DETERMINATION OF TRICHOTHECENES AND ZEARALENONE IN WHEAT AND DERIVED PRODUCTS BY LC-MS/MS: DEVELOPMENT OF A STANDARD METHOD WITHIN THE CEN MANDATE M/520

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Abstract–Results obtained within a project under theEuropean Commission standardization mandate M520/EN (item 4) are herein presented. Aim of the project was to develop and validate an analytical method for the simultaneous determination of nivalenol, deoxynivalenol and its acetyl derivatives, T-2 and HT-2 toxins, and zearalenone in cereals and cereal products by liquid chromatography - tandem mass spectrometry. The standardized method shall be applied to perform official control of the above food products, and to determine whether a production batch can be put on the market.

Keywords: trichothecenes,zearalenone, cereals and cereal products, standardization, LC-MS/MS

1. INTRODUCTION

The establishment of standardized methods of analysis is of utmost importance to guarantee a uniform application of the European legislation in all Member States. Standardized methods of analysis are an indispensable toolin guaranteeing a high level of food and feed safety.

At EU level, standardized methods of analysis are established by the CEN (European Committee for Standardization). With respect to mycotoxins, thirty-one standardized CEN methods are currently available. In particular for *Fusarium* toxinsare available two methods fordeoxynivalenol(DON) in cereals and cereal products (EN 15891:2010) and in feeds (EN 15791:2009) and two methods for zearalenone (ZEA) in cereal products (EN 15850:2010) and in feeds (EN15792:2009). None of thestandardized methods is based on liquid chromatography – mass spectrometry (LC-MS). Furthermore, no multi-analyte methods are available for the simultaneous determination of trichothecenes and zearalenoneeither within CEN or AOAC context.

However, the need of LC-MS methods for mycotoxin determination in control laboratories has been highlighted by a recent mandate by the European Commission (EC) for standardization of methods of analysis for mycotoxins in food (M520/EN) by which the Commission invited CEN to establish European Standards/Technical Specifications thatprovide standardized methods of analysis for mycotoxins in food. Six of the 11 methods of analysis listed in this mandate are specifically requested to be based on LC-MS.The work described herein addresses item 4 of the standardization mandate, aiming at optimizing and validating an analytical method suitable for the simultaneous determination of nivalenol (NIV), deoxynivalenol(DON)and itsacetyl derivatives (3-acetyl-DON and 15-acetyl-DON),T-2 and HT-2 toxins, and zearalenone(ZEA) in cereals and cereal products by liquid chromatography - tandem mass spectrometry (LC-MS/MS).

According to the mandate, methodstandardization included the following tasks:

1) single laboratory validation study to verify method analytical performances in the selected food commodities;

2) preparation and characterization of test materials for the collaborative study;

3) collaborative study, including a pre-trial exercise allowing the participant laboratories to familiarize with the method;

4) reporting and preparation of the final standard.

Activities carried out for single laboratory validation, preparation of test materials and pre-trial exercise will be presented and relevant results

will be discussed, whereas the full collaborative study is currently in progress.

2. PRINCIPLE

Trichothecenes (NIV, DON, 3-AcDON and 15-AcDON,T-2 and HT-2 toxins) and ZEAwere extracted from the commodities with a mixture of acetonitrile-water. The extract was filtered and evaporated to dryness. The residue was dissolved with a mixture methanol-water and applied to a polymeric solid phase extraction (SPE) column. The toxins were purified and concentrated on the column then released using methanol as eluent. Isotopically labelled mycotoxins (fully labelled ^{13}C mycotoxins)were added to the column eluate before evaporating it to dryness. After reconstitution of the dry extract with the injection solvent, the toxins were quantified by reversed phase high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS).

2. SINGLE LABORATORY VALIDATION

The selected food commodities for single-and inter-laboratory validationwere: unprocessed soft wheat, soft wheat flour, and cereal based snacks (crackers). Based on the available data on occurrence and dietary exposure, the above mentioned foods are among the major sources of intake of trichothecenes and ZEA in the EU.

To establish method performance characteristics of the candidate method (recoveries, repeatability, within laboratory reproducibilityand limits of quantification), full *in-house* validation was performed according to the IUPAC harmonized guidelines for single laboratory validation [2]. The method was validatedat three concentration levels, corresponding to 50% (low), 100% (medium), and 200% (high) EU maximum permitted or recommended levels for each targeted mycotoxin.

The validation experiments were carried out according to a 5-days nested design with two different batches of each food commodity(i.e. two wheat varieties, two wheat flour samples from different suppliers, two different cracker preparations). Samples were analysed on each day under repeatability conditions, resulting in 4 independent and complete analyses per day and 20 measurements in total per each spiking level.

Performance characteristics of the candidate methodare summarized in Table 1. Method performances resulted to be compliant with criteria for single laboratory validated methods for mycotoxin determination specified inCommission Regulation 401/2006/EC and in the document UNI CEN/TR 16059.

Table 1. Recoveries, repeatability (RSD_r), within laboratory reproducibility (RSD_{WLR}) and limits of quantification (LOQ) by single-laboratory validation of the candidate method

WHEAT				
	Recovery (%)	**RSD*r* (%)**	**RSD_{WLR} (%)**	**LOQ (µg/kg)**
NIV	90-93	3-6	3-6	50
DON	82-91	2-6	5-8	7
3AcDON	89-97	2-6	5-7	3
15AcDON	82-99	4-8	5-10	10
HT-2	78-109	9-30	11-29	3
T-2	91-101	5-10	6-9	1
ZEA	80-81	4-8	5-17	0.5
WHEAT FLOUR				
	Recovery (%)	**RSD*r* (%)**	**RSD_{WLR} (%)**	**LOQ (µg/kg)**
NIV	85-107	9-14	9-13	25
DON	93-97	14-20	18-24	15
3AcDON	89-99	13-21	19-23	3
15AcDON	98-112	14-19	11-26	10
HT-2	73-102	10-13	12-16	5
T-2	89-94	2-13	5-13	1
ZEA	80-102	7-14	10-13	0.5
WHEAT SNACKS				
	Recovery (%)	**RSD*r* (%)**	**RSD_{WLR} (%)**	**LOQ (µg/kg)**
NIV	75-80	4-9	7-13	25
DON	91-122	4-11	7-12	15
3AcDON	86-99	3-9	6-10	3
15AcDON	81-102	6-22	12-33	5
HT-2	76-96	3-7	19-26	1
T-2	77-90	3-8	17-22	0.5
ZEA	89-96	4-7	7-15	0.5

3. PREPARATION OF TEST MATERIALSFOR THE COLLABORATIVE STUDY

According to the mandate,unprocessed soft wheat, soft wheat flour and crackers materials were prepared for the collaborative study. For each food commodity, one blank material (containing mycotoxins at levels below the detection limit of the method), one blank material for spiking

purposes (recovery evaluation) and three contaminated materials (low, medium and high level) with target mycotoxins (NIV, DON, 3- and 15-acetylDON, T-2, HT-2, ZEA)were prepared. Desired mycotoxin levels in contaminated test materials should be within a contamination range encompassing EU maximum permitted levels (where applicable), therefore contamination levels of 50% (low), 100% (medium), and 200% (high) of EU maximum permitted or recommended levels were chosen.Since naturally contaminated materials containing all focused mycotoxins at desired levels were unavailable at the time of this study, preparation of contaminated test materials was performedapplying a previously developed protocol [3]. Briefly, wheat and wheat flour contaminated materials were prepared by mixing and homogenizingblank material with aqueous or organic culture extracts of *Fusarium* toxigenic species.

Contaminated crackers were prepared at laboratory scale by adding the appropriate amount of mycotoxin standard solutions to the water necessary for dough preparation.

Before distribution, test material homogeneity was verified according to ISO Guide 13528:2005. Furthermore, an isochronous stability study was carried out according to ISO Guide 35:2006 for each food commodity at each concentration level.

4. ORGANIZATION OF THE COLLABORATIVE STUDY

4.1Pre-Trial

Eleven laboratories from 9 different countries, listed in Table 2, were involved in the collaborative trial.

Table 2. Participant list

1	Barilla G.R. F.lli SpA (Italy)
2	EC-Joint Research Centre – IRMM (Belgium)
3	National Centre for Food (Spain)
4	RIKILT- Institute of Food Safety (The Netherland)
5	National Institute for Agricultural Research - INRA (France)
6	Agriculture and Agri-Food Canada (Canada)
7	Veterinary and Agrochemical Research Centre – CODA-CERVA (Belgium)
8	Istituto Zooprofilattico Sperimentale Umbria e Marche (Italy)
9	USDA-ASR (US)
10	Regional Agency for Environmental Protection(Apulia, Italy)
11	Regional Agency for Environmental Protection (Liguria, Italy)

Prior to the final validation study all laboratories had to participate in the pre-trial study, in order to familiarize with the correct execution of the method protocol. For these purposes, each participant received:

- one blank wheat flour sample, to be used for five determinations (two determinations as blank and three determinations for recovery check);
- onecontaminated wheat sample (to be analyzed as blind duplicate);
- twomixed mycotoxin spiking solutions in acetonitrile to be used for both spiking and calibration purposes;
- a mixed ^{13}C-mycotoxin solution in acetonitrile to be used as internal standard;
- columns for SPE clean up;
- method protocol in ISO/CEN format.
- reporting sheets.

Optimization of settings for LC and MS/MS detection was left to the participants. Each laboratory was free of using its own LC-MS/MS set up provided that certain minimum requirements (chromatographic resolution and signal-to-noise ratios) were met.

As an example, pre-trial results for DONare summarizedin Table 3.

Table 3. Precision and recovery data for deoxynivalenol in wheat

Samples	**1**[a]	**2**[b]
Year of pre-trial study	2016	2016
N. of laboratories	11	11
N. of laboratories retained after eliminating outliers	10	10
N. of outliers (laboratories)	1	1
Mean value, $\bar{x}$, µg/kg	710.9	1144.3
Repeatability standard deviation s_r, µg/kg	48.1	38.3
Repeatability relative standard deviation, RSD_r, %	6.8	3.3
Reproducibility standard deviation s_R, µg/kg	54.3	68.0
Reproducibility relative standard deviation, RSD_R, %	7.6	5.9
Recovery, %	94.8	-
HorRat value, according to [5]	0.45	0.38

[a]spiked wheat flour (750 µg/kg);[b]contaminated wheat sample.

Acceptable recoveries, repeatability and reproducibility data were obtained for all mycotoxins in the tested materials. Results were all within the criteria established by the European Commission for the acceptability of methods for mycotoxindetermination, proving the candidate method to be suitable for the full collaborative study.

4.2Collaborative Study

The collaborative trial has been planned according to the requirements of the IUPAC/AOAC international harmonized protocol [4].

In the collaborative trial, each participant will analyze a total of 15 samples (3 commodities× 5 samples) in blind duplicates for a total of 30 analyses. In particular, for each commodity one blank (containing mycotoxins at levels below the detection limit of the method), one spiked (for recovery evaluation) and three contaminated materials (low, medium and high level)will be provided (see section 3).

This final step will include results collection and statistical evaluation, establishment of performance characteristics (recovery, repeatability and reproducibility) at the levels of mycotoxin contamination.

Report of collaborative study results will be drafted in suitable format for publication as European standard. Results of the work will be discussed within CEN/TC 275/WG 5 “Biotoxins”. Technical comments given during meetings and during CEN enquiry stage of the draft standard will be evaluated and addressed.

5. CONCLUSIONS

Activities carried out for the standardization of a LC-MS/MS method for the simultaneous analysis of nivalenol, deoxynivalenol and its acetyl derivatives, T-2 and HT-2 toxins and zearalenone in cereals and cereal products have been described. Results obtained during the *in-house* validation indicated that the method was compliant with acceptability criteria set in the Commission Regulation 401/2006/EC and in the document UNI CEN/TR 16059. Preparation and characterization of test materials for the collaborative study have been described. Homogeneity and stability characterization according to ISO 13528:2005and ISO Guide 35:2006 guidelines revealed that produced materials were adequate for the collaborative study. Finally, pre-trial results indicated that the candidate method is suitable for the full validation process, through a collaborative study, that is actually in progress. The final standard is expected to be issued by the end of 2017.

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