JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of T-2 and HT-2 Toxins in Cereals Including Oats after Immunoaffinity Cleanup by Liquid Chromatography and Fluorescence Detection

ANKE TREBSTEIN,[†,‡] WALBURGA SEEFELDER,[§] UWE LAUBER,*[,†] AND HANS-ULRICH HUMPF[‡]

Chemisches und Veterinäruntersuchungsamt Stuttgart, Schaflandstrasse 3/2, 70736 Fellbach, Germany; Institut für Lebensmittelchemie, Westfälische Wilhelms-Universität Münster, Corrensstrasse 45, 48149 Münster, Germany; and Nestlé Research Center, Vers-Chez-Les-Blanc, 1000 Lausanne 26, Switzerland

A reliable method for the determination of T-2 toxin and HT-2 toxin in different cereals, including oats, as well as in cereal products was developed. After extraction with methanol/water (90/10, v/v) and dilution with a 4% NaCl solution, the toxins were purified with immunoaffinity columns, derivatized with 1-anthroylnitrile, separated by HPLC, and determined using fluorescence detection. Due to the unspecific derivatization reagents, validation parameters were matrix dependent: in the range 10−200 μg/kg, recovery rates of 74−120% with relative standard deviations between 0.5 and 20.3% were obtained. On average, the limit of quantitation was shown to be 8 μg/kg for each toxin. For naturally contaminated samples, comparable results were obtained when analysis was performed according to this method without derivatization as well as according to a method based on a SPE cleanup utilizing tandem mass spectrometric detection in both cases. Using aqueous acetonitrile as extractant resulted in incorrectly high toxin concentrations due to water absorption of dry samples and toxin accumulation in the organic phase in the subsequent phase separation of the extractant. Furthermore, when comparing the commercially available immunoaffinity columns for T-2 and HT-2 toxins, significant differences regarding capacity and cleanup performance were observed.

KEYWORDS: T-2 toxin; HPLC-FLD; analysis; cereals; immunoaffinity column; derivatization; matrix effects; HPLC-MS/MS

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by different genera of fungi that grow on agricultural commodities in the field and during storage (*1*). Trichothecene mycotoxins are a family of tetracyclic sesquiterpenoids divided into four groups, referred to as types A, B, C, and D, whose production in cereals has been reported for several *Fusarium* species, for example *F. sporotrichioides*, *F. poae*, and *F. acuminatum*. The mycotoxins T-2 toxin (T-2) and HT-2 toxin (HT-2) are predominantly found in oats, but other cereals may also contain these toxins (*2, 3*). The toxic effects of T-2 and its metabolite HT-2 in animals and cell cultures are inhibition of protein synthesis and mitochondrial function, immunosuppression, and general cytotoxicity (*3*). Because of a lack of data regarding exposure of consumers, maximum levels for T-2 and HT-2 have been discussed within the European Commission for about 5 years and are expected to be established in 2008 (*4*). T-2 and HT-2 toxins are important representatives of type-A trichothecenes owing to an isovaleryl group at the C-8 position (**Figure 1**). Thus, compared to type-B trichothecenes (e.g., deoxynivalenol), which are characterized by a carbonyl function at this position, T-2 and HT-2 are not detectable via UV or fluorescence devices (*5*).

Today, different methods for the determination of T-2 and HT-2 are used. In general, analysis via gas-chromatography (GC) with an electron capture detector (ECD), GC with mass spectrometric detection (MS), and high-performance liquid-chromatography (HPLC) with tandem mass spectrometry (MS/MS) are the most frequently applied techniques (*6*). When using methods based on GC, several problems have to be coped with. Matrix compounds and analytes adsorb to active sites in the injector as well as to the first part of the column, which leads to higher toxin responses in the presence of the matrix. Drifting responses of the detected trichothecenes and carry over or memory effects from previous samples were also observed (*7, 8*). Furthermore, an elaborate derivatization in the course of sample workup is necessary for GC analysis, in order to increase volatility and sensitivity of the analytes (*9*).

In recent years, probably because of a simplified sample preparation without derivatization, HPLC-MS/MS methods are

* To whom correspondence should be addressed. Phone: +49-711-3426-1002. Fax: +49-711-588176. E-mail: uwe.lauber@cvuas.bwl.de.
[†] Chemisches und Veterinäruntersuchungsamt Stuttgart.
[‡] Institut für Lebensmittelchemie.
[§] Nestlé Research Center.

10.1021/jf800316m CCC: $40.75
Published on Web 06/14/2008

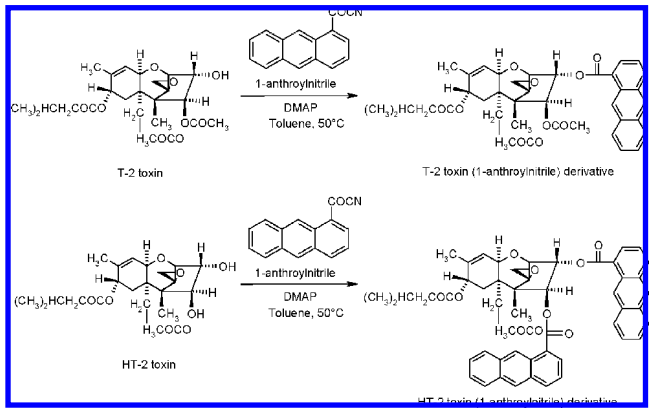

Figure 1. Scheme of the derivatization reaction of T-2 and HT-2 toxins with 1-anthroylnitrile in the presence of dimethylaminopyridine, according to Visconti et al. (*19*).

used more frequently (*10–12*). A critical point when using this detection method is the ionization of the analytes. Kloetzel et al. (*13*) described interfering effects of matrix compounds during the ionization of trichothecenes, which have to be overcome.

However, for laboratories without mass spectrometric equipment, no alternative to GC-ECD methods for T-2 and HT-2 analysis in cereals, especially in oats, exists. The structure of the T-2 and HT-2 molecules (i.e., the lack of any chromophore or fluorescent structure) makes it difficult to quantify these toxins. Derivatization procedures for T-2 and HT-2 using coumarin-3-carbonyl chloride, thus making flourescence detection possible, are reported by different research groups (*14–17*). Although the sensitivity of this method seems to be satisfying, its main disadvantage lies in a time-consuming synthesis of the reagent in the laboratory. In 2003, Pascale et al. (*18*) described the derivatization of T-2 with 1-anthroylnitrile in the presence of dimethylaminopyridine. This reaction was utilized for the precolumn derivatization of T-2 and the subsequent HPLC-fluorescence detection. The method was applied to extracts of wheat, corn, barley, oats, rice, and sorghum after immunoaffinity cleanup. Based on Pascale's method (*18*), 2 years later Visconti et al. (*19*) published an improved method, which was suitable for HT-2 quantitation as well. Reliable results were obtained applying the method to wheat, corn, and barley, while oats and oat containing products could not be analyzed due to interfering compounds eluting at the retention time of HT-2 (*19*). Therefore, the aim of this work was to develop a reliable HPLC-FLD method for the analysis of T-2 and HT-2 in cereals, especially in oats, using immunoaffinity cleanup and precolumn derivatization with 1-anthroylnitrile. In order to determine even the lowest toxin concentrations, the limit of detection (LOD) should be as low as possible but in any case well below 100 μg/kg for each toxin. Furthermore, different items (e.g., comparison of extraction solvents (methanol/water, acetonitrile/water)) including trials regarding recovery rates and different IAC brands should be investigated.

MATERIALS AND METHODS

Chemicals and Materials. Methanol (MeOH, hyper grade), acetonitrile (MeCN, gradient grade), sodium chloride (NaCl, p.a.), ammonia (25%, p.a.), and ammonium acetate (p.a.) were purchased from Merck (Darmstadt, Germany). For all experiments, ultrapure water (H_2O) provided by a Millipore Milli-Q-System (Billerica, MA) was used. Toluene (chromasolv Plus, HPLC grade, ≥ 99.9%), 4-dimethylaminopyridine, T-2 toxin (T-2), HT-2 toxin (HT-2), and silanized amber vials (4 mL) with green melamine resin screw-caps with PTFE-liner were purchased from Sigma (Taufkirchen, Germany). 1-Anthroylnitrile was purchased from Wako (Neuss, Germany). Cellulose filters MN 619 1/4 were obtained from Machery-Nagel (Dueren, Germany), glass microfiber filters (GF/A) from Whatman (Maidstone, U.K.). Immunoaffinity columns EASI EXTRACT T-2 and HT-2 (IAC 1) were from R-Biopharm Rhone Ltd. (Darmstadt, Germany), and immunoaffinity columns T-2 test HPLC (IAC 2) were purchased from VICAM (Watertown, MA). The SPE-columns Bond Elut Mycotoxin (BEM, 1000 mg) were obtained from Varian (Darmstadt, Germany). An oat quality control test material T-2234 was purchased from Central Science Laboratory (Sand Hutton, U.K.). For all experiments, immunoaffinity columns EASI EXTRACT T-2 and HT-2 (IAC 1) were used, unless stated otherwise.

Standard Stock Solutions and Derivatization Reagent. T-2 and HT-2 toxin stock solutions were prepared by dissolving the solid commercial toxins in acetonitrile, resulting in concentrations of 50 μg/mL for each toxin. Dimethylaminopyridine and 1-anthroylnitrile stock solutions were prepared in toluene at concentrations of 3.25 and 3.00 mg/mL, respectively. Dimethylaminopyridine and 1-anthroylnitrile working solutions (0.325 and 0.300 mg/mL, respectively) were prepared by 1:10 dilution of stock solutions with toluene.

APPARATUS

HPLC-FLD. HPLC analysis was performed using an Agilent 1100 series system consisting of a binary pump, degasser, column oven, autosampler, fluorescence detector, and Chemstation-Software (Agilent Technologies, Waldbronn, Germany). The separation of T-2 and HT-2 toxins was performed using a 150 mm × 4.6 mm i.d., 5 μm, Luna Phenyl-Hexyl column with a 4 mm × 3.0 mm i.d. guard column (Phenomenex, Aschaffenburg, Germany). The column temperature was set at 40 °C, and the injection volume was 100 μL. A binary gradient at a flow rate of 1 mL/min was performed as follows: 70% acetonitrile and 30% water as starting composition was kept constant for 5 min. Within 10 min, the acetonitrile content was increased to 85% and kept constant for 10 min. Within 2 min, the acetonitrile content was raised to 100% and kept constant for 5 min. To equilibrate the system, the initial composition was held for 8 min before injecting the next sample. The excitation and emission wavelengths of the fluorescence detector were set at 381 and 470 nm, respectively.

HPLC-MS/MS. The MS/MS-quantitation of T-2 and HT-2 was performed according to Kloetzel et al. (*13*) using an in-house validated multimethod, which includes the trichothecenes T-2 tetraol, nivalenol, deoxynivalenol, fusarenon X, neosolaniol, 15-acetyldeoxynivalenol, monoacetoxyscirpenol, 3-acetyldeoxynivalenol, T-2 triol, diacetoxyscirpenol, HT-2 toxin, T-2 toxin, and zearalenone.

HPLC Parameters. HPLC analysis was performed using an Agilent 1100 series system consisting of a binary pump, degasser, column oven, and autosampler (Agilent Technologies, Waldbronn, Germany). The separation of T-2 and HT-2 toxins was performed using a 250 mm × 2 mm i.d., 4 μm, Synergi Fusion RP 80A column with a 4 mm × 2.0 mm i.d. guard column (Phenomenex, Aschaffenburg, Germany). The column temperature was set at 25 °C, and the injection volume was 10 μL. Solvent A was 1.84 mM ammonia in water and solvent B was 0.92 mM ammonia and 0.13 mM ammonium acetate in acetonitrile. A binary gradient at a flow rate of 0.2 mL/min was performed as follows: within 25 min, changing solvent B from 20 to 70%. Then, the content of solvent B was lowered within 1 min to 20%, which was held for 10 min.

MS/MS Parameters. MS/MS was performed on an API 3000 triple quadrupole mass spectrometer equipped with a TurboIonSpray electrospray ionization (ESI) source (Applied Biosystems, Darmstadt, Germany) heated at 400 °C in the positive ionization mode. The ion spray voltage was set at 4200 V. As the nebulizer and auxiliary gas, zero grade air (8 arbitrary units and 8 L/min,

respectively) was used. Nitrogen served as the curtain gas (8 arbitrary units) and as the collision gas in quadrupole 2 (2.5×10^{-5} Torr corresponding to 4 arbitrary units). Quantitation was performed using multiple reaction monitoring (MRM) with a dwell time of 150 ms. The following transition reactions of T-2 and HT-2 with the respective declustering potential (DP), collision energy (CE), and cell exit potential (CXP) in brackets were recorded using the first mass transition for quantitation. T-2: *m/z* 484.4−245.4 (DP 26 V, CE 19 V, CXP 16 V), *m/z* 484.4−215.4 (DP 26 V, CE 25 V, CXP 14 V), *m/z* 484.4−185.4 (DP 26 V, CE 31 V, CXP 12 V). HT-2: *m/z* 442.2−215.0 (DP 26 V, CE 19 V, CXP 14 V), *m/z* 442.2−233.2 (DP 26 V, CE 17 V, CXP 16 V), *m/z* 442.2−197.2 (DP 26 V, CE 21 V, CXP 14 V). Data aquisition was carried out using Analyst 1.4.2 software (Applied Biosystems, Darmstadt, Germany).

SAMPLE PREPARATION

HPLC-FLD. *IAC Cleanup after Extraction with Methanol/Water.* By using an Ultra Turrax blender, 25 g of finely ground sample mixed with 2.5 g of NaCl were extracted with 100 mL of methanol/water (90/10; v/v) for 3 min. To ensure a complete extraction of the toxins, the samples were then shaken for 30 min at a speed of 230 rpm. The mixture was filtered through a MN 619 1/4 filter. A portion of the filtrate (7.5 mL) was diluted with 30 mL of 4% NaCl. To precipitate all proteins, the mixture was left to settle for 5 min, following 3 min of stirring and then again left to settle for 5 min. After filtration through a GF/A filter, 25 mL of the filtrate was passed through an immunoaffinity column at a flow rate of 1 drop/s. The IAC was washed with 20 mL of water and dried by rapidly passing air through it. The toxins were eluted in screw-cap amber vials with 1 mL of methanol. In order to ensure the complete release of toxins, the backflush technique (i.e., reversing the direction of the flow by generating a low pressure with a syringe) with the mentioned solvent was applied 3 times. Afterward, the IAC was rinsed with 1 mL of methanol. The combined eluates were evaporated in a heated aluminum block at 50 °C using a gentle stream of nitrogen.

IAC Cleanup after Extraction with Acetonitrile/Water. By using an Ultra Turrax blender, 25 g of finely ground sample mixed with 2.5 g of NaCl were extracted with 100 mL of methanol/water (90/10; v/v) for 3 min. To ensure a complete extraction of the toxins, the samples were then shaken for 30 min at a speed of 230 rpm. The mixture was filtered through a MN 619 1/4 filter. A portion of the filtrate (6.4 mL) was evaporated in a heated aluminum block at 50 °C using a gentle stream of nitrogen. The residue was reconstituted in 8.0 mL of acetonitrile/water (80/20; v/v). Part of the reconstituted mixture (7.5 mL) was diluted with 30 mL of 4% NaCl. The following steps were identical to those of the extraction with methanol.

Derivatization Procedure. The dried residues were dissolved in 100 μL of dimethylaminopyridine and 100 μL of 1-anthroylnitrile working solutions by vortexing for at least 1 min. The derivatization procedure was performed by heating for 20 min at 50 °C, followed by cooling for 15 min in an ice bath. The solvent was evaporated in a heated aluminum block at 50 °C using a gentle stream of nitrogen. The residue was redissolved in 1 mL of acetonitrile/water (70/30; v/v). A portion of this extract (100 μL) was injected into the HPLC-FLD.

HPLC-MS-MS. *IAC Cleanup.* In addition to the cleanup for fluorescence detection, another aliquot of each extract was treated identically for mass spectrometric detection. After evaporation of the IAC eluate in a heated aluminum block at 50 °C using a gentle stream of nitrogen, the residue was reconstituted in 0.5 mL of acetonitrile/water (20/80; v/v). The solution was mixed, and a 10 μL aliquot was used for HPLC-MS/MS analysis.

Bond Elut Mycotoxin (BEM) Cleanup. According to Kloetzel et al. (*20*), the samples were cleaned-up via solid phase extraction (SPE) using Bond Elut Mycotoxin columns. The finely ground sample (25 g) was extracted with 100 mL of a mixture of acetonitrile/water (80/20; v/v) by blending at a high speed for 3 min using an Ultra Turrax blender. The extract was filtered through a MN 619 1/4 filter. A portion of the filtrate (4 mL) was passed through a BEM column. An aliquot of 2 mL of the eluate was evaporated to dryness in a heated aluminum block at 50 °C using a gentle stream of nitrogen. The residue was reconstituted in 0.5 mL of acetonitrile/water (20/80; v/v). The solution was mixed, and a 10 μL aliquot was used for HPLC-MS/MS analysis.

Validation of the HPLC-FLD Method with Methanol/Water Extraction. For external calibration, standard solutions containing both T-2 toxin and HT-2 toxin were prepared at concentrations of 5 μg/mL, 100 ng/mL, and 10 ng/mL of each toxin by diluting the stock solutions in acetonitrile. Thirteen different concentration levels were prepared. Therefore, aliquots (corresponding to 1−420 ng and 0.8−336 μg/kg of each toxin, respectively) were placed in screw-cap amber vials, evaporated in a heated aluminum block at 50 °C using a gentle stream of nitrogen, and derivatized as described above. The resulting peak areas of the toxins were plotted against the concentrations. Calibration curves were calculated by linear regression. Because of the lack of blank matrices, recovery experiments were performed in triplicate (three independent extractions) using naturally contaminated oats, infant food, muesli, corn grits, and breakfast cereals. Corresponding toxin amounts were added to aliquots of the raw extract before cleanup via IAC, resulting in levels of 10, 50, 100, and 200 μg/kg of each toxin. The precision of the derivatization procedure was determined by triplicate derivatization of evaporated standard solutions at concentrations of 1, 10, 100, and 250 ng of each toxin. The precision of the complete method was determined by a 10-fold workup (10 independent extractions) of naturally contaminated oat and infant food samples. A commercially available Food Analysis Performance Assessment Scheme (FAPAS) oat quality control test material (T-2234) was worked up in duplicate (two independent extractions) in order to check the trueness of the validated method. The workup of this quality control test material included recovery experiments, which were also performed in duplicate by spiking 100 μg/kg of each toxin to aliquots of the raw extracts. All mentioned experiments were performed according to the method using extraction with methanol/water, IAC cleanup, and determination via HPLC-FLD after precolumn derivatization with 1-anthroylnitrile.

FURTHER EXPERIMENTS

Comparison of Different Cleanup (IAC, SPE) and Detection Systems (FLD, MS/MS). The same extracts of the samples used for validation experiments were cleaned-up in triplicate for HPLC-MS/MS detection without derivatization with 1-anthroylnitrile. Additionally, the same samples were cleaned-up in triplicate using Bond Elut Mycotoxin columns and detected via HPLC-MS/MS.

Comparison of Extraction Solvents. In addition to the validation experiments, the oat, muesli, and breakfast cereals samples as well as the FAPAS oat quality control test material

(T-2234) were cleaned-up according to the acetonitrile extraction procedure for HPLC-FLD, instead of using methanol, in triplicate and in duplicate, respectively.

Spiking Experiments (Direct, Extract) Using Blank and Naturally Contaminated Oat Samples and HPLC-FLD Detection. T-2 and HT-2 blank oat samples were directly spiked with about 150 μg/kg of each toxin and worked up in triplicate (three independent extractions) according to the method with methanol/water extraction, as well as according to the method with acetonitrile/water extraction. Additionally, to compare the spiking procedures, the sample was identically analyzed in triplicate (three independent extractions) but with the difference that the raw extracts, rather than the sample itself, were spiked at a level of 150 μg/kg of each toxin. Furthermore, three naturally contaminated samples were treated in the same way (direct and extract spiking, three independent extractions) with a spiking level of about 100 μg/kg of each toxin.

Comparison of IAC. Standard solutions of methanol/water (18/82; v/v) containing absolute toxin amounts of 10, 50, and 500 ng of each toxin were passed through IAC of both brands in triplicate each and prepared for fluorescence detection according to the validated method. Performance experiments were conducted in triplicate using a naturally contaminated oat sample. Therefore, corresponding toxin amounts at levels of 10, 50, 100, and 200 μg/kg of each toxin were spiked to aliquots of the raw extract before the cleanup via IAC of both brands, and the extracts were then prepared for fluorescence detection according to the validated method.

Statistical data for all experiments mentioned above was calculated using the software Valoo 2.1 (Analytik-Software, Leer, Germany).

RESULTS AND DISCUSSION

Preliminary Work. In general, methods based on derivatization of the analytes bring about challenges that have to be overcome. The derivatization process using 1-anthroylnitrile and dimethylaminopyridine is based on an esterification of hydroxyl groups (**Figure 1**). Because of this unspecific derivatization mechanism, not only the toxins T-2 and HT-2 but also many compounds originating from matrix as well as from solvents or from impurities of the reagents are potential reaction partners for 1-anthroylnitrile. In the first experiments where standard solutions were derivatized according to Visconti's method (*19*), chromatograms showing interfering peaks at the retention time of the toxins were obtained, thus making a reliable determination impossible. Experiments with different brands of toluene (all labeled "for residue analysis") as solvent for dimethylaminopyridine and 1-anthroylnitrile reagents resulted mainly in chromatograms of unsatisfying quality. Only one of the four tested brands of toluene resulted in chromatograms that were free of interfering signals at the toxins' retention time. In practice, even the chromatogram of a reagent blank (containing only dimethylaminopyridine and 1-anthroylnitrile) using this toluene led in general to a significantly higher base fluorescence as well as to a multitude of signals within the whole chromatographic process (**Figure 2**A). A chromatogram as shown in **Figure 2**B was obtained by the derivatization of standard solution. As Visconti et al. (*19*) observed, the clean-up of oat samples resulted in chromatograms with interfering matrix signals at the retention time of HT-2, making a reliable quantification for this toxin impossible. Hence, the method could not be validated for oats matrix. Recently new commercially available IAC for T-2 and HT-2 determination in cereals were launched together with a protocol based on the slightly modified method of Visconti et

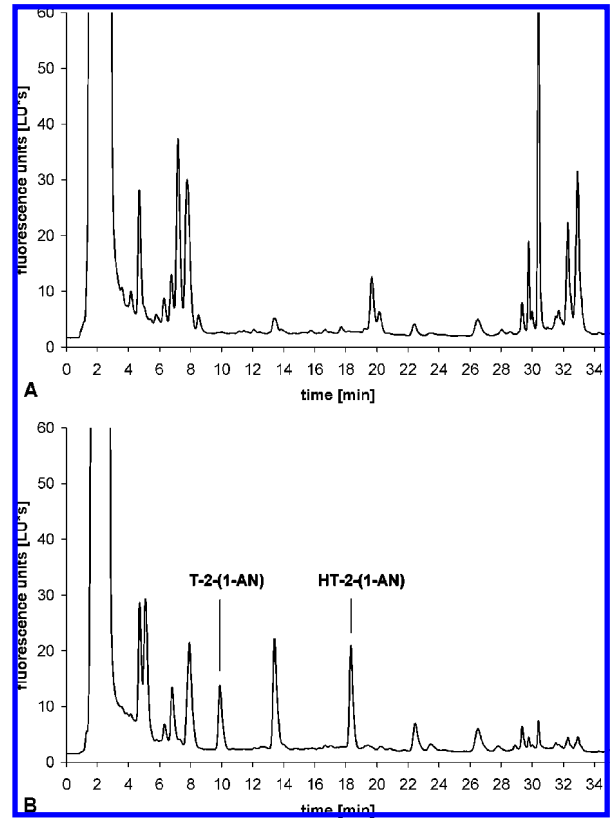

Figure 2. HPLC-FLD chromatograms of (**A**) a reagent blank (containing only 1-anthroylnitrile and dimethylaminopyridine) and (**B**) a standard solution of T-2 and HT-2 toxins (10 ng each) derivatized with 1-anthroylnitrile and dimethylaminopyridine.

al. (*19*). Application of this modified protocol to oat samples unfortunately led to no improvement in terms of purity of the chromatograms (**Figure 3**A). According to the protocols based on Visconti et al. (*19*), dilution of the extracts had to be performed using water, which resulted for most of the samples in turbid solutions. To precipitate oat-specific compounds (e.g. proteins) within this dilution, tests with different salts (e.g., sodium chloride) were performed. Interpretable chromatograms could be obtained by diluting the raw extract with a 4% sodium chloride solution instead of using water. To ensure the precipitation of proteins, the mixture was left to settle for 5 min after addition of 4% sodium chloride solution, followed by stirring for 3 min, and again left to settle for 5 min. Nearly all kind of matrices, including oats, were cleaned-up in this manner, giving reliable results. **Figure 3**B presents a chromatogram of an oat sample after clean-up including this step. Only with these improvements, the determination of toxin concentrations, which correspond to those obtained with our internal reference method (BEM-clean-up and quantitation via HPLC-MS/MS) (*20*), were acquired (**Figure 4**). It is evident that all hydroxyl group containing molecules compete with the toxins for the derivatization reagents. Easily accessible hydroxyl groups were derivatized better and faster than sterically hindered ones. Especially for HT-2, it was observed that the derivatization step was more critical due to the presence of a second hydroxyl group (**Figure 1**).

Pascale et al. (*18*) used 50 μL of each derivatization solution for the determination of only T-2 toxin in cereals including oats. The same volumes containing similar concentrations of reaction agents were used by Visconti et al. (*19*) for the derivatization of T-2 and HT-2 in cereals, with the exception of oats. Taking the high amount of oat-specific potential reaction partners for

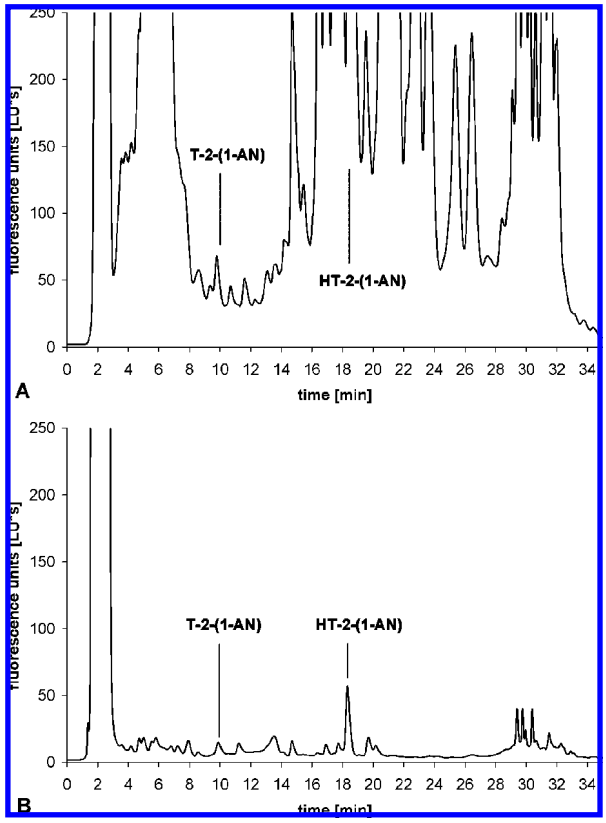

Figure 3. HPLC-FLD chromatogram of a naturally contaminated oat sample: (**A**) Cleanup according to the protocols of IAC-manufacturers—the dilution of the extract was performed using water; (**B**) Clean-up according to the validated method—the dilution of the extract was performed using 4% NaCl solution. The concentrations of T-2 and HT-2 toxins were 6.2 and 24.9 μg/kg, respectively.

1-anthroylnitrile into consideration, we tested volumes of 50 and 100 μL of each reagent for derivatization of the toxins extracted from different matrices (oats, infant food, breakfast cereals). Furthermore, the reaction time of 15 and 20 min was tested. Within the investigated matrices, there was no significant higher toxin response observable using higher volumes of derivatization reagents and/or more reaction time, but 3-fold trials showed lower standard deviations. In order to ensure a complete derivatization of toxins extracted from all considerable matrices, volumes of 100 μL of each reagent and a reaction time of 20 min were used for all experiments.

Validation of an HPLC-FLD Method for the Determination of T-2 and HT-2 Toxins after Methanol/Water Extraction, IAC Cleanup, and Precolumn Derivatization. Linearity for standard solutions with concentrations between 1 and 420 ng/mL (corresponding to 0.8−336 μg/kg) was confirmed for both toxins via a plot of the residuals. Correlation coefficients (R^2) ranged from 0.995 to 0.9999 and from 0.9980 to 0.9994 for T-2 and HT-2, respectively. To demonstrate the method's scope of application, a broad range of naturally contaminated matrices (oats, infant food, muesli, corn grits, and breakfast cereals) was cleaned-up according to the method stated above. The native contents of T-2 and HT-2 in these samples are given in **Table 1**. Statistics showed that the relative standard deviation (RSD_r, generated under repeatability = intralaboratory conditions) increased with decreasing quantities of the measured toxin content. This was mainly observable for the corn grits sample. However, even for this very lowly contaminated sample, the limits of RSD_r set by the EU to be ≤40% were fulfilled for T-2 (contamination level 50−250 μg/kg) and HT-2 (contamination level 100−200 μg/kg) (*21*). Due to a lack of oat- and oat-containing blank material for T-2 and HT-2, recovery trials were carried out with the same naturally contaminated materials mentioned above. Within this method, direct and extract spiking were verified to give comparable results, as described below. Therefore, the spiking solution was added to aliquots of the raw extracts. In the spiking range from 10 to 200 μg/kg of each toxin, reliable results were obtained (**Table 2**). The European Commission has set recovery limits for T-2 and HT-2: thus, recoveries should be in the range 60−130% at contamination levels of 50−250 μg/kg for T-2 and at 100−200 μg/kg for HT-2 (*21*). As a result, recovery rates of the validated method were in alignment with EU requirements. The limit of detection and the limit of quantitation (LOD, LOQ) were determined using standard solutions. They were based on a signal-to-noise ratio of 3/1 and 9/1, respectively. Limits were found to be theoretically clearly lower than 1 and 3 ng/mL (corresponding to 0,8 and 2,4 μg/kg), respectively. In practice, nontarget signals resulting from the derivatization process appeared close to the signals of T-2 and HT-2 in the chromatograms, even without matrix (**Figure 2**B). The composition of the matrix is mainly responsible for the abundance of signals. As per experience with this method, an average LOQ of 10 ng/mL (equivalent to 8 μg/kg) for each toxin is applicable in matrix samples. Depending on the matrix type, the LOD and LOQ may vary, resulting in slightly lower or higher values. However, based on the assumption of a future maximum limit of about 100−150 μg/kg (sum of T-2 and HT-2),the LOD and LOQ, even with slight variations from matrix to matrix, are acceptable. The RSD_r of the 3-fold derivatization of toxin standards was acceptable for all tested concentrations and both toxins (data not shown). With a value of ≤5%, the RSD_r for HT-2 was higher than for T-2 (≤2%), which correlates with the necessity to derivatize two hydroxyl groups in the HT-2 molecule instead of only one hydroxyl group as in the T-2 molecule. The precision of the complete method was performed 10-fold for oat and infant food samples. The mean contents ± SD (RSD_r) of the oat sample were 66.0 ± 3.4 μg/kg (5.2%) for T-2 and 187.5 ± 10.8 μg/kg (5.8%) for HT-2. The results for T-2 and HT-2 in the infant food sample were 5.0 ± 0.7 μg/kg (14.0%) and 40.3 ± 3.6 μg/kg (8.9%), respectively. In addition to recovery experiments, the trueness of the method was approved using a commercially available Food Analysis Performance Assessment Scheme (FAPAS) oat quality control test material (T-2234). This test material was a real food matrix with T-2 and HT-2 contents that have been sufficiently well-established from the results of laboratories participating in a proficiency test. The values have been derived as a consensus of a number of laboratories using a variety of methods. The mean values (corrected for recovery) for T-2 and HT-2 were 83.3 ± 18.3 and 113.4 ± 24.9 μg/kg, respectively, summarized from 41 (T-2) and 35 (HT-2) participating laboratories. The satisfactory range, which indicates the range between which results would have been awarded a satisfactory z-score in the proficiency test, was 46.6−120.0 μg/kg for T-2 and 63.5 - 163.3 μg/kg for HT-2, respectively. This broad range reflects the current situation regarding analysis of T-2 and HT-2 toxins. The values resulting from the newly developed method range slightly below but still close to the mean values of all participating laboratories (**Table 3**). Considering the performance data of the method, it is applicable for the determination of T-2 and HT-2 in all kind of cereals including oats. Thus, it provides an alternative to all GC as well as HPLC-MS/MS methods for the analysis of samples regarding compliance with future EU maximum limits.

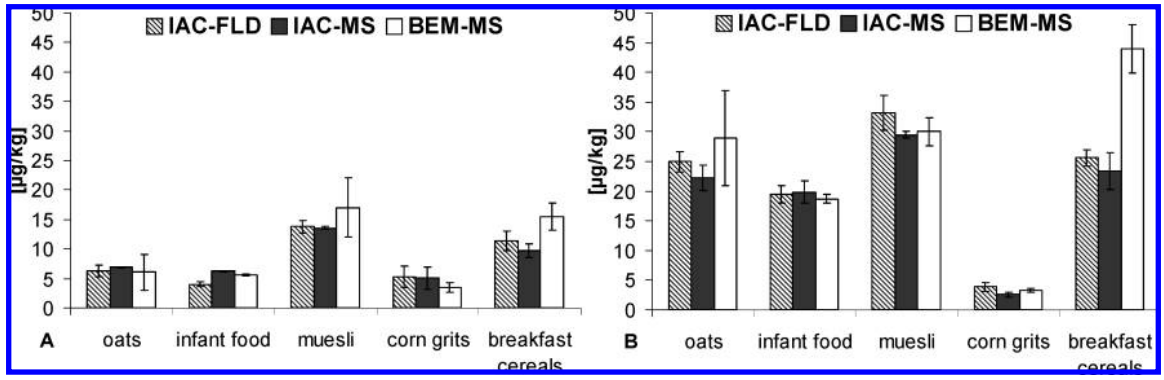

Figure 4. T-2 (**A**) and HT-2 (**B**) concentrations ± SD ($n = 3$) in different samples depending on cleanup and detection system. IAC-FLD and IAC-MS values are based on the very same extracts yielded after methanol/water extraction followed by FLD and MS/MS detection, respectively. BEM-MS values are based on acetonitrile/water extraction followed by MS/MS detection.

Comparison of Different Cleanup (IAC, SPE) and Detection Systems (FLD, MS/MS). In order to investigate the quality of derivatization via 1-anthroylnitrile, the extracts of the samples used for validation were worked up simultaneously for HPLC-MS/MS detection. The first and second bars in **Figure 4**A and **B** show the T-2 and HT-2 values determined after identical IAC cleanup, differing only in the type of detection: the first bar represents the fluorescence detection while the MS/MS detection is represented by the second bar. Within the variation of values, there is no difference between fluorescence and MS/MS detection, which confirms the reliability and quality of the precolumn derivatization used for the FL-detection. The third bar shows the values of an in-house validated method including a SPE cleanup using Bond Elut Mycotoxin columns with subsequent HPLC-MS/MS-detection based on Kloetzel et al. (*20*). While the T-2 values quantified with the three mentioned methods matched for all matrices, the HT-2 value of the breakfast cereals cleaned-up according to the BEM-protocol represented a trend to higher values. Beside the different cleanup principles (immunoaffinity chromatography versus solid phase extraction), the used extraction solvents (methanol/water versus acetonitrile/water) were probably the reason for this trend. Different research groups are convinced that, in general, a mixture of acetonitrile/water is a better alternative for T-2 and HT-2 extraction than methanol/water. The main problem concerning cleanup via immunoaffinity columns is the denaturation of the antibodies in the presence of even low concentrations of acetonitrile (3−5%) in comparison to methanol (15−20%) (*18, 19*).

Comparison of Extraction Solvents. In order to compare the obtained results using methanol/water extraction with those resulting from a method using acetonitrile/water extraction, an additional step had to be included in the latter. An aliquot of the raw extract had to be evaporated to dryness and reconstituted in methanol/water (90/10; v/v). The subsequent cleanup was then performed as described for the original method. Significantly higher results for both toxins were obtained for oats and especially for breakfast cereals after acetonitrile/water extraction but not for the investigated muesli sample. As presented in **Table 3**, the FAPAS oat quality control test material (T-2234) extracted with acetonitrile/water also resulted in higher toxin values in comparison to those determined via methanol/water extraction. This points out that, at least in some matrices, a more effective extraction is obtained using acetonitrile/water. As reported by Stroka et al. (*22*), the use of aqueous acetonitrile to extract aflatoxin B1 from dry samples like spices, infant formula, or animal feed can result in incorrect, higher toxin values. This is due to the water absorption of the investigated samples, resulting in phase separation with accumulation of the toxins in the organic phase. The author recommended the use of aqueous methanol for extraction because, for this solvent, the absorption effect was not observed (*22*). Taking this fact into consideration, the higher results after extraction with acetonitrile/water are doubtful and have to be verified by further investigations. The chromatograms resulting from the acetonitrile/water extraction were slightly better than those from methanol/water extraction regarding peak shape and resolution. This was probably due to less interfering compounds extracted by acetonitrile/water but also strongly depending on the matrix type.

Spiking Experiments (Direct, Extract) with Blank and Naturally Contaminated Oat Samples. To verify whether the higher results of the acetonitrile/water extraction were caused by the mentioned effect, we performed spiking trials with the only available oat blank sample, which was brought from New Zealand. Recovery rates for both toxins and extractants of about 96−105% were determined after spiking of the extracts. Interestingly, when the sample was spiked directly, we obtained significantly higher recovery rates for T-2 and HT-2 (118−130%) after acetonitrile extraction but not after methanol extraction. In the latter, recovery rates of about 100% were determined.

In order to test if the effect is also observable in naturally contaminated samples oats, muesli and breakfast cereals were worked up in the same way. For all three samples, the identical pattern was obtained—the recovery rates for both toxins determined after direct spiking and acetonitrile/water extraction were about 130% (data not shown). Stroka et al. (*22*) proposed salting out effects in matrices containing high amounts of water-soluble constituents as well as water absorbing effects being responsible for these higher values. In the case that this effect is the only reason for higher values obtained after acetonitrile/

Table 1. T-2 and HT-2 Concentrations ± SD ($n = 3$) in Different Samples after Methanol/Water Extraction, IAC Cleanup, and Determination via HPLC-FLD[a]

matrix	T-2 toxin		HT-2 toxin	
	content ± SD (µg/kg)	RSD_r (%)	content ± SD (µg/kg)	RSD_r (%)
oats	6.2 ± 1.0	16.2	24.9 ± 1.7	6.8
infant food	4.0 ± 0.4	10.0	19.4 ± 1.5	7.7
muesli	13.8 ± 1.0	7.2	33.1 ± 2.9	8.8
corn grits	5.3 ± 1.8	34.0	3.9 ± 0.7	17.9
breakfast cereals	11.3 ± 1.7	15.0	25.6 ± 1.4	5.5

[a] The values are not corrected for recovery (recovery rates are given in **Table 2**).

Table 2. T-2 and HT-2 Recovery Rates ± SD ($n = 3$) in Different Samples after Methanol/Water Extraction, IAC Cleanup, and Determination via HPLC-FLD[a]

		T-2 toxin		HT-2 toxin	
matrix	spiking level of each toxin (μg/kg)	recovery ± SD (%)	RSD_r (%)	recovery ± SD (%)	RSD_r (%)
oats	10	86.3 ± 11.4	13.2	99.2 ± 8.6	8.7
	50	86.9 ± 5.5	6.3	90.7 ± 4.0	4.4
	100	86.3 ± 2.3	2.3	93.9 ± 2.2	2.3
	200	89.7 ± 0.7	0.8	95.3 ± 0.5	0.5
infant food	10	90.4 ± 10.8	11.9	119.6 ± 17.3	14.5
	50	95.7 ± 4.2	4.4	107.3 ± 7.2	6.7
	100	95.4 ± 0.2	0.2	109.6 ± 2.7	2.5
	200	97.0 ± 2.8	2.9	112.4 ± 6.4	5.7
muesli	10	88.9 ± 12.6	14.2	101.2 ± 16.4	16.2
	50	96.3 ± 0.9	0.9	103.5 ± 0.8	0.8
	100	97.8 ± 0.7	0.7	109.2 ± 1.8	1.6
	200	97.3 ± 2.1	2.2	105.0 ± 3.4	3.2
corn grits	10	80.9 ± 9.1	1.1	98.8 ± 5.5	5.6
	50	86.6 ± 4.6	5.3	102.5 ± 2.8	2.7
	100	87.9 ± 3.4	3.9	100.2 ± 2.6	2.6
	200	90.8 ± 4.2	4.6	101.5 ± 4.8	4.7
breakfast cereals	10	74.3 ± 15.1	20.3	102.1 ± 2.3	2.3
	50	81.0 ± 8.8	10.9	95.5 ± 5.4	5.7
	100	89.0 ± 2.2	2.5	97.5 ± 1.6	1.6
	200	87.2 ± 3.0	3.4	93.6 ± 5.0	5.3

[a] Recovery rates were determined by spiking of the extract.

water extraction, the T-2 and HT-2 values corrected for recovery (determined for each single sample) should be identical for both extractants. However, taking recoveries into account, still higher results were observed. For two of the three tested matrices, the significance was confirmed (two-sided t-test, $p = 0.05$). This concluded that especially for HT-2 the efficiency of the acetonitrile/water extraction was better than those of the methanol/water extractant. According to Stroka et al. (*22*), the addition of salt can have a strong influence on the results. This fact was tested by comparison of toxin values and recovery rates after extraction with and without sodium chloride using a naturally contaminated oat sample (data not shown). The findings of Stroka et al. (*22*) could not be confirmed as there was no significant influence of sodium chloride observable. It is supposed that the composition of the matrix has a more distinct influence regarding the mentioned effect.

Actually, since recovery rates are strongly matrix dependent and can vary significantly within the same group of food (e.g., oats), a spiking of each sample to be analyzed would be necessary. Irrespectively, because of the absorbing effects, toxin values resulting from extraction with acetonitrile/water have to be corrected mandatorily for recovery rates (resulting from direct spiking of the same sample) in order to obtain reliable results. Otherwise, too high concentrations of T-2 and HT-2 will be determined. In practice, this procedure is unsuitable as there would be for example an increased need for standard substances, sample matrix, and time. As a precondition for validation of an appropriate method based on an extraction with acetonitrile/water, the absorbing effects have to be coped with.

Comparison of IAC. At the moment, immunoaffinity columns for cleanup of T-2 and HT-2 toxins are commercially available from two manufacturers. In order to compare these columns for using in combination with the validated method, the capacity as well as the cleanup performance in naturally contaminated and spiked samples were tested. Pascale et al. (*18*) determined the maximum capacity of the utilized IAC 2 only for T-2 toxin to be 1.6 μg, while Visconti et al. (*19*) determined this parameter for the sum of T-2 and HT-2 to be 1.4 μg. Within the IAC-capacity experiments, it can be concluded that IAC 1 gave about 90−100% recovery for both toxins at all tested concentrations. IAC 2 worked well for 10 and 50 ng of each toxin, but for 500 ng, the HT-2 recovery was only about 50% in comparison to 100% for T-2. The toxin contents in a naturally contaminated oat sample were 6.2 ± 1.0 μg/kg for T-2 and 24.9 ± 1.7 μg/kg for HT-2 after cleanup with IAC 1. Utilizing

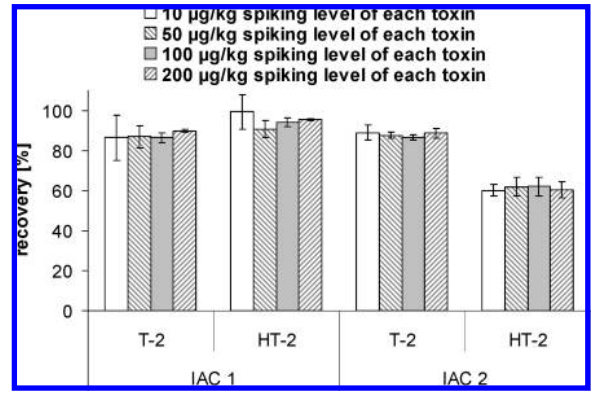

Figure 5. T-2 and HT-2 recovery rates ± SD ($n = 3$) depending on spiking level and used IAC determined in a spiked oat sample via HPLC-FLD.

Table 3. T-2 and HT-2 Concentrations ± SD ($n = 2$) in FAPAS Oat Quality Control Test Material (T-2234) after Different Extraction Procedures, IAC Cleanup, and Determination via HPLC-FLD[a]

	T-2 toxin			HT-2 toxin		
method	content ± SD (μg/kg)	z-score	recovery ± SD (%)	content ± SD (μg/kg)	z-score	recovery ± SD (%)
MeOH/H_2O extraction	72.4 ± 1.4	−0.6	96.5 ± 2.0	99.6 ± 3.9	−0.6	102.8 ±1.2
MeCN/H_2O extraction	83.7 ± 3.4	0.0	99.9 ± 0.7	114.5 ± 3.0	0.0	113.3 ± 4.1

[a] The values are corrected for recovery; recovery rates were determined by spiking of about 100 μg/kg of each toxin to the extract. The calculation of the z-score is based on the FAPAS proficiency test T-2234.

IAC 2 lower results for both toxins (4.9 ± 0.4 μg/kg for T-2 and 19.7 ± 1.0 μg/kg for HT-2) were obtained.

Recovery rates for the same sample spiked at four levels (10, 50, 100, and 200 μg/kg) are presented in **Figure 5**. This data revealed the high quality grade of both IAC regarding the determination of T-2 toxin. With IAC 1 a simultaneous determination of HT-2 is possible in the same quality, but using IAC 2 for the determination of HT-2 in naturally contaminated and spiked samples, the performance was unsatisfying. According to the results from the capacity experiments, the recovery rate at least for the lowest spiking level (10 μg/kg = 8 ng absolute) is expected to be about 100% when using IAC 2. However, all obtained recovery rates of HT-2 remained constant at about 60% independent of the spiking levels. It is assumed that, beside the lower capacity concerning HT-2, the antibodies of IAC 2 are obviously affected by matrix compounds. A satisfying determination of HT-2 according to the validated method is therefore only achievable using IAC 1.

ACKNOWLEDGMENT

We gratefully acknowledge the technical assistance of S. Brenner, S. Fochler, S. Maier, and S. Marschik.

LITERATURE CITED

(1) Bennett, J. W.; Klich, M. Mycotoxins. *Clin. Microbiol. Rev.* **2003**, *16*, 497–516.

(2) European Commission, Scientific Committee on Food. Opinion on Fusarium toxins Part 5: T-2 toxin, HT-2 toxin; http://ec.europa.eu/food/fs/sc/scf/out88_en.pdf (adopted May 30, 2001).

(3) Joint FAO/WHO expert committee on food additives (JECFA). Safety evaluation of certain food additives; WHO Food Additives Series 47; 2001; http://www.inchem.org/documents/jecfa/jecmono/v47je01.htm (accessed April 7, 2008).

(4) Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union 2006, *L364*, 5−24.

(5) Ueno, Y. *Trichothecenes: Chemical, Biological and Toxicological Aspects*; Elsevier: New York, 1983.

(6) 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.

(7) Pettersson, H.; Langseth, W. Intercomparison of Trichothecene Analysis and Feasibility to Produce Certified Calibrants and Reference Material—Method Studies, SMT4-CT96-2047 (Part I); European Commission: Brussels, Belgium, 2002.

(8) Pettersson, H.; Langseth, W. Intercomparison of Trichothecene Analysis and Feasibility to Produce Certified Calibrants and Reference Material—Homogeneity and Stability Studies Final Intercomparison, SMT4-CT96-2047 (Part II); European Commission: Brussels, Belgium, 2002.

(9) Langseth, W.; Rundberget, T. Instrumental methods for determination of nonmacrocyclic trichothecenes in cereals, foodstuffs and cultures. *J. Chromatogr., A* **1998**, *815*, 103–121.

(10) Biselli, S.; Hummert, C. Development of a multicomponent method for *Fusarium* toxins using LC-MS/MS and its application during a survey for the content of T-2 toxin and deoxynivalenol in various feed and food samples. *Food Addit. Contam.* **2005**, *22*, 752–760.

(11) 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* **2005**, *1062*, 209–216.

(12) Sulyok, M.; Krska, R.; Schuhmacher, R. A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples. *Anal. Bioanal. Chem.* **2007**, *389*, 1505–1523.

(13) Kloetzel, M.; Gutsche, B.; Lauber, U.; Humpf, H.-U. Determination of 12 type A and B trichothecenes in cereals by liquid chromatography-electrospray ionization tandem mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 8904–8910.

(14) Cohen, H.; Boutin-Muma, B. Fluorescence detection of trichothecene mycotoxins as coumarin-3-carbonyl chloride derivatives by high-performance liquid chromatography. *J. Chromatogr.* **1992**, *595*, 143–148.

(15) Jimenez, M.; Mateo, J. J.; Mateo, R. Determination of type A trichothecenes by high-performance liquid chromatography with coumarin-3-carbonyl chloride derivatisation and fluorescence detection. *J. Chromatogr., A* **2000**, *870*, 473–481.

(16) Mateo, J. J.; Mateo, R.; Hinojo, M. J.; Llorens, A.; Jimenez, M. Liquid chromatographic determination of toxigenic secondary metabolites produced by Fusarium strains. *J. Chromatogr., A* **2002**, *955*, 245–256.

(17) Dall'Asta, C.; Galaverna, G.; Biancardi, A.; Gasparini, M.; Sforza, S.; Dossena, A.; Marchelli, R. Simultaneous liquid chromatography-fluorescence analysis of type A and type B trichothecenes as fluorescent derivatives via reaction with coumarin-3-carbonyl chloride. *J. Chromatogr., A* **2004**, *1047*, 241–247.

(18) Pascale, M.; Haidukowski, M.; Visconti, A. Determination of T-2 toxin in cereal grains by liquid chromatography with fluorescence detection after immunoaffinity column clean-up and derivatization with 1-anthroylnitrile. *J. Chromatogr., A* **2003**, *989*, 257–264.

(19) Visconti, A.; Lattanzio, V. M. T.; Pascale, M.; Haidukowski, M. Analysis of T-2 and HT-2 toxins in cereal grains by immunoaffinity clean-up and liquid chromatography with fluorescence detection. *J. Chromatogr., A* **2005**, *1075*, 151–158.

(20) Kloetzel, 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.

(21) Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off. J. Eur. Union 2006, *L70*, 12−34.

(22) Stroka, J.; Petz, M.; Joerissen, U.; Anklam, E. Investigation of various extractants for the analysis of aflatoxin B1 in different food and feed matrices. *Food Addit. Contam.* **1999**, *16*, 331–338.

Received for review January 31, 2008. Revised manuscript received April 8, 2008. Accepted April 8, 2008. This work was financially supported by the German Federal Ministry of Food, Agriculture, and Consumer Protection (BMELV) through the Federal Institute for Agriculture and Nutrition (BLE), grant number 05 HS 001.

JF800316M