Journal of Chromatography, 629 (1993) 229–235
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 649

Multimycotoxin detection and clean-up method for aflatoxins, ochratoxin and zearalenone in animal feed ingredients using high-performance liquid chromatography and gel permeation chromatography

Catherine Dunne, Mary Meaney and Malcolm Smyth

School of Chemical Sciences, Dublin City University, Glasnevin, Dublin 9 (Ireland)

Louis G. M. Th. Tuinstra

State Institute for Quality Control of Agricultural Products, Bornesteeg 45, 6708 PD Wageningen (Netherlands)

(First received July 24th, 1992; revised manuscript received September 25th, 1992)

ABSTRACT

A sensitive and reliable method is described for the determination of aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A and zearalenone in animal feed ingredients. A multi-toxin extraction and clean-up procedure is used, with dichloromethane–1 *M* hydrochloric acid (10:1) being used for the extraction and gel permeation chromatography being used for the clean-up. The liquid chromatographic method developed for the separation of the six mycotoxins involves gradient elution with a reversed-phase C_{18} column and fluorescence detection. Recoveries, repeatability and reproducibility have been determined on maize, palm and wheat. The detection limits varied depending on the type of feed.

INTRODUCTION

The presence of mycotoxins in animal feeds has been of major concern for many years. A tolerance level of aflotoxin B_1, in groundnut, copra, palm-kernal, cotton seed, babassu, maize and products derived from the processing thereof is 20 μg/kg in the European Communities Regulations 1991. Many analytical procedures have been developed for their determination [1–5]. The majority of these are based on single extraction and single determinations. In more recent publications there is widespread interest in the developement of multi-toxin clean-up and determination to achieve a rapid, sensitive and more economical analysis. Hetmanski and Scudamore [6] used gel permeation chromatography (GPC) for clean-up of extracts prior to separation by reversed-phase HPLC for the analysis of aflatoxins B_1, B_2, G_1 and G_2. Langseth *et al.* [7] determined zearalenone and ochratoxin A in cereals and feed using one extraction step. The clean-up step used silica solid-phase extraction columns and the mycotoxins were eluted separately with different solvent mixtures. They were injected separately into a HPLC system under similar conditions. Hunt *et al.* [8] determined aflatoxins and ochratoxin A in food. The extract was purified using grooved thin-layer chromatography plates coated with silica gel. After chromatography any fluorescent bands were removed and examined by HPLC.

In this paper an extraction and clean-up method developed by Scudamore and Hetmanski [9] was

Correspondence to: L. G. M. Th. Tuinstra, State Institute for Quality Control of Agricultural Products, Bornesteeg 45, 6708 PD Wageningen, Netherlands.

0021-9673/93/$06.00

used with slight modifications. Dichloromethane–1 *M* hydrochloric acid (10:1) was used for the extraction. GPC was used for the clean-up. The HPLC method developed here, for the determination of the six mycotoxins, used gradient elution with two mobile phases: (A) water–methanol–acetonitrile (130:70:40) plus 1 m*M* nitric acid plus 1 m*M* potassium bromide; (B) 0.01 *M* phosphoric acid–acetonitrile (50:50).

Post-column derivatization with bromine was used to enhance the sensitivity of aflatoxin B_1 and G_1 in conjuction with fluorescence detection with pre-programmed wavelength change. Kok *et al.* [10] showed that the fluorescence intensity of aflatoxin B_1 and G_1 increased after the addition of bromine solution. The reaction is believed to be the bromination of the 8,9 double bond. Aflatoxin B_2 and G_2 do not react with bromine owing to the absence of the double bond. The fluorescent signal enhancement of the aflatoxin may be carried out by derivatization with on-line, electrochemically generated bromine. Bromine can be produced from bromide present in the mobile phase in an electrochemical cell after the column. The derivatization with bromine decreased the sensitivity of zearalenone. It was therefore necessary to inject the extract again without derivatization in order to determine zearalenone.

In brief the method described here allows the determination of aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A and zearalenone in animal feed using a multi-toxin extraction and clean-up and gradient elution with HPLC for the determination. Maize, palm and wheat were used for recovery, reproducibility and repeatability studies.

EXPERIMENTAL

Reagents

Aflatoxin B_1 and ochratoxin A were obtained from Calbiochem (San Diego, CA, USA). Aflatoxins B_2, G_1 and G_2 were obtained from Makor Chemicals (Jerusalem, Israel). Zearalenone was obtained from Carl Roth (KG 1975 Karlsruhe 21, Germany). All solvents were of analytical-reagent grade and were purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

The following stock solutions were prepared: (a) aflatoxin B_1, 1 μg/ml in chloroform, (b) aflatoxin B_2, 1 μg/l in chloroform, (c) aflatoxin G_1, 1 μg/ml in chloroform, (d) aflatoxin G_2, 1 μg/ml in chloroform, (e) ochratoxin A_1, 1 μg/ml in methanol, (f) zearalenone, 100 μg/ml in methanol.

Equipment

The GPC equipment consisted of a 60 mm × 6 mm I.D. glass column (Spectrum Medical Industries, Los Angeles, CA, USA) fitted with a 40–60 μm porous bed support and adjustable plunger packed with Bio-Beads SX-3 gel (Bio-Rad, Watford, UK). The gel was suspended in a mixture of dichloromethane–ethylacetate–formic acid (49.9:49.9:0.2) for one day before loading onto the glass column. The height of the column was 55 mm. A Waters (Milford, MA, USA) M-45 pump was used and a Waters WISP 710B automatic injector. A Gilson (Villiers-le-Bel, France) 202 fraction collector and 201-202 fraction controller were used for collecting fractions.

The HPLC equipment included a Gilson 305 and 302 pump, a Gilson 505 manometric module, Gilson 511B dynamic mixer, chromsphere RP-C_{18} column (Chrompack, Middelburg, Netherlands), KOBRA device (Lamers & Pleuger, Den Bosch, Netherlands) for generating bromine for the post-column derivatisation and a Perkin-Elmer LS4 fluorescence detector (Perkin-Elmer, Norwalk, CT, USA). An automatic Gilson 231 sample injector with a Gilson 401 dilutor was also used.

Other equipment included a Desaga flask shaker (Heidelburg, Germany) and a Büchi rotary evaporator (Switzerland).

Extraction

A 25-g portion of well mixed, finely ground sample was weighed into a 250-ml erlenmeyer flask. A 12.5-g amount of Celite (Johns-Manville, Denver, CO, USA), 12.5 ml 1 *M* hydrochloric acid and 125 ml dichloromethane were added. The flask was stoppered and shaken for 30 min before filtering the sample through a Whatman No. 1 filter paper into a 250-ml round-bottom flask. The residue in the filter paper was rinsed with 3 × 25 ml portions of dichloromethane. The combined filtrate and washings were evaporated to near dryness (*ca.* 0.5 ml) by rotary evaporation at 30°C. The residue in the flask was transferred to a 10-ml volumetric flask with at

least four rinses of dichloromethane, approximately 1 ml each time. A 5-ml volume of ethyl acetate and 0.02 ml formic acid were added and the solution was made up to the mark with dichloromethane.

Clean-up

Approximately 1 ml of the sample extract was filtered through a disposable 0.45-μm organic filter (Acrodisc CR PTFE; Gelman, Ann Arbor, MI, USA). A 200-μl volume of the filtrate was injected onto the GPC column using a WISP 710 B automatic injector. Dichloromethane–ethyl acetate–formic acid (49.9:49.9:0.20) mixture was passed through the column at 0.3 ml/min. One fraction from 25–45 min was collected. A 2-ml volume of water was added to the fraction which was stoppered and well shaken. The lower organic layer was passed through anhydrous sodium sulphate. The sodium sulphate was rinsed with 5 ml dichloromethane. The combined filtrate and washings were evaporated to near dryness (*ca.* 0.5 ml). The residue was taken up in water–acetone (85:15). This was well shaken and sonicated for 5 min and then filtered through a disposable organic filter before HPLC determination.

High-performance liquid chromatography

A gradient solvent system was used. The initial percentage of A was 100%. This was maintained for 8 min after injection. Over the next 5 min the percentage A was reduced to 30% and the percentage of B increased from 0% to 70% linearly. These were maintained at these levels for the following 14 min. The percentage A was then increased to 100% and the percentage B decreased to 0% linearly over the next 5 min and maintained for 8 min at which point the next injection could be made. The wavelengths of excitation and emission were changed as follows:

	Ex/Em
0–20.0 min	= 369/422 nm
20.0–24.0 min	= 335/500 nm
24.0–26.2 min	= 310/470 nm
26.2–38.9 min	= 335/500 nm
38.9–40.0 min	= 369/422 nm

RESULTS AND DISCUSSION

The extraction and clean-up method used was developed by Scudamore and Hetmanski [9]. Some minor modifications were made. Before the GPC injection, extracts were filtered through a 0.45-μm disposable filter in order to remove any suspended particles. The GPC column and injection volume were smaller than that used by Scudamore and Hetmanski [9]; however, these were reduced porportionally in our work. The flow-rate was also reduced porportionally. The solvent consumption is reduced due to the smaller injection volume and lower flow-rate, thus making the analysis more economical. Water–acetone was used to dissolve the mycotoxins for HPLC analysis as it had been used previously by Kok *et al.* [10] to determine aflatoxins in cattle feed. The dissolved mycotoxins were filtered through a 0.45-μm disposable filter in order to remove residue drops which did not dissolve. Residue drops were also present if acetonitrile–water (1:1) was used. This was the solvent used by Scudamore and Hetmanski [9]. The filtration did not affect recovery. It also ensured that a clean sample was injected onto the HPLC column.

The fraction in which the mycotoxins eluted from

TABLE I

RECOVERY OF MYCOTOXIN STANDARDS

Levels of mycotoxins used were 0.2 ng aflatoxins B_1, G_1 and G_2, 0.1 ng aflatoxin B_2, 1 ng ochratoxin A and 20 ng zearalenone. The analysis was repeated three times.

	Recovery (%)					
	B_1	B_2	G_1	G_2	Ochratoxin A	Zearalenone
Mean	99.1	96.5	100.5	98.5	74.0	87.3
S.D.	5.8	4.0	13.7	11.2	10.4	5.9
R.S.D. (%)	5.8	4.1	13.6	11.4	14.1	6.7

GPC was determined by monitoring the output, using fluorescence detection at appropriate wavelengths of excitation and emission, for each mycotoxin. Mycotoxins with a higher molecular mass eluted earlier, as expected. However, collecting from 25 to 45 min ensured that all mycotoxins of interest were included in the fraction.

Kok *et al.* [10] used a mobile phase of water–methanol–acetonitrile (130:70:40) plus 1 m*M* nitric acid and 1 m*M* potassium bromide to separate aflatoxins B_1, B_2, G_1 and G_2. By using a mobile phase of 180:70:40 water–methanol–acetonitrile, better separation of aflatoxins was achieved. In order to elute ochratoxin A and zearalenone the polarity of the mobile phase had to be decreased. The elution of ochratoxin A containing a carboxylic acid group also requires and acidic mobile phase [7]. The second mobile phase in the gradient elution system was 0.01 *M* phosphoric acid–acetonitrile (50:50). A similar mobile phase had been used by Howell and Taylor [11] for the determination of zearalenone and ochratoxin A.

Standard solutions containing known amounts of aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A and zearalenone, made up in the GPC mobile phase, were injected onto the GPC column, collected and determined by the HPLC method. Table I shows the recoveries obtained.

Typical chromatograms of samples spiked to contain aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A and zearalenone are shown in Figs. 1 and 2. The peak identified as zearalenone in the blank sample in Fig. 2 has the same retention time as that of the spiked zearalenone sample. The zearalenone peak also disappears when post-column derivatization is used as indicated in Fig. 1a, thus confirming the peak is zearalenone. Any change in baseline at 20, 24 and 26.2 min is due to a wavelength change. Although the post-column derivatization with bromine enhances aflatoxin B_1 and G_1, the zearalenone peak disappears under these conditions. This, however, can be used as a useful confirmation test for zearalenone, in particular with samples that contain interferences that coelute with zearalenone and give false positive results [12]. In order to determine zearalenone the extract must be re-injected without derivatization. To avoid this re-injection a second detector, set for the detection of zearalenone, may be placed after the column and before derivatization. The KOBRA cell also continues to influence the chromatogram for some time, *i.e.* a few hours, after it is switched off. In the setup used it is therefore not

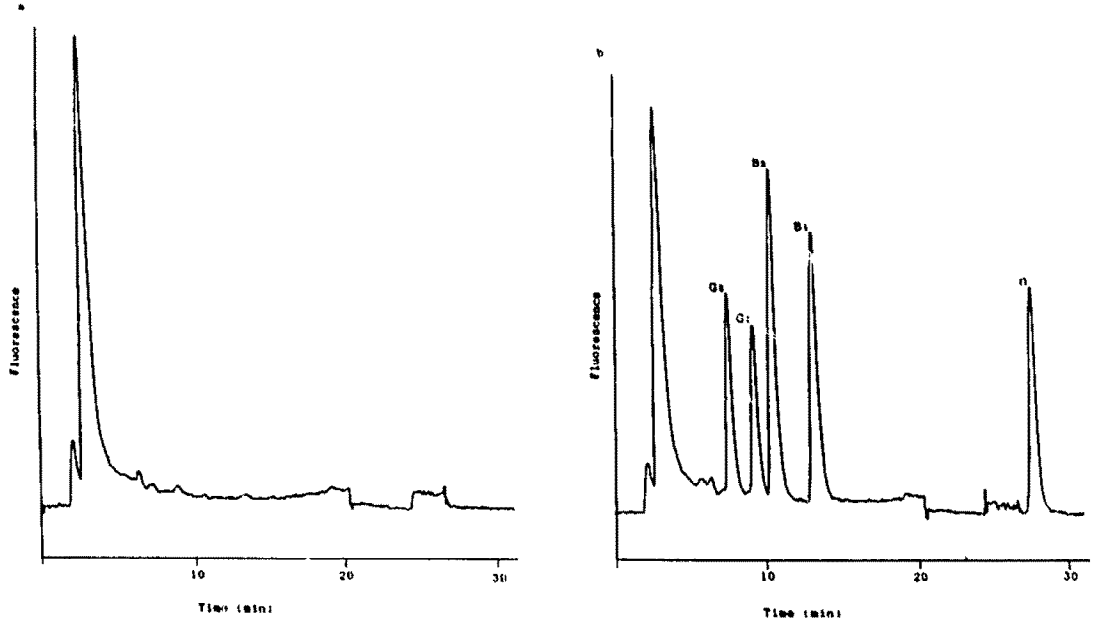

Fig. 1. Chromatograms of (a) maize samples and (b) maize sample spiked to contain 3.2 μg kg aflatoxin B_1, G_1 and G_2, 1.6 μg kg aflatoxin B_2, 16 μg kg ochratoxin A and 320 μg kg zearalenone with post-column derivatization. No zearalenone detected due to post-column derivatization.

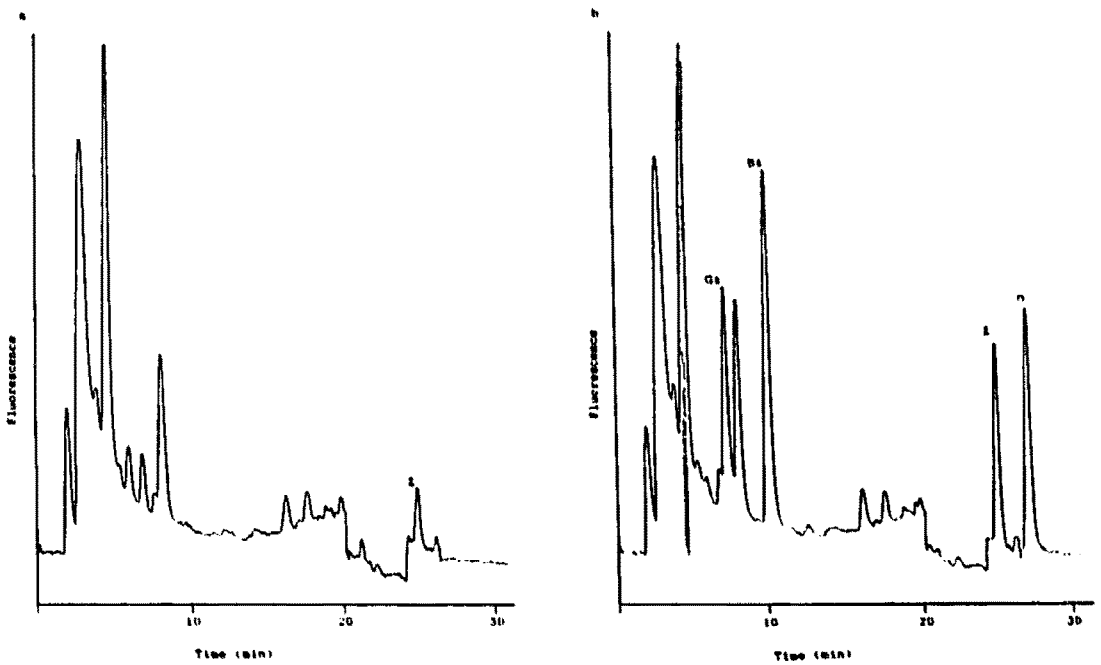

Fig. 2. Chromatograms (a) and (b) as in Fig. 1 without post-column derivatization.

possible to determine zearalenone immediately. The zearalenone should be determined first, before derivatization is carried out for the determination of the other mycotoxins, on the other hand the KOBRA cell may be bypassed. Post-column derivatization with bromine not only enhances the fluorescence intensity of aflatoxins B_1 and G_1 but also reduces, and in some cases completely diminishes, the fluorescence intensity of many interfering components.

Recoveries for spiked extracts of maize, palm and wheat are shown in Table II. Known amounts of standard mycotoxin solutions were added to extracts of maize, palm and wheat. Recoveries for zearalenone in maize and palm were low.

The reproducibility of the method was checked using different types of feed. Three feed ingredients were analyzed three times each for aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A and zearalenone, which were spiked onto the feed ingredient. The results are shown in Table III. The aflatoxin recoveries are greater than 73% for all feeds. The aflatoxin recoveries obtained for wheat had a tendancy to be higher than those obtained by Scudamore and Hetmanski [9], the ochratoxin A recoveries compare favourably and the zearalenone recoveries are

TABLE II

RECOVERY OF MYCOTOXINS FROM SPIKED EXTRACT

Levels of mycotoxins used the same as in Table I. N.D. = Not determined.

	Recovery (%)					
	B_1	B_2	G_1	G_2	Ochratoxin A	Zearalenone
Maize	93.4	102.4	97.3	97.9	N.D.	49.7
Palm	81.4	99.6	93.1	90.2	N.D.	17.2
Wheat	90.9	102.2	103.9	103.0	N.D.	71.6

TABLE III

REPRODUCIBILITY TEST ON THREE DIFFERENT SPIKED FEEDS

Levels of mycotoxins used were 3.2 μg/kg aflatoxins B_1, G_1, and G_2, 1.6 μg/kg aflatoxins B_2, 16 μg/kg ochratoxin A and 320 μg/kg zearalenone.

	Recovery (%)					
	B_1	B_2	G_1	G_2	Ochratoxin A	Zearalenone
Maize mean	96.3	101.0	102.6	102.6	77.6	24.6
S.D.	5.1	7.1	9.6	9.5	2.4	1.7
Palm mean	73.1	82.0	87.5	76.7	12.5	12.9
S.D.	7.3	5.5	10.5	10.3	3.7	0.9
Wheat mean	75.9	84.6	96.7	88.7	59.4	38.8
S.D.	5.6	2.6	7.3	12.2	12.6	9.0

much less than those obtained by Scudamore and Hetmanski [9]. The recoveries for palm are lower than other feed ingredients analysed, but this is due to the higher background interference especially in the case of aflatoxin B_2, G_1 and G_2 (refer to Fig. 3).

Repeatability of the method was checked using maize. Ten portions of feed ingredient from the same batch were spiked with aflatoxins, ochratoxin A and zearalenone. The results are shown in Table IV.

Detection limits for each mycotoxin are shown in Table V. These are based on the noise × 3 and are in ng levels. Taking the original feed ingredient and recovery into account the detection limits are quoted in μg/kg. This detection limit depends on the type of feed being analysed as the recovery varies from feed to feed. Therefore a range of values are included which take into account the different types of feed ingredient being analysed.

The detection limits are good, despite the poor

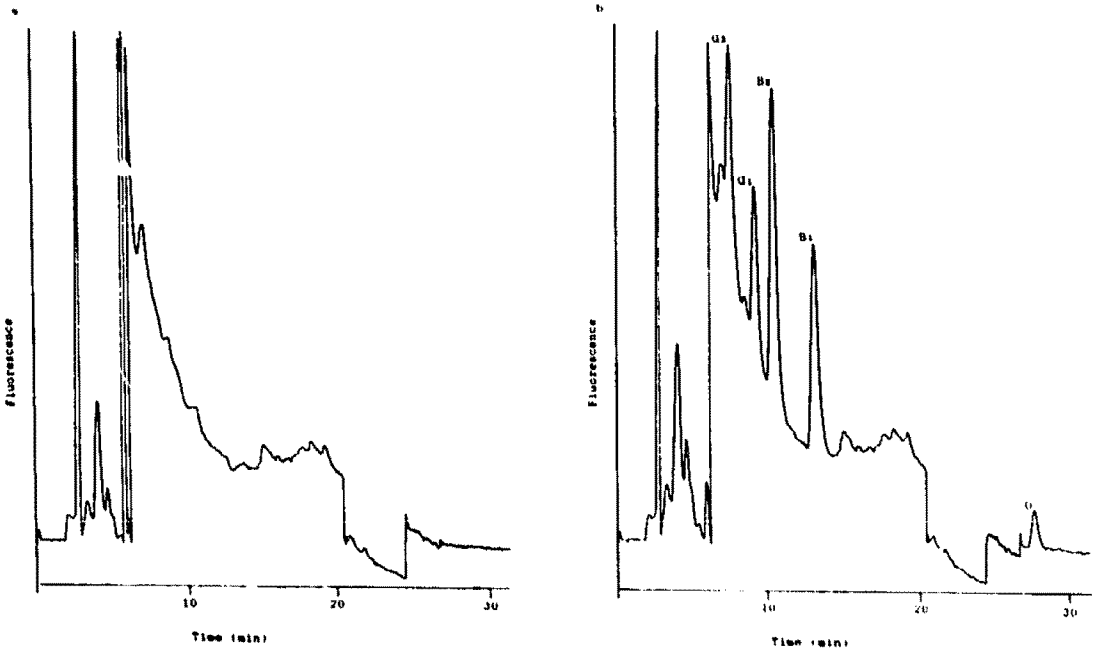

Fig. 3. Chromatograms of (a) palm sample and (b) palm sample, spiked to contain 3.2 μg kg aflatoxin B_1, G_1 and G_2, 1.6 μg kg aflatoxin B_2, 16 μg kg ochratoxin A, with post-column derivatization.

TABLE IV

REPEATABILITY TEST ON MAIZE (n = 10)

Levels of mycotoxins used the same as in Table III. N.D. = Not determined.

	Recovery (%)					
	B_1	B_2	G_1	G_2	Ochratoxin A	Zearalenone
Mean	102.7	105.6	108.2	106.5	73.1	N.D.
S.D.	4.1	4.9	5.8	4.6	3.8	N.D.
R.S.D. (%)	4.0	4.6	5.4	4.3	5.2	N.D.

TABLE V

LIMITS OF DETECTION

Mycotoxin	Detection limit	
	ng	μg/kg
B_1	0.009	0.144–0.197
B_2	0.003	0.048–0.058
G_1	0.016	0.256–0.334
G_2	0.013	0.208–0.271
Ochratoxin A	0.050	1.031–6.349
Zearalenone	0.619	25.525–76.775

recoveries in some cases for ochratoxin and zearalenone.

CONCLUSIONS

The HPLC method developed is fast, sensitive and economical. It allows the determination of six mycotoxins using one HPLC set up and gradient elution. The method is readily adaptable to automation. The clean-up procedure involving GPC lends itself to partial automation. Due to the low recoveries of zearalenone and improved or separate clean-up method is required. The reproducibility and detection limits of the overall method are good.

ACKNOWLEDGEMENTS

The authors thank the Commission of the European Communities for granting a research bursary, under the framework of the FLAIR programme, to enable this research project to be carried out at the State Institute for Quality Control of Agricultural Products (RIKILT)-DLO, Wageningen, Netherlands.

REFERENCES

1 W. E. Paulisch, E. A. Sizoo and H. P. van Egmond, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 957.
2 M. R. Smyth, D. W. Lawellin and J. Osteryoung, *Analyst*, 104 (1979) 73.
3 H. Cohen and M. LaPointe, *J. Assoc. Off. Anal Chem.*, 69 (1986) 957.
4 E. Josefsson and T. Möller, *J. Assoc. Off. Anal. Chem.*, 62 (1979) 1165.
5 P. M. Scott, T. Panalaks, S. Kanhere and W. F. Miles, *J. Assoc. Off. Anal. Chem.*, 61 (1978) 593.
6 M. T. Hetmanski and K. A. Scudamore, *Food Addit. Contam.*, 6 (1989) 35.
7 W. Langseth, Y. Ellingson, U. Nymoen and E. M. Økland, *J. Chromatogr.*, 478 (1989) 269.
8 D. C. Hunt, A. T. Bourden, P. J. Wild and W. T. Croby, *J. Sci. Food Agric.*, 29 (1978) 234.
9 K. A. Scudamore and M. T. Hetmanski, *Mycotoxin Res.*, in press.
10 W. Th. Kok, Th. C. H. van Neer, W. A. Traag and L. G. M. Th. Tuinstra, *J. Chromatogr.*, 367 (1986) 231.
11 M. V. Howell and P.W. Taylor, *J. Assoc. Off. Anal.Chem.*, 64 (1981) 1356.
12 N. Chamkasem, W. Y. Cobb, G. W. Latimer, C. Salmas and B. A. Clement, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 336.