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Development and validation of an LC–MS/MS method for the simultaneous determination of citrinin and ochratoxin a in a variety of feed and foodstuffs

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ABSTRACT

An ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI$^{+/-}$-MS/MS) method for the simultaneous analysis of citrinin (CIT) and ochratoxin A (OTA) in feed (chicken and pig) and food (cereal-based products, fruit, vegetable juices, nuts, seeds, herbs, spices, vegetarian and soy products, alcoholic beverages, baby food products and food supplements) was developed. The mycotoxins were extracted from these matrices using a QuEChERS-based extraction method without any further clean-up step. The samples were 5-fold concentrated. Final extracts were analyzed using a UPLC–MS/MS system and chromatographic separation was achieved by applying a gradient elution for a total run time of 10 min. Mycotoxins were quantified using an internal calibration via analyte/^{13}C-labeled internal standard ratio. The developed method was validated according to the criteria described in Commission Regulation No. 401/2006/EC and Commission Decision No. 2002/657/EC. Specificity, linearity, apparent recovery, limit of detection and quantification, intraday and interday precision, measurement uncertainty, matrix effect, and extraction efficiency were the parameters studied. Finally, 90 Belgian chicken and pig feed samples were analyzed, revealing the simultaneous presence of CIT (<LOQ – 3.90 μg/kg) and OTA (<LOQ – 5.60 μg/kg) in more than 50% of these products.

1. Introduction

Mycotoxins are secondary fungal metabolites and can contaminate various agricultural commodities, either before harvest or under post-harvest conditions [1]. They are known to cause serious toxic effects in humans and animals [2]. Mycotoxin exposure studies pointed out that exposure can reach worrying levels [3].

Mycotoxin analysis revealed that citrinin (CIT) and its metabolite dihydrocitrinone (HO—CIT) are frequently detected in human urine samples [4–8]. In Belgium, 59% of samples collected from adults (n = 239) contained CIT or HO—CIT in a range between 1.6 and 1494.3 pg/mL and 72% of samples collected from children (n = 155) contained these toxins in a concentration ranging from 2.0 to 415.7 pg/mL. The CIT presence in urine is worrying because of its toxicity [9], however, nowadays, the lack of research in CIT toxicity does not permit to predict the toxic effects of CIT from the CIT levels found in urine. CIT, which is produced by fungi of the genera *Aspergillus*, *Monascus* and *Penicillium* [10,11], affects the kidney function and results in necrosis of the distal tubule epithelium, and hence alters the function and degenerates several physiological processes of the renal tubules. Although the high exposure to CIT, limited data concerning its toxicity are available in literature, mycotoxin research has been more focused on other mycotoxins with a higher toxicity such as aflatoxins, or with a larger presence in food as deoxynivalenol. It is known that the consumption of cereals and cereal-based products is considered as the major contribution of dietary exposure to CIT [12]. Other common contaminated matrices are fruits, fruit and vegetable juices, medicinal and aromatic herbs and moldy cheeses [9].

CIT acts synergistically with ochratoxin A (OTA), also a nephrotoxic mycotoxin [13]. Both mycotoxins may be simultaneously produced by *Penicillium* and *Aspergillus* species and can co-occur

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in a wide variety of food and feedstuffs, mainly in cereals and cereal-based products [14,15]. OTA has been classified in group 2 B as a possible human carcinogen by IARC, whereas CIT is classified in group 3 as not classifiable as to its carcinogenicity to humans, because of limited evidence in animals [16]. Moreover, OTA analysis in urine revealed the concerning exposure to this toxin. For instance, Vidal et al. [17] found that only 9% of the Catalonian volunteers had levels below the tolerable daily intake (TDI) (17 ng/kg b.w./day) and an estimated exposure mean value of 139 ng of OTA/kg bw/day was predicted for the Italian population [18].

Until now, no legal maximum limits concerning CIT in food and feed are established, except for food supplements based on red yeast rice (RYR) powder, which have a defined legal maximum limit of 2 mg/kg [19]. For OTA, maximum levels for food and feed are set in Commission Regulation 1881/2006/EC [20] and Commission Recommendation 2006/576/EC [21] respectively.

Detection of mycotoxins can be achieved by various analytical methods as for instance, gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC) in combination with fluorescence and/or ultraviolet (UV) detection, and enzyme-linked immunosorbent assay (ELISA). However, the last 10 years, liquid chromatography-tandem mass spectrometry (LC–MS/MS) has become the universal approach for mycotoxin analysis, and it has been widely applied in various matrices both liquid and solid. On the other hand, the extraction procedure defined as "quick, easy, cheap, effective, rugged and safe" (QuEChERS) has been successfully tested for mycotoxins analysis [22]. Its simplicity, limited organic solvent usage and effectiveness for cleaning-up of complex samples has led to the wide use of it. The QuEChERS approach has been reported for the extraction of different mycotoxins in a variety of food matrices such as cereal-based products [23], beer [24] and spices [25], showing the large potential of this extraction technique.

Because CIT and OTA, both nephrotoxic contaminants, were commonly detected together in food [12,26] and were detected in urine samples from adults and children [7], it is important to investigate possible sources of intake of this mycotoxin. Therefore, although LC–MS/MS methods have already been developed to detect CIT and OTA separately [25,27,28], the aim of this study was to develop and validate an easy and reliable method such as QuEChERS for the simultaneous analysis of CIT and OTA by LC–MS/MS in feed and a large range of complex foodstuffs.

2. Materials and methods

2.1. Chemicals and reagents

CIT (Fig. 1) was purchased from Fermentek (Jerusalem, Israel) and OTA (Fig. 1) was obtained from Sigma-Aldrich (Bornem, Belgium). Stock solutions of 1 mg/mL were prepared in methanol. Liquid standards of ^{13}C-labelled OTA ($^{13}C_{20}$—OTA) and ^{13}C-labelled CIT ($^{13}C_{13}$—CIT) as internal standards (IS) in acetonitrile (ACN) were purchased from Biopure-Romer Labs (Tulln, Austria), with a concentration of 10.07 μg/mL and 10.6 μg/mL respectively. All stock solutions were stored at −20 °C. From the individual stock solutions, a CIT/OTA standard mixture of 10 μg/mL each and an $^{13}C_{13}$—CIT/$^{13}C_{20}$—OTA solution of 100 ng/mL each were prepared in a mixture of methanol, water and acetic acid (80:18:2, v:v:v). A Milli-Q SP Reagent water system (Merck Millipore, Darmstadt, Germany) was used to obtain ultrapure water. Methanol (MeOH, LC–MS grade, 99.95%) was procured from BioSolve BV (Valkenswaard, The Netherlands). Hydrochloric acid (HCl, 37%), ethyl acetate (EtAc, 99%) and ACN (HiPerSolv Chromanorm HPLC grade, 99.9%) were acquired from VWR International (Leuven, Belgium). Sodium chloride (NaCl, 99.9%), ammonium acetate (98%) and acetic acid glacial (HAc, 100%) were provided by Merck, whereas anhydrous magnesium sulfate (99.5%) was procured from Sigma-Aldrich.

2.2. Food and feed samples for validation study and commercially available Belgian feed samples

The following food groups were selected, based on occurrence data of CIT and OTA in literature [9,29–32]: cereal-based products, fruit and vegetable juices, nuts and seeds, herbs and spices, vegetarian and soy products, alcoholic beverages, baby food products and food supplements. Selected samples for method development and validation were wheat flour, apple juice, walnut, beer, sunflower seed, nutmeg (dried powdered spice), tofu, RYR food supplements and baby milk powder. For each commodity, only one package was bought. Expiration date, batch number and ingredients were saved in a log book. Additionally, to check the applicability of the developed method and to study the prevalence of CIT and OTA contamination, selected food products belonging to the mentioned groups (white bread, cornflakes, oatmeal, tortilla, white pasta, orange juice, tomato juice, curry powder, wine, cashew nuts, pistachio nuts, pumpkin seeds, chickpeas (canned), spiced tofu slices, and a valerian food supplement) were analyzed using the developed method. All food samples were bought in local supermarkets in Belgium.

For validation in feed, pig feed (pellets, 1 kg) was obtained from a Belgian feed producing company. Samples were homogenized, grinded (M20-grinder, Ika Werke, Staufen, Germany) and stored in aliquots of 4 g until analysis at −20 °C. The method was also applied on a chicken feed sample (maize).

The validated method for feed was used to analyze 90 commercially available feedstuffs (chicken (n = 38) and pig feed (n = 52)). The samples were collected from March 2017 until June 2017, from 6 different feed producing companies in Belgium. All pig feedstuffs were produced in pellet form, whereas the chicken feedstuffs consisted of a mixture of raw grains. Major components of the feed products were wheat and maize. Other important ingredients were beet pulp, barley, soybean meal, rapeseed meal and several oils (linseed, sunflower, palm kernel and soybean).

2.3. Sample preparation and extraction procedures

2.3.1. Apple juice and beer

A 4.00 ± 0.02 g homogenized sample was weighed. Recovery and calibration samples were prepared by fortifying (spiking) blank samples (as confirmed by a prior analysis) in a concentration of 10 μg/kg (recovery) and 0.25–100 μg/kg (calibration range) of CIT and OTA. The samples were left for a 30 min equilibration, then 10 mL of an acidified saline solution were added, consisting of 10% (m/v) NaCl in acidified water (1.6% HCl in H_2O:HAc, 99:1, v:v), followed by 20 mL of extraction mixture consisting of EtAC, ACN and HAc (75:24:1, v:v:v). Samples were shaken for 1 h at room temperature, as it was established by Kiebooms et al. (2016) [28], using an overhead shaker (Agilitec, J. Toulemonde and Cie, Paris, France). Subsequently, 6.0 ± 0.2 g of magnesium sulfate and 1.5 ± 0.1 g of NaCl were added. Samples were first agitated by hand for 30 s to avoid aggregation of the salts, followed by shaking using the overhead shaker for 3 min. Next, samples were centrifuged for 5 min at 4000 x *g*. An aliquot of 1 mL supernatant was transferred and evaporated to dryness under a gentle stream of nitrogen using a Turbovap LV Evaporator (Biotage, Charlotte, USA). Extracts were reconstituted in 0.2 mL of injection solvent (MeOH:H_2O, 50:50, v:v), vigorously vortexed for 1 min and subjected to centrifugation (Ultrafree® -MC centrifugal device, Millipore, Bedford, MA, USA) for 10 min at 5000*g*. From each sample, 45 μL of extract was added to 5 μL of a 100 ng/mL ^{13}C-labeled IS solution in an Eppendorf

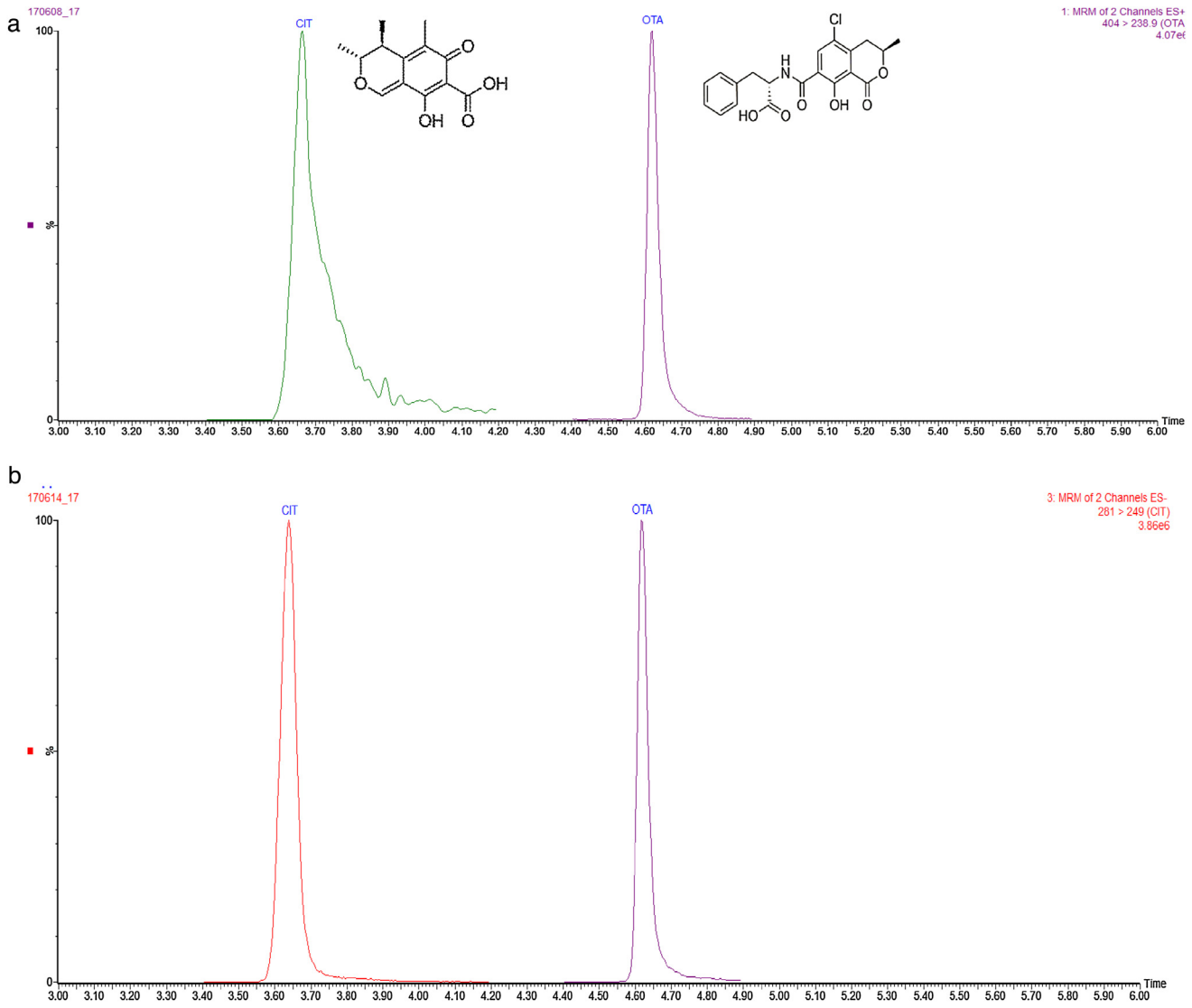

Fig. 1. (a) LC–MS/MS chromatogram (relative abundance in% versus time in min.) from a fortified pig feed sample (50 μg/kg) for citrinin (CIT) and ochratoxin A (OTA), without evaporation and reconstitution of the extract. Only one transition (quantifier ion) is shown. Chemical structures of the mycotoxins are shown on the right side of the corresponding peaks. (b) LC–MS/MS chromatogram from a fortified pig feed sample (50 μg/kg) for citrinin (CIT) and ochratoxin A (OTA), with evaporation and reconstitution of the extract. Only one transition (quantifier ion) is shown. A significant better peak shape and intensity was obtained for CIT by concentrating the sample, whereas a small improvement of the signal intensity for OTA was observed.

tube, followed by vortexing. IS was only added at the end of the procedure to reduce the cost, and after it was demonstrated that the extraction recovery on different quality control samples was acceptable, between 70 and 120%. Although small volumes were used (5 and 45 μL), the intraday and interday precision were not affected. Finally, mixtures were transferred into HPLC vials for analysis and 5 μL was injected into the LC–MS system. The samples were quantified using a matrix-matched calibration curve using blank samples (prepared by spiking samples before extraction in the same way as described above).

2.3.2. *Wheat flour, RYR food supplement, walnut, sunflower seed, nutmeg, tofu, baby milk powder and feed*

The extraction procedure for these matrices was similar to the procedure described above, with small modifications. For RYR, an aliquot (2.00 ± 0.02 g) of homogenized sample was weighed in a 50 mL extraction tube instead of 4 g. After adding the 10 mL of acidified saline solution, samples were left at room temperature for 15 min. Then, 20 mL of an extraction mixture consisting of ACN and HAc (99:1, v:v) and 10 mL of hexane were added to wheat flour, walnut, sunflower seed, nutmeg, tofu, baby milk powder, pig and chicken feed. The procedure was continued as previously described.

2.4. *LC–MS/MS analysis*

LC–MS/MS analysis was performed with an Acquity UPLC system coupled to a Waters Xevo® TQ-S triple quadrupole mass spectrometer (Waters Technologies, Zellik, Belgium) equipped with an electrospray interface. Masslynx and Targetlynx software 4.1 (Waters Corp., Milford, MA, USA) were used for data acquisition and processing. Chromatographic separation was achieved using an Acquity UPLC HSS T3 (1.8 μm x 2.1 × 100 mm) column (Waters, Milford, MA, USA). Column and autosampler temperature were set at 40 °C and 10 °C, respectively. Gradient elution was established with a mobile phase consisting of 0.05% acetic acid (v/v) and 5 mM ammonium acetate in water (eluent A) and MeOH (eluent B) at a flow rate of 0.4 mL/min. A run started with a linear increase of B from 30% to 90% during 6.5 min, with an instant increase to 100% B maintaining these conditions up to 9 min, after which column reequilibration took place for 1 min, resulting in a total run time of

Table 1
Mass spectrometric parameters for the analysis of citrinin (CIT) and ochratoxin A (OTA) and their ^{13}C-labeled internal standards ($^{13}C_{13}$—CIT and $^{13}C_{20}$—OTA).

Analyte	Retention time (min)	Precursor ion		Cone voltage (V)	Product ions *m/z* (collision energy)	
		m/z	Ion species		Quantifier	1st Qualifier
CIT	3.6	281.0	$[M+MeOH-H]^-$	50	249.0 (15 V)	205.0 (25 V)
$^{13}C_{13}$—CIT	3.6	294.0	$[M+MeOH-H]^-$	50	262.0 (15 V)	
OTA	4.6	404.0	$[M+H]^+$	35	238.9 (20 V)	221.0 (30 V)
$^{13}C_{20}$—OTA	4.6	424.2	$[M+H]^+$	35	249.8 (28 V)	

10 min. MS analyses were carried out using multiple reaction monitoring (MRM) with positive (OTA) and negative (CIT) electrospray ionization ($ESI^{+/-}$) (Table 1).

2.5. Method validation

The method was validated to meet the criteria of European Commission (EC) No. 2002/657 [33] and Commission Regulation (EU) No. 401/2006 [34] as guidance. Since no reference material was available, spiked blank samples of the corresponding matrix were used for the validation. Due to the fact that the feed and foodstuffs analyzed were very variable in composition, it was impossible to validate the method for every single commodity. Therefore, one blank feed product (pig feed) and one product per food category were selected for full validation. The selected food samples were wheat flour (cereal-based products), apple juice (fruit and vegetable juices), walnut, sunflower seed (nuts and seeds), beer (alcoholic beverages), nutmeg (herbs and spices), tofu (soy and vegetarian products), RYR food supplement (food supplements) and baby milk powder (baby food). Applicability of the method to other commodities within the same food group was tested by evaluating the apparent recovery. A set of parameters were used to evaluate method performance: specificity, linearity, apparent recovery (R_{app}), intraday (RSD_r) and interday precision (RSD_R), measurement uncertainty, limit of detection (LOD), limit of quantification (LOQ), extraction efficiency and matrix effects. According to Commission Decision (EC) No 2002/657, laying down the performance criteria of analytical methods, four identification points should be fulfilled to allow confirmation of the identity of the detected compound: 1 precursor and at least 2 product ions should be monitored, the relative intensities of the detected ions should correspond within accepted deviations to those of the calibration, detected ions should have a signal-to-noise ratio (S/N) of at least 3 and the relative retention time of the detected ions must range within a margin of 2.5%.

2.5.1. Specificity, linearity, LOD and LOQ

Specificity was determined by checking the presence of possible interfering chromatographic peaks in 15 representative blank samples and 5 samples fortified with other highly common mycotoxins found in the food and feed samples from Belgium as deoxynivalenol and fumonisin B1 at 20 μg/kg [35].

Linearity of 3 matrix-matched calibration curves (0.25–100 μg/kg) as well as calibration curves in neat solvent (0.1–50 ng/mL) was evaluated on three different days using the coefficient of determination (R^2) and confirmed by calculation of the goodness-of-fit coefficient (g) (%), which takes into account the difference between nominal value of the calibration curve and the calculated concentration (Eq. (1)). The coefficient g should be ≤ 10% for calibration curves with all spiked calibrator levels ≥10 μg/kg and ≤20% for calibration curves with spiked calibrator levels <10 μg/kg. [33,36,37]).

$$g = \sqrt{\left(\left(\sum (\%\text{deviation})^2\right)/(n-1)\right)} \quad (1)$$

With% deviation = (calculated concentration – nominal value)/(nominal value) × 100

LOD and LOQ (μg/kg) were determined according the guidelines of the International Conference of Harmonisation [38] using 2 different approaches, namely based on signal-to-noise (S/N) ratio and standard deviation of the y-intercept and the slope. Therefore, blank samples were spiked in decreasing concentrations within the range based on expected LOD and LOQ levels determined during method optimization (0.05–10 μg/kg). The samples were analysed as described above and the experiment was conducted in three independent replicates.

LOD was determined by a matrix-matched calibration curve (lower level equals lowest concentration for which S/N > 3 for both product ions and upper level equals 10 μg/kg). The standard deviation of the y-intercept as well as the slope of the curve were calculated using the linest function (Microsoft Excel, 2016). LOD equals 3.3 times the residual standard deviation of the regression line (standard deviation of the response) divided by the slope. Furthermore, detected peaks were visually inspected and a S/N of 3 is generally considered acceptable for the estimation of LOD.

LOQ equals 10 times the residual standard deviation of the regression line divided by the slope. Furthermore, detected peaks were visually inspected and the minimum level at which the analyte can be quantified with acceptable accuracy and precision was considered as LOQ. Another approach that was evaluated was the determination of the concentration of the corresponding peak with a S/N ratio of 10. All methods were compared and the strictest (i.e. highest) values were considered as LOD or LOQ.

2.5.2. Apparent recovery, intraday precision, interday precision and measurement uncertainty

Blank samples were spiked at three different concentration levels: 2.5 (low), 50 (medium) and 100 (high) μg/kg for feed and 0.5 or 1, 5 and 10 μg/kg for food matrices and analyzed in triplicate on three different days. Apparent recovery (R_{app}, %) is defined as the ratio between the measured concentration and the actual (spiked) concentration. The observed concentration was calculated in triplicate from a matrix-matched calibration curve. Intraday precision (repeatability) and interday precision (intermediate precision) were determined by calculating the residual standard deviation (RSD, %), respectively RSD_r and RSD_R using one-way ANOVA.

According to Commission Decision 2002/657/EC, precision values should not exceed the level calculated by the Horwitz equation (Eqs. (2) and (3)) were *C* represents the mass fraction expressed as a power of 10.

$$RSD_r = 2/3(2^{[1-0.5\log C]}) \quad (2)$$

$$RSD_R = 2^{[1-0.5\log C]} \quad (3)$$

Care should be taken with concentrations lower than 100 μg/kg, since the Horwitz equation can result in too high values (>20%). For these concentrations, precision values should be as low as possible [33].

An analytical result should be reported with respect to its measurement uncertainty. The combined standard uncertainty (u_c)

Table 2
Concentration range (μg/kg) of matrix-matched calibration curves for analysis of citrinin (CIT) and ochratoxin A (OTA) in the selected matrices, with corresponding LOD and LOQ (μg/kg).

Matrix	CIT			OTA		
	Range (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	Range (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
Beer	0.25–100	0.1	0.3	0.25–100	0.2	0.4
Apple juice	0.25–100	0.1	0.3	0.25–100	0.1	0.3
Sunflower seed	0.25–100	0.2	0.4	0.6–100	0.3	0.6
Walnut	5–100	2.5	5.0	2–100	1.0	1.9
Nutmeg	1–100	0.8	1.6	1–100	0.5	1.0
Tofu	1–100	0.4	0.8	0.50–100	0.5	0.9
RYR	1–3,000	1.0	2.0	1–3,000	0.6	1.1
Pig feed	1–250	0.3	1.0	1–250	0.3	0.6
Wheat flour	0.50–100	0.5	1.0	1–100	0.5	1.0
Baby milk powder	0.50–100	0.3	0.5	0.50–100	0.3	0.5

equals the positive square root of the interday precision and the bias of the analytical method, which comprises the uncertainty of the purity of the used standards ($U[C_{ref}]$), the accuracy of the bias (S_{bias}) and the root mean square of the bias (RMS_{bias}). The combined expanded measurement uncertainty, expressed as U, is obtained by multiplying the standard measurement uncertainty by a coverage factor (k = 2) and gives a range that contains the result with 95% confidence (Eq. (4)) [39].

$$U = 2\,xuc = 2\,x\sqrt{(RSD_R{}^2 + U[C_{ref}]^2 + S_{bias}{}^2 + RMS_{bias}{}^2)} \quad (4)$$

2.5.3. Extraction efficiency and matrix effects

Matrix effects were assessed following the approach described by Sulyok et al. [40]. Calibration curves for CIT and OTA in neat solvent ("standard") were constructed by plotting signal intensity against concentration. In the same way, calibration curves of CIT and OTA in extracts were made up by plotting signal intensity of the analyte in spiked samples before ("spiked") and after extraction ("spiked extract") *versus* spiked concentration. SSE (equation 5) and extraction efficiency (R_E, equation 6) were calculated by using the slopes of the constructed curves.

$$SSE = Slope_{spike\ extract}/slope_{standard} \times 100 \quad (5)$$

$$R_E = slope_{spiked}/slope_{spike\ extract} \times 100 \quad (6)$$

2.6. Applicability of the developed method to matrices belonging to the same food groups

In a small preliminary study, the validated method was tested on different kinds of foodstuffs belonging to the selected food groups: white bread, cornflakes, oatmeal, tortilla, white pasta, orange juice, tomato juice, curry powder, wine, cashew nuts, pistachio nuts, pumpkin seeds, chickpeas (canned), spiced tofu slices, and a valerian food supplement. Samples were fortified at 3 different concentrations (2.5, 50 and 100 μg/kg) for evaluation of apparent recovery. A "common" matrix-matched calibration was used for each commodity group, based on table A from Commission Regulation (EU) No. 519/2014 [41] (supplemental Table S. 1 and Table S. 2), meaning that calibration curves constructed in the food products for the validation study were also used for quantification of CIT and OTA in other foodstuffs belonging the same food commodity group. For instance, a calibration curve in wheat flour was constructed for quantification of CIT and OTA in white bread, cornflakes, oatmeal, tortilla and white pasta.

2.7. Statistical analysis

Data processing and calculations were performed using Microsoft Office Excel 2016 and IBM SPSS Statistics 21.

3. Results and discussion

3.1. Optimization of the LC–MS/MS conditions

The optimization was obtained through a 30 s MS spectrum in positive (ESI^+) and negative ion (ESI^-) mode. A precursor ion for each analyte was selected and cone voltages were optimized. For CIT and its ^{13}C-labeled IS, promoting the formation of the $[M+MeOH-H]^-$ adducts led to higher signal intensities, hence these MeOH adducts were chosen as precursor ions and they were obtained in ESI^-. For OTA and $^{13}C_{20}$—OTA, the molecular ion obtained in the ESI^+ mode was selected. Next, to investigate the most optimal product ions for the MRM transitions, different collision energies were applied. Two product ions were selected and used in the final MS method. An Acquity UPLC HSS T3 [1.8 μm, 2.1 mm × 100 mm] chromatographic column was tested, based on previous in-house experiments. The use of this column led to acceptable peak shapes, hence no other chromatographic columns were tested. The optimal LC–MS/MS conditions for each analyte (retention time, cone voltage, collision energy, precursor and product ions) were tuned to achieve the best signal (Table 1). These results are in accordance with previously reported methods for separate determination of CIT and OTA in food and feed by LC–MS/MS [28,42,43].

3.2. Optimization of sample preparation

Although there are different techniques for purifying a single mycotoxin from crude samples (immunoaffinity columns (IAC), solid-phase extraction (SPE), QuEChERS, . . .) [44], a direct and simple method for the purification of multiple mycotoxins (CIT and OTA) was challenging because of their diverse chemical structures and properties. SPE techniques have been widely used, however, the purification is tedious and time consuming. IAC techniques reduce matrix effects and are highly selective, but they are less suitable for the determination of different compounds. The QuEChERS method has been successfully applied in many products for mycotoxin determination [45–48]. Moreover, a QuEChERS procedure presents several advantages over the more traditional methods of analysis since a high sample throughput in a short time is possible, the method is applicable to multiple mycotoxins and moreover, solvent usage and waste are smaller, and reagents are less expensive than the use of other SPE techniques [7,49,50].

Based on literature and physicochemical properties of the target compounds, different mixtures of MeOH:H_2O and ACN:H_2O (both 50:50; 70:30 or 90:10, v/v) were evaluated first, yielding the best results for ACN:H_2O 70:30 (v/v). An example of a chromatogram is given in supplementary material, Figure S. 1. However, since EtAc and acidified extraction solvents were reported to improve recovery for CIT [9,51], an extraction mixture consisting of a saline

aqueous solution acidified with HCl and an organic solution of EtAc:ACN:HAc (75:24:1, v/v/v) was used. Indeed, the pKa values of CIT and OTA are 3.6 and 4.4 as weak acids, respectively. After adding the aqueous solution to dry foodstuffs, samples were left for 15 min at room temperature, in order to hydrate them prior to extraction. This step allows the matrix to swell and weakens interactions of the toxin with matrix compounds, assisting in efficient extraction [52]. For fatty commodities like cookies and feed, a defatting step with hexane was included to obtain cleaner extracts. An additional overnight freezing out step (–20 °C) was also tested on extracts [25], resulting in only a slight improvement of response with no further added value (supplemental Figure S. 2). Two approaches were evaluated for further clean-up of the sample extracts prior to LC–MS/MS analysis. The first protocol simply consisted of filtering 0.5 mL of the extract using an centrifugal filter device and in the second method, 5 mL of the extract was first evaporated to dryness and reconstituted in 1 mL of injection solvent (H_2O:MeOH, 50:50 v/v), after which the sample was filtered through an centrifugal filter device. The latter method was preferred since better peak shapes and improved sensitivity for CIT were obtained compared to the first method (Fig. 1a and 1b). Moreover, concentrating the sample led to higher sensitivity and cleaner aliquots to inject in the LC–MS system, as interferences were removed by evaporation to dryness.

3.3. Method validation

The LC–MS/MS method was validated for CIT and OTA in different matrices: pig feed, wheat flour, apple juice, nutmeg, sunflower seed, walnut, RYR, baby milk powder, beer and tofu, following the criteria mentioned in Commission Regulation (EC) No. 401/2006 [34] and Commission Decision (EC) No. 2002/657 [33].

3.3.1. Specificity, linearity, LOD and LOQ

Good specificity means that an analytical method is able to differentiate between the target analyte and other compounds or interferences [33]. No interference peaks (S/N > 3) were detected in the blank samples in the range of the retention time ± 2.5% for all target analytes, confirming the specificity of the method.

Linearity in predefined ranges was evaluated by the coefficient of determination R^2 and goodness-of-fit. Coefficient of determination of above 0.995 were obtained for most matrices, indicating good linearity. For CIT, the lowest obtained R^2 value was 0.991 (in wheat flour). Linear calibration curves were calculated for all matrices, except for walnuts and sunflower seeds, for which quadratic curves were constructed. For OTA, 0.991 was the lowest found R^2 value (in walnut). Linear calibration curves were also used for all matrices, except for sunflower seed, for which a quadratic curve was constructed. Weighted least-squares regression (WLSLR) with an optimal weighing factor ($1/x$ or $1/x^2$) was used to compensate the observed heteroscedasticity. This improved accuracy in the lower concentration range [53]. A satisfying result for goodness-of-fit (<20%) was obtained for both CIT and OTA in all matrices. A plot was constructed of the residuals *versus* concentration, and the error was randomly distributed around the concentration axis for all matrices (graphs for each matrix are given in supplementary materials, Figure S5–13), indicating the absence of proportional and systematic errors.

Results for LOD and LOQ, calculated and compared using different methodologies as described above, are summarized in Table 2. The strictest value was chosen for these parameters.

3.3.2. Apparent recovery, intraday precision, interday precision and measurement uncertainty

All validation parameters are summarized in Table 3. According to the followed guidelines (EC 401/2006), recoveries were within the acceptability ranges, which are 70–120% and 70–110% for CIT and OTA respectively [34]. All values for RSD_r and RSD_R were below 20%, showing good precision for all matrices.

The expanded measurement uncertainty (*U%*) data, calculated according to equation 4, were satisfactory.

3.3.3. Extraction efficiency and matrix effects

Due to the large variability of the analyzed samples, a wide range of matrix effects was expected. Matrix effects are caused by competition between analyte and co-eluting (non-detected) matrix compounds with ions formed in the LC–MS/MS interface [54]. The ionization of the compound of interest can either be enhanced or suppressed (signal enhancement or suppression, SSE), depending on conditions in which the ion formation takes place. Analyte quantification can be strongly affected by matrix effects, so these cannot be ignored during method development.

Extraction efficiency was determined by plotting signal intensity against concentrations for samples spiked before and after extraction. The ratio of the slopes gives the extraction efficiency, which was acceptable for all matrices (60–120% for CIT, 60–110% for OTA).

For CIT, strong signal suppression (SSE <10%) was observed in nutmeg, walnut and sunflower seed, while signal suppression was limited in wheat flour and tofu (SSE >70%). On the other hand, the signal was enhanced in beer (SSE of 125%). Non-parallelism of the calibration curves, constructed by plotting signal intensity *versus* concentration of the spiked extracts, confirms the presence of matrix effects (Fig. 2 and Figure S. 3 in supplementary material). Concerning OTA, the SSE values ranged between 78 and 99% for all matrices, except for nutmeg (20.8%). Details of extraction recovery and matrix effects are summarized in the supplementary materials (Table S. 3). Use of a stable isotope labeled mycotoxin as IS can satisfyingly compensate matrix effects [55]. Indeed, by plotting response ratio (area analyte divided by area IS $^{13}C_{13}$—CIT or $^{13}C_{20}$—OTA) instead of area against concentration, the slopes observed were not significantly different, as confirmed by an ANOVA test ($p > 0.05$) (Fig. 3 and Figure S. 4 in supplementary material). Hence, the compensation of matrix effects by using a ^{13}C-labeled IS, indicates that a calibration curve in neat solvent can be used for quantification in future experiments. However, as the IS were added after extraction to avoid high costs, they did not compensate for losses during extraction. Therefore, recovery should always be evaluated and data corrected accordingly.

3.4. Applicability of the validated methods to other feed and food commodities of the same food group

Apparent recovery for CIT and OTA was determined in selected foodstuffs and chicken feed. Apparent recoveries ranged from 70 to 112% for CIT, with an outlier of 61% for curry, and between 70 and 110% for OTA (details are shown in supplementary material, Table S. 4). The recovery ranges are within the criteria proposed by EC 401/2006 [34].These acceptable validation results allow to conclude that the general QuEChERS procedure developed in this study is applicable to analyze CIT and OTA in many different matrices.

3.5. Analysis of commercially available Belgian feedstuffs

In total, 90 feed samples (38 broiler chicken feed samples and 52 pig feed samples) were analyzed using the validated LC–MS/MS method to evaluate the occurrence of CIT and OTA (Table 4). To summarize, CIT was detected in 45% of the chicken feed samples, of which 4 above the LOQ in an average concentration (± SD) of 2.0 ± 1.3 μg/kg. OTA was detected in 61% of the same samples, of which 17 samples above the LOQ in an average concentration of 1.3 ± 1.3 μg/kg. For pig feed, 51% were positive for CIT of which 14 samples were contaminated above the LOQ-level, with

Table 3
Results for accuracy, expressed as apparent recovery (Rapp), repeatability (RSDr), intermediate precision (RSD_R) and measurement uncertainty (U) for citrinin (CIT) and ochratoxin A (OTA) in selected matrices. n.q.: non quantifiable.

Matrix	CIT					OTA				
	Concentration (μg/kg)	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)	Concentration (μg/kg)	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)
Beer	0.5	110	2.2	10.0	31	0.5	110	2.5	2.1	8
	5	98	5.2	3.3	16	5	92	6.7	7.3	24
	10	94	3.6	6.2	16	10	90	2.8	1.1	8
Apple juice	0.5	89	2.5	9.5	4	0.5	92	1.0	7.1	4
	5	97	2.2	5.2	9	5	96	2.2	2.2	7
	10	94	1.2	4.5	4	10	95	1.2	7.0	8
Sunflower seed	0.5	94	8.1	5.0	24	0.5	87	1.1	7.6	27
	5	84	4.8	3.2	12	5	93	1.8	6.6	15
	10	101	1.3	2.2	2	10	94	3.3	5.6	15
Walnut	0.5	n.q.	n.q.	n.q.	n.q.	1	93	12.1	5.1	28
	5	104	8.0	2.3	21	5	99	2.6	4.2	12
	10	102	6.0	4.0	16	10	100	4.2	5.0	16
Nutmeg	1	86	17.6	6.1	39	1	103	6.6	3.6	39
	5	96	12.5	17.8	35	5	91	6.0	2.4	12
	10	80	13.2	6.0	26	10	94	10.3	0.1	17
Tofu	1	91	0.4	7.6	15	1	89	10.4	4.4	28
	5	98	0.9	17.9	24	5	97	2.4	5.2	11
	10	100	1.9	0.6	4	10	101	2.3	0.4	5
RYR	5	86	2.9	13.9	39	1	91	4.6	7.7	27
	10	92	2.7	12.5	34	10	110	4.0	6.5	15
	100	105	0.5	9.6	25	100	94	6.0	5.9	12
Pig feed	2.5	108	6.2	4.4	24	2.5	107	3.6	5.0	28
	50	95	2.0	1.1	13	50	97	1.0	0.4	10
	100	99	1.3	0.7	5	100	100	1.3	0.6	5
Wheat flour	0.5	120	4.1	1.1	9	0.5	121	1.2	0.6	3
	5	75	3.7	0.7	6	5	74	2.6	0.9	3
	10	80	4.2	2.6	10	10	81	4.9	1.9	8
Baby milk powder	0.5	70	9.0	8.0	25	0.5	71	3.0	3.0	7
	5	79	21.0	11.0	5	5	77	2.0	0.6	5
	10	99	8.0	7.0	27	10	95	4.0	2.7	11

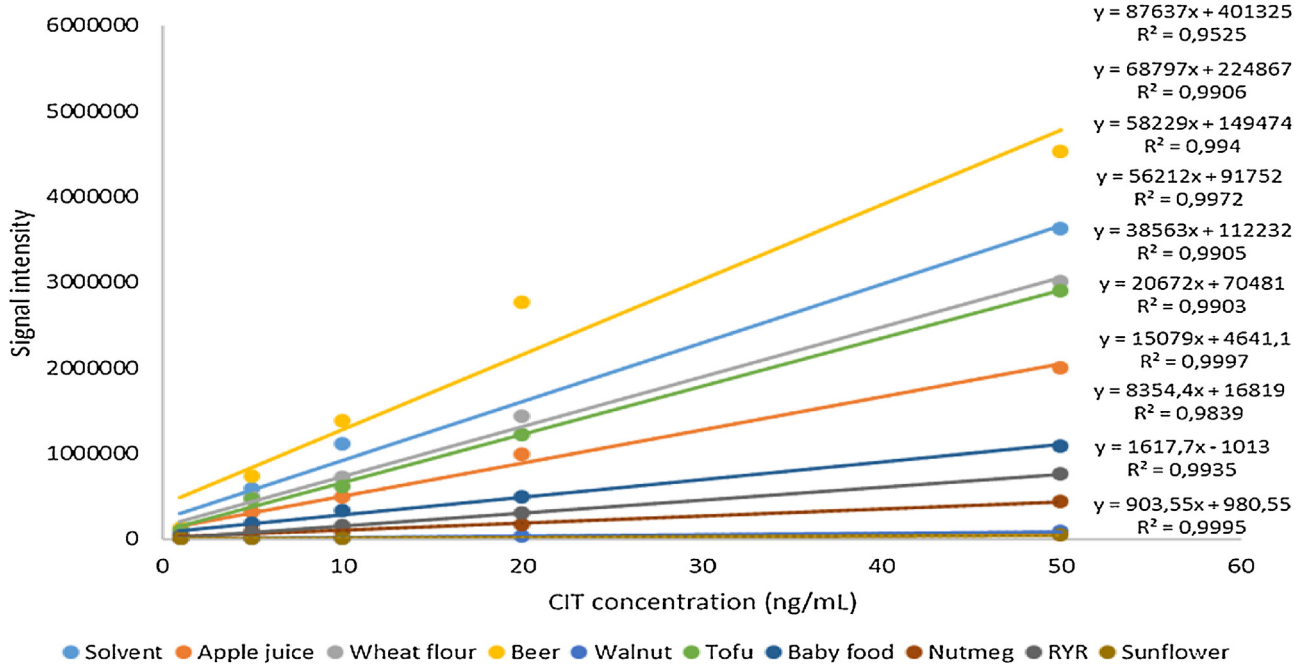

Fig. 2. Calibration curves for citrinin (CIT) in neat solvent and in various matrices illustrating matrix effects (signal suppression for all matrices, except for beer). Strong signal suppression was observed for walnut and sunflower seed.

an average concentration of 1.6 ± 0.8 μg/kg OTA was found in 92%, with 33 samples above the LOQ with an average concentration of 1.4 ± 1.1 μg/kg. CIT and OTA co-occurred in 61% of the positive (> LOD) samples. In 19% of the positive feed samples, CIT and OTA co-occurred in a concentration above LOQ.

Compared with the few studies reporting the presence of CIT in feed, CIT is a common mycotoxin in feed, as demonstrated in our survey. Other studies reported higher contamination levels of CIT, since Pleadin et al. [56] found > 62% of positive calf and pig feed samples with an average concentration of > 23 μg/kg, in Croatia and

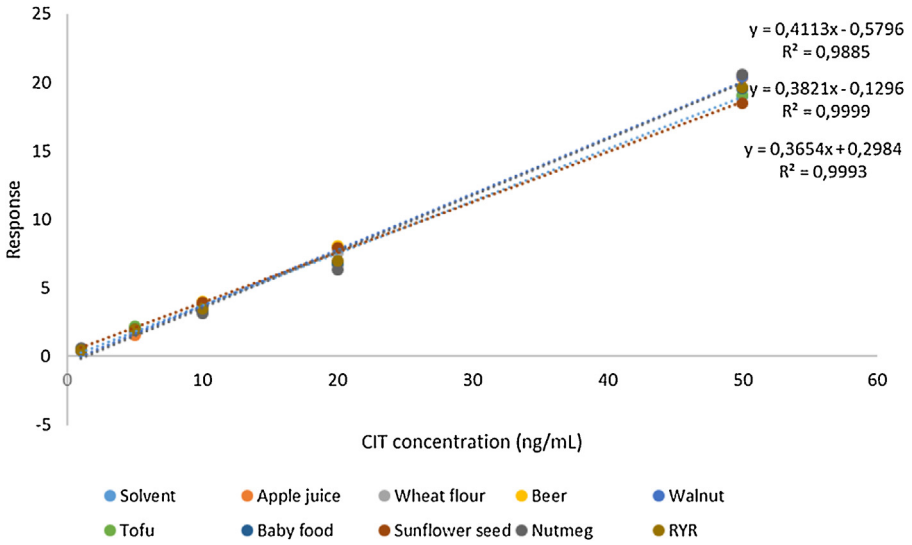

Fig. 3. Response of citrinin (CIT) plotted against spiked extract and neat solvent concentration (ng/mL). By using an ^{13}C-labeled internal standard, matrix effects are fully compensated. Only curve equations are displayed for matrices with the highest and lowest slopes (respectively walnut and sunflower seed) and for the curve in neat solvent (middle equation).

Table 4
Occurrence of citrinin (CIT) and ochratoxin A (OTA) in Belgian chicken and pig feed samples, expressed as positive samples > LOD. Average concentrations are shown with respect to their standard deviation (SD), maximum concentrations are shown with respect to their measurement uncertainty (U).

Feed	Toxin	Positive samples (%)	Average concentration ± SD* (μg/kg)	Max. concentration ± U (μg/kg)
Chicken (n = 38)	CIT	45	2.0 ± 1.3	3.9 ± 0.9
	OTA	61	1.3 ± 1.3	5.4 ± 1.5
Pig (n = 52)	CIT	51	1.6 ± 0.8	3.7 ± 0.9
	OTA	92	1.4 ± 1.1	5.6 ± 1.6

*Of samples above LOQ.

Bosnia & Herzegovina (n = 67). Kononenko et al. [57] found 8.8% of positive samples with a maximum of 182 μg/kg in Russian chicken and pig feed samples (n = 1700). As no legislation exists with regard to maximum levels, the high prevalence of CIT in feed could cause toxicological problems to animals because of its nephrotoxic ability. It is therefore recommended to set maximum levels based on occurrence and toxicity data.

Concerning OTA, its presence in feed is even more common than CIT. However, the results obtained were below the maximum levels recommend for chicken (100 μg/kg) and pig feed (50 μg/kg) [21]. Other studies reported higher concentrations, for instance, Abidin et al. [58] found 82% of poultry feed samples with OTA present in an average concentration of 122 μg/kg. Apart from induction of different pathological changes in the animals, residues of OTA in specifically the kidney, could pose a public health concern when such tissues are being consumed by humans.

Differences between CIT and OTA presence were not detected among the chicken and pig feed samples ($p > 0.05$), which corresponds to other studies. Indeed, Pleadin et al. found similar CIT concentrations in pig and cow feed. Some mycotoxin concentration differences could be detected depending on the cereal composition, for instance oat was shown to be the least contaminated cereal with CIT [56].

It is clear that CIT and OTA, although in low concentrations, co-occur in Belgian feed samples. Co-occurrence of mycotoxins in feed and food is an important research subject because the effects of co-exposure on human and animal health remain unclear. Most of the mycotoxin mixtures lead to additive or synergistic effects, which can cause even more health-related issues for humans and animals upon consumption of mycotoxin-contaminated food or feed [59].

4. Conclusion

An LC–MS/MS method for quantitative analysis of CIT and OTA in feed and food was developed. The developed method was validated according the criteria described in Commission Regulation No. 401/2006/EC and Commission Decision No. 2002/657/EC. Specificity, linearity, apparent recovery, limit of detection and quantification, measurement uncertainty, matrix effect, extraction efficiency and intraday and interday precision were successfully validated for each matrix (pig feed, wheat flour, apple juice, walnut, beer, sunflower seed, nutmeg, tofu, RYR food supplements and baby milk powder). The method can be used for a wide variety of food products, with slight adaptations per matrix. Application of the validated method on feedstuffs revealed co-occurrence of CIT and OTA in 61% of the Belgian feed samples (positive samples above the LOD). This implies that further research is needed to estimate the internal exposure to these mycotoxins in broiler chickens and pigs, and especially CIT, in Belgium.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2018.10.039.

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