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Semi-automated solid-phase extraction method for studying the biodegradation of ochratoxin A by human intestinal microbiota

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ABSTRACT

A simple and rapid semi-automated solid-phase (SPE) extraction method has been developed for the analysis of ochratoxin A in aqueous matrices related to biodegradation experiments (namely digestive contents and faecal excreta), with a view of using this method to follow OTA biodegradation by human intestinal microbiota. Influence of extraction parameters that could affect semi-automated SPE efficiency was studied, using C18-silica as the sorbent and water as the simplest matrix, being further applied to the matrices of interest. Conditions finally retained were as follows: 5-mL aqueous samples (pH 3) containing an organic modifier (20% ACN) were applied on 100-mg cartridges. After drying (9 mL of air), the cartridge was rinsed with 5-mL H_2O/ACN (80:20, v/v), before eluting the compounds with 3× 1 mL of MeOH/THF (10:90, v/v). Acceptable recoveries and limits of quantification could be obtained considering the complexity of the investigated matrices and the low volumes sampled; this method was also suitable for the analysis of ochratoxin B in faecal extracts. Applicability of the method is illustrated by preliminary results of ochratoxin A biodegradation studies by human intestinal microbiota under simple *in vitro* conditions. Interestingly, partial degradation of ochratoxin A was observed, with efficiencies ranging from 14% to 47% after 72 h incubation. In addition, three phase I metabolites could be identified using high resolution mass spectrometry, namely ochratoxin α, open ochratoxin A and ochratoxin B.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced as a metabolite by several fungi of the genera *Aspergillus* and *Penicillium* [1,2]. It has received particular attention due to its nephrotoxic, immunotoxic, teratogenic and carcinogenic effects (it is considered as a possible carcinogen for humans (group 2B) by the International Agency for Research on Cancer) [3]. It is of health concern due to its widespread occurrence in food and feed chain [4]; thus, assessing the fate of this contaminant during digestion is of prime importance. Surprisingly, even though it is now well established that OTA is efficiently degraded in the rumen (with major role of protozoa as well as bacteria as recently reported) [5–7], little is known about its fate during human digestion. So far, based on *in vitro* studies, it is considered to be partly absorbed by the small intestinal epithelium but, during digestion, OTA bioaccessibility may differ greatly depending on the food matrix as illustrated by a recent study focusing on the bioacessibility of OTA from food during *in vitro* digestion experiments [8]. Consequently, OTA may directly reach the human colon during digestion; also, part of absorbed OTA is excreted in the bile as conjugates, and such compounds are prone to release free OTA in the large intestine upon hydrolysis as reported [9]. Hence, OTA may exert its toxicity in the colon or be further subjected to microbial degradation as the human gut microbiota is now well-accepted to play a key-role during digestion. To the best of our knowledge, only one study reported its partial degradation by the human gut microbiota (derived from a Japanese health adult) [10].

In order to be able to investigate the metabolic fate of OTA in the presence of human gut microbiota, we developed a rapid and simple suitable analytical method aimed at analysing OTA with satisfactory recoveries in matrices of interest (namely digestive contents and faecal excreta); ochratoxin B (OTB) was considered along with OTA during this analytical development as it may be interesting to investigate its biodegradation as well in the future since this mycotoxin is also of health concern and present in the food chain [11]. In recent years, several analytical methods have been reported for OTA in biological matrices, such as urine [12–16], blood [14,16,17], or faeces [18]. Most developed methods

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use manual solid-phase extraction or immunoaffinity columns to selectively retain OTA and remove matrix interferent compounds. However they present several drawbacks: manual SPE is tedious and time-consuming, entailing personal costs; immunoaffinity columns are quite expensive, not reusable and present limited lifetime. In addition, OTB was found to interfere with OTA antibodies on immunoaffinity columns [19]. Consequently we found interesting to develop a rapid and simple semi-automated SPE method, using common C18-silica cartridges, in order to offer a rapid and cheap method to be used for monitoring OTA biodegradation; such reversed-phase cartridges were reported to be efficient for the analysis of OTA in aqueous beverages (wine, beer) [20–23]. For the same reason, classical liquid chromatography-fluorescence detection (HPLC-FLD) was used to determine OTA in the final extracts, to avoid the high acquisition and operation costs related to the coupling of liquid chromatography to mass spectrometry [20].

The analytical method was assessed based on parameters of limits of detection and quantification, linearity and precision as well. Recoveries and limits of quantification were determined in the different aqueous matrices of interest (digestive contents and faecal excreta). To illustrate the applicability of the method, we also present preliminary results of OTA biodegradation by human intestinal microbiota under simple *in vitro* conditions.

2. Materials and methods

2.1. Chemicals and reagents

Crystalline OTA and solution of OTB ($50\,mg\,L^{-1}$ in benzene/acetic acid 99:1, v/v) were obtained from Sigma (Saint-Quentin-en-Yvelines, France). A first stock solution of OTA was prepared by dissolving 1 mg in 1 mL HPLC-grade acetonitrile to yield a concentration of $1\,g\,L^{-1}$. Working standard solutions of analytes were prepared by dilution with mobile phase in the concentration range of 20–4000 $\mu g\,L^{-1}$. They were used over a period of 3 months and stored at −20 °C. Deionized water was produced with a Milli-Q system from Millipore (Saint-Quentin-en-Yvelines, France). HPLC-grade methanol (MeOH), acetonitrile (ACN), acetic acid and tetrahydrofuran (THF) were all from Carlo Erba (Val-de-Reuil, France). Analytical quality acetone and dichloromethane (DCM) were also from Carlo Erba. Quantification of compounds was performed by HPLC-FLD using an external calibration.

2.2. Samples

Faeces were collected from rats fed on a standard rodent diet, and frozen at −20 °C until analysis. Frozen faeces were lyophilized, homogenized with a mortar and pestle, and 1 g was transferred into a 200-mL Erlenmeyer flask. A volume of 100 mL sterile phosphate buffer ($0.2\,mol\,L^{-1}$, pH 7.0) containing yeast extract ($0.2\,g\,L^{-1}$) (PYE buffer) was added and samples were sonicated for 30 min. The particulate material was removed by centrifugation for 10 min at $8000 \times g$ (at 4 °C). 1 mL of the supernatant was then used for subsequent SPE experiments.

Fresh stools from three healthy female volunteers (40–60 years old) were collected (named "Microbiota 1", "Microbiota 2" and "Microbiota 3"). All donors were on a Western-type diet and none had history of digestive pathology nor had received antibiotics for the last 3 months. About 2 g of fresh stools were transferred into an anaerobic glove box, where they were diluted 100-fold in sterile phosphate buffer ($0.2\,mol\,L^{-1}$, pH 7.0) with added yeast extract ($0.2\,g\,L^{-1}$) (PYE buffer) and thoroughly homogenized with an Ultra-Turrax blender.

Colon suspensions were taken from the descending colon vessel of the simulator of the human intestinal microbial ecosystem (SHIME) (from the LabMET, Ghent University, Belgium); aliquots of 1 mL were used for further SPE experiments. This dynamic model of the human gastrointestinal tract consists of a succession of three compartments (kept at 37 °C) simulating (i) the stomach and small intestine, (ii) the ascending colon, and (iii) descending colon, respectively [24]. The first compartment is based on the fill-and-draw principle to simulate different steps in food uptake and digestion, with peristaltic pumps adding a defined amount of SHIME feed solution [25] and pancreatic and bile liquid to the stomach-small intestine compartment, and emptying the compartment after specified intervals. The two last compartments are continuously stirred with constant volume and pH control, the pH being maintained in the range 5.6–5.9 and 6.5–6.9 in the ascending and descending colon, respectively, by the addition of $0.1\,mol\,L^{-1}$ HCl or $0.1\,mol\,L^{-1}$ NaOH. Both colon compartments contained an *in vitro* cultured microbiota that was isolated from human faeces. There was no gas exchange between the different vessels and the headspace of the culture system was flushed once a day for 15 min with N_2 to ensure anaerobic conditions.

2.3. Biodegradation batch experiments

Human faecal suspensions in PYE buffer were supplemented with OTA to give final concentrations of 100 or $2.5\,\mu mol\,L^{-1}$. 15 mL fractions were distributed in glass vials and incubated outside the anaerobic glove box in a shaking water bath (150 rpm) at 37 °C for 72 h. Vials were previously tightly closed with butyl rubber stoppers and sealed with aluminium caps to maintain the anoxic environment originating from the glove box. In order to assess the extent of bacterial transformation, a number of control samples were included in the experimental setup. Firstly, sterile PYE buffer supplemented with OTA was carried out to check the stability of the substrate over time. Secondly, resting-cell suspensions non-spiked with OTA were analysed to check the absence of co-eluting peaks as well as the absence of native OTA in the faeces. Finally, resting-cell suspensions were autoclaved (120 °C, 20 min) before the addition of OTA to ascertain that the disappearance of the substrate could be attributed to the metabolic activity of viable cells and not to a passive adsorption on bacterial cell walls [26]. All experiments were performed in duplicate.

Incubation media were sampled at intervals of 24 h by sterile puncture through the butyl rubber stoppers. Samples (2 mL) were centrifuged ($8000 \times g$, 10 min, 4 °C), and the supernatants were kept at −20 °C until SPE treatment and HPLC-FLD analysis. Additional analysis using LC–MS/MS was also performed to identify possible metabolites.

2.4. Sample preparation and SPE equipment

Experiments were carried out using the semi-automated SPE system ASPEC GX-271 (Gilson, Villiers-le-Bel, France) equipped with a 10-mL syringe and 401 Dilutor, programmed by Trilution LH software. C18-silica cartridges were obtained from Chromoptic (Courtaboeuf, France). Experimental conditions were as follows, unless otherwise stated in the text: cartridges (100 mg, 1 mL) were conditioned with 3 mL MeOH followed by 3 mL Milli-Q water adjusted to pH 3; then 5 mL of aqueous samples (pH adjusted to 3) containing an organic modifier (20% ACN) were applied (at $1.5\,mL\,min^{-1}$). After drying with 9 mL of air, the cartridge was rinsed with 5 mL of H_2O/ACN (80:20, v/v), before eluting the compounds with 3× 1 mL of MeOH/THF (10:90, v/v) (at $1.5\,mL\,min^{-1}$). The eluate was evaporated to near 200 µL with nitrogen before being further diluted to 500 µL with mobile phase. The final volume of each extract was determined by weighing (in preliminary

experiments, we checked that there was no significant matrix effect on the weight of the final extract).

2.5. Determination of the SPE conditions

In order to find the best SPE conditions for aqueous samples, various parameters were tested such as sample pH and volume, sorbent mass, nature of rinsing solvent, volume and composition of the eluting solvent. Extraction efficiency was assessed by determining percentage recoveries from spiked aqueous samples at the individual 100 $\mu g\,L^{-1}$ level with OTA and OTB. Comparison of mean recoveries between two experiments was carried out using the Student's *t*-test (by previously assessing the equality of variances with the Fisher's test). For comparison of more than two experiments, a one-way ANOVA test was used (at a α risk of 0.05). When ANOVA indicated significant differences, experiments were compared in pairs with the Tukey (HSD) multiple comparison test. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using XLSTAT software (version 2010.3.05). Data are expressed as mean values ± standard deviation (SD) ($n = 3$ unless otherwise stated).

2.6. HPLC-fluorescence analysis

The HPLC-fluorescence system was routinely used as the analytical tool to quantify target contaminants in the SPE extracts. It consisted of a Waters 717 plus autosampler and a Waters 1525 high-pressure gradient binary pump connected in series with a Waters 2996 diode array detector (DAD) and a Waters 2475 fluorescence detector (FLD). Data analysis was achieved using the Millenium software. Separation of the selected compounds was performed on a Cogent Bidentate C18-silica column (150 mm × 4.6 mm i.d., 4 μm particle size, MicroSolv Technology, France) maintained at 30 °C, with a pre-column filled with C18-silica material (30 mm × 2.1 mm i.d., 3 μm particle size, Supelco, France). Injection volume was 20 μL *via* a Rheodyne injection valve. Gradient elution conditions were as follows: ACN/H_2O/acetic acid (49.5:49.5:1, v/v/v) for 10 min followed by a 20 min ramp to 100% ACN; this solvent was maintained for 10 min before returning back to the initial conditions. The total flow-rate was 0.8 $mL\,min^{-1}$. Detection of ochratoxins was performed at 333 and 460 nm as excitation and emission wavelengths, respectively.

2.7. LC–high resolution mass spectrometry analysis

Samples from batch experiments were qualitatively analysed for possible metabolites. The system consisted of an Accela pump with a solvent degasser and an Accela AS autosampler. The LC separation was performed with a Cogent Bidentate C18 column (100 mm × 2.1 mm i.d., 4 μm particle size, MicroSolv Technology, France). Mobile phase consisted in solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). A gradient was run from 100% A for 5 min, switching to 100% B over 15 min and maintaining 100% B for 5 min before returning back to the initial conditions. The total flow-rate was 250 $\mu L\,min^{-1}$ and the injection volume 10 μL. The MS system was a LTQ Orbitrap Velos Thermo Scientific MS equipped with a heated electrospray ionization interface. The interface parameters were set at the following values: source voltage, 3.50 kV; sheath gas flow rate (N_2), 30 (arbitrary units); auxiliary gas flow rate (N_2), 20 (arbitrary units); capillary temperature, 275 °C. Spectra were recorded in dependent scan mode with scan event 1 recorded in HRMS full scan mode with a resolution of 100,000 uma (mass range: 100–900 uma), scan event 2 in the ion trap MS^2 on parent mass from list, and scan events 3, 4, 5 in IT MS^3, all in both positive and negative modes. The whole system was driven by Xcalibur 2.1 software and data processing was carried out with Metworks version 1.3.

2.8. Method performance: calibration, matrix effects and validation

Calibration curves were obtained by HPLC-FLD analysis of standard solutions prepared in mobile phase in the range 30–800 $\mu g\,L^{-1}$ for quantification during SPE optimization, and in the range 0.2–800 $\mu g\,L^{-1}$ for evaluating the instrumental system performance. Calibration curves were fitted by linear least-square regression. In order to investigate for matrix effects on calibration, standard addition of OTA and OTB into SPE extracts was also performed at three levels. Matrix effects were evaluated by comparing for each compound the slopes of linear calibration curves from these standard addition experiments with those obtained based on the analysis of the calibration standards at the same concentration levels; a deviation of the slopes above 10% was considered as reflecting a matrix effect. LOD and LOQ were estimated, respectively, as the concentration corresponding to three and ten times the mean signal (peak height) of the background noise for blanks ($n = 10$); they were also confirmed experimentally. Precision was evaluated by measuring the within-day ($n = 5$) and between-days ($n = 15$) repeatability, expressed by means of the relative standard deviations (RSDs) on peak areas at two different concentrations (50 and 400 $\mu g\,L^{-1}$).

3. Results and discussion

As previously stated, the aim of this study was to develop a simple SPE procedure using the ASPEC SPE automate for quantitative recovery of OTA in aqueous matrices of interest for further studying OTA biodegradation by the human intestinal microbiota. We found interesting to include OTB in the SPE method development, as it is also frequently present in food along with OTA.

3.1. Optimization of the SPE procedure

We used the strategy of one-variable-at-a-time optimization approach, because of its easy implementation and interpretation of results; the factors evaluated include sample pH and volume, sorbent mass, composition of the eluting and washing solvents.

3.1.1. Sample pH and volume

The sample pH plays an important role in the SPE extraction efficiency when dealing with ionisable compounds like OTA. Considering its pK_a values (4.4 and 7.5), we tested two distinct pH values (3.0 and 6.5) for water samples using 5 mL spiked water/ACN (80:20, v/v) sample, 100 mg sorbent, and elution with 3× 1 mL MeOH/THF (10:90, v/v) (no rinsing). A statistical difference was observed for both OTA and OTB ($P < 0.05$), with the undissociated form (pH 3.0) being preferably retained on the C18-silica sorbent (mean recoveries: 89.5% and 81.1% for OTA and OTB, respectively) as compared to their ionic form (pH 6.5) (mean recoveries: 76.4% and 37.9% for OTA and OTB, respectively). pH values lower than 3.0 were not investigated because of the possible solubility of silica which could be the source of interfering peaks at the beginning of the chromatogram [27]; besides, at low pH values OTA degradation may occur (by acid catalysed hydrolysis, leading to phenylalanine and ochratoxin α) [28]. Accordingly, in further experiments sample pH was adjusted to 3.0 before SPE.

We evaluated the breakthrough volume by percolating increased volumes of water samples (containing 20% ACN) spiked with constant amounts of the studied compounds on the 100 mg sorbent (elution conditions: 3× 1 mL MeOH/THF (10:90, v/v), no rinsing). Increasing the volume till 50 mL led to significant losses of

both ochratoxins ($P < 0.05$). Indeed, 66% and 20% losses of, respectively, OTB and OTA occurred when loading till 50 mL of sample. This effect could be due to a shift in the adsorption/desorption equilibrium, favouring desorption from the cartridge with increased sample volume. As best recoveries were observed with 5-mL samples, this volume was finally retained.

3.1.2. Sorbent mass

The sorbent mass was investigated in the range of 25–100 mg using 5 mL spiked water/ACN (80:20, v/v) sample and elution with 3× 1 mL MeOH/THF (10:90, v/v) (no rinsing). With 25-mg cartridges, recoveries were significantly lower for OTB ($P < 0.05$), probably due to a sample volume exceeding the breakthrough volume in that case. No significant effect was observed for OTA ($P > 0.05$), this compound being more retained on the sorbent as compared to OTB. Overall, recoveries for OTB were improved with the higher sorbent mass, and 100 mg was selected for further experiments.

3.1.3. Eluting and washing solvents

Different eluting solvents were tested, namely ACN, DCM and MeOH/THF (10:90, v/v) using 5 mL spiked water/ACN (80:20, v/v) sample percolated on 100-mg cartridge and elution with 3× 1 mL eluting solvent (no rinsing). Better recoveries (along with acceptable SDs) were obtained with MeOH/THF (10:90, v/v) (mean recoveries in the range 81–90%, as compared to 70–91% and 68–75% with ACN and DCM, respectively). To further investigate the volume of the eluting solvent necessary for complete elution of analytes, cartridges were eluted with 6× 1 mL of MeOH/THF (10:90, v/v). Fractionations of the elution revealed no further enhancement in recoveries above 3× 1 mL of MeOH/THF (10:90, v/v); thus, the retained elution conditions were finally 3× 1 mL with MeOH/THF (10:90, v/v).

To reduce as much as possible matrix effects for complex aqueous samples (such as colon suspensions) and to protect the HPLC column, we tested different washing solvents using H_2O/ACN mixtures (5 mL), with percentages of ACN up to 50%, and with water pH adjusted to 3 to prevent elution of the analytes of interest (other conditions: 5 mL spiked water/ACN (80:20, v/v) sample; 100-mg cartridge; elution with 3× 1 mL MeOH/THF (10:90, v/v)). The use of 50% of ACN was unacceptable due to almost complete loss for ochratoxins. Even though overall better recoveries were obtained without any washing step (average recoveries of 85%), we decided to include a rinsing step using 5 mL of H_2O/ACN (80:20, v/v), as losses were still moderate under such conditions (around 5%, being similar to those observed using rinsing with water alone). Such a rinsing step is recommended when dealing with complex matrices (such as colon suspensions), to remove matrix compounds that may reduce the performances of the analytical column over time.

3.2. Instrumental system performance

Quality parameters such as limits of detection (LOD) and quantification (LOQ), precision and linearity were evaluated for the HPLC-FLD system. Within-day repeatabilities were near 2.1% for both compounds, and between-days repeatabilities around 5.5–6.0%, showing thus acceptable reproducibility. Calibration curves were established using least-square linear regression from analysis of external calibration standard solutions at concentrations ranging from 0.2 to 800 $\mu g\,L^{-1}$; R^2 values were higher than 0.9996, which indicates acceptable linearity of the response. For both ochratoxins, no matrix effect occurred. LOQs were estimated to be 0.0232 and 0.0386 ng injected for OTA and OTB, respectively; this compares favourably with a previous study reporting a LOQ of 0.065 ng injected for OTA using HPLC-FLD [29].

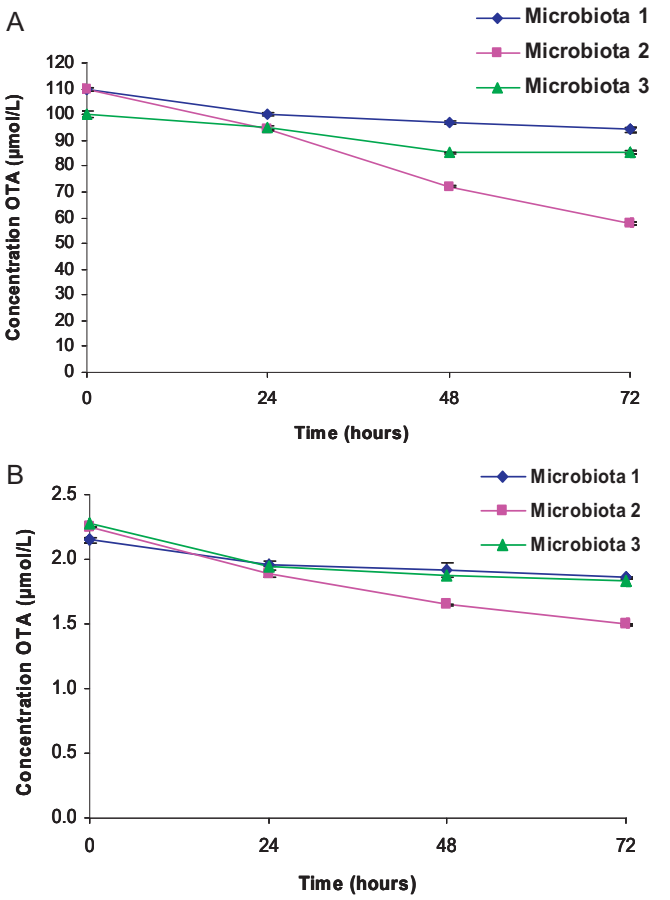

Fig. 1. Degradation kinetics of OTA during anaerobic incubation with faecal suspensions derived from three different donors, at initial OTA concentration of 100 $\mu mol\,L^{-1}$ (A) and 2.5 $\mu mol\,L^{-1}$ (B).

3.3. Applicability to various aqueous matrices

Aqueous matrices of interest for further biodegradation studies (namely faecal extracts and colon suspensions from the SHIME) were spiked to estimate the recoveries of the studied compounds in the presence of matrix interferences. As depicted in Table 1 average recoveries were similar in faecal extracts as compared to water, with values in the range 90–92 and 70–77% for OTA and OTB, respectively. For colon suspensions, the analytical method was still suitable in the case of OTA (even though a slight decrease was noted) but important losses of OTB were encountered. As OTB was less retained on the C18-silica sorbent as compared to OTA, losses of OTB could be explained by this compound being carried along with matrix constituents during the SPE step. Nevertheless, as our aim was to further investigate only OTA biodegradation due to its major occurrence in food and its higher toxicity, we decided to go on with that developed semi-automated SPE method.

For all these aqueous matrices, LOQs were also evaluated by analysing SPE extracts from unfortified samples ($n = 7$). Results are summarized in Table 1. LOQ values for OTA were near 1 $\mu g\,L^{-1}$, which is acceptable taking into account the complexity of the matrices considered and the low sample volumes used (*i.e.* 1 mL). This compares favourably with values reported in another study for OTA in urine (0.9 and 1.5 $\mu g\,L^{-1}$ depending on the method used) [15]. This sensitivity is sufficient to use our method in biodegradation studies. Increasing the sample volume before SPE should lower LOQ values for the overall method, but sampling batches over time should be as low as possible to maintain the batch volume quite constant. LOQ values for OTB were near twice the values for OTA;

Table 1
Recoveries and limits of quantification achieved for OTA and OTB in aqueous matrices of interest for biodegradation studies.

Compounds	Water		Faecal extract		Colon suspension	
	Recovery (%) ± SD	LOQ ($\mu g L^{-1}$)	Recovery (%) ± SD	LOQ ($\mu g L^{-1}$)	Recovery (%) ± SD	LOQ ($\mu g L^{-1}$)
OTB	70.8 ± 1.5	1.88	77.1 ± 1.1	2.22	26.1 ± 6.4	2.29
OTA	90.1 ± 0.8	0.64	91.9 ± 0.5	1.25	86.5 ± 2.6	1.44

this could be due to the earlier elution of OTB from the analytical column, so that it was less efficiently separated from non-retained matrix interferents.

3.4. Application to OTA degradation by human intestinal microbiota

Incubation of OTA in the presence of human intestinal microbiota revealed biodegradation of this contaminant as shown in Fig. 1. A greater degradation occurred with the faecal inoculum "Microbiota 2", with average OTA degradation of 47% and 34% at, respectively, 100 and 2.5 $\mu mol L^{-1}$. When similar experiments were performed with faecal material inactivated prior to incubation, no decrease in OTA concentration was observed, neither with sterile PYE buffer supplemented with OTA. Hence, it can be assumed that the degradation observed is attributed to the metabolic activity of the human intestinal microbiota. The different efficiencies of faecal microbiota to degrade OTA illustrate the interindividual variability in the microbial community and activity; possible interindividual differences in OTA degradation by gastrointestinal microbial was also suggested [16]. Interindividual differences in microbial metabolic activities are not uncommon: a striking example is the microbial conversion of the dietary phytoestrogen daidzein into the biologically more active equol [30,31].

OTA metabolites were investigated by way of LC–HRMS analyses. Three phase I metabolites, namely ochratoxin α (OTα), OTB and open ochratoxin A (OP-OTA), could be identified in samples after 24 h of incubation for all faecal microbiota tested. Their identity was confirmed by the extraction of the accurate mass of the $[M+H]^+$ and $[M+Na]^+$ adducts from high resolution full mass spectra (see chromatograms presented in Fig. 2). OTα has been frequently reported as the main metabolite, especially in human urine and blood [12,16]; it was also reported as a microbial metabolite of OTA in rumen and in rat gastrointestinal tract [6,9,18]. A few studies mentioned the formation of OP-OTA, but in animals only to our knowledge [9,32,33]. On the opposite, OTB has been rarely reported as a metabolite, again in animals only [2,9,34]; its presence in our samples was confirmed both by analysis of a standard and standard addition in the extracts. In any case, this is the first time that these metabolites are detected *in vitro* as microbial metabolites derived from human faeces. Thus, OTA could be metabolized by the human gut microbiota through the hydrolysis of the peptidic bond (formation of OTα), the dechlorination of the isocoumarin moiety (formation of OTB) or the opening of the lactone ring (formation of OP-OTA) as illustrated by Fig. 3; as expected with such *in vitro* incubations, phase II metabolites remained undetected. OTα and OTB have been reported to be less toxic than OTA [35], whereas OP-OTA has been recently reported to be more toxic [34]. As a consequence, processes that lead to the conversion of OTA into OTα and OTB are considered to be one of the main routes for OTA detoxification.

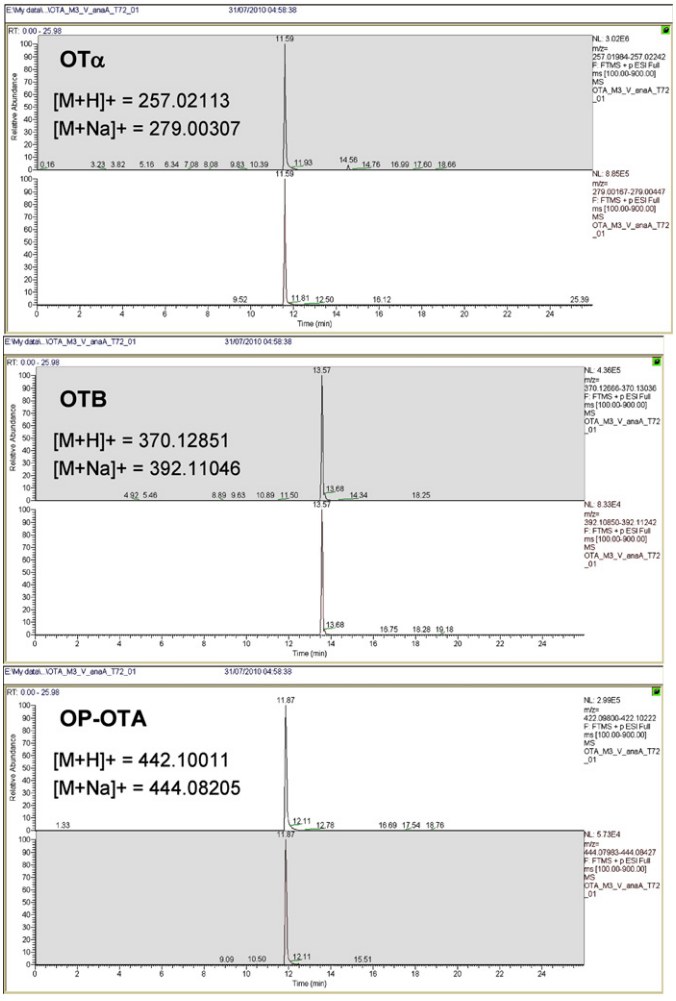

Fig. 2. Extracted ion chromatograms of the $[M+H]^+$ and $[M+Na]^+$ adducts of OTα, OTB and OP-OTA from their respective high resolution full mass spectra.

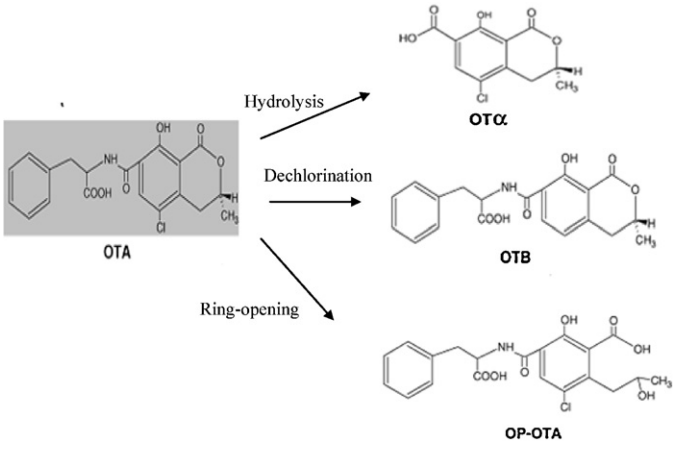

Fig. 3. OTA biodegradation pathway by the human gut microbiota according to the metabolites identified in this work.

4. Conclusion

A fast, simple, convenient and reliable sample preparation procedure has been developed for the extraction of OTA from faecal extracts and colon suspensions using a semi-automated SPE apparatus; such a semi-automated method increases sample

throughput and reduces exposure to potential hazardous compounds. Acceptable LOQs and calibration curves could be obtained considering the high level of complexity of the matrices considered. The proposed method has been successfully applied to follow the biodegradation of OTA by human intestinal microbiota *in vitro*. Partial degradation of OTA was observed; this biodegradation was further confirmed by the identification of three phase I metabolites (OTα, OTB and OP-OTA) using LC–HRMS analysis. In the future, we intend to look at the formation of these metabolites in more physiologically relevant conditions, namely in the SHIME and in human microbiota associated rats.

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