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Research Article

Evaluation of Solid Phase Extraction NH2 for Determination of Enniatins (A, A1, B, B1) and Beauvericin in Cereals by UPLC-MS/MS

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Abstract

We report herein, for the first time, the application of NH_2 solid-phase extraction NH_2 (NH_2-SPE) cartridge for cleanup of enniatins (ENA, ENA_1, ENB, ENB_1) and Beauvericin (BEA) in cereals. Samples were sequentially extracted with a solution containing 80% acetonitrile and 0.1% formic acid and the extracts were cleaned up with NH_2-SPE cartridges before being analyzed by Ultra-High Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS/MS). A CORTECS C18 column was used for separation of enniatins and beauvericin. Mass spectrometric analysis was conducted at the Positive Electrospray Ionization (ESI+) Mode with Multi-Reactions Monitoring (MRM). Very good linear relationships between spiked levels of ENA, ENA1, ENB, ENB1, BEA in cereals and mass spectra were observed with regression coefficients of 0.995 to 0.999 and Detection Limits (LOD) range of 0.01 to 0.1 $\mu g\ kg^{-1}$. Clean up with NH_2-SPE cartridge gave more satisfactory recoveries ranging from 80.9% to 109.8% than with QuEChERS method that gave recoveries between 63.5% to 96.6%. Application of the NH_2-SPE cartridge for the determination of enniatins and beauvericin showed detection levels ranging from 0.01 $\mu g\ kg^{-1}$ to 127.87 $\mu g\ kg^{-1}$ in corn and 0.01 $\mu g\ kg^{-1}$ to 116.59 $\mu g\ kg^{-1}$ for wheat.

Introduction

Enniatins (ENA, ENA1, ENB, ENB1) and Beauvericin (BEA) are a group of mycotoxins, which are cyclic hexadepsipeptides consisting of alternating hydroxy acid and N-methylamino acid residues. BEA has phenyl substituents on the N-methylamino acid residue whereas ENs has various aliphatic substituents at the same positions [1]. They are produced by several species of Fusarium such as Fusarium verticillioides, Fusarium proliferatum, Fusarium subglutinans, Fusarium oxysporum, Fusarium poae, and Fusarium avenaceum, which are known to contaminate cereals and cereal by-products [2]. These mycotoxins were called emerging mycotoxins for which little knowledge was available in the past. Recently, more research is focused at determining their occurrences and potential health effects [3].

Methods reported for determinations of ENs and BEA include HPLC-DAD analysis [4], stable isotope analysis [5], and UPLC-MS/MS analysis [6,7]. Various approaches have been proposed for extraction and clean up of mycotoxins, which are the critical steps in determining recoveries of mycotoxins. Extraction chemicals either used independently or in combination, include acetonitrile, formic acid and methanol [8]. A clean up step is typically performed on extracts [4,6,9]. QuEChERS (quick, easy, cheap, effective, rugged and safe) method have gained considerable attention for extraction of mycotoxins, including ENs and BEA [10,11] because of its simplicity. QuEChERS, however, does not perform clean up steps. As a result, it may cause damage to chromatographic column and the MS instrument and may influence recovery. SPE [12] is commonly used for clean up and used dispersive solid-phase extraction for water samples [13]. Jestoi, et al. [14] used SPE C8 column for clean-up of ENs and BEA, which resulted in mean recovery ranges of 76~82%, 55~66%,71~80%, 57~103%, and 68~116%, for BEA, ENA, ENA_1, ENB and ENB_1, respectively. However, some recoveries were low. Mycotosep@ 224 combined with silica SPE column (Supel-clean LC-Si, Supelco) was applied for cleaning up BEA [15] and was found to be well suited for extracting only minor amounts ($\mu g\ kg^{-1}$) in corn and corn meal. SPE C18 and SPE CN have also been used for purification of ENs, BEA and other mycotoxins [16,17], but recovery results were unsatisfactory. Rubert et al. [17] compared the efficiency and efficacy of four different extraction procedures. Matrix solid phase dispersion, QuEChERS, and solid-liquid extraction gave recoveries of ENA, ENA_1, ENB, ENB_1, and BEA ranging from 67.1~74.1%, 70.5~77.3%, and 80.1~90.1%, respectively. SPE C18 clean up method resulted in low recoveries. To our knowledge, amino (NH_2) columns, due to their strong polarity, are widely used in food residue extractions and separations but have not been used for purification of ENs and BEA. In this study, we used NH_2 column for

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purifying 26 mycotoxins, including ZEN, OTA, FBs, ENs and BEA. Herein, we report a method for determination of ENs and BEAs with a focus on the efficiency and efficacy of extraction and cleanup by NH_2 SPE. Moreover, we present ENs and BEAs determinations for 26 wheat, 167 corn and 25 rice samples collected in 2015 from fields in Henan, Hubei, Yunnan and Sichuan provinces in China, and from various supermarkets.

Material and Methods

Chemical and reagents

HPLC grade methanol, acetonitrile, and formic acid were purchased from Thermo Fisher Scientific (Shanghai, China). SPE NH_2 column was purchased from Agilent (USA). Purified water was produce by a Mill-Q system (Millipore, Billerica, MA, USA). Other reagents were of analytical grade. The standards of ENA, ENA_1, ENB, ENB_1 and BEA were purchased from Sigma-Aldrich (USA).

Preparation of standard solutions

The stock solutions of ENA, ENA1, ENB, ENB1 and BEA were prepared in methanol at a concentration of 100 mg L-1 and kept at -18°C in darkness. Working standard solutions were prepared immediately before use by diluting the stock solution with methanol/water (50:50, v/v) at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 50.0 μg L-1. Blank samples were prepared to minimize matrix effects by spiking working standard solution to mycotoxin-free cereals.

Sample preparation

Finely milled samples (2 ± 0.05 g) were weighed and placed into a 50 mL centrifuge tube. A 20 mL extraction solution of acetonitrile/water (80:20, v/v) containing 0.1% formic acid was added. The sample was vortexed for 30 s, put in an automatic thermostatic cultivation shaker (Yiheng Technology Co., LTD, Shanghai, China) for 30 minutes, and filtered into a 50 mL centrifuge tube.

For the cleanup step, SPE NH_2 column was pre-washed with 5 mL acetonitrile. Extracts (2 mL) were loaded onto the washed cartridge and eluted by adding 2 mL methanol. The cleaned up eluents were evaporated to dryness at 55°C under an N_2 stream and the dried residues were dissolved in 1.0 mL of methanol/water (1:1, v/v), vortexed for 1 min, and then filtered through 0.22 μm nylon filter (Membrana, Germany) for analysis.

UPLC-MS/MS analysis

The analyses of mycotoxins were performed using UPLC-MS/MS (XEVO-TQ, Waters, USA). The separation column was CORTECS C18 (100 × 2.1 mm, 1.6 µm, Waters, USA) and the flow rate was 250 μL min^{-1}. Volumes of strong wash (90% MeOH) and weak wash (10% MeOH) solvents were 100 μL and 600 μL, respectively. Mobile phases consisted of (A) MeOH and (B) 0.5% formic acid water solution. The gradient elution was as follows: 5% A, and rapidly increased to 85% A in 4.5 minutes, then slowly increased to 100% within 4.8 minutes, then linearly lowered to 5% A in 8.5 minutes. The column temperature was maintained at room temperature and the sample temperature was 20°C. The injection volume was 5.0 μL. Data processing was performed with MassLynx 4.0 software (Waters, USA).

The mass spectrometric (MS/MS) detection was carried out at the positive mode using electrospray ionization (ESI+). For infusion experiments, 0.1 mg L-1 of the mycotoxins standards dissolved in MeOH solution was used at a flow rate of 25 μL min^{-1}. The capillary voltage was 2.5 kV. Nitrogen was used as cone, nebulizing and desolvation gas. The source and desolvation temperatures were set at 110°C and 500°C, respectively. Cone and desolvation gas flow were maintained at 20 L h^{-1} and 800 L h^{-1}, respectively. The collision gas flow rate was 0.17 mL min^{-1}. The analysis of the mycotoxins was performed in Multiple Reactions Monitoring (MRM) mode. Information on the respective mycotoxins and the optimum cone voltage and collision energy are shown in Table 1.

Results and Discussion

Evaluation of matrix effect

Matrix Effects (ME) are unavoidable in detection. At present, literatures report the use of internal standards, such as Zearalanone (ZEA) and Deepoxy-Deoxynivalenol (DOM) [18] or isotope-labeled standards [19] to minimize matrix effects. Although the use of internal standard may result in good linear relationship and high precision, choosing the appropriate one in a multi-component analysis is often difficult and expensive. The response of the target mycotoxins can be suppressed or enhanced on account of the interfering matrix components. The ME calculated as 100× (1- area of mycotoxin standard in blank sample / area of mycotoxin standard in solvent) of different blank wheat, corn and rice samples are shown in Table 2. It can be observed that the signal suppression effect was very prominent

Table 1: UPLC-MS/MS acquisition parameters for enniatins and beauvericin.

Mycotoxins	Retention time (min)	Parent ion (m/z)	Daughter ion (m/z)	Dwell time (s)	Cove voltage (V)	Collision energy (V)
ENA	7.66	682.29	210.0(Q)	0.005	48	26
			228.0(q)	0.005	48	28
ENA_1	7.50	668.28	99.9(Q)	0.005	48	60
			210.0(q)	0.005	48	26
ENB	7.30	640.18	195.9(Q)	0.005	46	24
			213.9(q)	0.005	46	26
ENB_1	7.45	654.26	99.9(Q)	0.005	46	54
			195.9(q)	0.005	46	24
BEA	7.36	784.24	243.9(Q)	0.005	48	28
			262.0(q)	0.005	48	26

Note: Q: quantitative ion, q: qualitative ion.

Table 2: Calibration curves, linear ranges, correlation coefficients (r), LODs, LOQs, and ME of ENs and BEA.

Matrix	Mycotoxins	Calibration curve	Linear range (μg/kg)	r	LOD (μg/kg)	LOQ (μg/kg)	ME (%)
wheat	ENA	Y=13611.8X+5695.25	0.04~100	0.9998	0.10	0.4	-91.1
	ENA_1	Y=8862.07X+8001.94	0.01~100	0.9998	0.01	0.05	-89.9
	ENB	Y=9520.86X+17039.2	0.01~100	0.9998	0.01	0.05	-90.6
	ENB_1	Y=7140.00X+3812.5	0.01~100	0.9999	0.01	0.05	-88.8
	BEA	Y=4492.0X+1533.88	0.01~ 40	0.9995	0.01	0.05	-91.7
corn	ENA	Y=11966.0X+1333.36	0.1~100	0.9993	0.10	0.4	-89.2
	ENA_1	Y=9628.2X±8145.22	0.04~100	0.9991	0.04	0.1	-89.5
	ENB	Y=10265.2X+426.14	0.04~100	0.9996	0.04	0.1	-90.8
	ENB_1	Y=6535.65X±1278.84	0.04~100	0.9998	0.04	0.1	-88.8
	BEA	Y=4492.0X±3272.31	0.04~100	0.9991	0.04	0.1	-89.8
rice	ENA	Y=14482.3X+10622.7	0.1~100	0.9994	0.10	0.4	-89.8
	ENA_1	Y=8203.18X+3631.36	0.04~100	0.9998	0.04	0.1	-90.4
	ENB	Y=10658.2X+703.74	0.04~100	0.9999	0.04	0.1	-90.5
	ENB_1	Y=5104.59X+3428.13	0.04~100	0.9992	0.04	0.1	-90.2
	BEA	Y=3858.85X+1725.76	0.04~100	0.9998	0.04	0.1	-90.6

for ENs and BEA, with percentage ranging from 88.8% to 91.7% and this suppression effect is far beyond the tolerable range of +20% to -20% [11]. The signal suppression effect in barley was reported to be at 77.9~110.1% [20]. Matrix effects caused by different cereals were significant for most of the mycotoxins. Blank matrix preparation of standard curve reduces the impact of the matrix interference in parallel and improves the accuracy of analysis.

Calibration curves

The calibration curves were evaluated using a blank sample of wheat, corn and rice spiked with a series of concentrations: 0.01, 0.04, 0.4, 1, 4, 10, 20, 40, 100, 200 μg L^{-1}. It was constructed by plotting the peak areas (y) against the concentration of analytes (x). Highly linear relationships were achieved with linear regression coefficients (r) of 0.9991 to 0.9999 (Table 2).

Comparison of Limit of Detection (LOD) and Limit of Quantification (LOQ)

LODs and LOQs were calculated from spiked blank samples at the lowest spiking level (3-fold and 10-fold the S/N, for LOD and LOQ, respectively) based on the MRM chromatograms as shown in Figure 1. The obtained results were listed in Table 2. The LOD and LOQ values ranged from 0.01 to 0.1 μg kg^{-1} and 0.05 to 0.4 μg kg^{-1}, respectively.

The different cleanup procedures produce various results of LOD. A study reported methanol extraction from cereals and derived products from Tunisia giving LOD for ENA, ENA_1, ENB, ENB_1 and BEA of 215, 140, 145, 165 and 170 μg kg^{-1} and LOQ of 600, 400, 400, 500 and 500 μg kg^{-1}, respectively [21]. Another method used direct injection in maize and maize silage extracts without any tedious and laborious clean up procedures. The LOQ was 13 ng g^{-1} for BEA and 17, 34, 24, and 26 ng g^{-1} for ENA, ENA_1, ENB, ENB_1, respectively [22]. There was no apparent change in LOD when SPE C8 column was used [6]. The calculated LOQ for BEA and ENA, ENA_1, ENB, and ENB_1 were 0.2, 0.2, 0.7, 0.9, and 1.5 mg kg^{-1}, respectively [6]. When using LC-MS/MS with atmospheric pressure chemical ionization and without further treatment of sample extracts from grain, the LOD and LOQ were 3.0 μg kg^{-1} to 10 μg kg^{-1} for BEA, ENA, ENB and ENB_1 and 4.0 μg kg^{-1} to 13 μg kg^{-1} for ENA_1 [9]. Moreover, On-Line Thermospray-Mass Spectrometry (LC/TSP/MS) was used to analyze BEA, which resulted in a very low detectable limit of 1 ng and an S/N of 5:1 [23]. Overall, the LOD was obviously decreased about one order of magnitude when using SPE NH_2 compared to other methods reported in literatures.

Table 3: Recovery values of different spiked levels of ENs and BEA in cereals.

Mycotoxins	spiked level (μgkg^{-1})	wheat		corn		rice	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
ENA	1	85.7	7.0	83.2	4.3	84.8	12.1
	10	85.0	5.5	83.0	1.7	82.5	1.4
	50	90.2	12.5	97.3	1.0	87.0	0.7
ENA_1	1	104.7	10.3	88.7	7.4	84.8	1.8
	10	109.8	1.3	81.7	2.7	82.1	1.2
	50	99.3	6.8	88.0	2.6	96.6	1.0
ENB	1	88.7	2.1	100.0	6.1	87.3	2.9
	10	92.1	3.6	84.9	1.7	82.6	3.0
	50	105.7	5.2	108.5	1.0	103.1	7.2
ENB_1	1	99.0	4.5	96.2	8.9	91.3	2.8
	10	80.9	2.3	80.6	1.4	92.2	3.2
	50	101.5	3.1	85.9	2.7	106.0	2.7
BEA	1	87.2	1.8	93.7	4.6	94.0	5.4
	10	98.2	5.9	107.5	3.7	83.8	0.9
	50	98.7	5.6	88.2	7.1	83.0	4.6

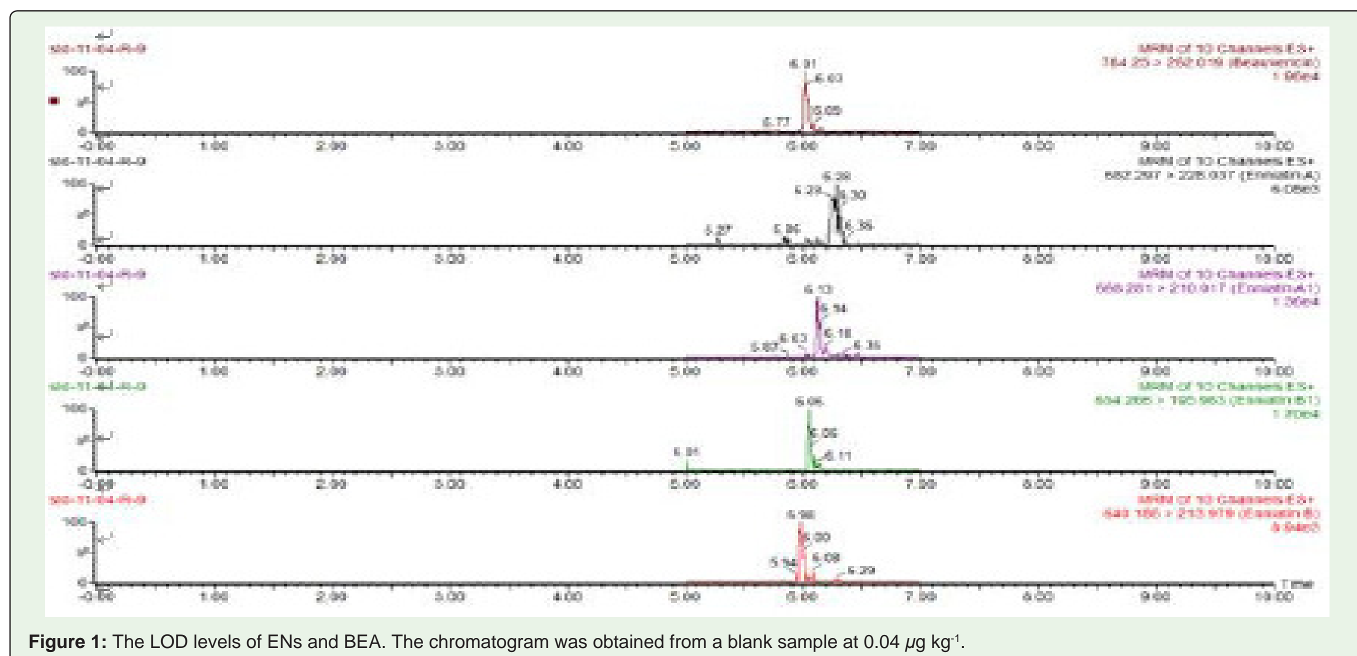

Figure 1: The LOD levels of ENs and BEA. The chromatogram was obtained from a blank sample at 0.04 μg kg^{-1}.

Method precision and accuracy

Method precision was evaluated for intra-day and inter-day repeatability for six times in wheat matrices. The Relative Standard Deviations (RSDs) for ENA, ENA_1, ENB, and ENB_1 were 2.6, 2.9, 8.6, 4.1, 6.0%, respectively and 8.2, 8.6, 6.9, 11.1% for BEA (Table 3). The results showed that the RSDs for the inter-day repeatability study were obviously higher than those of intra-day. Nonetheless, these values were still below 15% and within the allowable range. Method accuracy was evaluated by recovery of standard mycotoxins that were spiked to blank matrices at three different concentrations (Table 3). The recovery values were within 80.9 to 109.8% with RSDs of 0.7~12.5%. The results demonstrated that the method applied was highly accurate and precise.

Comparison of recovery for different extraction and clean-up methods

Based on the recovery results (Table 4), SPE NH_2 clean up were superior to QuEChERS method (established in our laboratory), showing increased recoveries of 10.9~36.0%. It may be due to the use of amino column (NH_2), which has strong polarity, promoting better adsorption of impurities while preserving target compounds, thereby significantly reducing the matrix interference. The same clean up using NH_2 SPE has validated that strong binding with fumonisins and ochratoxin A in sorghum led to unsatisfactory recovery [18]. No purification methods using amino SPE have been found in any relevant literature.

Use of SPE C8 column for cleanup of mycotoxins showed low recoveries of ENs [14]. The mean recoveries for BEA, ENA, ENA_1, ENB, and ENB_1 were 76~82%, 55~66%, 71~80%, 57~103%, and 68~116%, respectively [14]. With QuEChERS, poor recoveries (60~87%) were observed when C18 and CN cartridges were used for dried fruit [16]. Matrix solid-phase dispersion extraction used in wheat grain showed a recovery of 71~78% [24].

The use of SPE cartridge HLB and C18 resulted in poorer recovery of 74.5, 75.1, 72.5, 109.7% for ENA, ENA_1, ENB_1 and BEA, respectively [11]. Moreover, recovery of in human breast milk using QuEChERS extraction and UHPLC-HRMS detection was at 73~82% [25]). Satisfactory results (99.0~114.0%) validated based on QuEChERS extraction were observed in barley and malt [19]. These literatures suggest that satisfactory results could be obtained for all the ENs and BEA when SPE NH_2 cleanup is performed.

Determination of ENs and BEA

The developed method was applied for the determination of ENs and BEA in 26 wheat, 167 corn, and 25 rice samples from supermarkets. Frequency and the number of ENs and BEA detected

Table 4: Recovery values of different spiked levels of ENs and BEA in cereals.

Mycotoxins	spiked level (μg kg^{-1})	Wheat Recovery (%)		Corn Recovery (%)		Rice Recovery (%)	
		QuEChERS our laboratory	SPE	QuEChERS our laboratory	SPE	QuEChERS our laboratory	SPE
ENA	50	63.5	90.2	63.5	97.3	69.3	87.0
ENA_1	50	64.9	99.3	63.7	88.0	74.2	96.6
ENB	50	69.7	105.7	96.6	108.5	79.3	103.1
ENB_1	50	65.0	101.5	73.1	85.9	77.3	106.0
BEA	50	71.7	98.7	74.8	88.2	67.9	83.0

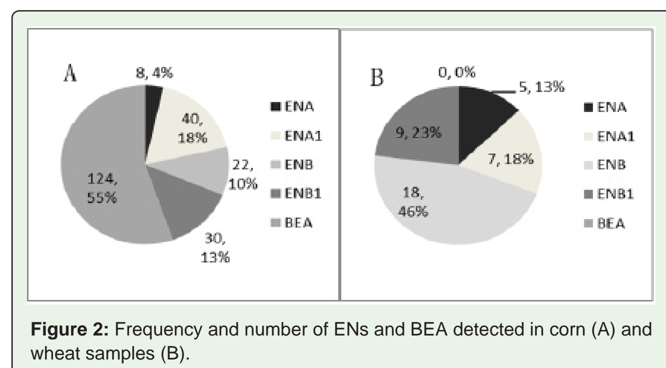

Figure 2: Frequency and number of ENs and BEA detected in corn (A) and wheat samples (B).

in corn and wheat is shown in Figure 2. All mycotoxins were not detected in rice. However, a survey showed that commercial rice samples in Morocco could be contaminated with ENs (50%) and BEA (75.7%) [4].

Corn samples were contaminated with ENA (4%), ENA_1 (18%), ENB (10%), ENB_1 (13%), BEA (55%) at a range of 0.13 to 88.93 $\mu g\ kg^{-1}$, 0.01 to 88.01 $\mu g\ kg^{-1}$, 0.01 to 1.96 $\mu g\ kg^{-1}$, 0.01 to 127.87 $\mu g\ kg^{-1}$ and 0.01 to 116.59 $\mu g\ kg^{-1}$, respectively. The main toxin in corn was BEA. Our results indicate that BEA may exist ubiquitously in maize crops; hence, more attention should be given to storage conditions to minimize contamination.

Wheat samples were contaminated with 13%, 18%, 46%, and 23% of ENA, ENA_1, ENB and ENB_1, respectively. The contamination levels ranged from 0.12 to 1.11 $\mu g\ kg^{-1}$, 0.12 to 1.48 μg kg-1, 0.12 to 21.22 $\mu g\ kg^{-1}$, 0.25 to 1.97 $\mu g\ kg^{-1}$ and 0.01 to 116.59 $\mu g\ kg^{-1}$, for ENA, ENA_1, ENB and ENB_1, respectively. Results showed that ENB was obviously higher than the ENA in wheat samples. Similar results were also reported in wheat grain [24]. In Tunisia, higher amount of ENA1 compared to other ENs were reported probably due to the climatic conditions [21]. BEA was not detected in durum wheat samples from Italy [26].

Based on our results, it can be confirmed that ENs and BEA represent a risk for cereals. Therefore, it is suggested that national standards for detection and maximum tolerable daily intake should be established. Moreover, strengthening supervision and identification of potential risks is highly recommended.

Conclusions

A liquid chromatography-mass spectrometric method was developed and validated to determine Fusarium mycotoxins BEA and ENs (A, A_1, B, B_1) in grain samples. Using NH_2 column for ENs and BEA may provide a simple and cost-effective method of purification. Experiments were conducted to compare recovery and LODs with other methods reported in literatures. It can be concluded that the efficiency and efficacy of modified SPE clean up demonstrate superior performance over QuEChERS and other purification methods in various cereals. Considering its advantages, the proposed method could be utilized for monitoring and examining potential risk of mycotoxins in cereals by analytical laboratories. It could also provide the basis for establishing national standards.

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