JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quantification of Zearalenone in Various Solid Agroenvironmental Samples Using D_6-Zearalenone as the Internal Standard

NICCOLO HARTMANN,[†] MARIANNE ERBS,[†,‡] FELIX E. WETTSTEIN,[†] CORINNE C. HOERGER,[†] RENÉ P. SCHWARZENBACH,[§] AND THOMAS D. BUCHELI*,[†]

Agroscope Reckenholz-Tänikon Research Station ART, Reckenholzstrasse 191, CH-8046 Zürich, Switzerland, and Swiss Federal Institute of Technology Zürich, Universitätsstrasse 16, CH-8092 Zürich, Switzerland

Because of its pronounced estrogenicity, zearalenone may be of concern not only in the aqueous but also in the terrestrial environment. Therefore, we developed several analytical methods to quantify zearalenone in different solid matrices of agroenvironmental relevance (i.e., plant organs, soil, manure, and sewage sludge). The use of D_6-zearalenone as the internal standard (IS) was essential to render the analytical method largely matrix-independent because it compensated for target analyte losses during extract treatment and ion suppression during ionization. Soil and sewage sludge samples were extracted with Soxhlet, whereas plant material and manure samples were extracted by liquid solvent extraction at room temperature. Absolute recoveries for zearalenone were 70−104% for plant materials, 105% for soil, 76% for manure, and 30% for sewage sludge. Relative recoveries ranged from 86 to 113% for all matrices, indicating that the IS was capable to largely compensate for losses during analysis. Ion suppression, between 8 and 74%, was in all cases compensated by the IS but influenced the method quantification levels. These were 3.2−26.2 ng/$g_{dryweight(dw)}$ for plant materials, 0.7 ng/g_{dw} for soil, 12.3 ng/g_{dw} for manure, and 6.8 ng/g_{dw} for sewage sludge. Plant material concentrations varied from 86 ng/g_{dw} to more than 16.7 μg/g_{dw}, depending on the organ and crop. Soil concentrations were between not detectable and 7.5 ng/g_{dw}, depending on the sampling depth. Zearalenone could be quantified in all manure samples in concentrations between 8 and 333 ng/g_{dw}. Except for two of the 85 investigated sewage sludge samples, zearalenone concentrations were below quantification limit.

KEYWORDS: D_6-Zearalenone; deuterated internal standard; resorcyclic acid lactones; mycotoxins; estrogenic; endocrine disruptors; *Fusarium*

INTRODUCTION

In the past, natural and anthropogenic endocrine disrupting chemicals (EDC) have mainly been studied in the aqueous environment (*1*). However, the aqueous fauna is not the sole group of organisms that is exposed to EDC. Soil organisms such as rodents and husbandry animals (*2*) also are potentially affected by such micropollutants. Sewage sludge contains EDC (*3, 4*) and is used for application on agricultural land as a fertilizer in many countries of the European Union and the U.S. (*5*). Animal excretion and application of manure on grassland fields are additional input sources of EDC to the terrestrial environment (*6*). Depending on the compound, other sources such as atmospheric deposition or compost application also add to the total loads of EDC to soil (*7*).

Less attention has been paid to naturally occurring estrogenic toxins such as the resorcyclic acid lactones (*3, 8*). Zearalenone, the main representative of the resorcyclic acid lactones, is produced by *Fusarium* species, which colonize a wide variety of crops such as wheat, corn, barley, or oats (*9*). Zearalenone is of particular concern due to its very high relative estrogen receptor affinity and estrogenic potency (*10, 11*). Its levels in animal feed and pet products reached up to several micrograms per gram (*12, 13*). Ingested zearalenone is either excreted directly or metabolized and then excreted via urine and/or faeces (*14*−*17*). Thus far, zearalenone contents in sewage sludge have not been investigated. Because of the occurrence of zearalenone in wastewater treatment plant (WWTP) in- and effluents (*18, 19*) and its affinity to organic carbon (data from own experiments), it is expected to be present also in sewage sludge. Hence, besides *Fusarium* infested crop fields (*20*),

* Corresponding author. Tel.: +41 44 377 73 42; fax: +41 44 377 72 01; e-mail: thomas.bucheli@art.admin.ch.
[†] Agroscope ART.
[‡] Current address: MDS Pharma Services Switzerland AG, Allmendstrasse 32, 8320 Fehraltorf, Switzerland.
[§] Swiss Federal Institute of Technology.

10.1021/jf8002448 CCC: $40.75
Published on Web 04/16/2008

manure and sewage sludge could be other potential input sources for zearalenone to the agricultural environment, especially to soil.

To accurately and precisely quantify the amounts of zearalenone expected to reach agricultural soils via *Fusarium* infested crops, manure, and sewage sludge, sensitive and robust analytical methods are required. Several methods for quantification of zearalenone in wheat were published (*21*−*23*). Cramer et al. (*24*) recently described a method quantifying zearalenone in cereal products using D_2-zearalenone as the internal standard (IS). Analytical methods also were developed for urine, faeces, and tissues (*17, 25*). The only analytical method for quantification in soil used fluorescence detection (*26*). To this end, no method is available for the quantification of zearalenone in sewage sludge and manure. HPLC-MS/MS is the state-of-the-art-technique for the analysis of organic compounds, such as zearalenone. In particular, for solid matrices, the crucial step in HPLC-MS/MS is the ionization of the analyte, which is affected by coextracted matrix compounds (*27*−*29*). Thus far, the only effective way to achieve precise and accurate results in the presence of matrix compounds is the use of isotope labeled IS, in our case, 6-fold deuterated zearalenone (D_6-zearalenone). One important objective of this work is to demonstrate that the use of a deuterated IS is an effective way to overcome matrix related problems and to compensate for sample losses during the analytical procedure. In the past, D_6-zearalenone was used in aqueous matrices (*29, 30*) and D_2-zearalenone in cereal products (*24*) for zearalenone quantification.

In this study, we present for the first time a series of accurate and precise analytical methods for several environmentally relevant types of samples. To our knowledge, this is the first time D_6-zearalenone has been used as an IS for the quantification of zearalenone in various solid matrices. Analytical methods were optimized and validated for corn flour, soil, and sewage sludge and validated for corn straw, wheat flour, wheat straw, and manure. Their application was demonstrated in real soil and plant samples from a local field study and in manure and sewage sludge samples from monitoring studies throughout Switzerland. Because of the similar analytical behavior, several metabolites of zearalenone also were monitored in all real samples using the procedure as described in Hartmann et al. (*29*) for various aqueous samples.

MATERIALS AND METHODS

Chemicals. Zearalenone [CAS No. 17924-92-4] (≥99%) was purchased from Sigma (Buchs, Switzerland). D_6-Zearalenone was prepared in our own laboratory by base-catalyzed hydrogen−deuterium exchange as described in Miles et al. (*31*). Hydrogen−deuterium exchange takes place at the positions C-3, C-5, C-5′, and C-7′. The purity of D_6-zearalenone was tested by injecting a pure IS solution, scanning for D_0- to D_6-zearalenone. The relative zearalenone amount was <0.1%. Ratios of the different grades of deuterated zearalenone did not change over time. Therefore, D_6-zearalenone was found to be a suitable IS, and the other deuterated zearalenone products did not influence quantification (*29*). Methanol, toluene, cyclohexane, acetone, and acetonitrile (all HPLC-grade) were purchased from Scharlau (Barcelona, Spain). Water was purified by a Milli-Q system from Millipore (Volketswil, Switzerland). N_2 (99.99995%) was purchased from PanGas (Dagmersellen, Switzerland). A zearalenone stock solution holding concentrations of 500 mg/L was prepared in pure methanol. The IS solution was prepared in methanol and had a concentration of 2 μg/mL. IS solutions contained deuterated resorcyclic acid lactone analogues (*29*) for the analysis of real samples but only D_6-zearalenone for method development. Aqueous calibration standards holding zearalenone equivalent to the concentration range of 0.5−100 and 100 ng/L IS were prepared in Milli-Q water from the methanol stock solution each time a new batch of samples was analyzed. Stock solutions and dilutions thereof were stored in the dark at −20 and 4 °C, respectively.

Sample Collection and Preparation. *Plant Material.* Wheat and corn plant materials were collected manually from a 0.2 ha field site at Zürich-Reckenholz, Switzerland. For method development and validation, 500 wheat and 50 corn plants were selected at harvest. Kernels of wheat and corn were separated from the rest of the plant, in the following referred to as straw. This material was naturally contaminated with zearalenone, and additional spiking thus was not necessary. Real samples were collected several times before and at the time of harvest as follows: 500 wheat plants and 50 corn plants were taken from randomly selected locations over the field and divided into their organs. Wheat plants were divided into kernels, spelts, stalks, stalks of the ears, and leaves. Similarly, corn plants were divided into kernels, stalks, spindles, leaves, and leaves of the spindles. Samples were stored at −20 °C. Before extraction, the samples were dried at 40 °C until weight constancy was achieved (but for at least 48 h), ground, and sieved to 0.5 mm using a ZM1 centrifuge mill (Retsch GmbH, Germany). Real samples were analyzed within 48 h after drying.

Soil. Soil samples were taken with a split tube core sampler (2.5 cm diameter). Fifteen to 20 samples were taken from randomly selected locations over the field site at Reckenholz and pooled to one composite sample. Topsoil (0−5 cm) samples were used for method development and validation. As these samples were naturally contaminated with zearalenone, additional spiking was not necessary. Real samples were taken in depths of 0−10, 10−20, and 20−40 cm. Further sample handling was as stated previously for the plant materials.

Manure. For method development and validation, 100 L of manure (swine/cattle, 1:1) was obtained from our research station in Tänikon (Switzerland) and then spiked with zearalenone to a resulting concentration of 100 ng/L. To account for real situations, the spiked manure aged for 96 h before further treatment. Real samples were taken from selected farms within the Swiss soil monitoring network (NABO). Because of the same origin and its similar composition, liquid manure (urine and faeces) and dung (faeces and straw material) were handled in the same way. In this study, the term manure stands for both liquid manure and dung. Before sampling, liquid manure containers at the selected farms were homogenized for 2 h using a stirring unit. Real samples were taken with a PVC pipe (5 m length and 7 cm diameter) for vertical sampling. Five samples were taken from different depths and filled in a 30 L bucket to form a composite sample. One liter of this composite sample was taken for analysis. This sampling device for liquid manure was applied in previous studies in Switzerland (*32*). Dung samples were taken manually. Ten samples were taken from randomly selected spots from the dung pile and pooled to one composite sample of 1 kg. The whole composite sample was used for analysis. Samples were handled as described previously for plant materials, except that they were dried at 105 °C for at least 96 h.

Digested Sewage Sludge. For method development and validation, 100 L of sewage sludge was taken from the WWTP Werdhölzli (Zürich, Switzerland) and then spiked with zearalenone to a resulting concentration of 100 ng/L. The spiked sludge was aged for 96 h before further treatment. Real samples were taken from 30 WWTP throughout the midlands of Switzerland. Selection criteria were the following: the WWTP had to be part of the existing monitoring networks such as SEA (Observation of the Metabolism of the Anthroposphere) (*33*), and possible zearalenone sources such as agricultural land, animal, and human excretion and wastewater from the feed and food industry had to be covered. Samples were taken at four different times from May 2006 to February 2007. Before sampling, the sludge holding tank at the selected WWTP was mixed using the stirring unit, obtaining a homogeneous sludge distribution. Real samples were taken manually (*33*) using a 1 L Niskin bottle. Three individual samples were then pooled to one composite sample. Further sample handling was as described previously for manure.

Extraction Method. *Plant Material.* In contrast to all other investigated matrices, a certified reference material was available for corn flour (zearalenone in corn flour: 60 ± 9 ng/$g_{\text{dry weight (dw)}}$; Biopure, Tullin, Austria). We therefore knew as to what zearalenone content

we needed to achieve and as to whether extraction was complete or not. The widely used solvent composition for corn flour extraction (acetonitrile/Milli-Q water (84:16, v/v)) (*34*−*36*) yielded a complete extraction after 2 h. This mixture also is frequently applied for wheat samples (*37*); therefore, this procedure was not further optimized but selected for all investigated corn and wheat materials. Liquid solvent extraction of 1 g of plant material with 50 mL of solvent was carried out on a SM-30 orbital shaker (Edmund Bühler GmbH, Hechingen, Germany).

Soil. Three different extraction methods were compared (i.e., liquid solvent, Soxhlet, and accelerated solvent extraction (ASE, ASE200, Dionex Corporation, Sunnyvale, CA)). ASE and Soxhlet extraction exhibited similar extraction rates and were superior to liquid solvent extraction. Because of the easier handling, Soxhlet extraction was preferred over ASE. Before extraction, soil samples were homogenized with a turbula (Turbula System Schatz, Willy A. Bachofen AG, Muttenz, Switzerland) for 15 min. Five grams of the sample was extracted with 150 mL of solvent in a 100 mL Soxhlet extractor. The optimization of extraction parameters was performed with aged soil gathered from the field site as described previously. Three replicates were extracted for every tested solvent, solvent mixture, and extraction duration. Different solvents (Milli-Q water, acetonitrile/Milli-Q water (84:16, v/v), acetonitrile, methanol, methanol/toluene (80:20, v/v), toluene, acetone/toluene (80:20, v/v), acetone, acetone/cyclohexane (80:20, v/v), and cyclohexane), ranging from nonpolar to polar were tested at 6 h extraction time. Extraction duration (6, 18, and 36-h) dependence was eventually tested for the most effective solvent.

Manure. Liquid solvent extraction was selected for manure because ASE and Soxhlet extraction both led to very dirty extracts that made further concentration steps almost impossible due to oily residues. Since an important component of manure is plant material, it was extracted with the same solvent mixture as used for plant material, and no solvent optimization was conducted. Extraction duration was tested at 2, 4, and 120 h. Two grams of manure was extracted with 50 mL of solvent mixture.

Digested Sewage Sludge. Sludge samples were extracted with Soxhlet. Liquid solvent extraction was much less effective, and ASE led to similar difficulties as described previously for manure. Before extraction, sludge samples were homogenized with the turbula for 15 min. Five grams of the sample was extracted with 150 mL of solvent. The optimization of extraction parameters was performed with spiked and aged sludge prepared as described previously. Optimization of extraction was performed as described previously for soil.

Extract Processing. *Plant Material.* The IS (50 μL, 2 ng/μL) was spiked to a 1 mL aliquot of the total extract. This subsample was transferred to a 10 mL microreaction vial (Supelco, Bellefonte, PA) and then evaporated to dryness with a gentle stream of nitrogen at 50 °C for approximately 10 min. The dried extract was reconstituted with 1 mL of acetone. For further cleanup, the extract was centrifuged (Labofuge 200, Heraeus Sepatech, GmbH, Osterode, Germany) at 4000 rpm for 5 min. The supernatant was transferred to a microreaction vial, evaporated to dryness again, and reconstituted with 300 μL of Milli-Q water/acetone (50:50, v/v). The percentage of acetone for reconstitution was tested between 20 and 80% with regard to chromatographic separation, peak shape, and resolution of the analytes. At 50% acetone, they were fully reconstituted, and neither separation nor peak shape were negatively influenced. The extracts were stored in the dark at 4 °C and analyzed (*29*) within 24 h. Prior to measurement, the extract was filtered with a syringe filter (13 mm Syringe Filter, 0.2 μm PTFE, BGB Analytik AG, Böckten, Switzerland) and transferred to a 350 μL amber glass vial.

Soil. The IS (50 μL, 2 ng/μL) was added to the total soil extract. The total extract was evaporated to 1 mL in a 12-fold parallel evaporator (Syncore Analyst, Büchi Labortechnik AG, Flawil, Switzerland), transferred to a 10 mL microreaction vial, and evaporated to dryness using a gentle stream of nitrogen at 50 °C for approximately 10 min. Reconstitution and further extract treatment were as described previously for plant material, except that the final filtration was only performed in the case of extract coagulation in the stored glass vial.

Manure. The IS (50 μL, 2 ng/μL) was spiked to a 5 mL aliquot of the total extract. This subsample was evaporated to dryness with a gentle stream of nitrogen at 50 °C for approximately 30 min. The dried extract was reconstituted with 1 mL of acetone and further treated as described previously for the plant material.

Digested Sewage Sludge. The IS (250 μL, 2 ng/μL) was added to the total sludge extract. The total extract was evaporated to 1 mL with the Syncore system, transferred to a 10 mL microreaction vial, and evaporated to dryness using a gentle stream of nitrogen at 50 °C for approximately 10 min. The dried extract was reconstituted with 1 mL of acetone and further treated as described previously for the plant materials.

Chromatographic Separation and Mass Spectrometric Detection. LC-MS/MS was performed on a Varian 1200L LC-MS instrument (Varian Inc., Walnut Creek, CA). The resorcyclic acid lactones were separated on a 150 mm × 2.0 mm, 3 μm Polaris Amide-C18 column (Varian Inc.) at room temperature by applying the following elution gradient: 0 min 0% B (100% A), 3 min 0% B, 4 min 40% B, 25.5 min 67.5% B, 26 min 100% B, 29 min 100% B, 30 min 0% B, and 35 min 0% B, with eluent A consisting of Milli-Q water/ACN (95:5, v/v) and eluent B consisting of Milli-Q water/ACN (5:95, v/v). Both eluents were buffered with 10 mM ammonium acetate (pH 6.8). The injection volume was 50 μL, and the mobile phase flow rate was 0.2 mL/min. Interface parameters of the LC-MS/MS were as follows: needle voltage −2000 V, nebulizing gas (compressed air) 4.21 bar, capillary voltage −54 V, drying gas (N_2, 99.5%) 300 °C and 1.59 bar, and shield voltage −600 V. Detection of the resorcyclic acid lactones was performed in the (−)ESI mode. The collision cell gas (Ar, 99.999%) pressure was 2.0 e^{-6} Torr, and the detector voltage was set to 2000 V. Detailed information about retention times, precursors, and product ions are given in Hartmann et al. (*29*). Although the analytical methods were optimized and validated for zearalenone only, the other resorcyclic acid lactones were monitored as well in all investigated samples.

Method Validation Parameters. Ion suppression was evaluated by comparing the analyte signals obtained from injection of the same amount of analyte dissolved in the final extract from the various matrices and in the respective pure solvent. Standard addition to the final extracts was carried out to yield concentrations equivalent to 5, 10, 25, 50, and 100 ng/mL. Curves were obtained by plotting measured analyte peak areas against corresponding analyte concentration levels in pure solvent and in extracted matrix, respectively. Linear regression was performed for each curve. The ion suppression (expressed in percent) was quantified as 1 minus the ratio between the slope of the curve obtained for the extracted matrix and the slope of the curve for the pure solvent.

Absolute recoveries over extraction, cleanup, and quantification were determined for zearalenone in all described matrices. They were spiked prior to extraction with 500 ng (plant material), 5 ng (soil), 100 ng (manure), and 25 ng (sewage sludge) zearalenone per gram of dry weight. In the case of corn flour, absolute recovery was obtained by the extraction of the reference material mentioned previously. The different spike levels were selected to match the expected native zearalenone concentration in each matrix. Five replicates were prepared for every matrix. The extraction was begun 24 h after spiking with zearalenone. IS was added prior to the analysis by HPLC-MS/MS. The absolute method recovery was defined as the ratio between the quantified and the spiked amount. Native amounts as determined in respective blank samples were accounted for. Relative recoveries over cleanup and quantification were obtained again for all matrices. Zearalenone spike levels and replicates were the same as described previously for the absolute recoveries, but the IS and zearalenone were spiked right after the extraction step in the extract or the aliquot thereof. The relative recoveries were defined as the ratio of the quantified and the spiked amounts. The precision of the analytical method was defined as the mean relative standard deviation of these five replicates at the chosen concentration level. For all matrices, the method quantification limit was calculated based on a signal-to-noise ratio of ten (S/N = 10) of spiked samples in the case of soil and sludge and of blank samples in the case of manure and plant material.

RESULTS AND DISCUSSION

Extraction. *Plant Materials and Manure.* Because of the good agreement between quantified and certified concentration of the corn flour reference material with a commonly used method (2 h of extraction with acetonitrile/Milli-Q water 84:

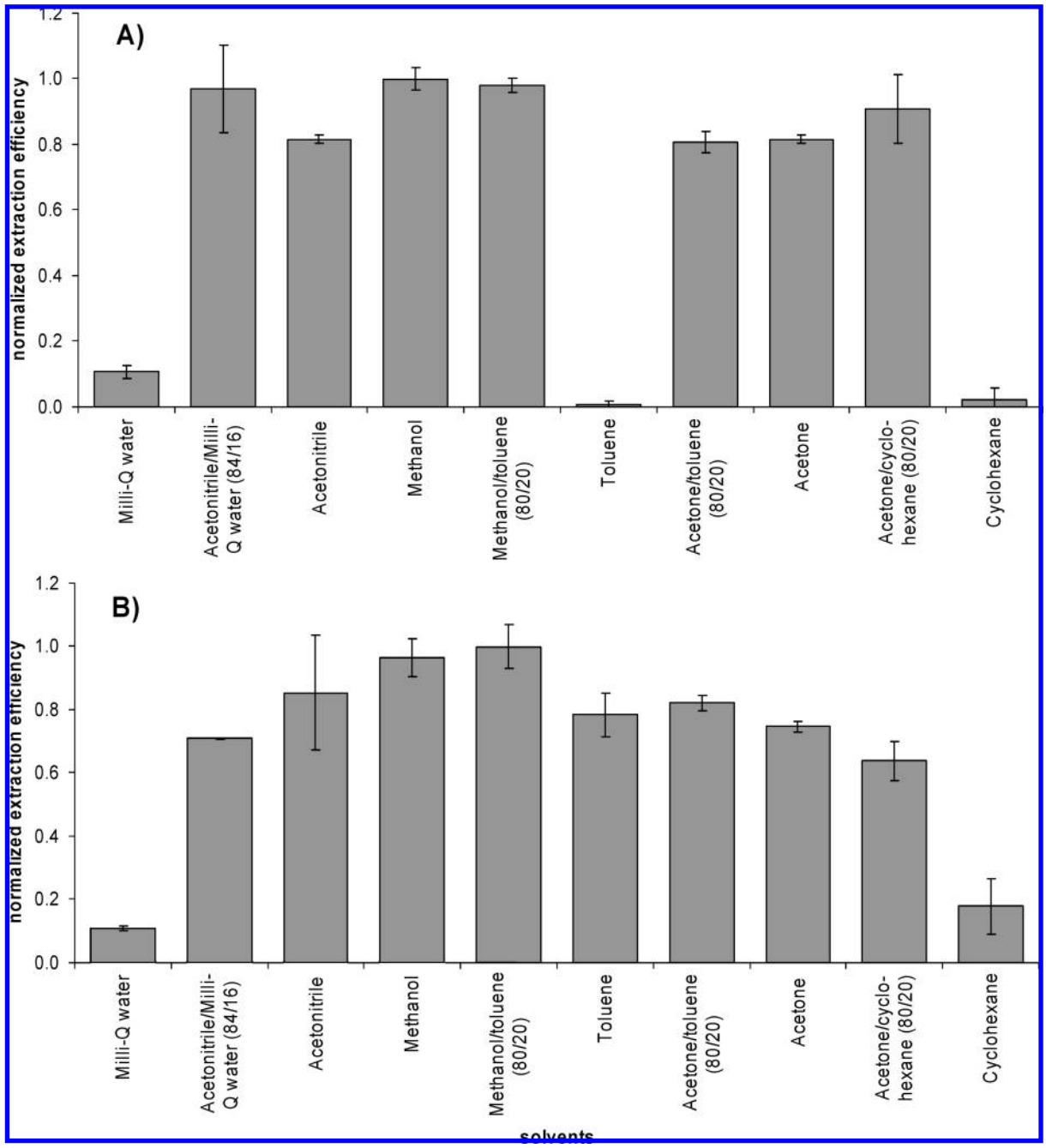

Figure 1. Normalized Soxhlet extraction efficiency from soil (**A**) and digested sewage sludge (**B**). Error bars represent standard deviations of three individual extractions.

16, v/v), further solvent and extraction time optimization for plant material was not performed. Because of the similarities of plant material and manure, the same solvent was chosen for the latter, and only the extraction time was optimized. The normalized extraction efficiency was 100 ± 15, 97 ± 7, and 87 ± 6% at 2, 4, and 120 h, respectively. Because of the very similar extraction efficiency at 2 and 4 h but a lower relative standard deviation at 4 h, method validation and real sample extraction were performed with acetonitrile/Milli-Q water (84:16, v/v) for 4 h by liquid solvent extraction.

Soil. **Figure 1A** shows the Soxhlet zearalenone extraction efficiency for several solvents and mixtures thereof. All numbers were normalized to the highest quantified concentration, which was 6.4 ng/g_{dw}. Nonpolar solvents such as toluene and cyclohexane were clearly inferior to polar solvents such as acetone, acetonitrile, methanol, or mixtures with 80% polar solvents. Maximum extractability was achieved with pure methanol. There was a clear difference in extraction efficiency between Soxhlet and simple liquid solvent extraction with acetonitrile/Milli-Q water (84:16, v/v) (data not shown here), probably due to a higher solvent temperature and repeated extraction cycles with fresh solvent during Soxhlet extraction. The optimization of the extraction time was performed with pure methanol. The normalized extraction efficiency was 82 ± 4, 100 ± 18, and 84 ± 18% at 6, 18, and 36 h, respectively. Consequently, the final Soxhlet extraction method chosen for method validation and real sample extraction was performed with pure methanol for 18 h.

Digested Sewage Sludge. The solvents yielding the best zearalenone extractabilities for soil (i.e., methanol and methanol/toluene (80:20, v/v)) were most efficient for sewage sludge as well (**Figure 1B**). Again, polar solvents performed somewhat

Table 1. Method Validation Parameters for Investigated Agroenvironmental Matrices[a]

matrix	ion suppression[b] (%)	absolute recovery (%)	relative recovery (%)	method precision[c] (%)	method quantification limit[d] (ng/g_{dw})
wheat flour	8	70 (9.1)[e]	107 (10.9)[e]	3	3.3
wheat straw	35	82 (1.7)[e]	97 (5.1)[e]	2	12.0
corn flour	15	104 (4.8)[f]	110 (17.1)[e]	9	3.2
corn straw	54	97 (13.5)[e]	106 (9.5)[e]	14	26.2
soil	8	105 (3.8)[g]	106 (4.4)[g]	9	0.7
manure	74	76 (3.5)[h]	86 (8.2)[h]	7	12.3
sewage sludge	49	30 (21.3)[i]	113 (1.7)[i]	7	6.8

[a] Numbers in parentheses show standard deviation of five replicates. [b] Between 5 and 100 ng/mL extract. [c] Relative standard deviation of five replicates of naturally contaminated (plant material and soil) and aged (manure and sewage sludge) samples. [d] S/N = 10. [e] Spiked with 1000 ng/g. [f] Reference material containing 60 ng/g. [g] Spiked with 5 ng/g. [h] Spiked with 500 ng/g. [i] Spiked with 25 ng/g.

better than nonpolar ones. The optimization of the extraction time was performed with methanol/toluene (80:20, v/v). The standardized extraction efficiency was 94 ± 7, 100 ± 1, and 89 ± 7% at 6, 18, and 36 h, respectively. The final method chosen for method validation and real sample analysis was Soxhlet extraction with methanol/toluene (80:20, v/v) for 18 h.

Method Validation Parameters. Ion suppression of zearalenone occurred in all investigated matrices but to a very different extent. Numbers ranged from 8 to 54% for plant materials and from 8 to 74% for environmental matrices (**Table 1**). For wheat flour, ion suppression was very similar to numbers reported by Zöllner et al. (*38*), whereas the level for corn flour was clearly lower than the reported 70% by Berthiller et al. (*23*). Interestingly, ion suppression in straw was considerably higher than in flour for both wheat and corn. Ion suppression levels for manure were considerably higher than reported by Songsermsakul et al. (*17*) for urine and faeces of horses.

Absolute method recoveries were determined in all matrices but at different concentrations as described previously. The lowest number (30%) was achieved for sewage sludge (**Table 1**). For all other investigated matrices, absolute recoveries were satisfactory with numbers above 70%. The absolute recovery obtained for soil (105%) was very similar to the one reported by Mortensen et al. (*26*). For manure, the absolute recovery of 76% was somewhat lower than that reported for horse faeces and urine (*17*). In sewage sludge, about 60% of the analyte remained in the discarded coagulated oily and solid extract fraction, as evidenced by the correspondingly lower IS signal when spiked after extraction as opposed to before analysis. This also indicates that extraction was almost complete and that losses of analyte primarily occurred during the following steps. Because the IS was spiked right after extraction for the quantification of real samples, the described analyte losses were compensated by respective losses of the IS. However, the method quantification limit rose due to less analyte per injection. The absolute recovery of 104% for corn flour deserves particular attention: as this number was obtained from a certified corn flour with a known zearalenone content, no further optimization of the extraction method was necessary.

Relative recoveries were established in all matrices at the same concentrations as for absolute recovery determination, except for corn flour. They ranged from 86% for manure to 113% for sewage sludge (**Table 1**). These numbers show very well that the IS behaved almost the same as zearalenone during all analytical steps after extraction, independent of the matrix. Therefore, analyte losses during extract processing such as in the case of sewage sludge and ion suppression were compensated by similar losses of D_6-zearalenone. This fact renders the analytical methods very robust to matrix variation and is in accordance with earlier results of different types of aqueous samples (*29*).

Table 2. Zearalenone Concentrations Quantified in the Investigated Agroenvironmental Matrices

matrix	*n* (no. of samples)	concentrations min (ng/g_{dw})	median (ng/g_{dw})	max (ng/g_{dw})
Plant material[a]				
wheat kernels (flour)	3	260	2228	2565
wheat organs[b] (straw)	10	86	1378	16653
corn kernels (flour)	4	270	368	399
corn organs[c] (straw)	9	126	1286	13767
soil	80	n.d.	0.4	8
Manure				
from swine	5	17	160	333
from cattle	6	24	90	197
mixed	7	8	40	118
cattle dung	12	21	41	70
sewage sludge	85	n.d.	n.d.	37

[a] Collected at time of harvest. [b] Investigated wheat organs were leaves, glumes, stalks, and stalks of the ears. [c] Investigated corn organs were leaves of the stalks, leaves of the spindles, spindles, and stalks.

The precision ranged from 1.8 to 13.5% (**Table 1**). These numbers lie well within acceptable values for solid matrices (*39*) and commonly reported ranges for plant materials (*22, 40*) and soil (*26*). The precision for manure is comparable to those reported for horse faeces and urine (*17*). Method quantification limits were between 0.7 ng/g_{dw} for soil and 26.2 ng/g_{dw} for corn straw and generally correlated with ion suppression (**Table 1**). These levels are higher than reported for plant materials (*23, 40*) and horse faeces (*17*) due to less specific cleanup steps. However, obtained method quantification limits were sufficiently low for our applications.

Note again that the extraction step was optimized only for corn flour, soil, and sewage sludge, whereas a common extraction solvent (acetonitrile/Milli-Q water (84:16, v/v)) was selected for other plant materials and manure. The satisfactory analytical figures of merit for all matrices (**Table 1**) justify this approach and indicate that our assumptions about similarities of the other plant materials and manure were appropriate.

Application to Real Samples. We are currently using the analytical methods presented here to study the zearalenone input and distribution on *Fusarium* infected wheat and corn fields at Reckenholz (*29, 30, 41*). Additionally, manure samples were investigated from the Swiss soil monitoring network (NABO), and sewage sludge samples were analyzed from our own monitoring network throughout Switzerland. **Table 2** shows a compilation of the measured zearalenone concentrations in these

matrices, which are discussed in more detail as follows. A further evaluation of the environmental distribution and relevance of zearalenone is carried out in Hartmann et al. (*20*).

Plant Materials. Depending on the plant organ, zearalenone concentrations (**Table 2**) in wheat samples ranged from 86 ng/g_{dw} to 16.7 μg/g_{dw}. Similar concentrations of 126 ng/g_{dw} to 13.8 μg/g_{dw} were quantified in corn. These ranges fit well with data found in the literature (*42*), although plant organs are usually not distinguished or specified. Our data show that zearalenone concentrations vary much within the different plant organs. Concentrations in some plant organs such as wheat spelts or corn spindles were significantly higher than in the kernels, which are removed by harvest. This indicates that, depending on the agricultural practice, a substantial zearalenone fraction remains on the field after wheat and corn harvest and is thus still potentially available to the environment.

Soil Samples. Zearalenone concentrations in the soil (**Table 2**) ranged from not detectable up to 7.5 ng/g_{dw}. Highest concentrations were quantified after a rain event at a time when the wheat plants were heavily infected by *Fusarium* fungi and in topsoil samples. For instance, in November 2005, zearalenone concentrations were 3.8 and 1.2 ng/g_{dw} and below the method quantification limit but above the method detection limit, at depths of 0−10, 10−20, and 20−40 cm, respectively. A decreasing zearalenone content with increasing sampling depth seems plausible and gives further credibility to the presented analytical methods, including sampling and sample preparation procedures.

Manure Samples. All investigated manure samples contained zearalenone, however, at very different levels. **Table 2** complies the zearalenone concentrations for different types of manure. Average zearalenone concentrations were between 50 and 150 ng/g_{dw} or between 2 and 8 ng/$g_{wetweight}$. From the daily manure production of cattle (55 L) and swine (4.4 L), an average zearalenone excretion per day can be calculated. These levels were between 25 and 350 μg/day/animal for swine and cattle, respectively. On the basis of the yearly amounts of manure applications and the corresponding areas (data obtained from the respective farmers), these amounts translate to an average zearalenone load of about 50−150 mg of zearalenone per hectare and year. In four out of the 30 investigated samples, α-zearalenol and/or β-zearalenol were quantified at levels between 12 and 179 ng/g_{dw}. The other 26 samples did not contain any detectable amounts of these metabolites, which are known to be excreted by farm animals (*43*). Corresponding to the literature (*14*), only α-zearalenol was found in swine manure, whereas β-zearalenol dominated in cattle manure.

Digested Sewage Sludge Samples. In total, 85 digested sewage sludge samples were analyzed from 30 WWTP between May 2006 and February 2007. In 24 out of these samples zearalenone was detected, but in only two cases were the levels high enough for quantification. These two zearalenone concentrations were 36.9 and 12.5 ng/g_{dw}. The respective WWTPs were located at Wenslingen (Canton of Basel-Land) and Märstetten (Canton Thurgau) and received wastewater via combined sewer systems from private households and surface runoff. They had the highest winter wheat area/inhabitants ratio, which indicates that *Fusarium* infested wheat fields were possibly responsible for this zearalenone occurrence. Using the equation $c_w = c_s/(f_{oc}K_{oc})$, hypothetical zearalenone concentrations in the corresponding wastewater effluent can be calculated. Including the f_{oc} values from the two sewage sludge samples (0.225 and 0.229, respectively) and a K_{oc} value of 4250 L/kg (own soil sorption experiments), hypothetical zearalenone concentrations in the wastewater effluents resulted in being 34 and 13 ng/L. These numbers correspond well with data reported in the literature (*18*).

ACKNOWLEDGMENT

We gratefully acknowledge A. Keller from the Swiss Soil Monitoring Network (NABO) for providing manure samples from different sampling stations throughout Switzerland. We thank T. Kupper (Swiss College of Agriculture), E. Vermeirssen (EAWAG), and P. Spohn (Office of Waste, Water, Energy, and Air, AWEL Zurich) for their support during the sewage sludge monitoring campaign. We are grateful to S. Vogelgsang and H. R. Forrer for collaboration in the field of *Fusarium* infection and to the field staff at ART for their great support at the field site.

LITERATURE CITED

(1) Sumpter, J. P. Endocrine disrupters in the aquatic environment: An overview. *Acta Hydrochim. Hydrobiol.* **2005**, *33*, 9–16.

(2) Boerjan, M. L.; Freijnagel, S.; Rhind, S. M.; Meijer, G. A. L. The potential reproductive effects of exposure of domestic ruminants to endocrine disrupting compounds. *Anim. Sci.* **2002**, *74*, 3–12.

(3) Mortensen, G. K.; Strobel, B. W.; Hansen, H. C. B. Degradation of zearalenone and ochratoxin A in three Danish agricultural soils. *Chemosphere* **2006**, *62*, 1673–1680.

(4) Lagana, A.; Fago, G.; Marino, A.; Santarelli, D. Liquid chromatography tandem mass spectrometry applied to the analysis of natural and synthetic steroids in environmental waters. *Anal. Lett.* **2001**, *34*, 913–926.

(5) Dolgen, D.; Alpaslan, M. N.; Delen, N. Agricultural recycling of treatment-plant sludge: A case study for a vegetable-processing factory. *J. Environ. Manage.* **2007**, *84*, 274–281.

(6) Kjaer, J.; Olsen, P.; Bach, K.; Barlebo, H. C.; Ingerslev, F.; Hansen, M.; Sorensen, B. H. Leaching of estrogenic hormones from manure-treated structured soils. *Environ. Sci. Technol.* **2007**, *41*, 3911–3917.

(7) Brandli, R. C.; Kupper, T.; Bucheli, T. D.; Zennegg, M.; Huber, S.; Ortelli, D.; Muller, J.; Schaffner, C.; Iozza, S.; Schmid, P.; Berger, U.; Edder, P.; Oehme, M.; Stadelmann, F. X.; Tarradellas, J. Organic pollutants in compost and digestate. Part 2. Polychlorinated dibenzo-*p*-dioxins and -furans, dioxin-like polychlorinated biphenyls, brominated flame retardants, perfluorinated alkyl substances, pesticides, and other compounds. *J. Environ. Monit.* **2007**, *9*, 465–472.

(8) Lagana, A.; Fago, G.; Marino, A.; Santarelli, D. Development of an analytical system for the simultaneous determination of anabolic macrocyclic lactones in aquatic environmental samples. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 304–310.

(9) Parry, D. W.; Jenkinson, P.; McLeod, L. *Fusarium* ear blight (scab) in small grain cereals—A review. *Plant Pathol.* **1995**, *44*, 207–238.

(10) Coldham, N. G.; Dave, M.; Sivapathasundaram, S.; Mcdonnell, D. P.; Connor, C.; Sauer, M. J. Evaluation of a recombinant yeast cell estrogen screening assay. *Environ. Health Perspect.* **1997**, *105*, 734–742.

(11) Matthews, J.; Celius, T.; Halgren, R.; Zacharewski, T. Differential estrogen receptor binding of estrogenic substances: A species comparison. *J. Steroid. Biochem.* **2000**, *74*, 223–234.

(12) Pittet, A. Natural occurrence of mycotoxins in foods and feeds—An updated review. *Rev. Med. Vet.—Toulouse* **1998**, *149*, 479–492.

(13) Placinta, C. M.; D'Mello, J. P. F.; Macdonald, A. M. C. A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. *Anim. Feed Sci. Technol.* **1999**, *78*, 21–37.

(14) Mirocha, C. J.; Pathre, S. V.; Robison, T. S. Comparative metabolism of zearalenone and transmission into bovine milk. *Food Cosmet. Toxicol.* **1981**, *19*, 25–30.

(15) Kleinova, M.; Zollner, P.; Kahlbacher, H.; Hochsteiner, W.; Lindner, W. Metabolic profiles of the mycotoxin zearalenone and of the growth promoter zeranol in urine, liver, and muscle of heifers. *J. Agric. Food Chem.* **2002**, *50*, 4769–4776.

(16) Elsharkawy, S. H.; Abulhajj, Y. J. Microbial transformation of zearalenone. 2. Reduction, hydroxylation, and methylation products. *J. Org. Chem.* **1988**, *53*, 515–519.

(17) Songsermsakul, P.; Sontag, G.; Cichna-Markl, M.; Zentek, J.; Razzazi-Fazeli, E. Determination of zearalenone and its metabolites in urine, plasma, and faeces of horses by HPLC-APCI-MS. *J. Chromatogr. B* **2006**, *843*, 252–261.

(18) Lagana, A.; Bacaloni, A.; De Leva, I.; Faberi, A.; Fago, G.; Marino, A. Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. *Anal. Chim. Acta* **2004**, *501*, 79–88.

(19) Bacaloni, A.; Cavaliere, C.; Faberi, A.; Foglia, P.; Samperi, R.; Lagana, A. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants. *Anal. Chim. Acta* **2005**, *531*, 229–237.

(20) Hartmann, N.; Erbs, M.; Wettstein, F. E.; Hoerger, C. C.; Vogelgsang, S.; Forrer, H. R.; Schwarzenbach, R. P.; Bucheli, T. D. Environmental exposure to estrogenic and other myco- and phytotoxins. *Chimia*. **2008**, in press.

(21) Krska, R.; Baumgartner, S.; Josephs, R. The state-of-the-art in the analysis of type-A and -B trichothecene mycotoxins in cereals. *Fresenius J. Anal. Chem.* **2001**, *371*, 285–299.

(22) Pallaroni, L.; Von Holst, C. Determination of zearalenone from wheat and corn by pressurized liquid extraction and liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. A* **2003**, *993*, 39–45.

(23) Berthiller, F.; Schuhmacher, R.; Buttinger, G.; Krska, R. Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2004**, *1062*, 209–216.

(24) Cramer, B.; Bretz, M.; Humpf, H. U. Stable isotope dilution analysis of the *Fusarium* mycotoxin zearalenone. *J. Agric. Food Chem.* **2007**, *55*, 8353–8358.

(25) Jodlbauer, J.; Zollner, P.; Lindner, W. Determination of zeranol, taleranol, zearalenone, and alpha- and beta-zearalenol in urine and tissue by high-performance liquid chromatography-tandem mass spectrometry. *Chromatographia* **2000**, *51*, 681–687.

(26) Mortensen, G. K.; Strobel, B. W.; Hansen, H. C. B. Determination of zearalenone and ochratoxin *A* in soil. *Anal. Bioanal. Chem.* **2003**, *376*, 98–101.

(27) Choi, B. K.; Hercules, D. M.; Gusev, A. I. LC-MS/MS signal suppression effects in the analysis of pesticides in complex environmental matrices. *Fresenius J. Anal. Chem.* **2001**, *369*, 370–377.

(28) Dijkman, E.; Mooibroek, D.; Hoogerbrugge, R.; Hogendoorn, E.; Sancho, J. V.; Pozo, O.; Hernandez, F. Study of matrix effects on the direct trace analysis of acidic pesticides in water using various liquid chromatographic modes coupled to tandem mass spectrometric detection. *J. Chromatogr. A* **2001**, *926*, 113–125.

(29) 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.

(30) Bucheli, T. D.; Wettstein, F. E.; Hartmann, N.; Erbs, M.; Vogelgsang, S.; Forrer, H. R.; Schwarzenbach, R. P. *Fusarium* mycotoxins: Overlooked aquatic micropollutants? *J. Agric. Food Chem.* **2008**, *56*, 1029–1034.

(31) 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 alpha-zearalanol (zeranol). *J. Agric. Food Chem.* **1996**, *44*, 3244–3250.

(32) Menzi, H.; Haldemann, C.; Kessler, J. Schwermetalle in Hofdünger—Ein Thema mit Wissenslücken. *Schweiz. Landw. Forschung* **1993**, *32*, 159–167.

(33) Kupper, T.; Berset, J. D.; Etter-Holzer, R.; Furrer, R.; Tarradellas, J. Concentration and specific loads of polycyclic musks in sewage sludge originating from a monitoring network in Switzerland. *Chemosphere* **2004**, *54*, 1111–1120.

(34) *Quantitative Method for DON, Aflatoxin B1, and Zearalenone*; Romer Labs, Inc.: Union, MO, 1994; Method MY 8402, Version 94.2.

(35) Silva, C. M. G.; Vargas, E. A. A survey of zearalenone in corn using Romer Mycosep 224 column and high performance liquid chromatography. *Food Addit. Contam.* **2001**, *18*, 39–45.

(36) Zollner, P.; Mayer-Helm, B. Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionization mass spectrometry. *J. Chromatogr. A* **2006**, *1136*, 123–169.

(37) Radova, Z.; Hajslova, J.; Kralova, J. Analysis of zearalenone in wheat using high-performance liquid chromatography with fluorescence detection and/or enzyme-linked immunosorbent assay. *Cereal Res. Commun.* **2001**, *29*, 435–442.

(38) Zöllner, P.; Leitner, A.; Berner, D.; Kleinova, M.; Jodlbauer, J.; Mayer, B. X.; Lindner, W. Improving LC-MS/MS analyses in complex food matrices, Part I—Sample preparation and chromatography. *LC GC* **2003**, *March*, 2–8.

(39) Kromidas, S. *Handbuch Validierung in der Analytik*; Wiley: New York, 2000.

(40) Zöllner, P.; Jodlbauer, J.; Lindner, W. Determination of zearalenone in grains by high-performance liquid chromatography-tandem mass spectrometry after solid-phase extraction with Rp-18 columns or immunoaffinity columns. *J. Chromatogr. A* **1999**, *858*, 167–174.

(41) Erbs, M.; Hoerger, C. C.; Hartmann, N.; Bucheli, T. D. Quantification of six phytoestrogens at the nanogram per liter level in aqueous environmental samples using C-13(3)-labeled internal standards. *J. Agric. Food Chem.* **2007**, *55*, 8339–8345.

(42) Zinedine, A.; Soriano, J. M.; Molto, J. C.; Manes, J. Review of the toxicity, occurrence, metabolism, detoxification, regulations, and intake of zearalenone: An oestrogenic mycotoxin. *Food Chem. Toxicol.* **2007**, *45*, 1–18.

(43) Seeling, K.; Danicke, S.; Ueberschar, K. H.; Lebzien, P.; Flachowsky, G. On the effects of *Fusarium* toxin-contaminated wheat and the feed intake level on the metabolism and carryover of zearalenone in dairy cows. *Food Addit. Contam.* **2005**, *22*, 847–855.

Received for review January 23, 2008. Revised manuscript received March 13, 2008. Accepted March 14, 2008. This work is part of the project "Environmental Exposure to Estrogenic Mycotoxins" within the National Research Program NRP50 "Endocrine Disruptors: Relevance to Humans, Animals, and Ecosystems" financed by the Swiss National Science Foundation. We thank G. Karlaganis and S. Müller (Swiss Federal Office for the Environment, FOEN) for financial support.

JF8002448