DETERMINATION OF OCHRATOXIN A IN FOOD BY LC-MS/MS

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Abstract

A liquid chromatographic – tandem mass spectrometry (LC-MS/MS) method for quantification of ochratoxin A (OTA) in foods was developed and validated. OTA is a mycotoxin which can contaminate food in semitropical and temperate climates. It has nephrotoxic, genotoxic, carcinogenic, teratogenic and neurotoxic potential. The chromatographic separation was performed on a Zorbax SB-C18 column with gradient elution using a mobile phase of acetonitrile and 0.1% (v/v) acetic acid in water at 48 ºC with a flow rate of 1 mL/min. The detection of OTA was performed in MRM mode using an ESI source in negative mode. The monitored transition was *m/z* 402 → (358 + 359 + 360). Samples of bread, dry fruit, breakfast cereals, spices, beer, cereals, and seeds were analyzed. OTA was extracted from the food samples in a mixture of ethyl acetate : methanol : acetic acid (95:5:0.5, v/v/v) after solubilization in aqueous acidic medium and cleanup on celite. The elaborated method showed a good linearity ($r > 0.998$), precision (CV < 10.0 %) and accuracy (bias < 9.7 %) over the range of concentrations studied (0.45-36 ng/mL) and a good sensitivity (LLOQ of 0.45 ng/mL). The run-time of chromatographic analysis was 18 min and the retention time of OTA 13 min. The developed and validated method is simple and efficient, and can be applied in the monitoring of food safety and assessment of exposure to OTA through food.

Rezumat

S-a elaborat şi s-a validat o metodă LC-MS/MS pentru dozarea ochratoxinei A (OTA) în alimente. OTA este o micotoxină care poate contamina alimentele în climatul semitropical şi temperat. Are potenţial nefrotoxic, genotoxic, cancerigen, teratogen şi neurotoxic. Separarea cromatografică s-a realizat pe o coloană Zorbax SB-C18 cu eluţie în gradient folosind ca fază mobilă acetonitril (A) şi acid acetic 0,1% în apă (v/v) la 48ºC cu

un debit de 1 mL/min. Detecţia OTA s-a efectuat în modul MRM folosind o sursă ESI (ionizare negativă). S-a monitorizat tranziţia *m/z* 402 → (358 + 359 + 360). S-au analizat probe de pâine, fructe uscate, cereale pentru micul dejun, mirodenii, bere şi seminţe. OTA s-a extras din probele de alimente într-un amestec de acetat de etil:metanol:acid acetic (95:5:0.5, v/v/v) după solubilizare în mediu apos acid şi purificare pe celite. Metoda elaborată a prezentat linearitate bună (r > 0.998), precizie (CV < 10.0 %) şi acurateţe (bias < 9.7 %) pe intervalul de concentraţii studiat (0.45-36 ng/mL) şi sensibilitate bună (LLOQ de 0.45 ng/mL). Timpul unei analize cromatografice a fost de 18 min, iar timpul de retenţie al OTA de 13 min. Metoda elaborată şi validată este simplă şi eficientă, şi poate fi aplicată în monitorizarea siguranţei alimentare şi evaluarea expunerii la OTA prin alimente.

Keywords: Ochratoxin A, mycotoxins, LC-MS/MS, food safety.

Introduction

Ochratoxin A (OTA) is a mycotoxin, a secondary fungal metabolite mainly produced by the fungi *Aspergillus ochraceus*, *Penicillium verrucosum* and *Penicillium viridicatum* [1, 2]. It can contaminate food in semitropical and temperate climates being detected mainly in cereal grains, green coffee beans, spices, dried fruit, beer and wines [3]. Furthermore, it passes through the food chain unchanged and a bioaccumulation of OTA was observed [4]. OTA has nephrotoxic potential through bioactivation. The presence of OTA in blood was associated with Balkan Endemic Nephropathy, a progressive renal pathology with an increased incidence in the Balkan countries, including Romania, and/or urinary system tumors [1, 2]. Several *in vitro* and *in vivo* studies on laboratory animals demonstrated its nephrotoxic, immunosuppressive, genotoxic, carcinogenic, teratogenic and neurotoxic properties [5]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA), on the basis of nephrotoxicity of OTA, established a provisional tolerable daily intake (TDI) for OTA of 14 ng/kg b.w./day. The maximum permissible limits for OTA approved by EU are 5 μg/kg in whole grain and 3 μg/kg for processed products [2, 6].

Several analytical methods are used for mycotoxin determination in foodstuff. Immunochemical methods such as ELISA determine many derivatives of a mycotoxin, depending of the cross-reactivity of the antibody, and can result in an over-estimation of contamination [7]. ELISA is currently more useful as a rapid screening tool [3]. Chromatographic methods are more selective and can determine each compound as single response [7] avoiding false positive results [8]. OTA can be analyzed in foodstuff by GC methods, but a derivatization is needed in this case because it is not volatile [3], by HPLC with fluorescence detection due to its native fluorescence [3, 8-11], or by LC-MS/MS, which is currently the method of

choice because it has considerable advantages on selectivity, specificity, sensitivity, and robustness [7, 12-14].

The aim of this work was to develop and validate a LC-MS/MS method to quantify OTA levels in some foodstuff. This method will be applied in the monitoring of food safety and the assessment of exposure to OTA through food.

Materials and Methods

Reagents

Ochratoxin A (OTA) was a reference standard from Fluka (code 32937). Acetonitrile of gradient-grade for liquid chromatography and methanol, ethyl acetate, chloroform, glacial acetic acid, phosphoric acid, sodium bicarbonate and celite of analytical-reagent grade were purchased from Merck KGaA (Darmstadt, Germany). Deionized water was obtained using a Milli-Q Water purification system (Millipore, Milford, MA, USA).

Apparatus

The following apparatus were used: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical Plus Balance (Mettler-Toledo, Switzerland); Vortex Genie 2 mixer (Scientific Industries, New York, USA); Ultrasonic bath Elma Transsonic 700/H (Singen, Germany); rotavapor Buchi R-205. The HPLC system used was an 1100 series Agilent Technologies model consisting of a G1312A binary pump, an in-line G1379A degasser, a G1329A autosampler, a G1316A column thermostat and an Agilent Ion Trap Detector 1100 VL.

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a Zorbax SB-C18 (100 mm x 3.0 mm i.d., 3.5 μm) column (Agilent Technologies) using a mobile phase of acetonitrile (A) and 0.1% (v/v) acetic acid in water at 48ºC with a flow rate of 1 mL/min and gradient elution: start at 10% A, 10% A → 47% A until 14.5 min, and 47% A → 10% A until 16.5 min. The detection of OTA was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer equipped with an electrospray ion (ESI) source, in negative mode (capillary 4500 V, nebulizer 60 psi (nitrogen), dry gas nitrogen at 12 L/min, dry gas temperature 325ºC). The ion transition *m/z* 402 → (358 + 359 + 360) was monitored.

Standard solutions

A stock solution of OTA (450 μg/mL) was prepared by dissolving an appropriate quantity of OTA in methanol. Two working solutions (450 ng/mL and 4.5 ng/mL, respectively) were prepared by appropriate dilution in a mixture of water : methanol (50:50, v/v). These solutions were used to

prepare standard solutions with the concentrations of 0.45, 0.90, 1.80, 4.50, 9.0, 18.0 and 36.0 ng/mL by appropriate dilution in water. Quality control (QC) samples of 0.90 ng/mL (lower), 4.50 ng/mL (medium) and 18.0 ng/mL (higher) were prepared by adding the appropriate volumes of working solutions to water. The resultant standard solutions and quality control samples were pipetted into 15 mL polypropylene tubes and stored -20ºC until analysis.

Food samples

The food samples were taken randomly from local markets in Romania in 2013. We analyzed samples of bread (n = 5), dry fruit (n = 6), breakfast cereals (n = 5), spices (n = 4), beer (n = 5), cereals (n = 8), and seeds (n = 13). All samples were stored in a refrigerator until analysis.

Sample preparation

To the amount of sample taken in work (25 g for bread, fruit, breakfast cereals, spices, seeds, 150 mL for beer, and 50 g for cereals) was added 0.1 M phosphoric acid (12.5 mL) and chloroform (125-150 mL). After stirring for 30-40 min using a magnetic stirrer, celite (5g) was added and the homogenized sample was filtered through a quantitative filter paper. Crude extract was extracted quantitatively with 1% sodium bicarbonate aqueous solution. OTA was re-extracted quantitatively from aqueous phase with a mixture of ethyl acetate – methanol – acetic acid (95:5:0.5, v/v/v). The organic phase was separated and evaporated to dryness in the rotavapor. Each residue was taken up in the mobile phase (150 µL), was transferred in autosampler vials and 20 µL were injected into the HPLC system.

Method validation

The specificity of the method was evaluated. The chromatograms obtained from six blank samples were compared with those obtained with the analyte at the lowest level of the calibration curve. The samples must not contain compounds that interfere with the analyte retention time. Therefore, the chromatograms of samples must not show peaks at the retention time of analyte. If they exist, their area should not be more than 20% of the analyte area at the limit of quantification.

The concentration of OTA was calculated automatically by the instrument data system using peak areas and the external standard method. The calibration curve model was determined by the least squares analysis: $y = b + ax$, weighted (1/y) linear regression, where y - peak area and x - analyte concentration. Five different series were analyzed. The accuracy (the difference between theoretical and entered concentrations) was calculated for each point of the calibration curve. The regression coefficient

must be > 0.98 and the accuracy must be < ±20% for the lowest calibration standard and < ±15% for the other concentrations.

The intra-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %) were determined by analysis of five different samples (n = 5) from each QC standards (at lower, medium and higher levels) on the same day. The inter-day precision and accuracy were determined by analysis on five different days (n = 5) of one sample from each QC standards (at lower, medium and higher levels). The precision and the accuracy are acceptable if their values are < ±15%.

The lower limit of quantification (LLOQ) was established as the lowest calibration standard with an accuracy and precision less than 20%.

Results and Discussion

We have studied the chromatographic behavior of OTA (Figure 1).

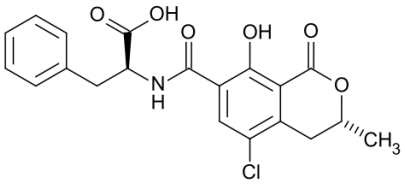

Figure 1.
Chemical structure of ochratoxin A (OTA).

Because OTA is found in foods at low concentrations, we have chosen a MS-MS method that provides selective and specific detection. The conditions for chromatographic separation of OTA were optimized and the best results were obtained using a mobile phase of acetonitrile (A) and 0.1% (v/v) acetic acid in water at 40 ºC with a flow rate of 1 mL/min. The optimal conditions of OTA ionization were then studied. Electrospray ionization (ESI) is often employed to ionize polar mycotoxins as OTA [7]. The molecular mass of OTA is 403.8 g/mol. The OTA molecule has a chlorine atom, with an isotopic distribution Cl35/Cl37 about 75/25, which would imply the presence of consecutive isotopic distributions. It also has two acidic groups, able to give protons (the carboxyl and phenol groups) and a group with a weak base character, able to accept protons (the substituted amide group). Negative ionization leads to a pseudo molecular ion with m/z of 402 (Figure 2).

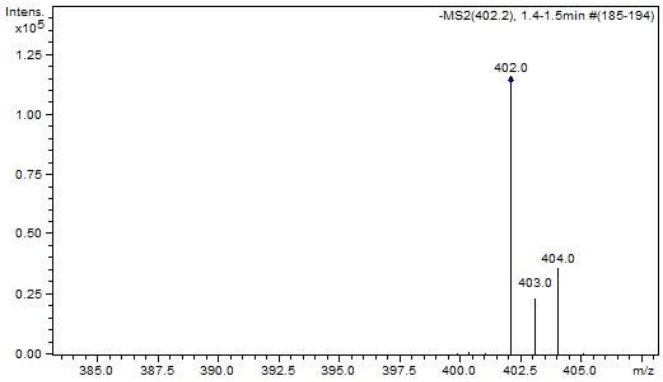

Figure 2.
MS spectrum of OTA obtained by ESI in negative mode.

By fragmentation results the principal ion m/z 358 (Figure 3) [14], and an isotopic distribution can be observed at values m/z 359 and 360, similar to that of the parent compound. The difference 402 – 358 suggests a fragmentation produced by decarboxylation and charge transfer. For positive ionization, pseudo molecular ion with m/z of 404 (Figure 4) is fragmented primarily into an ion having also m/z 358 [8], but another structure (Figure 5) [12].

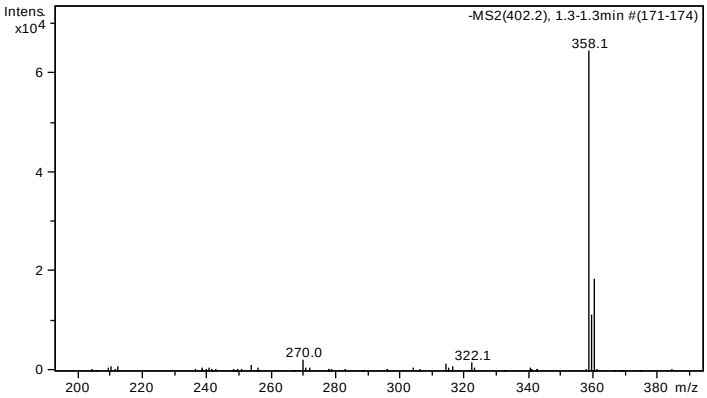

Figure 3.
MS/MS reactive spectrum of OTA by ESI in negative mode.

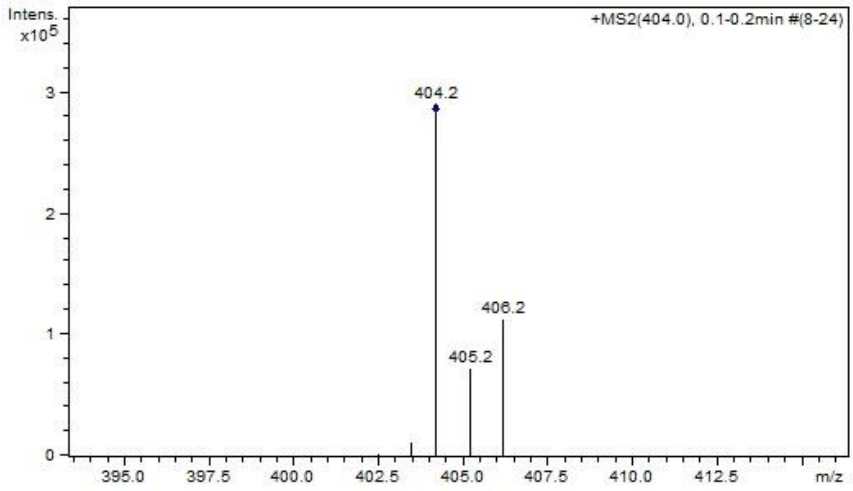

Figure 4.
MS spectrum of OTA obtained by ESI in positive mode.

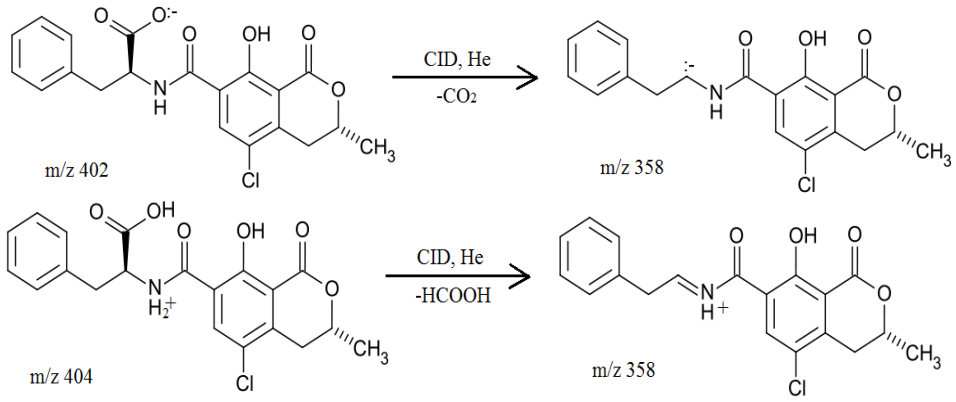

Figure 5.
Formation and structure of probable ion with m/z 358 resulted through fragmentation (CID – collision induced dissociations in the presence of helium) in the MS/MS reactive spectrum of OTA in negative mode (up) and in positive mode (down).

The influence of mobile phase on the yield of ionization was studied by testing more aqueous components: 0.1% (v/v) formic acid, 0.1% (v/v) acetic acid, 1 mM ammonium acetate, and 3 mM ammonium acetate. The best chromatographic signal was obtained with 0.1% (v/v) acetic acid in negative ionization. In these conditions, the retention time of OTA was 1.5 min and the run time of 2 min, but the sensitivity was low, 4.5 ng/mL. This method is applicable only in the screening of highly contaminated samples.

The sensitivity was increased 10 times by gradient elution at 48ºC. The run time was 20 min, the retention time of OTA 13 min (Figure 6), and the sensitivity was 0.45 ng/mL.

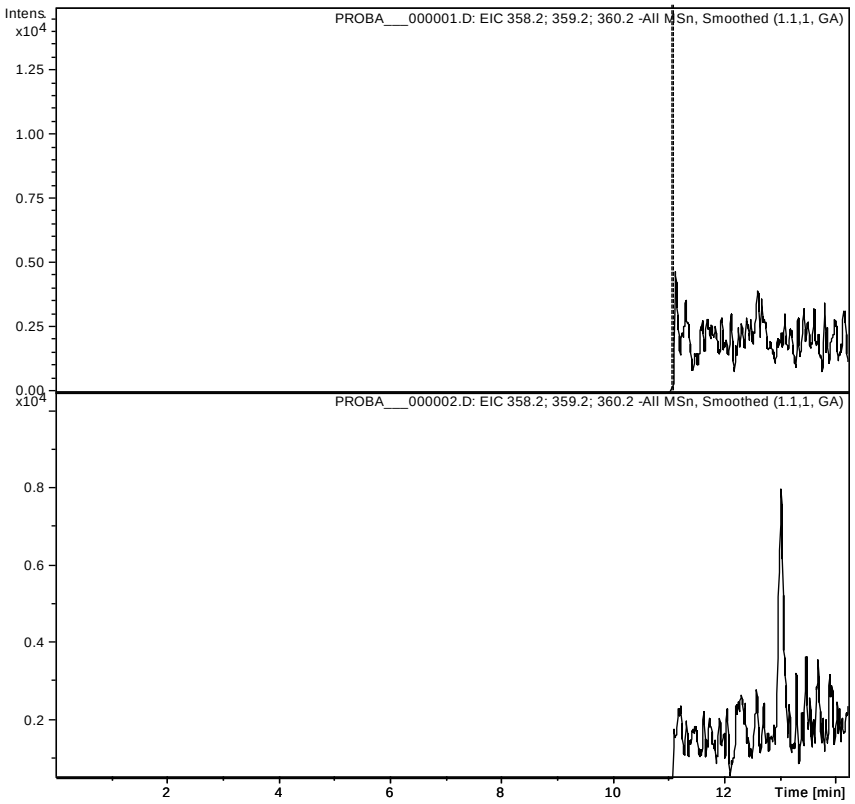

Figure 6.
Representative chromatograms of (up) a blank sample, and (down) a sample with OTA at LLOQ of analytical method (0.45 ng/mL); the retention time of OTA is 13 min.

Correct extraction and cleanup methods are essential for an efficient assay, as they are time consuming. All chromatographic methods used for OTA analysis from foodstuff usually include a liquid-liquid extraction (LLE) or a solid phase extraction (SPE) [13, 14]. Immunoaffinity columns [9, 11, 15] or molecularly imprinted polymer as selective sorbent of SPE [8, 16] are often used for cleanup in the extraction protocol of OTA. We chose a simple classical method consisting of solubilization of OTA in aqueous acidic medium, due to its polarity, cleanup on celite (diatomaceous earth) and LLE in a mixture of organic solvents: ethyl acetate – methanol – acetic acid (95:5:0.5, v/v/v). The residues obtained by evaporation to dryness were

then analyzed. The mode of sample preparation did not cause significant ion suppression, the limit of quantification (0.45 ng/mL) being sufficiently low to allow the analysis of OTA in foodstuff.

The developed and optimized LC-MS/MS method was validated in accordance with international regulations [17, 18]. Representative chromatograms of a blank sample and a sample with OTA at LLOQ are shown in Figure 7. No interfering peaks from the endogenous components of sample matrices were observed in the retention time of OTA.

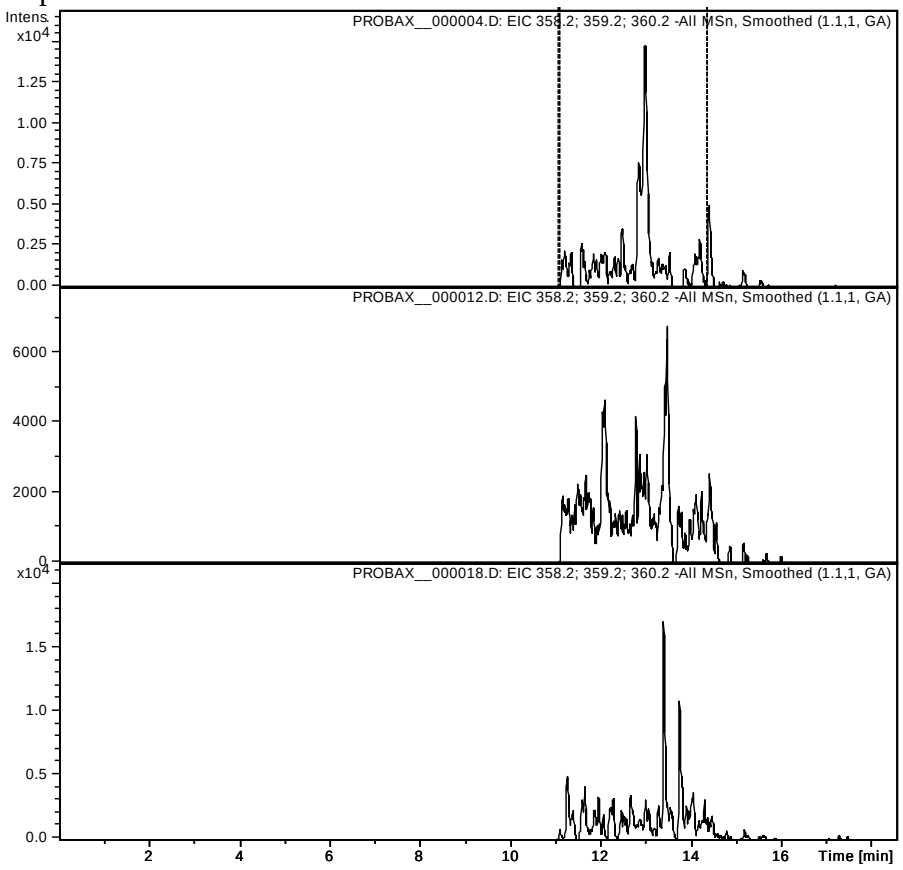

Figure 7.

Chromatograms of (up) a bread sample (found concentration: 1.88 ng/mL), (middle) a sample of breakfast cereals (found concentration: 0.59 ng/mL), and (down) a sample of spices (found concentration: 0.98 ng/mL).

The calibration curves were linear over the concentration range of 0.45 – 36.0 ng/mL with a correlation coefficient greater than 0.998, and with appropriate precision and accuracy. The values obtained for intra-day and inter-day precision and accuracy during the validation are shown in

Tables I and II, respectively. All values for accuracy and precision were within recommended limits.

Table I

The intra-day precision (CV %) and accuracy (bias %) for the measurement of OTA (the analysis of five different samples, n = 5)

Nominal concentration (ng/mL)	Found concentration mean		CV (%)	Bias (%)
	ng/mL	± SD		
0.45	0.42	0.02	4.9	-6.0
0.90	0.87	0.09	10.0	-2.8
4.50	4.72	0.24	5.1	4.8
18.00	17.34	0.19	1.1	-3.7

Table II

The inter-day precision (CV %) and accuracy (bias %) for the measurement of OTA (one analysis on five different days, n = 5)

Nominal concentration (ng/mL)	Found concentration mean		CV (%)	Bias (%)
	ng/mL	± SD		
0.45	0.41	0.02	5.1	-9.7
0.90	0.88	0.08	9.6	-2.8
4.50	4.85	0.12	2.5	7.8
18.00	19.00	1.74	9.1	5.6

In all food samples analyzed for OTA contamination, levels are below the maximum admitted limits for OTA approved by the EU (Table III). The highest levels of OTA were determined in bread and seeds. A very heterogeneous distribution of OTA in the products analyzed can be observed, as specified in the literature [4]. The results are in accordance with OTA levels reported in foodstuff analyzed in other countries such as Spain [19], Portugal [20], Croatia [21], Morocco [22].

Table III

Concentration of OTA (ng/kg) found in analyzed food samples

Sample	**n**	**Mean concentration**	**Concentration range**
Bread	5	6.26	3.80 - 11.28
Fruits	6	< LOQ	< LOQ
Breakfast cereals	5	1.06	< LOQ - 3.52
Spices	4	1.48	< LOQ - 5.90
Beer	5	< LOQ	< LOQ
Cereals	8	1.17	< LOQ - 4.67
Seeds	13	3.39	< LOQ - 18.42

Conclusions

We elaborated and validated a simple and efficient LC-MS/MS method for quantification of ochratoxin A (OTA) in foods. The method has a good sensitivity and allows the quantification of low levels of OTA, the order of ppb. This method was successfully applied for OTA determination in several food samples from the Romanian market. It can be also applied in the monitoring of food safety and assessment of exposure to OTA through food.

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