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Occurrence of Zearalenone-4-β-D-glucopyranoside in Wheat

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An LC-MS method was developed for the analysis of zearalenone-4-β-D-glucopyranoside and zearalenone in wheat (*Triticum aestivum*). The limit of determination for zearalenone-4-β-D-glucopyranoside and zearalenone was 10 μg/kg. The recovery rates were calculated to be 69% and 89% at a concentration of 100 μg/kg for zearalenone-4-β-D-glucopyranoside and zearalenone, respectively. Twenty-four Bavarian wheat samples from a 1999 harvest were analyzed. Zearalenone was present in 22 of 24 field samples, the levels ranged from 11 to 860 μg/kg. Zearalenone-4-β-D-glucopyranoside was found in 10 of the zearalenone positive samples (42%) at levels ranging from 17 to 104 μg/kg. The amounts of zearalenone-4-β-D-glucopyranoside were correlated to those of zearalenone ($r^2 = 0.86$, $b = 0.10$). After gastrointestinal hydrolyzation, zearalenone-4-β-glucopyranoside might be implicated in the development of a zearalenone-syndrome. Therefore, more attention should be focused on conjugated mycotoxins in food and feed.

KEYWORDS: *Zearalenone; zearalenone-4-β-D-glucopyranoside; wheat; LC-MS; conjugated mycotoxins*

INTRODUCTION

Zearalenone [6-10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone] is a nonsteroidal estrogenic fungal metabolite which is produced by different *Fusarium* species, e.g., *F. graminearum*, *F. culmorum*, and *F. semitectum* (*1*). The occurrence of zearalenone in feed has been implicated as a causal agent of hyperestrogenism in swine and may cause infertility and enlargement of the uterus and mammary glands (*3*−*5*).

Recent studies about the metabolism of zearalenone by cell suspension cultures of maize (*Zea mays*), wheat (*Triticum aestivum*), and different fungal species (*Rhizopus* sp., *Thamnidium elegans*, *Mucor bainieri*) showed that this metabolite is not only reduced to α- and β-zearalenol, but also conjugated to glucose (*2*, *6*−*8*). This zearalenone-4-β-D-glucopyranoside (**Figure 1**) is not determined by current zearalenone analysis (*9*−*11*). However, gastrointestinal digestion releases the aglucone zearalenone (*12*), which can contribute to the development of a mycotoxicosis.

As comparative analysis of cereals pretreated with or without β-glucosidase indicate the presence of zearalenone-4-β-D-glucopyranoside in field samples (*12*), the aim of this study was first to develop a method for the direct detection of this zearalenone-conjugate. Second, the occurrence of zearalenone-4-β-D-glucopyranoside in field samples should be verified and the share of this metabolite of the total content of zearalenone in wheat should be estimated.

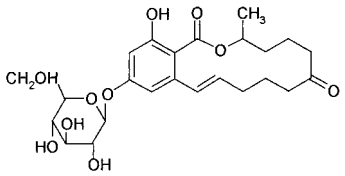

Figure 1. Structure of zearalenone-4-β-D-glucopyranoside.

MATERIAL AND METHODS

Chemicals. Zearalenone was purchased from Sigma Chemicals (Deisenhofen, Germany). All of the solvents used for extraction, cleanup, and liquid chromatography−mass spectrometry (LC-MS) were analytical grade. High-performance liquid chromatography (HPLC)-grade water was prepared using a Millipore Milli-Q purification system (Millipore, Eschborn, Germany). Florisil was obtained from Merck (Darmstadt, Germany).

Production of Zearalenone-4-β-D-glucopyranoside. Zearalenone-4-β-D-glucopyranoside was produced according to *Zill et al.* (*13*). A cell suspension culture of maize (Black Mexican Sweet) was incubated with zearalenone (25 mg/L) for 1 day. Cell culture without zearalenone was used as control. Cells were separated from the culture medium by filtration and 2.5 g of cells were placed in a 50 mL tube with 5 mL of methanol:water (4:1 v:v). After sonication (0.5 min), 20 mL of methanol:water (16:4 v:v) was added and the tube was shaken vigorously for one min. The sample was centrifuged (10 min, 4332*g*) and filtered through a glass microfiber filter (Whatman, Maidstone, UK). This step was repeated using 10 mL of methanol:water (8:2 v:v). The solvent was concentrated to the aqueous residue (approximately 3 mL) and extracted twice with ethyl acetate

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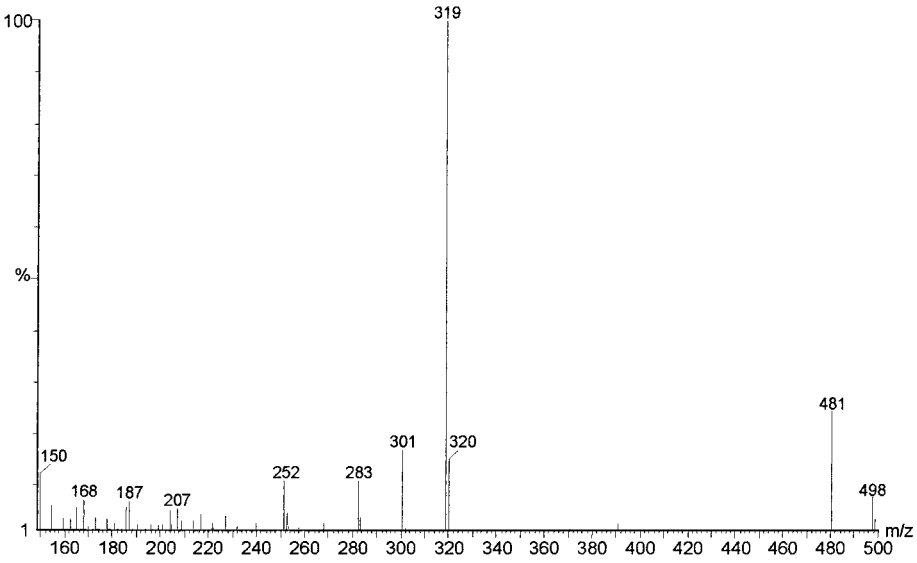

Figure 2. Mass spectrum (ESP+) of zearalenone-4-β-D-glucopyranoside (for MS conditions see text).

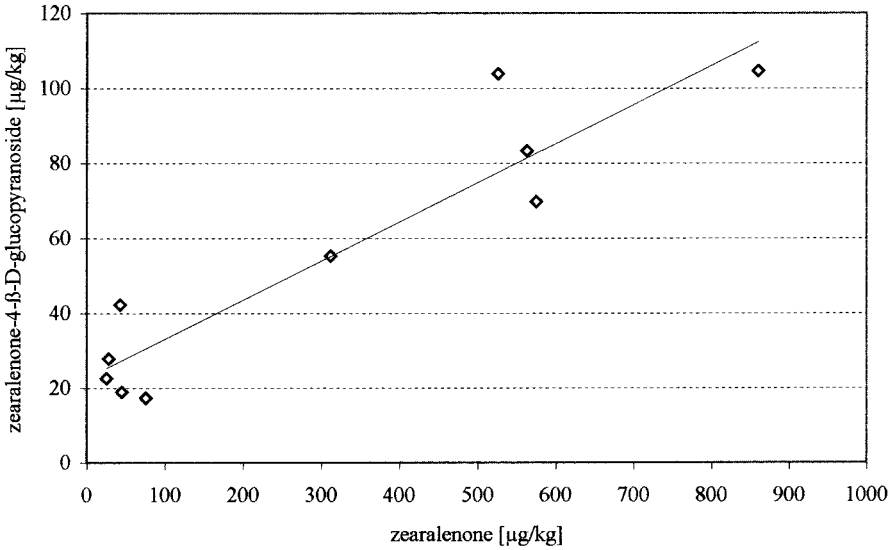

Figure 3. Correlation of zearalenone and zearalenone-4-β-D-glucopyranoside contents in wheat ($n = 10$, $r^2 = 0.86$, $b = 0.10$).

(10 mL). The combined ethyl acetate layers were evaporated to dryness and redissolved in 500 µL of methanol. The detection of zearalenone and zearalenone-4-β-D-glucopyranoside was performed by LC-MS. To prepare a zearalenone-4-β-D-glucopyranoside standard, the corresponding fraction (10.5−11.5 min) was collected (details are given below). For quantitation, an aliquot of this fraction (50 µL) was pretreated with 10 units of β-glucosidase from almonds (G-0395, Sigma Chemicals) and the amount of zearalenone released was analyzed by LC-MS.

Liquid Chromatography−Mass Spectrometry. The HPLC system (Waters 2690 Separations Module, Milford, MA) was connected to a quadrupole mass spectrometer (VG Platform 2) with an electrospray ionization source and a MassLynx data system (Micromass, Altrincham, UK). The conditions were as follows: column temperature, 30 °C; ionization mode, ESP^+; source temperature, 60 °C; scan rate, 1 scan/s (TIC); and cone voltage, 20 V.

To prepare a zearalenone-4-β-D-glucopyranoside standard, a normal-bore C_{18} column [Ultrasphere ODS, 150 × 4.6 mm i.d., 5 µm (Beckman, Krefeld, Germany)] was used. Solvent A consisted of water: 100% formic acid (99:1 v:v) and solvent B of acetonitrile. The gradient started at 10% B (2 min), increased linearly to 80% B (20 min) and was held for 4 min at a flow rate of 1.0 mL/min. A postcolumn splitting was arranged to achieve a flow of 10 µL/min to the source and 990 µL/min to waste and fractionation, respectively. The injection volume was 100 µL and the eluent was monitored in full scan mode (*m*/*z* 100−500).

For standard control and analysis of field samples the system was equipped with a narrow bore C_{18} column [Nucleosil 120-3, 125 × 2 mm i.d., (Macherey-Nagel, Düren, Germany)]. Solvent A consisted of water:100% formic acid (99:1 v:v) and solvent B of acetonitrile. The gradient started at 30% B (1 min), increased linearly to 80% B (13 min), and was held for 4 min at a flow rate of 0.2 mL/min. The injection volume was 10 µL. The eluent was split (1:20) and monitored in selected ion recording mode. Identification of zearalenone and zearalenone-4-β-D-glucopyranoside in spiked samples and field samples was based on retention time and relative peak area of selected ions [zearalenone: *m*/*z* 283, 301, 319 $(M + H)^+$; zearalenone-4-β-D-glucopyranoside: *m*/*z* 283, 301, 319, and 481 $(M + H)^+$]. For quantitation, the area of the quasi molecular ion peak (zearalenone: *m*/*z* 319 and zearalenone-4-β-D-glucopyranoside: *m*/*z* 481) was compared to that of an external standard.

Analysis of Wheat. *Extraction.* A 1.25 g portion of a 250 g ground sample (i.d. 0.5 mm) was diluted in 10 mL of

acetonitrile:water (21:4 v:v) and shaken vigorously for one min. The extract was centrifuged (10 min, 4332*g*) and an aliquot of 8 mL (equivalent to 1 g of grain) was concentrated to dryness.

Cleanup Chromatography. An extraction cartridge (12 mm i.d.) with frit was filled with 1 g of Florisil (Merck). The column was topped with a frit and conditioned with 5 mL of *n*-hexane. The extract was dissolved in 500 μL of methanol:*tert*-butylmethyl ether (50:450 v:v) and transferred onto the column. The column was washed with 5 mL of *n*-hexane and eluted with 5 mL of methanol:ethyl acetate (2.5:2.5, v:v). After evaporation of the solvent the residues were redissolved in 100 μL of methanol for LC-MS analysis.

Validation of Analysis. Spiking solutions were prepared in methanol at 1 μg/mL and 0.1 μg/mL of zearalenone and zearalenone-4-β-D-glucopyranoside. Spiked samples of wheat (1.25 g) were prepared by adding volumes of 125 μL of the standard working solutions to obtain concentrations of 10 ($n = 5$) and 100 μg/kg ($n = 5$); unspiked cereal samples ($n = 3$) were used as controls. The samples were analyzed and recovery rates were calculated.

Samples. Wheat samples ($n = 24$) were analyzed for zearalenone and zearalenone-4-β-D-glucopyranoside.

RESULTS AND DISCUSSION

The LC-MS analysis of the maize culture (25 mg/L zearalenone, added to the medium) showed a signal of the ion masses *m*/*z* 283, 301, 319, and 481 (**Figure 2**) at the retention time of 11.1 min. These masses correspond to $[M + H - C_6H_{10}O_5\text{-}2(H_2O)]^+$, $[M + H - C_6H_{10}O_5 - H_2O]^+$, $[M + H - C_6H_{10}O_5]^+$, and $[M + H]^+$ of zearalenone-4-β-D-glucopyranoside. For quantitation of zearalenone-4-β-D-glucopyranoside, an aliquot of the collected fraction was hydrolyzed by treating with β-glucosidase. The formation of the aglucone zearalenone, that was identified and quantified by LC-MS, is a further indication for the production of zearalenone-4-β-D-glucopyranoside by maize cells. Zearalenone-4-β-D-glucopyranoside could not be detected in the hydrolyzed sample. As the zearalenone-4-β-D-glucopyranoside fraction did not contain any zearalenone, an aglycone-free (< 0.1%) standard-solution was achieved.

The LC-MS method employed for the determination of zearalenone-4-β-D-glucopyranoside and zearalenone was linear in a range of 0.1−100 ng of standard substance per injection. The limit of determination in wheat samples was 10 μg/kg for zearalenone and zearalenone-4-β-D-glucopyranoside (signal-to-noise ratio 5:1). The recovery rates for zearalenone-4-β-D-glucopyranoside were calculated to be 69% at concentrations of 100 μg/kg and 67% for 10 μg/kg. The recovery rates for zearalenone were 89% and 32%, respectively.

Zearalenone was present in 22 of 24 field samples (92%), the levels ranged from 11 to 860 μg/kg. Zearalenone-4-β-D-glucopyranoside was detected in 10 of these samples (42%) at levels ranging from 17 to 104 μg/kg, all of them were zearalenone-positive. The amounts of zearalenone-4-β-D-glucopyranoside were correlated to those of zearalenone ($r^2 = 0.86$, $b = 0.10$) (**Figure 3**). This provides a first estimation of the share of this metabolite in the total content of zearalenone and zearalenone-derivatives in wheat.

The results obtained in this study demonstrate that zearalenone−**4**-β-D-glucopyranoside can occur in wheat in addition to free zearalenone. It is known that zearalenone-4-β-D-glucopyranoside is produced by wheat cell cultures contaminated by zearalenone (*2*). Therefore, we suggest that zearalenone-4-β-D-glucopyranoside is a product of the metabolism of zearalenone by wheat cells. As zearalenone-4-β-D-glucopyranoside can be easily hydrolyzed to zearalenone during digestion, releasing the free zearalenone (*12*) this compound can be implicated in the development of mycotoxicosis. Therefore more attention should be focused on conjugated mycotoxins of plant inhabiting fungi in food and feed.

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