National Research Council of Italy
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Report of the 2014 Proficiency Test for LC-MS(MS) multi-mycotoxin methods

Determination of DON, FB_1, FB_2, ZEA, T-2, HT-2, OTA, AFB_1, AFG_1, AFB_2, AFG_2 in maize

and

Determination of DON, ZEA, T-2, HT-2, OTA in wheat

Annalisa De Girolamo[a], Biancamaria Ciasca[a], Joerg Stroka[b], Stefanka Bratinova[b], Angelo Visconti[a] and Veronica M.T. Lattanzio[a]

[a] *Institute of Sciences of Food Production, National Research Council of Italy*
[b] *Institute for Reference Materials and Measurements, European Commission, Joint Research Centre*

MoniQA
Towards harmonisation of analytical methods to monitor and control quality and safety in the food chain supply

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European Commission
Joint Research Centre
Institute for Reference Materials and Measurements
http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

Contacts information

Veronica M.T. Lattanzio
Address: via G. Amendola 122/O, 70126 Bari, Italy
e-mail: veronica.lattanzio@ispa.cnr.it
Tel: +39.080.5929364
Fax: +39.080.5929374

Annalisa De Girolamo
Address: via G. Amendola 122/O, 70126 Bari, Italy
e-mail: annalisa.degirolamo@ispa.cnr.it
Tel: +39.080.5929351
Fax: +39.080.5929374

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Annalisa De Girolamo
Biancamaria Ciasca
Joerg Stroka
Stefanka Bratinova
Angelo Visconti
Veronica M.T. Lattanzio

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PT coordinators: Veronica M.T. Lattanzio and Annalisa De Girolamo

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1. Summary

This report presents the results of the 2014 Proficiency Test (PT) for determination of DON, FB_1, FB_2, ZEA, T-2, HT-2, OTA, AFB_1, AFG_1, AFB_2, AFG_2 in maize and for determination of DON, ZEA, T-2, HT-2, OTA in wheat. The main objective of this PT was to provide interested laboratories with an opportunity to test their multi-mycotoxin methods and to compare their results with those of other laboratories.

The PT was free of charge and was organized by ISPA-CNR in the framework of the Italian project S.I.Mi.S.A. (*PON02_00186_3417512*) and promoted by the MoniQA Association (www.moniqa.org). The S.I.Mi.S.A project addresses the wide area of Food Safety, in a context requiring continuous efforts to increase the safety level of food products, by a structured approach of advanced research, led by experts of international standing level. The MoniQA Association focuses on validation of and setting performance criteria/requirements for methods used to analyse foods and food products for safety and quality. MoniQA organizes, manages or supports international ring trials to validate methods for regulatory and surveillance purposes.

The contaminated maize and wheat test materials were produced and characterized by the ISPA-CNR and dispatched to the participants in June 2014. Each participant received two batches containing approximately 80 g of each test material with unknown levels of mycotoxins. Each participant was asked to analyze each sample twice by using its method of choice. The use of LC-MS(MS) methods, although not strictly required, was highly recommended, while the use of multi-mycotoxin methods was mandatory; however participants were not obliged to determine all toxins in each material, and let free to report only on those mycotoxins that could be simultaneously determined with their multi-mycotoxin methodology. Twenty-two participants from 10 countries registered for the exercise. Nineteen laboratories returned 22 sets of results for various combinations of analytes. Three laboratories returned two sets of results obtained by using two different methods for both contaminated maize and wheat. Fifty-five percent of laboratories analysed all the 11 targeted mycotoxins in maize, whereas 73% of laboratories analysed all the 5 targeted mycotoxins in wheat. The remaining laboratories reported results for different combinations of analytes in both matrices.

The assigned values (consensus values) were calculated according to ISO 13528:2005 whereas the target standard deviation was derived from the truncated Horwitz equation. No statistical evaluation was reported for AFB_2, AFG_2 in maize due to lack of sufficient quantitative data.

Laboratory results for determination of DON, FB_1, FB_2, ZEA, T-2, HT-2, OTA ,AFB_1 and AFG_1 in maize and for determination of DON, ZEA, T-2, HT-2 and OTA in wheat were rated with z-scores in accordance with ISO 13528 and the International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories.

The assigned values for maize test materials were 1264 µg/kg for DON, 1305 µg/kg for FB_1, 350 µg/kg for FB_2, 2.73 µg/kg for OTA, 54.4 µg/kg for T-2, 30.7 µg/kg for HT-2, 21.7 µg/kg for ZEA, 1.35 µg/kg for AFB_1 and 0.63 µg/kg for AFG_1.

The assigned values for wheat test materials were 1298 µg/kg for DON, 7.21 µg/kg for OTA, 8.26 µg/kg for T-2, 58.8 µg/kg for HT-2 and 148 µg/kg for ZEA.

2. Introduction

Mycotoxin contamination of agricultural food commodities and beverages poses a risk to human and animal health due to their toxic effects. Over 100 mycotoxins have been identified, although only a few of them present a significant source of food-borne illnesses and are of major concern worldwide. They are: aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2), ochratoxin A (OTA), fumonisins B_1 (FB_1) and B_2 (FB_2), deoxynivalenol (DON), zearalenone (ZEA), T-2 and HT-2 toxins (**Figure 1**) [1].

Mycotoxins can have toxic effects that range from acute to chronic symptoms. Some mycotoxins have been shown to be mutagenic, teratogenic, or/and carcinogenic. Symptoms of intoxications range from skin irritation to immunosuppression, hepatotoxicity, and nephrotoxicity [2]. In Europe, harmonized maximum levels for mycotoxins in foodstuffs have been specified in the Commission Regulation EC 1881/2006 [3], that has been further amended by the Regulation EC 1126/2007 for *Fusarium* toxins in maize and maize products [4], by Regulation EC 594/2012 for OTA in foodstuffs [5], by Regulations EC 165/2010 for aflatoxins in foodstuffs [6] and 1058/2012 for aflatoxins in dried figs [7]. Very recently, the Recommendation EC 165/2013 has been issued setting maximum recommended levels for the sum of T-2 (T-2) and HT-2 (HT-2) toxins in cereals and cereal products [8]. All these mycotoxins can occur in most cereals and can be retained in the relevant processed products (food/feed), with exception of fumonisins that can occur mainly in maize and are of concern only for maize and products thereof.

Effective and efficient analytical methods are required to identify and determine mycotoxins at legislated levels and enforce regulatory limits. In the recent decades several methods, mainly based on high-performance liquid chromatography (HPLC), have been developed and are extensively reviewed for the analysis of single mycotoxins or group of mycotoxins in food and feed [9-11]. Among them, multi-analyte methods have become the ones most required because several mycotoxins frequently occur in the same food product. Within this context the application of LC-MS(MS) techniques is being largely explored since it enables the simultaneous monitoring of different mycotoxins in one method. Moreover, it offers several advantages in terms of high selectivity and sensitivity, substantial reduction of sample treatment and reliable quantification and confirmation of identity at regulated levels [12]. Even though LC-MS(MS) methodologies for single or multiple mycotoxin determination are routinely used in control laboratories, to date none of official or standard methods approved by AOAC International or CEN (European Standardization Committee) is based on LC-MS.

Within the EU Network of Excellence MoniQA (www.MoniQA.eu) efforts have been made for method comparison and deeper understanding of performances of the available LC-MS(MS) methodologies for multiple-mycotoxin analysis. For these purposes in 2012 a proficiency test was conducted to benchmark laboratories using LC-MS(MS) for multi-mycotoxin analysis and to obtain information on currently used methodologies and related method performances [13-14]. The study involved 41 laboratories from 14 countries and was conducted for the simultaneous determination of up to 11 mycotoxins (aflatoxins, OTA, FB_1, FB_2, ZEA, DON, T-2 and HT-2) in spiked and contaminated maize. A robust and reliable method for simultaneous determination of 11 mycotoxins in maize could not be identified from this study, highlighting the need for more experimental work to set up a method suitable for interlaboratory validation.

However the need of standardized LC-MS methods for mycotoxin determination has been recently highlighted by a mandate by the European Commission (EC) for standardization of methods of analysis for mycotoxins in food (M/520 EN) by which the Commission invites CEN to establish European Standards/Technical Specifications that provide standardized methods of analysis for mycotoxins in food [15]. Six of the 11 methods of analysis listed in this mandate are specifically requested to be based on LC-MS/MS.

In this framework, a second PT was organised to check next to the laboratory performance the state-of-art of currently used multi-mycotoxin methods and their implementation in the respective laboratory.

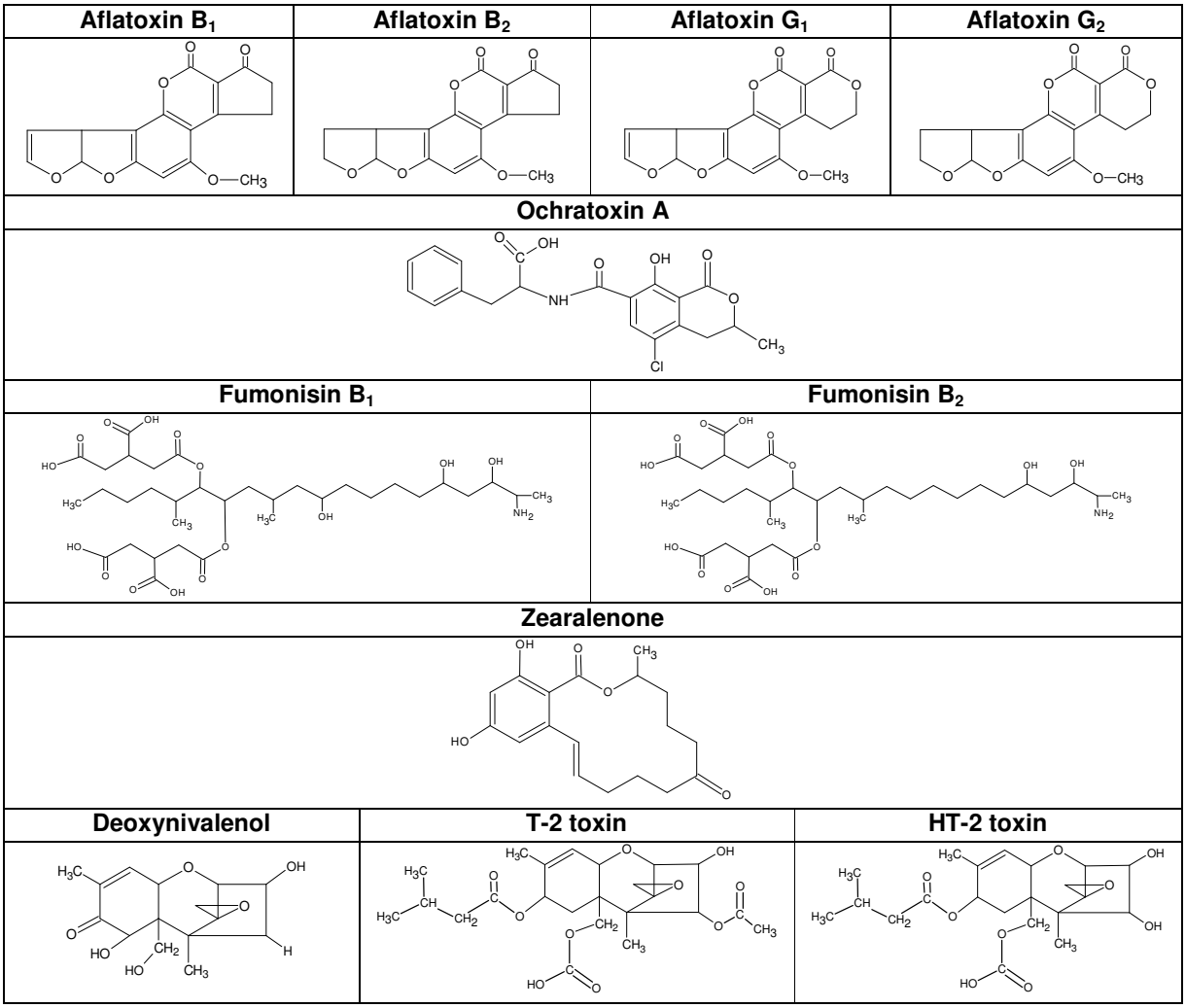

Figure 1. Chemical structure of the analytes in the proficiency test.

3. Scope

A PT is an effective procedure for quality assurance and performance verification in chemical analysis laboratories, providing a clear and a straightforward way of evaluating the accuracy (trueness and precision) of results obtained by different laboratories [16].

The main objective of this PT was to provide interested laboratories with an opportunity to test their multi-mycotoxin methods and to compare their results with those of other laboratories.

Test materials were maize contaminated with DON, FB_1, FB_2, ZEA, T-2, HT-2, OTA, AFB_1, AFG_1, AFB_2 and AFG_2, and wheat contaminated with DON, ZEA, T-2, HT-2 and OTA. All invited participants were asked to analyze each sample twice by using their method of choice. The use of LC-MS(MS) methods was not strictly required, even though it was highly recommended.

3.1 Confidentiality

In order to assure confidentiality, the identity of the laboratories were coded by a unique number between 1 and 21.

4. Time frame

The PT was free of charge and was organized in the framework of the project "New strategies for improvement of Food Safety: Prevention, Control, Correction" (S.I.Mi.S.A. PON02_00186_3417512, project of the Italian Ministry of Education, University and Research).

The S.I.Mi.S.A project addresses the wide area of Food Safety: in a context requiring continuous efforts to increase the safety level of food products, by a structured approach of advanced research, led by experts of international standing level. Participants were invited on 9th of May 2014 to take part to the PT, and, in case of acceptance, were asked to fill in a registration form [**Annexes 1** and **2**]. The deadline for registration was on 20th of May 2014. Potential participants were also contacted by an official announcement through the MoniQA website (www.MoniQA.org) and the International Association for Cereal Science and Technology website [**Annexes 3** and **4**]. The samples were dispatched to the participants on 16th of June 2014, whereas the reporting deadline was 31st of July 2014.

5. Material

5.1 Preparation

Maize test material: a maize sample naturally contaminated with approximately 8600 µg/kg FB_1 and 3600 g/kg FB_2 and a maize sample naturally contaminated with approximately 51500 µg/kg DON were mixed with a blank maize material to obtain about 28 kg of maize naturally contaminated with DON (1140 ± 290 µg/kg), FB_1 (1087 ± 81 µg/kg) and FB_2 (273 ± 81 µg/kg). Then, the obtained maize material was ground by an ultracentrifugal mill (ZM 200, Retsch) equipped with a 500 µm sieve, and homogenized by a mixer for 12 hours.

The homogenized sample was further fortified with culture extracts of mycotoxigenic species (deposited at the Institute of Sciences of Food Production collection, http://www.ispa.cnr.it/Collection) of *Fusarium graminearum* (producing DON and ZEA), *F. sporotrichioides* (producing T-2 and HT-2), *Aspergillus flavus* (producing AFB_1 and AFB_2), *A. ochraceous* (producing OTA), *A. parasiticus* (producing AFB_1, AFB_2, AFG_1 and AFG_2). Briefly, each fungal culture was dried, ground and extracted with extraction solvents specific for the produced mycotoxins according to relevant validated methods, i.e. EN 15851:2009 for aflatoxins [17]; Entelwise *et al.* (2000) [18] for OTA; MacDonald *et al.* (2005) for ZEA [19]; MacDonald *et al.* (2005) for DON [20]; Solfrizzo *et al.* (2011) for fumonisins [21]; Pascale *et al.* (2012) for T-2 and HT-2 toxins [22]. Aliquots of culture extracts were adequately diluted with mobile phase and analyzed by HPLC to measure their mycotoxin concentrations. To reach mycotoxin levels in maize material around the relevant regulatory limits, adequate amounts of fungal culture extracts were added to ground maize. The contaminated maize was passed through the ultracentrifugal mill (500 µm sieve), then homogenized by a mixer for 24 hours.

Wheat test material: a blank durum wheat sample was ground to a particle size < 500 µm, homogenized for 12 hours. To reach mycotoxin levels in wheat material around the relevant regulatory limits, adequate amounts of fungal culture extracts (*F. graminearum, F. sporotrichioides, A. ochraceous*) were added to the homogenized ground wheat. Then, the contaminated maize was passed through the ultracentrifugal mill (500 µm sieve) and homogenized by a mixer for 24 hours.

The two test materials were dispensed in plastic boxes (about 80 g each), that were labeled, sealed, and stored at -20 °C until dispatch or homogeneity or stability studies.

5.2 Homogeneity

For the study, 10 units of about 300 g of each test material were taken at systematic intervals from the filling sequence. Each unit of 300 g was divided in 6x50 g aliquots and analyzed in duplicate under repeatability conditions, by using the 6 reference methods for each mycotoxin or group of mycotoxins [17-22]. Homogeneity was evaluated according to ISO 13528:2005 [23], F-test and Harmonized International Protocol

[16] using the ProLab Software [24]. The necessary parameters for the test on homogeneity are the analytical precision (*standard deviation within bottles*) and the heterogeneity standard deviation (*standard deviation between bottles*). The *F*-test is used to determine whether the observed standard deviation between the units (containers) deviates significantly from the within unit measurements. If the differences between the mean values (from the replicates of each unit) do not differ from the within unit standard deviation, then it can be assumed that there is no significant heterogeneity and the sample homogeneity is accepted. For the homogeneity test according to ISO 13528:2005 [23], the standard deviation observed from the homogeneity test must be smaller than 0.3 *x* target standard deviation set for the PT, then the sample can be considered sufficiently homogenous. The target standard deviation for the homogeneity results and their statistical evaluation were obtained using the Horwitz equation corrected by Thompson, i.e. if the relative target standard deviation according to Horwitz is greater than 22 %, it is truncated to 22 %. The homogeneity results are displayed in **Table 1** for maize and **Table 2** for wheat. Both test materials showed sufficient homogeneity.

5.3 Stability study

Randomly selected units of the two candidate materials were submitted to accelerated ageing at temperatures between 4°C and 60°C over a total period of 1.5 months, as shown in **Table 3**, according to the so-called isochronous stability study [25]. A total of 26 bottles for each material were stored at -20°C (reference temperature), then 2 bottles per time were moved to the different temperatures after 0.25, 0.50, 1 and 1.5 month for a total of 24 bottles. All the units were analyzed at the end of month 1.5 under repeatability conditions together with 2 reference samples which were kept at -20°C over the whole period of the short-term stability study. Two independent extracts were obtained for each exposed bottle unit. Result assessment was performed according to ISO guide 35:2006 [26].

The evaluation of data was carried out by performing a linear regression on the experimentally determined concentrations of each mycotoxin (mean values) versus time (days). For a stable material, it is expected that the intercept is equal to the reference value, whereas the slope does not differ significantly from zero.

No significant trend was observed for the test samples at all temperature conditions (4ºC, 20°C and 60ºC) for the time span of the PT study. It was concluded that the two test materials were stable for at least 1.5 months following their preparation. **Annex 5** shows the raw data of the short-term stability study.

5.4 Distribution

All samples were packed in cardboard boxes and sent to every participant on 17 June 2014. The samples were mostly received within 3 days after dispatch.

Each participant received:

a) two plastic boxes each containing approximately 80 g of each test material;

b) an accompanying letter with instructions on sample handling and storage [**Annex 6**];

c) a material receipt form [**Annex 7**];

d) a report form and a detailed questionnaire on method description [**Annexes 8**].

The materials were shipped at room temperature; storage upon arrival was required to be at -18°C until the analysis was performed.

6. Instructions to participants

The laboratories were asked to report the results in µg/kg with one decimal place and to specify if results were corrected for the recovery of the method or not. In case of results corrected for recoveries, participants were asked to report the recovery. Each participant had to analyse each sample twice and to report each single value. The use of multi-mycotoxin methods was mandatory, however participants were not obliged to determine all toxins in each material, and were let free to report only on those mycotoxins that could be simultaneously determined with their multi-mycotoxin methodology. The use of LC-MS(MS) methods, although not strictly required, was highly recommended. However LC methods with fluorescence or UV detection were considered as well. Participants received a specific questionnaire intended to provide further information on the sample preparation, calibration, equipment, MS conditions and MS acquisition parameters. Participants were also asked to give general information on the exercise. A copy of the questionnaire is presented in **Annex 8**.

7. Approaches for statistical evaluation of results

7.1 General observations

Twenty-one laboratories from 10 countries registered for the exercise and were provided with the materials, with the exception of one participant that did not receive the parcel because it was rejected at customs.

7.2 Statistical evaluation of results

The statistical evaluation of the results was performed using the ProLab software [24]

7.2.1 Kernel density

The distribution of the results was checked by kernel density estimations for determining multimodality. Frequently analytical results from a proficiency study are not normally distributed or contain values from different populations giving rise to multiple distribution modes. These modes can be visualised by using Kernel density plots [28]. Kernel density plots were computed by the ProLab software from the analytical results by representing the individual numeric values each as a normalized Gaussian distribution centered on the respective analytical value. The sum of these normal distributions formed then the Kernel density distribution.

7.2.2 Assigned value

The consensus values were evaluated according to Algorithm A of ISO 13528:2005 [23] by using the ProLab software and were used as assigned values.

The results reported as “smaller than” (< values) were excluded from all calculations and no evaluation was done.

7.2.3 Target standard deviation

The target standard deviation (σ_p) determines the limits of satisfactory performance in a PT study. It should be set as a value that reflects best practices for the analysis in question. In most cases the Horwitz standard deviation is a good compromise, even though it does not reflect different levels of complexity of a given analytical method. For levels lower than 120 µg/kg the Horwitz standard deviation predicts less meaningful estimates and a truncated Horwitz standard deviation is used [29]. The standard deviation of the reproducibility obtained according the collaborative trials can be considered as an alternative indicator of the best agreement between laboratories.

The σ_p of each mycotoxin evaluated in the maize and wheat materials of this PT study was derived from the truncated Horwitz equation. However, the σ_p was also calculated using the standard deviation of the reproducibility according to the Algorithms A+S of ISO 13528:2005 [23]. Both σ_p values were evaluated using the ProLab software.

7.2.4 z-scores

Individual laboratory performance was expressed in terms of *z*-score in accordance with ISO 13528:2005 [23] and the IUPAC Protocol [16] and calculated by the following Equation (1).

$$(1) \quad z = \frac{x_{lab} - X_{assigned}}{\sigma_p}$$

where:

x_{lab} is the mean of the two measurement results reported by a participant.

$X_{assigned}$ is the assigned value (robust mean).

σ_p is the standard deviation for proficiency assessment.

The z-score compares the participant's deviation from the reference value with the target standard deviation accepted for the proficiency test (σ_p). Interpretation of z-scores was as follows:

$\|z\| \leq 2$	satisfactory result
$2 < \|z\| \leq 3$	questionable result
$\|z\| > 3$	unsatisfactory result

7.2.5. Youden Plots

Youden plots are a graphical technique for analyzing PT data when each laboratory has run test samples in duplicate or for at least 2 identical sample/analyte combinations. It is a simple but effective method for comparing both the within laboratory variability and the between-laboratory variability.

The Youden plot displays a combined graphic of the results of one analyte in two different test materials. Such a presentation allows identifying systematic effects in the laboratory-specific deviations for both matrices. It gives an immediate idea of the dominating sources of error (random or biased) in the results. Laboratories having results in the upper left or lower right hand corner of the diagram have analyses dominated by random error. On the other hand, laboratories having results close to the 45° line shown in the plot, but far away from the assigned value have results dominated by systematic error.

8. Results

8.1 Preliminary considerations

Eighteen laboratories returned 2 sets of results for various combinations of analytes. Three laboratories (i.e. Lab. 9, Lab. 10 and Lab 17) returned two sets of results obtained by using two different methods for both contaminated maize and wheat. These results were considered as being from independent laboratories for statistical evaluation (i.e. Lab. 9A and 9B, Lab. 10A and 10B, Lab 17A and 17B). Fifty-five percent of laboratories analysed all the 11 targeted mycotoxins, followed by another 9% that analysed 10 mycotoxins. The remaining laboratories reported results for a restricted combination (from 2 to 9 analytes). In the case of

wheat, 73% of laboratories analysed all the 5 targeted mycotoxins, followed by another 10% that analysed 4 mycotoxins. The remaining laboratories reported results for one or a combination of 2-3 mycotoxins.

For some mycotoxins few participants reported results as "less than the detection or quantification limits of the used method". This was mainly observed for mycotoxins occurring at low levels in the materials (i.e. aflatoxins and zearalenone in maize and T-2 in wheat).

As requested, most of the laboratories reported two replicate results under repeatability conditions. The participation of the laboratories was regarded as satisfactory concerning the number of received results (86% of participation).

The set of results returned for maize were 20 for DON, OTA and AFB_1, 19 for ZEA, 18 for T-2, 17 for HT-2, AFB_2 and AFG_1, 16 for FB_1, 15 for FB_2 and 8 for AFG_2, depending on group of mycotoxins analysed. The set of results returned for wheat were 20 for DON and 19 for ZEA, HT-2, OTA and T-2. The results reported as "smaller than" (< values) were excluded from all calculations and no evaluation was done. Furthermore, the results of T-2 and HT-2 reported by laboratory 1 and those of OTA reported by laboratory 17A for both maize and wheat materials were excluded from the statistical evaluation due to problems encountered by the participants with calibration curves and mycotoxin quantification.

According to the IUPAC [16] protocol, when the number of participants is smaller than about 15, the statistical uncertainty on the consensus (identified as the standard error) will be undesirably high, and the information content of the *z*-scores will be correspondingly reduced. In order to allow participants whose methods had sufficient measurement capacity (not met by participants reporting <LOD or <LOQ) a judgement of their results, also smaller number sets were evaluated. However the associated uncertainty of the performance benchmarking was rather high and results should be evaluated in view of this fact. The final set of quantitative results considered for statistical evaluation were 20 for DON, 16 for FB_1, OTA and AFB_1, 15 for FB_2 and T-2, 11 for ZEA and HT-2, and 9 for AFG_1 in maize and 20 for DON, 19 for ZEA, 15 for HT-2, 14 for OTA and 8 for T-2 in wheat. No statistical evaluation was reported for AFG_2 and AFB_2 in maize due to lack of sufficient experimental data.

A summary of the laboratories test results for each mycotoxin with their repeatability standard deviation is shown in **Figures 2-10** for maize and **Figures 11-15** for wheat. The upper/lower red lines represent the upper/lower tolerance limits determined by the target standard deviation, while the green area represent the confidence interval of the assigned values, calculated from the robust standard deviation of the PT for the respective measurand/matrix combination.

8.2 Kernel density plots

Kernel density plots for maize are shown in **Figures 16-24**, whereas those for wheat are shown in **Figures 25-29**.

8.3 Laboratories performance and z-scores

The assigned values for maize test materials were 1264 µg/kg for DON, 1305 µg/kg for FB_1, 350 µg/kg for FB_2, 21.7 µg/kg for ZEA, 54.4 µg/kg for T-2, 30.7 µg/kg for HT-2, 2.73 µg/kg for OTA, 1.35 µg/kg for AFB_1 and 0.63 µg/kg for AFG_1 (**Table 4**). The assigned values for wheat test materials were 1298 µg/kg for DON, 148 µg/kg for ZEA, 58.8 µg/kg for HT-2, 8.26 µg/kg for T-2 and 7.21 µg/kg for OTA (**Table 5**).

The z-scores results calculated with both σ_p values (truncated Horwitz standard deviation and reproducibility standard deviation) are reported in **Tables 6-14** for maize and **Tables 17-21** for wheat. Single data for AFB_2 and AFG_2 in maize are reported in **Tables 15** and **16**. A graphical distribution of z-scores is shown in **Figures 30-31**.

Youden plots presented in **Figures 32-36** show good correlation for DON and ZEA (correlation coefficients 0.5 and 0.7, respectively) but no correlation for OTA, T-2 and HT-2.

The overall performance for individual mycotoxin in each material was evaluated taking into account the results submitted (**Figures 37-38**). The blue bars represent the number of laboratories able to identify the mycotoxins; the red ones denote the number of laboratories that quantified the mycotoxins and the green bars the number of laboratories that quantified the mycotoxins within the tolerance limits.

The overall performance of the laboratories regarding all mycotoxins in maize and wheat is shown in **Table 22**. A laboratory was considered successful for the whole interlaboratory test if at least 80% of the z-scores were within the tolerance limits and at least 80 % of the mycotoxins had z-scores between the tolerance limits. Based on this evaluation, only 23% of laboratories satisfied this criterion.

8.4 Evaluation of the questionnaire

All laboratories that reported results (19 laboratories), submitted their questionnaires. Among them three laboratories provided two set of results obtained by using different methodologies. A total of 22 filled in questionnaires were collected. A summary of experimental details and evaluation of questionnaires is presented in the **Annex 9**.

General overview of the reported answers showed that participants mainly used LC-MS/MS (n=21), one participant used LC-HRMS, one participant used GC-MS (for DON and ZEA), and one used HPLC with fluorescence detection (for OTA and aflatoxins).

The majority of laboratories (73%) used mixtures of acetonitrile-water for extraction. Other laboratories used methanol-water mixtures (18%), one laboratory used isopropyl alcohol-water-acetone mixture (4.5%) and another one used acetonitrile-water to extract aflatoxins and methanol-water to extract OTA (4.5%) (**Figure 39**).

Extraction was mainly carried out by shaking (73%) or by blending (18%). The remaining laboratories used vortex or accelerated solvent extraction.

Fifty percent of laboratories analysed the crude extract; the others cleaned-up the extract prior to the analysis (37%), used Quick Easy Cheap Effective Rugged Safe (QuEChERS)-like approach (9%), or used a mixed approach (4%) (i.e. the sample extract was split in two aliquots, one was directly analysed by LC-MS/(MS) and the other was purified before analysis depending on the mycotoxin) (**Figure 40**).

The majority of laboratories (55%) used internal standard calibration mode using stable isotope labelled standards. Among them 8 laboratories used standard calibration (calibration solutions prepared in neat solvents), and 4 laboratories used matrix assisted calibration (calibration solutions prepared in blank matrix extract). The other laboratories (45%) used external calibration using native standard mycotoxins. Among them 6 laboratories used standard calibration, and 4 used matrix assisted calibration.

Fifty-four percent of laboratories reported recovery values for mycotoxins (**Annex 9**).

All participants found the instructions adequate (**Annex 9**).

Table 1. Results of the homogeneity study for maize.

Mycotoxins	Mean (µg/kg)	Analytical SD (µg/kg)[a]	Heterogeneity SD (µg/kg)[b]	Target SD (µg/kg)[c]	F-test[d]	ISO 13528[e]
DON	1221	67.4	26.1	190	OK	OK
FB_1	1062	108	0.00	168	OK	OK
FB_2	303	56.4	0.00	58.1	OK	OK
ZEA	21.6	3.84	1.38	4.75	OK	OK
T-2	54.1	5.31	0.00	11.9	OK	OK
HT-2	22.5	2.62	0.00	4.94	OK	OK
OTA	2.58	0.78	0.00	0.57	OK	OK
AFB_1	1.19	0.18	0.00	0.26	OK	OK
AFG_1	0.05	0.02	0.00	0.01	OK	OK
AFB_2	0.21	0.03	0.00	0.05	OK	OK
AFG_2	0.04	0.01	0.00	0.01	OK	OK

[a]Within bottle standard deviation; [b]between bottle standard deviation; [c]target standard deviation calculated using corrected Horwitz equation; [d]check for significant heterogeneity; [e]check for sufficient homogeneity.

Table 2. Results of the homogeneity study for wheat.

Mycotoxins	Mean (µg/kg)	Analytical SD (µg/kg)[a]	Heterogeneity SD (µg/kg)[b]	Target SD (µg/kg)[c]	F-test[d]	ISO 13528[e]
DON	1266	39.0	18.6	195	OK	OK
ZEA	149	11.8	8.06	31.7	OK	OK
T-2	4.91	0.72	0.00	1.08	OK	OK
HT-2	50.9	3.23	0.00	11.2	OK	OK
OTA	5.34	0.37	0.00	1.17	OK	OK

[a]Within bottle standard deviation; [b]between bottle standard deviation; [c]target standard deviation calculated using corrected Horwitz; [d]check for significant heterogeneity; [e]check for sufficient homogeneity.

Table 3. Accelerated ageing of exposed samples to perform an isochronous stability study

Ageing (months)	Storage temperature			
	-20°C	**+4°C**	**+20°C**	**+60°C**
0.25		X	X	X
0.50		X	X	X
1		X	X	X
1.5	X	X	X	X

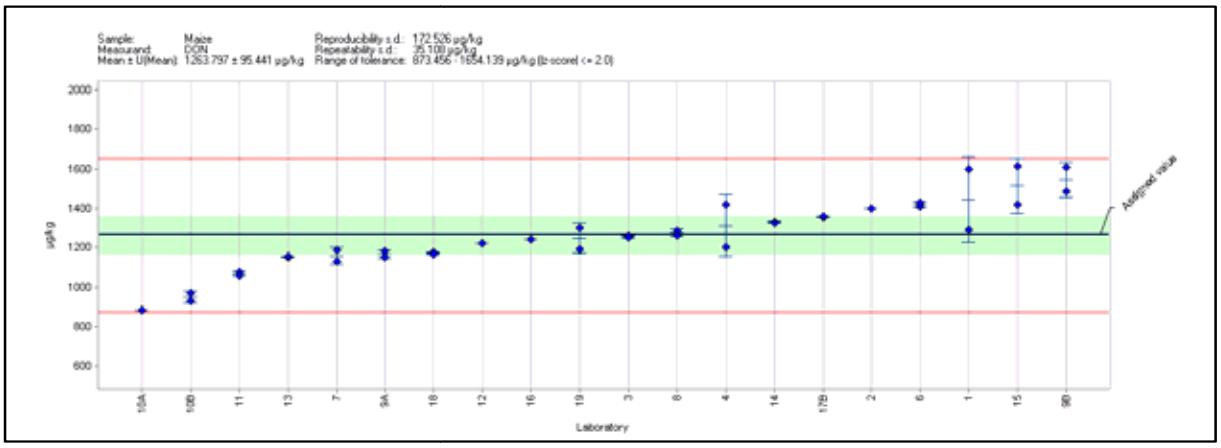

Figure 2. Summary graph of the laboratory's test results for deoxynivalenol in maize

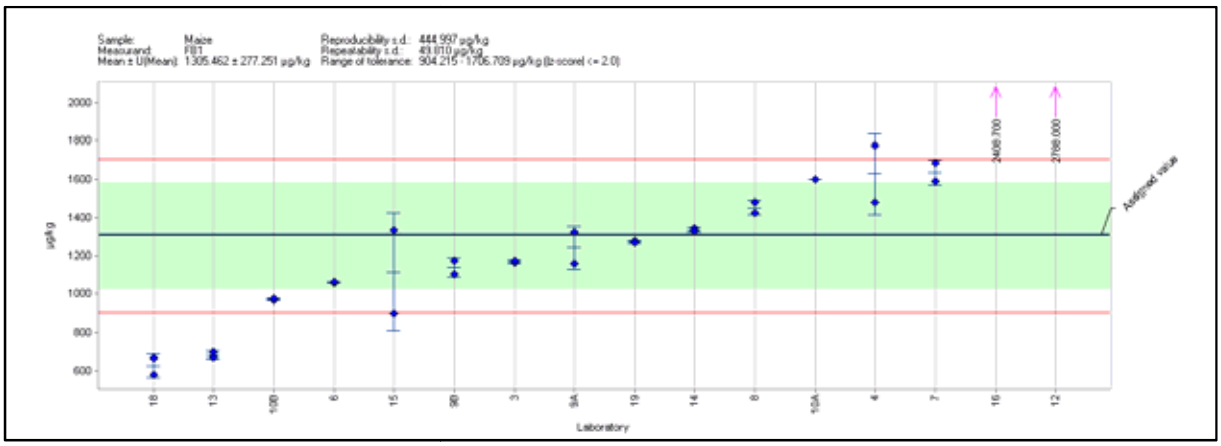

Figure 3. Summary graph of the laboratory's test results for fumonisin B_1 in maize

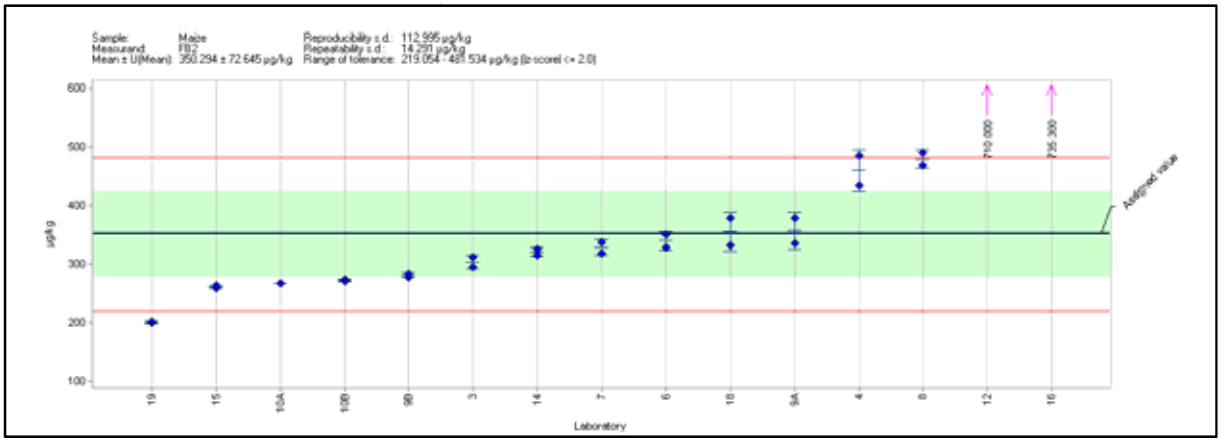

Figure 4. Summary graph of the laboratory's test results for fumonisin B_2 in maize

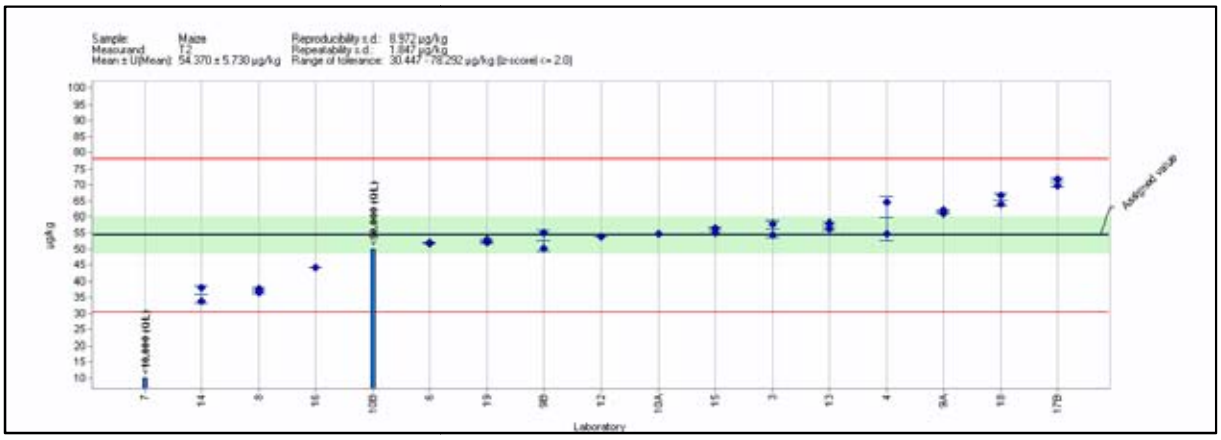

Figure 5. Summary graph of the laboratory's test results for T-2 toxin in maize

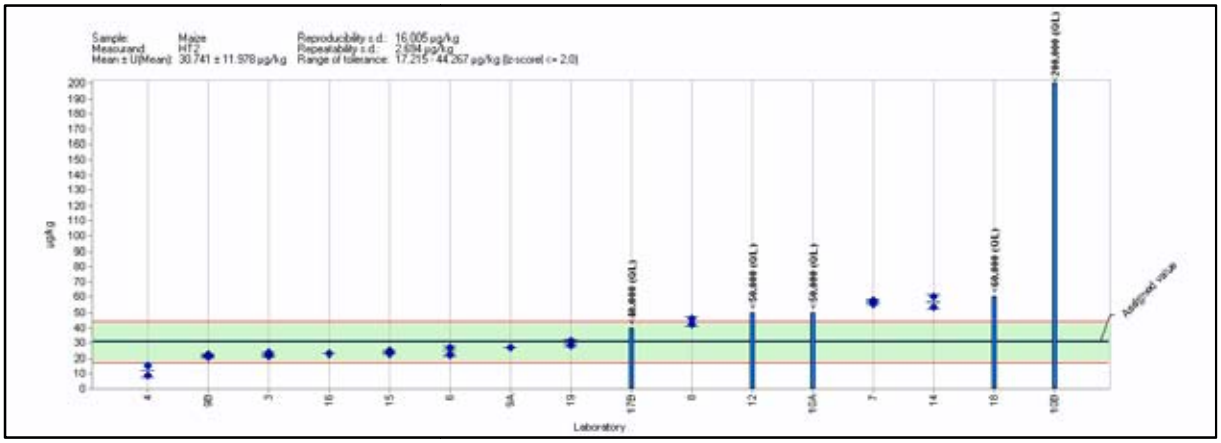

Figure 6. Summary graph of the laboratory's test results for HT-2 toxin in maize

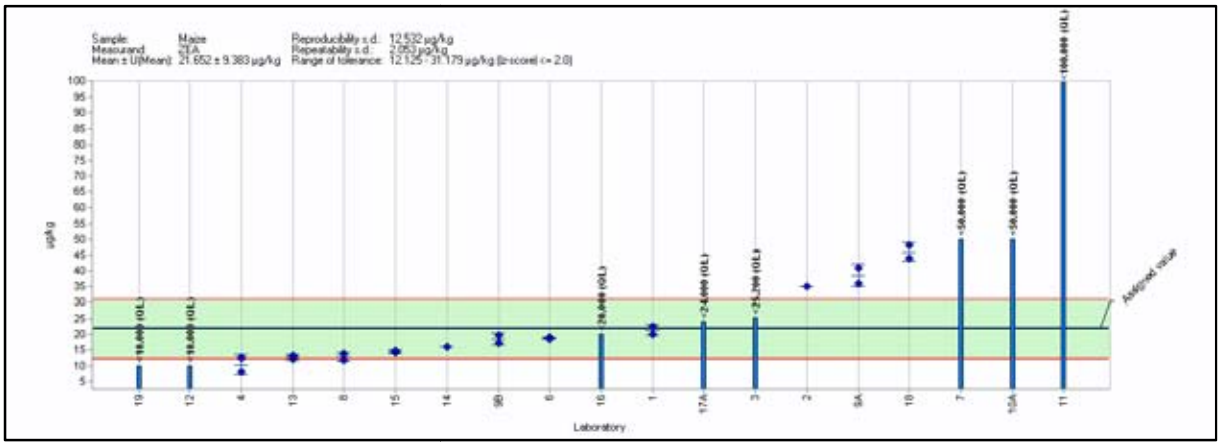

Figure 7. Summary graph of the laboratory's test results for zearalenone in maize

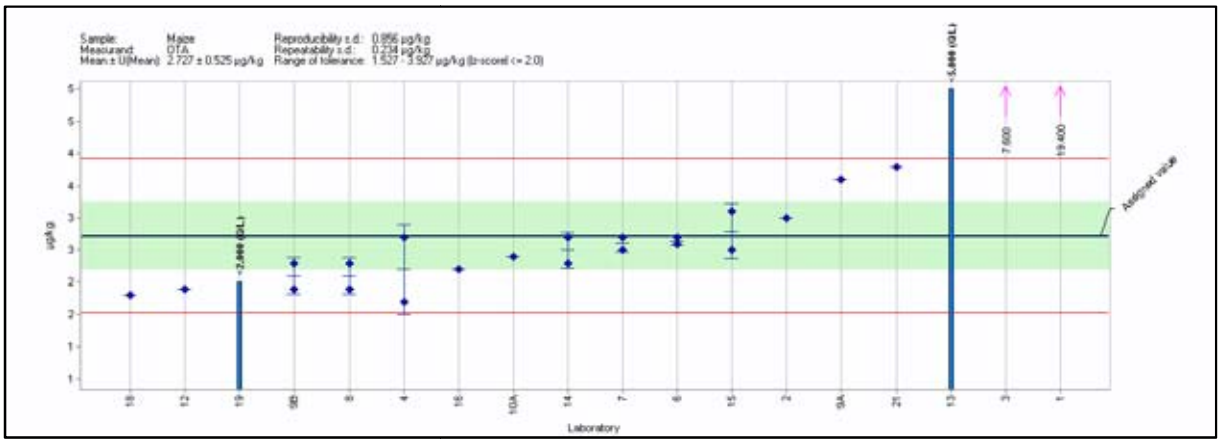

Figure 8. Summary graph of the laboratory's test results for ochratoxin A in maize

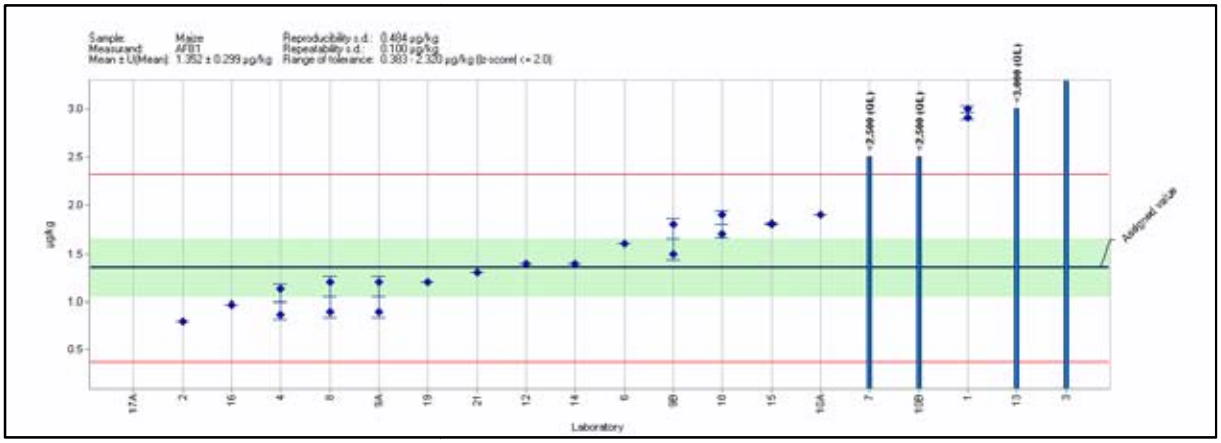

Figure 9. Summary graph of the laboratory's test results for aflatoxin B_1 in maize

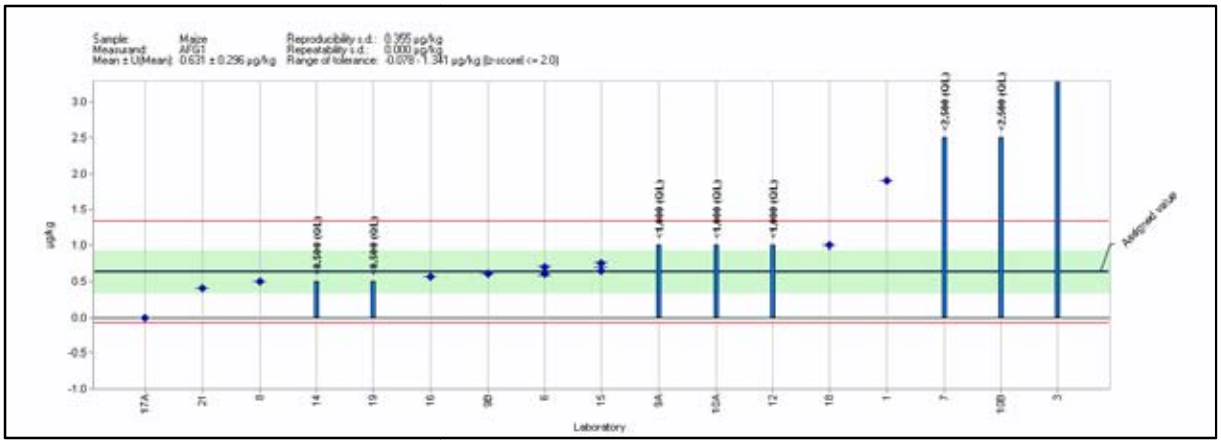

Figure 10. Summary graph of the laboratory's test results for aflatoxin G_1 in maize

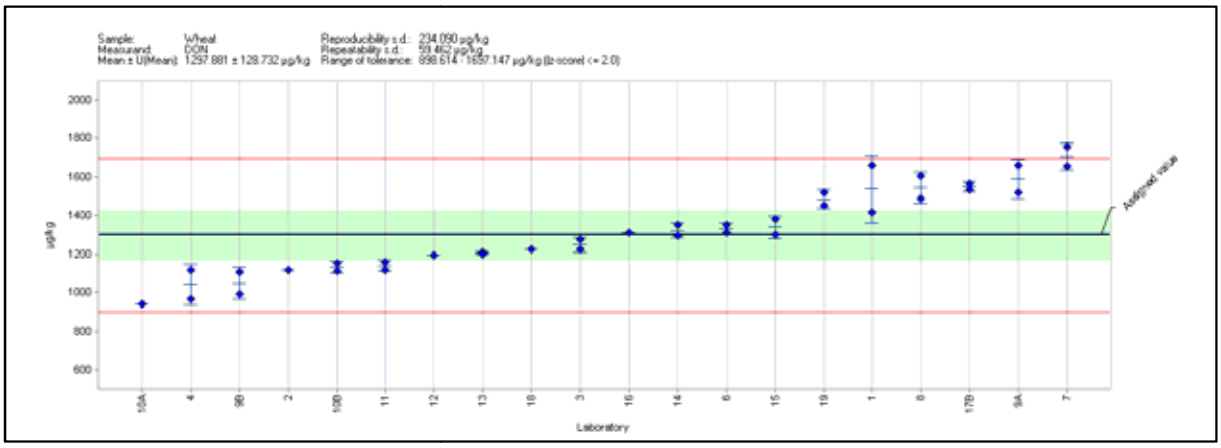

Figure 11. Summary graph of the laboratory's test results for deoxynivalenol in wheat

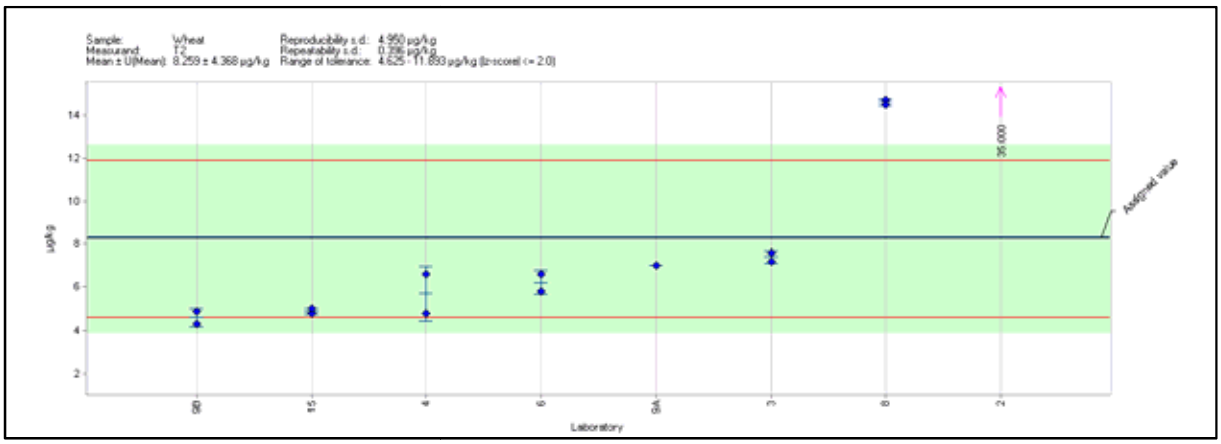

Figure 12. Summary graph of the laboratory's test results for T-2 toxin in wheat

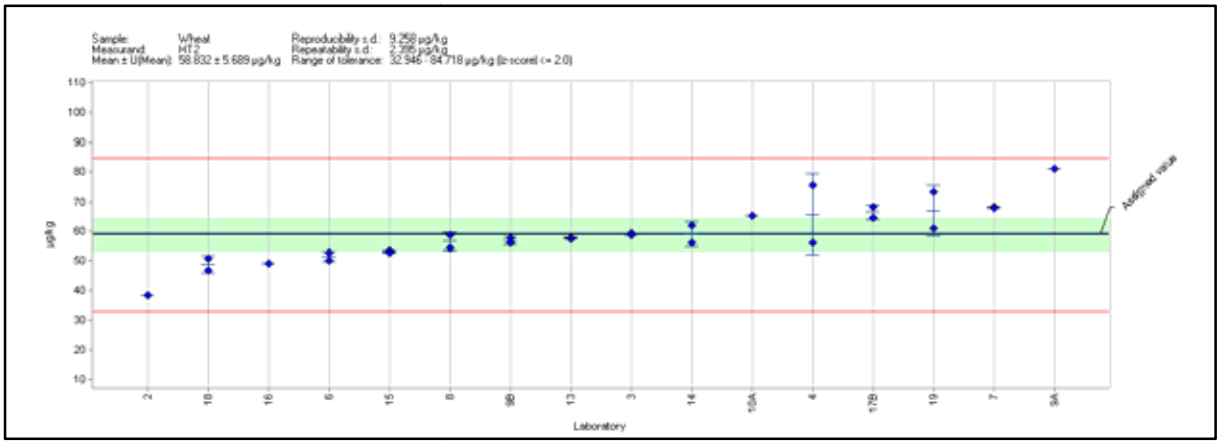

Figure 13. Summary graph of the laboratory's test results for HT-2 toxin in wheat

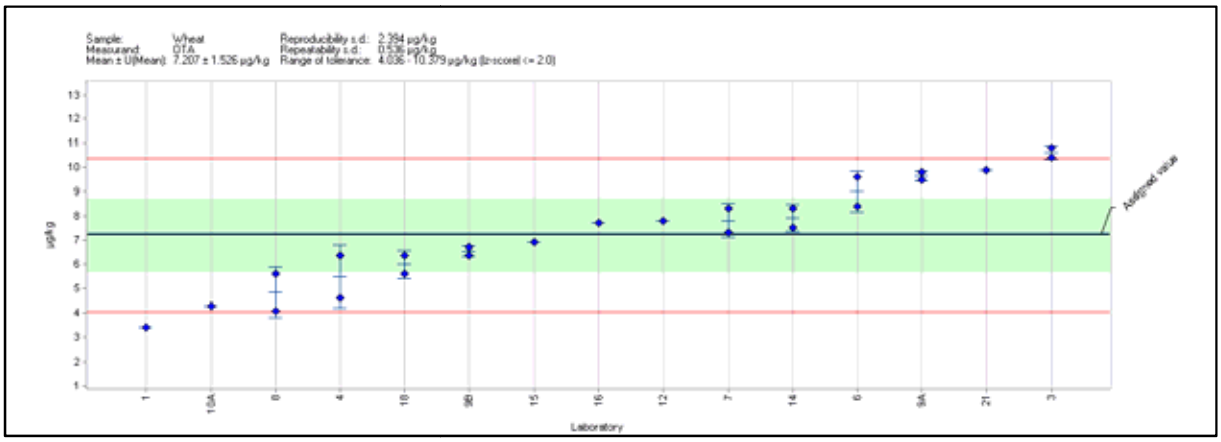

Figure 14. Summary graph of the laboratory's test results for ochratoxin A in wheat

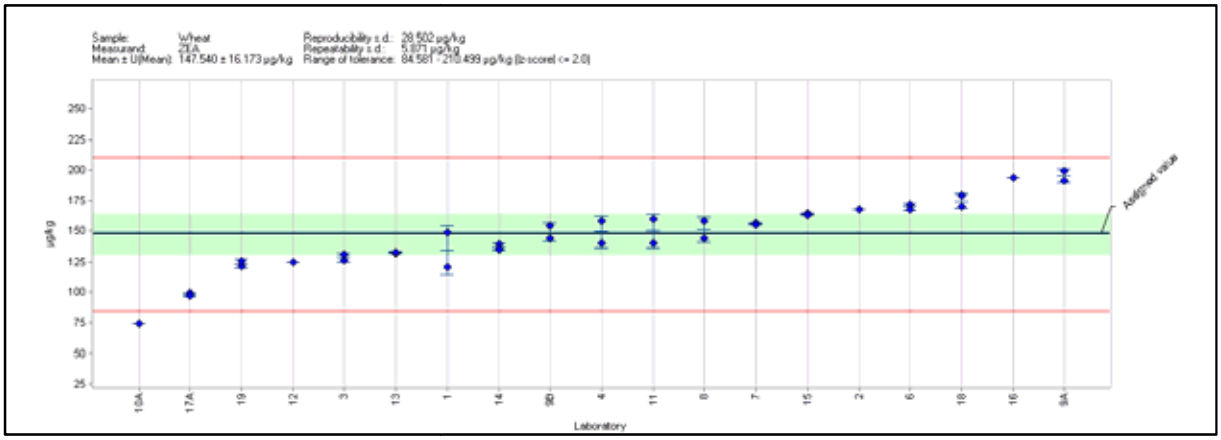

Figure 15. Summary graph of the laboratory's test results for zearalenone in wheat

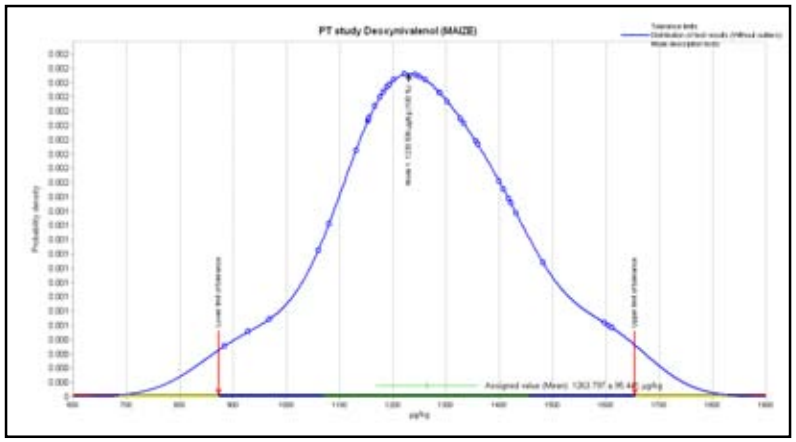

Figure 16. Kernel density plot for deoxynivalenol in maize

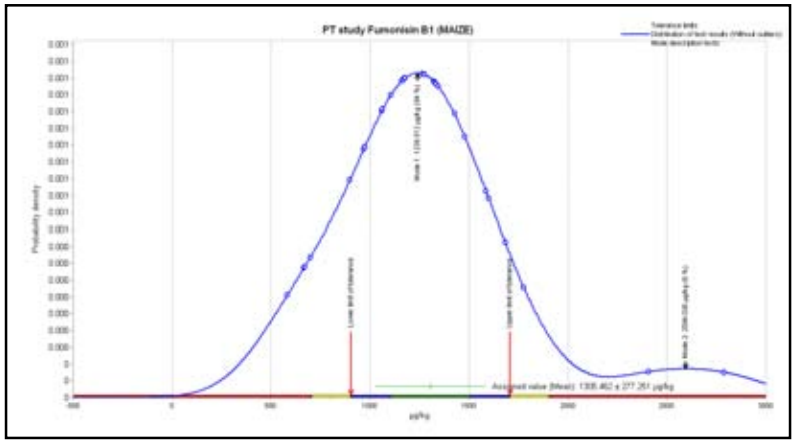

Figure 17. Kernel density plot for fumonisin B_1 in maize

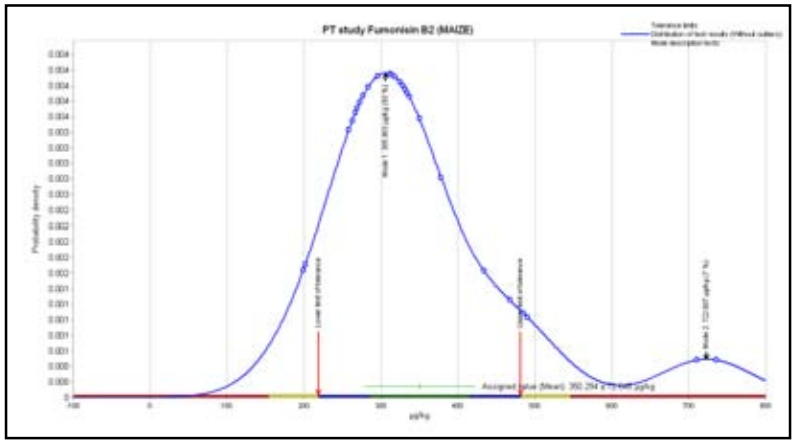

Figure 18. Kernel density plot for fumonisin B_2 in maize

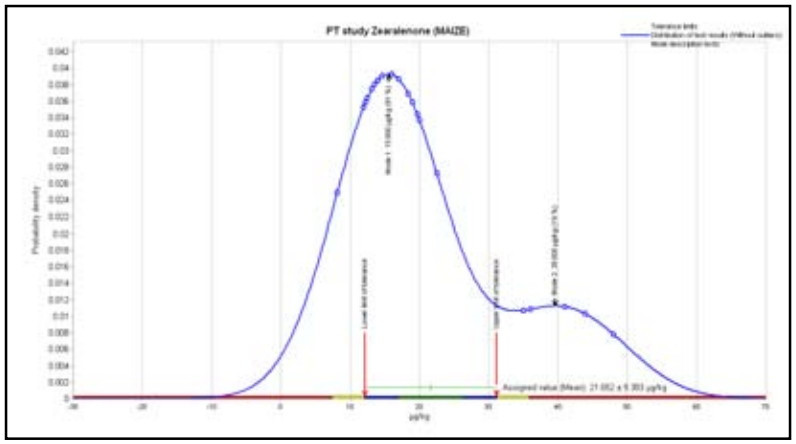

Figure 19. Kernel density plot for zearalenone in maize

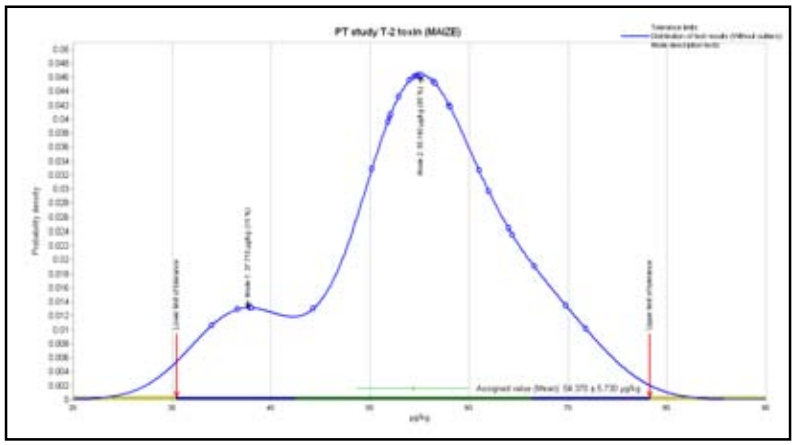

Figure 20. Kernel density plot for T-2 toxin in maize

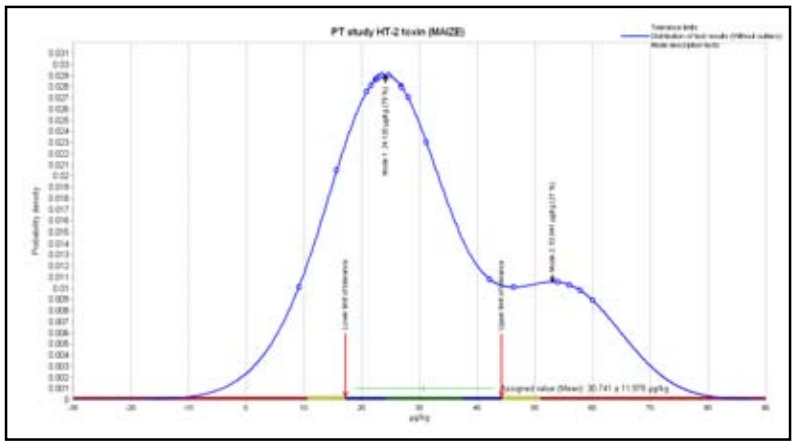

Figure 21. Kernel density plot for HT-2 toxin in maize

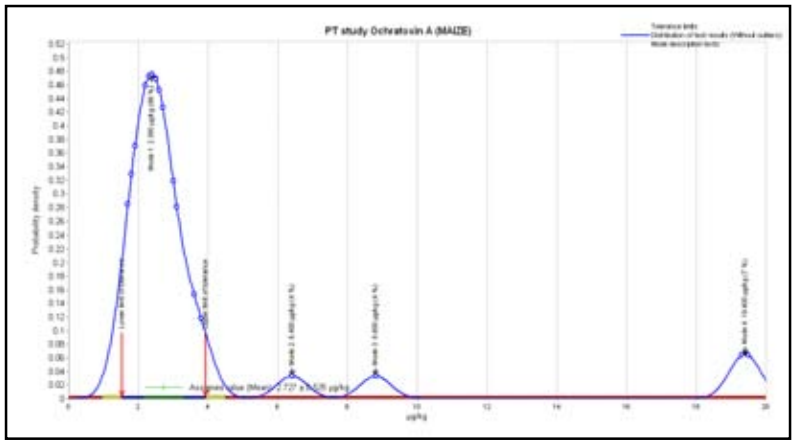

Figure 22. Kernel density plot for ochratoxin A in maize

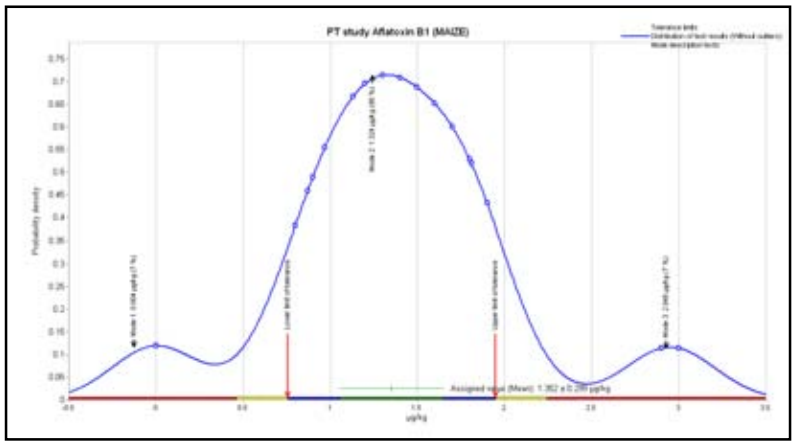

Figure 23. Kernel density plot for aflatoxin B_1 in maize

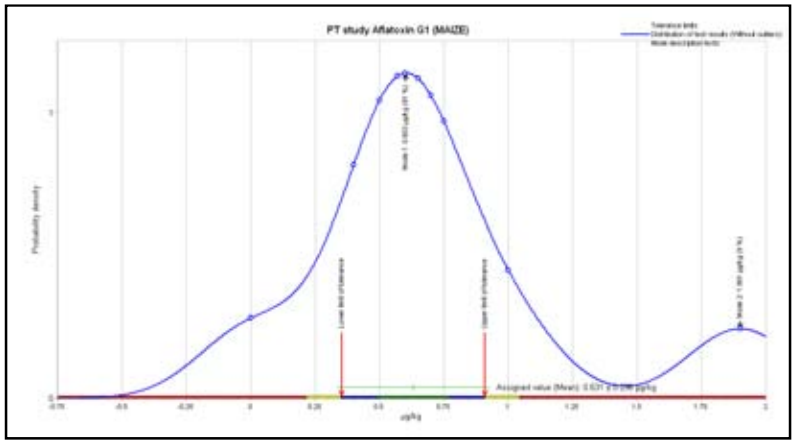

Figure 24. Kernel density plot for aflatoxin G_1 in maize

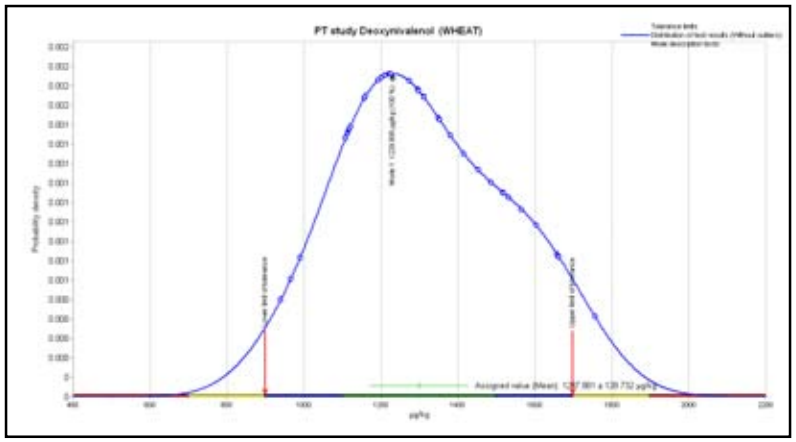

Figure 25. Kernel density plot for deoxynivalenol in wheat

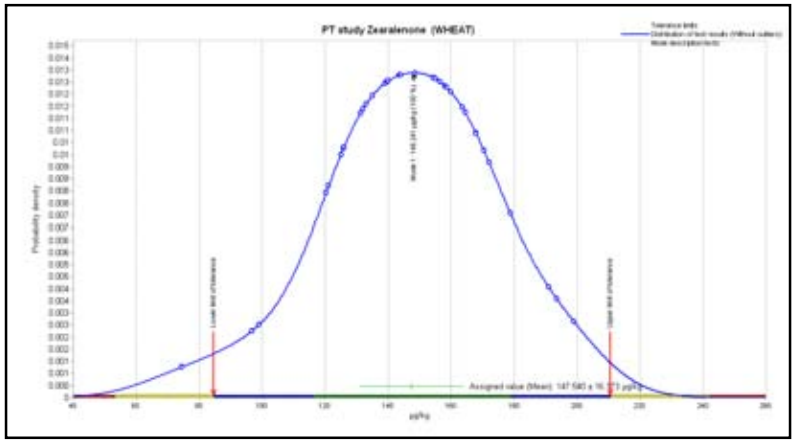

Figure 26. Kernel density plot for zearalenone in wheat

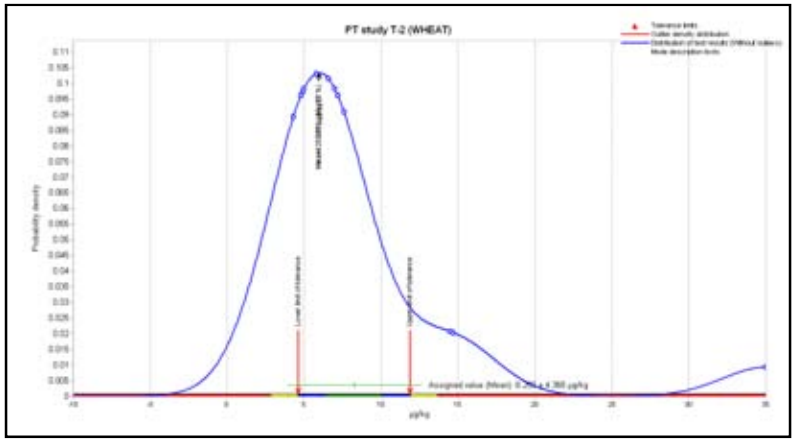

Figure 27. Kernel density plot for T-2 toxin in wheat

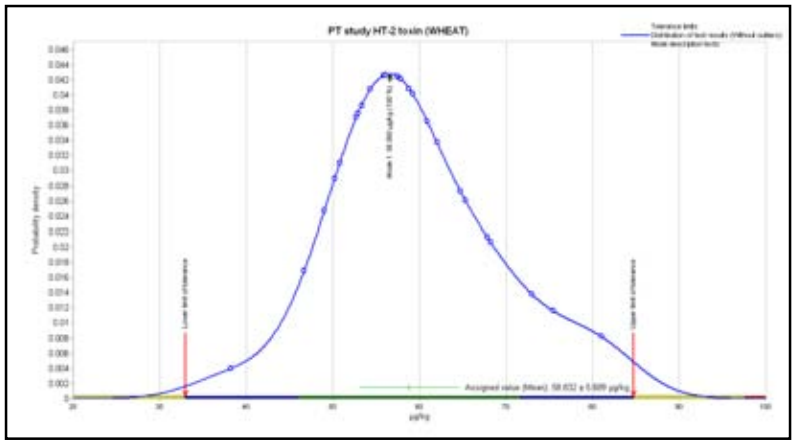

Figure 28. Kernel density plot for HT-2 toxin in wheat

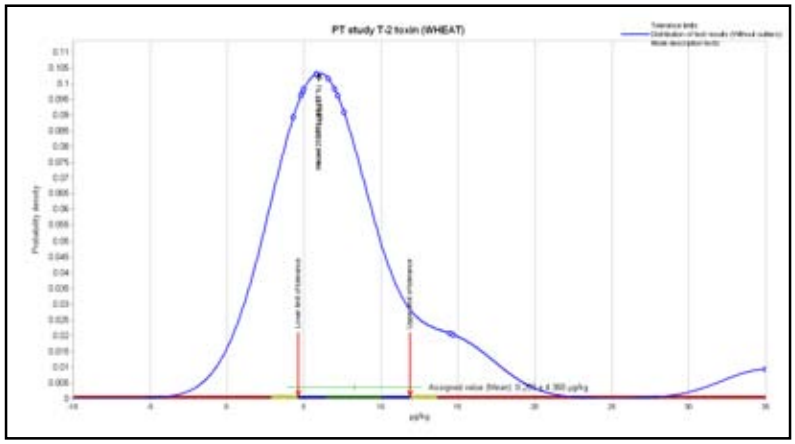

Figure 29. Kernel density plot for ochratoxin A in wheat

Table 4. Summary statistics for deoxynivalenol (DON), fumonisins B_1 (FB_1) and B_2 (FB_2), ochratoxin A (OTA), T-2 toxin (T-2), HT-2 toxin (HT-2), zearalenone (ZEA), aflatoxins B_1 (AFB_1), G_1 (AFG_1), B_2 (AFB_2) and G_2 (AFG_2) in maize

	DON	FB_1	FB_2	OTA	T-2	HT-2	ZEA	AFB_1	AFG_1	AFB_2	AFG_2
Number of participants (according to the design)	22	22	22	22	22	22	22	22	22	22	22
Number of submitted results	20	16	15	20	18	17	19	20	17	17	8
Number of quantitative results	20	16	15	17	16	12	11	16	9	4	4
Number of outliers	-	-	-	1	1	1	-	-	-	-	-
Number of results after removal of outliers	20	16	15	16	15	11	11	16	9	4	4
Arithmetical mean (µg/kg)	1256	1383	377	3.92	54.0	31.2	22.2	1.37	0.70	0.24	0.10
Median (µg/kg)	1252	1257	328	2.55	54.7	24.7	18.4	1.35	0.60	0.13	0.10
Minimal value (µg/kg)	883.4	581.0	198.8	1.7	34.0	9.1	8.1	0	0	0	0
Maximal value (µg/kg)	1611.2	2788.0	735.3	71.8	60.0	19.5	48.0	3.0	1.9	0.7	0.2
Assigned value (µg/kg)	1264	1305	350	2.73	54.4	30.7	21.7	1.35	0.63	-- [a]	--
Target standard deviation (µg/kg) (σ_p according to truncated Horwitz)	195	201	65.6	0.60	12.0	6.76	4.76	0.30	0.14	--	--
Relative target standard deviation (%) (σ_p according to truncated Horwitz)	15.4	15.4	18.7	22	22	22	22	22	22	--	--
Reproducibility standard deviation (µg/kg)	173	445	113	0.86	8.97	16.0	12.53	0.48	0.36	--	--
Relative reproducibility standard deviation (%) (σ_p according to truncated Horwitz)	13.7	34.1	32.3	31.4	16.5	52.1	57.9	35.8	56.2	--	--
Lower limit of tolerance (µg/kg)	873	904	219	1.53	30.5	17.2	12.1	0.76	0.35	--	--
Upper limit of tolerance (µg/kg)	1654	1707	482	3.93	78.3	44.3	31.2	1.95	0.91	--	--
Number of laboratories with mean outside of tolerance limits	-	4	3	2	-	3	4	2	3	--	--

[a] too few laboratories.

Table 5. Summary statistics for deoxynivalenol (DON), ochratoxin A (OTA), T-2 toxin (T-2), HT-2 toxin (HT-2) and zearalenone (ZEA) in wheat.

	DON	OTA	T-2	HT-2	ZEA
Number of participants (according to the design)	22	22	22	22	22
Number of submitted results	20	19	19	19	19
Number of quantitative results	20	16	9	17	19
Number of outliers	-	1	1	1	-
Number of results after removal of outliers	20	15	8	16	19
Arithmetical mean (µg/kg)	1300	7.19	10.7	58.9	146
Median (µg/kg)	1279	7.70	6.60	58.3	149
Minimal value (µg/kg)	939.6	3.4	4.3	38.2	74.5
Maximal value (µg/kg)	1756.1	10.8	35.0	81.0	199.0
Assigned value (µg/kg)	1298	7.21	8.26	58.8	148
Target standard deviation (µg/kg) (σ_p according to truncated Horwitz)	200	1.59	1.82	12.9	31.5
Relative target standard deviation (%) (σ_p according to truncated Horwitz)	15.4	22.0	22.0	22.0	21.3
Reproducibility standard deviation (µg/kg)	234	2.39	4.95	9.26	28.5
Relative reproducibility standard deviation (%) (σ_p according to truncated Horwitz)	18.0	33.2	59.9	15.7	19.3
Lower limit of tolerance (µg/kg)	899	4.04	4.63	32.9	84.6
Upper limit of tolerance (µg/kg)	1697	10.4	11.9	84.7	211
Number of laboratories with mean outside of tolerance limits	1	2	3	-	1

Table 6. Results of analysis and *z*-scores for deoxynivalenol (DON) in maize

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1	1288.6	1597.1	1442.9	0.9	1.0
2	1400.0	-[a]	1400.0	0.7	0.8
3	1260.8	1250.0	1255.4	0.0	0.0
4	1201.8	1421.8	1311.8	0.2	0.3
6	1408.0	1431.2	1419.6	0.8	0.9
7	1188.7	1130.7	1159.7	-0.5	-0.6
8	1288.0	1261.0	1274.5	0.1	0.1
9A	1482.0	1606.0	1544.0	-0.5	-0.6
9B	1182.0	1154.4	1168.2	1.4	1.6
10A	883.4	-	883.4	-1.9	-2.2
10B	968.3	928.3	948.3	-1.6	-1.8
11	1080.0	1060.0	1070.0	-1.0	-1.1
12	1222.0	-	1222.0	-0.2	-0.2
13	1152.9	1156.3	1154.6	-0.6	-0.6
14	1333.0	1327.0	1330.0	0.3	0.4
15	1417.6	1611.2	1514.4	1.3	1.5
16	1241.3	-	1241.3	-0.1	-0.1
17A	--[b]	--	--		
17B	1355.7	1359.4	1357.6	0.5	0.5
18	1175.4	1166.7	1171.1	-0.5	-0.5
19	1302.4	1193.0	1247.7	-0.1	-0.1
21	--	--	--		

The results are written as reported by the laboratories. [a]Second replicate result not reported; [b]not analysed .

Table 7. Results of analysis and *z*-scores for fumonisin B_1 (FB_1) in maize

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1	--[a]	--	--		
2	--	--	--		
3	1163.2	1177.2	1170.2	-0.7	-0.3
4	1479.2	1777.0	1628.1	1.6	0.7
6	1060.1	1064.0	1062.1	-1.2	-0.5
7	1682.8	1587.0	1634.9	1.6	0.7
8	1426.0	1479.0	1452.5	0.7	0.3
9A	1162.0	1322.0	1242.0	-0.3	-0.1
9B	1104.3	1174.1	1139.2	-0.8	-0.4
10A	1600.0	-[b]	1600.0	1.5	0.7
10B	968.3	974.8	971.6	-1.7	-0.8
11	--	--	--		
12	2788.0	-	2788.0	7.4	3.3
13	668.8	697.0	682.9	-3.1	-1.4
14	1344.0	1326.0	1335.0	0.1	0.1
15	1333.7	899.3	1116.5	-0.9	-0.4
16	2408.7	-	2408.7	5.5	2.5
17A	--	--	--		
17B	--	--	--		
18	667.0	581.0	624.0	-3.4	-1.5
19	1275.5	1265.9	1270.7	-0.2	-0.1
21	--	--	--		

The results are written as reported by the laboratories. [a]Not analysed; [b]second replicate result not reported.

Table 8. Results of analysis and *z*-scores for fumonisin B_2 (FB_2) in maize

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1	--[a]	--	--		
2	--	--	--		
3	295.2	311.6	303.4	-0.7	-0.4
4	484.4	433.6	459.0	1.7	1.0
6	349.8	328.0	338.9	-0.2	-0.1
7	318.5	336.6	327.6	-0.3	-0.2
8	490.0	467.0	478.5	2	1.1
9A	334.0	378.0	356.0	0.1	0.1
9B	276.8	283.1	280.0	-1.1	-0.6
10A	266.8	-[b]	266.8	-1.3	-0.7
10B	272.9	269.4	271.2	-1.2	-0.7
11	--	--	--		
12	710.0	-	710.0	5.5	3.2
13	--	--	--		
14	325.0	315.0	320.0	-0.5	-0.3
15	258.2	262.4	260.3	-1.4	-0.8
16	735.3	-	735.3	5.9	3.4
17A	--	--	--		
17B	--	--	--		
18	378.0	331.0	354.5	0.1	0.0
19	198.8	201.5	200.2	-2.3	-1.3
21	--	--	--		

The results are written as reported by the laboratories. [a]Not analysed; [b]second replicate result not reported.

Table 9. Results of analysis and *z*-scores for zearalenone (ZEA) in maize

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1	20.0	22.5	21.3	-0.1	0.0
2	35.0	-[a]	35.0	2.8	1.1
3	<25.2	<25.2			
4	12.5	8.1	10.3	-2.4	-0.9
6	19.0	18.4	18.7	-0.6	-0.2
7	<50	<50			
8	11.9	13.6	12.8	-1.9	-0.7
9A	36.0	41.0	38.5	3.5	1.3
9B	17.0	19.7	18.4	-0.7	-0.3
10A	<50	-[a]			
10B	--[b]	--	--		
11	<100	<100			
12	<10	-[a]			
13	12.2	13.1	12.7	-1.9	-0.7
14	16.0	16.0	16.0	-1.2	-0.5
15	14.0	14.7	14.4	-1.5	-0.6
16	<20	-			
17A	<24	-			
17B	--	--	--		
18	48.0	43.9	46.0	5.1	1.9
19	<10	<10			
21	--	--	--	-0.1	0.0

The results are written as reported by the laboratories. [a]Second replicate result not reported; [b]not analysed .

Table 10. Results of analysis and *z*-scores for T-2 toxin (T-2) in maize

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1[a]	143.2	146.5	144.9	7.6	10.1
2	--[b]	--	--		
3	54.4	58.0	56.2	0.2	0.2
4	64.4	54.8	59.6	0.4	0.6
6	51.8	52.1	52.0	-0.2	-0.3
7	<10	<10			
8	37.9	36.6	37.3	-1.4	-1.9
9A	61.0	62.0	61.5	0.6	0.8
9B	55.0	50.2	52.6	-0.1	-0.2
10A	54.7	-[c]	54.7	0.0	0.0
10B	<50	<50			
11	--	--	--		
12	54.0	-	54.0	0.0	0.0
13	56.4	58.1	57.3	0.2	0.3
14	34.0	38.0	36.0	-1.5	-2.0
15	55.2	56.6	55.9	0.1	0.2
16	44.3	-	44.3	-0.8	-1.1
17A	--	--	--		
17B	71.8	69.8	70.8	1.4	1.8
18	66.6	64.0	65.3	0.9	1.2
19	52.0	52.9	52.5	-0.2	-0.2
21	--	--	--		

The results are written as reported by the laboratories. [a]Outlier and excluded from statistical evaluation; [b]not analysed; [b]second replicate result not reported.

Table 11. Results of analysis and *z*-scores for HT-2 toxin (HT-2) in maize

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1[a]	73.6	76.2	74.9	6.5	2.8
2	--[b]	--	--		
3	21.6	23.6	22.6	-1.2	-0.5
4	9.1	15.7	12.4	-2.7	-1.1
6	22.6	26.8	24.7	-0.9	-0.4
7	57.8	56.0	56.9	3.9	1.6
8	42.2	46.3	44.3	2	0.8
9A	27.0	27.0	27.0	-0.6	-0.2
9B	22.4	20.9	21.7	-1.3	-0.6
10A	<50	-[c]			
10B	<200	<200			
11	--	--	--		
12	<50	-			
13	--	--	--		
14	60.0	54.0	57.0	3.9	1.6
15	24.7	22.9	23.8	-1.0	-0.4
16	23.0	-	23.0	-1.1	-0.5
17A	--	--	--		
17B	<40	<40			
18	<60	<60			
19	31.2	28.0	29.6	-0.2	-0.1
21	--	--	--		

The results are written as reported by the laboratories. [a]Outlier excluded from statistical evaluation; [b]not analysed; [b]second replicate result not reported.

Table 12. Results of analysis and *z*-scores for ochratoxin A (OTA) in maize

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1	19.3	19.5	19.4	27.8	19.5
2	3.0	-[a]	3.0	0.5	0.3
3	8.8	6.4	7.6	8.1	5.7
4	1.7	2.7	2.2	-0.9	-0.6
6	2.6	2.7	2.7	-0.1	-0.1
7	2.7	2.5	2.6	-0.2	-0.1
8	1.9	2.3	2.1	-1	-0.7
9A	3.6	3.6	3.6	1.5	1.0
9B	2.8	2.2	2.5	-1	-0.7
10A	2.4	-	2.4	-0.5	-0.4
10B	<2.5	<2.5			
11	--[b]	--	--		
12	1.9	-	1.9	-1.4	-1.0
13	<5	<5			
14	2.3	2.7	2.5	-0.4	-0.3
15	2.5	3.1	2.8	0.1	0.1
16	2.2	-	2.2	-0.9	-0.6
17A[c]	119.9	119.8	119.9	195.3	136.2
17B	--	--	--		
18	1.8	1.8	1.8	-1.5	-1.1
19	<2	<2			
21	3.8	-	3.8	1.8	1.3

The results are written as reported by the laboratories. [a]Second replicate result not reported; [b]not analysed; [c]outlier excluded from statistical evaluation.

Table 13. Results of analysis and *z*-scores for aflatoxin B_1 (AFB_1) in maize

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1	2.90	3.00	2.95	5.4	3.3
2	0.80	-[a]	0.80	-1.9	-1.1
3	<9.2	<9.2			
4	1.13	0.87	1.00	-1.2	-0.7
6	1.60	1.60	1.60	0.8	0.5
7	<2.5	<2.5			
8	1.20	0.90	1.05	-1	-0.6
9A	1.70	1.70	1.70	-1	-0.6
9B	1.80	1.50	1.65	1	0.6
10A	1.90	-	1.90	1.8	1.1
10B	<2.5	<2.5			
11	--[b]	--	--		
12	1.40	-	1.40	0.2	0.1
13	<3	<3			
14	1.40	1.40	1.40	0.2	0.1
15	1.80	1.81	1.81	1.5	0.9
16	0.97	-	0.97	-1.3	-0.8
17A	--	--	--	-4.5	-2.8
17B	--	--	--		
18	1.90	1.70	1.80	1.5	0.9
19	1.20	1.20	1.20	-0.5	-0.3
21	1.30	-	1.30	-0.2	-0.1

The results are written as reported by the laboratories. [a]Second replicate result not reported; [b]not analysed.

Table 14. Results of analysis for aflatoxin G_1 (AFG_1) in maize

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1	1.9	1.9	1.90	9.1	3.6
2	--[a]	--			
3	<4.9	<4.9			
4	--	--			
6	0.7	0.6	0.65	0.1	0.1
7	<2.5	<2.5			
8	0.5	0.5	0.50	-0.9	-0.4
9A	0.6	0.6	0.60		
9B	<1	<1		-0.2	-0.1
10A	<1	-[b]			
10B	<2.5	<2.5			
11	--	--			
12	<1	-			
13	--	--			
14	<0.5	<0.5			
15	0.75	0.65	0.70	0.5	0.2
16	0.57	-	0.57	-0.4	-0.2
17A	--	--		-4.5	-1.8
17B	--	--			
18	1.00	1.00	1.00	2.7	1.0
19	<0.5	<0.5			
21	0.40	-		-1.7	-0.7

The results are written as reported by the laboratories. [a]Not analysed; [b]second replicate result not reported.

Table 15. Results of analysis for aflatoxin B_2 (AFB_2) in maize

Laboratory code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)
1	<0.2	0.70
2	--[a]	--
3	<22	<22
4	--	--
6	<0.6	<0.6
7	<2.5	<2.5
8	<0.5	<0.5
9A	0.20	0.10
9B	<1	<1
10A	<1	-[b]
10B	<2.5	<2.5
11	--	--
12	<1	-
13	--	--
14	<0.5	<0.5
15	<0.5	<0.5
16	<0.2	-
17A	--	--
17B	--	--
18	<1	<1
19	<0.5	<0.5
21	0.10	-

The results are written as reported by the laboratories. [a]Not analysed; [b]second replicate result not reported.

Table 16. Results of analysis for aflatoxin G_2 (AFG_2) in maize

Laboratory code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)
1	<0.2	2.20
2	--[a]	--
3	<4.1	<4.1
4	--	--
6	<0.8	<0.8
7	<2.5	<2.5
8	--	--
9A	0.20	-[b]
9B	<2.5	<2.5
10A	<1	-
10B	<2.5	<2.5
11	--	--
12	<1	-
13	--	--
14	<0.5	<0.5
15	<0.5	<0.5
16	<0.2	-
17A	--	--
17B	--	--
18	<1	<1
19	<1	<1
21	0.10	-

The results are written as reported by the laboratories. [a]Not analysed; [b]second replicate result not reported.

Table 17 Results of analysis and *z*-scores for deoxynivalenol (DON) in wheat

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1	1661.1	1414.7	1537.9	1.2	1.0
2	1116.0	-[a]	1116.0	-0.9	-0.8
3	1273.2	1222.0	1247.6	-0.3	-0.2
4	1117.5	965.9	1041.7	-1.3	-1.1
6	1311.6	1352.8	1332.2	0.2	0.1
7	1657.0	1756.1	1706.6	2.0	1.7
8	1486.0	1603.0	1544.5	1.2	1.1
9A	1517.0	1662.0	1589.5	1.5	1.2
9B	989.8	1107.9	1048.9	-1.2	-1.1
10A	939.6	-	939.6	-1.8	-1.5
10B	1112.9	1155.9	1134.4	-0.8	-0.7
11	1160.0	1120.0	1140.0	-0.8	-0.7
12	1193.0	-	1193.0	-0.5	-0.4
13	1211.0	1200.1	1205.6	-0.5	-0.4
14	1349.0	1295.0	1322.0	0.1	0.1
15	1380.1	1298.9	1339.5	0.2	0.2
16	1310.7	-	1310.7	0.1	0.1
17A	--[b]	--	--		
17B	1532.1	1564.9	1548.5	1.3	1.1
18	1224.6	1224.6	1224.6	-0.4	-0.3
19	1517.9	1452.8	1485.4	0.9	0.8
21	--	--	--		

The results are written as reported by the laboratories. [a]Second replicate result not reported; [b]not analysed.

Table 18. Results of analysis and *z*-scores for zearalenone (ZEA) in wheat

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1	148.7	120.4	134.6	-0.4	-0.5
2	168.0	-[a]	168.0	0.6	0.7
3	126.0	131.2	128.6	-0.6	-0.7
4	139.9	158.5	149.2	0.1	0.1
6	172.1	167.9	170.0	0.7	0.8
7	156.6	155.2	155.9	0.3	0.3
8	144.2	158.2	151.2	0.1	0.1
9A	199.0	191.0	195.0	1.5	1.7
9B	154.4	143.6	149.0	0	0.1
10A	74.5	-	74.5	-2.3	-2.6
10B	--[b]	--	--		
11	140.0	160.0	150.0	0.1	0.1
12	125.0	-	125.0	-0.7	-0.8
13	132.0	133.0	132.5	-0.5	-0.5
14	139.0	135.0	137.0	-0.3	-0.4
15	164.5	163.5	164.0	0.5	0.6
16	193.7	-	193.7	1.5	1.6
17A	99.0	96.8	97.9	-1.6	-1.7
17B	--	--	--		
18	178.9	170.5	174.7	0.9	1.0
19	121.2	125.9	123.6	-0.8	-0.8
21	--	--	--		

The results are written as reported by the laboratories. [a]Second replicate result not reported; [b]not analysed.

Table 19. Results of analysis and *z*-scores for HT-2 toxin (HT-2) in wheat

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1[a]	159.1	158.3	158.7	7.7	10.8
2	38.2	-[b]	38.2	-1.6	-2.2
3	59.2	58.8	59.0	0	0.0
4	56.0	75.4	65.7	0.5	0.7
6	50.2	52.7	51.5	-0.6	-0.8
7	68.2	67.8	68.0	0.7	1.0
8	58.8	54.3	56.6	-0.2	-0.2
9A	81.0	81.0	81.0	1.7	2.4
9B	57.7	55.9	56.8	-0.2	-0.2
10A	65.3	-	65.3	0.5	0.7
10B	<200	-			
11	--[c]	--	--		
12	<50	-			
13	57.8	57.4	57.6	-0.1	-0.1
14	56.0	62.0	59.0	0	0.0
15	53.3	52.9	53.1	-0.4	-0.6
16	49.0	-	49.0	-0.8	-1.1
17A	--	--	--		
17B	68.2	64.7	66.5	0.6	0.8
18	50.8	46.7	48.8	-0.8	-1.1
19	73.0	60.9	67.0	0.6	0.9
21	--	--	--		

The results are written as reported by the laboratories. [a]Outlier excluded from statistical evaluation; [b]Second replicate result not reported; [c]not analysed.

Table 20. Results of analysis and *z*-scores for ochratoxin A (OTA) in wheat

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1	<2	3.4	3.4	-2.4	-1.6
2	--[a]	--	--		
3	10.8	10.4	10.6	2.1	1.4
4	4.6	6.4	5.5	-1.1	-0.7
6	9.6	8.4	9.0	1.1	0.7
7	7.3	8.3	7.8	0.4	0.2
8	1.7	1.5	1.6	-1.5	-1.0
9A	9.5	9.8	9.7	1.5	1.0
9B	6.4	6.7	6.6	-0.4	-0.3
10A	4.3	-[b]	4.3	-1.8	-1.2
10B	<2.5	-			
11	--	--	--		
12	7.8	-	7.8	0.4	0.2
13	<10	<10			
14	7.5	8.3	7.9	0.4	0.3
15	6.9	6.9	6.9	-0.2	-0.1
16	7.7	-	7.7	0.3	0.2
17A[c]	155.3	154.2	154.8		
17B	--	--	--		
18	6.4	5.6	6.0	-0.8	-0.5
19	<1	<1			
21	9.9	-	9.9	1.7	1.1

The results are written as reported by the laboratories. [a]Not analysed; [b]second replicate result not reported; [c]Outlier excluded from statistical evaluation.

Table 21. Results of analysis for T-2 toxin (T-2) in wheat

Lab. code	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Mean (µg/kg)	z-score (Horwitz equation)	z-score (Reproducibility SD)
1[a]	18.8	19.5	19.2	6.0	2.2
2	35.0	-[b]	35.0	14.7	5.4
3	7.2	7.6	7.4	-0.5	-0.2
4	6.6	4.8	5.7	-1.4	-0.5
6	5.8	6.6	6.2	-1.1	-0.4
7	<20	<20			
8	14.7	14.5	14.6	3.5	1.3
9A	7.0	7.0	7.0	-0.7	-0.3
9B	4.3	4.9	4.6	-2	-0.7
10A	<50	-			
10B	<50	<50			
11	--[c]	--	--		
12	<50	-			
13	<10	<10			
14	<10	<10			
15	4.8	5.0	4.9	-1.8	-0.7
16	<10	-			
17A	--	--	--		
17B	<10	<10			
18	<30	<30			
19	<10	<10			
21	--	--	--		

The results are written as reported by the laboratories. [a]Outlier excluded from statistical evaluation; [b]Second replicate result not reported; [c]not analysed.

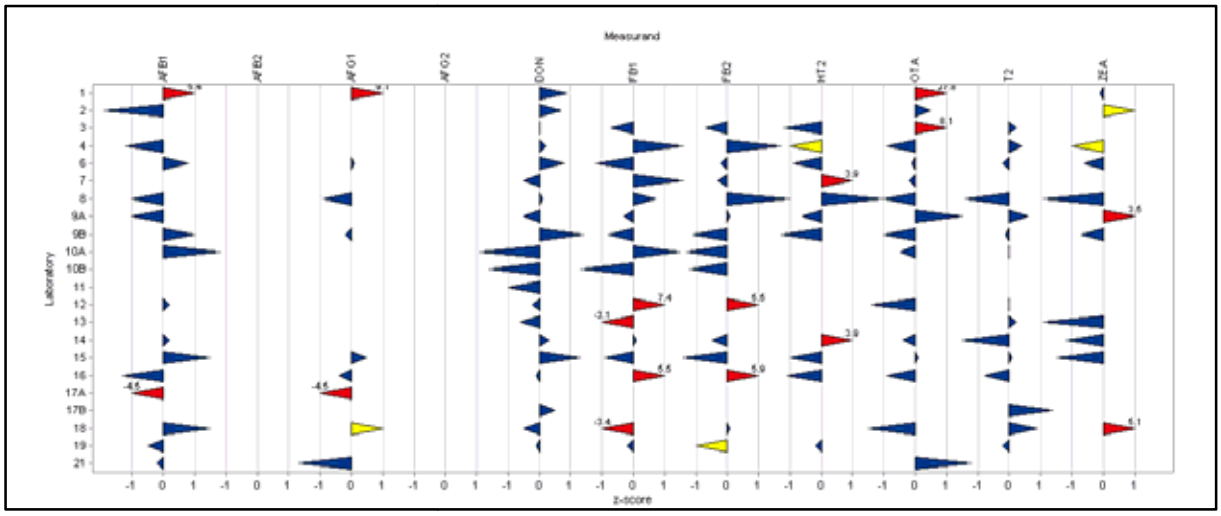

Figure 30. Summary graph of the laboratory's z-scores for all mycotoxins in maize. Target standard deviation calculated according to the truncated Horwitz equation.

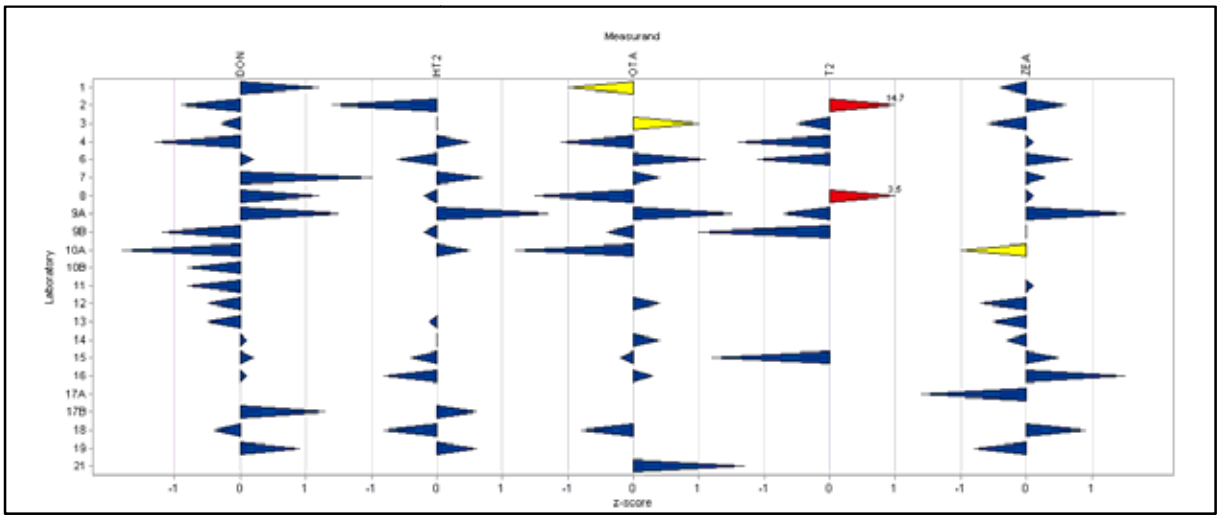

Figure 31. Summary graph of the laboratory's z-scores for all mycotoxins in wheat. Target standard deviation calculated according to the truncated Horwitz equation.

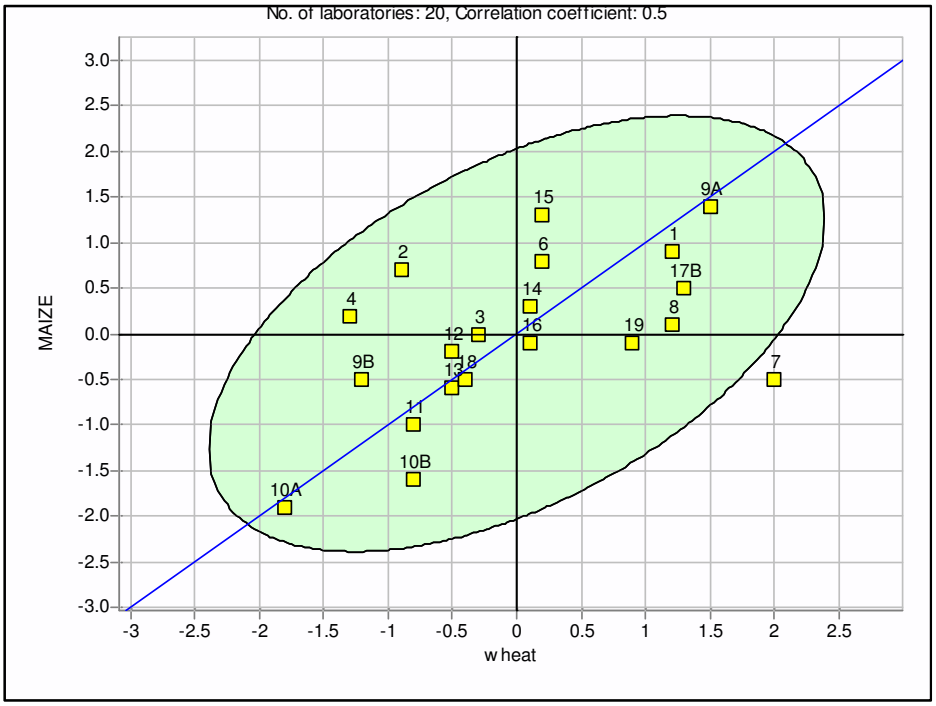

Figure 32. Youden Plot of DON z-scores in wheat against DON z-scores in maize

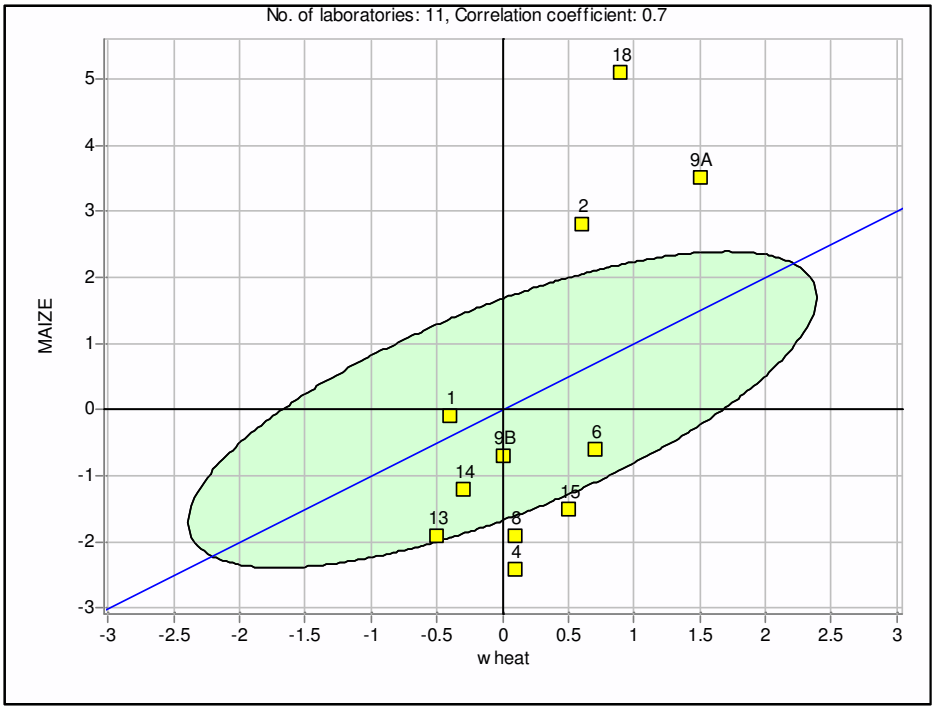

Figure 33. Youden Plot of ZEA z-scores in wheat against ZEA z-scores in maize

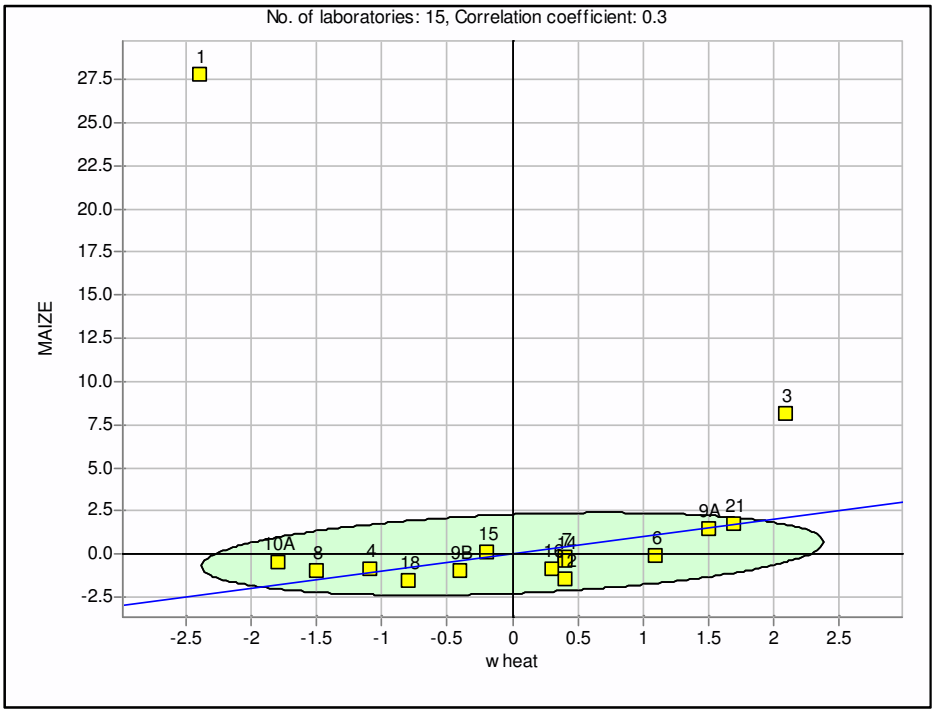

Figure 34. Youden Plot of OTA z-scores in wheat against OTA z-scores in maize

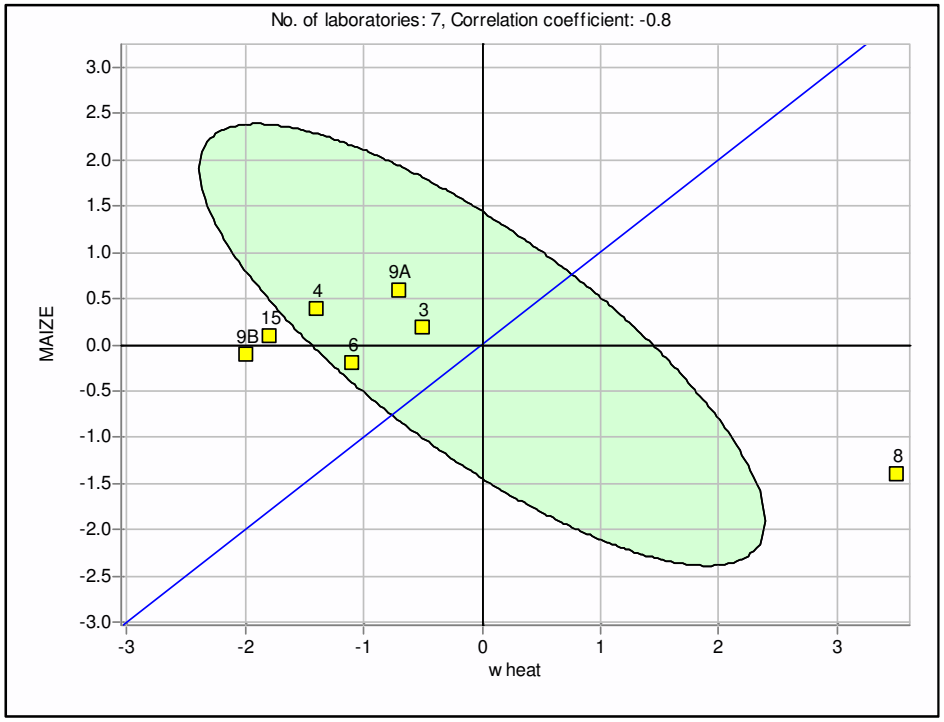

Figure 35. Youden Plot of T-2 z-scores in wheat against T-2 z-scores in maize

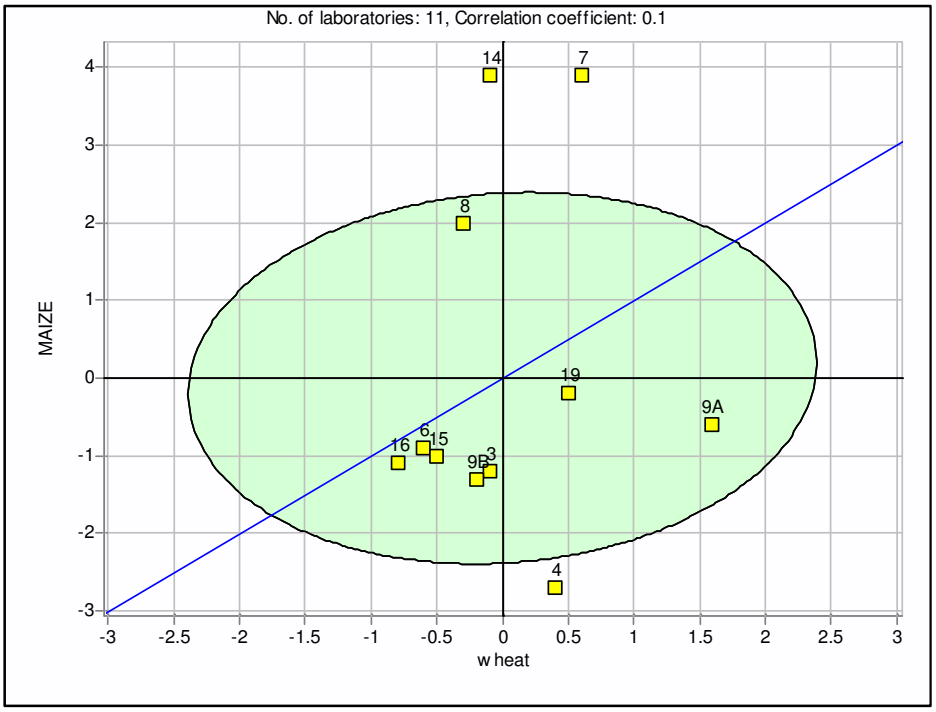

Figure 36. Youden Plot of HT-2 z-scores in wheat against HT-2 z-scores in maize

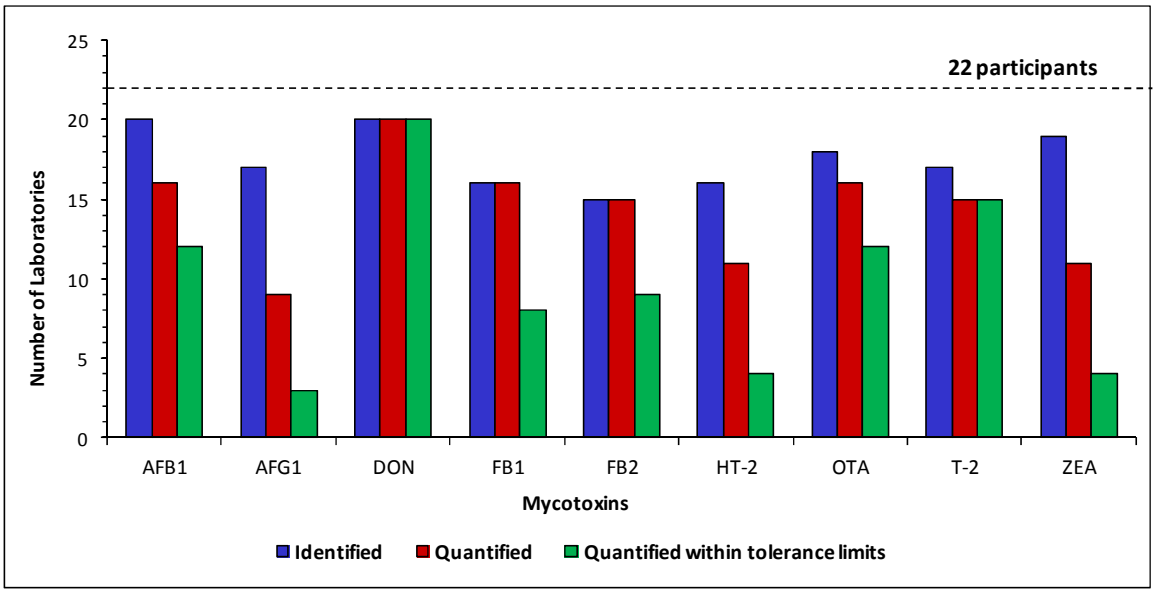

Figure 37. General overview obtained for each mycotoxin in maize

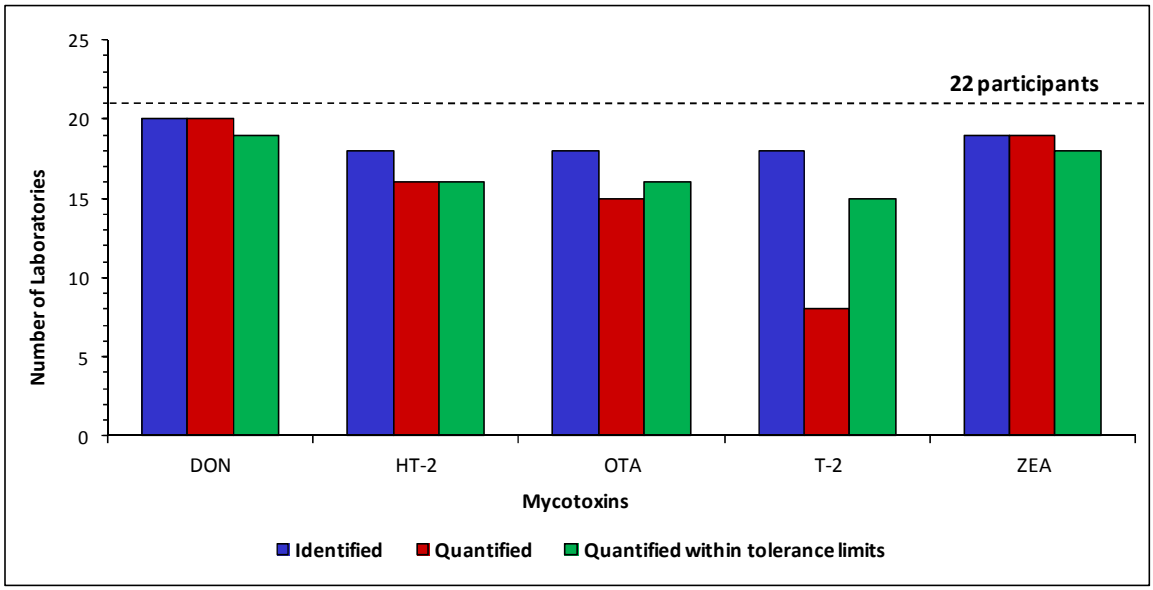

Figure 38. General overview obtained for each mycotoxin in wheat

Table 22. Overall performance of the laboratories in the identification and quantification of mycotoxins in maize and wheat

Lab code	Mycotoxins combinations in the two test samples			Mycotoxins			Successful
	Within tolerance limits	Total[a]	Percentage	Within tolerance limits	Total[b]	Percentage	
1	4	16	29 %	2	9	22 %	No
2	6	16	43 %	5	9	56 %	No
3	9	16	64 %	6	9	67 %	No
4	11	16	79 %	8	9	89 %	No
6	14	16	100 %	9	9	100 %	Yes
7	8	16	57 %	6	9	67 %	No
8	13	16	93 %	9	9	100 %	Yes
9A	12	16	86 %	8	9	89 %	Yes
9B	14	16	100 %	9	9	100 %	Yes
10A	9	16	64 %	7	9	78 %	No
10B	4	16	29 %	3	9	33 %	No
11	3	16	21 %	2	9	22 %	No
12	7	16	50 %	5	9	56 %	No
13	6	16	43 %	4	9	44 %	No
14	11	16	79 %	8	9	89 %	No
15	14	16	100 %	9	9	100 %	Yes
16	10	16	71 %	7	9	78 %	No
17A	1	16	7 %	1	9	11 %	No
17B	4	16	29 %	3	9	33 %	No
18	9	16	64 %	7	9	78 %	No
19	8	16	57 %	6	9	67 %	No
21	4	16	29 %	3	9	33 %	No

[a]11 mycotoxins (DON, FB_1, FB_2, ZEA, T-2, HT-2, OTA, AFB_1, AFG_1, AFB_2, AFG_2) in maize and 5 mycotoxins (DON, T-2, HT-2, OTA, ZEA) in wheat; [b]9 different mycotoxins, i.e. DON, FB_1, FB_2, ZEA, T-2, HT-2, OTA, AFB_1, AFG_1 considered for statistical evaluation.

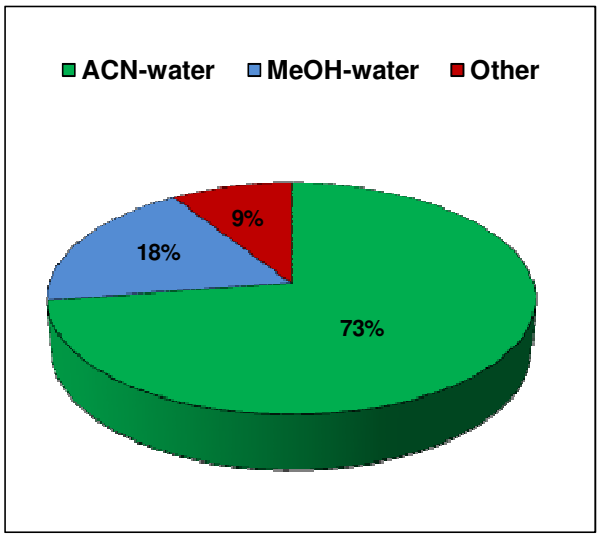

Figure 39. Extraction solvents used in the PT study by participant laboratories. *Abbreviations used: ACN = acetonitrile; MeOH = methanol*

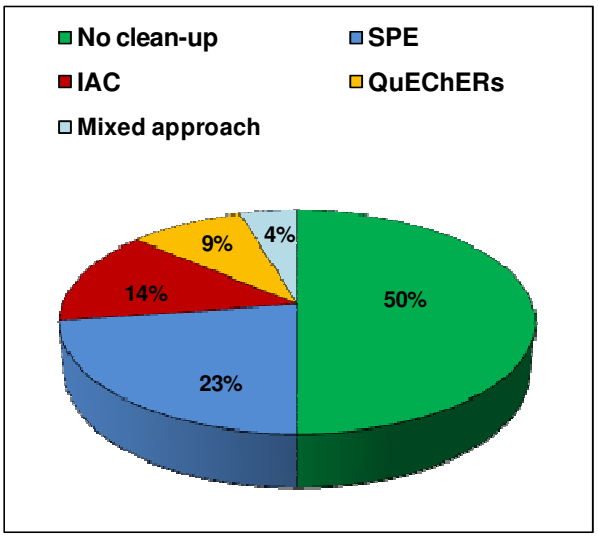

Figure 40. Sample extract preparation used in the PT study by participant laboratories. *Abbreviations used: SPE = solid phase extraction; IAC = immunoaffinity column; QuEChERs = Quick Easy Cheap Effective*

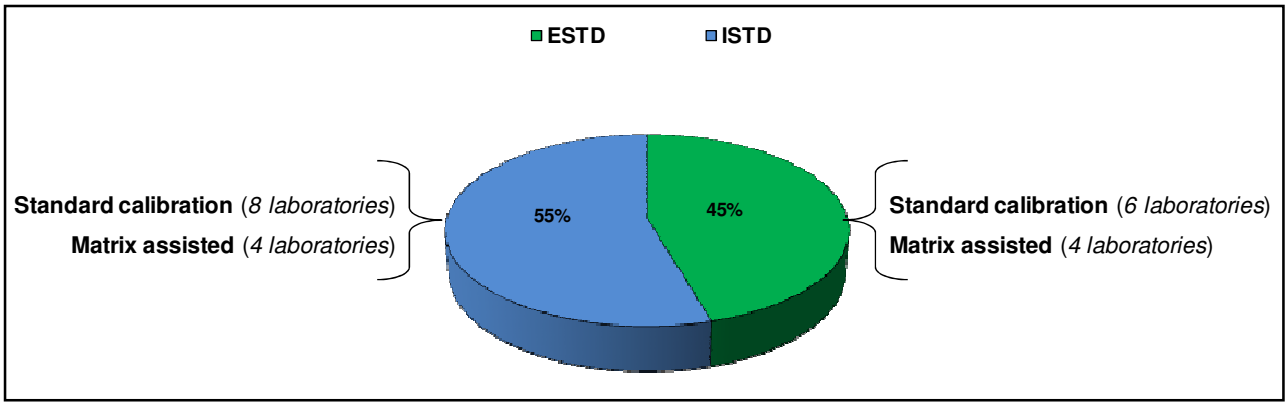

Figure 41. Quantification mode used in the PT study by participant laboratories. *Abbreviations used: ESTD = external calibration (neat solvent); ISTD = internal standard calibration*

9. Conclusions

As a conclusion of this PT study for LC-MS(MS) multi-mycotoxin methods in maize and wheat it could be concluded that:

a) The participation of the laboratories was regarded as satisfactory concerning the number of received results (86% of participation)
b) Fifty-five percent of laboratories analysed all the 11 targeted mycotoxins in maize, whereas 73% of laboratories analysed all the 5 mycotoxins in wheat. The remaining laboratories reported results for a different combination of mycotoxins (from 2 to 10 in maize and from 1 to 4 in wheat).
c) The assessment of laboratories on the base of their z-scores indicated that only 23% of laboratories were considered successful for the whole interlaboratory test.
d) The majority of laboratories used mixtures of acetonitrile-water or methanol-water mixtures for mycotoxins extraction.
e) Fifty percent of laboratories analysed the crude extract; the other cleaned-up the extract prior to the analysis.
f) The majority of laboratories used the internal standard calibration mode using stable isotope internal standards (^{13}C) for mycotoxins determination; the other laboratories used external calibration mode using native standard mycotoxins.

10. Acknowledgements

This work was carried out with the financial support of the Project MIUR – PON02_00186_3417512, “New Strategies for Improvement of Food Safety: Prevention, Control, Correction” (S.I.Mi.S.A).

The organizers of the study acknowledge the valuable technical assistance of Roberto Schena (ISPA-CNR) for the preparation of test materials and all the laboratories participating in the exercise (**Table 23**).

Table 23. Participant laboratories

Organization	Country
Barilla G.R. F.lli SpA	Italy
Bonassisa Lab	Italy
EC-Joint Research Centre - IRMM	Belgium
University of Natural Resources and Applied Life Sciences (IFA-Tulln)	Austria
RIKILT-Institute of Food Safety, Natural Toxins and Pesticides	Netherlands
University of Bari Aldo Moro	Italy
Veterinary and Agrochemical Research Centre, CODA-CERVA	Belgium
Food & Environment Research Agency	United Kingdom
Virginia Polytechnic Institute and State University	USA
NofaLab	Netherlands
AGES GmbH, National Reference Lab for Mycotoxin	Austria
Romer Labs Singapore Pte Ltd	Repubblica di Singapore
Romer Labs Diagnostic GmbH	Austria
LVA GmbH	Austria
Max RubnerInstitut	Germany
Canadian Grain Commission (CGC)	Canada
Southern African Grain Laboratory NPC (SAGL)	South Africa
China Grain Products Research & Development Institute Cereal Testing & Analysis Section	Taiwan

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Annexes

Annex 1. Invitation letter

National Research Council of Italy
INSTITUTE OF SCIENCES OF FOOD PRODUCTION

MoniQA
www.moniqa.org

Multi-mycotoxin PT

Invitation letter

Dear Colleagues,

It is our pleasure to invite you to participate in a multi-mycotoxin proficiency test (PT) organized by **ISPA-CNR** in the framework of the project "New Strategies for Improvement of Food Safety: Prevention, Control, Correction" (S.I.Mi.S.A. PON_02_00186_3417512, project of the Italian Ministry of Education, Universities and Research) and promoted by the **MoniQA** Association (Monitoring and Quality Assurance in the Total Food Supply Chain, www.moniqa.org).

The two materials involved in the study are unprocessed wheat flour and unprocessed maize flour contaminated with the mycotoxins included in the Commission Regulation 1881/2006/EC (and relevant amendments) as reported below:

wheat flour:
- ochratoxin A
- deoxynivalenol
- zearalenone
- T-2 and HT-2 toxins

maize flour:
- ochratoxin A
- deoxynivalenol
- zearalenone
- T-2 and HT-2 toxins
- fumonisins B_1 and B_2
- aflatoxins B_1, B_2, G_1, G_2

The main objective of the PT is to provide interested laboratories with an opportunity to test their multi-mycotoxin methods and to compare their results with those of other laboratories. The use of LC-MS(MS) methods, although not strictly required, is highly recommended. However LC methods with fluorescence or UV detection will be considered as well.
Participants will be asked to complete a comprehensive questionnaire to provide details of the applied method. This will enable us to feed back information to all participants , not only on their own proficiency, but also on currently used methodologies for multi-mycotoxin analysis and method related performances.
Each participant will be provided with one sample of each material (ca. 80 g each) and will be asked to report the results of 2 independent analyses for each material. Participants are not obliged to determine all mycotoxins in each material, and are free to report only on those mycotoxins that can be simultaneously determined with their multi-mycotoxin methodology. However the use of multi-mycotoxin methods able to determine simultaneously two mycotoxin groups at least (e.g. trichothecenes and aflatoxins, or trichothecenes and ochratoxin A, etc.) is mandatory.

The PT is free of charge and the time schedule is as follows:
- deadline for registration: 20/05/2014
- shipping of samples: 15/06/2014

National Research Council of Italy
INSTITUTE OF SCIENCES OF FOOD PRODUCTION

MonIQA

- deadline for submitting the results: 31/07/2014
- draft report: 30/10/2014
- final report: 30/11/2014

For further information about the procedure of the proficiency test, please contact Veronica Lattanzio (email: veronica.lattanzio@ispa.cnr.it) and Annalisa De Girolamo (email: annalisa.degirolamo@ispa.cnr.it).

If you wish to participate please complete the enclosed registration form and send it by email to Veronica Lattanzio and Annalisa De Girolamo.

Annex 2. Registration form

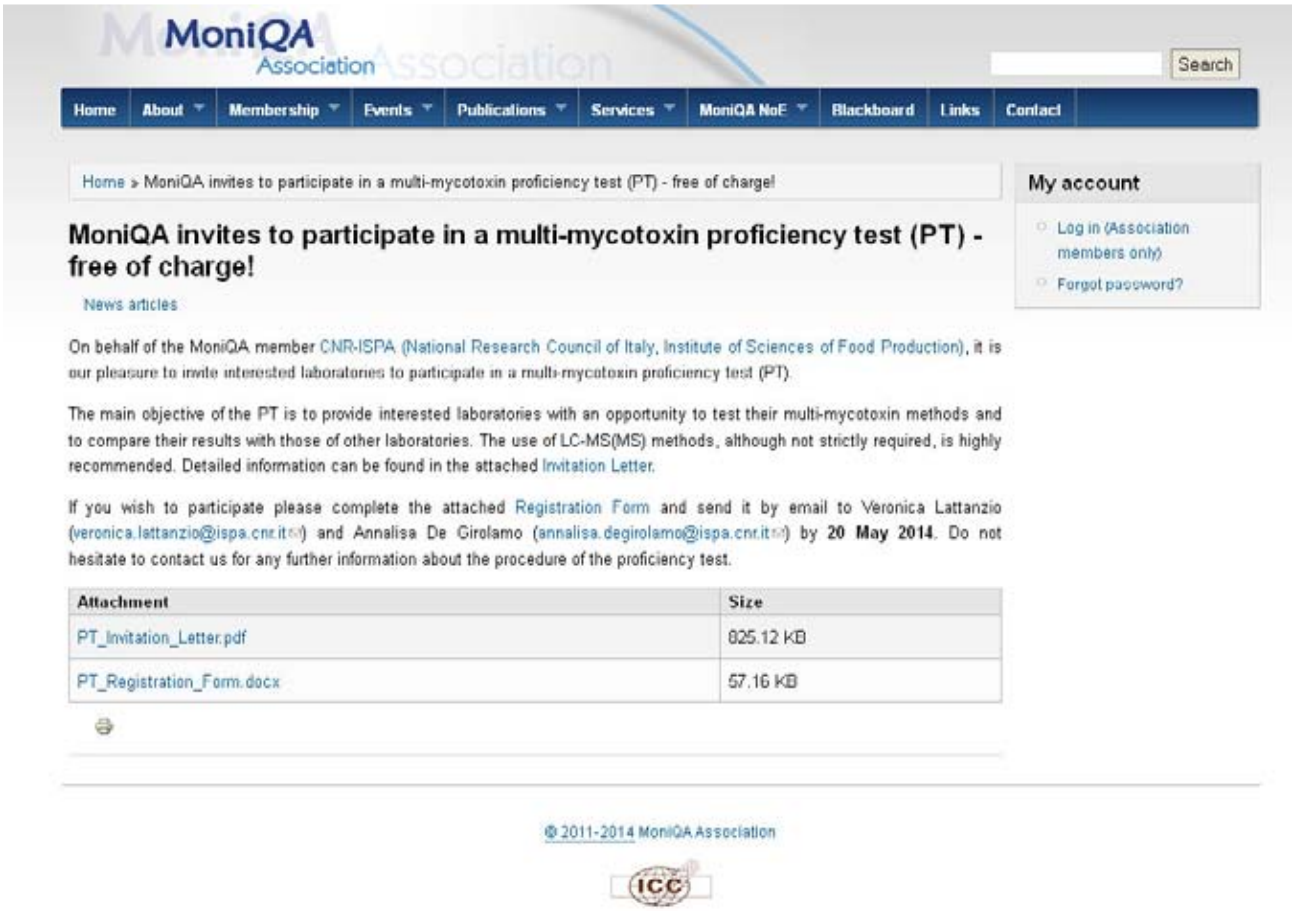
National Research Council of Italy
INSTITUTE OF SCIENCES OF FOOD PRODUCTION

MoniQA
www.moniqa.org

Multi-mycotoxin Proficiency Test
Registration Form
Participant Laboratory (name of the Institution and relevant acronym if present)
Contact Person(s) *Name* *Email address* *Tel /Fax*
Delivery Address
Method(s) that will be used and relevant combination of mycotoxins (please specify if you will use LC-MS(MS) or HPLC-UV/FLD method)

Please fill in and return the registration form by email to Veronica Lattanzio (veronica.lattanzio@ispa.cnr.it) and Annalisa De Girolamo (annalisa.degirolamo@ispa.cnr.it).

Annex 3. MoniQA Association promotion



https://www.moniqa.org/news/cnr_ispa_proficiency_test_may_2014#attachments

Annex 4. ICC promotion

https://www.icc.or.at/news/cnr_ispa_proficiency_test_may_2014

Annex 5. Stability Study

Table A5.1. Raw experimental data of the stability testing for DON in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			1116	940	1006	908	1033	870
0.50			1057	1068	1071	854	1028	739
1			1397	867	1202	870	1068	902
1.5	766	945	985	1041	683	977	1148	1084

Table A5.2. Raw experimental data of the stability testing for FB_1 in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			1636	1242	2032	1598	1904	2031
0.50			1521	1828	1764	1764	1649	1636
1			2249	1585	1623	1802	1955	1419
1.5	1674	1725	1993	1725	1725	1751	1470	1297

Table A5.3. Raw experimental data of the stability testing for FB_2 in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			510	386	596	505	576	331
0.50			468	564	522	492	465	467
1			688	491	522	540	554	402
1.5	530	514	565	493	511	492	474	441

Table A5.4. Raw experimental data of the stability testing for ZEA in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			9.45	5.05	10.2	5.40	11.2	5.51
0.50			11.3	8.90	10.4	5.44	7.60	7.49
1			12.2	5.09	15.1	6.80	9.45	9.01
1.5	5.80	6.78	9.23	8.36	3.56	8.58	12.7	9.01

Table A5.5. Raw experimental data of the stability testing for T-2 in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			25.3	23.2	18.5	12.3	18.3	9.23
0.50			22.8	12.6	33.7	12.8	29.7	34.5
1			31.7	34.2	44.7	39.2	47.7	36.1
1.5	17.6	21.7	14.7	38.1	33.4	10.6	48.0	21.4

Table A5.6. Raw experimental data of the stability testing for HT-2 in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			25.0	22.1	28.6	29.7	23.1	23.6
0.50			20.1	23.4	18.0	30.0	17.9	19.9
1			33.7	13.4	17.1	24.8	21.2	17.5
1.5	34.4	33.3	24.2	15.6	21.7	26.2	17.0	21.8

Table A5.7. Raw experimental data of the stability testing for OTA in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			1.34	1.84	1.86	1.93	1.76	1.86
0.50			1.32	1.95	1.64	1.84	1.65	1.99
1			1.95	1.95	1.73	1.99	2.10	0.28
1.5	1.92	1.87	1.87	1.67	1.64	1.49	1.85	1.58

Table A5.8. Raw experimental data of the stability testing for AFB_1 in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			1.54	1.56	1.36	1.30	1.65	1.56
0.50			0.96	1.35	1.21	1.59	1.53	1.17
1			1.25	1.31	1.43	1.45	1.36	1.36
1.5	1.07	1.07	1.27	1.29	1.48	1.33	1.08	1.28

Table A5.9. Raw experimental data of the stability testing for AFB_2 in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			0.05	0.07	0.06	0.06	0.07	0.07
0.50			0.05	0.07	0.06	0.07	0.08	0.06
1			0.06	0.06	0.07	0.07	0.06	0.06
1.5	0.05	0.05	0.05	0.06	0.08	0.07	0.06	0.07

Table A5.10. Raw experimental data of the stability testing for AFG_1 in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			0.30	0.26	0.14	0.32	0.25	0.26
0.50			0.21	0.27	0.18	0.19	0.26	0.25
1			0.31	0.27	0.29	0.26	0.26	0.28
1.5	0.25	0.22	0.35	0.19	0.20	0.26	0.23	0.26

Table A5.11. Raw experimental data of the stability testing for AFG_2 in maize test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			0.01	0.02	0.03	0.02	0.02	0.02
0.50			0.01	0.02	0.02	0.02	0.02	0.02
1			0.03	0.02	0.02	0.02	0.02	0.03
1.5	0.02	0.02	0.02	0.02	0.03	0.01	0.02	0.02

Table A5.12. Raw experimental data of the stability testing for DON in wheat test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			1160	758	1291	1267	1200	813
0.50			1456	951	1515	1298	1618	1283
1			1342	1512	1338	1342	1279	1220
1.5	1113	1211	1247	1425	1267	1693	1425	1476

Table A5.13. Raw experimental data of the stability testing for ZEA in wheat test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			86.0	53.9	106	120	120	116
0.50			32.0	72.0	89.2	122	135	33.0
1			78.7	57.9	129	97.0	49.0	55.8
1.5	103	114	53.2	115	65.7	77.2	81.9	124

Table A5.14. Raw experimental data of the stability testing for T-2 in wheat test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			8.94	8.54	8.94	8.90	9.47	9.02
0.50			8.98	8.76	9.38	8.77	8.65	8.84
1			8.60	9.02	8.34	8.96	8.98	8.79
1.5	8.79	8.96	8.67	8.87	8.93	8.77	9.09	8.99

Table A5.15. Raw experimental data of the stability testing for HT-2 in wheat test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			17.1	14.4	17.1	18.8	22.7	20.8
0.50			22.1	20.6	27.9	12.9	15.5	36.8
1			23.4	9.5	9.46	18.1	29.2	20.0
1.5	16.1	21.7	24.4	16.6	25.2	29.8	27.6	13.7

Table A5.16. Raw experimental data of the stability testing for OTA in wheat test material. Mycotoxin concentrations are expressed in µg/kg.

Ageing (months)	Storage temperature							
	-20°C		+4°C		+20°C		+60°C	
	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate	1st replicate	2nd replicate
0.25			2.92	2.24	2.81	3.60	3.57	3.35
0.50			2.82	3.07	3.62	2.84	3.75	3.49
1			3.92	2.99	2.67	3.31	3.52	2.72
1.5	2.73	3.43	3.39	4.00	3.33	3.93	3.90	2.96

Annex 6. Accompanying Letter

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MoniQA
www.moniqa.org

Multi-mycotoxin PT

Cover Letter

Bari, 16 June 2014

Dear Partner,

We are announcing the opening of the multi-mycotoxin proficiency test (PT) organized by **ISPA-CNR** in the framework of the Italian project **S.I.Mi.S.A.** and promoted by the **MoniQA Association** (www.moniqa.org).
We thank you for joining the study and ask you, in order to obtain consistent results, to please follow all instructions included in the documents you received.

In particular, you should note the following:

1. Please check that the content of the parcel is complete and undamaged. A **Receipt form** is enclosed in the parcel; please fill out and e-mail it back to us (veronica.lattanzio@ispa.cnr.it and annalisa.degirolamo@ispa.cnr.it).
2. Please store the two test materials at -18°C until the analysis. Let materials to reach ambient temperature before use.
3. In the parcel you will find your participation code (**LAB ID**): please use it in all following communications.
4. All samples should be homogenized before taking the test portion to perform the analyses.
5. Analyse each test material twice. In case you should encounter any problem during the analysis, please contact us for a replacement of the sample.
6. A **Results Report form** is attached to this e-mail. Once you have carried out all the analyses, please, fill out the Results Report Form by reporting your results and the method you used to analyse the two test materials and e-mail it back to us (veronica.lattanzio@ispa.cnr.it and annalisa.degirolamo@ispa.cnr.it) by the end of the study.

Should you have comments or questions, please, do not hesitate to contact us at veronica.lattanzio@ispa.cnr.it and annalisa.degirolamo@ispa.cnr.it.
The deadline for submitting the results is **31/07/2014** which gives a time of 4 weeks for the experiments.
We are looking forward to year from you.

With kind regards
Veronica Lattanzio and Annalisa De Girolamo

Annex 7. Acknowledgement of receipt form

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MoniQA
www.moniqa.org

MATERIALS RECEIPT FORM

Contact person and Laboratory:	

NOTE: upon receipt store test materials at -18°C until the analysis

Please fill the table below

Date of the receipt:	
CONTENTS of PARCEL	
Wheat test material is missing or damaged. I require a replacement.	□ YES □ NO
Maize test material is missing or damaged. I require a replacement.	□ YES □ NO

Please return the completed form by e-mail to Veronica Lattanzio veronica.lattanzio@ispa.cnr.it) and Annalisa De Girolamo (annalisa.degirolamo@ispa.cnr.it) or by fax (+39 080 5929374).

Sede Istituzionale: Via Amendola, 122/O – 70126 Bari (Italy); Tel. 080 5929365, Fax 080 5929374
U.O.S.: Lecce (Tel. 0832 422600), Milano (Tel. 02 50316685), Sassari (Tel. 079 233466), Torino (Tel. 011 6709230)

Annex 8. Results report form and questionnaire

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REPORT SHEETS

Following the analyses of test materials, please complete this form reports and return them to the co-ordinators by e-mail to:

veronica.lattanzio@ispa.cnr.it

annalisa.degirolamo@ispa.cnr.it

by July 31, 2014

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1. RESULTS OF ANALYSES.

Lab. Code: _____

Please report the results with one decimal point, f.i. 1250.0 µg/kg kg and specify if results were corrected for the recovery of the method or not. In case of results corrected for recoveries, please report the recovery.

Mycotoxin	Maize Test Material (µg/kg)	Wheat Test Material (µg/kg)
DON		
FB_1		
FB_2		
ZEA		
T-2 toxin		
HT-2 toxin		
OTA		
AFB_1 AFG_1		
AFB_2		
AFG_2		

*Date:*_____

*Signature.*_____

2

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2. QUESTIONNAIRE ON METHOD DETAILS

Lab. Code: _____

Please describe the method used for simultaneous determination of target mycotoxins in test materials. We would appreciate a method description containing as many details as possible. In particular, please give details requested in the following tables.

NOTE: Save properly all the data concerning this trial because we may ask for certain chromatograms during the results evaluation phase.

2.1 SAMPLE PREPARATION

Test sample size (g)
Volume and composition of the extraction solvent mixture
Extraction mode (*blending, shaking, sonication, etc.*), **temperature and extraction time**
Extract centrifugation and/or filtration
Extract dilution (if any), *please specify the dilution factor and the solvent used for dilution*
Extract volume subjected to the clean up procedure (if any)

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Clean up procedure: *if a cleanup procedure was employed, please specify the type of cleanup (immunoaffinity column, solid phase extraction column, liquid-liquid partitioning, QuEChERS, etc.), and details of the procedure (sample extract preparation before cleanup, washing conditions, toxins elution conditions)*

Volume and solvent composition of the final purified extract

If the method is published please give the complete reference reference *(alternatively please provide the limits of detection and quantification of the method and the approach used to quantify them)*

2.2 CALIBRATION

Please describe all steps in the preparation of calibration solutions *(use of standard calibration, matrix assisted calibration, isotope labelled mycotoxins)*

2.3 EQUIPMENT INFORMATION

Please specify the brand name and model of LC pump, autosampler, MS(MS) detector and eventual additional detector (UV or FLD):

LC Pump

AUTOSAMPLER

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MS Detector
Additional Detector (UV, PDA, FL)

2.4 LC CONDITIONS

LC column characteristics: type, manufacturer, dimensions, particles size. If a precolumn was used please specify its characteristics
Flow rate and composition of the LC mobile phase used.
Volume (µL) and equivalent matrix amount (mg) of injected sample extract

2.5 MS CONDITIONS

ION SOURCE ⊔ ESI ⊔ APCI ⊔ other (specify):
DETECTOR ⊔ single quadrupole ⊔ triple quadrupole ⊔ time of flight ⊔ Orbitrap ⊔ ion trap ⊔ other (specify):
ACQUISITION MODE ⊔ Full scan ⊔ Selected Ion Monitoring (SIM) ⊔ Selected Reaction Monitoring (SRM) ⊔ Product Ion Scan ⊔ other (specify):

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2.6 MS ACQUISITION PARANETERS

According to your instrument pease select the appropriate table below and complete it.

Table 2.6.1. SRM/MRM or full scan product ion spectra parameters

Mycotoxin	Precursor ion (m/z)	Adduct[a]	Product ion(s) (m/z)[b]
DON			
AFG_2			
AFG_1			
AFB_2			
AFB_1			
HT-2 toxin			
T 2 toxin			
FB_1			
FB_2			
OTA			
ZEA			

[a] *Please specify, e.g. $[M+H]^+$, $[M+NH_4]^+$, $[M-H]^-$, etc.*
[b] *Please mark with an asterisk the quantifier ion*

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Table 2.6.2 Low resolution NS parameters (full scan, SIN)

Mycotoxin	Diagnostic ion(s)[a]	Adduct[b]
DON		
AFG_2		
AFG_1		
AFB_2		
AFB_1		
HT-2 toxin		
T-2 toxin		
FB_1		
FB_2		
OTA		
ZEA		

[a] *Please mark with an asterisk the quantifier ion*
[b] *If the molecular ion is included among the diagnostic ions please specify the adduct, e.g. $[M+H]^+$, $[M+NH_4]^+$, $[M-H]^-$, etc.*

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Table 2.1.3 High resolution MS

Mycotoxin	Diagnostic ion (s)[a]	Adduct[b]
DON		
AFG_2		
AFG_1		
AFB_2		
AFB_1		
HT-2 toxin		
T-2 toxin		
FB_1		
FB_2		
OTA		
ZEA		

[a] Please mark with an asterisk the quantifier ion
[b] If the molecular ion is included among the diagnostic ions please specify the adduct, e.g. $[M+H]^+$, $[M+NH_4]^+$ $[M-H]^-$, etc.

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3. GENERAL COMMENTS TO THE EXCERCISE

Were the instructions and questionnaire adequate? If not please suggest the additional instructions and questions you would have liked to be asked for.
Please, repor: any cifficulties and/or observations conceming this Prcficiency Test.

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Annex 9. Experimental details

Table A9.1. Summary of information on analytical methodologies reported in the questionnaire (I)

Lab Code	Mycotoxin analyzed	Test sample size (g)	Extraction solvent mixture	Solvent to sample ratio	Extraction mode	Extraction time (min)
1	DON, ZEA, T2/HT2, OTA, AFs	10	ACN/H_2O (84:16, v/v)	10	Blending	2
2	DON, ZEA, T-2/HT2, OTA, AFs	5	ACN/H_2O/Formic Acid (79:20:1, v/v/v)	2	Shaking	90
3	All	5	ACN/H_2O/Formic Acid (79:20:1, v/v/v)	4	Shaking	60
4	All	5	ACN/H_2O/Formic Acid (79:20:1, v/v/v)	4	Shaking	60
6	All	20	ACN/H_2O/Formic Acid (79:20:1, v/v/v)	4	Shaking	90
7	All	2.5	H_2O/ACN 1% acetic acid (7.5:10, v/v/v)	7	Shaking	30
8	All	10	1st extraction: H_2O 2nd extraction: MeOH/H_2O (60:40, v/v)	10	Blending	4
9A	All	4	H_2O/IprOH/Acetone/AcOH (7.5:2.5:7.3:0.2, v/v/v/v)	4.4	Shaking	60
9B	All	5	ACN/AcOH/H_2O (80:2:18, v/v/v)	4	Shaking	60
10A	All	25 25	MeOH/H_2O (70:30, v/v) for DON, ZEA, T-2, HT-2 MeOH/H_2O (60:40, v/v) for AFs, OTA, FBs	4 4	Blending Blending	3 3
10B	All	5	ACN/H_2O/AcOH (79:20:1, v/v/v/v)	4	Shaking	120
11	DON, ZEA	1	ACN/H_2O (86:14, v/v)	8	Shaking	60
12	All	10	MeOH/H_2O (80:20, v/v)	6	Shaking	60
13	All	25	ACN/H_2O/Acetic Acid (79:20:1, v/v/v)	4	Shaking	120
14	All	25	ACN/H_2O (50:50, v/v)	4	Shaking	60
15	All	10	ACN/H_2O/Formic Acid (70:30:0.1, v/v/v)	8	Shaking	60
16	All	10	ACN/H_2O/Formic Acid (84:16:1, v/v/v)	2	Vortex, ultrasonic bath	-
17A	ZEA, OTA, AFs	10	ACN/H_2O (80:20, v/v)	10	Shaking	60
17B	DON,T-2,HT-2	10	ACN/H_2O (80:20, v/v)	10	ASE at 80°C	45
18	All	20	ACN/H_2O/Acetic Acid (75:25:1, v/v/v)	5	Shaking	20
19	All	5	1st extraction: MeOH/H_2O (80:20, v/v) 2nd extraction: MeOH/H_2O (20:80, v/v)	8	Shaking Shaking	60 30
21	OTA, AFs	10 for AFs 20 for OTA	MeOH/H_2O (80:20, v/v) for AFs ACN/H_2O (60:40, v/v) for OTA	10 for AFs 5 for OTA	Blending Blending	2 2

DON, deoxynivalenol; ZEA, zearalenone; OTA, ochratoxin A; AFs, aflatoxins; ACN, acetonitrile; MeOH, methanol; H_2O, water; AcOH, acetic acid; ASE, accelerated solvent extraction; IprOH, isopropyl alcohol.

Table A9.1. Summary of information on analytical methodologies reported in the questionnaire (II)

Lab Code	Clean up type	Calibration mode	Injected matrix (mg)	LC column	MS detection mode
1	SPE	ISTD (^{13}C mycotoxins) +ESTD	100	Kinetex C18 (100 × 2.10 mm, 2.6 µm) (Phenomenex)	HRMS (3 MS/MS ions)
2	No clean-up	ESTD	5.00	Zorbax Eclipse Plus (100 x 2.1 mm, 1.8 µm) (Agilent)	SRM
3	No clean-up	ISTD (^{13}C mycotoxins + matrix assisted)	1.25	Ascentis Express C18 (100 x 2.1 mm, 2.7 µm) (Supelco)	SRM
4	No clean-up	ISTD (^{13}C mycotoxins + matrix assisted)	0.50	Ascentis Express CX18 (75 x 2.1 mm, 2.7 µm) (Supelco)	SRM
6	No clean-up	ESTD	0.63	Gemini C18 (150 x 4.6 mm, 5 µm) (Phenomenex)	SRM
7	QuEChERs-*like* (liquid-liquid partition)	ISTD (matrix assisted, 1 level)	0.63	Ultra Aqueous C18 (100 x 2.1 mm, 3 µm) (Restek)	SRM
8	IAC (multi-antibody)	ISTD (^{13}C mycotoxins) + ESTD	25.0	Gemini C18 (150 x 2.1 mm, 5 µm) (Phenomenex)	SRM
9A	QuEChERs-*like* (liquid-liquid partition)	ISTD (^{13}C mycotoxins) + ESTD	2.00	Kinetex XDB (100 × 4.6 mm, 2.6 µm) (Phenomenex)	SRM
9B	No clean-up	ISTD (13C mycotoxins) + ESTD	1.00	Acquity UPLC - BEH C18 (2.1 x 100 mm, 1.7 µm) (Waters)	SRM
10A	IAC (multi-antibody)	ESTD	0.13 (neutral run) 0.25 (acidic run	Acquity UPLC – HSS T3 (100 x 2.1 mm,1.8 µm) (Waters)	SRM
10B	No clean-up	ESTD	0.13 (neutral run) 0.25 (acidic run)	Acquity UPLC – HSS T3 (100 x 2.1 mm,1.8 µm) (Waters)	SRM
11	SPE	ESTD (matrix assisted)	0.50	GC colum: HP-5MS (30 x 0.25 mm, 0.25 µm)	SIM
12	No clean-up	External standard calibration	0.08	Acquity UPLC – HSS T3 (100 x 2.1 mm,1.8 µm) (Waters)	SRM
13	No clean-up	ISTD (^{13}C mycotoxins)	0.50	Gemini C18 (150 x 4.6 mm, 5 µm) (Phenomenex)	SRM
14	SPE	ISTD (13C mycotoxins) + ESTD	2.50	Gemini C18 (150 x 4.6 mm, 5 µm) (Phenomenex)	SRM
15	SPE for AFs No clean-up for the others	ISTD (^{13}C mycotoxins) +ESTD	1.90	Kinetex C18 (100 × 3 mm, 2.6 µm) (Phenomenex)	SRM
16	No clean-up	ISTD (^{13}C mycotoxins) + ESTD	1.90	Zorbax Eclipse Plus C18 (100 x 2.1 mm, 5 µm) (Agilent)	SRM
17A	No clean-up	ESTD (matrix assisted)	0.33	Luna Phenyl-Hexyl, (150 x 2 mm, 5 µm) (Phenomenex)	SRM
17B	SPE	ESTD (matrix assisted)	3.33	Luna Phenyl-Hexyl, (150 x 2 mm, 5 µm) (Phenomenex)	SRM
18	No clean-up	ISTD (^{13}C mycotoxins) + ESTD	0.31	Kinetex C18 (50 × 2.1 mm, 2.6 µm) (Phenomenex)	SRM
19	No clean-up	ESTD (matrix assisted)	0.63	Acquity UPLC BEH C18 (50 x 2.1 mm, 1.7 µm) (Waters)	SRM
21	IAC	ESTD	5.00 for AFs, 80.0 for OTA	Cosmosil 5C 18-AR for AFs or 6C 18-AR for OTA (250 x 4.6 mm, 5 µm) (Agilent)	FLD

SPE, solid phase extraction; IAC, immunoaffinity column; QuEChERs , Quick, Easy, Cheap, Effective, Rugged Safe; ISTD, internal standard; ESTD, external standard; HRMS, high resolution mass spectrometry; SRM, selected reaction monitoring; SIM, selected ion monitoring; FLD, fluorescence detector.

Table A9.1. Summary of information on analytical methodologies reported in the questionnaire (III)

Lab code	DON		FB_1		FB_2		ZEA		T-2		HT-2		OTA		AFB_1		AFG_1		AFB_2		AFG_2	
	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)
1	-[a]	-					-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	0.2	-
2	-	-					-	-					-	-	-	-						
3	16.9	-	4.7	-	2.2	-	25.2	-	3.4	-	11.8	-	6.7	-	9.2	-	4.9	-	22	-	4.1	-
4	80.16	-	50.8	-	30	-	10	-	8	-	12.8	-	2	-	1.01	-						
6	-	-	-	62	-	72	-	-	-	-	-	72	-	-	-	62	-	60	0.6[a]	-	0.8[a]	-
7	-	-	-	-	-	-	50	-	20	-	-	-	-	-	2.5	-	2.5	-	2.5	-	2.5	-
8	20	82	5	77	5	77	10	70	5	79	5	79	1	71	0.5	74	0.5	76	0.5	76	0.5	76
9A	-	85	-	95	-	95	-	85	-	85	-	85	-	85	-	100	1	100	1	100	2.5	100
9B	-	-	-	70	-	80	<17	-	-	-	<21	-	2.5	-	-	-	<0.6	-	<0.1	-	<0.2	-
10A	-	88	-	71	-	89	50	-	-	95	50	90	-	61	-	43	1	51	1	45	1	50
10B	-	98[b]	-	57[b]	-	67[b]		93[b]	50	105[b]	200	108[b]	2.5	100[b]	2.5	95[b]	2.5	107[b]	2.5	102[b]	2.5	110[b]
11	-	74[c]					100	-														
12	-	-	-	-	-	-	10	-	-	-	50	-	-	-	-	-	1	-	1	-	1	-
13	80	-	1000	-	1000		20	-	80	-	160		20	-	10	-	10	-	4		16	
14	-	99	-	125	-	102	-	97	-	79	-	125	-	124	-	116	0.5	109	0.5	89	0.5	109
15	-	85		101	-	121	-	120	-	109	-	101	-	104	-	109	-	110	0.5	112	0.5	109
16	-	-	-	-	-	-	20	-	-	-	-	-	-	-	-	-	-	-	0.2	-	0.2	-
17A	40	94					48	118	40	93	40	95	30	207	180	113	1500	117	900	109	1500	109
17B	40	94					48	118	40	93	40	95	30	207	180	113	1500	117	900	109	1500	109
18	30	114	-	-	-	-	20	95	30	105	60	119	0.6	101	1	97	1	95	1	95	1	95
19	100	94	20	80	20	68	20	-	20	90	20	94	2	-	1	77	1	-	1	-	2	-
21											-	-	0.3[d]	-	0.2[d]	-	0.2[d]	-	0.1[d]	-	0.1[d]	-

Mycotoxins not analysed by participants are shared in gray. LOQ, limit of quantification; R, recovery. [a]not reported; [b]according to Sulyok et al, 2006. Rapid Communications in Mass Spectrometry 20, 2649-2659; [c]according to Khatibi et al., 2014. Toxins, 6, 1155-1168; [d]limit of detection.

Table A9.1. Summary of information on analytical methodologies reported in the questionnaire (IV)

Lab code	DON		ZEA		T-2		HT-2		OTA	
	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)	LOQ (µg/kg)	R (%)
1	-[a]	-	-	-	-	-	-	-	2	-
2	-	-	-	-	-	-	-	-	-	-
3	20.5	-	23.1	-	4.6	-	11.6	-	7.5	-
4	80.16	-	10	-	8	-	12.8	-	2	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	20	-	-	-	-	-
8	20	82	10	70	5	79	5	79	1	71
9A	-	85	-	85	-	85	-	85	-	85
9B	-	-	-	-	< 4.3	-	-	-	-	-
10A	-	88	-	-	50	95	-	90	-	61
10B	-	89[b]		102[b]	50	92[b]	200	94[b]	2.5	86[b]
11	-	-	-	-						
12	-	-	-	-	50	-	50	-	-	-
13	80	-	20	-	80	-	160	-	20	-
14	-	99	-	97	10	79	-	125	-	124
15	-	94	-	82	-	109	-	110	-	88
16	-	-	-	-	10	-	-	-	-	-
17A	40	94	48	118	40	93	40	95	30	207
17B	40	94	48	118	40	93	40	95	30	207
18	30	114	20	95	30	105	60	119	0.6	101
19	100	76	20	85	20	-	20	102	2	-
21							-	-	0.3[c]	-

Mycotoxins not analysed by participants are shared in gray. [a]not reported; [b]according to Sulyok et al, 2006. Rapid Communications in Mass Spectrometry 20, 2649-2659; [c]limit of detection.

Annex 10. Evaluation of the questionnaires

Lab code	Where the instructions and questionnaire adequate	Please report any difficulties and/or observations concerning this PT
1	YES	NO
2	YES	NO
3	YES	The instrumental sequence for the analysis of the maize sample (PT287) stopped during the night (during the calibration). The sequence was continued in the day after, apparently without any analytical consequences. The result obtained for ZEA in sample 287 (maize) was 15.8 $ugkg^{-1}$, which was reported as <LOQ, although it is above the LOD. The results for OTA might be affected by an increased error as there is some carryover in the analytical instrument.
4	YES	Problems with sensitivity of the MS
6	YES	Participants should be asked about the origin of their standards and the way they dilute and store them. Providing a third sample that is simply a mixture of the analytes with unknown concentration in LC-compatible solvent could reveal whether any unacceptable result reported by a participant could derive from using a spoiled standard for calibration.
7	YES	NO
8	YES	NO
9A	YES	NO
9B	YES	Also detected 3/15 acetyl deoxynivalenol, beauvericin and neosolaniol in the maize material.
10A	YES	NO
10B	YES	NO
11	YES	NO
12	YES	The only observation we make, is that when we want to insert the numbers in table 2.6.1 there were some difficulties by inserting. It was hard to select and write in them.
13	YES	NO
14	Too detailed	NO
15	YES	NO
16	YES	NO
17A	YES	NO
17B	YES	NO
18	YES	NO
19	YES	NO
21	YES	NO