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Assessing the hydrolytic fate of the masked mycotoxin zearalenone-14-glucoside – A warning light for the need to look at the "maskedome"

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ABSTRACT

Masked mycotoxins are plant metabolites of mycotoxins that contaminate food and feed. They pose health concern as the shortage of toxicological data forces the lack of regulation worldwide. The present work investigated the toxicological relevance of the masked mycotoxin zearalenone-14-glucoside. *In vitro*, it shows a lower toxicity in respect to the parent compound. However, the major risks related to the consumption of masked mycotoxins depend on the possibility to undergo hydrolysis. Therefore, the hydrolysis and further transformation of zearalenone-14-glucoside in bovine blood and blood components (i.e. plasma, serum and serum albumin) were monitored using LC/MS-MS analysis to gain insights on the possible systemic fate. Hydrolysis was observed in all matrices, and both cell-dependent and –independent contributions were pointed out. Moreover, further metabolism was observed in the whole blood as zearalenol isomers were found. Serum albumin was identified among the active components, and the protein-ligand interaction was investigated *via* computational analysis. The blood has been pointed out as possible district of reversion and further activation of zearalenone-14-glucoside, and a similar fate cannot be excluded for other masked mycotoxins. Therefore, the systemic hydrolysis should be evaluated beside the absorption, bioavailability and bioaccessibility to deeply understand the toxicity of masked mycotoxins.

1. Introduction

Mycotoxins are toxic low-molecular weight molecules naturally produced as secondary metabolites by filamentous fungi on crops. They can accumulate in a wealth of agricultural commodities, food and feed as a consequence of the infection of susceptible crops pre-harvest and/or raw materials post-harvest upon incompliant storage practices (Pitt et al., 2013). The dietary intake of mycotoxins in human and animal poses serious hazard for health and wellbeing as they cause a number of illnesses, physiological alterations and dysfunctions (Wu et al., 2014). The nature and severity of such altered conditions mostly depends on the chemical type, exposure route and the levels of contamination. Many countries have established regulations and recommendations for some mycotoxins (e.g. zearalenone, deoxynivalenol, aflatoxins, fumonisins, ochratoxin A and patulin in the EU) in order to reduce the possible exposure of consumers and livestock (for Europe: EC No, 1881/2006, EU No 165/2010, EU No 105/2010). However, regulatory actions typically address the parental compounds (i.e. the molecules chosen for representing the various chemical classes), neglecting the wealth of modified forms commonly found as co-contaminants in food and feed (Smith et al., 2016). This mostly depends on the shortage of toxicological data available so far for modified mycotoxins, since regulation must base on solid scientific dataset.

According to the classification proposed by Rychlik (Rychlik et al., 2014), the class of modified mycotoxins groups chemically modified (e.g. compounds formed upon thermal treatments) and biologically modified compounds (e.g. metabolites of plants, animals, fungi and microorganisms). Chemical modification may have huge effects on the biological activity on both positive and negative directions (*vide infra*). Nonetheless, the modulation of toxic activity by means of chemical (bio)transformation is completely overlooked

Abbreviation: ZEN, zearalenone; ZEN-14-Glc, zearalenone-14-glucoside; BSA, bovine serum albumin; FBS, fetal bovine serum; DON, deoxynivalenol; DON-3-Glc, deoxynivalenol-3-glucoside.

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in the regulatory actions up to now. As a consequence, the current regulations might not comply with the real scenario since part of the toxicologically active molecules might be not considered in the setting of tolerable contamination levels. In this respect, the European Food Safety Authority (EFSA) recently pinpointed the need to consider the ratio of bioactivated forms on the detoxified ones in the setting of appropriate health based guidance for foodborne mycotoxins (EFSA., 2016). Accordingly, steps toward a more comprehensive approach assessing also the toxicological relevance of modified forms shall be moved in the near future for setting more appropriate limits of tolerable contamination. Plant metabolites of mycotoxins, and in particular the phase-II metabolites (namely, masked mycotoxins; (Rychlik et al., 2014);) must be considered in toxicological studies as they can enter the food and feed production chain at significant levels.

The present work focused on the masked mycotoxin zearalenone-14-glucoside (ZEN-14-Glc). In particular, it was aimed at gaining further insight into the toxicological relevance in order to define a more informed scenario for risk assessment. Zearalenone (ZEN) is a mycotoxin produced by fungi belonging to *Fusarium* spp., mainly *F. culmorum* and *F. graminearum*, upon infection of small grains and maize pre-harvest (Marin et al., 2013). It may contaminate several cereal-based products worldwide including flour, malt, soybean and beer. ZEN raises major concern on account of the xenoestrogenic activity in human and animal by means of the binding and activation of the estrogen receptors (ERs) (Ehrlich et al., 2015). The onset of sexual disorders, anabolic effects, development alterations, and subsequent cancerogenicity are indeed linked to the contamination of food and feed by ZEN (Massart and Saggese, 2010; Schoevers et al., 2012; Pazaiti et al., 2012). ZEN can be converted massively by plants into several glycoconjugates. Among the masked form, ZEN-14-Glc can contribute to the total amount of mycotoxin up to 30% (Berthiller et al., 2013). It may accumulate in the edible parts of infected plants and enters the food and feed chain, thus raising a safety issue. For instance, it has been found among the most abundant ZEN congeners in some cereal and cereal-based foods in Belgium and Finland (De Boevre et al., 2013; Nathanail et al., 2015). A clear statement about the safety of ZEN-14-Glc for living organisms cannot be done due to the few data collected so far in respect to the LADME/Tox paradigm (i.e. *T*oxic effects of foodborne compounds upon *L*iberation from food/feed during digestion, *A*bsorption, *D*istribution, *M*etabolism and *E*xcretion).

Up to now, the possible hazard of ZEN-14-Glc for human and animals has been supposed on the basis of the hydrolysis to the ZEN aglycone observed after digestion in mammals (De Boevre et al., 2015), upon cleavage by human microbiota (Dall'Erta et al., 2013) and by breast cancer cell culture (Dellafiora et al., 2016). On the other side, the addition of glucoside proved to disrupt significantly the capability to interact with the ERs (Poppenberger et al., 2006). In this sense, the conjugation of ZEN seems an effective detoxifying pathway, as observed for other glycosylated mycotoxins (Frizzell et al., 2015; Pierron et al., 2015). Therefore, the extent of hydrolysis under *in vivo* conditions (if any) is a critical point to determine the real toxicological role of ZEN-14-Glc (Dellafiora et al., 2016) and the various districts where the hydrolysis may take place become relevant objects of investigation. Among them, the hydrolysis at the level of the systemic circulation may have a key role for the delivery of freely available toxic aglycones. Contextually, the hydrolysis of ZEN-14-Glc in the bloodstream is an appropriate object of investigation as the absorption of glycosylated molecules upon oral exposure can be possible (Broekaert et al., 2016; Mullen et al., 2004, 2006). Nevertheless, for masked mycotoxins, the investigation of absorption, plasma dosage and stability in the systemic circulation *in vivo* is challenging. Indeed, the lack of commercial availability and metrological infrastructures for these compounds force to resort to the in-home synthesis – which is not affordable for large-scale purposes – and hampers the standardization of procedures (Dellafiora and Dall'Asta, 2016). For this reason, the upstream assessment of hydrolysis and further (bio)transformations by blood and blood components beforehand *in vitro* can be a straightforward strategy to point out the need for further and more detailed investigations *in vivo*. In this framework, for the first time the present study addressed the possible hydrolytic fate of ZEN-14-Glc at the systemic level to gain general insights on the mechanisms of action possibly responsible of eliciting toxicity. Specifically, the hydrolysis of ZEN-14-Glc and the further transformation in known xenoestrogens by bovine plasma, fetal bovine serum (FBS), whole blood and serum albumin has been investigated through LC/MS-MS analysis. Furthermore, the interaction with serum albumin has been investigated at a molecular level by means of *in silico* modeling.

2. Materials and methods

2.1. Chemicals and samples

ZEN-14-Glc (specifically, the 14-β-D-glucoside isomer) was synthesized as previously described (Dall'Erta et al., 2013). The identity and structure of ZEN-14-Glc were confirmed by nuclear magnetic resonance (data not shown). The purity check ruled out the presence of relevant amount of ZEN in the stock solution of ZEN-14-Glc (Fig. 1S; supporting material).

ZEN (10 mg, crystalline) was purchased from RomerLabs (Tulln, Austria). The fetal bovine serum (FBS) was purchased from EuroClone® SpA (Milan, Italy), as viable alternative of adult serum in order to reduce the volume of collected blood. Bovine serum albumin (BSA; lyophilized powder, purity ≥ 96%), trypsin, α-ZEL, β-ZEL, solvents, salts and other general-use chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Blood samples for whole blood and plasma preparations were taken from the coccygeal vein of an adult dry cow and collected into commercially available anticoagulant-treated tubes containing Lithium Heparin as anticoagulant (BD Vacutainer® – Disposable Vacutainer, Green Cap - Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). No further treatments were done for whole blood preparation, while sample for plasma was obtained by collecting supernatant upon centrifugation at 3500*g* for 15 min.

2.2. Assessment of hydrolysis

2.2.1. Exposure and extraction

FBS, bovine plasma and bovine whole blood were treated with ZEN-14-Glc 1 μM for 6 and 24 h to assess the hydrolysis and further transformation phenomena. In addition, since serum albumin may show different (pseudo)enzymatic activities (*vide infra*), ZEN-14-Glc was incubated also with a BSA solution (1 mM in PBS buffer 8 M, pH 7.4) for 6, 18 and 24 h to investigate a possible role in the hydrolysis. All the incubations were carried out in 150 μL and they were gently mixed at 37 °C until the extraction for the LC/MS-MS analysis. The extraction was done by adding 450 μL of a methanol:ethanol solution (50:50), followed by 10 min on ice and then centrifuged for 10 min at 4 °C and 15000 g. Supernatants were retrieved and stored at −20 °C until the LC/MS-MS analysis.

2.2.2. LC/MS-MS analysis

The reduction of ZEN-14-Glc and the formation of ZEN, α-ZEL and β-ZEL were assessed by means of LC/MS-MS analysis. In particular, supernatants were analyzed using a UHPLC (Dionex Ultimate 3000) system coupled with a triple quadrupole mass

spectrometer (TSQ Vantage) (Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an ESI interface. All the analyses were performed on a RP-C18 Kinetex column (2.6 μm, 100A; 100 × 2.10 mm; Phenomenex, Torrance, CA, USA) using bi-distilled water (A) and methanol (B) both acidified with 0.2% of acetic acid as eluents. The injection volume was 3 μL. The gradient started at 20% B and reached 90% B in 7.5 min with a flux of 0.3 mL/min. Then it was kept isocratic for 1.5 min (9 min), by increasing the flux at 0.4 mL/min. From 9 to 12 min the flux was kept at 0.4 mL/min with 100% B. Finally, from 12 to 16 min flux decreased to 0.3 mL/min with 20% B. The total run time was 16 min. Compounds under investigation were monitored under negative ionization mode (spray voltage = 3500 V), with the capillary temperature at 270 °C, while the vaporizer temperature was kept at 200 °C. The sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units.

Detection was carried out using multiple reactions monitoring (MRM) mode. The following transitions were used for the compounds monitoring: 317 → 131 (CE = 34 eV) and 317 → 175 (CE = 28 eV) for ZEN, 479 → 317 (CE = 22 eV) for ZEN-14-Glc, and 319 → 129 (CE = 34 eV) for α-ZEL and β-ZEL.

Calibration curves (1/x weighted) for both ZEN and ZEN-14-Glc quantifications were prepared for BSA treatment in the concentration range 0.01–0.5 μM. Further details are reported in the Supplementing materials.

2.2.3. Qualitative assessment of ZEN-14-Glc transformation

The zero-time controls (t_0s) for each matrix were collected to obtain the unreacted initial reference values of molecules amount for comparing the fluctuations among the different treatment times. The t_0s were obtained by skipping the incubation time and extracting immediately (see above). As the same concentration of ZEN-14-Glc was used for all matrices, ZEN-14-Glc peak areas obtained at t_0s were used as normalization factors, thereby comparing roughly the decrease of ZEN-14-Glc amount among the various matrices.

The efficacy of the matrices in releasing ZEN was compared instead by normalizing the peak areas of ZEN with respect to the peak areas of the internal standard (i.e. ZEN 0.05 μM) obtained by spiking blank matrixes (further details are reported in the supporting material; Table 1S).

2.3. Analysis in silico

To model the interaction between ZEN-14-Glc and BSA the protein-ligand complex formation was evaluated by using the coupling of docking simulations and proper rescoring procedures. Specifically, the coupling of GOLD, as docking software, and HINT (Hydropathic INTeraction) (Kellogg and Abraham, 2000), as rescoring function, was chosen on the basis of previous studies demonstrating the higher reliability of HINT respect to other scoring functions and the efficacy as re-scoring function to predict ligands interaction with several protein targets (Cozzini and Dellafiora, 2012; Dellafiora et al., 2013, 2014, 2015a, 2015b). The HINT score (HS) provides empirical and quantitative evaluation of protein-ligand interaction, as a sum of all single atomic contributions. Since they correlate with the free energy of binding (Cozzini et al., 2002), HS correlates with the thermodynamic favor of protein-ligand interaction thus allowing the identification of preferred mode and site of binding (Dellafiora et al., 2015c).

The model for the BSA was derived from the Protein Data Bank (http://www.rcsb.org) structure having PDB code 4Or0 (Bujacz et al., 2014). The processing of protein structure and ligands, and docking simulations and rescoring procedure were performed in accordance to Ehrlich and co-workers (Ehrlich et al., 2015).

The ligand binding site was defined by using the Flapsite tool of the FLAP software (Baroni et al., 2007), while the GRID algorithm (Carosati et al., 2004) was used to investigate the corresponding pharmacophoric space. The DRY probe was used to describe the potential hydrophobic interactions, while the sp2 carbonyl oxygen (O) and the neutral flat amino (N1) probes were used to describe the hydrogen bond acceptor and donor capacity of the target, respectively. All 3D images were obtained using the software PyMol version 1.7 (http://www.pymol.org), while 2D sketch was obtained by using LigPlot + software (Laskowski and Swindells, 2011).

2.4. Statistical analysis

Each experiment was performed in technical triplicate over two different days (n = 6). Data are expressed as the mean ± standard deviation (SD). Data were statistically compared by one-way ANOVA ($\alpha = 0.05$), using IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, NY).

3. Results

3.1. Hydrolysis of ZEN-14-Glc

The variation of ZEN-14-Glc amount and the subsequent formation of ZEN after 6 and 24 h of treatments with bovine plasma, FBS and adult whole blood were qualitatively investigated. Furthermore, for each matrix the formation of the reduced ZEN metabolites α- and β-zearalenol (α-ZEL and β-ZEL, respectively) was investigated after 24 h of treatment. In all treatments, the reduction in the amount of ZEN-14-Glc and the release of ZEN were observed (Fig. 1), thus stating the hydrolytic activity over ZEN-14-Glc by whole blood, fetal serum and plasma.

More in details, the highest reduction of ZEN-14-Glc peak areas in respect to the t_0 was observed at 24 h treatment in FBS (Fig. 1). The trend was different when the release of ZEN is considered. Specifically, as shown in Fig. 2, all matrices showed time-dependent activity ($p < 0.001$), but the highest amount of free ZEN was actually found in whole blood ($p < 0.001$). No difference in terms of free ZEN amount were found instead between FBS and plasma at both the treatment time ($p = 0.173$).

Concerning the formation of α-ZEL and β-ZEL, it is worth noting that it was significantly observed upon exposure to the whole blood only (Fig. 3).

Keeping in mind that various (pseudo)enzymatic activities are ascribed to the serum albumins (including the glucuronidasic one) (Fanali et al., 2012), the hydrolytic activity of BSA was quantitatively investigated at 6, 18 and 24 h treatments. An appreciable and time-dependent reduction of ZEN-14-Glc's concentration and the subsequent formation of ZEN were observed, with a decrease of ZEN-14-Glc abundance close to 20% at 24 h (Fig. 4; further details are reported in the supporting material, Table 2S).

The stability of ZEN-14-Glc under the experimental conditions (i.e. in PBS buffer 8 M, pH 7.4, 37 °C for 24 h), but in the absence of BSA, was checked in order to rule out the BSA-independent hydrolysis. Appreciable hydrolysis was not found (further details are in the supporting material; Fig. S2).

3.2. Analysis of binding

The analysis of binding between BSA and ZEN-14-Glc was carried out by means of molecular modeling techniques. In particular, the possible interaction with two main drug binding sites (i.e. Sudlow's site I and II; Fig. 5A) was assessed by using a procedure based on docking simulations and rescoring procedures (Ehrlich et al., 2015). As reported previously, the procedure proved to be a reliable analytical tool for estimating the free energy of protein-

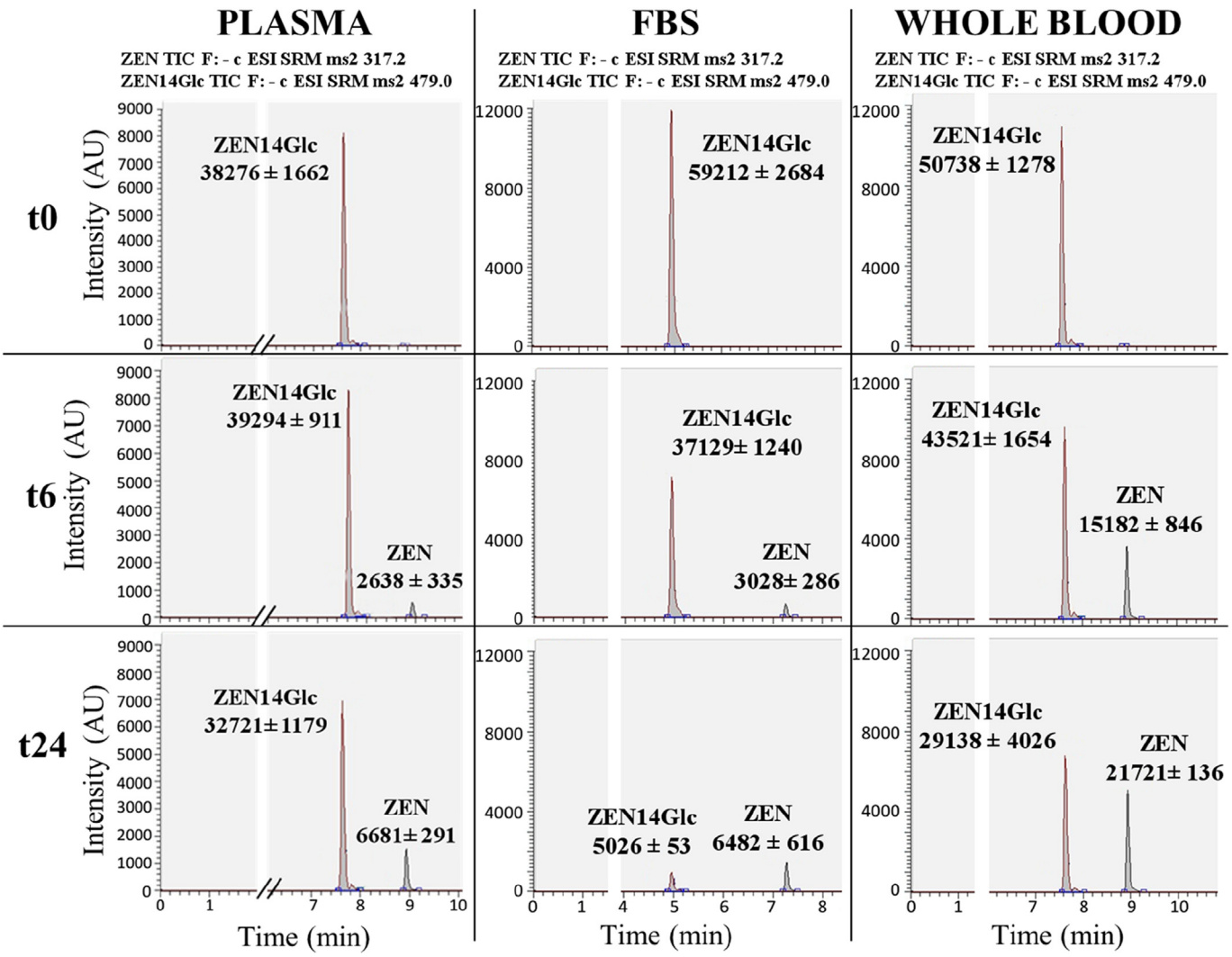

Fig. 1. Abundance of ZEN-14-Glc and ZEN in plasma, FBS and whole blood upon 6 and 24 h treatments. Peaks areas are expressed in bold as the mean value of two independent experiments ± standard deviation. The time-dependent reduction on ZEN-14-Glc abundance and formation of ZEN were found in all matrices.

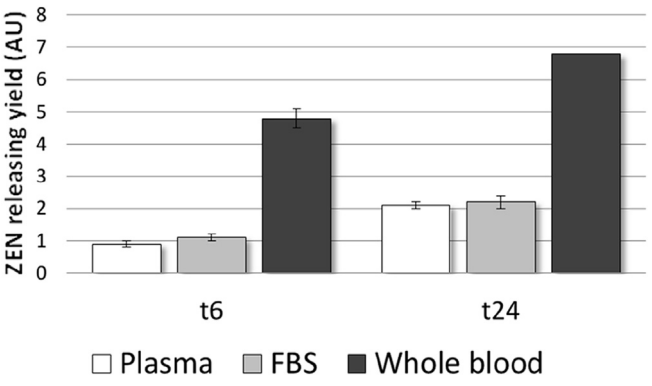

Fig. 2. Releasing yield of ZEN by plasma, FBS and whole blood. The time-dependent efficacy of matrices in converting ZEN-14-Glc to ZEN was expressed in arbitrary units (AU) as ZEN releasing yield. It has been calculated as the ten times ratio between the peaks areas of ZEN released upon the various treatments and the peak area of ZEN 0.05 μM in each blank matrix to correct matrix effects (further details are reported in supporting materials; Table 1S).

ligand complex formation and for predicting the architectures of binding, as well (Cozzini and Dellafiora, 2012; Cozzini et al., 2002; Dellafiora et al., 2015c). Thus it is suitable to hypothesize the mode of binding of ZEN-14-Glc within BSA's binding sites. Nonetheless, a fit-for-purpose validation aimed at assessing the case-specific reliability was done. In particular, the r-warfarin (a well-known ligand for serum albumin) was used as reference since both site and mode of binding were structurally resolved (Petitpas et al., 2001). In our calculation the r-warfarin properly scored the higher value within the Sudlow's site I (Table 1), thus pinpointing it as the site of interaction in accordance to experimental data reported in the literature. Also the binding architecture was calculated correctly, as revealed by comparing the computed pose with the crystallographic ones available so far (Fig. 5B). The case-specific reliability of computational procedure was stated accordingly.

Concerning ZEN-14-Glc, the interaction with the Sudlow's site I was positively scored, while steric interferences cause negative score within the Sudlow's site II (Table 1). Thus, the Sudlow's site I was identified as a possible site of interaction. Pharmacophoric analysis of the Sudlow's site I showed that the most part of the hydrophobic space was buried within the pocket, while the solvent exposed space at the entry was more hydrophilic (Fig. 5C). Concerning the architecture of binding, the glucoside was oriented toward the outside of the pocket, while the other part of the

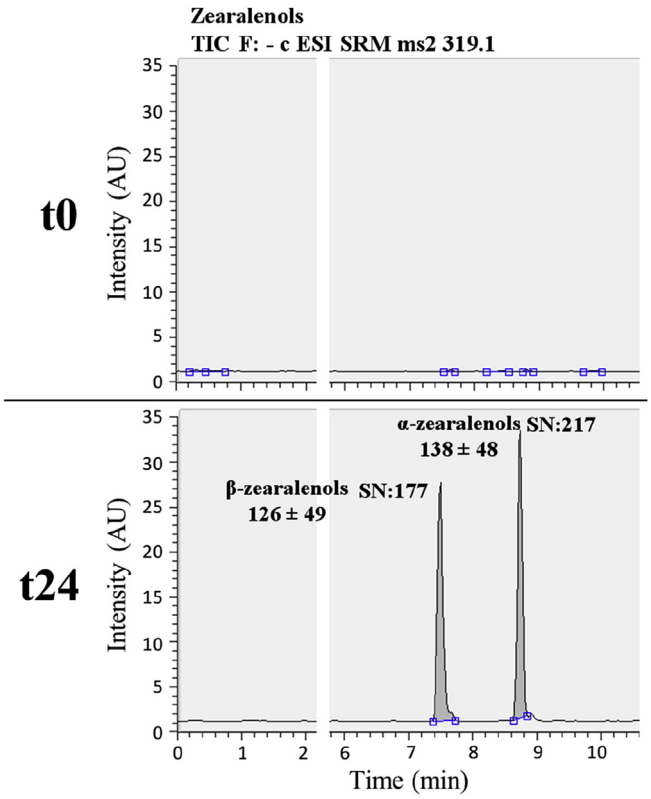

Fig. 3. Abundance of α-ZEL and β-ZEL in whole blood at 0 and 24 h of treatment. Peaks areas are expressed as the mean value of two independent experiments ± standard deviation. The formation of α-ZEL and β-ZEL was found.

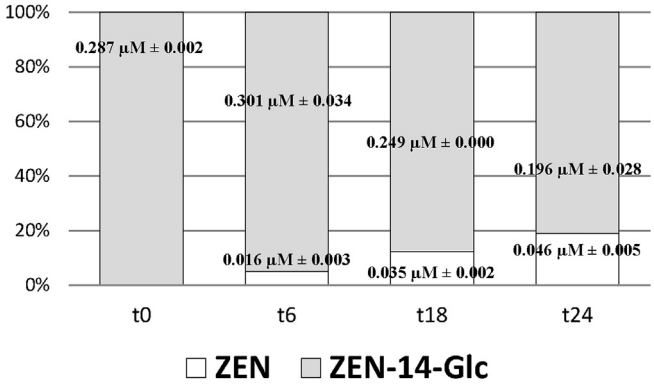

Fig. 4. The hydrolysis of ZEN-14-Glc and the subsequent release of ZEN upon exposure to BSA after 0, 6, 18 and 24 h treatments. Concentrations are expressed as the mean value of two independent experiments ± standard deviation (further details are reported in the Supporting material).

molecule was buried within the most hydrophobic part of the binding site (Fig. 5D). Notably, as shown in Fig. 6, the glucoside group occupied a polar and solvent-exposed region known for interacting with glucose molecules and undergoing glycation reactions (Wang et al., 2013).

4. Discussion

The work presented herein is framed within the study of the mechanisms of actions of masked mycotoxins that are potentially involved in the toxic outcomes *in vivo*. Specifically, the time-dependent capability of whole blood and the blood components FBS and plasma to hydrolyze and further (bio)transform ZEN-14-Glc to ZEN, α-ZEL and β-ZEL was investigated from a qualitative point of view.

We found that both cell-free (FBS and plasma) and cell-containing (whole blood) matrices were able to release ZEN from ZEN-14-Glc, in spite of a different yield. When the decrease of ZEN-14-Glc amount is monitored, the highest reduction of peak areas in respect to the t_0 was observed in FBS at 24 h. However, the trend is different when the amount of free ZEN is considered. The highest amount of free ZEN was actually found upon treatment with the whole blood, while it was found at comparable levels in FBS and plasma. Concerning the transformations occurred in FBS, at 24 h treatment the observed decrease of ZEN-14-Glc amount was not congruent with the observed amount of freed ZEN. This can be due to unknown mechanisms that may transform ZEN-14-Glc and/or ZEN in a time- and matrix-dependent manner. The higher release of ZEN found in the whole blood treatments may be explained instead taking into account the additive effects of cell metabolism and proteins activity. Consistently, it is worthy to note the formation of further reduced metabolites and, in particular, of the highly estrogenic α-ZEL in whole blood. The bioactivation of ZEN is commonly considered due to the liver metabolism (Malekinejad et al., 2006), but our results indicated the systemic circulation as a possible additional district of metabolism and activation of ZEN and congeners never considered before. Therefore, red blood cells, white blood cells, and other cells of the immune system might have a role in modulating the toxic outcomes of ZEN and congeners *in vivo*.

Serum albumin may show various (pseudo)enzymatic activity – including the glucuronidasic activity – and it is among the most abundant proteins in all matrices herein considered. Therefore, the hydrolytic activity of BSA over ZEN-14-Glc was assessed to investigate more in depth the non-cellular components possibly involved in the release of ZEN. BSA showed hydrolytic activity with a conversion yield close to 20% at 24 h. Accordingly, it was identified among the possible cell-independent components involved in the hydrolysis of ZEN-14-Glc. To the best of our knowledge, the glucosidasic activity of BSA was never reported before. However, the involvement of other blood proteins in the systemic transformation of ZEN-14-Glc cannot be excluded throughout. In addition, a purity degree ≥96% is declared for the commercial BSA in use. So, a possible additive effect due to the presence of other unknown proteins/enzymes as impurity of BSA stock cannot be excluded at all. Nonetheless, the close molecular investigation of BSA-mycotoxin interaction through molecular modeling identified a possible site of interaction which may provide a molecular explanation for the observed activity. In particular, the binding with two main drug binding sites of serum albumin (namely, the Sudlow's sites I and II) was assessed and the Sudlow's site I was identified as a possible binding site. The calculated binding architecture showed the glucoside exposed outside the pocket within an environment rich in arginine, while the rest part of molecule was buried within the pocket. Even if the mechanism is not clarified yet, it is worth noticing that glucoside occupied the sub-site involved in the glycation reaction of serum albumins. Glycation involves the non-enzymatic addition of reducing sugars to free amine groups on a protein (typically, arginine and lysine side chains) to form a reversible Schiff base, followed by the formation of more stable Amadori products (Anguizola et al., 2013). Therefore, the capability of the surrounding environment to link covalently saccharides might be involved in the release of ZEN aglycone.

As abovementioned, the present work addressed the molecular mechanisms possibly involved in the toxic outcomes of ZEN-14-Glc. In this framework, bovine blood and blood components have been chosen as a proof-of-concept model to investigate the possible

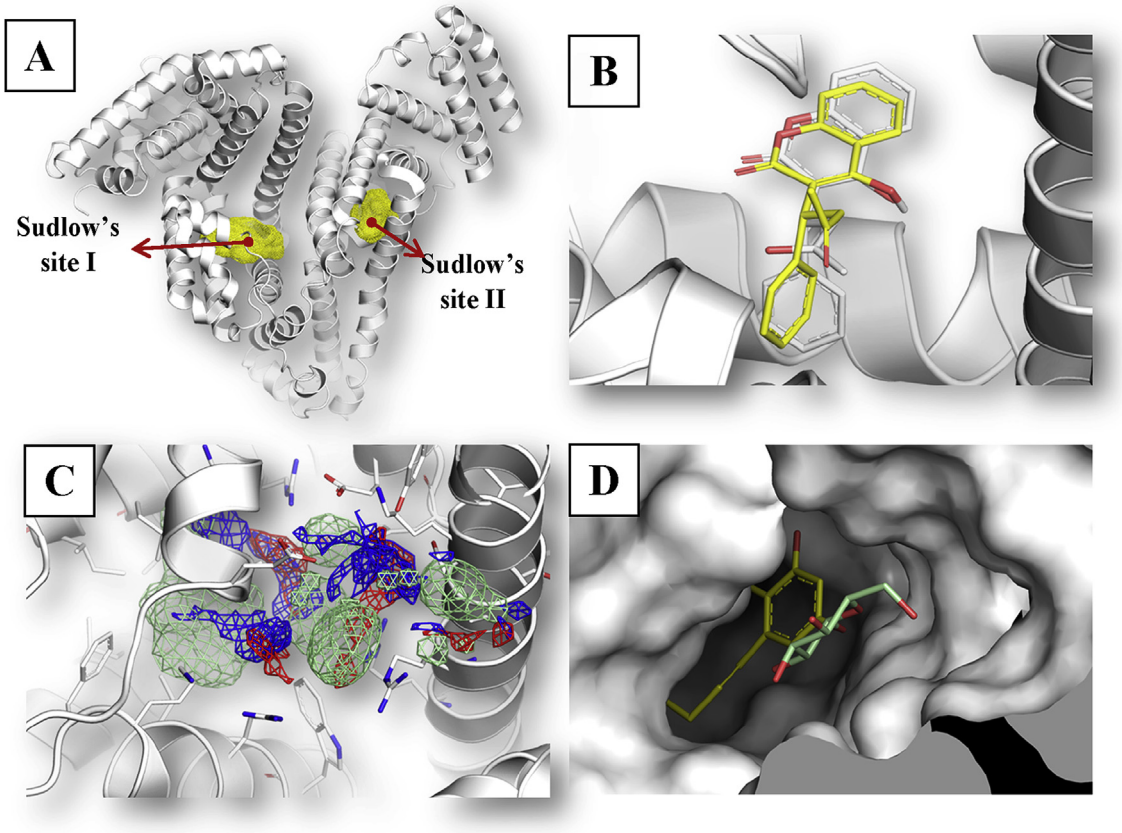

Fig. 5. Analysis of the BSA's binding sites and binding architectures of r-warfarin and ZEN-14-Glc within the Sudlow's site I. **A**. Ribbon-tube representation of the tertiary structure of BSA. The two drug binding sites are represented in yellow mash. **B**. Comparison between the calculated (yellow) and crystallographic (white) binding poses of r-warfarin from the PDB structure 1H9Z within the Sudlow's site I (Petitpas et al., 2001). **C**. Pharmacophoric analysis of the Sudlow's site I. Green, blue and red meshes identify regions sterically and energetically favorable for hydrophobic, H-bond donors and H-bond acceptors groups, respectively. **D**. Binding pose of ZEN-14-Glc. The glycoside (green colored) protruded outside the entry of the pocket. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Computational scores of the interaction with BSA's binding sites.

Compound	Sudlow's site I	Sudlow's site II
r-warfarin	**908**	565
ZEN-14-Glc	**1991**	−2239

Note → Scores and thermodynamic favors of complex formation are proportionally linked (Cozzini et al., 2002). The highest score for each compound (in bold) points the predicted site of interaction.

systemic fate as route for delivering toxicologically active byproducts. Albeit ZEN elicits xenostrogenic response in cattle, bovines are counted among the resistant species to the stimulation by ZEN and congeners. Indeed, bovine hepatic metabolism of ZEN produces prevalently β-ZEL (less toxic), instead of α-ZEL (more toxic) as in the most susceptible species (e.g. pigs) (Minervini and Dell'Aquila, 2008). Also, ZEN-14-Glc *per se* might play a minor role in ruminants in respect to other mammals as the pre-systemic transformation by rumen may reduce the absorbable fraction of intact glycosylated mycotoxins. Nevertheless, our results indicated for the first time the possible systemic hydrolysis and further activating metabolism of ZEN-14-Glc in a species prone to ZEN-mediated toxicity. For this reason, the systemic release of toxicologically active metabolites may be counted among the possible mechanisms of action eliciting the toxicity *in vivo* of ZEN-14-Glc. Monogastric species deserve major concern as the absence of rumen may lead to a more consistent absorbable fraction of intact glycosylated mycotoxins. Furthermore, different species may differentially release aglycones at the pre-systemic level, as shown for DON-3-Glc (Broekaert et al., 2016). Hence, the absorbable fraction of intact glycosylated mycotoxins may be species-dependent. On this basis, dosage, stability and (bio)transformation of ZEN-14-Glc in the systemic circulation should be assessed in each species of interest (including human) to deeply understand the mechanisms of toxic action.

5. Conclusion and future perspective

Taken together, our results pointed the toxicological relevance of ZEN-14-Glc as it may concur to the foodborne load of xenoestrogens upon hydrolysis and further bioactivation also at the level of bloodstream. Therefore, ZEN-14-Glc should be counted in defining the tolerable contamination levels for ZEN and congeners. Moreover, the systemic circulation was identified as a possible district of transformation not only for ZEN-14-Glc, but also for ZEN itself, since the further formation of α-ZEL and β-ZEL was observed. Therefore, the (bio)transformation in the bloodstream should be further assessed beside the absorption, bioavailability and bioaccessibility to better understand the *in vivo* mode of action of ZEN and modified forms.

For years, masked mycotoxins have been considered a quite homogenous class of functionally inactive compounds in respect to the toxicity of parent compounds. However, the most recent advances indicate that masked mycotoxins are not so functionally

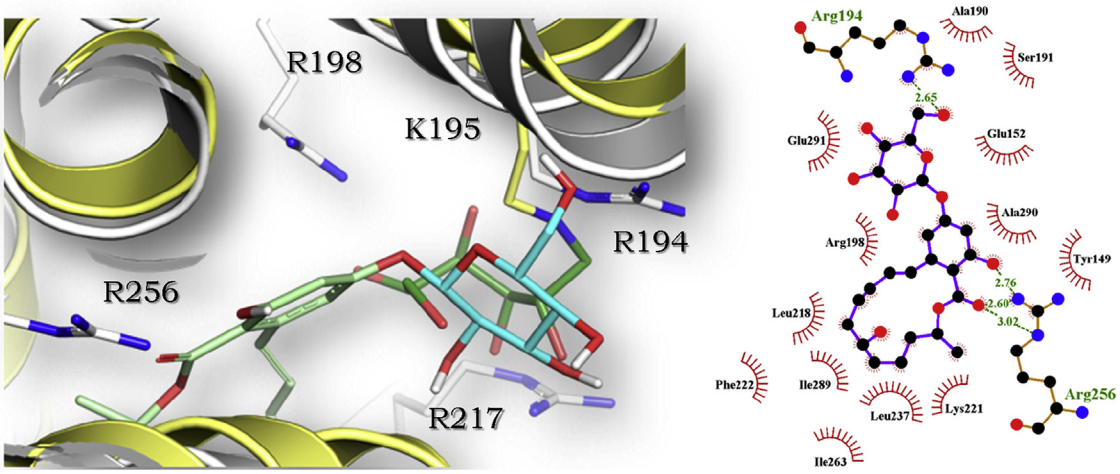

Fig. 6. Binding architecture of ZEN-14-Glc. **A**. Close-up of ZEN-14-Glc binding to BSA in comparison to the glycated human serum albumin (PDB code 4IW2; (Wang et al., 2013)). BSA (yellow) and human serum albumin (white) are represented in cartoon. Ligands and amino acid side chains are represented in sticks. Saccharidic moieties are colored in cyan (14-*O*-glucose of ZEN-14-Glc) and dark green (glycation of serum albumin). **B**. The 2D schematic representation of binding. Dotted lines indicate polar contacts and distances are expressed in Å. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

homogenous and the "safety" may strongly depend on the chemical type of toxin and the species exposed. The hazard for health mostly relies on the possibility to undergo hydrolysis *in vivo*, and in some cases masked mycotoxins may be reservoirs of toxicants able to release active aglycones at various districts before being excreted. This seems to be the case of ZEN-14-Glc; and also other glycosides might undergo the same route.

In this view, the single-dose consumption of naturally contaminated food and feed likely causes lower systemic concentration of ZEN-14-Glc in respect to that investigated herein. As a matter of fact, this study aimed at gaining insights on the possible molecular mechanisms eliciting toxicity. Such an investigation is framed within hazard identification studies that may not rely on the real-world conditions in terms of species-specific dose, route, duration and time of exposure. Nevertheless, human and animal diets may expose to a wealth of glycosylated mycotoxins (namely, the "maskedome"), whose absorption and systemic hydrolysis pose health concern as a whole, also on the account of the possible bioaccumulation upon chronic exposure. Therefore, the hydrolytic fate of the "maskedome" should be carefully evaluated to properly estimate the exposure to functionally active mycotoxins and to assess the real risk for health, thereby supporting appropriate regulatory actions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2016.11.013.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2016.11.013.

Conflict of interest

The authors declare that they have nothing to disclose.

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