Journal of Chromatography, 328 (1985) 271–278
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 699

RAPID AND SENSITIVE DETERMINATION OF ZEARALENONE IN CEREALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

TOSHITSUGU TANAKA*, AKIHIKO HASEGAWA and YUKIO MATSUKI
Division of Food Chemistry, Public Health Research Institute of Kobe City, 4-6, Minatojimanaka-machi, Chuo-ku, Kobe 650 (Japan)
and
UNG-SOO LEE and YOSHIO UENO
Department of Toxicology and Microbial Chemistry, Faculty of Phamaceutical Sciences, Science University of Tokyo, 12 Ichigaya-Funagawara-machi, Shinjuku-ku, Tokyo 162 (Japan)
(First received February 12th, 1985; revised manuscript received March 5th, 1985)

SUMMARY

Zearalenone, an estrogenic mycotoxin of *Fusarium* species, in cereals can be extracted with acetonitrile–water (3:1), purified on a Florisil column, resolved by high-performance liquid chromatography (HPLC) with a Nucleosil 50-10 column using 90% water-saturated chloroform–cyclohexane–acetonitrile–ethanol (50:15:2:1) and quantitated by fluorescence measurement. This method is rapid, simple and reproducible, and detects zearalenone in wheat, barley, corn and other cereals with picogram sensitivity. A combination of this HPLC method with a gas–liquid chromatographic method for trichothecenes may be applied to the simultaneous detection of *Fusarium* mycotoxins (zearalenone, nivalenol and deoxynivalenol) in cereals.

INTRODUCTION

Fusarium graminearum (*Gibberella zeae* in the sexual stage), one of the plant pathogenic fungi in wheat and other cereals, produces toxic metabolites such as nivalenol (NIV), deoxynivalenol (DON) and zearalenone (ZEN). Consequently, humans are exposed to serious danger when ingesting these mycotoxins directly or as residues in animal tissues[1–3]. The determination of these toxins in foods and animal feedstuffs is therefore important for the protection of human health.

In earlier[4,5], we reported thin-layer chromatographic (TLC) methods for the detection and quantitation of NIV and related trichothecenes. Subsequently we developed a sensitive and rapid method for the simultaneous detection of NIV and DON in cereals by gas–liquid chromatography (GC) with electron-capture detection (ECD)[6].

Recently, Rosen and Rosen[7] proposed a method that allows the detection of three trichothecenes (T-2 toxin, HT-2 toxin and diacetoxyscirpenol) and ZEN in

0021-9673/85/$03.30

corn. The identities of these four mycotoxins were achieved by GC combined with mass spectrometry–selected ion monitoring of three ions characteristic of the trimethylsilyl (TMS) derivatives at levels of 20 ng/g for diacetoxyscirpenol, HT-2 and ZEN and 50 ng/g for T-2 toxin. However, the detection limit of TMS-derivatized ZEN in GC analysis is much lower than that in high-performance liquid chromatographic (HPLC) analysis with fluorescence detection, and further, ZEN is often detected together with NIV and DON in cereals.

During the course of a survey of the natural occurrence of NIV and DON in cereals, we have demonstrated that ZEN was recovered from the same extract prepared for trichothecene analysis. In this paper, we describe a method that allows the detection of ZEN in cereals with picogram sensitivity. The procedures for the extraction and purification are the same as adopted for NIV and DON, and then ZEN was quantitated by HPLC with fluorescence detection.

EXPERIMENTAL

Apparatus

The HPLC apparatus consists of a Shimadzu Model 4A liquid chromatograph (Shimadzu, Japan) with a 20-μl loop injection valve (Rheodyne Model 7125), a fluorescence detector (Shimadzu Model RF-530) and a data processer (Shimadzu Model C-R3A).

The chromatographic separation was carried out using a 30 cm × 4 mm I.D. porous silica gel column (Nucleosil 50-10; Nihon Chromato Works, Japan).

The mobile phase proposed by Pons and Franz[8] for aflatoxin analysis was slightly modified, and the standard elution solvent was 90% water-saturated chloroform–cyclohexane–acetonitrile–ethanol (50:15:2:1); 90% water-saturated chloroform was prepared by adding 10% of chloroform to water-saturated chloroform. The column temperature and solvent flow-rate were set at 30°C and 1.0 ml/min, respectively. Separation was performed at ambient temperature (25°C).

The fluorescence spectrophotometer was set at an emission wavelength of 460 nm and the excitation wavelength was 276 nm.

ZEN in positive samples was confirmed by mass spectral (MS) analysis as follows. The sample solution was linearly spotted at 1 cm from the lower edge of pre-coated silica gel 60 TLC plates (E. Merck, Darmstadt, F.R.G.) and the plate was developed with chloroform–acetone (9:1) until the front reached a height of 10 cm. ZEN gives a blue fluorescent band at R_F 0.63 under UV light (254 nm). The zone corresponding to ZEN was scraped off and eluted with chloroform. The chloroform elute was filtered and evaporated nearly to dryness for MS analysis using a Hitachi Model M-80A mass spectrometer system and a Hitachi Model M-003 mass spectrum analyser (Hitachi, Tokyo, Japan). The MS analysis was performed with a direct-sample inlet system at 70 eV.

Chemicals and reagents

ZEN, analytical-reagent grade and HPLC-grade solvents and Florisil-PR were purchased from Wako (Osaka, Japan). Florisil was activated by drying for 2 h at 130°C before use.

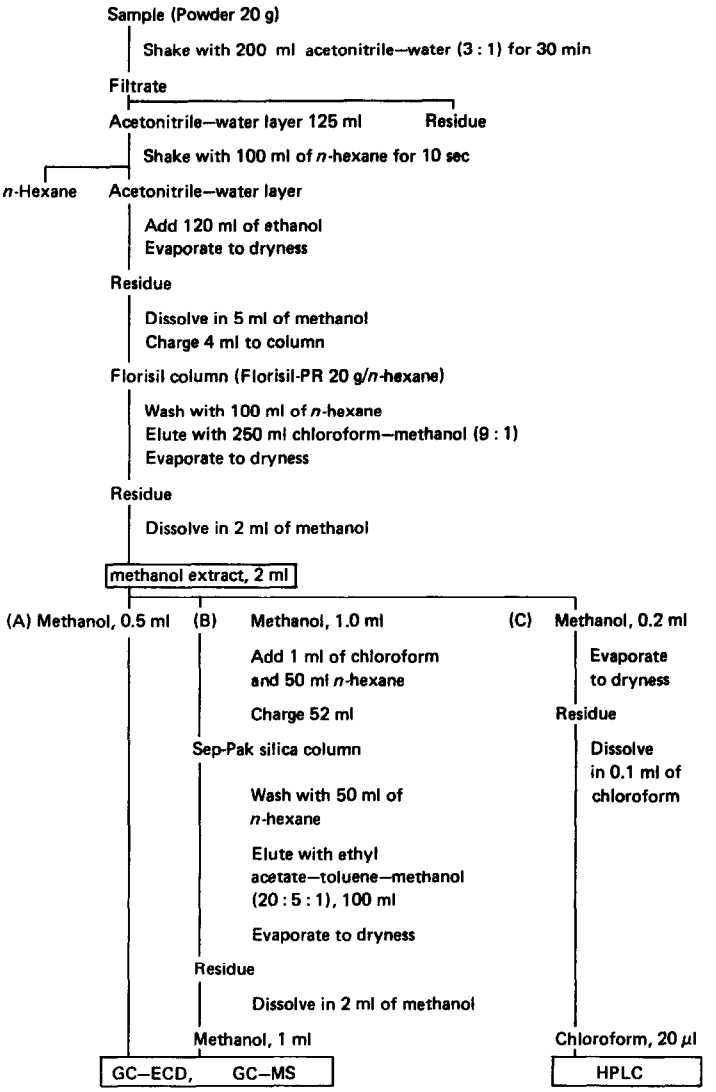

Fig. 1. Procedures for analysis of cereals for zearalenone, nivalenol and deoxynivalenol.

Preparation of samples

The extraction and column cleanup of ZEN from cereals were carried out by the method proposed for analyses of NIV and DON[6], as summarized in Fig. 1. Samples weighing 20 g each were finely ground and poured into 300-ml separating funnels. After adding 200 ml of acetonitrile–water (3:1), the funnels were shaken for 30 min on an automatic shaker. The extracts were filtered through filter-papers (No. 2; Toyo Roshi, Japan) and the filtrates (125 ml) were transferred into 300-ml separating funnels containing 100 ml of *n*-hexane, shaken for 10 sec and the upper *n*-hexane layers were discarded. After adding 120 ml of ethanol to the aqueous ace-

tonitrile layers, the mixtures were dried on a rotary evaporator at 45°C under reduced pressure.

For cleanup of the extracts, a chromatographic column (30 × 2.2 cm I.D.) was filled with 10 g of anhydrous sodium sulphate followed by 40 ml of *n*-hexane. A slurry of 20 g of Florisil in *n*-hexane was packed over above the sodium sulphate layer and, when the Florisil had settled, 15 g of sodium sulphate were added on top. Excess of *n*-hexane was drained to the top of the column. The extracts to be analysed were dissolved in 5 ml of methanol and 4-ml portions were charged to the column. After draining the excess of methanol and washing the column with 100 ml of *n*-hexane, ZEN, NIV and DON were eluted with 250 ml of chloroform–methanol (9:1) at a flow-rate of 5 ml/min. After evaporating the solvents, the residues were dissolved in 2 ml of methanol and 0.2 ml (C in Fig. 1) was subjected to ZEN analysis by HPLC as described below. The remaining methanol extract, 0.5 ml (A in Fig. 1) and 1.0 ml (B in Fig. 1), were used for analysis of the trichothecenes by GC–ECD and GC–MS techniques respectively, as reported elsewhere[6].

HPLC quantitation

The methanol extract, 0.2 ml (C in Fig. 1), was transferred into a 5-ml screw-capped vial and evaporated to dryness under a stream of nitrogen on an aluminium block heated at about 40°C. After adding 0.1 ml of chloroform to the vial, the latter was sealed with a screw-cap and the residue was dissolved by shaking. A portion (20 μl) was analysed by HPLC.

The samples (20 μl) were injected into the HPLC system under the same conditions used for preparing calibration graphs (Fig. 3), with the fluorescence emisson and excitation wavelengths set at 460 and 276 nm, respectively. The peak area of ZEN was measured by comparing it with that of the standard. The concentrations of ZEN were determined from the calibration graph.

RESULTS AND DISCUSSION

Selection of detection wavelength

HPLC analysis of ZEN has been reported by several authors[9–11], but no detailed examination of the selection of the detection wavelength has been performed. In order to define the optimal conditions for ZEN analysis by HPLC, we examined nine combinations of three emission and three excitation wavelengths in the HPLC analysis of ZEN. As summarized in Table I and Fig. 2, the highest fluorescence peak of ZEN was observed with emission at 460 nm and excitation at 276 nm. Therefore, we adopted these conditions for ZEN analysis by HPLC.

Mobile phases for ZEN analysis in HPLC

Among numerous mycotoxins, aflatoxin B_1 is of greatest concern because of its potent carcinogenicity. HPLC with a mobile phase composed of water-saturated chloroform–acetonitrile[8] has been widely used for the detection of aflatoxin B_1 in foodstuffs. Therefore, we adopted this system for ZEN analysis with a silica gel column. However, the successive use of water-saturated chloroform as proposed[8] resulted in a poor resolution of ZEN and therefore, instead of using water-saturated chloroform, we added 10% of chloroform to this water-saturated chloroform. The

TABLE I

COMPARISON OF FLUORESCENCE PEAK AREAS OF ZEARALENONE AT VARIOUS DETECTION WAVELENGTHS

Values are peak areas for 2 ng of zearalenone.

Emission wavelength (nm)	*Peak area (μV sec × 10^4)*		
	*276 nm**	*300 nm**	*316 nm**
450	4.3	2.5	2.1
460	4.6	2.6	2.3
470	4.3	2.4	2.1

* Excitation wavelength.

resulting 90% water-saturated chloroform was mixed with the other components. Preliminary experiments revealed that the addition of cyclohexane gave rise to a sharp resolution of ZEN from interfering materials in cereals, and ethanol was added to control the rate of elution of ZEN in HPLC. With an elution solvent consisting of 90% water-saturated chloroform–cyclohexane–acetonitrile–ethanol (50:15:2:0.5), ZEN was detected at 9 min, whereas with the same solvent system containing a high proportion of ethanol (50:15:2:1) ZEN gave a sharp peak at 7 min. Hence we propose the latter solvent system as the standard mobile phase for the HPLC analysis of ZEN in cereals.

Linearity and detection limit

The relationship between peak area and amount of ZEN injected was linear for each ZEN over the range 0.2–20 ng, as shown in Fig. 3. Twenty-eight injections

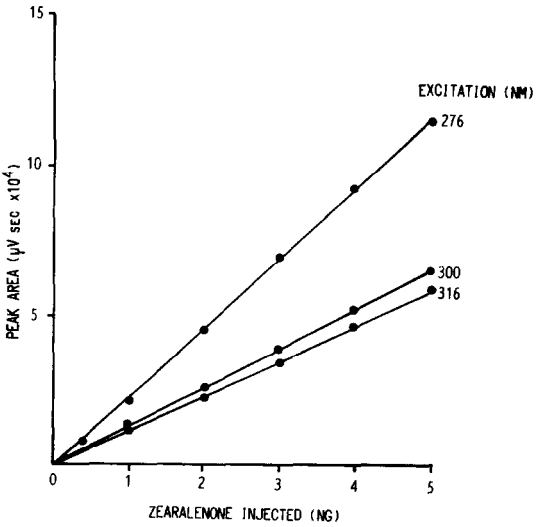

Fig. 2. Plot of fluorescence detector response for zearalenone peak area *vs.* amounts (ng) of zearalenone standard for excitation at three different wavelengths.

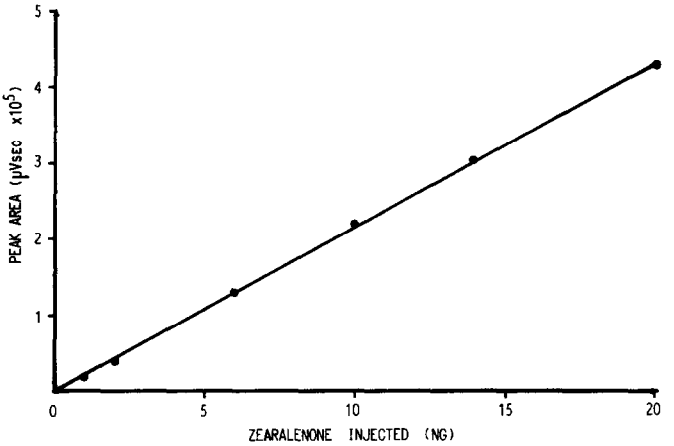

Fig. 3. Linearity of fluorescence detector response for zearalenone standards. Each point is the average of three measurements. Column: Nucleosil 50-10 porous silica gel (30 cm × 4 mm I.D.). Mobile phase: 90% water-saturated chloroform–cyclohexane–acetonitrile–ethanol (50:15:2:1). Detection: excitation 276 nm, emission 460 nm.

of 20 μl of ZEN standards were made over a 3-day period by a loop injection valve accurate to ±2%.

To establish the detection limit of ZEN in cereals, we subjected a 20-g sample to Florisil column chromatography, ZEN was recovered in 2 ml of the methanol extract and a 0.2-ml portion of this methanol extract was dissolved in 0.1 ml of chloroform. From this solution, 20 μl were subjected to HPLC analysis (process C in Fig. 1). Considering that 0.1 ml of the final chloroform solution is equivalent to 1 g of grain and 0.2 ng of ZEN is the lowest level for linearity (Fig. 3), the detection limit of ZEN in cereals is 1 ng/g in the proposed HPLC analysis with fluorescence detection. This detection limit is higher than those previously reported[7,9–11].

Recovery of ZEN spiked to cereals

Wheat, barley and corn grains, spiked with 50 and 500 ng/g of ZEN, were analysed by the proposed method as in Fig. 1 (C). As summarized in Table II, the recovery of ZEN added to wheat, barley and corn averaged more than 85, 93 and 92%, respectively.

TABLE II

RECOVERY (%) OF ZEARALENONE SPIKED TO VARIOUS CEREALS

Values are means ± standard deviations (%) of triplicate determinations.

Cereal	*Zearalenone spiked (ng/g)*	
	50	*500*
Wheat	87.1 ± 9.3	85.1 ± 5.9
Barley	92.5 ± 6.1	93.6 ± 7.3
Corn	94.7 ± 7.9	91.9 ± 7.4

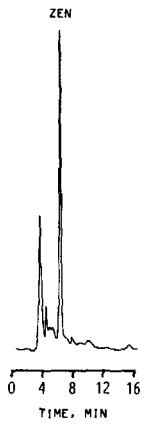

Fig. 4. HPLC profile of wheat (sample No. H-17) naturally contaminated with 36 ng/g of zearalenone.

Application of the HPLC method for ZEN in cereals

The proposed method was applied to the determination of ZEN in naturally polluted barley and wheat. Twenty-eight samples of barley were collected from farms in Korean provinces in 1983, and 18 scab wheat samples were collected from Hokkaido (Japan) harvested in 1984. Fig. 4 shows the HPLC profile of sample No. H-17 from Hokkaido polluted with 36 ng/g of ZEN. As shown in Table III, 21 out of 28 Korean barley samples (75%) were positive for ZEN, with an average level of 110 ng/g. The highest contamination was found in sample No. R-23, containing 1581 ng/g of ZEN. In Hokkaido, all samples were positive for ZEN, with an average level of 189 ng/g. The highest contamination (sample No. H-13) was 706 ng/g of ZEN. TLC analysis of ZEN-positive samples revealed the presence of a blue spot at R_F 0.63 under UV light (254 nm), and MS analysis gave *m/z* 318, 300, 270, 204 and 188. This confirms the presence of ZEN in cereals. These findings indicate that the Korean barley and Japanese wheat were contaminated with 100–200 ng/g of ZEN.

In an earlier paper[6], we described how the methanol extract after acetonitrile–water extraction and treatment on a Florisil column was subjected to GC–ECD and GC–MS analysis for NIV and DON (Fig. 1, A and B). In this paper, we propose that the same methanol extract is useful as the starting material for the HPLC analysis of ZEN. Therefore, we conclude that the combination of the present

TABLE III

NATURAL OCCURRENCE OF ZEARALENONE IN CEREALS

Cereal	*Source*	*No. of samples (positives/examined)*	*Zearalenone (ng/g)**	
			Average	*Range*
Wheat	Japan (Hokkaido)	18/18	189	8–706
Barley	Korea	21/28	110	N.D.**–1581

* Uncorrected for method recovery.

** N.D. = Not detected (< 1 ng/g).

HPLC method for ZEN and the GC method for NIV and DON, summarized in Fig. 1, is a powerful tool for simultaneous analyses of ZEN, NIV and DON in cereals.

ACKNOWLEDGEMENTS

We are grateful to Dr. Y. Matsui (College of Dairying, Ebetsu, Hokkaido, Japan) for the sampling of wheat in Hokkaido. This experiment was partly aided by the Ministry of Health, Japan.

REFERENCES

1 Y. Ueno (Editor), *Trichothecenes —Chemical, Biological and Toxicological Aspects*, Elsevier, Amsterdam, Oxford, New York, 1983.
2 H. Kurata and Y. Ueno (Editors), *Toxigenic Fungi—Their Toxins and Health Hazards*, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984.
3 C. J. Mirocha, S. V. Pathre and T. S. Robinson, *Food Cosmet. Toxicol.*, 19 (1981) 25.
4 S. Takitani, Y. Asabe, T. Kato, M. Suzuki and Y. Ueno, *J. Chromatogr.*, 172 (1979) 335.
5 A. Sano, Y. Asabe, S. Takitani and Y. Ueno, *J. Chromatogr.*, 235 (1982) 257.
6 T. Tanaka, A. Hasegawa, Y. Matsuki, K. Ishii and Y. Ueno, *Food Addit. Contam.*, (1985) in press.
7 R. T. Rosen and J. D. Rosen, *J. Chromatogr.*, 283 (1984) 223.
8 W. A. Pons, Jr. and A. Franz, Jr., *J. Ass. Offic. Anal. Chem.*, 60 (1977) 89.
9 T. E. Moller and E. Josefsson, *J. Ass. Offic. Anal. Chem.*, 61 (1978) 789.
10 P. M. Scott, T. Panalaks, S. Kanhere and W. F. Miles, *J. Ass. Offic. Anal. Chem.*, 61 (1978) 593.
11 H. Schweighardt, J. Bohm, A. M. Abdelhamid and J. Leibetseder, *Chromatographia*, 13 (1980) 447.