JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 6535−6546 **6535**

Synthesis of Four Carbon-13-Labeled Type A Trichothecene Mycotoxins and Their Application as Internal Standards in Stable Isotope Dilution Assays

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The first stable isotope dilution assay (SIDA) for the simultaneous quantitation of the most abundant type A trichothecenes in foods and feeds was developed. Synthesis of carbon-13-labeled T2-toxin, HT2-toxin, diacetoxyscirpenol, and monoacetoxyscirpenol was accomplished by [$^{13}C_2$]-acetylation of T2-triol and scirpentriol, respectively. Scirpentriol was prepared from diacetoxyscirpenol by complete alkaline hydrolysis and subsequently was converted to [$^{13}C_6$]-triacetoxyscirpentriol by peracetylation with [$^{13}C_4$]-acetic anhydride. The latter compound was selectively hydrolyzed using ammonium hydroxide to give [$^{13}C_4$]-diacetoxyscirpenol and [$^{13}C_2$]-monoacetoxyscirpenol in reasonable yields. Analogously, [$^{13}C_6$]-T2-triacetate was prepared from T2-triol and subjected to controlled hydrolysis to yield [$^{13}C_4$]-T2-toxin and [$^{13}C_2$]-HT2-toxin. All synthesized products were characterized by NMR and MS experiments. Using the prepared isotopically labeled standards, SIDAs were developed for the quantitation of type A trichothecenes in food and feeds. The mycotoxins were quantified by LC−single and tandem MS after cleanup on multifunctional columns. The method revealed good sensitivity with low detection and quantification limits along with excellent recovery and good precision in interassay studies. Food samples were analyzed using the developed SIDA and showed substantial contamination of oat products with T2-toxin and HT2-toxin. Diacetoxyscirpenol was detected on potatoes, whereas monoacetoxyscirpenol was not present in the analyzed samples.

KEYWORDS: T2-toxin; HT2-toxin; diacetoxyscirpenol; monoacetoxyscirpenol; LC-MS/MS; stable isotope dilution assay; trichothecenes

INTRODUCTION

Trichothecenes are mycotoxins produced by various fungi of the anamorphic genus *Fusarium*. Although about 145 different species of *Fusarium* are known, only about 20 of these are able to produce trichothecenes as secondary metabolites (*1*). All *Fusarium* species are common soil fungi, but the trichothecene-producing species especially are virulent plant pathogens, which infect a number of cereal grains such as wheat, oats, and maize. *Fusarium* molds are predominantly present in zones with moderate climate, and the species *F. graminearum*, *F. culmorum*, *F. sporotrichoides*, *F. poae*, and *F. equiseti* especially are almost ubiquitous on grains and responsible for mycotoxin contamination and lower crop yield (*2*).

Trichothecenes are tetracyclic sesquiterpenes bearing a spirocyclic epoxide moiety. The basic trichothecene structure is biosynthesized from farnesyl diphosphate, a common intermediate in terpenoid biosynthesis, followed by various rearrangements (*3*), and subsequently is functionalized and esterified to give a group of more than 170 different substances, which are classified into four different types, A, B, C, and D (*4*). Type A trichothecenes differ from those belonging to type B by the absence of a carbonyl group at C-8 and hydroxylation at C-7. The type A group itself can be differentiated into two families, namely, the T2 family with hydroxylation at C-8 and the scirpenol family, which is completely devoid of any functionalities at C-8. Structures of common type A trichothecenes are shown in **Figure 1**.

Trichothecenes belonging to group A especially are highly toxic and have been responsible for the outbreak of severe toxicoses both in animals and in humans. For example, poisoning of dairy cattle with about 20% deaths was traced back to a contamination of 2 mg/kg T2-toxin in the feed (*5*). Human toxicoses were observed in the years 1942−1947, when parts of the population of the district of Orenburg in west Siberia became ill after consuming products from grains that had remained unharvested in the field during the winter. The main symptoms of this disease were vomiting, diarrhea, skin inflammation, multiple hemorrhage, and, in the last stage, leukopenia, due to which it was termed alimentary toxic aleukia (ATA). Subsequent examination revealed that the cereals consumed were contaminated by a toxic strain of *F. sporotrichoides* producing significant amounts of T2-toxin (*6*), which itself evokes ATA-like symptoms when given to cats (*7*). In this

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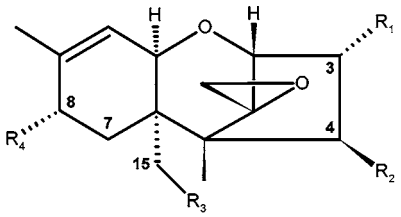
10.1021/jf061347+ CCC: $33.50
Published on Web 08/03/2006



trichothecene	R_1	R_2	R_3	R_4
T2-triol	OH	OH	OH	$OCOCH_2CH(CH_3)_2$
HT2-toxin	OH	OH	OAc	$OCOCH_2CH(CH_3)_2$
T2-toxin	OH	OAc	OAc	$OCOCH_2CH(CH_3)_2$
T2-triacetate	OAc	OAc	OAc	$OCOCH_2CH(CH_3)_2$
scirpentriol	OH	OH	OH	H
monoacetoxyscirpentriol	OH	OH	OAc	H
diacetoxyscirpentriol	OH	OAc	OAc	H
triacetoxyscirpenol	OAc	OAc	OAc	H

Figure 1. Structures of type A trichothecenes.

regard, oral LD_{50} values for type A trichothecenes are available for T2-toxin [5.2 mg/kg of body weight (BW), rats; 1.8 mg/kg of BW, day-old chick], HT2-toxin (6.3 mg/kg of BW, day-old chick), and diacetoxyscirpenol (7.3 mg/kg of BW, rats; 23 mg/kg of BW, mice) (*4*), showing decreased toxicity with reduced number of acetyl groups.

Although the toxicological data are still inadequate, a temporary tolerable daily intake (tTDI) value of 0.06 μg/kg of BW/day for the sum of T2-toxin and HT2-toxin was suggested by the Scientific Committee on Food (SCF) (*8*). This value is much lower than those for the type B trichothecenes deoxynivalenol (1 μg/kg of BW/day) and nivalenol (0.7 μg/kg of BW/day), mirroring the increased toxicity of the type A trichothecenes. The SCF also issued the statement that there is a need for more information about the occurrence of T2-toxin and HT2-toxin as well as of other trichothecenes in food commodities. In particular, the low sensitivity of the current analytical methods has raised doubts about the reliability of the data used for estimation of the tTDI (*8*).

In the past, analysis of type A trichothecenes has routinely been performed by gas chromatography with either flame ionization detection (*9*), electron capture detection (*10*), or mass spectrometric detection (*11*). However, due to matrix interference and the inevitable derivatization step for preparing the trimethylsilyl or fluoroacetyl derivates, which is time-consuming and sometimes incomplete, this technique today has lost its initial importance (*2*). HPLC-UV methods, which are routinely applied to the determination of type B trichothecenes, cannot be used for type A trichothecenes due to lack of a UV-absorbing moiety in the molecular structure. To overcome this drawback, determination of T2-toxin and HT2-toxin by high-performance liquid chromatography with fluorescence detection after precolumn derivatization (*12*) has been reported, but the additional sample preparation step precluded a wider acceptance of this method up to now.

As a consequence of low UV absorption, a number of methods for the determination of trichothecenes has been developed using HPLC with mass spectrometric detection (*13*−*15*). As recent investigations showed that remarkable matrix effects occur during LC-MS analysis, the use of an internal standard has been strongly recommended (*16*). The latter authors used de-epoxy-deoxynivalenol, which was added after cleanup. Other structurally similar internal standards such as neosolaniol (*17*) and verrucarol (*18*, *19*) have also been reported. Regarding the ideal structure of internal standards, there is general consensus that the use of isotopically labeled internal standards can compensate best not only for losses of analytes during sample preparation but also for any kind of matrix effect. Recently, the syntheses of ^{13}C isotopically labeled internal standards for the type B trichothecenes 3-acetyldeoxynivalenol, 15-acetyldeoxinivalenol, and 4-acetylnivalenol and their application to LC-MS/MS analysis of food samples have been reported by our group (*20*). Moreover, the most important type B trichothecene, deoxynivalenol, is commercially available as a fully carbon-13-labeled standard, which has been extensively tested (*21*). Besides these, routes to other labeled type B trichothecenes had been reported (*22*). However, no labeled standards currently are available for type A trichothecenes. Continuing our research on the field of stable isotopically labeled trichothecenes, we undertook the synthesis of carbon-13-labeled standards for four type A trichothecenes and their application in LC-MS and LC-MS/MS analysis of food and feed samples.

MATERIALS AND METHODS

Chemicals and Reagents. Trichothecene reference compounds T2-triol, T2-toxin, HT2-toxin, diacetoxyscirpenol, and 15-monoacetoxyscirpenol were purchased as solids from Sigma-Aldrich (Steinheim, Germany). A solution of reference substances containing inter alia diacetoxyscirpenol, HT2-toxin, and T2-toxin was from Coring Systems (Gernsheim, Germany). [1,1′,2,2′-$^{13}C_4$]-Acetic anhydride, 99%, was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA); acetone p.a., acetonitrile Lichrosolv, aqueous ammonium hydroxide p.a. 25%, formic acid p.a. 98−100%, anhydrous pyridine 99.8%, and isooctane (2,2,4-trimethylpentane) were purchased from Sigma-Aldrich. Water for HPLC was purified by a Milli-Q-system (Millipore GmbH, Schwalbach, Germany).

Syntheses of [$^{13}C_4$]-T2-toxin and [$^{13}C_2$]-HT2-toxin. *Synthesis of [$^{13}C_6$]-3,4,15-T2-triacetate.* 3,4,15-T2-triol (3 mg, 7.8 μmol) was dissolved in dry acetone (80 μL) before dry pyridine (10 μL) and [$^{13}C_4$]-acetic anhydride (10 μL, 102 μmol) were added. The reaction mixture was allowed to stand at room temperature for 48 h followed by evaporation of the solvent to dryness and redissolving the residue in acetonitrile/water (3+7, v+v, 1 mL). Separation by preparative HPLC revealed only one major peak at a retention time of 19.2 min, which was pooled from 10 runs and lyophilized to give the title compound in a yield of 85% (3.4 mg, 6.6 μmol). The structure of [$^{13}C_6$]-3,4,15-T2-triacetate was assigned by ^1H NMR spectroscopy in $CDCl_3$ (**Table 1**) as well as by an additional COSY experiment and GC-MS studies.

GC-MS (CI+) of the title compound in isooctane (relative intensities in parentheses): *m*/*z* 515.4 (15%), 453.4 (15%), 413.3 (20%), 351.2 (60%), and 289.2 (100%).

Hydrolysis of [$^{13}C_6$]-3,4,15-T2-triacetate. [$^{13}C_6$]-3,4,15-T2-triacetate (3 mg, 5.8 μmol) was dissolved in acetonitrile (100 μL), and aqueous ammonium hydroxide (1 mL, 1 mol/L) was added. Subsequently, the solution was kept for 15 min at 60 °C on a water bath. Then, formic acid (100 μL, 10 mol/L) was added before separation of the reaction mixture by preparative HPLC. The chromatogram showed three major peaks at 14.5 (peak 1), 17.0 (peak 2), and 19.4 (peak 3) min in an area ratio of 2:3:3 based on UV measurement at 195 nm. All peaks were pooled from 12 runs and lyophilized. Peak 3 was identified as residual [$^{13}C_6$]-3,4,15-T2-triacetate by cochromatography with the starting material. On the basis of ^1H NMR (**Table 1**) and COSY experiments, peak 1 was identified as [$^{13}C_2$]-15-T2-monoacetate ([$^{13}C_2$]-HT2-toxin) and peak 2 as [$^{13}C_4$]-4,15-T2-diacetate ([$^{13}C_4$]-T2-toxin). LC-MS (ESI+) studies of [$^{13}C_2$]-HT2-toxin revealed signals at *m*/*z* (%) 449.2 (80), 444.3 (100), 427.2 (50), 325.1 (50), and 263.1 (90), and LC-MS/MS (ESI+) studies (CE = 20 V) of the precursor ion at *m*/*z* (%) 449.2 (100) resulted in product ions at *m*/*z* (%) 347.2 (85) and 285.0 (60). Analogously, [$^{13}C_4$]-T2-toxin showed signals at *m*/*z* (%) 493.2 (100) and 488.2 (40) in LC-MS (ESI+) experiments, whereas LC-MS/MS studies (CE = 20 V) of *m*/*z* (%) 493.2 (100) resulted in product ions at *m*/*z* (%) = 391 (25) and 329.1 (18). Yields were 21% (500 μg, 1.2 μmol) for [$^{13}C_2$]-HT2-toxin and 33% (900 μg, 1.9 μmol) for [$^{13}C_4$]-T2-toxin.

Synthesis of [$^{13}C_4$]-Diacetoxyscirpenol and [$^{13}C_2$]-Monoacetoxyscirpenol. *Synthesis of 3,4,15-Scirpentriol.* 4,15-Diacetoxyscirpentriol

Table 1. ^{1}H NMR Data of Carbon-13-Labeled Trichothecenes of the T2 Group[a]

position	[$^{13}C_6$]-3,4,15-T2-triacetate	[$^{13}C_4$]-4,15-T2-diacetate ([$^{13}C_4$]-T2-toxin)	[$^{13}C_2$]-15-T2 monoacetate ([$^{13}C_2$]-HT2-toxin)	T2-triol
2	3.86 1H, d $J_{2,3} = 4.89$	3.70 1H, d $J_{2,3} = 4.89$	3.63 1H, d $J_{2,3} = 4.99$	3.59 1H, d $J_{2,3} = 4.69$
3	5.19 1H, m	4.16 1H, m	4.25 1H, m	4.25 1H, m
3-OH		3.14 1H, d $J = 2.74$	2.19 1H, d $J = 4.50$	2.18 1H, d $J = 4.50$
3-acetyl-[$^{13}CH_3$]	2.09 3H, dd $^{2}J = 6.84$, $^{1}J = 129.85$			
4	5.92 1H, m	5.32 1H, m	4.45 1H, dd $J = 2.55$, $J = 8.71$	4.39 1H, dd $J = 2.54$, $J = 8.02$
4-OH			1.72 1H, s	1.77 1H, s
4-acetyl-[$^{13}CH_3$]	2.17 3H, dd, $^{2}J = 6.85$, $^{1}J = 129.89$	2.15 3H, dd, $^{2}J = 6.85$, $^{1}J = 129.89$		
7a	2.36 1H, dd $J_{7,8} = 5.87$, $^{2}J = 15.16$	2.39 1H, dd $J_{7,8} = 5.87$, $^{2}J = 15.16$	2.36 1H, dd $J_{7,8} = 5.77$, $^{2}J = 15.16$	2.35 1H, dd $J_{7,8} = 6.07$, $^{2}J = 14.97$
7b	1.87 1H, d $^{2}J = 15.10$	1.92 1H, m	1.96 1H, d $^{2}J = 15.16$	1.93 1H, d $^{2}J = 15.26$
8	5.30 1H, d $J_{7,8} = 5.58$	5.29 1H, d $J_{7,8} = 5.68$	5.31 1H, d $J_{7,8} = 5.58$	5.46 1H, d $J_{7,8} = 5.38$
10	5.75 1H, d $J_{10,11} = 5.77$	5.80 1H, d $J_{10,11} = 5.96$	5.75 1H, d $J_{10,11} = 5.87$	5.78 1H, d $J_{10,11} = 5.86$
11	4.23 1H, d $J_{11,10} = 5.77$	4.37 1H, d $J_{11,10} = 5.87$	4.19 1H, d $J_{11,10} = 5.86$	4.03 1H, d $J_{11,10} = 5.28$
13a	3.06 1H, d $^{2}J = 3.91$	3.06 1H, d $^{2}J = 3.91$	3.02 1H, d $^{2}J = 3.81$	3.00 1H, d $^{2}J = 3.92$
13b	2.82 1H, d $^{2}J = 3.92$	2.80 1H, d, $^{2}J = 3.92$	2.77 1H, d, $^{2}J = 3.82$	2.78 1H, d, $^{2}J = 4.01$
14	0.74 3H, s	0.81 3H, s	0.81 3H, s	0.87 3H, s
15a	4.34 1H, d $^{2}J = 12.52$	4.37 1H, d $^{2}J = 12.52$	4.30 1H, d $^{2}J = 12.52$	3.81 1H, dd $^{2}J = 12.23$, $J = 8.02$
15b	4.12 1H, d $^{2}J = 12.52$	4.08 1H, d $^{2}J = 12.62$	4.04 1H, d $^{2}J = 12.52$	3.57 1H, dd $^{2}J = 12.92$, $J = 6.17$
15-OH				1.98 1H, d $J = 2.44$
15-acetyl-[$^{13}CH_3$]	2.06 3H, dd $^{2}J = 6.85$, $^{1}J = 129.60$	2.04 3H, dd $^{2}J = 6.85$, $^{1}J = 129.60$	2.05 3H, dd $^{2}J = 6.84$, $^{1}J = 129.50$	
16	1.75 3H, s	1.75 3H, s	1.75 3H, s	1.75 3H, s
18/19	2.15 3H, m	2.15 3H, m	2.15 3H, m	2.11 3H, m
20/21	0.97 6H, m	0.97 6H, m	0.96 6H, m	0.99 6H, m

[a] Chemical shifts, δ (TMS); coupling constants, J (Hz).

(3 mg, 10.6 μmol) was dissolved in acetonitrile (100 μL), and the resulting solution was added to aqueous ammonium hydroxide (1 mL, 1 mol/L). Hydrolysis was carried out for 2 h at 60 °C on a water bath. Then, the reaction mixture was neutralized with formic acid (100 μL, 10 mol/L) and separated by preparative HPLC. The chromatogram showed three major peaks at retention times of 9.5 (peak 1), 23.0 (peak 2), and 26.1 (peak 3) min in an area ratio of 96:28:1 based on UV measurement at 195 nm. Peaks 2 and 3 were assigned to 15-monoacetoxyscirpentriol and residual 4,15-diacetoxyscirpentriol, respectively, by cochromatography with reference substances. Peak 1 was pooled from 12 runs and lyophilized to give a white powder. LC-MS (ESI+) studies revealed signals at *m*/*z* (%) 283.1 (50), 265.1 (100), 247.0 (50), 229.0 (25), and 217.0 (60). NMR data obtained by a ^{1}H NMR and a COSY experiment of peak 1 in $CDCl_3$ are summarized in **Table 2** and allowed the assignment to 3,4,15-scirpentriol, which was obtained in an overall yield of 73% (2.2 mg; 7.7 μmol).

Synthesis of 3,4,15-[$^{13}C_6$]-Triacetoxyscirpentriol. 3,4,15-Scirpentriol (2 mg, 7.1 μmol) was dissolved in dry acetone (80 μL) before dry pyridine (10 μL) and [$^{13}C_4$]-acetic anhydride (10 μL, 102 μmol) were added. The mixture was kept at room temperature for 48 h followed by rotary evaporation of the solvent and redissolving the residue in acetonitrile/water (1+9, v+v, 1 mL) before it was separated by preparative HPLC. The chromatogram showed only one major peak at 29.5 min, which was pooled from 10 runs and lyophilized to give the title compound in a 91% yield (2.7 mg, 6.5 μmol). The structure of [$^{13}C_6$]-3,4,15-triacetoxyscirpentriol was assigned by ^{1}H NMR spectroscopy in $CDCl_3$ (**Table 2**) along with an additional COSY experiment and GC-MS studies. GC-MS (CI+) of the title compound in isooctane (relative intensities in parentheses): *m*/*z* (%) 415 (60), 353 (100), 291 (50), and 229 (34).

Synthesis of [$^{13}C_2$]-Monoacetoxyscirpenol and [$^{13}C_4$]-Diacetoxyscirpenol. [$^{13}C_6$]-3,4,15-Triacetoxyscirpentriol (2.5 mg, 6.0 μmol) was dissolved in acetonitrile (100 μL). Aqueous ammonium hydroxide (1 mL, 1 mol/L) was added, and hydrolysis took place for 15 min at 60 °C on a water bath. The solution was neutralized with formic acid (100 μL, 10 mol/L) and separated by preparative HPLC. Three major peaks appeared in the chromatogram at retention times of 22.9 (peak 1), 25.8 (peak 2), and 28.9 (peak 3) min in an area ratio of 2:5:2 based on UV measurement at 195 nm. All peaks were pooled from 12 runs and lypohilized. Peak 3 was assigned to residual [$^{13}C_6$]-3,4,15-triacetoxyscirpentriol by cochromatography with the starting material. ^{1}H NMR (**Table 2**) and COSY experiments (solvent $CDCl_3$) of peaks 1 and 2 allowed structural assignment as [$^{13}C_2$]-15-monoacetoxyscirpentriol and [$^{13}C_4$]-4,15-diacetoxyscirpentriol, respectively. LC-MS (ESI+) studies of [$^{13}C_2$]-15-monoacetoxyscirpentriol and [$^{13}C_4$]-4,15-diacetoxyscirpentriol showed signals at *m*/*z* (%) 365.1 (10), 349.1 (30), 344.2 (70), 309.1 (60), 306.1 (80), and 265.1 (100) and at *m*/*z* (%) 409.1 (20), 393.2 (80), 388.2 (100), 371.1 (20), 353.1 (30), 309.1 (60), and 291.1 (20), respectively. Yield was determined to be 25% (500 μg, 1.5 μmol) for [$^{13}C_2$]-15-monoacetoxyscirpentriol and 53% (1.2 mg, 3.2 μmol) for [$^{13}C_4$]-4,15-diacetoxyscirpentriol.

Preparation of Standard Solutions. Labeled trichothecenes as well as their unlabeled isotopologues were exactly weighed and dissolved in acetonitrile to give stock solutions of 100 μg/mL. Concentrations of the solutions of labeled and unlabeled substances were verified by comparison of their UV absorption at 195 nm. Working standards were prepared by diluting stock solutions to a final concentration of 1 μg/mL.

Sample Preparation and Cleanup. Food samples were purchased from local markets in quantities of 1 kg each. Before analysis, samples were thoroughly ground and intensively homogenized. Potatoes were washed, sliced, lyophilized, and finely ground. The homogeneous samples (1−3 g) were weighed in a 25 mL Erlenmeyer flask with stopper. Labeled standards in acetonitrile were separately added to give contents of 20−200 μg/kg depending on the toxin, and the flask was allowed to stand overnight to evaporate the solvent. Then, a mixture of water/acetonitrile (16+84, v+v, 15 mL) was added and stirred for 90 min. The suspension was filtered through a folded filter ($595^1/_2$, 125 mm diameter, Schleicher & Schuell, Dassel, Germany) and the complete filtrate was loaded on a MultiSep 225 Trich cleanup column (Romer Labs Inc., Union, MO). The solution was quickly passed through the column by suction (about 3 drops per second) and collected in a 25 mL flask. The purified solution was evaporated at 60 °C in vacuo, redissolved in mobile phase for LC-MS analysis (100 μL), and filtered through a 0.45 μm membrane (Schleicher & Schuell).

NMR. ^{1}H NMR and COSY experiments were performed on a 400 AMX NMR instrument (Bruker, Rheinstetten, Germany). ^{1}H NMR spectra were recorded at a ^{1}H resonance frequency of 400.132 MHz using the standard zg30 pulse program and 2048 scans for each experiment. Carbon-13 GARP-decoupling was applied with 2.5 kHz. The number of dummy scans was set at 64. For COSY experiments the cosygs pulse program was used with 64 scans each. TMS was used for calibration.

GC-MS. GC separation of polyacetylated trichothecenes was accomplished on a HP 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA), equipped with a 30 m × 0.25 mm i.d., 0.25 μm fused silica DB-5 capillary column. Helium was used as carrier gas at a flow of 2 mL/min. The compounds under study were applied as 10 ng/mL solutions in isooctane by the cold on-column technique at 115 °C. Two minutes after the sample (0.5 μL) had been injected, the temperature of the oven was raised to 200 °C at a rate of 15 °C/min. Immediately after reaching 200 °C, the temperature was further increased to 300 °C at a rate of 6 C/min and held for 10 min. The mass spectrometer was a Finnigan MAT-95S (Finnigan MAT, Bremen, Germany), which was run in the chemical ionization mode with isobutane as reagent gas at 150 eV ionization energy and ion source temperature of 200 °C.

LC-MS: HPLC Parameters. HPLC separation prior to MS analysis was performed on a Finnigan Surveyor Plus HPLC System (Thermo Electron Corp., Waltham, MA). A 150 × 2 mm i.d., 4 μm Synergi Polar-RP column (Phenomenex, Aschaffenburg, Germany) tempered at 30 °C and equipped with a C_{18} guard column (Phenomenex) was used as the stationary phase. The mobile phase consisted of variable mixtures of formic acid (0.1%, solvent A) and acetonitrile acidified with formic acid (0.1%, solvent B). The linear gradient program used for separation of all mycotoxins under study began with 30% B, which was held for 3 min and then increased to 100% B within 10 min. The column was washed for 2 min at 100% B before returning to initial conditions within 5 min. The subsequent equilibration time between two runs was set to 15 min. Flow rate was 0.2 mL/min, and the injection volume was 10 μL. The divert valve was used to cut off both the first 2 min of every run and the wash phase at 100% B into waste.

LC-MS: MS and MS/MS Parameters. The mass spectrometer consisted of a triple-quadrupole Finnigan TSQ Quantum Discovery (Thermo Electron Corp.). The ion source was used exclusively in the ESI positive mode. Spray voltage was set to 4500 V, sheath gas pressure was 38 mTorr, and auxiliary gas pressure was 8 mTorr. Capillary temperature was 340 °C and capillary offset 35 V. Source collision-induced dissociation (CID) was used with the collision energy set at 10 V. Full scan spectra for characterization of the synthesized products were recorded in quadrupole 3 using a mass range from *m*/*z* 200 to 550 and a scan time of 0.25 s. For LC-MS/MS experiments, the collision gas pressure in quadrupole 2 was 1 mTorr, scan time was 0.20 s, and peak width in quadrupoles 1 and 3 was adjusted to ±0.7 amu. Mass range was *m*/*z* 200−500.

Analysis of labeled as well as unlabeled trichothecenes was based on the measurement of sodium adduct ions ($[M + Na]^+$). Diacetoxyscirpenol (*m*/*z* 389.1), [$^{13}C_4$]-diacetoxyscirpenol (*m*/*z* 393.1), monoacetoxyscirpenol (*m*/*z* 347.1), and [$^{13}C_2$]-monoacetoxyscirpenol (*m*/*z* 349.1) were measured using single ion monitoring (SIM) with a peak width of ±0.2 amu. T2-toxin and HT2-toxin and their labeled analogues were measured using selected reaction monitoring (SRM) with mass transitions from $[M + Na]^+$ by applying corresponding collision energies as follows: T2-toxin (*m*/*z* 489.2 → *m*/*z* 387.1; 24 V); [$^{13}C_4$]-T2-toxin (*m*/*z* 493.2 → *m*/*z* 391.1; 24 V); HT2-toxin (*m*/*z* 447.2 → *m*/*z* 385.0; 22 V); [$^{13}C_2$]-HT2-toxin (*m*/*z* 449.2 → *m*/*z* 385.0; 22 V). Acquisition during the HPLC run was split into three segments. MonoacetoxyscirpenoI was measured in segment I (2−6.5 min), diacetoxyscirpenol and HT2-toxin were measured in segment II (6.5−13 min), and T2-toxin was measured in segment III (13−18 min).

HPLC-UV Parameters. HPLC-UV was performed on a HPLC system (BIO-TEK Instruments, Eching, Germany) equipped with a two-pump (type 552) gradient mixer (M 800), a Rheodyne injector, and a UV detector (type 535). A 250 × 3.0 mm, i.d., 4 μm Synergy Fusion

Table 2. ^{1}H NMR Data of Carbon-13-Labeled Trichothecenes of the Scirpenol Group[a]

position	[$^{13}C_6$]-3,4,15-triacetoxyscirpentriol	[$^{13}C_4$]-3,15-diacetoxyscirpentriol	[$^{13}C_2$]-15-monoacetoxyscirpentriol	scirpentriol
2	3.85 1H, d $J_{2,3} = 4.90$	3.71 1H, d $J_{2,3} = 4.89$	3.63 1H, d $J_{2,3} = 4.89$	3.62 1H, d $J_{2,3} = 4.89$
3	5.19 1H, m	4.15 1H, m	4.24 1H, m	4.22 1H, m
3-OH		3.13 1H, d $J = 2.84$	2.19 1H, d $J = 4.70$	2.20 1H, d $J = 4.79$
3-acetyl-[$^{13}CH_3$]	2.11 3H, dd $^2J = 6.95$, $^1J = 129.79$			
4	5.75 1H, m	5.15 1H, m	4.29 1H, dd $J = 2.64$, $J = 8.12$	4.43 1H, dd $J = 2.84$, $J = 8.32$
4-OH			1.77 1H, d $J = 8.11$	1.76 1H, s
4-acetyl-[$^{13}CH_3$]	2.14 3H, dd $^2J = 6.94$, $^1J = 129.90$	2.13 3H, dd $^2J = 6.84$, $^1J = 129.89$		
7a	2.04 1H, m	2.07 1H, m	2.06 1H, m	2.03 1H, m
7b	1.73 1H, m	1.77 1H, m	1.79 1H, m	1.74 1H, m
8	2.00 2H, m	1.97 2H, m	2.02 2H, m	1.97 2H, m
10	5.47 1H, d $J_{10,11} = 4.80$	5.54 1H, d $J_{10,11} = 5.18$	5.49 1H, d $J_{10,11} = 5.48$	5.51 1H, d $J_{10,11} = 4.20$
11	3.99 1H, d $J_{11,10} = 5.40$	4.11 1H, d $J_{11,10} = 5.38$	3.94 1H, m	3.94 1H, d $J_{11,10} = 5.67$
13a	3.07 1H, d $^2J = 3.96$	3.06 1H, d $^2J = 4.01$	3.03 1H, d, $^2J = 4.01$	3.03 1H, d $^2J = 3.91$
13b	2.79 1H, d $^2J = 3.96$	2.79 1H, d $^2J = 4.01$	2.76 1H, d $^2J = 3.91$	2.76 1H, d $^2J = 3.91$
14	0.77 3H, s	0.83 3H, s	0.83 3H, s	0.92 3H, s
15a	4.24 1H, d $J = 12.22$	4.18 1H, d $J = 12.15$	4.14 1H, d $J = 12.23$	3.80 1H, dd $J = 11.83$, $J = 4.89$
15b	4.08 1H, d $J = 12.31$	3.99 1H, d $J = 12.32$	3.91 1H, d $J = 12.33$	3.55 1H, dd $J = 11.74$, $J = 5.48$
15-OH				1.21 1H, m
15-acetyl-[$^{13}CH_3$]	2.06 3H, dd $^2J = 6.84$, $^1J = 129.70$	2.06 3H, dd $^2J = 6.84$, $^1J = 129.60$	2.06 3H, dd $^2J = 6.85$, $^1J = 129.61$	
16	1.73 3H, s	1.73 3H, s	1.73 3H, s	1.74 3H, s

[a] Chemical shifts, δ (TMS); coupling constants, J (Hz).

RP column (Phenomenex) was used as the stationary phase, and variable mixtures of acetonitrile (solvent B) and water (solvent A) were used as the mobile phase. Two different linear gradient programs were used for the analytical and preparative separation of substances of the scirpenol group, on the one hand, and the T2 group, on the other hand. Substances of the scirpenol group were successfully separated with a gradient starting with 10% B, which was held for 7 min, before the content of B was raised to 100% until 30 min after injection. The gradient used for the separation of members of the T2 group was programmed as follows: Initial conditions were 30% B, which was held for 3 min. Then, the content of B was raised to achieve 100% within 15 min after injection. In both cases 100% B was held for 2 min and then brought back to initial conditions within 1 min. The equilibration time before the next injection was set to 10 min. Flow rate was set to 0.4 mL/min, and UV absorption was monitored at 195 nm.

Lyophilization. Separated products were lyophilized on a Christ LOC-1 m Alpha 2-4 lyophylizator equipped with an external vacuum pump.

Calibration and Quantitation. Solutions of unlabeled and labeled trichothecenes were mixed in seven molar ratios ranging from 0.1 to 9 to give a total trichothecene concentration of 1 μg/mL for each trichothecene. Each mixture was analyzed by LC-MS, and a calibration curve was constructed from the peak area ratios versus the molar ratios. The calibration curves revealed good linearity, and the equations derived thereof using linear regression gave the respective response factors. For calculating the contents of trichothecenes in the samples, the response factors were multiplied by the peak area ratios obtained by stable isotope dilution assay (SIDA), and the results were corrected with the molar mass of the unlabeled compound to give the absolute mass. Calibration was also performed by adding the different mixtures to a type 405 wheat flour devoid of trichothecenes and subsequent cleanup as described above before LC-MS analysis. To check the obtained calibration, a reference solution of a different manufacturer was analyzed for T2-toxin, HT2-toxin, and diacetoxyscirpenol using LC-MS.

Stability of Labeled Standards. To prove the stability of the label under cleanup conditions, a mixture of labeled and unlabeled trichothecenes in acetonitrile was prepared and divided into two parts. The first half was analyzed by LC-MS directly after dilution with water, and the second half was added to a starch matrix and subjected to cleanup as described above. LC-MS/MS analysis was performed, and the differences in the ratios between labeled and unlabeled substances for these two measurements were calculated.

Detection and Quantification Limits. Detection and quantification limits were calculated according to the method suggested by Hädrich and Vogelgesang (*23*). As blank matrix devoid of any trichothecene contamination a type 405 wheat flour was chosen. Unlabeled as well as labeled trichothecenes were added in amounts as follows: T2-toxin, 5, 20, 35, and 50 μg/kg; HT2-toxin, 30, 100, 200, and 300 μg/kg; diacetoxyscirpenol, 5, 20, 35, and 50 μg/kg; monoacetoxyscirpenol, 50, 200, 350, and 500 μg/kg. As required by the method, each of the four addition assays was performed in triplicate and analyzed using SIDA as described before.

Precision. Naturally contaminated samples containing T2-toxin and HT2-toxin were analyzed in triplicate within 2 weeks. As no naturally contaminated material was available for diacetoxyscirpenol and monoacetoxyscirpenol, type 405 wheat flour (10 g) was suspended in acetonitrile (100 mL), which was spiked with diacetoxyscirpenol (50 μg/kg) and monoacetoxyscirpenol (500 μg/kg). After this suspension had been stirred for 2 h, the solvent was evaporated in vacuo followed by homogenization of the obtained flour and analysis in triplicate (2.5 g each) by SIDA as described before.

Recovery. Recovery of the analytes was examined at different spiking levels considering their limits of quantification. In detail, a wheat flour type 405 was fortified with T2-toxin and diacetoxyscirpenol at 20, 35, and 50 μg/kg contamination levels, with HT2-toxin at 100, 200, and 300 μg/kg levels, and with monoacetoxyscirpenol at 200, 350, and 500 μg/kg levels. Labeled standards were added, and SIDA was performed as described before.

RESULTS AND DISCUSSION

Synthesis of Carbon-13-Labeled Type A Trichothecenes. Isotopically labeled [$^2H_6$]-T2-toxin and [$^2H_3$]-HT2-toxin had already been synthesized in the late 1980s and successfully applied for the determination of T2-toxin and HT2-toxin in human blood using GC-MS/MS (*24*). Surprisingly, this approach has neither been used for the synthesis of other isotopically labeled trichothecenes nor applied to the analysis of T2-toxin and HT2-toxin in more complex matrices such as food, food products, and feeds. Besides the latter report, a synthesis of $^2H_3$-labeled T2-toxin by the hydrolysis of unlabeled T2-toxin to HT2-toxin followed by peracetylation with deuterated acetic anhydride and finally selective hydrolysis was described in the literature (*25*). However, deuterium in an α-position to a carbonyl group is highly susceptible to exchange with hydrogen in protic solvents. To obtain stable labeling, we replaced [$^2H_6$]-acetic anhydride used in the route of the latter authors by its 4-fold carbon-13-labeled analogue. As we further intended to synthesize labeled HT2-toxin, our synthesis started from T2-triol, which was peracetylated with [$^{13}C_4$]-acetanhydride to give its triacetate. The latter compound was characterized by NMR and GC-MS studies, which resulted in the following observations: compared to the ^{1}H NMR spectrum of T2-triol (**Table 1**), a downfield shift of the proton signals at C3, C4, and C15 occurred and new signals emerged from the three acetyl groups at the respective positions. The mass spectrum obtained by GC-MS (CI) showed the subsequent loss of [$^{13}C_2$]-acetic acid (Δ *m*/*z* 62) and isovaleric acid (Δ *m*/*z* 102) from the protonated molecular ion (*m*/*z* 515.4).

Hydrolysis of the resulting [$^{13}C_6$]-T2-triacetate with ammonium hydroxide yielded [$^{13}C_4$]-T2-toxin and [$^{13}C_2$]-HT2-toxin in one step, which easily could be separated by preparative HPLC. ^{1}H NMR spectra (**Table 1** and **Figure 2**) showed the reverse high-field shift of proton signals at C3 for T2-toxin and at C3 and C4 for HT2-toxin. This observation went along with the appearance of new signals, which were assigned to free hydroxy groups by COSY experiments. Following successive deacetylation, the number of acetyl signals in the spectra decreased. In general, ^{1}H NMR data were in good agreement with those described for T2-toxin and HT2-toxin in the literature (*26*, *27*).

Carbon-13-labeled diacetoxyscirpenol and monoacetoxyscirpenol were prepared using the same strategy as for the synthesis of T2- and HT2-toxin. As the deacetylated precursor scirpentriol was commercially not available, it was prepared from diacetoxyscirpenol by complete alkaline hydrolysis following a route described in the literature (*28*). Mass spectra of the product 3,4,15-scirpentriol obtained by LC-MS were not characteristic, showing repeated loss of water (Δ *m*/*z* 18) and formaldehyde (Δ *m*/*z* 30) from the protonated molecular ion (*m*/*z* 283.1). In the ^{1}H NMR spectrum of scirpentriol (**Table 1**) the positions of signals were found to be in accordance with literature data (*29*). Subsequently, scirpentriol was peracetylated to 3,4,15-triacetoxyscirpentriol using [$^{13}C_4$]-acetic anhydride. Mass spectra of [$^{13}C_6$]-triacetoxyscirpentriol were acquired by GC-MS experiments and showed threefold rapid loss of [$^{13}C_2$]-acetic acid (Δ *m*/*z* 62) from the protonated moleular ion (*m*/*z* 415). The ^{1}H NMR data of [$^{13}C_6$]-triacetoxyscirpentriol compared to those of scirpentriol (**Table 1**) revealed downfield shifts of proton signals at C3, C4, and C15 and emergence of new signals from the three acetyl groups. Taken together, the spectroscopic data were consistent with those reported for triacetoxyscirpentriol in the literature (*29*).

Hydrolysis of [$^{13}C_6$]-triacetoxyscirpentriol with ammonium hydroxide yielded [$^{13}C_4$]-diacetoxyscirpenol and [$^{13}C_2$]-monoacetoxyscirpenol, both of which were separated by preparative HPLC to obtain pure substances before NMR experiments were performed (**Table 2** and **Figure 3**). As expected, ^{1}H NMR spectra of [$^{13}C_4$]-diacetoxyscirpenol compared to those of [$^{13}C_6$]-triacetoxyscirpentriol revealed only two acetyl groups along with the signal of the proton at C-3 at high-field again and a new signal, which was assigned to the 3-OH by COSY experiments. Analoguous results were obtained from the ^{1}H NMR spectrum of [$^{13}C_2$]-monoacetoxyscirpenol. Only the signals of the protons at C-15 remained downfield along with the signal of only one acetyl-CH_3 being present in the spectrum, whereas two new signals were visible, which could be attributed to the 3-OH and 4-OH by COSY experiments.

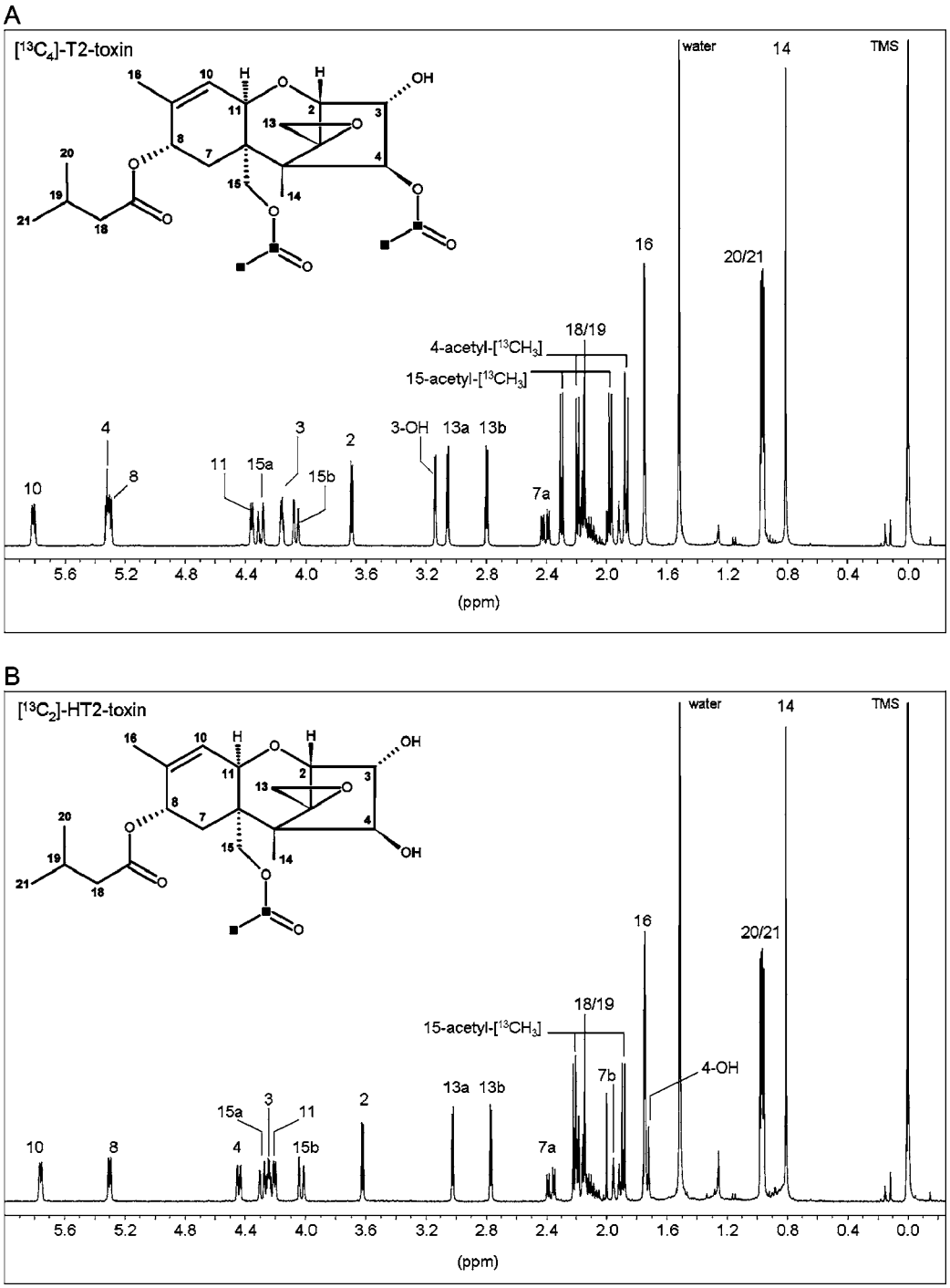


Figure 2. (**A**) ^{1}H NMR spectrum of [$^{13}C_4$]-T2-toxin; (**B**) ^{1}H NMR spectrum of [$^{13}C_2$]-HT2-toxin.

Special NMR Effects of ^{13}C-Labeled Trichothecenes. In ^{1}H NMR experiments of ^{13}C-enriched substances, a rare coupling phenomenon can be observed. ^{1}H and ^{13}C are both nuclei with a spin of $^{1}/_{2}$ and, therefore, are able to couple during NMR analysis. Due to the low natural abundance of ^{13}C, this coupling is negligible in common ^{1}H NMR spectroscopy in contrast to ^{13}C NMR experiments, which are usually ^{1}H-broadband decoupled. However, by introducing twofold carbon-13-labeled acetyl moieties into type A trichothecenes, a coupling between ^{13}C and ^{1}H has to be taken into consideration in the interpretation of ^{1}H NMR spectra. In this way, the three protons at the α position of any $^{13}C_2$-acetyl group did not appear as an usual singlet but showed ^{1}J coupling of 130 Hz with the α-^{13}C and ^{2}J coupling of 7 Hz with the ^{13}C-carbonyl, resulting in a broad double doublet. This characteristic multiplicity was observed in all ^{1}H NMR spectra of the synthesized acetyl-^{13}C-labeled type A trichothecenes. To verify that this effect was a mere coupling phenomenon, ^{13}C-decoupled ^{1}H NMR experiments were performed. By application of globally optimized alternating phase rectangular pulse (GARP) decoupling we found that the double doublets collapsed into singlets while other signals remained unaffected, as can be seen when standard ^{1}H NMR and the ^{13}C-decoupled ^{1}H NMR spectra of [$^{13}C_4$]-diacetoxyscirpenol are compared (**Figure 4**).

LC-MS. In ESI positive mode LC-MS spectra of all trichothecenes under study showed extensive adduct ion formation including sodium, ammonium, and potassium adducts even when pure water was used as solvent. Whereas in reference

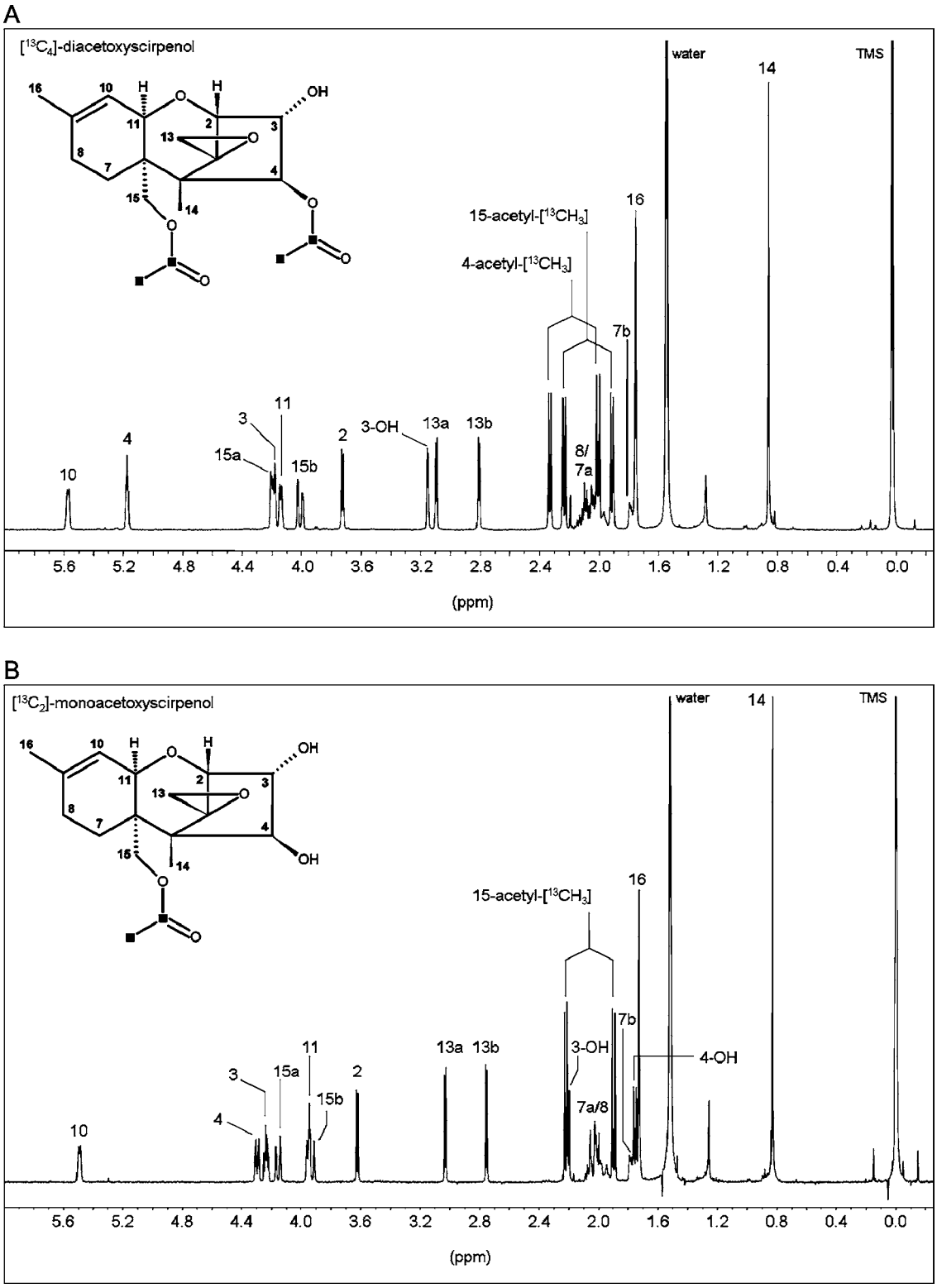


Figure 3. (**A**) ^{1}H NMR spectrum of [$^{13}C_4$]-diacetoxyscirpenol; (**B**) ^{1}H NMR spectrum of [$^{13}C_2$]-monoacetoxyscirpenol.

substances sodium adducts were prevalent, the synthesized labeled standards predominantly formed ammonium adducts due to residual ammonia from the synthesis. In any case, protonated molecular ions were obtained only in traces. Therefore, measurement of adduct ions was inevitable for LC-MS analysis of type A trichothecenes. Unfortunately, ammonium adducts did not show reproducible fragmentation in our instrument, which rendered MS/MS measurements ambiguous. In contrast to this, the sodium adducts for HT2-toxin (*m*/*z* 447.2) and T2-toxin (*m*/*z* 489.2) dissociated reproducibly and the latter showed loss of isovaleric acid (*m*/*z* 387.2 = 489.2 − 102) and twofold loss of acetic acid (*m*/*z* 327.2 = 489.2 − 102 − 60; *m*/*z* 267.0 = 489.2 − 102 − 60 − 60) in LC-MS/MS analysis (**Figure 5**). The labeled analogue (*m*/*z* 493.1) exhibited almost the same fragmentation pattern, leading toward *m*/*z* 391.0 (= 493.1 − 102) by loss of isovaleric acid and toward *m*/*z* 329.0 (= 493.1 − 102 − 62) as well as *m*/*z* 267.3 (= 493.1 − 102 − 62 − 62) by subsequent losses of [$^{13}C_2$]-acetic acid. The transition (*m*/*z*

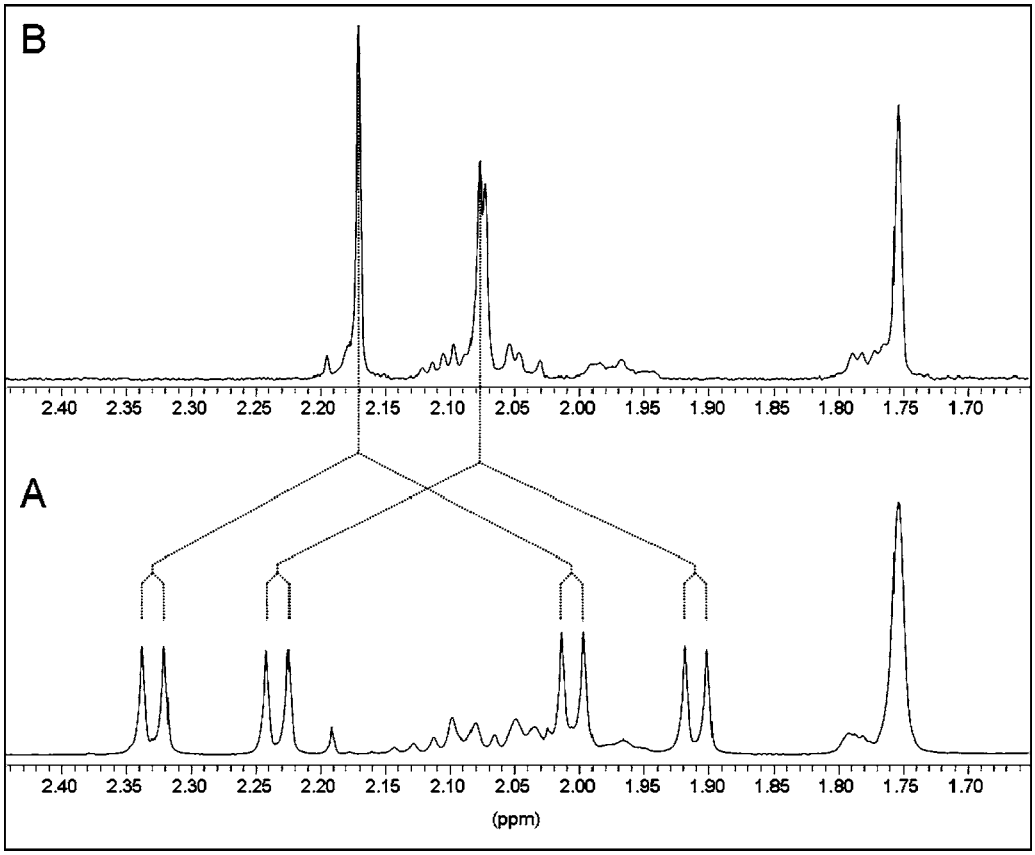


Figure 4. Comparison of a standard ^{1}H NMR spectrum of [$^{13}C_4$]-diacetoxyscirpenol (**A**) to its ^{13}C-decoupled ^{1}H NMR spectrum (**B**).

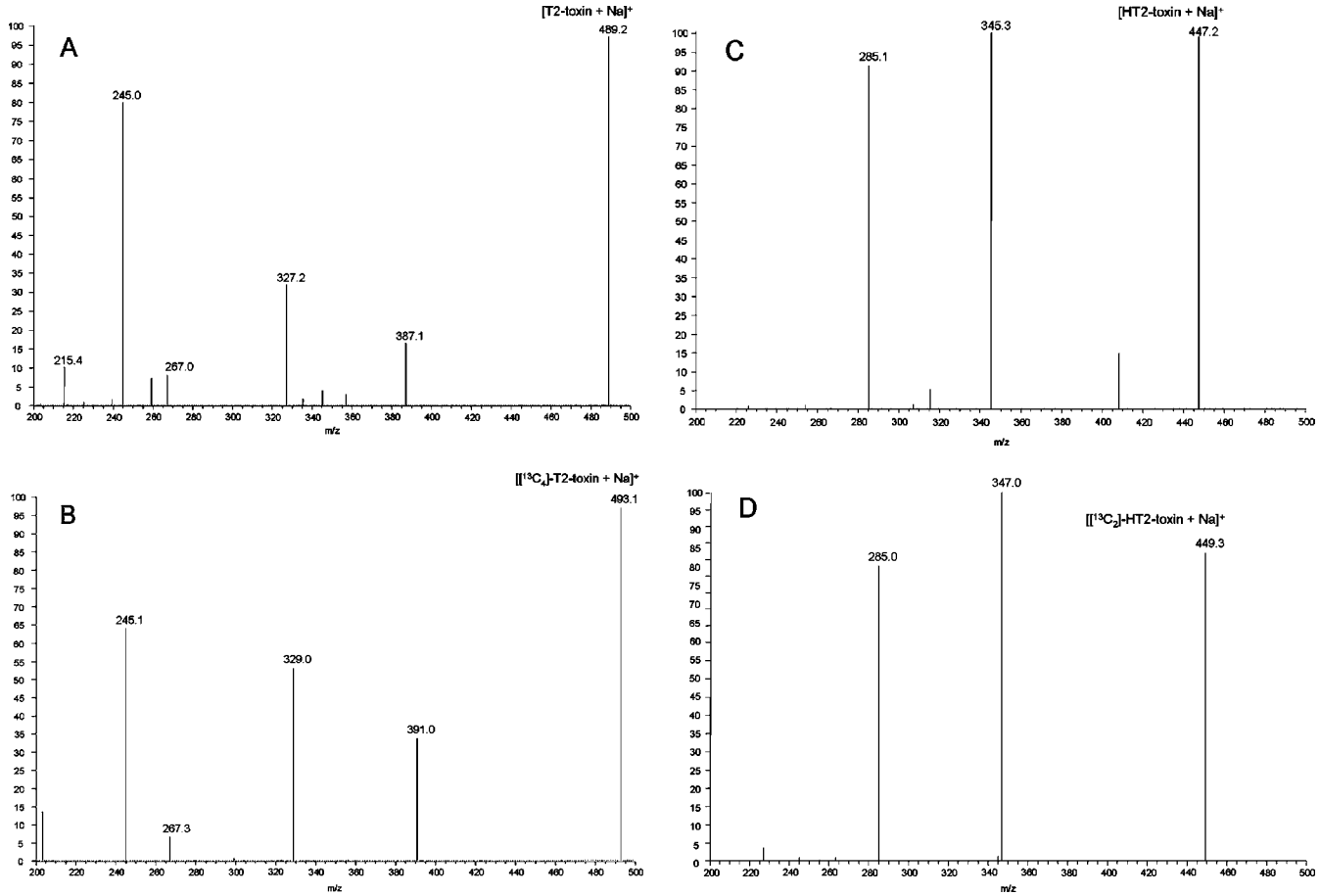


Figure 5. LC-ESI(+)-MS/MS (collision energy = 25 V) spectra of T2-toxin (**A**) and [$^{13}C_4$]-T2-toxin (**B**) and LC-ESI(+)-MS/MS (collision energy = 20 V) spectra of HT2-toxin (**C**) and [$^{13}C_2$]-HT2-toxin (**D**).

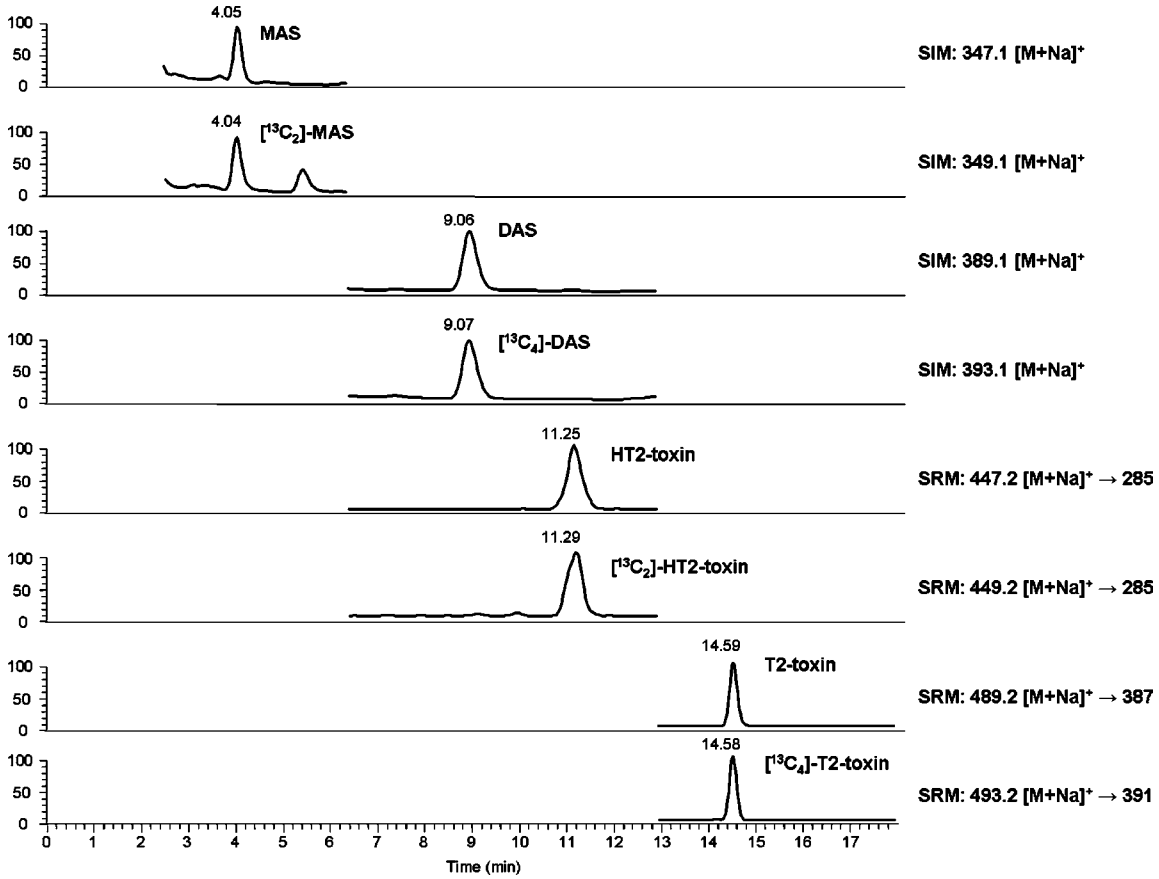


Figure 6. LC-ESI(+)-MS chromatogram of a wheat flour type 405 sample fortified with four type A trichothecenes.

Table 3. Contents of Type A Trichothecenes in Food and Feed Samples[a]

	T2-toxin (μg/kg)	HT2-toxin (μg/kg)	diacetoxyscirpenol (μg/kg)
oat flakes	50	100	(~ 1)
	20	–	–
	(~ 7)	–	–
	(~ 4)	–	–
	(~ 7)	–	–
	20	85	(~ 1)
oat grain	(~ 3)	–	–
oat cookies	–	–	(~ 1)
	–	–	(~ 1)
corn flour	(~ 3)	–	(~ 2)
corn grid	–	–	5
spelt grain	–	–	10
potatoes	–	–	4
	–	–	7[b]
horse feed	60	800	–
pig feed	–	–	10

[a] Monoacetoxyscirpenol was not detected in any sample. –, not detectable. ~, detectable, but below limit of quantification. [b] Content in μg/kg of dry mass.

489.2 → 387.0) was used for quantification of T2-toxin and (*m*/*z* 493.1 → 391.0) for [$^{13}C_4$]-T2-toxin, respectively. In the spectra of both T2-toxin and [$^{13}C_4$]-T2-toxin the fragment at *m*/*z* 245 was the most intense one, but was not chosen for quantification because its formation requires the exchange of sodium by a proton as carrier of the charge (*m*/*z* = 267 − 23 + 1), which was not reproducible and rendered this transition not suitable.

The MS/MS spectra (**Figure 5**) of the the sodium adduct of HT2-toxin (*m*/*z* 447.2) likewise showed the loss of isovaleric acid (*m*/*z* 345.3 = 447.2 − 102) followed by the loss of acetic acid (*m*/*z* 285.0 = 447.2 − 102 − 60). The sodium adduct of [$^{13}C_2$]-HT2-toxin (*m*/*z* = 449.3) fragmented analoguously, resulting in *m*/*z* 347.0 (= 449.3 − 102) and *m*/*z* 285.0 (= 449.3 − 102 − 62). For quantification, the transition (*m*/*z* 447.2 → 345.3) was used for HT2-toxin and the transition (*m*/*z* 449.3 → 347.0) for [$^{13}C_2$]-HT2-toxin.

In contrast to T2-toxin and HT2-toxin the toxins diacetoxyscirpenol and monoacetoxyscirpenol did not show any fragmentation either as ammonium or as sodium adduct ions and, therefore, could not be measured using MS/MS. Thus, we decided to use single MS in the SIM mode for the measurement of sodium adduct ions. In this way the additional problem emerged that the ammonium adduct of [$^{13}C_4$]-diacetoxyscirpenol (*m*/*z* 388.2) might interfere with the sodium adduct of diacetoxyscirpenol (*m*/*z* 389.2) during MS analysis. Therefore, the peak isolation width was set very narrow (±0.2 amu), which improved selectivity but also resulted in less sensitivity.

Calibration. Calibration curves for monoacetoxyscirpenol, diacetoxyscirpenol, HT2-toxin, and T2-toxin were linear in molar ratios ranging between 0.1 and 4 ($R^2 = 0.9968$), between 0.1 and 4 ($R^2 = 0.9955$), between 0.1 and 9 ($R^2 = 0.9991$), and between 0.1 and 9 ($R^2 = 0.9958$), respectively. The response factors (RF) for transferring the peak area ratio (*A*[standard]/*A*[analyte]) into the molar ratio (*n*[standard]/*n*[analyte]) were 2.4 for monoacetoxyscirpenol, 1.6 for diacetoxyscirpenol, 2.3 for HT2-toxin, and 1.1 for T2-toxin. The first three values are very unusual as in SIDAs RFs of around 1.0 are usually obtained. Possibly this effect is due to the different ratios of sodium to ammonium adducts in standard and analyte solutions, respectively. To check whether this ratio may be susceptible to

changes during cleanup, matrix calibration was performed. However, no changes of the RFs were found. Finally, a reference solution from a different manufacturer was analyzed to further verify the obtained calibration. Except for monoacetoxyscirpenol being not available from the second manufacturer, the expected amounts of the other three type A trichothecenes were found precisely.

Stability of Labeled Standards. During cleanup no change in the ratio between labeled and unlabeled substances occurred, which proved that the synthesized standards were stable.

Limits of Detection and Quantification. Limits of detection (LOD) and limits of quantification (LOQ) were determined according to the procedure suggested by Hädrich and Vogelgesang (*23*), which resulted in LODs of 3 μg/kg (T2-toxin), 30 μg/kg (HT2-toxin), 1 μg/kg (diacetoxyscirpenol), and 30 μg/kg (monoacetoxyscirpenol) and LOQs of 8 μg/kg (T2-toxin), 80 μg/kg (HT2-toxin), 4 μg/kg (diacetoxyscirpenol), and 80 μg/kg (monoacetoxyscirpenol). Thus, the developed SIDA was highly sensitive for analysis of diacetoxyscirpenol and T2-toxin and sufficiently sensitive for monoacetoxyscirpenol and HT2-toxin. The latter compounds are known to result in higher LODs compared to T2-toxin and diacetoxyscirpenol even when the most sensitive LC-MS/MS instruments were applied (*16*).

Recovery and Precision. Recovery was determined using different spiking levels. For all trichothecenes under study good and reproducible recoveries ranging between 90 and 130% were found, showing low standard derivations. In detail, recoveries were 127 $\pm$ 5% (spiking level = 20 μg/kg), 112 $\pm$ 2% (spiking level = 35 μg/kg), and 115 $\pm$ 7% (spiking level = 50 μg/kg) for T2-toxin. For HT2-toxin recoveries were 93 $\pm$ 16% (spiking level = 100 μg/kg), 107 $\pm$ 5% (spiking level = 200 μg/kg), and 99 $\pm$ 2% (spiking level = 300 μg/kg). Recovery values of 91 $\pm$ 6% (spiking level = 20 μg/kg), 90 $\pm$ 4% (spiking level = 35 μg/kg), and 91 $\pm$ 4% (spiking level = 50 μg/kg) were found for diacetoxyscirpenol. Monoacetoxyscirpenol was recovered in values of 112 $\pm$ 1% (spiking level = 200 μg/kg), 109 $\pm$ 3% (spiking level = 350 μg/kg), and 110 $\pm$ 1% (500 μg/kg). A representative chromatogram of a spiked wheat flour sample is shown in **Figure 6**. The single trichothecenes and their labeled counterparts are unequivocally visible and separated in their respective segments. CVs were determined in interassay precision studies (n = 3) and found to be 2.6% for diacetoxyscirpenol at 50 μg/kg, 3.1% for monoacetoxyscirpenol at 500 μg/kg, 5.4% for T2-toxin at 40 μg/kg, and 8.0% for HT2-toxin at 90 μg/kg.

Determination of Type A Trichothecenes in Food and Feed. To prove the suitability of the developed SIDA, different food samples from local markets with a focus on oats were analyzed (**Table 3**). In general, no contamination with monoacetoxyscirpenol was found above the LOD. In contrast, diacetoxyscirpenol occurred in a few samples and particularly was detectable in potatoes, but also a spelt grain and a corn grit sample contained distinct amounts of diacetoxyscirpenol. Whereas a sample of organically grown oat grain contained a very little amount of T2-toxin, in several oat flakes T2-toxin at levels >20 μg/kg was quantified. Contents of HT2-toxin were even higher and amounted to 100 μg/kg in one oat flakes sample. In pig feed consisting of different grains, smaller amounts of diacetoxyscirpenol were detected. In contrast to this, oat used for feeding horses was found to contain 60 μg/kg T2-toxin and 800 μg/kg HT2-toxin, thus indicating high contamination of grains used for animal nutrition.

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Received for review May 12, 2006. Revised manuscript received July 4, 2006. Accepted July 7, 2006.

JF061347+