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Determination of eight trichothecenes by gas chromatography–mass spectrometry after sample clean-up by a two-stage solid-phase extraction

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Abstract

A method is described for the determination of eight trichothecenes of type A and B in a variety of complex matrices including heavily moulded and pigmented cereals, whole cereal ears, cereal-based foods, mixed feeds and faeces from swine. Trichothecenes were determined as their trifluoroacetyl derivatives by gas chromatography with ion-trap mass spectrometry detection operating in chemical ionization mode. Isobutane was chosen as reactant gas and optimum parameters of measurement were determined. For sample preparation a clean-up procedure was developed using a combination of Florisil and cation-exchange cartridges for solid-phase extraction. Limits of detection and quantification as well as recoveries are described.

Keywords: Food analysis; Mycotoxins; Sample handling; Trichothecenes

1. Introduction

Trichothecenes represent a group of toxic fungal metabolites which are produced by strains of *Fusarium* and other species of fungi [1–3]. Some of these mycotoxins are now regarded as worldwide fungal contaminants of grain. The most frequent trichothecene in cereals seems to be deoxynivalenol (DON), but 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), nivalenol (NIV), fusarenon-X (FUS-X), HT-2 toxin (HT-2), T-2 toxin (T-2) and diacetoxyscirpenol (DAS) have also been found [4–9]. There is evidence that the occurrence of these toxic trichothecenes and other *Fusarium* toxins such as zearalenone is of concern to both livestock and human health [10–13].

A variety of chromatographic methods have been described for the determination of trichothecenes, including thin-layer (TLC), high-performance liquid (HPLC), supercritical fluid (SFC), and gas (GC) chromatography. Gas chromatography has been combined with flame ionisation (FID), electron-capture (ECD), or mass spectrometric (MS) detection. During the last years, immunological methods have also been developed [14,15]. Determination based on GC with ECD or MS detection probably are the methods most commonly used [16,17]. This may be due to the fact that GC analysis shows a greater sensitivity and specificity than HPLC and samples can be readily confirmed by GC–MS. Furthermore, due to the greater separating capacity of GC it should be possible to detect a number of different trichothecenes in the same extract. However, methods based on GC require selective and highly efficient sample preparation procedures [14].

Some of the clean-up methods described in the

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literature are feasible for the determination of trichothecenes of either the A-(HT-2, T-2, DAS) or the B-type (DON, 3-ADON, 15-ADON, NIV, FUS-X) [16–20]. Other clean-up procedures have been proposed which allow for the determination of trichothecenes of both groups in the same extract [21–24]. So far this approach has been restricted to cereal grains contaminated with trichothecenes following natural infection with fusaria.

The objective of the present study was to develop a clean-up procedure yielding extracts of sufficient high purity for subsequent GC–MS analysis from a variety of matrices with a high content of co-extracting substances. The sample preparation was aimed to cover a wide range of trichothecene concentrations. Matrices to be analysed included cereal grains artificially inoculated in the field with toxinogenic *Fusarium* strains. These samples originated from experiments which were intended to study the formation of trichothecenes during plant ripening. For this purpose whole ears from young plants with only low trichothecene contents but with high contents in chlorophyll were analysed. Furthermore, cereals inoculated with DON producing *Fusarium* strains according to our experience often contain high concentrations of this toxin in addition to low contents of other trichothecenes such as NIV. Further matrices were samples from experiments during which DON was fed to swine (faeces, mixed feeds, bedding material).

In addition to the clean-up procedure we describe parameters of the mass selective detector (ion-trap) used and of the complete method including recovery, detection limits and reproducibility.

2. Experimental

2.1. Chemicals and reagents

Mycotoxin standards were purchased from Sigma (Deisenhofen, Germany). Trifluoroacetic anhydride (TFAA) (derivatization reagent) was obtained from Pierce (Rockford, IL, USA). All other chemicals and solvents were of reagent grade and purchased from Merck (Darmstadt, Germany).

2.2. Sample preparation

Samples (grain, foodstuffs, feedstuffs) were ground to a particle size of about one mm with a laboratory mill, mixed and stored at −20°C. Faeces were homogenised with a known amount of water using a mixer.

2.3. Extraction

Extraction was carried out as described by Tanaka et al. [25]. An amount of 10 g of the ground sample was extracted for 30 min with 100 ml of acetonitrile–water (75:25, v/v) in a 250 ml screw-capped bottle using a rotary shaker. The extract was filtered through a folded filter. An aliquot (63 ml) of the filtrate was extracted for 10 s with 50 ml of hexane in a separating funnel. After addition of 60 ml of ethanol the aqueous phase was evaporated to dryness using a rotary evaporator at a maximum bath temperature of 40°C. The residue was dissolved in methanol, transferred to a 50 ml pear-shaped flask and evaporated to dryness with a rotary evaporator.

2.4. Clean-up

The residue was dissolved in 2.5 ml of methanol using ultrasonification, transferred to a 5-ml centrifuge tube and centrifuged for 10 min at 4000 rpm. Two ml of the supernatant were transferred to a 40-ml screw-capped centrifuge tube. After addition of 18.0 ml of ethylacetate centrifugation was performed for 10 min at 4000 rpm. The supernatant was removed.

The surface of a cartridge packed with Florisil (Varian, Mega Bond Elut Florisil, 5 g/20 ml) was covered with anhydrous sodium sulphate and the resin was conditioned with 20 ml of hexane. Then 12.0 ml of the supernatant were transferred to the column. The eluate was collected. Subsequently, elution was carried out with 30 ml of ethyl acetate–methanol (90:10). The two extracts were combined (42 ml) and evaporated to dryness in a rotary evaporator.

An additional step included a cation-exchange procedure using cartridges packed with a weak cation-exchange resin (Varian, Megabond Elut CBA, 1 g/6 ml). Conditioning of this cartridge included

the elution with 10 ml of 1 *M* acetic acid, followed after 15 min by elution with 20 ml of 0.05 *M* acetic acid and 10 ml of methanol–0.05 *M* acetic acid (30:70). The dried extract of the Florisil cartridge was dissolved in 3.0 ml of methanol followed by addition of 5.8 ml of 0.05 *M* acetic acid. The sample was transferred quantitatively to the cation-exchange cartridge and drained to its top. Then the cartridge was eluted with 30 ml of methanol–0.05 acetic acid (30:70). The combined eluates (38.8 ml) were evaporated to dryness in a rotary evaporator. The residue was dissolved in methanol and aliquots distributed to several reaction vials and evaporated to dryness under a gentle stream of nitrogen and stored at −20°C prior to GC–MS analysis.

2.5. Derivatization

Trifluoracetylation was carried out with TFAA according to Kientz and Verwej [26]. The residue, corresponding to 1 g of sample, was dissolved in 1.0 ml of methanol by brief ultrasonic treatment and an aliquot was transferred to a 5-ml screw-capped Reactivial ('Mini-Vial') with a PTFE-faced rubber liner. Using a heating block, the sample was evaporated to dryness under nitrogen at 40°C. Twenty to thirty mg of dried sodium hydrogen-carbonate and 300 μl TFAA were added to the dry residue. The Reactivial was closed and mixed for 10 s using a vortex mixer and then acetylated for 30 min at 80°C using a heating block. After cooling to room temperature, the Reactivial was unscrewed and the sample evaporated to dryness for about 15–20 min under nitrogen. The residue was extracted with 100 μl toluene (whirl-mix and short ultrasonic treatment). The resulting solution was extracted with 500 μl water. After 15 min the toluene layer was removed and dried by adding 5–10 mg anhydrous sodium sulphate and mixing for 10 s. After 15 min the toluene layer was transferred to an autosampler vial and diluted when necessary.

2.6. Equipment

As mass spectrometer a Magnum GC–MS (Finnigan, Bremen, Germany) was used. It consisted of a Finnigan MAT ion-trap detector ITD 800 interfaced to a Varian (Walnut Creek, CA, USA) Star 3400 CX gas chromatograph. The gas chromatograph was equipped with a Varian 1075 split/splitless injector and a CTC A 200S autosampler (CTC Analytics, Zwingen, Switzerland).

2.7. GC–MS

A DB-5 MS phase (30 m×0.25 mm, film thickness 0.25 μm) (J&W Scientific, Folsom, CA, USA) was used as a capillary column. The carrier gas was helium 5.0 (approximately 17 p.s.i., the carrier gas flow was optimised according to ion-trap conditions; 1 p.s.i.=6894.76 Pa). The temperature of the injection port was 260°C; the injection volume 1 μl corresponding to 10 μg–10 mg of sample depending on the trichothecene content. The mode of injection was splitless (valve 20 s closed), then split 60 ml min^{-1}. The column temperature, following an initial period of 90°C for 2 min, was increased to 275°C at 23° min^{-1}, held at 275°C for 2 min, then increased to 290°C at 30° min^{-1} and kept finally at 290°C for 15 min. The temperature of the transfer line was adjusted to 270°C. The mass spectrometer was operated in the chemical ionisation (CI) mode using isobutane (3.5) as reagent gas; the temperature of the ion trap was 190°C. The maximum ionisation time was 1500 μs, the maximum reaction time 80 ms, the ionisation level 25 u, the reaction level 40 u, the reagent ion eject level 250 u, the reagent ion eject adjust 100%, and the reagent reaction time 9000 μs. The scan range selected for full-scan data was 280 to 650 u.

3. Results and discussion

3.1. Derivatization

This procedure corresponds to that described by Kientz and Verwej [26]. These authors did not specify the amount of reagent needed. For derivatization of naturally contaminated samples Schwadorf and Müller [27] used 100 μl of reagent per sample. With extracts of inoculated and therefore heavily moulded cereals containing high concentrations of toxins and matrix components we found that a higher amount of TFAA (300 μl per reactivial) was required. Following routine analyses we

Table 1
GC–MS of trifluoroacetyl derivatives of eight trichothecene toxins (CI-mode with isobutane as reagent gas)

Toxin	Molecular ion $(M+H)^+$ (u)	Mass window	
		Main fragment (u)	Secondary fragment (u)
NIV	⟨697[a]⟩	583 (583–585)	469 (469–471)
DON	585	585 (585–587)	471 (471–473)
FUS	643	529 (529–531)	469 (469–471)
15-ADON	531	471 (471–473)	417(417–419)
3-ADON	531	531 (531–533)	471 (471–473)
DAS	463	403 (403–405)	289 (289–291)
HT-2	617	455 (455–457)	617 (617–619)
T-2	563	401 (401–403)	563 (563–565)

[a] Out of mass range of Ion-Trap Finnigan Magnum (10–650 u).
$(M+H)^+$: Protonated molecular ion (base peak).

observed that trichothecenes were destroyed during derivatization probably by destruction at active centres in the glass wall, which develop with frequent use of the vial. To control the derivatization reaction DAS was added in known amounts to the sample extracts before trifluoroacetylation. DAS is not used as an internal standard but for controlling the efficacy of the derivatization procedure. This toxin was chosen because the *Fusarium* strains used for artificial inoculation of our samples do not produce DAS.

3.2. Mass selective detection by ion trap

In mass spectrometry maximum sensitivity of measurement can be obtained if only few ions characteristic of the component of interest are produced in high yields during the ionisation reaction. By chemical ionisation less energy is transferred to the molecules of the sample and therefore less fragmentation is observed compared to electron ionisation. Mass spectrometers constructed as ion-trap detectors are suited for the determination of trichothecenes using chemical ionisation [27]. In order to obtain slight and stable fragmentation of the trifluoroacetyl derivatives of trichothecenes the proton affinity of the reactant gas must be high enough to guarantee soft ionisation [28,29]. On the other hand, the fragmentation must be strong enough to produce a measurable fragment of 583 u of the trifluoroacetyl derivative of nivalenol, because the mass of the molecule ion with 697 u exceeds the detection range of the Magnum MS (10–650 u) and therefore cannot be measured. We found isobutane to be the reactant gas of choice. It improved the stability of mass spectra compared to methanol as reactant gas even with complex matrices such as moulded or unripe samples and NIV can be measured with sufficient sensitivity.

Confirmation of the toxins was based on retention time and fragmentation pattern, quantification of each trichothecene on both the ion of the main fragment and of a second fragment as summarized in Table 1. Specific mass windows were chosen which allowed us to check the stability of fragmentation. A good reproducibility of fragmentation with differences between the two ions selected below 10% was achieved.

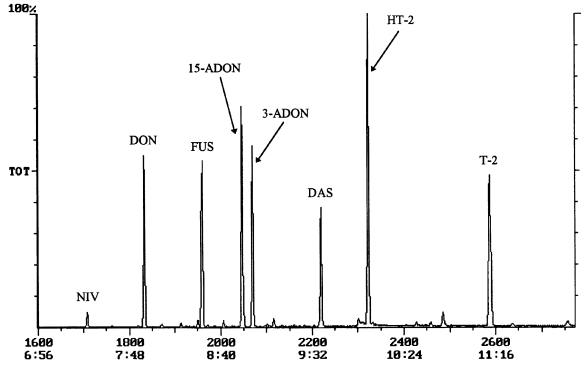

Fig. 1. Total ion chromatogram for eight trichothecenes, (standard, 2 ng each; trifluoroacetyl derivatives; CI-mode; reagent gas isobutane). Abscissa: scan number and retention time (min). Ordinate: relative intensity.

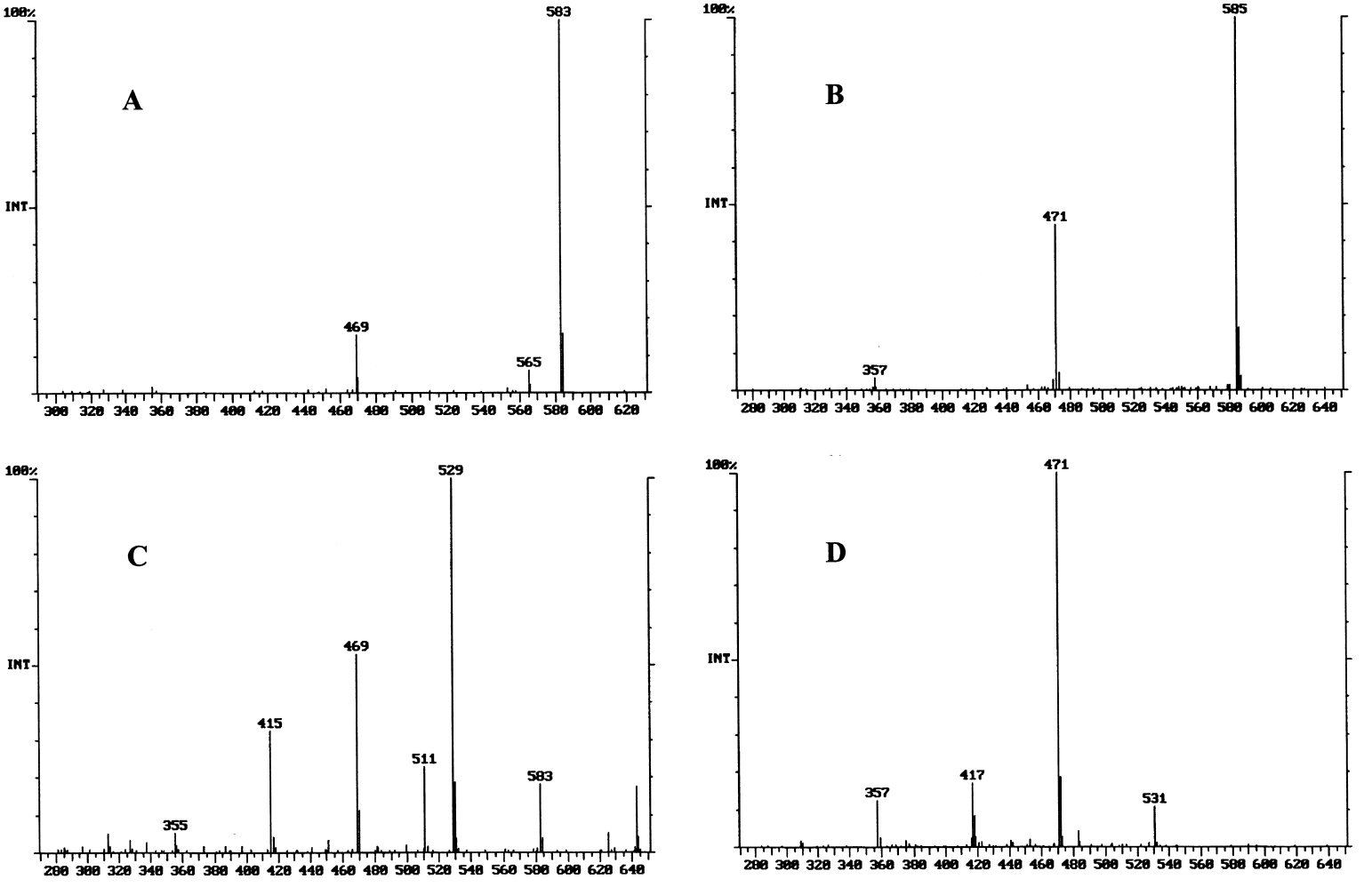

Fig. 2. Mass spectra of NIV (A), DON (B), FUS-X (C), 15-ADON (D), 3-ADON (E), DAS (F), HT-2 (G), T-2 (H), (trifluoroacetyl derivatives; CI-mode; reagent gas isobutane). Abscissa: mass/charge (m/z). Ordinate: relative intensity.

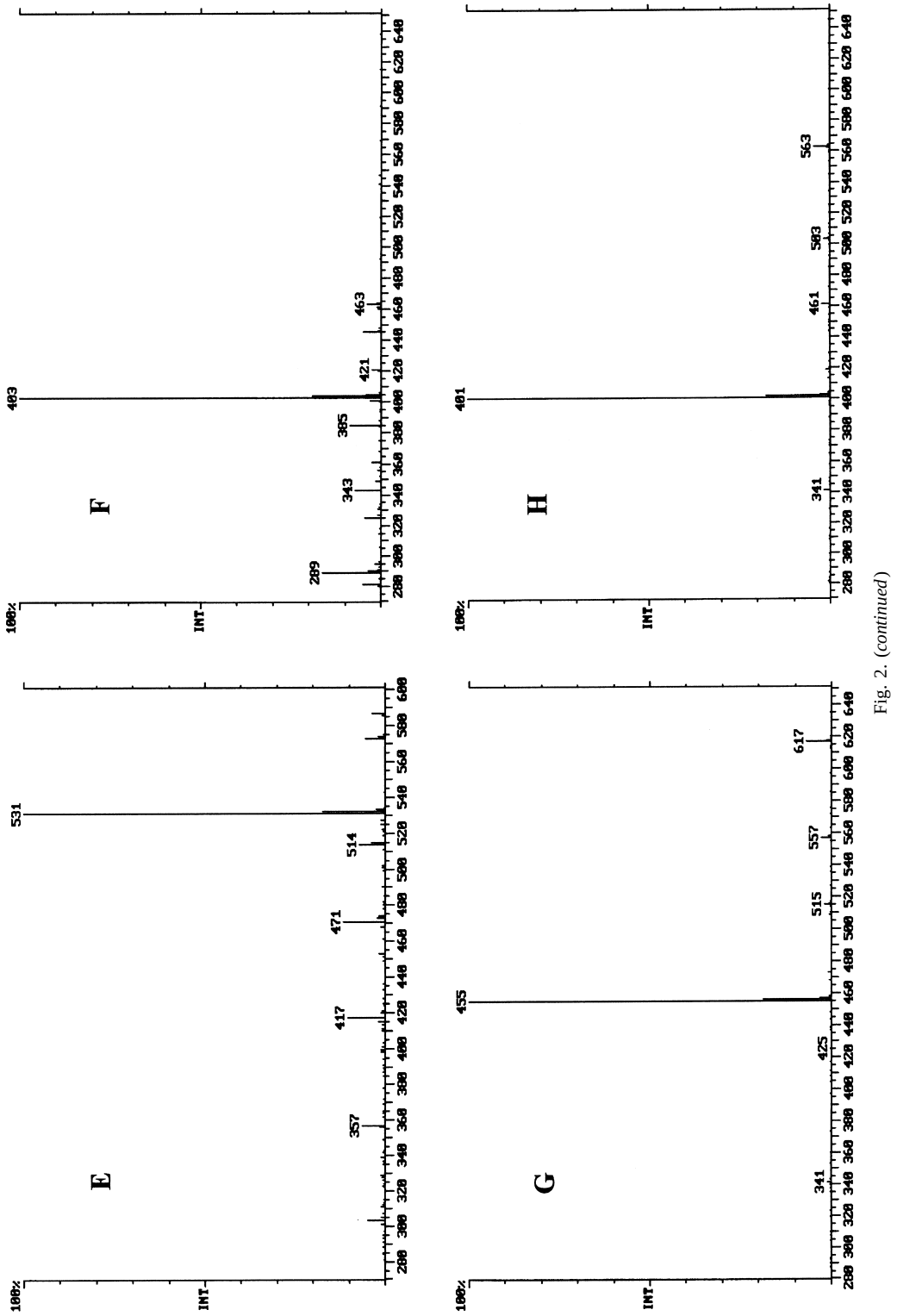

Fig. 2. (*continued*)

The total ion chromatogram of a mixture of eight trichothecenes is presented in Fig. 1. The mass spectra of trifluoroacetyl esters of these trichothecenes are presented in Fig. 2.

3.3. Clean-up procedure

The extraction was carried out as described by Tanaka et al. [25]. It was necessary to remove co-extracting matrix components from the solution as completely as possible. For this purpose, different clean-up steps were carried out. Trichothecenes differ with respect to their polarity. Among the trichothecenes determined in the current study nivalenol is the most polar one followed by deoxynivalenol, whereas T-2 toxin has the smallest polarity. Polarity of the other toxins of this group ranges between that of nivalenol and T-2 toxin.

During sample clean-up substances whose polarity is below that of T-2 toxin were removed by liquid–liquid extraction with hexane. Substances showing a higher polarity compared with NIV were removed by precipitation with ethyl acetate followed by solid-phase extraction using a Florisil cartridge. The subsequent clean-up procedure using a cation-exchange resin cartridge removed selectively sample components whose polarity is similar to that of the trichothecenes. Chlorinated solvents, often used in sample preparation [18,27,30], can be completely avoided.

Using this method trichothecenes of the A-as well as of the B-type can be determined in a variety of complex matrices such as heavily moulded and pigmented cereals, whole cereal ears, mixed feeds, faeces and different foods such as bread, noodles, rice and corn flakes. In all of these cases the clean-up procedure was sufficient for obtaining a transparent sample after derivatization and clearly separated peaks of trichothecenes using the specific mass windows for quantification. This is demonstrated in Figs. 3–6. Fig. 3 shows a chromatogram of a sample of triticale, naturally contaminated with NIV, DON and 3-ADON. Fig. 4 shows a chromatogram of a sample of whole ears of wheat, with high amounts of chlorophyll and with a high concentration of NIV in addition to relatively low concentrations of DON and FUS-X. Even in this latter sample with a very complex matrix the peaks of the trichothecenes were measured without interference. Fig. 5 shows the chromatogram of a sample of whole bread naturally contaminated with DON; Fig. 6 a chromatogram of a sample of faeces from swine spiked with eight trichothecenes.

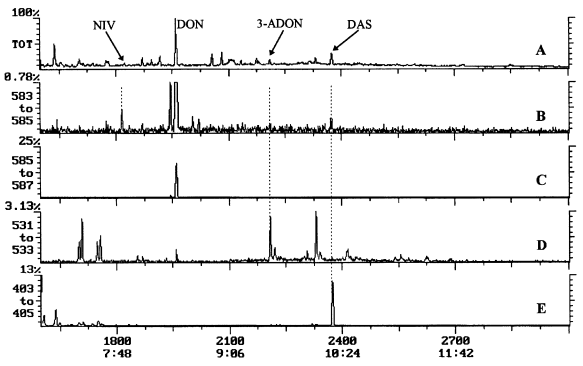

Fig. 3. Triticale sample containing 49 μg kg^{-1} NIV, 970 μg kg^{-1} DON and 68 μg kg^{-1} 3-ADON. Total ion chromatogram (A); mass window 583–585, main fragment NIV (B); mass window 585–587, main fragment DON (C); mass window 531–533, main fragment 3-ADON (D); mass window 403–405, main fragment DAS (E). Sample aliquot for derivatization corresponding to 1000 mg. (Trifluoroacetyl derivatives; CI-mode; reagent gas isobutane). Abscissa: scan number and retention time (min). Ordinate: relative intensity.

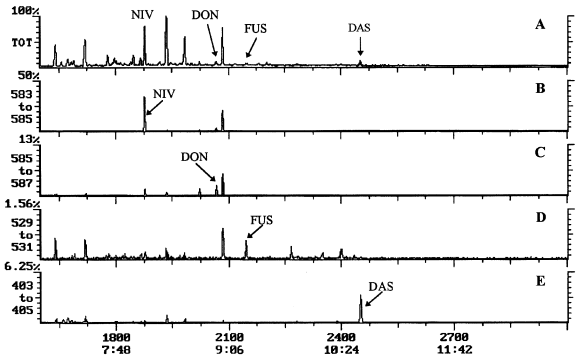

Fig. 4. Sample of whole wheat ears with a high content of chlorophyll containing 38 500 μg kg^{-1} NiV, 1000 μg kg^{-1} DON and 900 μg kg^{-1} FUS-X. Total ion chromatogram (A); mass window 583–585, main fragment NIV (B); mass window 585–587, main fragment DON (C); mass window 529–531, main fragment FUS-X (D); mass window 403–405, main fragment DAS (E). Sample aliquot for derivatization corresponding to 100 mg. (Trifluoroacetyl derivatives; CI-mode; reagent gas isobutane). Abscissa: scan number and retention time (min). Ordinate: relative intensity.

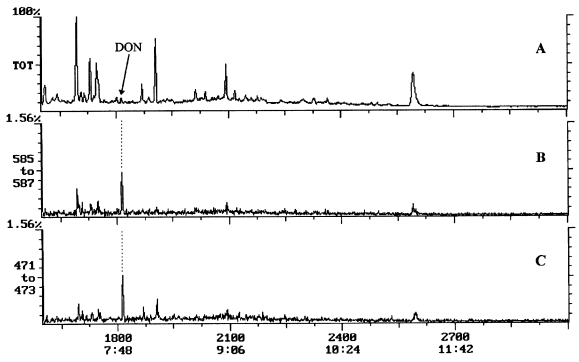

Fig. 5. Sample of naturally contaminated whole bread containing 50 μg kg^{-1} DON. Total ion chromatogram (A); mass window 585–587, main fragment DON (B); mass window 471–473, secondary fragment DON (C). Sample aliquot for derivatization corresponding to 1000 mg. (Trifluoroacetyl derivatives; CI-mode; reagent gas isobutane). Abscissa: scan number and retention time (min). Ordinate: relative intensity.

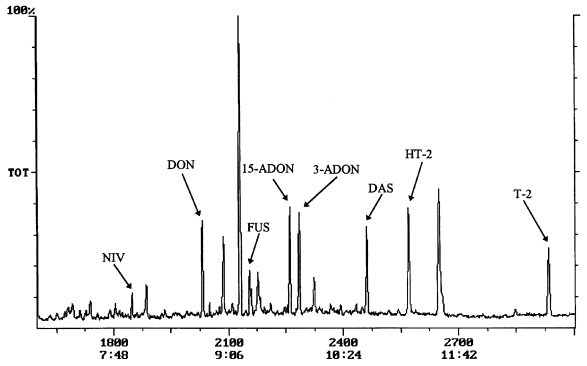

Fig. 6. Sample of faeces from swine, spiked with eight trichothecenes (600 μg kg^{-1} each). Total ion chromatogram. Sample aliquot for derivatization corresponding to 500 mg. (Trifluoroacetyl derivatives; CI-mode; reagent gas isobutane). Abscissa: scan number and retention time (min). Ordinate: relative intensity.

3.4. Detection limits, recoveries and reproducibility

Detection and quantification limits were determined according to DIN 32645 [31] using reference substances without matrix. As shown in Table 2, detection limits were between 20 and 120 pg, corresponding to 2–12 μg kg^{-1} of sample. Quantification limits were between 60 and 380 pg, corresponding to 6–38 μg kg^{-1} of sample. These results are in agreement with detection limits reported in the literature. Bergsjö et al. [32] were able to detect 10 μg kg^{-1} of trichothecene with GC–ECD measurement of samples of mixed feed and naturally contaminated oats. Wegmüller and Steiner [23] found detection limits of 20–60 μg kg^{-1} of sample for naturally contaminated milling products and foodstuffs with GC–MS.

To determine the recovery and reproducibility of the complete method known amounts in methanol solution of each of the trichothecenes tested were added to 10 g of samples of wheat, rice and whole bread. The material was treated as described previously. Recoveries are summarized in Table 3. In wheat spiked with 500 μg kg^{-1} of each of the eight trichothecenes recoveries ranged from 73 to 91% with standard deviations between 4.0 and 11.7%. After spiking of rice and whole bread with 100 μg kg^{-1} each comparable recoveries were obtained.

Table 2
Detection- and quantification limits (DIN 32 645) of trifluoroacetyl derivatives of eight trichothecene (Cl-mode with isobutane as reagent gas)

Toxin	Detection limit		Quantification limit	
	pg absolutely injected	μg kg^{-1}	pg absolutely injected	μg kg^{-1}
NIV	120	12	380	38
DON	70	7	230	23
FUS	60	6	210	21
15-ADON	70	7	220	22
3-ADON	50	5	160	16
DAS	70	7	220	22
HT-2	50	5	180	18
T-2	20	2	60	6

Table 3
Recovery of eight trichothecene toxins from wheat, rice and whole bread

Toxin	Wheat		Rice	Whole bread
	+50 μg kg^{-1} (n=4)	+500 μg kg^{-1} (n=4)	+100 μg kg^{-1} (n=4)	+100 μg kg^{-1} (n=4)
NIV	74±7.8	74±10.1	71±8.5	107±6.2
DON	98±7.7	83±4.7	84±4.8	95±9.8
FUS	120±15.6	82±9.6	91±2.5	116±9.7
15-ADON	90±14.4	73±5.9	87±5.8	108±4.2
3-ADON	110±3.4	88±4.8	89±8.5	108±7.5
DAS	110±7.6	91±11.7	101±2.5	102±5.1
HT-2	74±6.6	85±4.0	101±6.3	109±6.2
T-2	110±10.1	87±9.1	97±7.0	97±4.8

Samples spiked with toxin concentrations indicated. Values represent mean recovery and standard deviation in percentage of the toxin amount added.

This is in good agreement with recoveries reported in the literature. Scott et al. [33] added 200–1000 μg kg^{-1} of NIV and DON to ground corn and flour and found recoveries of NIV ranging from 70 to 108% and of DON 75 to 128%. Schwadorf and Müller [27] reported recoveries of NIV, DON, 3-ADON, DAS, HT-2 and T-2 of 78 to 89% from cereals spiked with 200 and 500 μg kg^{-1}. Recoveries from wheat spiked with 50 μg kg^{-1} ranged from 74 to 120% and standard deviations up to 15.6% (Table 3).

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