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Zheng Han, Zhiyong Zhao, Suquan Song, Gang Liu ...+7 more authors

Institutions: Ghent University, Shanghai Jiao Tong University, Huazhong Agricultural University, Zhejiang University

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Authors: Zheng Han, Zhiyong Zhao , Suquan Song , Gang Liu , Jianxin Shi, Jingbo Zhang, Yucai Liao , Dabing Zhang, Sarah De Saeger, Aibo Wu

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Establishment of an isotope dilution LC-MS/MS method revealing kinetics and distribution of co-occurring mycotoxins in rat

Zheng Han [a], Zhiyong Zhao [a], Suquan Song [a, b], Gang Liu [a], Jianxin Shi [c], Jingbo Zhang [d], Yucai Liao [d], Dabing Zhang [c], Sarah De Saeger [b], Aibo Wu [a *]

[a] Laboratory of Quality & Safety Risk Assessment for Agro-products (Shanghai, Ministry of Agriculture), Institute for Agri-food Standards and Testing Technology, Shanghai Academy of Agricultural Sciences, 1000 Jinqi Road, Shanghai 201403, P. R. China

[b] Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Gent, Belgium

[c] School of Life Science and Biotechnology, Bor Luh Food Safety Center, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, P. R. China

[d] College of Plant Science and Technology, Huazhong Agricultural University, No.1 Shizishan Street, Hongshan District, Wuhan, Hubei 430070, P. R. China

* To whom correspondence may be addressed.

Phone: +86-21-62202875; Fax: +86-21-62203612

Email: wuaibo@saas.sh.cn

Abstract

An isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with a fast sample preparation using homemade clean-up cartridges was developed for simultaneous determination of co-occurring mycotoxins exemplified with aflatoxin B1 (AFB1) and T-2 toxin (T-2) in representative biomatrices of rat plasma, heart, liver, kidney,

spleen, lung and brain in a total run time of 7 min. The established approach using stable internal standards of [$^{13}C_{17}$]-AFB1 and [$^{13}C_{24}$]-T-2 was extensively validated by determining the specificity, linearity ($R^2 \geq 0.9990$), sensitivity (lower limit of quantitation at 0.05 ng mL^{-1}), accuracy (70.9%-107.7%), precision (RSD≤14.2%) and stability (≥70.8%). Based on this methodological advance, the subsequent kinetics and tissue distribution after oral administration of 0.5 mg kg^{-1} b.w. of both AFB1 and T-2 in rats were thoroughly studied. As revealed, both AFB1 and T-2 were rapidly eliminated with the half-life time ($t_{1/2}$) in plasma of 8.44±4.02 h and 8.12±4.05 h, respectively. Moreover, AFB1 accumulated in all organs where the highest concentration was observed in liver (1.34 μg kg^{-1}), followed by kidney (0.76 μg kg^{-1}). Notably, only low levels of T-2 were observed in spleen (0.70 μg kg^{-1}) and in liver (0.15 μg kg^{-1}). The achieved data as supporting evidence would substantially promote the practical application of the proposed LC-MS/MS method for *in vivo* toxicokinetics and toxicity studies of co-occurring mycotoxins imitating natural incidence in rat system.

Keywords: Co-occurring mycotoxins; LC-MS/MS; Isotope dilution; Rat

Introduction

Mycotoxins, as the toxic secondary metabolites produced by various mold species, can contaminate many agricultural commodities in the field or during storage [1-3]. Typically, aflatoxin B1 (AFB1) and T-2 toxin (T-2), as frequently co-occurring mycotoxins in natural incidence, are produced by *Aspergillus* and *Fusarium* species, respectively [4-6]. AFB1 is a highly toxic and carcinogenic compound that causes disease in livestock and humans, and has been classified in group I as human carcinogen [7-9]. Due to its hepatotoxic and hepatocarcinogenic properties, the content of AFB1 in foods is strictly restricted in many countries [10]. T-2 can cause both acute and chronic diseases such as vomiting, diarrhea, skin irritation, weight loss, feed refusal and nausea, as well as neural disturbances and abortion [11-

[13]. Even so, up to now, no regulatory levels on public have been set for T-2 worldwide.

Practically, humans can be co-exposed to two representative mycotoxins of AFB1 and T-2. First, the same food can be infected or invaded by different mycotoxigenic molds resulting in the co-occurring AFB1 and T-2 even at high concentrations in many geographic origins under optimum environmental conditions [14-16]; Second, co-occurring mycotoxins can reach humans through the various diets and also, through milk, meat and eggs from livestock and poultry animals fed with different mycotoxins contaminated feedstuffs [17-19]. The toxic effects, appearing in consumers exposed to co-occurring mycotoxins, are surely in accordance to the possible interactions of concomitantly occurring mycotoxins, which might be antagonistic, additive, or synergistic on different occasions [20]. Nevertheless, knowledge regarding to the influence of co-occurring AFB1 and T-2 on kinetics and distribution is still unknown to date, where the main technical obstacle is the scare of simple, accurate and highly sensitive means capable of quantifying target mycotoxin molecules from complicated biological samples. Therefore, development and validation of desirable analytical methods for the simultaneous determination of co-occurring AFB1 and T-2 in a variety of biomatrices are very valuable, which are also the reliable tools for the kinetic and distribution studies to reveal the bidirectional effects of co-occurring AFB1 and T-2 in experimental animal system, minimizing the use of laboratory animals and the cost of analysis, with the premise of accuracy and precision.

Various methods have been employed to individually analyze single mycotoxin of either AFB1 or T-2 in biological fluids and tissues. These generally include radioactivity, thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) combined with different detectors [8, 21-28]. Radioactivity is very sensitive, which can be utilized in mass balance and tissue distribution studies, but not suitable for kinetic analysis due to the serious interferences of the extensive metabolites [29]. TLC and HPLC coupled to UV or fluorescence

detectors (FLD) could supply acceptable sensitivity in premise of tedious derivation process due to the weak chromophore of analyzed mycotoxins. Since the latest decade, liquid chromatography tandem mass spectrometry (LC–MS/MS) plays a pivotal role in trace analysis of multiple mycotoxins because of its high sensitivity and selectivity [30-32]. However, molecules originating from the sample matrix that co-elute with the compounds of interest may have significant impact on the ionization process causing ionization suppression/enhancement, and consequently on the accuracy of the method. Namely, the two main limiting features when detecting multiple mycotoxins in different biomatrices are the variety of the physico-chemical properties of the involved mycotoxins and the complex composition of sample matrix. Hitherto, although several LC-MS/MS methods have already been developed for simultaneous determination of AFB1 and T-2 in food and feedstuffs [7], no uniform method with sufficient simplicity, relative rapidity and high sensitivity has been reported, which could be applicable for different biomatrices i.e., plasma, heart, liver, spleen, lung, kidney and brain, with the complicated substances as interferences.

In the present study, a simple and sensitive LC-MS/MS method based on a combination of isotope dilution and fast sample preparation using homemade clean-up cartridges was developed and then validated for simultaneous quantitation of co-occurring AFB1 and T-2 in rat plasma, heart, liver, spleen, lung, kidney and brain. The utmost advantage of this proposed analytical method is that identical conditions can be utilized for analyzing AFB1 and T-2 in all the above mentioned biomatrices. Furthermore, the small amount of sample needed, the low volume of solvents used and the fact that immunoaffinity columns (IAC) are not required for this assay, making it more rapid, economical and practical for analyses of AFB1 and T-2 in such small animal experiments. This method was then successfully applied to the kinetics and tissue distribution studies after oral administration of co-occurring AFB1 and T-2 in rat system, demonstrating its potential for toxicological or toxicokinetic studies.

Experimental section

Safety precautions

AFB1 and T-2 are toxic substances, which should be manipulated in solution, avoiding the formation of dust and aerosols. FPP3 masks and nitrile gloves were used when all related procedures were carried out.

Chemicals and reagents

The standards of AFB1 (molecular weight (MW), 312), T-2 (MW, 467) and their internal standards (ISs) [$^{13}C_{17}$]-AFB1 (MW, 329), [$^{13}C_{24}$]-T-2 (MW, 491) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemical structures of AFB1 and T-2 are shown in Fig. S-1 (Supplementary data). Acetonitrile and methanol, purchased from Merck (Darmstadt, Germany), were both HPLC grade. Milli-Q quality water (Millipore, Billerica, MA, USA) was used throughout the whole analysis. All other reagents were of analytical grade.

Silica gel (Product No. 236799), active carbon (Product No. C3345), alumina neutral (Product No. 199974), alumina base (Product No. 199443), florisil (Product No. 220736) and kieselguhr (Product No. D3877) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Polypropylene SPE empty tubes (3 mL) and frits were from Shenzhen Biocomma Biotech CO, LTD (Shenzhen, China). High quality poly (9, 9-diethylfluorene) (PDEF) syringe filters (0.22 μm pore size, 13 mm diameter) were supplied by Millipore.

Apparatus

LC-MS/MS (TSQ QUANTUM ULTRA, Thermo Scientific, Brookfield, USA) using selected

reaction monitoring (SRM) mode was used for AFB1 and T-2 analyses in different biomatrices. Separation was performed on a Thermo Hypersil Gold column (100 mm×2.1 mm, 3.0 μm) at 35 °C, with a mobile phase flow rate of 0.35 mL min^{-1}. Water (containing 0.25 mmol L^{-1} ammonium acetate and 0.05% formic acid) (A) and methanol (containing 0.25 mmol L^{-1} ammonium acetate and 0.05% formic acid) (B) were used as the mobile phase. A linear gradient elution program was applied as follows: initial 30% B, 3 min 100% B, 3.8 min 100% B, 4 min 30% B, and hold on for a further 3 min for re-equilibration, giving a total run time of 7 min. The injection volume was 5.0 μL (full loop). The mass spectrometer was operated with an electrospray source in positive ionization mode (ESI^+). The following settings were used for MS/MS conditions: spray voltage, 4 kV; vaporizer temperature, 300 °C; sheath gas pressure, 30 psi; aux valve flow, 30 arb; capillary temperature, 350 °C. Data were acquired and processed by Xcalibur software (Thermo Scientific, Brookfield, USA).

Standard solutions

Accurately weighed solid portions (1.0 mg) of AFB1 and T-2 were dissolved in 2 mL of pure ethanol, and the solution was diluted with water to prepare a mixture of AFB1 and T-2 at a concentration of 0.1 mg mL^{-1} in 20% of ethanol aqueous solution, which was used for the oral administration of rats.

For LC-MS/MS analysis, solid portions of AFB1 and T-2 were individually dissolved in acetonitrile to prepare 0.1 mg mL^{-1} of stock solution, and stored at -20 °C under darkness. The stock solution was diluted step by step with the mixture of acetonitrile and water containing 10 mmol L^{-1} ammonium acetate (20/80, v/v) to prepare work solution. The stock solutions of the two ISs ($[^{13}C_{17}]$-AFB1 and $[^{13}C_{24}]$-T-2) were directly used the purchased product and diluted with the same mixed solution to 50 ng mL^{-1}. All work solutions were prepared

immediately before use.

Animals

Male Sprague–Dawley (SD) rats, weighing 200±20 g, were purchased from Fudan University Laboratory Animal Center (Shanghai, China). The study was approved by the Animal Ethics Committee of Shanghai Academy of Agricultural Sciences.

On the day of arrival, the animals were randomly distributed to polycarbonate cages with stainless steel covers for one week to allow acclimatization to the environmental conditions: 12 h day/night cycle, temperature 25±2 ˚C, standard diet and water.

For the kinetics studies in plasma, six rats were administrated one single dose of 0.5 mg/kg b.w. AFB1 and T-2 by gavage with the volume of about 1 mL of ethanol aqueous solution of pure ethanol in water (20/80, v/v, 0.1 mg mL^{-1}) depending on the weight of the rat itself. Blood samples (300 μL) were collected via the caudal vein catheter prior to dosage (0 min) and at 0.083, 0.167, 0.5, 1.0, 1.5, 2.0, 2.5, 5, 7.5, 10.0, 12.0 and 24.0 h thereafter with minor modifications according to the previous protocols [33]. The blood samples were immediately transferred to heparinized tubes and centrifuged at 4000 g for 5 min. All plasma samples were pipetted and stored at -20 °C until analysis.

For the tissue distribution studies, fifty four rats were randomly divided into nine groups (n = 6) and administrated an oral dose of 0.5 mg/kg b.w. AFB1 and T-2 by gavage. After euthanizing by cervical dislocation prior to dosage (0 h) and at 0.5 h, 1 h, 2 h, 4 h, 12 h 24 h, 48 h and 72 h thereafter, the tissues of heart, liver, spleen, lung, kidney and brain were excised and blot dried. In order to minimize the differences generated from various cell types in the organs and take a representative sample, the whole tissues were individually homogenized with normal saline (m/v, 1/3), and the homogenates were stored at −20 ˚C until analysis.

For investigation of tissue accumulation, six rats received a daily administration of 0.25 mg/kg b.w. AFB1 and T-2 by oral gavages during 20 days, while another six rats were administrated with normal saline as control. The animals were euthanized 24 h after the last administration, and the tissues were dealt as described above.

Preparation of homemade clean-up cartridges

Silica gel (0.09 g) was accurately weighed into a 3 mL hollow SPE cartridge and was shaken to compact the silica gel. Then, 0.01 g of florisil was added. After shaking, florisil was then covered by a frit at the end to ensure the upper surface smooth and flat.

Sample pretreatment

To the plasma/tissue homogenates (100 μL), 10 μL of IS solution (50 ng mL^{-1}) was added, followed by the addition of 700 μL of acetone for participation of proteins. The mixture was vortex-mixed for 1 min and transferred into SPE cartridges. The cartridges were eluted with 3 mL of methanol. The collected eluent was evaporated to dryness by a gentle stream of nitrogen gas at 40 °C. The residue was reconstituted in 100 μL mixture of acetonitrile and water containing 10 mmol L^{-1} ammonium acetate (20/80, v/v), passed through the PDEF syringe filters and was ready for injection. Samples with concentrations out of the linear range were appropriately diluted with blank plasma/tissue homogenates and 100 μL of the dilution was selected and processed as described above.

Method validation

The method was thoroughly validated on a serial of characteristics of specificity, linearity, sensitivity, extraction recovery, accuracy and precision. Matrix effects as well as the stability of AFB1 and T-2 in plasma and tissue homogenates were also determined.

Specificity was demonstrated by comparing chromatograms of blank plasma/tissue homogenates, plasma/tissue homogenates spiked with AFB1 and T-2, and plasma/tissue homogenates collected after the oral administration.

Calibration curves of seven data points were plotted in the range of 0.05–100 ng mL^{-1} in solvent, plasma, heart, liver, spleen, lung, kidney and brain, respectively, and 5 ng mL^{-1} of each IS was included in each solvent. The sensitivity was evaluated by determining the lower limit of detection (LLOD) and lower limit of quantitation (LLOQ). The LLOD and LLOQ were defined as the concentrations of AFB1 and T-2 that yielded a signal-to-noise (S/N) $\geq$3 and $\geq$10, which were both determined by decreasing the spike concentrations in various biomatrices, respectively.

Extraction recovery was assessed using a generic SPE extraction procedure with four spiked levels (0.05, 1, 10, 100 ng mL^{-1}). Recovery data were determined by comparing the peak areas (n = 6, each concentration) of AFB1 and T-2 obtained from plasma/ tissue homogenates spiked before extraction with that from plasma/ tissue homogenates spiked after extraction. Matrix effects were assessed by comparing the slope of the standard addition plot with the slope of the standard calibration plot with a concentration range of 0.05–100 ng mL^{-1} [30].

Accuracy was evaluated in the blank plasma and tissue homogenates employing the method of standard addition. The homogenized samples were spiked with LLOQ, low, intermediate and high levels of AFB1 and T-2 (0.05, 1, 10, 100 ng mL^{-1}) in sextuplicate. The spiked samples were pretreated and analyzed by the established LC-MS/MS method. Accuracy was expressed as the percentage of mean calculated concentrations vs actual concentrations. Precision was evaluated by determining the same spiked samples in one day (intra-day

precision) and the samples spiked with the same concentrations in four consecutive days (inter-day precision).

The stability of AFB1 and T-2 in plasma and tissue homogenates was investigated by analyzing extracted samples at two spiked levels of 1 and 10 ng mL^{-1} stored at room temperature (RT) for 8 h, at –20°C for two weeks and three successive freeze–thaw cycles. Stability was assessed by comparing the mean concentrations of AFB1 and T-2 in the stored samples with those of the freshly prepared ones.

Data analysis

All kinetic parameters were processed by Drug and statistics (DAS) software, version 2.0 (Shanghai, China). The parameters including peak concentration (Cmax), half-life time ($t_{1/2}$), time of maximum plasma concentration (Tmax), area under the curve (AUC_{0-t}), area under the curve from zero to infinity ($AUC_{0-\infty}$), total body clearance (CLz/F), mean residence time (MRT_{0-t}) and mean residence time from zero to infinity ($MRT_{0-\infty}$) were calculated. Data for all response variables were reported as mean ± SD. A significance level (α) of 0.05 was selected.

Results and discussion

Optimization of the LC-MS/MS conditions

The MS/MS conditions were firstly optimized for AFB1 or T-2 by individual injection of each standard solution (500 ng mL^{-1}). Identification of precursor ions was performed in the full scan mode by recording *m*/*z* from 100 to 800 in both ESI^+ and ESI^- mode. The results

showed that the responses of $[M+H]^+$ ions generated from AFB1 and $[^{13}C_{17}]$-AFB1 under ESI^+ mode were obviously higher than their $[M-H]^-$ ions generated under ESI^- mode. Then, 313.2 (*m/z*) and 330.2 (*m/z*) were selected as the precursor ions for AFB1 and $[^{13}C_{17}]$-AFB1, respectively. For T-2 and $[^{13}C_{24}]$-T-2, the responses of the $[M+Na]^+$ ions generated under ESI^+ mode were significantly higher than those of the other ions, i.e., $[M+H]^+$, $[M+NH_4]^+$ and $[M-H]^-$, generated under ESI^+ or ESI^- mode. As a consequence, 489.7 (*m/z*) and 513.7 (*m/z*) were selected as the precursors for T-2 and $[^{13}C_{24}]$-T-2, respectively. Based on the confirmation of precursor ions, two product ions for each precursor ion were selected according to the optimal selectivity and highest sensitivity for the target compounds. Collision energies were selected according to the responses of the product ions. The MS/MS spectrometry of AFB1 and T-2 are shown in Fig. S-1 (Supplementary data). The final selection of precursor ions, product ions and collision energies is shown in Table 1.

A mixture of methanol and water was used as the mobile phase for separation of AFB1 and T-2. After optimization, a satisfactory separation for AFB1 and T-2 was generated by linear gradient elution. In order to achieve high sensitivity of analysis, the ionization efficiency was investigated by further optimization of the composition of the mobile phase. Since ESI^+ was employed in the present study, the acidic conditions would be beneficial to the ionization of the analytes. Therefore, water containing 0.05% formic acid, water containing 10 mmol L^{-1} ammonium acetate, water containing 10 mmol L^{-1} ammonium formate, and water containing 0.25 mmol L^{-1} ammonium acetate and 0.05% formic acid were compared. Results of multiple injections indicated that the responses of AFB1 and T-2 were substantially improved and higher sensitivity was subsequently obtained when 0.05% formic acid and 0.25 mmol L^{-1} ammonium acetate were added compared to other additives. Under such situation, nice peak shapes and satisfactory separation efficiency were also achieved.

Development of the homemade clean-up cartridges

In general, matrix effects were directly related to an insufficient sample clean-up and might be reduced by simply injecting smaller volumes or diluting the sample, which seriously influence the sensitivity of the method, and were therefore inappropriate in the present study. In literature, IAC columns, Multisep multifunctional cartridges and Mycosep multifunctional cartridges are frequently used for mycotoxin purification [31, 32, 34]. However, no commercially available cartridges were reported with acceptable capability for simultaneous determination of AFB1 and T-2 in plasma and different tissue homogenates.

In the present study, six commercially available normal-phase materials, which were commonly used for the purification of the analytes in the previous studies, i.e., silica gel, active carbon, alumina neutral, alumina base, florisil and kieselguhr, were tested for their purification efficiencies. First, the recovery performance of all candidates was evaluated by purifying mixed standard solutions (1.5 ng mL^{-1}) with the cartridges filled with one single material (0.1 g). The mixed solutions (100 μL) were passed through the cartridges and eluted with 3 mL of methanol. The eluent was collected and dried by nitrogen gas at 40 °C. The residues were re-dissolved in 100 μL of mixed solution of acetonitrile and water containing 10 mmol L^{-1} ammonium acetate (20/80, v/v). As indicated in Fig. 1, silica gel and florisil showed satisfactory recoveries (>80%). An orthogonal design L_9 (3^4) was conducted to optimize the ratio of silica gel to florisil, quantity of total materials and quantity of the elution solvent. The factors and levels were designed as shown in Table S-1 (Supplementary data). The mixed solution was purified by the homemade clean-up cartridges prepared according to the orthogonal table. The combinations of levels and factors were optimized by screening of nine treatments and each treatment was done in triplicate. 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 significance difference (LSD, a=0.05) test. All computations were made by employing the statistical software (SAS, version 8.2). As shown in Online Resource 2, all of the three factors had significant effects on AFB1 and T-2 purification since the F values were all greater than the critical F value (19, P=0.05). The total content of AFB1 and T-2 was highest when the ratio of silica gel to florisil was selected as 9/1, quantity of total materials was 0.1 g and quantity of the elution solvent was 3 mL. Consequently, a simple sample purification approach based on the homemade clean-up cartridges was developed. Afterwards, spiked plasma and different tissue homogenates were further tested. It could be obviously seen from the results that the sensitivities were significantly improved for AFB1 and T-2 when the spiked extracts were purified with homemade mixed cartridges. On the other hand, some impurities, i.e., pigment and protein, which could reduce the lifetime of the analytical columns, were eliminated by the SPE cartridges. Satisfactory purification efficiencies evaluated by determining the matrix effects and the presence of interference peaks, and high recoveries were generated, supporting the strong ability of the homemade clean-up cartridges for the purification of co-occurring AFB1 and T-2 in different biomatrices.

Method validation

The method was selective for plasma and six different tissue homogenates since no interference peaks appeared at the retention time of AFB1 or T-2 in blank samples, also indicating that no AFB1 or T-2 existed in the regular rat feed. Chromatograms of blank plasma (a) and liver (d), blank plasma (b) and liver (e) spiked with AFB1 and T-2 (50 ng mL^{-1}), and rat plasma (c) and liver samples (f) at 0.5 h after oral administration of AFB1 and T-2 in rat are shown in Fig. 2.

The calibration curves for liquid solvent and all biomatrices constructed by isotope dilution method showed good linearity (R^2 > 0.9990) over the concentration range of 0.05-100 ng mL^{-1} (Table 2). For both mycotoxins in plasma and tissue homogenates, the LLOD and LLOQ were 0.01 and 0.05 ng mL^{-1}, respectively (Table 2), which were desirable and obviously lower than those obtained in bibliography via UHPLC-FLD approaches [35].

The observed matrix effects without ISs correction ranged from 73.0 to 105.8% for AFB1 and from 74.9 to 88.6% for T-2, suggesting that matrix effects in quantitative analysis could not be ignored. In order to establish an accurate method suitable for determination of co-occurring AFB1 and T-2 in different matrices, ISs were needed to correct the recovery losses during the ionization process. [$^{13}C_{17}$]-AFB1 and [$^{13}C_{24}$]-T-2 were selected, and the matrix effects were then calculated. The results showed that the extents of signal suppression/enhancement (SSE) were in the range of 90.2-108.9% for AFB1 and 90.9-100.0% for T-2, respectively, demonstrating that signal suppression/enhancement could be eliminated by the two ISs (Table 3).

The extraction recoveries of AFB1 and T-2 at LLOQ, low, intermediate and high concentrations (0.05, 1, 10 and 100 ng mL^{-1}) were in the range of 51.6-111.3% for AFB1 and 68.9-103.8% for T-2, respectively (Table 4). In order to minimize the losses of AFB1 and T-2 during the extraction process on some occasions, the isotope ISs were utilized in the present work. As a consequence, desirable accuracy of the method ranged from 70.9 % to 107.7% for AFB1 and from 72.4% to 108.3% for T-2 was obtained (Table 4). Values for the precision were no more than 12.3% (intra-day) and 13.4% (inter-day) for AFB1, and 11.6% (intra-day) and 14.2% (inter-day) for T-2, respectively (Table 5).

The stability of AFB1 and T-2 in rat plasma and tissue homogenates was fully evaluated. As summarized in Table 6, the results of short-term, freeze–thaw and long-term stabilities showed that all the samples were stable under these conditions, indicating there were no

stability-related problems during the routine and large-scale analysis of bulk samples. Comparatively, this proposed simple uniform LC-MS/MS method for simultaneous determination of AFB1 and T-2 in different biomatrices showed higher sensitivity and faster sample preparation, as well as more accuracy aided by isotope ISs than the previously reported methods.

In vivo kinetics and distribution studies

The oral dose for administration was ascertained based on the data from some pilot experiments and previous literatures [29, 36], and was obviously lower than the reported median lethal dose (LD_{50}) of AFB1 (5.5-17.9 mg/kg b.w. by oral) or T-2 (0.9 mg/kg b.w. by intravenous) in rat. The developed LC-MS/MS method was employed for kinetics investigation of co-occurring AFB1 and T-2 in rat plasma after oral administration at a dose of 0.5 mg/kg b.w. The concentration–time profiles are presented in Fig. 3. The calculated kinetic parameters expressed as mean ± SD are shown in Table 7. After oral administration, the highest concentration of AFB1 (Cmax= 16.58±1.05 ng mL^{-1}) was observed with Tmax being 0.17 min. In previous studies, the obtained time-to-peak for AFB1 in rat was in the range of 2-3 h [37, 38]. These existed differences might be due to two possibilities. First, different analytical techniques were utilized. The total radioactivity employed in the previous studies might misidentify the target analytes with the metabolites and other impurities [29], while LC-MS/MS with high selectivity was developed in the present study and could eliminate the false positive results; second, the co-occurring AFB1 and T-2 might accelerate the distribution of AFB1 showing the additive toxic effects [20]. The concentrations of T-2 in rat plasma were lower than that of AFB1, with a Cmax of only 0.53±0.08 ng mL^{-1}. As previously reported, only about 2% of the dose appeared in the effluent during the metabolism experiment of

tritiated T-2 toxin (2.3 and 230 pg) in vascularly autoperfused jejunal loops of rats [29]. Similarly, it could be concluded in this study that T-2 toxin was susceptible to liver and intestinal first-pass effects, so that its absolute bioavailability might be negligible following oral administration.

The concentration–time profiles in different tissues analyzed at 0-72 h after oral administration are shown in Fig. 4. The results indicated that AFB1 underwent a rapid distribution in the tissues. Within 1 h after administration, highest concentrations of AFB1 were reached in all of the target tissues. Then, AFB1 was rapidly eliminated and disappeared within 24 h. Interestingly, AFB1 was also detected in brain homogenate, demonstrating that AFB1 could efficiently cross the blood–brain barrier. For T-2, very low concentrations were observed in heart, liver, spleen, lung, kidney, and disappeared within 4 h in all tissues, also indicating its low absolute bioavailability. As to tissue accumulation of AFB1 and T-2 (Fig. 5), the highest concentration of AFB1 was observed in liver (1.34±0.02 μg kg^{-1}), followed by kidney (0.76±0.03 μg kg^{-1}), which might be related to its intensively hepatotoxic and carcinogenic effects. AFB1 was also detected in heart, brain, spleen and lung, indicating that AFB1 could also accumulate in these tissues. Comparatively, only very low concentrations of T-2 were observed in spleen (0.70±0.06 μg kg^{-1}), possibly causing immunosuppressive activity, and then in liver (0.15±0.02 μg kg^{-1}), suggesting that the accumulation effect of T-2 is weak and spleen is the main accumulation organ of T-2. More importantly, the results demonstrated in the kinetic studies in plasma, as well as tissue distribution and tissue accumulation of both toxins could provide valuable references for revealing the real mechanism of the toxicity on humans. In further investigations, the well known metabolites such as the AFB1 epoxide formed in the liver and HT-2 formed from T-2, or even unknown metabolites in aqueous media will be studied to more clearly elucidate the metabolism of co-occurring AFB1 and T-2.

Conclusions

A LC-MS/MS approach was specifically developed for simultaneous determination of co-occurring mycotoxins exemplified with AFB1 and T-2 in plasma and different tissues of heart, liver, spleen, lung, kidney and brain. The homemade clean-up cartridges and isotope ISs as combinatorial means were utilized together to eliminate the matrix effects, thus ensuring the accuracy and precision of the method. Full validation indicated that the well-established method with a total running of 7 min for each sample was highly sensitive, selective, fast, economic and proved to be applicable for multi-component analysis in different biomatrices in presence of interferences. These methodological advances guarantee the successful application for the kinetics study in plasma, and investigations on tissue distribution and accumulation after oral administration with two co-occurring mycotoxins in rat system. The kinetics parameter values, tissue distribution and accumulation data obtained in the present study might be helpful to predict the toxicokinetics and toxicity of co-occurring AFB1 and T-2 in animals and humans. In addition, the analytical method proposed in this work will benefit the subsequent *in vivo* evaluation on interaction of co-occurring mycotoxins, and provide the direct evidences imitating the natural incidence of co-occurring mycotoxin contaminants in cereal crops and foods through various dietary exposures.

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Supporting Information

Supporting information associated with this article can be found, in the online version.

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Figure Captions

Fig. 1 Comparison of the recovery performance of all candidate adsorbent materials by purifying mixed standard solutions (1.5 ng mL^{-1}) with the SPE cartridges filled with one kind of material

Fig. 2 SRM chromatograms of blank plasma (a) and liver (d), blank plasma (b) and liver (e) spiked with AFB1 and T-2 (50 ng mL^{-1}), respectively, and rat plasma (c) and liver samples (f) at 0.5 h after oral administration of AFB1 and T-2 in rat

Fig. 3 Mean plasma concentration–time curves of AFB1 (a) and T-2 (b) following the oral administration of co-occurring AFB1 and T-2 (0.5 mg/kg b.w.) in rat (n=6)

Fig. 4 The concentration–time profile of AFB1 (a) and T-2 (b) after oral administration of AFB1 and T-2 (0.5 mg/kg b.w.) in different tissues of rat (n=6)

Fig. 5 The tissue accumulation of co-occurring AFB1 and T-2 after 20 days of AFB1 and T-2 (0.25 mg/kg b.w.) administration by oral gavage in rat (n=6)

Table 1 Precursor ions, product ions and collision energies for the analytes.

Names	Precursor ion(m/z)	Primary product ion(m/z)	Collision energy(eV)	Secondary product ion(m/z)	Collision energy(eV)
AFB1	313.2 ($+H^+$)	285.1	24	241.2	32
[$^{13}C_{17}$]-AFB1	330.2 ($+H^+$)	301.0	20	251.9	30
T-2	489.7 ($+Na^+$)	387.3	23	245.2	26
[$^{13}C_{24}$]- T-2	513.7 ($+Na^+$)	406.4	22	344.3	24

Table 2 Calibration curves of AFB1 and T-2 in liquid solvent and seven different matrices.

Mycotoxin	Matrices	Slope	Intercept	R^2	Range (ng mL^{-1})	Sensitivity (ng mL^{-1}/ng g^{-1a})

						LLOD	LLOQ
AFB1	Solvent	1.12	0.36	0.9997	0.05-100	0.01	0.05
	Plasma	1.14	0.07	0.9991	0.05-100	0.01	0.05
	Heart	1.20	0.09	0.9999	0.05-100	0.01	0.05
	Liver	1.21	0.40	0.9999	0.05-100	0.01	0.05
	Spleen	1.10	0.27	0.9997	0.05-100	0.01	0.05
	Lung	1.22	0.14	0.9995	0.05-100	0.01	0.05
	Kidney	1.21	1.44	0.9990	0.05-100	0.01	0.05
	Brain	1.01	0.89	0.9993	0.05-100	0.01	0.05
T-2 toxin	Solvent	0.022	0.009	0.9999	0.05-500	0.01	0.05
	Plasma	0.022	0.034	0.9990	0.05-500	0.01	0.05
	Heart	0.020	0.007	0.9999	0.05-500	0.01	0.05
	Liver	0.022	0.002	0.9998	0.05-500	0.01	0.05
	Spleen	0.020	0.012	0.9998	0.05-500	0.01	0.05
	Lung	0.021	0.009	0.9996	0.05-500	0.01	0.05
	Kidney	0.020	0.004	0.9998	0.05-500	0.01	0.05
	Brain	0.020	0.032	0.9993	0.05-500	0.01	0.05

[a] ng mL^{-1} and ng g^{-1} refer to the LLOQ and LLOD values of AFB1 and T-2 in plasma and tissues, respectively.

Table 3 The extents of signal suppression/enhancements (SSEs) calculated using the isotope ISs or not (%, n=3).

Matrices	With ISs		Without ISs	
	AFB1	T-2	AFB1	T-2
Plasma	101.8±5.1	100.0±4.5	85.1±3.2	87.8±3.4
Heart	107.1±6.7	90.9±3.6	73.0±4.1	75.1±7.2
Liver	108.0±2.4	100.0±7.8	100.9±10.1	88.6±5.6
Spleen	98.2±4.3	90.9±4.3	105.8±3.2	80.9±6.4
Lung	108.9±3.2	95.5±5.6	99.4±2.9	82.7±2.9
Kidney	108.0±5.6	90.9±6.7	85.2±3.5	81.5±7.1
brain	90.2±6.8	90.9±8.4	94.4±6.1	74.9±4.6

Table 4 Recovery tests in different matrices (n=6).

Matrices	Spiked level	AFB1		T-2	
		Accuracy (Mean %)	Extraction recovery (Mean %)	Accuracy (Mean %)	Extraction recovery (Mean %)
Plasma	High level [a]	102.3±7.3	56.2±3.4	95.2±4.1	71.7±3.4
	Intermediate level [b]	107.1±5.1	56.9±4.3	95.6±3.3	69.3±3.2
	Low level [c]	97.5±3.1	59.1±5.1	94.3±4.0	74.1±4.5
	LLOQ[d]	101.3±6.5	64.9±3.1	96.4±8.3	78.0±3.1
Heart	High level	98.2±4.2	79.3±4.8	84.6±4.6	77.5±2.9
	Intermediate level	95.5±6.2	69.2±3.2	83.7±1.9	70.3±5.6
	Low level	82.5±2.0	80.1±3.9	84.1±3.9	68.9±6.7
	LLOQ	90.7±11.2	82.1±5.6	88.7±7.0	73.4±3.6
Liver	High level	84.7±3.3	60.0±4.8	89.3±6.3	88.9±3.8
	Intermediate level	93.0±2.0	55.7±4.4	92.5±6.0	83.7±3.4
	Low level	101.9±6.6	59.9±4.1	90.8±6.5	96.8±5.6
	LLOQ	97.1±7.1	56.0±3.9	89.8±8.0	92.2±6.7
Spleen	High level	90.1±2.7	60.7±5.7	85.8±6.3	82.0±6.9
	Intermediate level	102.9±2.2	57.4±3.1	91.3±5.1	82.4±4.5
	Low level	100.1±4.1	57.0±1.9	96.8±8.4	93.0±4.8
	LLOQ	93.9±4.3	51.6±2.8	90.3±7.6	80.5±7.4
Lung	High level	91.3±6.4	54.4±3.2	92.0±2.9	84.2±6.1
	Intermediate level	98.7±4.7	52.5±3.3	108.3±7.8	91.9±5.5
	Low level	98.6±6.8	55.5±4.9	92.2±8.2	90.3±5.2
	LLOQ	100.9±5.0	54.8±2.0	84.2±3.9	84.9±5.4
Kidney	High level	95.9±2.0	61.2±1.1	101.8±8.8	86.7±5.5
	Intermediate level	107.7±7.0	64.1±2.3	83.3±6.2	80.4±6.7
	Low level	94.2±8.2	58.8±4.5	103.8±9.8	98.3±3.4
	LLOQ	86.5±8.8	55.0±3.2	98.2±11.6	98.0±4.3
Brain	High level	76.4±3.2	95.2±3.3	72.4±1.9	89.9±2.3
	Intermediate level	70.9±3.6	103.8±4.8	86.7±2.1	98.6±3.4

	Low level	89.9±5.8	111.3±5.4	85.9±3.2	103.8±5.7
	LLOQ	84.9±6.2	108.9±7.8	83.6±5.6	103.7±6.1

[a] High level was designed as 100.0 ng mL^{-1} ;
[b] Intermediate level was designed as 10.0 ng mL^{-1};
[c] Low level was designed as 1.0 ng mL^{-1};
[d] LLOQ was designed as 0.05 ng mL^{-1}.

Table 5 The intra- and inter-day precision tests of AFB1 and T-2 in different matrices (n = 6).

Matrices	Spiked level	AFB1		T-2	
		Inter-day (RSD %)	Intra-day (RSD %)	Inter-day (RSD %)	Intra-day (RSD %)
Plasma	High level [a]	6.6	7.1	2.8	4.3
	Intermediate level [b]	3.0	4.8	3.9	3.5
	Low level [c]	7.2	3.2	6.5	4.2
	LLOQ [d]	8.4	6.4	10.2	8.6
Heart	High level	8.1	4.3	6.8	5.4
	Intermediate level	7.3	6.5	8.1	2.3
	Low level	5.1	2.4	7.9	4.6
	LLOQ	6.2	12.3	11.9	7.9
Liver	High level	3.2	3.9	2.1	7.1
	Intermediate level	4.9	2.1	7.3	6.5
	Low level	2.3	6.5	6.5	7.2
	LLOQ	5.6	7.3	8.6	8.9
Spleen	High level	2.1	3.0	3.1	7.3
	Intermediate level	6.5	2.1	8.7	5.6
	Low level	7.4	4.1	11.6	8.7
	LLOQ	8.6	4.6	14.2	8.4
Lung	High level	2.9	7.0	4.6	3.2
	Intermediate level	3.6	4.8	5.6	7.2
	Low level	4.5	6.9	7.2	8.9
	LLOQ	8.2	5.0	7.9	4.6
Kidney	High level	8.6	2.1	7.2	8.6
	Intermediate level	13.4	6.5	8.7	7.5
	Low level	10.9	8.7	9.1	9.4
	LLOQ	11.4	10.2	13.9	11.6
Brain	High level	4.3	4.2	4.9	1.9
	Intermediate level	6.5	5.1	2.3	2.1
	Low level	7.2	6.4	6.5	3.2
	LLOQ	10.1	7.3	7.8	5.6

[a] High level was designed as 100.0 ng mL^{-1};
[b] Intermediate level was designed as 10.0 ng mL^{-1};
[c] Low level was designed as 1.0 ng mL^{-1};
[d] LLOQ was designed as 0.05 ng mL^{-1}.

Table 6 Stability of AFB1 and T-2 (n = 6).

Toxins	Matrices	Concentration (ng mL^{-1})	Concentration of stored sample / Concentration of freshly prepared sample (mean ± SD %)		
			Short-term stability (at RT for 8h)	Freeze–thaw stability	Long-term stability (at -20 ℃ for 2 weeks)
AFB1	Plasma	1	93.2±3.1	90.1±2.4	84.9±3.4
		100	91.2±4.1	96.8±3.2	76.5±3.8
	Heart	1	95.1±3.6	78.8±3.4	86.6±5.2
		100	94.2±3.2	83.0±2.4	80.6±4.1
	Liver	1	95.2±4.5	70.8±4.6	87.9±6.4
		100	91.9±2.1	88.9±6.4	92.5±5.9
	Spleen	1	93.1±5.6	78.2±2.5	89.5±4.1
		100	91.6±3.4	79.3±3.4	80.3±3.1
	Lung	1	92.5±2.4	85.0±5.9	84.4±3.2
		100	95.6±3.8	83.0±4.5	82.2±6.7
	Kidney	1	97.5±4.6	74.3±6.5	80.7±5.1
		100	82.1±4.4	77.6±6.4	79.8±5.7
	Brain	1	92.8±3.3	79.8±5.6	80.8±6.4
		100	95.5±1.9	83.1±6.2	75.2±4.1
T-2	Plasma	1	82.2±10.6	91.5±2.9	83.8±2.9
		100	83.7±2.9	94.4±3.4	83.6±3.6
	Heart	1	82.4±8.2	92.5±3.4	90.1±3.6
		100	86.0±4.9	95.6±4.2	92.8±4.1
	Liver	1	84.7±7.1	96.5±3.4	84.1±2.9
		100	83.5±5.2	94.6±4.7	88.0±3.1
	Spleen	1	94.8±7.2	84.9±3.2	80.8±3.7
		100	85.3±6.7	82.6±2.7	84.9±5.1
	Lung	1	83.5±3.1	86.1±4.3	79.3±5.6
		100	95.4±4.9	83.1±2.9	84.4±3.2
	Kidney	1	88.7±4.6	79.9±4.6	78.9±6.4
		100	86.7±5.8	82.1±3.4	81.3±6.2
	Brain	1	82.4±4.2	81.6±4.5	77.2±7.2
		100	81.1±2.9	84.7±6.2	76.6±3.1

Table 7 Pharmacokinetic parameters of AFB1 and T-2 (0.5 mg/kg b.w.) after oral administration in rat (n = 6).

Pharmacokinetic parameters	Unit	AFB1	T-2
AUC(0-t)	μg /L*h	36.24±0.69	0.60±0.03
AUC(0-∞)	μg /L*h	40.84±3.65	0.98±0.24
MRT(0-t)	h	5.24±0.37	3.43±0.14
MRT(0-∞)	h	8.93±3.33	10.75±5.52
$t_{1/2}$	h	8.44±4.02	8.12±4.05
Tmax	h	0.17±0.00	0.17±0.00
CLz/F	L/h/kg	16.50±1.49	105.70±23.63

Cmax	μg /L	16.58±1.05	0.53±0.08

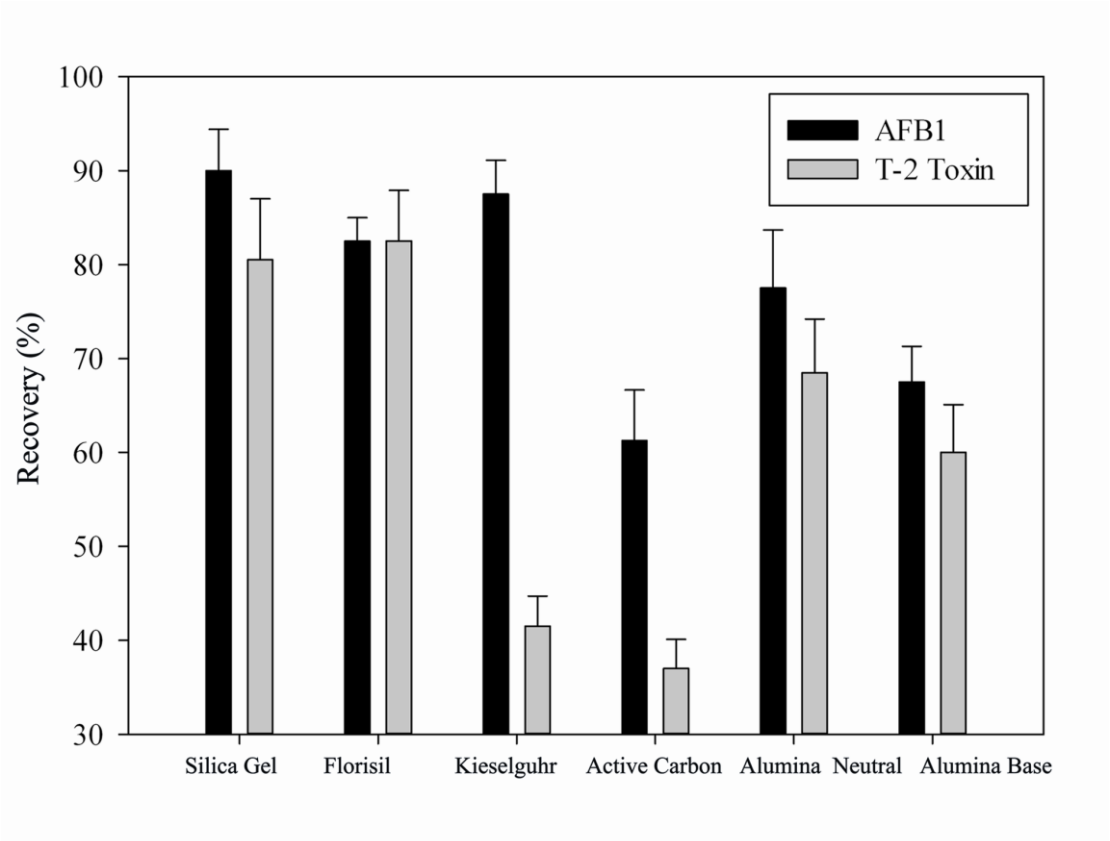

Fig. 1 Comparison of the recovery performance of all candidate adsorbent materials by purifying mixed standard solutions (1.5 ng mL^{-1}) with the SPE cartridges filled with one kind of material

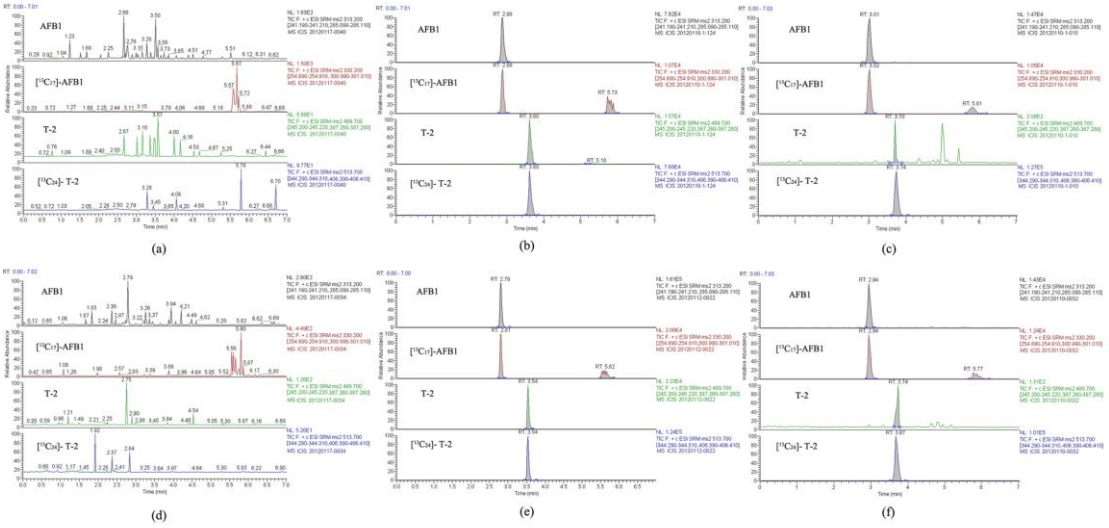

Fig. 2 SRM chromatograms of blank plasma (a) and liver (d), blank plasma (b) and liver (e) spiked with AFB1 and T-2 (50 ng mL^{-1}), respectively, and rat plasma (c) and liver samples (f) at 0.5 h after oral administration of AFB1 and T-2 in rat

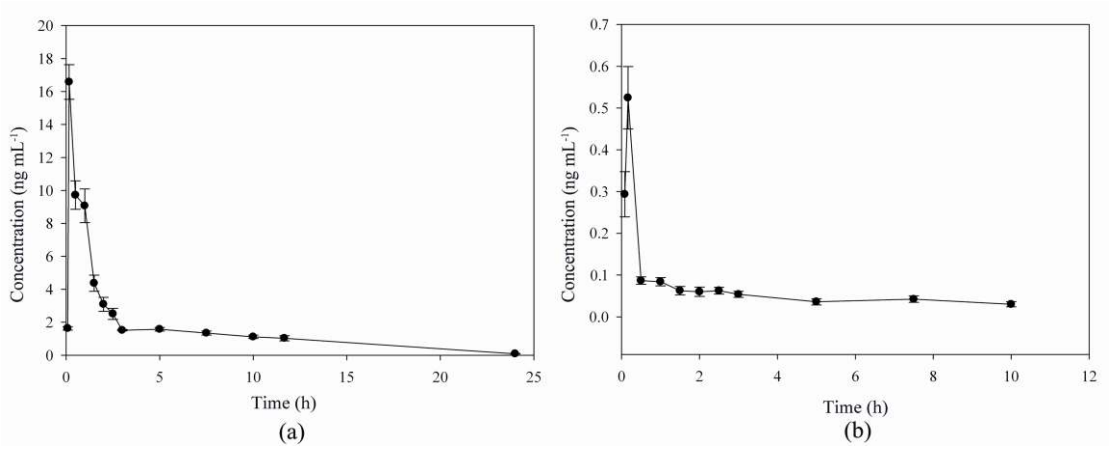

Fig. 3 Mean plasma concentration–time curves of AFB1 (a) and T-2 (b) following the oral administration of co-occurring AFB1 and T-2 (0.5 mg/kg b.w.) in rat (n=6)

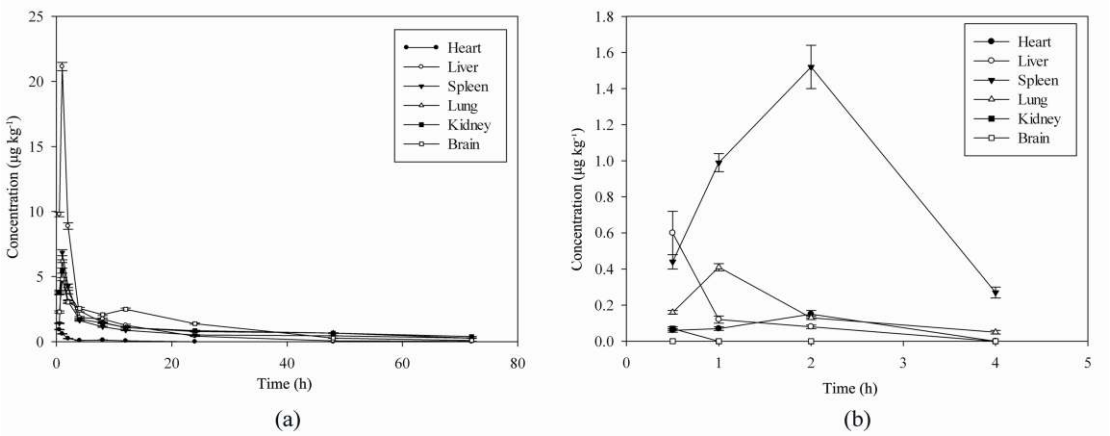

Fig. 4 The concentration–time profile of AFB1 (a) and T-2 (b) after oral administration of AFB1 and T-2 (0.5 mg/kg b.w.) in different tissues of rat (n=6)

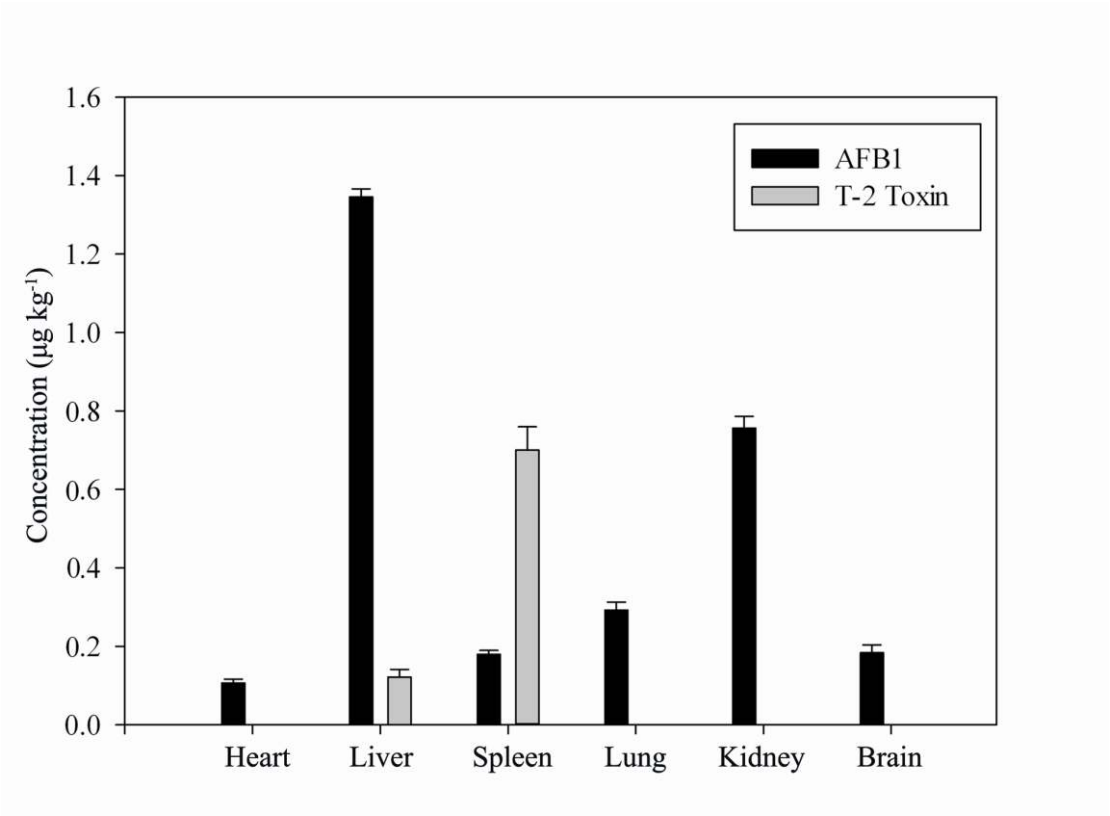

Fig. 5 The tissue accumulation of co-occurring AFB1 and T-2 after 20 days of AFB1 and T-2 (0.25 mg/kg b.w.) administration by oral gavage in rat (n=6)

Supporting Information

Supporting information for the article "Establishment of an isotope dilution LC-MS/MS method revealing kinetics and distribution of co-occurring mycotoxins in rat" by Zheng Han, Gang Liu, Zhiyong Zhao, Jingbo Zhang, Yucai Liao, Jianxin Shi, Dabing Zhang, Suquan

Song, Sarah De Saeger, Aibo Wu.

Corresponding Author:
Aibo Wu, Institute for Agri-food Standards and Testing Technology, Shanghai Academy of Agricultural Sciences, 1018 Jinqi Road, Shanghai 201403, P. R. China. Phone: +86-21-62202875; Fax: +86-21-62203612; Email: wuaibo@saas.sh.cn.

Captions

Fig. S-1. Chemical structures of AFB1 (a) and T-2 (b) and the MS/MS spectrometry of AFB1 (c) and T-2 (d).

Table S-1 Factors, levels and the results of the orthogonal experiment $L_9(3^4)$.

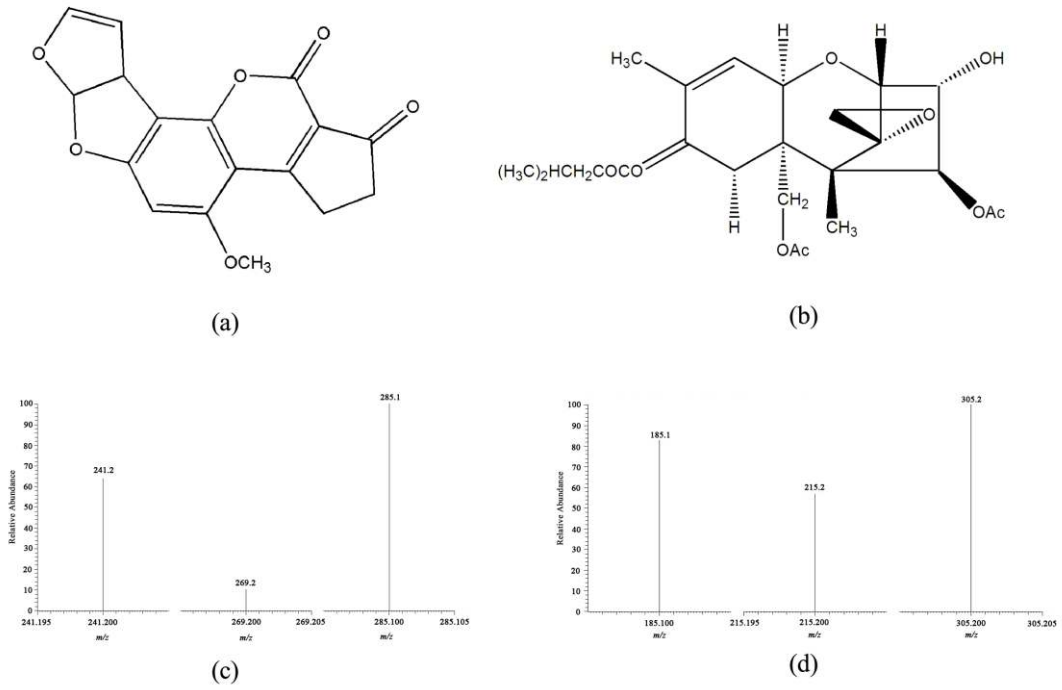

Fig. S-1. Chemical structures of AFB1 (a) and T-2 (b) and the MS/MS spectrometry of AFB1 (c) and T-2 (d).

Table S-1 Factors, levels and the results of the orthogonal experiment $L_9(3^4)$.

Levels	Factors			
	1 Ratio (silica gel/ florisil)	2 Quantity of total materials	3 Quantity of elution solvent (mL)	4 Blank

		(mg)	
1	9/1	0.1	1
2	7/3	0.3	2
3	5/5	0.5	3

Run	1	2	3	4	Total contents (ng mL^{-1})
1	1	1	1		2.269
2	1	2	2		2.077
3	1	3	3		2.466
4	2	1	2		2.262
5	2	2	3		2.066
6	2	3	1		1.267
7	3	1	3		2.518
8	3	2	1		1.318
9	3	3	2		1.822
$\bar{X}_1$ (ng mL^{-1})	2.271	2.350	1.618	2.052	
$\bar{X}_2$ (ng mL^{-1})	1.865	1.820	2.054	1.954	
$\bar{X}_3$ (ng mL^{-1})	1.886	1.852	2.350	2.015	
Range	0.406	0.530	0.732	0.098	

Factors	Sum of SSE^2	Degree of freedom	F	Critical Value of F	Significance
Ratio	0.313	2	20.867	19.000	*
Quantity of total materials	0.529	2	35.267	19.000	*
Quantity of elution solvent	0.813	2	54.200	19.000	*
Error	0.01	2			