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LC–MS/MS characterization of the urinary excretion profile of the mycotoxin deoxynivalenol in human and rat

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ABSTRACT

The understanding of mycotoxins transfer to biological fluids is challenged by the difficulties in performing and replicating *in vivo* experiments as well as the lack of suitable methods of analysis to detect simultaneously a range of chemically different metabolites at trace levels. LC–MS/MS has been used herein to study the urinary excretion profile of the mycotoxin deoxynivalenol in human and Wistar rat. Deoxynivalenol and deoxynivalenol glucuronide were found in both human and rat urines, whereas de-epoxydeoxynivalenol and its glucuronide conjugate were only detected in rat urine. The presence of two deoxynivalenol glucuronide isomers in Wistar rat urine has been shown for the first time. Structure confirmation of the detected metabolites was provided by the analysis of fragmentation patterns. A solid phase extraction clean up procedure allowing recoveries in the range 72–102% for deoxynivalenol, de-epoxydeoxynivalenol, and their glucuronide conjugates was optimized. A multiple reaction monitoring method for the simultaneous determination of all investigated metabolites was elaborated allowing the direct detection of deoxynivalenol metabolites without the hydrolysis step. Deoxynivalenol urinary levels in the range 0.003–0.008 μg/ml were detected in healthy human subjects, whereas deoxynivalenol and de-epoxynivalenol levels between 1.9–4.9 μg/ml and 1.6–5.9 μg/ml, respectively were found in *administered* rat urine. These findings emphasize the relevance of the highly selective and sensitive LC–MS/MS technique for the direct detection and characterization of deoxynivalenol metabolites in complex biological matrices.

1. Introduction

Deoxynivalenol (DON) is the most frequently occurring type-B trichothecene primarily associated with *Fusarium graminearum* and *Fusarium culmorum,* both important plant pathogens causing various blights, root rots and wilts [1,2]. Surveys on the occurrence of DON showed it to be a world-wide contaminant of grains such as wheat, barley, oats, rye, maize, and relevant derived foods [3,4]. This widespread contamination is a problem of concern in public health since DON is responsible for a wide range of disorders in animals, including haematic and anorexic syndromes, as well as neurotoxic and immunotoxic effects, and has been found to inhibit DNA, RNA and protein synthesis [2,3]. Taking into account new available data on toxicity of DON and its acetyl derivatives (3-acetyl-DON and 15-acetyl-DON), occurrence in raw cereals and finished products, and effects of processing the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recently updated the evaluations of this mycotoxin. The committee decided to convert the provisional maximum tolerable daily intake (PMTDI) to a group PMTDI of 1 μg/kg bw per day for DON and its acetyl derivatives since acetylated forms are converted to DON *in vivo* and therefore contribute to the total DON induced toxicity [5].

When reviewing the available studies on DON transfer in animals [6], the metabolic pathway appears quite complex. This complexity is well reflected by the urinary excretion pattern of this toxin. Besides the unchanged toxin, a range of urinary metabolites has been identified in animal studies. A de-epoxy metabolite (DOM-1) has been identified in rat urine as the result of the metabolism of gut micro-organisms [7]. The epoxy group is the major responsible for the toxicity of trichothecenes [2,8]. Animals with ability to de-epoxidate may therefore be less sensitive to trichotecenes than animals lacking this ability. The occurrence of DOM-1 in human urine has been poorly investigated [9,10]. The other main metabolic transformation of trichothecenes in animals is glucuronide conjugation. Glucuronidation enhances water solubility facilitating the excretion with the urine. Besides DOM-1,

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several more polar metabolites were found to be present in urine of rats administered with ^{14}C-DON [11]. Meky et al. [9] indirectly confirmed the presence of DON-glucuronide in humans since higher DON concentrations were found in urine samples after treatment with β-glucuronidase.

The understanding of mycotoxins transfer to biological fluids is challenged by the lack of suitable methods of analysis to detect simultaneously a range of chemically different metabolites at trace levels. One of the most common approaches used for metabolite identification is a combination of radioisotopic counting and chromatographic techniques following the fate of administered ^{14}C-DON [9,11–13]. When performed, quantitative determination is based on indirect measurements of the sum of DON and its glucuronide derivative after β-glucuronidase hydrolysis [9,14]. HPLC with UV detection is routinely used for quantitative analysis of DON and its acetylated derivatives with good accuracy and precision [15]. However, given the rather non-specific UV absorption of DON and its derivatives at 200–225 nm, an immunoaffinity column clean up is required to obtain chromatograms sufficiently free from interfering peaks. The lack of glucuronide retention ability of available immunoaffinity columns [9] makes this approach not applicable for the direct detection of DON, DOM-1 and their glucuronides. Gas chromatography–mass spectrometry (GC–MS), which has the sensitivity and selectivity to detect and identify toxin metabolites, requires hydrolysis of glucuronide conjugates and derivatization [16,17]. Liquid chromatography coupled with mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS/MS) offers suitable sensitivity, selectivity and possibility to get structural information and potentially allows the direct detection and characterization of DON metabolites. The use of LC–MS for the determination of DON and DOM-1 in pig urine has been reported by Razzazi-Fazeli et al. [18]. Only one report is available on the LC–MS detection of glucuronide conjugates of DON and DOM-1 together with unconjugate forms in cow urine and hen exctreta [19]. Based on the use of single quadrupole instruments, these studies provided limited structural information. However they clearly illustrated the potential of LC–MS for the simultaneous and direct detection of de-epoxide and conjugated DON metabolites in complex biological matrices eliminating the need of a hydrolysis step, and avoiding the hazard of handling radioactive reagents.

Aim of the present study was to evaluate LC–MS/MS as a tool to investigate on the kind of urinary metabolites of the mycotoxin deoxynivalenol in human and rats. A detailed structural characterization of the metabolites of interest is also given on the basis of their fragmentation patterns. Differences in the urinary metabolite profile in human and Wistar rat have been evidenced, furthermore the presence of two DON glucuronide isomers in rat urine has been shown for the first time.

2. Methods

2.1. Chemicals and reagents

Acetonitrile, methanol (both HPLC grade) and glacial acetic acid were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Ammonium acetate (for mass spectrometry) was from Sigma–Aldrich (Milan, Italy). Solid commercial standard of DON and certified calibrant solution (50 μg/ml in acetonitrile) of DOM-1 were purchased from Biopure Referenzensubstanzen GmbH (Tulln, Austria). β-Glucuronidase (*Escherichia coli,* type IX-A) was from Sigma–Aldrich (Milan, Italy). Phosphate buffered solution at pH 7.4 (PBS) was prepared by dissolving commercial phosphate buffered saline tablets (Sigma–Aldrich, Milan, Italy) in distilled water. Strata-X solid phase extraction (SPE) columns (60 mg/3 ml) were obtained from Phenomenex (Torrance, CA). HPLC syringe filters (0.45 μm) were from Alltech (Deerfield, IL, USA).

2.2. Preparation and analysis of contaminated feed

Contaminated feed was prepared by mixing 1.5 kg of grounded control feed (rat and mouse standard feed) with 1.5 l of a *F. graminearum* extract containing 34 mg/l of DON. Then, contaminated feed was frozen and freeze-dried for 72 h. The freeze-dried material was extracted and purified in triplicate according to the procedure described by Lattanzio et al. [20] to confirm the final level of DON (33.85 ± 1.96 mg/kg).

2.3. Urine samples

Human urine samples were collected from two Italian healthy adult volunteers, namely a 30-year-old woman and a 45-year-old man, with a standard Italian diet. This was assumed to be a potential exposure to DON through the consumption of contaminated cereals based on the SCOOP Report 2003 [4]. Volunteers were asked to collect first-voided urine in the early morning. Informed consent was obtained from all donors. The period of urine specimen collection was from October 2006 to May 2007.

Eight young male Wistar-albine rats were maintained at the animal facility of the Universidad National de Rio Cuarto, Argentina. The animal experimental design was approved by the ethical committee for animal experiments in Rio Cuarto (Argentina). Body weights of rats ranged from 250 to 300 g. The animals were housed singly in metabolic cages and allowed to acclimate for 24 h under controlled conditions of temperature (20 °C) and light (12-h light/dark cycle). During the acclimatising period rats were fed with a standard diet and water *ad libitum*. 24-h urine were collected from each rat and used as control urine (blank urine). Then, rats were fed for 4 days with a 40 g/day of contaminated feed. Urine samples from each rat were collected over the 4-day period and stored at −20 °C. The mean DON intake of rats was 3.57 ± 1.53 mg/kg bodyweight/day.

2.4. Standard toxin solutions

Stock solutions of DON were prepared by dissolving the solid commercial toxin in acetonitrile at concentration of 1000 μg/ml, whereas for DOM-1 the certified calibrant solution (50 μg/ml in acetonitrile) was used. Mixed diluted standard solutions of DON and DOM-1 were prepared in acetonitrile at concentrations 10 μg/ml, 1 μg/ml and 0.1 μg/ml, by mixing DON stock solution with DOM-1 certified calibrant solution. These solutions were used for spiking experiments and to prepare calibrant solutions for matrix-matched calibration.

2.5. Urine clean up on Strata-X columns

Human and rat urine samples were purified on Strata-X column by slightly modifying the protocol described by Murphy and Huestis [21]. The column was attached to a vacuum manifold (Supelco, Bellefonte, PA, USA), preconditioned with 2 ml methanol followed by 2 ml water prior to use. 0.4 ml urine sample was added with 1.5 ml of 2% phosphoric acid in 10 mM ammonium acetate and mixed well. The diluted urine solution was applied to Strata-X column. The column was washed with 2 ml 0.1% phosphoric acid and dried by passing air. The analytes were eluted with 2 ml of 5% aqueous ammonia in methanol. The eluate was evaporated to dryness under air stream at 50 °C. The residue was reconstituted with 0.1 ml of LC mobile phase (methanol/water, 10/90 v/v containing 5 mM ammonium acetate).

Table 1
MRM parameters for the determination of DON, DOM-1, DON-G1 (12.8 min), DON-G2 (38.8 min) DOM-1-G.

Analyte	Precursor ion	Q1 (*m*/*z*)	Q3 (*m*/*z*)	DP (V)	EP (V)	CE (V)	CXP (V)
DON	$[DON + CH_3COO]^-$	355.1	295.0	−23	−6	−17	−2.5
			265.1			−20	−2.1
			59.0			−36	−6.8
DOM-1	$[DOM\text{-}1 + CH_3COO]^-$	339.1	279.0	−15	−3	−10	−4
			249.1			−20	−6
			59.0			−20	−7
DON-G1 (12.8 min)	$[DON\text{-}G + NH_4]^+$	490.2	297.2	+10	+1	+20	+3
			249.2			+20	+3
			231.1			+20	+3
DOM-1-G	$[DOM\text{-}1\text{-}G + NH_4]^+$	474.2	281.2	+10	+1	+30	+3
			233.1			+40	+3
			130.1			+40	+3
DON-G2 (38.8 min)	$[DON\text{-}G + NH_4]^+$	490.2	177.1	+10	+1	+40	+3
			103.1			+50	+3
			89.0			+50	+3

Q1: first quadrupole; Q3: third quadrupole; DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential.

Ten microliters (equivalent to 40 μl urine) were analyzed by LC–MS/MS.

2.6. Recoveries from Strata-X columns

For recovery experiments from Strata-X columns, blank rat urine samples ($n = 3$) were spiked at level of 1 μg/ml of standard both DON and DOM-1, then cleaned up through Strata-X columns according to the procedure described in Section 2.5. The amounts of recovered toxins were calculated by using one-point matrix-matched calibration.

To evaluate DON-G and DOM-1-G recoveries, urine samples ($n = 6$) from DON exposed rats were purified on Strata-X columns according to the above procedure. After drying the eluates, $n = 3$ aliquots were reconstituted in 0.1 ml of LC mobile phase then analyzed by LC–MS/MS. Other $n = 3$ aliquots were reconstituted in 0.4 ml of blank rat urine and cleaned up a second time through Strata-X columns. The dried residues were reconstituted in 0.1 ml mobile phase and analyzed by LC–MS/MS. Recoveries of DON-G and DOM-1-G were evaluated by comparing relevant peak areas in samples after the first and the second clean up. To confirm the suitability of the developed procedure, recoveries of DON and DOM-1 were also evaluated using the same procedure.

2.7. β-Glucuronidase hydrolysis

For hydrolysis experiments, human urines (1 ml) were added with 1 ml phosphate buffer (pH 7.4) containing 4500 U β-glucuronidase. Control samples were diluted with PBS only. Samples were incubated for 18 h at 37 °C. After incubation, 1 ml of sample (equivalent to 0.5 ml urine) was added with 2 ml of 2% phosphoric acid in 10 mM ammonium acetate and cleaned up on Strata-X column according to the procedure described in Section 2.5. After drying the purified sample was reconstituted with 0.1 ml of LC mobile phase (methanol/water, 10/90 v/v containing 5 mM ammonium acetate). Ten microliters (equivalent to 50 μl hydrolyzed urine) were analyzed by LC–MS/MS.

Given the high DON and DOM-1 content, urines (8 ml) from DON exposed rats were extracted with 4 volumes of ethyl acetate before incubation. Organic phases were pooled together, dried (40 °C, air stream), reconstituted with 1 ml of LC mobile phase, and analyzed by LC–MS/MS. This procedure allowed to extract about 98% of DON and DOM-1 present in the sample, whereas glucuronides were not detected in the organic phase. Aliquots of 1 ml of the remaining aqueous fraction were added with 1 ml phosphate buffer (pH 7.4) containing 4500 U of β-glucuronidase, then incubated and purified as described above for human urine.

2.8. LC–MS/MS analysis of DON, DOM-1, DON-G, DOM-1-G

2.8.1. Instrumentation

LC–MS/MS analyses were performed on a Q TRAP® MS/MS system, from Applied Biosystems (Foster City, CA, USA) equipped with an APCI (atmospheric pressure chemical ionization) interface and a 1100 Series micro-LC system comprising a binary pump and a microautosampler from Agilent Technologies (Waldbronn, Germany).

2.8.2. LC parameters

The analytical column was a Synergi Hydro® (150 mm × 3 mm, 4 μm particles) (Phenomenex, USA), preceded by an Aqua C18 guard column (4 mm × 2 mm, 10 μm particles) (Phenomenex, USA). The flow rate of the mobile phase was 400 μl/min, while the injection volume was 10 μl. The column effluent was directly transferred into the APCI interface, without splitting. Before LC–MS/MS analysis all samples were filtered through 0.45 μm membrane filter. Eluent A was water and the eluent B was methanol, both containing 5 mM ammonium acetate. Gradient elution was performed by changing the mobile phase composition as follows.

For full scan and single ion monitoring (SIM) analysis, the proportion of eluent B was linearly increased from 1% to 20% in 30 min, kept constant for 20 min, then increased to 80% in 1 min, and kept constant for 4 min. The column was re-equilibrated with 1% eluent B for 10 min.

For multiple reaction monitorinr (MRM) analysis, after 5 min of 5% eluent B, the proportion was linearly increased to 20% in 1 min, kept constant for 24 min, then increased to 80% in 1 min, and kept constant for 4 min. The column was re-equilibrated with 1% eluent B for 7 min.

2.8.3. MS parameters

For MS analyses, the APCI interface was used with the following settings: temperature (TEM) 450 °C, respectively. Curtain gas (CUR), nitrogen, 30 psi; nebulizer gas (GS1), air, 50 psi; auxiliary gas (GS2), air, 30 psi; corona discharge needle current (NC), −3 or +3 μA, for negative and positive ion mode, respectively. Full scan chromatograms were acquired in the range 50–800 amu. When operating in MRM mode, 3 transitions were monitored for each compound, with a dwell time of 100 ms. MRM parameters are reported in Table 1. Limits of detection (LOD, signal-to-noise ratio

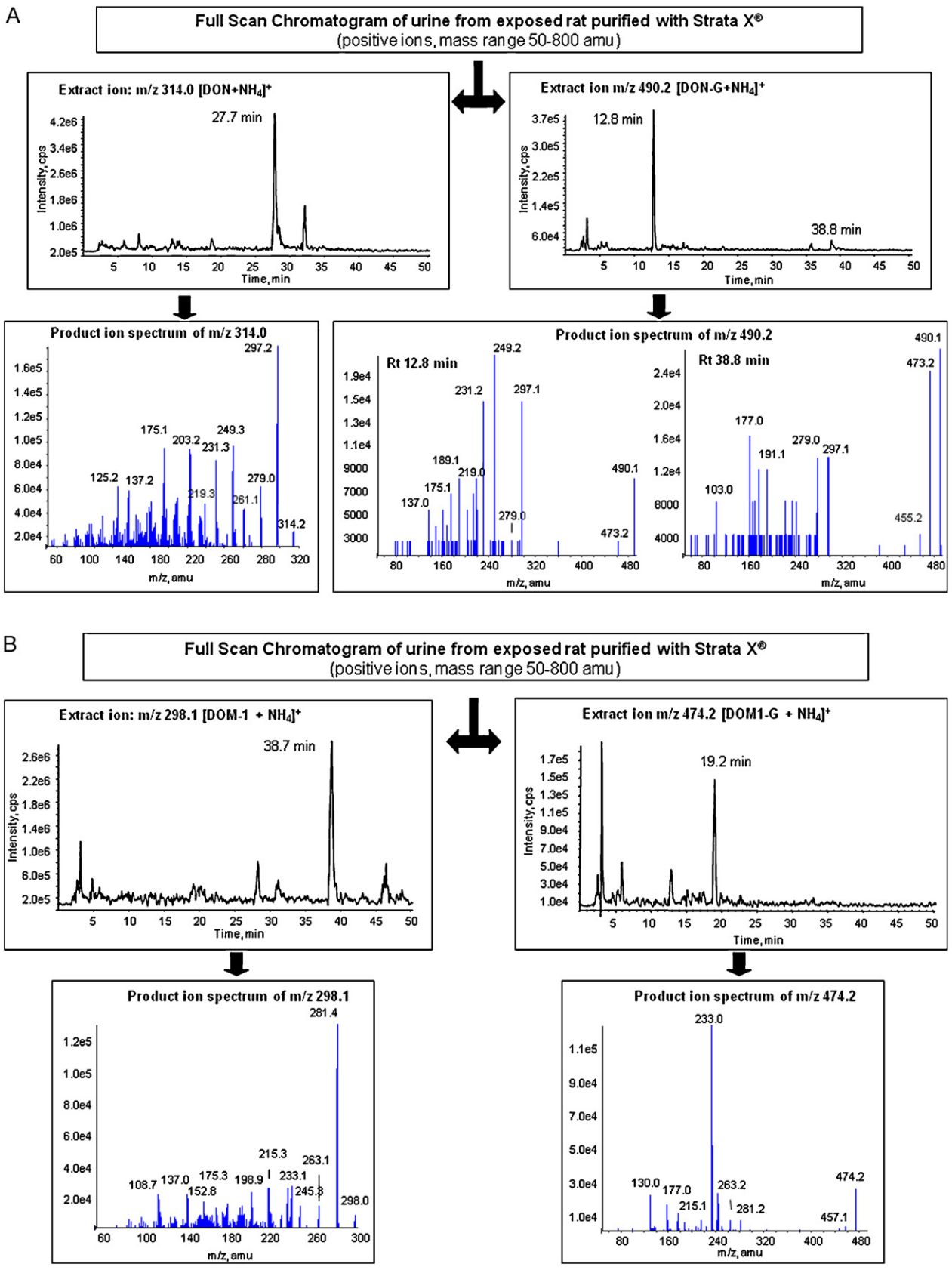


Fig. 1. Identification and characterization of DON metabolites in rat urine. (A) Extract ion profile and LC–MS/MS spectra of $[DON + NH_4]^+$ (m/z 314.0) and the two isomers $[DON\text{-}G + NH_4]^+$ (m/z 490.2) eluting at 12.8 and 38.8 min. (B) Extract ion profile and LC–MS/MS spectra of $[DOM\text{-}1 + NH_4]^+$ (m/z 298.1) and $[DOM\text{-}1\text{-}G + NH_4]^+$ (m/z 474.2).

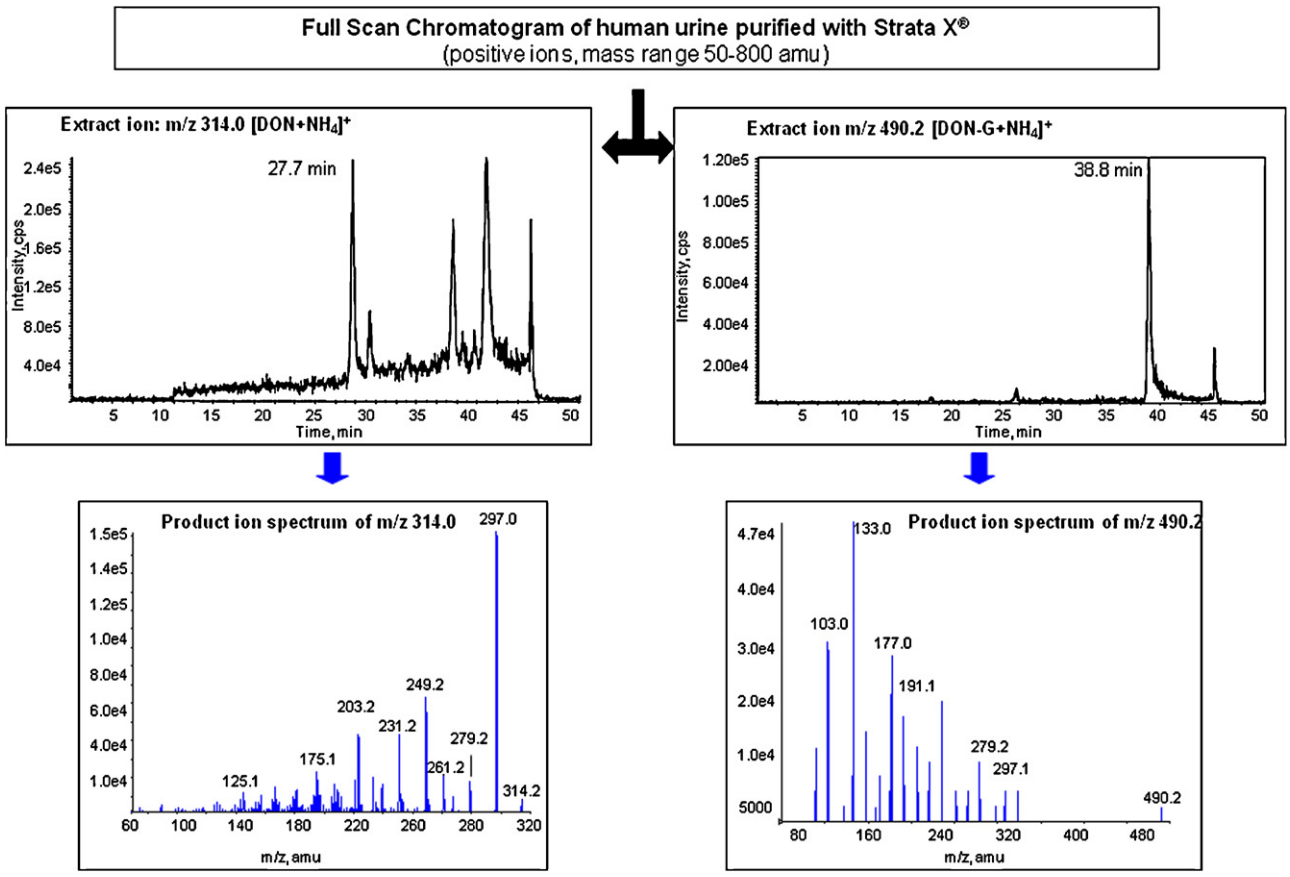


Fig. 2. Identification and characterization of DON metabolites in human urine. Extract ion profile and LC–MS/MS spectra of $[DON + NH_4]^+$ (m/z 314.0) and $[DON\text{-}G + NH_4]^+$ (m/z 490.2).

3:1) were 2 ng/ml for DON and 1 ng/ml for DOM-1. Limits of quantification (LOQ, signal-to-noise ratio 10:1) were 6 ng/ml for DON and 3 ng/ml for DOM-1. LOD and LOQ values refer to urine samples purified onto Strata-X columns according to the procedure described in Section 2.5 and were experimentally determined by spiking blank urine samples at different levels down to the lowest detectable concentration.

2.8.4. One-point matrix-matched calibration for quantitative determination of DON and DOM-1

For recovery experiments and analysis of naturally contaminated urine samples, calibrant solutions for matrix-matched calibration were prepared in both human and rat blank urine cleaned up through Strata-X columns (as described in Section 2.5) by adding an appropriate amount of DON and DOM-1 mixed standard solution to the methanolic eluate before drying. In hydrolysis experiments, calibrant solutions for matrix-matched calibration were prepared by treating blank urine from both humans and rats with β-glucuronidase as described in Section 2.7, then after clean up of the incubated sample onto Strata-X, the methanolic eluate was spiked with an appropriate amount of mixed DON/DOM-1 standard solution before drying.

3. Results

3.1. Sample preparation for DON metabolite investigation

SPE cartridges were evaluated for urine purification and concentration in order to extract the whole pool of DON metabolites. The suitability of Strata-X SPE cartridges for these purposes was demonstrated at a first stage by the LC–MS detection of characteristic ions of DON, DOM-1 and their glucuronide conjugates in the purified urine samples. Details are discussed in Section 3.2.

Then, recovery experiments were carried out to ensure that all metabolites were quantitatively extracted. Recoveries of DON and DOM-1, evaluated by using commercially available authentic standards, were 93% and 88% with relative standard deviations (RSD) of 4% and 7%, respectively. Due to the lack of commercial standards, recoveries of glucuronide derivatives were evaluated by using urines from DON exposed rats and by comparing peak areas of the relevant molecular ions $[DON\text{-}G + H]^+$ and $[DOM\text{-}1\text{-}G + H]^+$ (acquired in SIM mode) before and after the clean up. Glucuronide recoveries resulted to be 72% (RSD 11%) for DON-G and 102% for DOM-1-G (RSD 5%). DON and DOM-1 recoveries, calculated by using the same approach, resulted to be 95% (RSD 7%) and 100% (RSD 1%). These data, in agreement with those obtained using authentic standards, confirmed the suitability of the applied procedure for isolation of DON, DOM-1 and the relevant glucuronide conjugates.

3.2. Identification and characterization of DON metabolites in rat urine

For metabolite investigation, urine samples from DON exposed rat was cleaned up on Strata-X column then analyzed by LC–MS and LC–MS/MS. Levels of DON and DOM-1 in the investigated samples were in the ranges 1.9–4.9 μg/ml and 1.6–5.9 μg/ml, respectively.

To detect molecular ions corresponding to potential DON metabolites full scan chromatograms of rat urine samples were first acquired (Fig. 1A and B). The extracted ion chromatogram at m/z 314.0, corresponding to the adduct $[DON + NH_4]^+$, showed a main peak at 27.7 min, corresponding to the retention time of stan-

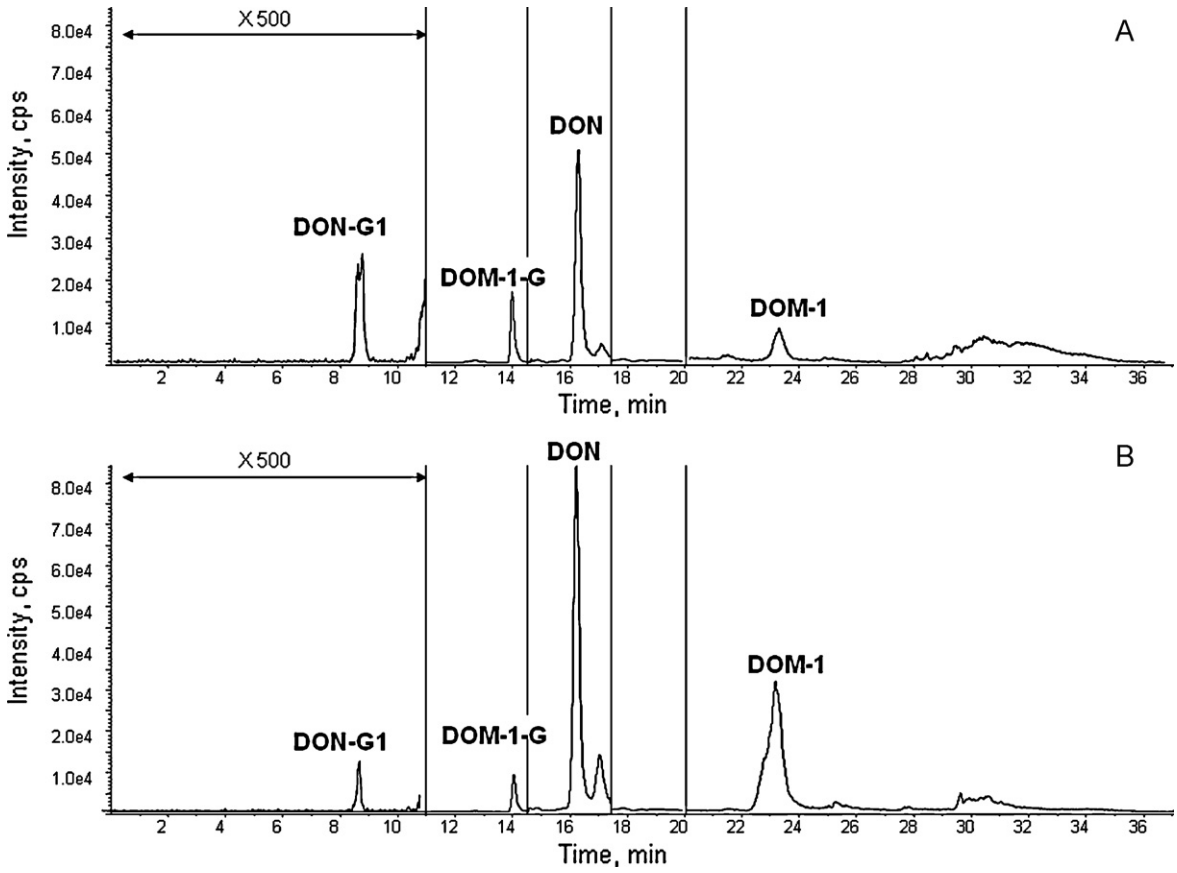


Fig. 3. TIC (sum of MRM transitions) chromatograms of a rat urine sample before (A) and after (B) hydrolysis with β-glucuronidase.

dard DON. Mass spectrum (not shown) at this retention time also showed a peak at *m*/*z* 297.0 corresponding to the ion $[DON + H]^+$. Interestingly, the extracted ion chromatogram of *m*/*z* 490.2, corresponding to $[DON\text{-}G + NH_4]^+$ showed two relevant peaks at retention times of 12.8 (DON-G1) and 38.8 min (DON-G2). Fig. 1A also shows the LC–MS/MS spectra of molecular ions $[DON + NH_4]^+$ (*m*/*z* 314.0) and the two detected ions $[DON\text{-}G + NH_4]^+$ (*m*/*z* 490.1). Fragmentation patterns of $[DON + NH_4]^+$ and $[DON\text{-}G + NH_4]^+$ eluting at 12.8 (DON-G1) min were almost overlapping, both exhibiting characteristic fragment ions of the trichothecene moiety, i.e. *m*/*z* 297.1, 279.0, 249.3, 231.3, 175.1, 137.2 [19,20]. All these fragments were also detected in the MS/MS spectrum of standard DON. Different fragmentation pattern was obtained, by applying the same collision energy value, for the compound eluting at 38.8 min (DON-G2). Besides the peaks at *m*/*s* 473.2 and 455.2 due to the subsequent neutral losses of NH_3 and water, respectively, this compound showed two fragment ions attributable to the trichothecene moiety, i.e. *m*/*z* 297.1 and 279.0 and three peaks at *m*/*z* 191.1 ($C_6H_7O_7$), 177.0 ($C_6H_9O_6$) and 103.0 attributable to the glucuronide residue. Results of LC–MS/MS analyses suggest therefore the presence of two DON glucuronide isomers eluting at different retention times. The MS/MS spectra of the compound eluting at about 33 min, as well as those of other minor peaks observed in extracted ion chromatograms of *m*/*z* 314.0 and 490.2 (Fig. 1A), did not show any fragment attributable to DON or structurally related compounds.

Since levels of DOM-1 in the analyzed sample were comparable to those of DON, the presence of a glucuronide conjugate of DOM-1 was also investigated (Fig. 1B). The extracted ion chromatogram of *m*/*z* 298.1, corresponding to the adduct $[DOM\text{-}1 + NH_4]^+$, showed a peak at 38.7 min. Both retention time and fragmentation of this compound were identical to those of the authentic standard. Furthermore, by extracting in the full scan chromatogram the mass 474.2, corresponding to the molecular mass of the ion $[DOM\text{-}1\text{-}G + NH_4]^+$ one peak was obtained at retention time of 19.2 min. The MS/MS spectrum of the ion of *m*/*z* 474.2 showed a fragment of *m*/*z* 457.1 ($[DOM\text{-}1\text{-}G + H]^+$) and main fragments at *m*/*z* 281.2, 233.1 also present in the MS/MS spectrum of standard DOM-1. Furthermore, fragments of *m*/*z* 191.1, 177.0, 133.1 originating from the glucuronide moiety were also present, thus confirming the molecular structure of DOM-1-G.

3.3. Identification and characterization of DON metabolites in human urine

To investigate the presence of DON metabolites in human urine, urine samples were collected from adult healthy subjects. MRM analyses showed the presence of DON at levels ranging from 0.003 to 0.008 μg/ml whereas DOM-1 was not detected in any of the collected samples.

Results of LC–MS and LC–MS/MS investigation of DON metabolites are reported in Fig. 2 DON was detected as ammonium adduct and confirmed by the analysis of MS/MS fragmentation pattern as shown above for rat urine. In the extracted ion chromatogram of *m*/*z* 490.2, corresponding to the adduct $[DON\text{-}G + NH_4]^+$, only one peak at 38.8 min was observed (DON-G2). This compound showed a fragmentation pattern similar to that obtained in rat urine sample, with peaks at *m*/*z* 297.1 and 279.0 relevant to the DON moiety and more prominent peaks at *m*/*z* 191.1, 177.0, 133.0, 103.0 originating from the glucuronide residue. The DON-G isomer eluting at 12.8 min (DON-G1), and DOM-1-G were not detected.

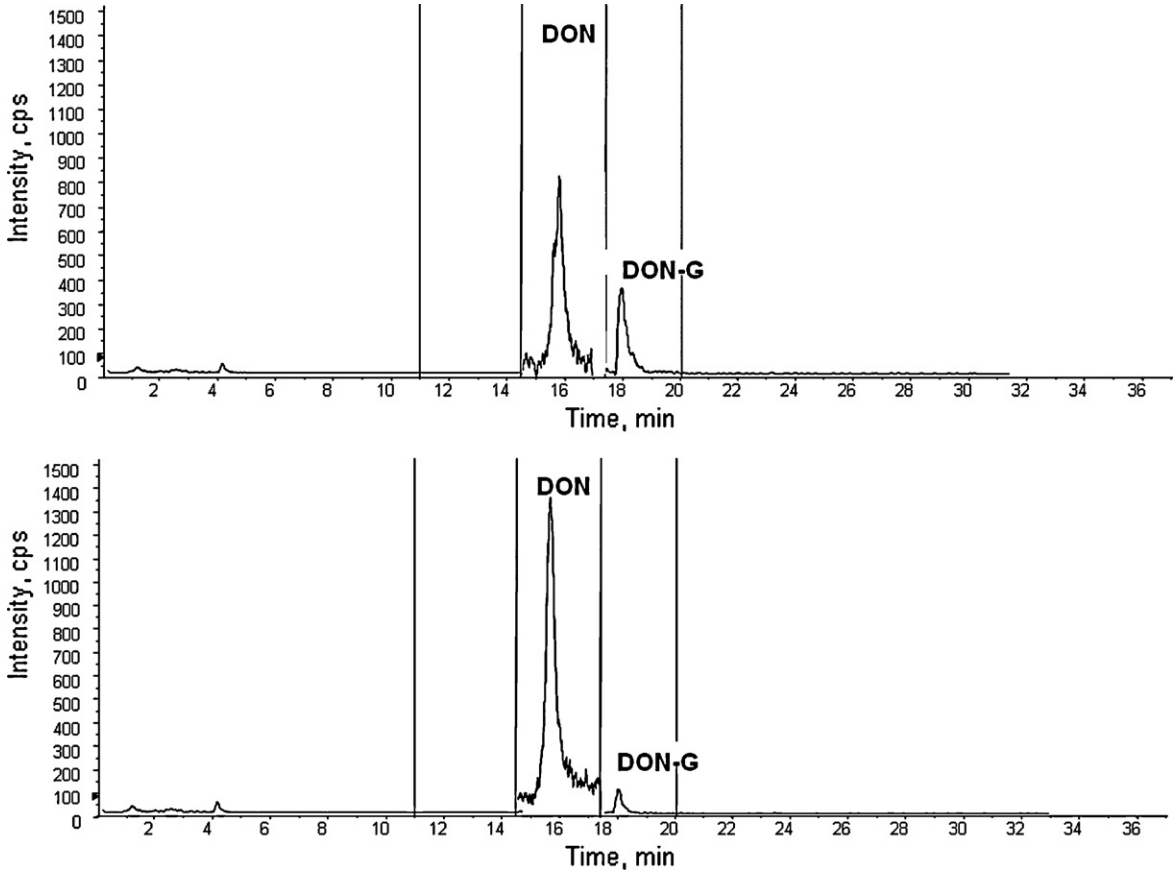


Fig. 4. TIC (sum of MRM transitions) chromatograms of a human urine sample before (A) and after (B) hydrolysis with β-glucuronidase.

3.4. MRM detection of DON, DOM-1, DON-G and DOM-G

A MRM method for the detection of DON, DOM-1, DON-G1, DON-G2 and DOM-1-G (Table 1) was elaborated for screening purposes and for the analysis of hydrolyzed urine samples (see following paragraph). MRM transitions for the detection of DON and DOM-1 were obtained by direct infusion experiments performed with standard toxins. MRM transitions and relevant collision energy values for the detection of glucuronide derivatives were chosen by acquiring LC–MS/MS spectra of DON-G1, DON-G2, and DOM-1-G at different collision energy values. Given the different MS/MS behavior (see Fig. 1) of the two DON-G isomers, different transitions were chosen for these compounds.

3.5. Structure confirmation by hydrolysis with β-glucuronidase

To further confirm the structure of glucuronide derivatives, rat and human urines were treated with β-glucuronidase. The initial DON and DOM-1 content of the rat urine sample used for hydrolysis experiments was 4.9 μg/ml and 1.6 μg/ml, respectively. Extremely high LC–MS/MS signals were observed for DON and DOM-1 in rat urine, as compared to those of glucuronides (see Fig. 3), therefore enzyme incubation was performed after removing most of DON and DOM-1 from the samples by extraction with ethyl acetate. This allowed to measure the increase of the chromatographic signal to quantify the release of DON and DOM-1 during the hydrolysis.

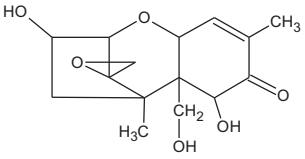
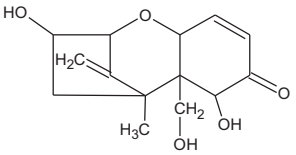
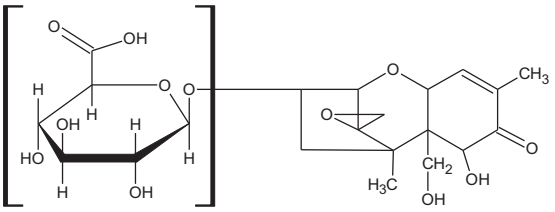
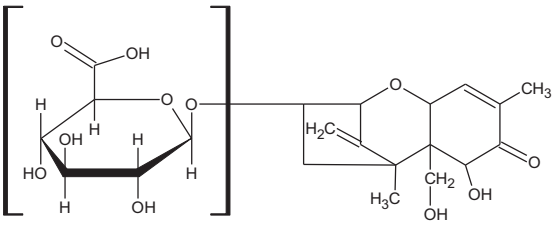
Chromatographic profiles of rat urine sample before and after incubation with β-glucuronidase are shown in Fig. 3. The β-glucuronidase treatment resulted in a DON increase of 0.350 (±0.042) μg/ml (corresponding to 0.557 μg/ml of DON glucuronide hydrolyzed), while a 44% (RSD 21%) decrease of the peak area of DON-G1 isomer was observed. Given the intensities of glucuronide peaks in the analyzed samples, it was only possible to quantify the decrease of the isomer DON-G1. The DOM-1 increase resulted in 1.850 (±0.211) μg/ml (corresponding to 3.012 μg/ml of DOM-1-G hydrolyzed), while a 16% (RSD 6%) decrease was observed for the peak area of DOM-1-G. Even though the hydrolysis was not quantitative, the decrease of glucuronide peaks and the simultaneous increase of DON and DOM-1 peaks confirmed the presence of glucuronide conjugates of both DON and DOM-1 in rat urine.

The structure of DON glucuronide in human urine was confirmed in the same way. The initial DON content in human urine sample used for hydrolysis experiments was 0.004 μg/ml. Following β-glucuronidase treatment, the DON content resulted 0.007 (±0.0005) μg/ml, corresponding to 0.004 μg/ml of DON glucuronide hydrolyzed. By comparing peak areas of DON-G2 in the chromatograms of samples before and after incubation, a decrease of peak area of 27% (RSDr 12%) was calculated. Chromatographic profiles of the urine sample before and after the incubation with β-glucuronidase are shown in Fig. 4. As observed for rat urine, the hydrolysis was not quantitative, but the increase of DON peak in conjunction with the decrease of DON-G2 peak confirmed the presence of a glucuronide derivative in human urine.

4. Discussion

Although the presence of de-epoxide and glucuronide conjugate of DON in biological fluids was previously reported, very limited MS data are available in the literature [19,22]. In the present work, LC–MS/MS has been investigated as a direct method to analyze for

Table 2
Summary of structural information and qualitative DON metabolite profile, in human and rat urine, obtained by analysis of MS/MS spectra and β-glucuronidase hydrolysis.

	Molecular structure	Characteristic ions (m/z)		Presence in urine	
				Rat	Human
DON		355.1 295.0 265.1 59.0	Negative ions	Yes	Yes
DOM-1		339.1 279.0 249.1 59.0	Negative ions	Yes	No
DONG1*		490.2 297.2 249.2 231.1	Positive ions	Yes	Yes
DONG2*		490.2 177.1 103.1 89.0	Positive ions	No	Yes
DOM-1-G*		474.2 281.2 233.1 130.1	Positive ions	Yes	Yes

* The position of glucuronide moiety is indicative since the exact site of glucuronidation cannot be determined by MS/MS spectra.

DON and its metabolites in urine samples. A summary of structural and qualitative information on DON metabolites in human and rat urine obtained by LC–MS/MS analysis is reported in Table 2. After sample pre-concentration on SPE cartridges, the whole set of DON metabolites could be detected in rat urine, whereas only DON and DON glucuronide were found in human urine. The fragmentation patterns (MS/MS spectra) provided detailed structural information for all toxin metabolites (even for those present at trace levels) without the need to isolate them. Differences in the urinary excretion profiles between human and rat were identified.

In agreement with previous findings [9,23], the metabolites detected in human urine were only DON and DON glucuronide, whereas neither DOM-1 nor its glucuronide conjugate were found. Structural confirmation of DON glucuronide in the investigated samples was obtained by β-glucuronidase hydrolysis. Although the hydrolysis was not quantitative, an increase of 1.7-fold of DON content was observed in human urine, confirming that DON glucuronide conjugate represents a significant portion of excreted DON in humans. Meky et al. [9] observed an increase of 1.2–2.8-fold of DON content when hydrolyzing human urine samples with a DON content at levels comparables to those reported in this study (in the range 0.004–0.018 μg/ml). Little information about gastrointestinal de-epoxidation of trichothecene in humans is available in the literature, except for a study by Eriksen and Petterson [23] that observed lack of de-epoxidation of type-B trichothecenes incubated with human excreta. Likewise, DOM-1 was not detected in this study also after β-glucuronidase treatment of human urine. Low levels of DOM-1 (in the range 0.0002–0.0028 μg/ml) in human urine has been recently reported [10].

With respect to urinary excretion profile in rats, comparison of our findings with previously reported studies was quite hard since the available data on DON metabolism in rats are conflicting. DOM-1 was first identified in urine from Wistar rats by Yoshizawa et al. [24], further studies confirmed the presence of this metabolite in excreta of oral administered PVG rats [7,11]. On the other hand, DOM-1 was not detected in urine from Sprague-Dawley rats, that were found to contain a significant portion of DON glucuronide [9]. *In vitro* studies showed no metabolic activity, neither DON de-epoxydation nor glucuronidation, in rat liver fractions from male rats [25]. Another report describes the biotransformation of DON to its glucuronide in the isolated perfused rat liver [16]. In this study both DON and DOM-1 were detected together with their glucuronide conjugates in urine from orally administered Wistar rats, revealing a complex excretion pattern in which de-epoxidation and glucuronidation routes are involved. This study shows for the first time the presence of two different DON glucuronide conjugate forms in Wistar rat urine.

Data from the literature are not homogeneous, indicating that metabolic routes could be influenced by several parameters such as the animal species, the method of administration or the level of toxin exposure. Variations in metabolic profile of trichothecene mycotoxins following oral or intravenous administration were found in Sprague-Dawley rats [26]. Differences in glucuronidation activity between Sprague-Dawley, Fischer 344, and Wistar rats, were evidenced and attributed to differences in β-glucuronidase levels in the examined strains [27].

On the other hand, the analytical approach to detect and quantify a range of different metabolites in such a complex matrix plays an important role. Even though several approaches have been pro-

posed, to date there is no validated method for the analysis of deoxynivalenol metabolites in urine. The most frequently used procedure is based on indirect measurements of the sum of DON and its glucuronide derivative after β-glucuronidase hydrolysis [9,14]. However, a standardized method based on hydrolysis that guarantees a quantitative conversion is currently not available and there is no harmonization between published protocols [9,14,22].

In this view, it appears to be quite important the availability of highly sensitive and selective analytical methods enabling the direct measurements of the whole set of DON metabolites in biological fluids even at trace levels, avoiding indirect measurements. The poor selectivity of HPLC with UV reading makes this technique not adequate for these purposes. Available GC–MS methods for DON metabolite analysis require both glucuronide hydrolysis and sample derivatization [16,17]. The present work showed how using LC–MS/MS it is possible to overcome these drawbacks and to obtain detailed structural information of investigated compounds. Moreover, although quantitative determination was out of the scope of the manuscript, a significant improvement in terms of detection limits and selectivity, i.e. an higher degree of analyte identification, has been obtained with such advanced technique, as compared with previous reports based on LC–MS [18,19].

A protocol for the laboratory synthesis of a standard DON glucuronide has been reported [22], although the compound is not commercially available. The availability of commercial standard would open future perspectives for the direct quantitative determination of DON and its metabolites including conjugate forms. This would make the method applicable to monitoring programmes providing a complete and realistic framework of exposure levels and relevant metabolic routes.

5. Conclusions

The urinary excretion profile of the mycotoxin deoxynivalenol in human and rat has been investigated by LC–MS/MS. Structure elucidation of the detected metabolites has been provided by the analysis of their fragmentation patterns, and confirmed by β-glucuronidase hydrolysis. Exploiting MS/MS data, a MRM method for the simultaneous detection of all investigated metabolites has been elaborated and applied for the analysis of naturally contaminated samples before and after β-glucuronidase treatment. A solid phase extraction clean up procedure allowing satisfactory recoveries for DON, DOM-1, and their glucuronide conjugates has been optimized. Differences in the urinary metabolite profile in human and rat have been evidenced, and the presence of two DON glucuronide isomers in Wistar rat urine has been shown for the first time. The reported results demonstrate the suitability of LC–MS/MS technique for the direct detection and characterization of DON metabolites.

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