Quantitative Determination and Structure Elucidation of Type A- and B-Trichothecenes by HPLC/Ion Trap Multiple Mass Spectrometry

Urs Berger, Michael Oehme,* and Fabian Kuhn

Organic Analytical Chemistry, University of Basel, Neuhausstrasse 31, CH-4057 Basel, Switzerland

A method is presented for the quantification and structure confirmation of trichothecenes in wheat by high-performance liquid chromatography combined with multiple mass spectrometry (MS^n). Nine type A- and B-trichothecenes were determined (nivalenol, deoxynivalenol, fusarenon-X, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, neosolaniol, diacetoxyscirpenol, HT-2 toxin, and T-2 toxin). Extraction was carried out with acetonitrile/water. The extract was purified on a MycoSep column. Quantification was based on an internal standard and atmospheric pressure chemical ionization in the positive ion mode. Recoveries from spiked wheat were in the range of 80−106% at levels of 500 ppb. The limits of quantification for the whole method were between 10 and 100 ppb. Ion adduct formation with ammonium and acetate ions and MS^n experiments provided information about substitution and fragmentation behavior of the mycotoxins. A scheme has been established for the partial structure elucidation of type A- and B-trichothecenes in fungal cultures.

Keywords: *HPLC/multiple MS; type A- and B-trichothecenes; quantification; Fusarium cultures; structure elucidation*

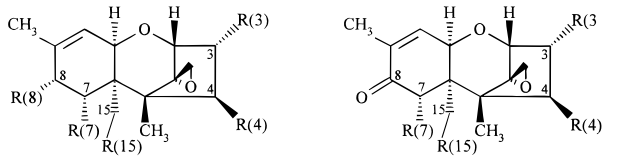
INTRODUCTION

Trichothecenes are sesquiterpenoid mycotoxins, mainly produced by various species of *Fusarium* fungi. Such fungi invade agricultural products, particularly grains, worldwide. More than 140 different trichothecenes have been isolated from fungal cultures (Grove, 1993), and this number is still increasing. Trichothecenes are subdivided into four different groups. Type B-trichothecenes differ from type A by the presence of a α,β-unsaturated carbonyl group (see Figure 1). These two types comprise about 100 of the isolated toxins. Type C are characterized by an additional epoxide, and type D are macrocyclic trichothecenes. The diversity of trichothecenes leads to a wide range of toxic effects in animals and humans such as feed refusal, vomiting, anemia, hemorrhage, and immunosuppression (Prelusky et al., 1994). However, only a limited number of the known compounds have been identified in *Fusarium*-infected crops.

Deoxynivalenol (DON) is most frequently detected in grain samples, but also nivalenol (NIV), fusarenon-X (F-X), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), neosolaniol (NEO), diacetoxyscirpenol (DAS), HT-2 toxin (HT-2) and T-2 toxin (T-2) can be found (Yoshizawa and Jin, 1995; Patel et al., 1996; Müller et al., 1997) and were therefore included in the presented method. Their structures are given in Figure 1.

Much effort has been focused on the development of quick and reliable methods for the quantification of trichothecenes. Several reviews about applied chromatographic techniques have been published (Gilbert, 1993; Scott, 1995; Langseth and Rundberget, 1998; Lin et al., 1998). Separation was based on thin-layer, high-performance liquid (HPLC) and high-resolution gas chromatography (HRGC). In recent years, newer techniques such as supercritical fluid chromatography (Young and Games, 1992, 1993) and immunoassays (Park and Chu, 1996) were proposed as well. At present, the most frequently applied method is HRGC following derivatization combined with either electron capture or MS detection.

This paper describes a method for the determination of nine of the most important type A- and B-trichothecenes by HPLC combined with ion trap mass spectrometry. Quantification was based on the addition of an internal standard to the sample prior to extraction. Details are given about the method performance including the linear range of the mass spectrometric detection, recoveries, and limits of determination as well as determination of blanks. Furthermore, based on the 10



Type A Trichothecenes							Type B Trichothecenes					
	MW	R(3)	R(4)	R(7)	R(8)	R(15		MW	R(3)	R(4)	R(7)	R(15)
NEO	382.2	OH	OAc	H	OH	OAc	**NIV**	312.1	OH	OH	OH	OH
DAS	366.2	OH	OAc	H	H	OAc	**DON**	296.1	OH	H	OH	OH
HT-2	424.2	OH	OH	H	i-Val	OAc	**F-X**	354.1	OH	OAc	OH	OH
T-2	466.2	OH	OAc	H	i-Val	OAc	**3-ADON**	338.1	OAc	H	OH	OH
VOL	266.2	H	OH	H	H	OH	**15-ADON**	338.1	OH	H	OH	OAc

MW: Molecular weight
OAc: acetyl
i-Val: *iso*-valeryl

Figure 1. Structures of the investigated type A- and B-trichothecenes.

* Corresponding author (phone, +41 61 639 23 01; fax, +41 61 639 23 00; e-mail, oehme@ubaclu.unibas.ch).

10.1021/jf9904012 CCC: $18.00
Published on Web 09/18/1999

trichothecenes in Figure 1 plus T-2 triol and acetyl T-2, a scheme was established that allowed unequivocal identification of known compounds and at least partial elucidation of the structure of unknown trichothecenes by on-line MS/MS and ion adduct formation with ammonium acetate.

MATERIALS AND METHODS

Materials. Trichothecene standards of the following certified purity were purchased from Sigma Chemie (Buchs, Switzerland): >97.5% (HT-2), 98% (T-2 triol), ≥98% (verrucarol, T-2), 99% (F-X, 15-ADON), ≥99% (NIV, NEO), and >99% (DON, 3-ADON, DAS). Acetyl T-2 was synthesized by reacting 200 μg of T-2 triol with eight drops of acetic anhydride (99.5%, Fluka Chemie AG, Buchs, Switzerland) and eight drops of pyridine (99.8%, Fluka Chemie AG, Buchs, Switzerland) at 0 °C for 90 min. Hydrocortisone (purum, >97%) was obtained from Fluka Chemie AG (Buchs, Switzerland), and analytical grade ammonium acetate was from E. Merck AG (Darmstadt, Germany). Acetonitrile (190 far-UV) was provided by Romil Ltd. (Cambridge, England), and methanol (pestipur) was provided by SDS (Peypin, France). Water was obtained from an Elgastat Maxima HPLC water purification unit (Elga Ltd., Bucks, England). Helium of 99.996% and nitrogen of 99.995% purity were used (Carbagas, Switzerland).

MycoSep 227 trichothecene cartridges and final cleanup columns 216 (Romer Labs Inc., Union, MO) were obtained from Coring-System Diagnostix GmbH (Gernsheim, Germany). They contain various adsorbents such as charcoal, Celite, and alumina. For filtration of the extracts, folded cellulose filters of medium porosity were used (Catalog No. 311845, Schleicher & Schuell, Feldbach, Switzerland).

The wheat flour used for the interlaboratory comparison test was prepared from *Fusarium*-infected and contaminated wheat batches (Pettersson, 1998). Rice samples were autoclaved and inoculated with *Fusarium* cultures (Langseth et al., 1999). Both the wheat and the rice samples were provided by the Veterinary Institute in Oslo (Norway). Commercial wheat flour, which contained only minor amounts of DON (estimated to 18 ppb), was used for recovery experiments. The lowest spike level of DON was ca. five times higher than the content of the wheat.

Sample Preparation. Wheat kernels were ground to pass a 1-mm sieve. For quantification, 15 μg of the internal standard verrucarol in 150 μL of methanol was added to the ground wheat in an open flask 15 h before extraction. A total of 1.5 μg of the recovery standard hydrocortisone in 150 μL of methanol/water (1:3) was transferred to the sample extract before HPLC separation.

Extraction and Cleanup. A total of 10 g of the milled and well-mixed sample was extracted with 40 mL of acetonitrile/water (84:16) in a 500-mL flask on a wrist-action shaker for 120 min. The extract was filtered through a cellulose filter, and 4 mL of the filtrate was slowly pressed through a MycoSep 227 column. An additional 7 mL of acetonitrile/water (84:16) was filled in the culture tube of the MycoSep 227 cartridge and pressed through the column. A final cleanup column 216 was conditioned with 6 mL of acetonitrile/water (84:16) that was discarded. The combined eluate of the MycoSep 227 column was transferred to the final cleanup column 216. This column was eluted with 12 mL of acetonitrile/water (84:16). Concentration of the entire eluate was carried out in a V-shaped flask on a heated aluminum block at 45 °C using a gentle flow of nitrogen. At a volume of 0.5−1 mL, the solvent was changed to methanol/water (1:3) by repeated addition of methanol and water and reconcentration. In this way, evaporation to dryness could be avoided. The volume of the final clear extract was 0.5−1 mL.

In contrast to the procedure described above, extracts of rice cultures were cleaned up on the MycoSep 227 cartridge without the rinse step. Approximately 8 mL of filtrate was pressed through the column, and 4 mL of the eluate was purified further on a final cleanup column 216.

Separation and Quantification. A low-pressure binary gradient HPLC pump (Rheos 4000, Flux Instruments, Basel, Switzerland) was employed. The samples were injected with a Valco Cheminert valve equipped with a 5- or 20-μL loop. HPLC separation was carried out on a C18 normal density phase (Nucleosil, 120 Å pore size, 3 μm particles, 125 mm column length, 2 mm i.d., Macherey-Nagel, Oensingen, Switzerland). The flow rate of the mobile phase was 250 μL/min. A linear binary gradient was applied changing from 25% to 98% methanol in water from injection to 12 min, followed by 5 min rinsing with 98% methanol. Then the methanol content was lowered to 25% within 1 min, and the column was reequilibrated with this eluent for 6 min.

An ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) was used in the positive ion mode employing atmospheric pressure chemical ionization (APCI(+)). Mass spectra were registered in the full-scan mode (mass range 150−550 u). The following instrument parameters were applied: heater temperature, 230 °C; nitrogen sheath gas flow, 40 arbitrary units (corresponding to ca. 400 mL/min); ionization current of corona discharge, 1.5 μA. Voltages of the following devices were optimized by the autotune program to achieve maximum transmission of the $[M + H]^+$ ion of 3-ADON: heated capillary; tube lens offset; octapole 1 offset; inter octapole lens; octapole 2 offset; octapole rf amplitude. The temperature of the capillary between the ionization chamber and the first vacuum stage was held at 150 °C.

The presence of trichothecenes in real samples was confirmed by retention time and fragmentation pattern. Quantification was carried out using the mass chromatograms of the $[M + H]^+$ ions (internal and recovery standard, DON, F-X, 3-ADON, DAS, and HT-2) or the fragment ions *m*/*z* 294.9 + 312.7 (NIV), *m*/*z* 261.1 (15-ADON), *m*/*z* 334.8 (NEO), and *m*/*z* 334.9 + 466.6 (T-2).

For ion adduct experiments, ammonium acetate (50 mM in water) was added via a T-piece between the HPLC column and the ionization chamber at a flow rate of 5 μL/min resulting in a final concentration of ca. 1 mM. Ammonium adducts were registered in the positive mode, and acetate adducts were registered in the negative ion mode (APCI(−)). MS^n experiments were performed by repeated collision-induced decays in the trap applying a relative collision energy between 25% and 35%.

RESULTS AND DISCUSSION

Internal and Recovery Standard. Verrucarol (VOL, see Figure 1) is a semi-synthetic trichothecene obtained by the hydrolysis of macrocyclic precursors. Up to now, VOL has not been found naturally. It was selected as internal standard (ISTD) and added to the ground wheat prior to extraction. Losses during cleanup and fluctuations of MS performance were corrected by quantifying the trichothecenes relative to the ISTD. Hydrocortisone (HYC) has a structure, molecular weight, and polarity comparable to trichothecenes. It was employed as a recovery standard (RSTD) and added before separation. This allowed us to control the recovery of the ISTD and the performance of the extraction and cleanup procedure.

Extraction and Cleanup. The applied cleanup procedure allowed an almost complete removal of the sample matrix and a interference-free MS detection (see Figure 2). NIV and DON as the most polar trichothecenes and T-2 as the least polar trichothecene showed the poorest recovery rates after extract purification and volume reduction. Compared to other working groups (Weingaertner et al., 1997; Langseth and Rundberget, 1998) who reported recoveries for the MycoSep 225 column of around 66% for NIV and >80% for T-2, we first found only 40% ($n = 2$) for NIV and 61% ($n = 2$) for T-2 with the MycoSep 227 column at a spiking level

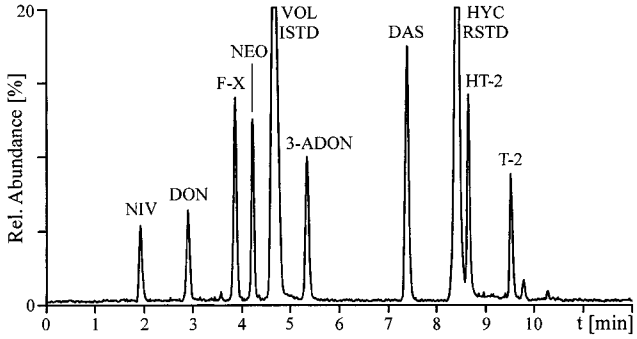


Figure 2. Base peak chromatogram (*m*/*z* 290−550) of the extract from wheat flour spiked with eight type A- and B-trichothecenes and the applied internal standard. The recovery standard was added before separation. Spike levels: NIV, 1190 ppb; DON, 728 ppb; F-X, 456 ppb; NEO, 456 ppb; VOL, 2000 ppb (relative abundance: 100%); 3-ADON, 226 ppb; DAS, 244 ppb; HYC, 2000 ppb (relative abundance: 79%); HT-2, 436 ppb; T-2, 504 ppb. For further details see Materials and Methods.

of 500 ppb. By using an additional rinse, the recovery rates were increased to ≥80% for all trichothecenes under investigation (see Table 1) without increasing the matrix background. The final cleanup column 216 removes any further remaining sample matrix without increasing significantly the processing time since it also replaces the filtration step necessary after the MycoSep 227 column.

Evaporation of the extract to dryness led to losses in the range of 14−30% ($n = 2$) for the different trichothecenes and a correspondingly poor repeatability. A change of the solvent to methanol/water during extract concentration solved this problem. Up to 15 samples can be extracted and purified with the described procedure and equipment by one person within 8 h.

HPLC Separation and Detection by APCI Ion Trap MS. The short chromatographic separation time of 12 min (24 min including flushing and reconditioning) allows a high sample throughput. Figure 2 shows the HPLC chromatogram of an extract from wheat flour spiked with eight type A- and B-trichothecenes at levels in the 226−1190 ppb range including the ISTD and RSTD. Absolute retention time variations for seven separations within 8 h were between 4 s for DAS (retention time average, 7.42 min) and 12 s for NIV (retention time average, 1.98 min). All employed trichothecenes except 3- and 15-ADON were baseline separated. 3- and 15-ADON coeluted but could be distinguished by different fragment ions in their mass spectra. However, mixtures of 3- and 15-ADON rarely occur in naturally contaminated wheat.

Chemical ionization mass spectrometry is a sensitive method for the detection of trichothecenes since the limited fragment formation allows low detection limits. $[M + H]^+$ was the predominant ion for most mycotoxins in the APCI(+) mode, but also $[M + NH_4]^+$ and occasionally $[M + CH_3OH + H]^+$ adducts could be detected. NIV and T-2 showed the strongest fragmentation.

HPLC/MS Performance Validation. A total of 25 ng of VOL (ISTD) was injected sequentially 10 times to evaluate the instrument repeatability. A relative standard deviation (RSD) of the signal area of 2.5% was obtained that increased to 10% ($n = 3$) at the 0.1 ng level. Therefore, sample extracts containing amounts of trichothecenes lower than 200 ppb were injected in duplicate, and the results were averaged.

Instrumental detection (signal-to-noise ratio (S/N) of 3/1) and quantification limits (S/N 10/1) were determined with pure reference substances (see Table 1). The obtained limits of detection (20−120 pg) and quantification (70−400 pg) are comparable to those reported for HRGC/MS methods (Schwadorf and Müller, 1991; Schollenberger et al., 1998).

The instrument response was linear between the quantification limits and 30 ng injected. The correlation coefficient r^2 for a calibration curve of eight measuring points was between 0.9983 and 0.9999 for the nine trichothecenes. The absolute variation of the relative response factors was within ±10%.

Performance of the Whole Method. Blank values for the whole method except extraction were equal to the detection limits. Extraction blanks could not be determined since no wheat was available that did not contain detectable amounts of DON. Repeatability was tested for all trichothecenes with spiked wheat at levels between 30 ppb and 10 ppm. The maximum relative standard deviation (RSD, 5.1%, $n = 3$) was found for 3-ADON and F-X at 50 ppb. Values for all trichothecenes at a level of 500 ppb are given in Table 1 (see RSD in recovery experiments). For DON, repeatability was also tested with three different naturally contaminated wheat samples. Average concentrations of 153 ppb (RSD 8.3%, $n = 4$), 533 ppb (RSD 1.1%, $n = 4$) and 2.72 ppm (RSD 1.4%, $n = 3$) were found. Recovery rates were calculated relative to the RSTD. The recoveries of the trichothecenes spiked into wheat at a level of 500 ppb are presented in Table 1 together with detection and quantification limits of the entire method. The recovery of the ISTD at a concentration of 1.5 ppm was 96% (RSD 3.0%, $n = 9$). Quantitative results were corrected by recovery difference of the corresponding toxin. At levels below 10−100 ppb, quantification was hampered by a

Table 1. Limits of Detection (LOD, Signal-to-Noise (S/N) 3/1) and Quantification (LOQ, S/N 10/1) for HPLC/MS and the Whole Method as Well as Recovery Rates for Wheat Spiked with 500 ppb of Each Trichothecene ($n = 3$)

compound	NIV	DON	F-X	NEO	3-ADON	15-ADON	DAS	HT-2	T-2
				HPLC/MS					
LOD (pg)	120	100	40	60	30	100	20	30	40
LOQ (pg)	400	300	120	200	100	300	70	100	120
				Whole Method					
LOD (ppb)	10	6	3	4	3	10	1	1	3
typical LOQ (ppb)[a]	100	50	40	50	25	100	20	10	60
				Spiked Wheat					
recovery (%)	80	86	91	106	98	102	102	90	87
RSD (%)[b]	3.1	3.6	3.4	0.5	2.6	2.5	4.9	1.7	4.6

[a] Definition: S/N ≥ 10/1, relative standard deviation for three replicates <10%, and recovery between 80% and 120%. [b] Relative standard deviation.

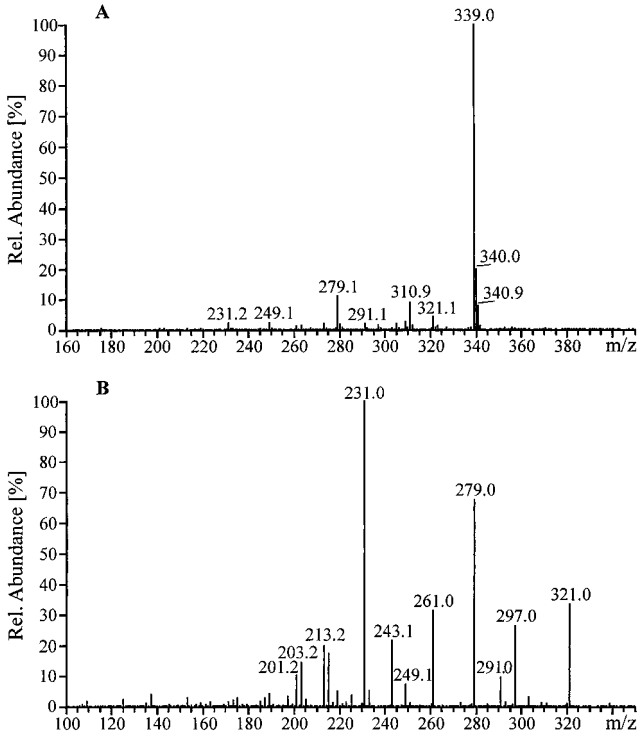


Figure 3. MS spectrum of 3-ADON (A) and the corresponding MS^2 spectrum of *m/z* 339 (B).

poorer repeatability (RSD >10%, $n = 3$) or recovery rates outside the range of 80−120%. The achieved detection and quantification limits of the whole method (see Table 1) are in good agreement with those for HRGC/electron capture detection (Croteau et al., 1994) and HPLC/UV detection (Lauren and Greenhalgh, 1987; Stratton et al., 1993; Jimenez and Mateo, 1997).

Interlaboratory Comparison Test Sample. The comparability of our method with others was evaluated with a contaminated wheat sample that had been employed in an intercomparison test (Pettersson, 1998). Fourteen results in that test were generated by HRGC/electron capture detection or HRGC/MS and one by an enzyme-linked immunosorbent assay (ELISA). Our results for DON (316 ppb), 3-ADON (23 ppb), HT-2 (295 ppb), and T-2 (250 ppb) differed 21% or less from the reported means (Pettersson, 1998). We also detected NIV, but below its quantification limit of 100 ppb (intercomparison mean, 88 ppb).

Advantages of HPLC/MS. Compared to HRGC, HPLC separation does not require derivatization of the trichothecenes; therefore, it involves less sample preparation time. Type B-trichothecenes contain a suitable chromophore for HPLC/UV detection, but type A-trichothecenes do not, which increases detectability problems as also an intercomparison showed. Pettersson (1998) concluded: "The method problems (inability to obtain straight calibration curves, memory effects, matrix interferences and matrix response enhancement) revealed during and after the intercomparison are serious ...". The presented HPLC/MS method is quick, is sensitive, and overcomes the mentioned problems. Linear calibration curves were obtained, and no memory effects were observed. Wrong positive results are also omitted since the MS spectrum allows a safe identification of trichothecenes. Furthermore, quantification is based on mass chromatograms, which eliminates matrix response enhancement.

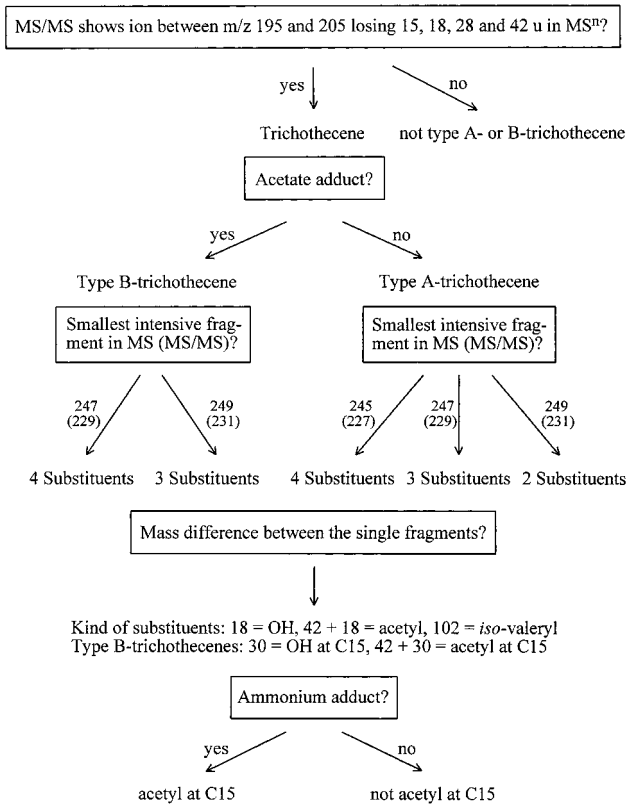


Figure 4. Identification scheme based on 12 type A- and B-trichothecenes.

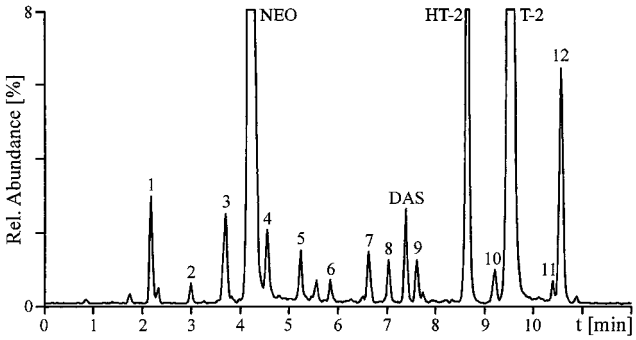


Figure 5. Base peak chromatogram (*m/z* 290−550) of the extract from rice infected by *F. sporotrichioides*. Relative abundance of NEO, 100%; T-2, 73%; and HT-2, 13%. Structure proposals for compounds **1**−**12** are given in Table 2.

MS^n Spectra. MS^2−MS^6 was applied to study fragment formation by collision-induced decay (CID) in the ion trap. All fragments were detected in their protonated form. Fragment ions observed in the MS spectra were generated during primary evaporation and ionization. Nevertheless, apart from few exceptions, the same fragments were formed by both processes.

Fragmentation by CID led to the loss of neutral molecules, preferably from the oxygenated substituents (except the carbonyl oxygen) and probably also from the epoxide ring. Compared to type A-trichothecenes, type B-trichothecenes easily lost the C15 side chain, resulting likely in a quinoic structure followed by rearrangement into a stable aromatic alcohol. Losses of the following mass units were observed (possible interpretations are given in parentheses): 18 u (H_2O from OH groups), 28 u (CO from the epoxide ring), 30 u (CH_2O from the epoxide ring or the OH group at C15 for type B-trichothecenes), 42 u (CH_2CO from acetyl), 60 u (CH_3-

Table 2. Identification of the Compounds 1−12 Marked in Figure 5

compd	Rt[a] (min)	MW[b]	ammonium adduct	substituents	trichothecene structure (proposal)
1	2.19	340	−	3 OH, 1 OAc[c]	15-deacetyl NEO
2	2.99	382	−	2 OH, 2 OAc	4,8-diacetoxy T-2 tetraol
3	3.70	340	+	3 OH, 1 OAc	4-deacetyl NEO
4	4.55	382	−	2 OH, 2 OAc	4,8-diacetoxy T-2 tetraol
5	5.23	382	+	2 OH, 2 OAc	iso-NEO or 7,8-dihydroxycalonectrin
6	5.84	366	+	1 OH, 2 OAc	7- or 8-hydroxycalonectrin
7	6.63	424	+	1 OH, 3 OAc	8-acetyl NEO
8	7.04	482	+	1 OH, 2 OAc, 1 OH-iso-Val[d]	3′-hydroxy T-2
9	7.62	382	−	3 OH, 1 iso-Val	T-2 triol[e]
10	9.21	464	+	1 OH, 2 OAc, 1 red. iso-Val	T-2 with reduced iso-Val
11	10.41	450	+	2 OAc, 1 iso-Val	deoxy T-2
12	10.57	508	+	3 OAc, 1 iso-Val	acetyl T-2[e]

[a] Retention time. [b] Molecular weight. [c] Acetyl. [d] iso-Valeryl. [e] Identity proven.

COOH from acetyl) and 102 u ($(CH_3)_2CHCH_2COOH$ from iso-valeryl). The same fragmentations were observed for plasmaspray and dynamic fast-atom bombardment HPLC/MS (Kostiainen, 1991), thermospray HPLC/MS (Rajakylä et al., 1987), and CI-tandem MS (Kostiainen, 1989). The order of fragmentation was only to some degree influenced by the overall substitution. This made an identification of positions difficult.

On-line HPLC/MS^2 provided information about all present substituents. Since hydroxy groups were cleaved off as water and esters as their corresponding acids, every lost substituent generated a double bond equivalent. Depending on the number of substitutents (two, three, or four) and the loss of the epoxide ring as 30/28 u, the type A-trichothecene VOL formed backbone structures of 200/202 u, DAS of 198/200 u and NEO, HT-2, T-2, T-2 triol, and acetyl T-2 of 196/198 u. Type B-trichothecenes with three and four substituents generated fragments of 200/202 u (DON, 3- and 15-ADON) and 200 u (NIV and F-X, 198 u was not observed). Additional collision energy was required to break the three-ring backbone resulting in the loss of 15, 18, 28, and 42 u. This fragmentation sequence was typical for all type A- and B-trichothecenes. The MS and MS^2 spectra of 3-ADON shown in Figure 3A,B illustrate the described fragmentation processes.

Ion−Adduct Formation with Ammonium Acetate. Traces of ammonium generally present in the background generated a $[M + NH_4]^+$ adduct with an abundance of 10−50% relative to the base ion $[M + H]^+$ for trichothecenes with an acetyl group at C15. A further postcolumn addition of ammonium acetate formed mass spectra containing almost exclusively the $[M + NH_4]^+$ ion. Furthermore, detection limits were lowered by a factor of 2, 4, 8, 8, and 12 for DAS, HT-2, NEO, 15-ADON, and T-2, respectively. This gain of sensitivity for NEO, 15-ADON, and T-2 was partly due to the suppression of fragmentation. On the other hand, trichothecenes without an acetyl group at C15 were hardly detectable in the APCI(+) mode after increasing the ammonium level. These findings are in accordance with the observed $[M + NH_4]^+/[M + H]^+$ ratios for monoacetoxyscirpenol, acetyl T-2, iso T-2, and T-2 triol when applying ammonia CI (Kostiainen, 1989). At present it is not clear why only a strong ammonium adduct is formed when an acetyl group is present at C15.

In the APCI(−) mode, the addition of ammonium acetate resulted in MS spectra of type B-trichothecenes showing solely a $[M + \text{acetate}]^-$ ion adduct. As a result, the S/N ratios increased by a factor of 3, 5, 5, 10, and 12, respectively, for 3-ADON, 15-ADON, F-X, NIV, and DON. The acetate ion probably added at the α,β-unsaturated ketone of the type B-trichothecenes. Type A-trichothecenes did not form anions neither in the presence nor in absence of ammonium acetate.

Structure Elucidation of Type A- and B-Trichothecenes in Fungal Cultures. The results from the MS^n experiments and ion−adduct studies allowed us to develop a structure elucidation scheme for the trichothecenes. This is based on measurements of 12 different type A- and B-trichothecenes and presented in Figure 4. It enabled identification of compounds separated by HPLC as trichothecenes. Furthermore, the type (A or B) could be determined as well as the number and kind of substituents. In some cases, this approach allowed elucidation of the complete structure of trichothecenes produced by fungal cultures. It was applied to extracts from three different rice samples infected by *Fusarium equiseti*, *F. graminearum*, and *F. sporotrichioides*, respectively (Langseth et al., 1999). Several type A- and B-trichothecenes were produced by *F. equiseti*, among them NIV, F-X, 15-acetylnivalenol, and DAS. Eleven more signals in the chromatogram were identified as type A-trichothecenes with four or five hydroxy and acetyl groups. *F. graminearum* produced predominantly DON and 3-ADON but also seven type A-trichothecenes with four substituents each, among them one with acetyl groups at C3, C4, and C7 and an iso-valeryl group at C8. None of these toxins formed an ammonium adduct.

The base peak chromatogram of the extract of *F. sporotrichioides* is shown in Figure 5. NEO, DAS, HT-2, T-2, and 12 more type A-trichothecenes were detected. The characterization of the unknown toxins and proposals for their structures are given in Table 2. The identities for compounds **9** (T-2 triol) and **12** (acetyl T-2) were confirmed by comparing retention time, MS, and MS^2 spectra with those of the standards. The findings are in good agreement with earlier identification of trichothecenes formed by *F. sporotrichioides* given in the review by De Nijs et al. (1996).

ACKNOWLEDGMENT

We thank the Veterinary Institute in Oslo (Norway) for introducing us into this challenging subject and supplying us with fungal cultures on rice as well as the wheat sample from the intercomparison. Many thanks also to Martin Biniossek for exploring the possibilities of HPLC/MS of trichothecenes at an early stage and giving valuable comments to the manuscript. The financial support by the Swiss Science Foundation under Projects 20-50474.97 and 20-53546.98 is gratefully acknowledged.

LITERATURE CITED

Croteau, S. M.; Prelusky, D. B.; Trenholm, H. L. Analysis of Trichothecene Mycotoxins by Gas Chromatography with Electron Capture Detection. *J. Agric. Food Chem.* **1994**, *42*, 928−933.

De Nijs, M.; Rombouts, F.; Notermans, S. *Fusarium* Molds and their Mycotoxins. *J. Food Saf.* **1996**, *16*, 15−58.

Gilbert, J. Recent Advances in Analytical Methods for Mycotoxins. *Food Addit. Contam.* **1993**, *10*, 37−48.

Grove, J. F. Macrocyclic Trichothecenes. *Nat. Prod. Rep.* **1993**, *10*, 429−448.

Jimenez, M.; Mateo, R. Determination of Mycotoxins Produced by *Fusarium* Isolates from Banana Fruits by Capillary Gas Chromatography and High-Performance Liquid Chromatography. *J. Chromatogr. A* **1997**, *778*, 363−372.

Kostiainen, R. The Characterization and Analysis of Trichothecenes by Chemical Ionization and Tandem Mass Spectrometry. Thesis, University of Helsinki, 1989.

Kostiainen, R. Identification of Trichothecenes by Thermospray, Plasmaspray and Dynamic Fast-Atom Bombardment Liquid Chromatography−Mass Spectrometry. *J. Chromatogr.* **1991**, *562*, 555−562.

Langseth, W.; Rundberget, T. Instrumental Methods for Determination of Nonmacrocyclic Trichothecenes in Cereals, Foodstuffs and Cultures. *J. Chromatogr. A* **1998**, *815*, 103−121.

Langseth, W.; Bernhoft, A.; Rundberget, T.; Kosiak, B.; Gareis, M. Mycotoxin Production and Cytotoxicity of *Fusarium* Strains Isolated from Norwegian Cereals. *Mycopathologia* **1999**, *144*, 103−113.

Lauren, D. R.; Greenhalgh, R. Simultaneous Analysis of Nivalenol and Deoxynivalenol in Cereals by Liquid Chromatography. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 479−483.

Lin, L.; Zhang, J.; Wang, P.; Wang, Y.; Chen, J. Thin-Layer Chromatography of Mycotoxins and Comparison with other Chromatographic Methods. *J. Chromatogr. A* **1998**, *815*, 3−20.

Müller, H.-M.; Reimann, J.; Schumacher, U.; Schwadorf, K. *Fusarium* Toxins in Wheat Harvested During Six Years in an Area of Southwest Germany. *Nat. Toxins* **1997**, *5*, 24−30.

Park, J. J.; Chu, F. S. Assessment of Immunochemical Methods for the Analysis of Trichothecene Mycotoxins in Naturally Occurring Moldy Corn. *J. Assoc. Off. Anal. Chem. Int.* **1996**, *79*, 465−471.

Patel, S.; Hazel, C. M.; Winterton, A. G. M.; Mortby, E. Survey of Ethnic Foods for Mycotoxins. *Food Addit. Contam.* **1996**, *13*, 833−841.

Pettersson, H. Intercomparison of Trichothecene Analysis and Feasibility to Produce Certified Calibrants and Reference Material. Directorate-General, Science, Research and Development: Brussels, 1998; EUR 18214 EN; ISBN 92-828-3224-4.

Prelusky, D. B.; Rotter, B. A.; Rotter, R. G. Toxicology of Mycotoxins. In *Mycotoxins in Grain, Compounds other than Aflatoxins*; Miller, J. D., Trenholm, H. L., Eds.; Eagan Press: St. Paul, MN, 1994; pp 359−403.

Rajakylä, E.; Laasasenaho, K.; Sakkers, P. J. D. Determination of Mycotoxins in Grain by High-Performance Liquid Chromatography and Thermospray Liquid Chromatography−Mass Spectrometry. *J. Chromatogr.* **1987**, *384*, 391−402.

Schollenberger, M.; Lauber, U.; Terry Jara, H.; Suchy, S.; Drochner, W.; Müller, H.-M. Determination of Eight Trichothecenes by Gas Chromatography−Mass Spectrometry after Sample Cleanup by a Two-Stage Solid-Phase Extraction. *J. Chromatogr. A* **1998**, *815*, 123−132.

Schwadorf, K.; Müller, H.-M. Determination of Trichothecenes in Cereals by Gas Chromatography with Ion Trap Detection. *Chromatographia* **1991**, *32*, 137−142.

Scott, P. M. Mycotoxin Methodology. *Food Addit. Contam.* **1995**, *12*, 395−403.

Stratton, G. W.; Robinson, A. R.; Smith, H. C.; Kittilsen, L.; Barbour, M. Levels of Five Mycotoxins in Grains Harvested in Atlantic Canada as Measured by High Performance Liquid Chromatography. *Arch. Environ. Contam. Toxicol.* **1993**, *24*, 399−409.

Weingaertner, J.; Krska, R.; Praznik, W.; Grasserbauer, M.; Lew, H. Use of MysoSep Multifunctional Clean-up Columns for the Determination of Trichothecenes in Wheat by Electron-Capture Gas Chromatography. *Fresenius J. Anal. Chem.* **1997**, *357*, 1206−1210.

Yoshizawa, T.; Jin, Y.-Z. Natural Occurrence of Acetylated Derivatives of Deoxynivalenol and Nivalenol in Wheat and Barley in Japan. *Food Addit. Contam.* **1995**, *12*, 689−694.

Young, J. C.; Games, D. E. Supercritical Fluid Chromatography of *Fusarium* Mycotoxins. *J. Chromatogr.* **1992**, *627*, 247−254.

Young, J. C.; Games, D. E. Analysis of *Fusarium* Mycotoxins by Supercritical Fluid Chromatography with Ultraviolet or Mass Spectrometric Detection. *J. Chromatogr. A* **1993**, *653*, 374−379.

Received for review April 23, 1999. Revised manuscript received July 21, 1999. Accepted July 26, 1999.

JF9904012