

Analysis of Fourteen Mycotoxins in Chili Peppers in Guizhou Province Combining DES-DLLME and UHPLC-MS/MS and Their Risk Evaluation

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Abstract Mycotoxins exist widely in food and have a serious impact on human health. At present, most detection methods of mycotoxins are costly and time-consuming. Most of these methods are aimed at detecting a single type of mycotoxin, and the efficiency is not high. On this basis, in this study, QuEChERS-deep eutectic solvent liquid-liquid microextraction was applied to extract and enrich 14 mycotoxins in chili peppers from the concept of green chemistry. A simple, time-consuming and environment-friendly multi-flux pretreatment method was established, and 100 chili pepper samples were randomly sampled from farmers' markets and supermarkets in major urban areas of Guizhou Province for detection, and risk assessment was carried out according to the detection results.

Key words Chili pepper; Mycotoxin; DES-DLLME; UHPLC-MS/MS

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Guizhou Province is characterized by few plains, many mountains and high humidity. It has established twelve ecological agricultural systems with local characteristics, including chili peppers with Guizhou characteristics. At present, chili pepper production in Guizhou Province is in the transition stage from workshop production to mechanized and industrial production, and most of the products are mainly rough-processed, which makes it difficult to effectively control the product quality. The quality of products is random and accidental, and there is a safety hazard of mycotoxin pollution. Mycotoxins are a series of secondary metabolites produced by mold, the molecular weight of which is usually less than 1 000 Da^[1]. According to statistics, more than 400 mycotoxins have been found in the world^[2], and the mycotoxins with major hazards can be divided into aflatoxin, ochratoxin, zearalenone and patulin. At present, many analytical methods have been used to detect and quantify mycotoxins in food systems. High performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) has the characteristics of simplicity, rapidity, sensitivity and high throughput, and gradually become the main method for analysis of mycotoxins. With the continuous development and wide application of detection techniques, the gap of performance indexes of various detection techniques is narrowing day by day. However, sample pretreatment is the key of sample analysis method. Because the mycotoxin content is low and the sample matrix is

complex, commonly used specific pretreatment methods mainly include immunoaffinity column and molecular imprinting column purification. Although the purification effect is good, the treatment cost is high and takes a long time, and only one or one type of mycotoxins can be extracted and purified, resulting in high cost. At present, common pretreatment methods are often used for simultaneous determination of multiple mycotoxins, including solid-phase extraction (SPE)^[3], liquid-phase extraction^[4] and so on. These methods all have disadvantages in different degrees. For example, SPE process is generally time-consuming and expensive, and a lot of organic solvents are needed in the extraction process, so it does not belong to the category of green pretreatment. Although the liquid-liquid extraction method is simple to operate, it consumes a lot of organic solvents, pollutes the environment and easily causes the loss of the target material. The emergence of liquid-phase microextraction technique has solved this problem^[5]. The emergence of QuEChERS technique, which is quick, easy, cheap, effective, rugged and safe, has attracted a lot of attention. This technique can not only greatly reduce the consumption of reagents, but also is simple to operate and has a high recovery value. In this study, based on the concept of green analytical chemistry, combining the advantages of QuEChERS and liquid-phase microextraction technique, a pretreatment method for testing 14 mycotoxins in chili peppers was established, including aflatoxin, ochratoxin and zearalenone. A series of DESs including natural compounds such as thymol and decanoic acid were used as liquid-phase microextraction agents. The mycotoxins in spices were first extracted by the QuEChERS technique, then extracted and enriched by DESs, and detected by tandem mass spectrometry. Finally, a simple, time-consuming and environment-friendly pretreatment and detection method was preliminarily established for the pre-treatment and detection of various fungal toxins in spices.

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Experimental Part

Preparation of mycotoxin standard working solution

First, 1 mg of pure solid of mycotoxin standard was dissolved in a brown storage bottle filled with 10 ml of methanol to obtain a stock solution with a concentration of 100 µg/ml. Next, the prepared solution was then diluted with 1% formic acid acetonitrile stepwise. The prepared standard solutions should be stored in a refrigerator at -20 °C and protected from light. Before use, they should be taken out and placed at room temperature for 1 h.

Preparation of DES

First, two materials needed for the preparation of DES, thymol and decanoic acid, were weighed and mixed in a glass liquid storage bottle at a certain molar ratio. They were continuously stirred by a magnetic stirring heater at 80 °C until they formed a transparent solution, and then, they were cooled to room temperature and stored in the dark. All DESs were set to be valid for one month.

Extraction of mycotoxins from chili peppers by QuEChERS-DES/DLLME

After crushing a chili pepper sample, 1 g (±0.005 g) was accurately weighed and added into a 50 ml polypropylene centrifuge tube. Next, 5 ml of pure water was added, and vortex-mixed with the sample for 2 min. After standing for 30 min, 5 ml of acetonitrile solution containing 1% formic acid was added, and the obtained extraction system was vortex-mixed for 5 min. Next, 4.0 g of anhydrous magnesium sulfate and 1.0 g of sodium chloride were added into the test tube, which was immediately shaken and vortex-mixed for 2 min. Next, centrifugation was performed at 5 000 r/min for 10 min to obtain the supernatant for later use.

Subsequently, 2 ml of the supernatant was added in a 10 ml polypropylene test tube, added with 6 ml of deionized water and 200 µl of DES solution in sequence. Next, the test tube was

shaken vigorously for 30 s, and centrifuged at 5 000 r/min for 2 min. After layering, the upper DES phase was filtered with a 0.22 µm PTFE filter, and the filtrate was added and diluted 0.5 ml for analysis.

LC-MS/MS analysis conditions

The liquid chromatography part used a BEH C₁₈ (2.1 mm × 100 mm × 1.7 µm) column (Waters, USA) as the liquid chromatography column. The separation was performed at a flow rate to 0.3 ml/min with a volume of single injection of 2.0 µl, and the total running time was 12.0 min. The mobile phase A was water containing 0.2 mM NH₄F, and B was acetonitrile. Gradient elution was adopted, and the elution procedures were as follows: 0–3 min; 10%–30% B, 3–8.4 min; 30%–60% B, 8.4–8.5 min; 60%–100% B, 8.5–10.5 min; 100% B, 10.5–10.51 min; 100%–10% B, 10.51–12 min; 10% B. The column temperature was 40 °C. The main parameters of MS were as follows: AJS ESI ionization source, operating in both 4.0 kV positive mode and 3.5 kV negative mode, with a detector gain of 4 for both positive and negative modes, a drying gas temperature of 300 °C, a sheath gas temperature of 350 °C, a drying gas flow at 10 L/min, an ion source gas pressure at 35 psi, and a flow rate of sheath gas at 11 L/min. High-purity nitrogen was used as collision gas. Nitrogen was supplied by Genius AE32 nitrogen generator (Peak Scientific, UK). All quantitative data were obtained in positive mode under MRM mode, and the set residence time was 10 ms. Two kinds of ion transitions were used in analyte analysis, and the one with the highest intensity was selected as the quantitative ion, and their intensity ratio was used for analyte identification. Table 1 describes the specific MS parameters used for structure quantification and confirmation. Fig. 1 shows the typical chromatogram of mycotoxins in chili pepper substrate.

Table 1 Retention time, molecular formulas and basic MS/MS parameters of 14 mycotoxins

Mycotoxin	RT//min	Molecular formula	Ion mode	Parent ion//m/z	Daughter ion//m/z	Fragmentor//v	CE//V
OTA	6.33	C ₂₀ H ₁₈ ClNO ₆	[M + H] ⁺	404.2	221.0 *	101	40
					239.0		24
OTB	5.25	C ₂₀ H ₁₉ NO ₆	[M + H] ⁺	370.2	205.0 *	101	20
					187.0		40
AFB1	5.42	C ₁₂ H ₁₄ O ₅	[M + H] ⁺	313.1	241.0	137	45
					285.0 *		25
AFB2	5.00	C ₁₇ H ₁₄ O ₆	[M + H] ⁺	315.0	259.0	147	30
					287.1 *		30
AFG1	5.01	C ₁₇ H ₁₂ O ₇	[M + H] ⁺	329.2	200.0 *	133	45
					243.0 *		30
AFG2	4.59	C ₁₇ H ₁₄ O ₇	[M + H] ⁺	331.1	189.0 *	147	45
					313.1		25
AFG2	4.59	C ₁₇ H ₁₄ O ₇	[M + H] ⁺	331.1 1	89.0 *	147	45
					313.1		25
AFM2	3.88	C ₁₇ H ₁₄ O ₇	[M + H] ⁺	331.1	259.0	125	25
					273.1 *		25
ZEN	8.11	C ₁₈ H ₂₄ O ₅	[M - H] ⁻	317.2	175.1	147	25
					273.1		20

(Continued)

Results and Discussion

Method verification

The method was verified by evaluating the detection limit (LOD), quantitative limit (LOQ), accuracy, linearity and precision, and the results are shown in Table 2. In order to reduce the interference of matrix effects on the test results, matrix standard was used for quantitative analysis in this study. In this study, the detection limit was 10 times signal-to-noise ratio, and the quantitative limit was 3 times signal-to-noise ratio.

In order to evaluate the accuracy of the method, 14 mycotoxins with three different levels of 1, 5 and 20 $\mu\text{g}/\text{kg}$ were added to the blank matrix, and the recovery and accuracy were evaluated. The results are shown in Table 3. The average recovery values of all mycotoxins tested were in the range of 60%–95%, and the inter-day relative standard deviation (RSD) was lower than 15%. It shows that the method in this study is suitable for the determination of mycotoxins in chili peppers.

Table 2 Determination of matrix effects, linear ranges, LOQ and LOD of 14 mycotoxins in chili peppers by external standard method

Mycotoxin	ME//%	Linear range	LOQ	LOD	Standard curve	R ²
OTA	-85.73	0.5–100	0.5	0.2	$Y = 591.19X + 472.56$	0.9999
OTB	-83.26	0.5–100	0.5	0.2	$Y = 368.07X + 1334.7$	0.9996
AFB1	-73.00	0.1–100	0.1	0.05	$Y = 277.53X - 569.57$	0.9999
AFB2	-60.03	0.1–100	0.1	0.05	$Y = 345.21X + 254.7$	0.9998
AFM1	-68.55	0.1–100	0.1	0.05	$Y = 333.79X - 40.808$	0.9998
AFM2	-48.27	0.1–100	0.1	0.05	$Y = 491.08X + 784.93$	0.9994
AFG1	-60.90	0.5–100	0.5	0.2	$Y = 403.13X + 868.86$	0.9994
AFG2	-56.43	0.5–100	0.5	0.2	$Y = 330.72X + 566.13$	0.9997
ZEN	-77.10	1.0–100	1.0	0.5	$Y = 125.43X - 277.79$	0.9921
α -ZEL	-80.28	1.0–100	1.0	0.5	$Y = 176.13X + 177.88$	0.9993
β -ZEL	-82.79	1.0–100	1.0	0.5	$Y = 153.77X + 89.158$	0.9996
ZAN	-78.73	1.0–100	1.0	0.5	$Y = 78.425X + 163.89$	0.9980
α -ZAL	-69.04	1.0–100	1.0	0.5	$Y = 307.75X + 140.67$	0.9994
β -ZAL	-65.99	1.0–100	1.0	0.5	$Y = 290.08X + 171.74$	0.9982

Table 3 Determination of recovery and RSDs of 14 mycotoxins in chili peppers

Mycotoxin	Recovery at three levels			RSDs at three levels		
	1 $\mu\text{g}/\text{kg}$	5 $\mu\text{g}/\text{kg}$	20.0 $\mu\text{g}/\text{kg}$	1 $\mu\text{g}/\text{kg}$	5 $\mu\text{g}/\text{kg}$	20.0 $\mu\text{g}/\text{kg}$
OTA	73.4	77.5	85.5	11.3	7.3	11.5
OTB	67.6	76.1	85.8	11.0	10.4	7.8
AFB1	78.1	74.3	70.6	10.8	7.5	9.8
AFB2	84.0	76.4	87.0	9.8	6.9	7.2
AFM1	83.4	76.0	87.8	10.5	7.8	9.2
AFM2	80.7	83.5	77.9	6.1	11.1	9.7
AFG1	70.2	83.5	77.7	9.2	6.1	9.1
AFG2	75.5	69.7	75.1	8.5	8.2	11.4
ZEN	62.0	79.7	75.6	10.1	7.7	11.1
α -ZEL	67.1	65.4	86.1	10.7	10.8	6.6
β -ZEL	80.3	66.0	71.4	9.0	11.3	9.5
ZAN	84.8	69.7	83.2	10.7	9.1	6.6
α -ZAL	63.6	70.4	84.8	9.7	11.2	9.4
β -ZAL	82.5	77.5	68.2	8.6	7.5	10.5

Comparing the pretreatment cost of this method with the standard method of GB5009 series, the cost of reagents and consumables required for the pretreatment of 14 mycotoxins in a sample in this study was less than 10 RMB, while those of the standard method was about 900 RMB. Therefore, the cost of reagents and consumables can be saved by at least 90% in this word. It took about 1 h to determine 14 mycotoxins in a sample by this experimental method, while it took more than 9 h by GB5009 series method, so the time cost can be saved by about 90%.

Determination of actual samples

The method was applied to the determination of 100 chili pepper samples in the markets from 10 main urban areas in Guizhou Province, including Renhuai (6 samples, No. (LJ) 01-06), Tongren (6 samples, No. (LJ) 07-12), Chishui (6 samples, No. (LJ) 13-18), Kaili (6 samples, No. (LJ) 19-24), Fuquan (6 copies, No. (LJ) 25-30), Duyun (6 copies, No. (LJ) 31-36), Qianxi (6 samples, No. (LJ) 37-42), Liupanshui (6 samples, No. (LJ) 43-48), Panzhou (6 samples, No. (LJ)

49-54), Xingyi (6 samples, No. (LJ) 55-60), Xingren (6 samples, No. (LJ) 61-66), Bijie (6 samples, No. (LJ) 67-72), Zunyi (6 samples, No. (LJ) 73-78), Guiyang (16 samples, No. (LJ) 79-88), Qingzhen (6 samples, No. (LJ) 89-94), and Anshun (6 samples, No. (LJ) 95-100). It was found that among the 100 chili pepper samples, the detection rates of AFB1, OTA, OTB, ZEN and α -ZEL were 19%, 7%, 4%, 2% and 1%, respectively, and the rest mycotoxins were not detected. The total number of detected samples accounted for 30%, and among them, there were three samples containing two toxins at the same time, and all others contained only one mycotoxin. The detected concentration range of AFB1 was 0.050 1 – 2.140 0 $\mu\text{g}/\text{kg}$, and that of OTA was 0.29 – 6.30 $\mu\text{g}/\text{kg}$. The detected concentration range of OTB was 0.282 – 0.340 $\mu\text{g}/\text{kg}$. The detected concentration of α -ZEL was 22.3 $\mu\text{g}/\text{kg}$. The detected concentrations of ZEN were 1.19 and 9.7 $\mu\text{g}/\text{kg}$, respectively. The detection results are shown in Table 4.

Table 4 Detection results of 14 mycotoxins in actual samples ($\mu\text{g}/\text{kg}$, $n = 100$)

Mycotoxin	Detection rate//%	Between LOD and LOQ//%	Mean $\mu\text{g}/\text{kg}$	Maximum value// $\mu\text{g}/\text{kg}$
OTA	7	3	0.121	6.3
OTB	4	4	0.012	0.34
AFB1	19	9	0.094	2.14
AFB2	ND	ND	ND	ND
AFM1	ND	ND	ND	ND
AFM2	ND	ND	ND	ND
AFG1	ND	ND	ND	ND
AFG2	ND	ND	ND	ND
ZEN	2	ND	0.109	9.7
α -ZEL	1	ND	0.223	22.3
β -ZEL	ND	ND	ND	ND
ZAN	ND	ND	ND	ND
α -ZAL	ND	ND	ND	ND
β -ZAL	ND	ND	ND	ND

Risk assessment of 14 mycotoxins in chili peppers in Guizhou Province

According to the risk assessment methods and reference guidelines of contaminants in food, the tolerable daily intake of aflatoxin [TDI, $\mu\text{g}/(\text{kg} \cdot \text{d})$] per kilogram of body mass for humans is 1 $\text{ng}/(\text{kg} \cdot \text{d})$ ^[6-7], so the tolerable daily intake for an adult is 60 ng. The daily tolerance intake of zearalenone is 0.20 $\mu\text{g}/(\text{kg} \cdot \text{d})$ 8 proposed by Joint Expert Committee on Food Additives (JECFA). In 1998, the Food Science Committee under the European Community proposed that the tolerable daily intake of ochratoxin A should be 5 $\text{ng}/(\text{kg} \cdot \text{d})$ 9. At present, the information about the intake of chili pepper and pepper in China is unknown, so according to the risk assessment of aflatoxin in spices by Ali *et al.*, the dietary exposure of mycotoxin was calculated by taking 13 g of spices every day, based on the average weight of 60 kg. The formula used to calculate the daily dietary exposure of each person in this study is as follows:

$$EDI = R \times F/bw \quad (1)$$

In the formula, *EDI* is daily dietary exposure, $\mu\text{g}/(\text{kg} \cdot \text{d})$; *R* is the residual concentration of food mycotoxin, $\mu\text{g}/\text{kg}$; *F* is food consumption, kg/d ; and *bw* is body weight. The weight of China standard person was taken as 60 kg for calculation.

According to the formula, the daily dietary exposure of chili pepper were calculated for 100 samples: AFB1: 0.020 4 $\text{ng}/(\text{kg} \cdot \text{d})$, OTA: 0.026 2 $\text{ng}/(\text{kg} \cdot \text{d})$, OTB: 0.002 6 $\text{ng}/(\text{kg} \cdot \text{d})$, ZEN: 0.023 6 $\text{ng}/(\text{kg} \cdot \text{d})$, and α -ZEL: 0.048 3 $\text{ng}/(\text{kg} \cdot \text{d})$. The daily dietary exposure of pepper were calculated for 100 samples: AFB1: 0.045 3 $\text{ng}/(\text{kg} \cdot \text{d})$, OTA: 0.062 0 $\text{ng}/(\text{kg} \cdot \text{d})$, and ZEN: 0.034 5 $\text{ng}/(\text{kg} \cdot \text{d})$.

The food safety index (*IFS*)^[11] was used to describe the risk of mycotoxin residues, and the calculation formula is shown as Formula 2. $IFS \leq 1$ means that the dietary risk is at an acceptable safety level, and the smaller the value, the smaller the risk; $IFS < 1$ means that the detected mycotoxins will not cause harmful effects on human health, and the safety status is acceptable; $IFS > 1$ indicates that the dietary risk is at an unacceptable level, and the greater the value, the greater the risk.

$$IFS = R \times F/SI \times bw \quad (2)$$

In the formula, *IFS* is food safety index; and *SI* was the acceptable daily intake (ADI) of a mycotoxin. The aflatoxin, ochratoxin and zearalenone in the sampled chili peppers and peppers were calculated, all showing $IFS < 1$, which indicated that mycotoxins in chili peppers from Guizhou Province would not cause harmful effects on human health.

Conclusions

QuEChERS-DES-liquid-liquid microextraction was adopted for pretreatment, and 14 mycotoxins in spices were determined. The linear range of the ochratoxin and AFG type was 0.5 – 100 $\mu\text{g}/\text{kg}$, and the detection limit and quantitative limit were 0.2 and 0.5 $\mu\text{g}/\text{kg}$, respectively. The linear range of other mycotoxins was 0.1 – 100 $\mu\text{g}/\text{kg}$, and the detection limit and quantitative limit were 0.1 and 0.05 $\mu\text{g}/\text{kg}$, respectively. The linear range of the zearalenone type was 1 – 100 $\mu\text{g}/\text{kg}$, and the detection limit and quantitative limit were 1 and 0.5 $\mu\text{g}/\text{kg}$, respectively. The average recovery of mycotoxins tested was between 60% and 90%, and the inter-day *RSD* was less than 15%. This method was used to test 100 chili pepper samples from main urban areas of Guizhou Province, and the total detection rate was 30%. The detection rates of AFB1, OTA, ZEN and α -ZEL were 19%, 7%, 2% and 1%, respectively. Also, 100 pepper samples were detected, and the total detection rate was 28%. The detection rates of AFB1, OTA and ZEN were 16%, 13% and 1%, respectively. The risk of the test results was evaluated, and concluded to be low.

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medium and glucose-added culture medium, and the reason why the data of the latter was slightly larger than that of the former might be that the strains in glucose-added culture solution grew better, so the degradation rate was better.

Efficient and stable triethylamine-degrading strains SHN6-1, SHN6-2 and SHN6-3 were identified, and these three strains had also been isolated and purified. Then, the 16S rDNA fragments of these three strains were amplified by PCR and sequenced. Through PCR sequencing, it was determined that these three strains of bacteria were all of the same species, named *Microbacterium arthrospira* CC-VM-Y (T).

Conclusions and Discussion

In this study, sludge and water samples were taken from a sewage treatment plant in Tangshan City. Triethylamine-degrading bacteria were primarily screened and re-screened. Through the enrichment and isolation of triethylamine-degrading bacteria, the degradation rate of triethylamine in water was determined by bromophenol blue spectrophotometry, and three strains with high degradation rates were selected, namely, SHN6-1, SHN6-2, and SHN6-3. The three strains were amplified by PCR and sequenced. Finally, they were found to be the same strain, named *M. arthrospira* CC-VM-Y (T).

Triethylamine is a typical organic small molecular substance which is degradation-resistant in wastewater, and there is little

research on its degrading microorganisms. This study aimed to screen new strains that can degrade triethylamine efficiently, so as to help sewage treatment plants purify water better and reduce the effects of harmful substances in sewage to the surrounding environment and the development of aquaculture. Microbial resources are huge and charming. We should make use of modern advanced science and technology to make microbial resources better serve aquaculture and all mankind. Further exploration and discovery are needed for other uses of microbial resources by future generations.

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