A Simple Method for Determination of Deoxynivalenol in Cereals and Flours

FILIP KOTAL and ZUZANA RADOVÁ

Department of Food Chemistry and Analysis, Institute of Chemical Technology, Prague, Czech Republic

Abstract

KOTAL F., RADOVÁ Z. (2002): **A simple method for determination of deoxynivalenol in cereals and flours**. Czech J. Food Sci., **20**: 63–68.

An effective and fast method for determination of deoxynivalenol (DON) in cereals and flours has been developed. The immunoaffinity column was used for the isolation of DON from wheat, corn, rice and flour extract. The determination was carried out by using the HPLC/UV method. The limit of detection was 0.02 mg/kg. The recoveries for the assay range 0.1 to 2 mg/kg were generally higher than 80%, ranging from 83 to 96% with an average relative standard deviation of 3.8%. The trueness of the method using the DON test – HPLC column was established by use of certified reference material CRM 379. The certified value was 0.67 mg/kg. The result obtained from three replicates was 0.68 ± 0.05 mg/kg. The corresponding confidence interval at 95% probability ranged from 0.63 to 0.73 mg/kg. A comparative study of the DON test^{TM} – HPLC/UV and the Mycosep 225 – GC/ECD methods was carried out. Six naturally contaminated wheat samples were analysed by both methods. Linear regression analysis demonstrates that DON test^{TM} – HPLC is a statistically significant predictor of the GC/ECD method using the Romer Mycosep 225 column.

Keywords: mycotoxins; deoxynivalenol; HPLC; immunoaffinity chromatography

Mycotoxins in grains, flours and feedstuffs have been a potential hazard to the health of people and the performance of farm animals (PESTKA & BONDY 1990).

One of the world significant mycotoxins seems to be a deoxynivalenol (vomitoxin).

DON is a trichothecene mycotoxin produced by *Fusarium* moulds genera. Its biosynthesis runs during the growth of infected grain in the field. This mycotoxin occurred worldwide in wheat, barley, corn, rice and oats (BHAT *et al*. 1989). In many countries a legal limit of 2 ppm of DON in cereals is established. This level of contamination in feedstuffs can cause serious health problems and diseases. Even at low levels, deoxynivalenol may cause animals to refuse feed or, at higher levels, induce vomiting leading to growth depression, increased susceptibility to infections, diarrhoea and haemorrhage (HSU *et al*. 1972). DON has been implicated as a factor in the human disease ATA (alimentary toxic aleukia).

Current methods used for deoxynivalenol determination include thin layer chromatography (TLC) (GILBERT 1993; FERNANDEZ *et al*. 1994), liquid chromatography (LC) (BAUER *et al*. 1987; SANO *et al*. 1987), gas chromatography (GC) with electron capture detection (ECD) (SCOTT *et al*. 1981) or mass spectrometry (MS) (GILBERT *et al*. 1985), and RIA and ELISA methods (USLEBER *et al*. 1992; WOLF-HALL *et al*. 1996). Advantages and disadvantages of each method depend on its capability to separate impurities from the analytes, the time of sample preparation and economic aspects. TLC is a fast and low cost method, but selectivity is not sufficient for quantitative determination. GC/ECD is a suitable method for trichothecene determination. A derivatisation procedure is necessary prior to the injection into a chromatograph. The GC/MS method is a research non-screening method operated with expensive apparatus and is usually used for the confirmation of results. HPLC seems a quick and simple method for DON determination. For determination UV detection in the wavelength range of 214–229 nm is often used (BAUER *et al*. 1987; SANO *et al*. 1987). Electrochemical detection could also be used. The determination of trichothecene mycotoxins, which do not absorb in the UV range by using the HPLC method, is possible after the

reaction with a suitable derivatisation agent. One of the possibilities of chemical modification is a conversion to p-nitrobenzoylderivates which absorb in UV at a wavelength of 254 nm (BAUER *et al*. 1987; SANO *et al*. 1987). Another possibility of chemical modification of the structure is a preparation of diphenylindenonsulphonylesters with a maximum of absorption at the wavelength of 278 nm or coumarin-3-carbonyl-chloride derivatives that absorb at the wavelength of 292 nm.

Another possibility is fluorescent detection, which allows the determination of DON and NIV in naturally contaminated cereal samples at the level of 2 ppm. For the separation of trichothecenes by the HPLC method, the C_{18} columns are most frequently used. The mobile phases consist of methanol:water, acetonitrile:water or acetonitrile:water:acetic acid in various volume ratios.

A very important part of the method appears to be a clean-up procedure of cereal extract and toxin preconcentration. The most common clean-up procedures use absorption chromatography on the Florisil (MÜLLER & SCHWANDORF 1991), alumina:charcoal:celite (ROMER 1986; GILBERT *et al*. 1992), silica gel (SCOTT *et al*. 1981) and Mycosep 225 (RADOVÁ *et al*. 1998; WEINGAERTNER *et al*. 1997; BERGER *et al*. 1999) columns. The advantage of alumina–charcoal–celite methods is their capability to remove the colour and fluorescent compounds. These compounds are bound to the column whereas the trichothecenes run through the column without sorption. The aromatic compounds are predominantly retained on charcoal contrary to the aliphatic ones with identical molecular weight (ROMER 1986). The quality of active charcoal is frequently discussed as the critical point of the clean-up procedure. Based on the experiments, most authors recommend active charcoal Darco G 60 produced by J. T. Baker (GILBERT *et al*. 1992; STRATTON *et al*. 1993). Celite is added to avoid the breakthrough of active charcoal to the eluate (BAUER *et al*. 1987; ROOD *et al*. 1988). Other isolation methods include supercritical fluid extraction, gel permeation (BETINA 1985) or immunoaffinity chromatography. Most methods of isolation as described above provide a mixture of analytes and co-extracts in different ratios. Only the immunoaffinity chromatography is a specific method of clean-up procedures, which selectively separates DON from the matrix interfering substances. The principle of the separation is a selective sorption of deoxynivalenol from the cereal extract to the antibody bound to the carrier. The impurities are removed from the cartridge without retention.

The aim of this study was to develop a simple method for determination of DON in various cereals. The immunoaffinity column VICAM DONtest™ was applied for isolation of deoxynivalenol from wheat, rice, corn and flour extract. The HPLC/UV method was used for the determination of DON. The trueness of the method was determined by the analysis of certified reference material CRM 379 (wheat DON contaminated sample). The range of applicability was determined by using spiked cereals at levels of contamination from 0.1 to 2 mg/kg.

After an analysis of naturally contaminated wheat samples, the DON assay results obtained by DON test™ – HPLC/ UV and GC/ECD – Mycosep 225 were compared.

MATERIAL AND METHODS

Materials and Reagents

The mycotoxin standards of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) were supplied by Sigma – Aldrich (Germany). Stock solutions of trichothecenes were prepared in acetonitrile from commercial standards (c = 100 mg/ml) and stored at –20°C. The working standards solutions ranging from 0.1 µg/ml to 5 µg/ml were prepared in acetonitrile.

The DON test – HPLC immunoaffinity column and microfibre filter were supplied by VICAM (USA). A fluted filter from Filtrak (Germany) was used. Analytical grade acetonitrile, methanol, polyethylene glycol PEG 8000 were obtained from Merck (Germany). Blank cereal samples (wheat grain, maize, rice and wheat flour) were obtained from a local store. Certified reference material CRM 379 (wheat flour containing deoxynivalenol residues 0.67 ± 0.02 mg/kg) was supplied by the European Community Bureau of Reference (CBR, Belgium). Contaminated wheat samples were obtained from the Research Institute of Crop Production (Prague, Czech Republic).

Instruments. The liquid chromatograph Hewlett Packard HP 1100 model (Agilent, Germany) equipped with quaternary pump, autoinjector and UV detector was used with a stainless steel reverse phase 150 × 4.6 mm, 3 mm particle size C_{18} Supelco HPLC column (Supelco, USA).

Analytical Procedures

Extraction. 10 g of grained sample was placed into ultraturax and then 40 ml redestilled water and 2 g polyethylene glycol were added. The mixture was stirred for 1 min. The extract was filtered through a fluted filter and then through a microfibre filter.

Clean-up by immunoaffinity chromatography. 1 ml of the final extract, corresponding to 0.25 g of the original material was placed into the DON test column. 10 ml of redistilled water was used for column washing. The elution of DON was done by 1 ml of methanol. The elution solvent was removed by a gentle stream of nitrogen and redissolved in 300 µl mobile phase.

HPLC chromatography. 50 µl of the samples were injected into the HPLC column heated to 30°C. The used mobile phase consisted of acetonitrile:water solution (10:90, v/v). The flow rate was 0.6 ml/min. Deoxynivalenol was determined at a wavelength of 218 nm by use of a UV detector.

RESULTS AND DISCUSSION

Method of DON Determination

The aim of the study was to develop an easy method for routine determination of DON in cereals. The HPLC method for DON determination was used. This is a common method. Deoxynivalenol absorbs in the UV spectra range. The maximum is around 218 nm.

DON (a relatively polar trichothecene) is fast excluded from the C_{18} analytical column if the less polar mobile phase is used. The optimum mobile phase consisting of the acetonitrile–water (10:90, v/v) was finally used for determination. The increasing temperature of the column moderately speeded up the elution of the analyte from the column. Owing to a future analysis of real matrices, the higher retention time was preferred.

The 30°C column temperature was chosen as an optimum. At the flow rate 0.6 ml/min, the analytes DON, 3-ADON and 15-ADON were sufficiently separated (Fig. 1).

The calibration curves for each trichothecene were linear in the range of 1–100 ng per injection (r^2 ranged from 0.992 to 0.998).

Immunoaffinity Cartridge Determination

The wheat extracts spiked by DON were used for determination of the immunoaffinity cartridge maximum capacity. The recoveries of the DON loaded into the cartridge increased linearly until its concentration was higher than 10 mg/kg. The amount of DON in a spike corresponds with the absolute amount of 2.5 µg of DON loaded into the cartridge. Despite the declared value 3.3 µg of DON, the recoveries at higher spikes than 10 mg/kg decreased (see Table 1 for more details).

Table 1. Recoveries of DON in wheat at high spike levels

Spike level (mg/kg)	Recovery (%)	RSD (%)
5	92.4	2.4
10	76.3	4.3
15	73.7	0.5

It should be noticed that the experimentally determined capacity was rather lower than the value indicated in the supplior manuel. It may happen that the amount of deoxynivalenol in naturally contaminated samples exceeds this limit level. In this case it is reasonable to dilute the cereal extract before loading into the cartridge.

Clean-up Procedure Development

The deoxynivalenol absorbs in the UV range in which most of the impurities from the cereal matrix have a significant UV maximum. When UV detection is used, an extensive clean-up procedure of the cereal extract is necessary. Most of the common clean-up procedures provide the eluates containing mixtures of analytes together with some impurities. For this reason the immunoaffinity clean-up (selective separation of DON) was tested. During the clean-up procedure, all steps recommended by the cartridge supplier were followed. After slow loading the cereal extract into the cartridge, the cartridge was washed with water to remove the organic impurities other than deoxynivalenol, which is bound to a specific antibody. We discovered that the recommended 5 ml of water was not enough to remove all impurities from the column after loading the wheat, rice, flour and corn extract. For wheat, rice and flour testing, 10 ml of water was the appropriate volume. In the case of corn extract, 30 ml was a necessary amount of water to remove all impurities from the cartridge. Fig. 2 shows the influence of wash water volume on the impurities profile of the elution fraction.

The organic solvents have an adverse effect on the antibody activity. The elution of DON was performed by 1 ml of methanol.

The optimised method was applied to the analysis of deoxynivalenol content in cereal samples. Fig. 3 shows the comparison of the elution profile of the blank wheat sample and the spiked wheat sample (level of contamination 1 mg DON/kg).

Method Evaluation

The detection limit of deoxynivalenol was determined by the analysis of the low-level spiked wheat extract. The

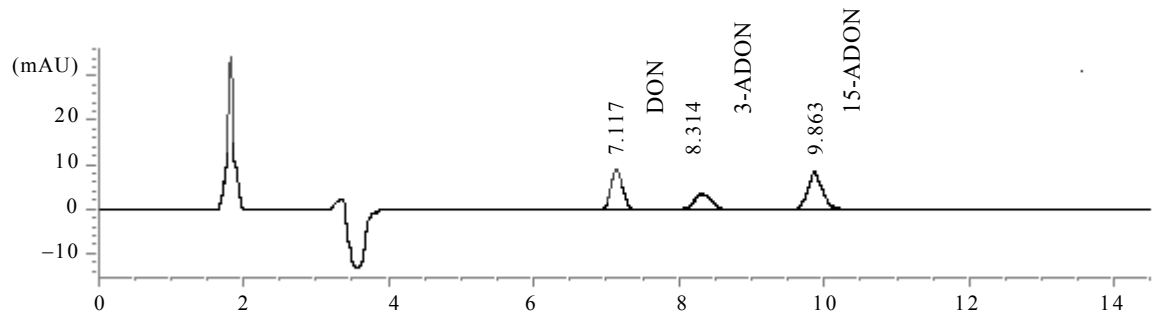

Fig. 1. A chromatogram of the standard mixture of analytes DON, 3-ADON and 15-ADON analysed in optimum HPLC conditions. The injection corresponds to 0.1 µg of each toxin

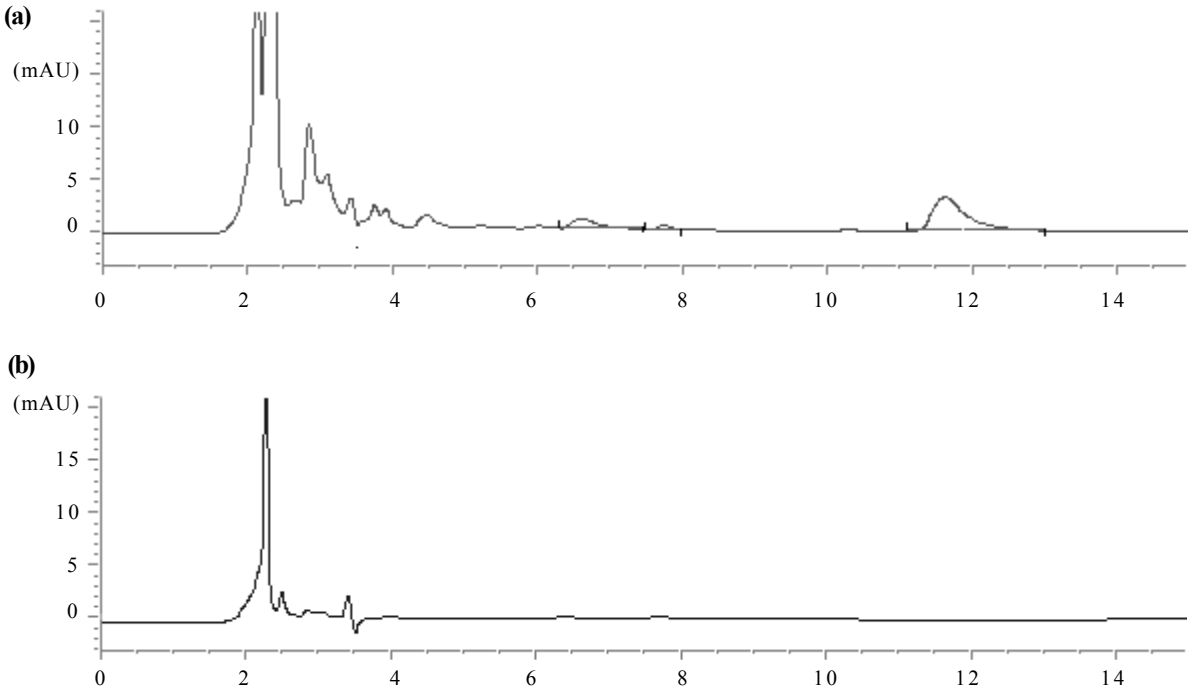

Fig. 2. A comparison of elution profiles of blank wheat samples (the injection corresponds to 41.7 mg of sample) prepared by using (a) 5 ml and (b) 10 ml in the wash column procedure

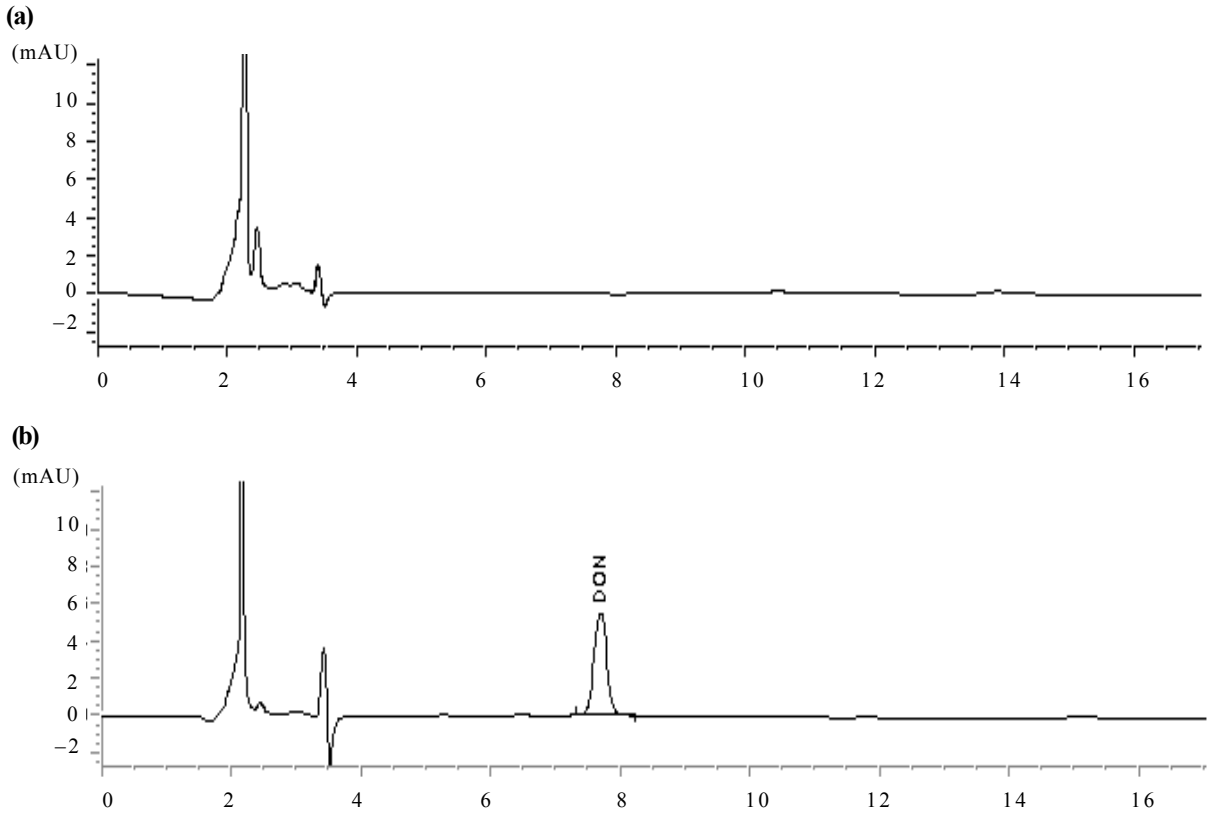

Fig. 3. A comparison of elution profiles of (a) blank wheat sample and (b) spiked wheat sample (level of contamination 1 mg DON per kg, the injection corresponds to 41.7 mg of sample and 41.7 µg of DON in the spiked sample)

Table 2. Recoveries of DON (in % ± relative standard deviation) for cereal samples determined by HPLC method (n = 5)

Cereals	Contamination level		
	0.1 mg/kg	1 mg/kg	2 mg/kg
Wheat	95.6 ± 4.9	85.4 ± 2.9	83.3 ± 1.0
Rice	93.6 ± 6.2	88.9 ± 4.2	84.1 ± 2.1
Corn	94.2 ± 5.5	86.1 ± 4.3	85.2 ± 2.9
Flour	95.2 ± 4.5	96.1 ± 3.1	–

As the limit for flour in the Czech Republic is 1 mg/kg, the 2 mg/kg contamination level was not investigated

LOD defined as the lowest amount reproducibly detected with at least 3:1 (signal to noise ratio) was 0.02 mg/kg.

The recovery of the method was determined by using cereal samples spiked with deoxynivalenol standard solution (c = 5 µg/ml). The resultant contamination levels were 0.1, 1.0 and 2.0 mg/kg. As shown in Table 2, the recoveries were generally higher than 80%, ranging from 83 to 96%. The precision of the method was determined from five replicates of DON determinations. The RSD values were not higher than 6.2%.

The trueness of the method using the immunoaffinity cartridge was established by the determination of DON content in certified reference material CRM 379. The value obtained from three replicates was 0.68 ± 0.05 mg/kg. The corresponding confidence interval at 95% probability ranged from 0.63 to 0.73 mg/kg. The certified value was 0.67 ± 0.02 mg/kg.

Comparative Study

Two alternative methods for the determination of DON content were compared. The first method consisted of the clean-up procedure using Mycosep 225 columns and GC/ECD determination of trifluoroacetylated deoxynivalenol BERGER *et al.* (1999). The second was the HPLC/UV method described above. The performance characteristics of both methods were compared. LOD values for DON were 0.02 mg/kg. The percentage recoveries at the 1 mg DON per kg level ranged from 85 to 96% for different cereals in the case of the HPLC method as shown in Table 3. In the case of the GC/ECD method, the recoveries ranged from 91.5 to 97.4%.

The time consumption of the HPLC and GC analysis was evaluated. Both methods consisted of extraction, clean-up and determination. The HPLC/UV (VICAM) results were obtained in 90 min. The time to obtain results from the GC/ECD (Mycosep) method was six times longer. The profiles of impurities in the elution fractions were also compared. In the elution fraction obtained by the GC/ECD method, more impurities appear than in the elution fraction from the immunoaffinity cartridge. The GC/ECD method is often used for a multi-trichothecene analysis. The HPLC method is suitable for DON determination only.

Table 3. Comparison of the results obtained by GC/ECD and HPLC/UV methods for naturally infected wheat samples

Wheat variety	GC/ECD	HPLC/UV
Boka	0.53	0.50
Šárka	0.88	0.82
Versailles	2.21	2.41
Hana A 33	2.24	1.75
Saskia	2.88	2.81
Bruta A	2.75	2.34

Naturally contaminated wheat samples with residues of deoxynivalenol were analysed using both methods. Comparable results were obtained (see Table 3 for more details). Linear regression analysis yielded the regression equation $Y = 1.017\ X + 0.114$, a slope of 1.017 with a 95% confidence interval (0.91, 1.07) and r^2 value 0.964 demonstrating that the HPLC/UV method is a statistically significant predictor of the GC/ECD multiresidual method using Romer Mycosep 225 column as a clean-up procedure prior to the determination of trichothecenes in cereals.

CONCLUSIONS

The above-described HPLC method for determination of deoxynivalenol in cereals provides a precision of the results of DON content. This method appears as an alternative to other methods that use the Romer Mycosep 225 column for the sample clean-up of cereal extracts. The method is easy to apply in routine laboratory practice. It is a low cost method and only a common apparatus is necessary. The method is suitable for the investigation of DON content in the toxicologically significant range corresponding to 0.1 to 10 mg/kg.

References

BAUER J., GARIES M., DETZLER W. (1987): Zum Metabolismus von Trichothecene beim Swein. Tierarzt. Umsch., **42**: 56.

BERGER U., OEHME M., KUHN F. (1999): Quantitative determination and structure elucidation of type A- and B-trichothecenes by HPLC/ion trap multiple mass spectrometry. J. Agr. Food Chem., **47**: 4240–4245.

BETINA V. (1985): Thin layer chromatography of mycotoxins. J. Chromatogr., **334**: 242–245.

BHAT R.V., BEEDU S.R., RAMAKRISHNA Y., MUNSHI K.L. (1989): Outbreak of trichothecene mycotoxicosis associated with consumption of mould-damaged wheat production in Kashmir Valley. Lancet, **1**: 35–37.

FERNANDEZ C., STACK M.E., MUSSER S.M. (1994): Determination of deoxnivalenol in 1991 U.S. winter and spring wheat by high performance thin layer chromatography. J. Assoc. Off. Anal. Chem., **77**: 628.

GILBERT J. (1993): Recent advances in analytical methods for mycotoxins. Food Addit. Contam., **10**: 37–48.

GILBERT J., SHARMAN M., PATEL S., BOENKE A., WAGSTAFFE P. J. (1992): Deoxynivalenol in wheat and maize flour reference materials. 1. An intercomparison of methods. Food Addit. Contam., **1**: 119–135.

GILBERT J., STARTIN J.R., CREWS C. (1985): Optimisation of conditions for the trimethylsilylation of trichothecene mycotoxins. J. Chromatogr., **319**: 376–381.

HSU I.C., SMALIEY E.B., STRONG F.M., RIBELIN W.E. (1972): Identification of T-2 toxin in moldy corn associated with a lethal toxicosis in dairy cattle. Appl. Microbiol., **24**: 685–690.

MÜLLER M.H., SCHWANDORF F. (1991): Determination of trichothecenes in cereals by gas chromatrography with ion trap detection. Chromatographia, **32**: 167.

PESTKA J.J., BONDY G.S. (1990): Alteration of immune function following dietary mycotoxin exposure. Can. J. Physiol. Pharmacol., **68**: 125.

RADOVÁ Z., HOLADOVÁ K., HAJŠLOVÁ J. (1998): Comparison of two clean-up principles for determination of trichothecenes in grain extract. J. Chromatogr. A, **829**: 259–267.

ROMER T.R. (1986): Use small charcoual/alumina clean-up columns in determination of trichothecenes in wheat by electron capture gas chromatography. J. Assoc. Off. Anal. Chem., **69**: 699.

ROOD H.D., BUCK W.B., SWANSON S.P. (1988): Diagnostic screening method for the determination of trichothecene exposure in animals. J. Assoc. Off. Anal. Chem., **71**: 493.

SANO A., SATOSHI M., SUZUKI M., TAKITANI S. (1987): High-performance liquid chromatographic method for determining trichothecene mycotoxins by post-column fluorescence derivatization. J. Chromatogr., **410**: 427–436.

SCOTT P.M., KANHARE R.S., LAU P.Y (1981): Gas chromatography with electron capture and mass spectrometric detection of deoxynivalenol in wheat and other grains. J. Assoc. Off. Anal. Chem., **64**: 1364.

STRATTON G.W., ROBINSON A.R., SMITH H.C., KITTILSEN L., BARBOUR M. (1993): Levels of five mycotoxins in grains in Atlantic Canada as measured by high performance liquid chromatography. Arch. Environ. Contam. Toxicol., **24**: 399–409.

USLEBER E., RENZ V., MÄRTELBAUER E. (1992): Studies on the application of enzyme Immunoassay the fusarium mycotoxins deoxynivalenol, 3-acetyldeoxynivalenol and zearalenon. J. Vet. Med., **39**: 617–627.

WEINGAERTNER J., KRSKA R., PRAZNIK W., GRASSERBAUER M., LEW H. (1997): Use Mycosep multifunctional clean-up columns for the determination of trichothecenes in wheat by electron capture gas chromatography. Fresenius J. Anal. Chem., **357**: 1206–1210.

WOLF-HALL C.E., BULLERMAN L.B. (1996): Comparison of thin layer chromatography and enzyme-linked immunosorbent assay for detection and quantification of deoxynivalenol in corn and wheat. J. Food Protect., **59**: 438–440.

Received for publication October 18, 2001

Accepted after corrections December 17, 2001

Souhrn

KOTAL F., RADOVÁ Z. (2002): **Jednoduchá metoda stanovení deoxynivalenolu v obilninách a mouce**. Czech J. Food Sci., **20**: 63–68.

Byla vyvinuta rychlá a efektivní metoda pro stanovení deoxynivalenolu (DON) v obilninách a mouce. Pro izolaci DON ze pšenice, kukuřice, rýže a mouky byla použita imunoafinitní kolona. Stanovení obsahu bylo provedeno metodou HPLC/UV. Metoda byla validována. Byl stanoven limit detekce 0,02 mg/kg. Recovery pro DON bylo ověřeno v koncentračním rozsahu 0,1–2 mg/kg pro všechny matrice, pro pšenici byl ověřen rozsah 0,1–15 mg/kg. Hodnoty recovery byly v intervalu 0,1–2 mg/kg vyšší než 80 % s průměrnou standardní relativní odchylkou 3,8 %. Správnost metody byla testována pomocí certifikovaného referenčního materiálu CRM 379. Certifikovaná hodnota byla 0,67 mg/kg. Průměrná hodnota obsahu DON ze tří paralelních stanovení byla 0,68 ± 0,05 mg/kg. Byla provedena srovnávací studie metod využívající kolonu DON test^{TM} a HPLC/UV koncovku a kombinaci GC/ECD a kolony Mycosep 225 pro izolaci DON. Oběma metodami byl stanoven obsah deoxynivalenolu v šesti vzorcích kontaminované pšenice. Výsledky lineární regresní analýzy ukazují, že výsledky dosažené metodou DON test^{TM} – HPLC jsou statisticky významné vzhledem k výsledkům dosaženým metodou GC/ECD – Mycosep 225.

Klíčová slova: mykotoxiny; deoxynivalenol; HPLC; imunoafinitní chromatografie

Corresponding author:

Ing. FILIP KOTAL, Údolní 649/75, 147 00 Praha 4, Česká republika

e-mail: filip.kotal@quick.cz