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## LC-MS/MS determination of phase II metabolites of the mycotoxin zearalenone in the model plant *Arabidopsis thaliana*

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**LC-MS/MS determination of phase II metabolites of the mycotoxin zearalenone in the model plant *Arabidopsis thaliana***

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Manuscripts

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3 **LC/MS/MS determination of phase II metabolites of the**  
4 **mycotoxin zearalenone in the model plant *Arabidopsis thaliana***  
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## Abstract

The biotransformation products of zearalenone (ZON), a *Fusarium* mycotoxin, were elucidated using the model plant *Arabidopsis thaliana*. After treatment of plant seedlings with 50 $\mu$ M ZON both the liquid media and the plant extracts were analysed with LC/MS/MS. An array of 17 different metabolites, most prominently glucosides, malonylglucosides, dihexose- and hexose-pentose-disaccharides of ZON,  $\alpha$ -Zearalenol and  $\beta$ -Zearalenol were detected in the samples. Time courses for the different ZON-metabolites were recorded and give a closer insight into the metabolism kinetic. A scheme proposing the ZON metabolism in *A. thaliana* is given. The aspect of food safety regarding the (potential) occurrence of masked mycotoxins in agricultural commodities is discussed.

## Keywords

mycotoxins, conjugated mycotoxins, masked mycotoxins, zearalenone, metabolism, mass spectrometry, *Arabidopsis thaliana*

## Introduction

Mycotoxins (reviewed in Hussein and Brasel 2001, Bennet and Klich 2003), poisonous secondary metabolites of moulds, can be released into food and feed after the growth of certain fungi on plant commodities. Living plants, however, are able to metabolize these xenobiotics (Wallnöfer et al. 1996, Cole and Edwards 2000). Humans and animals that consume parts of the contaminated plants (e.g. cereals, nuts, raisins, etc.) are therefore not just exposed to the native mycotoxins, but also to altered forms. Little is known about the occurrence, bioavailability and metabolism of these compounds.

Zearalenone (ZON) is a *Fusarium* mycotoxin with strong estrogenic activity. The main concern about this substance is caused by its ability to bind to estrogen receptors in

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3 mammalian cells (Bennet and Klich 2003). It was shown in 1988 that ZON can be  
4 transformed to  $\alpha$ -zearalenol ( $\alpha$ ZOL),  $\beta$ -zearalenol ( $\beta$ ZOL) and ZON-4-glucoside (ZON-4-  
5 Glc) by plant enzymes in maize cell suspension cultures (Engelhardt et al. 1988). Also  $\alpha$ ZOL-  
6 4-glucoside ( $\alpha$ ZOL-4-Glc) and  $\beta$ ZOL-4-glucoside ( $\beta$ ZOL-4-Glc) were detected in such  
7 cultures (Engelhardt et al. 1999). A mini-survey of 24 wheat samples revealed that for 22  
8 samples ZON contamination was above the lower limit of quantification and for 10 of these  
9 samples (42%) also ZON-4-Glc could be quantified (Schneweis et al. 2002). The ZON-4-Glc  
10 fraction was in the range of approximately 10-20% of the ZON content. The only plant  
11 metabolites of mycotoxins that have been subject to animal studies are ZON-4-Glc and ZON-  
12 4-sulfate (ZON-4-Sulf). ZON-4-Glc is completely cleaved to ZON during digestion in swine  
13 (Gareis et al. 1990, Gareis 1994), while ZON-4-Sulf is easily cleaved by acid and in rats  
14 (Plasencia and Mirocha 1991). Gareis coined the term “masked mycotoxins” to emphasize  
15 substances that are usually not detected in routine analysis but contribute to the total  
16 mycotoxin content. The picture further complicates when non-extractable bound residues are  
17 taken into account. Experiments with radiolabelled  $^{14}\text{C}$ -ZON in maize cell suspension cultures  
18 indicated that over 50% of the initial radioactivity was incorporated as insoluble “bound  
19 residue” in the plant matrix, which still might be partly bioavailable (Engelhardt et al. 1999).  
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46 *Arabidopsis thaliana* or mouse-ear cress is a small flowering plant that is widely used as a  
47 model organism in plant biology. *Arabidopsis* is a member of the *Brassicaceae* family, which  
48 includes cultivated species such as cabbage and radish. *Arabidopsis* is not of major agronomic  
49 significance, but it offers important advantages for basic research in genetics and molecular  
50 biology (<http://www.arabidopsis.org>). The plants possess a small genome (about 125Mb) that  
51 has been fully sequenced since 2000, a rapid life cycle and they are easy to cultivate also in  
52 restricted space. It has been shown that *Arabidopsis* is susceptible to the cereal ear blight  
53 fungal pathogens *Fusarium graminearum* and *Fusarium culmorum* (Urban et al. 2002).  
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3 Due to the steady advancement of analytical instrumentation during time, nowadays very  
4 selective and sensitive techniques are available. Liquid chromatography coupled to tandem  
5 mass spectrometry (LC-MS/MS) has become state of the art in metabolism research (Levsen  
6 et al. 2005).  
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15 This current work reports on an assay for monitoring metabolic transformation of ZON in  
16 plants, using *A. thaliana* as model plant and a QTrap LC/MS/MS (Applied Biosystems)  
17 instrument for identification and quantification of the metabolites. After treatment of plant  
18 seedlings with ZON in liquid medium, both the medium as well as plant extracts were  
19 analyzed for metabolites. The multiple reaction monitoring mode was chosen for analysis  
20 because of its high selectivity and sensitivity. Masses of potential analytes were calculated  
21 from known metabolic reactions. The identities of detectable metabolites were confirmed with  
22 enhanced product ion (MS/MS) and MS<sup>3</sup> scans.  
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## 34 35 36 **Material and Methods**

### 37 38 *Chemicals and Materials*

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41 Murashige and Skoog basal salt mixture and MS-grade ammonium acetate were purchased  
42 from Sigma (St. Louis, US), agar and DMSO from Fluka (Buchs SG, Switzerland). Sucrose  
43 was obtained from Agrana (Vienna, Austria), 12% sodium hypochlorite solution from Roth  
44 (Karlsruhe, Germany) and solid potassium hydroxide from VWR (Vienna, Austria). LC-grade  
45 methanol was purchased from J.T. Baker (Deventer, The Netherlands). Water for use as LC  
46 mobile phase was purified successively by reverse osmosis and a Milli-Q plus system from  
47 Millipore (Molsheim, France). ZON,  $\alpha$ ZOL,  $\beta$ ZOL, and zearalanone (ZAN) were purchased  
48 from biopure Referenzsubstanzen GmbH (Tulln, Austria). ZON-4-Glc was synthesized  
49 (Scharnhorst 2003) according to a modified protocol from (Grabley et al. 1992). ZON-4-Sulf  
50 was isolated from *Fusarium graminearum* inoculated rice (Plasencia and Mirocha 1991).  
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3 Authentic  $\alpha$ ZOL-4-Glc and  $\beta$ ZOL-4-Glc were available from fermentation mixtures after  
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5 treatment of transgenic yeast with  $\alpha$ ZOL or  $\beta$ ZOL (Poppenberger et al. 2006).  
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#### 10 *Treatment of A. thaliana with ZON and sample preparation*

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12 Seeds of *A. thaliana*, accession Columbia, were sterilized in aqueous sodium hypochlorite  
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14 solution (5%) for three minutes, rinsed with sterile water twice, stratified for 48h and  
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16 germinated on Murashige and Skoog agar plates (Murashige and Skoog 1962) containing  
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18 4.5% (m/v) sucrose at 22°C under continuous light (approx. 70 $\mu$ E/m<sup>2</sup>/s). After ten days  
19  
20 approximately 40 seedlings per sample were transferred to 20 mL liquid Murashige and  
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22 Skoog medium containing 2.5% (m/v) sucrose and shaken at 60 rpm for another three days  
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24 for acclimatization. In each experiment the ZON concentration was adjusted to 50 $\mu$ mol/L  
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26 (15.9 mg/L) by adding 100  $\mu$ l of a 10 mM ZON stock solution (in 10% DMSO, 0.01N KOH)  
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28 to 20mL liquid medium. Seedlings were harvested at different time periods (0, 30min, 2h, 5h,  
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30 12h, 24h, n=3 per time point) after treatment with a single dose of ZON.  
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39 After harvest, the seedlings were weighed, rinsed with 20 mL water per sample and  
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41 homogenized in 4 mL acetonitrile/water (75/25, v/v) using an Ultra-Turrax<sup>®</sup> T25 mixer (IKA,  
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43 Staufen, Germany) at 24000 rpm for 1 min. Extracts were centrifuged at 5300 rpm for 10 min  
44  
45 to remove insoluble cell wall components, and then filtered through a paper filter (Roth,  
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47 Karlsruhe, Germany). Each liquid medium was combined with the washing water obtained  
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49 after rinsing of the seedlings and diluted 1:1 (v/v) with methanol to ensure that ZON is  
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51 completely dissolved. Matrix blanks (both plant medium and plant extracts) to which no ZON  
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53 was added were prepared accordingly. The samples were stored at 4°C in the dark until  
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55 analysis by HPLC-MS/MS. Quantification was performed using external calibration with  
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57 mixed standards containing 10, 30, 100, 300 and 1000 $\mu$ g/L of ZON,  $\alpha$ ZOL,  $\beta$ ZOL, ZON-4-  
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59 Sulf and ZON-4-Glc in mobile phase. Samples were diluted 1:10 (or 1:20 for time point 0)  
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3 with mobile phase if the respective concentration exceeded 1000 $\mu$ g/L and measured again.  
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5 Recoveries for ZON,  $\alpha$ ZOL,  $\beta$ ZOL, ZON-4-Sulf and ZON-4-Glc in the plant medium were  
6  
7 elucidated by spiking diluted Murashige and Skoog medium (1:1 (v/v) with methanol) with  
8  
9 10, 30, 100, 300 and 1000 $\mu$ g/L of the mentioned analytes, before measurement with LC-  
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11 MS/MS.  
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#### 14 15 16 17 *LC parameters*

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19 HPLC separation was achieved on a Thermo Aquasil C18 column (100mm x 4.6mm, 3 $\mu$ m) at  
20  
21 25 $^{\circ}$ C applying a methanol/5mM aqueous ammonium acetate linear gradient from 30%  
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23 methanol to 90% methanol. After an initial hold time of 0.5min, 90% methanol were reached  
24  
25 after 7.5min and kept constant until 10min. Afterwards the column was re-equilibrated with  
26  
27 30% methanol until the end of the run at 15min. 25 $\mu$ L aliquots were injected into the HPLC-  
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29 MS/MS. A flow rate of 0.75mL/min was chosen.  
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#### 34 35 36 *MS parameters*

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38 ZON and its metabolites were detected and characterized with a 2000 QTrap MS/MS  
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40 instrument equipped with a Turbo Ionspray (electrospray) source. This instrument uses a  
41  
42 triple quadrupole ion path and is capable of performing all of the conventional tandem  
43  
44 quadrupole modes as well as several high sensitivity ion trap mass spectrometer scans using  
45  
46 the final quadrupole as a linear ion trap (enhanced modes). To monitor for assumed  
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48 metabolites, the instrument was operated in the MRM mode. MRM transitions (dwell time:  
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50 25ms) were monitored in a single LC run to screen for numerous conjugates (Tab.1). MS  
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52 settings were as follows: curtain gas 20psi (137.9kPa), source temperature 400 $^{\circ}$ C, source gas  
53  
54 25psi (172.4kPa), auxiliary gas 65psi (448.2 kPa), collision induced dissociation (CID) gas 6  
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56 (arbitrary units, corresponding to the pressure in the collision cell), ionspray voltage -4200V.  
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3 MRM intensities were optimized for standard substances, resulting in the following  
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5 parameters: ZON  $m/z$  317.1  $\rightarrow$   $m/z$  130.9 (declustering potential (DP)  $-51V$ , collision energy  
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7 (CE)  $-38eV$ ),  $\alpha$ ZOL and  $\beta$ ZOL  $m/z$  319.1  $\rightarrow$   $m/z$  129.9 (DP  $-71V$ , CE  $-48eV$ ), ZAN  $m/z$   
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9 319.1  $\rightarrow$   $m/z$  275.1 (DP  $-56V$ , CE  $-16eV$ ), ZON-4-Sulf  $m/z$  397.2  $\rightarrow$   $m/z$  317.1 (DP  $-46V$ ,  
10  
11 CE  $-18eV$ ), ZON-4-Glc  $m/z$  479.1  $\rightarrow$   $m/z$  317.1 (DP  $-31V$ , CE  $-22eV$ ),  $\alpha$ ZOL-4-Glc and  
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13  $\beta$ ZOL-4-Glc  $m/z$  481.1  $\rightarrow$   $m/z$  319.1 (DP  $-31V$ , CE  $-22eV$ ). For all other potential  
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15 metabolites, ions with  $m/z$  ratios corresponding to  $[M-H]^-$  of the assumed conjugates were  
16  
17 allowed to pass the first quadrupole (Q1) for fragmentation in Q2. Q3 was mainly set to the  
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19  $m/z$  value of deprotonated ZON ( $m/z$  317.1) or  $\alpha$ ZOL and  $\beta$ ZOL ( $m/z$  319.1) respectively.  
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21 Default values of DP  $-30V$ , and CE  $-30eV$  were used to monitor the ZON and ZOL  
22  
23 conjugates. In case of detection of chromatographic peaks, MS/MS spectra were recorded in  
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25 the enhanced product ion (EPI) mode (linear iontrap (LIT) fill time 50ms, LIT scan speed  
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27 1000amu/s). Also  $MS^3$  spectra were recorded for confirmation (data not shown).  
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## 36 Results and Discussion

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38 *A. thaliana* was treated with 50 $\mu$ M ZON (about 15.9mg/L) for 0, 0.5, 2, 5, 12 and 24h in  
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40 triplicates. None of the screened analytes were detected in the matrix blanks (medium and  
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42 plant extracts), which were treated in the same manner as the samples but to which no ZON  
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44 has been added.  
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51 In the media ZON and its metabolites ZON-4-Glc, ZON-4-Sulf,  $\beta$ ZOL and to a lesser extent  
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53 also  $\alpha$ ZOL were detected after treatment with ZON. For all of these metabolites standards  
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55 were available, which showed identical retention times, MS/MS and MS/MS/MS spectra (data  
56  
57 not shown) and proved the identities of the detected metabolites. Spiking experiments at  
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59 different concentrations in blank media revealed no significant matrix effects for the  
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components mentioned above (data not shown). Recoveries for all analytes were close to

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3 100% at all concentrations. A typical MRM chromatogram of the medium after treatment of  
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6 *Arabidopsis* with ZON for 24h is shown in Fig. 1A.  
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10 In the plant extracts also  $\alpha$ ZOL-Glc (at low levels) and  $\beta$ ZOL-Glc were unambiguously  
11 identified (besides ZON,  $\alpha$ ZOL,  $\beta$ ZOL, ZON-4-Glc and ZON-4-Sulf), but their concentration  
12 are only given in relative values due to the lack of known concentrations of the authentic  
13 standards. Fig. 1B shows a MRM chromatogram of a cell extract after 24h ZON exposure to  
14 *A. thaliana*. Furthermore, the chromatographic peaks, corresponding to the MRM transitions  
15 of malonyl-glucosides (ZON-MalGlc,  $\alpha$ ZOL-MalGlc,  $\beta$ ZOL-MalGlc), dihexose- (ZON-  
16 DiHex,  $\alpha$ ZOL-DiHex,  $\beta$ ZOL-DiHex) and hexose-pentose-disaccharides (ZON-HexPent,  
17  $\alpha$ ZOL-HexPent,  $\beta$ ZOL-HexPent) of ZON,  $\alpha$ ZOL and  $\beta$ ZOL were detected, although often in  
18 low levels (especially for the  $\alpha$ ZOL derivatives). For  $\beta$ ZOL also a trihexose conjugate  
19 ( $\beta$ ZOL-TriHex) was measured. MS/MS (EPI) and MS<sup>3</sup> scans confirmed the presence of ZON  
20 or the ZOLs respectively in all metabolites, but it has to be pointed out that no standards were  
21 available for the malonylglucosides and the di- or trisaccharides. Further experiments (e.g.  
22 using LC-NMR or authentic standards) are therefore required to fully characterize the  
23 molecular structures of these compounds.  
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50 Since xylose conjugates are usually found as conjugation products of xenobiotics in plants  
51 (Vostrowsky and Hirsch 2005) we suggest the hexose-pentose-disaccharides which were  
52 detected for the first time in this study to be glucose-xylose conjugates. According to other  
53 detected plant metabolites (Cole and Edwards 2000, Levsen et al. 2005), the di- and tri-hexose  
54 conjugates are assumed to be di- and tri-glucosides respectively.  
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3 In the present study we could not verify the exact location of the conjugation site by LC-  
4 MS/MS, as the added moieties were cleaved first from the molecules after low-energy CID,  
5 which is in good accordance with other studies (Zaia 2003). It is therefore not certain whether  
6 the dihexose conjugates are 2,4-dihexosides (with one molecule of sugar (glucose) conjugated  
7 to each of the phenolic groups of ZON) or oligosaccharides. ZON-2,4-diglucoside has been  
8 shown to be a microbial transformation product of ZON (El-Sharkawy 1989). The detection  
9 of a trihexose conjugate of  $\beta$ ZOL but the absence of a ZON trihexoside derivative suggests  
10 that also the non-phenolic hydroxy-group of  $\beta$ ZOL could be conjugated to a hexose in the  
11 found metabolite. Additional (enzymatic and NMR spectroscopy) experiments are planned to  
12 confirm the structures of the dihexoside- and the hexose-pentose-disaccharides.  
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29 Time course experiments with points at 0, 30min, 2h, 5h, 12h and 24h were performed as  
30 well. While after 0 min all of the added ZON was recovered in the medium, after 24h almost  
31 all available ZON (>97%) was metabolized. Fig. 2A summarizes the concentrations of the  
32 detected metabolites in the medium over time.  
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41 Phase I metabolism led to  $\alpha$ ZOL and  $\beta$ ZOL, which were further conjugated very similarly to  
42 ZON in phase II metabolism. Intracellular (plant extract) concentrations (Fig. 2B) of ZON,  
43  $\alpha$ ZOL and  $\beta$ ZOL but also of ZON-4-Glc and ZON-4-Sulf increased during the first few hours  
44 (often about 5h) to reach a maximum but then declined as the substances were further  
45 processed to ZON-DiHex, ZON-MalGlc and ZON-HexPent. It has to be pointed out here, that  
46 matrix effects were not investigated for plant extracts and the yields of the chosen extraction  
47 method are unclear. For this reason Fig. 2B contains the signal intensities of ZON,  $\alpha$ ZOL,  
48  $\beta$ ZOL, ZON-4-Glc and ZON-4-Sulf divided by the response factors of the respective analytes  
49 as measured in standard solution.  
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3 Fig. 2C shows the relative signal intensities of ZON-DiHex, ZON-MalGlc and  $\beta$ ZOL-Glc as a  
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5 function of the incubation time which reached a maximum at about 12h before the  
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7 concentrations also declined. Additional time points at 36h and 48h (not shown) revealed that  
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9 the concentration for these substances (as well as for ZON-HexPent and  $\beta$ ZOL-DiHex)  
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11 remained roughly constant between 24h and 36h and then declined further.  
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17 [Insert figures 2A, 2B, 2C about here]  
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22 The variety of the identified ZON metabolites, in total 17 different substances, is summarized  
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24 in Fig. 3. The proposed pathway shows metabolic activation (phase I) and conjugation (phase  
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26 II) processes. The later occurrence of malonylglucosides and disaccharides suggests that they  
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28 derive from the respective monoglucosides, which is also in agreement with the metabolism  
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30 of other xenobiotics in plants (Cole and Edwards 2000).  
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36 [Insert figure 3 about here]  
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41 As part of their metabolism, plants are capable of transforming xenobiotics into a huge variety  
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43 of conjugated forms. Nonetheless, usually just the parent mycotoxin is measured in routine  
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45 analysis of feed and food. It has been shown that some of these conjugates can be cleaved  
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47 easily by the gut microflora of swines (Gareis et al. 1990), releasing the precursor toxin. The  
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49 total available amount of mycotoxins in foodstuff, therefore, might currently be  
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51 underestimated.  
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56 It has to be stressed that *A. thaliana* is in no way an agricultural crop. In different plants the  
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58 metabolism of a given xenobiotic might look very different. Still, the ease of use of *A.*  
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60 *thaliana* plants combined with its known genome sequence allows a more general

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3 consideration of the interactions that occur between plants and mycotoxins. Genes responsible  
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5 for certain metabolic pathways can be identified and looked for in other, more economically  
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7 important, plants. Also a variety of (available) mutants of *A. thaliana* can be compared in  
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9 their metabolic behaviour towards various xenobiotics with the wild-type plants using LC-  
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11 MS/MS. Regarding the aspect of food safety, we want to screen wheat and maize for the  
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13 occurrence of ZON and other mycotoxin plant metabolites in the very near future. Also the  
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15 structural identities of the metabolites found will be confirmed by LC-NMR.  
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## 22 **Conclusion**

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24 This work shows the great capability of plants to metabolize xenobiotic compounds. We  
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26 utilized a method for the determination of ZON bio-transformation products in *A. thaliana*.  
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28 Seventeen different metabolites of ZON have been identified to occur in ZON treated  
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30 *Arabidopsis* plants. Known transformation products include ZON-4-Glc, ZON-4-Sulf,  $\alpha$ ZOL,  
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32  $\beta$ ZOL,  $\alpha$ ZOL-Glc and  $\beta$ ZOL-Glc. Dihexosides, hexose-pentosides and malonylglucosides of  
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34 ZON,  $\alpha$ ZOL and  $\beta$ ZOL are described for the first time.  
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Table 1. Common conjugations after metabolic transformation with the according mass shift after H-substitution (changed after Levsen et al. 2005)

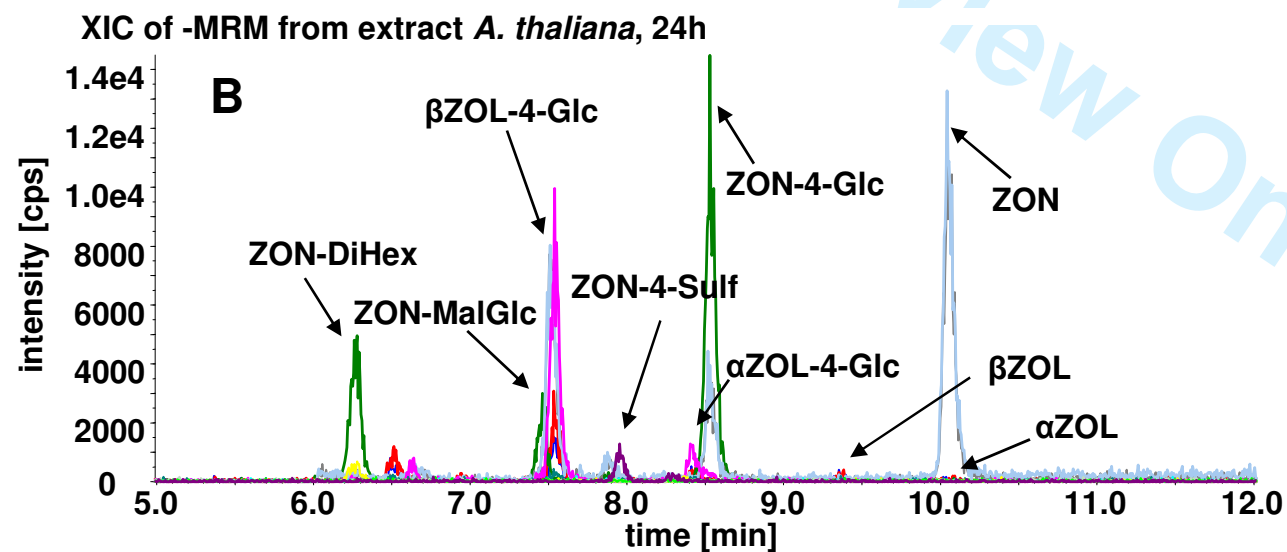
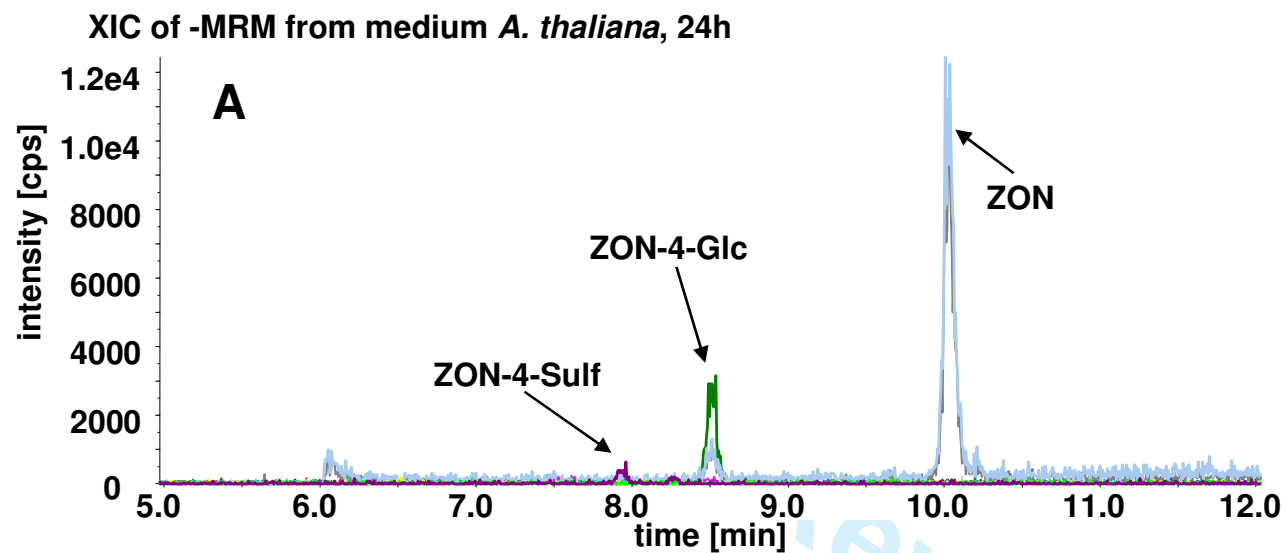
Conjugation	Mass shift (amu)
Methylation	14
Acetylation	42
S-Methylation	46
Glycine (Gly)	57
Sulfatation (Sulf)	80
Cysteine (Cys)	119
N-Ac-Cys	161
Glucose (Glc)	162
Cys-Gly	176
Glucuronic acid	176
N-Ac-Glc	203
Glc-Sulfate	242
$\gamma$ -Glu-Cys	248
Malonyl-Glc	248
Glc-Xyl	294
Glutathione	305
Glc-Glc	324
Acetyl-Glc-Glc	366
Malonyl-Glc-Glc	410
Glc-Glc-Glc	486

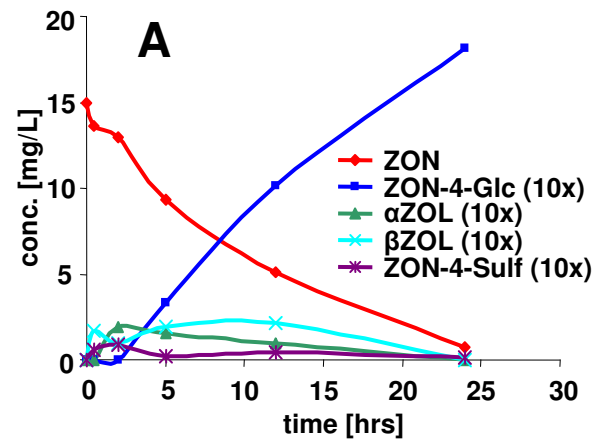
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6 Fig. 1. Total ion current LC-MS/MS (MRM) chromatograms of the growth medium (A) and  
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8 the cell extract (B) of a single *A. thaliana* seedling (10 days old) that was exposed to 50 $\mu$ M  
9  
10 ZON for 24h.  
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15 Fig. 2. (A) Concentrations of ZON, ZON-4-Glc, ZON-4-Sulf,  $\alpha$ ZOL and  $\beta$ ZOL found in the  
16  
17 growth medium, depending on the time of exposure of *A. thaliana* to 50 $\mu$ M ZON. (B)  
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19 Relative signal intensities divided by the response factors of ZON, ZON-4-Glc, ZON-4-Sulf,  
20  
21  $\alpha$ ZOL and  $\beta$ ZOL found in the cell extracts, depending on the time of exposure of *A. thaliana*  
22  
23 to 50 $\mu$ M ZON. (C) Relative signal intensities of ZON-DiHex, ZON-MalGlc and  $\beta$ ZOL-Glc  
24  
25 found in the cell extracts, depending on the time of exposure of *A. thaliana* to 50 $\mu$ M ZON.  
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32 Fig. 3. Proposed biotransformation pathway of ZON in *A. thaliana*, covering both phase I and  
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34 II metabolism.  
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