

PERFORMANCE EVALUATION OF LC-MS/MS METHODS FOR MULTI-MYCOTOXIN DETERMINATION IN MAIZE AND WHEAT BY PROFICIENCY TESTINGS

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Abstract – Two international Proficiency Testings (PTs) have been conducted in 2014 for the simultaneous determination of deoxynivalenol, fumonisins, zearalenone, T-2 and HT-2 toxins, ochratoxin A and aflatoxins in maize and of deoxynivalenol, zearalenone, T-2 and HT-2 toxins and ochratoxin A in wheat, respectively, by using LC-MS methods. The overall performance of participating laboratories to the PTs and the trend in multi-mycotoxin determination by LC-MS in maize over the years 2011-2014 are presented.

Keywords: proficiency testing, LC-MS, multi-mycotoxin, z-score, laboratory performance

1. INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by filamentous fungi produced under a wide range of climatic conditions on agricultural commodities both in the field and during storage [1]. Mycotoxin contamination of agricultural food commodities and beverages can pose serious risks to human and animal health due to their toxic effects [2]. Mycotoxins of major concern worldwide are: aflatoxins B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁) and G₂ (AFG₂), ochratoxin A (OTA), fumonisins B₁ (FB₁) and B₂ (FB₂), deoxynivalenol (DON), zearalenone (ZEA), T-2 (T-2) and HT-2 (HT-2) toxins. Harmonized maximum permitted levels for mycotoxins in foodstuffs have been established in the European Union and at international levels. 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, have been developed for the analysis of single mycotoxins or group of mycotoxins in food and feed [3-4]. Among them, multi-analyte methods have become the ones most required because more mycotoxins frequently co-occur in the same product. Within this context the application of liquid chromatography coupled with mass spectrometer detectors (LC-MS) 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 [5]. 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 [6]. Several PTs programs for mycotoxins are available in Europe focusing mainly on the determination of single mycotoxins 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 used methodologies and related method performances. The study involved 42 international participants and aimed to the determination of DON, FB₁, FB₂, ZEA, T-2, HT-2, OTA, AFB₁, AFG₁, AFB₂ and AFG₂ in contaminated and spiked maize [7]. In this framework, the ISPA-CNR organised other two multi-mycotoxin PTs in 2014 (ISPA-2014-PTs) for the determination of DON, FB₁, FB₂, ZEA, T-2, HT-2, OTA, AFB₁, AFG₁, AFB₂ and AFG₂ in maize and DON, ZEA, T-2, HT-2 and OTA in wheat, respectively, by LC-MS.

In this paper we present an evaluation of performances of laboratories participants in the ISPA-2014-PTs and an evaluation of the trend of laboratory performances in LC-MS methods for multi-mycotoxin determination in maize over the years 2011-2014.

2. DESIGN OF THE ISPA-2014-PTs

The ISPA-2014-PTs involved 18 laboratories from 10 countries, including public and private laboratories, universities and public research facilities (Table 1). The use of LC-MS multi-mycotoxin methods was mandatory, however participants were not obliged to determine all toxins in each material (maize and wheat), and were let free to report only on those mycotoxins that they could simultaneously determine by their multi-mycotoxin methodology.

Table 1. Participating Laboratories to the ISPA-2014-PTs

1	Romer Labs Diagnostic GmbH (Austria)
2	LVA GmbH (Austria)
3	University of Natural Resources and Applied Life Sciences (Austria)
4	AGES GmbH, National Reference Lab for Mycotoxin (Austria)
5	EC-Joint Research Centre – IRMM (Belgium)

6	EC-Joint Research Centre – IRMM (Belgium)
7	Veterinary and Agrochemical Research Centre (Belgium)
8	Canadian Grain Commission (CGC) (Canada)
9	Max Rubner Institut (Germany)
10	Barilla G.R. F.lli SpA (Italy)
11	Bonassisa Lab (Italy)
12	University of Bari Aldo Moro (Italy)
13	Romer Labs Singapore Pte Ltd (Republic of Singapore)
14	Southern African Grain Laboratory NPC - SAGL (South Africa)
15	RIKILT-Institute of Food Safety (The Netherlands)
16	NofaLab (The Netherlands)
17	Virginia Polytechnic Institute and State University (USA)
18	Food & Environment Research Agency (United Kingdom)

2.1. Preparation of materials

Test materials were maize contaminated with DON, FB₁, FB₂, ZEA, T-2, HT-2, OTA, AFB₁, AFG₁, AFB₂ and AFG₂, and wheat contaminated with DON, ZEA, T-2, HT-2 and OTA. Since naturally contaminated materials containing all focused mycotoxins at the EU legislated levels were unavailable at the time of this study, preparation of contaminated test materials was performed applying a previously developed protocol by fortifying wheat and maize with culture extracts of mycotoxigenic species of *Fusarium* and/or *Aspergillus* [7]. Before distribution, materials were tested for their homogeneity according to ISO Guide 13528:2015 [8]. Furthermore, an isochronous short-term stability study was carried out according to ISO Guide 35:2006 for each test material [9].

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

2.2 Statistical evaluation of ISPA-2014-PTs results

The assigned values (robust means) were calculated according to the Algorithm A of ISO 13528:2015 [8]. Results reported as “smaller than detection or quantification limits” were excluded from all statistical calculations.

The target standard deviation of each mycotoxin evaluated in the maize and wheat materials was derived from the truncated Horwitz equation corrected by Thompson as reported [6].

Individual laboratory performance was expressed in terms of z-score in accordance with ISO 13528:2015 [8]. Interpretation of z-scores was as follows: $|z| \leq 2$, acceptable result; $2 < |z| \leq 3$, questionable result; $|z| > 3$, unsatisfactory result.

3. ANALYTICAL PROCEDURES USED IN THE ISPA-2014-PTs

Eighteen participants returned two sets of results for various combinations of analytes. Three participants returned two additional sets of results obtained by using two different LC-MS methods for both contaminated maize and wheat. These results were considered for statistical evaluation as being from independent laboratories. An overall set of 21 results was obtained for both materials.

The most preferred procedures were based on acetonitrile-water extraction, direct injection without extract clean-up (“dilute and shoot”) and internal standard calibration (ISTD) (Fig. 1). All laboratory participants, but one, used triple quadrupole mass analyzers for mycotoxin detection in single reaction monitoring mode (SRM). One laboratory used an hybrid quadrupole-Orbitrap™ mass analyzer performing full scan-data dependent MS/MS analysis.

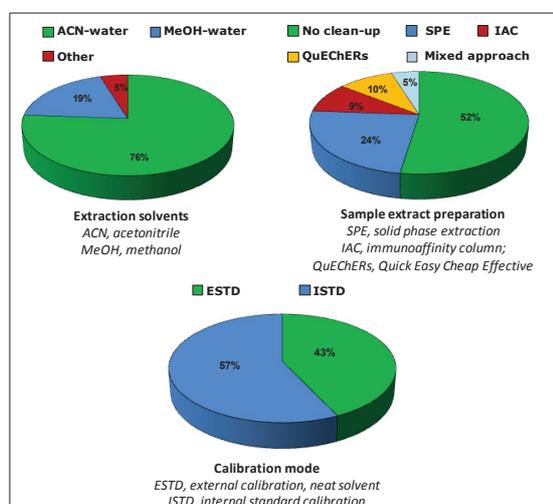


Fig. 1. Extraction solvents, sample extract preparation and calibration mode used by participant laboratories in the ISPA-2014 PTs.

4. LABORATORIES RESULTS AND PERFORMANCE EVALUATION

Fifty-seven percent of participants were able to analyze the eleven targeted mycotoxins in

maize while 71% of participants analysed the five targeted mycotoxins in wheat. For mycotoxins occurring at low levels in the test materials (i.e. AFB₂, AFG₁ and AFG₂ in maize and T-2 toxin in wheat) few participants reported results as less than the detection (LOD) or quantification limits (LOQ) of the used method.

The assigned values for analysed mycotoxins in maize and wheat are reported in Table 2.

A representative summary graph of the laboratory's z-scores for OTA in maize is shown in Fig. 2. Eighty-five percent of laboratories provided acceptable z-scores for maize while 91% of laboratories provided acceptable z-scores for wheat.

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 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. In total, six laboratory participants for maize and ten laboratories for wheat obtained 80% of acceptable z-scores for at least 80% of the measurands. For both matrices, the main common experimental parameters were the extraction solvent (acidified acetonitrile-water mixtures), the calibration mode (ISTD with labelled standards) and injected matrix equivalent (≤ 2.5 mg).

Table 2. Assigned values for targeted mycotoxins in maize and wheat test materials

Mycotoxin	Assigned value ($\mu\text{g}/\text{kg}$)	
	Maize	Wheat
DON	1264	1297
FB1	1306	- ^a
FB2	350	-
OTA	2.62	7.00
T-2	54.4	8.26
HT-2	30.7	58.8
ZEA	21.7	148
AFB ₁	1.40	-
AFB ₂	Too few values	-
AFG ₁	0.70	-
AFG ₂	Too few values	-

^anot included

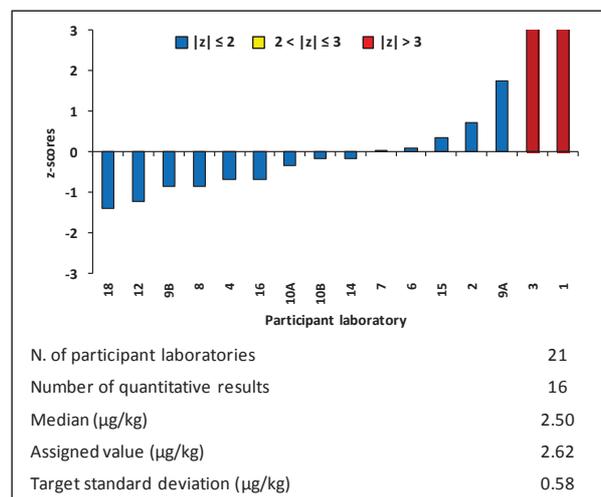


Fig. 2. Representative summary graph of the laboratory's z-scores calculated for OTA in maize

5. TREND IN MULTI-MYCOTOXIN ANALYSIS BY LC-MS/MS

Considering the similarities between the PTs coordinated by ISPA-CNR in 2011 and in 2014 in terms of targeted mycotoxins and test material (i.e. maize), a critical evaluation of results has been carried out by comparing results obtained for maize in both PTs. Although levels of mycotoxins in maize test material used in the ISPA-2011-PT were higher than those found in maize used in the present 2014 PT, an evident improvement of the acceptable z-scores (from 61% to 85%) was observed over the years (Fig. 3).

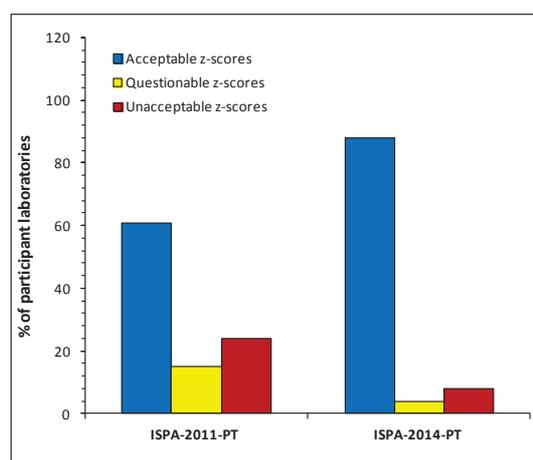


Fig. 3. Trend of z-scores results calculated in the ISPA-2011-PT and ISPA-2014-PT for the overall mycotoxins in maize

Several factors could have contributed to this positive trend of laboratories performances such as an overall improved knowledge and management of factors affecting reliability of LC-

MS analysis as well as the increased availability of highly sensitive and selective mass spectrometers.

6. CONCLUSIONS

The outcomes of the two ISPA-2014-PTs provided valuable information on the performances of LC-MS multi-mycotoxin methods for maize and wheat. Furthermore, the assessment of the trend of multi-mycotoxin determination in maize by LC-MS has indicated an improvement of laboratory performances over the years 2011-2014, thus suggesting that LC-MS methods can be successfully used as reliable tools for the simultaneous determination of groups of mycotoxins in cereals.

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