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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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40 **Abstract**

41 A reliable and rapid method for the determination of multiple mycotoxins was developed using a
42 QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) based extraction procedure in highly
43 pigmented and complex spice matrices, namely red chilli (*Capsicum annum* ssp.), black and white
44 pepper (*Piper nigrum* ssp.). High-performance liquid chromatography tandem mass spectrometry
45 (HPLC-MS/MS) was used for the quantification and confirmation of 17 chemically diversified
46 mycotoxins. Different extraction procedures were studied and optimized in order to obtain better
47 recoveries. Mycotoxins were extracted from the hydrated spices using acidified acetonitrile (1%
48 formic acid), followed by partitioning with NaCl and anhydrous $MgSO_4$; excluding the use of
49 dispersive-solid phase extraction. Significant matrix effect was compensated using the matrix
50 matched calibration curves. Electrospray ionization at positive mode was applied to simultaneously
51 detect all the mycotoxins in a single run time of 20 min. Multiple reaction monitoring mode,
52 choosing at least two abundant fragment ions per analyte was applied. Coefficients of determination
53 obtained were in the range of 0.9844 to 0.9997. Recoveries (ranging from 75 to 117%) were in
54 accordance with the performance criteria required by the European Commission. Intra-day
55 reproducibility ranged from 4 to 22% for most of the mycotoxins. The limit of quantification ranged
56 from 2.3 to 146 µg kg^{-1}. The validated method was finally applied to screen mycotoxins in ten of
57 each spice matrix. Aflatoxins, ochratoxin, fumonisins, sterigmatocystin and citrinin were among the
58 detected analytes. Positive findings were further confirmed using relative ion intensities. The
59 potentiality of the method to be used for confirmatory purposes according to Commission Decision
60 2002/657/EC was assessed.

61 Keywords: Mycotoxins, spices, QuEChERS, LC-MS/MS, red chilli, pepper

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 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 µg kg^{-1} and 10 µg 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 µg kg^{-1} in *Capsicum* spp. and 15 µg 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 µg 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 µg 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)^{o}C except FB2 and FB3 which were stored at 4^{o}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 μg mL^{-1}), OTA and ROQ C (1.0 μg mL^{-1}), STERIG (0.625 μg mL^{-1}), T-2, HT-2, NEO, 3-ADON and 15-ADON (2.5 μg mL^{-1}), DON, FB1, FB2, FB3, AME and CIT (5 μg mL^{-1}). The standard mixtures were prepared in MeOH, stored at (-20)^{o}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 aluminum 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 μg/kg) of ZAN internal standard (IS) was added. After

leaving the samples for an hour for equilibration, 5 mL water was added and mixed with a vortex for 1 min. Samples were left for soaking for further 30 min. Thereafter, 5 mL of the extraction solvent (MeCN/1% formic acid v/v) was added and after a brief shaking, samples were extracted using an end-over-end shaker (Agitelec, J. Toulemonde and Cie, Paris, France) for 20 min. Subsequently, 2.0 ± 0.05 g of the pre-weighed $MgSO_4$ anhydrous salt and 0.5 ± 0.01 g of NaCl were added and the tube was capped immediately (a brief hand shaking immediately after the addition of salts was performed to prevent agglomeration of the salts). The tubes were then vortexed for 2 min and centrifuged at 4000x-g for 7 min. Shaking and centrifugation was carried out in order to induce phase separation and mycotoxins partitioning. Finally, an aliquot of the supernatant MeCN layer was subjected for centrifuge filtration at 10000x-g for 3 min. After filtration an aliquot was transferred to the vials for LC-MS/MS analysis.

2.5 Instrumental conditions

2.5.1 HPLC apparatus and conditions

Liquid chromatography was performed using a waters ACQUITY ultra-performance liquid chromatography (UPLC ^{TM}) system. The analytical column used was a Symmetry C18, 5 µm, 150 x 2.1 mm (Waters; Zellik, Belgium) and the guard column was a Sentry, 3.5 µm, 10 x 2.1 mm (Waters; Zellik, Belgium), with a flow rate of 0.3 mL min^{-1}. An aliquot of 10 µL sample extract was injected into the chromatographic system. The partial loop mode was used as an injection technique. Volumes of weak wash (10% MeOH) and strong wash (100% MeOH) solvents were 500 µl each. The column and sample temperature were maintained at room temperature and 10°C, respectively. Mobile phase A was MeOH/water (20/80 v/v) and mobile phase B was MeOH/water (90/10 v/v), both contained 5mM ammonium formate and 0.1% formic acid. A gradient elution programme starting with 50% B was maintained for 2 min. From 2 to 10 min it linearly increased to 100% B. Over further 5 min, the gradient was kept unchanged at 100% B. In 1 min the gradient switched to

50% B and was equilibrated at the initial mobile phase conditions for further 4 min before the start of next injection. Total run time was 20 min.

2.5.2 MS/MS apparatus and conditions

Mass spectrometry (MS/MS) was performed with a Quattro Premier^{TM} XE tandem quadrupole mass spectrometer (Waters; Milford, MA, USA). The MS was operated at electrospray ionization in positive mode (ESI+). For infusion experiments, 10 ng μL^{-1} of the mycotoxin standards dissolved in mobile phase B were used at a flow rate of 10 $\mu L\ min^{-1}$. The capillary voltage was set at 3.5 kV. Nitrogen was used as cone, nebulizing and desolvation gas. Cone voltage was defined for each analyte separately (Table 1). Extractor cone voltage (V) was 3. Source temperature was 350°C. The desolvation temperature was set at 130°C. The cone and desolvation gas flow were maintained at 50 L hr^{-1} and 800 L hr^{-1}, respectively. First quadrupole settings (Q1): low mass resolution (LM1) was 14, high mass resolution (HM1) was 14 and ion energy 1 was 0.1. Collision cell settings (Q2): entrance was -1 and exit 0.0. Third quadrupole settings (Q3): low mass resolution (LM2) was 13.5, high mass resolution (HM2) was 13.5 and ion energy 2 was 1.5. Multiplier voltage was 650 V. Collision gas flow was set at 0.2 mL min^{-1}. Analysis of the mycotoxins was performed in multiple reaction monitoring (MRM) mode. For each mycotoxin, at least one precursor ion and two fragment/product ions were monitored. The most abundant product ion was selected for quantification and the second intense one for qualification. The quantification and qualification ion transitions of the respective mycotoxins and the optimum collision energies (collision energy 1 and collision energy 2) and cone voltages were programmed (Table 1). For data acquisition and processing, Masslynx and Quanlynx software 4.0 (Waters) were used.

2.6. Matrix effect evaluation

The matrix effect (ME) was evaluated by comparing the peak responses of the standard mycotoxins (n=3) spiked in the extraction solvent with the spiked spice extracts at six concentration levels for

each analyte. A standard mixture of mycotoxins was prepared using the individual stock and working standard solutions at the following concentrations for determining the ME: AFB1, AFB2, AFG1 and AFG2 (0.5 µg mL^{-1}), OTA and ROQ C (1.0 µg mL^{-1}), STERIG (0.625 µg mL^{-1}), T-2, HT-2, NEO, 3-ADON, 15-ADON, DON, FB1, FB2, FB3, AME and CIT (2.5 µg mL^{-1}). To prepare the spice extracts with AFs concentrations 5, 10, 20, 40, 100 and 150 µg L^{-1}, following volumes of the spice extracts 495, 490, 240, 230, 200 and 175 µL were spiked with 5, 10, 10, 20, 50, 75 µL of the standard mixture, respectively. The ME was calculated via the formula: ME (%) = (A2-A1/A1)*100, where A1 is the average area of the mycotoxin standard in solvent (MeCN/formic acid (99/1 v/v)) at a specific concentration and A2 is the average area of the mycotoxin standard in blank spice extract at the same concentration. In this way it was possible to compare the positive or negative ME, that is an increase or decrease of the detector response, respectively.

2.7 Method validation study

The multi-mycotoxin analytical method optimized for the three different spices was validated using spiked blank spice samples. Several samples were analysed in advance to obtain a sample that is free of analyte at the particular retention time (tR) of the analyte. We were able to get the blank samples for both peppers. However, for red chilli it was rather difficult to get a Sri Lankan sample free from AFB1. Matrix components of the spices may differ between regions hence, to resemble the similar matrix complexity the selected Lankan chilli sample was preferred to use throughout the validation study, with the blank subtraction for AFB1. A set of performance characteristics that were in compliance with the recommendations and guide lines defined by the Commission Decision 2002/657/EC [37] and Regulation EC/401/2006 [38] were evaluated. Validation parameters assessed were, linearity, recovery, limit of detection (LOD), limit of quantification (LOQ), repeatability (intra-day precision; RSDr), reproducibility (inter-day precision; RSDR) and specificity.

2.7.1. Calibration curves, linearity, LOD, LOQ and recovery

Linearity was evaluated using matrix matched calibration (MMC) curves, by spiking blank samples at six concentration levels for the three different spice matrices. Peak area was used as analyte response. Calibration curves were constructed by plotting the peak areas (y) versus the concentration of analytes (x). The concentration ranges used for this study were*:* AFs (5-40 μg kg^{-1}); OTA and ROQ C (10-80 μg kg^{-1}); T-2, HT-2, NEO, 3-ADON and 15-ADON (50-250 μg kg^{-1}), STERIG (6.25-75 μg kg^{-1}), FB1, FB2, FB3, AME and CIT (100-600 μg kg^{-1}). Calculations were performed on the average peak areas (n=6); relative standard deviations (RSDs), calibration curve equations and the determination coefficients (R^2) for each mycotoxin were determined.

LOD and LOQ were determined using the MMC curves. LODs were determined as the concentration corresponding to the blank response plus three times the standard error of the y-intercept [25]. The linest function of the Microsoft excel 2010 program was used. LOQ equaled the concentration corresponding to the blank response plus six times the standard error of the y-intercept; which is two times the LOD. For each of the analyte, the calculated LODs and LOQs were also verified by the S/N ratio which should be more than 3 and 10 according to the IUPAC settings [25]. The validation experiments that were used to calculate the LODs and LOQs were utilized also to calculate the recovery of the method. IUPAC defines the apparent recovery as the ratio of the predicted value obtained from the MMC curves divided by the actual/theoretical value [24].

2.7.2. Intra-day repeatability, inter-day reproducibility and specificity

Precision of the method was assessed by repeatability and intra-laboratory reproducibility experiments. Intra-day repeatability of the method was evaluated by spiking the mycotoxins standard solutions to the blank spice matrices at four different concentration levels (n=6) and analyzing in the same run of the day on the LC-MS/MS. Inter-day reproducibility of the method was determined by repeating this experiment consecutively for three different days for all the spice

matrices. Specificity of the method was performed by analyzing the blank samples and matrix interferences were checked close to the elution zone of each analyte.

3. Results and Discussion

3.1 Method development

3.1.1 Extraction solvent selection and evaluation of cleanup

In multiclass mycotoxin methods, the most critical step is the optimization of the extraction and clean up procedure, especially for complex matrices such as spices, which contain flavonoids, terpenes and alkaloids [21]. Following extraction solvent combinations (v/v) were initially investigated for achieving acceptable recoveries for each analyte from different matrices: MeCN (100%), MeCN/acetic acid (AA) (99/1), MeCN/water/AA (79/20/1) [23], MeOH/water/AA (79/20/1), MeOH/MeCN/AA (79/20/1), MeOH/ethyl acetate/water (70/25/5). Prior to any solvent extraction, the matrices were soaked in 5 mL water. Better liquid-liquid partitioning with salts was only obtained with the solvent combinations containing MeCN. Due to the absence of salt-induced partitioning with MeOH containing solvents, the extracts obtained were too dark in colour compared to the MeCN extract. When using pure MeCN as the extraction solvent, only AFB1, AFB2, AFG1, AFG2, OTA, T-2, HT-2, STERIG and ROQ C could be detected in the red chilli matrices and only AFs, T-2 and HT-2 could be recovered in both pepper matrices. In addition to the poor extraction of many other toxins in peppers, fumonisins recovery was very poor in both matrices. Addition of 1% AA (MeCN 99/1 v/v) to the extraction solvent increased the recovery of all the above said mycotoxins, but the sensitivity was low. Further, the apparent recovery for OTA (40 μg kg^{-1}) in both pepper matrices exceeded the acceptable range (142%) specified under EC performance criteria [38]. Instead of AA, addition of 1% formic acid to MeCN (99/1 v/v) therefore was investigated on toxins extraction and chromatographic performances. Inclusion of formic acid helped in better recovery of fumonisins (FB1, FB2 and FB3) with improved peak responses compared to AA in the solvent. Significant increase in fumonisins response was obtained for red

chilli compared to both peppers. Moreover, peak responses for all the AFs and STERIG were significantly higher in all the three spices with the addition of FA. However, the responses were lower in case of HT-2 in all the spices, OTA in both peppers and T-2 in black pepper with this solvent combination. Since spices were soaked in water in the beginning, addition of water to the solvent mixture was eluded.

Additionally, the extraction efficiency of DON, 3-ADON, 15-ADON, AME and CIT were assessed using this solvent. Extraction of DON was found not reproducible in all the matrices, so only qualitative identification was possible. DON is highly polar so separation of the aqueous phase probably had a negative effect on its extraction. However, comparatively better results were obtained with the DON derivatives in all the spices studied, possibly due to their slightly lower polarity. AME was only extractable from red chilli. Strong ion suppressions for AME were observed in pepper matrices even at very high spiking concentration. Peaks for CIT were more uniform in chilli than in peppers. Since addition of formic acid helped to extract most toxins the solvent combination MeCN/formic acid (99/1 v/v) was selected as the best solvent of compromise for the extraction of mycotoxins in all the three spices.

Since the QuEChERS method was initially developed for fruits and vegetables which contain plenty of water, it is generally recommended to add water in the beginning to dry food products. Therefore, water was added to dry spices in order to hydrate them prior to extraction. Soaking the spice in water could help to swell the matrix and weaken the interactions of the analyte with the matrix components and assist in efficient extraction. Different ratios of water to extraction solvent combinations were investigated (10/15, 10/10, 10/5, 5/15, 5/10, 5/5 v/v mL) per 1g of the spice matrix. Salt partitioning was observed in all the cases. A comparison of peak responses between the 10/15 and 5/5 ratio is shown in Fig. 1. Highest responses were observed with 5/5 (v/v mL) water to solvent ratio for most of the analytes although these were comparable to the response obtained in 10/5 (v/v mL) for AFG2, AFG1, AFB2, T-2 and ROQ C. Reduced water to solvent ratio helped to

detect mycotoxins at lower concentrations (5-10 μg kg^{-1} of aflatoxins and OTA) with acceptable recoveries (72-121%) in all the spice matrices.

3.1.2 Effect of freezing out and decolourization

Concentrating the analyte in order to increase the sensitivity was evaluated. However, this resulted in dark and turbid residues probably because of the co-extracted etheric oils. Application of a freezing out step resulted in increased responses but only for some toxins, T-2 (36%) in chilli, AFG2 (93%), T-2 (37%) and STERIG (44%) in black pepper and AFG1 (67%) and STERIG (46%) in white pepper hence, the extra analytical time was found not worth. The conventional QuEChERS implying a dispersive-SPE cleanup step, using adsorbents like graphitized carbon black (GCB) or primary secondary amine (PSA) as sorbent materials was also investigated. GCB (5 mg mL^{-1} extract) removed almost all of the pigments and produced very clear extract, but very poor signals were obtained with most of the analytes. PSA (50 mg mL^{-1}) produced a clear extract with black pepper and red chilli, but further evaluation was ignored since its nature (amino group) to bind the fumonisins (carboxylic acid) and influence on fumonisins recovery [39]. Zinc acetate (125 mg mL^{-1}) removed pigments, but the recovery of aflatoxins were affected. Although the pigments could not be completely removed with salt partitioning only, it resulted in a clear and transparent extract. Chromatograms of four different mycotoxins (OTA, T-2, ROQ C and STERIG) obtained in white pepper with and without salt addition is shown in Fig. S-1. Finally, it was decided to continue the extraction as described in section 2.4. The life time of the column was not affected by this extract.

3.1.3 Optimization of the chromatographic conditions

In most of the QuEChERS based mycotoxin analytical methods ammonium formate was used [2,36,39] as mobile phase additive. In the initial stages of method development the mobile phases A: 80/20 water/MeOH (v/v) and B: 90/10 MeOH/water (v/v) both containing 5mM ammonium formate were assessed. Compared to other mycotoxins in this study, ionization of the fumonisins

was lower in all the matrices. Since fumonisins are highly ionic, having four tri-carboxylic groups in their molecular structure it is reported that acidic chromatographic conditions could improve their ionization [23]. Hence, addition of formic acid to the mobile phases was evaluated on the chromatographic performances of each analyte. With the slight acidification of mobile phase, significant increase in ionization was obtained for most of the mycotoxins, except OTA (Fig. 2). In addition to the ionization intensity, peak uniformity was also improved with acidified mobile phase. Hence, mobile phases with 0.1% formic acid were selected.

All the mycotoxins were eluted with good selectivity and MS sensitivity in a gradient run of 10 min. Cleaning and re-equilibration steps included further 10 min. The order of peak elution of all the examined mycotoxins is shown in a chromatogram obtained with red chilli [Fig. S-2]. Except 3-ADON and 15-ADON, all other mycotoxins have shown good peak resolution. The ADONs were found to co-elute however, by the application of MS/MS, chromatographic separation of the two ADONs was not essential, as it will express two different fragment patterns in the collision cell [6].

Selection of the MS conditions was initially based on the in-house developed multi-mycotoxin method [25], but other multi-mycotoxin methods developed on different matrices [2,22-27,33-36] were referred to adjust the MS conditions and select different MRM transitions during the course of the study. Optimized MS conditions are shown in Table 1. The fragment ions reported in other multi-mycotoxin analytical methods were investigated in our spice matrices. In some occasions more than two fragment ion transitions were programmed in the MRM for additional confirmation, as strong ME was encountered in spices. Apart from the selection of two fragment ions, the relative ion intensity (peak area secondary ion/peak area primary ion*100) of the two transitions was additionally assessed to meet the identification criteria [37]. The relative ion intensities of the standards were compared with that of matrix samples. MRM chromatograms obtained with a standard mycotoxin mixture are shown in Fig. S-3. At positive ESI, protonated molecular ions $[M+H]^+$ were formed as precursor ions for most of the analytes. In the case of NEO, T-2 and HT-2

ammonium adducts $[M+NH_4]^+$ were formed as precursor ions. Ammonium adducts formation of these type A-trichothecenes was due to the ester groups in their structure [40].

The formation of AFB2 fragment ions m/z 287.2 and 259.2 could be similar as explained by Liau et al. [41]. Fragment ion m/z 241.2 of AFB1 formation can be explained by the removal of $-C_3H_4O_2$ from the precursor ion. The loss of group $-C_3H_2O_3$ from the AFG1 precursor ion will lead to the formation of the fragment m/z 245.2. The lactone ring is more prone to fragmentation than the difuranic ring in all the aflatoxins studied. The fragment ions formation from OTA (m/z 239, 358.2 and 221) could be postulated as similar to Lau et al. [42]. These ions were also reported in previous studies with similar order of intensity, regardless of the different ionization conditions used.

The most intense fragment ion of FB1 (m/z 704.4) was due to the loss of a water molecule $[M+H-H_2O]^+$, while the qualification ion (m/z 352.4) corresponds to the elimination of two tri-carboxylic acid (TCA) from the side chains and a concomitant loss of a water molecule $[M+H-2TCA-H_2O]^+$. Moreover, the fragment ion m/z 334.4 was also observed due to an extra elimination of a water molecule [43], which corresponds to the hydroxyl group of the fumonisin backbone. However, latter ion (m/z 334.4) was reported as the quantitation ion for FB1 in several other multi-mycotoxin methods [27,33,36,39] in contrast to our observation. The FB1 fragment ion m/z 528 $[M+H-H_2O-TCA]^+$) was selected as the confirmation ion in QuEChERS extract of maize silage [36], was only the fourth largest intense ion in our conditions. Both FB2 and FB3, the structural analogues of FB_1 produced similar fragment ions (both 16 amu lower than the corresponding FB1 fragments). Both of these analytes have been reported to produce following fragments: m/z 688.5 $[M+H-H_2O]^+$, m/z 354 $[M+H-2TCA]^+$, m/z 336.5 $[M+H-H2O-2TCA]^+$ and m/z 318 $[M+H-2H2O-2TCA]^+$. Therefore, double peaks were observed for both of these analytes in their respective MRM chromatograms. However, they were separated by finding these different fragment ions corresponding to their tR. FB1 is more polar than FB2 and FB3, as it has one more hydroxyl group, thus it has the lowest tR. Though, the structurally related FB2 and FB3 having the same molecular weight, the elution of FB3

occurs a minute ahead of FB2 because the polarity of FB3 is higher than FB2 as it could be assessed from the position of –OH group in their structures [33]. In FB3 it is close to the electron dense TCA moiety hence, it will tend to influence more the overall polarity of the molecule in contrast to its presence close to the alkyl group as on FB2. Hence, an elution order of FB1, FB3 and finally FB2 was obtained (Fig. S-2, S-3). The fragment ions of CIT, m/z 233.2 and m/z 205.4 could be due to the dismissal of a water molecule and -COOH group, respectively. Fragmentation of AME could be proposed in the following way, m/z 258.2 $[M+H-H_2O]^+$ and m/z 199.3 $[M+H-CH_3-OCH_3-CO]^+$.

3.2 Evaluation of matrix effect

Matrix effects are common problems that occur when using LC-MS or MS/MS, and thus have an adverse effect on the analytical results. The response of the target compound can be enhanced or suppressed due to the interfering matrix components, which is commonly known as signal suppression/enhancement effect (SSE). The ME of different spices on different analytes is shown [Fig. 3]. It can be seen that the signal suppression effect was very prominent for 75% of the analytes in pepper.

A range in between (-20) to +20% ME or SSE in between 0.8 to 1.2 was considered as tolerable [2]. Values outside this range indicate severe ME. It can be seen that, OTA in black pepper and OTA, STERIG, ROQ C and NEO in red chilli are the only analytes close to the tolerable range of ME. Signals for AFs in all the matrices were suppressed by 37 to 68%. A very strong ion suppression effect was observed on T-2, HT-2, STERIG, AME and ROQ C in black and white pepper (65-85%). In the study of Amate et al. [21], 67% of the compounds had a strong signal suppression effect on black pepper, and it was stated as the most critical matrix. All the three types of fumonisins showed an enhancement effect regardless of the type of spices. Ion enhancement of fumonisins was also reported in beer samples [33] extracted with QuEChERS approach.

409 Nevertheless, to compensate these significant ME and to improve the linearity, reliability and
410 accuracy of the analytical results MMC curves were used. Moreover, the residual co-extractives
411 were determined gravimetrically, by weighing the dried residue after evaporating the solvent under
412 N_2. The mean (n=5) residual co-extractives obtained after QuEChERS extractions, 8.94±0.99,
413 10.69±0.99, 9.37±1.59 mg mL^{-1}, in white pepper, black pepper and red chilli, respectively, showed
414 that there were no significant differences between different spices. However, the ME was found to
415 be significantly different for some analytes between matrices. It is noticeable with STERIG and
416 AME, on which both peppers had a significant ion suppression (80%) while chilli had 17% and
417 50% of ion enhancement, respectively. It is conceivable that in addition to the amount of matrix
418 components, the types of matrix components will also have an impact on analyte results.

419 A selective sample preparation to eliminate the matrix components is rather difficult and may risk
420 significant losses of some trace analytes. Finding appropriate IS in a multi-component analysis is
421 often challenging. A single IS cannot compensate the encountered matrix effects, as it would be
422 different with each analyte on each spice. The deuterated or isotopically labeled standard could
423 have been used for each analyte, but it was avoided considering the cost of multi-toxin analysis.
424 ZAN (IS) was used only for quality control purpose throughout our study; to additionally ensure the
425 constant retention time during the analysis. Moreover, no significant differences were obtained for
426 most of the analytes, when comparing the recoveries calculated using the peak areas and the relative
427 peak areas (analyte peak area divided by the peak area of ZAN). As an example in red chillies, the
428 recoveries (mean ± SD) of AFG2, AFG1, AFB2 and AFB1 obtained at 40 μg kg^{-1} using the relative
429 peak areas were 110±4, 116±11, 102±6, 103±16, respectively. Most of these values are also within
430 the acceptable recovery range of the required performance criteria [38], though ZAN is an inapt IS
431 in this case. Nevertheless, the previous studies state that the ME might not be completely eliminated
432 and it is already an established fact that ESI is more prone to ME than atmospheric pressure
433 chemical ionization (APCI) [44]. Finally, the validation parameters for each spice matrix were
434 determined based on the absolute peak area from the respective MMC curves.

435 **3.3 Method validation**

436 Method validation was performed in terms of linearity, repeatability, reproducibility, LODs, LOQs
437 and selectivity for all the three different spice matrices. A cut-off (CO) concentration level for each
438 analyte (a limit to distinguish high and low contamination level) was decided prior to validation by
439 analyzing some spice samples. For AFB1 and other AFs, a CO concentration of 10 µg kg^{-1} was
440 fixed, since contaminations of most of the samples analysed were close to this value. Hence,
441 validation of very low concentrations (less than 0.5 times of CO) was considered unnecessary. For
442 the non-detected ones, rather higher CO (200 µg $kg^{-1}$for all the fumonisins) values were determined
443 based on the judgment on the MLs set on other foods (Eg., Fumonisins in unprocessed maize is
444 2000 µg kg^{-1} and for maize flour it is 1000 µg kg^{-1}) [45].

445 **3.3.1 Linearity, LOD, LOQ and recovery**

446 MMC curves developed on different blank spice matrices were linear over the working
447 concentration ranges in all of the studied mycotoxins. Residual plots of each mycotoxins were
448 assessed to ensure the linearity of the model. Calibration curves fitted by linear regression showed
449 coefficients of determination (R^2) ranging from 0.9844-0.9997 in red chilli, 0.9890-0.9988 in black
450 pepper and 0.9903-0.9995 in white pepper [Table 2]. These statistical measures explain how well
451 the future outcomes could be predictable by our model on each of the spice matrices.

452 The LOD values of the tested mycotoxins ranged from, 1.2 to 73 µg kg^{-1} in red chilli, 2.0 to 33 µg
453 kg^{-1} in black pepper and 1.3 to 44 µg kg^{-1} in white pepper . The LOQs of different spices ranged
454 from 2.3 to 146 µg kg^{-1} [Table 2]. The highest LOQs obtained were for CIT in both black pepper
455 and red chilli and in white pepper it was FB3. The lowest LOQs obtained by this method were for
456 AFs, followed by OTA in all the spice matrices. The LOQs for both of these toxins meet the
457 regulatory limits set by the EC [16,17]. Validation for total AFs as a whole may not be very
458 essential, since the LOQs of the highly toxic AFB1 meets the EU ML of 5 µg kg^{-1} and a decision on

459 a lot could be made. Therefore, this simple and straightforward method allows the assessment of the
460 compliance of spices with the EU MLs. Moreover, the LOQs were quite satisfactory with other
461 toxins like fumonisins, when comparing with the regulations in cereals [15]. The method allows to
462 simultaneously analyse several chemically diverse mycotoxins on a matrix of analytical complexity
463 and achieving too high sensitivity is highly challenging with such simple and cost-effective
464 extraction method. Improving the method sensitivity may be needless, when the method LOQs
465 meets already the MLs of the regulated toxins. Time-consuming and costly SPE clean-ups possibly
466 can improve the method sensitivity, however it may limit the scope of the target analytes.

467 Mean recoveries for all the tested mycotoxins were in the range of 75 to 117% (Table 3), within the
468 acceptable range of required performance criteria [38]. According to the Commission Decision
469 2002/657/EC [37], "trueness" means the closeness of agreement between the average value
470 obtained from a large series of test results and an accepted reference value. Trueness can be
471 determined using the certified reference materials (CRM). It is acceptable to assess the trueness of
472 measurements through recovery of additions of known amounts of the analyte(s) to a blank matrix,
473 if no CRMs are available. Therefore, the term apparent recovery was found suitable to use in place
474 of trueness as we used the spiked spice samples. This is also stated as total recovery of a method
475 [24]. The recoveries obtained with our method were comparable with those described in a very
476 recent publication for red chilli and black pepper [28]. However, the LOQs reported were higher
477 (4.8 to 120 μg kg^{-1}) compared to our method for all the toxins, except AFG1.

478 **3.3.2 Intra-day repeatability and inter-day reproducibility**

479 Relative standard deviations (RSD) were calculated under intra-day repeatability (RSDr) and inter-
480 day reproducibility (RSDR) conditions. The results are summarized in Table 3. RSDr values were
481 within the acceptable range of <20% for almost all the analytes matching with the performance
482 criteria requirement of the EC [38]. However, RSDr for OTA was 24% in black pepper.
483 Comparatively, higher RSDr (up to 22-36%) and RSDR (up to 22-41%) values were obtained for

ADONs in different spice matrices. RSDR values were high for NEO in both red chili (23%) and white pepper (29%) and also for FB3 (26%) in red chilli. The higher variability of the ADONs and NEO could be due to the higher polarity of these mycotoxins compared to others as stated earlier. The salt induced partitioning could have hindered its adequate transfer to the MeCN phase. Poor performance criteria of the polar DON was also observed with this extraction, so it was kept out of the methods scope for validation and can only be determined qualitatively.

3.3.3 Specificity

The power of discrimination between the analyte and closely related matrix components, known as specificity [37] of the proposed method was evaluated by analyzing blank samples. The absence of any chromatographic signal close to the retention time as of the target compounds indicated the absence of any matrix interferences, despite the high complexity of the matrices. Carry-over of the analytes from the previous sample was evaluated by analyzing the mobile phases after the highest spiked sample. No carry-overs was observed, as there were no peaks detected in the elution zone of the analytes of interest.

4. Application of the method on real samples

Following the optimization and validation of the analytical method, it was applied on 30 commercially available samples collected from Sri Lanka. For each spice matrix, ten samples were analyzed to determine the contamination levels. The results of the analyses are summarized in Table 4. MMC curves were developed for each spice matrix for accurate quantification of mycotoxins. Apart from the selection of two MRM transitions, the relative ion intensity of the real samples was compared with that of the spiked samples as additional selectivity criteria [37]. Out of the ten red chilli powders six samples were found to be contaminated with AFB1 in the range of 5.1 to 35 μg kg^{-1}, exceeding the EU ML of 5 μg kg^{-1} [16]. Moreover, three out of the ten red chilli samples were contaminated simultaneously with four different toxins namely, AFB1, STERIG, OTA and FB2.

Earlier 65% of the chilli samples were found to contain more than one toxin from a Spanish study which included AFs, OTA and zearalenone [11]. OTA was found in four red chillies with a range of 7.0 to 27 μg kg^{-1} (mean concentration 13.3 μg kg^{-1}). Chromatograms of some contaminated spice samples are shown in Fig. 4. AFBI and AFG2 were detected only in one white pepper sample. 50% of the red chilli samples were positive for FB2. Incidence of FB2 in red chilli was not reported elsewhere till date. Concentrations of AFB1 and OTA found in each of the black pepper sample were above the EU ML [16,17]. Considering, the Indian black pepper samples 54% of them were positive for OTA in the range of 10-51 μg kg^{-1} according to Thirumala-Devi et al. [46]. Moreover, CIT was also detected in one black pepper sample (<LOQ). Meanwhile, highest STERIG contamination was found in white pepper, ranging from 15 to 36 μg kg^{-1}. However, compared to the black pepper, overall AFs and/or OTA contamination in white pepper is much lower. Despite the reported inhibitory action of the pepper pungent principle piperine on fungal growth and mycotoxin production [47], contamination in pepper seems inevitable. It should be noted that many previous studies failed to detect several other mycotoxins except AFs and OTA in different spices [12-14,21]. This study revealed that apart from these 'classical' mycotoxins associated with spices other toxicologically relevant mycotoxins were also found to be present.

5. Conclusion

A simple quantitative method based on a QuEChERS extraction approach, for simultaneous determination of multi class mycotoxins in three spices using HPLC-MS/MS was developed and successfully validated. The scope of the QuEChERS technique was further extended by its application on the extremely complex spices for mycotoxin analysis. MS/MS detection increased the confidence of analyte identification in spices. Significant matrix effect was successfully compensated using matrix matched calibration curves. The method LOQ meets the maximum levels of the two regulated toxins aflatoxins and ochratoxin A in spices hence, it can be used for the purpose of enforcement of the proposed EU MLs. It is an effective tool for quantitative screening of

diverse mycotoxins in spices and it can be useful for exposure assessment studies. This time saving and cost efficient method is also very flexible and new compounds like pesticide residues can be added easily. The clean chromatograms obtained on real sample matrices indicate the reliability of the method for confirmatory purposes.

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607 **Figure captions**

608 **Fig. 1.**

609 Peak responses obtained at one concentration level for different mycotoxins with two different
610 water to solvent ratios (10/15 and 5/5): (1) white pepper , (2) black pepper and (3) red chilli.

611 **Fig. S-1.**

612 MRM chromatograms obtained in white pepper: (A) with and (B) without salt partitioning, for (1)
613 OTA, (2) T-2, (3) ROQ C and (4) STERIG at 100 μg kg^{-1}.

614 **Fig. 2.**

615 Peak responses obtained in red chilli (n=3), without and with the addition of formic acid in mobile
616 phases (AFG2, AFG1, AFB2 and AFB1 at 20 μg kg^{-1}, STERIG at 25 μg kg^{-1}, T-2 and HT-2 at 50
617 μg kg^{-1}, OTA and ROQ C at 20 μg kg^{-1}, FB1, FB2 and FB3 at 80 μg kg^{-1}; other mycotoxins were
618 not tested)

619 **Fig. S-2.**

620 Chromatograms showing order of peak elution of mycotoxins in red chilli matrix (normalized
621 chromatograms). 1) NEO, 2) 3-ADON, 3) 15-ADON, 4) AFG2, 5) AFG1, 6) AFB2, 7) AFB1, 8)
622 ROQ C, 9) HT-2, 10) CIT, 11) FB1, 12) T-2, 13) FB3, IS) ZAN, 14) OTA, 15) FB2, 16) STERIG
623 and 17) AME.

624 **Fig. S-3.**

625 MRM transitions, quantification and qualification ions obtained with a mycotoxin standard mixture:
626 AFs at 12.5 μg kg^{-1}, STERIG at 15.625 μg kg^{-1} OTA and ROQ C at 25 μg kg^{-1}, T-2, HT-2, 3-
627 ADON, 15-ADON and NEO at 62.5 μg kg^{-1} and all other toxins at 125 μg kg^{-1}.

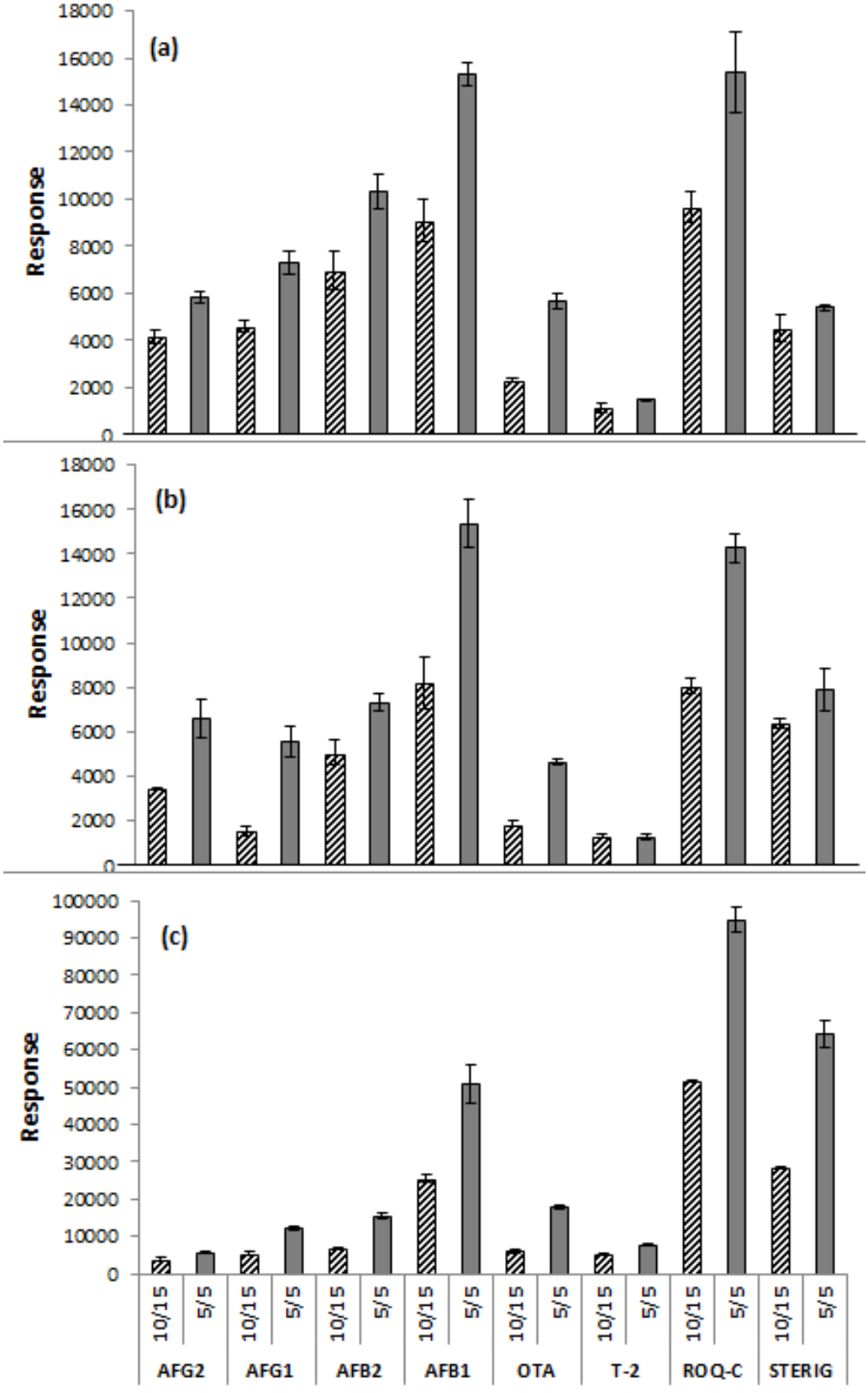
628 **Fig. 3.**

629 Matrix effects of different spices on the response of each mycotoxin. The concentration range used
630 for the ME evaluation were, AFB1, AFB2, AFG1 and AFG2 (5-150 μg L^{-1}), OTA (10-300 μg L^{-1}),
631 STERIG (6.25-187.5 μg L^{-1}), T-2, HT-2, ROQ C, NEO, 3-ADON, 15-ADON, FB1, FB2, FB3,

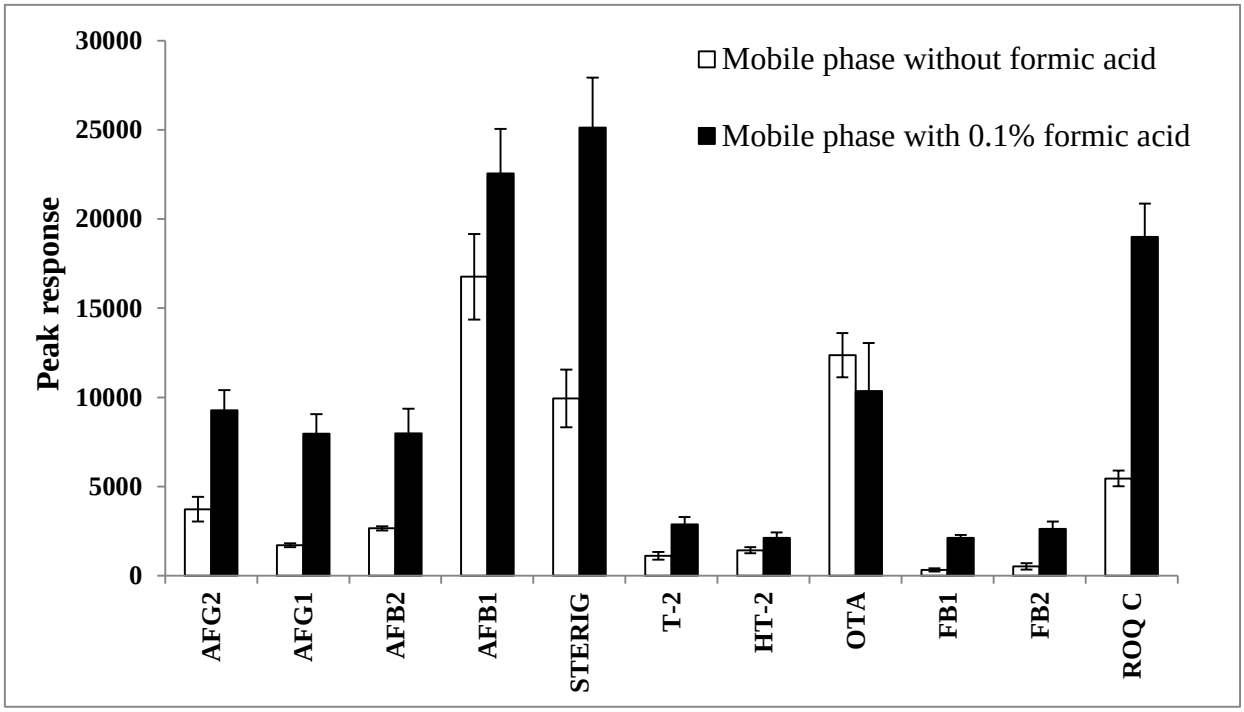
AME and CIT (25-750 µg L^{-1}). A tolerance level of matrix effect is shown between the two dashed lines.

Fig. 4.

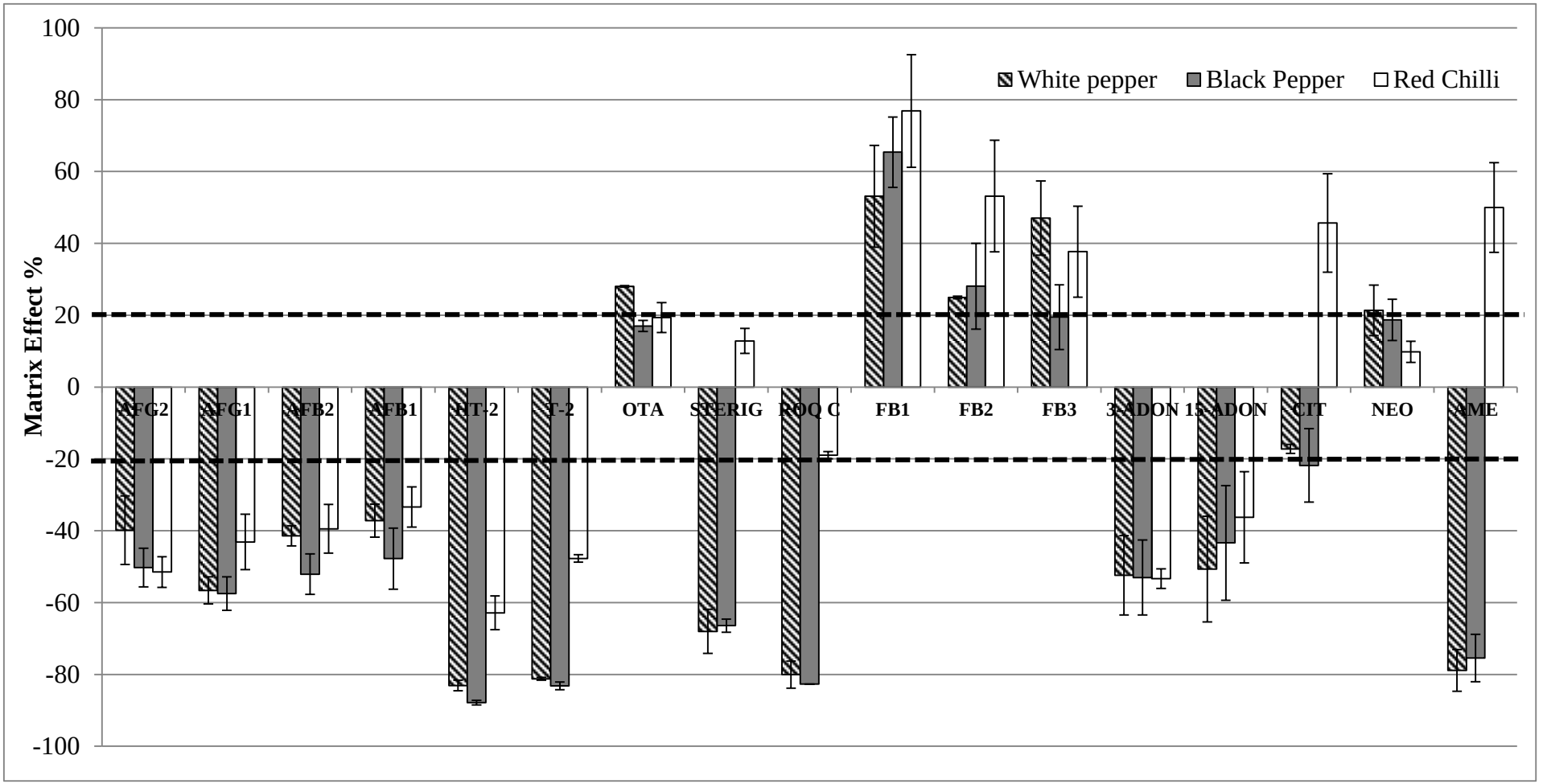
LC-MS/MS chromatograms of some positive spice samples: a) white pepper contaminated with 36 µg kg^{-1} STERIG; red chilli with b) 18 µg kg^{-1} AFB1 and c) <LOQ FB2 and d) black pepper with 134.5 µg kg^{-1} FB1. Quantification and confirmation transitions are shown for all the contaminated samples.


(a)
Response
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
(b)
Response
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
(c)
Response
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
10/15
5/5
AFG2
AFG1
AFB2
AFB1
OTA
T-2
ROQ-C
STERIG

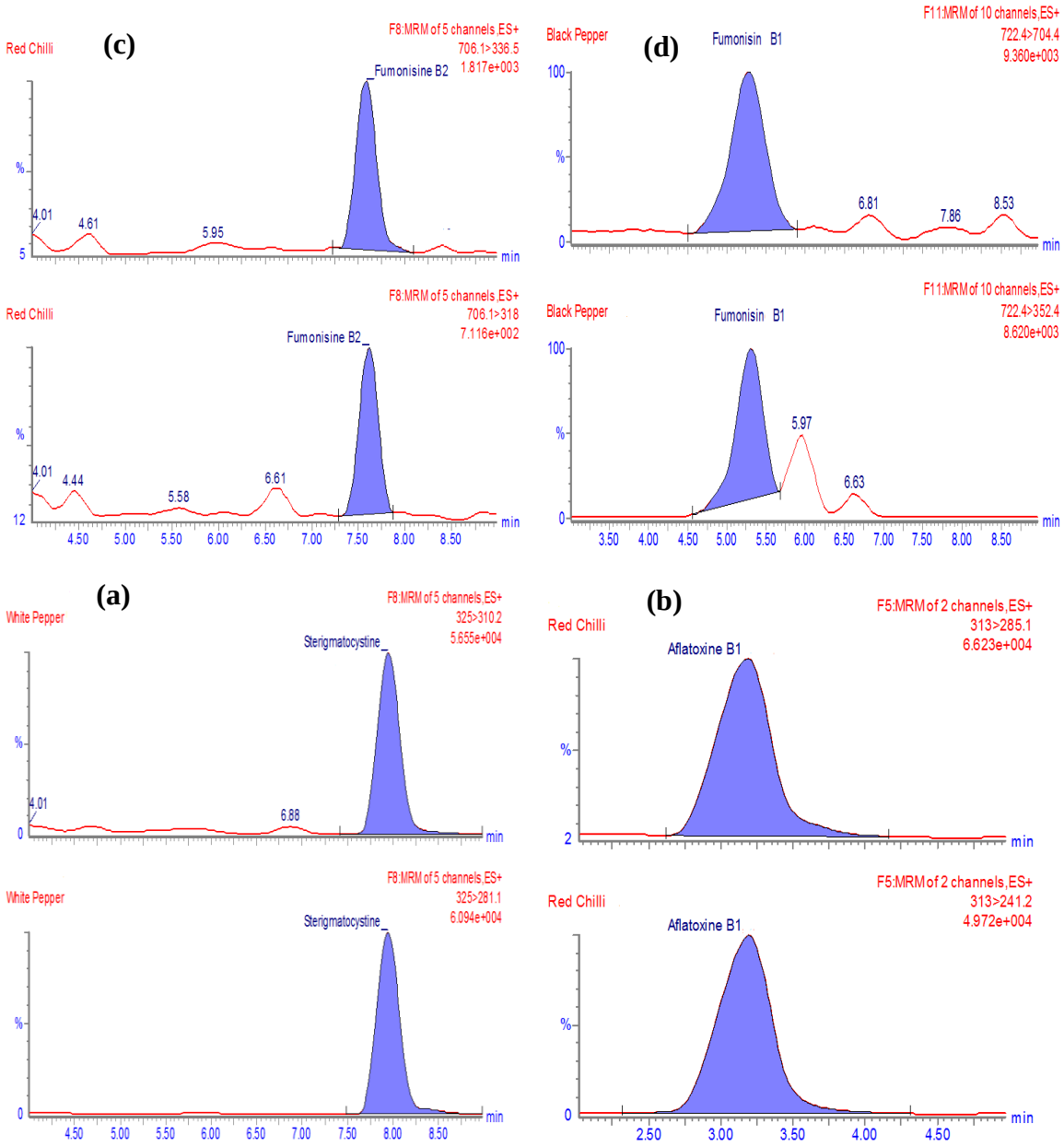
639 **Fig. 1.**


30000
25000
20000
15000
10000
5000
0
Peak response
Mobile phase without formic acid
Mobile phase with 0.1% formic acid
AFG2
AFG1
AFB2
AFB1
STERIG
T-2
HT-2
OTA
FB1
FB2
ROQ C

640 **Fig. 2.**


100
80
60
40
20
0
-20
-40
-60
-80
-100
Matrix Effect %
White pepper
Black Pepper
Red Chilli
AFG2
AFG1
AFB2
AFB1
HT-2
T-2
OTA
STERIG
ROQ C
FB1
FB2
FB3
3-ADON
15-ADON
CIT
NEO
AME

641 **Fig. 3.**


(c)
Red Chilli
F8:MRM of 5 channels,ES+
706.1>336.5
1.817e+003
Fumonisine B2
4.01
4.61
5.95
min
F8:MRM of 5 channels,ES+
706.1>318
7.116e+002
Fumonisine B2
4.01
4.44
5.58
6.61
(d)
Black Pepper
Fumonisin B1
F11:MRM of 10 channels,ES+
722.4>704.4
9.360e+003
6.81
7.86
8.53
F11:MRM of 10 channels,ES+
722.4>352.4
8.620e+003
5.97
6.63
(a)
White Pepper
F8:MRM of 5 channels,ES+
325>310.2
5.655e+004
Sterigmatocystine
4.01
6.88
F8:MRM of 5 channels,ES+
325>281.1
6.094e+004
(b)
Red Chilli
F5:MRM of 2 channels,ES+
313>285.1
6.623e+004
Aflatoxine B1
F5:MRM of 2 channels,ES+
313>241.2
4.972e+004

642 **Fig. 4.**

643 **Table 1.**

644 Parameters for the mass spectrometric detection of mycotoxins including analyte retention time
645 (tR), precursor ions, molecular ion, cone voltage, quantification ions (Quant), qualification ions
646 (Qual),collision energy 1 (CE1) and collision energy 2 (CE2)

Mycotoxin	tR (min)	Precursor ion (m/z)	Molecular ion	Cone voltage (V)	Quant (m/z)	Qual (m/z)	CE1 (eV)	CE2 (eV)
AFG2	2.23	331.0	$[M+H]^+$	53	313.1	245.2	30	25
AFG1	2.50	329.0	$[M+H]^+$	45	243.0	311.2	25	20
AFB1	3.23	313.0	$[M+H]^+$	51	285.1	241.2	24	36
AFB2	2.88	315.0	$[M+H]^+$	51	287.2	259.2	27	30
OTA	7.41	403.9	$[M+H]^+$	25	239.0	358.2	22	20
T-2	6.09	484.1	$[M+NH_4]^+$	30	215.0	185.1	20	18
HT-2	5.03	442.2	$[M+NH_4]^+$	20	263.1	215.0	13	13
STERIG	7.96	325.0	$[M+H]^+$	47	310.2	281.1	25	36
ROQ C	4.33	390.0	$[M+H]^+$	40	193.2	322.2	26	21
FB1	5.28	722.4	$[M+H]^+$	56	704.4	352.4	29	36
FB2	7.55	706.0	$[M+H]^+$	50	336.5	318.0	35	29
FB3	6.55	706.1	$[M+H]^+$	54	688.5	354.0	34	31
CIT	5.63	250.9	$[M+H]^+$	32	233.2	205.4	17	26
AME	8.74	272.9	$[M+H]^+$	57	258.2	199.3	26	30
3-ADON	2.26	339.2	$[M+H]^+$	24	231.2	261.4	12	10
15-ADON	2.26	339.2	$[M+H]^+$	24	137.2	203.2	10	12
NEO	1.64	400.1	$[M+NH_4]^+$	26	185.0	305.3	19	12
DON	1.58	297.1	$[M+H]^+$	26	249.2	231.2	15	10
ZAN (IS)	7.23	321.0	$[M+H]^+$	27	303.3	189.2	13	19

647

Table 2.

Limit of quantification (LOQ) and coefficients of determination (R^2) obtained for black pepper, white pepper and red chilli.

Mycotoxin	Black pepper		White pepper		Red chilli	
	LOQ (µg/kg)	R^2	LOQ (µg/kg)	R^2	LOQ (µg/kg)	R^2
AFG2	4.5	0.9985	2.5	0.9995	3.3	0.9992
AFG1	5	0.9973	3.8	0.9989	4.7	0.9976
AFB2	4	0.9984	3.7	0.9994	3	0.9993
AFB1	4	0.9988	3.1	0.9993	2.3	0.9994
T-2	47	0.9935	18	0.9991	20	0.9989
HT-2	42	0.9948	27	0.9978	23	0.9967
FB1	64	0.9969	82	0.995	64	0.997
FB2	68	0.9965	82	0.9951	64	0.997
FB3	43	0.9986	88	0.9916	80	0.9952
OTA	13	0.997	12	0.9986	4.2	0.9997
STERIG	8	0.9973	16	0.9937	11	0.9946
ROQ C	14	0.9964	4.7	0.999	17	0.9948
CIT	65	0.9988	84	0.9948	146	0.9844
NEO	32	0.997	77	0.9903	37	0.996
3-ADON	47	0.9935	40	0.9954	42	0.9948
15-ADON	61	0.9891	76	0.9954	46	0.9937
AME	Not detectable in both peppers				53	0.9979

650 **Table 3.**

651 Intra-day repeatability (RSDr), inter-day reproducibility (RSDR) expressed as relative standard
652 deviation (%) and apparent recovery (%) obtained for black pepper, white pepper and red chilli at
653 two concentration levels for each mycotoxin.
654

Mycotoxin	Concentration (µg kg^{-1})	Black pepper			White pepper			Red chilli		
		RSDr %	RSDR %	Apparent recovery %	RSDr %	RSDR %	Apparent recovery %	RSDr %	RSDR %	Apparent recovery %
AFG2	5	14	20	117	12	20	111	18	19	91
	40	9	8	100	4	7	100	10	13	93
AFG1	5	8	15	108	16	11	99	12	20	103
	40	6	4	101	7	6	99	10	8	103
AFB2	5	16	16	84	10	9	109	10	11	78
	40	4	8	100	4	7	99	20	14	102
AFB1	5	6	16	79	14	4	95	5	10	104
	40	7	8	102	4	7	99	20	13	102
T-2	50	20	23	106	10	23	95	12	16	103
	200	9	34	83	8	10	99	13	10	99
HT-2	50	25	17	98	16	12	112	11	17	106
	200	9	8	101	7	7	101	9	10	98
FB1	100	8	22	103	16	14	106	23	20	101
	400	7	31	83	11	7	102	6	8	102
FB2	100	18	15	105	17	18	99	9	11	106
	400	11	9	101	4	4	102	7	6	101
FB3	100	22	15	107	7	18	108	23	26	109
	400	9	7	102	6	15	102	12	14	98
OTA	20	24	18	103	21	18	104	15	13	98
	80	10	8	98	5	9	104	7	7	104
STERIG	12.5	13	21	97	9	15	94	5	16	107
	50	9	6	102	9	7	100	4	4	101
ROQ C	20	12	13	103	8	7	102	11	14	113
	80	8	7	101	7	7	93	8	6	100
CIT	100	7	10	106	11	10	101	6	19	103
	400	5	4	101	10	8	102	1	6	97
NEO	50	15	17	102	15	29	75	20	23	99
	200	16	30	85	8	13	105	12	9	99
3-ADON	50	22	15	110	12	41	111	22	30	91
	200	22	15	102	5	7	100	10	12	102
15-ADON	50	36	33	102	19	22	100	20	13	89
	200	16	19	100	4	6	100	15	15	97
AME	100	Not detectable in both peppers						9	13	101
	200							8	9	99

Table 4.

Frequency (ratio of positives/number of samples) and mean contamination (μg kg^{-1}) of different mycotoxins found in red chilli, black pepper and white pepper samples collected from Sri Lanka.

Type of spice	AFG2	AFB2	AFB1	OTA	STERIG	FB1	FB2	CIT
Red chilli (n=10)[a]	ND [b]	2/10; <LOQ [c]	3/10; <LOQ 6/10; 18	2/10; <LOQ 4/10; 13	4/10; <LOQ	ND	5/10; <LOQ	ND
Black pepper (n=10)[a]	1/10; 5.7	ND	1/10; 11	3/10; < LOQ 1/10; 48	5/10; <LOQ	1/10; 134.5	ND	1/10; <LOQ
White pepper (n=10)[a]	1/10; 2.6	1/10; <LOQ	1/10; 4.9	ND	4/10; 24 4/10; <LOQ	ND	ND	ND

[a,] no of samples
[b,] not detected
[c,] limit of quantification

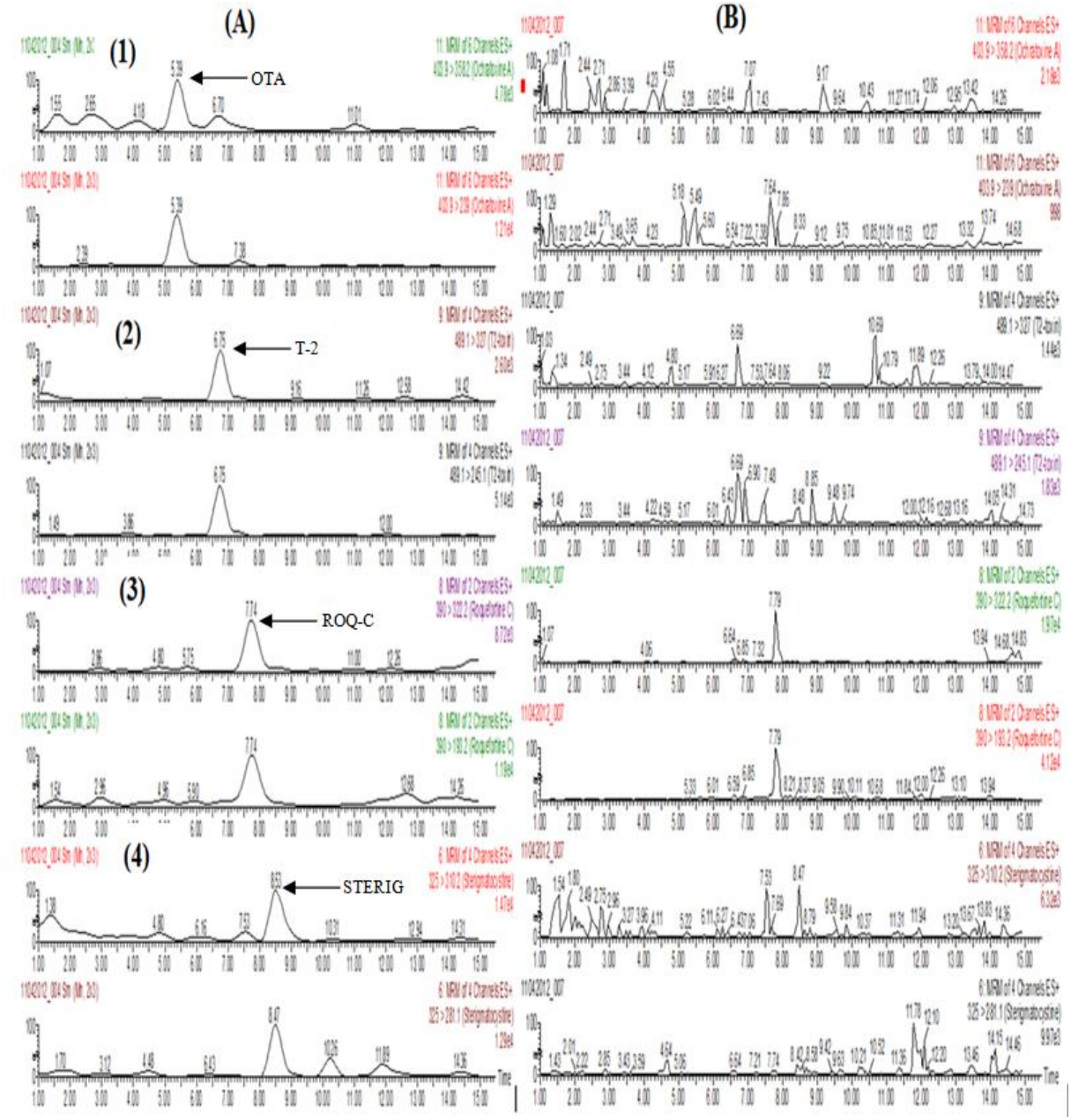


Fig. S-1.

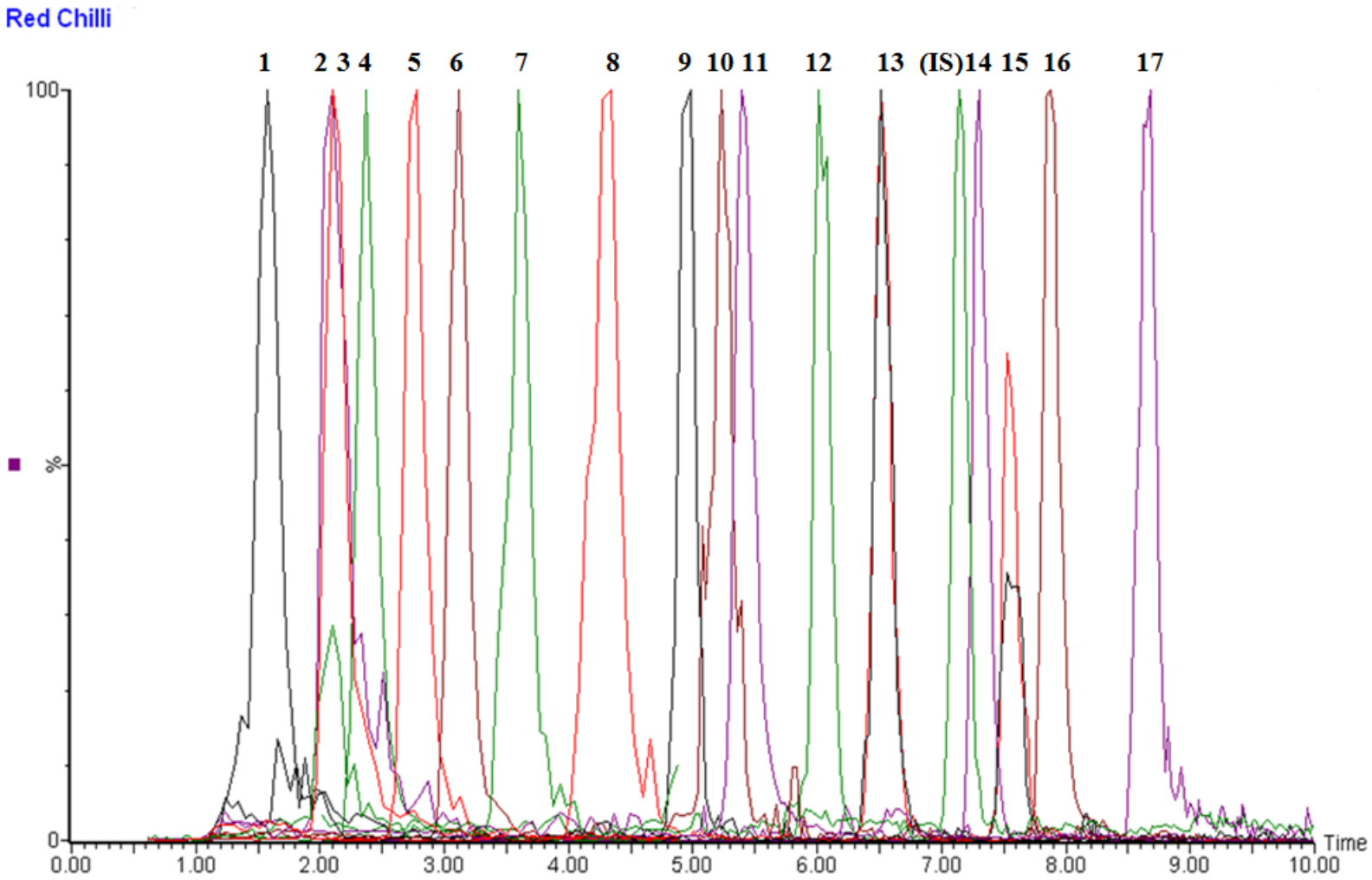
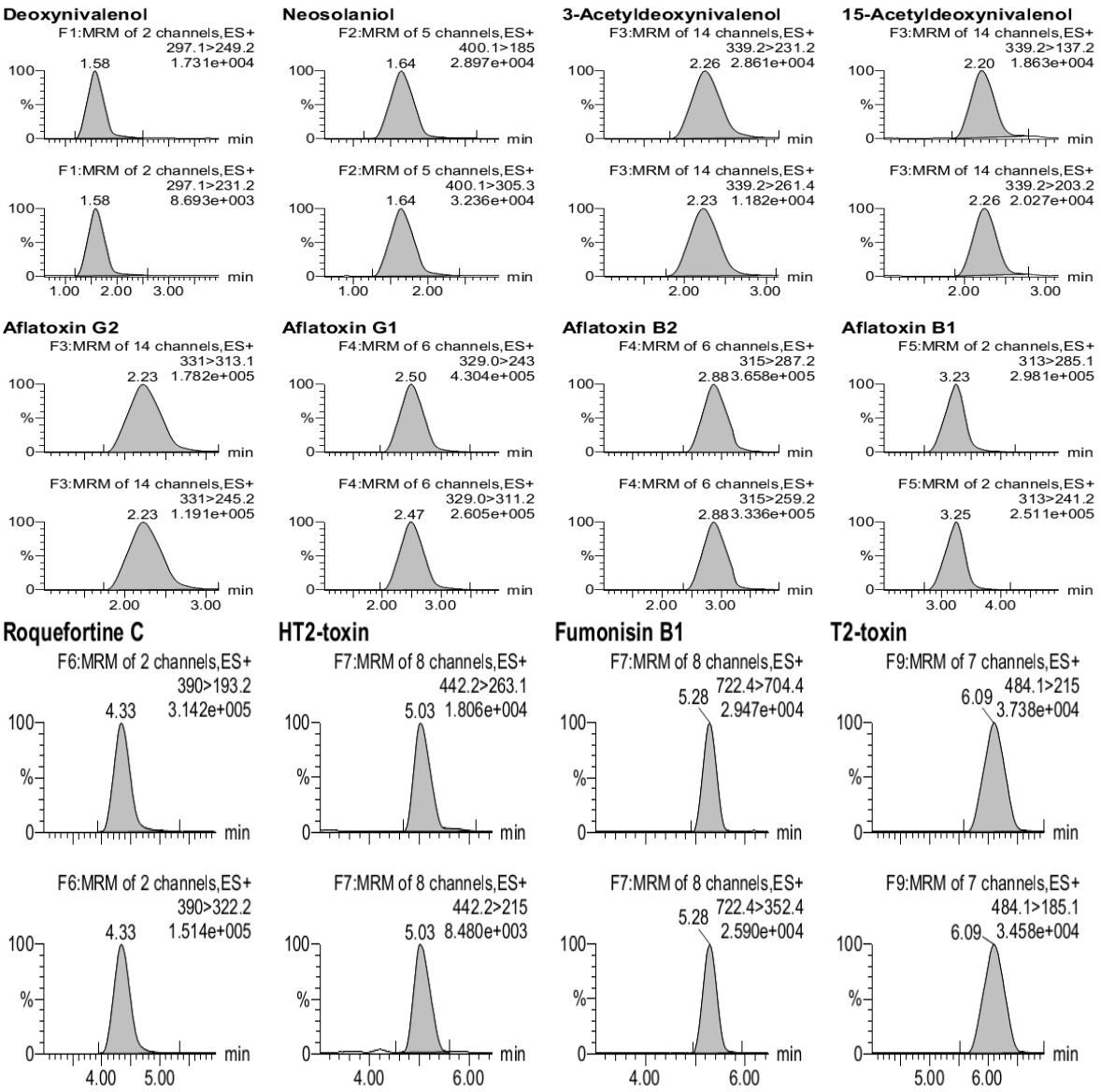

Red Chilli
1
2 3 4
5
6
7
8
9
10 11
12
13
(IS)14
15
16
17
100
%
0
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
Time

Fig. S-2.


Deoxynivalenol
F1:MRM of 2 channels,ES+
297.1>249.2
1.58
1.731e+004
F1:MRM of 2 channels,ES+
297.1>231.2
1.58
8.693e+003
Neosolaniol
F2:MRM of 5 channels,ES+
400.1>185
1.64
2.897e+004
F2:MRM of 5 channels,ES+
400.1>305.3
1.64
3.236e+004
3-Acetyldeoxynivalenol
F3:MRM of 14 channels,ES+
339.2>231.2
2.26
2.861e+004
F3:MRM of 14 channels,ES+
339.2>261.4
2.23
1.182e+004
15-Acetyldeoxynivalenol
F3:MRM of 14 channels,ES+
339.2>137.2
2.20
1.863e+004
F3:MRM of 14 channels,ES+
339.2>203.2
2.26
2.027e+004
Aflatoxin G2
F3:MRM of 14 channels,ES+
331>313.1
2.23
1.782e+005
F3:MRM of 14 channels,ES+
331>245.2
2.23
1.191e+005
Aflatoxin G1
F4:MRM of 6 channels,ES+
329.0>243
2.50
4.304e+005
F4:MRM of 6 channels,ES+
329.0>311.2
2.47
2.605e+005
Aflatoxin B2
F4:MRM of 6 channels,ES+
315>287.2
2.88
3.658e+005
F4:MRM of 6 channels,ES+
315>259.2
2.88
3.336e+005
Aflatoxin B1
F5:MRM of 2 channels,ES+
313>285.1
3.23
2.981e+005
F5:MRM of 2 channels,ES+
313>241.2
3.25
2.511e+005
Roquefortine C
F6:MRM of 2 channels,ES+
390>193.2
4.33
3.142e+005
F6:MRM of 2 channels,ES+
390>322.2
4.33
1.514e+005
HT2-toxin
F7:MRM of 8 channels,ES+
442.2>263.1
5.03
1.806e+004
F7:MRM of 8 channels,ES+
442.2>215
5.03
8.480e+003
Fumonisin B1
F7:MRM of 8 channels,ES+
722.4>704.4
5.28
2.947e+004
F7:MRM of 8 channels,ES+
722.4>352.4
5.28
2.590e+004
T2-toxin
F9:MRM of 7 channels,ES+
484.1>215
6.09
3.738e+004
F9:MRM of 7 channels,ES+
484.1>185.1
6.09
3.458e+004
100
%
0
min

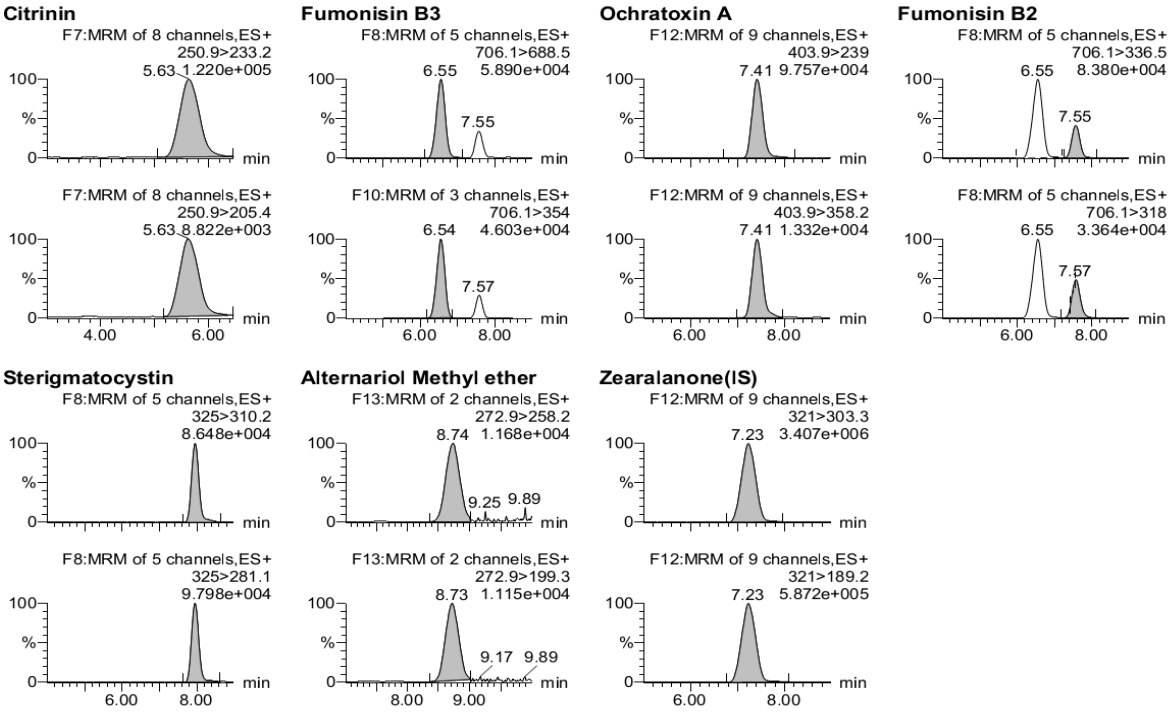

Citrinin
F7:MRM of 8 channels,ES+
250.9>233.2
5.63 1.220e+005
F7:MRM of 8 channels,ES+
250.9>205.4
5.63 8.822e+003
Fumonisin B3
F8:MRM of 5 channels,ES+
706.1>688.5
6.55 5.890e+004
7.55
F10:MRM of 3 channels,ES+
706.1>354
6.54 4.603e+004
7.57
Ochratoxin A
F12:MRM of 9 channels,ES+
403.9>239
7.41 9.757e+004
F12:MRM of 9 channels,ES+
403.9>358.2
7.41 1.332e+004
Fumonisin B2
F8:MRM of 5 channels,ES+
706.1>336.5
6.55 8.380e+004
7.55
F8:MRM of 5 channels,ES+
706.1>318
6.55 3.364e+004
7.57
Sterigmatocystin
F8:MRM of 5 channels,ES+
325>310.2
8.648e+004
F8:MRM of 5 channels,ES+
325>281.1
9.798e+004
Alternariol Methyl ether
F13:MRM of 2 channels,ES+
272.9>258.2
8.74 1.168e+004
9.25 9.89
F13:MRM of 2 channels,ES+
272.9>199.3
8.73 1.115e+004
9.17 9.89
Zearalanone(IS)
F12:MRM of 9 channels,ES+
321>303.3
7.23 3.407e+006
F12:MRM of 9 channels,ES+
321>189.2
7.23 5.872e+005
100
%
0
min

Fig. S-3.