JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Stable Isotope Dilution Analysis of the *Fusarium* Mycotoxin Zearalenone

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Zearalenone is a secondary metabolite produced by molds of the *Fusarium* genus. Beside its nonsteroidal molecular structure, zearalenone has estrogenic activity and can disrupt the function of the endogenous hormone 17β-estradiol in animals and possibly in humans. It can frequently be found in all major cereal grains as well as in processed food. Because of the estrogenic properties of zearalenone and its metabolites, legal regulations are installed in the European Union setting maximum levels in cereals and cereal products. Routine analysis of zearalenone in various commodities is carried out by HPLC with fluorescence detection, but due to the development of multi-mycotoxin methods and the reduced sample cleanup, HPLC-MS/MS has become a fast and efficient alternative. However, to achieve a reliable quantitation with this technique suitable internal standards are required. This paper reports the synthesis of stable isotope labeled 3,5-d_2-zearalenone (ZON) as internal standard for stable isotope dilution analysis. Furthermore, a method for the analysis of zearalenone by HPLC-MS/MS using 3,5-d_2-zearalenone as IS has been developed. Fifteen cereal products from the German retail markets were analyzed, of which seven contained ZON in levels from 4.9 to 45.0 μg/kg.

KEYWORDS: Zearalenone; HPLC-MS/MS; isotope dilution mass spectrometry; *Fusarium*; mycotoxin; zearalanone

INTRODUCTION

Mycotoxins are fungal secondary metabolites produced by molds during their growth period. They have various adverse effects such as acute toxicity, carcinogenicity, or hormonal properties making them serious health risks for both humans and animals. So far, more than 400 different mycotoxins are known, of which aflatoxins, ochratoxin A, and the *Fusarium* toxins are of special importance.

In the scope of cereals and cereal products, the mycotoxins most often found are deoxynivalenol, fumonisins, and zearalenone, all produced by *Fusarium* fungi, as well as ochratoxin A, which is produced by molds of *Penicillium* and *Aspergillus* genera.

Zearalenone (**Figure 1**) is a nonsteroidal secondary fungal metabolite with estrogenic activity, produced mainly by *Fusarium graminearum* and *Fusarium culmorum*. It can be found throughout the world in cereal grains such as maize, wheat, barley, oats, and rye, as well as in processed cereal products and milk (*1*, *2*). Zearalenone and some of its derivatives have been shown to competitively bind to estrogen receptors in cell culture as well as in animal experiment (*3*). In farm animals, especially in swine, higher levels of zearalenone in feed show anabolic properties and have adverse effects on reproductive performance (*3*, *4*). Zearalenone has a low acute toxicity after oral administration. LD_{50} values ranging from 4000 to 20000 mg/kg of body weight were recorded for mice, rats, and guinea pigs (*5*). In 1993 the International Agency for Research on Cancer (IARC) classified zearalenone as group 3 (not classifiable as to its carcinogenicity to humans) (*6*). The Scientific Committee on Food (SCF) concluded that the safety of zearalenone could be evaluated on the dose that had no hormonal effects in pigs, the most sensitive species, and established a temporary TDI of 0.2 μg/kg of body weight per day (*1*). Maximum limits for zearalenone are set up by the European Union ranging from 20 μg/kg for food for infants and small children to 200 μg/kg for unprocessed maize and maize flour (*7*).

Due to these regulations, a special requirement for quick and reliable methods to determine zearalenone in cereals and cereal-based food is given (*8*). Currently, several methods are established including thin layer chromatography (TLC) (*9*), gas chromatography coupled with flame ionization detection (GC-FID) (*10*) or mass spectrometric detection (GC-MS) (*11*), enzyme-linked immunosorbent assay (ELISA) (*12*), and high-performance liquid chromatography coupled with fluorescence detection (HPLC-FLD) (*13*). TLC is used as a cheap screening method for cereals and corn, but allows no precise quantitation and has a poor detection limit. The use of GC coupled with various detectors always requires a laborious sample preparation including a critical derivatization step prior to analysis. ELISA

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10.1021/jf0717283 CCC: $37.00
Published on Web 09/20/2007

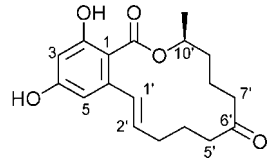

Figure 1. Structure of zearalenone.

tests allow a fast screening of samples, but often give higher results due to cross reactions, which cannot be reproduced by other chromatographic methods (*12, 13*). The determination of zearalenone by HPLC-FLD is until now the standard analytical technique. However, it is often necessary to use costly immunoaffinity columns prior to separation on HPLC and detection by FLD to reach low limits of detection (*14*). In the past decade, analysis of zearalenone by HPLC-MS/MS has been established as an analytical method that allows determination at highest sensitivity and specifity with only little sample cleanup required prior to analysis (*15*). A further advantage of this technique is the possibility of multi-mycotoxin analysis. Several multimethods for the determination of zearalenone with other *Fusarium* toxins in a single run have been published (*16, 17*).

However, a major problem of HPLC-MS/MS analysis is the matrix, which occurs during ionization of the analyte and leads to signal suppression or enhancement. The intensity of the matrix effect depends on sample type and purity and can hardly be predicted. To compensate for this effect, different strategies are reported. Matrix calibration compensates the effect caused by the sample matrix by adding a similar matrix to the calibration standards. However, this technique is often laborious, as for every sample type a respective calibration curve is required (*15*). An alternative to the matrix calibration is the use of suitable internal standards (IS). In the literature, structural analogues of zearalenone, such as zearalanone (*16*) or zearalanol (*17*), are described for this purpose. However, the structural differences between the internal standard and the analyte may cause differences in extraction behavior as well as in chromatographic retention and ionization efficiency. These differences might limit the compensation of matrix effects. A better internal standard would be a stable isotope labeled analyte that chemically acts as the analyte itself and has nearly the same retention time and ionizability. Recently, stable isotope labeled zearalenone has been applied as internal standard for the analysis in environmental samples by HPLC-MS/MS (*19*). However, the labeling techniques described for zearalenone yield a mixture of unlabeled zearalenone to d_9-zearalenone, with the highest relative amount of 38% for d_6-zearalenone. Due to this mixture, the fragmentation pattern can be inhomogeneous, and high absolute amounts of IS are required.

Here we report the synthesis of stable isotope labeled 3,5-d_2-zearalenone (ZON) with high isotopic purity and its use as internal standard for the analysis of food samples.

MATERIALS AND METHODS

General Remarks. All solvents and reagents were purchased from VWR (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany) in gradient grade or reagent quality. Zearalenone was produced by inoculating rice media with *Fusarium graminearum* (DSM 4528) and grown for 21 days at room temperature, followed by extraction with methanol/water (70:30, v/v). Further purification was achieved using centrifugal partition chromatography with a solvent system of water/methanol/acetonitrile/*tert*-butyl methyl ether/ethyl acetate/pentane (4:3:3:3:4) followed by crystallization. Water for HPLC separation was purified with a Milli-Q Gradient A 10 system (Millipore, Schwalbach, Germany).

GC-MS. Electron impact (EI) GC-MS data were acquired on a HP6890 series gas chromatograph and a HP5973 mass spectrometer (Hewlett-Packard/Agilent, Böblingen, Germany) after derivatization of the compounds with 200 μL of *N,O*-bis(trimethylsilyl)acetamide and addition of 300 μL of *tert*-butyl methyl ether. Data acquisition was carried out with the Chemstation software (Agilent). Chromatographic separation was performed on a 60 m $\times$ 0.25 mm i.d. fused silica, 0.25 μm methyl silicone coating, J&W Scientific DB-1 column (Agilent) using helium at 1.3 mL/min as carrier gas. The injector temperature was set at 260 °C, the injection volume was 1 μL, and the split was 1:10. The column temperature was held initially at 120 °C for 1 min and then programmed at 5 °C/min to 270 °C and then at 15 °C/min to 320 °C, which was held isothermally for 10 min. The transfer line was heated at 320 °C. The mass spectrometer was operated in the electron impact mode (EI, 70 eV electron energy) with a source temperature of 230 °C and the quadrupole heated at 150 °C. Mass spectra were acquired in the full scan mode ranging from *m/z* 40 to 800 with a scan rate of 2.0 scans/s.

NMR Spectroscopy. All NMR experiments were carried out on a Bruker DCX-400 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany). Signals are reported in parts per million referenced to TMS. For structural elucidation, in addition to the 1D-^{1}H- and ^{13}C experiments, further 2D NMR experiments were performed: gradient selected correlation spectroscopy (gs-COSY), heteronuclear multiple bond correlation (HMBC), and heteromolecular multiple quantum correlation (HMQC) spectroscopy. Pulse programs for the experiments were taken from the Bruker software library.

Synthetic procedures. *Zearalenone-6′-[1,3]dioxolane.* Zearalenone (60.7 mg, 190 μmol) was added to a solution of ethylene glycol (3 mL) and *p*-toluenesulfonic acid monohydrate (72.0 mg, 380 μmol) in toluene (40 mL). The reaction mixture was refluxed for 3.5 h and the reaction water removed via azeotropic distillation. After cooling, the solution was extracted twice with NaOH (0.1 M, 20 mL). The aqueous layer was neutralized with 1 M hydrochloric acid to pH 7 and extracted twice with *tert*-butyl methyl ether. The combined organic layers were dried over anhydrous sodium sulfate and evaporated to yield crude zearalenone-6′-[1,3]dioxolane (64.9 mg, 179 μmol, 94% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.39 (3H, d, $J_{13',10'} = 6.3$ Hz H-13′), 1.60–1.85 (10H, m, H-4′, H-5′, H-7′, H-8′, H-9′), 2.30 (2H, m, H-3′), 3.90–3.98 (4H, m, H-1″, H-2″), 4.95 (1H, m, H-10′), 5.86 (1H, ddd, $J_{H\text{-}1',H\text{-}2'} = 15.6$ Hz, $J_{H\text{-}2',\ H\text{-}3'A} = 7.1$ Hz, $J_{H\text{-}2',\ H\text{-}3''B} = 6.1$ Hz, H-2′), 6.33 (1H, d, $J_{3,5} = 2.6$ Hz, H-3), 6.40 (1H, d, $J_{5,3} = 2.6$ Hz, H-5), 7.10 (1H, d, $J_{1',2'} = 15.6$ Hz),7.43 (1H, s, br, 3-OH), 12.03 (1H, s, 5-OH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.3 (C-13′), 20.4 (C-8′), 22.0 (C-4′), 31.4 (C-3′), 31.8 (C-7′), 35.8 (C-9′), 36.3 (C-5′), 64.2 (C-1″), 64.5 (C-2″), 74.1 (C-10′), 102.3 (C-3), 104.1 (C-1), 108.6 (C-5), 112.7 (C-6), 132.2 (C-1′), 133.1 (C-2′), 143.9 (C-6), 161.0 (C-4), 165.0 (C-2), 171.7 (C-12′).

ESI-MS: negative mode, *m/z* 361 $[M - H]^-$. MS/MS (−35 V): *m/z* (%) 361 (100), 160 (83), 131 (47), 174 (37), 317 (36), 132 (19), 133 (18), 333 (11), 273 (11), 161 (11); positive mode, *m/z* 363 $[M + H]^+$. MS/MS (+20 V): *m/z* (%) 284 (100), 302 (65), 283 (59), 346 (43), 301 (29), 320 (25), 266 (21), 345 (15), 265 (12), 256 (11), 319 (10), 363 (4).

3,5-d_2-Zearalenone-6′-[1,3]dioxolane. To 50.2 mg of zearalenone-6′-[1,3]dioxolane (139 μmol) filled in a 4 mL screw-cap vial was added a solution of 55.0 mg of potassium carbonate (0.4 mmol) in 4 mL of deuterium oxide, and the suspension was stirred for 48 h at 50 °C.

The reaction mixture was transferred into a separation funnel, diluted with 10 mL of water, acidified with hydrochloric acid (1.0 mol/L), and extracted with *tert*-butyl methyl ether (20 mL) twice. The combined organic layers were dried over sodium sulfate, and the solvent was removed. The next step was carried out without further purification.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.40 (3H, d, $J_{13',10'} = 6.3$ Hz H-13′), 1.58–1.83 (10H, m, H-4′, H-5′, H-7′, H-8′, H-9′), 2.33 (2H, m, H-3′), 3.91–3.98 (4H, m, (H-1″, H-2″), 5.02 (1H, m, H-10′), 5.89 (1H, ddd, $J_{H\text{-}1',H\text{-}2'} = 15.6$ Hz, $J_{H\text{-}2',\ H\text{-}3'a} = 7.1$ Hz, $J_{H\text{-}2',\ H\text{-}3'b} = 6.1$ Hz, H-2′), 7.14 (1H, d, $J_{1',2'} = 15.6$ Hz), 11.97 (1H, s, 5-OH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.3 (C-13′), 20.5 (C-8′), 22.0 (C-4′), 31.4 (C-3′), 31.9 (C-7′), 35.8 (C-9′), 36.3 (C-5′), 64.2 (C-1″), 64.5 (C-2″), 74.1 (C-10′),

102.2 (t, C-3), 104.0 (C-1), 108.3 (t, C-5), 112.7 (C-6), 132.2 (C-1′), 133.1 (C-2′), 143.9 (C-6), 161.0 (C-4), 165.0 (C-2), 171.7 (C-12′).

ESI-MS: negative mode, *m/z* 363 $[M - H]^-$. MS/MS (−35 V): *m/z* (%) 363 (100), 162 (69), 132 (38), 176 (28), 131 (28), 319 (27), 335 (24), 129 (24), 189 (17), 175 (17), 133 (17), 345 (14),275 (14), 274 (14), 134 (14); positive mode, *m/z* 365 $[M + H]^+$. MS/MS (+20 V): *m/z* (%) 285 (100), 303 (79), 347 (44), 321 (31), 167 (30), 284 (23), 267 (19), 302 (14), 259 (12), 257 (12), 295 (10), 233 (14).

3,5-d_2-Zearalenone. A total of 35.2 mg of 3,5-d_2-zearalenone-6′-[1,3]dioxolane (96 μmol) was dissolved in MeOH (3 mL) and a solution of ammonium chloride (1.0 M, 1 mL) and stirred at 50 °C for 2 h. The cleavage product was purified on a 250 × 16 mm i.d., 4 μm semipreparative Synergi Fusion column (Phenomenex, Aschaffenburg, Germany) using water as solvent A and methanol as solvent B, delivered by two Varian ProStar 210 HPLC solvent delivery modules (Varian, Darmstadt, Germany) The flow rate was 10 mL/min, and the following gradient was applied: 0 min, 30% B; 30 min, 70% B. After each run, the column was washed with 100% B and equilibrated for 10 min at the starting conditions. 3,5-d_2-ZON was collected after peak detection on a Varian ProStar 325 UV–vis detector set at 273 nm. After evaporation of the organic solvent, the solution was lyophilized, yielding 27.3 mg of 3,5-d_2-ZON (85 μmol, 86% yield).

^{1}H NMR (400 MHz, MeOD): δ 1.37 (d, 3H, $J_{11',10'} = 6.1$ Hz, H-11′),1.54 (m, 1H, H-4a′), 1.59–1.66 (2H, m, H-9a′, b′), 1.72–1.81 (m, 2H, H-8a′,b′), 2.05 (1H, m, H-4b′), 2.09–2.22 (2H, m, H-3a′, H-7a′), 2.24–2.38 (2H, m, H-3b′, H-5a′), 2.65 (1H, m, H-7b′), 2.85 (1H, ddd, H-5b′, $J_{5a'-b'} = 18.8$ Hz, $J_{5b'-4b'} = 11.8$ Hz, $J_{5b'-4a'} = 2.5$ Hz), 5.0 (1H, m, H-10′), 5.71 (1H, ddd, $J_{1',2'} = 15.2$, $J_{2',3b'} = 10.2$, $J_{2',3a'} = 4.0$, H-2′), 6.98 (1H, dd, $J_{1',2'} = 15.3$, $J_{1',3b'} = 1.3$, H-1′). ^{13}C NMR (100 MHz, MeOD): δ 19.6 (C-11′), 20.6 (C-4′), 21.9 (C-8′), 30.7 (C-3′), 34.5 (C-9′), 36.1 (C-5′), 42.4 (C-7′), 73.0 (C-10′), 101.2 (t, C-3), 102.7 (C-1), 107.8 (t, C-5), 131.6 (C-2′), 133.0 (C-1′), 143.2 (C-6), 162.4 (C-4), 164.8 (C-2), 171.3 (C-12′), 212.3 (C-6′).

ESI-MS: negative mode, *m/z* 319 $[M - H]^-$. MS/MS (−35 V): *m/z* (%) 133 (100), 177 (98), 132 (47), 176 (43), 318 (39), 151 (32), 149 (30), 189 (27), 150 (21), 188 (20), 128 (20); positive mode, *m/z* 321 $[M + H]^+$. MS/MS (+30 V): *m/z* (%) 189 (100), 285 (61), 205 (55), 187 (55), 188 (51), 267 (29), 243 (28), 191 (27), 207 (21), 186 (21), 257 (20).

Stock Solutions. The internal standard (IS), 3,5-d_2-zearalenone was dissolved in MeCN (1 mg/mL) and diluted to a concentration of 10 μg/mL. The solution was stored at −18 °C and was stable over several months. No re-exchange of the deuterium atoms was observed under these conditions. A stock solution of zearalenone (1 mg/mL) was prepared in the same way and diluted to a final concentration of 10 μg/mL.

Sample Preparation. Twenty-five grams of ground food sample from the German retail market (tortilla chips, corn flakes, pasta, oats) was weighed into an Erlenmeyer flask. One microgram of 3,5-d_2-zearalenone (40 μg/kg) was added as IS, and 100 mL of MeCN/water (80:20, v/v) was added as extraction–solvent. After homogenization with an Ultra Turrax TB25B (Janke & Kunkel, IKA, Staufen, Germany) for 3 min at 20000 rpm, the extract was filtered to yield a clear solution. Further sample cleanup was carried out according to the method of Klötzel et al. (*18*) by passing 4 mL of the extract through a Varian Bond Elut Mykotoxin cartridge. The received eluate was directly used for HPLC-MS/MS analysis without solvent change.

Recovery Determination. The recovery of the method was checked by spiking a corn flakes sample containing no detectable zearalenone with 5, 50, and 300 μg/kg zearalenone, respectively. The samples were spiked with the IS 3,5-d_2-zearalenone (40 μg/kg), and the cleanup was carried out according to the procedure described above.

The column recovery was determined by spiking a corn flakes sample containing no detectable zearalenone with 50 μg/kg of the zearalenone standard. The extraction and purification were carried out as described above, but without the addition of IS. An aliquot (1000 μL) of the eluate of the Varian Bond Elut Mykotoxin cartridge was spiked with 10 μg of the internal standard 3,5-d_2-zearalenone in an autosampler vial prior to HPLC-MS/MS-analysis. All recovery experiments were carried out in duplicate.

Calibration Curves. Calibration solutions were prepared as follows. Aliquots of standard solutions of zearalenone and 3,5-d_2-zearalenone (10 μg/mL) were mixed in various concentration ratios (zearalenone/3,5-d_2-zearalenone 1:10 up to 10:1). The mixtures were analyzed in the MRM mode (each concentration was injected three times). The resulting peak area ratios were plotted against the concentration ratios.

HPLC-MS/MS. ESI mass and product ion spectra were acquired on an API 4000 QTRAP mass spectrometer (Applied Biosystems, Darmstadt, Germany) with direct flow infusion. For ESI, the ion spray voltage was set at −4500 V in the negative mode and at 5500 V in the positive mode. The MS/MS parameters were dependent on the compounds, detecting the fragmentation of the $[M - H]^-$ or $[M + H]^+$ molecular ions into specific product ions after collision with nitrogen (4×10^{-5} torr). Both quadrupoles were set at unit resolution. For HPLC-ESI-MS/MS analysis, an Agilent 1100 series HPLC was linked to the mass spectrometer. Data acquisition was carried out with the Analyst 1.4 software (Applied Biosystems). Chromatographic separation was carried out on a 150 × 2.0 mm i.d., 4 μm Synergi Fusion column (Phenomenex, Aschaffenburg, Germany) using a linear binary gradient. The injection volume was 5 μL and the flow rate 250 μL/min; solvent A was acetonitrile and solvent B water. The following gradient was used: 0 min, 60% A; 8 min, 100% A; 10 min, 100% A. After each run, the column was equilibrated for 10 min at the starting conditions. For HPLC-MS/MS, the mass spectrometer was operated in MRM mode, detecting negative ions. Zero grade air was used as nebulizer gas (30 psi) and heated at 350 °C as turbo gas for solvent drying (50 psi). Nitrogen served as curtain gas (20 psi) and collision gas (4.2×10^{-5} torr). The following transition reactions were monitored for a duration of 150 ms each. Declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) are given in parentheses: zearalenone, *m/z* 317 → 175 (DP −105 V, CE −35 V, CXP −13 V), *m/z* 317 → 131 (DP −105 V, CE −42 V, CXP −13 V); 3,5-d_2-zearalenone, *m/z* 319 → 177 (DP −105 V, CE −35 V, CXP −13 V), *m/z* 319 → 133 (DP −105 V, CE −42 V, CXP −13 V).

RESULTS AND DISCUSSION

The use of HPLC-MS/MS for the simultaneous determination of ZON together with further *Fusarium* toxins has been successfully established in the past several years (*17*, *18*). The respective procedures usually use cleanup columns consisting of rather nonselective adsorbents such as charcoal, silica, and alumina oxide for matrix removal. For the analysis of zearalenone the cleanup was carried out on a Varian Bond Elut Mykotoxin cartridge. The good cleanup properties of this column for the simultaneous analysis of multiple *Fusarium* mycotoxins have previously been described (*18*). However, due to the broad spectrum of polarities found among the group of *Fusarium* mycotoxins, the column material cannot be ideal for all compounds, which results in a partial binding of analytes to the column. For zearalenone, the recovery of the cleanup on the Varian Bond Elut Mykotoxin cartridge ranges between 65 and 80%.

To compensate for the loss during cleanup and matrix effects during HPLC-MS/MS analysis, suitable internal standards, such as stable isotope labeled analytes, are required. These substances show the same chemical and physical properties, and thus matrix effects can be easily compensated. The use of stable isotope labeled zearalenone has been successfully applied for the analysis of zearalenone in metabolic studies using GC-MS (*22*) as well as for its determination in aqueous environmental samples by HPLC-MS/MS (*19*). For both studies, the isotopic labeling of zearalenone was achieved by alkaline deuterium exchange yielding a spectrum of nonlabeled to 9-fold deuterium labeled zearalenone with a maximum relative amount of 38% for d_6-zearalenone (*19*). The positions favored for deuterium/hydrogen exchange are C-3, C-5, C-5′, C-7′ (*22*). Using the method described, it is nevertheless not possible to achieve a

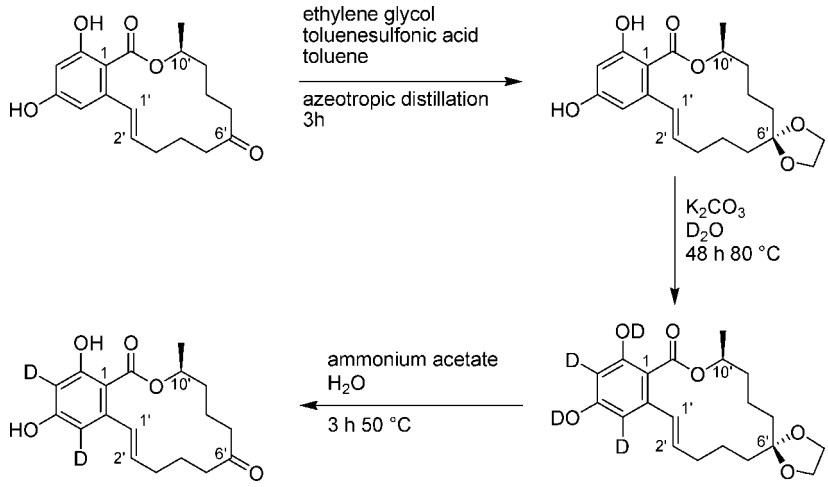

Figure 2. Synthesis of 3,5-d_2-zearalenone.

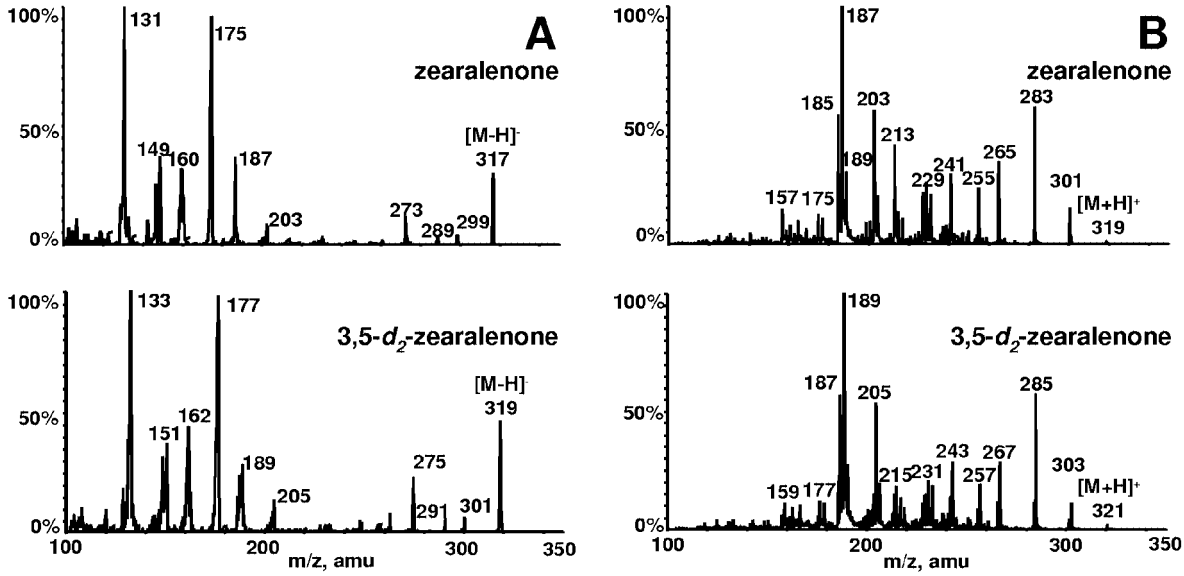

Figure 3. ESI-MS product ion spectra of zearalenone and 3,5-d_2-zearalenone in the (**A**) negative and (**B**) positive mode (collision energy, −35 V, respectively +30 V).

full deuterium labeling at all four carbon atoms. Whereas the deuterium/hydrogen exchange at C-3 and C-5 is quantitative, the exchange at C-5′ and C-7′ remains incomplete, probably due to steric hindrance.

To synthesize zearalenone with a high isotopic purity, a selective deuterium/hydrogen exchange at C-3 and C-5 is required without affecting the protons at positions C-5′ or C-7′. A successful procedure for the selective exchange of the protons at C-3 and C-5 was the formation of zearalenone-6′-[1,3]dioxolane (**Figure 2**). This cyclic acetal is stable under alkaline conditions but can easily be cleaved at lower pH. A deuterium/hydrogen exchange under alkaline conditions, as described by Miles et al. (*22*), can then be carried out to achieve a full deuterium labeling at C-3 and C-5. Due to the protective group formed with the carbonyl moiety at position 6′, no deuterium/hydrogen exchange at position 5′ or 7′ is possible. The following cleavage of the dioxolane in aqueous solution at pH 5 and purification by preparative HPLC yielded 3,5-d_2-zearalenone with 97% purity (NMR, GC-MS). A comparison of the mass spectrometric fragmentation patterns of the synthesized 3,5-d_2-zearalenone and the zearalenone standards demonstrates the successful incorporation of the two deuterium atoms. Whereas for unlabeled zearalenone the two most intense product ions are found to be *m*/*z* 175 and 131, in the negative mode, the respective product ions for 3,5-d_2-zearalenone are 2 mass units higher at *m*/*z* 177 and 133. The same difference of 2 mass units is observed in the positive mode with *m*/*z* 283 and 187 for zearalenone and the corresponding *m*/*z* 285 and 189 for 3,5-d_2-zearalenone (**Figure 3**). The isotopic purity (deuterium labeling) was determined as 94.5% by HPLC-MS/MS. Further isotopes are d_1-zearalenone (5.4%) and nondeuterated zearalenone (0.1%).

Calibration. For quantitation and calibration in MS, mixtures of analyte and internal standard (IS, 3,5-d_2-zearalenone) were prepared. Whereas the IS concentration in the solution is the same, the concentration of analyte is varied from a ratio of 1:10 to a ratio of 10:1. Plotting the area ratio analyte to IS against the concentration ratio gives the calibration function used for the quantitation of zearalenone by 3,5-d_2-zearalenone.

$$y = ax^2 + bx + c \quad (1)$$

To quantitate the mycotoxin, two correction factors have to be introduced into the calibration equation (eq 1). First, as usual for most isotopic standards, a small amount of unlabeled analyte can be found in the IS solution; this means that the pure IS solution also causes a small signal for the analyte. A compensation of this effect is achieved by introducing the $+c$ term into the equation

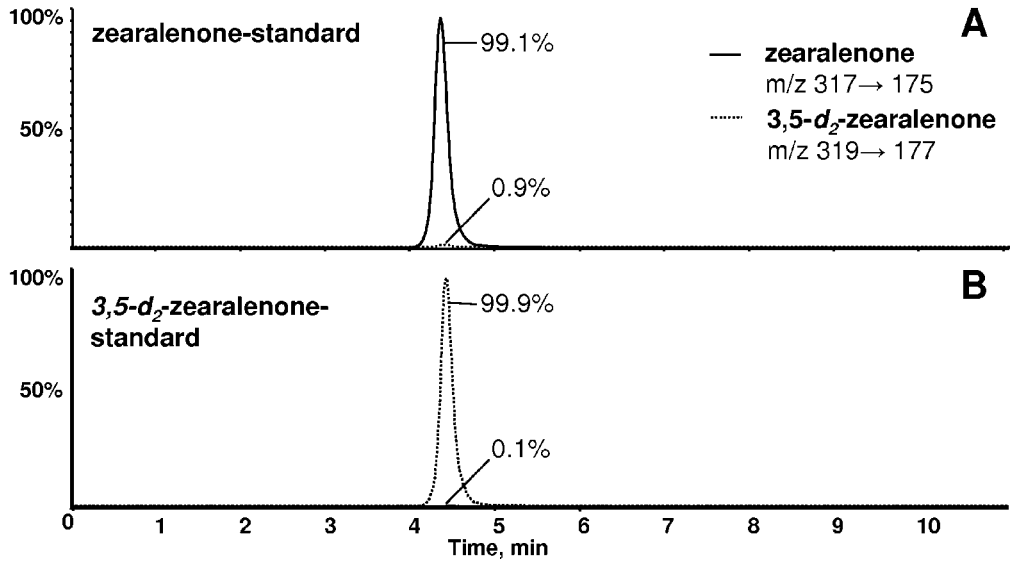

Figure 4. HPLC-MS/MS chromatograms of (**A**) zearalenone and (**B**) 3,5-d_2-zearalenone standard solutions. The corresponding area percentages are given in the chromatogram.

(eq 1), which shifts the calibration curve by the respective amount along the *y*-axis. In the case of 3,5-d_2-zearalenone, the simulation of unlabeled zearalenone is only 0.1% (**Figure 4**).

The second compensation factor is required due to the natural occurrence of the ^{13}C isotopomers of zearalenone. Besides the all-^{12}C-zearalenone with a monoisotopic mass of 318.2 Da, the $^{13}C_1$ isotopomer with a calculated percentage of 19.9% and a monoisotopic mass of 319.2 Da as well as the $^{13}C_2$ isotopomer with a percentage of 2.9% and a mass of 320.2 Da occurs in significant amounts. The latter isotopomer has nearly the same molecular mass as the internal standard, and depending on the position of the two ^{13}C atoms, shows also the same mass transition as 3,5-d_2-zearalenone in quantitative HPLC-MS/MS.

This means that in this case the analyte simulates a small amount of IS. To quantify this effect, a stock solution of zearalenone was analyzed by HPLC-MS/MS, and the intensities of the analyte transition $[M - H]^-$ *m/z* 319→133 and the IS transition $[M - H]^-$ *m/z* 319→133 were determined. As shown in **Figure 4**, a signal of 0.9% intensity was observed for the latter transition and thus describes the simulation of 3,5-d_2-zearalenone by the analyte. This effect depends on the respective concentrations, as high amounts of analyte simulate more IS than lower. In a previous publication, we have already shown that a mathematical compensation of this overlap can easily be achieved by the introduction of a quadratic term, ax^2 into the equation (*21*). The received quadratic calibration function is generally well established and incremented in all modern mass spectrometry software as well as in spreadsheet software such as Microsoft Excel. According to eq 1, the following calibration function was calculated: $y = -0.00952x^2 + 1.34x + 0.0176$ ($r = 0.9999$).

Stability. The stability of the deuterium labeling at positions 3, 5, 5′, and 7′ in the zearalenone molecule has already been investigated by Hartmann et al. (*19*) and Miles et al. (*22*). They found no change during sample preparation or storage in aqueous solutions, but a slight change in the deuterium/hydrogen ratio was observed in metabolic studies.

We could show that the 3,5-d_2-zearalenone stock solution is stable for several months when stored at −18 °C in acetonitrile. Furthermore, no significant change in the isotopic ratio was observed during sample preparation. However, the stability of the deuterium labeling can be affected by extreme pH values as well as by heating in aqueous solutions at high temperatures and for long durations. This has to be considered during method development and cleanup.

Table 1. Concentrations of Zearalenone in Various Food Samples

sample	concentration of zearalenone, μg/kg ± SD
corn flakes 1	nd
corn flakes 2	13.6 ± 0.6
corn flakes 3	<4
corn flakes 4	<4
tortilla chips 1	6.9 ± 0.1
tortilla chips 2	7.8 ± 0.2
tortilla chips 3	17.0 ± 2.2
tortilla chips 4	13.6 ± 0.5
tortilla chips 5	4.9 ± 0.2
bread	nd
taco shells	45.0 ± 2.0
crackers	<4
oat flakes	nd
noodles 1	nd
noodles 2	<4

Sample Purification. Different cleanup procedures for zearalenone analysis are described in the literature, including immunoaffinity columns and different types of SPE cartridges. For the analysis of zearalenone we chose a procedure previously described by Klötzel et al. (*18*). Briefly, after homogenization and addition of the IS, samples were extracted using a MeCN/water mixture. The extract was filtered, and the cleanup was performed using a Varian Bond Elut Mykotoxin column. In contrast to the procedure of Klötzel et al., no further concentration step was required, and the eluate was directly analyzed by HPLC-MS/MS.

The chromatographic separation was carried out on a Phenomenex Synergi Fusion column, with a water/MeCN gradient. The recovery rates were determined in a corn flakes matrix containing no detectable zearalenone. The matrix was spiked with three different levels of zearalenone. For each concentration, two samples were worked up independently. The recovery rates were 104 ± 3, 103 ± 4, and 101 ± 2% for the spiking levels of 5, 50, and 300 μg/kg, respectively.

The column recovery was determined using a corn flakes matrix containing no detectable amount of zearalenone. The

matrix was spiked with 50 μg/kg zearalenone, and the cleanup procedure was carried out as described above, but without addition of IS. To the purified sample solution was added an amount of IS, corresponding to the eluate volume, and the sample was analyzed by HPLC-MS/MS. The column recovery rate was 75.7 ± 0.7%.

The signal-to-noise ratio (S/N) was determined for zearalenone in the least concentrated calibration solution. Five microliters of a solution containing 1 ng of zearalenone/mL (equaling 4 μg/kg of sample) gave an S/N of 175. Limits of quantitation were not determined as they are only of little importance in the surveillance of legal limits ranging from 20 to 200 μg/kg.

Using this method 15 food samples from the German retail market were analyzed in duplicate. The results are given in **Table 1**. Zearalenone was detected in all corn products except one sample, which was used for the recovery experiments. In the other samples only little or no zearalenone was detected.

This is the first report of a stable isotope dilution analysis of ZON in food samples using HPLC-MS/MS.

ACKNOWLEDGMENT

We thank A. Klusmeier-König for technical assistance.

LITERATURE CITED

(1) European Commission, Scientific Committee on Food. Opinion of the Scientific Committee On Food on *Fusarium* toxins, part 2: zearalenone, 2000 (http://europa.eu.int/comm/food/fs/sc/scf/out65_en.pdf, accessed June 10, 2007.

(2) Directorate-Generale Health and Consumer Protection. Reports on tasks for scientific cooperation, report of experts participating in Task 3.2.10: collection of occurrence data of *Fusarium* toxins in food and assessment of dietary intake by the population of EU member states, 2003 (http://europa.eu.int/comm/food/fs/scoop/task3210.pdf, accessed June 10, 2007.

(3) Kuiper-Goodman, T.; Scott, P. M.; Watanabe, H. Risk assessment of the mycotoxin zearalenone. *Regul. Toxicol. Pharmacol.* **1987**, *3*, 253–306.

(4) Bauer, J.; Heinritzi, K.; Gareis, M.; Gedek, B. Veränderungen am Genitaltrakt des weiblichen Schweines nach Verfütterung praxisrelevanter Zearalenonmengen. *Tieraerztl. Prax.* **1987**, *15*, 33–36.

(5) Hidy, P. H.; Baldwin, R. S.; Greasham, R. L.; Keith, C. L.; McMullen, J. R. Zearalenone and some derivatives: production and biological activities. *Adv. Appl. Microbiol.* **1977**, *22*, 59–82.

(6) *Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins*; IARC: Lyon, France, 1993.

(7) Commission regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Union* **2006**, *L364*, 524.

(8) Krska, J. Mycotoxins of growing interest, zearalenone. Presented at the Third Joint FAO/WHO/UNEP international conference on mycotoxins, Tunis, Tunisia, March 3–6, 1999; MYC-CONF/99/5d.

(9) Eppley, R. M. Screening method for zearalenone aflatoxin and ochratoxin. *J. Assoc. Off. Anal. Chem.* **1968**, *51*, 74–78.

(10) Hagler, W. M.; Mirocha, C. J.; Pathre, S. V.; Behrens, J. C. Identification of the naturally occurring isomer of zearalenol produced by *Fusarium roseum* 'Gibbosum' in rice culture. *Appl. Environ. Microbiol.* **1979**, *37*, 849–853.

(11) Rosen, R. T.; Rosen, J. D. Quantification and confirmation of four Fusarium mycotoxins in corn by gas chromatography–mass spectrometry–selected ion monitoring. *J. Chromatogr.* **1984**, *283*, 223–230.

(12) Hanschmann, G. Studies on mycotoxins in feed. Determination of zearalenone with conventional analytical and ELISA techniques: a methodology comparison. *VDLUFA-Schriftenreihe* **2003**, *58*, 684–686.

(13) Bennett, G. A.; Nelsen, T. C.; Miller, B. M. Enzyme-linked immunosorbent assay for detection of zearalenone in corn, wheat, and pig feed: collaborative study. *J. AOAC Int.* **1994**, *77*, 1500–1509.

(14) Krska, R.; Josephs, R. D.; Pettersson, H.; MacDonald, S. Preparation and certification of zearalenone mass concentration of two low-level maize reference materials. *J. AOAC Int.* **2004**, *87*, 892–908.

(15) Rosenberg, E.; Krska, R.; Wissiack, R.; Kmetov, V.; Josephs, R.; Razzazi, E.; Grasserbauer, M. High-performance liquid chromatography atmospheric–pressure chemical ionization mass spectrometry as a new tool for the determination of the mycotoxin zearalenone in food and feed. *J. Chromatogr., A* **1998**, *819*, 277–288.

(16) Pallaroni, L.; von Holst, C.; Eskilsson, C. S.; Bjoerklund, E. Microwave-assisted extraction of zearalenone from wheat and corn. *Anal. Bioanal. Chem.* **2002**, *374*, 161–166.

(17) Royer, D.; Humpf, H. U.; Guy, P. A. Quantitative analysis of Fusarium mycotoxins in maize using accelerated solvent extraction before liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry. *Food Addit. Contam.* **2004**, *21*, 678–692.

(18) Klötzel, M.; Lauber, U.; Humpf, H. U. A new solid phase extraction clean-up method for the determination of 12 type A and B trichothecenes in cereals and cereal-based food by LC-MS/MS. *Mol. Nutr. Food Res.* **2006**, *50*, 261–269.

(19) Hartmann, N.; Erbs, M.; Wettstein, F. E.; Schwarzenbach, R. P.; Bucheli, T. D. Quantification of estrogenic mycotoxins at the ng/L level in aqueous environmental samples using deuterated internal standards. *J. Chromatogr., A* **2007**, *1138*, 132–140.

(20) Green, T. W.; Wuts, P. G. *Protective Groups in Organic Synthesis*, 3rd ed.; Wiley: New York, 1999.

(21) Bretz, M.; Beyer, M.; Cramer, B.; Humpf, H. U. Stable isotope dilution analysis of the *Fusarium* mycotoxins deoxynivalenol and 3-acetyldeoxynivalenol. *Mol. Nutr. Food Res.* **2006**, *50*, 251–260.

(22) Miles, C. O.; Erasmuson, A. F.; Wilkins, A. L.; Towers, N. R.; Smith, B. L.; Garthwaite, I.; Scahill, B. G.; Hansen, R. P. Ovine metabolism of zearalenone to α-zearalanol (zeranol). *J. Agric. Food Chem.* **1996**, *44*, 3244–3250.

Received for review June 12, 2007. Revised manuscript received August 2, 2007. Accepted August 2, 2007.

JF0717283