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Optimization and validation of a quantitative liquid chromatography–tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices

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ABSTRACT

An LC–MS/MS "dilute and shoot" method for the determination of 295 fungal and bacterial metabolites was optimized and validated according to the guidelines established in the Directorate General for Health and Consumer Affairs of the European Commission (SANCO) document No. 12495/2011. Four different types of food matrices were chosen for validation: apple puree for infants (high water content), hazelnuts (high fat content), maize (high starch and low fat content) and green pepper (difficult or unique matrix). Method accuracy and precision was evaluated using spiked samples in five replicates at two concentration levels. Method trueness was demonstrated through participation in various proficiency tests. Although the method covers a total number of 331 analytes, validation data were acquired only for 295 analytes, either due to the non-availability of analytical standards or due other reasons described in this paper. Concerning the apparent recovery, the percentage of 295 analytes matching the acceptable recovery range of 70–120% lied down by SANCO varied from 21% in green pepper to 74% in apple puree at the highest spiking level. At the levels close to limit of quantification only 20–58% of the analytes fulfilled this criterion. The extent of matrix effects was strongly dependent on the analyte/matrix combination. In general, the lowest matrix effects were observed in apple puree (59% of analytes were not influenced by enhancement/suppression at all at the highest validation level). The highest matrix effects were observed in green pepper, where only 10% of analytes did not suffer from signal suppression/enhancement. The repeatability of the method was acceptable (RSD $\leq$ 20) for 97% of all analytes in apple puree and hazelnuts, for 95% in maize and for 89% in green pepper. Concerning the trueness of the method, *Z*-scores were generally between −2 and 2, despite a broad variety of different matrices. Based on these results it can be concluded that quantitative determination of mycotoxins by LC–MS/MS based on a "dilute and shoot" approach is also feasible in case of complex matrices.

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1. Introduction

Mycotoxins are defined as low-molecular-weight natural products produced as secondary metabolites by fungi. By definition, they are toxic to vertebrates and other animal groups in low concentrations, causing acute as well as chronic diseases [1]. Mycotoxins exhibit a great diversity in their chemical structure, which explains that their toxicities and target organs also vary [2]. Over the years, health concerns related to mycotoxins have increased [3] and several regulations have been set into force to control the maximum levels of mycotoxins in food and feed in many countries. For instance, the European Union has laid down maximum levels for certain mycotoxin-matrix combinations in Commission Regulation 1881/2006/EC [4]. Regulations are based on the evaluation of risk assessment (hazard and exposure) but also reflect agriculturally achievable levels in different foodstuffs. As exposure assessment is an important aspect of risk assessment, validated analytical methods and the implementation of analytical quality assurance are necessary to provide a reliable assessment on the toxin intake [5].

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The complexity of food samples together with the low concentrations at which contaminants usually occur require highly sensitive, selective and reliable analytical techniques [6].

During the last decade the coupling of liquid chromatography (HPLC or UHPLC) to tandem mass spectrometry (MS/MS) has enabled the development of highly selective, sensitive and accurate methods for mycotoxin determination. Several methods have been published for the identification and accurate quantification of single or chemically related mycotoxins in several matrices [7]. However, different classes of mycotoxins are often found to co-occur as (i) some fungal species are capable to produce different classes of mycotoxins and (ii) susceptible commodities can be affected by several fungi if the environmental conditions (temperature, water activity) favor their growth. Therefore, different analytical methods are often employed to cover all mycotoxins addressed by the regulations. The techniques used are based on TLC, HPLC-UV, HPLC-fluorescence frequently in combination with time consuming purification step or immunochemical methods such as ELISA [8].

The development of LC–MS/MS based multi-mycotoxin methods tries to overcome the need for sophisticated clean-ups and/or multiple analytical techniques, although the chemical diversity of mycotoxins is a big obstacle to be overcome [2]. For instance, extraction of a wide range of target compounds from a variety of matrices has to be realized. Studies on generic extraction methods for multiple contaminants in different food and feed matrices have demonstrated that mixtures of acidified water with organic solvents (methanol, acetonitrile or acetone) are the most suitable extraction solvents [9,10].

Every clean-up step and even a rather unspecific QuEChERS-like approach [11] limits the number of analytes as some of the target substances might not be amenable to the chosen procedure [12]. On the other hand, reducing the sample clean-up to a minimum (i.e. injection of raw extracts) will result in suppression or enhancement of the analyte response during the ionization process. The influence of these matrix effects is the major challenge in developing reliable quantitative multi-analyte methods [13]. Therefore, considerable efforts to control matrix effects should be carried out to obtain accurate results. The use of stable isotope dilution assays (SIDA) seems to be the best alternative to correct matrix effects. Several methods have been validated using isotopically labeled internal standards [14–16]. However, the limited availability of labeled internal standards for non-regulated toxins and the comparably high costs of isotopically labeled standards are the main drawbacks. Another common approach to deal with matrix effects is the compensation of the signal suppression/enhancement through the usage of matrix matched standards (i.e. blank sample extracts fortified with an appropriate amount of a multi-analyte standard). Here the availability of analyte-free samples (which is especially difficult for certain analyte/matrix combinations such as deoxynivalenol in maize) and repeatable extraction efficiencies as well as matrix effects for all individual samples of a given commodity are the major challenges [17–22].

To assure reliable quantification at a high level of trueness, in-house validation has to be performed, preferably according to international guidelines. The SANCO document for the development of multi-analyte methods in pesticides residue analysis recommends that at least one representative commodity from each commodity group shall be validated and evidence for fitness of purpose shall be provided [22]. This approach has been successfully applied in the field of pesticide analysis [23–25] but has hardly been employed for methods devoted to mycotoxins, for which most methods focus on single commodities (mainly grain-based matrices). However, a few examples can be found for multi-mycotoxin methods which have been validated for a wider range of matrices [9,10,18,26–28].

The aim of this work was to evaluate the performance of a multi-analyte method for mycotoxins and other fungal as well as bacterial metabolites. Furthermore, a validation procedure in accordance to SANCO No. 12495/2011 was developed and applied to four model matrices. The range of analytes finally covered a total of 295 secondary metabolites for which validation data are presented in four different matrices. The model matrices were chosen as representative commodities belonging to the respective commodity groups according to SANCO (each commodity group includes matrices of similar properties). Another aspect of selection was the relevance of the matrix with respect of mycotoxin contamination, i.e. the commodities which are commonly contaminated with mycotoxins. Therefore, apple puree for infants (high water content), hazelnuts (high fat content), maize (high starch or protein content, low fat content) and green pepper (complex matrix) were chosen. In case of the mycotoxins addressed by regulations, the comparability of the method was verified through the participation in proficiency tests.

2. Material and methods

2.1. Chemicals and reagents

LC gradient grade methanol and acetonitrile as well as MS grade ammonium acetate and glacial acetic acid (p.a.) were purchased from Sigma–Aldrich (Vienna, Austria). A Purelab Ultra system (ELGA LabWater, Celle, Germany) was used for further purification of reverse osmosis water.

Standards of fungal and bacterial metabolites were obtained either as gifts from various research groups or from the following commercial sources: Romer Labs®Inc. (Tulln, Austria), Sigma–Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausanne, Switzerland) and LGC Promochem GmbH (Wesel, Germany). Stock solutions of each analyte were prepared by dissolving the solid substance in acetonitrile (preferably), acetonitrile/water 1:1 (v/v), methanol, methanol/water 1:1 (v/v) or water. Thirty-four combined working solutions were prepared by mixing the stock solutions of the corresponding analytes for easier handling and were stored at −20 °C. The final working solution was freshly prepared prior to spiking experiments by mixing of the combined working solutions.

2.2. Samples

Four samples of different matrix complexity were chosen for the method validation. Apple puree was taken as a high water containing matrix. Matrices with high fat content were represented by hazelnuts, and cereals and high starch matrices by maize. Green pepper was used as a model matrix for the validation of “difficult and unique commodities” [22].

The following proficiency testing samples were used for the verification of the method trueness: (i) FAPAS® testing materials – peanuts (T01044), maize (T2246, T2262), cereals (T1786) and cereal breakfast (T2257) provided by The Food and Environment Research Agency (York, United Kingdom); (ii) Proficiency Testing Scheme samples – peanut cake (04-0231), peanut paste (02-1331, 04-1331), animal feed (02-3031, 03-3031, 04-3031), wheat (05-0631, 03-2331), wheat draff (02-2831), pepper (01-1031, 01-3231), raisins (02-3131), maize (04-0731, 05-0731, 03-0731) milk powder (04-0331), coffee (02-1731), baby food (01-3331, 01-3431), pistachio paste (03-1431), liquorice (01-3531), oat (02-2931) were obtained from Bipea (Gennevilliers, France); (iii) CODA-CERVA proficiency test (oat flour) from 2013 organized by the Belgian National Reference Laboratory for Mycotoxins in Food and Feed.

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