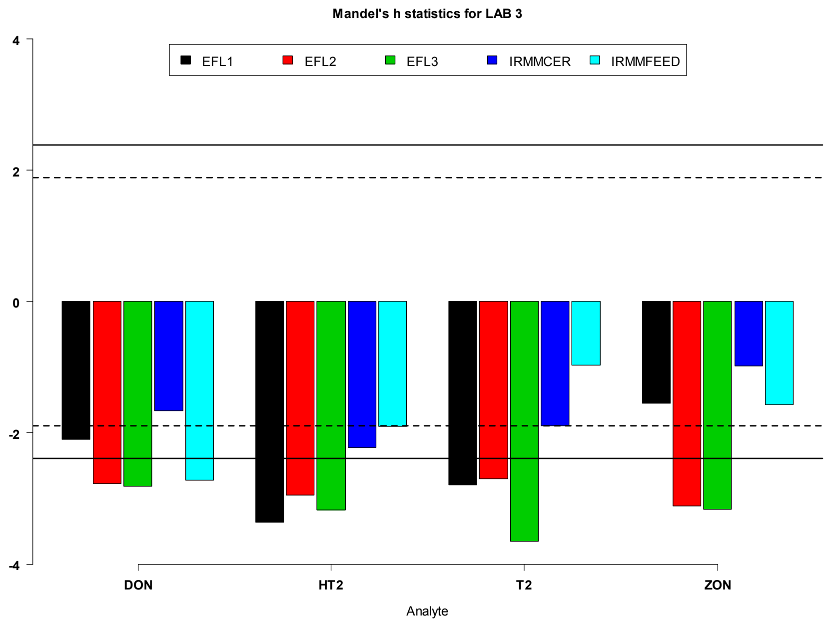

Figure 2: Mandel's h statistic for Lab 3 for all five materials grouped by analyte; solid line – 1% error probability, broken line – 5% error probability.

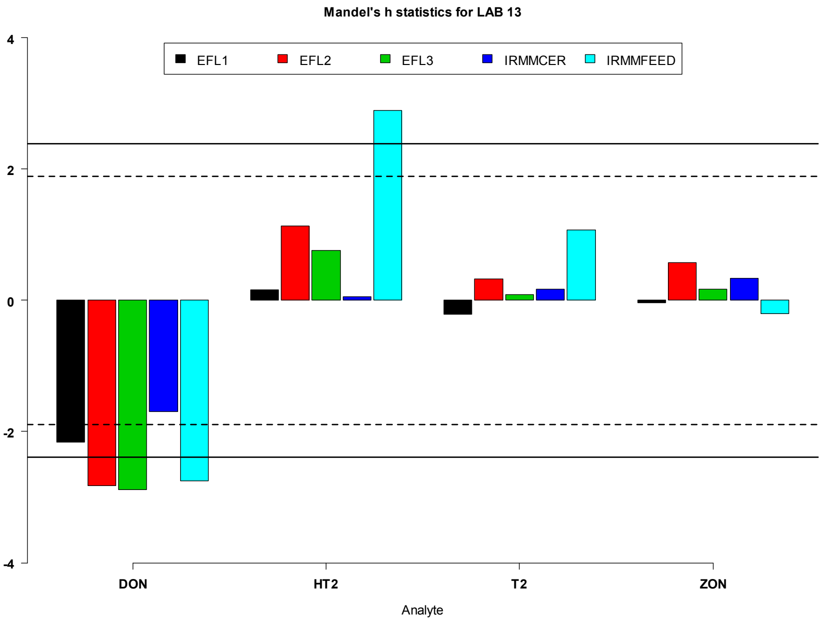

Figure 3: Mandel's h statistic for Lab 13 for all five materials grouped by analyte; solid line – 1% error probability, broken line – 5% error probability.

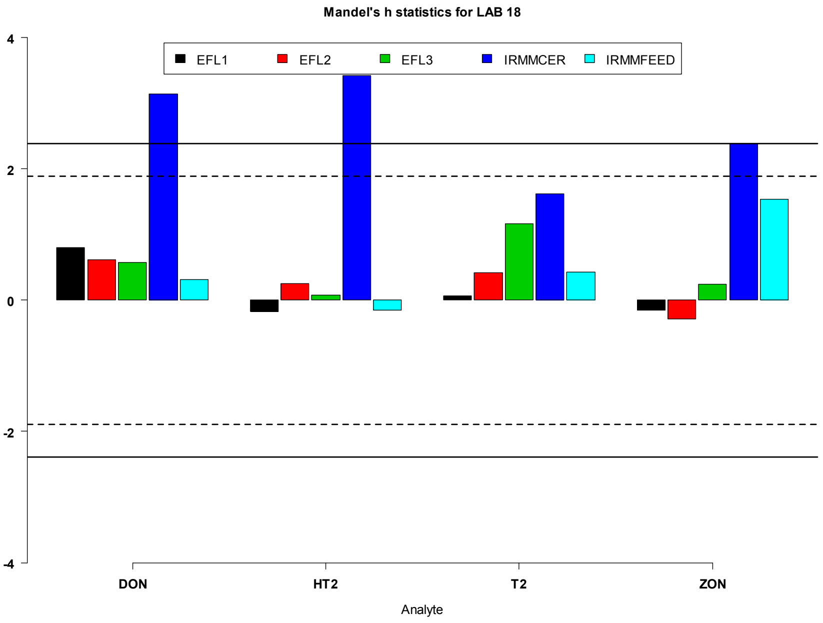

Figure 4: Mandel's h statistic for Lab 18 for all five materials grouped by analyte; solid line – 1% error probability, broken line – 5% error probability.

Statistical Evaluation

The data of the remaining 16 laboratories (15 for IRMMCER) were submitted to robust statistical methodology to determine overall means, repeatabilities, and reproducibilities as described above. Table 6 lists the results of this evaluation (see Annex E for the results of an evaluation applying parametric statistics). With the exception of T-2 toxin and ZON in IRMMCER and IRMMFEED the Horwitz ratios, an indicator of acceptable method performance if within the range of 0.5 to 1.5, tended to be near unity. This is also true for ZON in EFL3 whose contamination level was 1.8 times the highest calibration level. Even without diluting the injection solution to within the calibration range a reliable result was obtained which shows the utility of using isotope labelled ISTDs and that the linearity of the calibration function extends beyond the calibration range.

The contamination level of T-2 toxin in IRMMCER and IRMMFEED was so low that no reliable determination was possible. This was evidenced by the relative repeatability standard deviations of 27 and 35 %, respectively, which were about twice as large as the next highest value for T-2 toxin. For IRMMFEED the RSD_R of ZON was unacceptably high in contrast to EFL1 which had a comparable contamination level and RSD_r. An undetected inhomogeneity as cause can be excluded since due to the study design this would also have been indicated by an increased RSD_r of ZON in IRMMFEED.

Looking at Table 1 it is apparent that the composition of IRMMFEED is more complex than that of EFL1 begging the question whether the matrix is the cause of this discrepancy. In the method protocol (Annex A, Clause 5.12.3.) performance requirements for the analytical column are prescribed. One of these requirements is a resolution between two adjacent peaks of $R_s \geq 4$. Based on evaluation of chromatograms submitted by the reporting laboratories the compliance with this requirement was checked and seven of the 16 retained laboratories did not meet this requirement (vide supra). Comparing the group of laboratories meeting the resolution requirement with the group which did not shows no difference for the less complex material EFL1 but a significant difference for IRMMFEED (Figure 5). This difference between groups is the cause of the unusual shape of the respective mean & range plot (Annex D, Figure D 9) and the larger reproducibility standard deviation.

Trueness:

Because of the use of RMs an assessment of the trueness of the studied method can be made. Table 7 lists for all four analytes in the two materials the assigned values and their standard uncertainties next to the respective overall mean, the reproducibility standard deviation, the bias, and its significance. It can be seen that only DON in both materials and HT-2 toxin in the higher contaminated material showed a significant but small bias. For all other analyte / material combinations the bias was insignificant.

Reason for exclusion	**Excluded Laboratories**
Non-Compliance with protocol	2, 7, 17
Data inconsistencies	3 (altogether), 13 (altogether), 18 (just for material IRMMCER)

Table 5: List of laboratories excluded from evaluation and the reason for exclusion

Material	Labs total	Labs non-compl.	Labs ret'd	Mean	s_r	r	RSD$_r$	s_R	R	RSD$_R$	Hor Rat
DON											
EFL1	21	5	16	88.5	9.5	27	**11**	17.0	48	**19**	0.9
EFL2	21	5	16	250.0	13.6	38	**6**	33.3	93	**13**	0.7
EFL3	21	5	16	558.6	30.1	84	**5**	66.9	187	**12**	0.7
IRMMCER	21	6	15	135.8	8.2	23	**6**	23.0	64	**17**	0.8
IRMMFEED	21	5	16	281.8	19.9	56	**7**	33.1	93	**12**	0.6
HT-2											
EFL1	21	5	16	38.0	3.4	10	**9**	6.2	17	**16**	0.7
EFL2	21	5	16	49.1	3.4	10	**7**	12.0	34	**25**	1.1
EFL3	21	5	16	177.6	13.5	38	**8**	23.2	65	**13**	0.6
IRMMCER	21	6	15	53.1	8.1	23	**15**	12.4	35	**24**	1.1
IRMMFEED	21	5	16	22.0	3.3	9	**15**	6.3	18	**29**	1.3
T-2											
EFL1	21	5	16	12.1	1.7	5	**14**	3.9	11	**32**	1.5
EFL2	21	5	16	17.7	1.6	5	**9**	4.4	12	**25**	1.1
EFL3	21	5	16	50.3	3.1	9	**6**	6.5	18	**13**	0.6
IRMMCER	21	6	15	7.0	1.8	5	**27**	3.1	9	**44**	2.0
IRMMFEED	21	5	16	3.5	1.2	3	**35**	3.1	9	**88**	4.0
ZON											
EFL1	21	5	16	13.9	2.0	6	**15**	4.3	12	**31**	1.4
EFL2	21	5	16	30.5	2.9	8	**10**	6.0	17	**20**	0.9
EFL3	21	5	16	430.0	25.0	70	**6**	49.3	138	**12**	0.6
IRMMCER	21	6	15	3.4	1.1	3	**32**	3.3	9	**98**	4.4
IRMMFEED	21	5	16	15.9	1.7	5	**11**	10.4	29	**65**	3.0

Table 6: The robust performance characteristics for the five materials grouped by analyte; Labs total - total number of labs reporting, Labs non-compl. - Labs excluded for non-compliance or inconsistency, Labs ret'd - Labs retained in the calculations, Mean –overall mean value of retained labs, s_r –repeatability standard deviation, r –repeatability, RSD$_r$ –relative repeatability standard deviation, s_R –reproducibility standard deviation, R – reproducibility, RSD$_R$ – relative reproducibility standard deviation, HorRat – Horwitz Ratio.

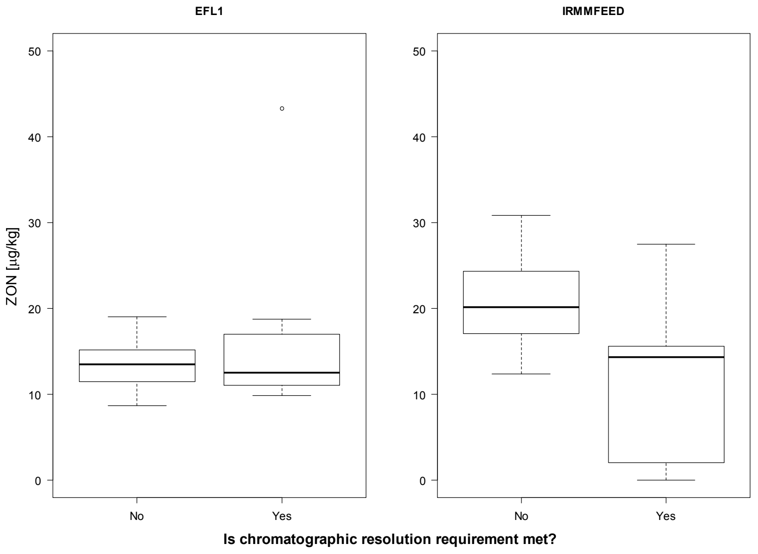

Figure 5: Box & Whisker plots of the distributions of reported ZON results of the retained laboratories grouped by whether the resolution requirement was met or not for material EFL1 (left panel) and IRMMFEED (right panel)

Measurement uncertainty estimation

According to ISO 21748:2010 [8] laboratories applying this method of analysis may use reproducibility and bias estimates established in this study to evaluate the combined uncertainty of their results as long as they have shown that their implementation of this method is consistent with the established performance.

Analyte	**Assigned Value**		**Study result**					
	x_a	$u(x_a)$	**Overall mean** $\bar{\bar{y}}$	s_R	$\hat{\delta}$	A	$\hat{\delta}-As_R$	$\hat{\delta}+As_R$
EFL2								
DON	282	13	250	33	-32	0.47	-47	-16
HT-2	51	3	49	12	-2	0.48	-8	4
T-2	18	1	18	4	0	0.47	-2	2
ZON	28	2	30	6	2	0.46	-1	5
EFL3								
DON	605	24	559	67	-46	0.46	-77	-15
HT-2	201	7	178	23	-23	0.45	-34	-13
T-2	52	2	50	6	-2	0.46	-5	1
ZON	445	8	430	49	-15	0.46	-38	7

Table 7: The assigned values and performance values of the study for the two reference materials

The first step in showing that a laboratory is implementing this method in agreement with the established performance characteristics is to investigate the laboratory component of bias and confirm that the latter is within the population of values represented in the collaborative study. This can be done by repeatedly measuring either a relevant certified reference material or, in absence of it, a relevant analyte-free test material spiked with known amounts of analytes (Sec. 6.4., Annex A). From these repeated measurements the laboratory mean m and its standard deviation s_w is computed. The number of repeats n should be larger than 8 (see Annex E for a derivation) to ensure that the uncertainty associated with this determination is small compared to the reproducibility standard deviation. The absolute difference $|\Delta_l|$ (laboratory mean m minus the expected value μ) is then compared with the sum of the between-laboratory standard deviation s^2_L, as determined in the collaborative study (Eq.3, see also Annex E), and the uncertainty of the bias determination s^2_w/n:

$$|\Delta_l| < 2\times\sqrt{s_L^2+\frac{s_w^2}{n}} \qquad (17)$$

Note that this procedure assumes that the uncertainty associated with the reference value is small compared to the uncertainty of the laboratory bias.

In a second step the laboratory needs to show that its repeatability is consistent with the data from the collaborative study. This can be achieved by replicate analysis of one or more relevant test materials and calculating the individual repeatability standard deviation s_i. The degrees of freedom should be larger than 15 (n>16) if practical, possibly by pooling results. Using an F-test the values of s_i and $\hat{s}_r$ (predicted for the same mass fraction from the collaborative study) should not be significantly different at a confidence level of 95%.

Compliance with the criteria above confirms that the laboratory is in agreement with the established performance (see Annex E for non-compliance) and that it may use s_R as its combined standard uncertainty. In any case, results for deoxynivalenol and HT-2 toxin obtained with this method of analysis should be corrected for the bias found in this study. The contribution of the uncertainty of these bias estimations is so small compared to s_R as to be negligible and maybe excluded from the uncertainty budget.

11. CONCLUSIONS

This study shows that LC-MS determination of the Fusarium toxins deoxynivalenol, HT-2 toxin, T-2 toxin, and zearalenone in unprocessed cereal and cereal-based compound feed without specific clean-up is fit for the purpose of enforcing existing or anticipated legislative limits. It exceeds existing legislation concerning precision [9] and shows only small biases which are significant for deoxynivalenol (-8%) and HT-2 toxin (-11%), and insignificant for T-2 toxin and Zearalenone.

That there are biases which in almost all instances are negative is to be expected. The extraction procedure is not exhaustive and the isotopologues are added after extraction because of which they can not account for any losses during extraction. That the biases are small and mostly insignificant can, we believe, be attributed to the extraction system. Because of the very limited miscibility of ethyl acetate and water and the addition of sodium sulphate the volume of the organic layer which is the preferred compartment of the analytes is well defined. This is more pronounced for zearalenone and T-2 toxin (large partitioning coefficient) than for deoxynivalenol and HT-2 toxin (small partitioning coefficients).

These low biases, or in other words apparent recoveries close to 100%, are in line with published work of single-laboratory validated methods. Sulyok et al. [10], using 2 mL of acetonitrile/water/acetic acid (79/20/1) to extract 0.5 g test material, reported apparent recoveries for the same analytes between 95% and 108% from wheat, and between 80% and 106% in maize with external calibration in neat solvent and no clean-up. Monbaliu et al. [11], using the same extraction system as Sulyok et al. but an elaborate clean-up, reported apparent recoveries from feed between 97% and 104.8% for a range of analytes including the analytes of this study. Those recoveries were determined with matrix-matched calibration and structurally related internal standards. A method of analysis using a more thorough extraction and isotopologues was published by Varga et al. [12]. Here the extraction was a two step process: 5 g of test material were extracted with 20 mL acetonitrile/water/formic acid (80/19.9/0.1, v/v/v). After centrifugation the supernatant was transferred to a new tube and 20 mL acetonitrile/water/formic acid (20/79.9/0.1, v/v/v) were added to the residue for a second extraction. The supernatants of both extraction were combined, mixed, and an aliquot was spiked with isotopologues before injection. With this setup apparent recoveries between 96% and 103% were reported.

The use of isotopologues of the analytes added to an aliquot of the sample extract strikes a good compromise between cost and benefit. Benefits are control of matrix effects and increased repeatability which is comparable to more traditional HPLC-UV and HPLC-fluorescence methods. Horwitz ratios were near unity with the exception of analytes at very low contamination levels. We attribute this to the well-defined and simple extraction procedure. The results of zearalenone in material IRMMFEED, which was the most complex material in this study, showed the importance of proper chromatographic resolution.

Comparing the performance parameters from robust statistics with those of a classical parametric approach shows no prominent differences. This should be seen as prove of the viability of robust statistics alleviating the statistician and the study director of the burden to detect and dismiss outliers.

To help laboratories meet the requirement of legislation [9] to report measurement uncertainties a description of how to estimate the combined uncertainty of measurement results of this method of analysis is provided. Method bias, a contributing factor to measurement uncertainty, depends, among many other influencing factors, also on the quality of the reference materials used for the calibration. For this study a mixed reference material was provided by the study organizer. Laboratories applying this method will have to ensure that well characterized reference materials of known purity are used for calibration.

12. ACKNOWLEDGEMENTS

We thank all the participating laboratories (listed in Annex C, Table C6 & C7) for their efforts in this collaborative trial. Furthermore, we want to express our gratitude to Carsten Mischke for his invaluable technical assistance and Beatrice de la Calle for her helpful comments.

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Annex A

The method protocol:

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements (Geel)
Food Safety and Quality

Method of analysis for the simultaneous determination of Deoxynivalenol, HT-2 toxin, T-2 toxin, and Zearalenone in unprocessed cereals and cereal-based compound animal feeds

!! Important !!

Read the Introduction and the following method protocol carefully before applying it. There might have been changes to previous versions you might have received.

!! Important !!

INTRODUCTION:

The rationale behind this method of analysis was to have an easy-to-apply protocol for enforcement of legislative limits for unprocessed cereals and recommended limits for compound animal feed for most of the regulated or soon-to-be regulated Fusarium toxins. Therefore, compromises were made with regards to limit of detection and quantification. We realize that lower limits of detection and of quantification are achievable but are not necessary for the purpose of this method.

The Fumonisins were left out since they are the only regulated ionic Fusarium toxins at neutral pH. Adding them would have increased the complexity of the method and, by that, decreased the chances of executing a successful collaborative study. They might by added at a later time.

During the time the method was developed there was a shortage in production of acetonitrile which affected availability and prizes. For this and other reasons, other extraction solvent systems were tested. It showed that a binary system with Ethyl acetate / water led to good extraction yields and lesser matrix effects than other more commonly used systems. A large solvent-to-sample ratio without subsequent concentration was sufficient for an adequate working range because of the sensitivity of selected reaction monitoring (SRM) with triple quadrupole mass spectrometers and of the latest generation of high mass-accuracy single MSs.

We forwent a clean-up of the extract as a possible source of error and instead focused on proper LC separation and control of possible matrix effects through the use of stable-isotope labeled analogues of the analytes. For the recommended mobile phase Methanol was chosen as organic modifier and formic acid at 0.1% as additive to keep the mobile phase as generic as possible.

We realize that test portion sizes of only 2 g are against the believe that large test portions are needed to avoid erroneous results due to sample inhomogeneities. With proper test material preparation this is not the case. The additional effort needed to mill the material to particle sizes < 500 µm and mixing it to homogeneity is, from our point-of-view, small against the benefits of saving large volumes of organic solvents.

We also realize that reconstituting dried down extracts containing T-2 toxin and/or Zearalenone necessitates a high organic solvent content and injection solution with high organic content might lead to peak broadening for early eluting analytes. No peak broadening of Deoxynivalenol was observed in our set-up because the aforementioned sensitivity of the MSs allows and the use of small particle-size analytical columns requires small injection volumes

The use of masses instead of concentrations in the model equation might be unfamiliar for some but it helps to keep the model equation simple. And it really is only a thing of familiarity. Neither the quantification software of your instrument nor your PC cares whether the units of a number are a mass or a concentration. All that matters is that the correct number was entered.

1. SCOPE

This method of analysis is applicable to the determination of Deoxynivalenol (DON) in the range of 200 µg/kg to 2560 µg/kg, HT-2 toxin (HT2) in the range of 25 µg/kg to 400 µg/kg, T-2 toxin (T2) in the range of 15 µg/kg to 240 µg/kg, and Zearalenone (ZON) in the range of 50 µg/kg to 240 µg/kg in unprocessed rice, wheat, oat, maize and soy or mixtures thereof. Legislative limits for unprocessed cereals as laid down in European legislation [1] or anticipated limits, being under discussion, fall within these ranges.

NOTE: These working ranges are applicable to the environment at IRMM. They will most likely change in a final version of this protocol and need not be applicable to the situation in your laboratory.

2. NORMATIVE REFERENCES

None

3. PRINCIPLE

Two gram of finely ground and homogeneous test material is suspended in water. After addition of 16.0 mL ethyl acetate the sample is agitated for 30 min. Then sodium sulphate is added to facilitate phase separation and after 10 to 20 min the sample is centrifuged to pellet particulate matter at the bottom of the extraction tube. The organic phase is transferred to a clean vial for possible storage. 500 µL of the organic phase, an equivalent of 1/16th of the test portion, are mixed with stable-isotope labeled analogues of the analytes and evaporated to dryness in deactivated glass vials. Adding the isotopically labeled analogues to an aliquot of the extract is a compromise between best accuracy and acceptable costs. After reconstitution of the dry extract with 250 µL of organic mobile phase modifier, addition of 250 µL of water, and thorough mixing the analytes are quantified with a LC-MS system.

4. REAGENTS

4.1. Water (deionized)

4.2. Water (LC-MS grade)

4.3. Methanol (LC-MS grade)

WARNING — Methanol is hazardous and handling shall be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) shall be worn.

4.4. Methanol (p.a.)

WARNING — Methanol is hazardous and handling shall be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) shall be worn.

4.5. Ethyl acetate (p.a.)

WARNING — Ethyl acetate is hazardous and handling shall be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) shall be worn.

4.6. Formic acid (98-100%)

WARNING — Formic acid is hazardous and handling shall be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) shall be worn.

4.7. Acetonitrile (LC-MS grade)

WARNING — Acetonitrile is hazardous and handling shall be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) shall be worn.

4.8. Sodium sulfate

anhydrous, granulated

4.9. Deoxynivalenol (DON)

WARNING — Deoxynivalenol is highly toxic. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.10. HT-2 toxin (HT2)

WARNING — HT-2 toxin is highly toxic. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.11. T-2 toxin (T2)

WARNING — T-2 toxin is highly toxic. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.12. Zearalenone (ZON)

WARNING — Zearalenone is highly toxic. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.13. $^{13}C_{15}$-Deoxynivalenol ($^{13}C_{15}$-DON)

WARNING — $^{13}C_{15}$-Deoxynivalenol is highly toxic. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.14. $^{13}C_{22}$-HT-2 toxin ($^{13}C_{22}$-HT2)

WARNING — $^{13}C_{22}$-HT-2 toxin is highly toxic. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.15. $^{13}C_{24}$-T-2 toxin ($^{13}C_{24}$-T2)

WARNING — $^{13}C_{24}$-T-2 toxin is highly toxic. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.16. $^{13}C_{18}$-Zearalenone ($^{13}C_{18}$-ZON)

WARNING — $^{13}C_{18}$-Zearalenone is highly toxic. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.17. Multitoxin stock solution:

A mixture containing Deoxynivalenol (4.9), HT-2 toxin (4.10), T-2 toxin (4.11), and Zearalenone (4.12) in neat acetonitrile (4.7) at relevant concentrations.

NOTE: Compare a new stock solution against the old one by adding 25 µL of each into separate deactivated vials (5.6) and proceeding as described in "Test solution" (6.3).

NOTE: 3.2 µg/mL DON, 0.5 µg/mL HT-2 toxin, 0.3 µg/mL T-2 toxin, and 0.3 µg/mL ZON in neat acetonitrile have shown to work well. This solution is stable for three months in the dark at 2-8 °C.

NOTE: If Sec. 6.4. "Spiking procedure" is executed at least 6 mL of the stock solution are needed.

4.18. Multitoxin working solution:

Dilute Multitoxin stock solution (4.17) with Methanol (4.4) such that the resulting concentration in the working solution is applicable to the calibration range of the different compounds. Only prepare enough volume for one full calibration.

NOTE: Adding 188 µL of the Multitoxin stock solution to a 3 mL volumetric flask and making up to the mark with methanol will result in a solution containing 0.2 µg/mL DON, 0.031 µg/mL HT-2 toxin, 0.019 µg/mL T-2 toxin, and 0.019 µg/mL ZON in methanol/ acetonitrile (94/6, v/v).

4.19. Multi ISTD stock solution:

A mixture containing $^{13}C_{15}$-DON (4.13), $^{13}C_{22}$-HT-2 toxin (4.14), $^{13}C_{24}$-T-2 toxin (4.15), and $^{13}C_{18}$-ZON (4.16) in neat acetonitrile (4.7) at the same concentrations as the respective native compounds in the Multitoxin stock solution (4.17).

NOTE: This solution is stable for three months in the dark at 2-8 °C.

4.20. Calibration:

To six deactivated glass vials (5.6) add different volumes of the Multitoxin working solution (4.18) such that six equidistant calibration levels across the calibration range result. Proceed as described in Sec. 6.3. "Test solution".

NOTE: Table 1 below shows example calibration levels using the solutions described in the notes above.

NOTE: Once it has been shown that there is linearity the number of levels may be adjusted to local needs and requirements.

Table 1: Calibration solutions

Volume of Multitoxin working solution (4.18.) [µL]	**Total mass of analyte per vial [ng]**			
	DON	**HT-2**	**T-2**	**ZON**
25	5	0.78	0.48	0.48
180	36	5.6	3.4	3.4

Volume of Multitoxin working solution (4.18.) [µL]	Total mass of analyte per vial [ng]			
	DON	HT-2	T-2	ZON
335	67	10	6.4	6.4
490	98	15	9.3	9.3
645	129	20	12	12
800	160	25	15	15

4.21. Quality control material

An appropriate material with natural contamination or fortification of the tested mycotoxins which is sufficiently stable.

5. APPARATUS

5.1. Mill

Single mill or multiple mills capable of comminuting test materials to particle sizes of < 500 µm. The recommended way is to mill the laboratory sample to a particle size of ca. 1 mm and after sufficient homogenization proceed with a subsample of 50 g to the final particle size.

5.2. Mixer

Capable of sufficiently homogenizing the comminuted test materials.

NOTE: a tumble mixer that uses a folding action either through moving paddles or fins, or an end-over-end movement has shown to work well.

5.3. Conical polypropylen screw-cap centrifuge tubes 50 mL with caps

5.4. Volumetric flasks

3, 5, and 10 mL

5.5. Pipettors

Adjustable 10-100 µL and adjustable 100-1000 µL, properly calibrated.

5.6. Deactivated glass vials

Silanized glass vials, f.i. 4 mL 45x14.7 mm.

5.7. Auto Liquid Sampler (ALS) vials

Of appropriate size for the Auto Liquid Sampler in use.

5.8. Shaker or Sonicator

5.9. Evaporator

Capable of maintaining a stable temperature in the range of 30 - 60 °C with a constant flow of dry nitrogen.

5.10. Centrifuge

Capable of generating a relative centrifugal force (RCF) of 3000 *g.*

5.11. Syringe filter: 0.2 µm Nylon

5.12. LC-MS:

5.12.1. Solvent delivery system:

Capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

5.12.2. Auto liquid sampler (ALS):

Capable of injecting an appropriate volume of injection solution with sufficient accuracy, cross-contamination below 0.1%.

5.12.3. Analytical column:

Capable of separating the four analytes with the following performance:

Peak asymmetry factor at 10% height: **0.9<As<1.4**; minimum apparent retention factor for any of the four analytes: **k ≥ 2**; minimum plate number for any of the four analytes: **N ≥ 1200**; minimum resolution between two adjacent analyte peaks: $\mathbf{R_s \geq 4}$.

5.12.4. Mass spectrometer:

An instrument capable of either performing selected reaction monitoring (SRM) or high-accuracy (sub 5 ppm mass accuracy) single MS measurements with a sufficiently wide dynamic range. Any ionization source giving sufficient yield may be employed.

6. PROCEDURES

6.1. Sample preparation

It is important that the laboratory receives a laboratory sample which is truly representative and has not been damaged or altered during transport or storage. Laboratory samples should be taken and prepared in accordance with European legislation where applicable. [2][3] The laboratory sample should be finely ground and thoroughly mixed using a mill (5.1.) and a mixer (5.2.) or another process for which complete homogenization has been demonstrated before a test portion is removed for analysis.

In all instances everything should be at room temperature before any kind of manipulation takes place.

6.2. Extraction

Some of the steps described below are more critical for the accuracy of the results than others. These steps are marked as such and should be carried out with the necessary attention.

- For the test portion weigh 1.9 to 2.1 g of the homogeneous sample into a conical polypropylene screw-cap tube (5.3.), round and record the weight to the second decimal (**the accuracy of this weight is critical for the accuracy of the final result!**).
- Add 7.2 to 8.8 mL of deionized water (4.1).

- Vortex thoroughly until test portion is completely suspended.
- Add 16.0 mL of ethyl acetate (4.5., **the accuracy of this volume is critical for the accuracy of the final result!**).
- Extract for 27 to 33 min in a sonicator or by vigorously shaking (5.8).
- Add between 7.2 and 8.8 g of sodium sulfate (4.8.).
- Instantly shake hard for 5 s.
- Let stand for 10 to 20 min.
- Centrifuge (5.10.) at RCF 3000 for at least 1 min to aid settlement of particulate matter and phase separation.
- If wanted for possible repeats: Transfer the extract (organic layer) into clean glass vial for storage of up to 7 days at 2 to 10 °C in the dark.
- Transfer 500 µL of the extract (organic layer) into a deactivated glass vial (5.6.) for further processing (**the accuracy of this volume is critical for the accuracy of the final result!**).

6.3. Test solution

- Add 25 µL of the Multi ISTD stock solution (4.19.) to the aliquot of the extract and/or the calibration solutions (4.20) (**the accuracy of this volume is critical for the accuracy of the final result!**).
- Dry down the aliquot of the extract and/or the calibration solutions in an evaporator (5.9.) with a gentle stream of dry nitrogen at 60 °C.
- Add 250 µL of the organic mobile phase modifier to the dry residue for reconstitution.
- Vortex thoroughly for at least 10 s.
- Add 250 µL deionized water (4.1.) to the reconstituted extract.
- Vortex thoroughly for at least 5 s.
- Transfer the test solution into an ALS vial (5.7.); if solution is turbid it may be filtered through a syringe filter (5.11.).

> **NOTE: It has been shown that even very turbid samples can be injected without any negative effects on the life time of column and LC provided that appropriate in-line filters or guard columns are used.**

6.4. Spiking procedure

If recovery needs to be determined execute the following in duplicate:

To three times 2 g of a material free of DON, HT2, T2, and ZON add three different volumes of the Multitoxin stock solution (4.17) such that 3 contamination levels across the calibration range result. Distribute the solutions evenly over the materials, mix to further distribute the spike, and leave for a minimum of 5 h to a maximum of 18 h. Proceed to Sec. 6.2. “Extraction” second step.

NOTE: Addition of 360, 980, and 1600 µL of the Multitoxin stock solution (4.17) with the concentrations described in the note has been shown to work well.

7. MEASUREMENTS

The LC-MS system must meet the requirements laid out in clause 5.12 and sub clauses.

7.1.LC conditions

Choose an analytical column, mobile phase, gradient settings, and injection volume that let you meet the requirements in clause 5.12.3 (for examples see Annex A).

7.2.MS conditions

Choose an ion source with sufficient ionization yield for the four analytes and ion source settings such that a stable spray is achieved.

Choose for each analyte an appropriate parent ion (adducts of the molecule with a Proton, Sodium, Ammonium, etc. in positive mode, or deprotonation, etc. in negative mode). If more than one ion of the parent is detectable choosing the strongest is a good starting point. But one must be aware that the choice of parent ion will affect repeatability and, by that, LOD and LOQ.

If SRM will be used select two daughter ions in the MS/MS spectrum of each chosen parent ion. Set up SRM transitions with these parent/daughter ion combinations (for SRM example see Annex A MS conditions).

If a high mass-accuracy MS will be used calculate the exact mass of your chosen parent ion and use this exact mass for your data analysis.

The chosen MS settings must be such that for a cereal mix (containing possibly small amounts of soy) with a contamination of ca. 90 µg/kg DON, 30 µg/kg HT-2 toxin, 10 µg/kg T-2 toxin, and 10 µg/kg ZON, prepared acc. to Sec. 6, signal-to-noise ratios of larger than 20 are obtained (see Annex B).

7.3. Batch composition

Always start a batch of measurements with a reagent blank run to prove non-contamination of the system. Then inject the calibration solutions once again followed by a reagent blank to check for possible carry-over. Subsequently inject the test solutions in duplicate. At the end of the batch reinject the calibration solutions for a second run.

7.4. Peak identification

When using SRM identify the analyte peaks in the test solution by plotting the extracted ion currents of the analyte and its respective labeled analogue and then A) comparing the retention time of the analyte with the retention time of the respective labeled analogue (difference must be smaller than 0.25 times peak width (FWHM)),and B) comparing the ratio of the two measured transitions with that of a calibration solution of comparable signal intensity.

When using high mass-accuracy MS identify the analyte peaks in the test solution by plotting the extracted ion currents of the analyte and its respective labeled analogue using their exact masses plus minus a mass window of 5 ppm and then comparing the retention time of the analyte with the retention time of the respective labeled analogue (difference must be smaller than 0.25 times peak width (FWHM)).

For example chromatograms see Annex B.

7.5. Determination of DON, HT2, T2, and ZON in calibration or test solutions

Inject aliquots of the calibration and/or test solutions (6.3.) onto the column using identical conditions. For each injection calculate the ratio of the peak area of the analyte divided by the peak area of the respective labeled analogue. These peak area ratios will be used in all subsequent calculations

7.6. Calibration

Plot the peak area ratios of all the measured calibration solutions against the corresponding total masses in the calibration solution of DON, HT2, T2, and ZON separately. Do not use means of the multiple injections! With weighted least-square regression over all data estimate slope and possible intercept of each of the four calibration functions (DON, HT2, T2, ZON). Check for significance of the intercept and for linearity (use e.g. a residuals vs fitted-values plot).

8. DETERMINATION OF MASS FRACTION

To calculate the mass fractions ($w_{An,S}$) of a specific analyte in the test portion use the following model equation:

$$w_{An,S} = \left(\frac{\overline{R}}{\beta_1} - \frac{\beta_0}{\beta_1} \right) \times \frac{m_{ISTD,S}}{m_{ISTD,C}} \times \frac{V_{EtOAc}}{V_{Aliq} \times m_S} \qquad (1)$$

with

$w_{An,S}$ = mass fraction of analyte in the test portion;

$\overline{R}$ = Mean of the peak area ratios of replicate injections;

β_1 = slope, estimated with weighted least-square regression from calibration data (7.5.);

β_0 = intercept, estimated with weighted least-square regression from calibration data (becomes zero if not significant (see 7.5.));

$m_{ISTD,S}$ = mass of the labeled analogue in the test solution;

$m_{ISTD,C}$ = mass of the labeled analogue in the calibration solution;

V_{Aliq} = Volume of the aliquot taken from the raw extract;

V_{EtOAc} = Volume of the ethyl acetate used for extraction;

m_S = mass of the test portion.

Under the assumption that test and calibration solutions are treated identically (same volume of Multi ISTD stock solution added, $m_{ISTD,S} = m_{ISTD,C}$) the model equation reduces to:

$$w_{An,S} = \left(\frac{\overline{R}}{\beta_1} - \frac{\beta_0}{\beta_1} \right) \times 1 \times \frac{V_{EtOAc}}{V_{Aliq} \times m_S} \qquad (2)$$

The term in parentheses is the total mass of the analyte in the test solution so the reduced model equation may be written as:

$$w_{An,S} = m_{An,S} \times \frac{V_{EtOAc}}{V_{Aliq} \times m_S} \qquad (3)$$

For a test portion of 2.0 g, 16.0 mL of ethyl acetate, and a 0.5 mL aliquot of the extract the second term becomes 16 and equation (4) may be written as:

$$w_{An,S} = m_{An,S} \times 16 \text{ [µg/kg]} \quad (4)$$

Because of the use of peak area ratios the total volumes of the test or calibration solutions and the injected volumes have no direct influence on the result and do not appear in the model equation.

9. REFERENCES

1. European Commission, *Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance).* Official Journal of the European Union, 2006. **L 364**: p. 5–24.
2. European Commission, *Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (Text with EEA relevance).* Official Journal of the European Union, 2006. **L 70**: p. 12–34.
3. European Commission, Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed (Text with EEA relevance). Official Journal of the European Union, 2009. **L 54**: p. 1-130.

Annex A

Example 1:

With a LC-MS system consisting of two Shimadzu LC-20AD pumps, Thermo Scientific Accela Auto Liquid Sampler, and a Thermo Scientific TSQ Quantum Ultra MS with IonMax HESI2 interface the following settings have shown to satisfy the performance requirements and provide overall acceptable results (see Annex B Figure 1&2 for chromatograms).

LC conditions

- Dwell volume: 60 µL
- Injection volume: 5 µL full loop
- Column Supelco Ascentis Express C18, 75 x 2.1 mm, particle size 2.7 µm fused-core
- Column temperature: 40 ºC
- Flow rate: 0.3 mL/min
- Mobile phase A: 0.1% formic acid (4.6.) in water (4.2.)
- Mobile phase B: 0.1% formic acid (4.6.) in methanol (4.3.)

NOTE: The mobile phase was chosen to be very generic. It is permissible to add Ammonium ions to the mobile phase if this leads to suppression of sodiation and you want to measure the ammonium adducts!

Table 2: Gradient settings

Run time [min]	% B
0	8
2	57
6	61
6.1	95
7.6	95
7.7	8
8.7	8

MS conditions

The run is divided in to four segments around the four analyte peaks. The following ion transitions in "selected reaction monitoring" mode are measured:

Item	Segment 1	Segment 2	Segment 3	Segment 4
Run time	0-2.6	2.6 – 4.1	4.1 – 4.9	4.9 – 8.7
Analyte	DON + $^{13}C_{15}$-DON	HT2 + $^{13}C_{22}$-HT2	T2 + $^{13}C_{24}$-T2	ZON + $^{13}C_{20}$-ZON
Adduct	Protonated	Sodium	Sodium	Deprotonated
Transitions (Collision Energy)	297->231 (16), 297->249 (13), 312->263 (9), 312->276 (9)	447->285 (22), 447->345 (20), 469->300 (19), 469->362 (18)	489->245 (30), 489->327 (25), 513->260 (26), 513->344 (23)	317->131 (25), 317->175 (22), 335->185 (26), 335->290 (21)
Tube Lens	80	110	140	80
Polarity	Pos	Pos	Pos	Neg
Spray Voltage [V]	2800	2800	2400	2000
Vaporizer Temperatur [°C]	350	350	350	350
Sheath Gas Pressure [arbitrary units]	30	30	30	30
Aux Gas Pressure [arbitrary units]	10	10	10	10
Transfer Capillary Temperature [°C]	320	320	320	320

Example 2:

With a LC-MS system consisting of a HP1100 HPLC and a Micromass Quattro Ultima PT with ESI interface the following settings have shown to satisfy the performance requirements and provide overall acceptable results (see Annex B Figure 3&4 for chromatograms).

LC conditions

- Dwell volume: the original static mixer was replaced by a low-volume peek mixing Tee
- Injection volume: 5 µL
- Column Supelco Ascentis Express C18, 75 x 2.1 mm, particle size 2.7 µm fused-core
- Column temperature: 40 ºC
- Flow rate: 0.3 mL/min
- Mobile phase A: 0.1% formic acid (4.6.) in water (4.2.)
- Mobile phase B: 0.1% formic acid (4.6.) in methanol (4.3.)

Table 3: Gradient settings

Run time [min]	% B
0	8
0.67	50
8	67
8.01	95
9.5	95
9.51	8
11.5	8

MS conditions

The run is divided in to four segments around the four analyte peaks. The following ion transitions in “selected reaction monitoring” mode are measured:

Item	Segment 1	Segment 2	Segment 3	Segment 4
Run time	0 – 4.0	4.0 – 6.2	6.2 – 7.2	7.2 – 11.5
Analyte	DON + $^{13}C_{15}$-DON	HT2 + $^{13}C_{22}$-HT2	T2 + $^{13}C_{24}$-T2	ZON + $^{13}C_{20}$-ZON
Adduct	Protonated	Sodium	Sodium	Deprotonated
Transitions (Collision Energy)	297->231 (18), 297->249 (18), 312->263 (18), 312->276 (18)	447->285 (21), 447->345 (18), 469->300 (17), 469->362 (17)	489->245 (24), 489->327 (21), 513->260 (20), 513->344 (19)	317->131 (18), 317->175 (18), 335->185 (18), 335->290 (18)
Cone Voltage	50	85	80	60
Polarity	Pos	Pos	Pos	Neg
Spray Voltage [V]	2500	2500	2500	2500
Desolvation Temperatur [°C]	350	350	350	350
Desolvation Gas Flow [L/h]	700	700	700	700
Cone Gas Flow [L/h]	100	100	100	100
Source Temperature [°C]	120	120	120	120

Example 3:

With a LC-MS system consisting of an Agilent 1200 SL HPLC and an Applied Biosystems/ MDSciex API4000 with Turbospray interface the following settings have shown to satisfy the performance requirements and provide overall acceptable results (see Annex B Figure 5 for chromatogram).

LC conditions

- Injection volume: 30 µL
- Column Phenomenex Luna C18, 150 x 4.6 mm, particle size 5 µm
- Column temperature: 40 ºC
- Flow rate: 0.3 mL/min
- Mobile phase A: Water/ Methanol/ Formic Acid (950/ 50/ 0.025, v/v/v), 1 mmol/L Ammonium carbonate
- Mobile phase B: Methanol (4.3.)

Table 4: Gradient settings

Run time [min]	% B
0	0
5	80
6.9	80
7	100
10	100
10.1	0
13	0

MS conditions

The following transitions were monitored:

Analyte	MS1	MS 3	Polarity
DON	295.000	265.000	**negative**
DON	295.000	138.000	**negative**

DON C13	310.200	279.000	**negative**
DON C13	310.200	145.000	**negative**
ZON	317.000	131.000	**negative**
ZON	317.000	175.000	**negative**
ZON C13	335.000	185.000	**negative**
ZON C13	335.000	290.000	**negative**
HT2	442.000	263.000	**positive**
HT2	442.000	215.000	**positive**
HT2 C13	464.000	340.000	**positive**
HT2 C13	464.000	322.000	**positive**
T2	484.000	215.000	**positive**
T2	484.000	185.000	**positive**
T2 C13	508.000	322.000	**positive**
T2 C13	508.000	260.000	**positive**

Annex B

Example chromatograms:

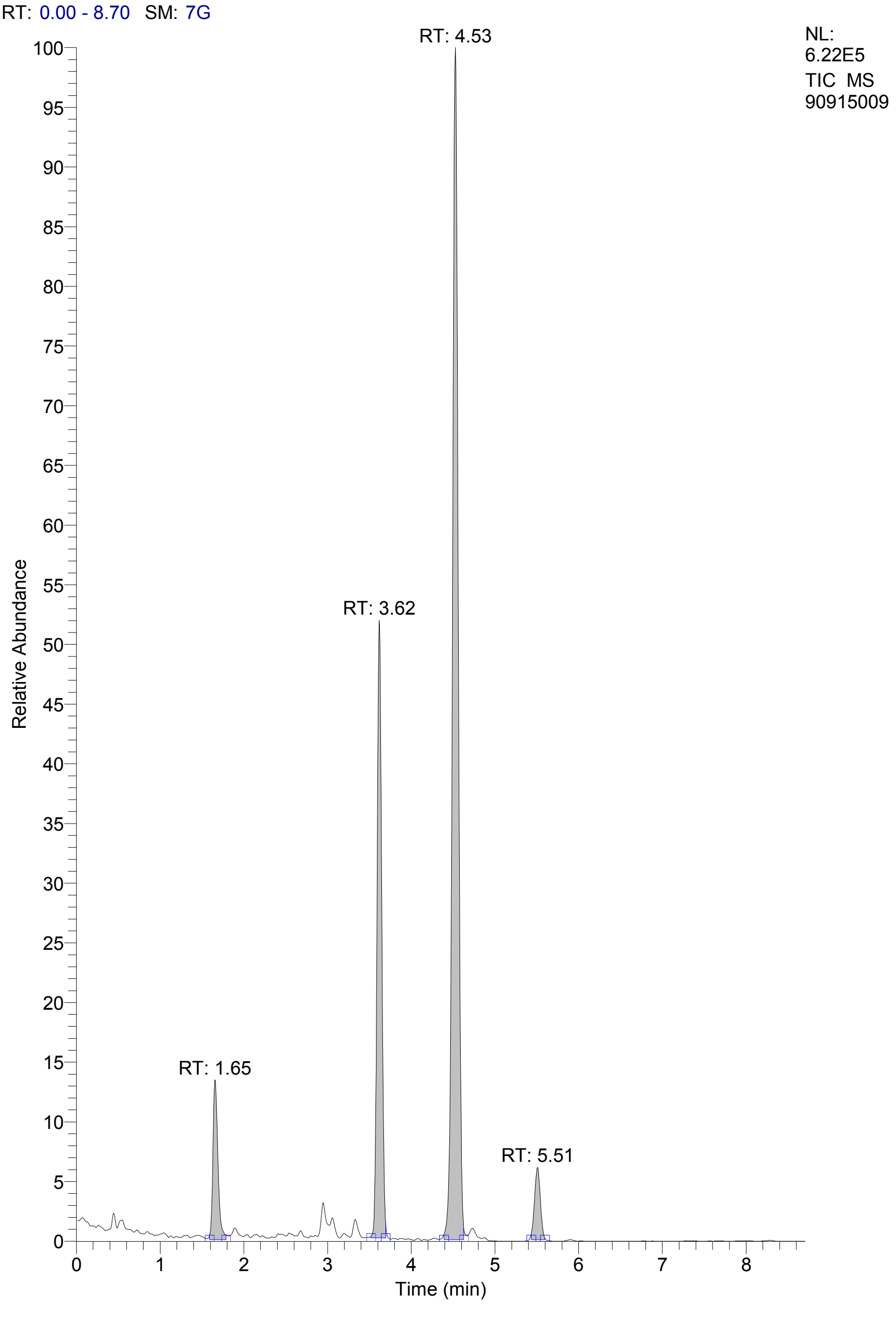

Figure 1: Total Ion Current (TIC) of a QC sample with ca. 90 µg/kg DON (RT 1.65), 30 µg/kg HT-2 toxin (RT 3.62), 10 µg/kg T-2 toxin (RT 4.53), and 10 µg/kg ZON (RT 5.51); the peak area is mostly representing the ^{13}C-labelled ISTDs. Acquired with settings Example 1 in Annex A.

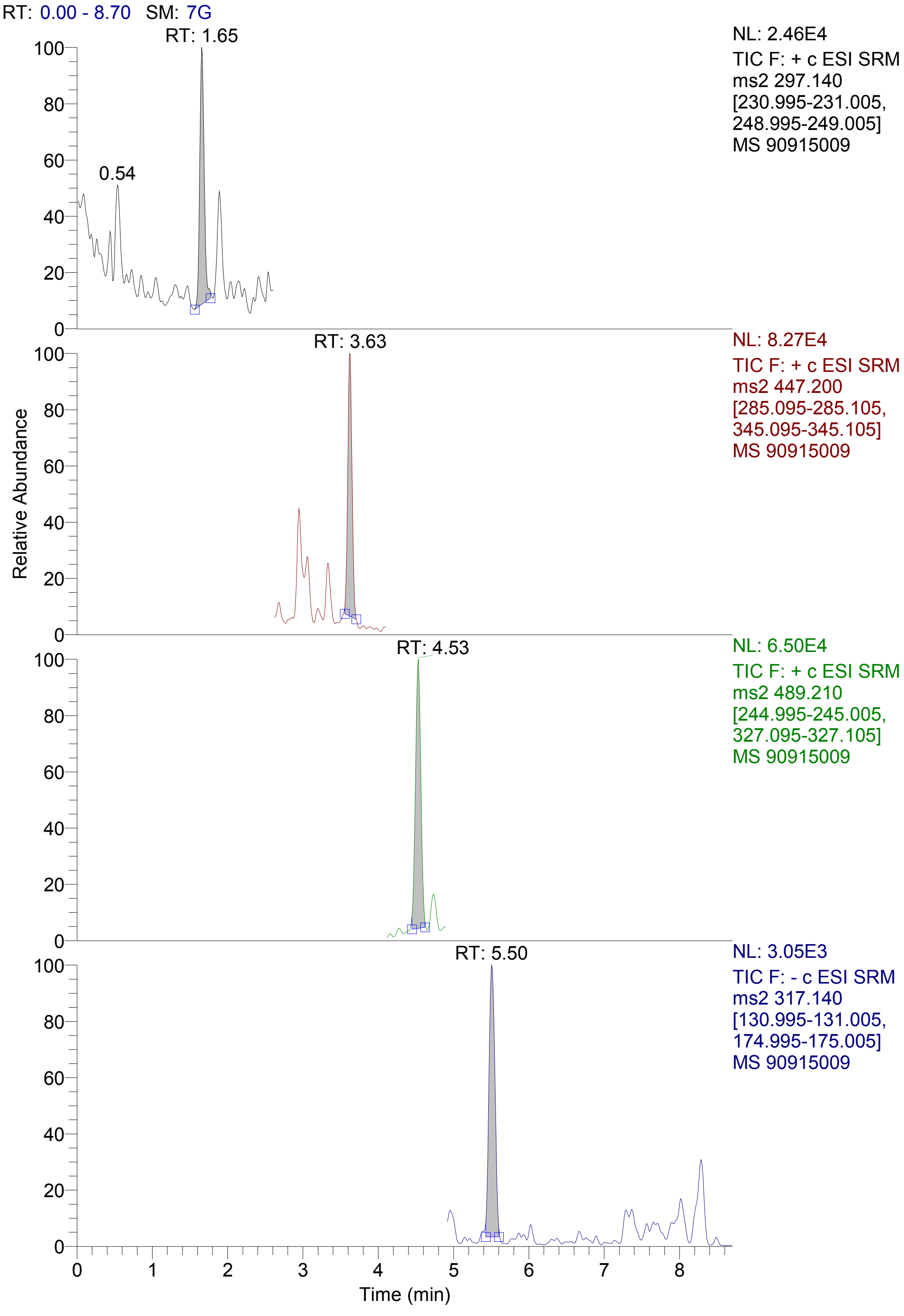

Figure 2: Extracted Ion Currents (XIC) of the same QC sample as above; the ion traces represent the transitions of the native analytes; for identification see caption of Fig. 1. Acquired with settings Example 1 in Annex A.

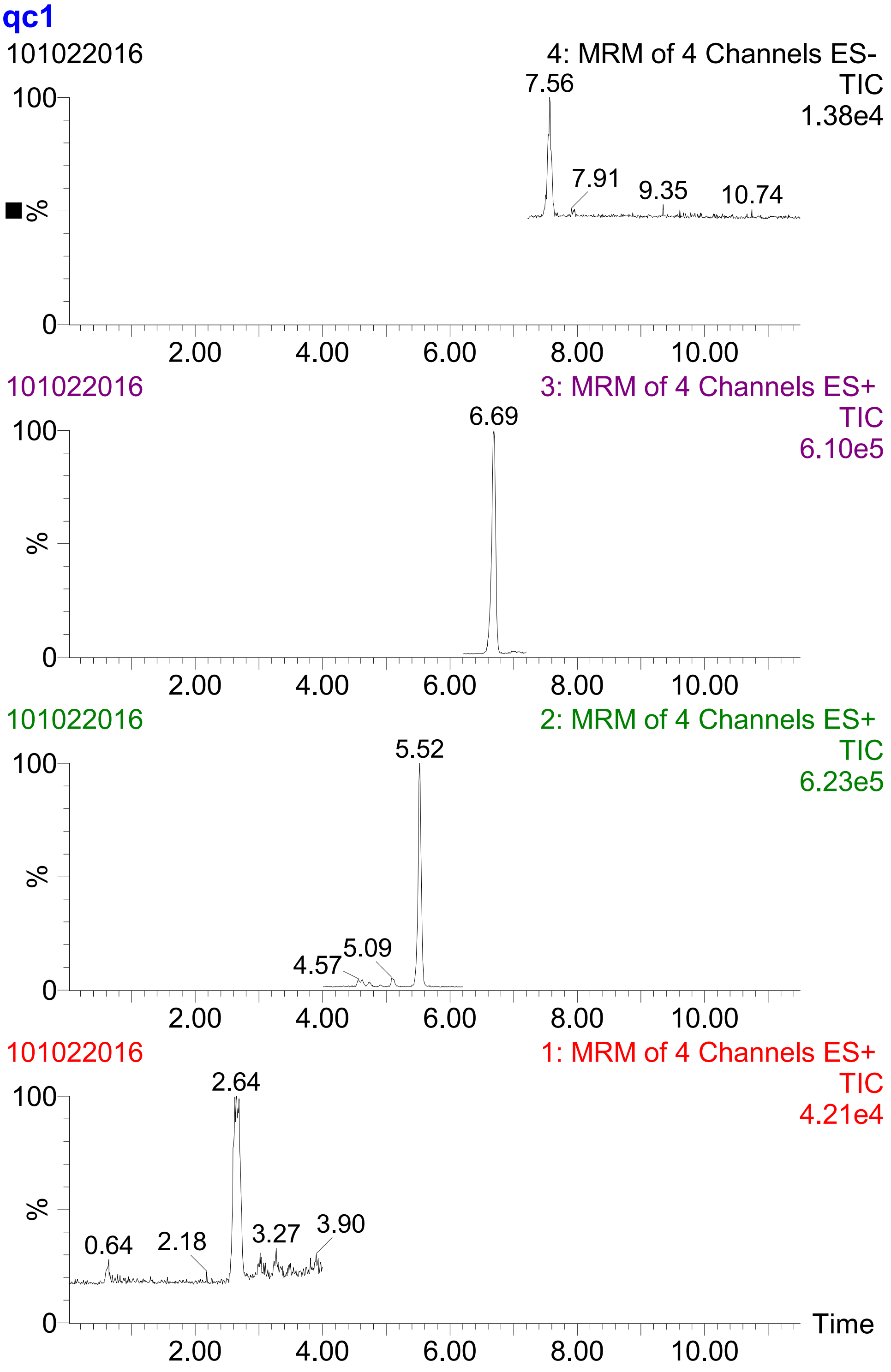

Figure 3: Total Ion Current (TIC) of the same QC sample as in Fig. 1. Acquired with settings Example 2 in Annex A

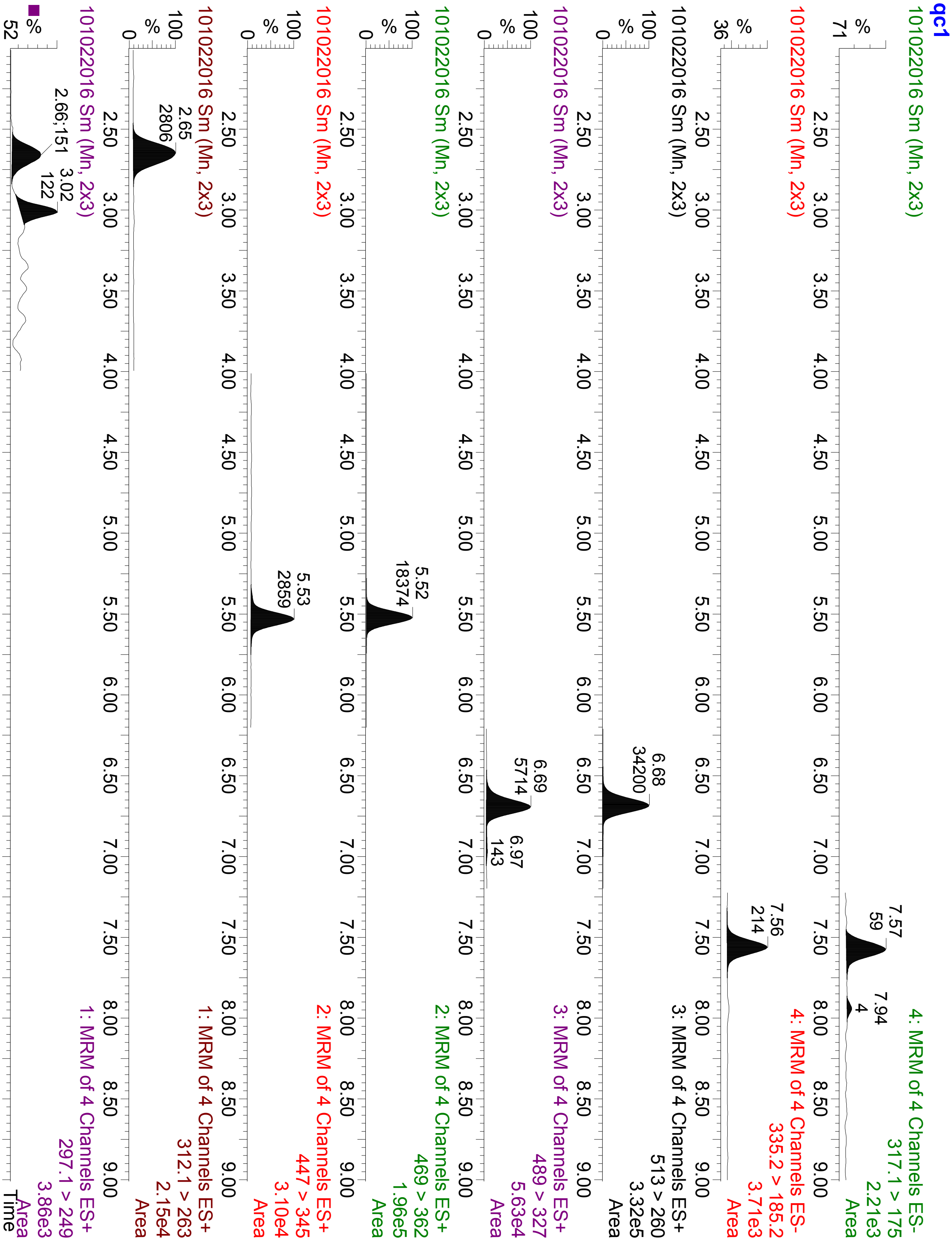

Figure 4: Extracted Ion Currents (XIC) of the same QC sample as in Fig. 1. The traces for the native analyte and the respective labeled analogue are right above each other. Acquired with settings Example 2 in Annex A

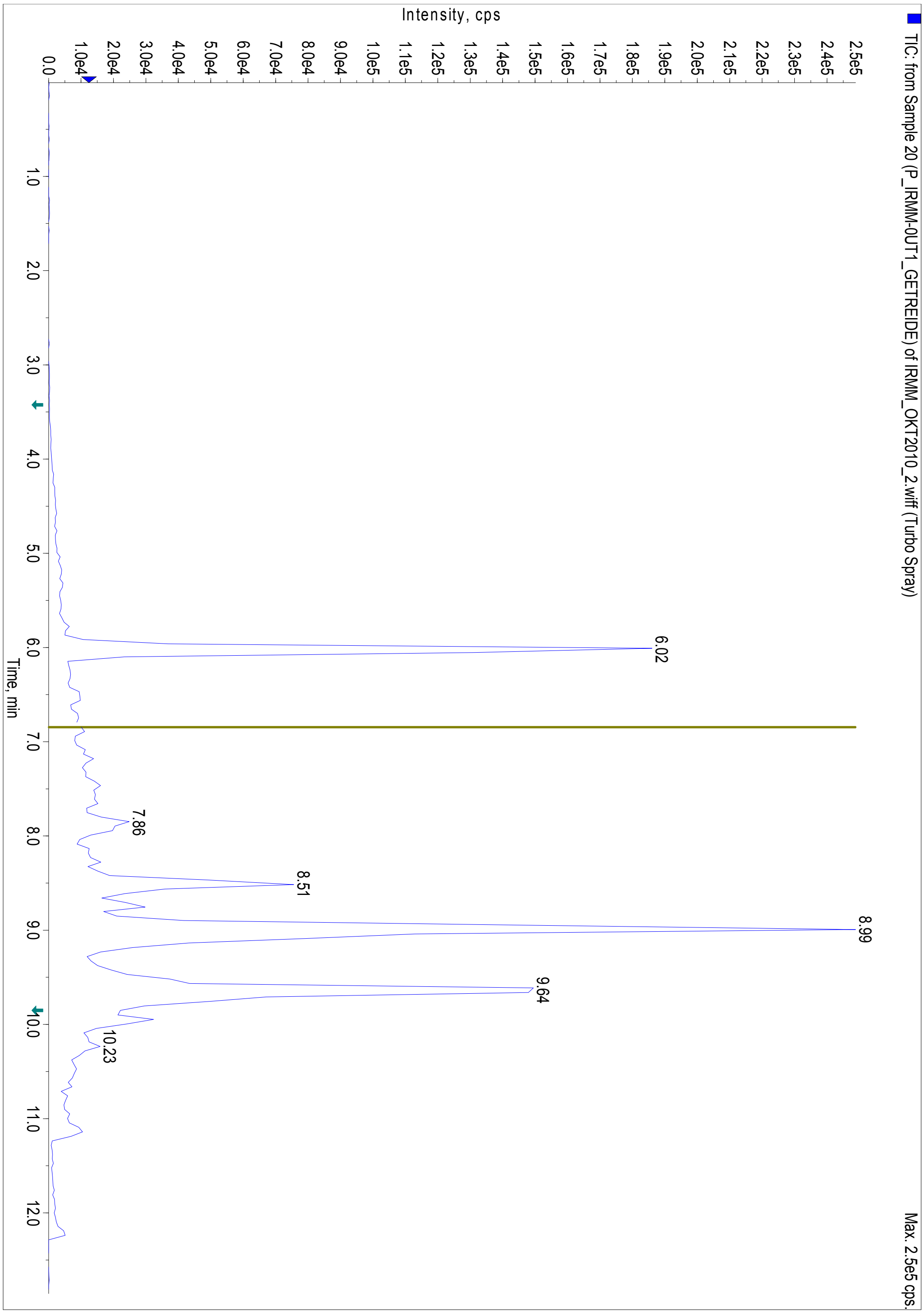

Figure 5: Total Ion Current (TIC) of the same QC sample as in Fig. 1. Acquired with settings Example 3 in Annex A

Annex B

The uncertainty budget

Annex B / The uncertainty budget

For each analyte and material three randomly selected test units were tested twice each. The following tables list the uncertainty components per analyte and material:

Deoxynivalenol					
Terms of Eqs. 13 & 15	**EFL2**		**EFL3**		**Units**
	Value	**Standard uncertainty *u***	**Value**	**Standard uncertainty *u***	
$w_{c,i}$	1.630	0.03897	3.577	0.08551	µg/g
$\overline{R}$	0.9276	0.04585	0.8871	0.02106	
$m_{c,i}$	0.1741	0.0004200	0.1873	0.0004300	g
$m_{ISTD,SB}$	0.1244	0.0004200	0.1357	0.0004300	g
$m_{ISTD,CB}$	0.1227	0.0004200	0.1334	0.0004300	g
$m_{smp,i}$	0.9982	0.00001630	0.9999	0.00001630	g
$\overline{w}_{s,i}$	0.2815	0.004489	0.6050	0.01174	µg/g
F_{BS}	1	0.01210	1	0.02147	
x_a	0.282	0.013	0.605	0.024	µg/g

Table B 1: the uncertainty budget for deoxynivalenol in material EFL2 and EFL3; the first six rows show exemplary values of one of the multiple determinations; the last three rows display the combined results of all determinations

HT-2 toxin					
Terms of Eqs. 13 & 15	**EFL2**		**EFL3**		**Units**
	Value	**Standard uncertainty *u***	**Value**	**Standard uncertainty *u***	
$w_{c,i}$	0.3155	0.004952	1.143	0.01793	µg/g
$\overline{R}$	1.153	0.02300	1.142	0.02500	
$m_{c,i}$	0.1400	0.00004800	0.1724	0.00004800	g
$m_{ISTD,SB}$	0.1387	0.00004800	0.1312	0.00004800	g
$m_{ISTD,CB}$	0.1384	0.00004800	0.1432	0.00004800	g
$m_{smp,i}$	0.9895	0.00001630	1.022	0.00001630	g
$\overline{w}_{s,i}$	0.05092	0.001695	0.2014	0.002147	µg/g
F_{BS}	1	0.001736	1	0.006004	
x_a	0.051	0.0024	0.201	0.0064	µg/g

Table B 2: the uncertainty budget for HT-2 toxin in material EFL2 and EFL3; the first six rows show exemplary values of one of the multiple determinations; the last three rows display the combined results of all determinations

T-2 toxin					
Terms of Eqs. 13 & 15	**EFL2**		**EFL3**		**Units**
	Value	**Standard uncertainty u**	**Value**	**Standard uncertainty u**	
$w_{c,i}$	0.1263	0.002198	0.3353	0.005835	µg/g
$\overline{R}$	0.7799	0.04210	1.097	0.02065	
$m_{c,i}$	0.1881	0.00004800	0.1382	0.00004800	g
$m_{ISTD,SB}$	0.2939	0.00004800	0.1597	0.00004800	g
$m_{ISTD,CB}$	0.2923	0.00004800	0.1550	0.00004800	g
$m_{smp,i}$	1.006	0.00001006	1.000	0.00001630	g
$\overline{w}_{s,i}$	0.01798	0.0007799	0.05204	0.0006054	µg/g
F_{BS}	1	0.0006497	1	0.001342	
x_a	0.018	0.0010	0.052	0.0015	µg/g

Table B 3: the uncertainty budget for T-2 toxin in material EFL2 and EFL3; the first six rows show exemplary values of one of the multiple determinations; the last three rows display the combined results of all determinations

Zearalenone					
Terms of Eqs. 13 & 15	**EFL2**		**EFL3**		**Units**
	Value	**Standard uncertainty u**	**Value**	**Standard uncertainty u**	
$w_{c,i}$	0.3246	0.004246	4.435	0.05799	µg/g
$\overline{R}$	0.9882	0.03502	0.9574	0.01164	
$m_{c,i}$	0.09174	0.0003800	0.1074	0.0003800	g
$m_{ISTD,SB}$	0.1877	0.0003800	0.2217	0.0003800	g
$m_{ISTD,CB}$	0.1940	0.0003800	0.2267	0.0003800	g
$m_{smp,i}$	0.9987	0.00001630	1.005	0.00001630	g
$\overline{w}_{s,i}$	0.02850	0.0007647	0.4456	0.001312	µg/g
F_{BS}	1	0.001196	1	0.007663	
x_a	0.028	0.0014	0.446	0.0078	µg/g

Table B 4: the uncertainty budget for zearalenone in material EFL2 and EFL3; the first six rows show exemplary values of one of the multiple determinations; the last three rows display the combined results of all determinations

Annex C

The individual results and participating laboratories

Annex C / The individual results and participating laboratories

EFL1:										
LAB_ID	**Code**	DON	HT-2	T-2	ZON	**Code**	DON	HT-2	T-2	ZON
1	**5071**	94.39	33.22	13.21	7.96	**7992**	90.54	39.41	12.49	9.37
2	**5348**	138.81	34.48	15.81	15.38	**4035**	117.92	33.72	14.10	17.34
3	**3855**	1.74	0.00	0.00	2.75	**7604**	2.65	2.95	1.49	2.78
4	**2374**	86.59	35.89	11.90	10.28	**7881**	79.83	37.21	11.30	9.72
5	**8878**	0.00	42.14	19.95	44.06	**8938**	0.00	35.84	13.95	42.56
6	**1124**	68.34	40.74	17.77	18.22	**4294**	64.17	43.46	17.77	19.79
7	**4188**	110.80	41.68	12.00	14.56	**7073**	99.44	36.56	9.84	22.64
8	**5259**	64.68	28.96	6.95	16.83	**5636**	94.56	31.42	6.45	17.15
9	**4617**	84.19	57.98	20.25	32.82	**9406**	84.23	52.25	13.05	0.00
10	**1319**	67.76	36.32	9.92	10.96	**5756**	78.08	37.28	9.68	13.44
11	**9741**	131.37	48.19	16.26	14.14	**2986**	149.38	45.64	12.92	14.37
12	**3293**	104.02	46.96	12.82	19.28	**9850**	90.29	36.39	9.85	12.78
13	**5677**	0.00	29.60	9.60	14.40	**9910**	0.00	45.60	12.00	14.40
14	**1306**	87.40	36.87	13.12	17.22	**4103**	100.00	33.62	12.66	20.28
15	**2773**	101.50	41.15	8.49	11.00	**4038**	97.00	44.80	9.36	11.15
16	**3105**	87.76	36.27	11.24	11.73	**3188**	105.63	31.72	13.38	9.67
17	**4514**	106.17	32.25	12.92	0.00	**6758**	91.12	37.52	14.16	19.76
18	**6040**	110.34	33.44	11.00	12.17	**1501**	113.61	34.95	12.79	14.85
19	**8390**	84.61	39.48	12.01	4.08	**9525**	95.53	38.44	11.60	20.89
20	**4475**	87.85	30.70	9.25	12.20	**9333**	77.20	45.00	13.45	13.55
21	**1471**	109.25	20.92	6.16	8.56	**4696**	69.78	20.88	5.85	11.21

Table C 1: All reported results of material EFL1 sorted by laboratory identification; the two code columns show the sample codes of the blind duplicates

EFL2:										
LAB_ID	**Code**	DON	HT-2	T-2	ZON	**Code**	DON	HT-2	T-2	ZON
1	**4299**	240.59	35.56	18.97	27.67	**8498**	266.84	42.35	18.15	20.92
2	**5306**	268.68	47.61	23.94	30.30	**9319**	276.15	46.48	22.42	28.83
3	**5761**	4.44	4.15	2.84	3.55	**9712**	3.84	4.72	2.89	3.74
4	**9457**	234.97	50.02	17.48	25.72	**9755**	220.38	48.51	17.34	23.55
5	**4739**	195.77	45.53	22.34	44.18	**8920**	211.10	46.28	21.94	46.72
6	**1208**	219.30	56.52	30.18	38.39	**5462**	214.41	50.89	25.44	34.12
7	**1611**	226.00	55.36	17.04	34.88	**5797**	250.08	48.72	15.36	37.92
8	**9310**	318.66	33.11	10.70	42.67	**9530**	304.54	31.90	7.96	39.19
9	**3845**	264.08	76.09	0.00	44.09	**9837**	260.92	59.57	20.68	25.22
10	**8010**	252.00	52.88	17.04	32.80	**1449**	238.40	50.96	17.60	37.04
11	**7163**	311.39	55.73	17.23	31.43	**8358**	342.30	52.99	18.68	31.05
12	**5628**	212.66	23.16	17.30	24.36	**7496**	249.81	22.42	17.89	28.98
13	**4268**	0.00	58.40	24.00	24.00	**9773**	0.00	69.60	14.40	44.80
14	**3525**	238.68	45.66	21.38	27.97	**4615**	249.48	48.79	20.81	34.43
15	**5082**	275.00	55.85	14.70	28.40	**9238**	264.50	56.85	16.70	28.75
16	**3593**	250.20	51.63	20.80	28.13	**6374**	259.46	47.26	20.39	25.48
17	**9083**	237.62	44.20	20.91	19.80	**9925**	250.53	53.14	21.88	22.86
18	**3915**	289.36	48.86	21.14	26.74	**1719**	267.36	53.34	18.24	27.77
19	**8535**	258.31	58.70	20.57	30.43	**8543**	247.57	48.03	16.01	30.11
20	**2138**	261.50	65.00	19.45	30.20	**4158**	247.50	71.10	17.40	28.95
21	**8987**	199.84	36.52	11.58	23.31	**6979**	217.88	36.28	11.19	25.39

Table C 2: All reported results of material EFL2 sorted by laboratory identification; the two code columns show the sample codes of the blind duplicates

EFL3:										
LAB_ID	**Code**	DON	HT-2	T-2	ZON	**Code**	DON	HT-2	T-2	ZON
1	**1355**	541.96	142.22	44.34	390.53	**9680**	590.06	153.67	45.20	500.07
2	**7529**	614.13	175.69	48.19	390.15	**2130**	617.92	171.16	46.11	375.92
3	**1434**	14.34	13.44	6.31	54.17	**4438**	13.67	13.11	6.22	51.29
4	**1911**	498.86	163.34	47.06	382.09	**8241**	482.64	170.56	43.59	400.97
5	**2713**	766.63	195.32	56.21	650.26	**5386**	558.05	190.93	53.74	722.02
6	**2540**	510.02	169.13	56.98	446.33	**6726**	447.18	191.08	48.88	467.27
7	**1155**	539.44	174.72	46.72	449.20	**7033**	498.64	178.64	43.84	424.24
8	**4101**	575.32	158.92	48.45	363.85	**9545**	588.11	123.10	41.55	368.99
9	**7228**	545.90	190.10	47.54	465.07	**6789**	556.09	183.76	61.71	457.43
10	**9872**	521.60	162.00	49.92	359.20	**5565**	530.40	180.00	45.28	384.80
11	**7810**	602.10	184.33	54.83	467.25	**6492**	779.02	177.40	50.65	440.49
12	**1861**	510.47	280.39	57.86	376.59	**4457**	500.80	305.67	59.06	431.88
13	**1485**	0.00	188.80	52.00	604.80	**6916**	0.00	230.40	44.00	255.20
14	**2229**	528.63	175.51	46.99	438.98	**5860**	540.36	171.36	45.75	396.17
15	**3022**	596.50	186.00	53.25	463.00	**6582**	597.50	204.50	52.45	453.00
16	**2134**	579.13	199.70	48.97	448.93	**7713**	563.78	177.62	47.02	434.53
17	**4689**	522.49	139.94	47.85	345.65	**7644**	531.78	141.83	44.46	383.44
18	**6253**	614.32	170.72	61.60	450.96	**9208**	602.88	180.00	58.39	425.20
19	**2066**	541.82	177.61	51.95	442.77	**7368**	554.73	212.22	56.39	435.77
20	**1139**	561.50	180.00	45.10	455.00	**9742**	631.00	192.50	46.60	492.50
21	**3461**	496.18	117.28	33.13	256.16	**7298**	453.02	110.15	30.13	238.73

Table C 3: All reported results of material EFL3 sorted by laboratory identification; the two code columns show the sample codes of the blind duplicates

IRMMCER:										
LAB_ID	**Code**	DON	HT-2	T-2	ZON	**Code**	DON	HT-2	T-2	ZON
1	**9298**	145.24	45.83	6.60	2.33	**9531**	135.02	41.97	10.88	2.82
2	**1370**	176.66	51.69	13.11	8.13	**1545**	179.82	61.88	10.13	7.02
3	**7576**	0.00	0.00	0.00	0.00	**7416**	4.71	4.36	0.85	0.50
4	**7514**	137.82	53.64	5.56	2.85	**8545**	143.01	61.62	12.54	7.57
5	**4986**	0.00	55.69	10.22	0.00	**8824**	0.00	55.69	10.32	0.00
6	**2650**	109.46	53.90	15.05	12.58	**6493**	110.08	60.16	14.49	12.55
7	**2450**	150.16	60.64	5.20	5.12	**3941**	152.48	56.48	7.44	4.48
8	**5117**	172.36	54.69	8.86	4.42	**6818**	149.37	30.95	0.52	2.78
9	**1038**	107.15	60.26	0.00	0.00	**7902**	131.08	79.39	0.00	0.00
10	**8048**	122.80	59.92	4.80	3.60	**8518**	124.80	61.84	5.28	5.20
11	**3758**	196.94	52.25	6.32	0.00	**7418**	210.33	57.70	7.20	0.00
12	**8700**	135.86	27.04	7.18	7.06	**8803**	139.30	37.86	7.12	4.93
13	**8268**	0.00	44.80	6.40	6.40	**8559**	0.00	66.40	9.60	4.80
14	**4376**	143.06	44.43	8.64	9.91	**5424**	139.32	53.09	8.26	9.10
15	**2866**	159.00	51.95	3.52	3.67	**9559**	145.50	58.70	4.18	2.99
16	**4783**	151.05	52.46	10.23	2.16	**9924**	155.12	50.84	7.56	0.89
17	**4807**	155.70	41.87	7.16	0.00	**5671**	154.74	44.19	12.11	0.00
18	**1123**	616.48	216.40	21.64	19.94	**6742**	155.00	52.20	4.96	7.98
19	**5635**	146.50	62.47	7.20	6.65	**9851**	142.82	57.34	5.43	4.58
20	**1489**	147.50	53.05	4.99	0.00	**4063**	119.50	76.50	8.73	0.00
21	**5467**	106.26	26.08	5.62	1.18	**1961**	112.05	38.52	3.60	3.66

Table C 4: All reported results of material IRMMCER sorted by laboratory identification; the two code columns show the sample codes of the blind duplicates

IRMMFEED:										
LAB_ID	**Code**	DON	HT-2	T-2	ZON	**Code**	DON	HT-2	T-2	ZON
1	**1813**	248.18	13.98	12.37	17.55	**4173**	267.55	17.11	12.76	26.43
2	**3735**	378.68	27.76	8.08	31.18	**8929**	300.20	20.07	7.11	21.90
3	**6616**	4.36	1.45	0.71	0.00	**5689**	0.00	3.80	1.36	0.00
4	**3997**	280.64	19.23	3.94	14.76	**8237**	282.64	19.67	3.73	18.16
5	**4261**	217.42	19.38	10.78	0.00	**4740**	325.50	18.13	6.60	0.00
6	**3173**	228.10	31.28	0.00	26.39	**6323**	232.27	35.27	0.00	26.96
7	**2193**	286.24	15.28	5.04	26.00	**6020**	263.36	18.88	5.36	33.44
8	**1959**	316.28	28.57	4.64	27.53	**4340**	302.34	25.74	2.93	27.45
9	**9907**	326.20	47.03	0.00	0.00	**5095**	274.49	0.00	0.00	0.00
10	**7948**	252.80	8.80	2.40	12.24	**6445**	268.80	10.08	3.04	14.00
11	**5161**	422.05	22.44	0.00	20.95	**8266**	396.61	29.96	0.00	19.25
12	**7393**	348.46	42.03	6.16	16.47	**8325**	362.82	41.63	5.24	18.93
13	**3054**	0.00	56.00	8.00	27.20	**9047**	0.00	50.40	7.20	0.00
14	**3557**	279.84	19.74	5.06	18.82	**9797**	254.22	17.58	0.00	19.56
15	**2943**	287.50	16.60	3.52	15.65	**8800**	317.50	21.00	5.86	15.50
16	**6752**	290.91	18.21	3.60	15.28	**8140**	291.79	17.68	2.61	14.75
17	**5965**	263.52	18.30	5.31	5.54	**7823**	270.01	17.19	4.46	2.63
18	**5355**	276.08	22.62	4.12	16.50	**6766**	306.56	19.50	6.92	45.17
19	**5870**	280.14	18.60	4.23	3.02	**9539**	254.50	34.62	5.03	1.04
20	**4339**	274.50	20.05	0.00	11.75	**8949**	259.00	25.80	0.00	13.00
21	**2316**	268.47	21.91	4.06	8.97	**4968**	234.00	19.22	2.11	19.64

Table C 5: All reported results of material IRMMFEED sorted by laboratory identification; the two code columns show the sample codes of the blind duplicates

The participating laboratories sorted by nationality. The order is not related to the laboratory IDs in the tables above.

Contact person		Laboratory	Address			Country
Philippe	Debongnie	CODA-CERVA Unit Toxins & Natural Substances	Leuvensesteenweg 17	3080	Tervuren	Belgium
Gary	Neumann	Health Canada Health Products and Food Program	510 Lagimodiere Boulevard	R2J 3Y1	Winnipeg Province: Manitoba	Canada
Steve	Clegg	University of Guelph Laboratory Services Division	95 Stone Road West	N1H8J7	Guelph, ON	Canada
Alena	Honzlova	State Veterinary Institute Jihlava	Rantirovská 93	586 05	Jihlava	Czech Republik
Yvonne	Simonsen	Danish Veterinary and Food Administration Feed Laboratory	Skovbrynet 20	2800	Kgs. Lyngby	Denmark
Anri	Aallonen	Ramboll Finland ltd Ramboll Analytics	Niemenkatu 73	15140	LAHTI	Finland
Marie-Paul	Herry	Laboratoire SCL de Rennes	26 rue Antoine Joly	35000	RENNES	France
Benedikt	Brand	Staatliches Veterinäruntersuchungsamt Arnsberg	Zur Taubeneiche 10-12	59821	Arnsberg	Germany
Christian	Struck	Chemisches und Veterinäruntersuchungsamt Münsterland-Emscher-Lippe	Joseph-König-Str. 40	48147	Münster	Germany

Annex C / The individual results and participating laboratories

Contact person		**Laboratory**	**Address**			**Country**
Ebru	Ates	Thermo Fisher Scientific Food Safety Response Center	Im Steingrund 4-6	63303	Dreieich	Germany
Gudrun	Hanschmann	STAATLICHE BETRIEBSGESELLSCHAFT FÜR UMWELT UND LANDWIRTSCHAFT	Gustav-Kühn-Straße 8	04159	Leipzig	Germany
John	Keegan	Public Analyst's Laboratory	Sir Patrick Dun's, Lower Grand Canal Street		Dublin 2	Ireland
Veronica	Lattanzio	Institute of Sciences of Food Production - National Research Council (CNR)	via G. Amendola, 122/O	70126	Bari	Italy
Guntis	Cepurnieks	BIOR	Lejupes Street 3	1076	Riga	Latvia
Robert	Kosicki	Kazimierz Wielki University Department of Experimental Biology Mycotoxin Analytical Laboratory	Chodkiewicza 30	85-064	Bydgoszcz	Poland
Asun	Suarez	Laboratori Agencia Salut Publica Barcelona	Av Drassanes 13-15	8001	Barcelona	Spain
Alexey	Solyakov	Statens Veterinärmedicinska Anstalt Enhet för kemi, miljö och fodersäkerhet	Travv 20	751 89	Uppsala	Sweden
Susan	MacDonald	The Food and Environment Research Agency	Sand Hutton	YO41 1LZ	York	United Kingdom
Chia-Ding	Liao	Center for Food Safety and Applied Nutrition (CFSAN) / U.S. FDA	5100 Paint Branch Parkway	MD 20740	College Park	USA

Annex C / The individual results and participating laboratories

Contact person		Laboratory	Address			Country
Jack C.	Cappozzo	NCFST IIT	6502 S. Archer Rd.	IL 60501	Summit-Argo	USA
Susie: Yuan	Dai	Office of the Texas State Chemist, Texas A&M University	445 Agronomy Rd	TX 77843	College Station	USA

Table C 6: Invited laboratories ordered by Country; the order is not related to laboratory identification in any of the figures or tables

Contact person		Laboratory	Address			Country
Horst	Klaffke	Bundesinstitute für Risikobewertung, Abt. 83	Thielallee 88-92	14195	Berlin	Germany
Simone	Staiger	Eurofins WEJ Contaminants GmbH	Neulaender Kamp 1	21079	Hamburg	Germany
Theo	de Rijk	RIKILT- Institute of Food Safety	Akkermaalsbos 2	6708 WB	Wageningen	The Netherlands
Susan	MacDonald	The Food and Environment Research Agency	Sand Hutton	YO41 1LZ	York	United Kingdom
Dionisis	Theodosis	LGC Limited,	Queens Rd	TW11 0LY	Teddington	United Kingdom

Table C 7: Participating laboratories of the pilot study ordered by Country; the order is not related to laboratory identification in any of the figures or tables

Annex D

Graphs

The following plots depict the Mandel's *h* statistics per analyte for all laboratories and test materials:

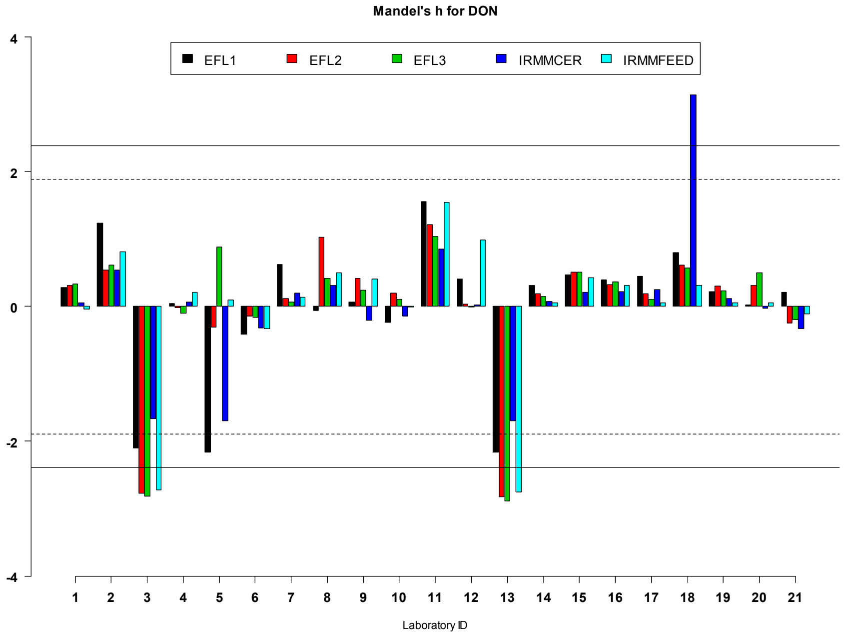

Figure D 1: Plot of Mandel's h statistic for DON in all five materials grouped by laboratory

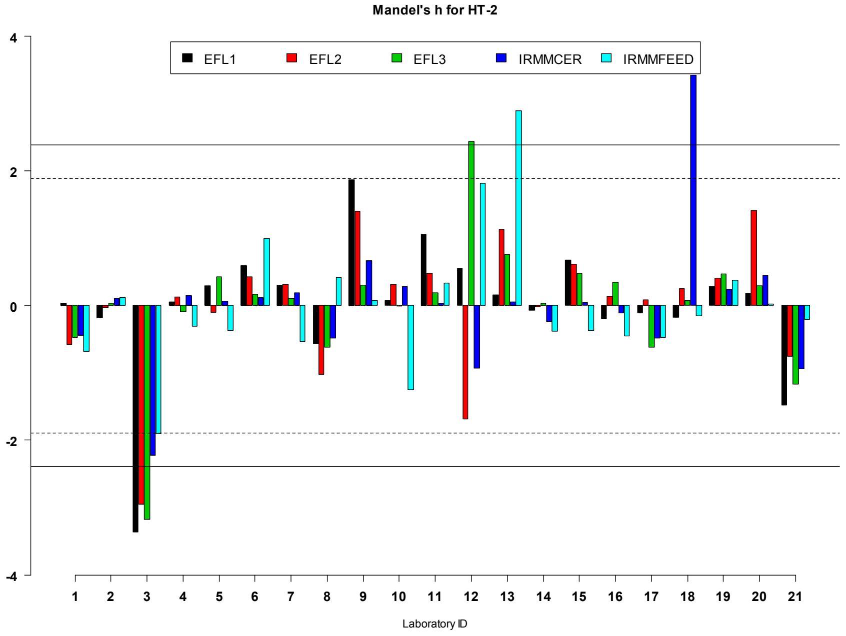

Figure D 2: Plot of Mandel's h statistic for HT-2 in all five materials grouped by laboratory

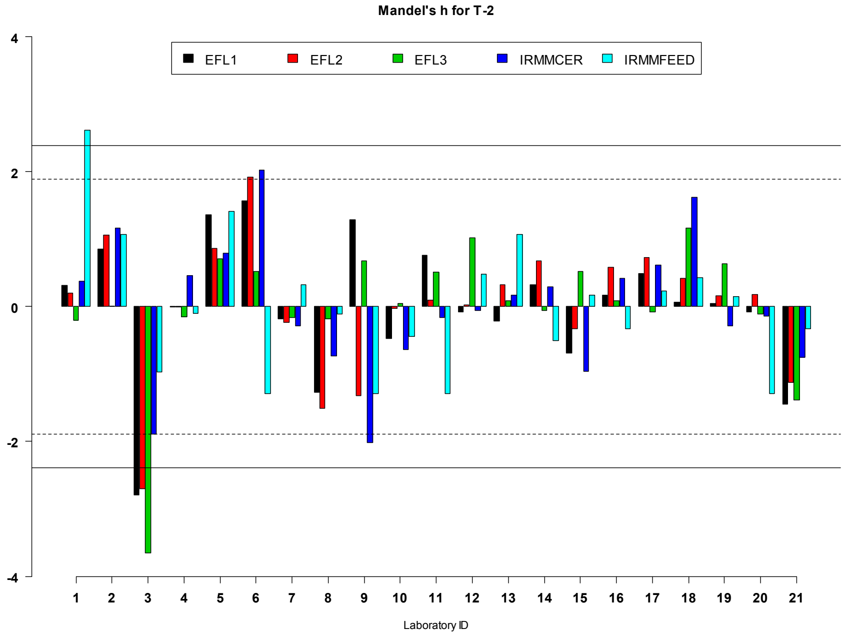

Figure D 3: Plot of Mandel's h statistic for T-2 in all five materials grouped by laboratory

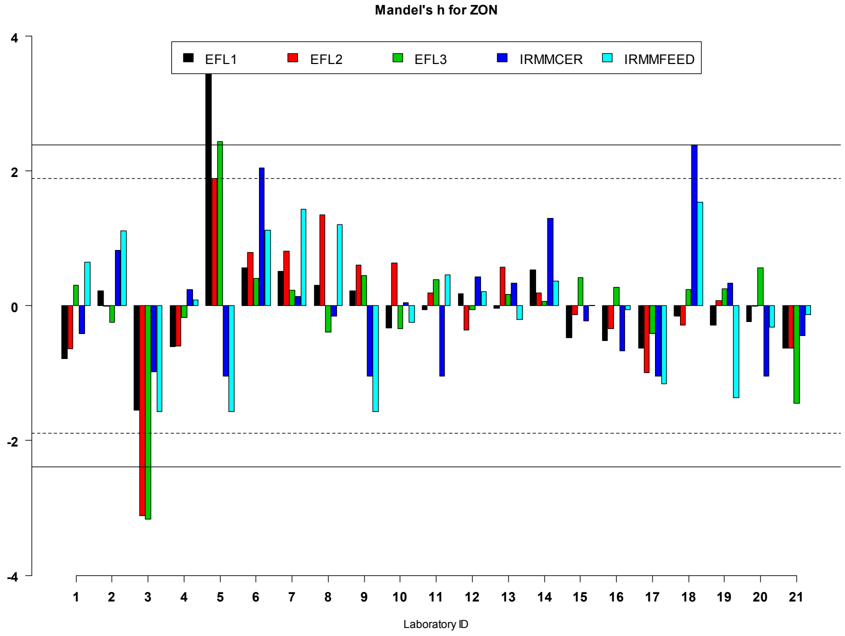

Figure D 4: Plot of Mandel's h statistic for ZON in all five materials grouped by laboratory

The following graphs depict the mean and range of the duplicate determinations per laboratory sorted by increasing mean in the five test materials:

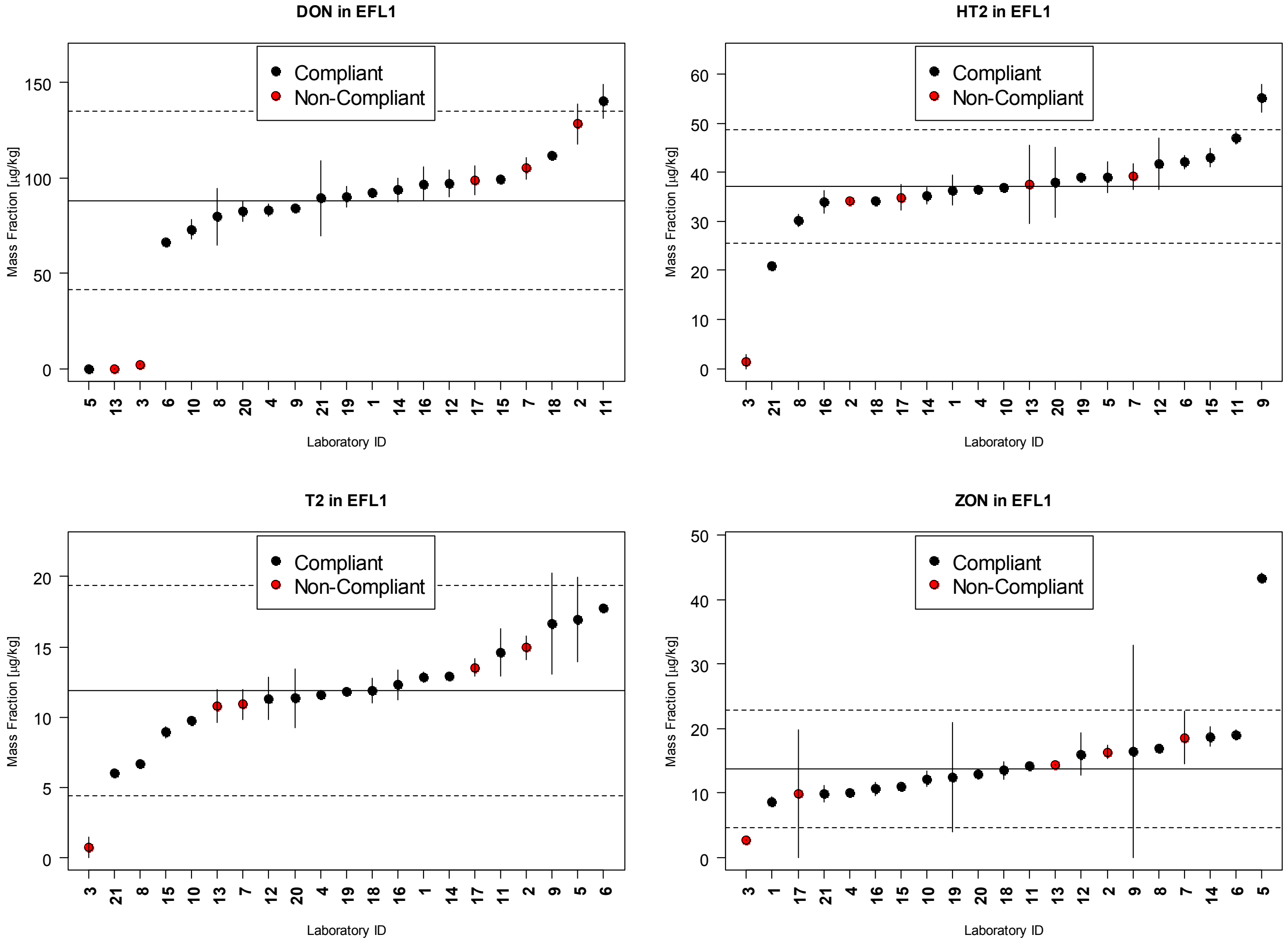

Figure D 5: Mean & Range plots of the four analytes in material EFL1; circles depict the mean of the blind duplicates per laboratory, vertical lines the range of the blind duplicates per laboratory, the solid horizontal line represents the robust overall mean and the broken horizontal lines the expanded robust reproducibility standard deviation (coverage factor of 2 for ~95% confidence)

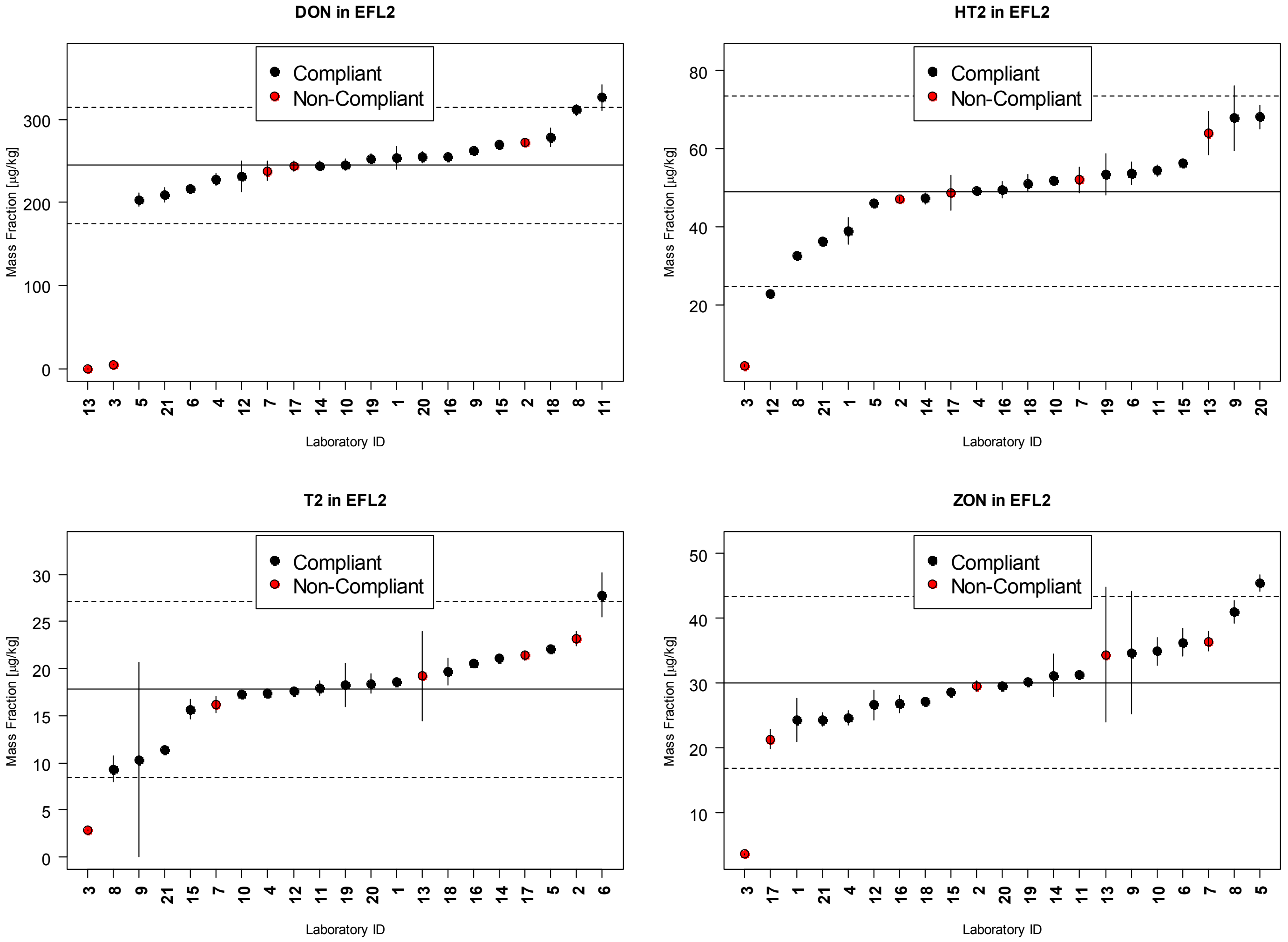

Figure D 6: Mean & Range plots of the four analytes in material EFL2; circles depict the mean of the blind duplicates per laboratory, vertical lines the range of the blind duplicates per laboratory, the solid horizontal line represents the robust overall mean and the broken horizontal lines the expanded robust reproducibility standard deviation (coverage factor of 2 for ~95% confidence)

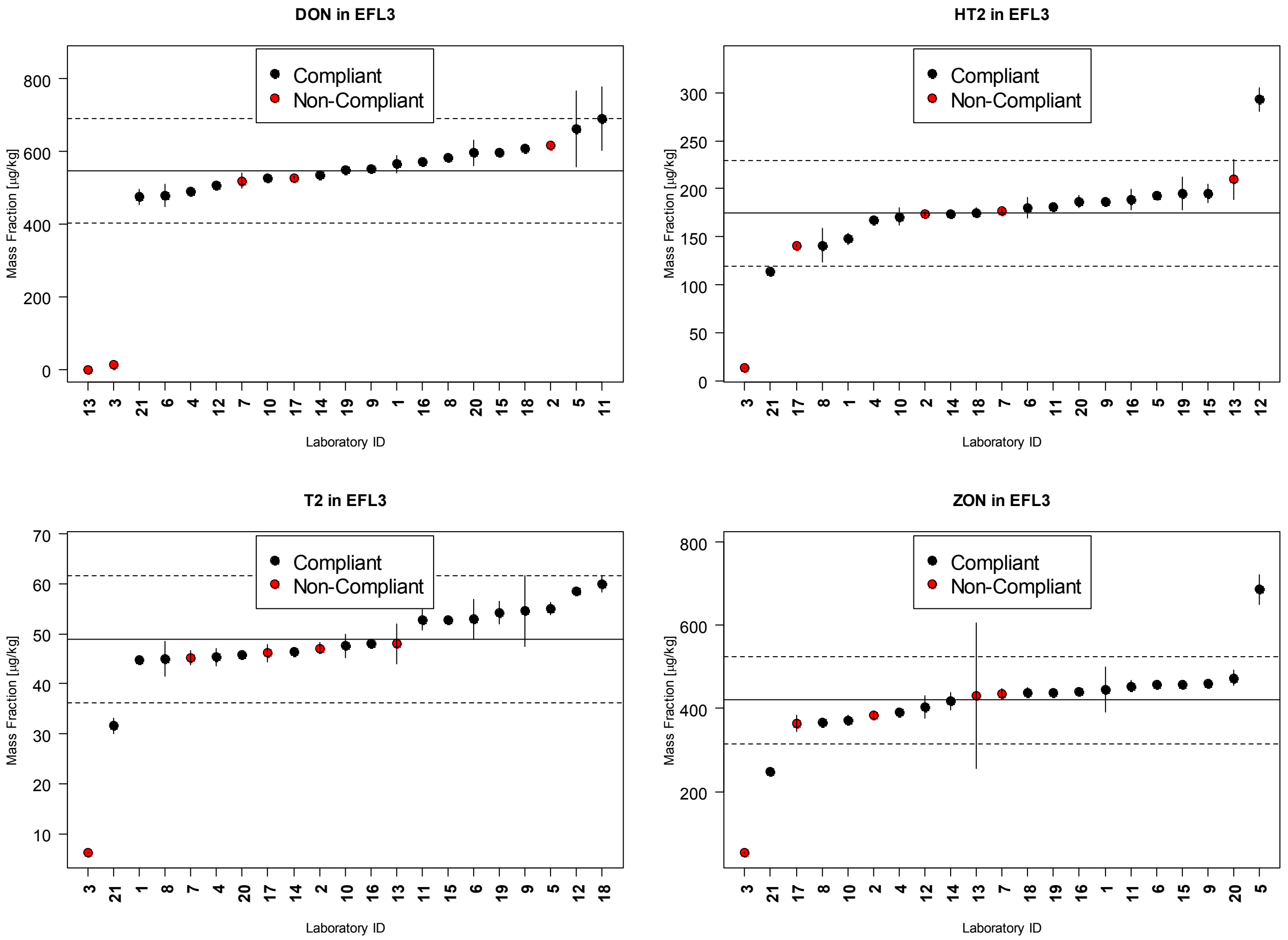

Figure D 7: Mean & Range plots of the four analytes in material EFL3; circles depict the mean of the blind duplicates per laboratory, vertical lines the range of the blind duplicates per laboratory, the solid horizontal line represents the robust overall mean and the broken horizontal lines the expanded robust reproducibility standard deviation (coverage factor of 2 for ~95% confidence)

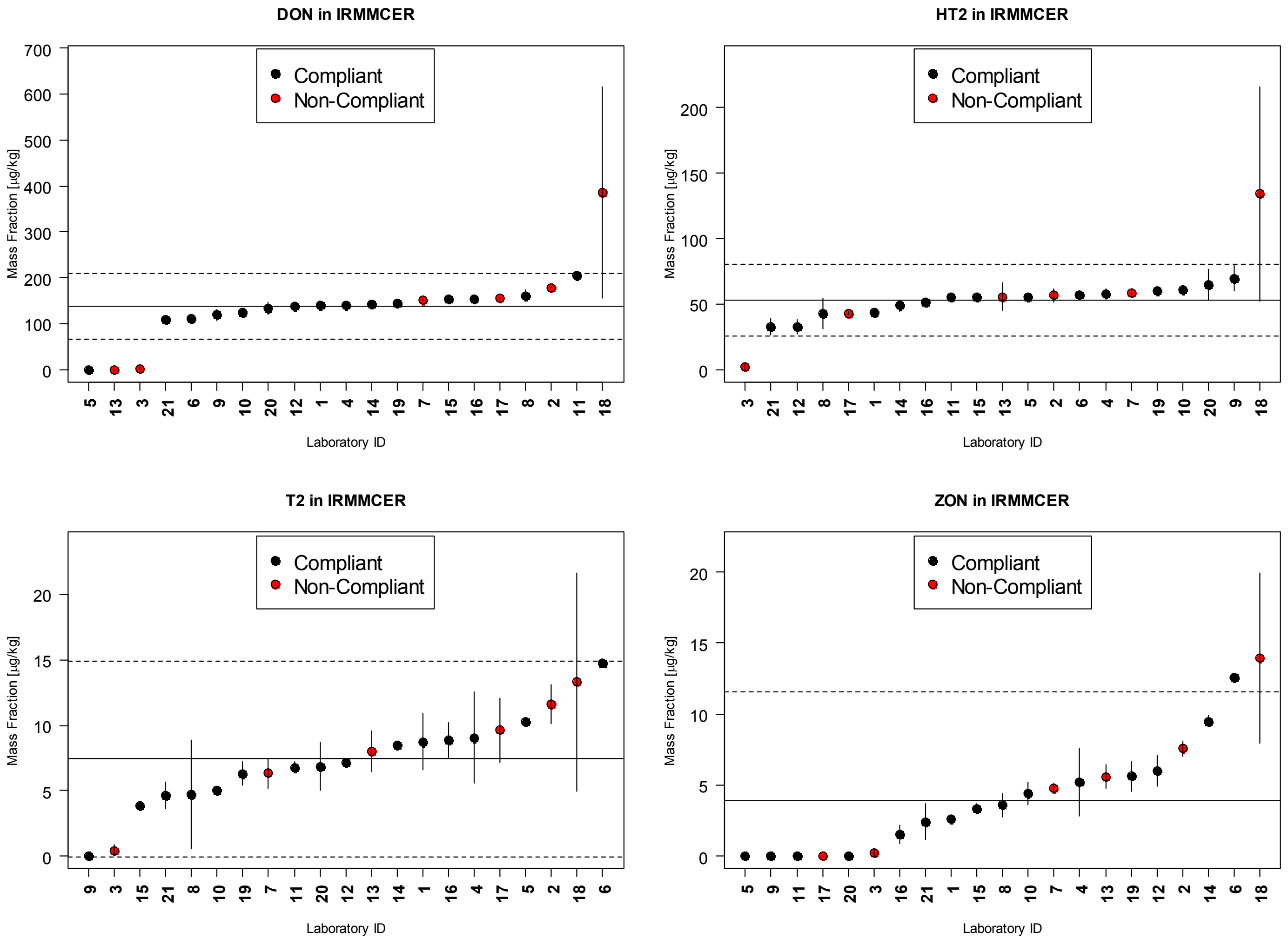

Figure D 8: Mean & Range plots of the four analytes in material IRMMCER; circles depict the mean of the blind duplicates per laboratory, vertical lines the range of the blind duplicates per laboratory, the solid horizontal line represents the robust overall mean and the broken horizontal lines the expanded robust reproducibility standard deviation (coverage factor of 2 for ~95% confidence)

Annex D / Graphs

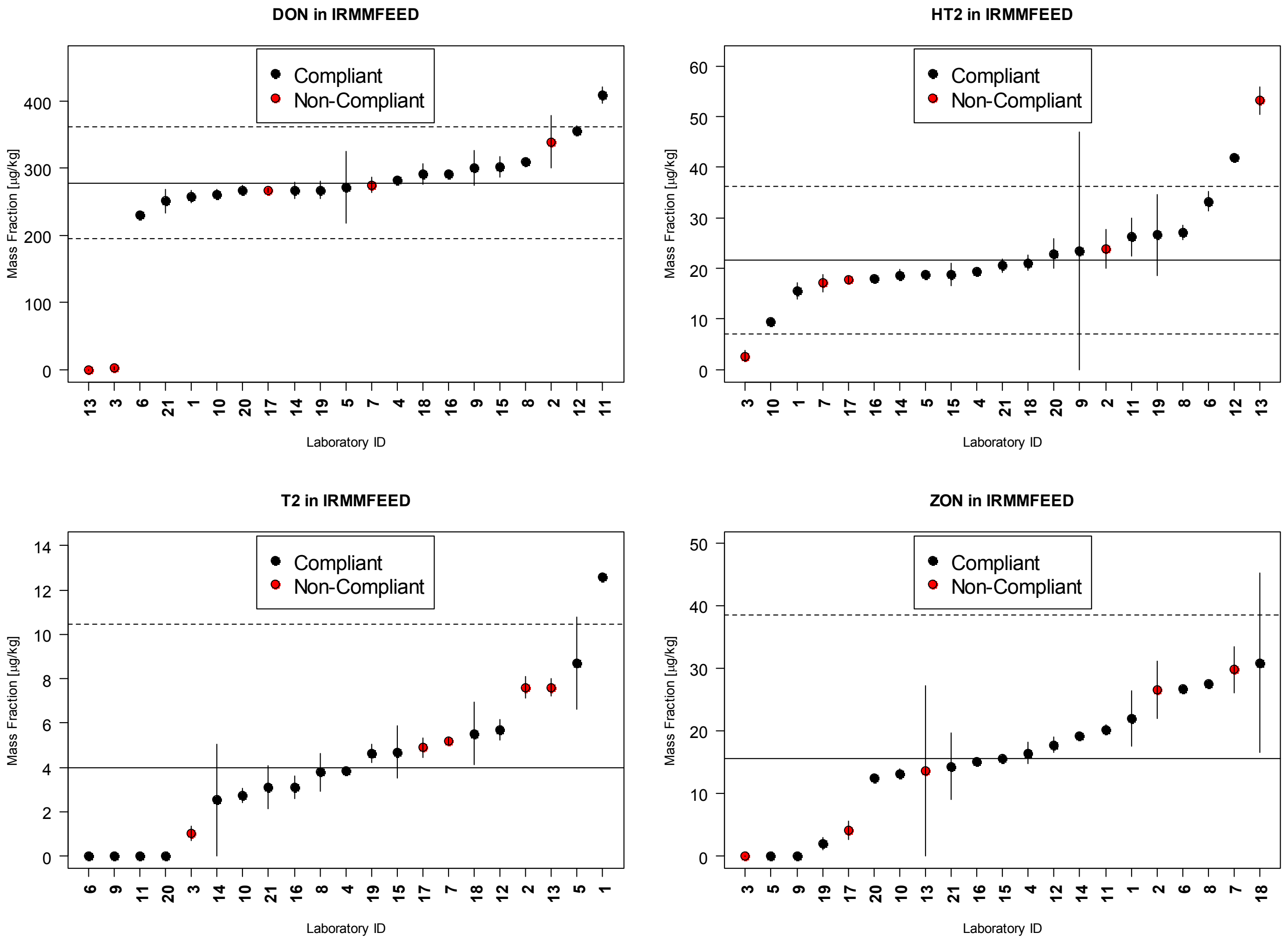

Figure D 9: Mean & Range plots of the four analytes in material IRMMFEED; circles depict the mean of the blind duplicates per laboratory, vertical lines the range of the blind duplicates per laboratory, the solid horizontal line represents the robust overall mean and the broken horizontal lines the expanded robust reproducibility standard deviation (coverage factor of 2 for ~95% confidence)

Annex E

The classical statistical evaluation / Measurement uncertainty estimation

Annex E / the classical statistical evaluation / Measurement uncertainty estimation

The performance characteristics of the method with the classical approach of outlier removal are listed below. Outlier removal was performed as described in the AOAC Guideline [1]. Only values for which the Cochran or Grubbs tests indicated a probability $p<0.01$ were removed.

Material	Labs total	Labs non-compl.	Labs outl.	Labs ret'd	Mean	sr	r	RSDr	sR	R	RSDR	Hor Rat	Labs rem'd
DON													
EFL1	21	5	2	14	88.5	11.3	32	**13**	14	40	**16**	0.7	5,11
EFL2	21	5	0	16	252.6	12.9	36	**5**	35	97	**14**	0.7	
EFL3	21	5	0	16	561.5	52.8	148	**9**	72	202	**13**	0.7	
IRMMCER	21	6	1	14	140.7	9.4	26	**7**	25	70	**18**	0.8	5
IRMMFEED	21	5	3	13	275.2	17.6	49	**6**	26	74	**10**	0.5	5,11,12
HT-2													
EFL1	21	5	2	14	38.1	4.1	11	**11**	5	15	**14**	0.6	9,21
EFL2	21	5	0	16	48.7	4.2	12	**9**	12	34	**25**	1.1	
EFL3	21	5	1	15	173	12.5	35	**7**	25	69	**14**	0.7	12
IRMMCER	21	6	0	15	52.5	8.3	23	**16**	12	34	**23**	1.1	
IRMMFEED	21	5	2	14	22.3	2.5	7	**11**	8	23	**37**	1.7	9,19
T-2													
EFL1	21	5	0	16	12.1	2.1	6	**17**	4	10	**30**	1.4	
EFL2	21	5	1	15	18.2	1.5	4	**9**	4	12	**24**	1.1	9
EFL3	21	5	0	16	49.7	3.6	10	**7**	7	21	**15**	0.7	
IRMMCER	21	6	0	15	7	2.4	7	**34**	4	11	**53**	2.4	
IRMMFEED	21	5	0	16	3.8	1.4	4	**38**	4	10	**93**	4.2	
ZON													
EFL1	21	5	3	13	13.4	1.8	5	**13**	4	10	**27**	1.2	5,9,19
EFL2	21	5	1	15	30.8	2.5	7	**8**	6	18	**21**	0.9	9
EFL3	21	5	2	14	430	27.8	78	**7**	39	110	**9**	0.5	5,21
IRMMCER	21	6	0	15	3.8	1.2	3	**33**	4	11	**99**	4.5	
IRMMFEED	21	5	1	15	14.8	2.7	8	**18**	9	25	**59**	2.7	18

Table E 1: The classical performance characteristics for the five materials grouped by analyte; Labs total - total number of labs reporting, Labs non-compl. - Labs excluded for non-compliance, Labs outl. - Labs removed because of outlying results, Labs ret'd - Labs retained in the calculations, Mean - mean value of retained labs, s_r – repeatability standard deviation, r – repeatability, RSD_r – relative repeatability standard deviation, s_R – reproducibility standard deviation, R – reproducibility, RSD_R – relative reproducibility standard deviation, HorRat – Horwitz Ratio, Labs rem'd – IDs of the removed laboratories

1. AOAC Official Methods Program, ed. *Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a method of Analysis*. AOAC Official Methods Program. Vol. 78(5). 2002, J. AOAC Int. .

Measurement uncertainty estimation / compliance testing (according to ISO 21748:2010):

Laboratory bias:

The laboratory bias is determined as:

$$\left|\Delta_l\right| = m - \mu \qquad \text{(E. 1)}$$

with m being the mean mass fraction of n determinations of a test material of known contamination (CRM or other reference material) with standard deviation s_w, and μ being the expected mass fraction of the reference material. To ensure that the uncertainty of the laboratory bias determination is small compared to the reproducibility standard deviation a minimum number n of replications is needed.

$$\sqrt{\frac{s_w^2}{n}} < 0.2 s_R \qquad \text{(E. 2)}$$

Rearranging for n and replacing s_R with $\hat{s}_R$ (see Eq. 8) and s_w with $\hat{s}_r$ (see Eq. 7) the following relationship can be derived:

$$n > \frac{\left(a_r + b_r w\right)^2}{0.04\left(a_R + b_R w\right)^2} \qquad \text{(E. 3)}$$

The values to calculate n for the different analytes can be found in Table E 2.

Whether the laboratory bias $|\Delta_l|$ is compliant with the laboratory bias component s_L of the collaborative study is tested as follows (Note that this procedure assumes that the uncertainty associated with the reference value is small compared to uncertainty of the laboratory bias):

$$\left|\Delta_l\right| < 2 \times \sqrt{s_L^2 + \frac{s_w^2}{n}} \qquad \text{(E. 4)}$$

Since the tested reference material has most likely a different contamination level than the test materials of the collaborative study and s_L has a dependency on the mass fraction provisions must be made for this. In analogy to Eqs. 7 & 8 s_L can be expressed as a function of the mass fraction w. For the data from this study a first order model with fixed term showed to be sufficiently accurate:

$$\hat{s}_L = a_L + b_L w \qquad \text{(E. 5)}$$

With w replaced by m Eq. E. 4 can be rewritten as:

$$|\Delta_l| < 2 \times \sqrt{(a_L + b_L m)^2 + \frac{s_w^2}{n}} \qquad \text{(E. 6)}$$

The relationship above compares the laboratory bias with a 95% confidence interval consisting of the laboratory bias component of the collaborative study and the uncertainty of the laboratory bias determination. With the values from Table E 2 $\hat{s}_L$ can be calculated for any mass fraction within the working range and then Eq. E. 6 is used to determine whether the laboratory bias is compliant.

Analyte	a_R	b_R	a_r	b_r	a_L	b_L
DON	8.6	0.10	3.8	0.05	5.8	0.09
HT-2	3.5	0.13	1.5	0.07	4.4	0.08
T-2	2.8	0.08	1.2	0.04	2.5	0.07
ZON	4.3	0.10	1.0	0.06	4.2	0.09

Table E 2: the coefficients of the functional relationships between the precision estimates and the mass fraction; *a* represents a fixed intercept and *b* the coefficient of the mass fraction; the indices *R, r* and *L* represent the reproducibility, repeatability, and laboratory bias, respectively.

In the case of non-compliance (the laboratory bias is outside the 95% confidence interval) investigation of the cause of the excessive bias should be conducted and any identified causes should be eliminated.

Repeatability:

To show whether a laboratory is compliant with the repeatability standard deviation s_r determined during the collaborative study it needs to determine its individual repeatability standard deviation s_i with v_i degrees of freedom. This can be done by repeatedly measuring one suitable material or by pooling results from different materials. If results are pooled it must be ensured that the standard deviations are constant for different test items. Otherwise a general model like in Eqs. 7, 8, and E. 5 needs to be derived. In any case v_i should be no less than 15.

Once s_i has been determined an F-test will be used to compare it to $\hat{s}_r$ (Eq. 7 and Table E 2):

$$F = \frac{s_i^2}{\left(a_r + b_r w_i\right)^2} \quad \text{(E. 7)}$$

with w_i the mass fraction of the investigated test material and a_r and b_r from Table E 2.

As long as F is smaller than some critical value $F_{(1-\alpha/2, vi, vr)}$ with:

α = error of first kind (0.05 for a 95% confidence),

v_i = degrees of freedom of s_i (number of replicates minus one),

v_r = degrees of freedom of $\hat{s}_r$ (for this study 16 participating laboratories times two replicates times five materials),

the repeatability of the laboratory is in compliance with the repeatability of the collaborative study.

If this test shows a non compliance (F is larger than critical value) the laboratory has two options. The first option would be to investigate and eliminate the cause of s_i being significantly larger than $\hat{s}_r$. The second option would be to use s_i in place of s_r and recalculate s_R:

$$s'_R = \sqrt{s_L^2 + s_i^2} \qquad \text{(E. 8)}$$

The result will be a larger estimate for the reproducibility. The opposite case with s_i being significantly smaller than s_r may be dealt with in the same way leading to a smaller estimate of s_R.

Mean	s_r	s_R	s_L	MATERIAL
DON				
88.5	9.5	17	14.1	EFL1
250	13.6	33.3	30.4	EFL2
558.6	30.1	66.9	59.7	EFL3
135.8	8.2	23	21.5	IRMMCER
281.8	19.9	33.1	26.4	IRMMFEED
HT-2				
38	3.4	6.2	5.2	EFL1
49.1	3.4	12	11.5	EFL2
177.6	13.5	23.2	18.9	EFL3
53.1	8.1	12.4	9.4	IRMMCER
22	3.3	6.3	5.4	IRMMFEED
T-2				
12.1	1.7	3.9	3.5	EFL1
17.7	1.6	4.4	4.1	EFL2
50.3	3.1	6.5	5.7	EFL3
7	1.8	3.1	2.5	IRMMCER
3.5	1.2	3.1	2.9	IRMMFEED
ZON				
13.9	2	4.3	3.8	EFL1
30.5	2.9	6	5.3	EFL2
430	25	49.3	42.5	EFL3
3.4	1.1	3.3	3.1	IRMMCER
15.9	1.7	10.4	10.3	IRMMFEED

Table E 3: Mean mass fraction, repeatability standard deviation (s_r), reproducibility standard deviation (s_R) and laboratory bias component (s_L) for the five materials grouped by analyte

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Title: **LC-MS Based Method of Analysis for the Simultaneous Determination of four Mycotoxins in Cereals and Feed**

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Abstract

An LC-MS/MS based method of analysis to determine the four Fusarium toxins deoxynivalenol, HT-2 toxin, T-2 toxin, and zearalenone in cereals and cereal-based compound animal feed has been validated through a collaborative study. After extraction of the mycotoxins with ethyl acetate / water, and addition of sodium sulphate an aliquot of the organic phase was spiked with stable-isotope labelled isotopologues of the targeted analytes and dried down. The dry extract was then reconstituted with mobile phase and injected into a LC-MS. The described use of the isotopologues keeps costs down while still offering many of their benefits. This is evidenced by relative repeatability standard deviations (RSD_r) between 5 and 15 %. Exceptions were T-2 toxin at 7 µg/kg with 27%, and at 3.5 µg/kg with 35%, and zearalenone at 3.4 µg/kg with 32% RSD_r.

The tested contamination ranges were 88 to 559 µg/kg for deoxynivalenol, 22 to 178 µg/kg for HT-2 toxin, 3.5 to 50 µg/kg for T-2 toxin, and 3.4 to 430 µg/kg for zearalenone. For 10 of the 20 analyte / matrix combinations (four analytes in five matrices) Horwitz ratios between 0.6 and 0.9 were computed, for another six the ratios were below 1.5. The remaining four test samples were associated with Horwitz ratios between 2.0 and 4.4. They were the samples described above, two containing T-2 toxin and one zearalenone, plus one complex matrix sample containing zearalenone at a low contamination level. For this complex matrix sample we were able to show the importance of proper separation in LC-MS.

Because of the use of test materials having assigned reference values in this study trueness could be assessed. The observed biases were small and only significant for deoxynivalenol (-8%) and HT-2 toxin (-11%). For T-2 toxin and zearalenone they were insignificant. To facilitate the checking of compliance of a test result produced with this method with legislation a description on how to estimate measurement uncertainty based on these results is provided.

All of the above shows that the studied method is fit for the purpose of enforcing existing and anticipated legislative limits of the four Fusarium toxins deoxynivalenol, HT-2 toxin, T-2 toxin, and zearalenone in unprocessed cereals and cereal-based compound animal feed.

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