Journal of Chromatography B, 925 (2013) 46–53

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Combinatorial approach of LC–MS/MS and LC–TOF-MS for uncovering *in vivo* kinetics and biotransformation of ochratoxin A in rat

Zheng Han[a,e], Zhiyong Zhao[a], Jianxin Shi[b], Yucai Liao[c], Zhihui Zhao[a], Dabing Zhang[b], Yongning Wu[d], Sarah De Saeger[e], Aibo Wu[a,*]

[a] *Institute for Agri-food Standards and Testing Technology, Shanghai Academy of Agricultural Sciences, 1000 Jinqi Road, Shanghai 201403, PR China*
[b] *School of Life Science and Biotechnology, Bor Luh Food Safety Center, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, PR China*
[c] *College of Plant Science and Technology, Huazhong Agricultural University, No. 1 Shizishan Street, Hongshan District, Wuhan, Hubei 430070, PR China*
[d] *National Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention, 29 Nan Wei Road, Beijing 100050, PR China*
[e] *Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Gent, Belgium*

ARTICLE INFO

*Article history:*
Received 4 December 2012
Accepted 25 February 2013
Available online 1 March 2013

*Keywords:*
Ochratoxin A
Liquid chromatography–tandem mass spectrometry
Liquid chromatography coupled with time of flight mass spectrometry
Kinetics
Biotransformation

ABSTRACT

A combinatorial platform of liquid chromatography–tandem mass spectrometry (LC–MS/MS) and liquid chromatography coupled with time of flight mass spectrometry (LC–TOF-MS) has been developed to investigate the *in vivo* kinetics and biotransformation of ochratoxin A (OTA) in rats. The stable isotope dilution LC–MS/MS method was first validated by determining the linearity ($R^2 \geq 0.9990$), sensitivity (lower limit of quantitation of 0.05 ng mL$^{-1}$), accuracy (83.3–108.3), precision (RSD $\leq$ 15.6%) and stability ($\geq$75.0%), and was approved for the determination OTA in plasma, heart, liver, spleen, lung, kidney and brain with a run time of 7.0 min. Simultaneously, an LC–TOF-MS method could unambiguously identify the metabolites of OTA in a total run time of 14 min. The subsequent studies on kinetics and distribution after oral administration of 0.2 mg/kg b.w. OTA in rat indicated that OTA could reach a maximum value of 1932.4 ± 124.9 ng mL$^{-1}$ within 5 h due to its fast absorption, and then was slowly eliminated in plasma with a half-life time ($t_{1/2}$) of 75.6 ± 29.0 h. Results of tissue accumulation after a daily oral administration of 0.1 mg/kg b.w. OTA during 20 days showed that the highest concentration of OTA was observed in lung (95.9 ± 13.7 ng g$^{-1}$), followed by liver (76.0 ± 9.7 ng g$^{-1}$), heart (62.0 ± 4.2 ng g$^{-1}$) and kidney (55.7 ± 4.7 ng g$^{-1}$). Furthermore, three less toxic metabolites of OTA were clearly identified: Ochratoxin β (OTβ) and ochratoxin B (OTB) methyl ester were found in kidney and spleen, respectively, while phenylalanine was detected in heart and kidney. Thus, a possible metabolic pathway of OTA was proposed. The above achieved results justified that the application of combinatorial LC–MS/MS and LC–TOF-MS methods are valuable tools to uncover the kinetics and metabolism of OTA for the interpretation of toxicological findings in animals and extrapolation of the resulting data as reference to humans.



## 1. Introduction

Mycotoxins, natural contaminants present in cereal crops and feeds, have caught worldwide attention in the fields of food safety and public health. Dried fruit, cereals, coffee, beer, grape juice, wine and spices, are frequently consumed. Unfortunately, these products likely also contain a common mycotoxin, ochratoxin A (OTA), N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzo-pyran-7-yl)carbonyl]-,(R)-l-phenylalanine, which comes from ubiquitous fungal species such as *Aspergillus* and *Penicillium* [1–3]. OTA has been found to be carcinogenic in the kidney of mice and rats, in addition to causing numerous specific toxic effects, such as hepatotoxicity, teratogenicity and immunosuppressivity [2,3]. It has been speculated that OTA may be associated with chronic renal diseases in humans (Balkan endemic nephropathy, BEN, interstitial nephritis) and has been designated by the IARC as a possible human carcinogen (group 2B). With regard to animal experiments and epidemiological studies on humans, the Scientific Committee for Food (SCF) has established a tolerable daily intake (TDI) of 5 ng/kg bw/day. The European Union has set maximum limits for OTA, *e.g.*, 5 μg kg$^{-1}$ in raw cereals, 3 μg kg$^{-1}$ in all products derived from cereals and 10 μg kg$^{-1}$ in raisins [4].

OTA can be biotransformed into a series of metabolites in animals by hydrolysis, hydroxylation, oxidation, and conjugation reactions. Interpretation of toxicological findings in animals and extrapolation of the resulting data as reference to human requires

* Corresponding author. Tel.: +86 21 62202875; fax: +86 21 62203612.
*E-mail addresses:* wuaibo2007@yahoo.com.cn, wuaibo@saas.sh.cn (A. Wu).

1570-0232/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2013.02.028

knowledge of the kinetics and metabolic fate of OTA and its metabolites in plasma and different organs. It is therefore not surprising that numerous kinetic and metabolic studies have been performed on OTA in different animals, *i.e.*, rat, monkey, pig and poultry [5–10].

For quantitative analysis of OTA, methods based on whole body autoradiography [11], enzyme-linked immunosorbent assay (ELISA) [4,12] and high performance liquid chromatography with fluorescence detection (HPLC-FLD) [13–16] have been developed. Among these, HPLC-FLD is the most commonly used method for the analysis of OTA in rat plasma, kidney and liver, however, other organs, *i.e.*, spleen, heart, lung, were ignored, which might be due to the relatively low selectivity of HPLC-FLD, along with the complex components in various matrices. Currently, liquid chromatography combined with mass spectrometry is gradually playing an increasingly important role in the area of instrumental analysis, due to its enhanced performance, additional sensitivity and selectivity, and generation of information with a high degree of structural specificity. Several studies employing liquid chromatography coupled with quadrupole (LC–MS) or triple quadrupole mass spectrometry (LC–MS/MS) for the determination of OTA *in vivo* have been reported [10,17,18]. Hitherto, there is not any uniform method, which could be used to analyze OTA in different bio-matrices, *i.e.*, plasma, heart, liver, spleen, lung, kidney and brains, due to the complex matrices causing terrible interferences during analysis. Thus, there is in need of substantial advance on stable bioanalytical method which would be definitely appropriate for quantitative analysis of OTA in a variety of generally recognized biomatrices.

For qualitative analysis of OTA metabolites, radioactivity, thin-layer chromatography (TLC), nuclear magnetic resonance (NMR) and HPLC based analytical methods were also established [19–22]. However, the metabolites purified from the biomatrices or the commercial standards were needed in these previous studies, thus certainly making the procedures tedious and expensive. Recently, liquid chromatography coupled with time of flight mass spectrometry (LC–TOF-MS) has been proposed as a powerful approach for identification of unknown compounds [23,24]. Considering the significance of OTA metabolic pathways revealing the OTA biotransformation in animals, with valuable reference to possible side effects on human beings, LC–TOF-MS would be powerful analytical tool to identify the involved substances and thus elucidate the mechanisms of kinetics and biotransformation. However, up to now, no LC–TOF-MS method has been established for the metabolic study of OTA in any target biological matrices.

For all these reasons, the overall objective of the present work was to first develop a fast and accurate isotope dilution LC–MS/MS method with the capability of determining OTA in plasma, liver, heart, spleen, lung, kidney and brain under the same analytical conditions of sample preparation and running, as well as a subsequent LC–TOF-MS approach capable of identifying the metabolites of OTA without reference standards or tedious purification processes. The developed combinatorial approach was then applied for the study of the kinetics and biotransformation of OTA in plasma and different organs in rats after oral administration, to elucidate the kinetics and metabolic pathway of OTA in rats, which would be significantly helpful for toxicological evaluation of target mycotoxin as a single contaminant source.

## 2. Experimental

### 2.1. Chemicals and reagents

The standards of OTA and [$^{13}C_{20}$]-OTA (IS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile and methanol, purchased from Merck (Darmstadt, Germany), were both HPLC grade. Milli-Q quality water (Millipore, Billerica, MA, USA) was used throughout the analyses. All other reagents were of HPLC or analytical grade.

### 2.2. Apparatus

LC–MS/MS (TSQ Quantum Ultra, Thermo Scientific, USA) was utilized for the analysis of OTA. Separation was performed at 35 °C using a Thermo Hypersil Gold column (100 mm × 2.1 mm, 3.0 μm) with a linear gradient elution using (A) water (containing 0.25 mmol $L^{-1}$ ammonium acetate and 0.05% formic acid) and (B) methanol as the mobile phase. The elution program was as follows: 30% B (initial), 30–100% B (3 min), 100% B (0.8 min), 100–30% B (0.2 min), and hold on for a further 3 min for re-equilibration, giving a total run time of 7 min. The flow rate was 0.35 mL $min^{-1}$ and the injection volume was 5 μL (full loop). MS/MS detection was performed in positive electrospray ionization mode ($ESI^+$). The following settings were used for MS/MS conditions: vaporizer temperature, 300 °C; spray voltage, 4 kV; sheath gas pressure, 30 psi; aux valve flow, 30 arb; capillary temperature, 350 °C. Selected reaction monitoring (SRM) mode with two transitions was developed. The transitions (*m*/*z*) used for quantitation and qualitation were 404.2, 239.1 (collision energy of 22 eV) and 404.2, 221.0 (collision energy of 35 eV) for OTA, and 424.5, 250.1 (collision energy of 25 eV) and 424.5, 232.1 (collision energy of 36 eV) for IS, respectively. Data were acquired and processed by Xcalibur software (Thermo Scientific, USA).

Identification of the metabolites of OTA was performed on an Agilent Technologies 6224 LC–TOF-MS system (Victoria, US) in $ESI^+$. The analytes were separated on an Agilent Poroshell 120 EC-$C_{18}$ column (50 mm × 2.1 mm, 2.7 μm). The same mobile phase as for the LC–MS/MS was selected and the elution program was as follows: 60% B (initial), 60–95% B (8 min), 95% B (2.8 min), 95–60% (0.2 min), and hold on for a further 3 min for re-equilibration, giving a total run time of 14 min. The flow rate was 0.3 mL $min^{-1}$ and the injection volume was 5 μL. The optimized TOF conditions were: gas temperature, 350 °C; drying gas, 10 L $min^{-1}$; nebulizer, 40 psig; V cap, 4 kV; capillary, 0.029 μA; chamber, 2.2 μA; fragmentor, 175 V; skimmer, 65 V. The instrument was operated with the resolution of more than 20,000, and data were collected between *m*/*z* 100 and 1000, with an acquisition rate of 2 spectra/s. Purine (*m*/*z* 121.050873) and HP-0921 (*m*/*z* 922.009798) were selected as the references for the independent reference spray utilized. Calculation of the accurate mass of the analytes was performed by Masshunter Workstation software supplied with the instrument.

### 2.3. Standard solutions

A stock solution of 0.5 mg $mL^{-1}$ of OTA was prepared by dissolving 5.0 mg of OTA in 10 mL of ethanol and could keep stable for one month when it was stored at −20 °C in the dark. The stock solution was diluted with water to prepare the solution used for the oral administration of rats at a concentration of 0.1 mg $mL^{-1}$ in 20% of ethanol aqueous solution, while the standard solutions used for LC–MS/MS and LC–TOF-MS analyses were prepared by appropriate dilution of the stock solution with the mixture of acetonitrile and water containing 10 mmol $L^{-1}$ of ammonium acetate (20/80, v/v). The stock solution of the IS ([$^{13}C_{20}$]-OTA) was directly used as purchased and diluted with the same combined solution to 50 ng $mL^{-1}$. All work solutions were prepared immediately before use.

### 2.4. Animals

One hundred thirty two male Sprague–Dawley (SD) rats, weighing 200 ± 20 g were provided by Fudan University Laboratory Animal Center (Shanghai, China). The study was approved by the Animal Ethics Committee of Shanghai Academy of Agricultural

Sciences (Shanghai, China). The animals were randomly divided into twenty two groups ($n = 6$), and were kept in an environmentally controlled breeding room at a temperature of $25 \pm 2$ °C and a relative humidity of $50 \pm 10$%, and free access to diet and water until 12 h prior to experiments [25].

Twenty groups of male SD rats were selected and used for the kinetics and biotransformation study of OTA in plasma and tissues. Eighteen groups of animals were administered a single dose (0.2 mg/kg b.w.) of OTA by oral gavages with the volume of about 1 mL of 20% ethanol aqueous solution (0.1 mg mL$^{-1}$) depending on the weight of the rat itself, while the other two groups were used as the controls and received 1 mL of 20% ethanol aqueous solution by oral gavages. Serial blood samples (approximately 0.3 mL) of one group and one control were drawn in heparinized polythene tubes from the rats' caudal veins at the times 0, 0.083, 0.167, 0.5, 1.0, 2.0, 5, 7.5, 10, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, 336, 360, 384, 408, 432, 456 h with minor modifications according to the previous protocols [25]. The samples were immediately centrifuged at 4000 g for 5 min and the plasma was removed and then stored at −20 °C until analysis. In order to obtain the liver, heart, spleen, lung, kidney and brain samples, one control group and seventeen groups of rats were sacrificed by decapitation at 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 120, 168, 216, 264, 312, 360, 408 h. The selected tissues were excised, washed by normal saline and blot dried by absorbent paper. 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) by glass homogenizer (10 mL) at about 0 °C, and the homogenates were stored at −20 °C until analysis.

For investigation of tissue accumulation, one group of male SD rats received a daily administration of 0.1 mg/kg b.w. OTA by oral gavages during 20 days. For control samples, animals were administered by oral gavage with normal saline during the same time. The animals were sacrificed for tissues collection 24 h after the last administration, and the tissue homogenates were prepared as described above.

### 2.5. Sample pretreatment

To the plasma/tissue homogenates (100 μL), 10 μL of IS solution (50 ng mL$^{-1}$) methanol for precipitation of proteins. After 1 min vortex shaking, the mixture was centrifuged at 15,000 × *g* for 5 min. The supernatant was transferred into a 1.5 mL centrifuge tube and dried by nitrogen gas at 40 °C. The residue was reconstituted in 100 μL acetonitrile/water (20/80, v/v) containing 10 mmol L$^{-1}$ of ammonium acetate by shaking another 1 min, then passed through the syringe filters (0.22 μm) and ready for injection.

### 2.6. LC–MS/MS method validation

The analytical method was validated according to the following criteria: selectivity, linearity, sensitivity, recovery, matrix effects, precision (within- and between-day variability) and stability [25]. All these were studied individually for each matrix, *i.e.*, plasma, heart, liver, spleen, lung, kidney and brain.

The selectivity of the LC–MS/MS method was evaluated by comparing the blank samples (untreated with OTA), with blank samples spiked with OTA and with samples collected from OTA-treated rats. Calibration curves in solvent and seven different blank matrices (plasma, heart, liver, spleen, lung, kidney and brain) were prepared at eight concentration levels in the range of 0.05–100 ng mL$^{-1}$. The sensitivity was calculated as the lower limit of detection (LLOD) and lower limit of quantitation (LLOQ). LLOD and LLOQ were defined as the concentrations of OTA that yielded a signal-to-noise (S/N) ≥3 and ≥10, respectively, which were both determined by decreasing the spike concentrations in various biomatrices. Extraction recovery was determined by comparing the peak areas ($n = 6$, each concentration) of OTA obtained from plasma/tissue homogenates spiked before extraction with those spiked after extraction without IS added. The present study also compared the slope of the standard addition plot with the slope of the standard calibration plot to evaluate the signal suppression/enhancement (SSE) using the IS or not. In order to determine the accuracy of the method, fortification of plasma, heart, liver, spleen, lung, kidney and brain samples with known concentrations of OTA was performed. Each blank matrix was spiked with 0.1, 1, 10 and 100 ng mL$^{-1}$ of OTA in sextuplicate, and then pretreated as described above with 10 μL of IS solution (50 ng mL$^{-1}$) included. Precision was evaluated by determining the same spiked samples in one day (intra-day precision, $n = 6$, each concentration) and the samples spiked with the same concentrations in four consecutive days (inter-day precision, $n = 6$, each concentration). The short-term, long-term and freeze–thaw stability of OTA was evaluated by analyzing the OTA spiked samples ($n = 6$) just after preparation and stored at room temperature (RT) for 8 h, at −20 °C for two weeks and subjected to three freeze–thaw cycles. The results were expressed as the percentage of calculated concentration *vs.* theoretical concentrations.

### 2.7. Identification of the metabolites via LC–TOF-MS

After detection by LC–TOF-MS, the accurate mass of the analyte was calculated not only by the precursor ions of $[M+H]^+$, but also by $[M+Na]^+$ or by $[M+NH_4]^+$, and the precursor ions of the isotopical pattern were also confirmed with the peak spacing tolerance 0.0025 *m*/*z*, plus 7.0 ppm. The possible elemental compositions were assumed from the obtained accurate mass, and then, based on the previous knowledge, such as type and number of atoms, several impossible formulas were further ruled out. To ensure accuracy and precision, the references of purine (*m*/*z* 121.050873) and HP-0921 (*m*/*z* 922.009798) were sprayed through the analysis time, thus the software could perform ongoing correction of the exact mass of the analytes in the ESI$^+$ conditions.

### 2.8. Statistical data analysis

Statistical analysis was performed with drug and statistics (DAS) software, version 2.0 (Shanghai, China). A significance level ($\alpha$) of 0.05 was used.

## 3. Results and discussion

### 3.1. Optimization of LC–MS/MS conditions

The MS/MS conditions were firstly optimized for OTA and IS by identification of precursor ions performed in both ESI$^+$ and ESI$^-$ modes by direct injection of standard solutions (500 ng mL$^{-1}$). The base peak intensity of the positive ion was almost the same as that of the negative ion in the different ionization modes. The ESI$^+$ mode was selected because aqueous ammonia, which was the most favorable additive in the mobile phase utilized in the ESI$^-$ mode, was unstable and consequently caused stability problems to the analysis. As a result, *m*/*z* 404.2 and 424.5 ($[M+H]^+$) were selected as the precursor ions for OTA and the IS, respectively. Two product ions for each compound were selected. For OTA, 221.0 (*m*/*z*) and 239.1 (*m*/*z*) were selected as the product ions with collision energies of 35 eV and 22 eV, respectively, while 232.1 and 250.1 were the product ions for the IS with collision energies of 36 eV and 25 eV, respectively. Finally, a SRM mode with two transitions was developed for the analysis of OTA. Both transitions (from OTA and labeled OTA) are related as the difference is only a water fragmentation. The transition with the highest signal intensity was preferred for quantitation

and the other one with less intensity plus the ratio of abundances of the two transitions was used for confirmation.

Different mobile phases, *i.e.*, (1) methanol–water, (2) methanol–water containing 0.05% of formic acid, (3) methanol–water containing 10 mmol $L^{-1}$ of ammonium acetate and (4) methanol–water containing 10 mmol $L^{-1}$ of ammonium acetate and 0.05% of formic acid, were compared in the pilot test. Results of multiple injections indicated that the response of OTA was obviously improved and higher sensitivity (signal to noise) was obtained when solvent (4) was selected compared to other candidate solvents (Supplementary data, Fig. S-1). Under such situation, OTA and IS were eluted at 3.36 ± 0.02 min with nice peak shapes and no interferences in both transitions.

### 3.2. Optimization of the sample pretreatment method

The extraction profile (extraction solvent and the ratio of extraction solvent to sample) was evaluated by determination of the extraction recovery. In the present study, three extraction solvents, *i.e.*, methanol, acetonitrile and acetone, with different ratios of extraction solvent to sample, *i.e.*, 1:1, 3:1, 5:1; 7:1 and 9:1, were compared. To 100 μL of plasma samples, the candidate solvents were added, and then pretreated as described in sample pretreatment. As shown in Fig. S-2 (Supplementary data), all of the three solvents performed well (recovery >70%) as long as the ratio of extraction solvent to sample was higher than 1:1 for methanol and acetonitrile, and 3:1 for the acetone, respectively. Even though there was no significant differences between these candidate solvents, 300 μL of methanol was selected, which would constitute a more economic and ecological procedure. Then, the same approach was applied to the extraction of OTA from different tissue homogenates, *i.e.*, heart, liver, spleen, lung, kidney and brain. Satisfactory extraction recoveries were also achieved as indicated in Table 1, supporting the suitability of the developed sample pretreatment method for extraction and purification of OTA in different biomatrices. In addition, since most of small organic molecules can be easily dissolved in methanol, methanol was also suitable for the extraction of OTA metabolites from different organs.

### 3.3. LC–MS/MS method validation

The selectivity was evaluated by comparing the chromatograms of blank matrix, blank matrix spiked with OTA, and the samples collected after oral administration of OTA. As shown in Fig. S-3 (supplementary data), in both transitions no interference peaks at the retention time of OTA appeared, indicating that the method was selective.

The calibration curves using the isotope dilution method were created after the injection (5 μL) of each standard solution, *i.e.*, solvent, plasma, heart, liver, spleen, lung, kidney and brain matrices, respectively. As shown in Table 2, linear relationships and good coefficients of determination ($R^2 \geq 0.9990$) were obtained. The LLOD and LLOQ for OTA in plasma were 0.01 ng $mL^{-1}$ and 0.05 ng $mL^{-1}$, respectively, while in heart, liver, spleen, lung, kidney and brain they were expressed as 0.01 ng $g^{-1}$ and 0.05 ng $g^{-1}$, respectively. The obviously lower concentrations compared to the previous reports indicated that the sensitivity of the method met the requirements of determination of OTA in different biomatrices [13,14,26].

The extraction recoveries were in the range of 72.4–98.3% (Table 1), indicating a satisfactory sample pretreatment. The matrix effects were also evaluated by determination of the SSE. The results showed that negligible matrix effects were observed in plasma, heart, liver, lung, kidney and brain with the SSEs ranging from 96.5% to 117.6%. Compared to other organs, it might be more matrix effects of spleen for LC–MS/MS that caused the signal enhancement of 142.1%. The various extents of SSE impelled the use of a proper IS. In order to find out whether the IS could be used for correction of losses during the ionization process, SSE using [$^{13}C_{20}$]-OTA as the isotope IS was also investigated. The results showed that the SSEs were in an acceptable range of 103.4–118.6%.

**Table 1**
Recovery tests of ochratoxin A (OTA) in different matrices. Data for all response variables were reported as mean ± SD ($n$ = 6).

| Matrix | Spiked level | Accuracy (%) | Extraction recovery (%) |
|---|---|---|---|
| Plasma | LLOQ[a] | 96.4 ± 9.0 | 81.7 ± 3.2 |
| | Low level[b] | 94.3 ± 7.0 | 80.3 ± 4.1 |
| | Intermediate level[c] | 95.6 ± 7.5 | 84.1 ± 3.8 |
| | High level[d] | 95.2 ± 4.3 | 88.0 ± 5.1 |
| Heart | LLOQ | 88.7 ± 12.7 | 73.4 ± 6.2 |
| | Low level | 84.1 ± 10.0 | 78.9 ± 6.5 |
| | Intermediate level | 83.7 ± 8.6 | 80.3 ± 5.9 |
| | High level | 84.6 ± 10.8 | 77.5 ± 4.3 |
| Liver | LLOQ | 89.3 ± 6.3 | 88.5 ± 6.7 |
| | Low level | 92.5 ± 7.9 | 83.7 ± 7.1 |
| | Intermediate level | 90.8 ± 6.8 | 96.8 ± 6.5 |
| | High level | 89.8 ± 3.5 | 92.2 ± 5.2 |
| Spleen | LLOQ | 90.3 ± 12.1 | 80.5 ± 5.3 |
| | Low level | 96.8 ± 8.7 | 83.0 ± 7.6 |
| | Intermediate level | 91.3 ± 6.1 | 82.4 ± 2.1 |
| | High level | 85.8 ± 5.9 | 82.0 ± 3.4 |
| Lung | LLOQ | 92.0 ± 10.7 | 85.0 ± 4.6 |
| | Low level | 108.3 ± 5.2 | 90.3 ± 7.5 |
| | Intermediate level | 92.3 ± 5.2 | 91.9 ± 7.7 |
| | High level | 84.2 ± 3.8 | 94.1 ± 6.9 |
| Kidney | LLOQ | 101.8 ± 7.5 | 86.7 ± 7.2 |
| | Low level | 83.3 ± 9.8 | 80.4 ± 7.4 |
| | Intermediate level | 103.8 ± 9.9 | 98.3 ± 6.5 |
| | High level | 98.2 ± 6.0 | 98.0 ± 5.9 |
| Brain | LLOQ | 103.8 ± 8.2 | 83.6 ± 7.6 |
| | Low level | 103.9 ± 7.1 | 85.9 ± 3.2 |
| | Intermediate level | 98.6 ± 6.3 | 86.7 ± 3.4 |
| | High level | 89.9 ± 8.8 | 72.4 ± 7.1 |

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

The method accuracy at different concentrations is presented in Table 1 (range of 83.3–108.3%). Values for the intra- and inter-day precision determined at 0.05, 1, 10 and 100 ng $mL^{-1}$ are shown in Table 3. The RSDs were not above 15.6% in all cases. All these values indicated that the established method was accurate and reproducible for the determination of OTA in plasma, heart, liver, spleen, lung, kidney and brain.

The short-term stability (at RT for 8 h), freeze–thaw stability (three circles) and long-term stability (at −20 °C for 2 weeks) were thoroughly investigated and results are shown in Table 4. The obtained results clearly demonstrated that all the samples were stable under these conditions, indicating that there were no stability-related problems during the routine and large-scale analysis of samples.

### 3.4. Kinetics and distribution study in rat plasma and tissues

A plot of the concentration of OTA in plasma *vs.* time after oral administration of 0.2 mg/kg b.w. is shown in Fig. 1. The calculated kinetic parameters are expressed as mean ± SD (Table 5). After oral administration, OTA reached a maximum value of 1932.4 ± 124.9 ng $mL^{-1}$ within 5 h due to its rapid absorption, which has good agreement with previous report [27]. Then, the concentration of OTA was kept at relatively high level in plasma with a half-life time ($t_{1/2}$) of 75.6 ± 29.0 h. The slow elimination

**Table 2**
Eight calibration curves of ochratoxin A (OTA) in solvent and seven different matrices.

| Matrix | Slope | Intercept | $R^2$ | Range (ng mL$^{-1}$) | Sensitivity (ng mL$^{-1}$/ng g$^{-1}$ [a]) | |
|---|---|---|---|---|---|---|
| | | | | | LLOD | LLOQ |
| Solvent | 0.059 | 0.025 | 0.9995 | 0.05–500 | 0.01 | 0.05 |
| Plasma | 0.061 | 0.058 | 0.9991 | 0.05–500 | 0.01 | 0.05 |
| Heart | 0.063 | 0.185 | 0.9990 | 0.05–500 | 0.01 | 0.05 |
| Liver | 0.067 | 0.157 | 0.9991 | 0.05–500 | 0.01 | 0.05 |
| Spleen | 0.068 | 0.142 | 0.9992 | 0.05–500 | 0.01 | 0.05 |
| Lung | 0.068 | 0.111 | 0.9996 | 0.05–500 | 0.01 | 0.05 |
| Kidney | 0.065 | 0.156 | 0.9992 | 0.05–500 | 0.01 | 0.05 |
| Brain | 0.070 | 0.143 | 0.9993 | 0.05–500 | 0.01 | 0.05 |

[a] ng mL$^{-1}$ and ng g$^{-1}$ refer to the values of LLOD and LLOQ of OTA in plasma and organs, respectively.

of OTA might be due to its high binding affinity to serum proteins such as albumin, as well as enterohepatic recirculation of the toxin, substantially contributing to the development of its chronic effects [28]. In previous studies, the plasma half-life times were 10 days, 3 days and 103 h in the Fischer, Dark Agouti and female SD rats, respectively [8,27]. Different animal species also showed different half-life times, *i.e.*, $t_{1/2}$ was 0.68 h for carp, 6.7 h for quail, 72 h for pig and 510 h for monkey macaca. These markedly different plasma retention times of OTA might be due to some fundamental differences between these species concerning serum proteins since most of the circulating OTA is well known to bind to serum albumins [7]. Interestingly, the half-life time of experimentally orally ingested OTA was shorter than that of intravenously (i.v.) injected OTA as reported in a previous study [29]. Indeed, exposure of the toxin after oral administration was lower and systemic concentrations fell below the LLOQ faster than that after IV administration.

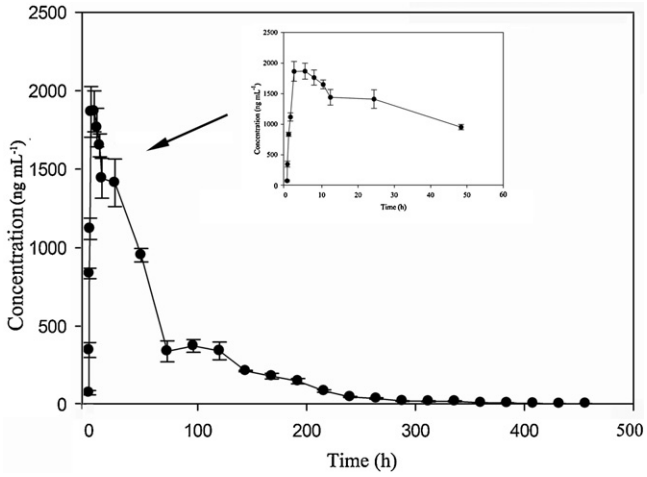


**Fig. 1.** Mean plasma concentration–time curve of ochratoxin A (OTA) following the oral administration of OTA (0.20 mg/kg b.w.) in rat. Data for all response variables were reported as mean ± SD ($n$ = 6).

**Table 3**
Intra- and inter-day precision tests of ochratoxin A (OTA) in different matrices ($n$ = 6).

| Matrix | Spiked level | Intra-day (RSD$_r$ %) | Inter-day (RSD$_R$ %) |
|---|---|---|---|
| Plasma | LLOQ[a] | 8.7 | 6.7 |
| | Low level[b] | 6.6 | 5.4 |
| | Intermediate level[c] | 7.2 | 6.2 |
| | High level[d] | 4.1 | 8.9 |
| Heart | LLOQ | 11.3 | 10.9 |
| | Low level | 8.4 | 3.8 |
| | Intermediate level | 7.2 | 6.7 |
| | High level | 9.1 | 5.2 |
| Liver | LLOQ | 5.6 | 11.6 |
| | Low level | 7.3 | 10.8 |
| | Intermediate level | 6.2 | 9.1 |
| | High level | 3.1 | 4.3 |
| Spleen | LLOQ | 10.9 | 13.6 |
| | Low level | 8.4 | 11.2 |
| | Intermediate level | 5.6 | 8.4 |
| | High level | 5.1 | 10.6 |
| Lung | LLOQ | 9.8 | 15.6 |
| | Low level | 5.6 | 5.9 |
| | Intermediate level | 4.8 | 8.9 |
| | High level | 3.2 | 7.1 |
| Kidney | LLOQ | 7.6 | 8.2 |
| | Low level | 8.2 | 7.8 |
| | Intermediate level | 10.3 | 6.4 |
| | High level | 5.9 | 3.9 |
| Brain | LLOQ | 8.5 | 7.8 |
| | Low level | 7.4 | 4.7 |
| | Intermediate level | 6.2 | 6.1 |
| | High level | 7.9 | 8.2 |

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

After oral administration of OTA, a rapid distribution in different rat tissues was observed (Fig. 2). With the tested OTA ingestion level (0.2 mg/kg b.w.), the concentrations in heart, liver, spleen, lung, kidney and brain were positively related to the plasma concentrations with delaying times due to the transfer of the toxin from the plasma to the target organs. For example, the highest concentrations of OTA were reached in all of the target tissues nearly at 4 h after oral administration. In general, kidney received most attention because

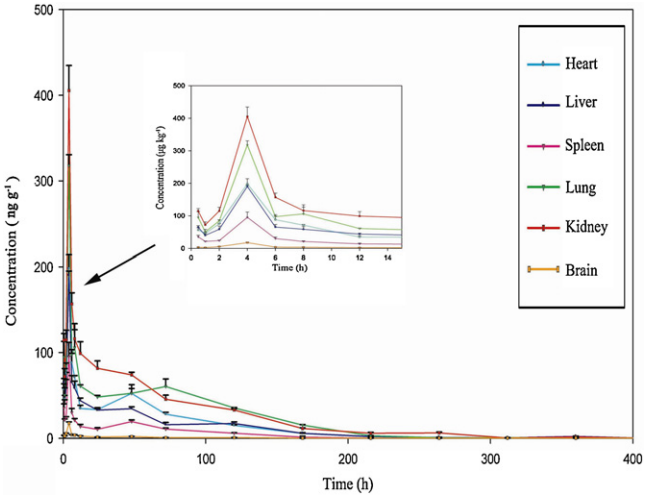


**Fig. 2.** The concentration–time profile of ochratoxin A (OTA) in the different rat tissues after oral administration of OTA (0.20 mg/kg b.w.). Data for all response variables were reported as mean ± SD ($n$ = 6).

**Table 4**
Stability of ochratoxin A (OTA) in different biomatrices. Data for all response variables were reported as mean ± SD ($n$ = 6).

| Matrix | Concentrations ($ng\,mL^{-1}$/$ng\,g^{-1}$[a]) | Recovery (mean ± S.D. %) | | |
|---|---|---|---|---|
| | | Short-term stability (at RT for 8 h) | Freeze–thaw stability (three circles) | Long-term stability (at −20 °C for 20 days) |
| Plasma | 1 | 88.4 ± 3.1 | 82.5 ± 5.6 | 90.2 ± 9.1 |
| | 100 | 76.2 ± 4.1 | 80.6 ± 2.8 | 88.4 ± 5.3 |
| Heart | 1 | 80.2 ± 2.9 | 82.7 ± 6.9 | 86.4 ± 8.9 |
| | 100 | 82.2 ± 3.1 | 91.6 ± 5.7 | 89.2 ± 3.3 |
| Liver | 1 | 82.0 ± 5.4 | 84.1 ± 10.2 | 78.4 ± 5.6 |
| | 100 | 85.6 ± 2.9 | 92.7 ± 8.1 | 81.3 ± 6.5 |
| Spleen | 1 | 82.7 ± 4.1 | 83.3 ± 6.1 | 85.9 ± 7.2 |
| | 100 | 80.9 ± 3.1 | 90.2 ± 5.9 | 88.4 ± 10.9 |
| Lung | 1 | 79.1 ± 3.8 | 91.5 ± 8.1 | 79.6 ± 5.8 |
| | 100 | 84.1 ± 5.3 | 92.2 ± 10.2 | 81.2 ± 9.5 |
| Kidney | 1 | 77.2 ± 6.4 | 89.2 ± 6.2 | 83.4 ± 5.2 |
| | 100 | 78.4 ± 6.5 | 86.3 ± 8.1 | 81.6 ± 6.9 |
| Brain | 1 | 75.0 ± 8.7 | 83.6 ± 5.4 | 80.2 ± 3.4 |
| | 100 | 82.6 ± 3.5 | 84.7 ± 6.9 | 88.5 ± 4.6 |

[a] $ng\,mL^{-1}$ and $ng\,g^{-1}$ refer to the concentrations of OTA in plasma and tissues, respectively.

of OTAs nephrotoxicity. As shown in Fig. 2, the highest concentrations of OTA among different tissues were observed in kidney, which was similar to the results of the tissue distribution of OTA in pig, chicken and goat [7]. This might be due to its re-absorption in all kidney segments, which may lead to the increase of its toxicity, *e.g.*, the disturbance of pH homeostasis in the renal papilla [26]. Interestingly, OTA was also detected in brain homogenate, demonstrating that OTA could efficiently cross the blood–brain barrier. Concentrations of OTA in different tissues were declined as the concentrations in plasma and were below the limit of detection at the last four sampling points (360 h). On the other hand, the slow elimination of OTA either from plasma or different tissues confirms previous observations on the kinetics of OTA transformation by cytochromes P450 and peroxidases *in vitro*, revealing that OTA biotransformation by these enzymes was very inefficient [30,31].

Tissue accumulation was also studied after a daily oral administration of 0.1 mg/kg b.w. OTA during 20 days (Fig. S-4, supplementary data). The highest concentration of OTA was observed in lung ($95.9 \pm 13.7\,ng\,g^{-1}$), followed by liver ($76.0 \pm 9.7\,ng\,g^{-1}$), heart ($62.0 \pm 4.2\,ng\,g^{-1}$) and kidney ($55.7 \pm 4.7\,ng\,g^{-1}$), which might be related to its toxic effects, *i.e.*, renal carcinogenic and hepatotoxic effects, as well as urinary tract tumors. The results of the lower accumulation of OTA in kidney compared to that in lung, liver and heart, where the similar results were also reported in the previous studies [32,33]. Apparently inconsistent to the case that kidney was commonly recognized as the targeted toxic organ of OTA. Indeed, Toxicity is not only driven by tissue distribution and pharmacokinetics, but also by organ-specific toxicodynamic effects. Furthermore, since the vascular part of the tissue is quite extensive, organs also contain blood and the concentration of mycotoxin is often related to the amount of blood present in the tissues [34]. The results shown in tissues might be related to the amount of vascular tissue in the organs. The higher concentrations of OTA in lung, heart and liver might be due to the relatively high distributions of the vessels, and thus could not infer that these organs are also the specific targets of OTA.

**Table 5**
Kinetic parameters of ochratoxin A (OTA) (0.20 mg/kg b.w.) after oral administration in rat. Data for all response variables were reported as mean ± SD ($n$ = 6).

| Kinetic parameters | Unit | OTA |
|---|---|---|
| AUC(0–$t$) | $\mu g\,h\,L^{-1}$ | 121,257.2 ± 8383.8 |
| AUC(0–∞) | $\mu g\,h\,L^{-1}$ | 121,739.8 ± 8497.9 |
| AUMC(0–$t$) | | 8,223,011.9 ± 595,385.0 |
| AUMC(0–∞) | | 8,504,394.4 ± 751,799.8 |
| MRT(0–$t$) | h | 67.8 ± 0.3 |
| MRT(0–∞) | h | 69.8 ± 1.6 |
| VRT(0–t) | $h^2$ | 4961.3 ± 131.1 |
| VRT(0–∞) | $h^2$ | 6034.2 ± 703.7 |
| $t_{1/2}$ | h | 75.6 ± 29.0 |
| $T_{max}$ | h | 4.8 ± 2.8 |
| CLz/F | $L\,h^{-1}\,kg^{-1}$ | 0.002 ± 0.0 |
| Vz/F | $L\,kg^{-1}$ | 0.178 ± 0.059 |
| $C_{max}$ | $\mu g\,L^{-1}$ | 1932.4 ± 124.9 |

### 3.5. Biotransformation study of OTA

The experimental mass of the parent (OTA) ions given by the LC–TOF-MS was $m/z$ = 404.0880 ($[M+H]^+$) and $m/z$ = 426.0705 ($[M+Na]^+$), which was less than a 5 ppm difference compared to the theoretical mass, 404.089 ($[M+H]^+$) and 426.0715 ($[M+Na]^+$), and the molecular formula proposed, $C_{20}H_{18}ClNO_6$, was the same as the theoretical molecular formula (Fig. 3a). OTA was identified in all biomatrices of heart, liver, spleen, lung, kidney and brain with the retention time of 6.26 min. According to the same principle, phenylalanine was identified in heart and kidney with the retention time of 0.93 min (Fig. 3c). Although phenylalanine might also be present in rat even without administration of OTA, no phenylalanine was detected by the established LC–TOF-MS in blank matrices of heart and kidney. Therefore, OTA could be transformed to phenylalanine in heart and kidney. In kidney, ochratoxin β (OTβ) with precursor ion of $m/z$ = 223.0634 and retention time of 7.25 min was also detected (Fig. 3b). In addition, after direct injection of the standard solution of OTA, no OTβ and phenylalanine observed, indicating that phenylalanine and OTβ were not the pre-existing impurities. Based on the identification of OTβ and phenylalanine, a possible metabolic pathway of OTA in kidney was proposed (Fig. 4). Firstly, OTA might be dechlorinated *in vivo* to transform to ochratoxin B (OTB), as reported in previous studies though not found in the present study [22,35]. Then, a hydrolysis reaction could occur and OTB was conversed to OTβ and phenylalanine. The site of the hydrolysis was tentatively supposed to be N-C9. This pathway was in great accordance to the literature, which has been reported that OTB can be transformed into OTβ by the liver microsomes of rats

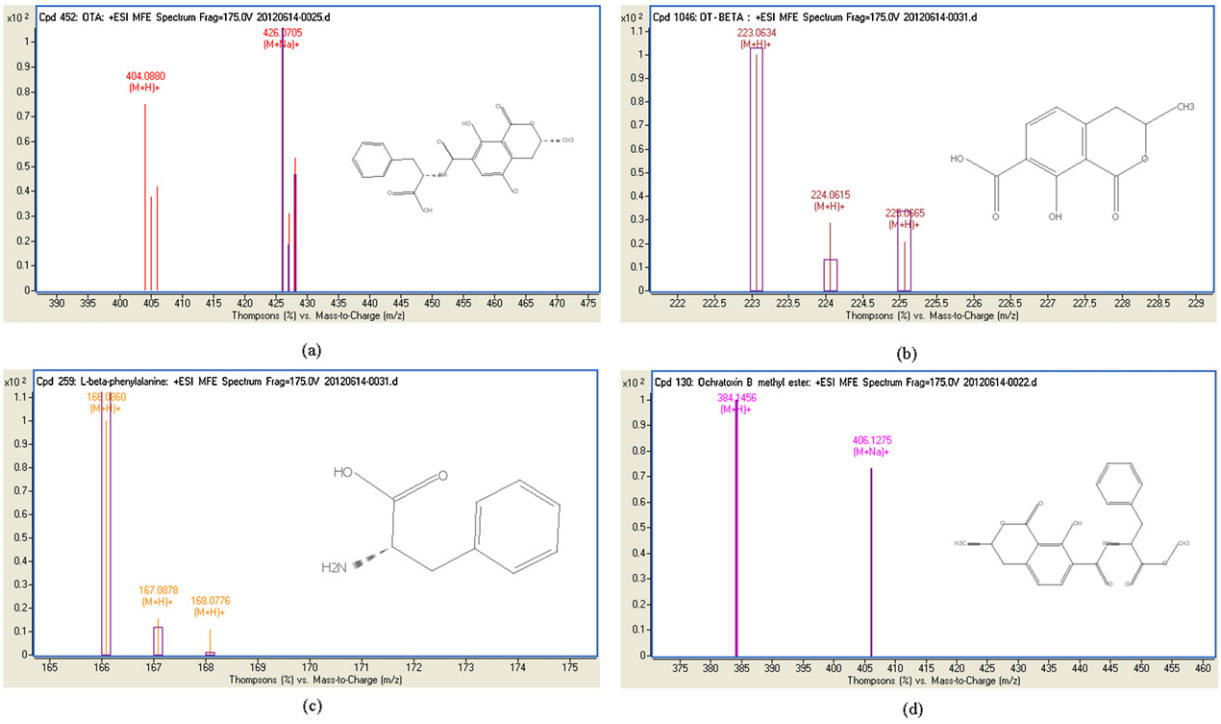


**Fig. 3.** Identification of ochratoxin A (OTA) and its metabolites by liquid chromatography coupled with time of flight mass spectrometry (LC–TOF-MS). (a) = OTA, (b) = ochratoxin β (OTβ), (c) = phenylalanine, and (d) = ochratoxin B (OTB) methyl ester.

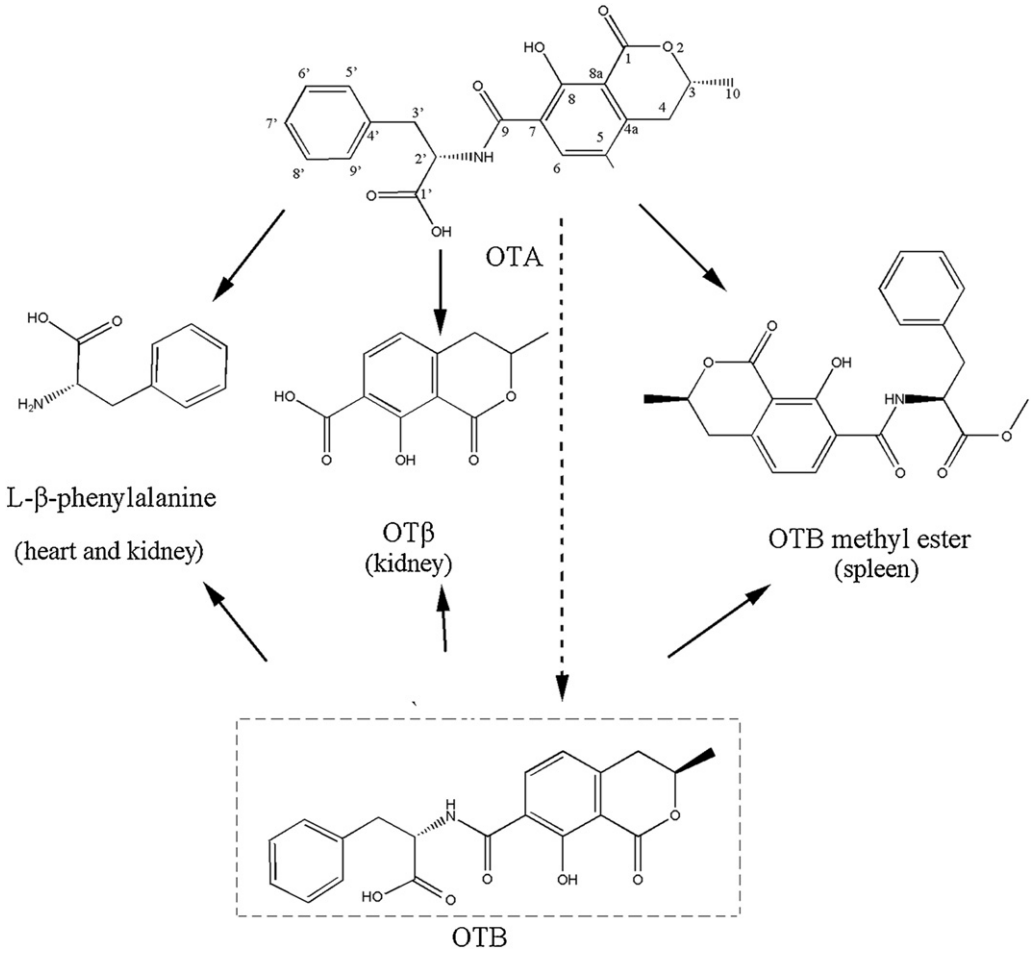


**Fig. 4.** The proposed metabolic pathway of ochratoxin A (OTA) in rat.

in the presence of NADPH *in vitro*, and OTβ was also the major metabolite excreted in the urine when male F344 rats were administered with OTB [22]. On the other side, OTA also might be directly metabolized to OTβ and phenylalanine in kidney. OTα, the direct hydroxyl metabolite of OTA, was not detected in any biomatrices, which was also in agreement with the findings from previous metabolism studies in rat, in which OTα was excreted mainly in feces and was detected as the only metabolite recovered from the caecum and large intestine of rat [21,36,37]. Another metabolization product named OTB methyl ester, which was reported in plants, was found in spleen with *m*/*z* 384.1456 ($[M+H]^+$) and 406.1275 ($[M+Na]^+$) and the retention time of 9.8 min. Compared to the molecular of OTA, OTβ, phenylalanine and OTB methyl ester can lead to a significant reduction in toxicity [36].

## 4. Conclusions

A combinatorial approach of LC–MS/MS and LC–TOF-MS were developed for quantitative and qualitative analysis of OTA and its metabolites in plasma, heart, liver, spleen, lung, kidney and brain in rat. Full validation of LC–MS/MS indicated that the well-established method with a total running of 7 min for each sample showed higher sensitivity and faster sample preparation, as well as more accuracy aided by isotope IS than the previously reported means. The firstly developed LC–TOF-MS method for identification of the metabolites of OTA in different biomatrices showed obvious advantages in terms of easy automatization and unambiguous analyte identification without any further time-consuming and error-prone confirmation steps with a total run time of 14 min. The combinatorial platform has been successfully applied to uncover the *in vivo* kinetics and biotransformation of OTA in rat.

## Acknowledgments

The authors highly appreciate the financial support for this work from the National Basic Research Program of China (2012CB720804, 2009CB118806), Shanghai Agriculture Commission Project (2011NO. 4-3), Shanghai Technical Standards Project (12DZ0502801), Shanghai Science Foundation for Youths (12ZR1448900) and the Chinese-Belgian Joint Project of BELSPO, Belgium (BL/02/C58) and MOST, China (2012DFG31840).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.02.028.

## References

[1] R. Vatinno, A. Aresta, C.G. Zambonin, F. Palmisano, J. Chromatogr. A 1187 (2008) 145.
[2] F. Karbancioglu-Guler, D. Heperkan, Anal. Chim. Acta 617 (2008) 32.
[3] M.W. Trucksess, P.M. Scott, Food Addit. Contam. 25 (2008) 181.
[4] X.H. Wang, T. Liu, N. Xu, Y. Zhang, S. Wang, Anal. Bioanal. Chem. 389 (2007) 903.
[5] H. Zepnik, W. Volkel, W. Dekant, Toxicol. Appl. Pharmacol. 192 (2003) 36.
[6] M.A. Stander, T.W. Nieuwoudt, P.S. Steyn, G.S. Shephard, E.E. Creppy, V. Sewram, Arch. Toxicol. 75 (2001) 262.
[7] D. Ringot, A. Chango, Y.J. Schneider, Y. Larondelle, Chem. Biol. Interact. 159 (2006) 18.
[8] S. Li, R.R. Marquardt, A.A. Frohlich, T.G. Vitti, G. Crow, Toxicol. Appl. Pharmacol. 145 (1997) 82.
[9] D.R. Dietrich, A.H. Heussner, E. O'Brien, Food Addit. Contam. 22 (1) (2005) 45.
[10] D. Milicevic, V. Juric, S. Stefanovic, T. Baltic, S. Jankovic, Arch. Environ. Contam. Toxicol. 58 (2010) 1074.
[11] R. Fuchs, L.E. Appelgren, S. Hagelberg, K. Hult, Poult. Sci. 67 (1988) 707.
[12] V. Lates, C. Yang, I.C. Popescu, J.L. Marty, Anal. Bioanal. Chem. 402 (2012) 2861.
[13] A. Vettorazzi, E. Gonzalez-Penas, L. Arbillaga, L.A. Corcuera, A. Lopez de Cerain, J. Chromatogr. A 1215 (2008) 100.
[14] L.A. Corcuera, M. Ibanez-Vea, A. Vettorazzi, E. Gonzalez-Penas, A.L. Cerain, J. Chromatogr. B 879 (2011) 2733.
[15] E.M. Guillamont, C.M. Lino, M.L. Baeta, A.S. Pena, M.I. Silveira, J.M. Vinuesa, Anal. Bioanal. Chem. 383 (2005) 570.
[16] L. Monaci, G. Tantillo, F. Palmisano, Anal. Bioanal. Chem. 378 (2004) 1777.
[17] R. Vatinno, D. Vuckovic, C.G. Zambonin, J. Pawliszyn, J. Chromatogr. A 1201 (2008) 215.
[18] M. Solfrizzo, L. Gambacorta, V.M. Lattanzio, S. Powers, A. Visconti, Anal. Bioanal. Chem. 401 (2011) 2831.
[19] M. Sieber, S. Wagner, E. Rached, A. Amberg, A. Mally, W. Dekant, Chem. Res. Toxicol. 22 (2009) 1221.
[20] F.C. Stormer, C.E. Hansen, J.I. Pedersen, G. Hvistendahl, A.J. Aasen, Appl. Environ. Microbiol. 42 (1981) 1051.
[21] O. Storen, H. Holm, F.C. Stormer, Appl. Environ. Microbiol. 44 (1982) 785.
[22] Q. Wu, V. Dohnal, L. Huang, K. Kuca, X. Wang, G. Chen, Z. Yuan, Curr. Drug Metab. 12 (2011) 1.
[23] R. Liu, Q. Jin, G. Tao, L. Shan, J. Huang, Y. Liu, X. Wang, W. Mao, S. Wang, J. Mass Spectrom. 45 (2010) 553.
[24] Q. Wu, L. Huang, Z. Liu, M. Yao, Y. Wang, M. Dai, Z. Yuan, Xenobiotica 41 (2011) 863.
[25] Y. Wu, Y. Zheng, N. Chen, L. Luan, X. Liu, J. Chromatogr. B 879 (2011) 443.
[26] A. Vettorazzi, I.F. de Troconiz, E. Gonzalez-Penas, L. Arbillaga, L.A. Corcuera, A.G. Gil, A.L. de Cerain, Food Chem. Toxicol. 49 (2011) 1935.
[27] P.G. Mantle, Food Chem. Toxicol. 46 (2008) 1808.
[28] S. Hagelberg, K. Hult, R. Fuchs, J. Appl. Toxicol. 9 (1989) 91.
[29] E. Petzinger, K. Ziegler, J. Vet. Pharmacol. Ther. 23 (2000) 91.
[30] J. Gautier, J. Richoz, D.H. Welti, J. Markovic, E. Gremaud, F.P. Guengerich, R.J. Turesky, Chem. Res. Toxicol. 14 (2001) 34.
[31] H. Zepnik, A. Pahler, U. Schauer, W. Dekant, Toxicol. Sci. 59 (2001) 59.
[32] A. Mally, W. Volkel, A. Amberg, M. Kurz, P. Wanek, E. Eder, G. Hard, W. Dekant, Chem. Res. Toxicol. 18 (2005) 1242.
[33] E. Rached, G.C. Hard, K. Blumbach, K. Weber, R. Draheim, W.K. Lutz, S. Ozden, U. Steger, W. Dekant, A. Mally, Toxicol. Sci. 97 (2007) 288.
[34] A. Garcia-Sanz, A. Rodriguez-Barbero, M.D. Bentley, E.L. Ritman, J.C. Romero, Hypertension 31 (1998) 440.
[35] V. Faucet-Marquis, F. Pont, F.C. Stormer, T. Rizk, M. Castegnaro, A. Pfohl-Leszkowicz, Mol. Nutr. Food Res. 50 (2006) 530.
[36] P. Galtier, Int. Agency Res. Cancer 1 (1991) 187.
[37] M.S. Madhyastha, R.R. Marquardt, A.A. Frohlich, Arch. Environ. Contam. Toxicol. 23 (1992) 468.