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Performance evaluation of LC–MS/MS methods for multi-mycotoxin determination in maize and wheat by means of international Proficiency Testing

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ABSTRACT

Two Proficiency Testings (PTs) involving eighteen laboratory participants from 10 Countries have been conducted in 2014 for the simultaneous determination of deoxynivalenol, fumonisins B_1 and B_2, zearalenone, T-2 and HT-2 toxins, ochratoxin A and aflatoxins B_1, B_2, G_1 and G_2 in maize and of deoxynivalenol, zearalenone, T-2 and HT-2 toxins and ochratoxin A in wheat, respectively. The aim of PTs was to check next to the laboratory performance the state-of-art of the LC–MS multi-mycotoxin methods used by participants. In addition, the trend of performances of LC–MS methods for multi-mycotoxin determination in maize together with method-related issues was assessed by comparing three PTs organised over the years 2011–2014. Data showed the improvement of laboratory performances with the overall acceptable z-scores that progressively increased from 59% in 2011 PT to 85% in 2014 PT, while the rate of unacceptable z-score decreased from 25% in 2011 PT to 11% in 2014 PT.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by filamentous fungi under a wide range of climatic conditions on agricultural commodities in the field and also during storage [1]. Mycotoxin contamination of agricultural food commodities and beverages poses a risk to human and animal health due to their toxic effects. Although over 300 mycotoxins have been identified, only a few of them can cause 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 toxin (T-2) and HT-2 toxin (HT-2). These mycotoxins have been shown to be mutagenic, teratogenic, or/and carcinogenic. Symptoms of intoxication range from emesis and 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 and its amendments [3]. Very recently, the Recommendation EC 165/2013 setting indicative levels for the sum of T-2 and HT-2 in cereals and cereal products has been issued [4]. All these mycotoxins can occur in most of cereals and can be retained in the relevant processed products (food/feed), with exception of fumonisins that are of concern only for maize and products thereof.

Effective and reliable 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 extensively reviewed for the analysis of single mycotoxins or group of mycotoxins in food and feed [5–7]. Among them, multi-analyte methods have become the ones most required because several mycotoxins frequently co-occur in the same product. Within this context the application of liquid chromatography–mass spectrometry (LC–MS) techniques is being largely explored since it enables the simultaneous monitoring of different mycotoxins. Moreover, it offers several advantages in terms of high selectivity and sensitivity, substantial reduction of sample treatment, and simultaneous quantification and confirmation of identity at regulated levels [8]. Even though LC–MS methodologies for

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single or multiple mycotoxin determination are routinely used in control laboratories, to date no official/standard methods for mycotoxins are based on LC–MS. The need of standardized LC–MS methods for mycotoxin determination has been recently highlighted by the M/520 mandate of the European Commission (EC) for standardization of methods of analysis for mycotoxins in food. With this mandate the European Commission invited the European Committee for Standardization (CEN) to establish European Standards/Technical Specifications and to launch a call for tender for the development of eleven standardized methods of analysis for mycotoxins in food. Six of them were specifically requested to be based on LC–MS [9].

A Proficiency Testing (PT) is an effective procedure for quality assurance and performance verification in chemical analysis laboratories, ensuring that laboratory validation and within-laboratories procedures are working satisfactorily [10]. Several PTs programs for mycotoxins are available in Europe, such as FAPAS (Food and Environment Research Agency, UK), BIPEA (France), DLA (Dienstleistung Lebensmittel Analytik GbR, Germany), DUCARES B.V. (The Netherlands), LGC Standards Proficiency Testing (UK) and Test Veritas S.r.l. (Italy). A detailed list of these schemes is available at https://www.eptis.bam.de/en/index.htm. The majority of PTs organised by these providers are focused on the determination of single mycotoxin or mycotoxins belonging to the same group (i.e. fumonisins or aflatoxins). In the year 2011 the Institute of Sciences of Food Production of the National Research Council of Italy (ISPA-CNR) co-ordinated the first international multi-mycotoxin PT (ISPA-2011-PT) to benchmark laboratories using LC–MS for multi-mycotoxin analysis and to obtain information on the used methodologies and related method performances [11,12]. The study involved 41 laboratories from 14 countries and was conducted for the simultaneous determination of up to 11 mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, ZEA, DON, T-2 and HT-2) in spiked and contaminated maize.

The Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC) operates as the European Union Reference Laboratory (EU-RL) for mycotoxins with the aim to facilitate the implementation of European legislation related to monitoring of mycotoxins in food and feed. One of its core tasks is to organise PTs among appointed National Reference Laboratories (NRLs), as stipulated in Regulation (EC) No 882/2004 of the European Parliament and of the Council [13]. In the year 2013 the JRC organised a PT (JRC-2013-PT) for the determination of DON, FB_1 and AFB_1 in maize. The study involved 71 participants, 21 of which used LC–MS for the simultaneous analysis of these three mycotoxins [14]. Other multi-toxin PTs are those organised by CODA-CERVA, a Belgian National Reference Laboratory for Mycotoxins in Food and Feed (http://www.coda-cerva.be). In this framework, the ISPA-CNR organised other two multi-mycotoxin PTs in 2014 (ISPA-2014-PTs) for the determination of DON, FB_1, FB_2, ZEA, T-2, HT-2, OTA, AFB_1, AFG_1, AFB_2 and AFG_2 in maize and DON, ZEA, T-2, HT-2 and OTA in wheat, respectively.

The aim of the present study is to evaluate the performance of participant laboratories of these PTs using LC–MS methods for the analysis of wheat and maize. Considering the similarities between the PTs carried out in 2011 and 2014 in terms of targeted mycotoxins and test material (i.e. maize), a further aim of the present study is to evaluate the possible trend of laboratory performances in LC–MS methods for multi-mycotoxin determination in maize.

2. Experimental section

2.1. Participation

The ISPA-2014-PTs were announced via e-mail and potential participants were also contacted by an official announcement through the MoniQA website (www.MoniQA.org). The subscription occurred in May 2014 and the studies were free of charge. Eighteen laboratories from 10 countries – Austria (4), Belgium (3), Canada (1), Germany (1), Italy (3), Republic of Singapore (1), South Africa (1), The Netherlands (2), United Kingdom (1), United States of America (1) – including public and private laboratories, universities and public research facilities expressed interest in participating (Table 1). For confidentiality, names of institutions and each laboratory participant were omitted and randomly assigned with a laboratory code. The use of multi-mycotoxin methods was mandatory, while participants were not obliged to determine all toxins in each material, and were let free to report only on those mycotoxins that they could simultaneously determine by their multi-mycotoxin methodology.

2.2. Test materials

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 analyse each sample twice by using their method of choice and to report each single value.

Table 1
Participating laboratories to the ISPA-2014-PT laboratories. Three laboratories provided 2 set of results for both maize and wheat.

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

2.2.1. Preparation of maize and wheat test materials

Maize samples naturally contaminated with DON, FB_1, FB_2, ZEA, T-2, HT-2, OTA, AFB_1, AFG_1, AFB_2 and AFG_2 and wheat samples naturally contaminated with DON, ZEA, T-2, HT-2 and OTA at the EU legislated levels were not found. To reach mycotoxin levels around the relevant regulatory limits (i.e. 750 μg/kg DON; 1000 μg/kg sum of FB_1 and FB_2; 100 μg/kg ZEA; 3 μg/kg OTA; 100 μg/kg sum of T-2 and HT-2, 4 μg/kg sum of AFB_1, AFG_1, AFB_2, AFG_2) samples of wheat and maize were collected by a local market and were fortified with culture extracts of mycotoxigenic species of *Fusarium* and/or *Aspergillus* (deposited at the Institute of Sciences of Food Production collection, http://www.ispa.cnr.it/Collection). In particular wheat samples were fortified with aqueous/organic extracts of cultures of *Fusarium graminearum* (ITEM 126 producing DON and ZEA), *Fusarium sporotrichioides* (ITEM 707 producing T-2 and HT-2) and *Aspergillus ochraceous* (ITEM 4211 producing OTA) on wheat, while maize samples were fortified with aqueous/organic extracts of cultures of *Aspergillus flavus* (ITEM 7828 producing AFB_1 and AFB_2) and *Aspergillus parasiticus* (ITEM 7531) producing AFB_1, AFB_2, AFG_1 and AFG_2) on maize. Before using, 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 [15]; Entwisle et al. (2000) [16] for OTA; MacDonald et al. (2005) for ZEA [17]; MacDonald et al. (2005) for DON [18]; Solfrizzo et al. (2011) for fumonisins [19]; Pascale et al. (2012) for T-2 and HT-2 toxins [20]. Aliquots of culture extracts were adequately diluted with mobile phase and analysed by HPLC with UV or fluorescence (FL) detection to measure their mycotoxin concentrations [15–20].

For maize test material, a maize sample (2.3 kg) naturally contaminated with FB_1 (8600 μg/kg), FB_2 (3600 μg/kg) and DON (2900 μg/kg) and a maize sample (0.4 kg) naturally contaminated with FB_1 (1200 μg/kg), FB_2 (230 μg/kg) and DON (6700 μg/kg) were mixed with an uncontaminated maize material (25 kg) to obtain a maize batch (approx. 28 kg) naturally contaminated with DON and fumonisins. Then, the obtained maize material was ground by an ultracentrifugal mill (ZM 200, Retsch) equipped with a 500 μm sieve, homogenized by rotation in a laboratory mixer (20 rpm) for 12 h and fortified with adequate volumes of culture extracts to achieve the target levels of mycotoxins. The contaminated materials were passed again through the ultracentrifugal mill and then homogenized by rotation in a laboratory mixer for further 24 h.

In the case of wheat test material, an uncontaminated durum wheat sample (30 kg) was ground by an ultracentrifugal mill (ZM 200, Retsch) equipped with a 500 μm sieve, homogenized by a mixer for 12 h and fortified with adequate volumes of culture extracts to achieve the target levels of mycotoxins. The contaminated wheat was then passed through the ultracentrifugal mill and homogenized by rotation in a laboratory mixer for 24 h.

Maize and wheat test materials were dispensed in plastic bottles each containing about 80 g. Bottles to be used for the homogeneity study were filled with about 300 g of material at systematic intervals from the filling sequence (one bottle every ten bottles). Then, all bottles were labelled, sealed, and stored at −20 °C until homogeneity or stability studies and dispatch.

2.3. Homogeneity testing

Homogeneity study was carried out according to the procedure described in ISO guide 35:2006 [21]. Each unit of 300 g was divided in 6 aliquots (50 g each) and analysed in duplicate under repeatability conditions as specified in the ISO guide 35:2006 [21], by using the appropriate reference method for each mycotoxin or group of mycotoxins [15–20]. Homogeneity was statistically evaluated according to ISO 13528:2015 [22] and *F-test*. The parameters considered for the test on homogeneity were the analytical precision (*within bottle standard deviation*, s_w) and the heterogeneity standard deviation (*between bottle standard deviation*, s_b). The *F-test* was used to determine whether the observed s_b deviated significantly from the s_w. The s_b was then compared to the standard deviation for proficiency assessment (σ). The σ values were obtained using the truncated Horwitz equation corrected by Thompson, i.e. if the relative target standard deviation according to Horwitz was greater than 22%, it was truncated to 22%. The samples were considered to be adequately homogenous if $s_b \leq 0.3\ \sigma$ [21,23].

2.4. Stability study

Randomly selected units of the two test materials were submitted to accelerated ageing at different temperatures (4 °C, 20 °C and 60 °C) over a total period of 1.5 months, according to the so-called isochronous stability study [21]. A total of 26 bottles for each material were stored at −20 °C (reference temperature), then 2 random bottles per time were moved to the different temperatures after 0.25, 0.5, 1 and 1.5 month for a total of 24 bottles. All the units were analysed 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. The analyses were carried out by using the appropriate reference method for each mycotoxin or group of mycotoxins [15–20]. Statistical results assessment was performed according to ISO guide 35:2006 using the *F-test* to test the trend for significance [21]. In particular, 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 is not differ significantly from zero.

2.5. Sample dispatching and instruction to participants

All samples were packed in plastic bottles and sent to each participant on 17 June 2014. Samples were mostly received within 3 days after dispatch. Each participant received: a) two plastic bottles each containing approximately 80 g of each test material; b) an accompanying letter with instructions on sample handling and storage; c) a material receipt form; d) a report form and a detailed questionnaire on method description (i.e. sample preparation, calibration, equipment, MS conditions and MS acquisition parameters). The materials were sent at room temperature; storage upon arrival was required to be at −18 °C until the analysis was performed.

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 value. Participants received a specific questionnaire intended to provide further information on the method and general comments on the exercise.

2.6. Statistical evaluation of PT results

The statistical evaluation of the results, in terms of homogeneity study, kernel density, assigned value, target standard deviation and z-scores was performed using the software ProLab Plus, v 2016.6.29.0 (QuoData GmbH, Dresden, Germany).

2.6.1. Kernel density

The distribution of the results was checked by kernel density estimations for determining if results were normally distributed or

contained values from different populations giving rise to multiple distribution modes (multimodality) [24].

2.6.2. Assigned value

The assigned values $\chi_{assigned}$ (robust means) were calculated according to the Algorithm A of ISO 13528:2015 [22]. Results reported as "smaller than detection or quantification limits" were excluded from all calculations and no further evaluation was done.

2.6.3. Target standard deviation

The target standard deviation (σ_p) of each mycotoxin evaluated in the maize and wheat materials of this PT study was derived from the truncated Horwitz equation corrected by Thompson as reported in Section 2.3 [23].

2.6.4. z-score

Individual laboratory performance was expressed in terms of *z*-score in accordance with ISO 13528:2015 [22] and calculated by the following Equation:

$$z = \frac{\chi_{lab} - \chi_{assigned}}{\sigma_p}$$

where:

χ_{lab} is the mean of the two measurement results reported by a participant;
$\chi_{assigned}$ is the assigned value (robust mean);
σ_p is the standard deviation for proficiency assessment derived from the truncated Horwitz equation.

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:

$|z| \leq 2$ acceptable result

$2 < |z| \leq 3$ questionable result

$|z| > 3$ unacceptable result

2.7. Comparison among PTs results for LC–MS multi-mycotoxin determination in maize

Results obtained for maize in the ISPA-2014-PT were compared with those obtained in the ISPA-2011-PT [11,12] and with those obtained in the JRC-2013-PT [14]. For this purpose only those participants analyzing simultaneously two or more mycotoxins belonging to a different chemical families and those using LC–MS methodologies were included in the statistical analysis. In particular, two participants of the ISPA-2011-PT analyzing only FB_1 and FB_2 or T-2 and HT-2, and 50 participants of the JRC-2013-PT that did not used LC–MS methodologies were excluded from the comparison. In total, the comparison included 39 participants of the ISPA-2011-PT, 21 participants of the JRC-2013-PT and 18 participants of the ISPA-2014-PT. For the comparison study a new statistical evaluation (kernel density, assigned value, target standard deviation, z-scores) was carried out for both 2011 and 2013 PTs according to Section 2.6. Results were compared in terms of number of mycotoxins analysed simultaneously, number of quantitative results provided by each laboratory and percentage of satisfactory, questionable and unacceptable z-scores.

A comparison was made also in terms of LC–MS methodologies used by the participants to the studies by considering the extraction solvent mixtures, the sample extract preparation (with or without clean-up) and the quantification mode (external or internal calibration).

3. Results and discussion

3.1. Homogeneity and stability of maize and wheat test-materials

Both maize and wheat test materials passed the homogeneity test and were considered appropriate for the Proficiency Testing (Table 2). Furthermore, the evaluation of data from the short-term stability study indicated that no significant trend was observed for the test samples at all temperature conditions (4 °C, 20 °C and 60 °C) 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.

Table 2
Results of the homogeneity study for deoxynivalenol (DON), fumonisins B_1 (FB_1) and B_2 (FB_2), zearalenone (ZEA), T-2 and HT-2 toxins, ochratoxin A (OTA) and aflatoxins B_1 (AFB_1), G_1 (AFG_1), B_2 (AFB_2) and G_2 (AFG_2) in maize and for DON, ZEA, T-2, HT-2 and OTA in wheat.

Matrix	Mycotoxins	Average (μg/kg)	s_w (μg/kg)[a]	s_b (μg/kg)[b]	σ (μg/kg)[c]	F-test[d]	ISO 13528[e]
Maize	DON	1221	67.4	26.1	190	Passed	Passed
	FB_1	1062	108	0.00	168	Passed	Passed
	FB_2	303	56.4	0.00	58.1	Passed	Passed
	ZEA	21.6	3.84	1.38	4.75	Passed	Passed
	T-2	54.1	5.31	0.00	11.9	Passed	Passed
	HT-2	22.5	2.62	0.00	4.94	Passed	Passed
	OTA	2.58	0.78	0.00	0.57	Passed	Passed
	AFB_1	1.19	0.18	0.00	0.26	Passed	Passed
	AFG_1	0.05	0.02	0.00	0.01	Passed	Passed
	AFB_2	0.21	0.03	0.00	0.05	Passed	Passed
	AFG_2	0.04	0.01	0.00	0.01	Passed	Passed
Wheat	DON	1266	39.0	18.6	195	Passed	Passed
	ZEA	149	11.8	8.06	31.7	Passed	Passed
	T-2	4.91	0.72	0.00	1.08	Passed	Passed
	HT-2	50.9	3.23	0.00	11.2	Passed	Passed
	OTA	5.34	0.37	0.00	1.17	Passed	Passed

[a] Within bottle standard deviation.
[b] Between bottles standard deviation.
[c] Standard deviation for proficiency assessment calculated using truncated Horwitz equation.
[d] Check for significant differences between bottles.
[e] Check for sufficient homogeneity.

3.2. Analytical procedures used in the PT

The eighteen participants returned 2 sets of results for various combinations of analytes. Three participants (i.e. Lab. 9, Lab. 10 and Lab 17) returned two additional sets of results obtained by using two different LC–MS methods for both contaminated maize and contaminated wheat. These results have been considered for statistical evaluation as being from independent laboratories and were indicated as Lab. 9A and Lab. 9B, Lab. 10A and Lab. 10B, Lab. 17A and Lab. 17B. An overall set of 21 results was obtained for both materials.

The majority of participant laboratories (76%) used mixtures of acetonitrile–water for extraction, followed by methanol–water mixtures (19%) and isopropyl alcohol–water–acetone mixture (5%). The majority of participant laboratories (76%) extracted samples by shaking for 30–120 min, the remaining laboratories used blending for 2–4 min (14%) or accelerated solvent extraction or vortex with ultrasonic bath (10%). Fifty-two percent of laboratories analysed the crude extract ("dilute and shoot"); the others cleaned-up the extract prior to the analysis by solid phase extraction (24%), immunoaffinity columns (9%), or used Quick Easy Cheap Effective Rugged Safe (QuEChERS)-like approach (10%). One laboratory (5%) used a mixed approach, i.e. split the sample extract in two aliquots, one was directly analysed by LC–MS/MS and the other was purified before analysis depending on the mycotoxin. The majority of laboratories (57%) used internal standard calibration mode using stable isotope labelled standards in combination with standard calibration (calibration solutions prepared in neat solvents) or matrix assisted calibration (calibration solutions prepared in blank matrix extract). The other laboratories (43%) used external calibration using native standard mycotoxins. Among them 4 laboratories used standard calibration, and 4 used matrix assisted calibration.

All laboratory participants, but one, used triple quadrupole mass analyzers for mycotoxin detection in single reaction monitoring mode (SRM). One laboratory used a hybrid quadrupole-Orbitrap™ mass analyzer performing full scan-data dependent MS/MS analysis.

3.3. Laboratories performance and z-scores

Fifty-seven percent of laboratories analysed all the 11 targeted mycotoxins in maize, followed by another 19% that analysed from 7 to 10 mycotoxins. The remaining laboratories reported results for a restricted combination (from 2 to 6 analytes). In the case of wheat, 71% of laboratories analysed all the 5 targeted mycotoxins while the remaining laboratories analysed from 1 to 4 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 μg/kg levels in the test materials (i.e. AFB_2, AFG_1 and AFG_2 in maize and T-2 toxin in wheat). All these qualitative data were excluded from all statistical calculations and no evaluation was done for AFB_2 and AFG_2 in maize (too few quantitative values). Furthermore, results of T-2 and HT-2 and OTA reported by Lab.1 and Lab 17A, respectively, for both maize and wheat materials were excluded from the statistical evaluation due to problems encountered by the participants with calibration curves (mycotoxin concentrations in test samples far below the calibration range) and mycotoxin quantification (wrong settings in the quantification method).

The IUPAC international harmonized protocol for the Proficiency Testing of analytical chemistry laboratories reports that serious limitations are induced by small group sizes of participants. 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 [10]. In order to allow participants whose methods had sufficient measurement capacity for a judgement of their results (not met by participants reporting < LOD or < LOQ), 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 ranged from 20 for DON to 7 for AFG_1 in maize and from 20 for DON to 8 for T-2 in wheat. No statistical evaluation was reported for AFG_2 and AFB_2 in maize because more than 80% of participants returned results as qualitative ones. Summary statistics for the target mycotoxins in maize and wheat analysed in the ISPA-2014-PTs are reported in Tables 3 and 4, respectively.

The assigned values for maize test material was calculated to 1264 μg/kg for DON, 1306 μg/kg for FB_1, 350 μg/kg for FB_2, 2.62 μg/kg for OTA, 54.4 μg/kg for T-2, 30.7 μg/kg for HT-2, 21.7 μg/kg for ZEA, 1.40 μg/kg for AFB_1 and 0.70 μg/kg for AFG_1 (Table 3). The assigned values for wheat test material was calculated to 1297 μg/kg for DON, 7.00 μg/kg for OTA, 8.26 μg/kg for T-2, 58.8 μg/kg for HT-2 and 148 μg/kg for ZEA (Table 4). In general, the majority of these results were comparable to those estimated with the homogeneity study for both maize and wheat (Table 2), thus indicating the accuracy of the LC–MS/MS methods used in the present

Table 3
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 analysed in the ISPA-2014-PT.

Statistical parameters	DON	FB_1	FB_2	OTA	T-2	HT-2	ZEA	AFB_1	AFG_1	AFB_2	AFG_2
Number of participant laboratories[a]	21	21	21	21	21	21	21	21	21	21	21
Number of submitted results	20	16	15	18	17	16	19	19	16	16	15
Number of quantitative results	20	16	15	15	15	11	11	14	7	2	2
Median (μg/kg)	1252	1257	328	2.50	54.7	24.7	18.4	1.35	0.60	0.50	1.20
Minimal value (μg/kg)	883	581	199	1.70	34.0	9.10	8.10	0.80	0.50	0.10	0.20
Maximal value (μg/kg)	1611	2788	735	19.5	71.8	60.0	48.0	3.0	1.90	0.70	2.20
Assigned value (μg/kg)	1264	1306	350	2.62	54.4	30.7	21.7	1.40	0.70	– [c]	–
Target standard deviation (μg/kg, σ_p)[b]	195	201	65.6	0.58	12.0	6.76	4.76	0.30	0.20	–	–
Reproducibility standard deviation (μg/kg)	173	445	113	0.74	8.97	16.0	12.5	0.48	0.30	–	–
Lower limit of tolerance (μg/kg)	873	904	219	1.46	30.4	17.2	12.1	0.76	0.38	–	–
Upper limit of tolerance (μg/kg)	1654	1707	482	3.77	78.3	44.3	31.2	1.95	1.10	–	–
Number of laboratories with mean outside of tolerance limits	0	4	3	2	0	3	4	1	1	–	–
Overall acceptable z-scores	85%										
Overall questionable z-scores	3%										
Overall unacceptable z-scores	11%										

[a] Three participant laboratories provided two additional sets of results.
[b] Calculated according to truncated Horwitz equation.
[c] Too few values.

Table 4
Summary statistics for deoxynivalenol (DON), ochratoxin A (OTA), T-2 toxin (T-2), HT-2 toxin (HT-2) and zearalenone (ZEA) in wheat analysed in the ISPA-2014-PT.

Statistical parameters	DON	OTA	T-2	HT-2	ZEA
Number of participant laboratories[a]	21	21	21	21	21
Number of submitted results	20	17	18	17	19
Number of quantitative results	20	12	8	16	19
Median (μg/kg)	1279	7.30	6.60	58.3	149
Minimal value (μg/kg)	940	3.40	4.30	38.2	74.5
Maximal value (μg/kg)	1756	10.8	35.0	81.0	199.0
Assigned value (μg/kg)	1297	7.00	8.26	58.8	148
Target standard deviation (μg/kg)[b]	200	1.50	1.82	12.9	31.5
Reproducibility standard deviation (μg/kg)	236	2.30	4.95	9.26	28.5
Lower limit of tolerance (μg/kg)	898	3.90	4.63	32.9	84.6
Upper limit of tolerance (μg/kg)	1696	10.1	11.9	84.7	211
Number of laboratories with mean outside of tolerance limits	1	2	3	0	1
Overall acceptable z-scores	91%				
Overall questionable z-scores	6%				
Overall unacceptable z-scores	3%				

[a] Three participant laboratories provided two additional sets of results.
[b] Calculated according to truncated Horwitz equation.

PTs, as compared to the HPLC reference methods used for the homogeneity study. A lot of qualitative results were reported for AFB_2 (88%) and AFG_2 (87%) in maize and T-2 in wheat (56%). In the case of T-2 in wheat it was observed that the reported LOQ or LOD values (from 10 μg/kg to 50 μg/kg) were higher than the calculated assigned value (8.26 μg/kg). This finding indicated that although the LC–MS/MS methods used by the participants showed a low sensitivity towards these toxins, they correctly estimated qualitatively the T-2 content in wheat. Probably the T-2 contamination level of the wheat material used in this study was too low to be quantitatively detected from these laboratories with their own LC–MS/MS methods. Indeed, the calculated assigned value of T-2 in maize was higher (54.4 μg/kg) and the mycotoxin was quantified by 88% of laboratories. A similar conclusion may also be drawn for AFB_2 and AFG_2 in maize.

A graphical distribution of z-scores, calculated with σ_p values (truncated Horwitz standard deviation), is shown in Fig. 1 for both maize and wheat. In the case of maize, 85% of laboratories provided acceptable z-scores, 3% of laboratories provided questionable z-scores and 11% of laboratories provided unacceptable z-scores. In particular, the acceptable z-scores ranged from 64% for ZEA to 100% for DON and T-2 (Table 3, Fig. 1). In the case of wheat, 91% of laboratories provided acceptable z-scores, 6% of laboratories provided questionable z-scores and 3% of laboratories provided unacceptable z-scores. The acceptable z-scores ranged from 63% for T-2 to 100% for HT-2. Good results were also obtained for DON and ZEA, both giving 95% of acceptable z-scores (Table 4, Fig. 1).

In order to individuate the best experimental conditions that gave the highest number of acceptable results for the simultaneous analysis of the 11 target mycotoxins in maize and the 5 mycotoxins

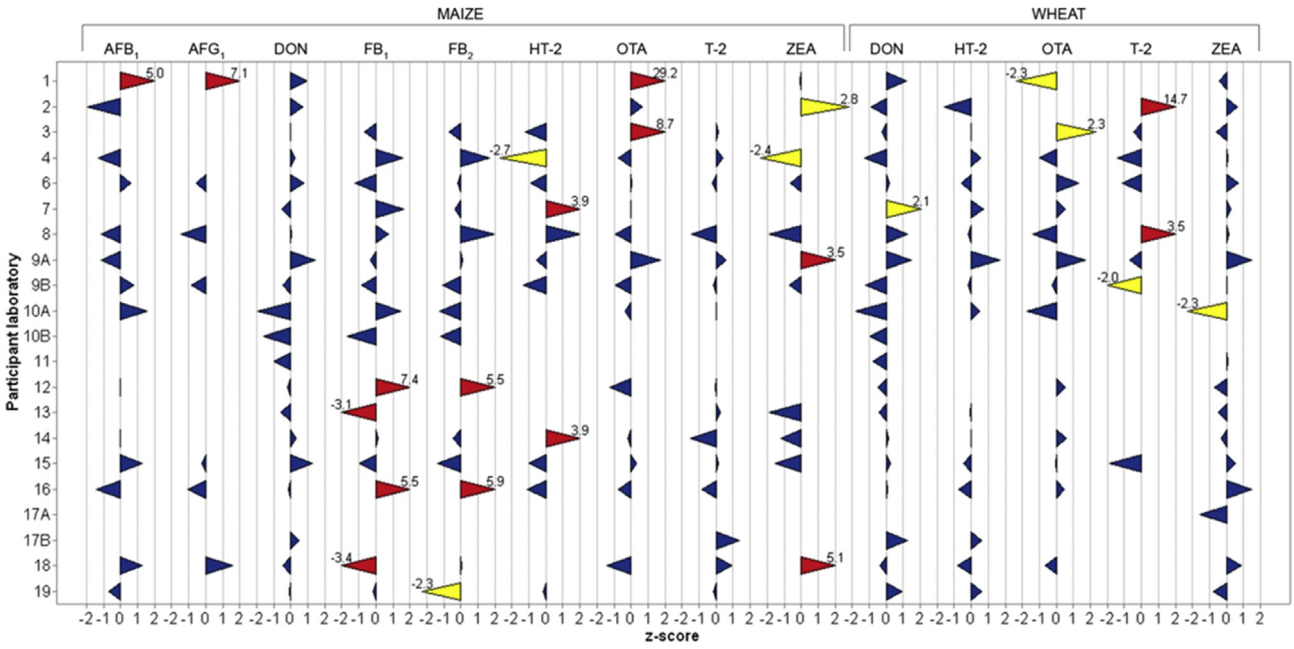

Fig. 1. Summary graph of the laboratory's z-scores for deoxynivalenol (DON), fumonisins B_1 (FB_1) and B_2 (FB_2), zearalenone (ZEA), T-2 and HT-2 toxins, ochratoxin A (OTA), aflatoxin B_1 (AFB_1) and B_2 (AFB_2) in maize and for (DON), zearalenone (ZEA), T-2 and HT-2 toxins and ochratoxin A (OTA) in wheat calculated in the ISPA-2014-PT. Blue triangles indicate acceptable z-score, yellow triangles indicate questionable z-scores and red triangles indicate unacceptable z-scores.

in wheat, the total number of analysed mycotoxins, the number of quantitative results and the percentage of acceptable z-scores provided by each laboratory were considered. According to the LAWA (German Working Group on Water Issues) mode of evaluation of PT results, a laboratory is considered successful for the whole interlaboratory test, if at least 80% of the z-scores are acceptable and at least 80% of the measurands have acceptable z-scores (i.e. $|z| \leq 2$). Although this mode of evaluation is considered very restrictive, its application allowed individuating the best procedures for the simultaneous analysis of the target mycotoxins in maize and wheat. By excluding AFG_2 and AFB_2 from the statistical evaluation due the relevant high number of qualitative results for these mycotoxins in maize, 6 laboratory participants for maize and 10 laboratories for wheat fulfilled these requirements. However, most of participant laboratories showed acceptable z-scores higher than 80% for those mycotoxins for which the laboratory delivered quantitative results (Table 5). The experimental conditions of the selected laboratories fulfilling the LAWA selection mode are summarized in Table 6. For both matrices, the main common experimental parameters were the extraction solvent (acidified ACN–water mixtures), the calibration mode (ISTD with labelled standards) and injected matrix equivalent (up to 2.5 mg). These results are in agreement with recent reviews on LC–MS multi-mycotoxin methods that report the use of ACN/water (acidified or not) mixtures as the preferred choice for multi-mycotoxin extraction [6,7,25]. However, systematic studies comparing the extraction efficiency of different solvent mixtures for multi-mycotoxin analysis have not been reported. This aspect will be further discussed in the following paragraph.

3.4. Trend in multi-mycotoxin determination in maize by LC–MS(/MS)

In the last 5 years different PTs have been organised for multi-mycotoxin determination in cereals by LC–MS. The first international PT was organised from the Institute of Sciences of Food Production of the National Research Council of Italy in the year 2011 (ISPA-2011-PT) and results were extensively reviewed by De Girolamo et al. [11]. The PT was organised within the EU Network of Excellence MoniQA (http://www.MoniQA.eu) that made several efforts for method comparison and deeper understanding of performances of the available LC–MS methodologies for multiple-mycotoxin analysis. The study involved 42 participants and aimed to the determination of DON, FB_1, FB_2, ZEA, T-2, HT-2, OTA, AFB_1, AFG_1, AFB_2 and AFG_2 in contaminated and spiked maize. The evaluation of results in relation to analytical parameters (i.e. extraction solvent, clean-up, calibration mode, injected matrix) used by each laboratory permitted to identify strengths and weaknesses of each parameter that could be useful in the development of a robust LC–MS method for the simultaneous determination of mycotoxins in maize. However it was not possible to identify a single procedure suitable for the simultaneous analysis of the 11 legislated mycotoxins in maize at European level [11]. In order to evaluate a possible positive trend of performances of LC–MS multi-mycotoxin methods in maize, ISPA-2011-PT results were compared to those of the ISPA-2014-PT. In a second step, the PT organised by the Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC) in the 2013 (JRC-2013-PT) for the determination of DON, FB_1 and AFB_1 in maize was also included in the evaluation of performance of LC–MS methods for the determination of these mycotoxins. This PT involved 71 participants, 21 of which used LC–MS/MS for the simultaneous analysis of these three mycotoxins [14]. To the best of our knowledge this is the first time that a comparison of PTs results for multi-mycotoxin determination by LC–MS has been made.

The statistical evaluation of results obtained in the ISPA-2011-PT has been reported elsewhere [11,12]. However, the removal from the original set of laboratories that did not analyse simultaneously mycotoxins belonging to different chemical group generated a new set of 39 data and the new statistical elaboration is summarized in Table 7. The graphical distribution of z-scores, calculated with σ_p values (truncated Horwitz standard deviation), is shown in Fig. 2. In general, levels of mycotoxins in maize test material used in the ISPA-2011-PT were higher than those found in

Table 5
Score count table for laboratories taking part to the ISPA-2014-PT.

Lab code	Maize			Wheat		
	Analysed mycotoxins	Quantitative results	Satisfactory z-scores (%)	Analysed mycotoxins	Quantitative results	Satisfactory z-scores (%)
1[a]	7	5	40	3	3	67
2	4	4	75	4	4	75
3	11	6	83	5	5	80
4	8	8	75	5	5	100
6	11	9	100	5	5	100
7	11	5	80	5	4	75
8	10	9	100	5	5	80
9A	11	8	88	5	5	100
9B	11	9	100	5	5	80
10A	11	6	100	5	4	75
10B	10	4	100	4	1	100
11	2	1	100	2	2	100
12	11	6	67	5	3	100
13	6	4	75	5	3	100
14	11	8	88	5	4	100
15	11	9	100	5	5	100
16	11	8	75	5	4	100
17A[b]	5	0	0	1	1	100
17B	3	2	100	3	2	100
18	11	8	75	5	4	100
19	11	6	83	5	3	100

[a] The quantitative results provided for T-2 and HT-2 toxins in both maize and wheat were excluded from the statistical evaluation due to problems encountered by the participant with calibration curves.
[b] The quantitative results provided for OTA in both maize and wheat were excluded from the statistical evaluation due to problems encountered by the participant with mycotoxin quantification.

Table 6
Method details of the best performing laboratories taking part to the ISPA-2014-PT.

Lab. code	Extraction solvent[a]	Clean-up[b]	Calibration mode[c]	Injected matrix (mg)
3, 4	Acidified ACN–H_2O	no	ISTD (^{13}C mycotoxins)	0.5
6	Acidified ACN–H_2O	no	ESTD	0.63
8	MeOH–H_2O	IAC (multi-antibody)	ISTD (^{13}C mycotoxins)	25
9A	Isopropyl alcohol–H_2O–acetone	QuEChERs-like (liquid-liquid partition)	ISTD (^{13}C mycotoxins)	2
9B	Acidified ACN–H_2O	no	ISTD (^{13}C mycotoxins)	1
14	ACN–H_2O	SPE	ISTD (^{13}C mycotoxins)	2.5
15	Acidified ACN–H_2O	SPE for AFs; No clean-up for the others	ISTD (^{13}C mycotoxins)	1.9
16	Acidified ACN–H_2O	no	ISTD (^{13}C mycotoxins)	1.9
18	Acidified ACN–H_2O	no	ISTD (^{13}C mycotoxins)	0.3

[a] ACN, acetonitrile; MeOH, methanol; H_2O, water.
[b] SPE, solid phase extraction; IAC, immunoaffinity column; QuEChERs, Quick Easy Cheap Effective.
[c] ESTD, external calibration (neat solvent); ISTD, internal standard calibration.

Table 7
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 analysed in the ISPA-2011-PT.

Statistical parameters	DON	FB_1	FB_2	OTA	T-2	HT-2	ZEA	AFB_1	AFG_1	AFB_2	AFG_2
Number of participant laboratories[a]	39	39	39	39	39	39	39	39	39	39	39
Number of submitted results	37	30	30	30	33	32	36	33	33	31	32
Number of quantitative results	37	30	29	30	21	32	36	32	32	22	22
Median (μg/kg)	576	2085	726	6.90	3.60	189	277	4.90	8.90	0.50	1.10
Minimal value (μg/kg)	45.9	884	93.4	1.60	0.70	38	3.70	0.90	0.50	0.10	0.20
Maximal value (μg/kg)	1512	6795	2814	13.2	107	725	720	11.8	18.2	1.70	3.30
Assigned value (μg/kg)	571	2177	885	7.37	7.41	181	274	5.03	9.12	0.53	1.08
Target standard deviation (μg/kg, σ_p)[b]	99.4	310	144	1.62	1.63	37.4	53.3	1.11	2.00	0.12	0.24
Reproducibility standard deviation (μg/kg)	0.52	934	529	3.82	7.72	41.2	126	3.17	3.17	3.17	3.60
Lower limit of tolerance (μg/kg)	373	1557	597	4.13	4.15	106	168	2.82	5.11	0.30	0.61
Upper limit of tolerance (μg/kg)	653	2518	1082	8.77	10.8	195	316	6.16	10.4	0.66	1.30
Number of laboratories with mean outside of tolerance limits	15	15	15	11	18	5	12	15	10	8	7
Overall acceptable z-scores	59%										
Overall questionable z-scores	16%										
Overall unacceptable z-scores	25%										

[a] Two participant laboratories provided an additional set of results.
[b] Calculated according to truncated Horwitz equation.

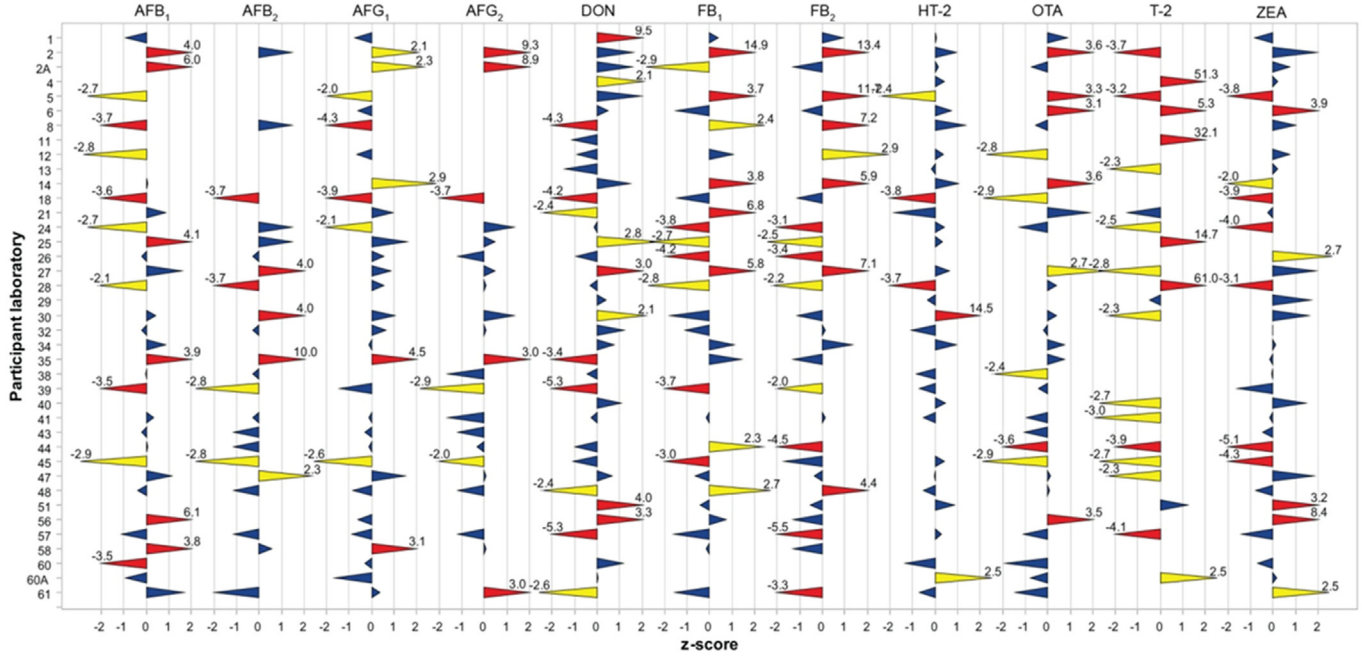

Fig. 2. Summary graph of the laboratory's z-scores for deoxynivalenol (DON), fumonisins B_1 (FB_1) and B_2 (FB_2), zearalenone (ZEA), T-2 and HT-2 toxins, ochratoxin A (OTA), aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) in maize calculated in the ISPA-2011-PT. Blue triangles indicate acceptable z-score, yellow triangles indicate questionable z-scores and red triangles indicate unacceptable z-scores.

maize used in the present 2014 PT, especially for HT-2, ZEA and aflatoxins. The overall acceptable z-scores were 59%, while the overall questionable and unacceptable z-scores were 16% and 25%, respectively. All these values were worse than those obtained in the ISPA-2014-PT (i.e. 85%, 3% and 11%, respectively, Table 3), clearly indicating a positive trend of results over the years. In particular, by looking at single mycotoxin z-scores, an improvement of acceptable z-scores from 2011 to 2014 was observed for DON (from 59 to 100%), FB_1 (from 50 to 75%), FB_2 (from 48 to 80%), OTA (from 63 to 87%), T-2 (from 14 to 100%), for AFB_1 (from 53 to 93%) and for AFG_1 (from 69 to 86%). (Fig. 3). For ZEA and HT-2 acceptable z-score were comparable, while no comparison was made for AFB_2 and AFG_2 because too few data were returned by participants in ISPA-2014-PT.

Some hypotheses could be done to explain this evident improvement of laboratory results. Over the past several years, there has been a pronounced shift towards the use of LC–MS techniques for mycotoxin detection, particularly in the context of multi-toxin methods, while the number of literature reports developing single-analyte LC–MS methods or LC-FLD or LC-UV methods has deeply decreased [6,7,25]. This shift to a broader use of LC–MS more than likely resulted in a deeper knowledge/experience in the use of these methodologies, mainly with respect to the choice of a suitable extraction solvent for the common extraction of

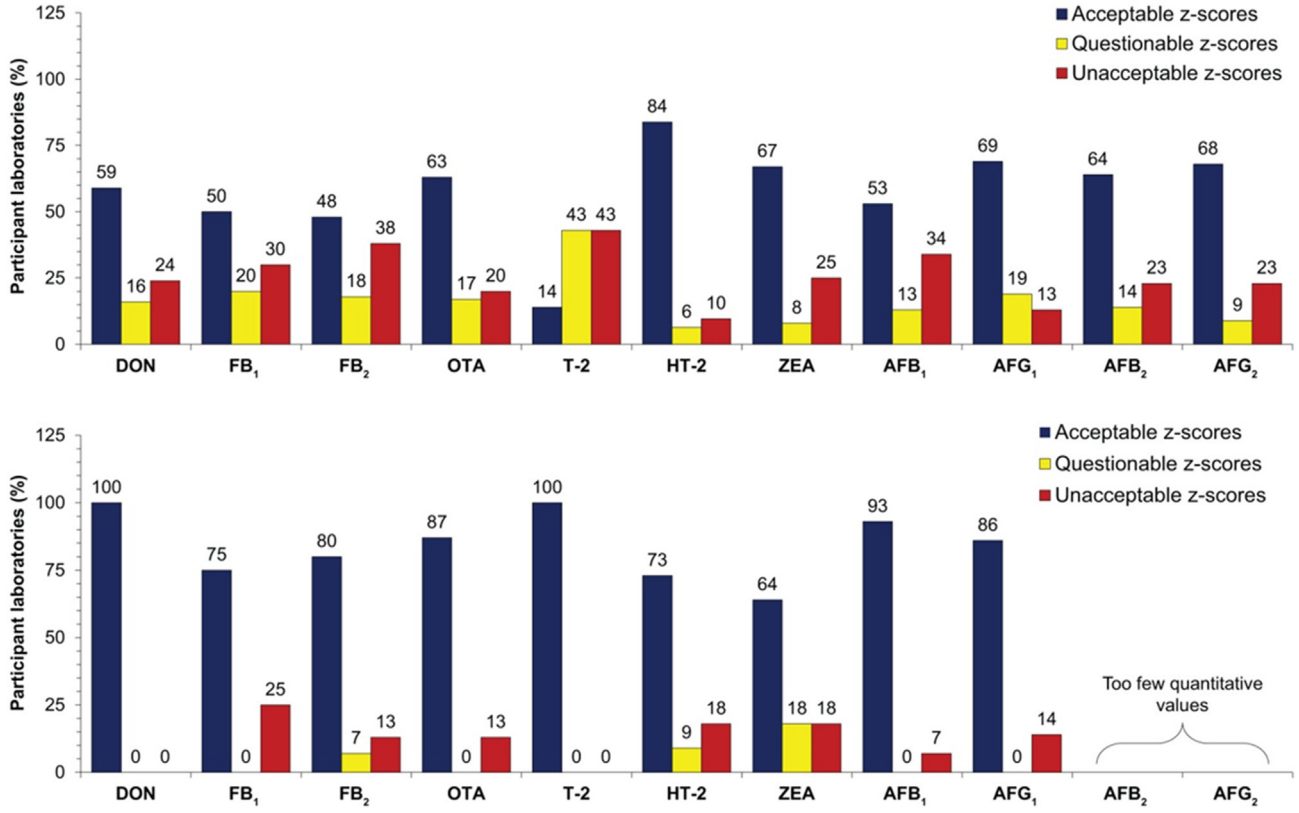

Fig. 3. Trend of z-scores results for deoxynivalenol (DON), fumonisins B_1 (FB_1) and B_2 (FB_2), zearalenone (ZEA), T-2 and HT-2 toxins, ochratoxin A (OTA), aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) in maize calculated in the ISPA-2011-PT and ISPA-2014-PT.

Table 8
Summary statistics for deoxynivalenol (DON), ochratoxin A (OTA), T-2 toxin (T-2), HT-2 toxin (HT-2) and zearalenone (ZEA) in maize analysed in the JRC-2013-PT.

Statistical parameters	DON	FB_1	AFB_1
Number of participant laboratories	21	21	21
Number of submitted results	21	21	21
Number of quantitative results	21	21	21
Median (μg/kg)	1077	4600	9.90
Minimal value (μg/kg)	830	78.0	3.50
Maximal value (μg/kg)	1825	12540	22
Assigned value (μg/kg)	1100	4260	8.90
Target standard deviation (μg/kg)[a]	173.5	547.9	1.96
Reproducibility standard deviation (μg/kg)	254.1	1981	2.30
Lower limit of tolerance (μg/kg)	753.1	3164	4.98
Upper limit of tolerance (μg/kg)	1447	5356	12.8
Number of laboratories with mean outside of tolerance limits	4	11	4
Overall acceptable z-scores	70%		
Overall questionable z-scores	12%		
Overall unacceptable z-scores	18%		

[a] Calculated according to truncated Horwitz equation.

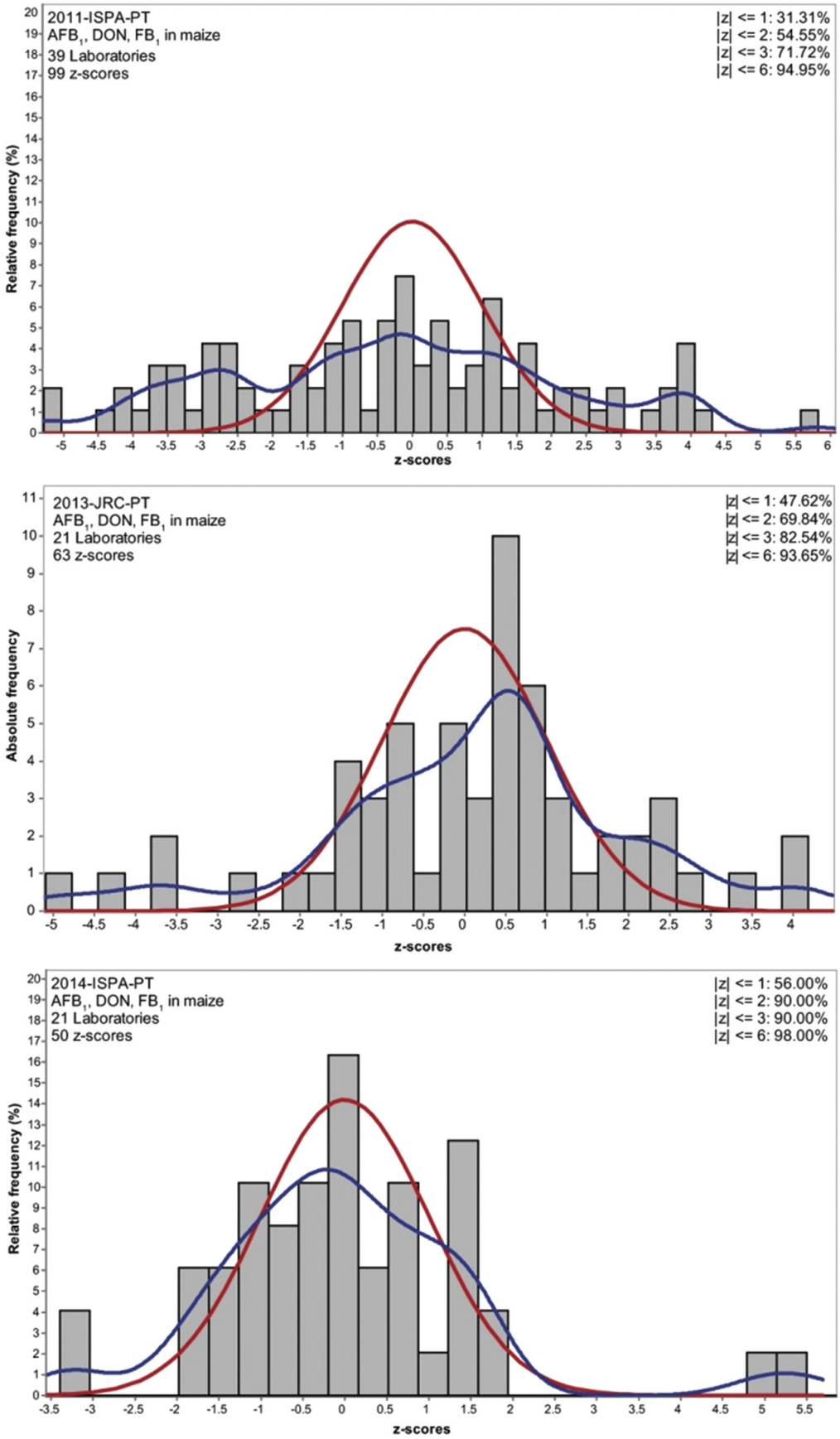

Fig. 4. Overall distribution of z-score results for deoxynivalenol (DON), fumonisins B_1 (FB_1) and aflatoxin B_1 (AFB_1) in maize calculated in the ISPA-2011-PT, JRC-2013-PT and ISPA-2014-PT. Expected z-scores for a normal distribution: 68.27% for $|z| \leq 1$, 95.45% for $|z| \leq 2$, 99.73% for $|z| \leq 3$ and 100% for $|z| = 6$.

all relevant mycotoxins, and strategies for effective compensation of instrument specific matrix effects.

Furthermore, a general simplification of sample preparation protocols (generally improving recovery rates and repeatability) has been observed, thanks to the increased availability, over the last five years, of highly sensitive and selective mass spectrometers making multi-analyte methods more accessible.

Finally, the improved confidence with multi-mycotoxin methods, resulting in an increased participation to PTs organised by different institutions worldwide, might also explain the overall improvement of laboratory performances.

To individuate the analytical conditions affecting the performance of the LC–MS methods used in the ISPA-2011-PT we focused our attention on the percentage of laboratories that reported questionable or unacceptable z-scores. The majority of them used the external standard calibration in neat solvent and injected more than 5 mg of matrix. It is well known that co-eluting matrix components may either enhance or suppress the ionization efficiency of the analytes and as a result could affect the reproducibility and accuracy of the results. Thus, the use of external standard calibration, even though coupled with extract dilution prior LC–MS analysis, in the ISPA-2011-PT did not eliminate or address efficiently matrix effects thus did not allow to obtain quantitative accurate results. In the case of the ISPA-2014-PT an improvement was observed due to a shift to internal standard calibration with isotope labelled internal standard or matrix assisted calibration (62% of participant laboratories) and the injection up to 5 mg matrix equivalent (91% of participant laboratories).

Furthermore, differently from the ISPA-2014-PT, the statistical evaluation of ISPA-2011-PT laboratory results in terms of number of acceptable z-scores and number of analysed mycotoxins did not allow to select common analytical parameters giving acceptable results. It is notable that the best performing laboratories (according to LAWA mode of evaluation) in the ISPA-2011-PT accounted only to 8% as compared to 29% in the ISPA-2014-PT, further supporting the positive trend of the LC–MS methods, as well as the improvement of laboratories performance over these years.

To support this claim we investigated on results obtained by laboratories that took part to both 2011 and 2014 ISPA-PTs and we found 11 matched participant laboratories, representing a valid cross section of analytical laboratories at each time. The majority of them (55%) increased the number of mycotoxins (from 4 up to 11) that were simultaneously analysed with their own methodology in the 2014-ISPA PT, while the remaining (45%) analysed 11 mycotoxins in both studies. Furthermore, although the analytical procedure used by these laboratories (i.e. extraction solvent used, clean-up mode, amount of injected matrix, and use of isotope labelled internal standards) was similar in the two studies, 64% of laboratories increased the number of acceptable z-scores (from 18% up to 100%), thus confirming an overall improved knowledge/experience in multi-mycotoxin methodologies based on LC–MS.

A further confirmation of the positive trend of LC–MS methodologies and laboratories performance over the years (from 2011 to 2014) was given by the comparison between results of JRC-2013-PT on maize (Table 8) and the two ISPA PTs. Although the JRC-2013-PT focused only on DON, FB_1 and AFB_1, the overall z-score results were intermediate to those of the two ISPA PTs, i.e. 70% of acceptable z-scores, 13% of questionable z-scores and 17% of unacceptable z-scores. In agreement with our conclusions for ISPA-2011-PT, most of laboratories giving unacceptable z-scores used external standard calibration, thus confirming the primary role of this analytical factor on LC–MS analytical methodologies for multi-mycotoxin analysis.

Fig. 4 shows the overall distribution of z-scores obtained for all mycotoxins by participant laboratories within the three PTs

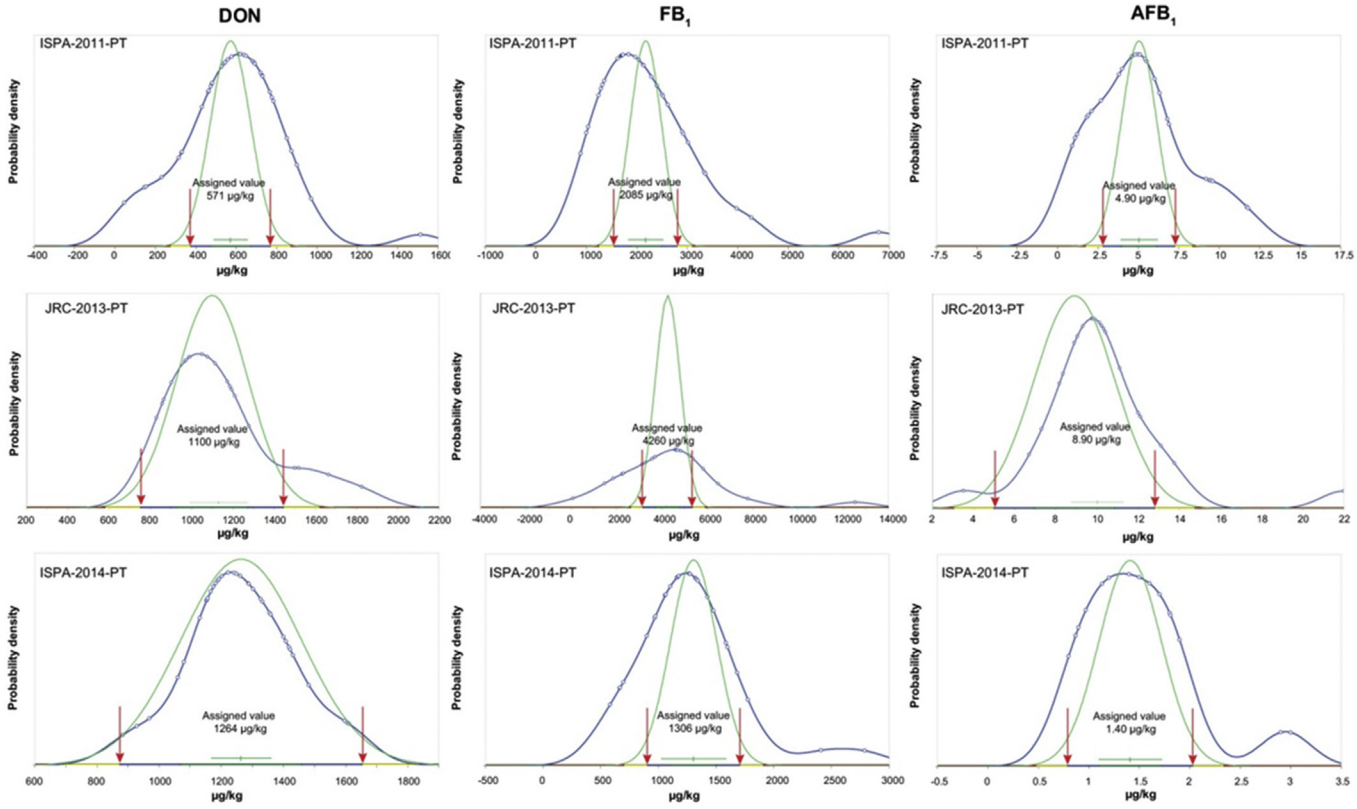

Fig. 5. Kernel density plots for deoxynivalenol, fumonisin B_1 and aflatoxin B_1 in maize in the ISPA-2011-PT (on the top), JRC-2013-PT (in the middle) and in the ISPA-2014-PT (on the bottom).

Abbreviations

ACN	acetonitrile
AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFG_1	aflatoxin G_1
AFG_2	aflatoxin B_2
CEN	European Committee for Standardization
DON	deoxynivalenol
ESTD	external calibration (neat solvent)
EU-RL	European Union Reference Laboratory
FB_1	fumonisin B_1
FB_2	fumonisin B_2
HT-2	HT-2 toxin
H_2O	water
IAC	immunoaffinity column
ISTD	internal standard
Lab.	participant laboratory
LAWA	German Working Group on water issues
LOD	limit of detection
LOQ	limit of quantification
MeOH	methanol
MoniQA	Monitoring and Quality Assurance in the Total Food Supply Chain
NRLs	National Reference Laboratories
OTA	ochratoxin A
PT	proficiency testing
QuEChERS	Quick Easy Cheap Effective Rugged Safe
SPE	solid phase extraction
s_b	between bottle standard deviation
s_w	within bottle standard deviation
T-2	T-2 toxin
ZEA	zearalenone
z	z-score
σ_p	target standard deviation

together with the kernel density estimation of the score distribution and the standard normal distribution. The percentage of z-scores expected for a normal distribution is 68.3% for z-score values between −1 and +1, 95.5% for z-scores values between −2 and +2, 99.7% for z-scores values between −3 and +3 and 100% for z-scores values between −6 and +6. The increasing of acceptable z-scores over the years is clearly evident; in particular, the percentage of z-scores increased over the years from 31.3% to 56.0% for z-score values between −1 and +1 and from 54.5% to 90% for z-score values between −2 and +2 (Fig. 4). This improvement of results was also evident by comparing the kernel density plots. For example, in the kernel density plots of DON, FB_1 and AFB_1 it can be seen that in the ISPA-2011-PT and 2013-JRC-PT the majority of the laboratories reported values far from the assigned value, while in the ISPA-2014-PT results were quite better and normally distributed around the assigned value (Fig. 5). The outcomes of ISPA-2014-PT provided valuable information on the performances of LC–MS multi-mycotoxin methods for maize and wheat. Furthermore, considering the high number of participants from 10 Countries, and different operational conditions, it can be considered a robust and representative interlaboratory study.

4. Conclusions

The following major conclusions can be highlighted: i) the evaluation of satisfactory results ($|z| \leq 2$) shows that the majority of laboratory participants had the ability to provide acceptable results for the simultaneous analysis of deoxynivalenol, fumonisins (B_1 and B_2), ochratoxin A, zearalenone, T-2 and HT-2 toxins and aflatoxins (B_1 and G_1) in maize and for deoxynivalenol, ochratoxin A, zearalenone, T-2 and HT-2 in wheat. However, laboratory performances were better for wheat analysis compared to maize analysis (91% *vs* 85%). This could be partly attributed to the lower number of mycotoxins to be analysed in wheat, that did not include aflatoxins and fumonisins, that present particular challenges due to the low contamination levels or difficulties in co-extraction with other mycotoxins, respectively; ii) a large variability of LOQ values was observed among laboratories, therefore only few laboratories were able to analyse AFB_2 and AFG_2 in maize and T-2 in wheat; a further improvement of LC–MS multi-mycotoxin methodologies sensitivity is still required for these mycotoxins in cereals; iii) the best performing laboratories used acidified ACN–water extraction, dilute and shoot injection without extract clean-up and internal standard calibration (^{13}C standards).

The trend toward the multi-mycotoxin determination in maize by LC–MS has been assessed for the first time by comparing three recent PTs. Laboratory performances (overall acceptable z-scores) increased from 59% in 2011-PT, to 70% in 2013-PT and to 85% in 2014-PT. The rate of unacceptable z-score decreased from 25% in 2011-PT, to 18% in 2013-PT and to 11% in 2014 PT. The improved LC–MS method's performances can be attributed to an overall improved knowledge and management of factors affecting reliability of LC–MS analysis, related to proper matrix effect compensation (mainly by using isotope labelled mycotoxins as internal standards), and to a general simplification of sample preparation protocols thanks to the increased availability of highly sensitive and selective mass spectrometers.

In summary, the findings of this study provide an advance towards the harmonization of an LC–MS method for the multi-mycotoxin analysis of unprocessed cereals.

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Abbreviations

ACN	acetonitrile
AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFG_1	aflatoxin G_1
AFG_2	aflatoxin B_2
CEN	European Committee for Standardization
DON	deoxynivalenol
ESTD	external calibration (neat solvent)
EU-RL	European Union Reference Laboratory
FB_1	fumonisin B_1
FB_2	fumonisin B_2
HT-2	HT-2 toxin
H_2O	water
IAC	immunoaffinity column

ISTD	internal standard
Lab.	participant laboratory
LAWA	German Working Group on water issues
LOD	limit of detection
LOQ	limit of quantification
MeOH	methanol
MoniQA	Monitoring and Quality Assurance in the Total Food Supply Chain
NRLs	National Reference Laboratories
OTA	ochratoxin A
PT	proficiency testing
QuEChERS	Quick Easy Cheap Effective Rugged Safe
SPE	solid phase extraction
s_b	between bottle standard deviation
s_w	within bottle standard deviation
T-2	T-2 toxin
ZEA	zearalenone
z	z-score
σ_p	target standard deviation

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