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Article

A Novel Method for Multiple Mycotoxins Analysis in Feedstuffs by Flow-Through Cartridge Purification and Ultra-High-Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry

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Abstract: A novel determination method using flow-through cartridge purification and ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) was developed for the identification and quantification of 30 mycotoxins (aflatoxin B_1, B_2, G_1, G_2, sterigmatocysin, T-2 toxin, diacetoxyscirpenol, roquefortine C, methylergonovine, ergocornine, lysergol, enniatin A, enniatin A_1, enniatin B, enniatin B_1, beauvericin, deoxynivalenol, 3-acetylDeoxynivalenol, 15-acetylDeoxynivalenol, patulin, verruculogen, neosolaniol, gliotoxin, HT-2 toxin, wortmannin, zearalenone, α-zearalenol, β-zearalenol, α-zearalanol, and β-zearalanol) in feedstuffs. Mycotoxins were extracted from sample by 0.1% formic acid aqueous solution/acetonitrile (16/84 v/v), then purified by a Cleanert® MC clean-up column. As a result, the pigments and other matrices were efficient removed from the sample extract solution. The purified eluate was collected, then evaporated and redissolved by 0.1% formic acid aqueous solution/acetonitrile (70:30 v/v) for UPLC-MS/MS detection. A BEH Shield RP_{18} chromatographic column was employed for separation of 30 mycotoxins in a total of 14 min. Multiple-reaction monitoring (MRM) mode was applied for qualitative and quantitative analysis, and matrix calibration curves obtained with the external-standard method was used for quantitation of target analytes. Under optimized conditions, the linearity range was from 2 to 1000 ng/g, and the limit of quantification of the developed method was from 2 to 50 ng/g. The recoveries of 30 mycotoxins spiked in urine samples were from 72.0% to 118.5%, and the relative standard deviation was below 20%. The method was also well approved by certified reference sample, and applied on the real feedstuff samples testing successfully.

Keywords: UPLC-MS/MS; mycotoxins; feedstuffs; flow-through; multiple analysis

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1. Introduction

Mycotoxins are a chemically diverse group of toxic secondary metabolites generated by fungi[1-2]. So far, there are 300-400 mycotoxins with identified chemical structures have been known [3], and they are mainly produced by fungus species of *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, and *Claviceps* [4]. Mycotoxins contamination may occur both in the field and during storage[5]. Cereals and cereal byproducts as important ingredients in animal feed are very subject to contamination by various mycotoxins, witch can cause acute or chronic poisoning in animals [6]. Clinical symptoms of mycotoxins intoxication include diarrhoea, vomiting, liver and kidney damage, pulmonary oedema, haemorrhaging and tumours [7, 8, 9]. Generally, under common conditions, mycotoxins usually occur in low concentrations leading to reduced animal performance and/or immune suppression without causing any obvious clinical symptoms [10, 11]. Regarding to animal, immune suppression can result in increased susceptibility to diseases and parasites to overt disease and death [12]. So, it is important to emphasize that multiple mycotoxins occur together at low concentrations [13]. This is not only due to the ability of various fungi to simultaneously produce a variety of mycotoxins, but also due to the fact that any given commodity is almost always infected by several different fungal species at once [14].

Although, only a very limited number of mycotoxins is subject to legal guidance and regular monitoring in animal feed industry, mainly includes afatoxins, deoxynivalenol, zearalenone, ochratoxin A, T-2 toxin and fumonisins. Considering the fact that many different mycotoxins are usually contaminated simultaneously in feedstuffs and cause synergistic or additive interactions [15], there is an increased concern of more information on all mycotoxins present in feed raw materials and finished feeds, even currently without limit regulations and at levels well below the legal limits [16]. Moreover, the occurrence of masked mycotoxins (mycotoxins covalently or non-covalently bound to matrix component), such as acetylated deoxynivalenol, have been gotten more and more attention for their bioavailability [17, 18].

High performance liquid chromatography coupled with mass spectrometry, especially tandem mass spectrometry (LC-MS/MS) is a valuable tool for obtaining an accurate picture of mycotoxins contamination pattern in feed and feed raw materials [19]. It can provide the highest sensitivity and specificity, detecting low levels of mycotoxins in various samples, reducing sample preparation and analysis time. This method has been widely applied in the determination of multi-mycotoxins in a large variety of samples, including grain [19, 20], spice [21], food [22], feed [23], beer [24], and urine [25, 26, 27]. The determination of multi-mycotoxins in feedstuff is a difficult task because the feed material is always a quite complex matrix, and the analysis requires the application of previous clean-up step in order to remove matrix substances. The purpose of this study is to develop and validate a novel fast multi-mycotoxin UPLC-MS/MS method for the simultaneous detection of 30 mycotoxins being parts of various species (including *Aspergillus*, *Fusarium*, *Penicillium,* and *Claviceps*) and its application in the analysis of naturally contaminated feedstuff samples.

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2. Materials and Methods

2.1 Apparatus

TQ UPLC-tandem mass spectrometer and Acquity BEH Shield RP_{18} column (100 mm × 2.1 mm, 1.7 μm) (Waters, USA), RVC 2-18 desktop vacuum centrifugal concentrator (CHRIST Inc., Germany), 3K15 high speed refrigerated centrifuge (Sigma, USA), D37520 high-speed centrifuge (Kendro Laboratory Products, Inc., USA), VX-ⅢImulti-tube vortexer (Beijing Targin Technology Co., Ltd, China) were used in this experiment.

2.2 Chemicals and Reagents

The information of certified analytical standards for the mycotoxins and the mixture stock solutions diluted by HPLC grade acetronitrile are listed in **Table 1**. The mixture stock solutions were subdivided in two different groups of concentration levels, according to their sensitivity in UPLC-MS/MS, and stored in amber vial keeping in a freezer at –20 °C. When it was used, an appropriate amount of solution was dried with the vacuum centrifugal concentrator and then dissolved in 0.1% formic acid aqueous solution/acetronitrile (70:30 v/v) to prepare mixture working solutions of different concentrations. Ultrapure water was obtained from a MilliQ Gradient System (Merck Millipore, China). Acetonitrile, methanol, ammonium acetate, and formic acid of high performance liquid chromatography grade were purchased from Fisher Scientific (USA). A commercial flow-through cartridge Cleanert® MC clean-up column was purchased from Bonna-Agela Technologies Company (China). Mycotoxins-free corn, wheat and cottonseed meal samples were kindly donated by the National Feed Quality Control Center (Beijing). The certificated reference corn sample naturally contaminated with multi-mycotoxins was obtained from Trilogy Analytical Laboratory (USA).

2.3 Sample Preparation

The feedstuff samples were kept in a dark, cool room at 4 ℃ until analysis. A 5-g ground sample of feedstuff and 20 mL of 0.1% formic acid aqueous solution/acetonitrile (16/84 v/v) were added into a 50-mL polypropylene centrifuge tube and were then vigorously shaken for 20 min by a multi-tube vortexer. After centrifuged at 8000 rpm for 10 min, 1 mL of the supernatant was applied to a Cleanert® MC cartridge with a nylon syringe filter on the outlet port. The flow was kept constant speed at a rate of about 1-2 drops/s. 500 μL of the purified eluate was evaporated to dryness at 50 ℃ in vacuum centrifugal concentrator, and was redissolved with 250 μL 0.1% formic acid aqueous solution/acetonitrile (70/30 v/v). Finally, the reconstituted solution was centrifuged for 10 min at 13000 rpm and the supernatant was then transferred into a conical bottom screw vial for UPLC-MS/MS analysis.

2.4 UPLC-MS/MS Analysis

Ultra-high-performance liquid chromatography (Acquity UPLC) coupled tandem mass spectrometer (XEVO TQ, Waters) was employed to detect mycotoxins with Multiple-reaction monitoring (MRM) mode. For the chromatographic conditions, the BEH Shield RP_{18} chromatographic column (Acquity UPLC, 100 mm × 2.1 mm; 1.7 μm; Waters) was employed for separation of 30 mycotoxins. The column temperature was 40 °C and flow rate was set at 0.3 mL/min. Mobile phase A was 0.1% formic acid and 0.5 mM ammonium acetate aqueous solution, and mobile

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phase B was 0.1% formic acid in methanol. The gradient profile were performed as follows: 0-2 min, 95% A; 2-4 min, 95%～80% A; 4 min-12 min, 80%～5% A; 12-12.1 min, 5%～1% A; 12.1-13 min, 1% A; 13-13.5 min, 1%～95% A; 13.5-14 min, 95% A. The injection volume was 2 μL.

A Xevo TQ tandem quadrupole mass spectrometer was operated in both positive and negative mode with the electrospray-ionization (ESI) source. The operating parameters were optimized under the following conditions: capillary voltage, 1.5kV (positive mode) and 2.5 kV (negative mode); ion source temperature, 150 °C; desolvation temperature, 400 °C; desolvation gas flow, 800 L/h; cone gas flow, 50 L/h (both gases were nitrogen); and collision-induced dissociation was performed using argon as collision gas at 3.5×10^{-3} mbar. MRM transitions, applied cone voltages, and collision energies are shown in **Table 2**. Instrument control, data acquisition and data processing were carried out with Masslynx V 4.1 software (Waters, MA, USA). Positive-ion monitoring mode was used to monitor mycotoxin nos. 1–25, and negative-ion monitoring mode was used to monitor mycotoxin nos. 26–30.

Table 1. Information of standards of 30 mycotoxins

No.	Analyte	Formula	CAS number	Purity (%)	Supplier	Concentration in stock solution (μg/mL)
1	Aflatoxin B_1 (AFB_1)	$C_{17}H_{12}O_6$	1162-65-8	99	Fermentek	1
2	Aflatoxin B_2 (AFB_2)	$C_{17}H_{14}O_6$	7220-81-7	99	Fermentek	1
3	Aflatoxin G_1 (AFG_1)	$C_{17}H_{12}O_7$	1165-39-5	99	Fermentek	1
4	Aflatoxin G_2 (AFG_2)	$C_{17}H_{14}O_7$	7241-98-7	99	Fermentek	1
5	Sterigmatocysin (STE)	$C_{18}H_{12}O_6$	10048-13-2	99	Fermentek	1
6	T-2 toxin (T-2)	$C_{24}H_{34}O_9$	21259-20-1	98	Fermentek	1
7	Diacetoxyscirpenol (DIA)	$C_{19}H_{26}O_7$	2270-40-8	99	Fermentek	1
8	Roquefortine C (RC)	$C_{22}H_{23}N_5O_2$	58735-64-1	99	Fermentek	1
9	Methylergonovine (MET)	$C_{19}H_{26}O_7$	2270-40-8	99	Fermentek	1
10	Ergocornine (ERG)	$C_{31}H_{39}N_5O_5$	564-36-3	98	Fermentek	1
11	Lysergol (LYS)	$C_{16}H_{18}N_2O$	602-85-7	99	sigma	1
12	Enniatin A (ENA)	$C_{36}H_{63}N_3O_9$	2503-13-1	99	sigma	1
13	Enniatin A_1 (ENA_1)	$C_{35}H_{61}N_3O_9$	4530-21-6	99	sigma	1
14	Enniatin B (ENB)	$C_{33}H_{57}N_3O_9$	917-13-5	95	sigma	1
15	Enniatin B_1 (ENB_1)	$C_{34}H_{59}N_3O_9$	19914-20-6	99	sigma	1
16	Beauvericin (BEA)	$C_{45}H_{57}N_3O_9$	26048-05-5	99	sigma	1
17	Deoxynivalenol (DON)	$C_{15}H_{20}O_6$	51481-10-8	99	Fermentek	10
18	3-AcetylDeoxynivalenol (3-AcDON)	$C_{17}H_{22}O_7$	50722-38-8	99	Fermentek	10
19	15-AcetylDeoxynivalenol (15-AcDON)	$C_{17}H_{22}O_7$	88337-96-6	99	Fermentek	10
20	Patulin (PAT)	$C_7H_6O_4$	149-29-1	98	Fermentek	10
21	Verruculogen (VER)	$C_{27}H_{33}N_3O_7$	12771-72-1	98	Fermentek	10
22	Neosolaniol (NEO)	$C_{19}H_{26}O_8$	36519-25-2	99	Fermentek	10

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23	Gliotoxin (GLI)	$C_{13}H_{14}N_2O_4S_2$	67-99-2	99	Fermentek	10
24	HT-2 toxin (HT-2)	$C_{22}H_{32}O_8$	26934-87-2	98	Fermentek	10
25	Wortmannin (WOR)	$C_{23}H_{24}O_8$	19545-26-7	99	Fermentek	10
26	Zearalenone (ZEN)	$C_{18}H_{22}O_5$	17924-92-4	99	sigma	10
27	α-Zearalenol (*α*-ZEL)	$C_{18}H_{24}O_5$	6455-72-8	98	sigma	10
28	β-Zearalenol (*β*-ZEL)	$C_{18}H_{24}O_5$	71030-11-0	98	sigma	10
29	α-zearalanol (*α*-ZAL)	$C_{18}H_{26}O_5$	26538-44-3	97	sigma	10
30	β-zearalanol (*β*-ZAL)	$C_{18}H_{26}O_5$	42422-68-4	97	sigma	10

2.5 Method Evaluation

A validation study was performed in terms of linearity, matrix effects, recovery and precision. The validation procedure for the method was carried out using spiked feedstuff samples with target mycotoxins. The coefficient of linearity was evaluated by assessing the signal responses of the target mycotoxin from matrix-matched calibration solutions prepared by spiking blank sample injection solution at six concentration levels, from 1 to 500 ng/g (1, 2.5, 5, 10, 25, 50 ng/g for nos. 1-16; 10, 25, 50, 100, 250, 500 ng/g for nos. 17-30), which correspond in the sample to 2 to 1000 ng/g (2, 5, 10, 20, 50, 100 ng/g for nos. 1-16; 20, 50, 100, 200, 500, 1000 ng/g for nos. 17-30) given the 2-times dilution factor. Recovery and precision (represented as the relative standard deviation, RSD %) experiments involved 5 replicate measurements that were carried out on the same day using the samples spiked with each mycotoxin at high, intermediate, and low levels.

Table 2. Retention time, precursor ion, product ions and optimized mass spectrometric parameters for targeted mycotoxins.

No.	Analyte	Retention time (min)	Cone voltage (V)	Precursor ion (*m/z*)	Quantification ion		Identification ion	
					Product ion (*m/z*)	Collision energy (eV)	Product ion (*m/z*)	Collision energy (eV)
1	AFB_1	8.25	46	313.1 $[M+H]^+$	241.0	36	284.9	22
2	AFB_2	7.92	44	315.2 $[M+H]^+$	287.1	26	259.0	28
3	AFG_1	7.73	38	329.1 $[M+H]^+$	242.9	26	200.0	40
4	AFG_2	7.39	48	331.1 $[M+H]^+$	189.0	40	245.0	30
5	STE	10.81	40	324.8 $[M+H]^+$	310.0	22	253.1	44
6	T-2	9.63	18	484.2 $[M+NH_4]^+$	305.3	22	185.1	12
7	DIA	8.07	20	384.2 $[M+NH_4]^+$	307.2	10	105.0	24
8	RC	7.89	36	389.9 $[M+H]^+$	193.0	26	108.2	40
9	MET	5.12	18	339.9 $[M+H]^+$	223.0	24	207.6	34
10	ERG	7.72	32	562.2 $[M+H]^+$	208.0	44	223.1	36

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11	LYS	4.32	10	254.8 [M+H]+	197.0	22	44.0	18
12	ENA	11.96	11	682.5 [M+H]+	210.1	26	228.1	26
13	ENA1	11.81	12	668.5 [M+H]+	210.1	24	196.1	28
14	ENB	11.46	13	640.5 [M+H]+	214.1	26	196.1	28
15	ENB1	1165	14	654.5 [M+H]+	196.1	24	210.1	22
16	BEA	11.82	15	784.4 [M+H]+	362.2	36	541.3	18
17	DON	4.38	18	296.9 $[M+H]^+$	249.1	12	231.1	14
18	3-AcDON	6.75	24	339.0 $[M+H]^+$	231.0	12	203.1	18
19	15-AcDON	6.75	18	338.6 $[M+H]^+$	137.0	18	261.1	10
20	PAT	2.29	28	154.9 $[M+H]^+$	70.9	12	80.9	8
21	VER	10.71	26	534.0 $[M+Na]^+$	392.1	14	435.2	16
22	NEO	6.09	18	400.2 $[M+NH_4]^+$	185.0	20	215.1	16
23	GLI	8.30	22	327.4 $[M+H]^+$	263.1	10	245.1	18
24	HT-2	9.03	14	442.2 $[M+NH_4]^+$	263.2	12	215.0	14
25	WOR	8.36	12	429.1 $[M+H]^+$	355.1	6	313.1	14
26	ZEN	10.83	38	317.2 $[M–H]^–$	175.1	26	187.1	26
27	α-ZEL	10.96	40	319.0 $[M–H]^–$	159.9	30	174.2	28
28	β-ZEL	10.34	38	319.0 $[M–H]^–$	159.9	30	174.2	24
29	α-ZAL	10.69	40	321.2 $[M–H]^–$	277.6	24	259.3	24
30	β-ZAL	10.11	38	321.2 $[M–H]^–$	259.3	28	161.2	24

3. Results and Discussion

3.1 Optimization of UPLC-MS/MS Conditions

The UPLC-MS/MS is a powerful separation and detection platform in multi-mycotoxins analysis. The chromatographic separation condition and MS parameters were important factors that needed to optimize in different instrument. In this study, the appropriate molecular ion peaks and ionization mode were selected after a full scan under the positive- and negative-ion modes. $[M+H]^+$or $[M+NH_4]^+$ were obtained under positive-ion mode for parts of mycotoxins (nos. 1-25), and $[M–H]^–$ was obtained under negative-ion mode for another parts of mycotoxins (nos. 26-30). Then, the parameters were further auto optimized using Intellistart function, and the characteristic ion pairs collected under MRM mode for

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each mycotoxin type were identified. Product ion with highest abundant was selected as the quantifier ion and the second highest abundant was selected as the qualifier ion. For 3-AcDON and 15-AcDON which are isomers, their respective unique ion fragments were used as product ions for monitoring.

Based on our previous study [28], most target mycotoxins had higher signal intensity under mobile phase with 0.1% formic acid aqueous solution (A) and 0.1% formic acid-methanol (B) than 0.1% formic acid aqueous solution (A) and 0.1% formic acid-acetonitrile (B). Moreover, adding 0.5 mM ammonium acetate into A, only the signal intensities of T-2, PAT, β-ZEL and β-ZAL were decreased slightly. But, for the other 26 target mycotoxins, the signal intensities were approximately increased 1.5 to 5.5 times. So, 0.1% formic acid and 0.5 mM ammonium acetate aqueous solution (A) and 0.1% formic acid-methanol (B) were employed as mobile phases. Furthermore, UPLC conditions (flow rate, elution gradient and time) were optimized to achieve high resolution, sensitivity, good peak shape and short analysis time with a suitable analyte separation. **Figure 1** shows the total ion chromatograms of the purified corn sample spiked with target mycotoxins.

3.2 Optimization of Clean-up and Analytical Conditions

Usually, sample preparation is the most crucial step in a multi-analytes detection method, because there are numerous co-extract impurities in the final extract, witch are assumed to cause strong matrix effects. Due to existence of matrix compounds, injection of feedstuff samples always results in lower analytical sensitivity and precision, and contamination of analytical system. Nowadays, flow-through cartridges (also known as multifunctional cartridges) have been widely applied for the analysis of mycotoxins. In this study, a Cleanert® MC cartridge was adopted to remove of matrix substances from the sample extract solution. First of all, the cartridge was examined and confirmed that any target mycotoxins was not absorbed on the it. After purification of Cleanert® MC cartridge, the extract solution was then transparent and colorless, and the sensitivities of target mycotoxins were significantly increased. **Figure 2** demonstrates the purification process and chromatograms of AFB_1 results from before and after purification. In general, the reconstitution solution always is employed as the initial mobile phase. However, in this study, the cartridge can not effectively remove the lipid from sample extract solution . So, the initial mobile phase (containing 5% methanol) is unable to dissolve the dried sample residue completely. To resolve this problem, 0.1% formic acid aqueous solution/acetronitrile (70:30 v/v) was adopted as the reconstitution solution, and small injection (2 μL) volume was employed to avoid solvent effect.

3.3 Evaluation of Matrix effect

The matrix effect was tested by comparing the responses between six level of standards in injection solvent and standards in matrix solutions. The slope ratios between matrix-matched and solvent calibration curves of the target mycotoxins were compared, and the range of 80%–120% was regarded as acceptable, otherwise meant matrix suppression or enhancement. **Figure 3** demonstrated significant different from one slope ratio to another. So, to get more accurate and precise results, matrix matched calibrations were used for the analysis.

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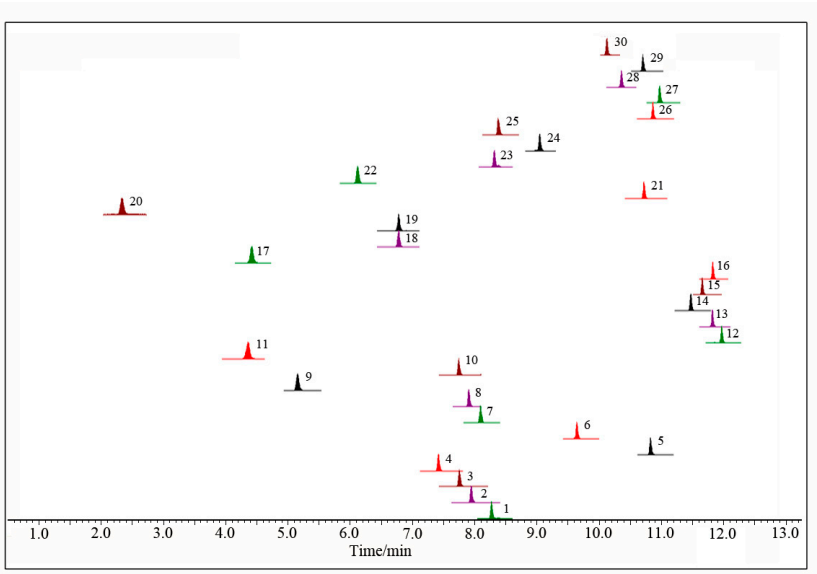

Figure 1. The total ion chromatograms of the purified corn sample spiked with target mycotoxins (10 ng/g for analyte nos. 1-16; 100 ng/g for17-30)

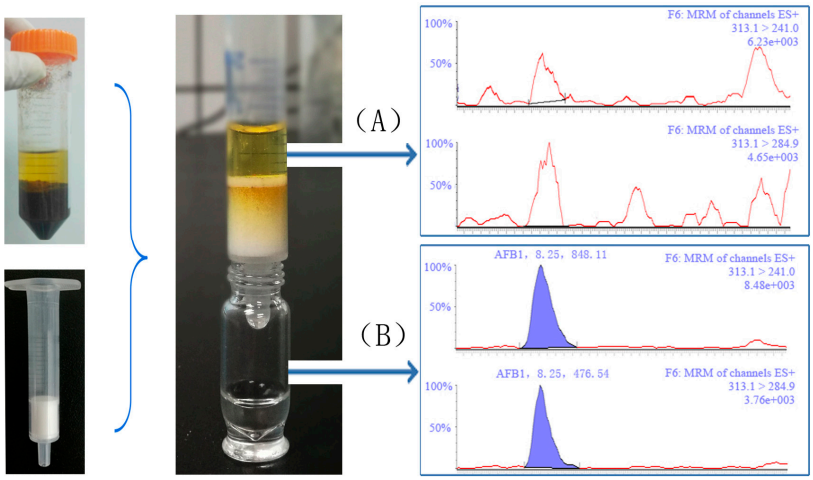

Figure 2. The purification process and chromatograms of AFB_1 results from before and after purification. The picture shows that the yellow cottonseed meal extract solution spiked with mycotoxins (2 ng/mL for nos. 1-16; 20 ng/mL) was purified by Cleanert® MC cartridge. The pigments was then absorbed in the cartridge, and the eluate was transparent and colorless. Take AFB_1 for example, the chromatograms of AFB_1 from extract solution was suppressed by matrix substances (A). Instead, the good chromatograms were obtained from the eluat (B).

3.4 Evaluation of Matrix Effect

Results of the method performance evaluation are summarized in **Table 3**. The method exhibited good linearity over the relevant working range, with R^2 of each mycotoxin >0.990. Limit of quantification (LOQs) for the target mycotoxins were calculated based on signal to noise ratio approach and found acceptable. The LOQs ranged from 2 to 50 ng/g, witch were acceptable because they were far

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below the China regulations for corresponding maximum levels of mycotoxins in animal feed. Recovery rates ranged from 72.0% to 118.5%, with the repeatability (as RSD %) ranging from 0.8% to 18.1%, showing the acceptable accuracy and precision of this method for all target mycotoxins.

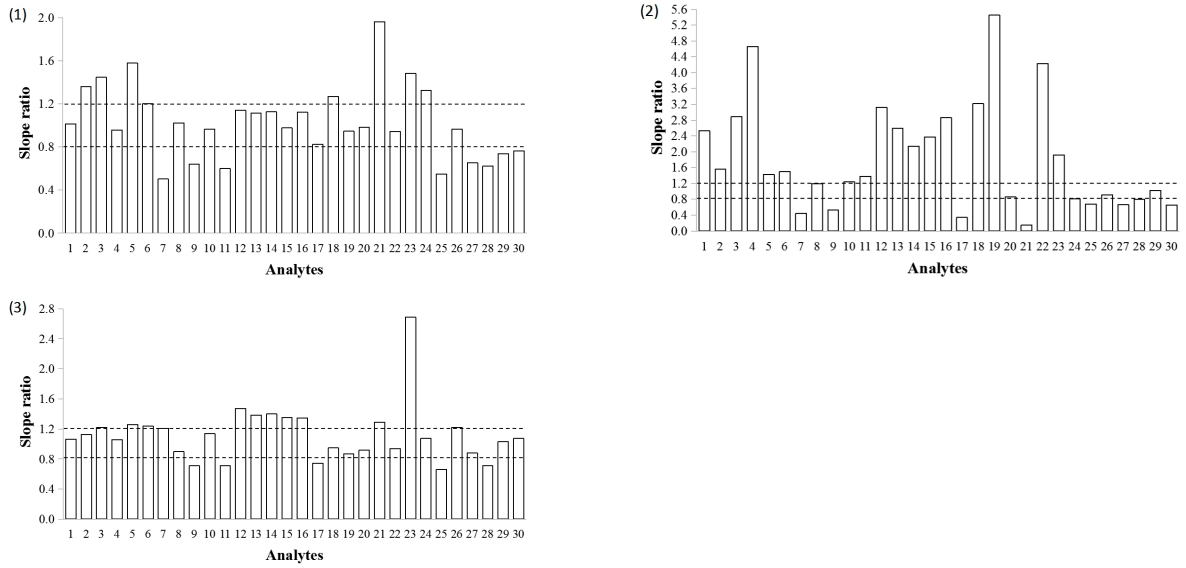

Figure 3. Slope ratios between matrix-matched and solvent calibration of the target mycotoxins: (1) corn; (2) wheat; (3) cottonseed meal.

3.5 Method Proficiency Testing

A certificated corn sample was applied to test the multi-mycotoxins method on all-in-one material and compare the results with the reference values. The corn was naturally contaminated with AFB_1, B_2, G_1, G_2, DON, T-2, HT-2, and ZEA, with the reference value of 18.8, 0.9, 2.4, ＜0.5, 2600, 263.7, 523.3, and 352.0 ng/g respectively. Correspondingly, the data gained by using the developed method were 19.5, ＜2, 2.2, ＜2, 2536, 248.5, 491.3, and 385.1 ng/g, witch all were in the acceptable range of 1sd. Thus the developed method can produce accurate results in accordance with the reference values.

Table 3 Performance parameters of method such as LOQ, linear range, average recoveries and RSD of 30 target mycotoxins in corn, wheat and cottonseed meal

No.	Analyte	LOQ (ng/g)	Linear range (ng/g)	Spiked levels (ng/g)	Recovery (%)	RSD (%, n = 5)
1	AFB_1	2	2-100	5, 20, and 50	90.4-110.6	1.7-10.7
2	AFB_2	2	2-100	5, 20, and 50	88.6-102.6	3.2-11.6
3	AFG_1	2	2-100	5, 20, and 50	87.7-108.2	2.3-13.6
4	AFG_2	2	2-100	5, 20, and 50	91.6-110.7	2.5-9.4
5	STE	2	2-100	5, 20, and 50	86.2-100.4	4.8-12.6

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6	T-2	5	5-100	5, 20, and 50	77.9-105.6	1.7-11.2
7	DIA	2	2-100	5, 20, and 50	88.6-103.6	1.3-18.2
8	RC	5	5-100	5, 20, and 50	84.4-95.6	2.6-11.3
9	MET	2	2-100	5, 20, and 50	83.2-108.1	1.6-9.9
10	ERG	2	2-100	5, 20, and 50	83.1-109.7	1.2-9.7
11	LYS	2	2-100	5, 20, and 50	72.0-105.3	2.6-12.4
12	ENA	2	2-100	5, 20, and 50	87.8-109.2	3.0-7.9
13	ENA1	2	2-100	5, 20, and 50	85.9-107.6	2.3-7.8
14	ENB	2	2-100	5, 20, and 50	81.8-112.7	2.2-11.0
15	ENB1	2	2-100	5, 20, and 50	89.1-111.4	2.6-11.0
16	BEA	5	5-100	5, 20, and 50	72.2-110.1	6.9-14.1
17	DON	50	50-1000	50, 200, and 500	82.3-104.8	1.4-10.6
18	3-AcDON	50	50-1000	50, 200, and 500	86.2-109.7	2.4-10.3
19	15-AcDON	50	50-1000	50, 200, and 500	97.3-115.4	1.6-17.4
20	PAT	50	50-1000	50, 200, and 500	93.3-113.0	2.2-6.8
21	VER	20	20-1000	50, 200, and 500	85.4-104.4	3.0-18.1
22	NEO	20	20-1000	50, 200, and 500	92.7-111.3	0.8-8.9
23	GLI	50	50-1000	50, 200, and 500	81.0-111.2	2.4-13.0
24	HT-2	50	50-1000	50, 200, and 500	89.4-111.4	2.7-11.4
25	WOR	50	50-1000	50, 200, and 500	87.3-107.5	3.7-16.1
26	ZEN	20	20-1000	50, 200, and 500	86.3-111.6	2.1-9.1
27	α-ZEL	50	50-1000	50, 200, and 500	84.5-114.7	4.3-13.9
28	β-ZEL	50	50-1000	50, 200, and 500	84.2-113.9	5.6-17.3
29	α-ZAL	50	50-1000	50, 200, and 500	90.8-118.5	6.5-17.6
30	β-ZAL	50	50-1000	50, 200, and 500	78.1-117.9	4.0-17.9

3.6 Application of the method on real samples

Eighty feedstuff samples including 20 corn, 20 DDGS, 10 wheat, 10 soybean meal, 10 cottonseed meal, 5 wheat bran, and 5 peanut meal samples were analyzed using the developed UPLC-MS/MS

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multi-mycotoxins method. 100% of corn samples, 100% of DDGS samples, 60% of wheat samples, 20% of soybean meal samples, 30% of cottonseed meal samples, 80% of wheat bran samples, 100% of peanut meal samples, and 64 out of 80 total samples (80%) were contaminated with at least one mycotoxin. But, the certain mycotoxins in most of samples were safety below the maximun limits (AFB_1 was 30–50, T-2 was 1000, DON was 1000, ZEA was 500 μg/kg) set by China. Only two corn samples and six DDGS samples contaminated with DON, three DDGS samples contaminated with ZEA, and three peanut samples contaminated with AFB_1 exceeded the maximun limits. For other target mycotoxins without proposed limit standards, there were varying levels of detection rates.

4. Conclusions

A novel simultaneous detection method for mycotoxins in feedstuffs by using a simple preparation method based on flow-through purification followed UPLC-MS/MS detection was presented. Thirty mycotoxins were well separated in noly 14 min applying an Acquity BEH Shield RP_{18} column with gradient elution. The performance parameters, including linearity, sensitivity, recovery and precision, presented that this method was suitable for multi-mycotoxins determination in feedstuffs. The method was also well examined by reference sample, and applied on the real feedstuff samples testing successfully.

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