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Comparison of two clean-up principles for determination of trichothecenes in grain extract

Z. Radová, K. Holadová, J. Hajšlová*

Department of Food Chemistry and Analysis, University of Chemistry and Technology, Technická 3, 166 28 Prague 6, Czech Republic

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Abstract

Two clean-up principles for the multi-determination of seven trichothecene toxins, deoxynivalenol, nivalenol, diacetoxyscirpenol, fusarenon-X, T-2 tetraol, HT-2 toxin and T-2 toxin in wheat extract are described. For clean-up of acetonitrile–water (84:16, v/v) extract either gel permeation chromatography (Bio-Beads S-X3 gel) or solid-phase extraction (combination of Florisil and C_{18}-silica gel SPE cartridges or a Romer Labs. MycoSep 225 column) were used. The MycoSep 225 column was chosen as the best alternative for clean-up of grain samples. Recovery of this procedure was tested on certified material. Derivatisation of analytes prior to the final determinative step was carried out by trifluoroacetic acid anhydride. Trifluoroacyl derivatives of the trichothecenes were separated by high-resolution capillary gas chromatography with electron-capture detection. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: Sample handling; MycoSep 225; *Fusarium*; Food analysis; Trichothecenes; Toxins

1. Introduction

Trichothecenes are secondary metabolites produced by some species of several fungal genera, most notably *Fusarium*. These substances compose a large group of mycotoxins identified in the natural environment. At present over 150 trichothecenes are known, which may be divided into two groups: non-macrocyclic and macrocyclic compounds [1,2]. Macrocyclic trichothecenes are commonly classified according to their chemical structures based on a 12,13-epoxytrichothec-9-ene ring system with various functional groups [2].

Trichothecenes of types A and B (see Fig. 1) were of interest in our study [3,4]. These compounds constitute an important health problem because of their possible occurrence in foods and feeds [2,5]. Trichothecenes such as deoxynivalenol, nivalenol and T-2 toxin are widespread, being most frequently found in cereals (wheat, corn, barley, oats and rye) [6–8].

For determination of trichothecenes, thin-layer chromatography (TLC) [9–11], high-performance liquid chromatography (HPLC) [12–14], supercritical fluid chromatography (SFC) [15] and enzyme-linked immunosorbent assay (ELISA) [16,17] have been applied. Nowadays capillary gas chromatography (GC) with either electron-capture detection (ECD) [4] or mass-selective detection (MS) [18] is widely used.

GC employing capillary columns offers a high-resolution separation (which is essential for multi-residue trichothecene analysis [19]) and good selec-

*Corresponding author. Tel.: +42-2-2431-4096, Fax: +42-2-2435-3185, E-mail: jana.hajslova@vscht.cz

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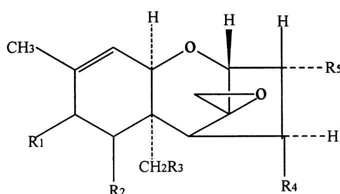

Type	Trichothecene	Abbreviation	R_1	R_2	R_3	R_4	R_5
A	HT-2 toxin	HT-2 tox.	$-O-COCH_2CH(CH_3)_2$	-H	-O-Ac	-OH	-OH
	T-2 toxin	T-2 tox.	$-O-COCH_2CH(CH_3)_2$	-H	-O-Ac	-O-Ac	-OH
	T-2 tetraol	T-2 tr.	-OH	-H	-OH	-OH	-OH
	Diacetoxyscirpenol	DAS	-H	-H	-O-Ac	-O-Ac	-OH
B	Deoxynivalenol	DON	=O	-OH	-OH	-H	-OH
	Nivalenol	NIV	=O	-OH	-OH	-OH	-OH
	Fusarenon-X	FUS-X	=O	-OH	-OH	-O-Ac	-OH

Fig. 1. Basic chemical structure of trichothecenes of types A and B.

tivity of the detection process compared to HPLC (although the demands for sample purity remain high even for GC).

Several clean-up procedures applicable for trichothecene analysis have been published. Most of them include adsorption chromatography on Florisil [20,21], silica gel [20,22] or charcoal–alumina column [23,24]. Alternatively, gel permeation chromatography (GPC) [22,25] has been employed.

In some studies, two clean-up steps have been combined for better purification of grain extract [20,26,27].

The aim of the presented study was to compare two clean-up strategies from the point of view of their suitability for the multi-determination of trichothecene toxins by high-resolution GC–ECD.

2. Experimental

2.1. Chemicals

2.1.1. Analytical standards

Trichothecene standards (99% purity, 1 mg) deoxynivalenol (DON), nivalenol (NIV), T-2 tetraol, fusarenon-X (FUS-X), diacetoxyscirpenol (DAS), HT-2 toxin and T-2 toxin were obtained from Sigma–Aldrich (Steinheim, Germany). Stock solutions of trichothecene standards were made up in acetonitrile from received commercial standards (0.2 mg/ml). Stock solution containing 5 μg/ml of each standard was prepared in acetonitrile.

2.1.2. Certified reference material

Certified reference material CRM 379 (wheat flour containing incurred deoxynivalenol residues, 0.67±0.02 mg/kg), tested under the supervision of the European Commission, Community Bureau of Reference (CBR, Brussels, Belgium), was used.

2.1.3. Reagents

Toluene, ethyl acetate, dichloromethane, methanol and acetonitrile (Merck, Darmstadt, Germany) were of HPLC-grade (99% purity), chloroform (analytical-reagent grade, Lachema, Prague, Czech Republic) was redistilled in glass prior to its use. Trifluoroacetic acid anhydride (TFAA) for GC analysis (99.5% purity) and sodium hydrogencarbonate (99.5% purity) (Merck) were used for derivatization.

2.2. Apparatus

2.2.1. Gel permeation chromatography

The equipment used for GPC consisted of a high-pressure pump HPP 4001 (Laboratorní Přístroje, Prague, Czech Republic); Rheodyne 4594, six-port injection valve, with a 2-ml external loop; and a steel separation column (50 cm×0.8 cm I.D.). The column was packed with Bio-Beads S-X3, 200–400 mesh (40–80 μm) (Bio-Rad Labs., Hercules, CA, USA) swollen in chloroform. Chloroform was used as the mobile phase (flow-rate 0.6 ml/min).

2.2.2. Solid-phase extraction

Solid-phase extraction (SPE) was performed using

a Dorcus vacuum manifold (Tessek, Prague, Czech Republic). C_{18}-silica gel cartridges (0.5 g, 52 μm average particles size) were obtained from Supelco (Bellefonte, PA, USA), Florisil SPE cartridges (1 g, 100 μm particle size) were supplied by J.T. Baker (Deventer, Netherlands) and MycoSep 225 columns were supplied by Romer Labs. (Union, MO, USA).

2.2.3. Gas chromatography

A HP-5890 series II gas chromatograph (Hewlett-Packard, Avondale, CA, USA) was equipped with a fused-silica capillary column HP-35 (30 m×0.25 mm I.D., 0.15 μm), an ECD system and an auto-sampler.

2.2.4. Derivatisation

Heating block and "MiniVials" with screw-cap PTFE-coated rubber septa from Alltech (Deerfield, IL, USA) were used.

2.3. Samples

Barley, oat, rye, wheat flour, corn and rice were obtained from a retail market.

Spiked sample (2000 μg/kg, 500 μg/kg or 300 μg/kg): blank sample was spiked with a mixture of trichothecenes in acetonitrile and left to dry for 30 min at ambient temperature. The residue of solvent was then evaporated by nitrogen.

2.4. Extraction

Ten g of blended homogenous representative wheat (or other grain) sample were extracted with 60 ml of acetonitrile–water (84:16, v/v) in a 250-ml flask for 1 h employing a rotary shaking machine. The supernatant was transferred to a 100-ml Erlenmeyer flask through folded filter paper. Extraction was repeated once more using 40 ml of acetonitrile–water (84:16, v/v) and shaking for 15 min. The combined extracts were made up to 100 ml with acetonitrile–water (84:16, v/v) and stored in a refrigerator at 4°C (max. two weeks) before purification.

2.5. Clean-up procedures

For purification of crude grain extracts, three experimental set-ups – GPC and two alternatives of SPE – were employed. Experiments were carried out under following conditions:

2.5.1. (A) Gel permeation chromatography

The aliquot (5 ml) of the grain extract was evaporated to dryness in a rotary vacuum evaporator, the residue was dissolved in 3 ml chloroform and then 2 ml of this solution (equivalent to 0.3 g of sample) were loaded onto the GPC column. A fraction 8–17 ml was collected and evaporated to dryness in the rotary vacuum evaporator.

2.5.2. (B) Solid-phase extraction

2.5.2.1. (B1) Florisil SPE cartridge followed by C_{18}-silica gel SPE cartridge

SPE cartridges were used to reduce interferences. For purification of wheat extract, a two-step clean-up procedure, a Florisil SPE cartridge followed by a C_{18}-silica gel SPE cartridge, was applied.

Florisil cartridge: Preconditioning was done with 4 ml of chloroform, followed by 4 ml of methanol and 4 ml of dichloromethane. An aliquot (5 ml) of crude grain extract was evaporated to dryness and re-dissolved in 2×1 ml of dichloromethane and loaded on the Florisil cartridge. To obtain target analytes, elution was carried out by 5 ml of chloroform–methanol (7:3, v/v). This eluate was further purified using a C_{18}-silica gel cartridge.

C_{18}-silica gel cartridge: Preconditioning was done with 3 ml of acetonitrile, followed by 3 ml of water and 3 ml of acetonitrile–water (84:16, v/v). The eluate from the Florisil cartridge was then evaporated to dryness, taken up in 2×0.5 ml of acetonitrile–water (84:16, v/v), and loaded on the C_{18}-silica gel cartridge. Analytes were eluted with 3 ml acetonitrile–water (2:8, v/v), solvent mixture was evaporated to dryness in the rotary vacuum evaporator.

2.5.2.2. (B2) Mycosep 225 column

The MycoSep 225 clean-up column consists of packing material, containing several adsorbents, such as charcoal, Celite, ion-exchange resins and others [33]. As illustrated in Fig. 2, the packing material is placed in a plastic tube in the space between filter discs with a rubber flange on the lower end containing a porous frit. When the column is pushed into

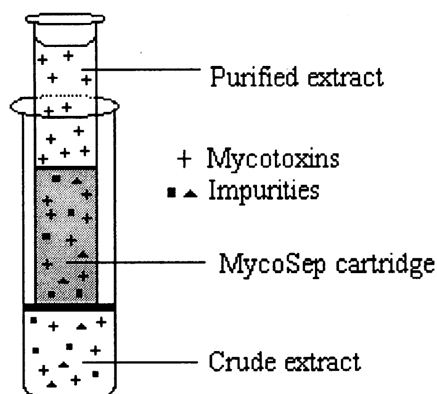

Fig. 2. MycoSep 225 clean-up column.

the test tube containing crude extract, the flange is sealed tight, thus the sample is forced through the packing material of the column. On the top of the plastic tube (i.e., above upper frit) the pure extract is collected (approximately 2.5 ml), see Fig. 2. A 2-ml volume of this purified extract was transferred using a micropipet to the round-bottom flask and concentrated to dryness in the rotary vacuum evaporator.

2.6. Derivatisation

The residue left after evaporation of solvent from purified analyte fraction was dissolved in ethyl acetate and transferred quantitatively to a 2-ml "MiniVial ". The solvent was evaporated to dryness using a gentle stream of nitrogen. A 100-μl volume of TFAA and about 10 mg of sodium hydrogencarbonate were added to the vial. After its capping it was heated for 20 min at 60°C. After cooling, the excess of derivatization reagent was evaporated by a gentle stream of nitrogen. Then 500 μl of toluene and 1 ml of redistilled water were added and this mixture was shaken for 1 min. A 300-μl volume of organic layer was transferred by syringe to autosampler vial and made up to 500 μl with toluene.

2.7. GC determination

The GC–ECD determination was carried out under the following conditions: nitrogen was used as a carrier gas with a flow-rate 1 ml/min and splitless injection mode (1 μl). The temperature of the splitless injection port was 250°C and the temperature of the detector 300°C. Column temperature program was: 80°C held for 2 min, 5°C/min to 150°C and 3°C/min to 207°C and 1.5°C/min to 250°C held for 5 min.

Table 1
GC linear calibration for trichothecenes (standards in the range 20–300 pg per injection)

Analyte	Coefficient of regression	R.S.D. (%)
NIV	0.996	8.5
T-2 tetraol	0.995	7.2
DON	0.999	3.8
FUS-X	0.997	8.1
DAS	0.994	10.2
HT-2 toxin	0.999	6.4
T-2 toxin	0.998	6.8

2.8. Calculations

Calculation of analytes content was performed using a ratio standard curve of trichothecene area versus trichothecene standard concentration. Six standard solutions, in concentrations ranging from 50 μg/kg to 2000 μg/kg, were used. Resulting response data were fit using a linear regression forced through the origin.

ECD responses were found to be linear ($r^2>0.99$) over the range 20–300 pg per injection of particular standard mixture of analytes (see Table 1).

3. Results and discussion

In our study, GC–ECD was employed for the quantitation of analytes. Due to a relatively polar nature, trichothecenes have to be volatilized prior to the GC run. Several agents provide appropriate derivatives. When TFAA is used for this purpose, detection sensitivity of individual trichothecenes depends on the number and position of hydroxyl functional groups available for acylation. The highest relative response is obtained for DON which has three hydroxyl groups amenable for trifluoroacetate esters formation (see Fig. 1). As expected, the more hydroxyl groups in the molecule, the greater is the detector response (see Fig. 3). Unlike trimethylsilyl (TMS) derivatives, which might be also considered,

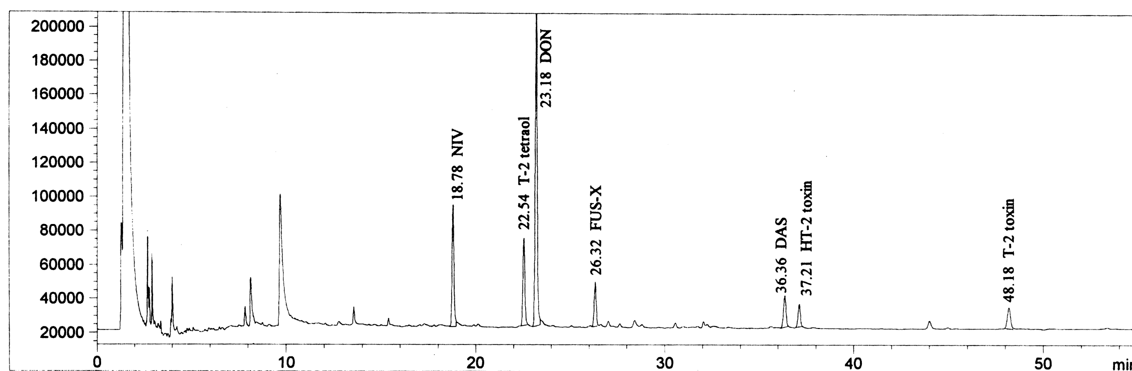

Fig. 3. Chromatogram of standard solution of trichothecenes; 72 pg injection of each analyte. x-Axis: Analysis time; y-axis: ECD response.

trifluoroacyl esters offer comparable detector sensitivity for both groups of trichothecenes (i.e., types A and B). Moreover, trifluoroacyl derivatives are less sensitive to the presence of moisture [21]. Thus for our purpose we used TFAA as derivatization agent.

Several extraction solvents (acetonitrile [28], methanol [29]) and/or their mixtures (methanol–water [30], acetonitrile–water [1], acetonitrile–ethyl acetate–water [21], chloroform–ethanol–water [31]) in different proportions have been employed for extraction of grain samples in published studies. In any case, due to the bulk coextracts typically contained in crude extracts obtained by relatively polar solvent mixtures, great demands were placed both on the efficiency of the clean-up step and on the performance of the GC separation, especially when a relatively non-specific detection technique such as ECD was employed.

A solvent mixture consisting of acetonitrile and water in the ratio 84:16 (v/v) was employed for extraction of wheat samples. Subsequent concentration of this water-containing mixture was facilitated because the boiling point of such azeotropic solution is lower. In the first set of experiments, the crude extract was purified by GPC on Bio-Beads S-X3 with chloroform as the mobile phase. Most of the coextracts (lipids and pigments) presented in sample were removed but there were still some matrix components left in the trichothecene fraction. These impurities were deposited in the injection port and the front part of the chromatographic column and caused successive losses of chromatographic resolution and deterioration of the overall performance of the GC system [32]. It was not therefore possible to determine reliably trichothecenes below spiking level 2 mg/kg. At this spiking level exceeding this value, good recoveries of trichothecenes (86–97%) were obtained.

Attempting to minimise time of the sample extract evaporation and simultaneously trying to reduce co-isolation of polar compounds from grain samples, non-aqueous mixture consisting of acetonitrile–methanol (1:1, v/v) was used for extraction of grain sample. Unfortunately, the amount of coextracts was not reduced significantly, similar problems as those mentioned above were encountered. The recoveries of polar analytes (type B, see Fig. 1) were even lower (72–88%).

In the further set of the experiments focused on optimisation of sample purification the extraction mixture acetonitrile–water (84:16, v/v) was used again, because of its suitability for isolation of analytes covering wide range of polarities. However, instead of GPC, other clean-up principles employing various types of SPE cartridges were tested. Unfortunately, none of the commonly used single cartridges (silica gel cartridge, C_{18}-silica gel cartridge, Florisil, EN-LiChrolut) provided efficient clean-up procedure. Therefore, two other alternatives, (i) combination of two SPE cartridges (Florisil cartridge followed by C_{18}-silica gel cartridge [33]) and (ii) a commercial MycoSep 225 column dedicated for trichothecene analysis, were tested.

The recoveries obtained by SPE-based clean-up procedures together with the limit of detection

Table 2
Performance characteristics of clean-up procedures and determinative steps in trichothecenes analyses

Analyte	Florisil+C_{18}-silica gel cartridges[c]:	MycoSep 225 column	
	Recovery (%) of clean-up procedure±R.S.D.[a] (%)	Recovery (%) of clean-up procedure±R.S.D.[a] (%)	LOD[b] (mg/kg)
NIV	19.9±1.2	84.9±4.0	0.05
T-2 tetraol	19.7±14.5	58.5±9.3	0.20
DON	37.5±7.3	75.5±6.7	0.02
FUS-X	27.0±2.0	80.5±14.2	0.20
DAS	80.1±24.8	78.1±19.2	0.20
HT-2 toxin	84.8±1.4	82.4±5.6	0.20
T-2 toxin	87.0±8.0	92.9±2.4	0.10

[a] Spiking level of crude extract: 0.5 mg/kg (n=6).
[b] Calculated as three-times the standard deviation of blank sample noise.
[c] LODs were not determined for GC employing this clean-up procedure.

(LOD) of GC–ECD are shown in Table 2. Although an efficient removal of coextracts was achieved by the ‘‘two cartridges’’ SPE method (see Fig. 4), unacceptably low recoveries for more polar trichothecenes (DON, NIV, T-2 tetraol and FUS-X) were obtained (see Table 2). The usage of the C_{18}-silica gel cartridge was proved to be a critical step of this clean-up procedure. Similar problems were encountered by Weingaertner et al. [34], who pointed to potential binding of analytes to active sites of cartridge. Only minor interferences were present in blank sample at retention times corresponding to DON and DAS.

As shown in Table 2, good recoveries and precision (R.S.D.) for the whole spectrum of analytes were obtained when clean-up of grain extract was carried out by the MycoSep 225 column (see Fig. 5). The spiking level of 0.5 mg/kg was chosen according to concentrations of trichothecenes in contaminated samples. The only exception was rather low recovery recorded for T-2 tetraol (58.5±9.3%). As proved by standard solution, this phenomenon was obviously due to the strong adsorption on polar sites of the packing material. As regards efficiency of purification, only negligible interferences were present in the blank sample at retention times corresponding to deoxynivalenol, nivalenol and diacetoxyscirpenol.

The applicability of the MycoSep 225 column for a clean-up of other cereals, e.g., barley, oats, rye, corn, wheat flour and rice were tested at spiking level 0.3 mg/kg, even lower than for wheat in previous experiments.

Recoveries determined for single trichothecenes are shown in Table 3. As shown in Figs. 6 and 7, the purified extracts of rice or corn sample contained

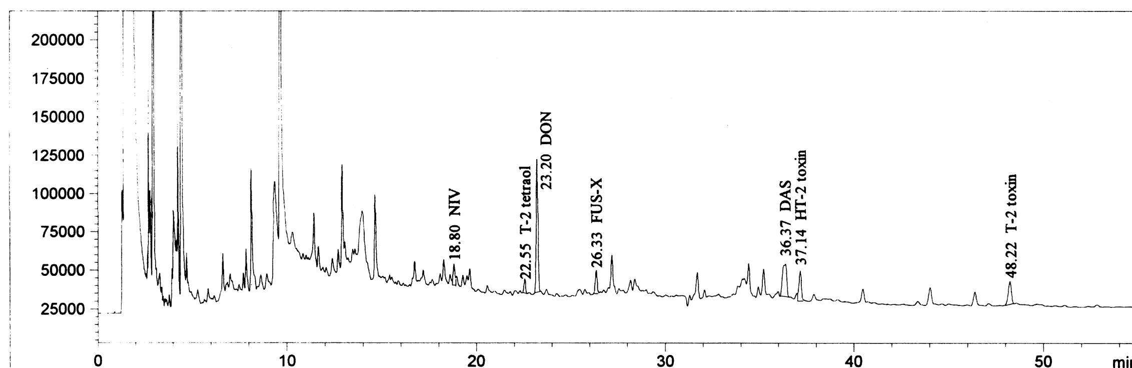

Fig. 4. Chromatogram of spiked wheat sample (0.5 mg/kg); clean-up on Florisil+C_{18}-silica gel cartridges; injection corresponds to 180 pg of each toxin in 0.36 mg sample. Axes as in Fig. 3.

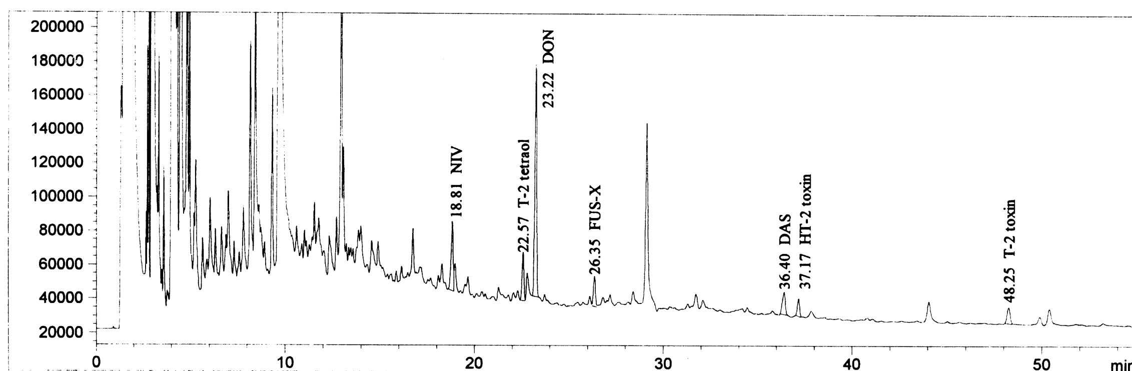

Fig. 5. Chromatogram of spiked wheat sample (0.5 mg/kg); clean-up on a MycoSep 225 column; injection corresponds to 72 pg of each toxin in 0.144 mg sample. Axes as in Fig. 3.

Table 3
Recoveries and R.S.D.s of trichothecenes from grain samples (spiking level 0.3 mg/kg of sample[a], $n=5$) obtained with the MycoSep 225 column clean-up procedure

Matrix	Recovery (%)±R.S.D. (%)						
	NIV	T-2 tetraol	DON	FUS-X	DAS	HT-2 toxin	T-2 toxin
Barley	89.8±26.6	63.2±22.9	94.6±16.8	77.7±15.0	×	85.5±6.4	102.3±5.3
Oats	62.7±20.7	111.0±27.2	77.9±16.9	83.2±9.5	×	106.8±3.4	113.4±6.9
Rye	79.4±30.8	56.1±20.9	71.2±4.4	67.2±10.6	×	105.7±11.9	109.6±8.4
Corn	67.2±30.5	52.3±31.2	64.6±8.7	64.1±5.4	139.5±5.9	108.2±14.1	107.2±8.4
Rice	106.2±21.3	69.5±30.9	115.3±11.8	105.2±18.1	×	95.6±4.0	104.4±0.6
Wheat flour	83.4±25.4	58.5±7.9	92.2±7.8	97.6±1.5	×	94.0±11.5	105.4±7.2

[a] Hygienic limits in Czech Republic for DON in grains, flour, rice and corn are 2.0 mg/kg, 1.0 mg/kg, 2.0 mg/kg and 2.0 mg/kg, respectively [35].

lower amount of interferences in comparison with other commodities. Unfortunately, for all the matrices except corn, the quantitation of DAS, which gives very low detector response (see Fig. 3), was at such low level impossible due to the presence of interfering peak in blank samples. The recovery of

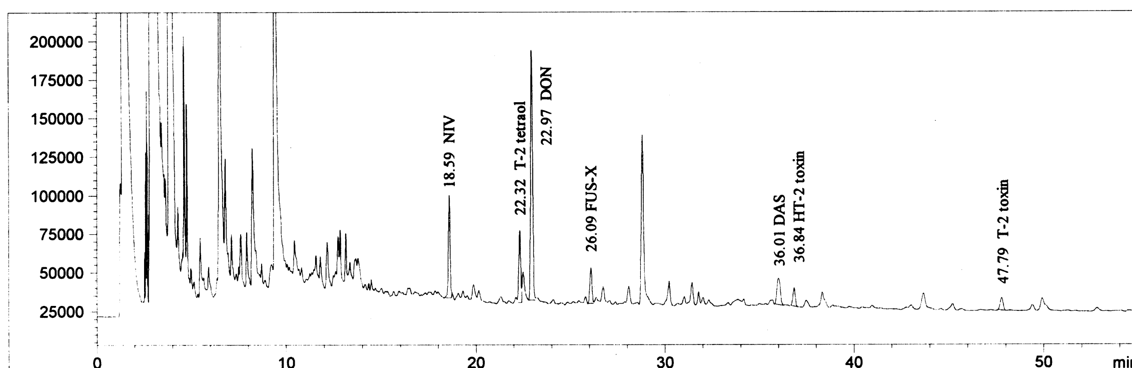

Fig. 6. Chromatogram of spiked rice sample (0.3 mg/kg); clean-up on a MycoSep 225 column; injection corresponds to 43.2 pg of each toxin in 0.144 mg sample. Axes as in Fig. 3.

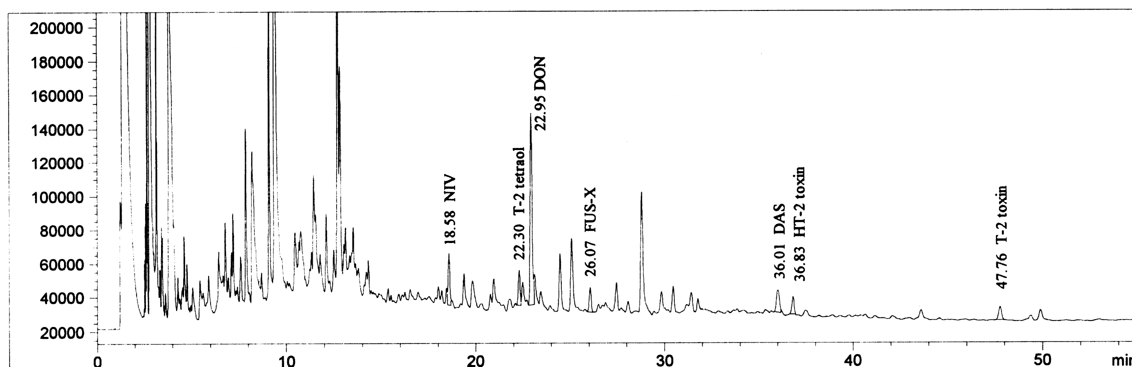

Fig. 7. Chromatogram of spiked corn sample (0.3 mg/kg); clean-up on a MycoSep 225 column; injection corresponds to 43.2 pg of each toxin in 0.144 mg sample. Axes as in Fig. 3.

DON, which is the most controlled trichothecene mycotoxin by legislation, ranged in grain samples from 64.6 to 115.3% with the relative standard deviation in the range 4.4 to 16.9%. The trueness of results obtained by procedure employing MycoSep 225 column was documented through the analysis of CRM 379. The analysis of the certified material showed a mean value of 0.671 mg/kg with a standard deviation of 0.029 mg/kg for five determinations. The corresponding confidence interval at 95% probability ranged from 0.642 to 0.700 mg/kg. The certified value was 0.673 mg/kg, determined and that certified as a mean value of six independent determinations by six laboratories.

4. Conclusions

GC–ECD analyses of six trichothecenes (determined as triflouracyl derivatives) in grains (wheat, barley, oat and rye), wheat flour, rice and corn were shown to be accurate enough even at relatively low levels of mycotoxins supposing an efficient purification step is involved. For this purpose, the GPC technique on Bio-Beads S-X3 gel was found not to be acceptable due to the poor separation of impurities interfering in subsequent GC run. SPE clean-up procedure on Florisil followed by C_{18}-silica gel cartridges was proved suitable for non-polar trichothecenes (HT-2 toxin and T-2 toxin). The comparison of a MycoSep 225 column and the "two cartridges" SPE clean-up procedures showed that more time and special laboratory equipment (vacuum manifold) were needed to obtain reliable results in the latter case. The method employing the one-step clean-up procedure by a MycoSep 225 column offers not only good recoveries of analytes representing various polarity classes and adequate precision at levels below common hygienic limits, but also a significant reduction of analysis time as well as reduction of the production of solvent waste. These features make the MycoSep 225 clean-up column very perspective for its use in routine analyses of trichothecenes in grains and related matrices.

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