Research Article

Combination of LC-MS² and GC-MS as a Tool to Differentiate Oxidative Metabolites of Zearalenone with Different Chemical Structures

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Received 25 October 2011; Accepted 20 February 2012

Academic Editor: Hakan Arslan

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Recent studies on the mammalian and fungal metabolism of the mycotoxin zearalenone (ZEN) have disclosed the formation of six regioisomers of monohydroxy-ZEN and its reductive metabolite zearalenol (ZEL). Hydroxylation occurs at the aromatic ring or at one of four positions of the aliphatic macrocycle. In addition, an aliphatic ZEN epoxide, its hydrolysis product, and other products were identified in fungal cultures. In this paper, we report the product ion spectra of the $[M-H]^-$ ions of 22 oxidative metabolites of ZEN and ZEL, obtained by LC-MS² analysis using a linear ion trap mass spectrometer with negative electrospray ionization. The MS² spectra exhibit qualitative and quantitative differences which allow a clear distinction of most metabolites. Moreover, GC-MS analysis of the trimethylsilylated metabolites yields electron impact mass spectra with numerous fragment ions which can be used as fingerprint to confirm the chemical structure derived by LC-MS² analysis.

1. Introduction

Zearalenone (ZEN) is a mycotoxin with the chemical structure of a β -resorcylic acid lactone (Figure 1), which is produced by *Fusarium* species and frequently found as a contaminant of food and feed [1–3]. Due to its pronounced estrogenic activity, the exposure to ZEN has been associated with endocrine disruptive effects in domestic animals, especially in pigs, and possibly in humans [1, 4]. As is the case with virtually all other mycotoxins, ZEN is not the only congener produced by the fungus, and several other fungal resorcylic acid lactones have previously been identified, in particular the α - and β -stereoisomers of zearalenol (ZEL, Figure 1) and of 5-hydroxy-ZEN and 10-hydroxy-ZEN [5]. More recently, ZEN-11,12-oxide, ZEN-11,12-dihydrodiol and cyclization products of the latter have been reported as fungal metabolites [5].

In addition to fungal metabolites of ZEN, numerous monohydroxylation products of ZEN and ZEL have recently

been disclosed as mammalian metabolites *in vitro* [7–9]. Depending on the species, either the aromatic ring or the aliphatic macrocycle were the preferred site for hydroxylation (Figure 1). Taken together, fourteen oxidative ZEN and ZEL metabolites are presently known as regioisomers, that is, differing in the position of the hydroxyl group. The total number of oxidative metabolites is even higher, as hydroxylation at each aliphatic position gives rise to two stereoisomers.

Although the numerous fungal and mammalian ZEN and ZEL metabolites can be well separated by HPLC on reversed-phase (RP) columns [5, 7–9], their identification is presently difficult because no authentic reference compounds are available for most of them. In the course of our past LC-MS² studies using a linear trap mass spectrometer [5, 8, 9], we observed that fragmentation of the $[M-H]^-$ ions obtained by electrospray ionization in the negative mode gives rise to product ion spectra which differ significantly between different regioisomers. Moreover, mass spectra obtained by GC-MS analysis of the trimethylsilyl derivatives

FIGURE 1: Chemical structures of ZEN and ZEL. Arrows indicate positions of hydroxylation in fungal (f) and mammalian (m) metabolites. Nomenclature according to [6].

exhibit multiple fragmentations upon electron impact ionization which are specific for the respective regioisomer and can thus be used for fingerprinting. Hence, we propose to use a combination of LC-MS² and GC-MS for the identification of oxidative ZEN metabolites in the analysis of food samples and body fluids.

2. Mass Spectrometry

2.1. LC-MS² Analysis. LC-MS experiments were performed using a LXQ Linear Ion Trap MSn system (Thermo Fisher Scientific Inc., Waltham, MA, USA) operated in the negative electrospray ionization (ESI) mode. Nitrogen was used as sheath gas and auxiliary gas with flow rates of 30.0 and 15.0 L/min, respectively. Spray voltage was 4.5 kV, and capillary temperature was 350°C. Ion optics were automatically tuned with a 10 μ M solution of ZEN in methanol. MS² of the [M-H]⁻ ions was conducted at CID 35 (35% of 5 V). The LC column was a 250 $\times$ 4.6 mm i.d., 5 μ m RP Luna C18 (Phenomenex, Torrance, CA, USA). Solvent A was water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. The solvent gradient was changing from 30% B to 100% B in 30 min. The initial 30% B were then reached within 1 min and kept for 5 min before the next injection. The flow rate was 0.5 mL/min.

2.2. GC-MS Analysis. A Finnigan GCQ capillary gas chromatograph equipped with a 30 m $\times$ 0.25 mm i.d., 0.25 μ m, 5% phenylmethyl MDN-5S fused-silica column (Supelco, Bellefonte, PA, USA), and coupled to an ion-trap detector was operated with electron impact (EI) ionization at 70 eV (Thermo Finnigan, Austin, TX, USA). Metabolites were obtained as HPLC fractions, using the same conditions as described above for LC-MS analysis. Individual fractions were concentrated *in vacuo* to remove the organic eluent, and the remaining aqueous phases were extracted twice with an equal volume of ethyl acetate. Extracts were evaporated to dryness under a stream of nitrogen and the residues dissolved in 20 μ L of *N,O*-bis(trimethylsilyl)trifluoroacetamide. After at least 3 h at 20°C, 1 μ L was injected, using the split-less mode for 90 s. The injection port temperature was 60°C at the time of injection and raised to 275°C at a rate of 8°C/s. The oven temperature was programmed from 60°C

(1 min hold) to 150°C at 30°C/min, then to 295°C at 10°C/min and held at 295°C for 25 min. The transfer line and ion source were kept at 275°C and 250°C, respectively. Helium was used as carrier gas with a flow rate of 40 cm/s. Mass spectra were scanned from *m/z* 50 to 800 at a rate of 0.5 s/scan.

3. Results

3.1. MS² of the [M-H]⁻ Ions Obtained by LC-MS. The chemical structures of the major monohydroxylated fungal and mammalian metabolites of ZEN were elucidated earlier [5, 8, 9]. The product ion spectra of their [M-H]⁻ ions as obtained by LC-MS² on our linear ion trap mass spectrometer are depicted in Figure 2. All hydroxy-ZENs share a [M-H]⁻ ion at *m/z* 333, which gives rise to several fragment ions of different intensities. Common fragmentations of the hydroxy-ZENs, which also occur in the parent ZEN molecule, are the loss of water or carbon dioxide from *m/z* 333 to yield *m/z* 315 or *m/z* 289, respectively. Combined loss of water and carbon dioxide leads to *m/z* 271. Other common fragments of most, but not all, hydroxy-ZENs have *m/z* 175 and *m/z* 149. The fragment at *m/z* 175 possibly arises through loss of the C-2 to C-9 portion of the aliphatic macrocycle, resulting in the formation of a six-membered ring retaining the C-1 carbonyl group and C-10, C-11, and C-12. Hydroxyl groups located at C-5, C-6, C-8, or C-9 are lost during this fragmentation, whereas hydroxyl groups at C-10, C-13, or C-15 are retained in the fragment, shifting its mass to *m/z* 191 (Figure 2). Other diagnostic fragment ions, which probably originate from compound-specific rearrangements, are *m/z* 263 and *m/z* 245 for 5-hydroxy-ZEN, *m/z* 216 for 6-hydroxy-ZEN, *m/z* 247 and *m/z* 187 for 8-hydroxy-ZEN, and *m/z* 203 for 9-hydroxy-ZEN. The effect of hydroxylation at C-9 is particularly striking, because fragment ions with *m/z* 289 and 315, which have high intensities for the other regioisomers, are almost absent. This indicates that hydroxylation at C-9 strongly favors cleavage of the bond between C-9 and C-10, leading to *m/z* 175, and cleavage of the bond between C-9 and C-8, leading to *m/z* 203. Although the two aromatic hydroxylation products of ZEN, that is, 13-hydroxy-ZEN and 15-hydroxy-ZEN, have most of the prominent fragment ions in common, pronounced differences in ion intensities

FIGURE 2: Product ion spectra of $[M-H]^-$ ions of the regioisomers of hydroxyl ZEN. The LC retention times (Rt) refer to the conditions described in Section 2.1.

allow their distinction; moreover, 15-hydroxy-ZEN exhibits a specific ion at m/z 201 (Figure 2).

In addition to the monohydroxylation products of ZEN, the respective hydroxylated metabolites of ZEL can be present in food samples and body fluids. MS² of the $[M-H]^-$ ions does not discriminate between the α - and β -stereoisomers of ZEL or their hydroxylated metabolites,

but the stereoisomers have different HPLC retention times. At present, only α - and β -ZEL are available as authentic reference compounds, whereas the stereochemistry of the hydroxyl ZELs is unknown. The MS² data and LC retention times of the hydroxy-ZELs are compiled in Figure 3.

As discussed above for the hydroxy-ZENs, the fragmentation of most hydroxy-ZELs is also dominated by the loss of

FIGURE 3: Product ion spectra of $[M-H]^-$ ions of the regioisomers of hydroxy-ZEL. The LC retention times (Rt) refer to the conditions described in Section 2.1.

water, carbon dioxide, and both, giving rise to the fragments at m/z 317, 291, and 273, respectively. Whereas 5-hydroxy-ZEL and 6-hydroxy-ZEL have very similar MS^2 of the $[M-H]^-$ ion, the MS^2 of 8-hydroxy-ZEL exhibits additional

fragment ions at m/z 189 and m/z 299, whereas 10-hydroxy-ZEL, 13-hydroxy-ZEL, and 15-hydroxy-ZEL have a small but diagnostic fragment ion at m/z 190 and differ sufficiently in the intensity of the other fragment ions to be differentiated.

FIGURE 4: Chemical structures of ZEN metabolites arising from epoxidation at C-11, 12 or further oxidation of hydroxylation products.

13-Hydroxy-ZEL and 15-hydroxy-ZEL can be easily distinguished by the intensity of m/z 291. 9-Hydroxy-ZEL exhibits a base product ion at m/z 175 and rather low intensities of the higher fragment ions, suggesting a highly unstable macrocycle as already observed for 9-hydroxy-ZEN (Figure 2).

In addition to hydroxylated metabolites of ZEN and ZEL, a few other fungal congeners of ZEN have been reported, arising from epoxidation of the aliphatic double bond and subsequent hydrolysis and rearrangement [5]. Moreover, oxidation products of some hydroxy-ZENs and hydroxy-ZELs may occur as minor mammalian metabolites. The structures of these miscellaneous ZEN metabolites are depicted in Figure 4 and their MS² data given in Figure 5.

ZEN-11,12-oxide has the same $[\text{M}-\text{H}]^-$ ion at m/z 333 as the monohydroxylated ZENs. Its MS² (Figure 5) is characterized by a multitude of fragment ions, many of which are also exhibited in the MS² of hydroxy-ZENs (Figure 2). A diagnostic ion, however, only observed with ZEN-11,12-oxide appears at m/z 277, possibly arising from the subsequent loss of two carbon monoxides from the $[\text{M}-\text{H}]^-$ ion. In contrast to the complex MS² of ZEN-11,12-oxide, the MS² of its hydrolysis product ZEN-11,12-dihydrodiol and its "half-acetal" are extremely simple and dominated by a fragment ion at m/z 165, which probably arises from the facile cleavage of the ester bond next to C-1 and the benzylic

bond between C-11 and C-12, thereby losing the C-2 to C-11 portion of the macrocycle. Just the opposite behaviour is observed for the cyclization products [5] of the ZEN-11,12-dihydrodiol (Figure 4), which form very stable $[\text{M}-\text{H}]^-$ ions. The quinones of 13-hydroxy-ZEN and 13-hydroxy-ZEL are characterized by dominant fragment ions at m/z 303 and 305, respectively, resulting from the release of carbon monoxide from the $[\text{M}-\text{H}]^-$ ions. This type of fragmentation also occurs with most hydroxy-ZENs and hydroxyl-ZELs, but only to a very small extent (Figures 2 and 3).

The LC-MS² data have also been compiled as a supplementary table for easy comparison (see Supplementary Materials available online at doi:10.1155/2012/472031).

3.2. MS of the Trimethylsilyl Derivatives Analyzed by GC-MS. The hydroxyl groups of ZEN and its monohydroxylated metabolites are very amenable to trimethylsilylation and give rise to EI spectra with numerous fragments upon GC-MS analyses. As shown earlier by Pfeiffer et al. [7], the EI spectrum of trimethylsilylated ZEN (Figure 6) is dominated by several fragments arising (i) from the molecular ion (m/z 462) by the loss of a methyl group from the TMS group at C-16 to yield m/z 447, (ii) the loss of water from the ester group to yield m/z 444, and (iii) the combined loss of a methyl group and water to yield m/z 429. Further

FIGURE 5: Product ion spectra of $[M-H]^-$ ions of miscellaneous fungal and mammalian ZEN metabolites.

fragmentation of m/z 444 by cleavage of the bond between C-8 and C-9 with loss of part of the aliphatic macrocycle leads to m/z 333, from which m/z 305 is derived by release of carbon monoxide. The EI spectrum of ZEN exhibits intense fragment ions both in the higher (m/z 447, 444, 429) and lower (m/z 333, 305) mass range.

Monohydroxylation of ZEN at any position shifts the molecular ion of the TMS derivative to m/z 550. A comparison of the various regioisomers of hydroxyl-ZEN shows that the site of hydroxylation markedly affects the fragmentation. Hydroxylation at C-5, C-6, C-8, and C-9 favours the loss of the macrocycle, as fragment ions in the higher m/z range

FIGURE 6: EI mass spectra of the TMS derivatives of ZEN and of the regioisomers of hydroxy-ZEN.

are virtually lacking, whereas fragment ions in the lower m/z range dominate but differ in their intensities (Figure 6). Moreover, each regioisomer appears to exhibit a specific fragment ion, that is, m/z 442 for 5-hydroxy-ZEN, m/z 361

for 6-hydroxy-ZEN, m/z 200 for 8-hydroxy-ZEN, and m/z 213 for 9-hydroxy-ZEN. In contrast, hydroxylation at C-10 or at the aromatic ring leads to high intensities of fragments in the high m/z range, that is, 535, 532, and 517, indicating

FIGURE 7: EI mass spectra of the TMS derivatives of regioisomers of hydroxy-ZEL.

increased stability of the macrocycle against fragmentation. Again, differences in intensities and diagnostic fragment ions, for example, m/z 532 and m/z 460 for 10-hydroxy-ZEN, m/z 421 for 13-hydroxy-ZEN, and m/z 445 for 15-hydroxy-ZEN allow distinction of these regioisomers.

Finally, the EI mass spectra of the TMS derivatives of the stereoisomers of ZEL and some hydroxylated ZELs are depicted in Figure 7. As expected, α -ZEL and β -ZEL have very similar spectra, dominated by the loss of trimethylsilyl-anol (90 amu) from the molecular ions, in addition to the fragmentation discussed above for ZEN. The very complex EI spectra of the few hydroxylated ZELs shown in Figure 7 appear to exhibit sufficient differences in ion intensities and, in part, diagnostic ions for differentiation. However, this statement is preliminary because the mass spectra of several regioisomers of hydroxy-ZEL are still lacking.

4. Discussion

Numerous oxidative products of the wide-spread mycotoxin ZEN have recently been described as mammalian and fungal

metabolites [5, 7–9]. Their chemical structures were elucidated by various approaches, for example, NMR spectroscopy, loss of deuterium from specifically labelled ZEN, selective enzymatic methods, and, in very few cases, chemical synthesis. For most of the novel ZEN metabolites, reference compounds are presently not available. In order to facilitate the detection and identification of these metabolites in food items, body fluids and other complex matrices, we have compiled their mass spectra obtained by LC-MS and GC-MS analysis in this paper. LC-MS with ESI or atmospheric pressure chemical ionization (APCI) has become the golden standard in mycotoxin analysis because of its sensitivity, selectivity, propensity for multitoxin analysis, simplicity of sample preparation, and lack of need for derivatization. ZEN and its metabolites contain at least two hydroxyl groups and are very suitable for ESI and APCI in the negative mode, leading to high yields of the $[M-H]^-$ ions without further fragmentation. Therefore, regioisomeric hydroxylation products can not be distinguished by their mass spectra in LC-MS analysis. However, fragmentation of the $[M-H]^-$

ions in an ion trap mass spectrometer gives rise to specific product ion spectra, which differ both in the intensities and, for most ZEN metabolites, also in the m/z values of some fragments, as demonstrated in this paper. The appearance of such “diagnostic” ions and differences in the intensities of the shared fragment ions should allow the assignment of the individual structure based on the MS^2 of the $[M-H]^-$ for most ZEN metabolites reported to date.

As an example, the MS^2 information provided in this paper is applied to the recent report by Reinen et al. [10] on the ZEN metabolites formed by bacterial cytochrome P450 mutants. Six oxidative metabolites, designated M1, M2, M3A, M3B, M4, and M5, were detected and the MS^2 , obtained by LC- MS^2 with a 3D ion trap mass spectrometer operated with negative APCI, depicted in their paper [10]. M3A and M3B were tentatively proposed to represent products of aromatic hydroxylation, that is, at C-13 and C-15, and no structures were proposed for the remaining metabolites other than hydroxylation at the aliphatic macrocycle. Comparison of the MS^2 depicted in their report with the MS^2 presented here strongly suggest that M1 is 8-keto-ZEL, M2 and M5 are the two stereoisomers of 8-hydroxy-ZEN, M3A is 5-hydroxy-ZEN, M3B is 10-hydroxy-ZEN, and M4 is 6-hydroxy-ZEN. This assignment is supported by the retention times of M1 to M5 in LC-MS. LC was conducted on C18 RP column both by Reinen et al. [10] and in our studies. Although methanol was used in the solvent gradient by Reinen et al. and acetonitrile by us, the sequence of elution of the six products was the same. For further confirmation of the assigned structures, the six metabolites should be isolated by analytical HPLC and the EI spectra of their TMS derivatives obtained by GC-MS analysis.

As a caveat, it should be pointed out that the MS^2 spectra of the $[M-H]^-$ ions in our study and that of Reinen et al. [10] were obtained with ion trap mass spectrometers. So far, no information is available on the product ion spectra of the hydroxylated ZEN and ZEL using other types of instruments, for example, triple quad mass spectrometers. However, even if such spectra are less suitable for identification of the regioisomers, the approach of collecting the metabolites after HPLC separation and subjecting them to GC-MS after trimethylsilylation remains a viable option, as EI mass spectra are known as highly reproducible.

Although the MS^2 of the $[M-H]^-$ ions allow to distinguish between regioisomers, the stereoisomeric forms remain unknown. For example, the MS^2 of the $[M-H]^-$ ions of α -ZEL and β -ZEL are identical. Also, in the report by Reinen et al. [10], M2 and M5 exhibit the same MS^2 and most likely represent the stereoisomers of 8-hydroxy-ZEN, but it is unclear which is the α -isomer and which is the β -isomer. Likewise, none of the other metabolites can be identified with respect to its stereochemistry. Because of the S-configuration of ZEN at C-3, the two stereoisomers of each regioisomer of the aliphatic hydroxylation products of ZEN represent diastereomers and have different HPLC retention times. Work is presently in progress in our laboratory to isolate the stereoisomers of each regioisomeric hydroxy-ZEN and to determine their configuration.

Abbreviations

APCI:	Atmospheric pressure chemical ionization
EI:	Electron impact
ESI:	Electrospray ionization
GC:	Gas chromatography
HO:	Hydroxyl
HPLC:	High-performance liquid chromatography
LC:	Liquid chromatography
MS:	Mass spectrometry
MW:	Molecular weight
RP:	Reversed phase
Rt:	Retention time
TMS:	Trimethylsilyl
ZEL:	Zearalenol
ZEN:	Zearalenone.

Acknowledgments

The authors thank Mrs. Doris Honig for expert help with the GC-MS analysis. This work has been supported by the Deutsche Forschungsgemeinschaft (Grant ME 574/32-1) and by the Research Program “Food and Health” of the Karlsruhe Institute of Technology (KIT).

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