

ANALYSIS OF ZEARELENONE IN WHEAT USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION AND/OR ENZYME-LINKED IMMUNOSORBENT ASSAY

Zuzana Radová, Jana Hajšlová* and Jana Králová

The Technical Institute of Chemistry Prague, Technická 3, 166 28 Prague

Ludmila Papoušková and Světlana Sýkorová

*Research Institute of Crop Production, Drnovská 507, 161 06 Prague 6, Czech Republic***Summary**

In the first phase of experiments, performance characteristics of two alternative analytical methods used for the determination of zearalenone and its metabolites were assessed in presented study. Extraction of ground grains with acetonitrile-water (84/16, v/v) mixture followed by clean-up using GPC BioBeads S-X3 soft gel were employed prior to HPLC (reversed phase column C₁₈). The use of ELISA method allowed direct, highly sensitive measurement of zearalenone levels in crude extracts. For ELISA method, extraction with methanol-water (70/30, v/v) without any clean-up was used. Compared to HPLC/FLD method, the precision of ELISA method was inferior.

The second part of study was concerned with toxic potential of two isolates of *Fusarium culmorum* (W. G. Sm.) Sacc. under field conditions. Analyses of wheat varieties tested for the content of zearalenone were carried out. Levels of zearalenone in wheat inoculated by more toxic isolate (B) ranged from 309.1 to 2162.7 µg/kg. These values were higher almost by 2 orders of magnitude compared to levels found in samples inoculated with isolate A. Wheat variety SPARTA was identified as the most resistant against fungal attack from all checked varieties.

Keywords: Zearalenone, GPC, HPLC, ELISA, *Fusarium culmorum* (W. G. Sm.) Sacc., wheat varieties

1. Introduction

Zearalenone (ZEN), a derivative of β-resorcyclic acid lactone [(E,S)-2,4-dihydroxy-6-(6'-oxo-10'-hydroxy-1-undecenyl)-benzoic acid-μ-lactone] is a natural toxin produced by *Fusarium spp.* It mainly occurs in infected corn, wheat, and other cereals, often together with trichothecene mycotoxins (deoxynivalenol – DON, nivalenol – NIV, etc.).

Reproductive disturbances and alterations in genital organs that have been observed in swine and rodents⁽¹⁾ are related to the capability of zearalenone to bind to the oestrogen receptors. ZEN is metabolized in intestine of animals yielding α-zearalenol [(E,R,S)-2,4-dihydroxy-6-(6',10'-hydroxy-1-undecenyl) benzoic acid-μ-lactone] and β-zearalenol [(E,S,S)-2,4-dihydroxy-6-(6',10'-hydroxy-1-undecenyl) benzoic acid-μ-lactone] (Fig. 1), which can be excreted to milk of lacting cows.⁽²⁾ In this way, these metabolites may enter human food chain.

For determination of ZEN in plant matrices, various techniques e.g. chromatographic methods such as thin-layer chromatography (TLC), gas chromatography (GC)-mass spectrometry (MS) and high-performance liquid chromatography (HPLC) or immunochemical method such as enzyme-linked immunosorbent assay (ELISA), have been applied.^(3,4) Nowadays, HPLC with fluorescence detection (HPLC/FLD) is the most widely used. In any case, methods aiming at the determination of multiple residues of mycotoxins typically require complicated clean-up procedures and are generally time consuming and costly. A number of SPE clean-up procedures have been described for the purification of crude extracts.^(5,6) In some studies comparison or combination of different purification steps is

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

described^(7,8,9). On the other hand, ELISA method (specific antibody for one mycotoxin) allows to be used for routine screening of the particular toxin in a large number of samples simultaneously.

In the presented paper, incidence of ZEN in wheat samples obtained from field trials was investigated. The main aim of project, within which this study was conducted, was to identify wheat varieties possessing high resistance against fungal infection. Two isolates of *Fusarium culmorum* (W. G. Sm.) Sacc. (typical producer of DON) were used for inoculation of wheat plants (spikelets) during flowering. To obtain reliable data on the levels of examined mycotoxin, optimization of isolation procedure (GPC clean-up step implemented) and subsequent validation of HPLC/FLD determinative step were carried out. The results obtained by this instrumental method were compared with data generated by the ELISA method.

The possible use of HPLC/FLD procedure for simultaneous determination of α -zearelenol and β -zearelenol was also tested.

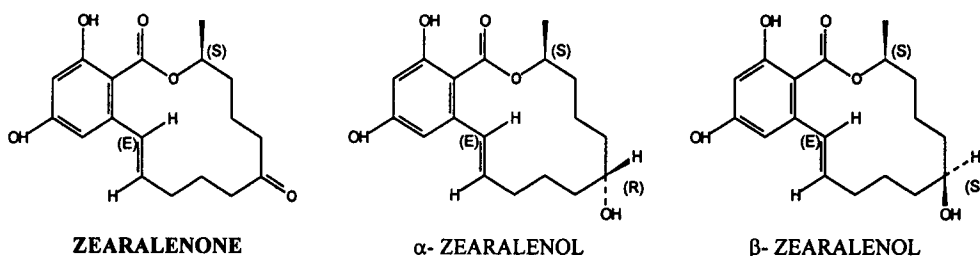


Figure 1. Chemical structures of zearelenone, α -zearelenol and β -zearelenol

2. Materials and Methods

2.1. Chemicals

2.1.1. Analytical standards

ZEN (99 % purity, 10 mg), α -zearelenol (99 % purity, 5 mg) and β -zearelenol (99 % purity, 5 mg) were obtained from Sigma-Aldrich (Steinheim, Germany). Stock solutions (0.5 mg/ml) were made up in acetonitrile from the commercial standards. Working stock solution containing 20 μ g/ml acetonitrile of each standard was prepared.

2.1.2. Reagents

Ethyl acetate, cyclohexane, acetonitrile (Merck, Darmstadt, Germany) were of HPLC-grade (99 % purity). Chloroform and acetone (analytical reagent grade, Lachema, Brno, Czech Republic) were redistilled in glass prior to use. All glassware was washed with detergent, rinsed with distilled water and then with acetone before use.

2.2. Apparatus

2.2.1. Gel permeation chromatography (GPC)

The automated GPC system Gilson (Aspec), consisting of a pump (Gilson 305 Master pump), a fraction collector, an automatic regulator of a loop Aspec XLI (controller keypad via RS232C), a microcomputer (software 731 PC via RS232C), a dilutor Gilson 401C (Gilson, France) and a stainless steel column 50 \times 0.8 cm I.D. (Tessek, Prague, Czech Republic) packed with Bio-Beads S-X3, 200-400 mesh (Bio-Rad Labs., Hercules, CA, USA) was used for clean-up of crude extracts. Chloroform was used as a mobile phase (flow rate 0.6 ml/min).

2.2.2. High-performance liquid chromatography (HPLC)

The high-performance liquid chromatographic system was composed of a Hewlett-Packard 1050 series quaternary pump system, a HP 1050 series autosampler, a HP 1046 A fluorescence detector

(FLD) (Hewlett-Packard, Avondale, CA, USA) and a LiChroCART 250-4 column (250×4 mm I.D.) with the sorbent LiChrospher 100 RP-C₁₈ (Merck, Darmstadt, Germany).

2.2.3. Enzyme-linked immunosorbent assay (ELISA)

Ridascreen Zearalenone kit (R-Biopharm GmbH, Darmstadt, Germany)

Microtiter plate spectrophotometer (EMAX, Molecular Devices, Corporation Sunnyvale, CA, USA)

Laboratory centrifuge K 24 (Janetzki, Germany)

Termostated water bath (EL-20R, Czech Republic)

Vortex Genie-2 (Scientific Industries Inc., USA)

2.3. Test material

2.3.1. Spiking contamination of samples

Blank (toxin-free) wheat samples were obtained from retail market. For recovery study, analysis of spiked wheat samples were carried out. Two hundred and fifty µl of working solution (20 µg/ml acetonitrile of each analyte) was added to 10 g of ground wheat sample (the spiking level was 500 µg/kg for each analyte). The sample was shaken in a stoppered bottle and solvent was allowed to evaporate overnight in exsiccator.

2.3.2. Samples inoculated with *Fusarium culmorum* (W. G. Sm.) Sacc.

Ten wheat cultivars (Arina, SG-U-466/Bona/, Bruta, Danubia, Hana, Samanta, Siria, Sparta, Šárka and Vlada) were chosen for their resistance to *Fusarium culmorum* tests. The set includes resistant, tolerant and susceptible wheat genotypes which were tested in the previous experiments⁽¹⁰⁾. Wheat plants were inoculated with 2 isolates of *Fusarium culmorum* (W. G. Sm.) Sacc. (referred as A and B) at mid-anthesis of middle spikelets according to Mesterházy⁽¹¹⁾ (the inoculum of these isolates was cultivated on sterilized kernels at 20 °C). Control plots at the same stage were sprayed with distilled water. Control and test plots were located in the same field. Field trials were realized at two locations (Ruzyně and Úhřetice) in Czech Republic: on the experiment fields of RICP in Prague - Ruzyně (340 m above sea level, sugar beet growing region, brown soils) and at Selgen a.s. Breeding Station Úhřetice (cca 100 km to east from Prague, 240 m above sea level, sugar beet growing region, degraded chernozem). In the field tests the effect of the inoculation on symptom reaction (SH), yield values reduction (grain number per ear, thousand grain weight - TGW and grain weight per ear - GWE) and predominant fusarium mycotoxin DON content were evaluated. (From the point of view of technological parameters and locations results will be discussed in the another paper.)

Prior to further processing, samples of wheat were homogenized by using a ZM100 mill (Retsch GmbH&Co.KG, Germany).

2.4. Sample preparation

2.4.1. Preparation of samples for HPLC analysis

Extraction

The extraction of ZEN and its metabolites was carried out by the same method as currently applied for isolation of trichothecenes (DON)⁽¹²⁾: 10 g of homogenous wheat sample (4 g of inoculated wheat sample) were extracted with 100 ml of acetonitrile-water azeotropic mixture (84/16, v/v) in a 250-ml flask for 1 h employing a rotary shaker. The supernatant was filtered through filter paper (No. 390) to 100-ml Erlenmeyer flask and stored in a refrigerator at 4 °C (max. two weeks). Before purification, 40 ml aliquot was evaporated to dryness on a rotary vacuum evaporator (RVO) and the residue was dissolved in 2 ml of chloroform.

Clean-up

The clean-up procedure was carried out by GPC employing Bio-Beads S-X3 and the mobile phase chloroform (flow rate was 0.6 ml/min). The injection volume of aliquot of crude extract dissolved in chloroform was 1 ml.

To determine elution curve of analytes, standard solution (1 µg/ml each analyte) was injected, 2 ml fractions of eluate were collected, evaporated to dryness in RVO, residues were dissolved in 0.5 ml acetonitrile and examined by HPLC/FLD. For determination of ZEN and its metabolites in wheat samples, 8-13 ml fractions were collected.

2.4.2. Preparation of samples for ELISA analysis (according to R-Biopharm instructions)

Ground sample (2 g) was placed in a screw cap glass vial, 10 ml methanol-water (70/30, v/v) was added and shaken for 10 min. The suspension was then filtered through folded paper filter, 2 ml of the filtrate were mixed with 2 ml of distilled water in a screw cup centrifuge vial, 3 ml dichloromethane were added and the mixture was shaken vigorously for 5 min, centrifuged for 10 min at 3500g and 15 °C. Upper aqueous layer was removed, dichloromethane layer was mixed shortly by vortexing and evaporated to complete dryness at 50-60 °C under nitrogen. The residue was redissolved in 1 ml of methanol (100 %) and mixed thoroughly, 1 ml of distilled water was added and mixed, then 1.5 ml of n-heptane was added and shaken for 5 min. After 5 min centrifuging (3500g at 15 °C) upper n-heptane layer was completely removed, methanol layer (100 µl) was diluted with 400 µl of sample dilution buffer. Fifty µl of aliquot was used in the assay.

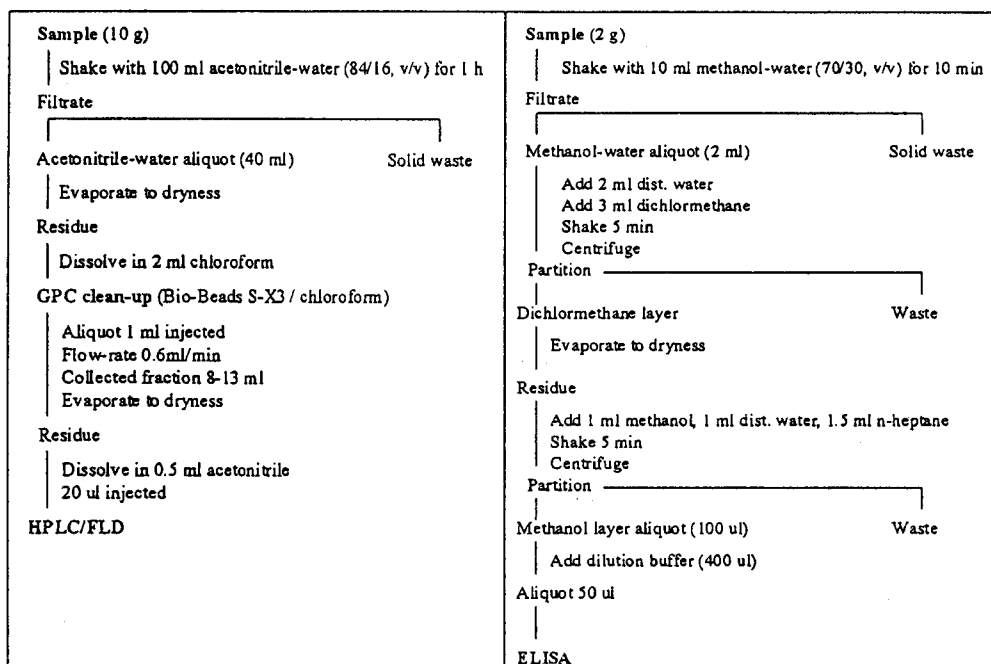


Figure 2. Flow charts of alternative procedures used for determination of zearalenone

2.5. Quantification**2.5.1. HPLC**

The HPLC/FLD determination was carried out under the following conditions: Mobile phase acetonitrile-water (40/60, v/v), flow rate 1 ml/min, column temperature 35 °C, fluorescence detector

$\lambda_{\text{ex}} = 235 \text{ nm}$ and $\lambda_{\text{em}} = 440 \text{ nm}$, 20 μl aliquot injected (corresponding to 160 mg of original sample, see procedure flow-chart Fig. 2).

2.5.2. ELISA (according to R-Biopharm instructions)

Fifty μl of each standard solution or prepared sample was added to separate duplicate wells of microtiter plate, then 50 μl of the diluted enzyme conjugate was loaded to each well. The plate was mixed thoroughly and incubated for 2 h at room temperature in the dark. After this time the liquid was poured out of the wells and the microwell holder taped upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. All wells were filled with 250 μl of distilled water and the liquid poured out again. This procedure was repeated two times more. After adding 50 μl of substrate and 50 μl of chromogen to each well, microtiter plate was mixed thoroughly and incubated for 30 min at room temperature in the dark. Finally, 100 μl of the stop reagent was added to each well, mixed and the absorbance was measured at 450 nm. The mean values of the absorbance values obtained for standards and the samples were divided by the absorbance value of the first standard (zero standard) and multiplied by 100. The zero standard is thus made equal to 100 % and the absorbance values are reported in percentage. The ZEN concentration in ng/kg corresponding to the extinction of each sample can be read from the calibration curve.

3. Results and discussion

Application of HPLC method employing fluorescence detection for analysis of ZEN in plant matrices has already been reported^(4,13,14). Regarding detection conditions, excitation wavelengths (236, 274, 276, 285 nm) in combination with various emission wavelengths (418, 440, 445, 460, 466 nm) were published in literature. In order to find out optimal parameters for ZEN analysis by HPLC, four FLD settings as regards wavelength combinations were tested. The highest fluorescence gains for detection of ZEN were observed at excitation wavelength 235 nm and emission wavelength 440 nm; which were then employed for all analyses of our wheat samples. It was also proved that these conditions were optimal for detection of main zearalenone metabolites. It should be noted that α - and β -zearalenols are not commonly present in moulded cereals, but they typically occur in milk from cows fed by fodder contaminated by ZEN. To be able to apply the HPLC/FLD procedure for examination of a wide range of matrices (not only wheat grains), the above zearalenone metabolites were also involved in the validation study.

FLD responses were found to be linear (coefficient of correlation >0.999) in the range $10^1 - 1.6 \times 10^2 \text{ ng}$ per injection of particular analyte. The repeatability of measurements expressed as relative standard deviation (RSD) was 2.6%, 2.7% and 6.4% (at 0.5 mg/kg concentration level) for ZEN, α -zearalenol and β -zearalenol, respectively.

To establish limit of detection (LOD) for ZEN in cereals, a software for HP 1100 HPLC instrument was used. Forty μl sample, which is a typical volume injected into HPLC system, corresponds under experimental conditions (see sample preparation - Fig. 2) to 64 mg of original matrix and to 4 ng of analyte (considering the lowest calibration point). The values of LOD calculated by this way, were then experimentally confirmed using purified extract of matrix spiked at the 50 $\mu\text{g}/\text{kg}$ level. LOD established for ZEN (7.5 $\mu\text{g}/\text{kg}$) is well below the hygienic limits set in several countries for this mycotoxin (for example, in France the regulation limit is 200 $\mu\text{g}/\text{kg}$ of cereals).

Regarding the ELISA method, specificity of the RIDASCREEN Zearalenon Test was documented by testing the cross-reactivity to structurally similar toxins, such as metabolites of the parent toxin. Cross-reaction was 41.6 %, 27.7 % and 13.8 % for α -zearalenol, zearalanol and β -zearalenol, respectively. The calibration curve was virtually linear in the 0.05 - 1.35 $\mu\text{g}/\text{kg}$ range. According to the test preparation record, the detection limit is 1.25 $\mu\text{g}/\text{kg}$ of ZEN for cereals/feed. In particular case, ELISA method was more sensitive for determination of target analyte compared to

HPLC/FLD. To get the same sensitivity, however, derivatisation (fluorescent labeling) of analytes can be done before HPLC analysis.

The accuracy assessment of both methods was based on analyses of spiked samples (spiking level 500 µg/kg, for preparation see chapter 2.3.1.). In the first phase of validation procedure, elution efficiencies (recovery) of analytes from GPC column and ELISA method were tested. One ml of mixed standard solution (1.0 µg/ml of each analyte) prepared in chloroform was loaded onto GPC column and then analytes were determined in 2 ml fractions of eluate. The "recoveries" of GPC step for ZEN and its metabolites were established using the same standard solution (fraction 8 – 13 ml collected). In the case of ELISA method, standard solution of ZEN (0.5 µg/ml) was analyzed and "recoveries", obtained in that way, are shown together with results for the HPLC method in Table I. Poor reproducibility of results obtained by ELISA can be attributed to losses of analyte that may occur during several reextraction and partition steps (see Fig. 2) involved in the sample preparation procedure.

Table I. Analyses of pure standards of mycotoxins by HPLC/FLD and ELISA: "recoveries" and relative standard deviations (% RSD in parenthesis, n=3)

	zearalenone		α-zearalenol		β-zearalenol	
	GPC + HPLC	ELISA	GPC + HPLC	ELISA	GPC + HPLC	ELISA
mixed standard soln.	98.2 (2.5)	87.8 (10.0)	99.8 (2.0)	-	103.7 (5.5)	-

In the second phase of validation process, several solvent mixtures, acetonitrile-water (84/16, v/v), methanol-water (70/30, v/v) and chloroform-ethanol (80/20, v/v) used for extraction of samples were compared. To determine target analytes, crude extracts were put through analytical methods as described in chapter 2.4.2, see flow-chart in Figure 2. For both used analytical methods, results were evaluated by comparing responses obtained from the samples with external calibration. As summarized in Table II, the extraction efficiencies of all three analytes were highest when acetonitrile-water (84/16, v/v) was used as extraction mixture, with recoveries from 83.5 % to 95.8 %. The interferences in HPLC chromatograms (see Fig. 4) due to the absence of matrix coextracts from wheat were low (only small signal in retention time of analyte α-zearalenol was recorded, but this metabolite can not occur in that type of matrix). Generally, good efficiency of GPC system in removing of matrix coextracts was demonstrated. It should be noted, that simultaneously with zearalenone, extraction of trichothecene mycotoxins (produced by the same *Fusarium* fungi) will occur. As long as their determination is required GC/FID, ECD, MS method or HPLC/UV, APCI-MS method can be used.

Table II. Recoveries and RSD (in %) of analytes spiked to wheat (500 µg/kg, n=3) *

	zearalenone		α-zearalenol		β-zearalenol	
	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA
methanol-water (7:3)	57.8 (2.1)	66.2 (4.8)	45.9 (3.8)	-	37.8 (10.2)	-
acetonitrile-water (84:16)	84.3 (1.6)	91.5 (8.0)	83.5 (0.5)	-	95.8 (0.8)	-
chloroform-ethanol (8:2)	79.7 (8.6)	81.8 (8.6)	142.9 (7.4)	-	18.3 (12.9)	-

* For analytical procedures, see chapters 2.4.1 and 2.4.2

As mentioned earlier, the extent of ZEN production under different field conditions was investigated in our experiments. Grain samples from ten wheat varieties inoculated with two isolates of *F. culmorum* (W. G. Sm.) Sacc. (see chapter 2.3.2.) were collected. Considering that the influence of the infection on yield was very distinctive (particularly in some susceptible cultivars) the deficiency of grain material after DON content determination occurred. For this reason the ZEN content determination was performed in select grain samples only.

For determination of ZEN content both tested methods were used. As shown in Table III, large differences in production of ZEN by the two isolates of *F. culmorum* (W. G. Sm.) Sacc. were

recognized. For isolate A, concentration of ZEN in grains ranged from 3.4 to 89.0 µg/kg (as determined by ELISA). ZEN contamination of grains from wheat varieties inoculated by isolate B was distinguishably higher: from 309.1 µg/kg to 2162.7 µg/kg, what documents high virulence of this isolate. Figure 3 shows the HPLC chromatogram of a purified extract from grains of wheat variety SIRIA (experimental field: Úhřetice, inoculation: isolate B).

Table III. Levels of zearalenone (µg/kg) in wheat samples as determined by two alternative procedures

Wheat variety	Location Ruzyně						Location Úhřetice					
	Isolate A		Isolate B		control		Isolate A		Isolate B		control	
	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA
ARINA	<25	18.3	-	-	<25	0.9	-	-	1095.4	954.1	-	-
SG-U-466 (BONA)	<25	8.5	2092.6	2162.7	<25	3.4	-	-	837.9	1192.9	33.7	31.2
ŠARKA	<25	33.8	-	-	<25	2.2	-	-	667.7	611.6	-	-
HANA	30.6	28.7	-	-	-	-	-	-	578.9	602.0	-	-
SIRIA	36.4	29.8	-	-	-	-	-	-	897.8	1095.9	-	-
DANUBIA	<25	89.0	-	-	-	-	-	-	386.5	622.4	-	-
SPARTA	<25	3.4	-	-	-	-	-	-	309.1	261.9	-	-
BRUTA	<25	18.4	-	-	-	-	-	-	1374.7	764.8	-	-
SAMANT A	<25	9.7	-	-	-	-	-	-	563.7	381.8	-	-
VLADA	<25	5.4	-	-	<25	1.8	-	-	439.5	738.4	-	-

- no samples

detection limit = 7 µg/kg (injection 40 µl)

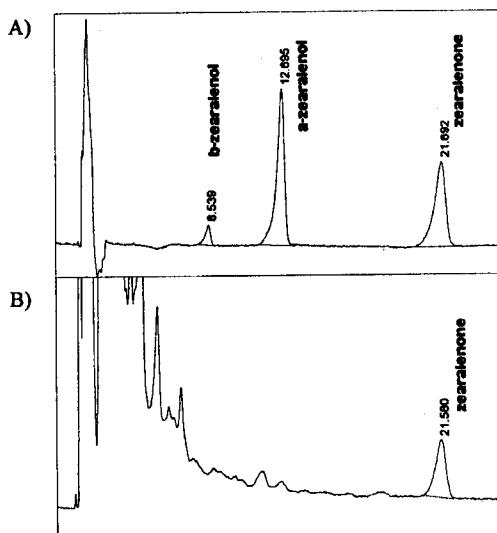


Figure 3. Chromatograms of:

A) standard solution (0.08 µg of each analytes injected)

B) contaminated wheat sample [SIRIA, isolate B, location Úhřetice – ZEN concentration 897.8 µg/kg (64 mg of wheat injected)]

Diffusion of fungal infection in the fields was clearly documented. Its extent depends also on climatic conditions. However, compared with levels of ZEN found in samples infected artificially, concentrations in the controls were significantly lower.

Regarding the quality of data generated by the two alternative methods, no statistically significant differences were recognized between the results obtained for samples in location Ruzyně.

As regards ZEN levels in examined wheat samples (inoculated at the same way) the highest mycotoxin levels were found in wheat varieties ARINA, SG-U-466 (BONA), SIRIA and BRUTA. These varieties seem to be less resistant to *Fusarium* fungi consequently allowing more extensive production of ZEN.

As shown in Table III [see wheat variety SG-U-446 (BONA) – isolate B], different levels of ZEN were found in the wheat varieties, inoculated with the same isolate and grown at two locations under different climatic conditions.

The detectable residues of ZEN were present also in grains obtained from "control" plots located close to the test plots where the inoculated wheats were grown.

However, poor agreement between the data obtained by HPLC and ELISA methods was encountered for wheat variety DANUBIA in both locations (see Table III). This may be attributed to the error caused by repeated dilutions of extract which are needed whenever highly contaminated samples are analyzed by the sensitive ELISA method (linearity range of calibration curve was relatively narrow).

4. Conclusions

An accurate determination of ZEN occurring in cereals at any concentration level exceeding 25 µg/kg (limit of quantification) can be obtained by HPLC/FLD method with clean-up step on gel Bio-Beads S-X3 implemented in this study. As long as lower concentrations of targeted mycotoxin are to be determined, the more sensitive ELISA method can be used (LOQ = 1.25 µg/kg). Comparing performance characteristics of both alternative procedures tested, better repeatability of measurements was obtained by HPLC/FLD method for spiked samples examined in this study. For the same level of target analyte, representing strongly contaminated wheat samples, several times higher relative standard deviations of ELISA method were obtained, probably due to need of multiple dilutions of the crude extract.

The high virulence of isolate B was clearly documented in this study. Examination of wheat grains obtained from experimental fields (*F. culmorum* (W. G. Sm.) Sacc.) isolates used for inoculation of spikelets for ZEN concentration enabled to identify wheat variety SPARTA as accumulating the least amounts of ZEN. It should be noted, that *Fusarium* fungi usually produce more than one mycotoxin. However, it is rather difficult to speculate on the extent of an overall contamination on the basis of determination of only one toxin. When the content of ZEN was compared with deoxynivalenol (DON) levels determined in the same samples in another, parallel pilot study no correlation between these fusarium toxins was found in grains of any examined varieties. The only exception was the variety SPARTA, which obviously resisted against fungal infection. The grains of this variety always contained the lowest levels of fusarium toxins.

Acknowledgements

Method development and HPLC/FLD analyses carried out by Department of Food Chemistry and Analysis, Institute of Chemical Technology, Prague, were funded by COST Action 835 "Agriculturally important toxigenic fungi" (2000). Experiments (field inoculation of wheat varieties and ELISA tests) carried out by Research Institute of Crop Production, Prague, were conducted within the project GA CR (Project No. 521/98/1019).

References

1. Long, G.G., Diekman, M., Tuite, J.F., Shannon, G.M., Vesonder, R.F., Amer. Vet. Res., 43 (1982) 1599
2. Warner, R., Ram, B. P., Hart L. P., and Pestka J., J. Agric. Food Chem. 34 (1986) 714
3. Dorner, J. W., Mycotoxins in Food: Methods of Analysis, in Handbook of Food Analysis (1997) Hogeschool Gent, Belgium
4. Richard, J. L., Bennett, G. A., Ross, P. F., Nelson, P. E., J. Anim. Sci. 71 (1993) 2563
5. Krska, R., Schuhmacher, R., Grasserbauer, H., Cer. Res. Comm., 25 (1997) 327-329
6. Wolf-Hall, Ch., E., Bullerman, L., B., J. of Food Prot., 59 (1996) 438-440
7. Langseth, W., Rundberget, T., J. Chromatog. A, 815 (1998) 103-121
8. Tacke, B., K., Casper, H., H., J. of AOAC Int., 79 (1996) 472-475
9. Weingaertner, J., Krska R., Fresenius J. Anal. Chem., 357 (1997) 1206-1210
10. Šíp, V., Stuchlíková, E., Czech J. Genet. Plant. Breed., 36 (2000) 49-58
11. Mesterházy, Á., Plant Breeding 98 (1987) 25
12. Radová, Z., Holadová, K., Hajšlová, J., J. Chromatog. A, 829 (1998) 259
13. Smith, J. E., Lewis C. V., Anderson J. G., Solomons, G. L., Mycotoxins in Human Nutrition and Health (1994) UER 16048 EN Agroindustrial Research Division
14. D'Mello, P. F., Duffus, C. M., Duffus, J. H., Mycotoxins. In Toxic Substances in Crop Plant (1991) Royal Society of Chemistry, Cambridge

Received 6 December, 2000, accepted 11 April, 2001