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Rapid analysis and identification of multi-class mycotoxins in *Morinda officinalis* by UFLC-ESI-MS/MS

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Abstract

A simple, rapid and cost-effective analytical method based on dilute-and-shoot pretreatment coupled with ultra fast liquid chromatography-tandem mass spectrometry (DAS-UFLC-MS/MS) has been developed for simultaneous quantification and identification of multi-class mycotoxins in *Morinda officinalis*, using a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP®). Mycotoxins were extracted with methanol/water/formic acid mixture and directly injected into the chromatographic system after a one-fold dilution with the initial mobile phase. One precursor ion and two product ions of all analytes were simultaneously characterized and quantified based on the scheduled multiple reaction monitoring-information-dependent acquisition-enhanced product ion (sMRM-IDA-EPI) mode with a turbo ion spray interface simultaneously operated in both positive and negative modes in one chromatographic run within 10 min. Matrix-matched calibration is recommended for reliable quantitation, with zearalanone as the internal standard. After careful optimization of the corresponding parameters, the DAS-UFLC-ESI-MS/MS method was validated to express satisfactory linearity (r>0.9900), sensitivity (limits of detection, 0.02 and 4.00 ng mL^{-1} and limits of quantification, 0.06-10 ng mL^{-1}), precision (intra-day and inter-day precision, <15%), stability (4.12-14.10%), repeatability (5.45-15.56%) and spiked recoveries (63.63-119.44%). The proposed method was applied for 40 *M. officinalis* samples, and two samples were detected with five classes of mycotoxins with concentrations below the regulatory maximum residue limits. This study highlighted the occurrence of multi-class mycotoxins in *M. officinalis*, which should be under safety control.

1. Introduction

Morinda officinalis (Bajitian in Chinese), the perennial root of *Morinda officinalis* How. (family Rubiaceae), has been grown widely in humid areas of tropical and subtropical regions for the past two thousand years.[1] It has been extensively used to support the entire body as crude drugs in oriental medicine in China and northeast Asia, with pharmacological and biological activities including reinforcing kidney function, strengthening the tendons and bones and relieving rheumatic condition,[2,3] for the treatment of impotence, osteoporosis and inflammatory diseases.[4] Meanwhile, *M. officinalis* is also broadly used as food ingredients in China.[5] Generally, the medicinal part of *M. officinalis* directly contacts with the soil for five or seven years in a warm and moist climate, which means that it is more vulnerable to be polluted by pre- and post-harvest fungi, leading to mildew and production of mycotoxins and other secondary metabolites.[6]

Mycotoxins are defined as secondary metabolites produced by a variety of fungi [7] in various matrices under a wide range of climatic conditions [8] and some of which are carcinogenic, mutagenic or teratogenic, causing severe health effects in both humans and animals and posing serious problems to the worldwide safety of food and traditional Chiniese medicine (TCM).[7-9] For example, aflatoxins are hepatotoxic and carcinogenic to humans,[10] inhibiting the synthesis of RNA and interfering the inductive style of specific enzymes. Ochratoxin A (OTA) has shown to be nephrotoxic and a possible human carcinogen.[10,11] As to two represent trichothecenes, HT-2 and T-2 toxins are immunosuppressive compounds and can inhibit the synthesis of protein, causing tissue necrosis, hemorrhage throughout the digestive tract.[12,13] As an estrogenic compound, zearalenone (ZON) can lead to hyperestrogenism and a variety of symptoms, which has been regarded as an important etiologic agent of intoxication in young children.[14] In reference to citrinin (CTN), it may result in nephropathies.[15] Fumonisins, such as FB_1 and FB_2, are a kind of hydrosoluble mycotoxins, expressing acute toxicity and potential carcinogenicity.[13] Usually, these mycotoxins can be present in commodities without being able to detect fungi associated with the toxins and vice versa[16]. Hence, necessary limited standards, as well as some analytical methods have been regulated by the European Union and many other countries.[17,18] However, validated methods for these mycotoxins in *M. officinalis* are scarce. Therefore, developing an efficient, highly sensitive, fast and multi-analyte method to measure these toxic mycotoxins in *M. officinalis* is indispensable.

Different analytical methods, such as thin layer chromatography (TLC),[19] enzyme-linked immunosorbent assay

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(ELISA),[20] or capillary electrophoresis (CE),[21] liquid chromatography (HPLC) and gas chromatography (GC) coupled to different detectors [22-24] have been proposed for mycotoxin determination. Owing to their various limitations, such as low resolution and sensitivity, [25] bad repeatability, [26] difficulty in performing gradient elution, [27] or large consumption of time and organic solvents, [28] inconvenience for simultaneous qualitative and quantitative analysis, [29] ultra fast liquid chromatography coupled to tandem mass spectrometry (UFLC-MS/MS) with superior efficiency, sensitivity and specificity [30] has been accepted as the main tool in the structural characterization, identification, and quantitative analysis of multi-class mycotoxins. Until now, triple quadrupole (QqQ) LC-MS/MS equipment has been widely employed for the quantitative analysis of mycotoxins. Although the sensitivity and selectivity of QqQ are satisfactory, the qualitative information for complementary structural elucidation of the analytes is missing. [31] This liability could be overcome with the hybrid QTRAP® mass spectrometer, which is appropriate for both quantification and confirmation of selected analytes. [32,33] Considering of the high selectivity provided by QTRAP® mass spectrometer, dilute-and-shoot (DAS) procedure was developed and validated for multi-mycotoxin analysis by injecting diluted crude sample extracts without further clean-up, in contrast to usual preparatory methods based on extraction/cleanup/pre-concentration steps for analysis of single toxin or multiple toxins belonging to the same group.

In this paper, followed by the extraction of eleven mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, CIT, ZON, T-2, HT-2, FB_1 and FB_2) using DAS procedure, we developed a simple, rapid, selective, sensitive and cost-effective ultra fast liquid chromatography-tandem mass spectrometry (DAS-UFLC-MS/MS) method for simultaneous quantification and identification of these multi-class mycotoxins in 40 batches of *M. officinalis* using a 5500 QTRAP® instrument based on positive and negative electrospray ionization ($ESI^{+/-}$) source and scheduled multiple reaction monitoring (sMRM) acquisition mode by searching in the appropriate retention time window. Special emphasis has been placed on the confirmation of positive mycotoxin findings. For this purpose, the signal intensity ratios of the two transitions (quantification and qualification) were calculated and compared to the two transitions obtained using fortified blank samples, with the extra confirmation tool of information dependent acquisition (IDA). To our knowledge, this was a practical strategy based on LC-QqQ_{LIT}-MS/MS targeted technique employing an IDA approach by sMRM as survey and EPI as dependent scan for screening, identifying and quantifying multi-class mycotoxins in *M*. *officinalis*, affording a lot of significant references and guidance for the analysis of classes of mycotoxins in other TCMs and more complicated matrices.

2. Materials and methods

2.1 Chemicals and reagents

All organic solvents including methanol and acetonitrile used for both sample extraction and chromatographic analysis were of HPLC grade and purchased from Burdick & Jackson (Morris, NJ, USA). Formic acid was analytical grade from Beijing Chemical Works (Beijing, China). Wahaha purified water (Wahaha, Hangzhou, China) was used. Stock solution of aflatoxins containing 2 μg of AFB_1, 2 μg of AFG_1, 0.5 μg of AFB_2, 0.5 μg of AFG_2 in 1 mL of acetonitrile, together with powders (1 mg for each) of OTA, ZON, FB_1, FB_2, CIT, HT-2 and T-2 toxins were purchased from Pribolab (Singapore). Their structures have been listed in Fig. 1. A multic-analyte working solution in acetonitrile was prepared at 120 ng mL^{-1} for AFB_1, 30 ng mL^{-1} for AFG_1, AFB_2 and G_2, 200 ng mL^{-1} CIT and OTA, 800 ng mL^{-1} for ZON and 1000 ng mL^{-1} for T-2, HT-2, FB_1 and FB_2. Zearalanone (ZAN), also bought from Pribolab, was selected as the internal standard, in order to improve the accuracy of quantification. 10 μg mL^{-1} working solution of ZAN was prepared in acetonitrile. All the stock, working and mixed standard solutions were stored in amber vials which were wrapped with parafilm and then stored at -20 °C until analysis. They were diluted with the initial mobile phase or the blank M. *officinalis* extract before being injected in chromatography system.

All glassware used was soaked in 5% aqueous sodium hypochlorite for several hours to destroy residual toxins before cleaning and reuse. After the analyses, all materials were decontaminated with 5% aqueous sodium hypochlorite solution.

2.2 Instrumentation

An ultra fast liquid chromatography (UFLC) system, which consisted of two LC-20ADXR pumps, a DGU-20 A3 degasser, an SIL-20AC auto-sampler and a CTO-20A column oven (Shimadzu, Japan) was used. Chromatographic separation of the eleven mycotoxins was performed on a SHISEIDO Capcell core C18 column (50 mm×2.1 mm, 2.7 μm) at a flow rate of 350 μL min^{-1}. The mobile phase combing eluent A (water slightly acidified with 0.1% formic acid) and eluent B (acetonitrile with 0.1% formic acid) was employed with gradient elution as follows: 0-2 min (80% A); 8 min (10% A); 10 min (10% A) and 10.01-12 min (80% A). 3 μL of the samples was injected and directly transferred into the ESI interface without split.

An Applied Biosystem 5500 QTRAP® hybrid triple quadrupole/linear ion trap (QqQ_{LIT}) mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) was coupled to the above UFLC system and equipped with the Turbo V ion Spray electrospray ionisation (ESI) interface performing in both positive and negative ionization mode $ESI^{+/-}$ by continuous positive/negative polarity switching (switching time of 0.02 s). Nitrogen (purity 99.999%) was used as the nebulizer (GS_1), heater (GS_2) and curtain (CUR) gas, as well as the collision activation dissociation (CAD) gas. In order to maximize the generated response of precursor ions of each targeted compound and to efficiently produce its characteristic fragment/product ions, operating parameters were optimized: capillary voltages of 5.5 kV (positive ionization mode) and -4.5 kV (negative ionization mode); ion source temperature, 550 °C; GS_1, 50 psi; GS_2 50 psi; CUR 35 psi; CAD medium and dwell time, 80 ms/scan. Tandem MS analyses were performed in sMRM acquisition mode by screening two fragmentation reactions per analyte in one chromatographic run. SMRM is defined as a MRM with the amount of time for detection that surrounds the rentention time for each transition. Analyte dependent sources settings and instrumental parameters for each sMRM transition including mass number of one precursor ion and two product ions, declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potentials (CXP) were optimized, respectively. Furthermore, to get synchronous supplementary confirmation of targeted analytes *via* the LC-QqQ_{LIT}-MS/MS, especially when trace concentration levels were required, IDA experiments were carried out to automatically trigger EPI scan, which allowed the two most intense product ions of each analyte

that exceeded 1000 counts per second (cps) through the MRM-EPI analysis with dynamic exclusion of the former ions for 15 s. Analyst 1.6 software (Applied Biosystems/MDS Sciex, Foster City, CA, USA) was used to control the instruments and data processing.

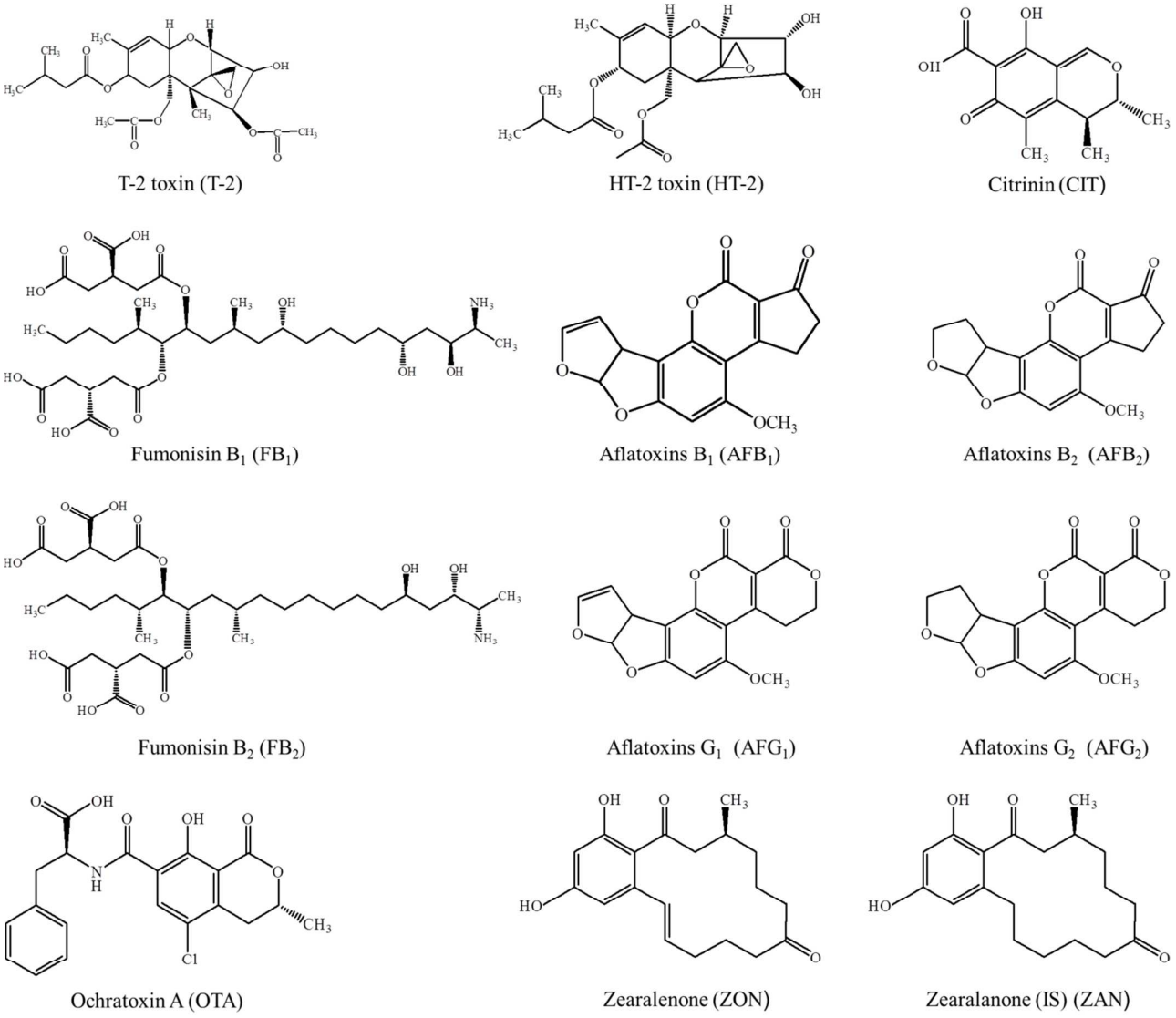

Figure 1. Chemical structures of the 11 analyzed mycotoxins and the internal standard.

2.3 Sample preparation

Random samples of 40 batches of *M. officinalis* were purchased or collected from different markets in Hainan province, China. The crude drugs of them were cultivated in Guangdong and Guangxi provinces. After harvest, they were processed into slices by different companies before entering the market. All samples were identified by Prof. Yulin Lin, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Peking Union Medical College, and were thoroughly triturated to obtain consistent particle size and to homogenize the sample. The subsample was collected in a ziplock bag and kept at -20 °C pending mycotoxins analysis. Samples were extracted and analyzed in triplicate.

Triturated sample (2.0 g ± 0.1 mg) was accurately weighed and transferred to teflon centrifuge tube (10 mL), and 5 mL of methanol/water (80: 20, *v*/*v*) containing 0.1% formic acid was added. Then, the tube was sealed and shaken with a vortex mixer for 3 min, followed by centrifugation at 12000 rpm for 10 min. Afterwards, the supernatant extract was one-fold diluted with the initial mobile phase, i.e. acetonitrile/water (20: 80, *v*/*v*). The final extract was amended with 20 µL of internal standard solution at 10 µg mL^{-1}. After mixing (30 s) by vortex, the solution was passed through a 0.22 µm syringe nylon filter and sealed in an auto-

sampler vial and then stored at -20 °C pending analysis. 3 µL of the filtrate were directly injected into the UFLC-MS/MS system.

2.4. Performance evaluation

Two MS/MS transitions were acquired for each analyte reaching four identification points with a defined sMRM transitions ratio for the developed method as indicated in the requirements for mass spectrometric analysis.[9,34] The principle of the sMRM is to monitor these transitions increasing the time that is available for acquiring one data point. It was therefore observed an enhancement of the selectivity and consequent improvement on the limit of detection (LOD) and quantification (LOQ). For each compound, the most abundant MRM transition was used for quantification while the other transition was used for confirmation. The criteria applied for the confirmation were: (1) signal for the two MRM transitions of each analyte should be identical in the extract and in the standard or matrix-matched solution; (2) Intensity ratio of the two MRM transitions should agree with the related value of an authentic standard within 30% relative deviation; (3) the relative retention time of the analyte in both sample and standard solution should be within maximum variation of 0.1 min.

The performance of the established method was evaluated for its reliability and accuracy on the blank (mycotoxins-free) *M. officinalis* sample spiked with targeted mycotoxins. According to the recommendations by the European Community[35] and the guidelines in other documents, [36-38] the UFLC-MS/MS method was validated in terms of specificity, accuracy, LOD and LOQ, precision, linearity, matrix effect as well as recovery.

3. Results and discussion

3.1. Method development

3.1.1 Optimization of sample preparation

For accurately and effectively detection of targeted toxicological important mycotoxins in complex matrices of *M. officinalis*, the use of a simple and practical sample preparation procedure is advisable to reduce the time and cost of analysis, especially when a sensitive and specific analytical technique such as UFLC-ESI-MS/MS [39] is introduced. However, the complicated matrix constituents in *M. officinalis* will bring in some interference on the extraction of mycotoxins of interest. Therefore, different extraction systems such as the mixtures of water/organic solvents (methanol and acetonitrile) at different ratios were tested. Repeated experiments showed that the composition of water/methanol (20:80, *v*/*v*) was preferred, which could obtain satisfactory recoveries (>70%) for all mycotoxins with the exception of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) and CIT. Then, different ratios of formic acid or acetic acid [9,23,39] was added to the extraction solvent, respectively, to improve the extraction recovery of aflatoxins and CIT. Consequently, 0.1% HCOOH was selected as a compromise for all analytes. As shown in Fig. 2, the final extraction system of water/methanol (20:80, *v*/*v*) with 0.1% formic acid was optimized, as it could result in the optimum extraction recoveries (>80%) for the eleven targeted mycotoxins from the fortified *M. officinalis* sample.

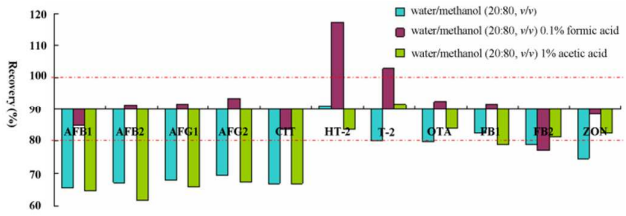

Figure 2. Effect of different solvent on the extraction efficiency of mycotoxins from *M. officinalis*.

3.1.2 Optimization of UFLC-MS/MS conditions

For quantitative purposes of multi-analyte using the UFLC-MS/MS method, baseline-separation of all analytes is important, which is based on the optimization of the chromatographic conditions and MS/MS parameters for each target. In this study, particular attention should be paid on the compromise between mobile phase composition and MS response to achieve excellent resolution and high sensitivity of the eleven mycotoxins.

The reported mobile phases consisted generally of a combination of acetonitrile or methanol and water to which some additives (formic acid or acetic acid or ammonium acetate) were added to improve the elution profile, peak resolution and signal intensity [39, 40-42]. Here, the mobile phase of 0.1% formic acid in water and acetonitrile could give a better resolution and sensitivity for all investigated analytes, compared with other compositions of water and acetonitrile or methanol containing ammonium acetate, acetic acid or formic acid. Short isocratic elution (2 min) then gradient elution was used to substantially eliminate matrix effects. The sMRM chromatograms and EPI spectrum of a negative *M. officinalis* spiked with mixed standard solution in Fig. 3 showed that the optimum chromatographic conditions ensured satisfactory separation within 8 min, despite of the relatively high complexity of the tested matrices.

The optimum mass spectrometric behaviors and ESI source parameters for the identification and quantification of the eleven analytes were explored by manual tuning mode through the syringe pump continuous infusion analysis of the individual standard solutions of each compound at 50, 100 or 200 ng mL^{-1} and IS at 100 ng mL^{-1}, depending on the sensitivity of the compounds using methanol/water (50: 50) as carrier solvent. Sensitivity of targeted analytes was checked by full scan of *m/z* from 50 to 800 in both positive and negative ionization modes. The relative intensity for the most abundant *m/z* was chosen as precursor ion to further evaluate the performance of ionization and the cone voltage value. The results showed that AFB_1, AFB_2, AFG_1, AFG_2, HT-2, T-2, FB_1, B_2, T-2, HT-2 and OTA in the form of $[M+H]^+$ under positive ionization mode and ZON in $[M-H]^-$ under negative ionization mode could get a higher MS response. Then, the selected precursor ion was dissociated with nitrogen by using different CEs to obtain the most suitable product ions with high intensity. Product ions resulting from non-specific losses (such as H_2O or CO_2 losses) were avoided. In this study, two characteristic product ions for each compound were decided. The product ion with the highest S/N ratio and intensity was chosen for quantification, whereas the other was used for confirmation. All the MS/MS parameters have been summarized in Fig. 3, most of which were in compliance with other reported papers. [7,43] In comparison, [44] the above-optimized UFLC-MS/MS conditions allowed 50% reduction of analysis time for each sample.

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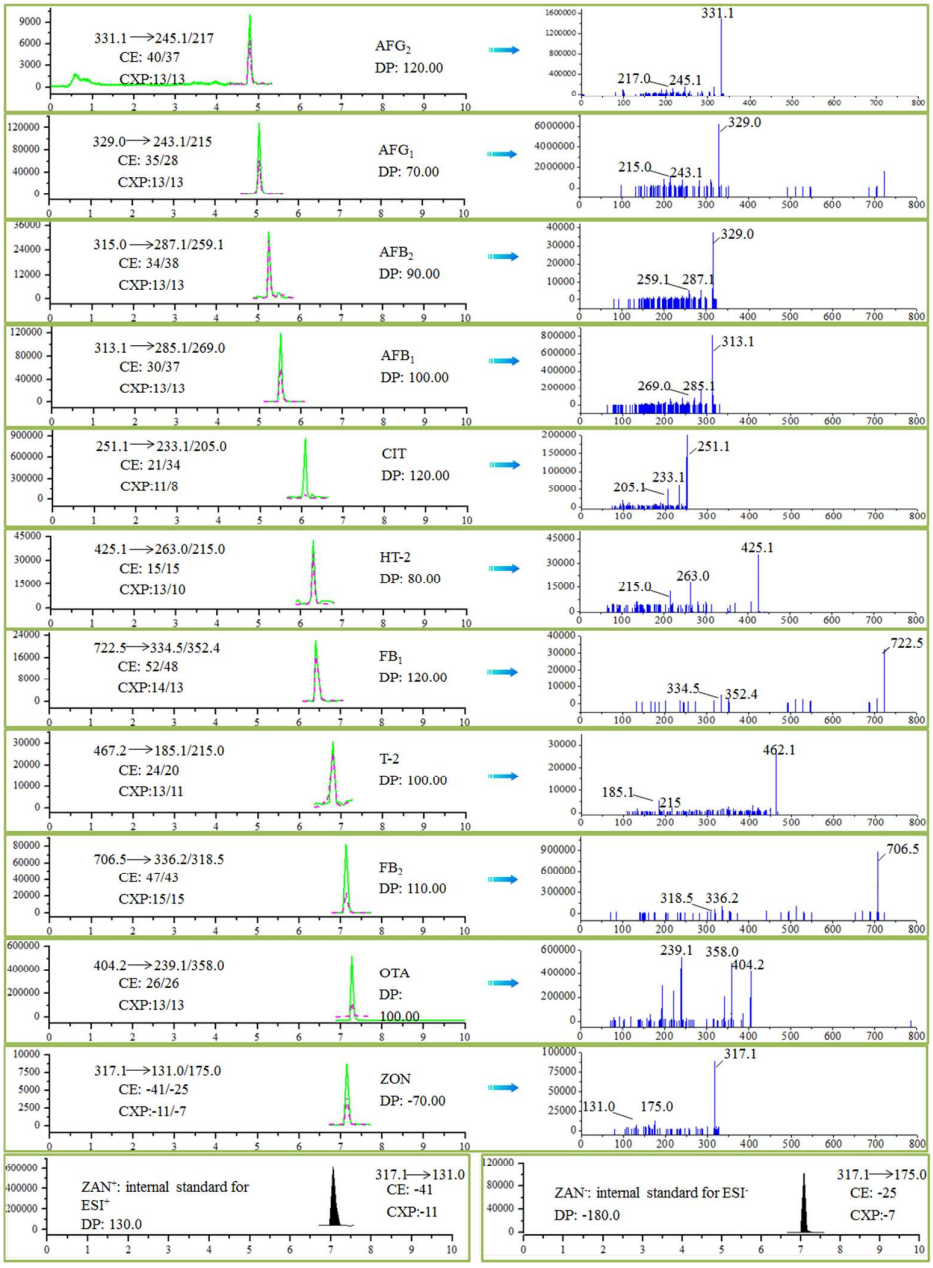

Figure 3. sMRM chromatograms, EPI spectrum and MS/MS conditions for the eleven mycotoxins in blank *M. officinalis* spiked with 6.0 μg kg^{-1} of AFB_1 and AFG_1; 1.5 μg kg^{-1} of AFB_2 and AFG_2; 10 μg kg^{-1} of CIT and OTA; 40 μg kg^{-1} of ZON; 50 μg kg^{-1} of T-2, HT-2, FB_1 and FB_2. Only ZON in negative mode;
Dp: Declustering potential; CE: Collision energy; CXP: Collision cell exit potential.

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3.1.3. Selection of internal standard

Usually, internal standard (IS) was applied to compensate for matrix effects and and sporadic variations in MS/MS analysis using the ESI source. Isotopically labelled ISs with similar structures, physical and chemical properties to the targeted analyte are commonly preferable,[39, 45] while, they are expensive and unavailable for every analyte. In this study, a structurally-related standard, ZAN was introduced as a substitute. The results expressed that no interference was found at the retention time of ZAN. Interestingly, ZAN could be ionized in both positive and negative ionization modes.[46] Because of similar chemical structures with the only difference of an additional double bond in ZON, ZAN partly co-eluted with ZON in the chromatographic system. Nevertheless, both of them could be accurately quantified under the MRM mode (*m/z* 317.1-174.9, 317.1-130.8 for ZON and *m/z* 319.1-205, 319.1-275 for ZAN). Finally, ZAN was selected as the internal standard for all the eleven mycotoxins in ESI^+ and ESI^- modes.

3.2 Assessment of matrix effect

One of the main problems in using ESI source for UFLC-MS/MS analysis is the presence of matrix effects (MEs), which may lead to signal suppression or enhancement of the target compounds, affecting the accuracy and precision of the results.[17] Injection of crude extracts of *M. officinalis* without any purification should take careful consideration of MEs caused by co-eluting compounds. For this, the calibration curve in solvent and matrix-matched calibration curves were prepared in the initial mobile phase and the blank *M. officinalis* extract, respectively. The signal suppression/enhancement (SSE) for each analyte was calculated as the percentage of the matrix-matched calibration slope divided by the solvent-based one in the whole identical calibration range by the following equation:

$$SSE\ (\%) = 100 * slope_{matrix\text{-}matched\ standard} / slope_{solvent\text{-}based\ standard}.$$

SSE of equal to 100 % indicates that matrix has no effect on the MS signal, meaning the absence of matrix effects, SSE<100% exhibits the suppression of the analyte signal, while SSE of >100% indicates signal enhancement. In usual, SSE of 70-120% is acceptable. The results of SSEs in Table 1 elucidated that MEs, especially the signal enhancement effect, were observed for most mycotoxins with the exception of OTA. Hence, matrix-matched calibration was used for compensating MEs and accurate quantitation of the targeted mycotoxins.

3.3 In-house method validation

Specificity. The specificity of the method was demonstrated by respective analysis of mixed standard solution as well as blank *M. officinalis* extract. No peaks were observed in any of the matrices at the same retention time as target mycotoxins, indicating good specificity of the established method.

Linearity. A series of calibration curve samples (concentration range of 0.12-30 ng μL^{-1} for AFB_1 and AFG_1, 0.03-7.5 ng μL^{-1} for AFB_2, 0.03-15 ng μL^{-1} for AFG_2, 0.2-50 ng μL^{-1} for CIT and OTA, 1.6 -400 ng μL^{-1} for ZON, 1-125 ng μL^{-1} for T-2, 2.5-250 ng μL^{-1} for HT-2 and 1-250 ng μL^{-1} for FB_1 and FB_2) were prepared by spiking the blank *M. officinalis* extract with the mixed standard working solutions, followed by a vortex mixing step and then injection to the analytical system. All matrix-matched calibration curves were constructed by plotting the peak area ratio of respective analyte to IS *versus* the amount of analyte within the above-described concentration ranges. As documented in Table 1, linear regression coefficients of $r \geq 0.99$ were obtained for all mycotoxins

Limit of detection/quantification. The limits of detection (LOD) and quantification (LOQ) were calculated by analyzing decreasing concentration of the spiked *M. officinalis* in triplicate to evaluate the sensitivity of the established method. LOD with signal-to-noise (S/N) ratio higher than 3 was established as the lowest concentration that could be detected, whereas, LOQ was the lowest concentration for which recovery and relative standard deviation (RSD) values were in accordance with the recommended ranges of accuracy and precision, with a S/N ratio of ≥10. The Analyst version 1.6 software was applied to calculate these limits of each mycotoxin. As could be seen in Table 1, the LODs were in the range of 0.01-1 ng mL^{-1} for all analytes and LOQs of 0.03-2.5 ng mL^{-1}, which were all lower than the maximum residue levels (MRLs) established by the European Union. Therefore, the presented method succeeded in sensitive quantitation of the eleven interesting mycotoxins within a wide concentration range.

Precision. The precision of the established method was evaluated in terms of intra- and inter-day validation by performing repeated analysis of blank *M. officinalis* extract spiked with 6.0 ng mL^{-1} for AFB_1 and AFG_1, 1.5 ng mL^{-1} for AFB_2 and AFG_2, 10 ng mL^{-1} for CIT and OTA, 40 ng mL^{-1} for ZON, 50 ng mL^{-1} for T-2, HT-2, FB_1 and FB_2. The intra-day precision from the same sample and technician using the same equipment and method was calculated by six consecutive injections on the same day, while the inter-day precision was based on independent results from six injections on six consecutive days, which were expressed as RSD using the relative peak area, in which the peak area of each analyte was divided by that of IS. RSDs for intra-day and inter-day precision were respectively in the range of 4.57-13.00% and 6.95-14.89%, which were below the values recommended by the European Union,[36] testifying to the good precision for the established method.

Reproducibility. Six portions of the same *M. officinalis* samples were spiked with 6.0 ng mL^{-1} for AFB_1 and AFG_1, 1.5 ng mL^{-1} for AFB_2 and AFG_2, 10 ng mL^{-1} for CIT and OTA, 40 ng mL^{-1} for ZON, 50 ng mL^{-1} for T-2, HT-2, FB_1 and FB_2 to estimate the within laboratory reproducibility. All the samples were then subjected to pretreatment and analysis as the above-mentioned procedure. above-mentioned procedure. The obtained data expressed as RSD

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Table 1. Method performance for eleven mycotoxins.

Mycotoxin	Linearity			SSE (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Precision		Stability	Recoveries (RSD, %)		
	Regression equation	*r*	ranges (ng mL^{-1})				Intra-day	Inter-day		High level [a]	Medium level [b]	Low level [c]
AFB_1	$y = 0.0112x - 0.00207$	0.9959	0.12-30	187.05	0.02	0.06	14.16	4.57	7.15	67.28 (7.18)	79.32 (3.59)	91.91 (12.13)
AFB_2	$y = 0.0126x + 0.0015$	0.9900	0.15-7.5	216.58	0.03	0.10	11.70	5.92	12.38	90.60 (4.78)	97.00 (11.04)	102.64 (5.83)
AFG_1	$y = 0.0105x + 0.000332$	0.9971	0.12-30	150.00	0.04	0.12	7.00	4.82	14.10	94.17 (5.32)	91.80 (6.37)	115.58 (7.34)
AFG_2	$y = 0.00225x + 0.00102$	0.9978	0.15-15	193.97	0.03	0.08	13.27	10.97	8.56	77.76 (4.67)	94.79 (11.34)	106.59 (12.53)
CIT	$y = 0.0641x + 0.108$	0.9992	0.2-50	168.24	0.05	0.20	7.47	7.07	7.63	70.28 (0.57)	75.78 (5.99)	77.64 (6.81)
HT-2	$y = 0.000402x + 0.00804$	0.9945	2.5-250	137.67	0.04	0.20	6.95	8.73	6.79	76.85 (10.24)	88.01 (12.33)	100.41 (8.06)
T-2	$y = 0.000308x + 0.00715$	0.9935	10-125	77.97	3.00	10.00	12.32	9.86	10.01	63.66 (11.05)	105.09 (8.74)	75.18 (5.86)
OTA	$y = 0.02x - 0.0015$	0.9916	1-50	175.48	0.35	1.00	14.89	9.13	9.55	90.70 (0.22)	100.34 (2.89)	89.15 (8.28)
FB_1	$y = 0.000225x + 0.00175$	0.9985	1-250	232.20	0.40	1.00	12.56	5.74	10.00	90.30 (10.91)	102.19 (8.97)	107.85 (5.26)
FB_2	$y = 0.000959x + 0.00251$	0.9994	2.5-250	209.39	0.50	2.00	10.19	12.94	4.42	63.63 (3.10)	119.44 (6.84)	80.32 (10.65)
ZON	$y = 0.002386x - 0.0105$	0.9980	16-400	225.09	4.00	10.00	9.13	13.00	4.12	101.90 (13.68)	102.30 (5.19)	100.59 (16.65)

[a] AFB_1, AFG_1 30 μg kg^{-1}; AFB_2, AFG_2 15 μg kg^{-1}; CIT, OTA: 100 μg kg^{-1}; ZON 1600 μg kg^{-1}; HT-2 500 μg kg^{-1}; T-2 1000 μg kg^{-1}; FB_1 and FB_2 250 μg kg^{-1};
[b] AFB_1, AFG_1 6 μg kg^{-1}; AFB_2, AFG_2 3 μg kg^{-1}; CIT, OTA: 20 μg kg^{-1}; ZON 320 μg kg^{-1}; HT-2 100 μg kg^{-1}; T-2 200 μg kg^{-1}; FB_1 and FB_2 50 μg kg^{-1};
[c] AFB_1, AFG_1 3 μg kg^{-1}; AFB_2, AFG_2 1.5 μg kg^{-1}; CIT, OTA: 10 μg kg^{-1}; ZON 160 μg kg^{-1}; HT-2 50 μg kg^{-1}; T-2 100 μg kg^{-1}; FB_1 and FB_2 25 μg kg^{-1};

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was in the range of 5.26-15.21% (10.19% on average), indicating satisfactory reproducibility.

Trueness. The trueness of the established method was determined by recoveries obtained from experiments conducted with mycotoxin-free *M. officinalis* samples. Nine portions of the same sample were spiked with the mixed standard solution at three different fortification levels (low, medium and high) of 1.2, 6.0, 12.0 ng mg^{-1} for AFB_1 and AFG_1, 0.3, 1.5, 3.0 ng mg^{-1} for AFB_2 and AFG_2, 2, 10, 20 ng mg^{-1} for CIT and OTA, 16, 40, 80 ng mg^{-1} for ZON, 10, 50, 100 ng mg^{-1} for T-2, HT-2, FB_1 and FB_2 in triplicate, according to the MRLs allowed by the European Commission. Then, the spiked samples were incubated for 1 h at room temperature to evaporate the solvent and establish the balance between matrix and mycotoxins. Afterwards, the nine samples were subjected to pretreatment and analyzed as the above-described procedure. Recovery (extraction efficiency) was assessed and expressed as:

Recovery (%) = mean measured concentration/spiked concentration*100 Results in Table 1 showed that the recoveries of all analytes ranged from 63.63% to 101.89% for high level, from 75.78% to 119.44% for medium level and from 75.18% to 115.58% for the low level. Usually, mean recoveries of 70-110% for concentrations from 1 μg kg^{-1} to 10 μg kg^{-1} and 80-110% for concentrations ≥ 10μg kg^{-1}, with RSD ≤ 20% are considered acceptable.[38] However, the acceptable range according to EU SANCO [36,47] is wider reaching 70-120% for mean recoveries. While in certain cases, recoveries outside this range may also be acceptable, typically for multiresidue methods. Hence, the trueness of the proposed method was acceptable.

3.3. Application of to real *M. officinalis* samples

The optimized and validated DAS-UFLC-ESI-MS/MS method was applied to analyze the mycotoxins of interest in 40 batches of *M. officinalis* samples. The occurrence and levels of these mycotoxins in all tested samples for this survey are summarized in Table 2. Only 2 out of 40 samples showed mycotoxin contamination by CIT, fumonisin (FB_2) and aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2), with residual levels below the regulatory MRLs suggested by EU. In addition, all positive findings were confirmed by accomplishment of the Q/q ratios with deviations within the limits established by the European Union as well as by agreement of retention time with deviations lower than 0.1 min respect to a reference standard.[35] Fig. 4 showed the MRM chromatogram of positive findings of mycotoxins in naturally contaminated *M. officinalis* samples. It could be concluded that for all positive findings, deviations of ion ratios and retention time were lower than 15% (ranging from 1.32-14.25%) and 0.1 min respectively, which were below the maximum tolerance accepted. However, no noticeable mildew could be seen on the surfaces of both positive samples, as shown in Fig. 4. From the analysis data and the degrees of mildew, it could be concluded that there is no necessary relationship between the contents of mycotoxins and the mildew degree.

Table 2. Occurrence and residual level of mycotoxins in 40 *M. officinalis* sample.

Sample No.	Origin [a]	Production date [b]	Mycotoxins detected	Mycotoxin residue level (μg kg^{-1})	MRL suggested (μg kg^{-1})
S1	Guangxi Province	20120506	ND [c]	-	
S2	Guangdong Province	20120422	ND	-	
S3	Guangdong Province	20111218	ND	-	
S4	Guangxi Province	20120316	ND	-	
S5	Guangdong Province	20120510	ND	-	
			ND	-	
S6	Guangdong Province	20120405	ND	-	
S7	Guangxi Province	20111115	AFB_1	0.3990	5.0 μg kg^{-1} for AFB_1
			AFB_2	0.5920	
			AFG_1	< LOQ	10.0 μg kg^{-1} for the sum of AFs [d]
			AFG_2	< LOQ	
			CIT	1.430	Not set
S8	Guangdong Province	20111006	ND	-	
S9	Guangdong Province	20110310	ND	-	
S10	Guangdong Province	20120116	ND	-	
S11	Guangxi Province	20120603	ND	-	
S12	Guangdong Province	20120611	ND	-	
S13	Guangdong Province	20120628	ND	-	

S14	Guangdong Province	20120305	ND	-	
S15	Guangdong Province	20120318	ND	-	
S16	Guangxi Province	20120510	ND	-	
S17	Guangdong Province	20120604	ND	-	
S18	Guangdong Province	20120302	ND	-	
S19	Guangdong Province	20120518	ND	-	
S20	Guangdong Province	20120511	ND	-	
			AFB_1	< LOQ	5.0 μg kg^{-1} for AFB_1
S21	Guangdong Province	20120810	AFG_1	< LOQ	10.0 μg kg^{-1} for the sum of AFs
			FB_2	1.0050	1000 μg kg^{-1} for the sum of of FBs [e]
S22	Guangdong Province	20120524	ND	-	
S23	Guangdong Province	20121008	ND	-	
S24	Guangdong Province	20110720	ND	-	
S25	Guangdong Province	20120923	ND	-	
S26	Guangdong Province	20121023	ND	-	
S27	Guangdong Province	20120702	ND	-	
S28	Guangdong Province	20120923	ND	-	
S29	Guangdong Province	20120803	ND	-	
S30	Guangdong Province	20120427	ND	-	
S31	Guangdong Province	20120622	ND	-	
S32	Guangxi Province	20120923	ND	-	
S33	Guangdong Province	20111115	ND	-	
S34	Guangdong Province	20110624	ND	-	
S35	Guangdong Province	20120803	ND	-	
S36	Guangdong Province	20120826	ND	-	
S37	Guangdong Province	20120920	ND	-	
S38	Guangdong Province	20120427	ND	-	
S39	Guangdong Province	20120831	ND	-	
S40	Guangxi Province	20120530	ND	-	

[a] Place where the crude drugs of M. *officinalis* were cultivated;

[b] The date when the crude drugs were processed into slices by some companies;

[c] Not detected;

[d] Including AFB_1, AFB_2, AFG_1 and AFG_2;

[e] Including FB_1 and FB_2;

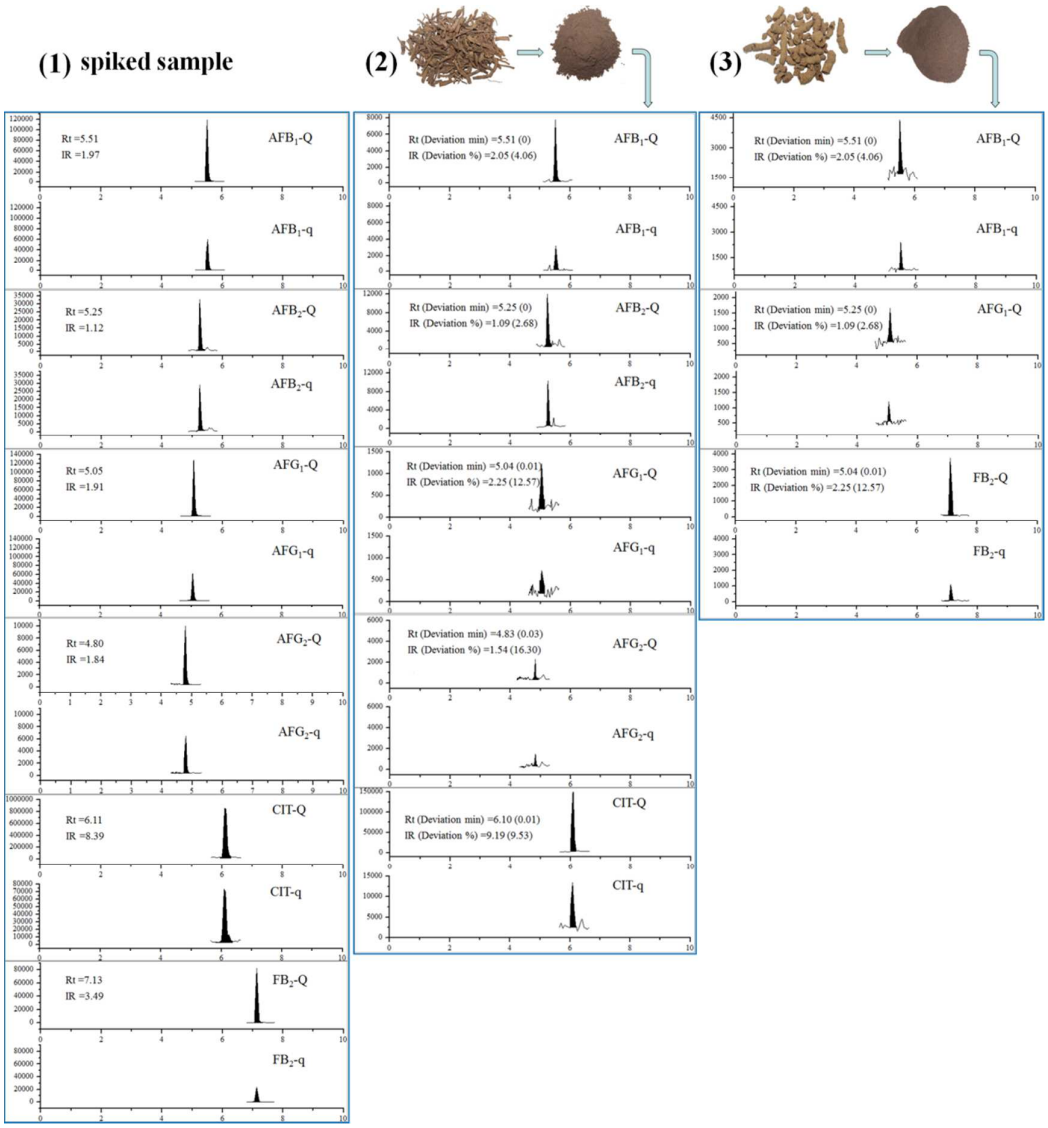

Figure 4. Confirmation of two positive *M*. *officinalis* samples by the accomplishment of IR, Rt and MRM chromatograms corresponding to: (1) spiked sample with 6.0 μg kg^{-1} of AFB_1 and AFG_1; 1.5μg kg^{-1} of AFB_2 and AFG_2; 10 μg kg^{-1} of CIT; 50 μg kg^{-1} of FB_2; (2) S7 sample positive for AFB_1, AFB_2, AFG_1, AFG_2 and CIT; (3) S21 sample positive for AFB_1, AFG_1 and CIT.
Rt: Retentation time; IR: Ion ratio; Q: Transition used for quantification; q: Transition used for confirmation

Conclusions

A simple and cost-effective multi-analyte DAS-UFLC-ESI-MS/MS method has been developed and validated for rapid screening, quantification and confirmation of 11 important mycotoxins in *M*. *officinalis*. The method allowed the unambiguous identification and concurrent quantification of analytes of different physicochemical properties within 8 min from the complex TCM matrices without any clean-up step. In addition, the fast positive-negative switching ionization mode allowed the simultaneous determination of positively and negatively ionized analytes in a single chromatographic run, avoiding extra injections. Matrix-matched calibration curves were established for compensating MEs with LOQs below the usual MRLs established by the EU regulation. Meanwhile, we also took other measures to compensate MEs including dilution of crude extract and the use of internal standard. The established method was systematically validated in terms of linearity, selectivity, stability, precision, recovery and repeatability. Afterwards, the developed method was successfully applied for quantitative detection and qualitative confirmation of the 11 multi-class mycotoxins in 40 batches of real *M*. *officinalis* samples and only two samples were detected with five-class of investigated mycotoxins including AFB_1, AFB_2, AFG_1, AFG_2, FB_2 and CIT, without necessary relationship between the contents of mycotoxins and the degree of mildew. CIT is a toxic metabolite produced by several filamentous fungi of the genera *Penicillium*, *Aspergillus* and *Monascus*, which has been known as a natural contaminant in cereal grains, foods and feedstuffs.[48] However, it was first found in *M*. *officinalis*, a kind of rhizomatic TCM, which belongs to Chinese dietary herbs with high content of carbohydrate. Furthermore, aflatoxins were detected in both of the positive samples, which

should arise enough attention on the safety of *M. officinalis* and related products. Meanwhile, this study held great promises for routine high-throughput analysis of more mycotixins in other TCMs in near future.

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