



Bioavailability, metabolism, and excretion of a complex *Alternaria* culture extract versus altertoxin II: a comparative study in rats

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Abstract

Despite the frequent infection of agricultural crops by *Alternaria* spp., their toxic secondary metabolites and potential food contaminants lack comprehensive metabolic characterization. In this study, we investigated their bioavailability, metabolism, and excretion in vivo. A complex *Alternaria* culture extract (50 mg/kg body weight) containing 11 known toxins and the isolated lead toxin altertoxin II (0.7 mg/kg body weight) were administered per gavage to groups of 14 Sprague Dawley rats each. After 3 h and 24 h, plasma, urine and feces were collected to determine toxin recoveries. For reliable quantitation, an LC–MS/MS method for the simultaneous detection of 20 *Alternaria* toxins and metabolites was developed and optimized for either biological matrix. The obtained results demonstrated efficient excretion of alternariol (AOH) and its monomethyl ether (AME) via feces (> 89%) and urine (> 2.6%) after 24 h, while the majority of tenuazonic acid was recovered in urine (20 and 87% after 3 and 24 h, respectively). Moreover, modified forms of AOH and AME were identified in urine and fecal samples confirming both, mammalian phase-I (4-hydroxy-AOH) and phase-II (sulfates) biotransformation in vivo. Despite the comparably high doses, perylene quinones were recovered only at very low levels (altertoxin I, alterperyleneol, < 0.06% in urine and plasma, < 5% in feces) or not at all (highly genotoxic, epoxide-holding altertoxin II, stemphylytoxin III). Interestingly, altertoxin I was detected in all matrices of rats receiving altertoxin II and suggests enzymatic de-epoxidation in vivo. In conclusion, the present study contributes valuable information to advance our understanding of the emerging *Alternaria* mycotoxins and their relevance on food safety.

Keywords *Alternaria alternata* · Emerging mycotoxins · In vivo metabolism · Liquid chromatography tandem mass spectrometry · ADME · Perylene quinone

Abbreviations

ADME	Absorption, distribution, metabolism, excretion
LC	Liquid chromatography

SRM	Selected reaction monitoring
MS	Mass spectrometry

Alternaria toxins

AA-III	Altenuic acid
ALT	Altenuene
ALP	Alterperyleneol
AME	Alternariol monomethyl ether
AME-3-Glc	Alternariol monomethyl ether-3-glucoside
AME-3-Sulf	Alternariol monomethyl ether-3-sulfate
AOH	Alternariol
AOH-3-Glc	Alternariol-3-glucoside
AOH-3-Sulf	Alternariol-3-sulfate
AOH-9-Glc	Alternariol-9-glucoside
ALS	Altenusin
AST	Altersetin
ATX-I	Altertoxin I
ATX-II	Altertoxin II

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isoALT	Isoaltenuene
STTX-III	Stemphytoxin III
TeA	Tenuazonic acid
TEN	Tentoxin

Solvents and chemicals

ACN	Acetonitrile
HAc	Acetic acid
MeOH	Methanol
NH ₄ Ac	Ammonium acetate
NH ₄ F	Ammonium fluoride

Introduction

Alternaria mycotoxins can contaminate a broad variety of agricultural crops intended for human consumption. These include cereals like wheat, barley, and oats, as well as tomatoes, peppers, sunflower seeds, and fruits (Ostry 2008; Scott 2001; Solfrizzo 2017). Commonly known as prevalent plant pathogens, fungal species belonging to the genus *Alternaria* are producing these toxins as secondary metabolites. Despite their considerable adverse effects demonstrated in vitro, no legally binding guidelines or maximum levels for food or feed exist in the European Union to date (EFSA 2016). The lack of both, comprehensive occurrence data and toxicological characterization of *Alternaria* toxins, is a reason for incomplete exposure and risk assessment. Particularly data providing thorough toxicological in vivo characterization is scarce. *Alternaria* spp. are ubiquitously found in the environment and capable of spreading and proliferating even at low temperatures. Hence, plant or crop infestation can occur pre- and post-harvest, i.e. in the orchards or during transport, storage as well as after food processing. In fact, crop spoilage results in considerable economic losses worldwide.

Due to their highly diverse chemical structures, *Alternaria* toxins show a wide range of biological effects comprising cytotoxicity, genotoxicity, mutagenicity, and estrogenicity. However, most studies published to date, focus on single or a low number of these toxic molecules. Even the most recent dietary exposure assessment performed by the European Food Safety Authority (EFSA (2016)) included only the four key toxins alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), and tentoxin (TEN). Available occurrence data were evaluated using the TTC approach (thresholds of toxicological concern) indicating that human exposure to AOH and AME may exceed the TTC of 2.5 ng/kg body weight, while this was not the case for TEN and TeA (TTC of 1500 ng/kg body weight). The comparatively low TTC value for AOH and AME derives from their genotoxic and mutagenic effects (Brugger et al. 2006) related to topoisomerase I and II poisoning (Fehr et al. 2009). Furthermore, these compounds demonstrated

estrogenic activity (Dellafiora et al. 2018; Lehmann et al. 2006). No genotoxic effects were described for TEN and TeA. TeA is reported to be acutely toxic to mice, rats and dogs (Griffin and Chu 1983; Pero et al. 1973), while TEN is a phytotoxin inhibiting photophosphorylation in chloroplasts (Arntzen 1972). The group of perylene quinones including altertoxin I (ATX-I), II (ATX-II), and III (ATX-III), alterperyleneol (ALP), and stemphytoxin III (STTX-III), gained increasing interest because of their high genotoxic potential. Especially ATX-II exhibited more potent genotoxic effects compared to AOH (Fleck et al. 2012; Schwarz et al. 2012).

Only limited data describing biotransformation of *Alternaria* toxins in plants and mammals is available in the literature. In general, biologically modified toxins often remain undetected by targeted analysis, while still contributing to the total contamination burden (Berthiller et al. 2013). Modified mycotoxins may still have toxicological impact and certain modifications can be reverted by hydrolysis during mammalian digestion. In phase-I metabolism, AOH and AME are hydroxylated at various positions as observed in microsomal incubations of rat, human, and porcine liver (Pfeiffer et al. 2007), in incubation with recombinant cytochrome P450 isoforms (Pfeiffer et al. 2008), and in rat liver slices (Burkhardt et al. 2011). In vivo, hydroxylated AOH metabolites have been identified in blood and feces of mice (Schuchardt et al. 2014). This study reported generally low systemic absorption and effective fecal (85–91%) and urinary (up to 9%) excretion rates of the orally administered radiolabeled AOH after 24 h. Similar results of poor absorption were reported for ¹⁴C-AME in Sprague Dawley rats (Pollock et al. 1982). Moreover, Sarkanj et al. (2018) reported AOH (up to 0.2 ng/mL) for the first time in human urine samples as new biomarkers of exposure. Catechols formed by hydroxylation of AOH and AME may be further methylated by catechol-O-methyltransferase (Pfeiffer et al. 2007; Tiessen et al. 2017), while AME was also reported to be demethylated by microsomes of rat, human, and porcine liver (Pfeiffer et al. 2007). However, AOH and AME are thermally and chemically rather stable in food matrices and therefore persistent during food processing (Estiarte et al. 2018; Siegel et al. 2010). Other dibenzopyrones are altenuene (ALT) and its isoform isoaltenuene (isoALT). Hydroxylation and methylation of catechols were confirmed also for these compounds in vitro (Pfeiffer et al. 2009a). However, no metabolism was observed for AOH, AME and ALT by human fecal microbiota (Lemke et al. 2016). Importantly, several conjugation products (phase-II metabolism) were reported for AOH and AME. Glucuronidation was confirmed for rat, human and porcine metabolism in vitro (Pfeiffer et al. 2009b), in Caco-2 cells (Burkhardt et al. 2009), in rat liver slices (Burkhardt et al. 2011), and mouse hepatoma cell lines (Burkhardt et al. 2012). Sulfation was reported for Caco-2 cells, rat liver cytosol (Burkhardt et al. 2009),

and rat liver slices (Burkhardt et al. 2011), but also the fungus *Alternaria alternata* itself is capable of synthesizing this metabolite (Soukup et al. 2016). With respect to plant metabolism, glycosylations including β -D-glycopyranosides and 6'-malonyl-glucopyranosides were identified in tobacco BY-2 suspension cell culture (Hildebrand et al. 2015) and glucosylations of the sulfate conjugates in tomato tissue (Soukup et al. 2016). Rapid and nearly complete urinary excretion was confirmed for the comparatively polar tenuazonic acid (TeA) (Asam et al. 2013).

Very little is known about metabolic pathways of perylene quinones. The highly reactive epoxide groups of ATX-II and STTX-III has been suggested to cause DNA adducts leading to their severe genotoxicity (Fleck et al. 2016; Schwarz et al. 2012). Chemical reactions with thiols such as glutathione have been reported and support this hypothesis (Fleck et al. 2014b; Jarolim et al. 2017). However, the precise mechanism of action has not been elucidated so far. In human cell lines (and tomato tissue in case of ATX-II), both compounds were found to be reduced to their alcohol analogues, ATX-I and ALP, respectively (Fleck et al. 2014a, b; Puntischer et al. 2019c). Moreover, significant degradation or chemical reactivity of ATX-II was determined in the presence of the natural food component and the flavonoid delphinidin (Aichinger et al. 2018) and in tomato matrix (Puntischer et al. 2019c).

The aim of the present study was to elucidate *Alternaria* toxin in vivo metabolism by mimicking oral exposure of rats. Biological effects induced by a complex *Alternaria* culture extract comprising 11 quantified *Alternaria* toxins were compared to the effects of respective amounts of the isolated altertoxin II. Consequently, a major focus of this work was the development of an LC–MS/MS method for the reliable quantitation of multiple *Alternaria* toxins in different biological matrices comprising rat plasma, urine and feces. The application of this assay to the samples obtained from this experiment revealed intriguing information about the biological absorption, potential systemic availability, and the excretion rates of *Alternaria* toxins and metabolites.

Materials and methods

Chemicals and reagents

LC–MS grade acetonitrile (ACN), methanol (MeOH), and water were purchased from Honeywell (Seelze, Germany), ammonia solution (25% in water), ammonium acetate and formate from Sigma Aldrich. For rice extraction and preparative HPLC, Milli-Q water, MeOH (HPLC grade) and acetic and formic acid (p.a.) from Sigma Aldrich (Steinheim, Germany) and *n*-hexane (p.a.) from Carl Roth GmbH (Karlsruhe, Germany) were used. Ethyl acetate (anhydrous, 99.8%) was purchased from Sigma Aldrich (Steinheim,

Germany). *Alternaria* toxin reference materials were acquired as follows: TeA and TEN were purchased from Sigma Aldrich (Steinheim, Germany), AOH and AME from Toronto Research Chemicals (Ontario, Canada) and ALS from Eubio (Vienna, Austria). ALT, isoALT and AA-III were kindly provided by Prof. Joachim Podlech (Institute of Organic Chemistry, Karlsruhe Institute of Technology, Germany) (Altemöller et al. 2006; Nemecek et al. 2013), AOH-3-Glc, AOH-9-Glc, AOH-3-Sulf, AME-3-Glc, and AME-3-Sulf were a gift from Dr. Hannes Mikula (Institute of Applied Synthetic Chemistry, Vienna University of Technology (TU Wien), Vienna, Austria) (Mikula et al. 2013). ATX-I, ATX-II, STTX-III and ALP were isolated from in-house-cultivated fungal cultures grown on rice by an optimized protocol based on Puntischer et al. (2019b) and Schwarz et al. (2012).

Stock solutions were prepared in MeOH at final concentrations of 10–500 $\mu\text{g/mL}$. Dibenzo- α -pyrone conjugates AOH-3-Glc, AOH-9-Glc, AOH-3-Sulf, AME-3-Glc, AME-3-Sulf were dissolved in water/ACN (20/80, v/v). By combining the individual stock solutions, multi-toxin working solutions containing all analytes were prepared freshly every 4 weeks resulting in concentrations of 1–60 $\mu\text{g/mL}$. All solid reference materials and solutions were stored at -20°C .

Alternaria culture extract

Long grain rice was artificially inoculated with the *A. alternata* strain DSM 62010 (DSMZ, Braunschweig, Germany) resulting in a heavily infested black culture after 21 days as reported by Puntischer et al. (2019b). After adding cold potassium chloride solution (0.15 mM, pH 3), the rice culture was extracted with ethyl acetate. The solvent of the combined organic phases was evaporated and the residual oily extract was stored at -20°C for further experiments.

Altertoxin-II isolation

The *Alternaria* toxin ATX-II was isolated from an *Alternaria* culture extract utilizing a semi-preparative HPLC-system including a Knauer Smartline 1000 (LPG) pump, a Knauer Manager 5000 controller, a manual injector, and a diode array detector (Agilent G1315D, with preparative flow cell). The instrument was operated with ClarityChrom Software, while the chromatographic data were acquired with ChemStation analysis software. The LC system included a Phenomenex Luna column (5 μ , 250 mm \times 21.2 mm), equipped with a Phenomenex SecurityGuard column. A binary gradient program, using water (pH 3, adjusted with formic acid) as eluent A and methanol as eluent B, with a constant flow rate of 14 mL/min was used: After 1 min with 55% B, eluent B was increased to 58% within the following 45 min. Subsequently, the system was purged for 20 min

with 100% B, before it was re-equilibrated at the starting conditions (55% B) for 20 min. Injection volumes were between 1 and 7 mL. The UV detector recorded chromatograms for the following wavelengths: 254 nm and wavelengths typically absorbed by perylene quinones: 262 nm, and 280 nm (Schwarz et al. 2012). The chromatographic peak at a retention time of 35–40 min was collected and identified as ATX-II using LC–MS/MS and HPLC–UV. After removing the methanol from the combined ATX-II containing eluent fractions by the use of a rotary evaporator, the remaining aqueous phase was extracted twice with cold chloroform using a separation funnel. Subsequently, the organic phase was again evaporated resulting in a yellowish residue, which was finally reconstituted in EtOH and immediately put to -20°C until further experiments. The chemical identity and purity ($>90\%$) of ATX-II was confirmed by LC–UV, LC–MS and $^1\text{H-NMR}$.

Experimental procedure

The animal study was approved by the Austrian Federal Ministry of Education, Science and Research (Austria, GZ: BMFWF-66.009/0120-WF/II/3b/2014) according to the animal welfare act 2012, BGBl. I Nr. 114/2012 (TVG 2012). Forty-two male Sprague Dawley rats, obtained from the Institute of Laboratory Animal Science and Genetics (Medical University of Vienna, Austria), were acclimated at the Center for Biochemical Research (Medical University of Vienna, Austria) for at least 14 days before the study was conducted. An overview of the animal study design is represented in Fig. S1. The rats were randomly assigned to one of the three test groups (each consisting of 14 rats) and received a single administration by gavage (Table 1). (1) The prepared *Alternaria* culture extract was administered to the “extract group” (50 mg/kg body weight, a dose determined as appropriate in a recently published pilot study (Puntscher et al. 2019b)). (2) Isolated ATX-II was administered to the ATX-II group (0.7 mg/kg body weight, corresponding to the amount quantified in the extract). (3) A solvent control not containing any *Alternaria* toxins was administered to the “control group”. For administration, extract and ATX-II doses were dissolved in a small amount of EtOH and sunflower seed oil resulting in a total administration volume of approximately 1 mL/300 g rat. All animals received the same amount of EtOH (90 μL /300 g body weight) and oil (900 μL /300 g body weight). Animals belonging to the control group received only the respective amount of the solvent EtOH in sunflower seed oil. The absence of *Alternaria* toxins in the respective the sunflower seed oil was confirmed by a validated LC–MS/MS method (sample #07 in Puntscher et al. 2019a). Directly after administration, the animals were placed into metabolic cages of the type no. 3700M071 (diameter 23 cm, height 18 cm; from Techniplast, Varese, Italy). Both, feed and

Table 1 Administrations and physiological observations (consumption of feed and water, excretion, body temperature, weight of animals). Significant differences between the results of the ATX-II and extract groups and the control group were calculated applying an unpaired heteroscedastic Student's *t* tests and are indicated by $*p<0.05$

Experimental groups	Administration per rat BW of 300 g				Consumed		Excreted		Change of			
	Sunflower seed oil (μL)	EtOH (μL)	ATX-II (mg)	Extract (mg)	Feed (g)	Water (mL)	Urine (mL)	Urinary creatinine (mg/dL)	Feces (dry mass)		Body temp. (%)	Body weight (g)
									g	%		
3 h												
Control	900	90	–	–	0.2±0.4	0.8±0.3	0.4±0.2	49±10	0.1±0.1	38±24	–0.5±0.6	–5.2±3.4
ATX-II	900	90	0.21	–	0.1±0.2	0.7±0.4	0.6±0.6	68±14	0.2±0.2	44±16	–0.4±0.6	–6.7±1.7
Extract	900	90	0.21 ^a	15	1.1±0.5*	1.6±2.1	0.9±0.4*	55±13	0.4±0.5*	45±13	–1.1±0.3	–4.8±2.0
24 h												
Control	900	90	–	–	20.4±1.5	26.7±3.9	8.2±2.3	53±7	3.8±1.3	41±3	–0.7±0.8	5.2±3.6
ATX-II	900	90	0.21	–	21.8±1.2	27.5±3.2	9.4±1.7	55±8	4.8±0.7	43±5	–0.8±0.6	2.1±3.4
Extract	900	90	0.21 ^a	15	20.9±2.2	26.3±2.9	9.7±3.0	55±13	4.2±0.6	42±3	–1.0±0.7	1.0±2.7*

^aATX-II amount comprised in the extract, not additionally administered

water were provided ad libitum. Collection funnels under the stainless steel grid floor of the metabolic cages allowed for the separated sampling of urine and feces. Body weight and temperature, as well as the consumption of water and feed during the experiment were documented for each rat (Table 1). Per day, six animals were treated: two from each administration group, of which one was euthanized after 3 and one after 24 h (see Fig. S1 for the study design). In total, seven animals of each group were killed after 3 h and seven after 24 h. Before euthanasia by cardiac puncture and cervical fracture, the animals were anesthetized by intravenous injection of ketamine (0.1 mL/100 g rat) and xylazine (0.1 mL/rat). Blood was collected directly from the heart using EDTA rinsed syringes. Aliquots were prepared for immediate hematology (1 mL in Vacuette, measured by an automated analyzer ADVIA 2120i, Siemens AG) and for separating plasma by centrifugation (4 °C, 2500 rcf, 10 min, by a Rotana 46 RC centrifuge, Hettich AG, Germany). All samples were directly put on dry ice for transport until storage at –80 °C.

Sample preparation

Before extraction, fecal samples were lyophilized (for 24 h) to allow for quantitative analysis normalized to dry matter. The resulting dry fecal pellets were homogenized using a FastPrep-24 5G™ High Speed Homogenizer (MP Biomedicals Life Sciences, USA). Subsequently, fine powder aliquots of 20 mg were suspended in water (4 °C, 200 µL). Urine and plasma samples (200 µL) were also aliquoted on ice.

Urine, plasma, and fecal suspension (4 °C, 200 µL) were extracted by the addition of precooled extraction solvent (800 µL, ACN/MeOH/HAc, 49.5/49.5/1, v/v/v, –20 °C). After thorough homogenization by vortexing and sonication (15 min in ice bath), the samples were stored at –20 °C overnight to allow for precipitation of proteins. Subsequently, the samples were centrifuged (4 °C, 14,000 rpm, 10 min), before evaporating the supernatants (850 µL) to dryness, using a vacuum concentrator (Labconco, Missouri, USA) set to 4 °C. The resulting residues were reconstituted in 170 µL solvent (ACN/water (1:1, v/v, precooled to 4 °C) by careful vortexing and sonication (15 min in ice bath). After a final centrifugation step (4 °C, 20,000 rcf, 15 min for urine and fecal samples, 30 min for plasma samples), the supernatants were transferred to amber HPLC vials and stored at 4 °C in the LC autosampler for analysis. Thereafter, all samples were stored at –80 °C. A fully ¹³C-labeled yeast extract was added to the plasma samples before extraction as internal standard for additional high-resolution mass spectroscopy experiments (Hermann et al. 2018).

If concentration results exceeded the provided calibration range, respective samples were diluted using ACN/water (50/50, v/v) and re-measured. This was the case for AOH,

AME, TeA, and AST levels in fecal samples obtained from the extract group (dilution by a factor 10). Analogously, urine samples required a dilution by a factor of 2500 to quantify TeA. Solvent-matched calibration standards were applied for toxin quantitation in these cases. While matrix effects in highly diluted urine were neglected, they were taken into account for the less diluted fecal matrix. Hereby, the obtained results were corrected considering the signal ratio of solvent-matched and diluted fecal-matrix-matched (dilution 1–10) calibration standards.

LC–MS/MS analysis

Alternaria toxin and metabolite identification and quantitation was performed on a high-performance liquid chromatography triple quadrupole mass spectrometer system (HPLC UltiMate 3000, TSQ Vantage) coupled by a heated electrospray ionization interface (all Thermo Scientific). Chromatographic separation was achieved by the UHPLC column Acquity UPLC HSS T3 (1.8 µm, 2.1 × 100 mm, Waters, Austria) equipped with a VanGuard pre-column (1.8 µm, 2.1 mm, Waters, Austria) and a binary elution at a flow of 0.4 mL/min. The most favorable chromatographic performance was obtained by the use of an aqueous NH₄F solution (0.3 mM) as eluent A and methanol as eluent B. The instrument's autosampler and column oven were set to a temperature of 4 °C and 35 °C, respectively. For analysis, a sample volume of 5 µL was injected. To reduce matrix effects and to allow for the baseline separation of isomeric target analytes an optimized multi-step gradient was applied as follows: during the first minute, eluent B was kept at 10%, before increasing it to 30% within half a minute. Eluent B was further increased linearly to 55% until 12 min and to 100% until 16 min. After 2.5 min at 100% of eluent B, the column was re-equilibrated with the initial conditions (10% B) between 18.6 and 20 min, resulting in a total run time of 20 min. To reduce the contamination of the ion source and the MS inlet by the most polar sample fractions, a divert valve was directing the LC flow to waste between 0.5 and 1.5 min.

Validation experiments

As specified in the Commission Decision (EC) No. 657/2002 (EC 2002) and the Eurachem Laboratory Guide for the validation of analytical methods (Magnusson 2014), the method's performance was evaluated by the determination of the following validation parameters: selectivity, linearity, matrix effects, extraction recovery, limit of detection (LOD), limit of quantification (LOQ), repeatability (intraday precision, RSD_i), and intermediate precision (interday precision, RSD_R). Target analytes were quantified based on the chromatographic peak area of two respective SRM transitions

with the most favorable signal-to-noise ratio for each matrix (quantifier and qualifier transitions for the quantitation and identification confirmation, respectively). Moreover, the ion ratios between these signals and their chromatographic retention time of according reference standards were considered as further identification criteria.

Due to a lack of certified reference materials, spiking experiments of representative blank matrices were conducted at two concentration levels (“low” and “high”) to evaluate the method’s performance. Therefore, the biological samples obtained by control group animals were pooled. In case of plasma, samples of 14 control rats (7 killed after 3 h and 7 after 24 h) were combined. For blank urine and lyophilized blank feces mixtures were derived from seven control rats. Urine and fecal samples collected after 24 h were considered as more representative. Moreover, only very limited amounts of these were obtained after 3 h.

To confirm the selectivity of the selected SRM transitions, the absence of matrix signals interfering or co-eluting with target analytes has been investigated by the comparison of respective blank and spiked samples. The calibration curves (5–7 concentration levels) in neat solvent and matched with matrix were evaluated for sufficient linearity by the respective regression coefficients (R^2). Furthermore, the ratio between the slopes of the regression lines (matrix-matched vs. solvent calibration) was used to determine matrix effects potentially causing signal suppression or enhancement (SSE). The extraction recovery (R_E) of each analyte was calculated as the ratio of the concentration in spiked blank matrix samples quantified by matrix-matched calibration and the according known spiking level. It was determined at two concentration levels: the lower spiking concentration (“low”) was chosen in the range of 2–5 times the limits of quantification of the respective analyte. The second concentration level (“high”) was set higher by a factor of five. The multi-toxin working solution (see “[Chemicals and reagents](#)”) was further diluted with methanol to provide for two individual spiking solutions, of which a volume of 25 μ L was used for spiking either low or high spiking levels. Blank urine and plasma aliquots (4 °C, 200 μ L) were directly spiked, while lyophilized fecal samples (4 °C, 20 mg) were spiked before adding water (4 °C, 200 μ L, see “[Sample preparation](#)”). Before proceeding with the sample preparation, the spiked samples were kept on ice for 15 min to allow the analytes to interact with the according matrix. The whole sample preparation was carried out at 4 °C as we intentionally developed the protocol to allow for untargeted metabolomics in exactly the same set of samples. The extraction of blank matrix and spiked samples were performed in triplicate for each matrix. During a period of 3 months, this procedure was repeated three times to complete a robust in-house validation giving three independent sample extraction batches and LC–MS/MS measurements. Moreover, the

repeatability (intraday precision, RSD_I), and intermediate precision (interday precision, RSD_R) were calculated from the standard deviations of the analyte concentrations quantified in the spiked samples. Based on the analysis of blank matrices spiked at the low concentration level as well as the lowest matrix-matched calibration levels, the LOD and LOQ values were manually estimated based on a signal-to-noise ratio of three and six, respectively.

Results and discussion

Physiological observations

No visually apparent health impairments of the animals were noted before the experiment was started. Their average body weight was 309 ± 27 g and the average body temperature was 38.2 ± 0.6 °C. An overview of parameters recorded during the experiment is given in Table 1. This includes details on administration, the animals’ consumption of feed and water, excretions, and changes in body temperature and weight gain. Statistical analysis was carried out applying unpaired heteroscedastic Student’s t tests. It was noted that rats of all groups lost weight 3 h post-administration (5.7 ± 2.6 g), and gained weight after 24 h. Usually, rats consume more feed and water during night hours. However, all animals which received the complex *Alternaria* extract gained significantly less weight after 24 h (weight gain of 1.0 ± 2.7 g, $p=0.043$) than animals from the ATX-II group (2.1 ± 3.4 g) and the control group (5.2 ± 3.6 g). These results are in line with those reported in our pilot study (Puntscher et al. 2019b). The animals’ body temperature of all groups decreased by 0.7 ± 0.6 °C and 0.8 ± 0.7 °C after 3 and 24 h, respectively. This effect is assumed to derive from the animal handling before the administration and may not be directly associated to the administration itself. However, body temperatures of rats that received the extract appeared slightly lower compared to the control group while not reaching significance ($p=0.051$). Moreover, rats of the extract group consumed more water ($p=0.195$) and significantly more feed ($p=0.013$) compared to the animals of the other two groups after 3 h. They also excreted significantly more urine and feces (calculations based on the fecal dry mass). The latter was of soft and mucous consistence and partly of intensive malodor. By examining the content of the gastrointestinal tract, rats of the extract group (after 3 h) appeared to suffer from partly severe digestion problems indicated by anomalous, mucous intestinal contents. One of these rats was suffering from diarrhea, which may be considered a result of the treatment. Most animals of the extract group (24 h) also demonstrated visibly enlarged Peyer’s patches in the small intestine. Peyer’s patches (PPs) are considered as key players of the mucosal immune host response toward antigens and

bacteria of the gut (Jung et al. 2010). Albeit there are large variations in size, shape and distribution of PPs between individuals, the observation of enlarged PPs in animals treated with *Alternaria* extract might be not neglected as this could be considered a potential sign of immune response.

A commercially available creatinine assay (Abcam, Cambridge, UK) was used for colorimetric determination of urinary creatinine levels. No significant differences were observed for urinary creatinine concentrations between animals of the different groups and time points (57 ± 12 mg/dL and 54 ± 10 mg/dL for 3 h and 24 h, respectively). This may suggest no apparent impairment of the renal function. Toxin concentrations in urine were not corrected for creatinine concentrations in the present study, because the aim was to determine the absolute amounts of toxins and metabolites excreted. As expected, higher relative standard deviations were observed for quantified excretions and consumptions after 3 h compared to after 24 h. For single animals, no urine or fecal samples were obtained after the short period and some rats did not consume feed or water.

Hematology

The parameters analyzed in the differential blood test (routine WBC differential) were generally within the normal ranges and included white blood cells (WBC), neutrophils (Neut), lymphocytes (Lymph), monocytes (Mono), eosinophils (Eos), basophils (Baso), large unstained cells (LUC), lobularity index (LI), mean peroxidase index (MPXI), white blood cell peroxidase (WBCP). No significant differences were observed between the test groups for both time points. A decrease of Neut levels was observed for all groups from $24.4 \pm 9.5\%$ after 3 h to $9.7 \pm 1.9\%$ after 24 h. This was suggested to be an effect of the animal handling and may not be related to the toxin administration.

Development of the analytical method

The aim of the present study was to develop a generic sample preparation suitable for both, targeted (quantitation of *Alternaria* toxins) and untargeted (metabolomics) analysis (data not included in this work). Therefore, biological samples were stored at -80°C until sample preparation and all further processing was carried out on ice.

To establish an adequate method allowing for the baseline separation of toxin isomers, a sufficient reduction of the challenging matrix effects, and a satisfying ionization of the target analytes by electrospray ionization, ten binary eluent systems were tested. Therefore, five aqueous eluents (A) including LC–MS grade water with 5 mM NH_4Ac (adjusted pH to 8 using NH_4OH ; 0.1% HAc; 0.1% HAc, 1% HAc, 0.1% formic acid) and 0.3 mM NH_4F have been combined with both ACN or MeOH (as eluents B). In all

combinations, MeOH demonstrated better chromatographic results than ACN. For method development, blank matrix extracts (urine, plasma, fecal samples) were spiked with low concentrations of a multi-*Alternaria*-toxin working solution (see “Chemicals and reagents”). In general, matrix effects were significantly different in each matrix. The most challenging matrix for most eluent combinations appeared to be plasma with signal suppressions of up to 100% and partly shifting and tailing peaks. The polar analytes TeA, AA-III and AOH- and AME-sulfates, as well as the included perylene quinones, demonstrated better peak shapes with basic eluents. However, the overall signal-to-noise ratio was most favorable with 0.3 mM NH_4F as eluent A (Preindl et al. 2019; Yanes et al. 2011). Despite the still interfering matrix suppression in fecal samples, the overall performance was most suitable using this eluent A and MeOH as eluent B. The elution gradient was further optimized to improve chromatographic separation of isomers.

Ionization and MS parameters were tuned by infusion of individual analytes in neat solvent into a constant LC flow. Up to five SRM transitions were evaluated for each analyte and matrix to determine the most favorable matrix-specific signal-to-noise ratios (Table S1). To increase the required number of chromatographic data points, a scheduled SRM acquisition of four segments was applied.

Method validation

The performance of the developed method was evaluated based on the analysis of spiked blank matrix samples at concentration levels in the low ng/mL or ng/g range. Respective parameters and results are reported in Table 2. As expected, the strict regulations outlined for the analytically less challenging food matrixes according to Commission Decision (EC) No. 657/2002 (EC 2002) were not met for all analytes. While the method performance demonstrated reproducible performance, extraction recoveries were limited by the sample preparation at low temperatures (below 5°C). This protocol was optimized for a dual analysis enabling broad coverage of endogenous metabolites as well for untargeted metabolomics analysis. Therefore, these conditions were considered as an acceptable compromise for this elegant two-in-one approach that has not been applied in mycotoxin research so far to the best of our knowledge. The method's specificity was confirmed by the measurement and comparison of respective blank matrix and spiked blank matrix samples. To assure the signal identity of more challenging analytes, additional SRM transitions were taken into account. This was the case for AOH-3-Sulf, ATX-I and ATX-II in urine and feces. Moreover, AOH, AME, TeA, and AST were identified in supposedly blank urine and fecal samples (AST just in feces) obtained from the control group of these experiments. Naturally contaminated rat feed was confirmed to

Table 2 LC–MS/MS performance parameters spiking levels, limits of detection (LOD) and quantification (LOQ), concentration range used for calibration (linear range confirmed within LOQ and highest calibration level), extraction recovery (R_E), intermediate precision (RSD_R , interday precision), repeatability (RSD_r , intraday precision), and signal suppression and enhancement (SSE)

Analytes	Spiking levels ^a (ng/mL) low/high	LOD ^{b,c} (ng/mL) P/U/F	LOQ ^{c,d} (ng/mL) P/U/F	Cal. range ^e (ng/mL)	$R_E^f \pm RSD_R^g$ (%)			RSD_r^h (%)			SSE ⁱ (%)		
					P	U	F	P	U	F	P	U	F
AOH	2.5/12.5	0.05/0.05/0.05	0.1/0.1/0.1	0.1–100	28 ± 12	43 ± 8	65 ± 19	4	8	11	111	62	42
AME	0.5/2.5	0.005/0.005/0.03	0.01/0.01/0.06	0.02–20	22 ± 9	31 ± 9	56 ± 18	4	8	7	96	66	19
ALT	15/75	0.2/8/2	0.4/16/4	0.6–600	62 ± 15	81 ± 10	83 ± 15	4	5	10	101	47	81
isoALT	15/75	0.2/8/2	0.4/16/4	0.6–600	65 ± 13	79 ± 13	86 ± 16	4	5	10	103	46	75
TeA	30/150	0.25/0.5/3 ^j	0.5/1/6 ^j	1.2–1200	117 ± 13	117 ± 17	89 ± 23	14	7	13	121	86	109
TEN	1/5	0.04/0.1/0.5	0.08/0.2/1	0.04–40	59 ± 18	67 ± 9	93 ± 12	6	7	15	99	83	55
AOH-3-Glc	2.5/12.5	0.05/1.5/0.3	0.1/3/0.6	0.1–100	93 ± 11	96 ± 11	80 ± 14	8	11	14	89	27	45
AOH-9-Glc	10/50	0.6/35/10	1.2/70/20	0.4–400	59 ± 25	92 ± 13	82 ± 23	3	11	16	90	26	55
AOH-3-Sulf	2.5/12.5	0.08/3/1	0.16/6/2	0.1–100	50 ± 17	84 ± 21	87 ± 13	4	15	7	98	46	4
AME-3-Glc	10/50	1.5/4/5	3/8/10	0.4–400	60 ± 16	87 ± 21	76 ± 28	7	20	20	117	101	107
AME-3-Sulf	2.5/12.5	0.1/0.2/0.3	0.2/0.4/0.6	0.1–100	38 ± 13	59 ± 11	81 ± 18	4	6	13	103	56	14
ATX-I	2/10	0.3/0.75/0.15	0.6/1.5/0.3	0.08–80	34 ± 7	60 ± 6	79 ± 20	4	10	43	105	80	36
ATX-II	2.5/12.5	0.75/3/50 ^k	1.5/6/100 ^k	0.1–100	22 ± 11	60 ± 17	— ^k	6	11	— ^k	102	94	30
ALP	2.5/12.5	0.4/3/1.5	0.8/6/3	0.1–100	38 ± 22	68 ± 15	95 ± 45	7	14	21	96	71	91
ALS	15/75	0.5/5/7	1/10/14	0.6–600	69 ± 9	82 ± 20	85 ± 62	8	15	57	110	14	9
AA-III	15/75	0.3/5/5	0.6/10/10	0.6–600	107 ± 6	93 ± 4	94 ± 9	12	7	11	101	59	97
AST	0.5/2.5	0.6/0.15/2	1.2/0.3/4	0.02–20	30 ± 20	36 ± 12	— ^k	8	4	— ^k	138	104	29
4OH-AOH	4/20	0.3/1/1.5	0.6/2/3	0.16–160	44 ± 25	52 ± 5	60 ± 17	7	7	15	112	56	54
4OH-AME	2/10	0.02/0.6/1	0.04/1.2/2	0.08–80	28 ± 10	45 ± 8	50 ± 12	5	5	6	114	82	39

^aSpiking levels (low/high), identical for the matrices plasma (P) and urine (U); dry blank feces (F) suspended in water (20 mg in 200 µL) was spiked at the same spiking levels, which accounts for a tenfold higher spiking concentration for the dry fecal matter

^bLimit of detection

^cValues in the following order: plasma (P), urine (U), feces (F)

^dLimit of quantitation

^eConcentration range for solvent- and matrix-matched calibration

^fExtraction recovery (%), calculated as the ratio of the toxin concentration quantified by matrix-matched calibration in the spiked blank matrix and the corresponding spiking level, averaged over the results of two spiking levels

^gIntermediate precision (intraday precision), calculated from standard deviations of the samples spiked at two levels (each in triplicate) and extracted and measured on three different days within the time of 3 months, averaged over the results of two spiking levels

^hRepeatability (intraday precision), calculated from standard deviations of the samples spiked at two levels and measured twice within the same sequence

ⁱSignal suppression and enhancement, calculated as the ratio of calibration curve slopes of the respective matrix and neat solvent

^j“Blank” urine contained low levels of AOH, AME, and TeA. LOD levels were estimated considering the whole calibration curve and its intersection with the x-axis

^kMatrix interferences for ATX-II and AST in feces caused high LODs and recoveries could not be determined

contribute additional exposure (see Table S2). However, the low amounts ($< \text{LOQ}$, $< 1\%$) were considered as neglectable with respect to validation. Matrix effects (SSE, e.g. the signal suppression and enhancement) were determined to be more severe in fecal matrix, followed by urine. The developed sample preparation utilized fourfold higher concentrations of fecal extracts compared to our previous study (Puntscher et al. 2019b). Urine extracts were analyzed in the volume of the original sample (no dilution). For the toxins ATX-II and AST, the analysis in feces was significantly affected by matrix effects and can therefore only be regarded as semi-quantitative. High noise levels resulted in high LOD levels for AST and ATX-II in fecal matrix. However, the determined low matrix effects for all analytes in plasma would allow for quantitation using solvent-matched calibration.

The majority of the analytes demonstrated reproducible and almost identical RTs for the different matrices. Even the polar compounds TeA, AA-III, ALS, AOH-3-Sulf and AME-3-Sulf demonstrated stable RTs within single batches, even though their chromatographic behavior was affected by matrix-related differences and minor pH changes of the eluent in different measurement sequences (reported in Puntscher et al. 2018). The linearity of the matrix- and solvent-matched calibration curves (covering at least three orders of magnitude) was verified by adequate regression coefficients (R^2) of 0.97–0.99. The achieved LOD and LOQ values were significantly lower compared to the analytical tools initially used for biomonitoring (Puntscher et al. 2019b). Even LOD values in the pg/mL range were achieved for certain toxins and demonstrate the high sensitivity. The method's performance proved to generate reproducible data and to be fit-for-purpose.

Determination of *Alternaria* toxins in rat biospecimen

Overall, 13 different *Alternaria* toxins and metabolites were identified in the analyzed rat samples, namely the dibenzopyrones AOH, AME, their respective 4-hydroxyl- and 3-sulfate-metabolites, and ALT, the perylene quinones ATX-I and ALP, as well as TEN, TeA, ALS, and AST. The quantified toxin concentrations in the respective biological matrices are reported in Table 3. Furthermore, percentual ratios are stated, representing the toxins' biological recoveries in the respective samples considering the administered dose. For the calculation of the toxin recovery in urine and feces, the volumes of collected urine and the dry masses of fecal samples were taken into account. The estimation of the animals' total plasma volume was based on the individual body weights at the time of blood sampling. According to Bogdanske et al. (2010), the plasma volume of laboratory rats can be approximated as $4.15 \text{ mL}/100 \text{ g}$ body weight.

Biological recovery of toxins determined below their respective LOQ values were calculated by assuming a toxin concentration of $\text{LOQ}/2$.

The analysis of samples obtained from the extract group revealed intriguing information regarding bioavailability, their prevalence in plasma, and the excretion efficiency after 3 and 24 h. Six *Alternaria* toxins were determined in plasma sampled after 3 h, namely TeA, AME-3-Sulf, ATX-I, ALP, ALS, and AST. Their concentrations declined after 24 h. In contrast to the identification of AOH and 4OH-AOH reported for mice plasma by Schuchardt et al. (2014), these compounds were not detected in any rat plasma sample in the present study. However, the mentioned mouse study conducted high oral administrations of $200\text{--}2000 \text{ }\mu\text{g/kg}$ body weight. Moreover, it was reported that the total radioactivity in plasma deriving from labeled AOH did not exceed 0.06% of the administered dose. Thus, the significantly lower (and more realistic) dose of AOH and AME in the present study ($32\text{--}39 \text{ }\mu\text{g/kg}$ body weight) was not assumed to be detectable by means of the developed method. Interestingly, AME-3-Sulf ($0.6 \pm 0.5 \text{ ng/mL}$) was determined in plasma sampled after 3 h, accounting for 0.06% of the administered AME. For biological recovery calculations of modified toxins determined in the analyzed samples, the respective molecular masses were considered. AOH and AME were mostly excreted without biological modification via feces after 24 h (89 and 142%, respectively) and via urine (0.1% after 3 h and 2.6–2.8% after 24 h for both toxins, see Fig. 1). Employing an AME extraction recovery of 70% (determined for the higher spiking level) instead of the averaged 56%, the calculated excretion rate for AME was 113%. However, the resulting low bioavailabilities and high fecal excretion rates ($> 85\%$ after 24 h) are in line with previous *in vivo* studies investigating exclusively AOH or AME (Pollock et al. 1982; Schuchardt et al. 2014). Considering respective phase-II metabolites, low amounts of AME-3-Sulf were also present in urine. After 3 and 24 h, 0.01% and 0.6% AME equivalents were determined, which is in line with Puntscher et al. (2019b). In fecal samples, levels reached 2% after 24 h. Moreover, AOH-3-Sulf was identified for the first time in rat urine accounting for the excretion of 0.8% of the administered parent toxin after 24 h. In urine sampled after 3 h, AOH-3-Sulf was detected as well, with an averaged signal below the LOQ. Two rats demonstrated AOH-3-Sulf at $8.4\text{--}8.5 \text{ ng/mL}$. Considering these results, the systematic absorption and metabolic modification of AOH and AME was confirmed. Moreover, hydroxylated metabolites of AOH (and AME) were determined in fecal samples after 24 h. 4OH-AOH accounted for 1% of AOH intake, while the signals for the more challenging analyte 4OH-AME were close to the detection limit and therefore not as reliable. As expected, no glucose-conjugates which typically are formed by plant metabolism, were identified in any sample. Due

Table 3 *Alternaria* toxins determined in the *Alternaria* culture extract (administered to extract group) and recovered toxin concentrations (ng/mL or ng/g) in rat plasma, urine, and feces sampled after 3 and 24 h. The percentual biological recoveries are given in parenthesis considering the respective administration dose. In case of non-quantifiable results (< LOQ), recoveries were calculated based on concentration levels of LOQ/2

Administration (µg/kg b.w.)	AOH	AME	4OH-AOH ^e	4OH-AME ^e	AOH-3-Sulf ^e	AME-3-Sulf ^e	ALT	TEN	TeA	ATX-I	ATX-II	ALP	STTX-III	ALS	AST
Blank	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ATX-II	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	707	n.d.	n.d.	n.d.	n.d.
Extract	39.3	32.3					39.2	1.2	29,848	496	707	630	1049	13.8	919
Plasma (ng/mL)	AOH	AME	4OH-AOH ^e	4OH-AME ^e	AOH-3-Sulf ^e	AME-3-Sulf ^e	ALT	TEN	TeA	ATX-I	ATX-II	ALP	STTX-III	ALS	AST
3 h															
Blank	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ATX-II	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ	< LOQ (0.01% ^d)	n.d.	n.d.	n.d.	n.d.	n.d.
Extract	n.d.	n.d.	n.d.	n.d.	n.d.	0.6 ± 0.5 (0.06%)	n.d.	n.d.	1504 ± 853 (0.22%)	7 ± 5.5 (0.06%)	n.d.	2.8 ± 0.7 (0.02%)	n.d.	1.8 ± 1.6 (0.57%)	24.3 ± 8.9 (0.12%)
24 h															
Blank	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ATX-II	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Extract	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	139 ± 228 (0.02%)	< LOQ (0.01%)	n.d.	< LOQ (0.01%)	n.d.	< LOQ (0.09%)	16.8 ± 12.7 (0.08%)
Urine (ng/mL)	AOH	AME	4OH-AOH ^e	4OH-AME ^e	AOH-3-Sulf ^e	AME-3-Sulf ^e	ALT	TEN	TeA	ATX-I	ATX-II	ALP	STTX-III	ALS	AST
3 h															
Blank	< LOQ	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2349 ± 2698	n.d.	n.d.	n.d.	n.d.	N/A	n.d.
ATX-II	< LOQ	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2546 ± 2712	2.9 ± 1.7 (0.001% ^d)	n.d.	n.d.	n.d.	N/A	n.d.
Extract	10.4 ± 13.4 (0.1%)	6.3 ± 7.4 (0.1%)	n.d.	n.d.	< LOQ (0.02%)	2.3 ± 1.8 (0.01%)	n.d.	0.6 ± 0.3 (0.1%)	2,242,757 ± 957,497 (20%)	12.4 ± 10.1 (0.01%)	n.d.	< LOQ (0.003%)	n.d.	N/A	< LOQ (0.0003%)
24 h															
Blank	< LOQ	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3384 ± 3741	n.d.	n.d.	n.d.	n.d.	N/A	n.d.
ATX-II	< LOQ	< LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2614 ± 4144	< LOQ (0.01% ^d)	n.d.	n.d.	n.d.	N/A	n.d.
Extract	35.7 ± 15 (2.8%)	26.3 ± 12.2 (2.6%)	n.d.	n.d.	13.4 ± 7.8 (0.78%)	8.7 ± 6.2 (0.63%)	n.d.	0.3 ± 0.1 (0.9%)	860,009 ± 267,290 (87%)	8.5 ± 5.7 (0.05%)	n.d.	< LOQ (0.03%)	n.d.	N/A	< LOQ (0.004%)
Feces (ng/g)	AOH	AME	4OH-AOH ^e	4OH-AME ^e	AOH-3-Sulf ^e	AME-3-Sulf ^e	ALT	TEN	TeA	ATX-I	ATX-II	ALP	STTX-III	ALS	AST ^e
3 h															
Blank	14.8 ± 7.2	6.4 ± 2.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	993 ± 1933	n.d.	n.d.	n.d.	n.d.	N/A	37 ± 22
ATX-II	15.8 ± 7.5	5.8 ± 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	36 ± 17	n.d.	n.d.	n.d.	n.d.	N/A	26 ± 12
Extract	25.9 ± 7.5 (0.3%)	12.3 ± 6.1 (0.2%)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	36,637 ± 45,322 (0.6%)	< LOQ (0.03%)	n.d.	n.d.	n.d.	N/A	494 ± 244 (0.4%)
24 h															
Blank	12.7 ± 3.9	3.9 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	77 ± 43	n.d.	n.d.	n.d.	n.d.	N/A	84 ± 73

Table 3 (continued)

Feces (ng/g)	AOH	AME	4OH-AOH ^e	4OH-AME ^e	AOH-3-Sulf ^e	AME-3-Sulf ^e	ALT	TEN	TeA	ATX-I	ATX-II	ALP	STTX-III	ALS	AST ^c
ATX-II	16.2 ± 5.7	3.5 ± 1.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	129 ± 161	< LOQ (0.07%) ^d	n.d.	n.d.	n.d.	N/A	37 ± 28
Extract	1090 ± 367 (89%)	1406 ± 485 (142%) ^a	7.8 ± 9.3 (1%)	n.d. ^b	n.d.	21.9 ± 25.1 (2%)	86 ± 37 (7%)	16.3 ± 4 (45%)	19,193 ± 4926 (2.1%)	568 ± 160 (4%)	n.d.	920 ± 451 (5%)	n.d.	N/A	12,489 ± 3384 (45%)

^aHigher extraction efficiencies of up to 70–75% were determined for AME in spiking level “high” (2.5 ng/mL), resulting in biological recoveries for AME in fecal samples of 105–115%

^bPutatively positive results in the range of the LOD were obtained

^cAST analysis in fecal matrix can only be considered as semi-quantitative. Despite the method's limitations, high fecal excretion rates can be suggested

^dRecoveries calculated based on the administered ATX-II equivalents (taking into account the respective molecular masses)

^eRecoveries calculated based on the administered parent toxin AOH or AME (taking into account the respective molecular masses)

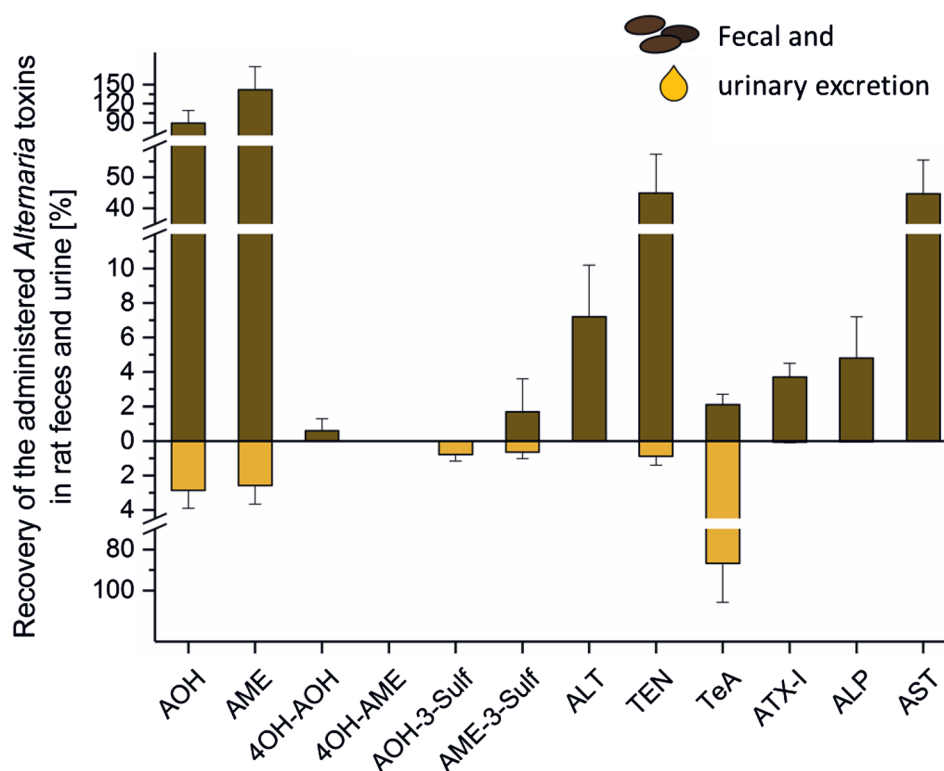
to the lack of respective reference standards, glucuronides, typical mammalian metabolites, were not investigated in this study.

ALT, another dibenzopyrone present in the extract at similar levels as AOH and AME, was detected only in fecal samples after 24 h. The concentration accounted for 7% of the administrated dose and was not determined before in the reported pilot study (Puntscher et al. 2019b). Its isomer isoALT was neither determined in the administered extract, nor in any of the analyzed biological samples. Highest excretion rates of TEN were found in fecal samples after 24 h (45%). Moreover, 0.1 and 0.9% were detected in urine after 3 and 24 h. TeA, which was present in the extract at comparably high concentrations, was recovered in all matrixes. Plasma levels reached 0.2% after 3 h and declined to 0.02% after 24 h. Urinary excretion was confirmed as the main excretion strategy with 20 and 87% after 3 and 24 h, respectively (see Fig. 1). Rapid and efficient urinary excretion was also reported for rats (Puntscher et al. 2019b) and humans (Asam et al. 2013). Only low TeA concentrations were determined in fecal samples (0.6 and 2% after 3 and 24 h, respectively).

Perylene quinones, which were administered at comparatively high levels, were either recovered at very low levels (ATX-I and ALP) or not at all (epoxide-holding analogues ATX-II and STTX-III). ATX-I and ALP were recovered in plasma (up to 0.06% after 3 h), in urine (up to 0.05% after 24 h) and in fecal samples (4–5% after 24 h, see Fig. 1). Due to interfering matrix effects, reliable quantitation of more than half of the ALP signals was not possible. However, these results are in line with previous in vivo (Puntscher et al. 2019b) and in vitro studies demonstrating the highly reactive potential and low persistency of epoxide-holding perylene quinones (Aichinger et al. 2018; Fleck et al. 2014a, b; Puntscher et al. 2019c). Of note, ATX-I was also determined in plasma, urine and fecal samples of rats receiving the isolated ATX-II (ATX-II group). The respective concentrations were lower by a factor 5–10 compared to the samples of the extract group and partly below the LOQ (in plasma and in urine after 24 h). However, these results clearly suggest de-epoxidation of ATX-II in vivo resulting in the formation of ATX-I as suggested in in vitro models (Fleck et al. 2014a, b) and for plant metabolism (Puntscher et al. 2019c) before. In general, the enzymatic reduction of epoxides by mammalian epoxide hydrolases is known as a major detoxification mechanism (Decker et al. 2009).

Low amounts of the toxins ALS (up to 0.6%) and AST (up to 0.12%) were recovered in plasma from rats receiving the extract. In respective urinary and fecal samples, the quantitation of ALS was not possible due to highly varying matrix effects between the individual samples. However, AST was determined in urine (< LOQ) and fecal samples. The method's performance was not adequate for quantitative

Fig. 1 Biological recovery of *Alternaria* toxins in fecal and urinary excretions of rats sampled 24 h after the administration of a complex *Alternaria* culture extract



analysis of AST in feces due to severe carryover effects and co-eluting matrix components. Despite these analytical challenges, the results indicate high fecal excretion rates of this barely studied fungal metabolite.

Moreover, low concentrations of single *Alternaria* toxins were determined in samples of the control and the ATX-II group. By LC–MS/MS analysis of sampled feed aliquots, this cereal-based product was identified as a contributing toxin source. In fact, AOH, AME, TeA, and AST (2.7, 1.4, 596, and 1.5 ng/g) were quantified in feed by standard addition (further details are reported in the supplementary material). This explains the contamination of rat feces sampled after 3 h, which was not expected due to gastrointestinal transit times of 15–24 h (Bove 2015).

Conclusions and outlook

In the present study, we report the investigation of the bioavailability, metabolism, and excretion of *Alternaria* toxins in Sprague Dawley rats (see Fig. 2). After the administration of a complex fungal culture extract comprising 11 determined and quantified *Alternaria* toxins (extract group) and the isolated highly genotoxic altertoxin II (ATX-II group), rat plasma, urine, and feces were sampled after 3 and 24 h and analyzed by a novel LC–MS/MS method. Extensive spiking experiments proofed the analysis to be reliable for these challenging biological matrices and therefore

fit-for-purpose. Up to ten partly modified toxins were determined in rat plasma, urine and fecal samples. Toxin concentrations in plasma were generally below 1% of the administered dose after 3 h (0.01–0.6%) and even lower after 24 h. While the dibenzopyrones AOH and AME showed a low bioavailability and high excretion rates within 24 h mostly via feces (> 89%), TeA was predominantly excreted via urine (87%). Biotransformed metabolites of AOH and AME were identified including 4OH-AOH (putatively also 4OH-AME), AOH-3-Sulf, and AME-3-Sulf, which were determined in fecal samples, but partly also in urine. Epoxide-holding perylene quinones ATX-II and STTX-III were not recovered in any sample, while their analogues ATX-I and ALP were determined in all matrixes at very low levels confirming their potential bioavailability. Moreover, ATX-I was identified in samples deriving from rats, which just received the isolated ATX-II. This may suggest chemical reduction during digestion or even enzymatic de-epoxidation mechanisms in vivo as a potential detoxification pathway.

This study contributes important insights in mammalian metabolism of *Alternaria* toxins and confirmed the relevance of their biologically modified forms. A putatively low systemic absorption does not exclude toxicological effects induced by acute or chronic exposure to these compounds. In this study, physiological aberrations such as digestive disturbance were observed for rats, which received the complex culture extract. With the identification of toxicologically relevant tissues and target organs, such as the gastrointestinal

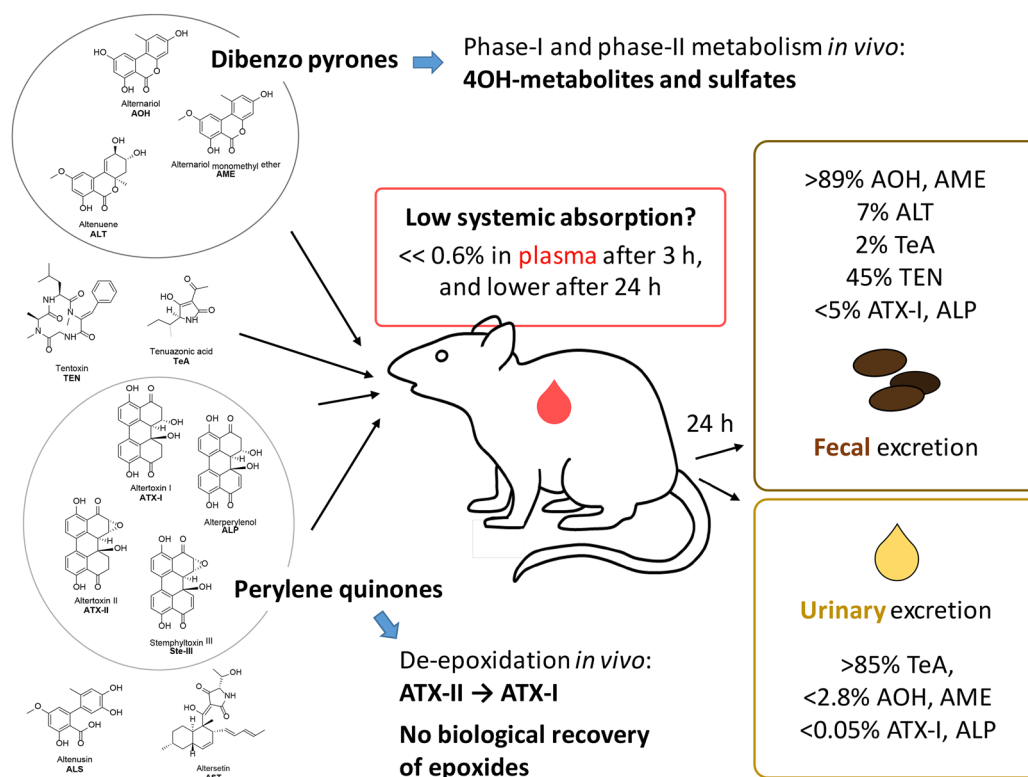


Fig. 2 Overview on the administration, absorption, metabolism, and excretion of *Alternaria* toxins in rats

tract and the liver, it is crucial to investigate toxicological endpoints in follow-up studies. Moreover, the establishment of suitable biomarkers of exposure in animal and human biofluids (human biomonitoring, HBM) is required for future risk assessments and a more reliable understanding of the impact of *Alternaria* toxins on global food safety.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical considerations According to the animal welfare act 2012, BGBl. I Nr. 114/2012 (TVG 2012), the present study (GZ: BMWFV-66.009/0120-WF/II/3b/2014) was approved by the Austrian Federal Ministry of Education, Science and Research (Austria).

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