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Determination of trichothecenes in wheat by capillary gas chromatography with flame ionisation detection

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Abstract

Trichothecenes are mycotoxins produced by several fungal genera. The *Fusarium* species, mainly, can contaminate a wide range of cereals used for human and animal consumption. Trichothecenes are associated with various adverse health effects in animals and humans. Deoxynivalenol (DON) and nivalenol (NIV) are the trichothecenes most commonly found worldwide, although 3-acetyldeoxynivalenol, fusarenon X, T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS) and neosolaniol are also found. They are included in the present study. For the determination of these trichothecenes in wheat, a method based on capillary gas chromatography (GC) with flame ionisation detection (FID) has been developed and validated. The trichothecenes are extracted from the sample matrix by acetonitrile/water (84/16, v/v). Two different Mycosep® clean-up columns are used to purify the extract. The extract is evaporated to dryness and the trichothecenes are derivatised to trimethylsilyl ethers at room temperature. The residue is dissolved in iso-octane and washed with water. The final extract is analysed for trichothecenes by GC with FID. Quantification is based on the internal standard α-chloralose. The average recoveries for the trichothecenes range from 79% for NIV to 116% for DAS. The limit of quantification is 75 μg/kg for each of the individual trichothecenes. The GC-FID method produced good results in an intercomparison study of trichothecene analysis within the European Union Standards, Measurements and Testing Programme. A survey was carried out in the Netherlands in 1999 to detect the presence of trichothecenes in imported wheat. A temporary tolerance limit of 500 μg/kg is in effect in the Netherlands for DON in cleaned wheat. Seven of the 22 wheat samples exceeded this limit; one exceeded the limit by more than 100%. Thirteen of the 22 wheat samples exceeded a proposed DON tolerance limit of 120 μg/kg for cleaned wheat. Indeed, 12 samples exceeded the limit by more than 100%. Besides DON, no trichothecenes were found in the wheat samples at levels above the limit of quantification.

Keywords: Trichothecenes; Wheat; DON; NIV; 3-AcDON; FusX; T-2; HT-2; DAS; NeoSol; GC; FID

1. Introduction

Trichothecenes are secondary metabolites produced by several fungal genera, but mainly by *Fusarium* species. The *Fusarium* species are probably the most prevalent toxin-producing species of the northern temperate regions. More than 140 trichothecenes are known, all of which are tetracyclic sesquiterpenoid compounds with a C12-C13 epoxy group. Trichothecenes are commonly divided into four groups according to their functional groups (A–D) (Eriksen & Alexander, 1998). Only the so-called A and B types (Fig. 1) are produced by *Fusarium* species and these are the subject of the present study. Type A trichothecenes are characterised by the presence of a hydrogen, a hydroxyl or an ester functional group at C8. Type B trichothecenes possess a carbonyl functional group at that position (Eriksen & Alexander, 1998).

Growth of *Fusarium* species and toxin production can occur at relatively low temperatures on agricultural commodities in the field or during storage. Therefore, trichothecenes are commonly found in cereals from moderate climatic zones (van Egmond & Speijers, 1999).

Placinta, D'Mello, and Macdonald (1999), provide an exhaustive review of the worldwide trichothecene

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contamination of cereal grains and animal feed. Deoxynivalenol (DON, also known as vomitoxin) and nivalenol (NIV) are the most commonly found trichothecenes in the world. To a lesser extent, 3-acetyldeoxynivalenol (3-AcDON), fusarenon X (FusX), T-2 toxin (T-2), HT-2 toxin (HT-2), diacetoxyscirpenol (DAS) and neosolaniol (NeoSol) are also found and are included in this study. Fig. 1 shows their structures.

The trichothecenes are associated with various adverse health effects in animals and humans. The most prominent toxic effects of the trichothecenes are inhibitory effects on the protein synthesis in mammalian cells, effects on cell membranes, immunotoxic effects, weak clastogenic effects, indications of neurotoxic effects and, for T-2 toxin, a possible carcinogenic effect (Eriksen & Alexander, 1998).

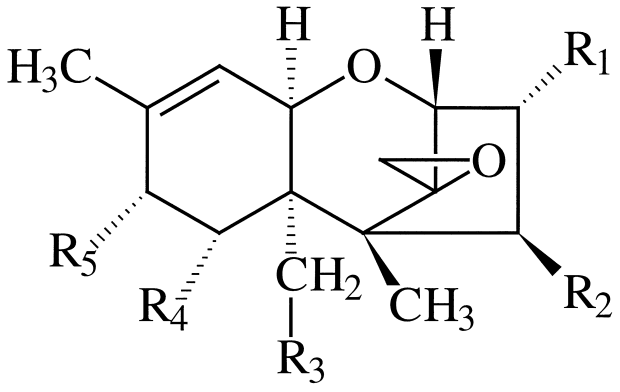
Pigs appear to be the domestic animals most sensitive to DON. The occurrence of more than 1 mg/kg DON in pig feed may result in a reduced feed intake and reduced weight gain (van Egmond & Speijers, 1999).

Vomiting is one of the earliest symptoms of trichothecenes intoxication in humans (van Egmond & Speijers, 1999).

There is no harmonised international regulation for trichothecenes, and only a few countries regulate some of the toxins. For DON, a tolerance limit of 120 μg/kg for cleaned wheat has recently been proposed in the Netherlands (Pieters, Fiolet, & Baars, 1999). This limit takes into consideration a provisional tolerable daily intake of 1.1 μg/kg body weight, which is based on the scientific literature on DON toxicity. In practice, a temporary DON limit of 500 μg/kg for cleaned wheat is in effect in the Netherlands.

The published data on the occurrence of trichothecenes in cereal grains and animal feed available on the Dutch market are very limited and only available for DON and NIV (de Nijs, Soentoro, Delfgou-van Asch, Kamphuis, Rombouts, & Notermans, 1996; Tanaka et al., 1990; Veldman, Borggreve, Mulder, & Lagemaat, 1992).

The methods that have been applied to identify the trichothecenes are thin-layer chromatography; high-performance liquid chromatography; supercritical fluid chromatography; capillary gas chromatography (GC) with either electron-capture detection (ECD), flame ionisation detection (FID) or mass-selective detection (MSD; Langseth and Rundberget, 1998), and enzyme-linked immunosorbent assay (Hart, Casper, Schabenberger, & Ng, 1998). At present, gas chromatographic methods with ECD or MSD are most commonly used (Langseth & Rundberget, 1998). The objective of the present study was to develop a GC method with FID to determine the amounts of DON, NIV, 3-AcDON, FusX, T-2, HT-2, DAS and NeoSol in wheat. FID was choosen because of its generally large linearity range and the ease and economy of its use. A survey was carried out in the Netherlands in the first half of 1999 to detect the quantities of trichothecenes in imported wheat (1998 harvest). The results of this survey are presented.



Type	Trichothecene	R1	R2	R3	R4	R5	Elemental formula	Molecular mass (amu)
A	Diacetoxyscirpenol	OH	$OCOCH_3$	$OCOCH_3$	H	H	$C_{19}H_{26}O_7$	366
A	Neosolaniol	OH	$OCOCH_3$	$OCOCH_3$	H	OH	$C_{19}H_{26}O_8$	382
A	T-2 Toxin	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$	$C_{24}H_{34}O_9$	466
A	HT-2 Toxin	OH	OH	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$	$C_{22}H_{32}O_8$	424
B	Deoxynivalenol	OH	H	OH	OH	=O	$C_{15}H_{20}O_6$	294
B	3-Acetyldeoxynivalenol	$OCOCH_3$	H	OH	OH	=O	$C_{17}H_{22}O_7$	338
B	Fusarenon X	OH	$OCOCH_3$	OH	OH	=O	$C_{17}H_{22}O_8$	354
B	Nivalenol	OH	OH	OH	OH	=O	$C_{15}H_{20}O_7$	312

Fig. 1. Structures of investigated types A and B trichothecenes.

2. Materials and methods[1]

2.1. Chemicals and reagents

All reagents were of analytical grade, unless otherwise specified. Trichothecenes standards were purchased from Sigma, and the internal standard α-chloralose was purchased from Dr. Ehrenstorfer Reference Materials. A combined solution containing 5 μg/ml of the internal standard and each of the trichothecenes in acetonitrile was prepared from the standards. An internal standard solution of 10 μg/ml in acetonitrile/water (84 + 16, v/v) was prepared from the standard. These solutions were kept refrigerated and were stable for at least 2 years (Pettersson, 2000).

2.2. Sampling and sample preparation

The Flour Millers Association of the Netherlands co-ordinated the sampling. Samples (about 500 g) were taken in January and February 1999 from wheat that originated from Germany, France, Canada, the United Kingdom and the Netherlands. The samples were ground in a laboratory mill (Romer Labs Inc.), mixed and stored at −18°C until analysis.

2.3. Extraction

A 25-g portion of a wheat sample to which 1.5 ml internal standard was added, was extracted overnight at room temperature with 200 ml of acetonitrile/water (84 + 16, v/v) in a 500-ml capped bottle, using a shaking machine (Bühler, SM B1). The extracts were stored in the dark at room temperature and were stable for at least 2 weeks. A 20-ml aliquot of the extract, to which 10 ml ethanol was added, was rotary evaporated (60°C) under reduced pressure. The dry residue was re-dissolved in 4 ml acetonitrile/water (84 + 16, v/v).

2.4. Clean-up and derivatization

The samples were cleaned up with Mycosep® 227 (consists of various adsorbents, including charcoal, Celite and ion-exchange resins) and Mycosep® 216 (consists of charcoal) columns (Romer Labs.). The stationary phase of a Mycosep® 227 column was repacked above the packing material of a Mycosep® 216 column (Fig. 2). Before use, the combined column was washed three times with 5 ml acetonitrile/water (84 + 16, v/v). The concentrated extract (4 ml) was put onto the combined Mycosep® column. The trichothecenes were eluted with 30 ml of acetonitrile/water (90 + 10, v/v), and the cleaned extract was rotary evaporated (60°C) under reduced pressure. The dry residue was re-dissolved in 2 ml acetonitrile and transferred to an autosampler vial. The extract was evaporated to dryness under nitrogen at 80°C with a heating block. Seventy-five microliters of tri-sil TBT [trimethylsilylimidazole-bis(trimethylsilyl)acetamide-trimethylchlorosilane (3:3:2), Pierce] was added to the dry residue. After the autosampler vial was flushed with N_2, it was closed, and the extract was derivatised to trimethylsilyl ethers in 15 min at room temperature. The autosampler vial was opened, and the extract was evaporated for 15 min at 60°C under nitrogen. The residue was re-dissolved in 0.5 ml iso-octane and washed with 1.0 ml water. The mixture was centrifuged, and the iso-octane layer was transferred to a new autosampler vial. The sample preparation procedure for determining the amounts of trichothecenes in wheat is shown schematically in Fig. 2. The final extract was analysed for trichothecenes by GC with FID under the experimental conditions specified in Table 1.

2.5. Equipment

Table 1 summarises the GC-FID equipment and the experimental conditions. In the first experiments, we used a single column, and it turned out that we had a co-eluting component on the place of the DON peak. This problem was overcome by using a combination of two columns (Table 1).

3. Method validation

3.1. Limit of detection and limit of quantification

With a test portion of 25 g of the wheat sample, the limit of detection for the tested trichothecenes is 25 μg/kg at a signal-to-noise ratio of 3. The limit of quantification for the tested trichothecenes is 75 μg/kg at a signal-to-noise ratio of 9.

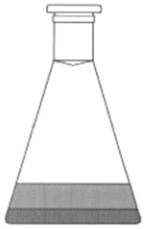
3.2. Linearity

A 5-point calibration curve was prepared for each of the tested trichothecenes (concentration range 70–1100 μg/kg). All calibration curves were checked for linearity on the basis of a linearity plot (van Trijp & Roos, 1991) and were linear.

3.3. Trueness

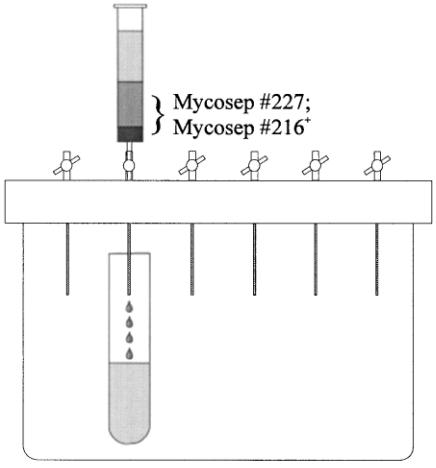
Four BCR-certified reference materials, CRMs 377, 378, 379 and 396 were analysed to determine the trueness of the method. Certified values are known only for DON. The results are summarised in Table 2. A chromatogram

[1] Reference in this paper to a company and/or product is for purposes of information and identification only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and the Environment, to the exclusion of others which may also be suitable.



Extraction:
Weigh about 25 g of sample.
Add 200 ml AcN/W; 84/16; v/v,
add 1.5 ml internal standard solution by weight.
Shake overnight.

Concentration:
Transfer 20 ml of the extract into an evaporation flask,
add 10 ml ethanol.
Evaporate to dryness, vacuum 105 hPa at 60 °C.
Dissolve the residue in 4 ml AcN/W; 84/16; v/v.



Clean-up:
Condition the clean-up column with
AcN/W; 84/16; v/v.

Transfer the extract to the column,
elute with 30 ml AcN/W; 9/1; v/v;
speed not less than 2 drops/second.

Concentration:
Evaporate the purified extract to dryness,
vacuum 105 hPa at 60 °C.
Dissolve the residue in 2 ml acetonitril and
transfer into an autosampler vial.

Evaporate to dryness under nitrogen at 80 °C.

Silylation:
Silylate the residue with 75 µl Tri-Sil TBT;
15 min. at room temperature.
Evaporate under nitrogen at 60 °C during 15 min.

Water treatment:
Dissolve the residue in 0.5 ml iso-octane and wash with
1.0 ml water; Vortex at maximum speed during 30 sec.
Centrifuge.
Transfer the upper layer (iso-octane) into an autosampler vial.
Inject 2 µl into GC.

Fig. 2. Sample preparation procedure for determination of trichothecenes in wheat.

of a silylated BCR CRM 379 test sample is presented in Fig. 3, together with a chromatogram of a silylated standard solution.

In the case of a positive finding, a second GC system with another column type was used for confirmation (see Table 1 for equipment and experimental conditions). This column showed a different elution profile; this way, it is possible to eliminate false positives.

3.4. Recovery

Recovery experiments were done by adding standards of the trichothecenes to a blank wheat sample. The level at which standards were added to the test portion was about 300 μg/kg. The sample was analysed on three separate days in triplicate. The results are summarised in Table 3.

Table 1
Test conditions for determining trichothecenes in wheat

Test GC-system: Gas chromatograph, Fisons 8160 with Fisons AS 800 autosampler	
Column type	DB-17 (100% dimethylpolysiloxane), 15 m, 0.32 mm ID, 0.15 μm film thickness combined with DB-1HT (50% phenyl–50% methylpolysiloxane), 30 m, 0.32 mm ID, 0.1 μm film thickness (J&W Scientific) with 2 m, 0.53 mm ID retention gap (Machery-Nagel).
Carrier gas	Hydrogen at 110 kPa. Linear velocity: 42 cm/s at initial temperature (115°C)
Internal standard	α-Chloralose
Injector	Cold on-column
Injection volume	2 μl
Detection	Flame ionisation detector (FID) at 300°C
Calculation	Internal standard
Column temperature programme	2 min at 115°C 5°C/min to 300°C 5 min at 300°C
Confirmation GC-system: Gas chromatograph, Fisons 8160 with Fisons AS 800 autosampler	
Column type	CP-Sil 19 CB (7% cyanopropyl–7% phenyl–86% dimethylpolysiloxane), 60 m, 0.25 mm ID, 0.15 μm film thickness (Chrompack)
Carrier gas	Hydrogen at 190 kPa. Linear velocity: 46.5 cm/s at initial temperature (115°C)
Internal standard	α-Chloralose
Injector	Cold on-column
Injection volume	2 μl
Detection	Flame ionisation detector (FID) at 300°C
Calculation	Internal standard
Column temperature programme	2 min at 115°C 5°C/min to 300°C 5 min at 300°C

Table 2
Results of the trueness experiments

BCR CRM	Composition	Certified value for DON (μg/kg)	Measured mean (μg/kg)	RSD (%)	Relative recovery (%)
377	Maize	< 50	< 75 ($n=1$)	–	–
378	Maize	430±40	377 ($n=2$)	0.9	88
379	Wheat	670±20	707 ($n=10$)	5.8	105
396	Wheat	< 50	< 75 ($n=1$)	–	–

Table 3
Results of repeatability and recovery experiments (spiking level about 300 μg/kg for all trichothecenes)

	Repeatability ($n=6$)			Recovery ($n=9$)		
	Mean (μg/kg)	S.D.	RSD (%)	Mean (%)	S.D.	RSD (%)
DON	276	1.0	0.4	96	6.9	7.1
3-AcDON	287	3.6	1.3	115	5.9	5.2
FusX	284	2.0	0.7	100	6.5	6.5
NIV	293	2.0	0.7	79	6.1	7.7
DAS	333	3.5	1.0	116	8.5	7.4
NeoSol	266	2.5	0.9	113	6.7	6.1
HT-2	265	1.3	0.5	111	5.1	4.6
T-2	295	3.2	1.1	113	6.7	5.9

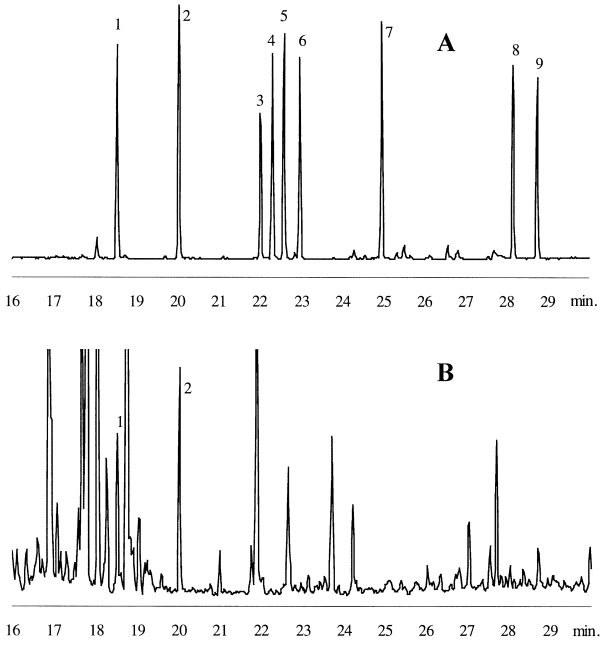


Fig. 3. Chromatograms of a silylated standard solution (about 600 μg/kg) of trichothecenes (A) and of a silylated BCR CRM 379 test sample (B). Peaks: 1, α-chloralose (internal standard.): 2, deoxynivalenol: 3, 3-acetyldeoxynivalenol: 4, fusarenon X: 5, nivalenol: 6, diacetoxyscirpenol: 7, neosolaniol: 8, T-2 toxin: 9, HT-2 toxin.

3.5. Repeatability

The repeatability of results with the method was established by repeatedly analysing ($n=6$) test portions of a wheat sample. Standards of trichothecenes, each at a level of about 300 μg/kg, were added to the wheat sample. The results are presented in Table 3.The repeatability of determining DON was also tested by analysing BCR CRM 379 ($n=10$). The result is presented in Table 2.

3.6. Interlaboratory comparison

The GC-FID method was used in an intercomparison study of trichothecene analysis within the European Union Standards, Measurements and Testing Programme with good results (Pettersson, 1998).

4. Results and discussion

The GC-FID method described was used to determine the trichothecene content of 22 wheat samples from the 1998 harvest. During the analytical sessions, duplicate determinations and recovery experiments were performed. Blanks (determination without test portion) were performed at regular intervals. A control sample (BCR CRM 379) was incorporated in each series of analyses as well. The results of these quality assurance experiments complied with the performance characteristics established for the GC-FID method.

Table 4
Trichothecene levels in wheat samples (μg/kg)

Country of origin and sample number	Trichothecene							
	DON	3-AcDon	FusX	NIV	DAS	NeoSol	HT-2	T-2
Germany 1	100	–[a]	–	–	–	–	–	–
Germany 2	1654	–	–	–	–	–	–	–
Germany 3	76	–	–	–	–	–	–	–
Germany 4	784	–	–	–	–	–	–	–
Germany 5	294	–	–	–	–	–	–	–
Germany 6	702	–	–	–	–	–	–	–
Germany 7	467	–	–	–	–	–	–	–
Germany 8	484	–	–	–	–	–	–	–
Germany 9	729	–	–	–	–	–	–	–
France 1	76	–	–	–	–	–	–	–
France 2	95	–	–	–	–	–	–	–
France 3	840	–	–	–	–	–	–	–
France 4	323	–	–	–	–	–	–	–
France 5	–	–	–	–	–	–	–	–
France 6	81	–	–	–	–	–	–	–
France 7	–	–	–	–	–	–	–	–
France 8	777	–	–	–	–	–	–	–
France 9	519	–	–	–	–	–	–	–
Canada 1	352	–	–	–	–	–	–	–
Canada 2	138	–	–	–	–	–	–	–
United Kingdom	–	–	–	–	–	–	–	–
The Netherlands	113	–	–	–	–	–	–	–

[a] –, Below limit of quantification (75 μg/kg).

The results of determining the trichothecene content of the wheat samples from the 1998 harvest are summarised in Table 4. No trichothecenes except DON were found in the wheat samples at levels above the limit of quantification.

Seven of the 22 wheat samples had DON levels above the temporary tolerance limit of 500 μg/kg in cleaned wheat, which is in effect in the Netherlands. Only one wheat sample exceeded the limit by more than 100%. Thirteen of the 22 wheat samples exceeded the proposed tolerance limit of 120 μg/kg for DON in cleaned wheat. Twelve of these samples exceeded the limit by more than 100%.

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