JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of 12 Type A and B Trichothecenes in Cereals by Liquid Chromatography–Electrospray Ionization Tandem Mass Spectrometry

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A new sensitive method for the simultaneous determination of 12 trichothecenes (deoxynivalenol, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon X, T-2 toxin, HT-2 toxin, neosolaniol, monoacetoxyscirpenol, diacetoxyscirpenol, T-2 triol, and T-2 tetraol) by liquid chromatography−electrospray ionization tandem mass spectrometry (LC−ESI−MS/MS) is presented. The development of the method and investigations on the matrix influence on the MS signal are described in particular. The matrix effect was thereby minimized by using an internal standard, a special mobile phase, and specific fragmentation parameters. The sample was extracted with acetonitrile/water (84:16, v/v), and the extract was cleaned up with a MycoSep 227 column. Quantification was based on the internal standard de-epoxy-deoxynivalenol. Calibration curves were linear between 16 and 1600 ng/g, and the limits of detection ranged from 0.18 to 5.0 ng/g. The developed method was applied for the determination of trichothecenes in 120 naturally contaminated wheat and oat samples.

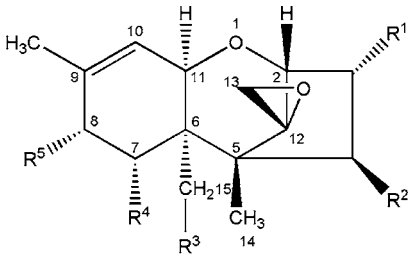
KEYWORDS: Trichothecenes; LC−MS/MS; food; cereals; analysis; matrix effects

INTRODUCTION

The mycotoxin-producing genus *Fusarium* is an important group of fungi, which grow on agricultural commodities in the field. Their growth may lead to contamination of grains with Fusarium toxins such as fumonisins, trichothecenes, and zearalenone. The occurrence of trichothecenes in a wide range of cereals, cereal-based food, and animal feedstuff has become of great concern worldwide in the last 20 years.

Trichothecenes are a large group of tetracyclic sesquiterpenoid mycotoxins produced by various *Fusarium* fungi. They are a group of over 150 different toxins and are classified in four groups: type A, B, C, and D. Type A trichothecenes include, e.g., T-2 toxin (T-2), HT-2 toxin (HT-2), neosolaniol, monoacetoxyscirpenol, diacetoxyscirpenol, T-2 triol, and T-2 tetraol, and differ from type B trichothecenes (deoxynivalenol, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and fusarenon X) by the absence of a carbonyl group at the C-8 position (*1*). Chemical structures of the trichothecenes investigated in this paper are shown in **Figure 1**. Deoxynivalenol and nivalenol are the most commonly found trichothecenes, followed by T-2, HT-2, and the deoxynivalenol-acetyl-derivates (*1*).

Trichothecenes are responsible for a wide range of toxicity symptoms in animals, causing toxic effects on skin and mucous surfaces, feed refusal, weight loss, vomiting, immunosuppressive and haemorrhaging effects, and inhibiting protein biosynthesis and RNA and DNA synthesis (*2*, *3*). The Scientific Committee



Trichothecene		MW	R^1	R^2	R^3	R^4	R^5
Type A							
Neosolaniol	(**1**)	382	OH	OAc	OAc	H	OH
HT-2 toxin (HT-2)	(**2**)	424	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
T-2 toxin (T-2)	(**3**)	466	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
T-2 triol	(**4**)	382	OH	OH	OH	H	$OCOCH_2CH(CH_3)_2$
T-2 tetraol	(**5**)	298	OH	OH	OH	H	OH
Monoacetoxyscirpenol	(**6**)	324	OH	OH	OAc	H	H
Diacetoxyscirpenol	(**7**)	366	OH	OAc	OAc	H	H
Type B							
Deoxynivalenol	(**8**)	296	OH	H	OH	OH	=O
3-Acetyl-DON	(**9**)	338	OAc	H	OH	OH	=O
15-Acetyl-DON	(**10**)	338	OH	H	OAc	OH	=O
Nivalenol	(**11**)	312	OH	OH	OH	OH	=O
Fusarenon-X	(**12**)	354	OH	OAc	OH	OH	=O

Figure 1. Chemical structures of type A and B trichothecenes.

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10.1021/jf051501c CCC: $30.25
Published on Web 10/12/2005

on Food (SCF) established a full tolerable daily intake (TDI) of 1 μg/kg bodyweight/day for deoxynivalenol and confirmed the TDI of 0.7 μg/kg bodyweight/day for nivalenol and the combined TDI of 0.06 μg/kg bodyweight/day for T-2 and HT-2 (*2*, *3*). In the German national food law, tolerance levels for deoxynivalenol, as low as 100 μg/kg in grains and grain products for infants, have been established in February 2004. Limits for deoxynivalenol will be applied by the European Union (EU) in June 2006. Further regulations in the μg/kg range for nivalenol, T-2, and HT-2 are currently under discussion by the EU authorities. To control these low limits, a sensitive and reliable method for the routine analysis of trichothecenes is required.

The first methods for the determination of trichothecenes in grains were based on thin-layer chromatography (TLC) (*4*, *5*) or gas chromatography (GC) with electron-capture detection (ECD) (*6*−*8*), flame-ionization detection (FID) (*9*), or mass-spectrometric detection (MS) (*10*−*12*). Because of the time intensive analysis, the use of toxic solvents such as chloroform, and very high limits of detection, TLC is not used any more. GC−MS analysis after derivatization is a highly sensitive method for the determination of both type A and B trichothecenes. The derivatization of the hydroxy groups is necessary to attain the volatility and sensitivity required for trace GC analysis. Almost all GC methods for determination of trichothecenes in food and cereals currently in use are based on derivatization forming trimethylsilyl (TMS) or fluoroacyl derivatives. However, because of matrix effects and the time-consuming derivatization procedure, GC methods are nowadays used more rarely (*13*). Alternatively, high-performance liquid chromatography (HPLC) methods with ultraviolet (UV) or fluorescence detection (FLD) have been used. In contrast to the B trichothecenes, which have a conjugated double bond, type A trichothecenes can only be detected by FLD after pre- or postcolumn derivatization. HPLC methods with ultraviolet detection are applicable only to B trichothecenes and require very effective cleanup procedures such as, e.g., immunoaffinity columns (*14*). For FLD, different pre- or postcolumn strategies have been established after derivatization with anthracene- or coumarin-carbonyl chlorides for A trichothecenes and methylacetoacetate for type B compounds (*15*−*19*). Nevertheless, the derivatization process requires a further step in the analysis, is time-consuming, and results usually in higher limits of detection.

Coupling of LC and MS provides a great opportunity for the analysis of mycotoxins. HPLC with MS detection eliminates the need for sample derivatization and tandem mass spectrometry and especially enables a very selective and sensitive detection. The first LC−MS methods for the determination of trichothecenes were based on fast-atom bombardment (FAB), thermospray, and plasmaspray ionization (*20*, *21*). Later, soft ionization techniques such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) have been used. The analysis of A trichothecenes by LC−APCI−MS has been reported by Razzazi-Fazeli et al. (*22*) and by LC−ESI−MS by Thimm et al. (*23*). Several methods for type B trichothecenes using ESI or APCI are described (*24*−*26*). Only a few LC−MS methods for the simultaneous determination of both type A and B trichothecenes exist in the literature (*27*−*31*). In these methods, ESI or APCI have been applied and only a range of different trichothecenes is determined, but no method for the simultaneous analysis of the 12 above-mentioned A and B trichothecenes has been published. Matrix effects in LC−MS are well-known, but there is a deficit in the literature on the investigation of the influence of interfering matrix in the LC−MS analysis of mycotoxins.

The aim of our study was to develop a reliable and sensitive LC−MS/MS method for the simultaneous determination of 12 type A and B trichothecenes (T-2, HT-2, neosolaniol, diacetoxyscirpenol, monoacetoxyscirpenol, T-2 triol, T-2 tetraol, deoxynivalenol, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and fusarenon X). In addition, the influence of matrix compounds during the LC−MS/MS analysis of trichothecenes was investigated in detail. The LC and MS parameters were optimized, and validation details including calibration graphs, limits of detection (LOD), recoveries, and instrument repeatability were determined. The validation of the method was carried out with the matrices durum, corn, and bread, and the method was applied to the analysis of 120 wheat and oat samples.

MATERIALS AND METHODS

Chemicals and Reagents. Trichothecene standards were purchased from Sigma (Taufkirchen, Germany), and de-epoxy-deoxynivalenol was purchased from Biopure (Tulln, Austria). Acetonitrile (HPLC grade), methanol (HPLC grade), ammonium acetate (puriss. p.a.), and ammonia solution 25% (analytical grade) were obtained from Merck (Darmstadt, Germany). Filter papers MN 619 1/4 from Macherey−Nagel (Dueren, Germany) were used for the filtration of the extracts. MycoSep 227 Trichothecene columns (Romer Labs Inc., Union, MO) were purchased from Coring System Diagnostix GmbH (Gernsheim, Germany). Deionized water was used for all procedures.

Sample Preparation. A total of 25 g of the finely ground sample was extracted with 100 mL of a mixture of acetonitrile/water (84:16, v/v) by blending at high speed for 3 min using an Ultra Turrax. The extract was filtered, and 5 mL of the filtrate was slowly pressed through a MycoSep 227 column. A total of 2 mL of the eluate was evaporated to dryness on a heated aluminum block at 50 °C using a gentle stream of nitrogen, and the residue was reconstituted in 0.5 mL of acetonitrile/water (1:4). A total of 50 μL of de-epoxy-deoxynivalenol solution [5 μg/mL in acetonitrile/water (1:4)] was added. The solution was mixed, and a 10 μL aliquot was used for LC−MS/MS analysis.

LC Parameters. LC analysis was performed using an Agilent 1100 series system consisting of a binary pump, a degasser, a column oven, and an autosampler (Agilent Technologies, Waldbronn, Germany). Analysis was done on a 250 × 2 mm i.d., 4 μm Synergi Fusion RP 80A column (Phenomenex, Aschaffenburg, Germany), and the column temperature was kept at 25 °C. The flow rate was set to 0.2 mL/min, and the injection volume was 10 μL. Solvent A was 0.00184 mM ammonia and 0.13 mM ammonium acetate in water (pH 7.4), and solvent B was acetonitrile. A linear binary gradient was applied changing from 20 to 70% solvent B within 25 min. Then, the content of solvent B was lowered to 20% within 1 min, and the column was re-equilibrated for 9 min.

MS Parameters. A Quattro Ultima tandem mass spectrometer (Micromass U.K. Limited, U.K.) was used in positive and negative ESI and APCI modes. The MS parameters were optimized in the scan mode by a constant infusion of each toxin standard into the LC flow using a syringe pump (Harvard apparatus, Holliston, MA) with a flow of 10 μL/min and a mixing tee. An acetonitrile/water (20:80, v/v) eluent was used for ESI, and a methanol/water (20:80, v/v) eluent was used for the chemical ionization. The APCI interface was used with a LC flow of 1 mL/min and with the following settings: corona discharge needle, 2 μA; cone voltage, 40 V; source temperature, 130 °C; desolvation temperature, 500 °C; desolvation and cone gas flows, 150 and 100 L/h; and collision energies, between 9 and 25 V. In the final method, the following ESI parameters were applied: LC flow, 0.2 mL/min; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation and cone gas flows, 600 and 250 L/h; capillary voltage, 3 kV; cone voltage, 30 V; and collision energies, between 8 and 23 V, depending upon the analyte. System control was carried out using Masslynx 4.0 software.

Determination of Ion Suppression and Enhancement. The LC−MS/MS instrument was used in ESI positive and negative modes. A standard solution of each toxin (5 μg/mL) was infused through a mixing

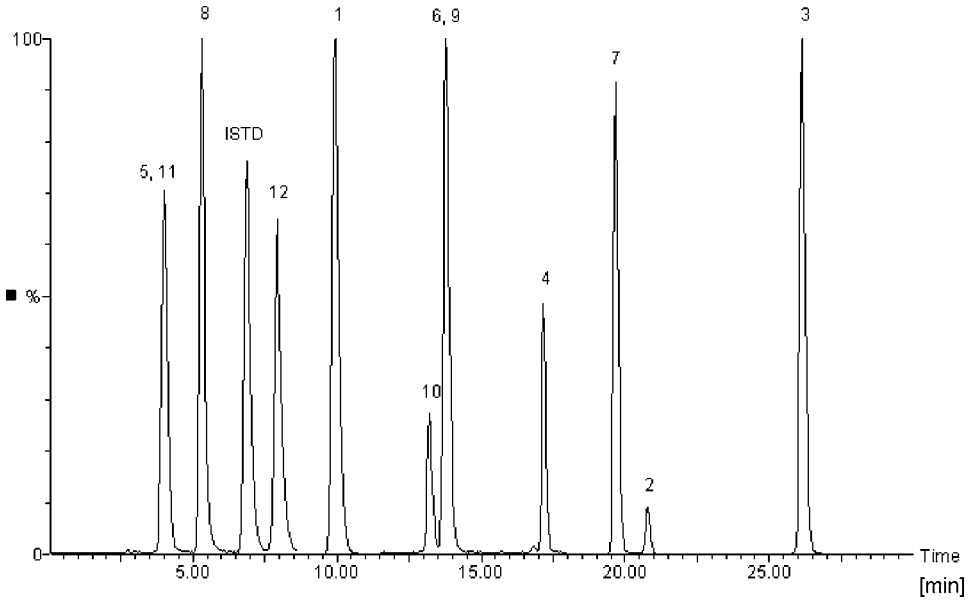

Figure 2. LC–ESI–MS/MS TIC of a durum sample spiked with 12 trichothecenes at a concentration level of 400 ng/g, cleaned up with Mycosep 227 columns (internal standard DED spiked at a concentration level of 400 ng/mL).

tee at a constant rate (10 μL/min) into the effluent flowing from the LC system (0.2 mL/min) to the mass spectrometer. After a steady baseline was obtained, a blank sample extract was injected into the LC system. Any eluted matrix compound that suppresses or increases ionization in the mass spectrometer will cause a drop or rise in the baseline (*32*).

Spiking and Recovery Experiments. Aliquots (100 μL) of a trichothecene standard solution containing T-2, HT-2, neosolaniol, diacetoxyscirpenol, monoacetoxyscirpenol, T-2 triol, T-2 tetraol, deoxynivalenol, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and fusarenon X were evaporated to dryness in vials on a heated aluminum block at 50 °C using a gentle stream of nitrogen. The residues were redissolved in either 500 μL of mobile phase or blank matrix extract (durum, corn, or bread extract cleaned up with Mycosep columns, respectively). A total of 50 μL of de-epoxy-deoxynivalenol solution (5 μg/mL) was transferred into each vial as ISTD and mixed, and 10 μL aliquots were injected into the LC−MS/MS system. The response of the solutions in mobile phase was set to 100%, and the response of the matrix solutions was calculated in % (all values corrected by the ISTD).

For testing the recovery, extracts of blank durum, corn, or bread samples were spiked with the above-mentioned trichothecene standard solution at levels of 150 and 400 ng/g, cleaned up with Mycosep columns, and analyzed. Three replicates were performed for each spiked sample extract.

Calibration Curves and Determination of the LOD. Mixed standard solutions of T-2, HT-2, neosolaniol, diacetoxyscirpenol, monoacetoxyscirpenol, T-2 triol, T-2 tetraol, deoxynivalenol, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and fusarenon X with concentrations between 16 and 1600 ng/mL were prepared with the mobile phase. To 500 μL of each solution, 50 μL of de-epoxy-deoxynivalenol solution (5 μg/mL) was added. The peak area of each toxin was plotted against the concentrations, and the calibration curves were calculated by linear regression. Matrix-assisted calibration curves were performed by spiking standard solutions of different concentrations (16−1600 ng/mL) to cleaned extracts of blank durum, corn, and bread samples. For the determination of the LOD, the signal-to-noise (S/N) ratio was calculated (peak to peak calculation with Masslynx 4.0 software) from the matrix-assisted calibration for each toxin and the LOD was determined by S/N to be more than 3:1. The limit of quantification (LOQ) is obtained by S/N to be more than 10:1.

RESULTS AND DISCUSSION

HPLC Separation. A range of methods for the determination of either type A or B trichothecenes are reported in the literature. Berger et al. (*28*), Biselli et al. (*30*), and Berthiller et al. (*31*) determined B trichothecenes and some A trichothecenes, but these methods do not for example include monoacetoxyscirpenol, T-2 triol, and T-2 tetraol. Besides, they did not perform a separation of 3-acetyldexynivalenol and 15-acetyldexynivalenol, because their aim was a fast chromatography method. Up to now, there is no published HPLC separation method for the 12 described trichothecenes in a single run, but there is a need for a confident and reliable analysis method for all important trichothecenes.

In our study, three different Phenomenex Synergi analytical columns (Hydro, Polar, and Fusion) were tested for separation efficiency and sensitivity. The Phenomenex Synergi Fusion column showed the lowest noise and a good separation efficiency and was therefore chosen for this study. Because it was not possible to separate all 12 toxins under isocratic conditions, a gradient changing from 20 to 70% acetonitrile was developed. **Figure 2** shows a LC−ESI−MS/MS total ion chromatogram (TIC) of a durum extract cleaned up with a Mycosep column and spiked with 12 type A and B trichothecenes at a level of 400 ng/g each, including the internal standard (ISTD) de-epoxy-deoxynivalenol. All toxins (including the ISTD), except nivalenol/T-2 tetraol and monoacetoxyscirpenol/3-acetyldeoxynivalenol, are baseline-separated. The coelution of nivalenol/T-2 tetraol and monoacetoxyscirpenol/3-acetyldeoxynivalenol does not affect the selectivity of the method, because these toxins have different masses and show different fragmentations. Absolute retention time variations for 10 injections within 48 h were between 3 and 6 s.

MS Method. The MS parameters (cone and capillary voltage, desolvation, nebulizer, and cone gas flows, desolvation temperature, mass resolutions, and collision energies) for all toxins were optimized in the ESI and APCI modes, each with positive and negative ionization. This was achieved by continuous infusion of a standard solution of each toxin into the MS/MS. Biselli et al. (*30*) and Berthiller et al. (*31*) used methanol to improve the sensitivity of their methods. We used acetonitrile/water in the ESI and methanol/water in the APCI mode. This is due to the fact that methanol from different manufacturers very often contained impurities, and therefore, we observed interferences in the ESI mode when using methanol. For the

comparison of the four ionization techniques positive and negative ESI and APCI, multiple reaction monitoring (MRM) methods were created in each mode containing the two most intensive fragmentations of each precursor ion. TICs of a toxin standard solution were recorded in each mode, and the S/N ratio was calculated for all signals in each extracted ion chromatogram. In the APCI negative mode, all toxins showed very poor sensitivity, and therefore, no MS/MS method could be created. For all type B trichothecenes, monoacetoxyscirpenol, T-2 triol, and T-2 tetraol, the response in the ESI negative mode was found to be more sensitive than in the positive mode using ESI or APCI. The best sensitivity for T-2, HT-2, neosolaniol, and diacetoxyscirpenol was obtained with positive electrospray ionization. Biselli et al. (*30*) reported similar results in 2004, whereas Berthiller et al. (*31*) found the best sensitivity with methanol and APCI using a QTrap LC−MS/MS system. Applying negative ionization, the precursor ions of all toxins were detected in their deprotonated form ($[M-H]^-$). With positive ionization, all mass spectra, except these of T-2, HT-2, neosolaniol, and diacetoxyscirpenol showed intensive $[M+H]^+$ signals. Very intensive ammonium adducts and small $[M+H]^+$ base peaks were obtained for T-2, HT-2, neosolaniol, and diacetoxyscirpenol, although no ammonia salts were added to the eluent. Traces of ammonium ions, generally present even in deionized water, generated these adducts. When small amounts of ammonium acetate were added to the eluent, the response of the four above-mentioned A trichothecenes in ESI positive mode could be increased 2−3 times.

Because the best sensitivity for deoxynivalenol, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon X, monoacetoxyscirpenol, T-2 triol, and T-2 tetraol was found in the negative ESI mode and for T-2, HT-2, neosolaniol, and diacetoxyscirpenol in the positive ESI mode, switching between the two modes and an adjusted mobile phase were necessary. Ammonium acetate had to be added to the eluent for the formation of ammonium adducts in the positive mode. Because of the formation of acetate adducts in the negative mode, which showed no specific fragmentation, the amount of ammonium acetate had to be very small. Furthermore, a neutral pH was needed for sensitive negative ionization; therefore, the minimum amount of ammonium acetate (0.13 mM in water) was added, and the pH was set to 7.4 with ammonia (0.00184 mM in water). In our MS method, we were not able to use either sodium adducts nor acetate adducts as precursor ions, as reported by Biselli et al. and Berthiller et al., respectively (*30*, *31*). With our instrument, no fragmentation of these adducts could be achieved. Nevertheless, a very sensitive and specific detection in the positive and negative mode was possible by switching between the ionization modes.

Table 1 shows the obtained precursor and product ions in ESI positive and negative modes. The bold-marked fragmentations were used for the final ESI MRM method. Fragmentations of the trichothecenes are characterized by the loss of 18 amu (H_2O), 28 amu (CO), 30 amu (CH_2O), and 42 amu (CH_2CO) from the substituents or the epoxide ring. Berger et al. (*28*) described the same or similar results obtained with an ion-trap mass spectrometer.

Matrix Effect Investigations. The LC−MS technique often shows matrix effects, especially in the case of complex samples, such as food. Lagana et al. (*24*), Berger et al. (*28*), and Biselli et al. (*30*) reported these effects but did not investigate them. In our studies, we carried out detailed experiments to demonstrate the matrix influence on the ionization.

When running extended sequences, a continuous rise in the MS response was noticed. A possible reason is the contamination of the interface with matrix compounds leading to a signal increase. Therefore, the use of an ISTD was applied. Verrucarol and de-epoxy-deoxynivalenol, a decomposition metabolite of deoxynivalenol, were tested for their applicability as ISTD. It was not possible to achieve a baseline separation of verrucarol and fusarenon X using different gradients. Although these two toxins showed different fragmentations, an interfering effect was observed for fusarenon X when using verrucarol as ISTD. Furthermore, the sensibility for verrucarol was 3 times lower than for de-epoxy-deoxynivalenol. With de-epoxy-deoxynivalenol as an ISTD, all toxins were baseline-separated and no interferences were noticed. The quantification of samples was performed by external standard calibration and correction of each value by the ISTD de-epoxy-deoxynivalenol. The analysis of sequences using this calculation showed that the effect of the "response rise" could be eliminated.

A possible reason for matrix effects is the influence of different matrix compounds such as carbohydrates on the analyte ionization. A detailed investigation of this influence was done by a constant infusion of a toxin standard solution into the LC flow and the injection of blank matrix samples. In this way, the retention time of the interfering matrix compounds was determined and the ion suppression or enhancement could be made "visible" in a chromatogram. **Figures 3** and **4** show such chromatograms for nivalenol and diacetoxyscirpenol in the bread matrix. The drop in the baseline at 2.5 min in **Figure 3** indicates a strong ion suppression region between 2 and 3 min. A slight drop at 4 min, which is the retention time of nivalenol, indicates a disturbance in the ionization process of nivalenol. In contrast to this result, no changes of the baseline at 20 min were observed in the chromatogram for diacetoxyscirpenol in **Figure 4**. The drop at 12 min does not influence the ionization of diacetoxyscirpenol, which has a retention time of 20 min. Except for nivalenol, no ion suppression or enhancement could be observed for the 12 toxins using this method to determine matrix effects. Usually, stable isotope-labeled standards are used to compensate the matrix effect. Such standards are not available commercially

Table 1. MS/MS Parameters for the Determination of 12 Trichothecenes in ESI Negative and Positive Mode[a]

	ESI negative			ESI positive		
toxin	Q1 *m/z*	Q3 *m/z*	CE (eV)	Q1 *m/z*	Q3 *m/z*	CE (eV)
8	**295 $[M-H]^-$**	**265**	**12**	297 $[M+H]^+$	249	12
8	**295 $[M-H]^-$**	**247**	**12**	297 $[M+H]^+$	231	14
11	**311 $[M-H]^-$**	**281**	**11**	313 $[M+H]^+$	295	8
11	**311 $[M-H]^-$**	**191**	**19**	313 $[M+H]^+$	247	10
9	**337 $[M-H]^-$**	**307**	**11**	339 $[M+H]^+$	279	11
9	**337 $[M-H]^-$**	**173**	**11**	339 $[M+H]^+$	231	13
10	**337 $[M-H]^-$**	**219**	**11**	339 $[M+H]^+$	321	8
10	**337 $[M-H]^-$**	**150**	**18**	339 $[M+H]^+$	261	11
12	**353 $[M-H]^-$**	**263**	**12**	355 $[M+H]^+$	337	10
12	**353 $[M-H]^-$**	**187**	**23**	355 $[M+H]^+$	247	12
2	465 $[M-H]^-$			**484 $[M+NH_4]^+$**	**245**	**14**
2	465 $[M-H]^-$			**484 $[M+NH_4]^+$**	**215**	**17**
3	423 $[M-H]^-$			**442 $[M+NH_4]^+$**	**263**	**13**
3	423 $[M-H]^-$			**442 $[M+NH_4]^+$**	**215**	**12**
1	381 $[M-H]^-$			**400 $[M+NH_4]^+$**	**305**	**13**
1	381 $[M-H]^-$			**400 $[M+NH_4]^+$**	**215**	**15**
7	365 $[M-H]^-$			**384 $[M+NH_4]^+$**	**307**	**12**
7	365 $[M-H]^-$			**384 $[M+NH_4]^+$**	**247**	**15**
6	**367 $[M+CO_2-H]^-$**	**201**	**9**	342 $[M+NH_4]^+$	107	15
6	**367 $[M+CO_2-H]^-$**	**59**	**13**	342 $[M+NH_4]^+$		
4	**381 $[M-H]^-$**	**101**	**20**	383 $[M+H]^+$		
5	**297 $[M-H]^-$**	**219**	**14**			
5	**297 $[M-H]^-$**	**201**	**18**			

[a] The bold-marked fragmentations were used for the final MRM method.

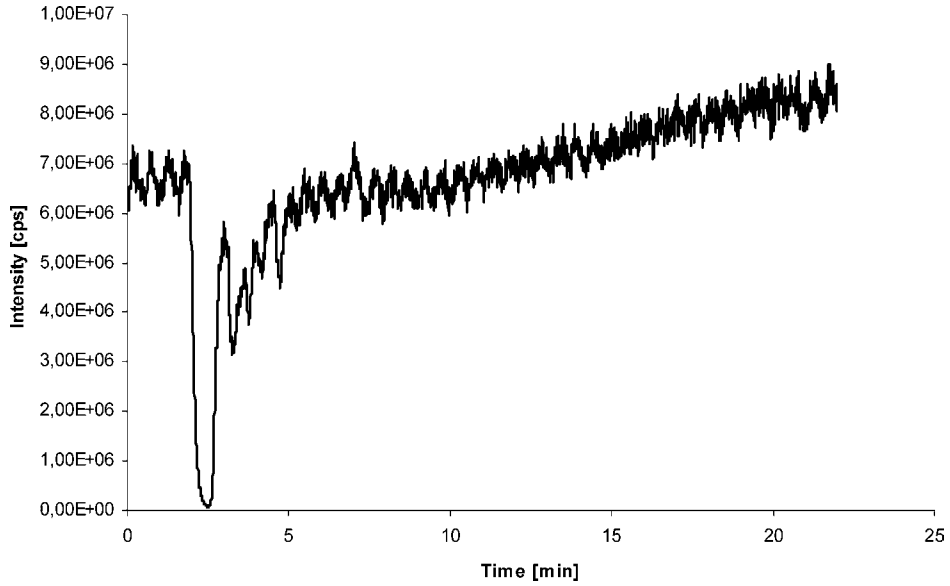

Figure 3. LC–MS/MS chromatogram (ESI negative) of a constant infusion of NIV after injection of a blank bread extract, cleaned up with Mycosep 227 columns.

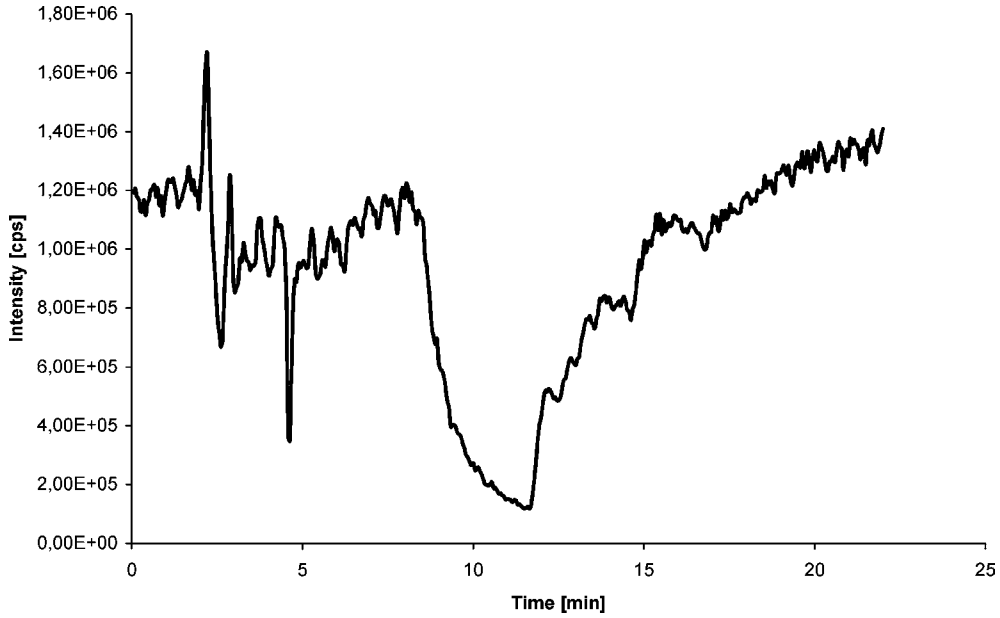

Figure 4. LC–MS/MS chromatogram (ESI positive) of a constant infusion of DAS after injection of a blank bread extract, cleaned up with Mycosep 227 columns.

for trichothecenes. Therefore, we investigated the use of de-epoxy-deoxynivalenol as an ISTD to eliminate this effect. The response difference between a standard calibration and a matrix-assisted calibration could be decreased from 23 to 12% when using an ISTD. For further investigations, the response of standard solutions in the eluent and matrix-assisted standard solutions with the same concentrations, calculated on ISTD, was compared. **Table 2** shows the results for the matrices durum, corn, and bread in % (the response of the standard solutions in the eluent was set to 100%), calculated on ISTD. Only T-2 and diacetoxyscirpenol showed a signal increase by 4−14%. The signal suppression of all other toxins was reduced from >35 to 1−20%. These results demonstrate that the matrix effect could not be eliminated completely, but it was minimized as far as possible by the use of an internal standard.

Calibration Curves and Determination of the LOD. For all tested mycotoxins, calibration graphs show very good correlation (r^2 between 0.9960 and 0.9996) in the matrices durum, bread, and corn. **Table 3** shows the linearity ranges for the tested compounds for durum. The calibration curves of all toxins, except T-2 and diacetoxyscirpenol, were linear in the range of 16−1600 ng/g. The calibration curves of T-2 and diacetoxyscirpenol were linear in the range of 16−1200 ng/g. This is due to the very high response of these two compounds. The LOD was defined as the concentration of the analyte in the corresponding matrix giving a S/N ratio of at least 3:1 (calculated from the extracted ion chromatogram). The LOQ is obtained by S/N to be more than 10:1. The LODs of all investigated trichothecenes are given in **Table 3**. The lowest LOD was found for T-2 triol in corn with 0.18 ng/g. The highest LOD was obtained for HT-2 in durum with 5 ng/g. These LODs are lower than those reported, e.g., by Razzazi-Fazeli et al. (*22*, *26*) with two different methods analyzing type A or B trichothecenes. They are also lower than those obtained by Berger et al. (*28*), although they determined the LOD with pure reference substances and not with the matrix. Biselli et al. (*30*)

Table 2. Calculated Response in % of Spiked Durum, Corn, and Bread Extracts Compared with the Response of Standard Solutions (Set to 100%)

	calculated response in %		
toxin	durum	corn	bread
8	97	92	92
11	89	94	80
9	97	97	93
10	94	98	93
12	93	95	89
3	114	105	106
2	95	83	85
1	96	83	88
7	111	104	105
6	95	91	91
4	99	94	95
5	93	85	85

Table 3. LOD, Linearity Ranges, and Correlation Coefficients for the Matrices Durum, Corn, and Bread, Cleaned Up with Mycosep 227 Columns

	LOD (ng/g)			
toxin	durum	corn	bread	linearity range (ng/g)
8	1.5	1.2	1.0	16–1600
11	1.0	2.1	1.8	16–1600
9	0.4	0.5	0.5	16–1600
10	0.8	1.2	1.1	16–1600
12	1.3	1.3	1.1	16–1600
3	0.2	0.2	0.2	16–1200
2	5.0	4.4	4.2	32–1600
1	0.6	0.7	0.6	16–1600
7	0.5	0.5	0.6	16–1200
6	2.0	2.7	3.5	16–1600
4	0.2	0.3	0.2	16–1600
5	1.8	2.8	1.5	16–1600

and Berthiller et al. (*31*) recently reported similar results using two different LC−MS/MS systems. The LODs of GC−MS methods for trichothecenes vary between 1 and 60 ng/g (*7*−*12*), and these of HPLC methods with either UV or FLD vary between 3 and 50 ng/g (*4*−*6*). Thus, the described LC−MS/MS method allows a very sensitive and simultaneous determination of type A and B trichothecenes in different matrices and enables the control of the planed EU limits for deoxynivalenol, nivalenol, T-2, und HT-2.

Performance Validation. Extracts of blank durum, corn, and bread samples spiked with 12 trichothecenes and the ISTD were injected sequentially 10 times within 72 h to evaluate the instrument repeatability. The obtained relative standard deviation (RSD) of the signal area was between 3.4 and 8.4%. The same extracts were injected after 5 and 10 days to determine the interday precision of the mass spectrometer. The precision values were between 5.4 and 9.5% and therefore smaller than those reported by Razzazi-Fazeli et al. (*22*).

For the verification of the results obtained when measuring sample extracts by LC−MS/MS, the same extracts ($n = 10$) were analyzed for deoxynivalenol with a HPLC method based on FLD after postcolumn derivatization. This HPLC method was validated in our laboratory for different matrices, and no matrix interferences were observed using FLD (*14*). The deviations of the HPLC−FLD and LC−MS/MS measurements for each sample ranged between 0.15 and 5.0%, and the values showed no significant difference (two-sided F and t tests, $p = 0.05$). These results point out the reliability and routine capability of the developed LC−MS/MS method.

Table 4. Recoveries and RSD in % Obtained for 12 Trichothecenes from Spiked Durum, Corn, and Bread Samples, after Cleanup with Mycosep 227 Columns at a Spiking Level of 150 ng/g ($n = 3$)

toxin	recovery (%) and RSD (%) ($n = 3$) durum	recovery (%) and RSD (%) ($n = 3$) corn	recovery (%) and RSD (%) ($n = 3$) bread
8	83 ± 2.4	85 ± 5.6	81 ± 2.2
11	59 ± 5.4	59 ± 9.4	53 ± 6.3
9	65 ± 8.0	70 ± 6.2	65 ± 1.4
10	79 ± 2.9	80 ± 3.6	74 ± 2.4
12	82 ± 2.4	80 ± 2.9	82 ± 2.2
3	88 ± 3.5	87 ± 3.7	89 ± 3.3
2	66 ± 1.9	74 ± 4.4	68 ± 1.9
1	82 ± 7.9	84 ± 4.8	80 ± 5.9
7	90 ± 1.9	92 ± 3.4	89 ± 2.3
6	85 ± 3.6	86 ± 2.3	80 ± 1.7
4	78 ± 6.5	77 ± 5.1	75 ± 4.2
5	60 ± 8.4	59 ± 6.6	55 ± 4.4

Table 5. Recoveries and RSD in % Obtained for 12 Trichothecenes from Spiked Durum, Corn, and Bread Samples, after Cleanup with Mycosep 227 Columns at a Spiking Level of 400 ng/g ($n = 3$)

toxin	recovery (%) and RSD (%) ($n = 3$) durum	recovery (%) and RSD (%) ($n = 3$) corn	recovery (%) and RSD (%) ($n = 3$) bread
8	75 ± 8.8	83 ± 7.9	73 ± 9.2
11	53 ± 16.5	54 ± 11.3	49 ± 12.5
9	71 ± 9.0	74 ± 7.8	72 ± 10.1
10	86 ± 1.3	83 ± 2.6	81 ± 2.3
12	69 ± 2.3	75 ± 5.7	73 ± 2.2
3	87 ± 4.7	85 ± 3.9	85 ± 2.5
2	76 ± 2.8	79 ± 3.4	71 ± 1.9
1	90 ± 5.4	93 ± 3.4	94 ± 5.7
7	95 ± 1.7	92 ± 1.2	94 ± 2.1
6	88 ± 1.6	90 ± 4.3	88 ± 1.2
4	80 ± 7.8	81 ± 5.7	79 ± 8.9
5	51 ± 14.2	48 ± 8.9	46 ± 7.6

To determine the recovery of the complete method, trichothecene standard solutions were added to extracts of blank durum, corn, and bread samples and the extracts were cleaned up with Mycosep columns ($n = 3$). The direct spiking of samples with standards for recovery experiments seems critical because spiked samples do not represent the conditions in natural contaminated samples. To determine only the performance of the Mycosep columns, the extracts were spiked and not the samples. In **Tables 4** and **5**, the recovery rates obtained for spiking levels of 150 and 400 ng/g, corrected by ISTD, are summarized. The recoveries ranged between 46 and 95%, showing the lowest values for nivalenol and T-2 tetraol. One possible reason for this might be the inadequate content of water in the extraction solution (84:16 MeCN/H_2O, v/v). To elute the polar compounds NIV and T-2 tetraol from the Mycosep column containing polar adsorbents such as alumina, a hydrophilic solvent is needed. When using more polar solvent mixtures such as 75:25 MeCN/H_2O, the recoveries of the polar toxins nivalenol and T-2 tetraol were raised from 50 to >75%. The extraction yield of deoxynivalenol from naturally contaminated samples ($n = 25$) was also higher by up to 40%, but the higher water content in the solvent resulted in coextraction of more matrix compounds and led to strong ion suppression in the MS analysis. Currently, we are investigating the extraction process, cleanup, and the effect of these parameters on the MS signal.

The developed method was applied for the determination of trichothecenes in naturally contaminated wheat ($n = 100$) and oat ($n = 20$) samples collected from different mills in Germany.

In wheat, only type B trichothecenes, especially deoxynivalenol, nivalenol, and 15-acetydeoxynivalenol, were found. The contents ranged between 10 and 3700 ng/g for deoxynivalenol and between 1 and 70 ng/g for nivalenol and 15-acetydeoxynivalenol. In contrast to wheat, most of the oat samples were contaminated with HT-2, T-2, nivalenol, and T-2 tetraol. Up to 200 ng/g T-2 tetraol were determined in these samples. These results show that it was important to develop a combined method for the analysis of all type A and B trichothecenes, because no LC−MS method for the determination of T-2 tetraol exists yet.

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Received for review June 24, 2005. Revised manuscript received September 14, 2005. Accepted September 14, 2005. We would like to thank the Landesstiftung Baden-Württemberg gGmbH for the financial support of this project (project: P-LS-E/MTP A5-13).

JF051501C