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Short communication

## Determination of trichothecenes in cereals

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### Abstract

An effective method for the determination of seven trichothecenes – deoxynivalenol (DON), nivalenol (NIV), T-2 tetraol (T-24), fusarenon-X (FUS-X), diacetoxyscirpenol (DAS), T-2 toxin (T-2), HT-2 toxin (HT-2) in cereals is presented. Gel permeation chromatography on Bio-Beads S-X3 was used for clean-up of acetonitrile–methanol extract. GC–ECD was used for identification and quantification of trifluoroacetylated trichothecenes. The limit of quantitation for the method was in the range 40–200 µg/kg. Recoveries at a spiking level of 2 mg/kg ranged from 76 to 100%. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Trichothecenes; Mycotoxins; Deoxynivalenol; Nivalenol; T-2 Tetraol; Fusarenon-X; Diacetoxyscirpenol; T-2 Toxin; HT-2 Toxin

### 1. Introduction

Trichothecenes are secondary metabolites produced by several fungal genera, most notably *Fusarium*, *Trichothecium*, *Stachybotrys* and *Myrothecium*. Chemical structures of trichothecenes are derived from a 12,13-epoxytrichothec-9-ene ring system. Individual groups of these mycotoxins differ in various functional groups. The type A compounds are characterised by the presence of a hydrogen, a hydroxyl or an ester functional group at C8, while the type B trichothecenes possess a carbonyl functional group at that position [1]. Fig. 1 shows basic structures of trichothecenes. At present 148 trichothecenes are known, of which 83 are non-macrocyclic and 65 macrocyclic compounds [2].

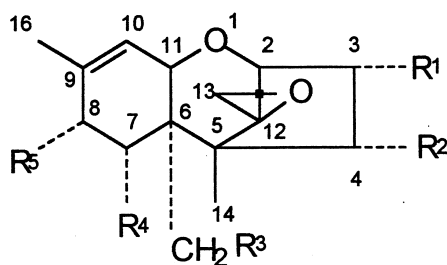
Trichothecenes such as deoxynivalenol, nivalenol

and T-2 toxin are spread worldwide. In central Europe, due to a moderate climate, fusariotoxins seem to be the most common mycotoxins in cereals such as wheat, corn, barley and oats.

These compounds have been known for a number of years to cause toxicosis in farm animals. In addition, they are associated with several human diseases. Alimentary toxic aleukia is characterised by vomiting, skin inflammation, diarrhoea, leukopenia, haemorrhaging and so on. Many fatal diseases were recorded in Russia and Central Europe, particularly in 1944 [3].

The correlation between the presence of certain trichothecene mycotoxins and adverse physiological responses indicates a need for specific methods for detection of these mycotoxins in various commodities. Chromatographic methods or, alternatively, enzyme-linked immunosorbent assays (ELISAs) are preferably used for determination of trichothecenes.

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Type	Trichothecene	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>
A	HT - 2 toxin	OH	OH	OCOCH <sub>3</sub>	H	OCOCH <sub>2</sub> CH(C H <sub>3</sub> ) <sub>2</sub>
	Diacetoxyscirpenol	OH	OCOCH <sub>3</sub>	OCOCH <sub>3</sub>	H	H
	T - 2 toxin	OH	OCOCH <sub>3</sub>	OCOCH <sub>3</sub>	H	OCOCH <sub>2</sub> CH(C H <sub>3</sub> ) <sub>2</sub>
	T - 2 tetraol	OH	OH	OH	H	OH
B	Nivalenol	OH	OH	OH	OH	=O
	Deoxynivalenol	OH	H	OH	OH	=O
	Fusarenon - X	OH	OCOCH <sub>3</sub>	OH	OH	=O

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

Analyses of these mycotoxins by thin-layer chromatography (TLC) typically lack either selectivity or sensitivity. Different separation systems and spray reagents are required to visualise and to characterise the type A and B compounds [4]. Difficulties, as regards sensitivity of detection, are encountered in high-performance liquid chromatography (HPLC) analysis when common UV detector is used because of the absence of a chromophore in the type A trichothecenes [5]. This problem could be solved by the application of LC–mass spectrometry (MS) [6].

In most of the available studies gas chromatography (GC) is employed for determination of trichothecenes. Application of high-resolution of capillary columns together with optimised ways of detection (electron-capture detection, ECD, MS) provides desired selectivity and sensitivity for analyses of the most common trichothecenes. In order to obtain sufficient volatility and enhanced detectability it is necessary to carry out derivatisation prior to GC [7,8].

A lot of clean-up procedures were developed. The methods are often time consuming and several purification steps are necessary. These procedures are concerned with one trichothecene or a group of trichothecenes of the same polarity.

The aim of the study is the development of a

simple gel permeation clean-up procedure as an alternative to solid-phase extraction (SPE) using alumina–charcoal or Florisil columns. This procedure combined with GC–ECD allows the determination of seven trichothecenes (type A and B) in cereals simultaneously.

## 2. Experimental

### 2.1. Chemicals and reagents

Mycotoxin standards: nivalenol, deoxynivalenol, T-2 tetraol, fusarenon-X, diacetoxyscirpenol, T-2 toxin, HT-2 toxin (Sigma–Aldrich, USA), individual stock solutions in ethyl acetate ( $c=50 \mu\text{g/ml}$ ) were stored at 4°C.

Internal standard: PCB No. 138 (Dr. Ehrenstorfer, Germany) in toluene ( $c=0.5 \mu\text{g/ml}$ ).

Derivatisation reagent: trifluoroacetic acid anhydride (TFAA) (Serva, USA).

Solvents: toluene, ethyl acetate, chloroform (Lachema, Brno, Czech Republic) acetonitrile, methanol (Merck, Germany), redistilled water.

Other chemicals: sodium bicarbonate (Merck), anhydrous sodium sulphate (Penta, Prague, Czech Republic). Bio-Beads S-X3 [poly(styrene–di-

vinylbenzene) copolymer, 200–400 mesh) (Bio-Rad, USA). Sample: wheat grain.

## 2.2. Instruments

Apparatus for GPC clean-up consisted of a HPLC pump HPP 5001 (Laboratorni Pristroje, Prague, Czech Republic), a stainless steel column (50 cm × 0.8 cm), sampling valve Rheodyne 7125 (Rheodyne, USA) with external injection loop – 1 ml, 2 ml, fused-silica capillary columns: NB-54 (5% phenyl)–(1% vinyl)–methylpolysiloxane (Nordion, Norway); NB-1701 (14% cyanopropylphenyl)–methylpolysiloxane (Nordion) and HP-5 (5% phenyl)–methylpolysiloxane (Hewlett-Packard, USA).

The gas chromatograph was a Hewlett-Packard Model HP 5890 II equipped with a split/splitless injector, HP Model 7673 autosampler and two  $^{63}\text{Ni}$  electron capture detectors (Hewlett-Packard). The mass selective detector, was a Hewlett-Packard Model HP 5872, the heating block was from Altech (USA) and the microfilter SR SLNSR025 NS was from Milex (USA).

## 2.3. Analytical procedure

### 2.3.1. Extraction

(A) Five g of representative sample of grained wheat were placed into an 100-ml Erlenmeyer flask and then 40 ml of a acetonitrile–methanol solvent mixture (1:1, v/v) were added. The flask was shaken for 1 h and then the suspension was filtered through a paper filter with 5 g of sodium sulphate.

(B) Five g of representative sample of grained wheat were placed into an 100-ml Erlenmeyer flask and then 40 ml of a acetonitrile–water solvent mixture (84:16, v/v) were added. The flask was shaken for 2 h and then the suspension was filtered through a paper filter with 5 g of sodium sulphate.

### 2.3.2. Clean-up by gel permeation chromatography (GPC)

Sample clean-up was performed by GPC on the column filled with Bio-Beads S-X3, which was swollen in chloroform. Mobile phase was chloroform, flow-rate was 0.6 ml/min.

Three ml of extract (A) or (B) were evaporated to dryness and redissolved in 3 ml of chloroform. The

extract was filtered through a microfilter and then injected onto the GPC column (via 1-ml or 2-ml injection loop). The first fraction (8 ml) was discarded. The second fraction (7 ml) was collected and then evaporated to dryness using a vacuum evaporator. The remainder was redissolved in 1 ml of ethyl acetate, placed into a 3-ml derivatisation vial and evaporated to dryness under a gentle stream of nitrogen.

### 2.3.3. Derivatisation

To the dried eluate in a 3-ml screw-cap vial 100  $\mu\text{l}$  of TFAA and 10 mg of sodium bicarbonate were added. The vial was placed in a heating block for 20 min at 60°C.

After cooling (5 min) the excess of derivatisation reagent was removed by a gentle stream of nitrogen. After addition of 300  $\mu\text{l}$  of toluene and agitation for 10 s 1 ml of water was added and the mixture was agitated again for 30 s. The upper toluene layer was collected and dried with 10 mg of anhydrous sodium sulphate. A 200- $\mu\text{l}$  aliquot was placed into the autosampler vial together with 50  $\mu\text{l}$  of internal standard solution (PCB 138,  $c=0.5 \mu\text{g}/\text{ml}$ ) and analysed by GC–ECD.

### 2.3.4. GC–ECD

Sample compounds were separated simultaneously on two fused-silica capillary columns NB-54 (50 m × 0.20 mm, 0.1  $\mu\text{m}$ ) and NB-1701 (50 m × 0.20 mm, 0.1  $\mu\text{m}$ ) that were inserted via a double hole ferrule into one injection port. The carrier gas was nitrogen, constant flow 0.5 ml/min and the make-up gas was nitrogen, constant flow 40 ml/min. Injection technique: splitless (period 2 min), 1  $\mu\text{l}$ , injector temperature:  $T_{\text{inj.}}=225^\circ\text{C}$ , detector temperature:  $T_{\text{det.}}=300^\circ\text{C}$ . The temperature program was as follows: initial period 80°C for 2 min, then 20°C/min to 175°C, 1°C/min to 245°C, 10°C/min to 270°C, held 10 min.

### 2.3.5. GC–MS

Analyses were carried out on fused-silica capillary column HP-5 (30 m × 0.25 mm, 0.25  $\mu\text{m}$ ). The carrier gas was helium, constant flow 0.6 ml/min. Injection technique: splitless (period 2 min), 1  $\mu\text{l}$ ,

injector temperature:  $T_{inj.}=250^{\circ}\text{C}$ , detector temperature:  $T_{det}=280^{\circ}\text{C}$ . The temperature program was as follows: initial period  $90^{\circ}\text{C}$  for 2 min, then  $10^{\circ}\text{C}/\text{min}$  to  $300^{\circ}\text{C}$ , held 10 min. Ionisation voltage: 70 eV.

### 3. Results and discussion

At the beginning of our study possible ways of trichothecene derivatisation and detection were considered. In principle, silylation, fluoroacetylation or fluorobutyration can be used for derivatisation of the OH groups contained in these mycotoxins. Each of these possibilities has some advantages and drawbacks. Heptafluorobutyration results in stable single derivatives (only in the case of NIV two tetraHFB – NIV products with identical molecular mass are formed) [10,11], however the reaction is rather time consuming (60–120 min) [9]. Trimethylsilylation is often used for derivatisation of type B trichothecenes. Trimethylsilyl (TMS) derivatives are easily prepared and suitable for MS detection [12]. The use of silylation is limited by the thermal stability of

derivatives, which may be decomposed in the injection port [13,14]. In our experiments, trifluoroacetylation was chosen as a derivatisation reaction aimed at conversion of analytes into volatile compounds. The use of TFAA as a derivatisation agent was preferred, because the reaction is quite rapid and a single reaction product is yielded. Removal of the derivatisation agent from the reaction mixture is easy and fast. Some authors [12,15,16] found TFA–trichothecenes to be stable for several days in a freezer at  $-20^{\circ}\text{C}$ . In our study, no changes in analyte content for a one-week period at laboratory temperature ( $20^{\circ}\text{C}$ ) were recorded.

Two types of detection techniques for determination of TFA derivatives of analytes were tested. The spectra obtained by the use of MS in the electron impact mode contained abundant fragments with low  $m/z$ , selective molecular ions or ions with high  $m/z$  had either low intensity or were absent. The prevalence of non-specific fragments in spectra of most derivatives avoided specific quantitation of trace amounts of analytes by this technique. Unfortunately, chemical ionisation, the use of which

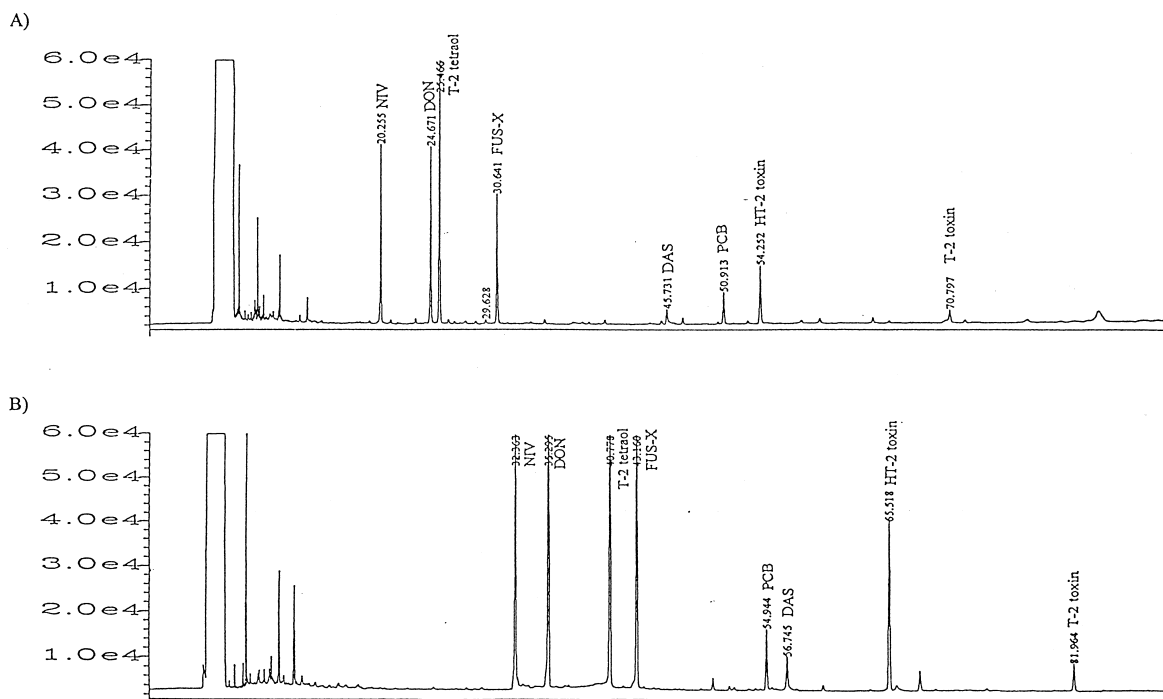


Fig. 2. GC separation of TFA derivatives on (A) NB-54 and (B) NB-1701 columns.

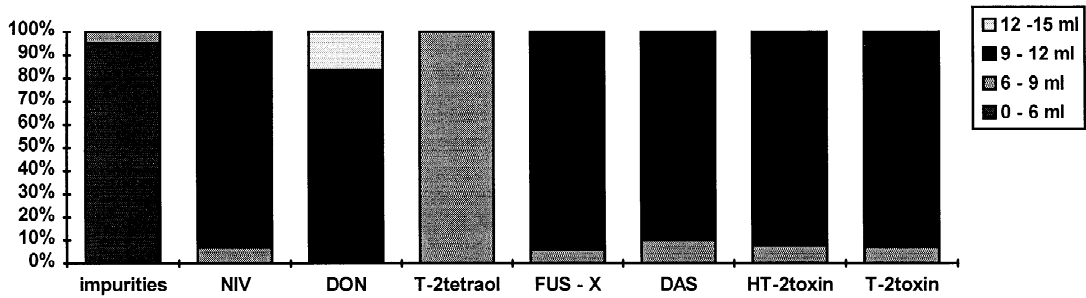


Fig. 3. Elution profile of trichothecenes and impurities.

was described in several studies [16,17], was not available in our laboratory.

ECD was therefore chosen as an alternative method of detection. To prevent separation problems as well as for confirmation purposes, a GC system consisting of two parallel columns with different polarities (NB-54 and NB-1701) was used for analy-

ses of samples. Similar columns were individually utilised for separation of TFA and HFB derivatives of trichothecenes in several published studies [18,19]. The calibration curve was linear in the range 40–800 pg per injection for each trichothecene ( $r^2$  ranging from 0.905 to 0.997). Fig. 2 shows chromatograms of a standard mixture of seven mycotox-

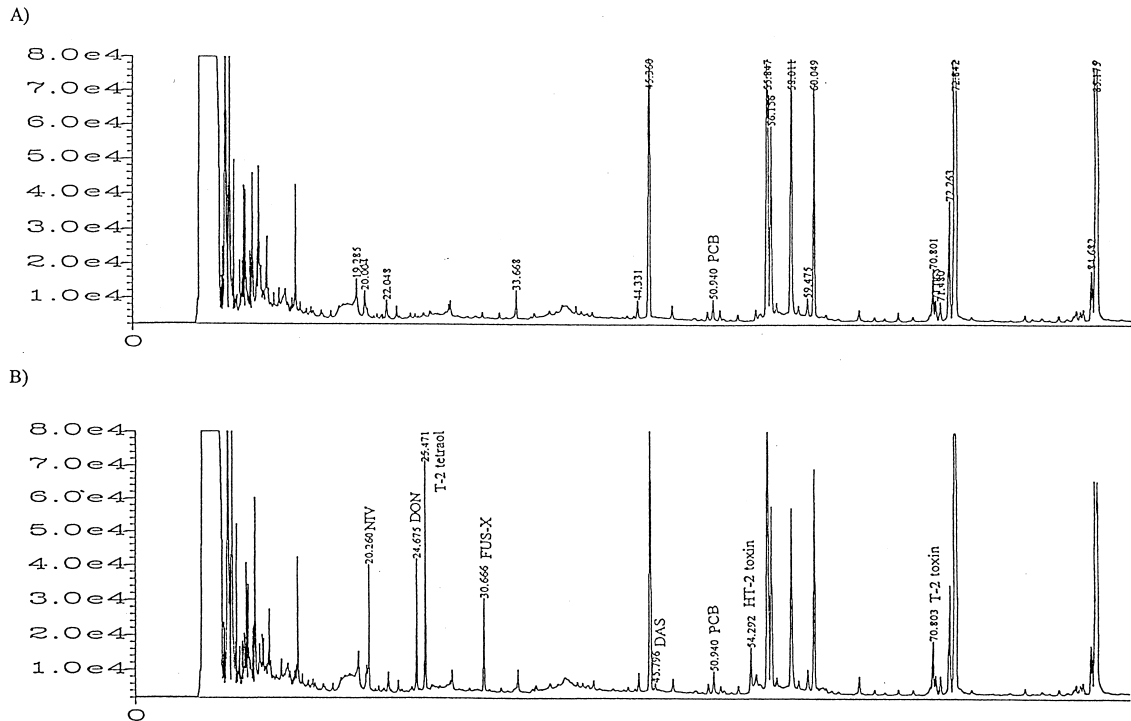


Fig. 4. Gas chromatogram of (A) blank wheat extract and (B) wheat extract spiked with trichothecenes obtained after separation on the NB-54 column.

ins. As can be seen, higher retention of analytes were recorded on the more polar NB-1701 column.

For extraction of trichothecenes from grain samples acetonitrile–methanol (1:1, v/v) and acetonitrile–water (84:16, v/v) [17,18,20] solvent mixtures were tested. After extractions transparent extracts were obtained. The absence of water in the extraction agent eliminated problems with evaporation.

The most common method of sample clean-up is the use of alumina–charcoal, Florisil or silica gel column [18,21–23]. In this study an alternative clean-up procedure was developed. It was carried out by means of GPC on the column filled with Bio-Beads S-X3. The method was optimised as follows: the first fraction (8 ml), which contained impurities, was removed. The second fraction (7 ml CHCl<sub>3</sub>) contained 78–100% of individual toxins was collected (see Fig. 3).

The only fraction, which was obtained after clean-up of acetonitrile–methanol (1:1, v/v) extract was analysed by GC–ECD. The fraction after clean-up of acetonitrile–water (84:16, v/v) extract was not ana-

Table 1

Recovery (mean±S.D., n=5) of trichothecenes from wheat spiked at a level of 2 mg/kg

Trichothecene	Recovery (%)	LOQ (µg/kg)
Nivalenol	87±3	40
Deoxynivalenol	88±9	50
T-2 tetraol	72±13	40
Fusarenon-X	78±4	100
Diacetoxyscirpenol	83±8	200
HT-2 toxin	105±6	100
T-2 toxin	91±5	200

lysed, because of coelution of waxes and other impurities. Table 1 shows recoveries of GPC method for spiked wheat.

GPC as described above, removes most impurities from wheat extract and in combination with GC appears to be a suitable method for analysis of wheat samples, which contain both types (A and B) of trichothecenes. Figs. 4 and 5 show chromatograms of wheat sample spiked by trichothecenes at a level of 2 mg/kg. Good separation of analytes was achieved on

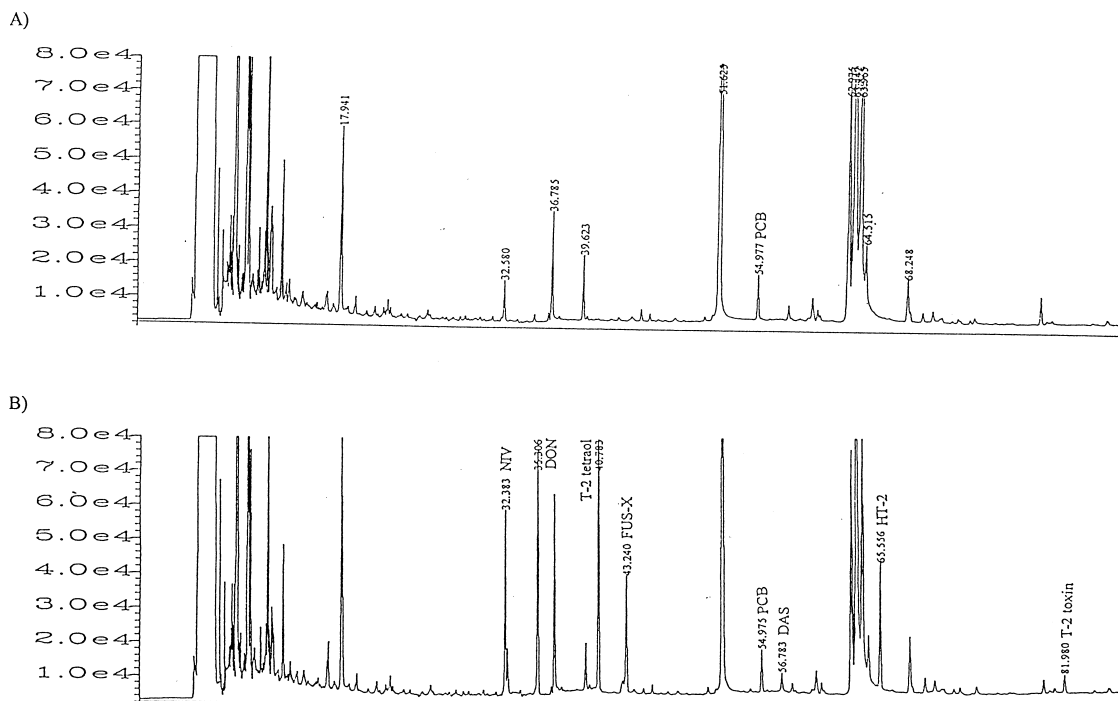


Fig. 5. Gas chromatogram of (A) blank wheat extract and (B) wheat extract spiked with trichothecenes obtained after separation on the NB-1701 column.

the both columns. The exceptions were NIV and T-2 toxin, which co-eluted with some interfering co-extract on the less polar NB-54 column. However, on the NB-1701 column NIV and T-2 toxin could be separated from these impurities. The quantitation limit (signal-to-noise ratio is 10) of this method ranged from 40 to 200 µg of trichothecenes/kg of sample (see Table 1).

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