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Simultaneous Multi- Residue Determination of Mycotoxins in Foods Using LC-MS/MS

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Abstract

This paper describes a method for simultaneous determination of eleven mycotoxins in chilli powder, dried fish, peanuts and rice using Liquid Chromatography-Mass Spectrometry (LC-MS/MS). The method is based upon a single extraction step followed by analysis of the diluted crude extract. The proposed method was applied for the simultaneous quantification of multi-residue mycotoxins in 80 samples. The results showed that four chilli powder and twelve peanut samples of a total 80 analyzed samples were contaminated with levels greater than the EC regulatory limit for AFB_1.

Keywords: Mycotoxins; LC-MS/MS; Multi-target analysis; Food samples

Introduction

Natural toxins in food are of increasing concern for human health. Among the natural toxins, mycotoxins pose a significant health risk within the tropical developing countries food supply chain [1]. Mycotoxins are produced by fungi (molds), such as Aspergillus, Fusarium, Penicillium and Alternaria, that are frequently present on agricultural products [2]. The contamination of foods and feeds by the major mycotoxins, e.g. aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2), ochratoxin A (OTA), fumonisins (FB_1 and FB_2), deoxynivalenol (DON), T-2, HT-2, and zearalenone (ZEA), has been recognized by the World Health Organization [3] as a significant source of food-borne illnesses. Due to the health risk these toxins pose to humans, mycotoxin content in food and feed are regulated by legislation in different national and international levels [4,5]. In particular, the European Commission [4] has established maximum permitted levels in foodstuff for direct human consumption of aflatoxins (AFB_1, 2-8 ng g^{-1}; total AF_S, 4-15 ng g^{-1}), OTA (3-10 ng g^{-1}), ZEA (75-400 ng g^{-1}), DON (500-1750 ng g^{-1}), FB_S (800-4000 ng g^{-1}); however, limits for T-2 and HT2 are currently under discussion. In detail, The European Commission [4] has set 5.0 ng g^{-1} for AFB_1 and 10.0 ng g^{-1} for total aflatoxins in spices; 2.0 ng g^{-1} for AFB_1 and 4.0 ng g^{-1} for total aflatoxins for peanuts and other oil seeds intended for direct human consumption. The limit was 5.0 ng g^{-1} and 10.0 ng g^{-1} for AFB_1 and total aflatoxins, respectively for dried fruits to be subjected to sorting, or other physical treatment, before human consumption. In cereals, the limit was 2.0 ng g^{-1} for AFB_1 and 4.0 ng g^{-1} for total aflatoxins, OTA (3 ng g^{-1}), DON (750 ng g^{-1}) and , ZEA (75 ng g^{-1}), respectively. In Malaysia, the regulatory limit for total aflatoxins in groundnut is 15 ng g^{-1}. The regulation for other foods and milk in Malaysian Food Act (1983) is 10 ng g^{-1} and 0.05 ng mL^{-1}, respectively [6] and specific limits for other types of mycotoxins have not been established yet.

The two most important environmental components favoring for mycotoxins production by molds are humid and hot conditions. The climate in Malaysia, with an average temperature (28 - 31°C) and humidity (60 - 80%), is conducive to growth of mycotoxins-producing molds. Therefore, monitoring mycotoxin levels in foodstuffs is necessary in Malaysia to ensure the quality of the food supply. Crops which are used in high amount in the Malaysian diet and frequently affected by mycotoxins include cereals [7-9] and spices [10,11]. Some of these materials are often eaten raw and can induce serious health problems. Sundried fish constitutes a major source of proteins in the diet of the people in many tropical countries, including Malaysia. The presence of different types of mycotoxins in fishes has been reported by Abdel-Wahhab & Kholif [12] and Hashem [13].

Currently about 400 compounds are recognized as mycotoxins and only few of them are listed by food legislation. Consequently, most of the existing analytical methods focus on these regulations. Analytical methods for mycotoxins determination are thin layer chromatography [14], enzyme linked immunosorbent assays [15], HPLC with diode array detector [16], HPLC coupled with fluorescence detector [17] and Gas chromatography coupled with electron capture [18]. All these methods almost using immunoaffinity and solid phase extraction techniques to improve the measurement of mycotoxins by removing interferences. These analytical methods used to foodstuffs samples to get results within hours or days. On the other hand, these methods are sufficiently selective for single target analysis in food and feed samples, but are unable of dealing with a large number of analytes with the high complexity of food matrices. LC-MS/MS techniques have been used in the last few years to overcome problems related with the complicated food matrices. The LC-MS/MS techniques capable to simultaneous determination of chemically diverse compounds at relatively low concentration levels. The most commonly used analysis of the multi-mycotoxin methods are the quadrupole based instruments, particularly the triple quadrupole analysers [19,20]. Therefore, LCMS/MS is gaining in importance in mycotoxin analysis with considering the high selectivity of MS detection with the reduce analysis time and cost. Several different types of LCMS mycotoxin methods for food and feed matrices have been introduces [21-25]. This research aimed to simultaneous determination of 11 mycotoxins in four food matrices by LC-ESI-MS/MS. Chilli powder, dried fish, peanuts and rice samples have been selected in this research due to comparatively high

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consumption by the Malaysian population.

Experimental Section

Chemicals and materials

Mycotoxins standards (AFB_1, AFB_2, AFG_1, AFG_2, OTA, DON, ZEA,) were obtained from Supelco® (USA) and FB_1, FB_2, T-2 and HT-2 toxins were supplied by Fermentek® (Israel). Ultrapure deionized water of 18.2 MΩ.cm resistivity was obtained from a water purification system (PURELAB Option-R, ELGA®, UK). HPLC grade acetonitrile (ACN) and methanol (MeOH), formic acid and ammonium acetate were obtained from Merck® (Germany).

Standard solutions

To obtain a stock solution of AFB_1 (75 ng ml^{-1}), AFB_2 (22.5 ng ml^{-1}), AFG_1 (75 ng ml^{-1}), AFG_2 (22.5 ng ml^{-1}), OTA (75 ng ml^{-1}), ZEA (1500 ng ml^{-1}), FB_1 (6000 ng ml^{-1}), FB_2 (6000 ng ml^{-1}), T-2 (1500 ng ml^{-1}), HT-2 toxin (1500 ng ml^{-1}) and DON (6000 ng ml^{-1}), 750 µL of aflatoxins mixed standard solution (AFB_1 and AFG_1, 1000 ng mL^{-1} and AFB_2, AFG_2 300 ng mL^{-1}) were mixed with 300 µL of DON standard solution (200,000 ng mL^{-1}), 1200 µL of FB_2 standard solution (50,000 ng mL^{-1}), 1200 µL of FB_1 standard solution (50,000 ng mL^{-1}), 150 µL of HT-2 toxin standard solution (100,000 ng mL^{-1}), 150 µL of T-2 toxin standard solution (100,000 ng mL^{-1}), 300 µL of ZEA standard solution (50,000 ng mL^{-1}), and 15 µL of OTA standard solution (50,000 ng mL^{-1}), in a 10 mL volumetric flask with methanol, respectively. This stock solution was then used to prepare the working standard solutions. All stock and working standard solutions were stored in amber screw cap bottles at -18°C. Ad hoc combined working standard mycotoxin solutions were made at different concentrations for sample spiking and LC-MS/MS calibration.

HPLC-MS/MS parameters

Detection and quantification were performed with a high performance liquid chromatography (Agilent 1200 series, USA) by injecting 20 µL of the sample on a reversed-phase C_{18} column (Zorbax, 2.1 mm × 50 mm, 1.8 µm particles), equipped with a security guard cartridge (2.1 mm × 12.5 mm, 2 µm particles) containing the same stationary phase as the column, at 40 °C. The column was eluted using a gradient flow (0.30 mL·min^{-1}) of two solvents A (H_2O) and B (MeOH). Then, the formic acid and ammonium acetate were added to both solvents. Concentrations in both solvents were set to 0.1% and 0.5 mM, respectively. The mobile phase was maintained at 60:40 (A:B, v/v) and then changed to a linear gradient to 90% solvent B over 5 min. Then 90% (B) decreased to 40% in 2 min. An isocratic elution of 40% (B) was then used for 6 min to re-equilibrate the column. The column was flushed after each triplicate sample by injecting 20 µL of acetonitrile/water (40:60, v/v). The LC system was coupled to a triple-quadrupole mass spectrometer (Agilent G6410) equipped with an electrospray ionization (ESI) probe. Positive ESI-MS/MS was performed in MRM mode. The two most abundant product ions per analyte were chosen for quantitative and confirmation purposes. The peak widths of precursor and product ions were maintained at 0.7 amu at the MRM mode. The capillary voltage was set at +4.0 kV, the nebulizer gas pressure at 50 psi and desolvation temperature at 350 °C. Nitrogen of 99.5% purity was generated by a Parker® generator (Parker Hannifin) and used for desolvation at 12 L min^{-1} flow rate. The collision cell gas was 99.999% pure nitrogen (MOX®, Malaysia). All data were acquired and analyzed using Agilent MassHunter Workstation® software version B.01.03 analyst data processing software (Agilent Corporation, MA, USA).

Food Sample Preparation

Sixty samples including chilli powder (n=20), dried fish (n=20) and peanuts (n=20) were purchased from local supermarkets, shops and open markets throughout Shah Alam city, while rice samples (n=20) were collected from private homes from Serdang city, Selangor State, Malaysia. The samples were stored at -18 °C until analysis. Samples were extracted according to the method of mycotoxin extraction described by [26] with some modifications. Sample spiking was carried out with appropriate amounts (17- 500 µL) of the stock standard mycotoxin solutions added to 2.5 g of finely ground sample matrices in 50mL Falcon® tubes with PTFE-lined screw-caps. The samples were vortexed for about 1 min and kept for half a day in a fume hood to allow slow evaporation of the solvent. The mixture was extracted with 10 mL of ACN/water (80:20, v/v) added with 0.1% formic acid on a MaxQ 4000® shaker (Barnstead®/Lab-Line®, USA) for 60 min and centrifuged (8944×g) for 10 min in an Eppendorf 5804 R® (Germany). Subsequently, 500 µL of the supernatant extract was diluted with the same amount of ultrapure water, filtered through a 0.45 µm nylon syringe filter (Millipore®, USA), and injected into the LC-MS/MS system without further pre-treatment. All samples were analyzed in triplicate and the average values were recorded. To investigate possible matrix effects, blank extracts from each type of food commodities (chilli powder, peanut, rice and dried fish) were diluted with the same amount of ultrapure water (1:1) and fortified for matrix-matched calibration. The concentrations of the analytes in the matrix-matched standards and external standards were matched at each level to the expected concentrations in the final diluted extract of the spiked samples. The LOD was determined from MRM chromatograms of spiked samples showing a signal-to-noise ratio (S/N) equal to 3. Similarly, the LOQ was obtained from chromatograms with an S/N of 10. The each analytical batch contained at least one reagent blank and one triplicate spiked sample fortified at LOQ level for each of the analytes.

Results and Discussions

Chromatographic conditions

A simple and rapid method for determining eleven mycotoxins was developed and validated for four different types of food commodities. The tandem mass spectrometry conditions were optimized for each analyte and combined in one multi-method using the ESI in positive ion source. The most important ESI parameters, which were capillary voltage, nebulizer pressure, drying gas flow rate and temperature were initially tuned to achieve maximum sensitivity. In the preliminary tests, a better response was obtained for ZEA as $[M+H]^-$ but due to co-elution of matrix components, it was monitored as $[M-H]^+$ in the positive mode of ESI. A good response was achieved since ZEA is ionisable with both polarities, as has also been demonstrated in previous studies [26,27]. In most of the ESI positive compounds abundant $[M+H]^+$ ions were present, except for HT-2 and T2 that ionised as $[M+NH_4]^+$. Thus, to improve ionization efficiency and sensitivity, 0.1% formic acid was added to the mobile phase for determination of mycotoxins as $[M-H]^+$ in the positive mode of ESI [25,26,28,29] . In addition, 0.5 mM ammonium acetate was added to the mobile phase allowing the detection of HT-2 and T2 molecular ions as ammonium adducts $[M+NH_4]^+$ [26,28,30,31].

Two product ion transitions were selected for quantitative and confirmatory purposes. The most abundant transition was used for quantification and the other one was employed for confirmation purposes. A dwell time of 30 ms was applied to obtain sufficient peak shape with monitoring 22 MRM transitions in positive ionization

mode. Furthermore, the collision energy was tuned for each analyte to get optimum sensitivity using nitrogen collision gas. Retention times of the corresponding compounds in external standard calibration and related parameters are shown in Table 1.

Different proportions of mobile phase at different flow rates were used for elution of mycotoxins in LC-ESI-MS/MS. Chromatographic conditions were optimized to provide both short retention times and adequate peak shapes. The best chromatograms for all analytes were obtained using a mobile phase with a gradient elution program consisting of (A) 0.5 mM ammonium acetate, 0.1% formic acid in water, and (B) 0.5 mM ammonium acetate and 0.1% formic acid in methanol at a flow rate of 0.3 ml min^{-1}. The mobile phase was maintained at 60:40 (A:B) and then changed with a linear gradient to 90% solvent B over 5 min. Then 90% (B) decreased to 40% in 2 min. An isocratic elution of 40% (B) was then used for 6 min to re-equilibrate the column. This approach co-elution cannot be avoided completely in a multi-target-analysis; however, this is normally of minor importance as these compounds show different MRM transitions in LC-MS/MS [31,32]. Among the various injection volumes studied (5-30 µL), the 20 µL injection enhances sensitivity to compensate for the limited detection sensitivity of the mass spectrometer.

Linearity, recovery, signal suppression/enhancement, detection and quantification limits

Linearity was evaluated by analyzing standard solutions and matrix-matched calibration curves at minimum six points in triplicate. The lowest and highest standard concentrations of the linear regression identify the valid range over which the method may be validated. To evaluate matrix effects, the signal suppression/enhancement (SSE) was calculated for all analytes in each matrix. The matrix effect was studied by comparing the slopes of the calibration curves in solvent and matrix. A 100% SSE value indicates that the matrix does not have any significant effect on the MS signal intensity. The percentage of the difference between these slopes is below 100% in case of signal suppression, wheras above 100% is indicative for signal enhancement. The data in Table 2 shows no signal enhancement in any cases, and ion suppression varies in these matrices. In view of these results, it is confirmed that these ion suppression distributions depend on both analyte and matrix together. Matrix-matched calibration curves for all studied compounds were linear over their respective working range in all sample matrices except for AFG_2 and AFG_1 in dried fish and chilli powder matrices. In these cases, no correlation between the analyte concentrations and peak areas could be established in the matrix matched-standards, although the matrix was found to be blank. Therefore, in this study, AFG_1 and AFG_2 could be detected but not quantified in these food products. For high levels of lipids, pigments and colored materials available in certain commodities (e.g; dried fish and chilli powder), nonpolar solvents such as hexane can be added before purification steps to remove lipid, pigment and colored material constituents. However, our method is based upon a single extraction step and does not intend to replace other established methods for single mycotoxin classes, which are capable to meet the requirements regarding sensitivity due to dedicated sample clean-up. Therefore, for optimization of the method, several different approaches were studied due to the complex nature of the matrices in order to increase recovery rates. Finally, the best results were achieved for an extraction for 60 min using a mixture of acetonitrile: water (80:20, v/v; 0.1% formic acid) in all analytes for the matrices selected.

The method was validated by spiking rice, dried fish, chilli powder and peanuts at three levels in triplicate. Spiking levels were 9, 12, and 15 ng g^{-1} for AFB_1, AFG_1 and OTA, 2.7, 3.6 and 4.5 ng g^{-1} for AFG_2 and AFB_2; 180, 240 and 300 ng g^{-1} for ZEA, T-2, and HT2; and 720, 960 and 1200 ng g^{-1} for DON and fumonisins (FB_1 and FB_2). Satisfactory results in the majority of the cases were obtained for all analytes and matrices, with an acceptable level of accuracy and precision. Recovery values ranged from 82 to 105% for all mycotoxins in the rice and peanuts matrices except for DON in peanuts (69%). The lowest overall recoveries, reaching from 59 to 83% were found in spiked chilli powder. However, low recoveries in dried fish were achieved in the case of AFB_1 (52%), AFB_2 (50%) and OTA (61%). The average recoveries were outside the preferred range of 70% to 100%. However, they were still accepted since it is often not possible to get high recovery rates for all of the chemically diverse analytes when using a generic extraction scheme for different of matrices [20]. The within-day repeatability of the method was satisfactory, with the measured RSD values typically below 15% with very few exceptions. Recovery and repeatability results of optimized analytical procedure are reported in Table 3.

LOD and LOQ were calculated for the sensitivity of the method. The reporting levels of the studied compounds in the different matrices are shown in Table 4. The values obtained for the LOQ are below the corresponding maximum concentrations of mycotoxins in foodstuff permitted in the European Commission [4]. However, higher LOQs were determined for DON, FB_1 and FB_2 (320 ng g^{-1}); whereas the lowest LOQ values were determined for AFB_1, AFB_2, AFG_1, AFG_2, OTA in rice and peanuts, which could be quantified at 0.9 -2 ng g^{-1} level.

To support these data, Figure 1 shows the MRM chromatograms of the spiked rice, peanuts and chilli powder, dried fish samples for all of test compounds at the LOQs concentration level. The retention times are consistent for all of the analytes in a matrix sample. However, it was not possible to avoid the shifting of retention times of the

Analyte	RT (min)	Precursor ion (m/z)	1^{ary} product ion (m/z)	2^{ary} product ion (m/z)	Collision energies (1^{ary}/2^{ary}) (eV)
Aflatoxin B1 (AFB1)	0.99	313.1 $[M+H]^+$	241.10	285.10	40/25
Aflatoxin B2 (AFB2)	0.96	315.1 $[M+H]^+$	259.10	287.10	30/30
Aflatoxin G1 (AFG1)	0.88	329.1 $[M+H]^+$	243.10	311.10	25/20
Aflatoxin G2 (AFG2)	0.85	331.1 $[M+H]^+$	245.10	313.10	30/25
Fumonisin B1 (FB1)	1.23	722.5 $[M+H]^+$	352.30	334.40	45/40
Fumonisin B2 (FB2)	4.41	706.5 $[M+H]^+$	336.20	318.30	35/35
HT-2 toxin (HT-2)	1.60	442.3 $[M+NH_4]^+$	263.20	215.10	10/10
Ochratoxin A (OTA)	4.02	404.2 $[M+H]^+$	239.10	221.10	25/30
T-2 toxin (T-2)	2.35	484.4 $[M+NH_4]^+$	215.10	305.00	25/15
Zearalenone (ZEA)	3.45	319.1 $[M+H]^+$	185.00	187.00	15/20
Deoxynivalenol (DON)	0.72	297.1$[M+H]^+$	249.00	231.0	10/15

Table 1: LC-MS/MS parameters for analytes including retention time (RT), precursor ions, primary (quantifier) and secondary (qualifier) product ions and respective collision energies.

Analyte	SSE (%)			
	Chilli powder	Rice	Peanuts	Dried fish
AFB1	56.56	41.60	36.89	40.26
AFB2	54.56	37.78	53.12	44.00
AFG1	ND	46.15	29.81	ND
AFG2	ND	34.45	26.96	ND
DON	84.67	77.61	29.98	41.85
FB1	90.18	59.00	67.63	71.30
FB2	63.18	63.81	53.26	52.36
HT-2	83.83	63.41	77.28	85.90
OCR	45.80	27.18	41.41	51.78
T-2	59.93	24.23	22.44	24.67
ZEA	70.42	34.74	31.68	52.76

ND: not detected

Table 2: Signal suppression/enhancement (SEE) for selected mycotoxins in different food matrixes.

Analyte	Spiked level (ng g^{-1})	Rice		Peanuts		Chilli powder		Dried fish	
		Avg.Rec (%)	RSD (%)	Avg.Rec (%)	RSD (%)	Avg. Rec (%)	RSD (%)	Avg. Rec (%)	RSD (%)
AFG2	2.7, 3.6, 4.5	102.78	13.59	98.140	6.78	ND	-	-	-
AFG1	9, 12, 15	97.57	2.54	85.079	22.39	ND	-	-	-
AFB2	2.7, 3.6, 4.5	100.84	6.75	97.29	9.75	58.95	13.19	50.20	10.47
AFB1	9, 12, 15	100.29	2.02	100.14	3.16	63.53	4.77	52.09	6.46
OTA	9, 12, 15	99.28	6.14	97.28	5.80	60.59	11.97	60.95	8.47
HT-2	180, 240, 300	105.06	7.40	98.240	7.78	64.20	5.85	93.33	5.83
T-2	180, 240, 300	101.28	8.16	86.43	9.15	71.99	5.44	97.44	3.46
FB2	720, 960, 1200	84.49	8.43	96.22	9.40	82.76	4.64	94.53	5.31
FB1	720, 960, 1200	92.48	4.38	91.97	6.30	81.67	5.51	93.32	5.19
ZEA	180, 240, 300	96.82	7.92	82.05	5.67	64.12	5.70	74.87	5.15
DON	720, 960, 1200	84.24	8.45	69.17	11.78	67.24	18.80	90.65	12.53

Table 3: Average recovery (%) and relative standard deviation (%) of LC-MS/MS method for simultaneous determination of mycotoxins.

Analyte	Rice & Peanuts		Chilli powder & Dried fish	
	LOD	LOQ	LOD	LOQ
AFG2	0.15	0.9	-	-
AFG1	0.5	1	-	-
AFB2	0.15	0.9	0.6	1.2
AFB1	0.5	1	1	3
OTA	0.5	2	1	3
HT-2	40	60	40	80
T-2	40	60	40	80
FB2	80	240	160	320
FB1	80	240	160	320
ZEN	40	60	40	80
DON	80	240	160	320

Table 4: Limits of detection and quantification (ng g-1) of the studied compounds in the different matrices.

targeted mycotoxins among different matrices. The slight differences in retention times observed between different matrices can be explained by the presence of the matrix, which may affect the behavior of the analytes. The maximum deviation in retention time for all of the test compounds between different matrices was less than ± 1.5%, which was considered to be acceptable for this study.

Analysis of real samples

Although food commodities in this research can be very different in composition, our objective was to develop a single method capable of measuring the occurrence of mycotoxins in these different matrices. Therefore, the proposed LC-ESI-MS/MS method was applied in the quantitation of eleven mycotoxins in 80 commercial samples of peanuts, rice, chilli powder and dried fish collected at Serdang and Shah Alam cities, Malaysia. The results showed that 10 rice samples (50%) of a total of 20 analyzed samples were contaminated with at least one aflatoxin at levels above the detection limits (0.15-1.3 ng g^{-1}). 95% and 65% of the peanut samples were contaminated with AFB_1 and AFB_2, concentration varying from 1.44 to 113.28 and 0.49 to 18.15 ng g^{-1}, respectively, whereas 60% of the peanut samples exceeded the MRLs set by European Commission [4] for AFB_1. Among these samples, 17 (85%), 13 (65%) and 18 (90%) out of 20 chilli powder samples were contaminated with AFB_1, AFB_2, and OTA, at levels above detection limits (1.05 - 11.29 ng g^{-1}), respectively. Only one chilli powder sample was contaminated with 48.3 ng g^{-1} of ZEA. Moreover, five dried fish samples (25%) showed the presence of OTA at levels ranging from 1.69 to 1.89 ng·g^{-1}. The findings supported that these foods are easily contaminated with *Aspergillus* species during storage. However, different contamination levels of other mycotoxins were also found in samples though these values were lower than LODs of this study.

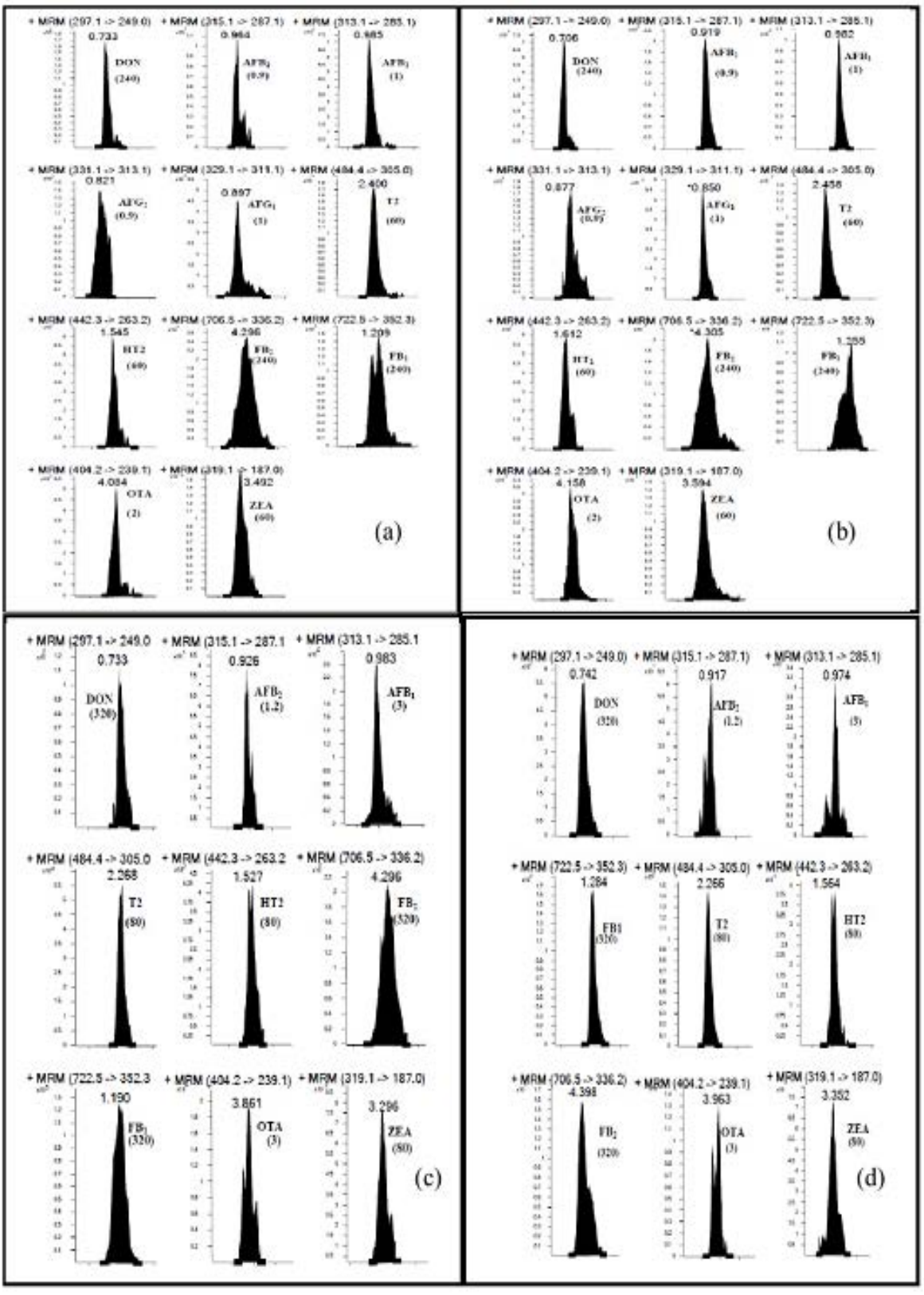

Figure 1: LC-MS/MS chromatograms for the targeted mycotoxins in (a) peanuts, (b) rice, (c) dried fish and (d) chilli powder, at the LOQ level validated (in brackets, expressed in ng g^{-1}).

Conclusion

The proposed method in this study in which the separation time is similar to the reported UHPLC method allows the simultaneous determination of analytes with very different physicochemical properties in a single chromatographic run in less than 5 minutes for four different food commodities. The method described in this study can be employed as a useful tool for contamination monitoring and determination of mycotoxins in food samples. Moreover, due to the straightforward sample prepration procedure, the method is time-saving and cost-efficient. However, this method did not detect AFG_1 and AFG_2 in chilli powder and dried fish samples, so that further development of the method is needed, especially if all the regulatory limits set by the EC need to be achieved. Considering the tropical weather in Malaysia, food products stored under such conditions are very susceptible to aflatoxin contamination. The high incidence of aflatoxins emphasize the need for regular monitoring and more stringent food safety system in order to control the mycotoxin at the lowest possible levels.

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