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Simultaneous Quantification of Aflatoxins, Ochratoxin A and Zearalenone in Cereals by LC-MS/MS

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ABSTRACT

The occurrence of mycotoxins in foods and feeds has long been recognized as a potential hazard for human and animal health due to its severe toxic and carcinogenic properties. Among various mycotoxins, aflatoxins (AFs), ochratoxin A (OTA) and zearalenone (ZON) are most important because of many contamination cases in the world. In this study, a simple and rapid multiresidue method to quantify AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON was developed. The simultaneous determination of these 6 mycotoxins was performed using immunoaffinity column for clean-up and liquid chromatography/electrospray tandem mass spectrometry (LC-MS/MS) for quantification. Cereal samples were extracted with 80% methanol followed by a Vicam AOZ^{TM} immunoaffinity column clean-up. Mycotoxins were eluted from the column with methanol and quantified using Selected Reaction Monitoring (SRM) mode in LC-MS/MS with an electrospray ionization (ESI) interface. A mobile phase of methanol-water was used. Average recoveries of AFs, OTA and ZON spiked in cereals ranged 65 - 95%, 66 - 83% and 69 - 86%, respectively. Good accuracy and precision results were also obtained in intra-day and inter-day analysis. The limit of quantitation (LOQ) of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON were 0.1, 0.2, 0.1, 0.3, 0.03 and 2 ppb, respectively. Thirty-five commercial cereal products including thirteen wheat samples, seven oat samples, six rice samples and nine corn samples were analyzed. The results showed that OTA was detected in four samples ranging from 0.05 to 0.07 ppb, and ZON was detected in two samples ranging from 2.73 to 4.73 ppb. This rapid, easy and highly efficient method was successfully developed. Six common mycotoxins could be analyzed in a single 20-min run. The method could be applied to routine cereal analysis, thus dramatically shortening the analysis time.

Key words: aflatoxin, ochratoxin A, zearalenone, cereals, LC-MS/MS

INTRODUCTION

Mycotoxins are known as carcinogenic, harmful to the health of humans and animals, and even caused huge economic loss[1]. It is necessary to develop a fast, sensitive and reliable analytical method for mycotoxins in cereals. Among the many mycotoxins, aflatoxins (AFs), ochratoxin A (OTA) and zearalenone (ZON) are most important because of their frequent and increasing occurrence in grain. The maximum residue levels of AFs, OTA and ZON have been set by many countries and some international authorities, including European Commission, indicating their importance[2,3].

Aflatoxins, produced by different *Aspergillus* species growing on agricultural commodities, were the first mycotoxins identified as potential health hazard. They were commonly found in areas with hot and humid climates[4]. The four main aflatoxins are AFB_1, AFB_2, AFG_1 and AFG_2. The International Agency for Research on Cancer (IARC) classified AFB_1 as a human carcinogen (Group 1)[5]. The AFs have hepatotoxic, immunosuppressive, teratogenic, mutagenic and carcinogenic effects[1,6]. Legal limits have been set for food and feed in many countries. US Food and Drug Administration has set the limit for total AFs at 20 μg/kg[2], where as the current limit for AFB_1 and total AFs established by European Commission are 2 μg/kg and 4 μg/kg, respectively[3]. Taiwan has set limits for total AFs in peanut and corn at 15 ppb, and other cereals at 10 ppb. In 2007, 32% of dried figs (4,917 samples) destined for export from Turkey to the European Union (EU) contained AFs in the range of 0.2 to 259.46 μg/kg and 9.8% of them exceeded the EU limits[7]. Cavaliere reported that among 48 marketed maize samples, 15 were found contaminated with AFs and 5 did not comply with EU legislation[8].

The next most studied mycotoxin is ochratoxin A (OTA), which was produced by several *Aspergillus* and *Penicillium* species in semitropical and temperate climates. OTA

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occurs in food commodities such as cereals, fruits, wine and coffee. OTA is a potent nephrotoxin and hepatotoxin as well as with teratogenic, mutagenic and carcinogenic effects[9-11]. It has been linked to Balkan Endemic Nephropathy and the development of tumors in the urinary tract in humans[12,13]. The IARC classified OTA as possible carcinogen for humans (Group 2B)[5]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommended the Provisional Tolerable Weekly Intakes (PTWI) for OTA of 100 ng/kg body weight, but the Scientific Committee on Food of the EU recommended a lower daily intake (5 ng/kg)[14,15]. Taiwan has set limit for OTA in rice and wheat at 5 ppb. In the past years, the contamination of foods with OTA worldwide has also been reported. One hundred samples of rice purchased from retail markets in Morocco were surveyed for the presence of OTA. 14 out of 100 samples exceeded the maximum level of 5 ng/g set by EU for OTA in cereals[16]. OTA was determined in 274 samples of dry pasta sold across Canada in 2004 to 2006. Incidence of contamination above 0.5 ng of OTA per g was 21, 18 and 66% in 2004, 2005 and 2006, respectively[17]. Among 60 cereal samples collected from Malaysian markets, 1 wheat and 2 rice samples were contaminated with levels greater than EU regulatory limit for OTA[18].

Zearalenone (ZON) is a mycotoxin produced mainly by fungi of the genus *Fusarium* in foods and feeds. It is frequently implicated in reproductive disorders of farm animals and hyperoestrogenic syndromes in humans[19]. ZON was also shown to be hepatotoxic[20], and may contribute to the increasing occurrence of cancer[21]. In 1993 IARC classified ZON as group 3[5]. ZON production has been reported on grains in the field, during harvest, commercial grain processing and storage[22,23]. It is prevalent in many cereals such as wheat, barley, oat, sorghum, corn and rice. In the past years, the contamination of foods with ZON has been reported in many countries and the levels varied between ppb and ppm[19,23]. Currently many countries have made specific regulations for ZON in foods with maximum tolerated levels ranging from 20 to 1000 ppb[19]. EU has the regulatory limits as follows: 200 ppb for unprocessed maize, 100 ppb for unprocessed cereals other than maize, 50 ppb for cereal snacks and breakfast cereals, 20 ppb for processed cereal-based foods and baby foods for infants and young children[24]. Besides, the tolerable intakes have also been estimated by JECFA as 0.5 μg/kg b.w./day[25]. Up to now, Taiwan has not set any regulatory limits for ZON.

Mycotoxins are commonly extracted from ground solid matrices by blending with aqueous methanol or aqueous acetonitrile. Purification of extracts is an important step in mycotoxin analysis, especially for the determination of trace levels. Solid-phase extraction (SPE) and immunoaffinity columns (IAC) are the most frequently used. Current analytical methods for mycotoxin include Enzyme-linked immunosorbent assay[26], thin-layer chromatography[27], gas chromatography[28], high-performance liquid chromatography[18,29] and liquid chromatograph-tandem mass spectrometry[8,30]. HPLC has been the main analytical method to determine mycotoxin in the past 20 years. But there were some disadvantages using HPLC such as time-consuming, matrix effect and derivatization required. LC-MS/MS is the most powerful tool for mycotoxin analysis because of its high sensitivity and selectivity. Moreover, simultaneous determination of mycotoxins can be achieved by LC-MS/MS. Several mycotoxins could be detected in a single run, which was suitable for high-throughput screening for mycotoxin analysis. The multiresidue method is quite important because co-occurrence of several mycotoxins was frequently found. LC-MS/MS provides reliable, selective, and quantitative data for multi-toxin, and thus can be applied in routine analysis.

The objective of this study was to develop a sensitive, accurate and reliable method using immunoaffinity column for clean-up and LC-MS/MS for simultaneous quantification of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON in cereals in a single run. In addition, the established method was applied to analyze these 6 mycotoxins contamination in cereal samples in Taiwan. The information is necessary and of high priority in order to protect the consumers from the risk of exposure to these toxins.

MATERIALS AND METHODS

I. *Materials*

(I) *Sample Collection*

Thirty-five commercial cereal samples were collected from supermarkets in Taiwan, including 13 wheat samples, 7 oat samples, 6 rice samples and 9 corn samples. The minimum sample size was 200 g.

(II) *Chemicals and Reagents*

The PBS buffer with 0.01% Tween-20 and immunoaffinity columns AOZ™ specific for AFs, OTA and ZON were purchased from Vicam (Watertown, MA, USA). Potassium chloride (reagent grade) was from J. T. Baker (Phillipsburg, NJ, USA). Acetic acid and formic acid were from Sigma-Aldrich (Hamburg, Germany). Acetonitrile and methanol (LC grade) used for the liquid chromatographic mobile phases were purchased from Merck (Darmstadt, Germany). Milli-Q plus water (Millipore, Bedford, MA, USA) was used throughout this study. AFs, OTA and ZON standard stock solutions of 1, 50 and 50 μg/mL, respectively, were acquired from Supelco (Bellefonte, PA, USA). The working standard solution (0.1 μg/mL for all) was prepared by diluting the stock solution in methanol.

II. *Methods*

(I) *Sample Preparation*

Cereal samples were milled before extraction. Five grams of test portion were weighted into a 50-mL centrifuge

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tube and extracted with 25 mL of 80% methanol. The mixture was blended and shaken for 30 min, and then centrifuged for 5 min at 3,000 rpm. The supernatant was filtered through Whatman No.1 filter paper. Ten mL filtrate was mixed with 10 mL PBS (containing 0.01% Tween-20).

(II) *Immunoaffinity Clean-Up*

Ten milliliter of filtrate (equivalent to 1 g matrix) was passed through an AOZ^{TM} immunoaffinity column at about 1 - 2 drops/s. Ten mL PBS (containing 0.01% Tween-20) was then passed through the AOZ^{TM} immunoaffinity column at the same speed. Ten mL water was used to wash the loaded immunoaffinity column at a steady flow rate. Mycotoxins were eluted with 1 mL methanol, followed by 1 mL water containing 0.1% acetic acid. The eluate was filtered through a 0.22 μm microfilter and collected in a clean vial for the subsequent LC-MS/MS analysis.

(III) *LC-MS/MS Analysis*

LC-MS/MS analysis was performed on a TSQ Ultra MS system (Thermo Electron Co., MA, USA) equipped with a Surveyor Plus LC pump (Model 68649), a Surveyor Plus autosampler (Model 76598) and an electrospray ionization (ESI) interface. Data acquisition was performed using an Xcalibur software system. Chromatographic separation was achieved using a XBridge^{TM} C18 column (2.1 × 150 mm, 3.5 μm, Waters, USA). Injection volume was 25 μL. The mobile phase, methanol-water (containing 0.1% formic acid), was pumped in at a flow rate of 0.3 mL/min. The ESI interface was operated in the positive ion mode for AF and OTA, negative ion mode for ZON. The parameters for ESI operation were as follows: capillary voltage, 4 kV; capillary temperature, 350°C; source CID, 2 mTorr; sheath gas pressure, 49 psi; ion gas pressure, 2 psi; scan width, 0.01 m/z; scan time, 0.05 s. Tube lens of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON were 139, 59, 108, 108, 111 and -171, respectively.

Quantitative determination of all compounds was carried in the selective reaction monitoring (SRM) mode. The molecular ions and fragments used are given in Table 1. The precursor ions of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON were 313, 315, 329, 331, 404 and 317 m/z, respectively. The product ions for quantitation of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON were 241, 243, 200, 245, 239 and 131 m/z, respectively. Collision energy of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON was 48, 44, 50, 40, 45 and 35 V, respectively.

(IV) *Validation of the Method*

Linearity of this method was verified by analyzing five standard solutions in the range of 0.05 - 10 ppb for AFB_1, 0.1 - 15 ppb for AFB_2, 0.05 - 10 ppb for AFG_1, 0.2 - 15 ppb for AFG_2, 0.01 - 2 ppb for OTA and 1 - 50 ppb for ZON. Each standard was analyzed in triplicate.

The intra-day precision and accuracy were determined at three various levels of standard solution in three replicates each. The inter-day precision and accuracy were determined at three levels of standard solution on three independent occasions. The levels of each mycotoxin were as follows: 0.05, 1, 10 ppb for AFB_1; 0.1, 3, 15 ppb for AFB_2; 0.05, 1, 10 ppb for AFG_1; 0.2, 3, 15 ppb for AFG_2; 0.01, 0.1, 2 ppb for OTA; 1, 5, 50 ppb for ZON. The precision was calculated as the relative standard deviation of the mean (RSD) with RSD (%) = (standard deviation of the mean / mean) × 100. The accuracy was calculated as the relative mean error (RME) with RME (%) = [(mean concentration – theoretical concentration) / theoretical concentration] × 100.

The recovery test was performed by adding known amounts of AFs, OTA or ZON standard to the sample. The actual and measured concentrations were then compared. Wheat, oat, rice and corn samples were spiked with AFs, OTA or ZON standard in triplicate. The concentrations of each spiked mycotoxin were as follows: 0.1, 1, 10 ppb for AFB_1; 0.2, 2, 10 ppb for AFB_2; 0.1, 1, 10 ppb for AFG_1; 0.3, 3, 10 ppb for AFG_2; 0.03, 0.5, 2 ppb for OTA; 2, 10, 50 ppb for ZON. The detection and quantification limits were determined as the concentration with peak area ratio of signal to noise (S/N ratio) no less than 3 and 10, respectively.

RESULTS AND DISCUSSION

I. *Method Optimization*

In this study, we compared different LC and MS

Table 1. Parent ions, daughter ions and collision energy for the determination of 6 mycotoxins

Mycotoxin	Precursor ion	Q1 (*m/z*)	Q3 (*m/z*)	CE (V)
AFB_1	$[AFB_1 + H]^+$	313	285	60
			241*	48
			213	33
AFB_2	$[AFB_2 + H]^+$	315	287	30
			259	43
			243*	44
AFG_1	$[AFG_1 + H]^+$	329	243	39
			215	44
			200*	50
AFG_2	$[AFG_2 + H]^+$	331	313	35
			245*	40
			217	19
OTA	$[OTA + H]^+$	404	358	21
			239*	45
			221	32
ZON	$[ZON – H]^+$	317	273	33
			175	36
			131*	35

*Transitions used for quantitation.
Q1 First quadrupole; Q3 third quadrupole; CE: collision energy.

conditions in order to find a simple and sensitive method suitable for the simultaneous determination of 6 mycotoxins. Initially various mobile phases were tested in an attempt to obtain the best resolution for AFs, OTA and ZON. The solvent system used was (A) methanol and (B) water. The best linear gradient program we found was the following: 0 min, (A) 50% and (B) 50%; 9 min, (A) 90% and (B) 10%. Hold (A) 90% and (B) 10% from 9 to 12 min, then hold (A) 50% and (B) 50% from 12 to 20 min. It is better to use methanol rather than acetonitrile as mobile phase A due to its higher resolution. The higher resolution also resulted from addition of 0.1% formic acid into water because of the increase of polarity. The most appropriate separation of 6 mycotoxins was achieved by using a Waters XBridge^{TM} C18 column at a flow rate of 0.3 mL/min compared to phenomenex Gemini-NX column, Biosil SP-ODS column, and Waters XBridge^{TM} Shield RP18 column. Using the conditions mentioned above, sharp peaks corresponding to AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON can be obtained at retention time of 3.4, 2.9, 2.6, 2.3, 10.6 and 10.3 min, respectively (Figure 1). The retention time was much shorter than that in the literatures[8,30]. Lattanzio *et al*. also developed simultaneous method of AFs, OTA and *Fusarium* toxins in maize by LC-MS/MS after multitoxin immunoaffinity cleanup[31]. The retention time of AFs, OTA and ZON were approximately 20, 40 and 50 min, respectively using the LC condition they developed. The retention time in this study was shorter compared to that in the literature[31], indicating this is an effective analytical method.

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were two popular ion source used in LC-MS/MS analysis. In this study, we found that better performance can be achieved using ESI rather than APCI because of the high-polarity of target compounds. ESI was also used in the literatures[30,32,33]. The ESI interface was operated in the positive ion mode for AFs and OTA, negative ion mode for ZON. We found better sensitivity of ZON by negative ion mode. In agreement with the literature, the negatively charged deprotonated molecular ion of ZON was found to be more abundant than the positively charged protonated ones[34]. The precursor and product ions we selected in this study were almost the same with those in the literatures[32,35,36]. In this study, 3 product ions were selected

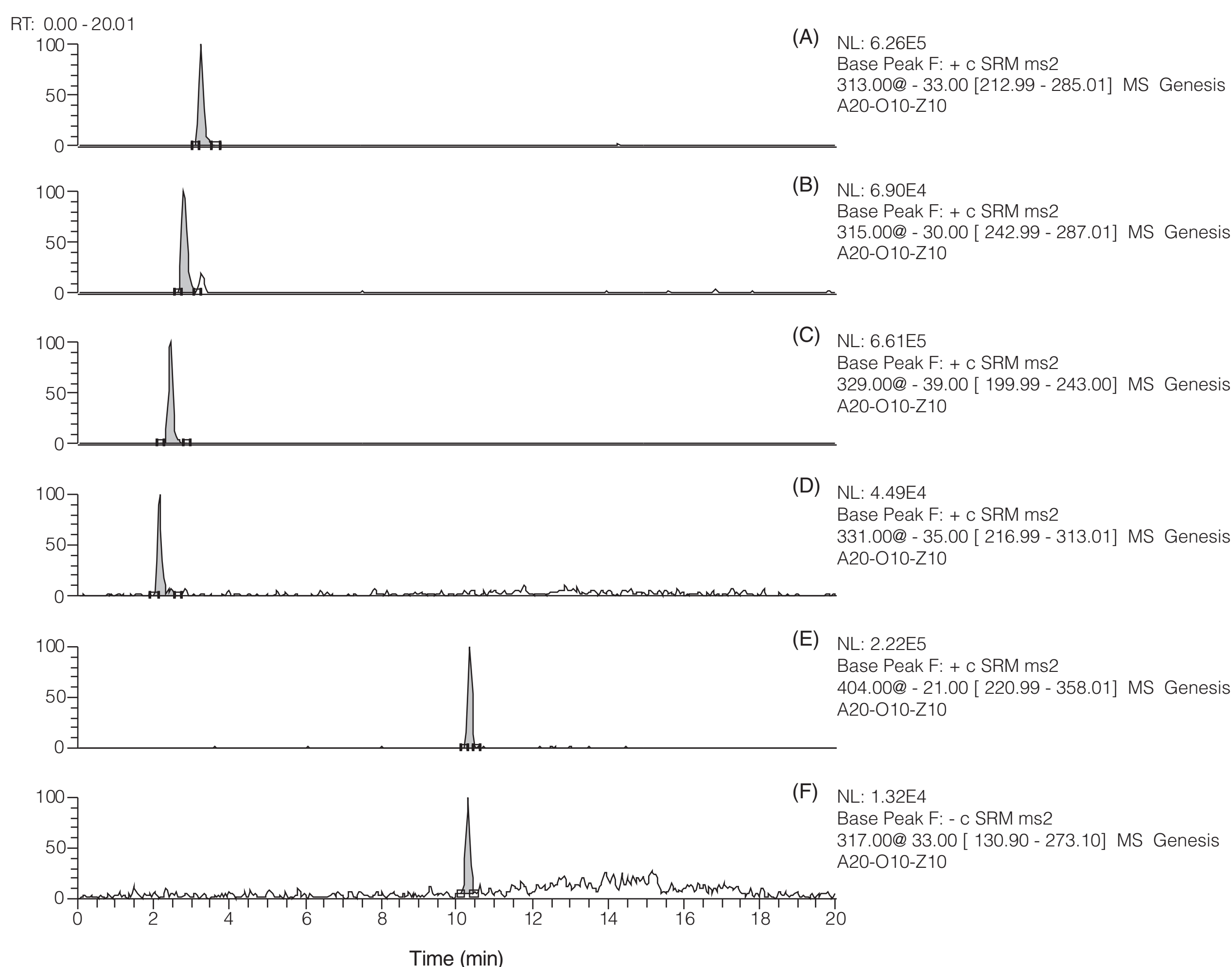

Figure 1. LC-MS/MS chromatogram of a wheat sample spiked with 6 mycotoxins. (A) AFB_1 50 ppb (B) AFB_2 15 ppb (C) AFG_1 50 ppb (D) AFG_2 15 ppb (E) OTA 25 ppb (F) ZON 25 ppb.

for each compound in SRM mode. The strongest product ion was used for quantitation. The optimum parameters such as capillary voltage, capillary temperature, gas pressure and collision energy were found and mentioned in Materials and Methods.

II. *Method Validation*

The proposed method for simultaneous determination of 6 mycotoxins was validated for the linearity, limit of detection/quantitation, precision, accuracy and recovery.

(I) *Linearity*

Standard curves were made in triplicate for each concentration in LC-MS/MS analysis. A good linearity was achieved in the concentration of 3, 6, 12.5, 25, 50 and 100 ppb (Figure 1). The regression equation and correlation coefficient of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON were y = 357568x + 43923 (R^2 = 0.9994), y = 61192x − 9265.5 (R^2 = 0.997), y = 388348x + 53030 (R^2 = 0.9985), y = 34578x + 13935 (R^2 = 0.9934), y = 953593x + 10343 (R^2 = 0.9998) and y = 8174.4x − 1698.1 (R^2 = 0.9992), respectively. All correlation coefficients (R^2) were above 0.995, indicating good linearity.

(II) *Limit of Detection (LOD) and Limit of Quantitation (LOQ)*

The LOD and LOQ were determined as the concentration with peak area ratio of signal to noise (S/N ratio) no less than 3 and 10 respectively. The LOD of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON were 0.05, 0.1, 0.05, 0.2, 0.01 and 1 ppb respectively. The LOQ of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON were 0.1, 0.2, 0.1, 0.3, 0.03 and 2 ppb respectively (Table 2). The LOQ of this method was lower than those reported in the literatures[8,35,36]. The LOD of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON were 0.5, 1.0, 1.0, 0.5, 1.0 and 10 ppb in maize slurry[35], respectively, which were much higher than those obtained in our study, indicating that this developed method has good detection limit capable of trace analysis.

There are several types of mass analyzers, including time-of-flight (TOF), Orbitrap and tandem mass. Zachariasova *et al.* used U-HPLC-orbitrapMS to analyze multiple mycotoxins in beer[37]. The LOD of AFB_1, AFB_2, AFG_1, AFG_2 and OTA were 0.5, 0.5, 1, 1 and 60 ppb, respectively, much higher than those obtained here using triple quadrupole tandem mass. It seems that triple quadrupole tandem mass has better detection limit.

The action limits of total AFs, OTA and ZON in food and feedstuff set by different countries were approximately 4 - 20, 2 - 50 and 20 - 1000 ppb, respectively. This method provided excellent sensitivity in the low ppb range which was well below the present guideline and maximum residue levels.

Table 2. Linear regression, LOD and LOQ obtained by LC-MS/MS

	Linear range (ppb)	R^2	LOD (ppb)	LOQ (ppb)
Aflatoxin B_1	0.05 - 10	0.9994	0.05	0.1
Aflatoxin B_2	0.1 - 15	0.9970	0.1	0.2
Aflatoxin G_1	0.05 - 10	0.9985	0.05	0.1
Aflatoxin G_2	0.2 - 15	0.9934	0.2	0.3
Ochratoxin A	0.01 - 2	0.9998	0.01	0.03
Zearalenone	1 - 50	0.9992	1	2

LOD: limit of detection; LOQ: limit of quantitation.

(III) *Precision and Accuracy*

The precision of this method, expressed as % RSD, and the accuracy expressed as % RME, were determined by analyzing three different concentrations of different mycotoxin standard solutions in three replicates. The inter-day precision and accuracy were determined at three different levels of concentrations on three independent occasions. The results were summarized in Table 3. The precision and accuracy were satisfactory with an overall value < 15% for all samples. It indicated high precision with RSD. of intra-day and inter-day test ranging from 0.9 to 11.1% and from -10.5 to 10.3%, respectively. Besides, it also showed high accuracy with RME of intra-day and inter-day test ranging from 1.8 to 10.7% and from -10.0 to 12.5%, respectively. Validation data such as precision and accuracy results were not shown in most research papers regarding mycotoxin analysis. Good precision and accuracy was obtained in this study compared to the literature[35]. Spanjer *et al.* reported that RSD of AFs, OTA and ZON were 17 to 21, 15 and 17%, respectively, which were higher than those in this study.

(IV) *Recovery*

This LC-MS/MS method was validated for different matrices, including wheat, oat, rice and corn. In order to control the validity of the method, recovery test was performed. The recovery was determined by measuring the concentration of spiked samples. To determine the average recovery of the complete method, AFs, OTA and ZON standard solutions were added to blank wheat, oat, rice and corn samples and the extracts were cleaned up using an immunoaffinity column (n = 3). The concentrations of spiked mycotoxin were from 0.03 to 50 ppb, different with each mycotoxin. As shown in Table 4, average recoveries of AFs, OTA and ZON from cereals spiked standard ranged from 65 - 95%, 66 - 83% and 69 - 86%, respectively. Higher recoveries (>80%) could be achieved when spiked level is high. The coefficient of variation (CV) of all the data were below 11% and most of them were below 5%, indicating good stability. Among 4 matrixes, higher recoveries in wheat and rice were found than those in oat and corn. Among 6 mycotoxins, lower recoveries were found in AFG_2 and OTA compared to others.

Table 3. Precision and accuracy data for the determination of 6 mycotoxins

Concentration (ppb)	Intra-day accuracy and precision			Inter-day accuracy and precision		
	Mean ± SD	RSD (%)	RME(%)	Mean ± SD	RSD(%)	RME(%)
Aflatoxin B_1						
0.05	0.046 ± 0.005	2.7	-7.0	0.054 ± 0.005	2.7	7.3
1	0.938 ± 0.065	6.1	-6.1	1.019 ± 0.062	5.4	1.9
10	9.896 ± 0.220	2.2	-1.0	9.855 ± 0.417	4.2	-1.5
Aflatoxin B_2						
0.1	0.108 ± 0.011	10.2	8.0	0.112 ± 0.012	10.7	12.0
3	3.052 ± 0.069	2.4	1.7	3.110 ± 0.101	3.4	3.7
15	15.231 ± 0.332	2.2	1.5	15.366 ± 0.510	3.3	2.4
Aflatoxin G_1						
0.05	0.045 ± 0.005	2.8	-9.1	0.045 ± 0.010	7.0	-10.0
1	0.964 ± 0.033	3.0	-3.6	0.939 ± 0.060	6.2	-6.1
10	9.816 ± 0.086	0.9	-1.8	10.241 ± 0.638	6.7	2.4
Aflatoxin G_2						
0.2	0.179 ± 0.064	11.1	-10.5	0.222 ± 0.042	6.7	11.2
3	3.156 ± 0.270	7.6	5.2	3.372 ± 0.232	6.1	12.4
15	14.440 ± 0.693	4.7	-3.7	14.262 ± 1.038	7.1	-4.9
Ochratoxin A						
0.01	0.010 ± 0.002	10.0	4.2	0.009 ± 0.002	9.7	-6.5
0.1	0.104 ± 0.006	5.3	3.7	0.103 ± 0.006	5.3	2.9
2	1.954 ± 0.046	2.3	-2.3	1.945 ± 0.074	3.8	-2.8
Zearalenone						
1	1.103 ± 0.058	6.4	10.3	1.125 ± 0.079	8.6	12.5
5	5.207 ± 0.255	5.1	4.1	5.363 ± 0.260	5.0	7.3
50	50.036 ± 0.685	1.4	0.1	48.951 ± 0.870	1.8	-2.1

The precision was calculated as the relative standard deviation of the mean (RSD) with RSD (%) = (standard deviation of the mean / mean) × 100. The accuracy was calculated as the relative mean error (RME) with RME (%) = [(mean - theoretical concentration)/ theoretical concentration] × 100.

Overall, the recovery results were satisfying.

This developed method with methanol extraction and clean-up procedure could be used for reliable analysis of AFs, OTA and ZON in cereal samples. Recently, solid-phase extraction (SPE) cartridges and antibody-based immunoaffinity columns (IAC) have become popular in clean-up for mycotoxin analysis. They are selective and time-saving. We found that AOZ^{TM} immunoaffinity column is a suitable tool for purifying AFs, OTA or ZON-contaminated cereal samples. The specificity of IAC should be higher than that of SPE. IAC are commercially available for AFs, OTA and ZON. The advantages of IAC are rapid clean up, provision of clean extracts due to the specificity of the antibody and applicability to complex matrices. Klotzel *et al*. compared the performance of IAC and SPE (Mycosep 227) for the determination of 12 type A and B trichothecenes in various kinds of cereals(30). The results showed that IAC had higher recoveries than Mycosep 227. The author analyzed ten naturally contaminated samples with both methods and determined the toxin content by LC-MS/MS. Higher values (23 to 57%) using IAC method were obtained in all cases, indicating better performance than SPE.

This reliable LC-MS/MS multi-toxin method could be applied to routine survey. High throughput mycotoxin analysis could be achieved since these 6 compounds could be detected simultaneously in less than 11 min. Compared to traditional HPLC method, there are many advantages of this LC-MS/MS method, such as high sensitivity, no derivatization required, simultaneous analysis and confirmation provided(38). By traditional HPLC method, each mycotoxin may need a single 20-min run and it may take 60 min to analyze AFs, OTA and ZON in total. By this LC-MS/MS multi-toxin method, the analysis time could be shortened from 60 to 20 min. The sample extraction and clean-up time could also be shortened by simultaneous treatment. The amount of solvent needed in analysis also greatly decreased.

Table 4. Recoveries of 6 mycotoxins in different samples

Spike level (ppb)	Recovery ± SD (%)			
	Wheat	Oat	Rice	Corn
Aflatoxin B_1				
0.1	74.64 ± 3.79	72.95 ± 6.09	85.33 ± 7.90	72.35 ± 8.40
1	82.31 ± 3.85	73.73 ± 4.54	86.85 ± 10.68	73.74 ± 4.65
10	86.84 ± 2.29	81.33 ± 3.72	86.48 ± 6.06	78.03 ± 3.60
Aflatoxin B_2				
0.2	88.31 ± 1.73	85.73 ± 1.63	87.44 ± 2.45	81.22 ± 1.41
2	85.24 ± 2.60	79.18 ± 2.51	84.52 ± 4.28	75.55 ± 3.20
10	87.03 ± 4.36	82.28 ± 4.52	88.61 ± 5.80	80.37 ± 4.05
Aflatoxin G_1				
0.1	80.81 ± 5.09	79.45 ± 5.61	84.05 ± 8.14	75.67 ± 3.11
1	87.79 ± 2.12	80.28 ± 3.65	92.81 ± 4.77	79.42 ± 3.07
10	94.14 ± 3.25	82.41 ± 5.51	94.73 ± 5.89	80.33 ± 4.15
Aflatoxin G_2				
0.3	73.38 ± 3.49	65.19 ± 7.76	73.38 ± 3.49	64.56 ± 5.26
3	76.92 ± 4.14	72.01 ± 3.44	75.22 ± 3.99	65.68 ± 3.56
10	83.31 ± 5.37	77.13 ± 5.35	75.89 ± 6.58	73.07 ± 7.54
Ochratoxin A				
0.03	72.63 ± 5.23	69.66 ± 7.35	72.84 ± 7.33	65.58 ± 1.88
0.5	77.32 ± 5.35	75.35 ± 6.90	76.10 ± 7.38	67.77 ± 4.62
2	82.63 ± 3.41	76.51 ± 3.24	77.93 ± 5.27	74.99 ± 2.24
Zearalenone				
2	79.24 ± 5.41	79.01 ± 5.42	74.86 ± 3.84	68.89 ± 6.26
10	85.57 ± 2.47	82.78 ± 4.14	83.38 ± 6.32	75.27 ± 4.33
50	80.25 ± 6.85	82.23 ± 6.05	81.28 ± 9.47	76.64 ± 3.13

In addition, detection limit was improved using LC-MS/MS multi-toxin method compared to traditional HPLC or LC-MS. The LOQ of AF using traditional HPLC were 0.2 - 1.07 ppb[39,40], higher than that in this study. Without derivatization process as in HPLC method, lower detection limit was achieved using this developed LC-MS/MS method. The LOQ of OTA in cereals using traditional method were 0.3 - 0.75 ppb[41,42], much higher than that in this developed method. The LOQ of ZON in cereals using traditional method were 10 - 25 ppb[43,44], higher than that in this study. Overall, the LC-MS/MS multi-method had many advantages and can thus be applied to routine mycotoxin analysis.

III. *Survey Results*

Presence of AFs, OTA and ZON in cereals is a hazard to human health in the world so that surveys have been done in many countries[7,8,16-18]. The developed method in this study was applied for the determination of AFB_1, AFB_2, AFG_1, AFG_2, OTA and ZON in 35 commercial samples collected from supermarkets in Taiwan. No AFs was detected in 35 cereal samples. OTA was detected in 3 of 13 (23.1%) wheat samples and 1 of 7 (14.3%) oat samples with OTA levels ranging from 0.05 to 0.07 ppb (Table 5), which were below the maximum permissible level of 5 ppb set by Department of Health in Taiwan. In addition, no OTA was detected in 6 rice samples and 9 corn samples. Overall, OTA was present in 4 of 35 (11.4%) cereal samples, and the OTA levels were very low compared to those reported in the literatures[16-18]. The LC-MS/MS chromatogram of one of OTA-contaminated wheat samples was shown in Figure 2 (A).

ZON was present in 1 of 6 (16.7%) rice samples and 1 of 9 (11.1%) corn samples with ZON level ranging from 2.73 to 4.73 ppb (Table 3). In addition, no ZON was detected in 13 wheat samples and 7 oat samples. Overall, ZON was present in 2 of 35 (5.7%) cereal samples, and the ZON levels were much lower than the maximum contents in cereals recommended by most countries. The LC-MS/MS chromatogram of one of ZON-contaminated corn samples was shown in Figure 2 (B).

Table 5. Survey results of AFs, OTA and ZON in different cereal samples

Samples	No. of samples	No. of AF-positive samples	No. of OTA-positive samples (%)	No. of ZON-positive samples (%)
Wheat	13	0	3 (23.1)[1]	0
Oat	7	0	1 (14.3)[2]	0
Rice	6	0	0	1 (16.7)[3]
Corn	9	0	0	1 (11.1)[4]
Total	35	0	4 (11.4)	2 (5.7)

[1]OTA content was 0.05 - 0.06 ppb.
[2]OTA content was 0.07 ppb.
[3]ZON content was 2.73 ppb.
[4]ZON content was 4.73 ppb.

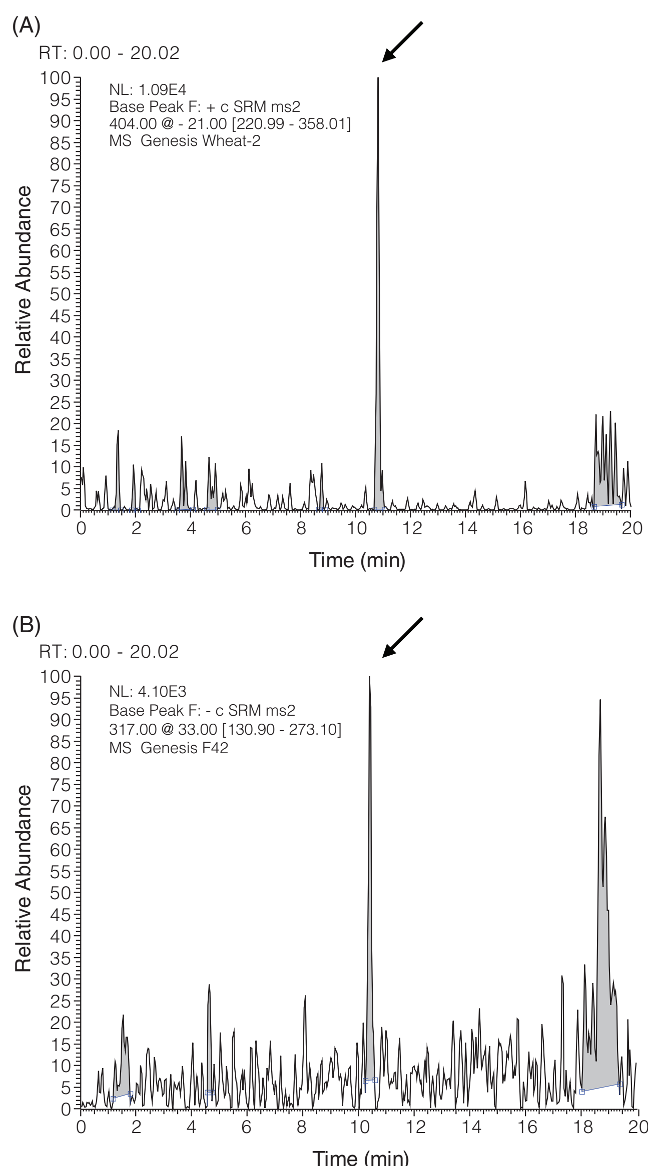

Figure 2. LC-MS/MS chromatograms of (A) OTA-contaminated wheat sample (0.06 ppb) and (B) ZON-contaminated corn sample (4.73 ppb).

According to the "Nutrition and Health Survey in Taiwan 1993 - 1996", the daily intake of starch from roots and tubers were 21.19 and 28.96 g for adult male and female, respectively. The average body weight of adult male and female was 64.3 and 54.5 kg, respectively. Based on the highest ZON level of 4.73 ppb found in this survey, the daily possible ZON intake was estimated as follows:

(The intake sum of starch from roots and tubers) × ZON level / average body weight

The results were 0.00156 and 0.00251 (μg/kg bw/day) for adult male and female, respectively. The ZON intakes were much lower than the tolerable intakes estimated by JECFA (0.5 μg/kg bw/day), indicating that ZON should not cause high risk to Taiwanese.

In addition, based on the highest OTA level of 0.07 ppb found in this survey, the weekly possible OTA intake was estimated as follows:

(The intake sum of starch from roots and tubers) × OTA level / average body weight

The results were 0.000161 and 0.000259 (μg/kg bw/week) for adult male and female, respectively. The OTA intakes were also much lower than the tolerable intakes estimated by JECFA (0.1 μg/kg bw/week), indicating that OTA should not cause high risk to Taiwanese.

CONCLUSIONS

The occurrence of mycotoxins in cereals is an emerging issue, and therefore a multi-mycotoxin LC-MS/MS method was developed and validated. The developed method is appropriate for the analysis of 6 common mycotoxins in cereals. The preparation procedure is quick and easy. The LC-MS/MS method in Selective Reaction Monitoring (SRM) detects all compounds in a single run with limits of quantitation (LOQ) between 0.03 and 2 ppb, which was far below regulated levels in different countries. The method was validated for the analysis of wheat, oat, rice and corn samples. It showed good linearity, accuracy, precision and recoveries. Six common mycotoxins could be analyzed in a single 20-min run. The method could be applied to routine cereal analysis. Thirty-five commercial cereal products in Taiwan were analyzed by this developed method. The results showed that OTA was detected in four samples ranging from 0.05 to 0.07 ppb, and ZON was detected in two samples ranging from 2.73 to 4.73 ppb.

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