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Development and validation of a liquid chromatography/atmospheric pressure photoionization-tandem mass spectrometric method for the analysis of mycotoxins subjected to commission regulation (EC) No. 1881/2006 In cereals

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ABSTRACT

A sensitive and reliable liquid chromatography/photoionization (APPI) tandem mass spectrometry method has been developed for determining nine selected mycotoxins in wheat and maize samples. The analytes were chosen on the basis of the mycotoxins under EU Commission Regulation (EC) No. 1881/2006, i.e., deoxynivalenol (DON), zearalenone (ZON), aflatoxins (AFs), and ochratoxin A (OTA), and considering the possibility of a near future regulation for T-2 and HT-2 toxins. Mycotoxins were extracted from samples by means of an one-step solvent extraction without any cleanup. The developed multi-mycotoxin method permits simultaneous, simple, and rapid determination of several co-existing toxins separated in a single chromatographic run, in which AFs, T-2 and HT-2 toxin are acquired in positive, while OTA, DON and ZON in negative mode. Although a moderate signal suppression was noticeable, matrix effect did not give significant differences at $p = 0.05$. Then, calibration in standard solution were used for quantitation. Based on the EU Commission Decision 2002/657/EC, the method was in-house validated in terms of ruggedness, specificity, linearity, trueness, within-laboratory reproducibility, decision limit (CCα) and detection capability (CCβ). For all the analytes, the regression coefficient r ranged between 0.8752 (DON in wheat) and 0.9465 (ZON in maize), biases related to mean concentrations were from −13% to +12% of the nominal spiking level, and the overall within-laboratory reproducibility ranged 3–16%; finally, CCα values did not differ more than 20% and CCβ not more than 42% from their respective maximum limit. Method quantification limits ranged from 1/20 (AFG1) to 1/4 (AFG2 and OTA) the maximum limit established by European Union in the Commission Regulation (EC) No. 1881/2006 and its subsequent amendments.

1. Introduction

Many cereals and other crops are susceptible to fungal attack either in the field or during drying, and subsequent storage. These fungi may produce as secondary metabolites diverse groups of naturally occurring, toxic chemical substances known as mycotoxins. The natural fungal flora associated with foods is dominated by three genera, i.e., *Aspergillus*, *Fusarium*, and *Penicillium* [1]. Contamination from *Fusarium* genus, e.g., deoxynivalenol (DON), T-2 and HT-2 toxin, can occur mainly pre-harvest, while contamination from *Aspergillus* and *Penicillium* genera, e.g., ochratoxin A (OTA) and aflatoxins (AFs), mainly post-harvest [2].

In terms of structural complexity, mycotoxins vary from simple C4-compounds, to complex substances [3,4]. When present in food in sufficiently high amounts, these fungal metabolites can have toxic effects that range from acute to chronic symptoms. Some mycotoxins were shown to be mutagenic, teratogenic, or/and carcinogenic. Mycotoxins may also cause developmental effects including birth defects, or affect the reproductive system, the immune system and specific target organs, or exhibit hormonal activity [1]. In addition to these diverse organ or site-specific actions, mycotoxins may affect the gastrointestinal system, cause skin irritation, have hematological effects and reduce growth [5]. Due to toxic effects on human and animals, the risk assessment of mycotoxins is of high relevance [5]. Over a hundred mycotoxins have been identified; however, only a few present significant food safety challenges. The International Agency for Research in Cancer (IARC) has classified AFs as carcinogenic to humans, while OTA and fumonisins B (FB) were classified as possibly carcinogenic. Trichothecenes (TRs) and zearalenone (ZON) were classified as non-carcinogenic but cause other adverse effects [6].

The frequent incidence of these toxins in agricultural commodities has a potentially negative impact on the health and economies of the affected regions. Generally, mycotoxins are stable chemical

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compounds and can neither be completely removed from the food supply nor destroyed during processing and heat treatment, thus, monitoring of this contaminants in food and in feed are important issues associated with public health, agricultural production, food processing, and trade. Especially in internationalization of food and feed trades, restrict regulation strategies have to be set to protect consumers from mycotoxin exposure. European Food Safety Authorities (EFSA) and US Food and Drug Administration (FDA) have set maximum levels (MLs) and guidelines for AFs, OTA, ZON, DON, FB1 and FB2 down to the ppb to ppt level in different food and feed products [7–9]. More recently, in Regulation (EC) No. 1881/2006 and Regulation (EC) No. 1126/2007, mycotoxin levels were set restrictively to: 4 $\mu g\,kg^{-1}$ for total AFs, and 2 $\mu g\,kg^{-1}$ for AFB1; 5 $\mu g\,kg^{-1}$ for OTA, 750 $\mu g\,kg^{-1}$ for DON, and 75 $\mu g\,kg^{-1}$ for ZON. Statutory regulations do not exist for T-2 and HT-2, yet. However, a selection of advisory and tolerance limits is available in the literature and from them we considered as possible maximum residue limit for T-2 and HT-2 the value of 50 $\mu g\,kg^{-1}$. Monitoring and control programs for mycotoxins in food and feed have been implemented in many countries, especially in the European Union (EU).

The analysis of mycotoxins is challenging as they are usually present in minute concentrations in complex sample matrices, and they may occur in various combinations produced by a single or by several fungal species. The fact that most mycotoxins are toxic at very low concentrations requires sensitive and reliable methods for their detection. The analytical methods for the identification and determination of mycotoxins in food, feed and biological samples should be accurate and should provide reliable data.

Although Enzyme-linked immunosorbent assays (ELISA) have been used for screening purposes as well as for sensitive quantification of mycotoxins in various samples, modern analysis of mycotoxins relies heavily on high-performance liquid chromatography (HPLC) with UV or fluorescence detection [10–15] and, more recently, mass spectrometry (MS). An important and critical step in the mycotoxin analysis is sample preparation and sample cleanup. Different strategies have been performed, including solid phase extraction (SPE), liquid–liquid extraction, supercritical fluid extraction, and accelerated solvent extraction (ASE) [16]. Multifunctional columns (MycoSep) [17] and selective or specific antibodies (immunoaffinity columns: IAC) for isolation and purification from the matrix compounds have also been extensively used. HPLC–MS and HPLC–MS/MS has, in many cases, revolutionized the analysis of mycotoxins and it has become an important analytical tool for routine analysis in complex matrices because of unambiguous analyte identification, accurate quantification and sensitivity. Since some mycotoxins are inserted in legislation there is the need to determine mycotoxins by routine analysis in different types of matrices in one single extract and, if possible, in a single LC–MS run. Thus, to enable reliable and fast risk estimation of mycotoxin intake and poisoning, the development of multi-mycotoxin methods with a common sample preparation and final analysis procedure is highly desirable. The number of such multitoxin LC–MS methods is still relatively limited due to the complexity of the biological matrices as well as the wide range of physical and chemical properties of mycotoxins, challenging both sample preparation and LC–MS detection. For the most complex food matrices some of the published multitoxin LC–MS methods rely basically on multiple but parallel or sequential sample preparation strategies of one sample followed by separate analysis of each isolated class of mycotoxins in separate LC–MS runs [18–26]. This procedure is required either due to insufficient chromatographic separation of mutually interfering analytes [27,28] or by mycotoxin specific MS sensitivity differences in the positive and negative ion mode [22,23,29].

Positive/negative polarity switching has been shown to be a proper tool to solve this latter problem within one LC–MS run whenever modern MS instrumentation with sufficiently rapid polarity switching capability is available or, alternatively, analytes are sufficiently separated from each other by LC that a limited number of positive and negative mode windows can be set up within one LC–MS run [22,29]. Recent assays are focused on typical *Fusarium* toxins as trichothecenes, ZON and its metabolites [19,22–24,29], sometimes including fumonisins [22,23,30,31], OTA and AFs [22,32].

Aside the problematic sample preparation of complex matrices, LC–MS analyses of heterogeneous mixtures of mycotoxins suffer in principal from dramatic differences of analyte ionization efficiencies [23,29,33]. These are influenced by various parameters, as physical and chemical properties of the analytes, the employed ionization source, the preferred ionization polarity, the LC elution solvent and the presence of disturbing matrix components. For this reason, MS sensitivity can hardly be kept stable over a wide LC elution zone and polarity range.

The most recent trend in sample preparation with LC–MS/MS analysis has been the injection of a diluted crude sample extract with no further cleanup. This approach was first applied in multi-mycotoxin analysis by Sulyok et al. [34,35], who analyzed 39 different mycotoxins in a variety of grains. Later, the authors expanded the method for semi-quantitative analysis of 87 fungal metabolites [36]. Several different types of LC–MS multi-mycotoxin methods for food and feed matrices have been published recently [20,27,29,34–42].

In this respect Nielsen, Smedsgaard and coworkers [43] monitored simultaneously up to 474 mycotoxins in fungal cultures in order to compile a data base for pharmaceutical high throughput screening or to identify individual *Penicillium* species by their mycotoxin patterns [44]. The majority of multitoxin LC–MS methods has been done in fungal cultures and grain and to less extent in cheese, milk and other foodstuff. However, due to matrix effect, appropriate sample preparation and chromatographic separation of analyte from matrix compounds seem to be necessary to insure accurate quantification as well as unambiguous identification.

Modern LC–MS instrumentation is mostly based on atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), which exhibits the common problem of matrix effect. Atmospheric pressure photoionization (APPI) is the latest interface introduced in the field of soft ionization techniques for coupling MS to liquid-phase separation systems [45]. The additional analytical capabilities offered by APPI-MS, with respect to ESI and APCI-MS, have been optimized to improve the detection limits of some classes of compounds. In particular some studies have shown that APPI can provide higher signal-to-noise ratios with respect to APCI [46,47] and that it is less prone to matrix effect than ESI [48]. The use of LC/MS with an APPI source for analyzing AFs in some foodstuffs has been reported in precedent papers [45,49]. Using APPI, detection limits for the investigated compounds were lower than by using ESI, due to a much lower noise and matrix effect.

The aim of this work was to develop and validate a simple method for several mycotoxins based on one-step solvent extraction followed by LC/APPI-MS/MS. The analytes were chosen on the basis of the mycotoxins under EU Commission Regulation (EC) No. 1881/2006 and considering the possibility of a near future regulation for T-2 and HT-2. By taking advantage of the high specificity of LC–MS/MS, a simple sample treatment not involving any cleanup step was developed.

We decided to investigate the behaviour of APPI source considering that: (1) some of these compounds were not included in the cited works; (2) the easy and fast sample preparation may require a kind of source less sensible to matrix effect than ESI one; (3) none of the proposed method employing APPI has been validated following the guidelines given in the Decision 2002/657/EC. Preliminary experiments showed that the APPI source did not give a response