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Multianalysis of 35 Mycotoxins in Traditional Chinese Medicines by Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry Coupled with Accelerated Solvent Extraction

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Supporting Information

ABSTRACT: A generic procedure, which involved accelerated solvent extraction and homemade cleanup cartridges, has been developed for the extraction and purification of 35 mycotoxins in various traditional Chinese medicine (TCM) matrixes, i.e., rhizomes and roots, seeds, flowers, and grasses and leaves, for subsequent analysis by ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). All target analytes could be simultaneously quantitated in less than 17 min per run, showing narrow symmetrical peaks. The developed method was also successfully applied in routine monitoring programs, which implied a significant reduction of both effort and time, to investigate the contamination of TCMs. Among 60 commercial TCMs analyzed, 50 were positive. The achieved data underpin the practical application of the UHPLC–MS/MS method as a valuable tool for the trace analysis of multiple mycotoxins in TCMs.

KEYWORDS: *mycotoxins, traditional Chinese medicine, UHPLC–MS/MS, accelerated solvent extraction, homemade cleanup cartridges*

INTRODUCTION

Mycotoxins, a series of secondary metabolites, are produced by various mold species growing on the plant origin product either in the field or during storage. Hitherto, approximately 300–400 mycotoxins have been found, which can be categorized into different groups on the basis of their different sources.[1] Aflatoxins, a family of structure-related mycotoxins, are produced as secondary metabolites by the spoilage of fungi *Aspergillus*, particularly *Aspergillus flavus* and *Aspergillus parasiticus*. The most important members, i.e., aflatoxin B1 (**1**), aflatoxin B2 (**2**), aflatoxin G1 (**3**), aflatoxin G2 (**4**), and aflatoxin M1 (**5**), have been classified in group I as human carcinogens by the International Agency for Research on Cancer (IARC). Sterigmatocystin (**26**), which is produced by the same fungi, also has direct carcinogenic activity. Trichothecenes represent a group of secondary metabolites produced by toxigenic *Fusarium* species, especially *Fusarium sporotrichioides*, *Fusarium poae*, and *Fusarium equiseti*. Acute and chronic ingestion of trichothecenes exemplified as deoxynivalenol (**10**) by humans and animals can elicit a variety of toxic effects, i.e., feed refusal, weight loss, vomiting necrosis of the myeloid tissue, and hemorrhage of visceral organs.[2] Ochratoxins are also very important secondary metabolites, which are related to many diseases, i.e., urothelial urinary tract tumors and renal intumescences. Zearalenone (**33**) and its derivatives, a group of phenolic compounds produced by several species of *Fusarium*, may damage the reproduction of mammals.

The widespread presence of fungi in the environment renders mycotoxins practically ubiquitous contaminants in food, feedstuff, and herbal drugs. To protect public health, one of the most effective means is to establish reasonable regulatory levels of these toxins on the basis of valid toxicological data. In the European Union (EU), the maximum contents are established at the following levels: 2 μg/kg for **1** and 4 μg/kg for total aflatoxins in peanut products, 1250 μg/kg for **10**, 100 μg/kg for **33**, and 5 μg/kg for ochratoxin A (**22**) in unprocessed cereals.[3]

Although the presence of these mycotoxins is potentially harmful, research has mainly focused on the study of food and feedstuff, and few papers have been published for monitoring the mycotoxins in traditional Chinese medicines (TCMs). As a consequence, limited information about the contamination levels of mycotoxins in TCMs has been obtained, and thus, scarce regulations have been established up to now. Moreover, the TCMs can be simultaneously infected by different toxigenic molds, which potentially results in the co-occurrence of multiple mycotoxins. The previous studies were only directed toward a single class of mycotoxins in TCMs.[4,5] Consequently, the same sample needs to be analyzed multiple times to cover all relevant analytes. Therefore, within the field of mycotoxin

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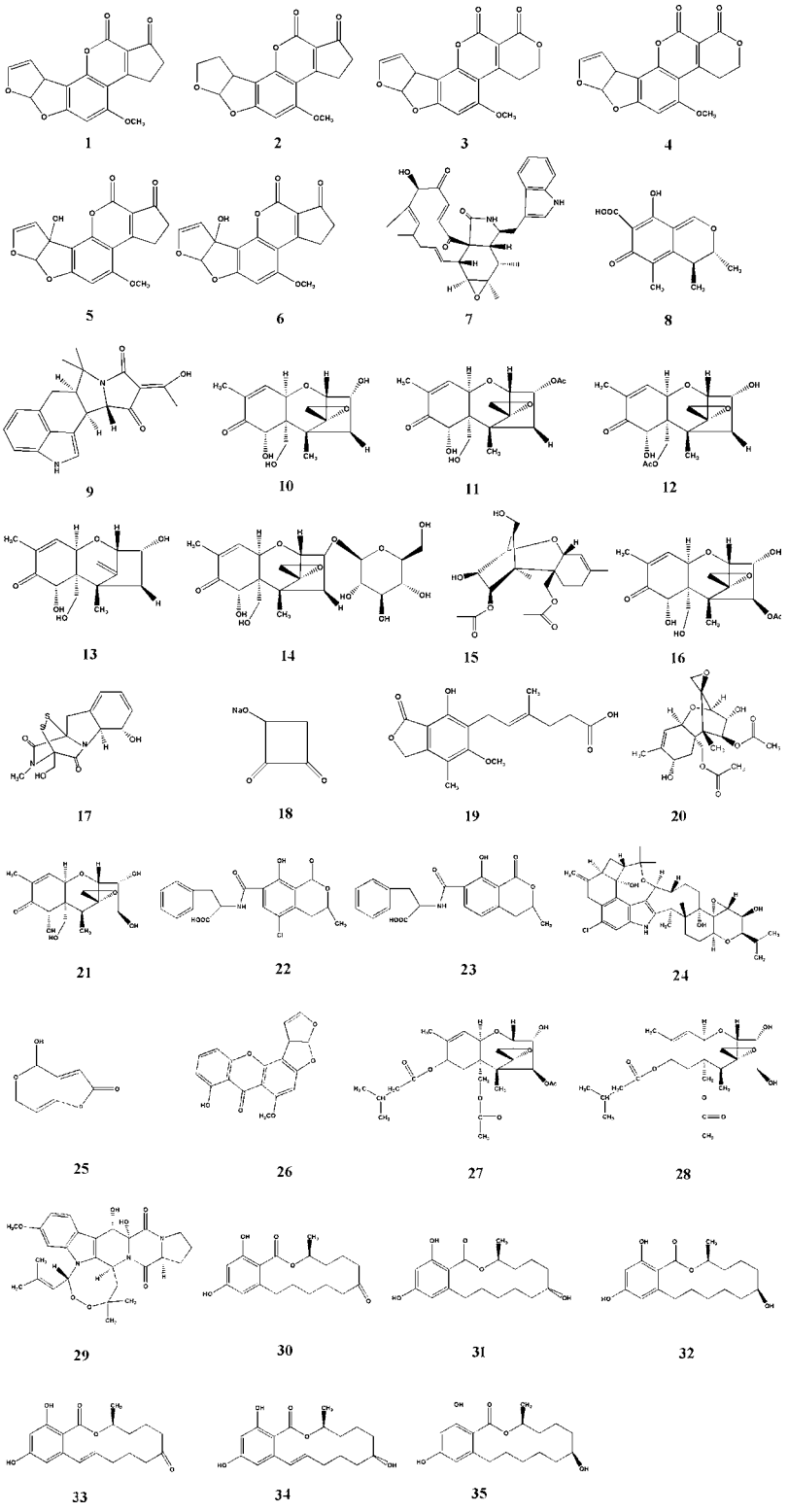

Figure 1. Chemical structures of the 35 mycotoxins investigated in the present study.

analysis in TCMs, a clear trend toward the use of multianalyte methods can be seen.

The increasing need for mycotoxin analysis of TCMs has promoted increasing research on the methods with the employment of adequate extraction and cleanup procedures. In some previous studies, mixtures of methanol/water (sometimes added with sodium chloride, acid, or alkali) or acetonitrile/water (acetonitrile 75−90%, v/v) with various extraction procedures, i.e., ultrasonic and homogenization, were commonly used.[4,6,7] The main drawbacks of these techniques are extensive sample manipulation and labor intensiveness. As more rapid and automated procedures are required because of the constant increase in the number of samples tested, interest in the incorporation of new technologies that are faster and

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amenable for automated sample handling and reduce exposure of laboratory technicians to toxic chemicals is ongoing. One of the most promising extraction techniques is accelerated solvent extraction (ASE). Due to its high efficiency, ASE has been employed for the extraction of **22** in rice and bread,[8,9] **33** in cereals,[10] fumonisins in corn-based baby food,[11] and **10** and fumonisins in wheat and corn.[12] For further purification and analyte enrichment, the extracts are submitted to solid-phase extraction (SPE) with a wide variety of sorbents, i.e., reversed-phase, strong anion exchange, immunoaffinity, or polymeric materials.[13,14] However, up to now, this field has been limited to a few surveys of mycotoxins in food and no method has been developed for the given application to TCMs due to the variety of the physicochemical properties of the mycotoxins, along with the complex composition of the sample matrix.

For quantitative analysis, methods based on thin-layer chromatography (TLC),[15] enzyme-linked immunosorbent assay (ELISA),[16,17] gas chromatography (GC),[18] and high-performance liquid chromatography (HPLC)[1,19,20] have been developed. The most frequently used technique is HPLC as it combines high precision with increasing sophisticated automation. After the introduction of ultra-high-performance liquid chromatography (UHPLC), better chromatographic resolution and improved sensitivity have been achieved for the analysis of the multicomponent mycotoxin contaminants.[21,22] In recent years, the availability of ionization sources has significantly improved the possibilities of employing liquid chromatography−tandem mass spectrometry (LC−MS/MS) in the mycotoxin analysis.[23−27] The methodology achieves its preferred status because it is perceived that MS/MS detection is highly selective and thus can effectively eliminate interferences. However, the accuracy of quantitative assays using LC−MS/MS may not be absolute. Results may be adversely affected by the ion suppression/enhancement caused by the sample matrix when the electrospray ionization (ESI) mode is utilized in the MS/MS detection.[28] The purpose of the current work is to develop an accurate analytical method which should not only cover a vast number of mycotoxins but also be applicable to different types of TCM matrixes to investigate the actual contaminant situations in representative TCMs in China.

MATERIALS AND METHODS

Chemicals and Reagents. The standards **1**, **2**, **3**, **4**, **5**, aflatoxin M2 (**6**), **22**, ochratoxin B (**23**), T-2 toxin (**27**), and HT2 toxin (**28**) were purchased from Alexisa (San Diego, CA). 3-Acetyldeoxynivalenol (**11**) and 15-acetyldeoxynivalenol (**12**) were purchased from Biopure (Tulln, Austria). Chaetoglobosin A (**7**), citrinin (**8**), cyclopiazonic acid (**9**), **10**, deepoxydeoxynivalenol (**13**), deoxynivalenol-3-glucoside (**14**), diacetoxyscirpenol (**15**), fusarenon X (**16**), gliotoxin (**17**), moniliformin (**18**), mycophenolic acid (**19**), neosolaniol (**20**), nivalenol (**21**), penitrem A (**24**), patulin (**25**), **26**, verruculogen (**29**), zearalanone (**30**), α-zearalanol (**31**), β-zearalanol (**32**), **33**, α-zearalenol (**34**), and β-zearalenol (**35**) were obtained from Sigma-Aldrich (St. Louis, MO). The chemical structures of the 35 mycotoxins are shown in Figure1. ^{13}C-**1**, ^{13}C-**10**, ^{13}C-**22**, ^{13}C-**27**, and ^{13}C-**33**, also purchased from Sigma-Aldrich, were used as internal standards (ISs).

Milli-Q-quality water (Millipore, Billerica, MA) was used during the whole analysis. Acetonitrile and methanol, both HPLC grade, were purchased from Merck (Darmstadt, Germany). Other solvents and chemicals were of HPLC or analytical grade. High-quality poly(9, 9-diethylfluorene) (PDEF) syringe filters (0.22 μm pore size, 13 mm diameter) were purchased from Millipore. Oasis HLB SPE cartridges (200 mg, 6 cm^3) were purchased from Waters Corp. (Milford, MA). Polypropylene SPE empty tubes (6 mL) and frits were purchased from Shenzhen Biocomma Biotech Co., Ltd.

UHPLC Conditions. UHPLC was performed using a Waters Acquity ultra-high-performance LC system (Waters). Separation was performed on a 150 mm × 2.1 mm i.d., 1.7 μm, Acquity UPLC BEH Shield RP18 column at 40 °C, with a mobile phase flow rate of 0.35 mL/min. The mobile phase consisted of (A) water (containing 0.25 mmol/L ammonium acetate and 0.2% aqueous ammonia) and (B) acetonitrile/methanol (80/20, v/v). A linear gradient elution program was applied as follows: 0 min 5% B, 4 min 18% B, 6 min 20% B, 8 min 20% B, 9 min 25% B, 11.5 min 60% B, 13 min 80% B, 14 min 100% B, 14.5 min 100% B, 14.8 min 5% B, and hold for a further 2.2 min for re-equilibration, giving a total run time of 17 min. The injection volume was 5.0 μL (partial loop with needle overfill).

Mass Spectrometry. The separated compounds were detected with a Waters XEVO TQ mass spectrometer (Waters). The following settings were used: source temperature, 150 °C; desolvation temperature, 500 °C. The cone and desolvation gas flows were 30 and 1000 L/h, respectively. Quantitation was performed in multiple-reaction monitoring (MRM) mode, and the conditions were optimized for each mycotoxin during infusion. Data acquisition and processing were performed using MassLynx v4.1 and Targetlynx (Waters).

Preparation of Standard Solutions. Accurately weighed solid portions of each mycotoxin (1 ± 0.05 mg) were dissolved in 10 mL of acetonitrile to prepare 0.1 mg/mL stock solutions, and the resulting solutions were stored at −20 °C in the dark. The work solutions were prepared by diluting each stock solution step by step with the combined solution (acetonitrile/water containing 0.2% formic acid, 20/80, v/v). The stock solutions of the ISs were directly used with the purchased products and diluted with the same combined solution to 50 ng/mL for ^{13}C-**1**, ^{13}C-**22**, and ^{13}C-**27** and 500 ng/mL for ^{13}C-**10** and ^{13}C-**33**. All work solutions were prepared immediately before use.

Preparation of Homemade Cleanup Cartridges. Silica gel (2 ± 0.010 g) was accurately weighed into a 6 mL hollow SPE cartridge, and the cartridge was shaken to compact the silica gel. Then the frit was put on the top layer to ensure that the surface was smooth and flat.

Samples. A total of 60 dried TCMs (500 g each), randomly collected from different regulated enterprises, were included in four types: (1) rhizomes and roots (Radix Angelicae Sinensis, Radix Paeoniae Alba, Rhizoma Coptidis, Radix Angelicae Dahuricae (two), Rhizoma Bolbostematis, Bulbus Fritillariae Thunbergii, Radix Paeoniae Rubra (two), Rhizoma Coptldis, Radix Puerariae Lobatae, Rhizoma Iridis Tectori, Caulis Dendrobii, Radix Bupleuri, Radix Polygoni Multiflori (two), Radix Et Rhizoma Salviae Mil Tiorrhizae (two), Radix Rumicis Japonici, Radix Astragali (two), Radix Scutellariae, Radix Rhizoma Glycyrrhizae); (2) seeds (Fructus Citri, Semen Persicae (three), *Citrus reticulata* Blanco, Fructus Aurantii, Semen Ziziphi Spinosae (two), Fructus Mume, Semen Armeniacae Amarum, Fructus Crataegi (two), Fructus Schisandrae Chinensis, Fructus Forsythiae, Spora Lygod II); (3) grasses and leaves (Herba Spanishneedles, Herba Ephedrae, Herba Lysimachiae (two), Herba Violae, Folium Isatidis, Herbal Moellendorfs Spidemoss (two), Herba Spreeding Hedyotis, Indigo Naturalis (two)); (4) flowers (Flos Carthami (five), Flos Chrysanthemi (two), *Solidagodecurrens* Lour., Flos Lonicerae Japonicae, Flos Carthami, Flos Albiziae). Information about the geographic origin of the samples was required and registered. Each TCM was milled into powders using a Romer analytical sampling mill (Romer Laboratories, Tulln, Austria) and maintained in zipper-top paper bags at −20 °C until the analysis time.

Sample Pretreatment. Mycotoxins were extracted from TCMs using the ASE method by an APLE-2000 (Beijing Titan Instruments Co., Ltd.). Nitrogen was obtained from an in-house nitrogen source to assist the pneumatic system and to purge the extraction cells.

A ground test portion of each TCM (2 g) was spiked with 100 μL of each IS solution. The spiked test portion was packed into an 11 mL pressure-resistant stainless steel extraction cell. Acetonitrile/water solution (84/16, v/v) (60% flush volume) was utilized as the elution solvent. The other conditions were set as follows: temperature, 70 °C; pressure, 10 MPa; preheating time, 2 min; static time, 300 s; purge time, 60 s; number of cycles, 2. The total volume of extract was about 18 mL, and the total extraction time was around 15 min.

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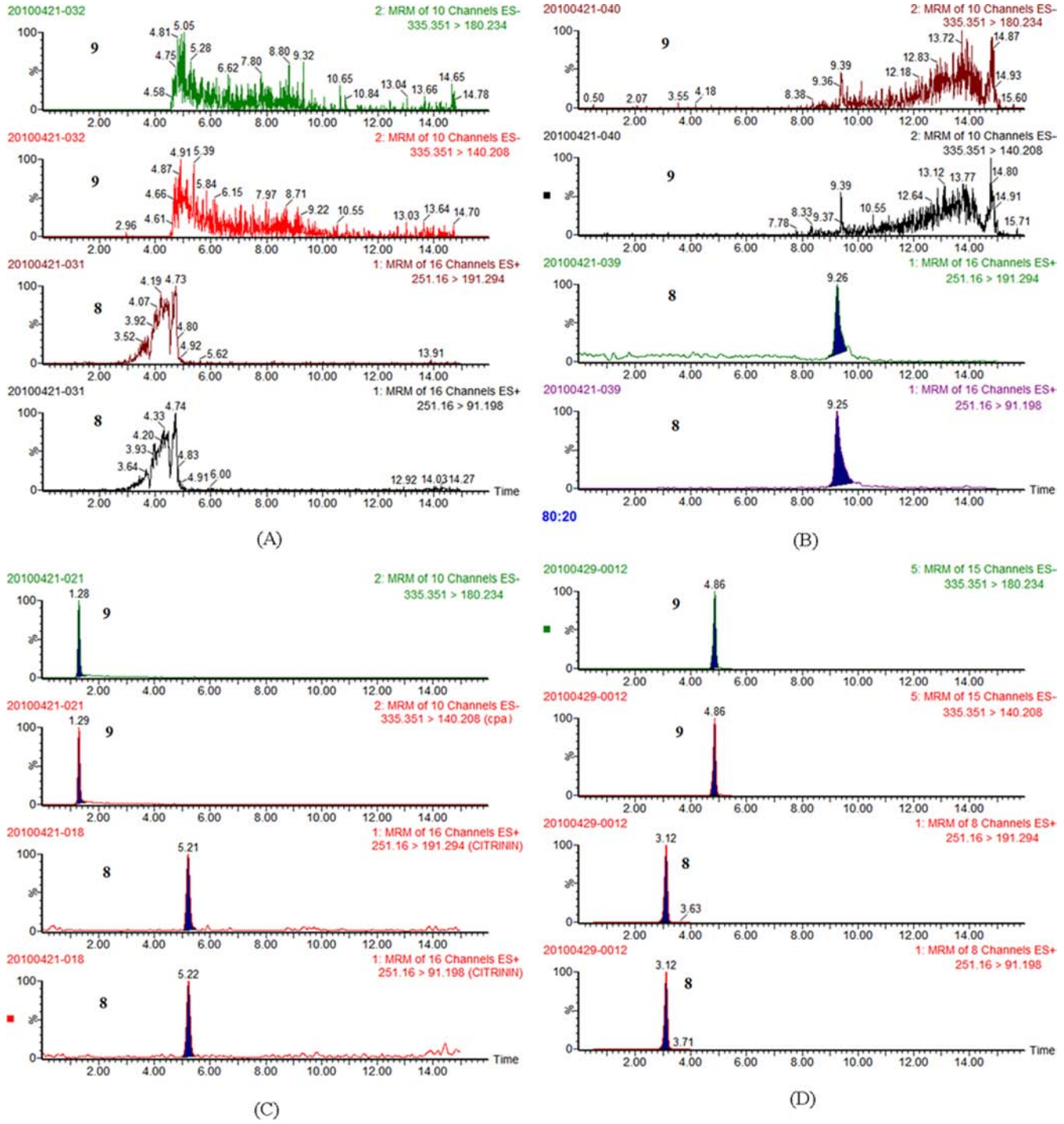

Figure 2. Comparison of the peak shapes and ionization efficiencies of citrinin (**8**) and cyclopiazonic acid (**9**) among four candidate weak elution mobile phases: (a) water containing 10 mmol/L ammonium acetate; (b) water containing 0.2% formic acid; (c) water containing 0.2% aqueous ammonia; (d) water containing 0.2% aqueous ammonia and 10 mmol/L ammonium acetate.

The ASE extract was transferred into a 50 mL round-bottomed flask and concentrated to about 5 mL with a rotatory evaporator at 50 °C and 120 mbar. Then the extract was transferred to a 10 mL test tube and dried by a gentle stream of nitrogen gas at 50 °C. The residue was redissolved in acetonitrile/water solution (84/16, v/v) to a volume of 5 mL. The solution was passed through the homemade cleanup cartridges and eluted with 3 mL of methanol. The effluents, including the loading solution and the eluent, were collected in a test tube and dried by nitrogen gas at 50 °C. The residue was redissolved by 200 μL of acetonitrile and then shaken for about 30 s by vortex, and 800 μL of the combined solution (acetonitrile/water containing 0.2% formic acid, 20/80, v/v) was added. The tube was then shaken for another 30 s briefly to mix the contents of the tube. Finally, the solution was passed through a 0.22 μm nylon filter and was ready for injection.

Cleanup of TCM Extraction by the Candidate Purification Materials. For normal-phase materials, the extractions were passed through the cartridges and eluted with 3 mL of methanol, and the effluents were combined and dried by nitrogen gas at 50 °C; for reverse-phase materials, the same standard solutions were diluted with distilled water until the content of acetonitrile was no higher than 5%. The solutions were passed through the reconditioned cartridges, and then 5 mL of water was passed. All targets were eluted with 6 mL of

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Table 1. MRM Transitions, Ionization Modes, Cone Energies, and Collision Energies for Various Mycotoxins[a]

mycotoxin	precursor ion (m/z)	cone energy (eV)	primary product ion (m/z)	collision energy (eV)	secondary product ion (m/z)	collision energy (eV)
1	313 (+H)	50	241	37	285	23
2	315 (+H)	50	287	26	259	30
3	329 (+H)	25	243	25	283	25
4	331 (+H)	50	245	30	257	30
5	329 (+H)	38	229	42	273	22
6	331 (+H)	36	313	18	259	22
7	529 (+H)	24	130	47	511	10
8	251 (+H)	26	91	41	191	23
9	335 (−H)	48	140	27	180	25
10	355 ($+CH_3COO^-$)	20	295	9	265	15
11	337 (−H)	24	307	15	173	9
12	337 (−H)	20	150	23	219	11
13	339 ($+CH_3COO^-$)	18	59	11	249	13
14	517 (+CH3COO$^-$)	26	457	15	–	–
	457 (−H)	36	–	–	427	17
15	384 ($+NH_4^+$)	20	307	11	105	31
16	413 ($+CH_3COO^-$)	18	353	11	263	17
17	327 (+H)	16	263	11	111	29
18	97 (−Na)	20	41	10	–	–
19	321 (+H)	22	207	21	159	41
20	400 ($+NH_4^+$)	18	185	21	215	19
21	371 ($+CH_3COO^-$)	20	281	15	311	9
22	404 (+H)	31	239	19	358	14
23	370 (+H)	24	205	23	187	37
24	636 (+H)	34	560	15	618	10
25	153 (−H)	18	–	–	81	11
	213 ($+CH_3COO^-$)	10	153	5	–	–
26	325 (+H)	42	310	23	253	41
27	484 ($+NH_4^+$)	22	185	19	215	19
28	442 ($+NH_4^+$)	16	263	11	145	25
29	534 (+Na)	50	392	13	191	21
30	319 (−H)	44	159	30	174	26
31	321 (−H)	44	259	24	91	36
32	321 (−H)	44	259	24	91	36
33	317 (−H)	44	175	30	131	24
34	319 (−H)	44	159	30	174	26
35	319 (−H)	40	205	22	107	30
^{13}C-**1**	330 (+H)	48	301	25	255	37
^{13}C-**10**	370 ($+CH_3COO^-$)	20	310	11	279	15
^{13}C-**22**	424 (+H)	26	250	25	232	37
^{13}C-**27**	509	20	229	17	323	15
^{13}C-**33**	335 (−H)	42	185	26	140	36

[a]A dash means no relative product ions (collision energies) have been found.

methanol at a rate of about 1−2 drops/s and then evaporated under a stream of nitrogen gas at 50 °C. All of the above residues were redissolved in 1 mL of acetonitrile/water containing 0.2% formic acid (20/80, v/v) solution and analyzed by the established UHPLC−MS/MS method.

Evaluation of Matrix Effects. The samples, showing the absence of the target compounds, for each type of TCM, i.e., rhizomes and roots, flowers, seeds, and grasses and leaves, were selected and carried through the whole sample preparation to prepare the blank matrixes. The standards of mycotoxins were added to the blank matrixes to prepare matrix-matched standards at 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, and 600 ng/mL. The standard solutions, with the same concentration sequence, were prepared in the combined solution (acetonitrile/water containing 0.2% formic acid, 20/80, v/v). The slope of the standard addition plot was compared with the slope of the standard calibration plot to calculate the signal suppression/enhancement (SSE), which was used for quantitative assessment of the matrix effects.

Method Validation. The standard solutions, with the concentration sequence of 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, and 600 ng/mL, were prepared in the combined solution (acetonitrile/water containing 0.2% formic acid, 20/80, v/v) and the four different blank matrixes, with 5 ng/mL for ^{13}C-**1**, ^{13}C-**22**, and ^{13}C-**27** and 50 ng/mL for ^{13}C-**10** and ^{13}C-**33**, included. The calibration curves were created using the isotope dilution method. The limit of detection (LOD) and limit of quantitation (LOQ) were determined by successive analyses of spiked matrixes with decreasing amounts of every standard until signal-to-noise ratios of 3/1 and 10/1 were reached, respectively. A recovery test was performed in the noncontaminated TCMs employing the method of standard addition at high, intermediate, and low levels. The noncontaminated TCMs were also employed for the intra- and interday precision analysis. The recoveries and precisions were calculated on the basis of the relative matrix-matched calibration.

Statistical Treatment. Statistical analysis was performed using Student's t test and one-way analysis of variance. Multiple comparisons of means were separated at $P < 0.05$ by the least significant difference

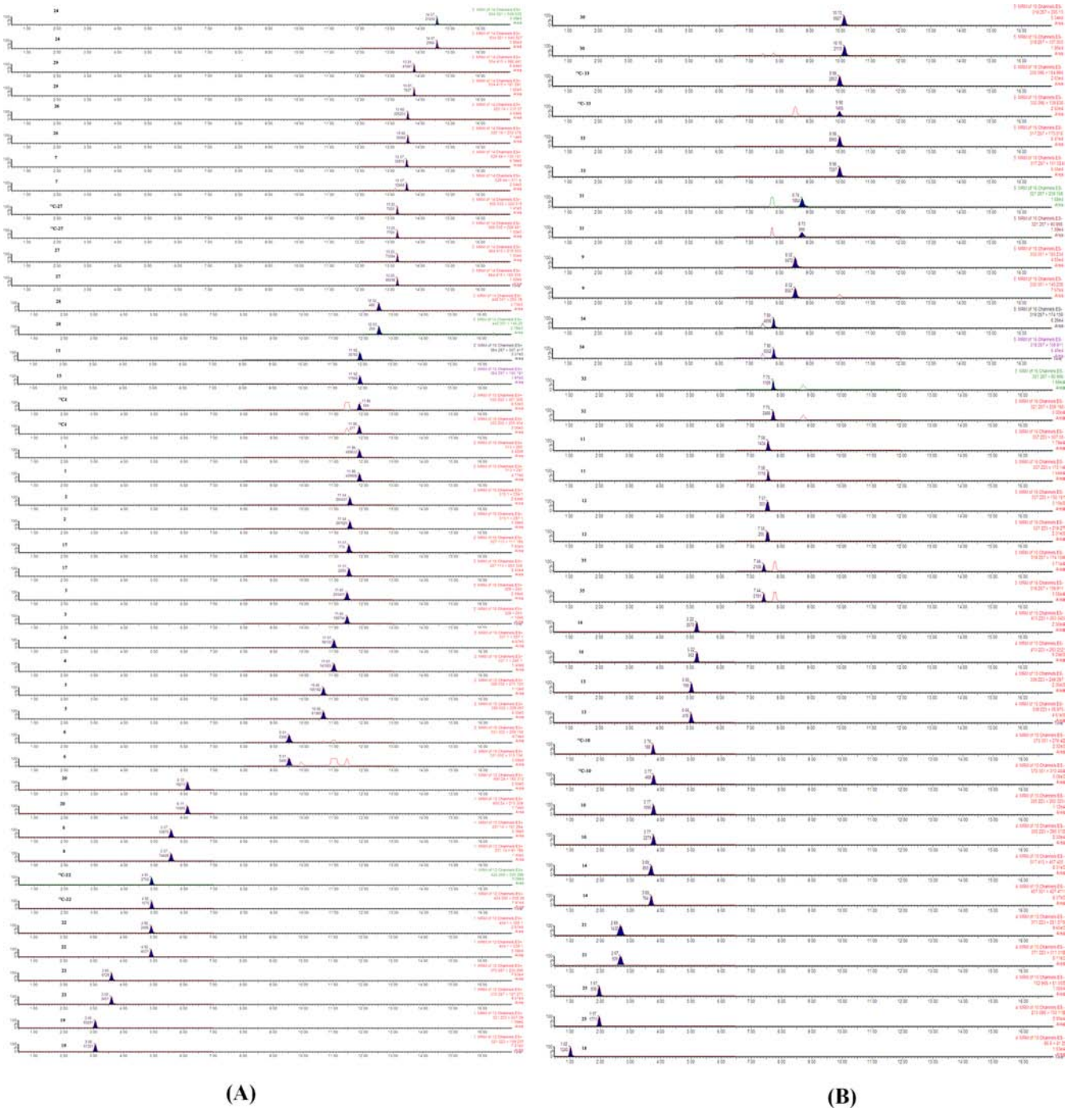

Figure 3. MRM chromatograms of 19 positive ions (A) and 16 negative ions (B) of mycotoxins in the standard solution. The concentrations were 10 ng/mL for the positive ions of mycotoxins and 50 ng/mL for the negative ones, while 5 ng/mL ^{13}C- aflatoxin B1 (^{13}C-**1**), ^{13}C-ochratoxin A (^{13}C-**22**), and ^{13}C-T-2 toxin (^{13}C-**27**) and 50 ng/mL ^{13}C- deoxynivalenol (^{13}C-**10**) and ^{13}C-zearalenone (^{13}C-**33**) were included.

(LSD; a = 0.05) test. All computations were made by employing statistical software (SAS, version 8.2).

RESULTS AND DISCUSSION

Optimization of UHPLC Conditions. In previous studies, ESI$^+$ analysis and ESI$^-$ analysis were performed.[1,6] These were time- and labor-consuming processes and are dealt with in the present study by optimizing the mobile phase. For the choice of the weak elution mobile phase, (a) water containing 10 mmol/L ammonium acetate, (b) water containing 0.2% ammonium formate, (c) water containing 0.2% aqueous ammonia, and (d) water containing 0.2% aqueous ammonia and 10 mmol/L ammonium acetate were compared. Results from an MS full scan of 35 kinds of mycotoxins showed that **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **17**, **19**, **22**, **23**, and **26** could generate corresponding $[M + H]^+$ ions under the ESI$^+$ mode and **9**, **30**, **31**, **32**, **33**, **34**, and **35** could generate corresponding $[M - H]^-$ ions under the ESI$^-$ mode. The responses of $[M + NH_4]^+$ ions generated from **15**, **20**, **27**, and **28** and $[M + Na]^+$ generated from **29** were obviously higher than those of the $[M + H]^+$ ions with all candidate mobile phases. However, the ionization efficiency of the type B trichothecenes (**10**, **11**, **12**, **13**, **14**, **16**, **21**) was quite

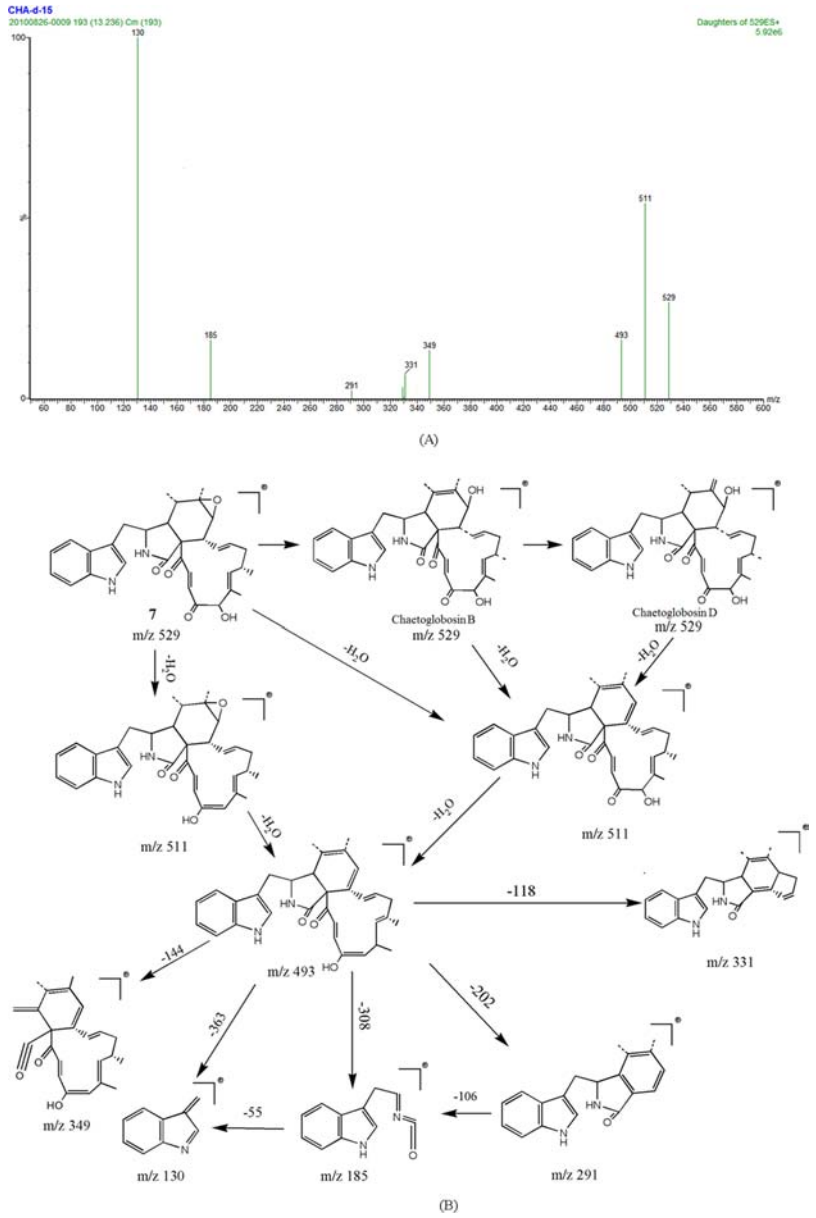

Figure 4. LC−(ESI$^+$)-MS/MS spectrum of chaetoglobosin A (**7**), chaetoglobosin B, and chaetoglobosin D (A), as well as their postulated fragmentation pathways (B).

poor, and the peak shapes of **8** and **9** were unsatisfactory when solution a was selected (Figure 2A). When using solution b, the peak shape of **8** was obviously improved; however, the other problems could not be solved (Figure 2B). When solution c (Figure 2C) or solution d (Figure 2D) was selected, all mycotoxins could obtain high ionization efficiency with good peak shapes. Alternatively, solution d was selected because the isomers (**31**, **32** and **34**, **35**) could not be separated and thus could not be accurately quantitated if solution c was used. After that, the concentration of ammonium acetate (0.25, 0.5, 1, 2, 5, 10 mmol/L) was optimized. Then the established UHPLC method was applied to analyze mycotoxin-spiked TCM extracts, i.e., Radix Angelicae Dahuricae (rhizome), Herbal Moellendorfs Spidemoss (grasses), Fructus Crataegi (seeds), and Flos Carthami (flowers). The results showed that all targets could be completely separated from the interferences in different matrixes. Finally, water containing 0.2% aqueous ammonia and 0.25 mmol/L ammonium acetate was selected as the mobile phase in the present study.

MS/MS Analysis. The MS/MS conditions were optimized for each mycotoxin by direct injection of the standard solution (500 ng/mL). Identification of precursor ions was performed in the full-scan mode by recording from m/z 100 to m/z 800 in both ESI$^+$ and ESI$^-$ modes. On the basis of the confirmation of precursor ions, two product ions for each precursor ion were selected according to the highest sensitivity and optimal selectivity for the target compounds. Cone voltages were selected according to the sensitivity of the precursor ions, and collision energies were chosen to give the maximum intensity of the fragment ions. The final selections of precursor ions, product ions, cone energies, and collision energies are shown in Table 1.

After optimization of the MS/MS parameters, the MRM mode with two transitions was developed. The transition with the best signal intensity was preferred for quantitation, and the

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less intense one, as well as the ratio of abundances between two transitions, was used for confirmation. MRM chromatograms of 19 positive ions and 16 negative ions of mycotoxins in the standard solution are shown in Figure 3.

Among the 35 mycotoxins, **7** has received more attention. After the standard solution of **7** had been stored under the room conditions for 3 days, another two peaks appeared both in the most intense transition used for quantitation and in the less intense transition used for qualification. To find out what the other two peaks represented, full-scan and daughter-scan modes were employed. After comparison of the LC−(ESI^+)-MS/MS spectra of three compounds, the same precursor ions and product ions were found (Figure 4A). In previous studies, chaetoglobosin B, chaetoglobosin C, and chaetoglobosin D were reported to have the same molecular formula, $C_{32}H_{36}O_5N_2$, and showed similar spectrometric properties, suggesting that they were isomeric with **7**. When **7** was treated with some chemicals (BF_3-etherate in $CHC1_3$ or triethylamine in methanol) or heated, chaetoglobosin B, chaetoglobosin C, and chaetoglobosin D formed.[29,30] According to the retention time in Figure 3A, the last peak was established as **7**. Considering the longer retention time of **7** compared to that of the other two compounds, its polarity might be the lowest. Therefore, the two peaks might represent chaetoglobosin B and chaetoglobosin C (two hydroxy groups) rather than chaetoglobosin C (no hydroxy group). The main fragmentation pathways of **7**, chaetoglobosin B, and chaetoglobosin C were also investigated as shown in Figure 4B, which obviously supported the speculation. Overall, the stock solution of **7** should be carefully stored at −20 °C in the dark, and the working solution should be prepared immediately before use; otherwise, it might be transformed to chaetoglobosin B or chaetoglobosin C, which would result in inaccurate quantitative analysis.

Optimization of the ASE Method. Six commonly used solvent systems, (a) acetonitrile/water (84/16, v/v), (b) methanol/water (84/16, v/v), (c) acetonitrile/water containing 1% sodium chloride (84/16, v/v), (d) acetonitrile/water (70/30, v/v), (e) acetonitrile/water/formic acid (79/20/1,v/v), and (f) acetonitrile/water/acetic acid (79/20/1,v/v), were compared in the pilot test. Extraction recovery was evaluated by comparing the peak areas of the relative mycotoxins spiked before extraction with those spiked after extraction in Radix Angelicae Dahuricae (rhizomes and roots) samples. The results showed that the highest extraction recoveries, ranging from 76.3% to 98.1%, were obtained when solvent a was selected. Similar good results were obtained when solvent a was applied to perform the extraction of the 35 mycotoxins in other TCMs, i.e., (1) flowers (Flos Carthami), (2) seeds (Fructus Crataegi), and (3) grasses and leaves (Herbal Moellendorfs Spidemoss). Finally, acetonitrile/water (84/16, v/v) was selected as the extraction solvent.

After optimization of the extraction solvent, the influence of the pressure, extraction time, and number of cycles was also studied to find the optimal conditions for the ASE method. An orthogonal design L_9 (3^4) was used. First, a spiked Radix Angelicae Dahuricae sample (10 μg/kg for positive ions and 50 μg/kg for negative ions) was selected and extracted following the procedure according to the orthogonal table, and each treatment was done in triplicate. The extraction efficiency was directly evaluated by testing the responses of the 35 mycotoxins in the extracts. The results showed that the pressure had a significant effect on the extraction of many mycotoxins: F values of **5**, **6**, **8**, **20**, **16**, **21**, **10**, **11**, **12**, and **25** were all greater than the critical F value (19, $P = 0.05$). Although its effect on the extraction efficiency for the other mycotoxins was not significant, 10 MPa was obviously the best choice. The number of cycles also played a vital role in the extraction of **1**, **14**, **22**, and **24**. The extraction efficiency was the highest for all mycotoxins when the number of cycles was three; however, the extraction efficiency of two cycles was almost the same as that of three cycles for all mycotoxins (>90%). On the basis of the consideration of solvent and time consumption, the number of cycles was standardized as two. Although the influence of the extraction time was not significant, the extraction efficiencies for most of the mycotoxins were highest when the extraction time was 300 s.

After selection of the three significant factors, some other conditions, such as the cell size and temperature, were also investigated. For optimization of the cell size, 11 and 22 mL cells were tried. For both sizes, the same quantity of spiked sample was added, and the results showed that no difference existed between the two types of cells. Alternatively, the 11 mL cell was selected as it required a lower quantity of solvent, which constitutes a more economical and ecological procedure. The ASE method was also optimized by changing the temperature (30, 50, 70, 90, and 110 °C). High temperature during the extraction process increased the solubility of the analytes and the extraction rate and decreased the viscosity and surface tension of the solvents. However, when the temperature was set above 70 °C, the extraction efficiency of some mycotoxins (**7** and **28**) decreased, probably due to the degradation or their reaction with matrix components. To ratify the optimal conditions, four spiked TCM samples, i.e., (1) rhizomes and roots (Radix Angelicae Dahuricae), (2) flowers (Flos Carthami), (3) seeds (Fructus Crataegi), and (4) grasses and leaves (Herbal Moellendorfs Spidemoss), were extracted with the established ASE method. It was shown that the extraction recoveries were more than 74.2% for all mycotoxins, indicating that the established method could be used for simultaneous extraction of the 35 mycotoxins in different TCMs.

To characterize and clarify the established extraction method, homogenization, ultrasonic, and ASE methods were compared for the sample extraction. A spiked Radix Angelicae Dahuricae sample (10 μg/kg for positive ions and 50 μg/kg for negative ions) was selected. The test was performed as follows: (a) The spiked samples were macerated with 10 mL of acetonitrile/water solution (84/16, v/v) for 40 min and then subjected to the ultrasonic procedure for 40 min. Subsequent steps were identical to those described above. (b) The spiked samples were macerated with 10 mL of acetonitrile/water solution (84/16, v/v) for 40 min and then homogenized for 5 min with an IKA T25 high-speed homogenizer (Ika-Werke GmbH, Staufen, Germany). Then the extracts were pretreated as described above. (c) The spiked samples were pretreated as described above. The results showed that the extraction recoveries of ASE were obviously higher than those of the other two extraction approaches, ranging from 76.3% to 98.1%. As the developed extraction method showed high recoveries combined with high sophisticated automation, it should be a suitable extraction method for the trace analysis of multiple mycotoxins in TCMs.

Development of the Homemade Cleanup Cartridges. Since there are too many categories of TCMs and target analytes, the applicability of some special SPE cartridges, i.e., Mycosep multifunctional cartridges or immunoaffinity car-

Table 2. Calibration Curves of the 35 Mycotoxins in Liquid Solvent and in Different TCM Matrixes

	liquid standard			seeds			flowers			rhizomes and roots			grasses and leaves			
mycotoxin	slope	intercept	R^2	slope	intercept	R^2	slope	intercept	R^2	slope	intercept	R^2	slope	intercept	R^2	range (ng/mL)
17	0.045	0.011	0.9991	0.041	−0.003	0.9995	0.063	0.083	0.9992	0.045	0.055	0.9991	0.042	0.017	0.9940	0.2−60
1	42.993	1.742	0.9991	53.320	8.322	0.9991	49.327	4.029	0.9995	39.324	26.690	0.9993	42.655	4.805	0.9992	0.2−60
2	26.155	3.384	0.9992	31.815	1.367	0.9991	27.954	−0.388	0.9991	19.302	13.658	0.9992	22.218	1.993	0.9996	0.2−60
3	21.707	2.082	0.9993	23.789	0.100	0.9994	12.843	−1.881	0.9992	10.453	2.720	0.9990	25.004	3.332	0.9997	0.2−60
4	12.646	4.641	0.9993	15.131	1.418	0.9991	8.439	−0.447	0.9990	7.808	1.105	0.9992	17.657	−1.254	0.9993	0.2−60
5	8.852	2.545	0.9992	12.227	−1.262	0.9996	12.449	0.045	0.9990	8.447	3.561	0.9996	13.863	1.978	0.9993	0.2−60
24	0.534	−0.095	0.9994	0.320	−0.020	0.9973	1.007	−0.249	0.9946	0.753	0.051	0.9980	0.291	−0.014	0.9958	0.2−20
28	0.003	0.005	0.9991	0.001	0.002	0.9926	0.002	0.004	0.9994	0.002	0.003	0.9991	0.003	0.006	0.9997	0.2−60
9	1.290	−0.435	0.9998	1.795	−1.820	0.9992	1.775	−1.725	0.9995	1.710	0.430	0.9992	1.700	−1.585	0.9990	2−600
10	5.200	0.295	0.9993	5.650	−7.290	0.9990	5.960	1.600	0.9997	5.210	−1.490	0.9992	6.075	20.165	0.9994	2−600
11	2.795	−0.560	0.9990	3.265	−3.035	0.9996	3.610	4.370	0.9997	3.555	−2.310	0.9991	3.150	0.780	0.9996	2−600
12	0.745	−0.745	0.9992	0.895	−0.030	0.9993	0.685	−0.660	0.9992	0.950	−0.945	0.9998	0.785	1.670	0.9993	2−600
13	0.305	−0.275	0.9993	0.415	0.705	0.9924	0.435	0.620	0.9995	0.380	0.945	0.9997	0.340	0.955	0.9993	2−600
14	1.775	1.385	0.9992	2.365	−1.710	0.9936	2.195	−3.175	0.9933	1.350	−0.700	0.9994	0.760	0.610	0.9964	2−600
8	1.177	2.417	0.9991	0.692	0.464	0.9991	0.436	1.647	0.9994	0.550	3.492	0.9991	1.264	0.077	0.9990	0.2−60
16	1.505	0.205	0.9994	1.900	−1.950	0.9993	1.760	−5.010	0.9971	1.475	0.490	0.9998	1.580	−1.390	0.9973	2−600
15	2.789	−0.239	0.9990	5.995	−0.279	0.9990	4.713	−0.482	0.9993	3.474	0.942	0.9994	3.927	0.204	0.9990	0.2−60
18	3.455	−1.135	0.9991	0.590	1.200	0.9969	1.680	−0.415	0.9993	1.730	0.165	0.9934	0.460	0.160	0.9990	2−600
26	11.039	0.494	0.9990	20.507	4.027	0.9992	20.987	−0.468	0.9991	17.270	3.583	0.9991	9.471	8.968	0.9990	0.2−20
6	0.399	−0.075	0.9993	0.469	−0.084	0.9991	0.569	0.012	0.9991	0.330	−0.001	0.9996	0.462	−0.104	0.9994	0.2−60
21	3.390	4.715	0.9995	3.305	−2.700	0.9991	2.435	8.845	0.9998	4.210	3.275	0.9985	3.455	−5.570	0.9992	2−600
29	2.980	−0.450	0.9991	1.930	−0.405	0.9947	3.010	−0.330	0.9990	1.185	−0.295	0.9970	2.030	0.005	0.9928	0.2−20
20	3.220	3.055	0.9995	6.090	0.510	0.9993	4.130	0.025	0.9992	4.590	0.810	0.9990	5.150	0.025	0.9990	0.2−60
23	1.116	0.073	0.9994	1.088	−0.064	0.9991	0.832	0.161	0.9993	1.291	−0.073	0.9993	1.160	−0.049	0.9999	0.2−60
25	0.170	−0.115	0.9995	0.105	0.290	0.9994	0.120	0.705	0.9992	0.110	−0.020	0.9993	0.150	−0.105	0.9966	2−600
7	0.308	0.340	0.9994	0.202	−0.042	0.9956	0.590	−0.077	0.9992	0.372	0.086	0.9999	0.257	−0.020	0.9945	0.2−20
19	0.694	0.019	0.9993	0.864	0.162	0.9993	0.539	0.180	0.9993	0.601	0.156	0.9992	0.910	0.975	0.9990	0.2−60
27	0.539	0.017	0.9991	0.555	0.014	0.9994	0.592	−0.012	0.9994	0.510	0.032	0.9994	0.525	0.056	0.9993	0.2−60
22	0.633	−0.007	0.9991	0.713	−0.044	0.9992	0.713	−0.035	0.9991	0.658	−0.033	0.9994	0.595	−0.031	0.9994	0.2−60
30	0.990	−0.955	0.9992	0.890	0.390	0.9991	1.045	−0.280	0.9994	0.850	0.025	0.9993	0.970	−0.685	0.9990	2−600
31	0.345	−0.260	0.9992	0.350	−0.040	0.9991	0.330	−0.240	0.9995	0.325	−0.115	0.9996	0.330	−0.240	0.9990	2−600
32	0.445	−0.115	0.9991	0.475	−0.480	0.9993	0.470	−0.170	0.9993	0.410	0.025	0.9991	0.415	0.205	0.9993	2−600
33	1.620	0.165	0.9993	1.630	−0.970	0.9992	1.540	−0.085	0.9990	1.465	0.685	0.9996	1.610	−1.510	0.9991	2−600
34	0.860	−0.170	0.9993	0.800	−0.350	0.9991	0.830	0.145	0.9995	0.765	−0.265	0.9990	0.800	−0.480	0.9991	2−600
35	0.390	−0.155	0.9994	0.395	−0.040	0.9994	0.450	−0.045	0.9991	0.350	−0.045	0.9991	0.345	−0.080	0.9996	2−600

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Table 3. Sensitivities of the Mycotoxins in Different TCM Matrixes (μg/kg)

	solvents		rhizomes and roots		flowers		seeds		grasses and leaves	
mycotoxin	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
1	0.01	0.02	0.01	0.02	0.01	0.03	0.01	0.02	0.01	0.02
2	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02
3	0.04	0.04	0.08	0.09	0.08	0.08	0.04	0.05	0.04	0.04
4	0.02	0.03	0.04	0.06	0.03	0.05	0.04	0.06	0.07	0.09
5	0.01	0.02	0.02	0.03	0.02	0.02	0.01	0.02	0.01	0.01
6	0.06	0.10	0.08	0.11	0.06	0.10	0.05	0.08	0.06	0.10
7	0.05	0.08	0.04	0.07	0.02	0.05	0.02	0.06	0.03	0.05
8	0.02	0.03	0.05	0.11	0.09	0.18	0.03	0.06	0.03	0.05
9	0.02	0.04	0.03	0.08	0.05	0.09	0.03	0.05	0.04	0.09
10	0.30	0.90	0.34	0.98	0.46	1.23	0.56	1.68	0.60	1.79
11	0.68	0.79	0.71	0.82	0.66	0.69	0.77	0.92	0.90	1.03
12	0.78	0.90	0.90	1.23	0.90	1.00	0.76	0.87	0.90	1.12
13	0.65	0.87	1.30	1.73	1.12	1.80	0.79	0.91	0.62	0.93
14	0.68	0.90	1.33	1.68	0.62	0.88	0.77	0.99	1.56	1.79
15	0.02	0.04	0.02	0.04	0.02	0.04	0.01	0.02	0.02	0.03
16	0.63	0.76	1.02	1.16	0.90	1.03	0.99	1.24	1.21	1.56
17	0.07	0.13	0.12	0.15	0.13	0.18	0.10	0.14	0.09	0.13
18	0.06	0.12	0.66	1.56	0.58	1.49	0.09	0.19	0.72	1.86
19	0.01	0.02	0.02	0.03	0.02	0.03	0.01	0.02	0.01	0.02
20	0.09	0.14	0.08	0.14	0.10	0.15	0.07	0.10	0.08	0.10
21	0.68	0.79	0.82	1.00	1.02	1.15	1.01	1.15	0.90	1.00
22	0.02	0.04	0.03	0.04	0.03	0.04	0.02	0.03	0.02	0.03
23	0.02	0.03	0.02	0.03	0.03	0.07	0.02	0.03	0.02	0.04
24	0.10	0.14	0.08	0.11	0.07	0.10	0.14	0.18	0.14	0.19
25	0.51	0.68	1.01	1.32	1.00	1.13	0.88	1.02	1.01	1.54
26	0.02	0.02	0.01	0.02	0.01	0.02	0.01	0.02	0.03	0.06
27	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02
28	0.12	0.13	0.14	0.18	0.18	0.19	0.16	0.17	0.11	0.13
29	0.02	0.03	0.16	0.19	0.04	0.06	0.14	0.19	0.05	0.08
30	0.28	0.80	0.31	0.33	0.33	0.38	0.32	0.34	0.37	0.38
31	0.57	0.80	0.57	0.79	0.80	1.02	0.54	0.76	0.90	1.14
32	0.42	0.65	0.50	0.77	0.51	0.82	0.69	0.90	0.61	0.88
33	0.28	0.30	0.31	0.32	0.35	0.37	0.32	0.34	0.36	0.43
34	0.19	0.47	0.23	0.57	0.30	0.87	0.29	0.74	0.29	0.90
35	0.37	0.79	0.40	0.81	0.18	0.32	0.48	0.95	0.62	1.04

tridges, to the analysis of the mycotoxins in different TCMs has yet to be proven and is probably not feasible. In the present study, six normal-phase materials, i.e., silica gel, Florisil, kieselgur, alumina base, alumina acid, alumina neutral, and two reversed-phase materials, i.e., Oasis HLB SPE cartridges and cartridges filled with RP C18 material, were tested for their purification efficiencies. First, we evaluated the recovery performance of all candidates by purifying mixed standard solutions (10 ng/mL for positive ions and 50 ng/mL for negative ions) with the cartridges filled with one material. The results showed that aflatoxins were absorbed by florisil and ochratoxins were absorbed by alumina base, alumina acid, or alumina neutral and could not be eluted by methanol, causing low recovery performance (<20%). The kieselgur was also not suitable because of the poor recovery performance (<20%) of **7**, **11**, **12**, **16**, **17**, **18**, **19**, **20**, and **25**. The recoveries of **18**, **21**, **22**, **23**, and **26** were also not satisfactory (<31.8%) when HLB or C18 was selected. Only silica gel showed good recoveries ranging from 78.9% to 84.3%. Then the spiked TCM extracts purified by silica gel were tested, and the recoveries were in the range of 71.8−82.3%. After comparison of the sensitivities of the mycotoxins in different TCM matrixes purified by different quantities of silica gel, a reliable cleanup cartridge was developed.

To characterize and clarify the purification method, the sensitivities of the 35 mycotoxins in the extracts of Fructus Crataegi purified with the homemade mixed cartridges or not were compared. It could be obviously seen from the results that the sensitivities were significantly improved for most of the mycotoxins when the spiked extracts were purified with homemade mixed cartridges. On the other hand, some impurities, i.e., pigment and tannin, which could reduce the lifetime of the analytical columns, were absorbed on the SPE cartridges. As the proposed cartridge showed satisfactory recoveries and purification efficiencies, the developed sample cleanup method was proved to be beneficial for the purification of TCM samples.

Evaluation of Matrix Effects. In the present study, the matrix effects were carefully accessed to ensure the accuracy of the method. Samples of Radix Angelicae Dahuricae, Flos Carthami, Fructus Crataegi, and Herba Moellendorfs Spidemoss were selected to prepare the blank matrixes of rhizomes and roots, flowers, seeds, and grasses and leaves, respectively. The results showed that the extents of SSEs were quite different for the four matrixes. The maximal extent was up to 191.7%,

Table 4. Contamination Levels of the 35 Mycotoxins in a Total of 60 Commercial TCMs Widely Used in China (μg/kg)

sample category	sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
rhizomes and roots	Radix Angelicae Sinensis	1.2	0.2	–	–	0.6	–	2.3	5.1	2.7	–	–	–	–	–	–	–	6.1	–
	Radix Paeoniae Alba	1.9	–	–	–	–	–	1.3	–	–	–	–	–	–	–	–	–	4.3	–
	Rhizoma Coptldis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Radix Angelicae Dahuricae	–	–	–	–	–	–	0.5	–	–	–	–	–	–	–	–	–	–	–
	Radix Angelicae Dahuricae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Rhizoma Bolbostematis	1.6	–	0.6	–	0.2	–	–	–	–	–	–	–	–	–	–	–	–	–
	Bulbus Fritillariae Thunbergii	–	–	–	–	–	–	2.1	–	36.1	–	–	–	–	–	–	–	2.3	–
	Radix Paeoniae Rubra	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.8
	Radix Paeoniae Rubra	–	–	–	–	–	–	0.3	–	2.2	–	–	–	–	3.4	–	–	–	–
	Rhizoma Coptldis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.3	–
	Radix Puerariae Lobatae	2.9	6.5	2.5	0.4	1.2	–	0.3	–	–	–	–	–	–	–	–	–	–	–
	Rhizoma Iridis Tectori	–	–	–	–	–	–	–	0.8	–	–	–	–	–	1.4	–	–	–	2.6
	Caulis Dendrobii	—	7.1	1.2	0.5	—	—	—	—	—	—	—	—	—	—	—	—	—	2.2
	Radix Bupleuri	—	—	—	—	—	—	1.2	—	—	—	—	—	—	—	—	—	—	—
	Radix Polygoni Multiflori	2.1	—	—	—	—	—	—	—	23.8	—	—	—	—	—	—	—	0.3	—
	Radix Polygoni Multiflori	—	—	—	—	—	—	—	—	0.5	—	—	—	—	—	—	—	0.9	—
	Radix Et Rhizoma Salviae Mil Tiorrhizae	1.9	0.9	—	4.8	—	—	3.4	—	—	—	—	—	—	—	—	—	—	—
	Radix Et Rhizoma Sophorae Tonkinensis	—	—	—	0.2	—	—	—	—	—	—	—	—	2.4	—	—	—	—	—
	Radix Rumicis Japonici	—	—	—	—	0.2	—	—	0.3	—	—	—	—	—	—	—	—	6.7	—
	Radix Astragali	—	—	—	—	—	—	0.5	—	—	—	—	—	—	—	—	—	—	—
	Radix Astragali	—	—	—	—	—	—	—	0.8	—	—	—	—	—	—	0.3	—	—	—
	Radix Scutellariae	—	—	—	—	—	—	0.3	—	—	—	—	—	—	—	—	—	—	—
	Radix Rhizoma Glycyrrhizae	9.8	6.2	2.3	—	1.2	—	—	—	3.4	—	—	—	—	—	—	—	—	—
seeds	Fructus Citri	—	—	0.3	—	—	—	1.4	—	—	—	—	—	—	—	—	—	—	—
	Semen Persicae	0.5	1.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Semen Persicae	0.9	—	—	—	—	—	0.4	—	—	—	—	—	—	—	—	—	—	—
	Semen Persicae	1.5	0.5	0.2	0.5	0.4	—	5.6	—	6.8	—	—	—	—	—	—	—	2.1	—
	C. reticulata Blanco	—	—	—	—	—	—	—	—	—	25.4	—	—	2.3	—	—	—	—	—
	Fructus Aurantii	—	—	—	—	—	—	—	—	2.5	—	—	—	—	—	—	—	—	—
	Semen Ziziphi Spinosae	1.6	—	0.8	1.5	—	—	—	—	—	—	—	—	—	—	0.3	—	—	—
	Semen Ziziphi Spinosae	1.9	1.5	—	—	—	—	—	—	4.9	—	—	—	—	—	0.3	—	0.5	2.1
	Fructus Mume	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2.0	—	—
	Semen Armeniacae Amarum	1.5	0.7	—	—	—	—	—	—	—	—	—	—	—	—	0.2	—	3.2	—
	Fructus Crataegi	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Fructus Crataegi	2.3	0.8	—	—	—	—	1.3	—	2.4	—	—	—	2.5	—	—	—	—	—
	Fructus Schisandrae Chinensis	–	2.3	–	–	–	–	–	–	–	–	–	–	–	1.2	–	–	–	–
	Fructus Forsythiae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Spora Lygod II	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
grasses and leaves	Herba Spanish-needles	0.2	–	–	–	–	–	–	–	–	–	–	–	–	–	1.9	–	–	–
	Herba Ephedrae	–	–	–	–	–	–	–	8.5	–	–	–	–	–	–	–	–	–	–
	Herba Lysimachiae	–	–	–	–	–	–	–	–	0.4	–	–	–	–	–	–	–	–	–
	Herba Lysimachiae	2.4	–	0.3	–	–	–	–	–	–	–	–	–	–	–	–	–	1.8	–

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Table 4. continued

sample category	sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	Herba Violae	0.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Folium Isatidis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Herbal Moellen-dorfs Spidemoss	–	–	–	–	–	–	–	–	–	–	–	–	–	18.2	–	–	–	–
	Herbal Moellen-dorfs Spidemoss	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Herba Spreeding Hedyotis	–	0.2	–	–	–	–	–	–	9.8	–	–	–	–	–	–	–	–	–
	Indigo Naturalis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Indigo Naturalis	–	–	–	–	–	–	–	–	0.3	–	–	–	–	–	–	–	–	2.7
flowers	Flos Carthami	0.3	–	–	0.2	–	–	0.6	–	2.8	–	–	–	–	6.5	0.2	–	–	–
	Flos Carthami	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Flos Carthami	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Flos Carthami	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Flos Carthami	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.5	–
	Flos Chrysanthe-mi	–	–	–	–	–	–	–	–	2.1	–	–	–	–	–	–	–	–	–
	Flos Chrysanthe-mi	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Solidago decurrens Lour.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Flos Lonicerae Ja-ponicae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.1	2.9
	Flos Carthami	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Flos Albiziae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.3

sample category	sample	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	total
rhizomes and roots	Radix Angelicae Sinensis	7.1	–	–	–	1.2	–	–	2.1	–	–	1.1	–	–	–	–	–	–	29.7
	Radix Paeoniae Alba	1.6	–	–	0.2	0.2	–	–	–	0.2	–	–	–	–	–	–	–	–	9.7
	Rhizoma Coptldis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Radix Angelicae Dahuricae	–	–	–	–	–	–	–	5.4	–	–	–	–	–	–	–	–	–	5.9
	Radix Angelicae Dahuricae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Rhizoma Bolbos-tematis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.3
	Bulbus Fritillariae Thunbergii	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	40.5
	Radix Paeoniae Rubra	22.7	0.8	–	–	–	–	–	–	0.4	–	–	–	–	–	–	–	–	27.7
	Radix Paeoniae Rubra	–	–	–	–	–	0.2	–	–	–	–	–	–	–	–	–	–	–	6.1
	Rhizoma Coptldis	6.1	–	–	0.4	–	–	–	–	–	–	–	–	–	–	–	–	–	8.8
	Radix Puerariae Lobatae	0.2	–	–	0.7	–	–	–	–	–	–	–	–	–	–	–	–	–	14.7
	Rhizoma Iridis Tectori	–	–	–	–	–	–	8.9	2.9	–	–	–	–	–	–	–	–	–	16.6
	Caulis Dendrobii	2.9	0.2	–	–	–	–	–	0.3	–	–	–	–	–	–	–	–	–	14.4
	Radix Bupleuri	0.2	–	–	–	–	–	–	10.8	–	–	–	–	–	–	–	–	–	12.2
	Radix Polygoni Multiflori	0.7	–	–	–	0.4	–	–	0.3	0.3	–	–	–	–	–	2.1	–	–	30.0
	Radix Polygoni Multiflori	2.1	–	–	–	–	–	–	0.6	–	–	0.2	–	–	–	–	–	–	4.3
	Radix Et Rhizoma Salviae Mil Tiorrhizae	-—	–	4.3	–	0.2	–	–	–	0.8	–	–	–	–	–	–	–	–	16.3
	Radix Et Rhizoma Sophorae Ton-kinensis	–	0.3	2.9	0.2	0.9	–	–	–	–	–	–	–	–	–	–	–	–	6.9
	Radix Rumicis Ja-ponici	6.9	0.3	–	–	–	–	–	2.3	–	–	–	–	–	–	–	–	–	16.7
	Radix Astragali	–	–	–	–	–	0.2	–	–	–	–	0.2	–	–	–	–	–	–	0.9
	Radix Astragali	–	–	–	–	–	–	–	0.4	–	–	0.2	–	–	–	–	–	–	1.7
	Radix Scutellariae	–	–	–	–	–	–	–	2.6	–	–	–	–	–	–	2.1	–	–	5.0
	Radix Rhizoma Glycyrrhizae	–	0.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	23.1

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Table 4. continued

sample category	sample	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	total
seeds	Fructus Citri	2.1	–	–	0.2	1.8	–	–	–	–	–	–	–	–	–	–	–	–	5.8
	Semen Persicae	3.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4.1
	Semen Persicae	–	–	–	–	–	–	–	0.3	–	–	–	1.4	–	–	4.4	1.5	–	8.9
	Semen Persicae	0.4	–	–	–	–	5.8	–	3.3	–	2.9	1.5	–	–	–	2.1	–	–	33.6
	C. reticulata Blanco	–	–	7.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	35.6
	Fructus Aurantii	–	–	–	–	–	–	–	7.6	–	–	–	–	–	–	–	–	–	10.1
	Semen Ziziphi Spinosae	7.3	–	–	–	–	0.5	–	0.3	–	–	–	–	–	–	–	–	–	10.7
	Semen Ziziphi Spinosae	1.5	–	3.7	–	–	–	–	0.2	–	–	0.3	–	–	–	–	–	–	16.9
	Fructus Mume	6.1	0.2	–	1.8	1.0	–	7.1	0.2	–	–	–	–	–	–	–	–	–	18.4
	Semen Armeniacae Amarum	–	–	–	–	–	–	–	1.2	–	–	–	–	–	–	2.7	–	–	9.5
	Fructus Crataegi	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Fructus Crataegi	0.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	9.5
	Fructus Schisandrae Chinensis	0.9	–	–	–	–	–	–	3.6	–	–	0.2	–	–	–	–	–	–	8.2
	Fructus Forsythiae	10.8	–	–	0.3	–	–	–	–	–	–	–	–	–	–	–	–	–	11.1
	Spora Lygod II	–	–	–	0.3	–	–	–	–	–	–	–	1.8	–	–	10.3	2.1	1.3	15.8
grasses and leaves	Herba Spanish-needles	–	–	–	0.2	0.2	–	–	–	–	–	–	–	–	–	–	–	–	2.5
	Herba Ephedrae	0.8	–	–	0.3	0.4	–	–	–	–	2.3	–	–	–	–	–	–	–	12.3
	Herba Lysimachiae	–	–	5.8	–	–	–	–	7.5	–	–	–	–	–	–	–	–	–	13.7
	Herba Lysimachiae	3.5	–	–	–	1.5	1.2	–	0.8	–	–	–	–	–	–	–	–	–	11.5
	Herba Violae	–	–	2.1	0.3	–	0.9	–	–	–	–	–	–	–	–	–	–	–	4.2
	Folium Isatidis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Herbal Moellendorfs Spidemoss	8.1	–	–	–	–	–	–	5.6	–	–	–	–	–	–	2.3	–	–	34.2
	Herbal Moellendorfs Spidemoss	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Herba Spreeding Hedyotis	0.9	–	–	0.2	–	–	3.2	0.8	–	–	–	–	–	–	–	–	–	15.1
	Indigo Naturalis	3.5	–	–	–	–	0.2	–	1.9	–	–	–	–	–	–	–	–	–	5.6
	Indigo Naturalis	3.3	–	–	–	–	–	–	9.6	–	–	–	–	–	–	–	–	–	15.9
flowers	Flos Carthami	–	–	–	–	–	1.1	–	0.6	–	–	–	–	–	–	–	–	–	12.3
	Flos Carthami	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Flos Carthami	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Flos Carthami	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Flos Carthami	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.5
	Flos Chrysanthemi	–	–	–	–	–	0.2	–	–	–	–	–	–	–	–	–	–	–	2.3
	Flos Chrysanthemi	–	–	–	1.1	0.4	–	–	–	–	0.2	–	–	–	–	–	–	–	1.7
	Solidago decurrens Lour.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Flos Lonicerae Japonicae	–	–	–	–	–	–	–	–	0.2	–	–	–	–	–	–	–	–	5.2
	Flos Carthami	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Flos Albiziae	–	–	–	–	–	–	–	0.2	–	–	–	–	–	–	–	–	–	2.5

while the minimal extent was only 22.0%, indicating that suitable ISs were needed. After comparison of the recoveries of all compounds in different matrixes, the ^{13}C-labeled compounds were selected as the ISs for the analytes whose recoveries were similar. Finally, ^{13}C-**1** was selected as the IS for **1**, **2**, **3**, **4**, **5**, **6**, **15**, **24**, and **26**, ^{13}C-**10** for **10**, **11**, **12**, **13**, **14**, **16**, **18**, and **21**, ^{13}C-**22** for **7**, **8**, **17**, **22**, and **23**, ^{13}C-**27** for **19**, **27**, and **28**, and ^{13}C-**33** for **9**, **20**, **25**, **29**, **30**, **31**, **32**, **33**, **34**, and **35**. In the next step, the SSE using ISs was also investigated to find out whether the ISs could be used for the correction of recovery losses during the ionization process. The results showed that the SSEs were in the range of 37.1−215.0%, indicating that the matrix effects could not be ignored even though the ISs were used. To guarantee a reliable quantitation, ISs and matrix calibration were combined using the contents of the mycotoxins in TCMs with the same kind of matrix calculated using the relative calibration.

Validation. The calibration curves were constructed using the isotope dilution method. Good linear relationships and coefficients of determination ($R^2 \geq 0.9990$) were obtained (Table 2). The LODs were in the range of 0.01−1.56 μg/kg, and the LOQ ranged from 0.01 to 1.86 μg/kg (Table 3).

Recovery was performed in the noncontaminated TCM matrix employing the method of standard addition. Each

sample was fortified 6-fold with 35 mycotoxins at high, intermediate, and low levels (50, 5, and 0.5 μg/kg for the 15 positive ions of mycotoxins, 15, 5, and 0.5 μg/kg for **20**, **24**, **26**, and **29**, 500, 50, and 5 μg/kg for the 16 negative ions) and analyzed by the established method. All recoveries were in the range of 71.4−119.3%.

Intra- and interday precisions for each mycotoxin at high, intermediate, and low concentration levels are thoroughly studied. Research has shown that the analytical variance (and standard deviation) increases with the concentration squared, and as a result the relative standard deviation (RSD) decreases. The RSD goes to infinity as the concentration approaches zero. The RSD is controlled by the functional relationship between variance and concentration. Therefore, in most cases, the RSDs were less than 15% at high and intermediate levels. Not surprisingly, the highest RSD worse than 20% occurred for the lowest levels for the interday precision, which also improved rapidly with increasing concentration levels.

To prove the trueness of the analytical method developed in the present study, several certified reference materials, supported by FAPAS and Romer Laboratories, were analyzed. Good consistent results are obtained, indicating that the current analytical method can be regarded as selective, robust, and accurate.

Method Application. The evaluated method was subsequently applied to determine the natural occurrence of the 35 mycotoxins in a total of 60 commercial TCMs widely used in China.

Among the pool of 60 TCMs, 50 were contaminated with mycotoxins (83.3% of incidence), ranging from 1.7 to 48.0 μg/kg (Table 4). A total of 24 TCMs (occurrence 40.0%) were contaminated with aflatoxins in the range of 0.2−19.5 μg/kg. The occurrence of aflatoxins in seeds (60.0%) was a little higher than that in rhizomes and roots (43.5%), but obviously higher than that in grasses and leaves (36.4%) or flowers (9.1%), indicating that the fungi might favor growing on the TCMs which are rich in starch and oil. The ranges (occurrence) of type A trichothecenes, type B trichothecenes, and **33** and its derivatives were 0.2−2.9 μg/kg (31.7%), 1.2−35.6 μg/kg (21.7%), and 2.1−15.5 μg/kg (11.7%), respectively. To our surprise, the ochratoxins, which were rarely found in TCMs in previous studies, were also detected in TCMs (incidence 28.3%) with a maximal content of 2.8 μg/kg. Some other mycotoxins, such as **7**, **9**, **17**, and **19**, also frequently existed in TCMs. The results revealed that most of the TCMs in the present study were contaminated with some mycotoxins, usually in low levels. Although there appeared to be a low risk to the consumers who occasionally used TCMs, the potential risks were associated with the accumulative effects.

Overall, the current study describes a reliable analytical method for simultaneous quantitative determination of 35 mycotoxins in 60 different TCMs. ASE was newly applied for the coextraction of multiple mycotoxins from different TCMs. Compared to the traditional extraction techniques, i.e., ultrasonic and homogenization, the optimal ASE method showed higher extraction efficiency. The extraction method, allowing for complete extraction by using a minimum amount of solvent in a short period, has been proved to be a powerful tool for extraction of a wide range of chemicals (from nonpolar to polar) from various TCM matrixes. For the purification of the extracts, a simple and cost-efficient solid-phase extraction-based cleanup method was established. This pretreatment procedure greatly improved the detection sensitivity and was suitable for all of the complex TCM matrixes. After optimization of the chromatographic conditions, the 35 mycotoxins could be simultaneously quantitated by UHPLC−MS/MS in less than 17 min in one run. Considering the satisfactory performance of the method and the concentrations that were investigated, it can be concluded that the present method made the multimycotoxin analysis faster and more sensitive, selective, and repeatable compared to some traditional approaches of ELISA, GC, and HPLC coupled with conventional sample preparation.

■ ASSOCIATED CONTENT

Supporting Information

Figure S-1, MRM chromatogram of chaetoglobosin A (**7**) after it was put under the room conditions for 3 days; Figure S-2, MRM chromatograms of a representative contaminated sample (Semen Persicae); Figure S-3, results of the orthogonal experiment for optimization of the conditions of the ASE method; Table S-1, factors and their levels of the orthogonal experiment L_9 (3^4); Table S-2, comparison of the extraction recoveries by different extraction approaches,; Table S-3, sensitivities of mycotoxins cleaned up with the homemade mixed cartridges or not (μg/kg); Table S-4, SSEs calculated with or without ISs; Table S-5, recovery tests of the mycotoxins in different TCMs; Table S-6, inter- and intraday precision of the UHPLC−MS/MS method (μg/kg); and Table S-7, results of the proficiency test on the determination of aflatoxin B1 (**1**), aflatoxin B2 (**2**), aflatoxin G1 (**3**), deoxynivalenol (**10**), ochratoxin A (**22**), and zearalenone (**33**) in several certified reference materials. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

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