DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE DETERMINATION OF MYCOTOXINS IN FOOD

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Abstract – For enforcement purposes and for reliable surveillance programs, the availability of validated methods for determining food contaminants with performance characteristics that meet certain minimum criteria is mandatory. A brief overview of recent activities carried out at the Institute of Sciences of Food Production, National Research Council of Italy (ISPA-CNR) on the development and validation of analytical methods for the determination of mycotoxins in food is presented.

Keywords: mycotoxins, method validation, LC-MS, rapid methods, Proficiency Testing.

1. INTRODUCTION

Mycotoxins are toxic metabolites produced by various fungal species belonging mainly at the genera *Aspergillus*, *Penicillium* and *Fusarium* that can contaminate several agricultural products, both in the field and during storage. The major mycotoxins that frequently occur in a wide variety of commodities and products are aflatoxins, ochratoxin A, trichothecenes (including deoxynivalenol, T-2 and HT-2 toxins), zearalenone and fumonisins. Foodstuffs and feedstuffs contaminated by these mycotoxins can cause serious risks to human and animal health due to their toxic effects. For example, aflatoxin B1 is carcinogenic to humans, ochratoxin A is a potent nephrotoxin and a possible human carcinogen, while zearalenone is an estrogen mimic. In order to protect human and animal health indicative or maximum permitted levels have been established for the major occurring mycotoxins in several commodities, both at European and international level. In addition, sensitive, reliable and accurate methods of analysis are required to fulfil regulatory requirements and to carry out reliable surveillance programs aimed to gather adequate information on the levels of exposure to these mycotoxins [1].

Several methods for mycotoxin determination have been developed. Chromatographic methods are commonly used including gas-chromatography (GC) and liquid chromatography (HPLC or UHPLC) coupled with ultraviolet or diode array (UV/DAD), fluorescence (FL) or mass spectrometry (MS) detectors. In addition, several commercial immunometric assays, such as enzyme-linked immunosorbent assay (ELISA), are frequently used for screening purposes [2, 3]. Recently, a variety of emerging methods have been proposed for the analysis of mycotoxins in several food matrices, mainly cereals, based on immunochromatography (i.e. lateral flow devices or dipsticks), fluorescence polarization (FP), infrared spectroscopy (FT-NIR), electronic nose (e-nose) and optical/electrochemical biosensors [4-6]. Such methods must be subjected to validation procedures in order to be sure that they produce accurate and precise results, both within- and between- laboratories.

In this paper, we present a brief overview of recent results obtained at the Institute of Sciences of Food Production, National Research Council of Italy (ISPA-CNR) on development and validation of analytical methods for the determination of mycotoxins in food by liquid chromatography-mass spectrometry and rapid methods.

2. VALIDATION OF ANALYTICAL METHODS FOR DETERMINING MYCOTOXINS

Method validation is the process of demonstrating that a method is suitable for its intended purposes. For quantitative methods the

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validation process aims to establish the performance characteristics of the method such as accuracy, trueness, precision, sensitivity, selectivity, limit of detection, limit of quantification, linearity range and ruggedness.

Single-laboratory and inter-laboratory validation should follow International or European harmonized guidelines. Furthermore, the use of Certified Reference Materials (CRMs) and the participation of laboratories in Proficiency Testing (PT) programs should ensure that single-laboratory validation and within-laboratory procedures are working satisfactorily.

2.1. Liquid chromatography - mass spectrometry methods

Liquid chromatography - tandem mass spectrometry (LC-MS/MS) is a powerful technique for simultaneous screening, identification, characterisation and quantitative determination of a large number of mycotoxins, including their modified forms. Accuracy, precision, and sensitivity of LC-MS/MS methods may vary depending on the mycotoxin, matrix and instrumental sensitivity/selectivity. Quantitative measurement of mycotoxins by LC-MS is often unsatisfactory due to matrix effects and ion suppression/enhancement.

Several LC-MS methods have been developed and validated at ISPA-CNR for the simultaneous determination of legislated mycotoxins in cereals and derived products. They are based on reversed phase-solid phase extraction columns (SPE Oasis HLB) or multi-toxin immunoaffinity columns (Myco6in1+) for clean-up of extracts prior to MS detection [7-10]. In addition, LC-MS/MS and liquid chromatography high-resolution mass spectrometry (LC-HRMS) based on Orbitrap technology have been applied to investigate the presence of modified mycotoxins in naturally contaminated cereals and *Fusarium langsethiae* fungal cultures. Molecular structure details obtained by measuring exact masses of main characteristic fragments with high mass accuracy led to the identification of a monoglucosyl derivative of T-2 toxin and two monoglucosyl derivatives of HT-2 toxin. In addition, two monoglucosyl derivatives of neosolaniol and one monoglucosyl derivative of diacetoxyscirpenol, two type-A trichothecenes, were identified and characterised by LC-MS/MS [11-13].

LC-MS methodologies for single or multiple mycotoxin determination are routinely used in control laboratories, however 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 European Commission (EC) and the European Committee for Standardization (CEN) has issued a call for tender for the development of standardized methods of analysis for mycotoxins in food. Six of them were specifically requested to be based on LC-MS/MS [14].

In order to check next to the laboratory performance the state-of-art of currently used multi-mycotoxin methods, two PTs involving 18 laboratory participants from 10 Countries have been recently organized by ISPA-CNR for the simultaneous determination of deoxynivalenol (DON), fumonisin B1 (FB1), fumonisin B2 (FB2), zearalenone (ZEA), T-2 toxin, HT-2 toxin, ochratoxin A (OTA) and aflatoxins (AFB1, AFB2, AFG1 and AFG2) in maize and for the simultaneous determination of DON, ZEA, T-2, HT-2 and OTA in wheat, respectively [15]. All laboratory participants but one used LC-MS methods based on triple quadrupole mass detector. Fifty-seven percent and 71% of participants were able to analyze the eleven targeted mycotoxins in maize and the five targeted mycotoxins in wheat, respectively. Acceptable z-scores ($|z| \leq 2$) accounted for 88% in maize and 91% in wheat. The most preferred procedures were based on acetonitrile-water extraction, direct injection without extract clean-up ("dilute and shoot") and internal standard calibration. Even though to date official methods based on LC-MS are not available for the simultaneous determination of all the EU legislated eleven mycotoxins in maize, a critical evaluation of results from PTs highlighted that LC-MS methods can be successfully used as reliable tools for the simultaneous determination of groups of mycotoxins [De Girolamo, unpublished].

2.2. Rapid methods

Immunoassays, including ELISA, lateral flow devices and dipsticks, have become very popular in mycotoxin screening. Screening methods are intended to be rapid and easy-to-use and do not

require skilled operators and expensive instrumentations. In the last years, several rapid immunoassay-based tests have been developed for the analysis of mycotoxins in food/feed commodities [4-6]. However, before using such methods their fitness for the intended purpose needs to be verified. Recently the European Commission has established criteria with which screening methods have to comply for use for regulatory purposes. Validation procedures by single laboratory and through collaborative trials were defined [16].

Lateral flow devices (LFDs), also called immunochromatographic strip tests, are rapid immunoassays based on the interaction between specific antibodies and antibody-coated dyed receptors, e.g., colloidal gold, that react with the analyte to form an analyte-receptor complex. Generally, LFDs have been developed for the determination of a single mycotoxin. A commercial lateral flow immunoassay for the determination of DON has been recently subjected to single laboratory validation according to the Commission Regulation (EU) No. 519/2014 for evaluating its analytical performances and its reliability to verify wheat compliance with EC maximum permitted level [17]. The obtained results indicated the tested assay as fit for the purpose of assessing DON contamination in wheat at regulatory level. The applicability of the validated immunoassay was demonstrated by analysis of naturally contaminated wheat samples and comparison of results obtained with a LC-MS confirmatory method [17].

A multiplex dipstick immunoassay for the simultaneous determination of ZEA, T-2 and HT-2 toxins, DON and fumonisins (sum of FB1 and FB2) in wheat, oats and maize has been recently developed at ISPA-CNR [18]. Analysis of naturally contaminated samples and the comparison with an LC-MS confirmatory method showed how the developed multiplex immunoassay can provide a reliable tool for rapid and simultaneous assessment of the presence/absence of the six major *Fusarium* toxins at levels close to the EU regulatory levels within 30 min. A collaborative study involving 12 laboratories for evaluating the performances of the multiplex dipstick immunoassay showed the test to be able to differentiate blank samples from samples contaminated at target mycotoxin levels with a false positive rate lower than 10% for ZEA, DON and fumonisins. The assay is rapid, inexpensive, easy-to-use and fit for the purpose of rapid screening of mycotoxins in cereals [19].

Fluorescence polarisation immunoassay (FPIA) is a homogenous assay based on the competition between the antigen and a fluorescently labelled antigen (tracer) for a specific antibody. The binding of the tracer to the antibody affects the rate of rotation of the tracer and increases the fluorescence polarisation value. The amount of bound tracer is inversely proportional to the amount of free analyte in the sample, as a result the polarisation value is inversely related to the analyte concentration. FP immunoassays are getting attention as screening tools in food safety control due to their simplicity, rapidity and cheapness. Recently, reliable FPIAs have been developed at ISPA-CNR for the rapid determination of mycotoxins in cereals, including T-2 and HT-2 toxins in unprocessed cereals (wheat oats, barley and rye), and in cereal-based products for direct human consumption, such as oat flakes, oats crispbread and pasta, OTA in wheat and DON in wheat bran and whole-wheat flour [20-23]. The overall time of analysis ranged from 10 to 15 minutes, depending on the matrix. The immunoassays were validated by using Certified Reference Materials (when available) and by comparison with in-house validated UHPLC-IAC methods (confirmatory methods) by analysing naturally contaminated samples. Validation results showed the FPIA to have accuracy and precision values similar to those obtained with LC methods.

3. CONCLUSIONS

LC-MS/MS is a technique widely used for the simultaneous determination of mycotoxins. However, validation studies indicated that matrix-assisted calibration, isotopically labelled internal standards and improved sample preparation are essential for an accurate determination. At present, no LC-MS methods are recognized as standard or official methods for mycotoxin detection, although LC-MS methods validated by inter-laboratory studies for the simultaneous determination of mycotoxins are highly required. Proficiency Testings for multi-mycotoxin LC-MS methods are a useful tool to provide insights on the used methodologies and related performances and could provide useful information for the optimisation and selection of methods to be used in inter-laboratory validation

studies. The Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC), a Directorate-General of the European Commission, operates as European Union Reference Laboratory (EURL) for mycotoxins. One of its core tasks is to organise PTs among appointed National Reference Laboratories (NRLs). In addition the EURL for mycotoxins aims to facilitate the implementation of European legislation related to monitoring of mycotoxins in food and animal feed. Several analytical methods have been validated by the EURL enabling official control laboratories to make use of methods with known performance. The majority of these methods have already become international standards (CEN, ISO, AOAC) or are in the process of becoming a standard.

Reference materials for quality control of mycotoxin methodologies are commercially available for the major mycotoxins, although they are quite limited. Certified Reference Materials of complex matrices, as well as multi-mycotoxin standards and multi-mycotoxin reference materials are highly required to assess quality of methods, especially when LC-MS methods are used.

The availability of rapid methods for mycotoxin analysis has been increasing in the last years. The advantages of these methods, with respect to conventional ones, are the easiness of operations and the rapidity of analysis together with their low cost. Harmonised validation guidelines for rapid methods are not always available. Recently, the European Commission has established criteria with which screening methods for mycotoxins, for which the result of the measurement is a numerical value, have to comply with when they are used for regulatory purposes (Commission Regulation No. 519/2014). Validation guidelines for binary test methods that do not give numerical values are currently under discussion by various standardization bodies (i.e. AOAC, EC, ISO). Recently the AOAC has drafted a guideline on this matter and methods that give binary results should be validated according to this guideline [24].

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REFERENCES

[1] A.G. Marroquín-Cardona, N.M. Johnson, T.D. Phillips and A.W. Hayes, “Mycotoxins in a changing global environment - A review”, *Food and Chemical Toxicology*, vol. 69, pp. 220–230, 2014.

[2] R. Krska, P. Schubert-Ullrich, A. Molinelli, M. Sulyok, S. MacDonald and C. Crews, “Mycotoxin analysis: an update”, *Food Additives & Contaminants: Part A*, vol. 25, n°. 2, pp. 152-163, 2008.

[3] J. Gilbert and M. Pascale, “Analytical methods for mycotoxins in the wheat chain”, in: Mycotoxin Reduction in Grain Chains, J.F. Leslie, A.F. Logrieco Editors, John Wiley & Sons, Inc., Ames, Iowa, USA, pp. 169-188, 2014.

[4] C.M. Maragos and M. Busman, “Rapid and advanced tools for mycotoxin analysis: a review”, *Food Additives & Contaminants: Part A*, vol. 27, n°. 5, pp. 688-700, 2010.

[5] V. Lippolis and C. Maragos, “Fluorescence polarisation immunoassays for rapid, accurate and sensitive determination of mycotoxins”, *World Mycotoxin Journal*, vol. 7, n°. 4, pp. 479-489, 2014.

[6] I.E. Tothill, “Biosensors and nanomaterials and their application for mycotoxin determination”, *World Mycotoxin Journal*, vol. 4, n°. 4, pp. 361-374, 2011.

[7] V.M.T. Lattanzio, B. Ciasca, S. Powers and A. Visconti, “Improved method for the simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in cereals and derived products by liquid chromatography-tandem mass spectrometry after multi-toxin immunoaffinity clean up”, *Journal of Chromatography A*, vol. 1354, pp. 139-143, 2014.

[8] V.M.T. Lattanzio, S. Della Gatta, M. Suman and A. Visconti, “Development and in-house validation of a robust and sensitive solid-phase extraction liquid chromatography/tandem mass

spectrometry method for the quantitative determination of aflatoxins B1, B2, G1, G2, ochratoxin A, deoxynivalenol, zearalenone, T-2 and HT-2 toxins in cereal-based foods", *Rapid Communications in Mass Spectrometry*, vol. 25, n°. 13, pp. 1869-1880, 2011.

[9] V.M.T. Lattanzio, M. Solfrizzo and A. Visconti, "Determination of trichothecenes in cereals and cereal-based products by liquid chromatography-tandem mass spectrometry", *Food Additives and Contaminants*, vol. 25, n°. 3, pp. 320-330, 2008.

[10] V.M.T. Lattanzio, M. Solfrizzo, S. Powers and A. Visconti, "Simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in maize by liquid chromatography/tandem mass spectrometry after multitoxin immunoaffinity cleanup", *Rapid Communications in Mass Spectrometry*, vol. 21, n°. 20, pp. 3253-3261, 2007.

[11] V.M.T. Lattanzio, A. Visconti, M. Haidukowski and M. Pascale, "Identification and characterization of new *Fusarium* masked mycotoxins, T2 and HT2 glycosyl derivatives, in naturally contaminated wheat and oats by liquid chromatography – high - resolution mass spectrometry", *J. Mass. Spectrom.*, vol. 47, pp. 466-475, 2012.

[12] V. M. T. Lattanzio, B. Ciasca, M. Haidukowski, A. Infantino, A. Visconti and M. Pascale, "Mycotoxin profile of *Fusarium langsethiae* isolated from wheat in Italy: production of type-A trichothecenes and relevant glucosyl derivatives", *Journal of Mass Spectrometry*, vol. 48, pp. 1291-1298, 2013.

[13] V.M.T. Lattanzio, B. Ciasca, V. Terzi, R. Ghizzoni, S. P. McCormick and M. Pascale, "Study of natural occurrence of T-2 and HT-2 toxins and their glucosyl derivatives from field barley to malt by high resolution Orbitrap mass spectrometry", *Food Additives & Contaminants: Part A*, vol. 32, n°. 10, pp. 1647-1655, 2015.

[14] European Commission, Mandate for standardisation addressed to CEN for methods of analysis for mycotoxins in food, M/520 EN, Brussels, 6 March 2013.

[15] A. De Girolamo, B. Ciasca, J. Stroka, S. Bratinova, A. Visconti, and V.M.T. Lattanzio, "Report of the 2014 Proficiency Test for LC-MS(MS) multi-mycotoxin methods", 2014 (*available on line: http://www.ispacnr.it/simisa-report-of-the-2014-proficiency-test-for-lc-msms-multi-mycotoxin-methods/)*

[16] Commission Regulation (EU) No 519/2014 of 16 May 2014 amending Regulation (EC) No 401/2006 as regards methods of sampling of large lots, spices and food supplements, performance criteria for T-2, HT-2 toxin and citrinin and screening methods of analysis, *Official Journal of the European Union*, L 147/29-43, 2014.

[17] V.M.T. Lattanzio, B. Ciasca, S. Powers and C. von Holst, "Validation of screening methods according to Regulation 519/2014/EU. Determination of deoxynivalenol in wheat by lateral flow immunoassay: A case study", *Trends in Analytical Chemistry*, vol. 76, pp. 137–144, 2016.

[18] V.M.T. Lattanzio, N. Nivarlet, V. Lippolis, S. Della Gatta, A.C. Huet, P. Delahaut, B. Granier and A. Visconti, "Multiplex dipstick immunoassay for semi-quantitative determination of Fusarium mycotoxins in cereals", *Analytica Chimica Acta*, vol. 718, pp. 99-108, 2012.

[19] V.M.T. Lattanzio, C. von Holst and A. Visconti, "Collaborative study for evaluating performances of a multiplex dipstick immunoassay for *Fusarium* mycotoxin screening in wheat and maize", *Quality Assurance and Safety of Crops & Foods*, vol. 6, n°. 3, pp. 299-307, 2014.

[20] V. Lippolis, M. Pascale, S. Valenzano, V. Pluchinotta, S. Baumgartner, R. Krska and A. Visconti, "A rapid fluorescence polarization immunoassay for the determination of T-2 and HT-2 toxins in wheat", *Analytical and Bioanalytical Chemistry*, vol. 401, pp. 2561-2571, 2011

[21] V. Lippolis, M. Pascale, S. Valenzano, A.C.R. Porricelli, M. Suman and A. Visconti, "Fluorescence Polarization Immunoassay for Rapid, Accurate and Sensitive Determination of Ochratoxin A in Wheat", *Food Analytical Methods*, vol. 7, n°. 2, pp. 298-307, 2014.

[22] S. Valenzano, V. Lippolis, M. Pascale, A. De Marco, C.M. Maragos, M. Suman and A. Visconti, "Determination of deoxynivalenol in wheat bran and whole-wheat flour by fluorescence polarization immunoassay", *Food Analytical Methods*, vol. 7, n°. 4, pp. 806-813, 2014.

[23] A.C.R. Porricelli, V. Lippolis, S. Valenzano, M. Cortese, M. Suman, S. Zanardi and M. Pascale, "Optimization and Validation of a Fluorescence Polarization Immunoassay for Rapid Detection of T-2 and HT-2 Toxins in Cereals and Cereal-Based Products", *Food Analytical Methods*, 2016 (*available on line: DOI 10.1007/s12161-016-0527-1*).

[24] AOAC International Guidelines for Validation of Qualitative Binary Chemistry Methods, *Journal of AOAC Int.*, vol. 97, n°. 5, pp. 1492-1495, 2014.