Journal of Chromatography, 603 (1992) 285–289
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 241

Short Communication

Automated liquid chromatographic determination of ochratoxin A in cereals and animal products using immunoaffinity column clean-up

Matthew Sharman, Susan MacDonald and John Gilbert

Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Colney Lane, Norwich NR 4 7UQ (UK)

(First received February 7th, 1992; revised manuscript received April 3rd, 1992)

ABSTRACT

The analysis of the fungal mycotoxin ochratoxin A in cereals and animal products is described using an immunoaffinity column clean-up and high-performance liquid chromatographic determination. The clean-up can be carried out manually or using a commercially available automated sample preparation system. The method has been applied to cereals such as wheat, rye and barley, unprocessed breakfast cereals and animal products such as pigs' kidneys and blood sausages. Recoveries ranged from 70–80% for spiked samples (10 μg/kg) and the method had a relative standard deviation of 1.3% ($n=8$) for the analysis of a wheat sample naturally contaminated at 13.7 μg/kg ochratoxin A and relative standard deviation of 3.0% ($n=8$) for a pig kidney sample spiked at 10 μg/kg ochratoxin A. The immunoaffinity approach was significantly faster than methods employing conventional chromatographic clean-up, and extracts were freer of co-extractives giving a limit of detection of 0.2 μg/kg.

INTRODUCTION

Ochratoxin A is a mycotoxin produced by *Aspergillus ochraceus* as well as by other fungi notably *Penicillium veridicatum*. The toxin has been found to occur in foods of plant origin, and through transfer from animal feeds can occur as a contaminant in edible animal tissues. Despite extensive data already in the literature concerning the occurrence of ochratoxin A there still exists a need for routine monitoring both to assess human exposure, and to test for compliance with regulations in countries such as Denmark [1]. Numerous methods have been published for the analysis of ochratoxin A using conventional column clean-up with high-performance liquid chromatographic (HPLC) end-determination [2] or using immunological methods such as enzyme-linked immunosorbent assay (ELISA) [3,4]. Many of these methods although individually effective do have the disadvantage of being only applicable to a limited number of matrices.

There is some advantage for laboratories that have large numbers of samples to analyse for a range of different mycotoxins to standardise procedures and thereby rationalise available instrumentation. For the analysis of aflatoxins in foods and animal feeds we have recently reported [5] a fully automated sample preparation and HPLC analysis procedure based on immunoaffinity column clean-up. An identical approach can be adopted for the

Correspondence to: Dr. J. Gilbert, Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Colney Lane, Norwich NR4 7UQ, UK.

0021-9673/92/$05.00

determination of ochratoxin A, offering the same advantages of automation, application to a range of differing matrices and allowing utilisation of the same equipment as for the aflatoxins.

Immunoaffinity columns comprise an anti-mycotoxin antibody bound to a gel material contained in a small plastic cartridge. Crude extract is forced through the column and the specific mycotoxin is left bound to the recognition site of the immunoglobulin. Extraneous material can be washed off the column with water and the mycotoxin in question can be recovered in purified form by liberating the bound analyte from the antibody with an elution solvent such as methanol or acetonitrile. Immunoaffinity columns are commercially available and have been routinely employed for determining aflatoxins B_1, B_2, G_1 and G_2 in nuts, nut products and dried fruit [6–8], as well as for determining aflatoxin M_1 in milk [9] and cheese [10]. These columns have the advantages of speed and simplicity compared to conventional clean-up and of high specificity thereby producing extracts free of interferences, although they have yet to be established by collaborative trial as satisfying requirements of official methods.

The simplicity of analysis using immunoaffinity columns makes this approach particularly amenable to automation, which in turn overcomes problems that are sometimes reported as being associated with sample loading onto the column such as poor recovery due to erratic flowrate through the columns. We previously described [5] a modification to the design of the plastic immunoaffinity cartridges to fit the rack of a commercially available automated sample preparation system (ASPEC). Changes to the software of the system which were made for aflatoxin analysis and which permitted conditioning, loading, washing and eluting operations were sufficiently flexible to allow the system to be readily extended for use for ochratoxin A analysis. The system described in this paper as previously for automated aflatoxin analysis, has been fully integrated with the HPLC determination, utilising fluorescence detection to determine ochratoxin A.

EXPERIMENTAL

Materials

Immunoaffinity columns (Easi-extract) for ochratoxin A were supplied by Biocode (York, UK). The manufacturer's data for the recovery from the columns was >85% when 100 ng of ochratoxin A were applied in a suitably diluted methanol extract, and a total capacity of greater than 2.7 μg of ochratoxin A when applied in phosphate-buffered saline (PBS).

Acetonitrile, methanol and chloroform were purchased from Rathburn (Walkerburn, UK). All water was deionised and distilled. Ochratoxin A was purchased from Sigma (Poole, UK) and buffer salts from BDH (Poole, UK). PBS was prepared from potassium chloride (0.2 g), potassium dihydrogenphosphate (0.2 g), anhydrous disodium hydrogenphosphate (1.16 g) and sodium chloride (8.0 g) added to distilled water (900 ml). After dissolution the pH was adjusted to 7.4 (with 0.1 *M* HCl or 0.1 *M* NaOH as appropriate) and the solution made to 1 l.

Sample preparation

Cereal samples. The sample (10.0 g) was weighed into a 100-ml tall form beaker and 40 ml of PBS–methanol (50:50) were added. The mixture was homogenised at high speed for 3–5 min with an Ultra Turrax homogeniser fitted with an 18N shaft to produce a slurry. The sample was filtered through Whatman 113V filter paper and an aliquot of the supernatant (15 ml) was diluted with PBS (35 ml) prior to affinity column clean-up.

Animal products. For pigs' kidneys the sample (10.0 g) was weighed into a 250-ml beaker, chloroform (100 ml) and 85% phosphoric acid (0.6 g) were added and the sample was homogenised as above. For black pudding (blood sausage) the sample (10.0 g) was similarly extracted but with the addition of water (15 ml). The kidney sample was filtered through a Whatman 113V filter paper, whilst the black pudding sample was centrifuged at 4500 *g* (4°C) for 10 min followed by filtration through a Whatman 113V filter paper into a separating funnel. In both cases the chloroform solution (50 ml) was partitioned with 1 *M* sodium hydrogencarbonate (100 ml), and an aliquot of the aqueous phase (45 ml) was diluted with water (15 ml), taking 50 ml

of the resultant solution for affinity column clean-up.

Manual immunoaffinity column clean-up. The immunoaffinity column was conditioned with PBS (20 ml) followed by the sample extract (50 ml) pushing both through the column at a steady flow of approximately 5 ml/min. The column was washed with distilled water (10 ml), then air dried by pumping air from a disposable syringe. The ochratoxin A was eluted from the column with 2.0 ml of methanol over 2 min, collecting in a 4-ml amber vial. The sample was subsequently diluted with water (2 ml) and thoroughly mixed prior to HPLC analysis.

Automatic clean-up and chromatography. The automated HPLC system (from Anachem, Luton, UK) consisted of a Gilson 307 isocratic pump, a Gilson ASPEC (an automatic solid-phase extraction system fitted with a Rheodyne 7010 injector and a Gilson 401 dilutor), and a Perkin-Elmer LC240 fluorescence detector (Beaconsfield, UK). The detector, the Gilson pump and the ASPEC were linked via a Gilson 506B system interface module to an IBM Model 30 personal computer. The use of Gilson 712 HPLC software allowed both collection of data and the control of the interfaced equipment.

The sequence of operations for the automated clean-up of samples using the ASPEC were as follows:

(a) Affinity column conditioned with PBS (20 ml) at 3.0 ml/min.
(b) Column loaded with sample extract (50 ml) at 3.0 ml/min.
(c) Column washed with water (10 ml) at 6 ml/min.
(d) Column dried with air (2 ml).
(e) Column eluted with methanol (0.5 ml) at 0.36 ml/min.
(f) Waiting period of 1 min.
(g) Column eluted with methanol (1.5 ml) at 0.36 ml/min.
(h) Remaining solvent recovered from column with air (3 ml).
(i) Total methanol eluate mixed by bubbling air (3 ml).
(j) Eluate (1.0 ml) transferred to a vial (2.0 ml).
(k) The remaining aliquot (1 ml) of methanol eluate diluted with 2% (v/v) acetic acid (2 ml) followed by further air mixing (3 ml).
(l) Automated injection (400 μl) of the diluted eluate onto HPLC by partial loop fill.

High-performance liquid chromatography

A Spherisorb ODS2 analytical column (5 μm particle size, 250 × 4.6 mm I.D.) was employed (held at 35°C) and this was protected by an inline filter (A315, Upchurch) fitted with a 2-μm frit (A101, Upchurch) and a C_{18} guard column (C752, Upchurch). The mobile phase of acetonitrile–water–acetic acid (99:99:2) was pumped at 1.0 ml/min. Detection was using a Perkin-Elmer LC240 fluorescence detector operated at an excitation wavelength of 333 nm and an emission wavelength of 477 nm.

RESULTS AND DISCUSSION

Although a single analytical procedure is desirable that can be used for both cereals and animal products, the simplified procedure that worked well for cereals using PBS–methanol extraction and then direct application of the extract onto the affinity column, gave low recoveries (*ca.* 50%) for animal products and problems with co-extractives. To increase recoveries for animal products it was necessary to use an acidified chloroform extraction solvent. To maintain solvent compatibility with the antibody and to remove some co-extractives an additional back-extraction step was needed prior to loading the extract onto the affinity column. The affinity column clean-up (manual or automated) and HPLC analysis was thereafter identical for cereal or animal product samples.

For the automated clean-up procedure the two-stage elution (stages e–g) increased the recovery of ochratoxin A from the immunoaffinity columns as the initial application of methanol permeated the gel, breaking the antibody–antigen binding, prior to final elution with the larger volume of methanol. Stage h purged all the remaining methanol from the column maintaining a quantitative recovery. Immunoaffinity columns were only used once before disposal although at least in theory multiple-use should be possible. Stage j in the sequence of operations for the ASPEC was introduced for two purposes. Firstly it minimised the amount of 2% acetic acid required in stage k and secondly it provided a spare portion of sample that could be kept as a reference to be analysed on a different instrument or at

a later date. This is of particular importance if a confirmation step such as the formation of the methyl ester is required. To avoid sample concentration by evaporation prior to HPLC, which can be time consuming and result in loss of analyte, the methanol eluate from the affinity column was diluted so that the aqueous composition of the solvent was approximately 20% greater than that of the HPLC mobile phase. This allowed a relatively large volume (400 μl) to be injected onto the HPLC without loss of column performance.

For the fluorescence detection of ochratoxin A the emission wavelength was optimised by obtaining a spectrum of a solution in HPLC mobile phase. At an excitation wavelength of 333 nm the maximum was found to be at 477 nm, which gave a 25-fold increase in sensitivity compared with a wavelength of 420 nm which has been frequently employed in other published methods.

Performance data for the use of affinity columns were obtained using the automated system. For a wheat sample that had been shown to contain <0.2 μg/kg ochratoxin A, spiked at 10 μg/kg the recovery averaged 74% ($n = 8$) with a relative standard deviation (R.S.D.) of 3.5%. As ochratoxin A in PBS gave a quantitative recovery from the affinity column it can only be assumed that recovery losses are occurring from the matrix possibly due to binding effects. For samples of pigs' kidney and of blood sausages again found to contain <0.2 μg/kg ochratoxin A in both, spiked at 10 μg/kg, the recoveries averaged 79% ($n = 8$) and 74% ($n = 8$) with R.S.D. of 3.0 and 6.0%, respectively. A naturally contaminated sample of pigs' kidney and blood sausages was not available but for a naturally contaminated sample of wheat containing 13.7 μg/kg ochratoxin A a R.S.D. of 1.3% was obtained on repeated analysis ($n = 8$). Ochratoxin A certified reference materials are not as yet available so it was not possible to demonstrate the accuracy of the method on naturally contaminated samples. The limit of detection of the method was found to be 0.2 μg/kg for both cereal and animal products, which was comparable to most other published methods and sufficiently low for the needs of food surveillance.

The major advantage of use of immunnoaffinity column clean-up for mycotoxin analysis is the ability to apply essentially the same procedure to differing matrices, to achieve high recoveries and to produce chromatograms essentially free of interferences. Typical chromatograms are shown in Fig. 1 for cereals and for pigs kidneys illustrating the high specificity of the clean-up and thus the ability to achieve low limits of detection. Animal products such as pigs' kidney and black pudding can frequently be difficult matrices to analyse by conventional methods because of co-extractives which are essentially removed with the affinity column procedure. To date in surveillance work several hundred retail samples of wheat, barley, maize, bran products, wholemeal breakfast cereals, pigs' kidneys and black pudding have been analysed using the immunoaffinity method without experiencing any matrix problems or interferences. The reliability of the automated system has been demonstrated and significant time-savings have been achieved compared with manual approaches.

The full flexibility of the automated ASPEC system can be utilised to allow for concurrent analysis

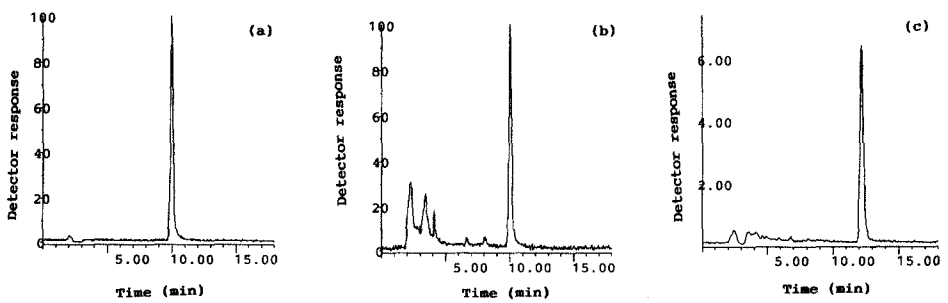

Fig. 1. HPLC chromatograms illustrating the analysis of ochratoxin A. Peaks for ochratoxin A normalised to full scale deflection in each chromatogram. (a) Standard equivalent to 20 μg/kg; (b) naturally contaminated wheat sample containing 13.7 μg/kg; (c) pigs' kidney spiked with 10 μg/kg ochratoxin A.

of samples for either aflatoxins or ochratoxin A. This has been achieved by modification of the system to incorporate column switching between an ODS2 column for ochratoxin analysis and an ODS1 column (with post-column derivatization) for aflatoxin analysis. By the use of the appropriate affinity columns and application of the differing sample extraction procedures the ASPEC can be utilised to carry out both mycotoxin analyses in an unattended mode of operation. For the future it is anticipated that automated multi-mycotoxin analyses will be possible as the range of affinity columns is extended further and through the development of multi-analyte columns using mixed antibodies.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the assistance of P. Arnold (Biocode Ltd.) for the provision of ochratoxin A affinity columns and M. Lindsay (Anachem Ltd.) for modification of the software for the ASPEC system.

REFERENCES

1 H. P. van Egmond, in M. Castegnaro, R. Plestina, G. Dirheimer, I. N. Chernozemsky and H. Bartsch (Editors), *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumors*, International Agency for Research on Cancer (IARC), Lyon, pp. 57–70.
2 World Health Organisation, *Environmental Health Criteria 105: Selected Mycotoxins: Ochratoxins, Trichothecenes, Ergot*, WHO, Geneva, 1990, pp. 30–32.
3 S. C. Lee and F. S. Chu, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 45.
4 M. R. A. Morgan, R. McNerney and H. W.-S. Chan, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 1481.
5 M. Sharman and J. Gilbert, *J. Chromatogr.*, 543 (1991) 220.
6 A. L. Patey, M. Sharman and J. Gilbert, *Food Add. Contam.*, 7 (1990) 515.
7 M. Sharman, A. L. Patey, D. A. Bloomfield and J. Gilbert, *Food Add. Contam.*, 8 (1991) 299.
8 A. A. G. Candlish, C. A. Haynes and W. H. Stimson, *Int. J. Food Sci. Technol.*, 23 (1988) 479.
9 D. N. Mortimer, J. Gilbert and M. J. Shepherd, *J. Chromatogr.*, 407 (1987) 393.
10 M. Sharman, A. L. Patey and J. Gilbert, *J. Chromatogr.*, 474 (1989) 457.