

Note

Application of an immunoaffinity column sample clean-up to the determination of aflatoxin M₁ in cheese

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Aflatoxin M₁ the hydroxylated metabolite of aflatoxin B₁ is found in milk and dairy products of animals that have consumed contaminated feed. Although Regulations control aflatoxin B₁ levels in the feeding stuffs there is still a need to monitor milk to check the effectiveness of these controls particularly concerning imported products. When aflatoxin M₁ contaminated milk is used to make other dairy products, the toxin is not destroyed and being associated with the casein fraction it is found for example at a 3-4 fold concentrated level in cheese¹. Although recent analysis^{2,3} of aflatoxin M₁ levels in European cheeses has shown a low incidence as well as generally low levels of contamination, it is nevertheless prudent from time-to-time for Regulatory Authorities to carry out monitoring of cheeses for aflatoxin M₁ as part of on-going food safety surveillance programs.

Analysis of cheese for aflatoxin M₁ involves solvent extraction, filtration and then a number of column or cartridge chromatographic clean-up stages prior to a high-performance liquid chromatographic (HPLC) or thin-layer chromatographic (TLC) determination^{4,5}. Although these established methods are effective showing both good recoveries and precision, with detection limits of the order of 0.01 µg/kg, the sample preparation stage is time-consuming and may depend on some prior experience of the analysis to obtain consistent results. As part of our evaluation of new techniques for sample preparation we have recently reported the use of immunoaffinity columns for sample clean-up in the analysis of aflatoxin M₁ in liquid and powdered milk⁶. This approach offered rapid sample throughput, good recovery and with HPLC as the determinative step a sample extract that was significantly cleaner than could be obtained with more conventional approaches. In this paper we report an extension of the use of immunoaffinity columns to the analysis of aflatoxin M₁ in cheese where similar advantages were obtained and with an even more significant time-saving.

EXPERIMENTAL

Materials

"Aflatoxin M₁ Easi-extract" immunoaffinity columns type TD 120 were obtained from Oxoid (Basingstoke, U.K.). Acetonitrile and methanol were purchased

from Rathburn (Walkerburn, U.K.). All water used was deionised distilled and, for HPLC, passed through a Milli-Q (Millipore, London, U.K.) purification system. Aflatoxin M₁ was from Sigma (Poole, U.K.) and buffer salts from BDH (Poole, U.K.).

Sample preparation

A sample of cheese (20 g) chopped into small pieces was weighed accurately into a 250-ml beaker to which was added chloroform (75 ml), saturated sodium chloride solution (1 ml) and Celite 545 (5 g). The mixture was homogenized with an IKA-Ultra-Turrax blender at high speed for 2–3 min to produce a slurry, which was subsequently filtered through a Whatman 113V filter paper into a round bottomed flask. The beaker was washed with chloroform (50 ml) and the washings filtered, finally squeezing the filter paper against the funnel to obtain maximum yield of extract. The chloroform extract was evaporated to dryness under vacuum at 30°C and to the residue was added methanol (1 ml), water (30 ml) and hexane (50 ml). After gentle swirling, the mixture was transferred to a separating funnel with washing (2 × 10 ml of water) and shaken for 10–15 s. The lower layer was collected and used in the affinity column stage of the sample clean-up.

Immunoaffinity column clean-up

The immunoaffinity column was washed with distilled water (10 ml) using a syringe at a flow-rate of about 2–3 ml/min. This was followed by the sample extract prepared as above (50 ml) and a further washing of distilled water (10 ml). The aflatoxin M₁ was then slowly eluted from the column with acetonitrile (2 ml) into a glass vial. The solvent was evaporated to near dryness by blowing-down with a gentle nitrogen stream, and was then redissolved in acetonitrile–water (1:1) to give a final volume of 250 µl. The extract was finally filtered through a 0.2-µm membrane before HPLC analysis.

Chromatography

The HPLC system consisted of a Varian 5500 ternary pump, a Rheodyne 7125 injector, and a Perkin Elmer LS-4 fluorescence detector set at 364 nm excitation and 434 nm emission. The detector was linked to a Trivector Trilab 2000 data station. A Spherisorb ODS 1 column (5 µm particle size, 250 × 4.9 mm I.D.) was employed, thermostatted at 35°C with a mobile phase of water–acetonitrile–methanol (60:10:30) at 0.7 ml/min. Sample extract (50 µl) was injected using a fixed loop.

RESULTS AND DISCUSSION

The immunoaffinity column used in the work in this paper was derived from monoclonal antibodies and was originally developed for reactivity against the B and G aflatoxins. The column was however found to have a good retention of aflatoxin M₁, and the manufacturer's data for cross-reactivity were B₁, 100%; B₂, 75%; M₁, 50%; and G₁, 38%.

Preliminary work on the extraction of the cheese samples was carried out using the AOAC extraction procedure for aflatoxin M₁ in dairy products (Sect. 26.090)⁷ based on a solvent mixture of acetone–water. Without further clean-up the crude

sample extract was directly loaded onto the immunoaffinity column. Although this simple approach was effective for detecting the presence of aflatoxin M_1 , the HPLC chromatograms showed more extraneous peaks than might reasonably have been expected and aflatoxin recoveries were highly variable. This suggested that in attempting to analyse very crude sample extracts containing a high solids content, some entrained or bound aflatoxin was being carried through the column, thus not being available for antibody binding. This would however only explain the variable recoveries and not the chromatographic interferences.

The approach finally adopted was based on the AOAC First Action Method (Sect 26.095)⁷ using a chloroform extraction and some liquid-liquid partitioning for a preliminary sample clean-up overcame both these initial difficulties. The method was found to take slightly longer than that for the analysis of aflatoxin M_1 in milk, but nevertheless represented a considerable time saving over the conventional approach to aflatoxin M_1 analysis in cheese. The recovery of the method for five replicate samples of one type of cheese spiked at 0.1 $\mu\text{g}/\text{kg}$ averaged 75% with a standard deviation of 10%. Analyses of eight other cheese types, which varied considerably both in fat and water content, gave recoveries after spiking at 0.1 $\mu\text{g}/\text{kg}$ in the range of 66 to 80%, but with one sample giving a recovery of only 55%. Calibration curves over the range 0 to 8.0 $\mu\text{g}/\text{kg}$ were linear with a correlation coefficient of 0.9989. The limit of detection of the procedure was demonstrated to be 0.005 $\mu\text{g}/\text{kg}$ at a signal-to-noise ratio of 5:1, by spiking a series of cheese samples with aflatoxin M_1 in the range 0.005 to 0.05 $\mu\text{g}/\text{kg}$.

In view of the large compositional differences in cheese types and the potential presence of interferences that might arise from artificially moulded cheeses, the performance of the columns was evaluated with a number of different varieties of cheeses from different countries of origin. In all cases the presence of aflatoxin M_1 could be detected in the cheeses and the naturally occurring levels which are shown in Table I were found to range from < 0.01 to 0.08 $\mu\text{g}/\text{kg}$. The HPLC chromatograms were in all cases essentially clean and no significant differences in chromatography were detected between the different cheese types. A typical chromatogram for a naturally

TABLE I

RECOVERIES AND RESULTS OF SMALL SURVEY OF AFLATOXIN M_1 IN RETAIL SAMPLES OF CHEESE

<i>Cheese type</i>	<i>Country of origin</i>	<i>Aflatoxin M_1 ($\mu\text{g}/\text{kg}$)</i>	<i>Recovery (%) (at 0.1 $\mu\text{g}/\text{kg}$ spike)</i>
Danish Blue	Denmark	0.03	75
Tilister	F.R.G.	0.08	72
Emmenthal	Switzerland	<0.01	55
Gouda	The Netherlands	0.05	80
El Mancho	Spain	<0.01	—
Gorgonzola	Italy	0.04	70
Gjetust	Norway	0.04	80
Jarlesberg	Norway	<0.01	80
Raclett	Switzerland	0.02	66
Red Leicester	U.K.	0.01	—
Cheddar	U.K.	0.04	—

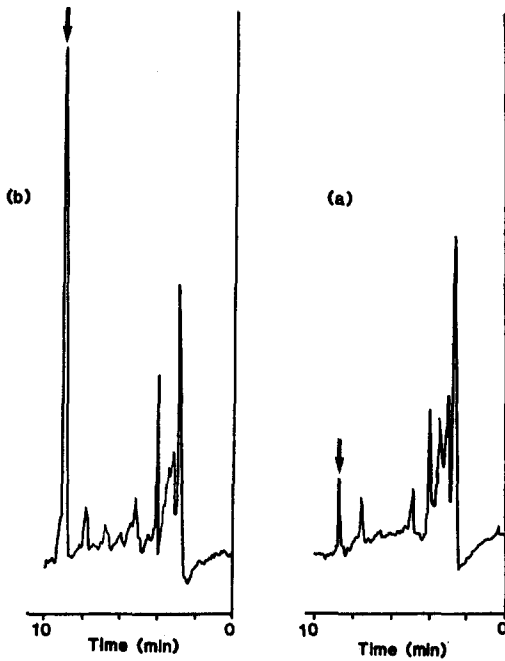


Fig. 1. HPLC of samples of cheese naturally contaminated with $0.02 \mu\text{g}/\text{kg}$ aflatoxin M_1 (a) and spiked with an additional $0.1 \mu\text{g}/\text{kg}$ (b). Detection: fluorescence at 364 nm excitation and 434 nm emission. Column: Spherisorb ODS 1 with a mobile phase of water-acetonitrile-methanol operated at 0.7 ml/min.

contaminated cheese is shown in Fig. 1 illustrating the presence of low levels of aflatoxin M_1 and the effect of spiking with an additional $0.1 \mu\text{g}/\text{kg}$. Confirmation of the presence of aflatoxin M_1 was carried out by reacting half the final extract from the cheese with trifluoroacetic acid leading to the loss of the M_1 peak in the chromatogram at a retention time of 8.8 min and the appearance of a new derivative peak at a retention time of 6.4 min.

CONCLUSION

An extension of use of affinity columns for aflatoxin M_1 for the analysis of cheese has been demonstrated as being effective with considerable time-saving over a more lengthy sample preparation. Further work is required to evaluate the potential application of these columns to other sample matrices such as eggs and animal tissue, and to assessing their potential for cross-reactivity with other aflatoxin metabolites with close structural similarities.

REFERENCES

- 1 R. E. Brackett and E. H. Marth, *J. Food Protect.*, 45 (1982) 549.
- 2 M. W. Trucksess and S. W. Page, *J. Food Protect.*, 49 (1986) 632.
- 3 G. Piva, A. Pietri, L. Galazzi and O. Curto, *Food Addit. Contam.*, 5 (1988) 133.

- 4 K. Hisada, H. Terada, K. Yamamoto, H. Tsubouchi and Y. Sakabe, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 601.
- 5 J. P. Bijl, C. H. van Peteghem and D. A. Dekeyser, *J. Assoc. Off. Anal. Chem.*, 70 (1987) 472.
- 6 D. N. Mortimer, J. Gilbert and M. J. Shepherd, *J. Chromatogr.*, 407 (1987) 393.
- 7 S. Williams (Editor), *Official Methods of Analysis*, Association of Official Analytical Chemists, Washington, DC, 14th ed., 1984, Nos. 26.090–26.095.