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Urinary levels of enniatin B and its phase I metabolites: First human pilot biomonitoring study

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ABSTRACT

Enniatins (Enns) are mycotoxins produced by *Fusarium* spp. and are widely distributed contaminants of cereals and derivate products. Among the different identified enniatins, Enn B is the most relevant analogue in cereals in Europe. Therefore, the aim of this study was to investigate for the first time the occurrence of Enn B and Enn B phase I metabolites in 300 human urine samples throughout an ultrahigh-performance liquid chromatography-high resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) methodology. Three different sample preparation procedures were evaluated and salting-out liquid-liquid extraction showed satisfactory validation results. Enn B was quantified in 83.7% of samples ranging from 0.006 to 0.391 ng/mL (average content: 0.016 ng/mL). In line with the *in vitro* observations with human liver microsomes, in the here analyzed samples the Enn B mono-oxygenated, *N*-demethylated and dioxygenated metabolites were tentatively found in 87.7%, 96.3% and 6.7% of samples. The data of this pilot biomonitoring survey indicate a frequent intake of enniatins in Italy, supporting further toxicological studies to provide better basis for understanding their potential effects in humans.

1. Introduction

Mycotoxins are toxic secondary metabolites produced under favorable conditions by species within the genera *Aspergillus*, *Penicillium*, *Fusarium, Alternaria* and *Claviceps.* These toxic compounds can cause adverse health effects in humans and animals consuming contaminated products. *Fusarium* species are common pathogens of cereal grains, animal feeds and food commodities and are probably the most prevalent toxin-producing fungi of the temperate regions of America, Europa and Asia (Santini et al., 2012; Tolosa et al., 2013). In these sense, governmental authorities have set maximum limits in some foodstuffs for mycotoxins with recognized adverse health effects as well as tolerable daily intakes (TDI) or provisional TDIs have been stablished for them by the Scientific Committee on Food and the Joint FAO/WHO Expert Committee on Food Additives (EC No 1881/2006). In the last decade attention to the risk posed to human and animal health has also been extended to the so-called emerging *Fusarium* mycotoxins including enniatins (Enns). Structurally, Enns are cyclohexadepsipeptides composed of alternating residues of three *N*-methyl amino acids, commonly valine, leucine and isoleucine, and three hydroxyl acids, typically hydroxyisovaleric acid (Prosperini et al., 2017). Several Enns analogues have been identified being the most relevant in cereals in Europe, in this order, Enn B, Enn B1, Enn A1 and Enn A. In Mediterranean countries, unprocessed and processed grains containing Enns in a concentration range from few μg/kg to over mg/kg levels were observed (Meca et al., 2010; Fraeyman et al., 2017). Unlike other *Fusarium* mycotoxins, no limits have been set for Enns up to know.

In 2014, the European Food Safety Authority (EFSA) concluded that there might be a concern with respect chronic exposure to Enns, but a risk assessment for dietary exposure was not possible because of the overall lack of toxicity data, and EFSA is still collecting occurrence data for a future risk assessment (EFSA, 2014). Regarding their toxicological characteristics, Enns have ionophoric properties through which Enns are capable of promoting the formation of stable lipophilic complexes with essential cations mono and divalent (Ca^{2+}, Na^{+}, K^{+}) and their transport through biological membranes, disrupting normal physiological concentrations. This ionophoric behavior seems to be related to their wide range of biological activity. *In vitro* studies have demonstrated their antimicrobial, insecticidal and phytotoxic effect (Sy-Cordero et al., 2012). Furthermore, Enns exerted a remarkable cytotoxic effect in several human and animal cells lines at very low micromolar range as recently reviewed by Prosperini et al. (2017). On contrary enniatins have been characterized as compounds with low toxicity *in vivo* (Escrivá et al., 2015), and it could be related to the

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toxicokinetic data of Enn B with liver microsomes that suggested fast and extensive metabolism, and this together with a rapid elimination appears to be mainly responsible for the low *in vivo* toxicity (Dornetshuber et al., 2009; Juan et al., 2014; Manyes et al., 2014).

As regards the bioavailability, among the different enniatin analogues, Enn B seems to have the highest oral absorption (followed by Enn B1, A1 and A) (Meca et al., 2012; Devreese et al., 2013; Fraeyman et al., 2016). As far as the metabolism is concerned, main phase I Enn B metabolites were determined after the incubation of this compound with rat, dog and human liver microsomes and the resulting metabolites were structurally characterized by liquid chromatography (LC) coupled to: ion trap mass spectrometry (ITMS), multiple-stage mass fragmentation (MS^n), time-of-flight mass spectrometry (Q/TOF) and high resolution mass spectrometry (HRMS) using an Orbitrap mass spectrometer (Faeste et al., 2011; Ivanova et al., 2011). This integrated approach allowed the tentative structure elucidation of 12 Enn B metabolites. The identified metabolites were the result of hydroxylation, carboxylation and *N*-demethylation processes. On the other hand, conjugative phase II metabolism was most likely irrelevant for Enn B (Faeste et al., 2011; Fraeyman et al., 2016).

There is currently few data regarding the detection of enniatins and their biotransformation products in tissues and biological fluids, complicating the evaluation of the potential risk related to exposure to enniatins. Recently, some authors investigated the occurrence of Enn B in human biological fluids and reported Enn B at a concentration range from ng/L to few μg/L (Gerding et al., 2015; Escrivá et al., 2017). Nonetheless, these research works did not investigate the occurrence of the tentative phase I Enn B metabolites. The characterization of metabolites is of important consideration in the safety and risk evaluation of food contaminants because they can influence the overall toxic profile (Rodríguez-Carrasco et al., 2014; Ivanova et al., 2017). Therefore, the aim of this study was to investigate for the first time the occurrence of Enn B and Enn B phase I metabolites in 300 urine samples from volunteers living in Southern Italy throughout an ultrahigh-performance liquid chromatography-high resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) methodology.

2. Material and methods

2.1. Chemicals, reagents and materials

Acetonitrile (AcN), methanol (MeOH), Ethyl acetate (EtOAc) and water for LC mobile phase (HPLC grade) were acquired from Merck (Darmstadt, Germany). Formic acid and ammonium formate were obtained from Fluka (Milan, Italy). Sodium chloride and C18 were obtained from Sigma Aldrich (Milan, Italy). Syringe filters with polytetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.2 μm) were provided by Phenomenex (Castel Maggiore, Italy).

Analytical standard of Enn B (> 95% HPLC purity) was purchased from Sigma–Aldrich (Milan, Italy). A stock solution (1 mg/mL MeOH) was prepared and working standard solutions were obtained by serial dilution of the stock and stored at −20 °C.

2.2. Sampling

In total 300 human first-spot morning urine samples from Italian population resident in Campania region (Southern Italy) were collected into sterile plastic vessels during January and February 2018. The following exclusion criteria were taken into consideration in this study: (i) only one member per family can participate; (ii) people who are exposed to a large amount of mycotoxins in a way other than food, such as farmers and veterinarians, were excluded; (iii) persons with severe problems with their liver, bile or kidney cannot participate due to the related risk for interferences with the metabolism of mycotoxins. The use of medication was not an exclusion criterion since there is limited information about possible interferences with mycotoxins. After collection, each urine sample was aliquoted and kept frozen at −20 °C until analysis for stability reasons. A written and approved informed consent was obtained from all volunteers. The informed consent for the mycotoxin evaluation in urine was in accordance to the Helsinki Declaration on ethical principles for medical research involving human subjects. This project was approved by the University of Naples Institutional human research Committee and the study purposes and procedures were justified and accepted for this study. The participants were not subjected to any diet restriction before and during the sampling. The sample size ($n = 300$) selected is consistent with previous pilot biomonitoring studies as well as by the recommendation of the International Federation of Clinical Chemists (IFCC) (Pérez et al., 2017).

All samples were anonymous but participants were asked to write down their gender and age in the vessel for sample classification purpose. The sampling try to maintain the gender parity (male: 45.7%, female: 54.3%). Three age groups were considered for statistical treatment of data: ≤ 30 years old ($n = 94$), from 31 to 59 years old ($n = 72$), and ≥60 years old ($n = 134$). Samples with undetectable levels of mycotoxins were used for spiking and recovery studies. On the other hand, the cereal consumption data were stablished based on age and gender according to the Guidelines for Healthy Italian Food Supply reported by the National Institute for Food Research and Nutrition (INRAN).

2.3. Sample preparation

The sample preparation was performed following a previously developed method slightly modified (Rodríguez-Carrasco et al., 2017). In short, 1.5 mL of sample was placed in a 2 mL Eppendorf Safe-Lock Microcentrifuge tube and centrifuged at 4000 rpm for 3 min. Then, 1 mL of the supernatant was placed into a 15 mL screw cap test tube with conical bottom and 1 mL of acetonitrile was added. The mixture was vortexed for 30 s and later a mixture of 0.3 g sodium chloride and 30 mg of C18 sorbent were added. The solution was vortexed again for 30 s and centrifuged at 4000 rpm for 3 min at 4 °C. Finally, the upper layer was collected and evaporated to dryness under gentle nitrogen flow at 45 °C, reconstituted with 0.5 mL of MeOH/H_2O (70:30 v/v), and filtered through a 0.2 μm filter prior to UHPLC-Q-Orbitrap HRMS analysis.

2.4. UHPLC-Q-Orbitrap HRMS analysis

Qualitative and quantitative profile of Enn B and Enn B phase I metabolites was obtained using Ultra High Pressure Liquid Chromatograph (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a degassing system, a Dionex Ultimate 3000 a Quaternary UHPLC pump working at 1250 bar, an auto sampler device and a thermostated (T = 30 °C) Luna Omega 1.6 μm (50 × 2.1 μm) column. Injection volume was of 5 μL. Eluent phase was formed as follows: phase A (H_2O in 0.1% formic acid and 5 mM ammonium formate), phase B (methanol in 0.1% formic acid and 5 mM ammonium formate). Analytes were eluted using a 0.4 mL/min flow rate with the following programmed gradient: 0–1 min—0% of phase B, 2 min–95% of phase B, 2.5 min–95% of phase B, 5 min–75% of phase B, 6 min–60% of phase B, 6.5 min–0% of phase B and then column was equilibrated for 1.5 min at 0% phase B. Detection was performed using a Q-Exactive mass spectrometer. Full scan data in positive mode was acquired at a resolving power of 70,000 FWHM at m/z 200. Ion source parameters in positive (ESI+) mode were: spray voltage 4 kV, sheath gas (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, capillary temperature 290 °C, S-lens RF level 50, auxiliary gas heater temperature 305 °C. Data analysis and processing were performed using the Xcalibur software, v. 3.1.66.10. For the compounds of interest, a scan range of m/z 100–800 was selected; the automatic gain control (AGC) was set at 1×10^6 and the injection time was set to 200 ms. Scan-rate was set at 2 scans/s.

Data were evaluated by the Quan/Qual Browser Xcalibur software, v 3.1.66.10. The accuracy and calibration of the Q Exactive Orbitrap LC-MS/MS was checked daily using a reference standard mixture obtained from Thermo Fisher Scientific. The mass tolerance window was set at 5 ppm.

2.5. Method validation

In-house validation was conducted following the EU Commission Decision 2002/657/EC (EC, 2002). The validation parameters evaluated included linearity, trueness, repeatability, within-laboratory reproducibility, limit of detection (LOD) and limit of quantitation (LOQ). Recovery experiments were performed by spiking blank urines samples with Enn B standard at four different concentrations (0.1, 0.5, 1 and 5 ng/mL). Since no suitable reference material was available, the trueness and selectivity was estimated by using recovery studies. The measurements were repeated on three days with three determinations per concentration level and expressed as relative standard deviation (RSD, %). Linearity was evaluated using standard solutions and matrix-matched calibrations by analyzing in triplicate at least six concentrations levels over a range of 0.001–5 ng/mL. Matrix-matched calibration was prepared by spiking extracts of blank samples with Enn B at similar concentrations than the calibration built in neat solvent. The slopes of the resulting linear calibration functions were compared and the signal suppression/enhancement (SSE) due to matrix effects was determined according to the following equation: SSE (%) = Slope matrix-matched calibration/Slope standard in solvent x 100. A SSE of 100% indicates that no matrix effect occurred in the concentration range investigated. A SSE above 100% revealed signal enhancement, whereas a SSE below 100% signal suppression. When HRMS is used, the concept of LOD and/or LOQ may be ambiguous because the common definition of these parameters are based on the signal-to-noise (S/N) ratio involving its measurement. LOD was defined as the minimum concentration where the molecular ion can be identified (mass error value below 5 ppm) and LOQ was set as the lowest concentration that allowed the concentration of the analyte to be determined with accuracy and precision ≤20%.

2.6. Statistical analysis

One-Way ANOVA (Robust Testing: Brown-Forsythe) was used for assessing homogeneity of variances. For comparison of categorical data, the Pearson Chi-square and Fisher exact test were evaluated. Data were examined in 95% confidence level, and *p*-value lower than 0.05 was assumed significant. All tests were carried out by using IBM SPSS 24.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Method development

3.1.1. Optimization of the Q-Orbitrap HRMS conditions

The optimization of the Enn B-dependent MS/MS parameters was performed via direct infusion of the analytical standard (diluted at 1 μg/mL) into the Q-Orbitrap system using a syringe injection at a flow rate of 8 μL/min. The MS instrument was operated in the positive electrospray mode (ESI+) as this was the most sensitive mode. The Q-orbital provides ion spectrum with accurate mass (< 5 ppm) and thus offering a higher level of confidence in analyte identification.

In literature twelve biotransformation products were detected and their structures tentatively identified by multiple high-performance liquid chromatographic/mass spectrometric analyses. The observed molecular masses reported in literature regarding amonium adducts of Enn B metabolites were targeted in Q-Orbitrap-HRMS to evaluate the presence or absence of these metabolites in the herein investigated human urine samples based on the accurate mass of the related ions. Data for retention times, accurate mass (experimentally measured ion mass) and mass accuracy for Enn B and its metabolites are shown in Table 1.

Table 1
Retention times, accurate mass and mass accuracy of Enn B and its phase I metabolites.

Compound	Retention time (min)	Molecular formula	Observed mass $[M + NH_4]^+$	Accuracy (Δ ppm)
Enn B	5.71	$C_{33}H_{57}N_3O_9$	657.44331	4.0
M1	5.39	$C_{33}H_{57}N_3O_{10}$	673.4373	2.0
M2	5.62		673.4377	3.0
M3	5.68		673.4364	2.0
M4	n.d.		673.4377	–
M5	n.d.		673.4369	–
M6	5.14	$C_{32}H_{55}N_3O_9$	643.4265	4.0
M7	5.25	$C_{31}H_{55}N_3O_9$	629.4120	2.5
M8	4.81	$C_{31}H_{53}N_3O_9$	687.4164	3.5
M9	n.d.		687.4160	–
M10	5.01		687.4169	2.0
M11	5.10		687.4157	2.5
M12	5.18		687.4161	1.5

3.1.2. Selection of the sample preparation

Analytical support to toxicokinetic studies include the handling of a large number of samples generated from these studies, and therefore the use of a simple and straightforward sample preparation procedure is of great importance. In this sense, method simplification and miniaturization is highly desirable in sample preparation of biological matrices, allowing the use of low sample and solvent volume, fast analysis, and high efficiency (Kabir et al., 2017). Lately, three different sample preparation procedures namely dilute and shoot (Gerding et al., 2015), dispersive liquid-liquid microextraction (DLLME) (Escrivá et al., 2015), and salting-out liquid-liquid extraction (SALLE) (Rodríguez-Carrasco et al., 2017) have been applied to for the determination of different mycotoxins in biological fluids.

In this work, the above mentioned protocols were adopted with slight modifications. Experiments were performed in blank urine samples spiked with Enn B at 1 ng/mL and carried out in triplicate and in three non-consecutive days. The followed protocols were:

(i) Dilute and shoot: To 50 μL of centrifuged urine 1.5 mL of AcN was added followed by a vortex mixing (15 s) and centrifugation (4000 rpm, 3 min, 4 °C). The clear supernatant was transferred into an autosampler vial and 5 μL were subsequently injected into the instrument.
(ii) DLLME: To 1 mL of centrifuged urine 0.3 g NaCl was added. After mixing, the mixture of 1 mL of AcN and 100 μL of EtOAc was quickly added. The mixture was vortexed for 1 min and centrifuged (4000 rpm, 3 min, 4 °C). The supernatant was transferred to a vial and evaporated to dryness under nitrogen flow at 45 °C. Afterwards, the dry extract was reconstituted with 500 μL of MeOH/water (70:30, v/v), filtered and 5 μL were injected into the instrument.
(iii) SALLE: To 1 mL of centrifuged urine 1 mL of acetonitrile was added. After mixing, the mixture of 0.3 g NaCl and 30 mg of C18 sorbent was added. The solution was vortexed and centrifuged (4000 rpm, 3 min, 4 °C). The upper layer was collected and evaporated to dryness under nitrogen flow at 45 °C. The dry extract was reconstituted with 500 μL of MeOH/water (70:30, v/v), filtered and 5 μL were injected into the instrument.

Results showed that the dilute and shoot procedure was not suitable for analyzing Enn B in the urine samples (mean recovery = 69%, RSD = 22%, *n* = 9). This procedure is less time consuming and more

Table 2
Method performance.

Parameters	R^2	SSE (%)	Recovery, % (RSD_R, %; $n=9$)				LOD (ng/L)	LOQ (ng/L)
			5 ng/mL	1 ng/mL	0.5 ng/mL	0.1 ng/mL		
Enn B	0.9994	67	92 (7)	97 (5)	84 (9)	75(13)	0.5	1.0

cost-effective but partially less sensitive (low signal intensity) than the other assayed protocols. The fact of diluting the sample could suppose a loss of analytical information taken into account the low levels (ppt-range) of Enn B reported in urine. In addition this method could clog and seriously contaminate the MS instrument as reported by other authors (Malachova et al., 2015). On the other hand, Enn B mean recoveries of 82% (RSD = 21%, $n=9$) and 97% (RSD = 5%, $n=9$) were obtained for DLLME and SALLE protocols, respectively. Therefore, to keep the sample preparation procedure as short as possible and based on the results obtained, SALLE protocol was selected in this study as optimal extraction protocol.

3.2. Method performance

The performance characteristics, including matrix effects, linearity, trueness, repeatability, inter-day precision, LOD and LOQ were obtained according to the guidelines set at Commission Decision 2002/657/EC (EC, 2002). Table 2 showed the method performance results. Signal suppression (67%) was observed for Enn B in the human urine. Therefore, matrix-matched calibration was used for quantification. A coefficient of determination (R^2) of 0.9994 was obtained within the linear range from 0.001 to 5 ng/mL. The carry-over was evaluated by a blank sample ($n=10$) just after the highest calibration sample. No carry-over was present since no peaks were detected in the same Enn B retention time zone. Satisfactory apparent recoveries, with mean values ranging from 75% for 0.1 ng/mL to 97% for 1 ng/mL of fortification were obtained. The RSD_r and RSD_R were ≤13% for all concentration levels assayed, indicating the suitability of the developed method. LOD and LOQ were calculated from matrix matched solutions and were 0.5 and 1.0 ng/L, respectively. The developed method successfully detected and quantitated Enn B at low ppt-range.

3.3. Occurrence of enniatin B in human urines

As it can be observed in Table 3, in this study Enn B concentrations were quantified in 251 out of 300 (83.7%) Italian human urine samples ranging from 0.006 to 0.391 ng/mL (average values: 0.016 ng/mL). Recently, Escrivá et al. (2017) reported levels of Enn B in urines from ten Spanish volunteers of up to 0.54 ng/mL. However, Serrano et al. (2015) reported absence of emerging *Fusarium* mycotoxins in urine and plasma from Italian volunteers. This absence could be justified taken into consideration both the limited sampling ($n=10$) and the lack of sensibility (instrumental LOQ reported: 5 ng/L). On the contrary, Gerding et al. (2015) reported levels of Enn B from 0.010 to 0.065 ng/mL in human urine samples ($n=287$) from different countries but at significant low incidence (4.5%) compared to the here reported data. Nonetheless, interregional differences in urinary mycotoxin excretion patters were previously observed in several pilot-scale studies conducted in population from Africa, Asia and Europe. Gerding et al. (2015) also reported interregional differences in their study being the percentages of Enn B positive samples: 14% in Germany ($n=50$), 3% in Haiti ($n=142$) and 2% in Bangladesh ($n=95$). The reason for interregional variation in mycotoxin excretion could be related to differences in nutritional habits and quality of consumed foodstuffs. In fact, the latest *per capita* annual consumption data reported by FAO (FAO, 2013) showed a significant variability in the consumption of wheat (Haiti: 16.11 kg, Bangladesh: 17.47 kg, Europe: 108.97 kg), which is one of the most susceptible cereal to *Fusarium* spp infection. Similarly, the differences observed in the Enn B incidence in human urines from Germans (14% positive samples, $n=50$) and Italians (83.7% positive samples, $n=300$) could be discussed taken into account the different consumption data in these populations, being the wheat consumption of Italians (146.33 kg/*per capita*) nearly twice that of the Germans (83.41 kg/*per capita*). Statistical analysis of data showed significant differences ($p \leq 0.05$) between the occurrence of Enn B (and Enn B metabolites) and the consumption of cereals by Italian population reported by the National Institute for Food Research and Nutrition. Similarly, significant differences ($p \leq 0.05$) were found between the occurrence of Enn B (and Enn B metabolites) and the age of participants (order of contribution: more than 60 years old, > from 30 to 60 years old, > lower than 30 years old) (Table 4). These results could be related with the age-associated reduction in renal function that might potentially affect mycotoxin metabolism in the liver, but further research is needed to clarify this issue. On the other hand, significant difference ($p \leq 0.05$) was not found between the occurrence of Enn B (and Enn B metabolites) and the gender of participants.

Table 3
Occurrence of Enn B and Enn B metabolites in the analyzed human urine samples ($n=300$).

Compound/Group	Incidence (%)	Range (ng/mL)	Mean[a] (ng/mL)
Enn B	83.7	0.006–0.391	0.016
M1	43.7	0.003–0.073	0.006
M2	67.0	0.002–0.070	0.011
M3	64.3	0.002–0.087	0.012
M4	*n.d.*	–	–
M5	*n.d.*	–	–
Monooxygenated group (M1-M5)	87.7	0.005–0.107	0.033
M6	11.7	0.004–0.012	0.007
M7	93.7	0.002–0.102	0.021
N-demethylated group (M6-M7)	96.3	0.002–0.102	0.020
M8	4.0	0.007–0.552	0.077
M9	*n.d.*	–	–
M10	1.7	0.001–0.105	0.006
M11	1.0	0.006–0.015	0.009
M12	0.3	0.011	0.011
Dioxygenated group (M8-M12)	6.7	0.001–0.552	0.051

[a] Mean values are based in positive samples only.

3.4. Urinary excretion pattern of enniatin B phase I metabolites

Regarding *in vivo* studies, very scarce data related to the metabolization pathways of Enn B are available in literature. Major Enn B phase I biotransformation products were tentatively identified in plasma of mice as well as in serum, liver, plasma and eggs samples from broiler chickens and hens fed with Enn B contaminated feed, and a good correlation with the previously reported *in vitro* data was found (Ivanova et al., 2014; Fraeyman et al., 2016; Rodríguez-Carrasco et al., 2016).

Table 4
Results of urinary concentrations of Enn B and its metabolites for different age groups.

Compound/Group	Incidence (%)	Range (ng/mL)	Mean[a] (ng/mL)
Volunteers ≤ 30 years old (n = 94)			
Enn B	74.4	0.006–0.083	0.015
Monooxygenated group (M1-M5)	85.5	0.007–0.093	0.029
N-demethylated group (M6-M7)	95.5	0.006–0.076	0.024
Dioxygenated group (M8-M12)	7.8	0.001–0.217	0.005
Volunteers > 30 and < 60 years old (n = 72)			
Enn B	83.9	0.005–0.078	0.013
Monooxygenated group (M1-M5)	87.6	0.005–0.081	0.030
N-demethylated group (M6-M7)	90.1	0.005–0.098	0.023
Dioxygenated group (M8-M12)	7.4	0.007–0.029	0.015
Volunteers ≥ 60 years old (n = 134)			
Enn B	89.9	0.006–0.391	0.019
Monooxygenated group (M1-M5)	89.1	0.006–0.107	0.037
N-demethylated group (M6-M7)	94.6	0.002–0.102	0.020
Dioxygenated group (M8-M12)	5.4	0.002–0.552	0.100

[a] Mean values are based in positive samples only.

The obtained results in human urine samples are shown in Table 3. In line with the *in vitro* observations with human liver microsomes (Faeste et al., 2011), in the here analyzed human urine samples were tentatively found monooxygenated (M1-M5), mono-and didemethylated (M6-M7) and dioxygenated metabolites (M8-M12). According to literature, the identified metabolites eluted in front of the parent compound due to they are more hydrophilic than Enn B, and it was confirmed in this study by their respective retention times in reversed-phase chromatography (Table 1). However, due to the fact that there are no standards of Enn B metabolites available, it was not possible to precisely quantify the metabolites in the samples. To overcome that, the matrix-matched calibration curve of the parent compound was used as an approach to quantify the detected metabolites. Monooxygenated metabolites were found ranging from 0.005 to 0.107 ng/mL (87.7% samples) whereas *N*-demethylated and dioxigenated metabolites were quantified in a range between 0.002 and 0.102 ng/mL (96.3% samples) and 0.001–0.552 ng/mL (6.7% samples), respectively (Table 3). Among the monooxygenated analogues, the main metabolites were, in this order, M2 > M3 > M1 whereas M4 and M5 were not detected in any analyzed samples. M7 was the most important *N*-demethylated metabolite. Similarly, the main metabolites among the dioxygenated metabolites were, in this order, M8 > M10 > M11 > M12, whereas M9 was not detected in any urine sample (Table 3). Significant differences were found among the occurrence of the Enn B metabolites and the concentrations found ($p \leq 0.05$, F = 11,202, *degree of freedom*: 32,267) being the combinations M2 + M3 + M7 and M1 + M2 + M3 + M7 the most important (frequency: 18.3% and 16.0%, respectively). Fig. 1 shows the chromatograms of a human urine sample containing Enn B at 0.017 ng/mL; mono-oxyganted Enn B metabolites (M1: 0.006 ng/mL; M2: 0.028 ng/mL; M3: 0.016 ng/mL); *N*-demethylated metabolites (M6: 0.011 ng/mL; M7: 0.024 ng/mL); and dioxygenated Enn B metabolite (M10: 0.001 ng/mL). The results above reported evidenced that the main metabolization route of Enn B in human occurs via monooxygenation and demethylation reactions. The data of this biomarker

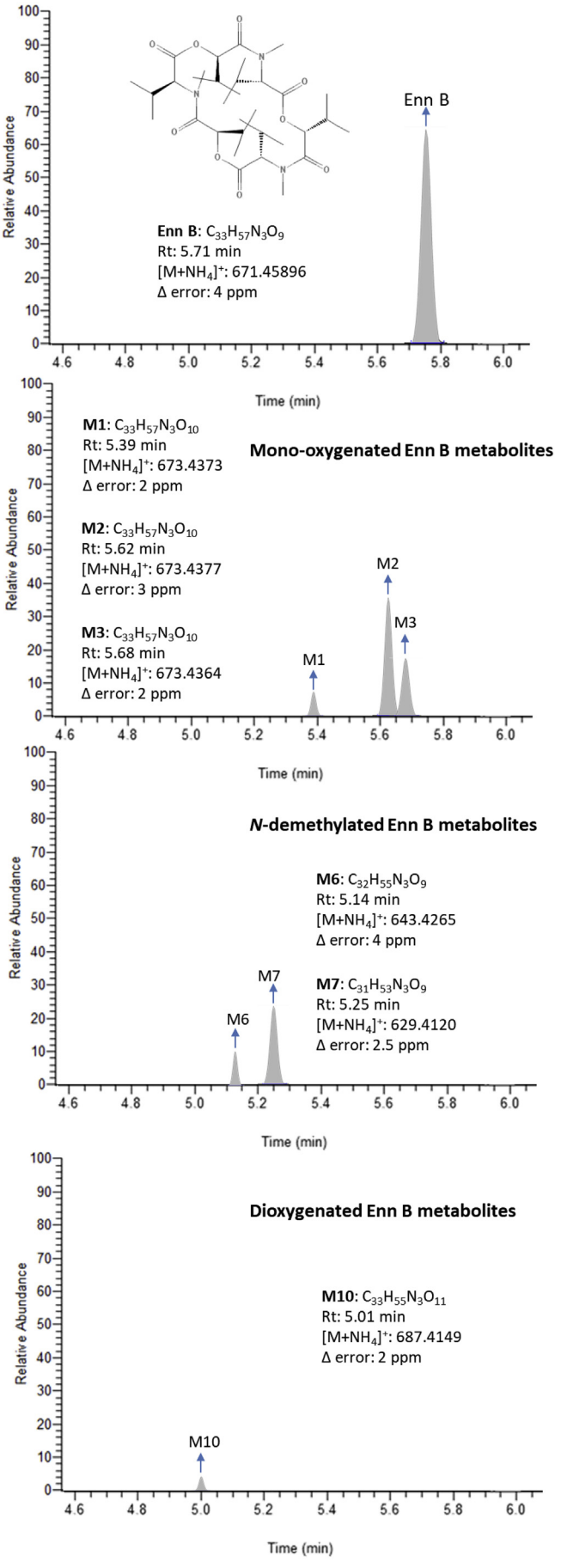

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Fig. 1. Chromatograms of a human urine sample containing Enn B at 0.017 ng/mL; mono-oxyganted Enn B metabolites (M1: 0.006 ng/mL; M2: 0.028 ng/mL; M3: 0.016 ng/mL); N-demethylated metabolites (M6: 0.011 ng/mL; M7: 0.024 ng/mL); and dioxygenated Enn B metabolite (M10: 0.001 ng/mL).

survey indicate a frequent exposure of enniatin B in Italy, supporting further toxicological studies to determine the impact of enniatins contamination in foodstuffs.

4. Conclusions

In this work a methodology based on a salting-out liquid-liquid extraction followed by an ultrahigh-performance liquid-chromatography-high resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) was in-house validated and then applied to 300 human urine samples to evaluate the occurrence of the *Fusarium* mycotoxin Enn B. Results showed an Enn B incidence of 83.7%, and the concentrations found ranged from 0.006 to 0.391 ng/mL (average content: 0.016 ng/mL). In addition, this study investigated the occurrence of the Enn B phase I metabolites in human urine samples for the first time. In line with the *in vitro* observations with human liver microsomes, in the here analyzed samples were tentatively found monooxygenated Enn B metabolites (83.7% samples, average content: 0.033 ng/mL), *N*-demethylated Enn B metabolites (96.3% samples, average content: 0.020 ng/mL), and dioxygenated Enn B metabolites (6.7% samples, average content: 0.051 ng/mL). Results showed that several Enn B metabolites belonging to the monooxygenated and *N*-demethylated groups were the predominant compounds found in urine samples. The characterization of metabolites is of important consideration in the safety and risk evaluation of food contaminants, and based on the results here obtained a frequent exposure of Enn B is highlighted in Italian population.

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