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DETERMINATION OF MYCOTOXINS IN GRAIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THERMOSPRAY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

An high-performance liquid chromatographic method is described for determination of deoxynivalenol, patulin, diacetoxyscirpenol, HT-2-toxin, T-2-toxin, zearalenone and ochratoxin A using a reversed-phase column and a diode-array detector. The extraction and purification steps and optimum chromatographic conditions are described. Detection limits and recoveries from spiked wheat samples were investigated. The combination of the high-performance liquid chromatographic system described together with a modern thermospray quadrupole mass spectrometer is a very specific and sensitive method for analyzing a wide range of mycotoxins in biological samples.
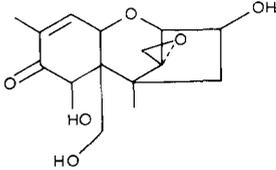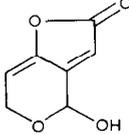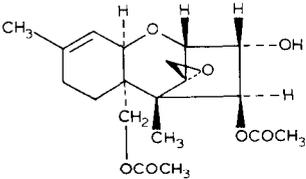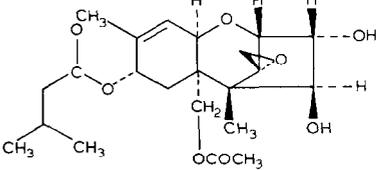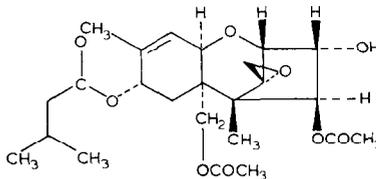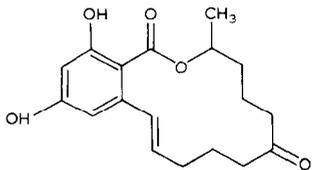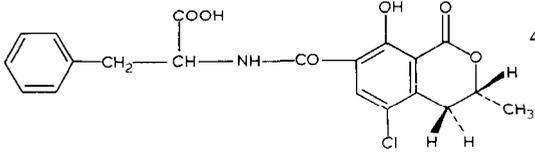
INTRODUCTION

Mycotoxins are secondary metabolites of various fungal species. The study of these toxins has become of the utmost importance, because many of them contaminate foods, and in some countries constitute an important health hazard for human and animal populations. Increasing awareness of the hazards posed by fungal toxins in foodstuffs and feeds has led to the development of a plethora of methods for their purification and analysis.

There have been many attempts to identify and estimate mycotoxins using thin-layer chromatography[1–3], gas chromatography[4–10], gas chromatography–mass spectrometry[11–16], high-performance liquid chromatography (HPLC)[17–25], supercritical fluid chromatography–mass spectrometry (SFC–MS)[26] and thermospray HPLC–MS[27].

HPLC has become the fastest growing technique available to the analytical food laboratory. The most serious limitation at the present time is the sensitivity and specificity of the detectors and the poor response of many compounds to them. Sev-

TABLE I

SOME PHYSICOCHEMICAL DATA

Name	Structure	Molecular weight	$\lambda_{max.}$ (nm)
Deoxynivalenol (DON)		296.0	218 (ethanol)
Patulin		154.0	276 (ethanol)
Diacetoxyscirpenol (DAS)		366.4	No UV abs.
HT-2-toxin		484.3	No UV abs.
T-2-toxin		466.5	187 (cyclohexane)
Zearalenone		318.4	236 (ethanol) 274 316
Ochratoxin A		403.8	210 (methanol) 330

eral mycotoxins, for example, exhibit weak absorbance in the UV region (see Table I), and detection at 190 nm is severely hampered by the background absorption of other sample constituents or by organic solvents introduced during gradient elution. The method presented here overcomes many of these limitations. Following a preliminary acetonitrile extraction of a wheat sample, the extract is cleaned up before the separation of mycotoxins by HPLC on a reversed-phase column.

The mass spectrometer is a highly specific and sensitive detector and the introduction of the recently developed thermospray (TSP) interfacing technique[28] has permitted measurement of a wider range of organic compounds than previously. Voyksner et al.[27] successfully used this technique for the HPLC–MS analysis of some Fusarium mycotoxins.

The aim of the present investigation was to develop a fast, sensitive and reliable analytical method applicable to the detection of a wide range of mycotoxins.

EXPERIMENTAL

Equipment and materials

The experiments were carried out with a Model 5000 liquid chromatograph (Varian Aerograph, Walnut Creek, CA, U.S.A.) and an HP 1040A diode-array detection system (Hewlett-Packard, Waldbronn, F.R.G.). The stainless-steel columns, 150 mm × 4.6 mm I.D., were slurry packed in 2-propanol–acetone, using methanol as the pressurizing solvent, with the following modified 5-μm silicas: Vydac 201 HSB 5 reversed-phase (The Separation Group, Hesperia, CA, U.S.A.), Nucleosil 5 C_{18} (Macherey-Nagel, Düren, F.R.G.) and Spherisorb S 5 ODS-2, (Phase Separations, Queensferry, U.K.). Bond Elut NH_2 extraction columns were obtained from Analytichem International (Harbor City, CA, U.S.A.) and Acro LC13 HPLC sample filters from Gelman Sciences (Ann Arbor, MI, U.S.A.). The mycotoxins were obtained from Sigma (St. Louis, MO, U.S.A.) and acetonitrile, HPLC-grade, from Rathburn Chemicals (Walkerburn, U.K.).

The TSP apparatus consisted of an HP 5988A mass spectrometer and an HP thermospray interface. The HPLC system was an HP 1090A. The interface closely resembles the Vestal design with the important, additional features of a negative heat ramp for gradient elutions and a filament for electron-assisted ionization.

Preparation of standard solutions

The toxins are readily soluble in polar organic solvents, such as methanol and acetonitrile, but when the toxins were dissolved in water–acetonitrile instead of pure acetonitrile the peak shape of each toxin was much sharper. This increased the column efficiency significantly and decreased the detection limits. The standard mycotoxin solutions were prepared by first dissolving the toxins in acetonitrile and then adding water until the acetonitrile–water ratio was 3:4.

Sample preparation

A 20-g amount of finely ground wheat was extracted with 150 ml ACN on an automatic shaker for 120 min. The extract was filtered through a PTFE membrane (0.45 μm) and the residue was washed with 100 ml of acetonitrile. The combined filtrates were evaporated to dryness, and the residue was dissolved in 3 ml *n*-hexane.

This was extracted with three 2-ml portions of acetonitrile–water (3:4). The *n*-hexane layer was discarded. The pooled acetonitrile–water extracts were evaporated and the residue was dissolved in 1 ml acetonitrile–water. A 250-μl aliquot was filtered through an Acro LC13 0.45-μm membrane and analysed for patulin, zearalenone and ochratoxin A (step 1) using the described chromatographic system. The remaining extract was evaporated to dryness, and the residue was dissolved in *n*-hexane. This was transferred to a 3-ml Bond Elut NH$_2$ extraction column, and washed with 10 ml of *n*-hexane. The mycotoxins DON, DAS, HT-2 and T-2 were eluted from the column with 3 ml of ethanol–*n*-hexane (1:1). The eluate was evaporated, and the residue was dissolved in 750 μl of acetonitrile–water and membrane-filtered prior to chromatography (step 2).

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The separation characteristics of DON, patulin, DAS, HT-2-toxin, T-2-toxin, zearalenone and ochratoxin A on several reversed-phase columns with water (pH 3, adjusted with phosphoric acid)–acetonitrile gradients as the mobile phase were investigated. These preliminary experiments indicated that the Vydac 201 HSB 5-μm reversed-phase material was superior in this application under these conditions. The resolution with the other column materials was often poor, *e.g.*, the Macherey-Nagel and Phase Separation materials did not resolve zearalenone and ochratoxin A although the other mycotoxins were quite well separated.

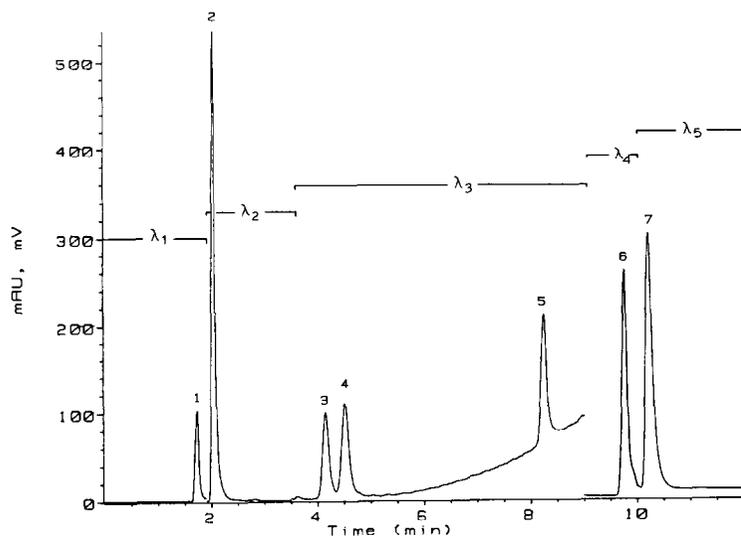We observed that the pH very strongly influences the retention behaviour of

Fig. 1. Chromatogram of a mycotoxin standard (300–600 ng of each) under optimized conditions (see text). Wavelengths: $\lambda_1 = 217$, $\lambda_2 = 275$, $\lambda_3 = 190$, $\lambda_4 = 237$ and $\lambda_5 = 210$ nm. Peaks: 1 = deoxynivalenol (DON); 2 = patulin; 3 = diacetoxyscirpenol (DAS); 4 = HT-2-toxin; 5 = T-2-toxin; 6 = zearalenone; 7 = ochratoxin A.

ochratoxin A but has no significant effect on the retention times of the other mycotoxins. Also, the addition of ammonium acetate to the eluent system, which is absolutely necessary for thermospray MS, has a very strong influence on the retention of ochratoxin A. The column temperature was maintained at 35°C in order to standardize the conditions. Fig. 1 shows a typical chromatogram under optimized conditions: Eluents: A, water (pH 3, phosphoric acid); B, acetonitrile; gradient from 40 to 70% B in 7 min, flow-rate 1 ml/min.

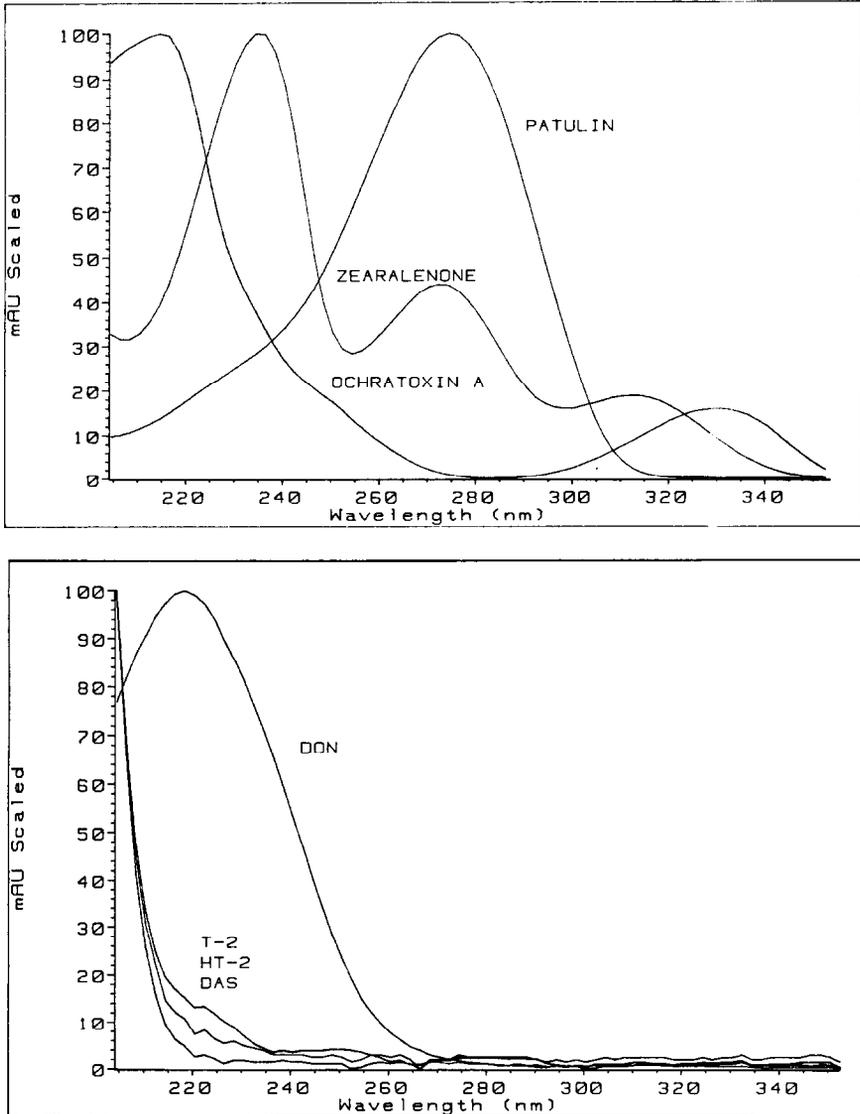The absence of conjugated unsaturation in diacetoxyscirpenol (DAS), HT-2-

Fig. 2. The UV spectra of each toxin taken during chromatography of the standard (Fig. 1).
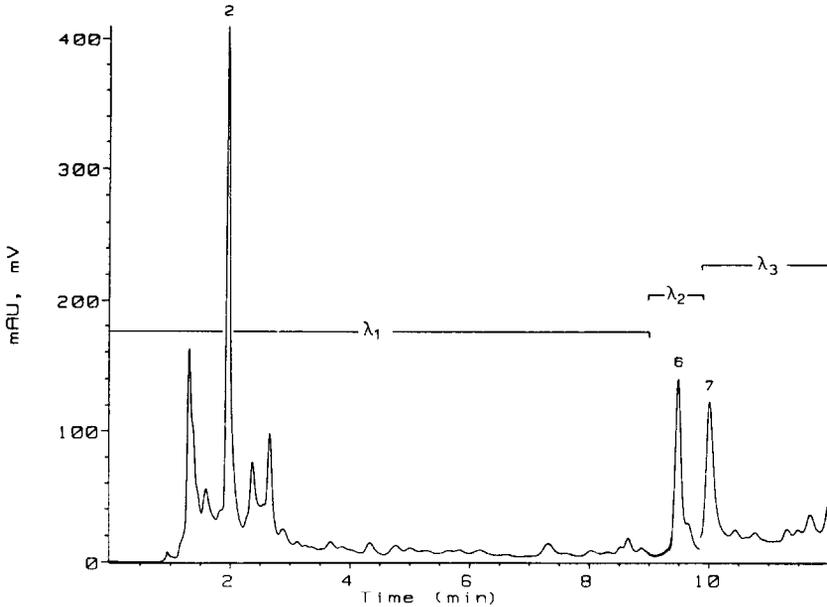
Fig. 3. Analysis of patulin (2, 314 ng), zearalenone (6, 375 ng) and ochratoxin A (7, 188 ng) in spiked wheat at wavelengths $\lambda_1 = 275$, $\lambda_2 = 237$ and $\lambda_3 = 210$ nm. Sample: preparation step 1.

toxin and T-2-toxin explains their weak UV absorbance and makes the analysis of these compounds very difficult. However, under the chromatographic conditions chosen, we were able to monitor the eluate for these compounds at 190 nm. DON, patulin, zearalenone and ochratoxin A were detected at 217, 275, 237 and 210 nm, respectively. The chromatograms for sample preparation steps 1 and 2 are shown in Figs. 3 and 4, respectively.
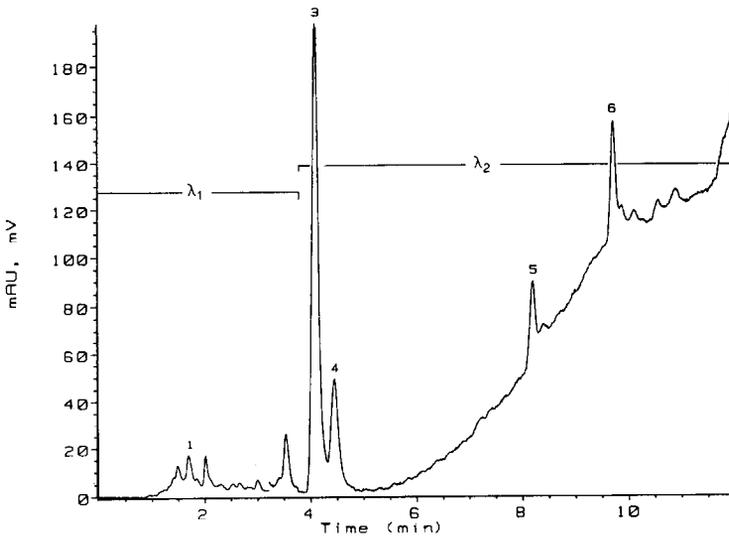

Fig. 4. Analysis of DON (1, 73 ng), DAS (3, 135 ng), HT-2-toxin (4, 353 ng) and T-2-toxin (5, 200 ng) in spiked wheat at wavelength $\lambda_1 = 217$, $\lambda_2 = 190$ nm. (step 2, see text). Peak 6 corresponds to zearalenone.

Linearity and detection limits

The relationships between the peak heights and concentration of each toxin were linear over the range 40–800 ng. The detection limits for the pure mycotoxins using the described chromatographic conditions varied from 1 to 20 ng per 10 μl, depending on the molar extinction coefficient of each compound. In real and in spiked wheat samples we were able to analyze reliably 50–200 ng per 10 μl of purified extract, representing a toxin concentration of 5–20 μg per 20 g of wheat.

Recoveries of mycotoxins from spiked wheat

Clean, mycotoxin-free, wheat samples were spiked with various amounts of mycotoxins and analyzed by the method described. The quantitative results are summarized in Table II. We have now extended this study to other grains, such as barley and rye and to animal feeds to determine whether our method has wider application. Poor results have been obtained in the case of the animal feeds.

Thermospray LC–MS analysis of mycotoxins

The operational temperatures at the tip of the probe and the source were held constant at 200 and 280°C respectively. The pressure in the analyzer was kept below $3 \cdot 10^{-6}$ Torr at a flow-rate of 1 ml/min. The mass spectrometer was optimized and calibrated with polypropylene glycol over the mass range 150–1000.

The samples were analyzed on the column mentioned earlier using water at pH 3 (acetic acid) and a gradient of 40–70% acetonitrile in 8 min. Spectra were measured with 0.001 and 0.05 mol ammonium acetate in the eluent. Fig. 5 shows the thermospray LC–MS reconstructed ion chromatogram of the standard mycotoxin sample. The retention time of ochratoxin A was very sensitive to the presence of ammonium acetate, whereas those of the other compounds were unaffected by it.

The spiked wheat sample (Fig. 5) gave results comparable to the standard with the exception of patulin. The background from the matrix limits the detection level of patulin, which has a relatively low mass ion (155, protonated molecular ion).

The mass spectra showed large differences depending on the concentration of ammonium acetate in the eluate. In thermospray LC–MS three competing ionization mechanisms are involved: the thermospray charge-transfer ionization, chemical ionization with plasma ions, which depend on the solvent composition and filament

TABLE II

RECOVERIES (%) OF MYCOTOXINS FROM SPIKED WHEAT SAMPLES

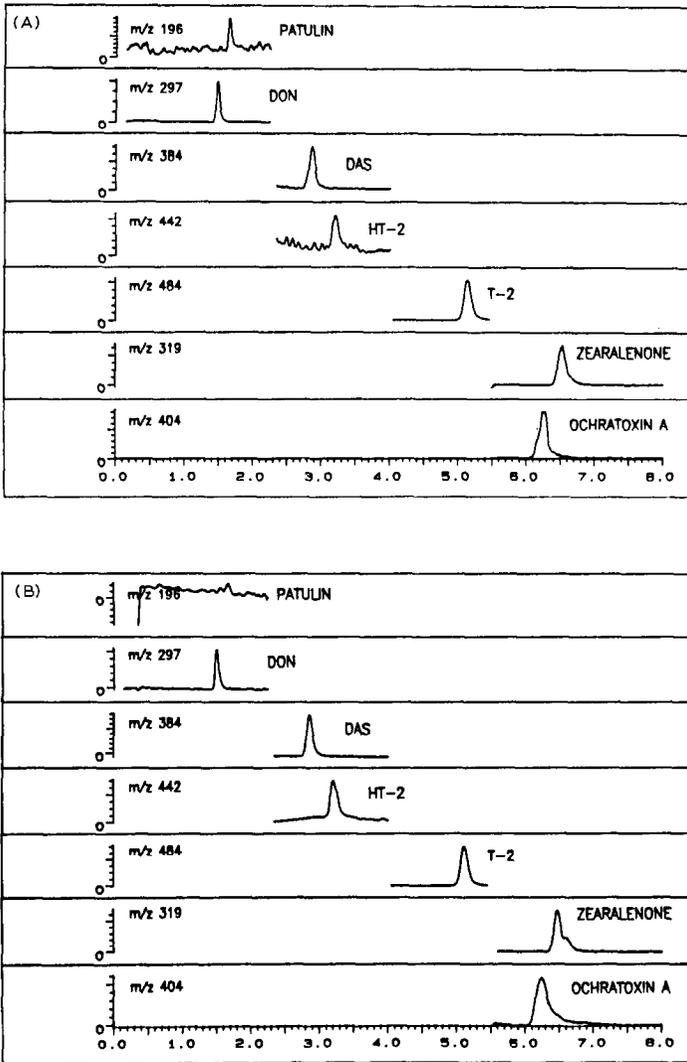| *Toxin*	*Sample*					*Mean*	*R.S.D. (%)*
	1	*2*	*3*	*4*	*5*		
DON	74	84	83	66	70	75.4	10.5
Patulin	76	81	83	75	77	78.4	4.5
DAS	88	91	83	90	85	87.4	3.8
HT-2	81	90	89	90	100	90.0	6.3
T-2	80	86	90	95	97	89.6	7.7
Zearalenone	65	68	82	60	79	70.8	13.1
Ochratoxin A	85	83	88	60	68	76.8	15.8

Fig. 5. The thermospray LC–MS reconstructed ion chromatograms of (A) a mycotoxin standard (30–60 ng of each) and (B) of wheat spiked with identical amounts of standard. Conditions as described in the text.

on/off mode and thermal ionization. It is not yet fully understood which one of these is dominant under any given conditions.

At very low ammonium acetate concentrations we can rule out the "thermospray ionization" which is the softest mechanism of the three. As the filament-on mode gave greater sensitivity, chemical ionization is the main mechanism. As a result, protonation and ammonium adduct formation occur, but there are also very specific fragmentation patterns for DAS, T-2-toxin and HT-2-toxin (Fig. 6). At higher ammonium acetate concentrations we noted an increase in sensitivity for all mycotoxins.

The spectra of DAS, T-2-toxin and HT-2-toxin showed a dominant $(M + NH_4^+)$ ion and no fragmentation. Fig. 7 compares the spectra of T-2-toxin under both conditions. Apparently the presence of an ester group favours the $(M + NH_4^+)$ formation,
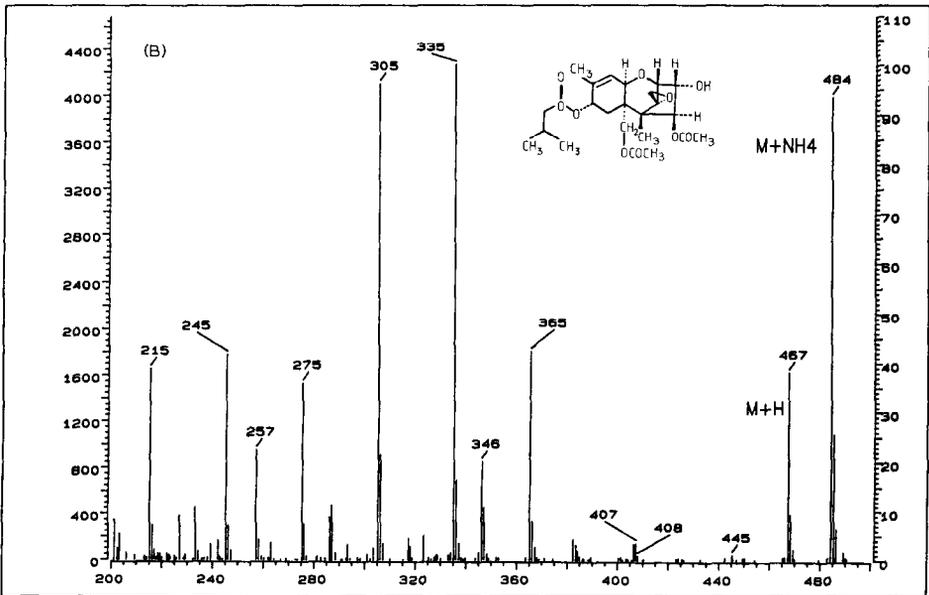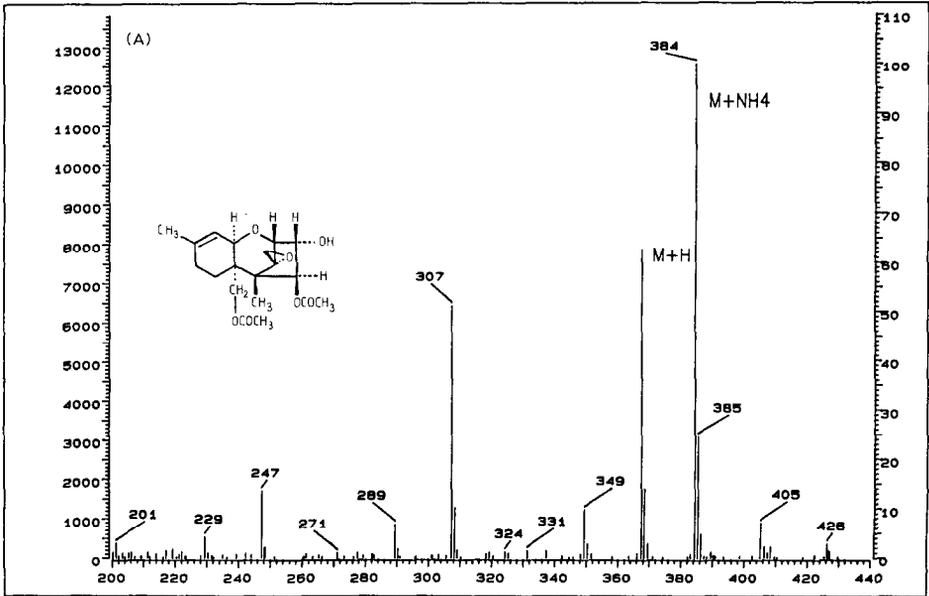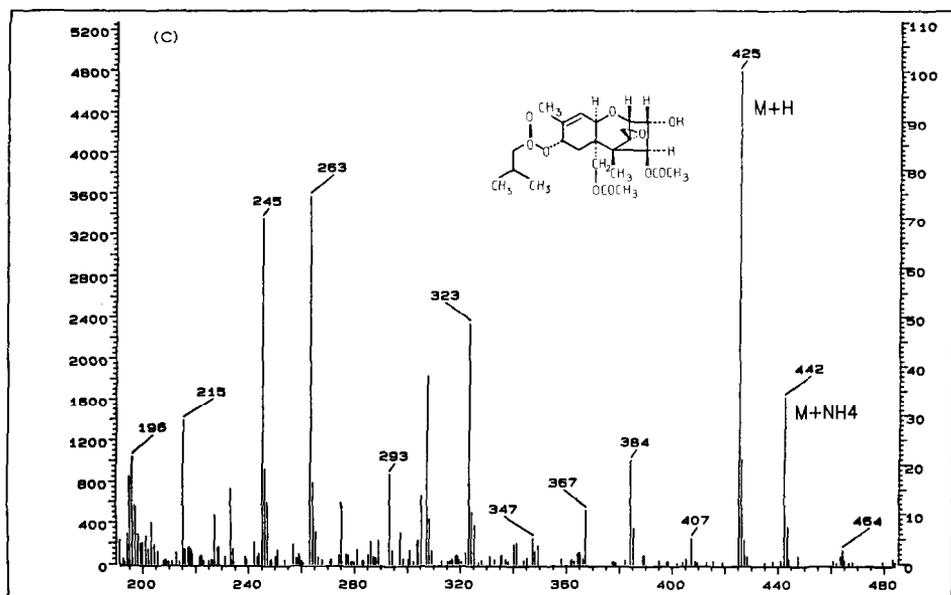

Fig. 6.

Fig. 6. The thermospray LC–MS positive ion spectra of (A) DAS, (B) T-2-toxin and (C) HT-2-toxin; filament on, 0.001 mol ammonium acetate.

rather than protonation by reaction with water. Evidence in support of this is provided by the observation of a much higher $(M + NH_4)$ adduction of T-2-toxin which contains one ester group more than the HT-2-toxin. The other compounds gave
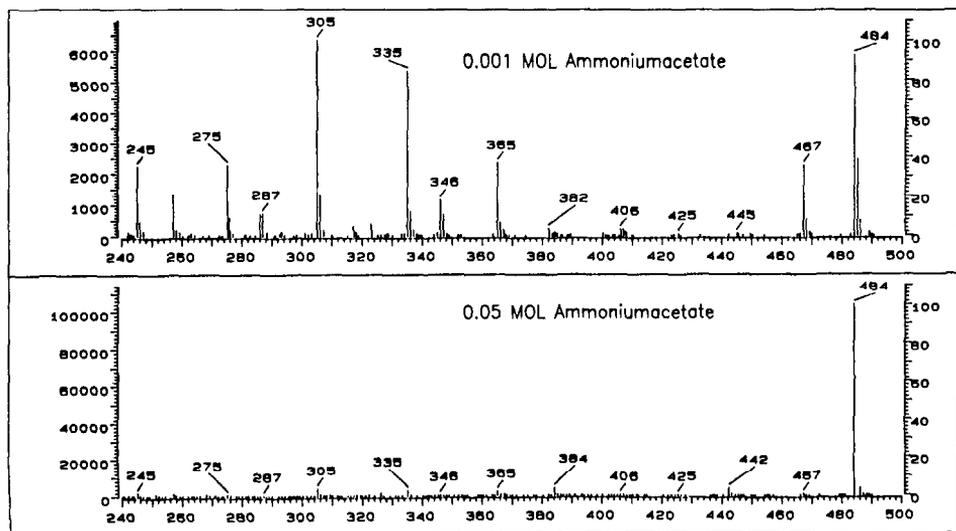

Fig. 7. Effect of the ammonium acetate concentration on the TSP LC–MS positive ion spectra of T-2-toxin.
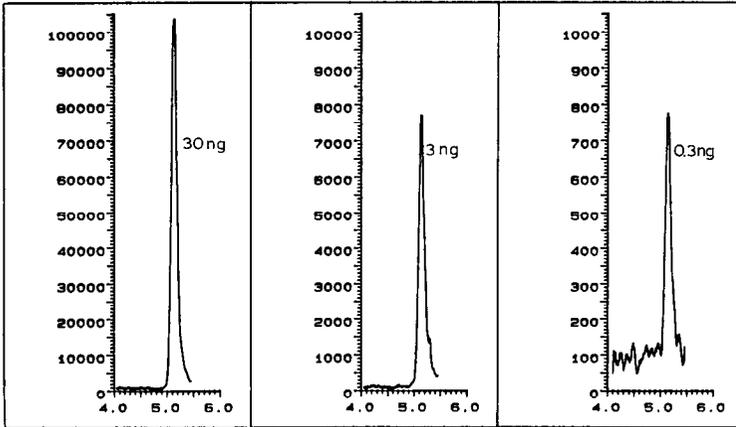
Fig. 8. Determination of the detection level of T-2-toxin.

simple spectra with protonated molecular ions and no fragmentation at both am-
monium acetate concentrations.

In the LC–MS system, T-2-toxin gave the strongest response. Based on the
results shown in Fig. 8, a signal-to-noise ratio (S/N) of 20 was obtained for 300 pg
injected into the column. With a S/N of 5 the detection level was 75 pg. Detection
levels for the other test compounds ranged from 100 pg to 1 ng. Relatively large
injection volumes (20–30 μl) did not adversely affect the linearity of the detector
response. It was therefore possible to measure mycotoxin concentrations ranging
from 3 to 40 ppb (ng/g).

A sample of the residue from a rye-milling process (Ruismyllyn Jäte) was an-
alyzed for T-2-toxin by the method described. Fig. 9 shows the results obtained with
a 10-μl sample of the extract, compared with a 500-pg standard. A rough estimation
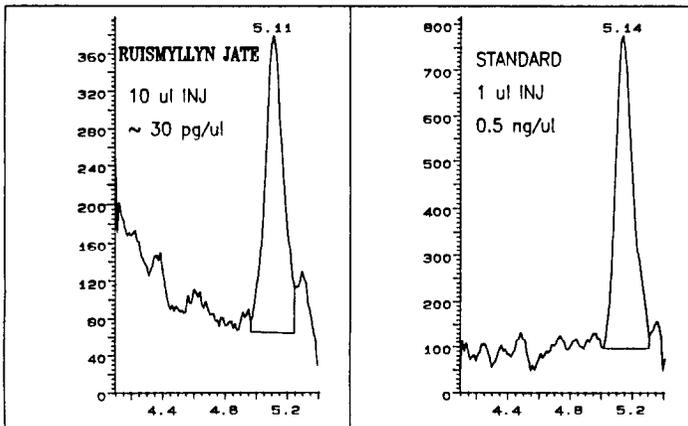gives a concentration of 30 pg/μl, equivalent to 1.5 μg/kg.

Fig. 9. Determination of T-2-toxin in the residue from a rye-milling process.

CONCLUSIONS

A novel HPLC method for the separation of mycotoxins has been described. The column, packed with Vydac 201 HSB 5-μm material, is stable, durable and superior to the other columns tested in this application under the specified conditions. The sample preparation is simple and the recoveries are acceptable. The most serious limitation of the method is the sensitivity of the available detectors.

Thermospray HPLC–MS, as demonstrated, shows great potential for the analysis of mycotoxins. By varying the electrolyte concentration, the analysis can be used for either qualitative or quantitative purposes. The additional ionization supplied by the filament at low concentrations of salt results in specific fragmentation patterns. At high salt concentration the sensitivity is increased by a factor of 5.

The sample preparation needed for the TSP–LC–MS determination of mycotoxins in grains and animal feeds is minimal (step 1) and background interference is negligible compared with UV detection.

ACKNOWLEDGEMENTS

REFERENCES

 1 S. Takitani, Y. Asabe, T. Kato, M. Suzuki and Y. Ueno, *J. Chromatogr.*, 172 (1979) 335.
 2 A. Sano, Y. Asabe, S. Takitani and Y. Ueno, *J. Chromatogr.*, 235 (1982) 257.
 3 M. W. Trucksess, S. Nesheim and R. Eppley, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 40.
 4 P. M. Scott, P.-Y. Lau and S. R. Kanhere, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 1364.
 5 B. G. Osborne and K. H. Willis, *J. Sci. Food Agric.*, 35 (1984) 579.
 6 T. Ilus, M.-L. Niku-Paavola and T.-M. Enari, *Eur. J. Appl. Microbiol. Biotechnol.*, 11 (1981) 244.
 7 C. O. Ikediobi, I. C. Hsu, J. R. Bamburg and F. M. Stron, *Anal. Biochem.*, 43 (1971) 327.
 8 H. Kamimura, M. Nishjima, R. Yasuda, K. Saito, A. Ibe, T. Nagayania, H. Ushiyama and Y. Nadi, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 1067.
 9 T. R. Romer, T. M. Boling and J. L. MacDonald, *J. Assoc. Off. Anal. Chem.*, 61 (1978) 801.
10 C. E. Kientz and A. Verweij, *J. Chromatogr.*, 355 (1986) 229.
11 R. T. Rosen and J. D. Rosen, *Biomed. Mass. Spectrom.*, 9 (1982) 443.
12 C. J. Mirocha, R. A. Pawlosky, K. Chatterjee, S. Watson and W. Hayes, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 1485.
13 E. Karppanen, A. Rizzo, S. Berg, E. Lindfors and R. Aho, *J. Agric. Sci. Finland*, 57 (1985) 195.
14 T. Krishnamurthy and E. W. Sarver, *J. Chromatogr.*, 355 (1986) 253.
15 J. D. Rosen, R. T. Rosen and T. G. Hartman, *J. Chromatogr.*, 355 (1986) 241.
16 R. Tiebach, W. Blaas, M. Kellert, S. Steinmeyer and R. Weber, *J. Chromatogr.*, 318 (1985) 103.
17 A. Visconti and A. Bottalico, *Chromatographia*, 17 (1983) 97.
18 R. Schmidt and K. Dose, *J. Anal. Toxicol.*, 8 (1984) 43.
19 R. Schmidt, E. Ziegenhagen and K. Dose, *J. Chromatogr.*, 212 (1981) 370.
20 G. M. Ware and C. W. Thorpe, *J. Assoc. Off. Anal. Chem.*, 61 (1978) 1058.
21 D. C. Hunt, A. T. Bourdon and N. T. Crosby, *J. Sci. Food Agric.*, 29 (1978) 245.
22 G. A. Bennett, O. L. Shotwell and W. F. Kwolek, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 958.
23 T. Tanaka, A. Hasegawa, Y. Matsuki, U.-S. Lee and Y. Ueno, *J. Chromatogr.*, 328 (1985) 271.
24 K. C. Ehrlich and L. S. Lee, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 963.
25 K. C. Ehrlich, L. S. Lee and A. Ciegler, *J. Liq. Chromatogr.*, 6 (1983) 833.
26 R. D. Schmith, H. R. Udseth and B. W. Wright, *J. Chromatogr. Sci.*, 23 (1985) 192.
27 R. D. Voyksner, W. M. Hagler, Jr., K. Tyczkowska and C. A. Haney, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 119.
28 C. R. Blackley and M. L. Vestal, *Anal. Chem.*, 55 (1983) 75.