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Development and validation of a QuEChERS based liquid chromatography tandem mass spectrometry method for the determination of multiple mycotoxins in spices

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ABSTRACT

A reliable and rapid method for the determination of multiple mycotoxins was developed using a QuEChERS (quick, easy, cheap, effective, rugged and safe) based extraction procedure in highly pigmented and complex spice matrices, namely red chilli (*Capsicum annum* ssp.), black and white pepper (*Piper nigrum* ssp.). High-performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) was used for the quantification and confirmation of 17 chemically diversified mycotoxins. Different extraction procedures were studied and optimized in order to obtain better recoveries. Mycotoxins were extracted from the hydrated spices using acidified acetonitrile (1% formic acid), followed by partitioning with NaCl and anhydrous $MgSO_4$; excluding the use of dispersive-solid phase extraction. Significant matrix effect was compensated using the matrix matched calibration curves. Electrospray ionization at positive mode was applied to simultaneously detect all the mycotoxins in a single run time of 20 min. Multiple reaction monitoring mode, choosing at least two abundant fragment ions per analyte was applied. Coefficients of determination obtained were in the range of 0.9844–0.9997. Recoveries (ranging from 75% to 117%) were in accordance with the performance criteria required by the European Commission. Intra-day reproducibility ranged from 4% to 22% for most of the mycotoxins. The limit of quantification ranged from 2.3 to 146 $\mu g\,kg^{-1}$. The validated method was finally applied to screen mycotoxins in ten of each spice matrix. Aflatoxins, ochratoxin, fumonisins, sterigmatocystin and citrinin were among the detected analytes. Positive findings were further confirmed using relative ion intensities. The potentiality of the method to be used for confirmatory purposes according to Commission Decision 2002/657/EC was assessed.

1. Introduction

Mycotoxins are a group of naturally occurring toxic chemical substances, produced by different fungal species, which can cause illness or even death due to their toxigenic, carcinogenic, mutagenic and teratogenic effects [1]. Though more than 400 mycotoxins are known till date [2], only few of them are of major concern because of their potent toxicity. They are aflatoxins (AFB1, AFB2, AFG1 and AFG2), ochratoxin A (OTA), fumonisins (FB1, FB2), deoxynivalenol (DON), zearalenone (ZEN), T-2 and HT-2 toxins [3]. Because of their great structural diversity, they can cause a variety of toxic effects in humans as well as in animals, a syndrome generally referred to as mycotoxicosis. AFB1 and other naturally occurring aflatoxins (AFs) have been classified as group 1 human carcinogen because of their role in aetiology of liver cancer whereas OTA and fumonisins are classified as probable human carcinogens in group 2B [4,5]. Meanwhile, trichothecenes and zearalenone were classified to be non-carcinogenic [6]. Mycotoxins are generally produced from the fungal genera of *Aspergillus, Fusarium, Penicillium* [2] and *Alternaria*, either in field or during storage [7]. Approximately, 5–10% of agricultural products worldwide are spoiled by fungi, to the extent that crops cannot be consumed by human or animals. Furthermore, FAO estimates that more than 25% of the agricultural produce is contaminated by mycotoxins [8].

Spices are valued for their distinctive flavours, colours, aromas and are among the most versatile and widely used ingredient in

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food preparations and processing. They are also well known for their medicinal and preservative purposes [9]. Spices are mainly cultivated in developing countries with tropical and/or semi tropical climates and exported worldwide. High temperature, high rainfall and relative humidity in these growing areas are highly conducive for fungal proliferation and mycotoxin production. Apart from the climatic conditions, lack of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) are of great concern in developing countries.

In terms of world trade value, the leading spices are black pepper (*Piper nigrum L.*) and chilli (*Capsicum annum L.*) [10]. They are also the most common spices used in culinary worldwide hence, they were chosen for this study. Paprika (non-pungent) and chilli (pungent) are produce of *Capsicum* spp. fruits from the night shade family of *Solanaceae* [11]. Black Pepper, known as "king of spices", is the dried mature peppercorns. White pepper is produced by removing the outer pericarp of the ripened red pepper berries through a process known as "retting". Among various spices, chilli and pepper have been reported as the spices most frequently contaminated with AFs and OTA [11–14].

Most countries have set stringent regulatory requirements on the level of mycotoxins permitted in traded commodities [15]. According to the latest Commission Regulation No 165/2010 [16] the stipulated EU maximum level (ML) in spices for AFB1 is 5 $\mu g\,kg^{-1}$ and 10 $\mu g\,kg^{-1}$ for total AFs (sum of AFB1, B2, G1 and G2). In addition to AFs, only OTA is currently regulated by EU for spices. The ML for OTA is 30 $\mu g\,kg^{-1}$ in *Capsicum* spp. and 15 $\mu g\,kg^{-1}$ for all other spices [17]. As from 2015, a lower ML also for *Capsicum* spp. is foreseen [18]. Meanwhile, maximum AFs levels of 10–20 $\mu g\,kg^{-1}$ are agreed for the commercial transactions within the international spice trade [19]. In 2007, the Scientific Committee of the Federal Agency for the Safety of the Food Chain (FASFC) in Belgium decided the necessity for further research into "silent carriers" of mycotoxins like spices, spice extracts and food supplements [20].

Analysis of mycotoxins is challenging as they are often present at low concentrations in complex matrices. Current analytical methods for the determination of AFs and/or OTA in spices include the use of thin layer chromatography, immuno affinity chromatography, enzyme linked immuno sorbent assay and high performance liquid chromatography (HPLC) [11,24]. To date, several liquid chromatography tandem mass spectrometry (LC–MS/MS) based methods using solid phase extraction (SPE) cleanup are available for multiple mycotoxin analysis for various food and feed commodities [22–27]. However, multi-mycotoxin methods for spices are lacking. Amate et al. [21] introduced a multi-analyte method for spices, which included pesticide residues, aflatoxins and dyes. Very recently, some existing extraction methods were assessed for multi-residue analysis in paprika and black pepper [28]. The aim of the present study was to develop a simple, selective and reliable method based on the QuEChERS extraction approach for the determination of multiple mycotoxins in spices using LC–MS/MS. Although the QuEChERS method introduced by the USDA scientists in early 2003 [29] has been extended in the analysis of veterinary drug residues [30], antibiotics [31], acrylamide [32] and mycotoxins [33–36] in different matrices, to our knowledge this is the first publication describing a QuEChERS method for the quantitative determination of multiple mycotoxins in spices using LC–MS/MS.

2. Experimental

2.1. Chemicals and reagents

LC–MS grade absolute methanol (MeOH) and analytical grade acetonitrile (MeCN) were purchased from VWR International (Zaventem, Belgium). Formic acid ULC–MS grade (99%) was supplied by Bio Solve B.V. Ammonium formate (±99%) was obtained from Sigma–Aldrich, Steinheim. Formic acid analytical grade (98–100%) and sodium chloride (±99.5%) were from Merck (Darmstadt, Germany). Magnesium sulphate anhydrous (±99%) was purchased from nacalai tesque Inc. (Gentaur; Kyoto, Japan). Ultrafree®-MC centrifugal filter devices (0.22 μm) were obtained from Millipore (Bredford, MA, USA). Water was purified (18 MΩ) on a Milli-Q Plus apparatus (Millipore; Brussels, Belgium). All other chemicals and reagents used were of analytical grade.

2.2. Mycotoxins standards

Mycotoxins reference standards namely, deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), neosolaniol (NEO), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), ochratoxin A (OTA), fumonisin B1 (FB1), fumonisin B2 (FB2), HT-2 toxin (HT-2), alternariol methyl ether (AME), zearalenone (ZEN), sterigmatocystin (STERIG) and zearalanone (ZAN) were purchased from Sigma–Aldrich (Bornem, Belgium). NEO was obtained as solution (100 $\mu g\,mL^{-1}$) in MeCN. T-2 toxin (T-2) was purchased from Biopure (Tulln, Austria). Fumonisin B3 (FB3) was supplied by Promec Unit (Tygerberg, South Africa). Roquefortine C (ROQ C) was purchased from Enzo Life Science (Lorrach, Germany). FB2 and FB3 standards at a concentration of 1 $mg\,mL^{-1}$ were prepared in MeCN/water (50/50, v/v). Stock solutions of DON, 3-ADON, 15-ADON, AFB1, AFB2, AFG1, AFG2, OTA, FB1, HT-2, T-2, ZEN, STERIG, ZAN and ROQ C were prepared in MeOH at a concentration of 1 $mg\,mL^{-1}$. Stock solution of AME (1 $mg\,mL^{-1}$) was prepared in MeOH/dimethylformamide (60/40, v/v). All the stock solutions were stored for maximum one year at (−20)°C except FB2 and FB3 which were stored at 4 °C.

From the individual stock standard solutions, working solutions were prepared by diluting them in MeOH. A standard mixture of mycotoxins was prepared using the individual stock and working standard solutions at the following concentrations: AFB1, AFB2, AFG1 and AFG2 (0.5 $\mu g\,mL^{-1}$), OTA and ROQ C (1.0 $\mu g\,mL^{-1}$), STERIG (0.625 $\mu g\,mL^{-1}$), T-2, HT-2, NEO, 3-ADON and 15-ADON (2.5 $\mu g\,mL^{-1}$), DON, FB1, FB2, FB3, AME and CIT (5 $\mu g\,mL^{-1}$). The standard mixtures were prepared in MeOH, stored at (−20)°C and renewed every 2 months. Necessary precautions were taken to avoid photo-degradation of the light sensitive mycotoxins, such as wrapping the standard solutions and the extracts with aluminium foil and by storing them in dark.

2.3. Samples

The spice samples of black pepper, white pepper and red chilli were collected from Sri Lankan markets. Different forms of spices include whole pepper, crushed pepper, pepper powder, whole chilli, chilli flakes and chilli powder. The samples were packed air-tight in low density poly ethylene (LDPE) and transferred to Belgium. Samples were stored at room temperature until analysis.

2.4. Sample preparation

Samples were extracted using a modified QuEChERS based approach. A very simple and straightforward extraction procedure was applied. All the different forms of spices were finely ground using a universal mill (grinder) (M20 IKA®-WERKE; Staufen, Germany). Finely ground and homogenized spice sample of 1.0 ± 0.05 g was weighed in a 50 mL extraction tube. The sample was spiked with a mycotoxins standard mixture containing standard mycotoxins at different concentrations. A fixed concentration (500 $\mu g\,kg^{-1}$) of ZAN internal standard (IS) was added. After leaving the samples for an hour for equilibration, 5 mL water