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Journal of Chromatography A, 795 (1998) 297–304

JOURNAL OF CHROMATOGRAPHY A

Determination of trichothecene mycotoxins in wheat by use of supercritical fluid extraction and high-performance liquid chromatography with diode array detection or gas chromatography with electron capture detection

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Received 15 July 1997; accepted 10 September 1997

Abstract

The extraction behaviour of the *Fusarium* mycotoxin deoxynivalenol (DON) and some related type B trichothecenes from spiked seasand, spiked wheat flour and naturally contaminated wheat flour with modified supercritical CO_2 has been investigated and optimized under several conditions. The extraction fluid was decompressed over a solid-phase trap and the amount of deposited analytes was determined by HPLC–diode array detection (DAD) or GC–electron capture detection (ECD) without any further clean-up. Recovery rates as high as 90.1±10.7% were achieved for spiked wheat samples and 53.0±3.2% for naturally contaminated samples. The performance of the optimized supercritical fluid extraction (SFE) method was compared with an already well established analytical method employing extraction on a rotary shaker in combination with Mycosep clean-up.

Moreover, the SFE procedure developed for naturally DON contaminated wheat was employed for the simultaneous extraction of 5 type B trichothecenes by GC–ECD.

This work represents the first successful approach in obtaining an SFE-method for the extraction of *Fusarium* mycotoxins from wheat with reasonable recoveries and good precision. © 1998 Elsevier Science B.V.

Keywords: Supercritical fluid extraction; Mycotoxins; Trichothecenes; Deoxynivalenol

1. Introduction

The determination of mycotoxins in food and animal feed is of great importance because of their variety of toxic effects on humans and animals. Trichothecenes are a group of over 148 mycotoxins with a common tetracyclic 12,13-epoxytrichothec-9-ene ring system (Fig. 1), which is toxic due to the epoxide group. Many of the trichothecenes are produced by a diverse variety of fungi imperfecti, especially by the genus *Fusarium*, which is known to attack various cereals [1,2].

Generally, the quantitative analysis of these compounds involves their extraction from the sample matrix, followed by laborious clean-up procedures. For the final separation and quantification step several chromatographic methods are applied includ-

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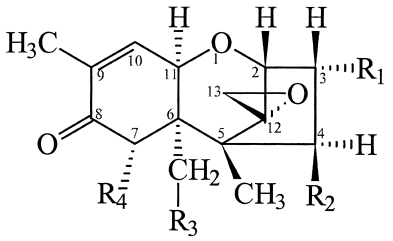
0021-9673/98/$19.00
PII S0021-9673(97)00944-8



	R_1	R_2	R_3	R_4
3-Acetyldeoxynivalenol (3-AcDON)	OAc	H	OH	OH
15-Acetyldeoxynivalenol (15-AcDON)	OH	H	OAc	OH
Deoxynivlenol (DON)	OH	H	OH	OH
Fusarenon X (FUS-X)	OH	OAc	OH	OH
Nivalenol (NIV)	OH	OH	OH	OH

Fig. 1. Structures of type B trichothecenes.

ing thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) with UV- or fluorescence detection [3], gas chromatography (GC) with electron capture detection (ECD) or mass selective detection (MS) [4–6] and supercritical fluid chromatography (SFC) coupled with MS or flame ionization detection (FID) [7]. Immunological methods such as enzyme linked immunosorbent assays (ELISA) or radio immuno assays (RIA) are also used for the analysis of these mycotoxins [8].

However, the main problem of trichothecene analysis is not detection but sample pretreatment for obtaining a selective and fast extraction of the mycotoxins from the matrix. The commonly used extraction and clean-up procedures require time-consuming solvent extractions with high consumption of organic solvents generally followed by solid-phase extraction (SPE) employing silica gel, florisil, C_{18} phases and others for sample purification.

Supercritical fluid extraction (SFE) is a useful alternative to these conventional techniques for extraction and sample purification in which a great amount of potentially hazardous solvents are employed. In contrast to the use of these solvents, supercritical CO_2 is nontoxic, nonflammable and chemically inert. Furthermore, by means of modern SFE-approaches both extraction and clean-up can be performed in one step [9,10].

Supercritical fluids generally have low viscosities (e.g. 10^{-3}–10^{-4} g/cm/s), high diffusivities (e.g. 10^{-3}–10^{-4} cm^2/s) and moderate densities (e.g. 0.3–0.8 g/cm) which lead to more rapid and efficient extractions because of the combination of liquid-like solvating and gas-like mass transfer properties. Hence, SFE has been shown to be a quantitative and rapid method for extracting nonpolar components from a lot of different sample matrices. Modifications of the extraction selectivity can be simply achieved by changing the temperature or pressure during extraction [10]. Moreover, moderately polar compounds can be dissolved in supercritical CO_2, especially at higher densities or by addition of polar modifiers (e.g. methanol), because the solvent strength of a supercritical fluid increases with increasing density. However, in particular for polar substances, as e.g. mycotoxins, the recoveries obtained with CO_2-SFE are generally very poor (<20%) [11]. One of the few studies on SFE of mycotoxins deals with the extraction of aflatoxin B1 from corn. The recoveries obtained from incurred compound feed were only 4–14% even when extraction periods of 30 to 90 min were employed. Higher recoveries up to 86% were only achieved with excessive extraction times of 6 to 7 h [11–14].

Only two publications can be found in literature which deal with SFE of *Fusarium* mycotoxins [15,16]. However, no values for the obtained DON recoveries are given in the work which deals with SFE of DON [15].

The goal of this study was to develop an analytical method for the determination of DON in wheat based on SFE and HPLC–DAD/GC–ECD end-detection. For this purpose several SFE parameters (*p*, *T*, *t*, flow, thimble packing, analyte trapping, etc.) had to be optimized. In addition, the DON recoveries obtained by SFE were compared with the recoveries obtained with a well established method employing classical solvent extraction and SPE clean-up as routinely applied in our laboratory for the analysis of trichothecenes.

2. Experimental

2.1. Chemicals

Crystalline 3-AcDON, 15-AcDON, FUS-X and NIV from Sigma, Vienna, Austria and DON from

Promochem, Wesel, Germany were dissolved in methanol (HPLC gradient grade, LiChrosolv, Merck, Vienna, Austria) to obtain a solution which was used for spiking experiments. To prepare DON standard solutions for HPLC analysis, pure DON was dissolved in water (mono-distilled)–acetonitrile (HPLC grade, Donauchem, Vienna, Austria) (95:5, v/v). The naturally DON contaminated wheat used in this work was preanalyzed with the described reference method (c=22.84±2.36 mg/kg).

The wheat was ground with a Retsch mill, type SM 1, Vienna, Austria with 1.00- and 0.25-mm sieves. The ground wheat was dried at least 2 h at 100°C to remove water which might act as a modifier during SFE.

2.2. Supercritical fluid extraction

Supercritical fluid extractions were performed by using modified carbon dioxide (5.2-SFC quality, AGA, Vienna, Austria on an SFE-Module (type HP 7680 T, Hewlett-Packard Vienna, Austria). The modifier was added to the CO_2 with a T-connected HPLC pump (type HP 1050, Hewlett–Packard, Vienna, Austria) prior to the cryogenic high-pressure pump of the SFE apparatus. Solvents used as SFE modifiers or rinse solvents (p.a. grade) were purchased from Baker, Vienna, Austria with the exception of methanol (HPLC gradient grade, LiChrosolv, Merck, Vienna, Austria). Analyte trapping was carried out with solid-phase traps, either prepacked with stainless steel balls, ODS hypersil and florisil (Hewlett–Packard, Vienna, Austria) or a self-made silica trap (silica gel 60, Merck, Vienna, Austria). The rinse solvents were degassed in an ultrasonic bath (type 2210, Branson, Vienna, Austria).

The extracts were evaporated to dryness under a stream of nitrogen at 50°C and redissolved in 500 μl of water–acetonitrile (95:5 v/v) for HPLC analysis or derivatized for GC analysis.

2.3. Conventional solvent extraction and clean-up (Reference method)

The conventional solvent extraction and clean-up was performed according to Weingaertner et al. [5]. 25 g of the ground wheat were weighed into a 250-ml Erlenmeyer flask and extracted with 100 ml acetonitrile–water (84:16 v/v) for 80 min on a shaker (type 3017, GFL, Vienna, Austria). After filtrating the extract, an aliquot of about 8 ml was transferred to a glass culture tube and was purified by pushing the flange end of the Mycosep #225 column (Romer Labs, Union, MO, USA) into the culture tube until about 3 ml had passed the column. Then 2 ml of this clear extract were transferred to vials and evaporated to dryness under a gentle stream of nitrogen at 50°C. The residue was redissolved in 500 μl of water–acetonitrile (95:5, v/v) for HPLC analysis or derivatized for GC analysis.

2.4. Analysis by high-performance liquid chromatography

After centrifugation at 5000 r.p.m. for about 15 min with a centrifuge (type GS 15, Beckman, Vienna, Austria), aliquots (500 μl) of the extracts containing DON were directly analyzed by HPLC.

2.4.1. HPLC equipment

An HPLC (type HP 1090 series II, Hewlett–Packard, Vienna, Austria) equipped with a diode array detector (DAD HP 1096 A, Hewlett-Packard, Vienna, Austria) and HP data processing software was used.

2.4.2. HPLC column

The employed analytical column was a reversed-phase ODS hypersil column (100×2.1 mm, 5 μm, Hewlett–Packard, Vienna, Austria) series connected with a reversed-phase ODS hypersil guard column (20×2.1 mm, 5 μm, Hewlett–Packard, Vienna, Austria).

2.4.3. HPLC conditions

The HPLC operated with a gradual carrier of water–acetonitrile (H_2O–AcN) pumped at 0.4 ml/min. The HPLC pump was programmed as follows: start H_2O–AcN (95:5, v/v), increase to H_2O/AcN (90:10, v/v) during 5 min, held for 0.5 min, followed by a return to H_2O–AcN (95:5, v/v) during 2 min and finally held for 4.5 min. Injections were made in duplicate and the injection volume was 20 μl. The detection wavelength used was 220 nm. Quantification was done by comparison of the peak area of DON against a calibration curve of the peak area obtained with authentic standard solutions.

2.5. Analysis by gas chromatography

The analysis by GC was performed similar to the procedure described by Weingaertner et al. [5] and Scott et al. [6]. The dry residue was derivatized for 20 min at 30°C after adding 50 μl of TRI–SIL–TBT and shaking on a tube shaker. Then 500 μl of isooctane and 1 ml of buffer solution (mixture of 50 ml 0.1 *M* potassium phosphate and 29 ml of 0.1 *M* sodium hydroxide, pH 7) were added and mixed for 1 min on a tube shaker. After phase separation an aliquot of 100 μl of the iso-octane phase containing the analytes were transferred to a GC vial and filled up to 1 ml with iso-octane, which was directly analyzed by GC.

2.5.1. GC equipment

Separation and detection were performed on a GC (type HP 5890 series II, Hewlett–Packard, Vienna, Austria) equipped with a ^{63}Ni electron capture detector and split/splitless autoinjector (type HP 7673, Hewlett–Packard, Vienna, Austria) and HP data processing software.

2.5.2. GC column

The employed analytical column was a capillary column (type HP 5, 30 m×0.32 mm, 0.25 μm, Hewlett–Packard, Vienna, Austria).

2.5.3. GC conditions

The GC operated in the splitless mode with helium as carrier gas at a flow-rate of 2 ml/min and nitrogen as detector gas with a flow of 80 ml/min. The injector and detector temperatures were adjusted to 250°C and 320°C. The GC oven was programmed as follows: start at 80°C, held for 2 min, increase to 182°C at 26°C/min, followed by an increase to 196°C at 1°C/min with a holding time of 19 min, followed by raising the temperature to 285°C at 25°C/min with a final hold for 4 min.

3. Results and discussion

3.1. General behaviour

The ability to extract analytes from a matrix by SFE depends on a variety of factors such as analyte–matrix interactions, the location of the analyte within the matrix, the porosity of the matrix, the vapour pressure, the diffusion coefficient of the analyte in the supercritical fluid and many other complex experimental variables. But the main variable is the solubility of the analyte in the supercritical CO_2, which is controlled by their polarity and the fluid density. The density is a function of temperature and pressure. Moreover, a small amount of a modifier (organic solvent, e.g. methanol) added to the CO_2 is able to enhance the solvating power and the polarity of the supercritical fluid. In addition, it is assumed that the role of modifer is to compete with the analyte for sites on the matrix or rather to disrupt the analyte–matrix interactions [9].

3.2. Behaviour of spiked inert matrix

Initial extractions of DON spiked inert matrix were performed to investigate the relationships and the influences of these variables. A 5 g amount of seasand (p.a. grade, Fluka, Vienna, Austria) was spiked with 250 μl of a solution containing 201 μg/ml resulting in a spiking level of 10.05 mg/kg. SFE of 5 g seasand conducted at 148 bar and 70°C at a density of 0.5 g/ml gave the highest DON recovery of 90.1±0.9% by use of CO_2 modified with 2% methanol. The extraction was carried out in dynamic mode for 25 min with a fluid flow-rate of 1.5 ml/min by use of a ODS Hypersil solid-phase trap to collect the analyte after decompression. Under the same conditions SFE of DON spiked wheat flour lead up to 7.5±0.8% recovery only, owing to the obviously stronger analyte–matrix interactions and different surface properties.

3.3. Behaviour of spiked wheat

In the next set of experiments 1 g of DON spiked wheat flour (25.1 mg/kg spiking concentration) was extracted with modifed CO_2. The temperature was kept straight above the critical temperature (40°C) at nearly highest possible pressure (318 bar) to obtain a maximum density (0.92 g/ml) and polarity. Preliminary extractions showed that maximum extraction efficiency could be achieved by employing the dynamic mode for 60 min with a fluid flow-rate of 2.0 ml/min and a preceding static mode of 15 min presumably due to the disruption of the strong analyte–matrix interactions.

3.3.1. Effect of the modifier

Due to the polar structure of DON, many modifiers were tested to increase the polarity of the supercritical fluid and thus to remove the analyte from the active sites of the matrix. The values obtained with the well established method was referred to a 100% recovery for the naturally contaminated samples. For the spiked wheat samples the best recoveries (90.1±10.7%) were obtained by use of 3% modifier consisting of a mixture of acetonitrile–methanol (2:1, v/v) (Fig. 2). Using 3% methanol yielded lower recoveries of 70.0% but with a better precision (R.S.D.=5.0% for $n=3$).

SFE modified with water and water-based mixtures was also tested. In all cases where water was present the recoveries decreased dramatically, until no DON was detectable. To check whether the extraction was exhaustive or if there was still a remaining amount of analytes bound to the matrix, the samples already extracted by SFE were additionally extracted according to the reference method. However, only low recoveries (<5%) were obtained when applying this second extraction procedure. We tentatively explain the loss of DON by hydration or decomposition of the analyte during the SFE in the presence of water.

3.3.2. Effect of the trapping performance

The solid-phase trap within the SFE system serves to collect the analyte after completed extraction and decompression of the supercritical fluid. Four different trap materials, silica, florisil, stainless steel balls and ODS Hypersil, were tested. Fig. 3 indicates that better recoveries can be achieved by employing polar chemically active sorbent traps due to the more efficient absorption of the polar DON. Especially, the use of the self-made silica solid-phase trap gave the best recoveries. For preparation, a suspension of 890 mg silica gel 60 (Merck, Vienna, Austria) in $CHCl_3$ (p.a., Baker, Vienna, Austria) was filled continuously into an empty SFE-trap, which was connected to a low-pressure chamber. However, it turned out that the trap can only be used for the retention of the analyte due to its chemical affinity to the compound of interest and not to remove interfering compounds. The adsorbed DON could be quantitatively eluted from the trap with 1.5 ml of methanol.

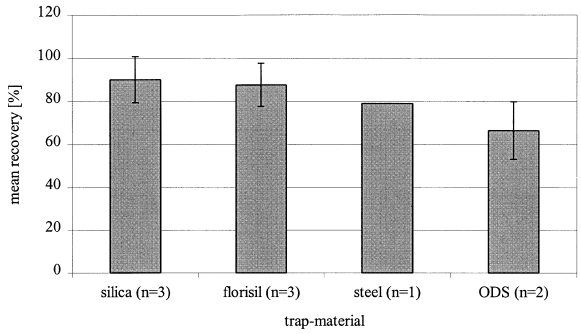


Fig. 3. Effect of different trap materials on the recovered concentration of DON.

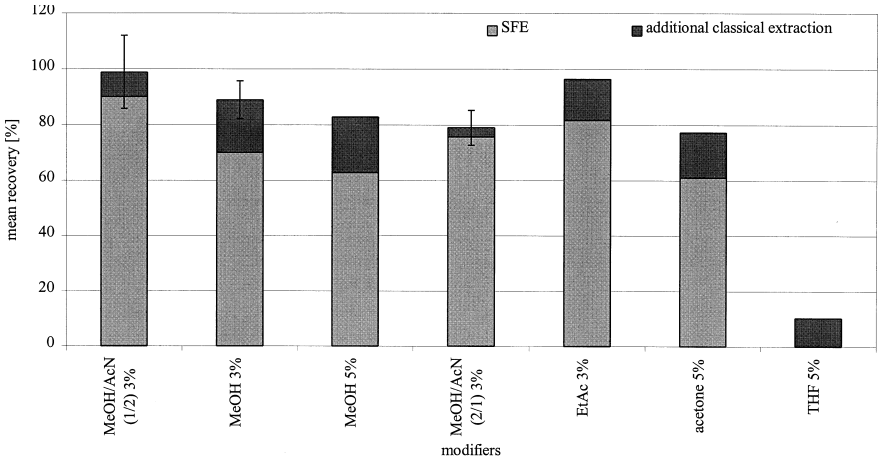


Fig. 2. Effect of different modifiers on the recovered concentration of DON extracted from 1 g of spiked wheat.

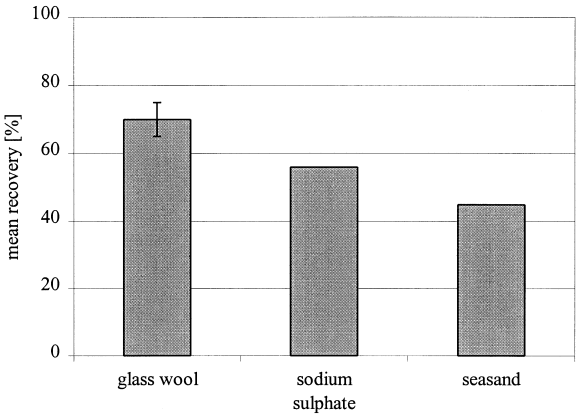


Fig. 4. Effect of different thimble-filling materials on the extraction efficiency of DON.

3.3.3. Influence of the extraction cell

The orientation of the extraction thimble, the fluid direction and the thimble void volume are parameters, which also have an influence on the extraction efficiency. In our preliminary experiments, the thimble was filled with a mixture of the wheat flour sample and an inert matrix (e.g. seasand) or drying agent (e.g. sodium sulphate) to make the analyte better accessible to the supercritical fluid by increasing the sample surface [17]. Moreover, obviously due to contrary effects as e.g. back mix effects and circular flows, low recoveries were obtained. As shown in Fig. 4 the best recoveries were achieved when filling the thimble with the wheat flour sample and glass wool in layers (covering the wheat sample on both sides with layers of glass wool).

Compared to the conventional mixing of the sample with sea-sand as it is recommended by Taylor et al. [9], the use of glass wool gave 45% better recovery. After the optimization of all these parameters, a recovery of 91.1±10.7% was finally achieved for the 1 g of spiked wheat samples.

Finally these optimized SFE parameters (Table 1) were applied to 1 g of naturally contaminated wheat flour. However, only 5.0% DON could be recovered, which shows again the great influence of the analyte–matrix interaction especially for SFE. In a further set of experiments the SFE conditions were optimized for naturally DON contaminated wheat flour.

3.4. Behaviour of naturally contaminated wheat

According to the optimized parameters found for the spiked wheat, we started with a temperature of 40°C at a pressure of 318 bar with a density of 0.92 g/ml. Trapping was carried out with a silica solid-phase trap, as described above.

To obtain better recoveries, a longer static extraction time was employed subsequent to the dynamic extraction mode which includes the on-line addition of the modifier. In addition, modifier was added to the sample in the thimble prior the extraction process in order to improve a swelling of the matrix and to thus accelerate the solute diffusion out of the matrix and to support the disruption of the analyte-matrix interactions.

The optimum conditions were found (Table 1) with a dynamic mode of merely 15 min using a fluid flow-rate of 2.0 ml/min and 3% methanol as modifer and a longer preceding static mode of 30 min with an in-cell addition of 500 μl methanol applied to the sample before running the SFE. It should be noted that 1 g wheat flour is not able to absorb more than 500 μl methanol. When less than 500 μl methanol were employed the extraction yields decreased. Fig. 5 shows the HPLC chromatograms obtained after

Table 1
Optimized extraction conditions for the SFE of DON from wheat

Chamber temperature:	40°C	Pressure	318 bar
Density:	0.92 g/ml		
Extraction fluid:	CO_2+modifier[a]	Fluid flow-rate:	2.0 ml/min
Nozzle temperature:	85°C	Trap temperature	85°C
Trap packing:	silica	Solvent flow-rate:	1.0 ml/min
Rinse solvent:	methanol	Solvent volume:	1.5 ml
Nozzle temperature:	55°C	Trap temperature:	55°C

[a] see Section 2.2.

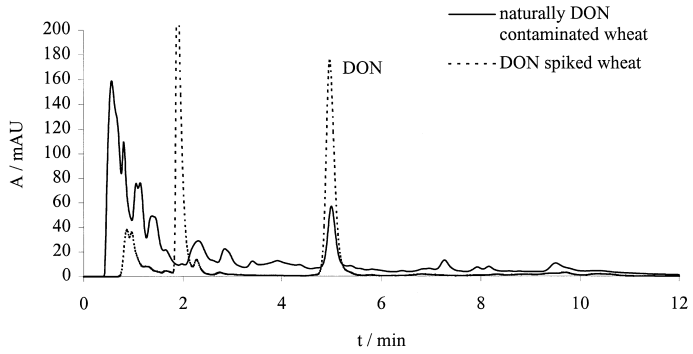


Fig. 5. HPLC chromatograms of the SFE-extracts of naturally contaminated wheat containing 14.0 μg/ml DON and spiked wheat containing 44.7 μg/ml DON.

SFE of a DON spiked wheat (dotted line) and a naturally contaminated wheat (full line) monitored at 220 nm. It can be seen that there are no interferences from the remaining matrix components at the retention time of DON although no further clean-up has been applied. This clearly demonstrates the strength of SFE compared to classical extraction techniques based on solid–liquid extraction, which require at least one further clean–up step to obtain similar chromatograms [5].

3.4.1. Performance characteristics

By means of the SFE conditions mentioned above the highest DON recoveries of 53.0±3.2% by extracting naturally DON contaminated wheat flour were obtained. For the determination of the mean recovery of the method, SFE experiments with wheat flour naturally contaminated with DON at four concentration levels (2.35, 5.73, 11.42 and 16.75 mg/kg) were employed. The statistical evaluation of the analytical data was performed according to the guidelines of the German norm DIN 32645 (Table 2). In this norm the limit of detection (LOD) and the limit of quantification (LOQ) are derived from the y-intercept and its confidence interval after linear regression [18]. The sensitivity of the developed SFE method is only limited by the use of the UV detection. When GC–ECD after derivatization with TRI-SIL-TBT [5] is employed subsequent to the SFE procedure, an LOD of 250 μg/kg can be achieved which meets the requirements of several guidelines in Europe and the USA which regulates the maximum DON concentration in wheat [19].

Table 2
Performance characteristics of the SFE method

Sensitivity	48.442 mAU/s/g/μg
Method standard deviation	0.87 mg/kg
Relative method standard deviation	9.6%
Limit of detection (LOD)	1.6 mg DON per kg wheat
Confidence interval of LOD	1.07–3.68 mg/kg
Limit of quantification (LOQ)	5.35 mg/kg

The GC–ECD chromatogram (Fig. 6) of an SFE extract of wheat spiked with the five trichothecenes deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol and fusarenon X demonstrates that the described SFE procedure, which has been developed for naturally DON contaminated wheat is also well suited for the simultaneous determination of several DON related compounds.

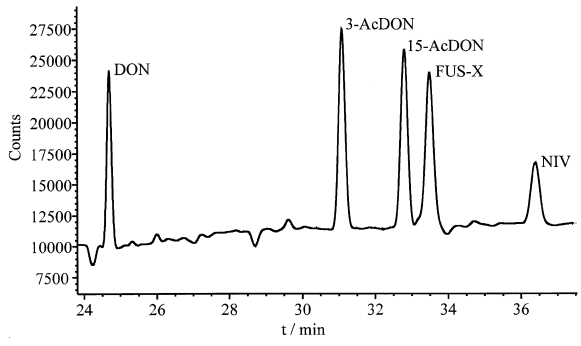


Fig. 6. GC chromatogram of an SFE-extract of wheat spiked with DON, 3-AcDON, 15-AcDON, NIV and FUS-X.

Table 3
SFE versus classical extraction: comparison of cost/analysis

	Reference solvent extraction method	SFE method
Extraction/clean-up time per sample (min)	110	70
Costs of material per sample (US$)	11	9.5
Total costs per sample (US$)	68	46

3.4.2. Economy calculation

The economy calculation (Table 3) shows that, besides its unsurpassable environmental features, the developed SFE procedure is less time-consuming and less expensive than the well established extraction procedure described before. The total costs are calculated in consideration of personnel costs of 31 US$ per hour. In addition, the use of hazardous solvents is reduced to a minimum. However, it has to be pointed out that this calculation does not consider the costs for the purchase of an SFE-apparatus (≈20.000–30.000 US$).

4. Conclusion

In this work the optimization of an SFE procedure for the extraction of DON and DON related compounds from wheat samples is described for the first time. While SFE of DON spiked inert matrix and spiked wheat yielded recoveries >90%, the best results achieved for naturally DON contaminated wheat were 53.0% with a precision of R.S.D.=3.2% for $n=5$.

However, the results achieved with this SFE method are much more promising than previously published results on the SFE of the even less polar aflatoxins. Due to the simplicity of the method SFE seems to be ideally suited for the screening of trichothecenes, such as DON. The tremendous improvement of the extraction efficiency for DON and the good reproducibility of the method indicates, that the potential of SFE for the extraction of more polar substances as e.g. mycotoxins, is probably greater than expected. However, further investigations are required to verify this conclusion.

Acknowledgements

R. Krska wishes to thank Keith Bartle for fruitful discussions and his hospitality at the University of Leeds. This work was only possible due to the support of the ERASMUS-Program of the EC.

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