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Automated determination of aflatoxin B_1 in cattle feed by two-column solid-phase extraction with on-line high-performance liquid chromatography

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ABSTRACT

A method has been developed for the determination of aflatoxin B_1 in cattle feed which is an automated version of the method which was ring-tested by the European Community. The method uses a two-column clean-up with intermediate solvent evaporation and is coupled on-line to a high-performance liquid chromatographic system. The automated and manual methods are compared with regard to repeatability, accuracy and recovery of the analyte. Both methods have comparable repeatability at a concentration of 5 μg/kg of aflatoxin B_1; however, due to the lower recovery of the automated method, it differs significantly from the manual method with respect to accuracy. Nevertheless, the method may be used for screening purposes due to its good reproducibility.

INTRODUCTION

The presence of aflatoxin B_1 in feed stuffs for dairy cows may give rise to the occurrence of its metabolite, aflatoxin M_1, in their milk. As about 2% of the ingested aflatoxin B_1 is excreted in the milk as the suspected carcinogen aflatoxin M_1, high levels of aflatoxin B_1 in cattle feed pose a threat to human health. For this reason there is an official tolerance level for aflatoxin B_1 in cattle feed in the European Community (EC) of 10 μg/kg. However, this tolerance level does not result in a low enough concentration of aflatoxin M_1 in the milk produced. Therefore, in The Netherlands, manufacturers of cattle feed have agreed to attempt to have less than 5 μg/kg in their products.

For the control of this level a method, which was recently ring-tested by the EC [1], will become the official method. This method uses a two-column solid-phase extraction (SPE) with Florisil and RP-C_{18} cartridges [2,3]. Initially the extract is applied to a Florisil cartridge. Following elution, the solvent is evaporated and the residue is redissolved. This extract is applied to the RP-C_{18} cartridge. The resulting extracts can be analysed by high-performance liquid chromatography (HPLC) with post-column derivatisation [2–4]. However, this method is laborious and time-consuming and therefore does not have a very high sample turnover.

In this paper an automated method of analysis is described for aflatoxin B_1 in cattle feed based on the same principles as the EC ring-tested method.

EXPERIMENTAL

The automated clean-up was performed using an ASPEC system (Gilson, Villiers le Bel, France) with a 100-mg disposable extraction column (DEC), which was equipped with a module for on-line evaporation (Meyvis, Bergen op Zoom, Netherlands). The evaporation module consisted of a thermostated container for the collection vessels and a three-way valve to supply nitrogen via the ASPEC needle. The standard software of the ASPEC system is suitable for the application of a straightforward clean-up using one SPE column. The software necessary to perform a more complicated two-column clean up was developed at the RIKILT and is available from the authors or Meyvis & Co. The ASPEC system was equipped with an extra solvent rack to contain the washing and eluting solvents.

Florisil (100 mg) and RP-C_{18} (50 mg) DECs were obtained from Analytichem (Harbor City, CA, USA).

The ASPEC system was coupled on-line to an HPLC system consisting of a Waters 590 pump (Waters, Milford, MA, USA), a 200 × 3.0 mm Chromsphere RP-C_{18} column (Chrompack, Middelburg, Netherlands), a 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). The excitation wavelength was 369 nm and emission was measured at 422 nm.

An aflatoxin B_1 standard solution in chloroform (25 μg/ml) was obtained from the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, Netherlands). Calibration standards were prepared by dilution of this standard solution to obtain solutions containing 0.4, 0.8, 1.6 and 2.4 mg/ml, corresponding to 100, 200, 400 and 600 pg of aflatoxin B_1 in the injected volume, respectively. Aflatoxicol was used as an internal standard to determine the relative retention time; it was not used for quantification.

All reagents were purchased from Merck (Darmstadt, Germany) and were of analytical-reagent grade. Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

The HPLC eluent was a mixture of 1300 ml of water, 700 ml of methanol and 400 ml of acetonitrile to which were added 286 mg of potassium bromide and 152 μl of 85% nitric acid.

Samples were prepared as described elsewhere [2] to obtain sample extracts in chloroform, corresponding to 0.2 g of product per ml of extract. Blank samples were spiked with aflatoxin B_1, resulting in sample extracts corresponding to 5 and 10 μg/kg. Development and testing of the method was mainly carried out using a standard solution of aflatoxin B_1 containing 1 ng/ml, which corresponds to 5 μg/kg for a true sample extract when 100% recovery of the analyte is attained.

RESULTS AND DISCUSSION

To achieve automation of the analysis of aflatoxin B_1 using an ASPEC system, the manual method [2] had to be scaled down by approximately a factor of 10. To achieve this, a 100-mg Florisil-containing DEC and a DEC containing 50 mg RP-C_{18} were used. All volumes were decreased by a factor of 10. Scaling down is necessary because the elution volume is limited to 3.5 ml when using an ASPEC instrument. Scaling down almost always makes the procedure more critical and is not always a linear process. Therefore the elution profiles of aflatoxin B_1 had to be determined and possible breakthrough and other recovery losses had to be investigated for the redesigned procedure.

Figs. 1 and 2 show the elution profile of aflatoxin B_1 as a function of eluent composition on the Florisil and RP-C_{18} DEC respectively, obtained by fractionating the effluent of the DECs into nine 350-μl portions. It can be seen that the elution of aflatoxin B_1 from the Florisil DEC shows severe tailing. This cannot be significantly decreased by increasing the eluent strength (Fig. 1). Therefore a mixture of 5% water in acetone was used as the eluent in all experiments. However, this choice is more or less arbitrary as there is no significant effect from the eluent composition. Elution profiles on the RP-C_{18} DEC show a significant influence of the eluent composition. To achieve the maximum recovery within the allowable elution volume of 3.5 ml, the acetone content had to be increased, compared with the manual method, from 15 to 20%.

Unfortunately, the overall recovery was poor. Further investigation indicated that the Florisil clean-up gave rise to an approximate 50% loss of

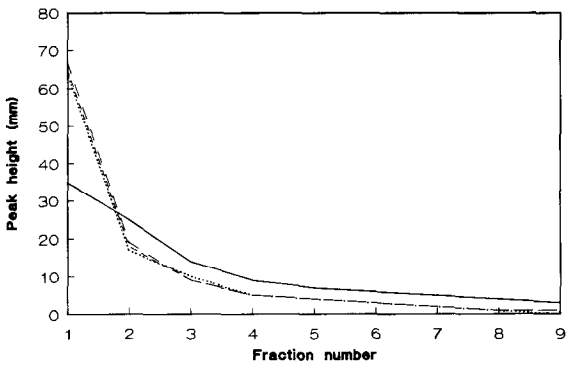

Fig. 1. Fraction number and peak height of aflatoxin B_1 chromatographed on the Florisil column with eluents of various water–acetone compositions: (—) 2:98; (- - -) 3:97; (– – –) 4:96); (. . . . .) 5:95. The effluent from the Florisil column was collected in 350-μl portions.

the analyte. The subsequent RP-C_{18} clean-up adds another 20% loss of the remaining analyte, resulting in an overall recovery of approximately 40% (Table I). It is obvious that the recovery needs improvement as recovery values of 80–90% can easily be achieved using the manual method. The loss of aflatoxin B_1 from the Florisil clean-up was studied more closely.

No breakthrough of the analyte is observed during the application of the sample, nor during the washing of the Florisil DEC. Repeated elution of the Florisil DEC did not show any additional aflatoxin B_1 in the effluent. Therefore the low recovery on the Florisil clean-up is probably due to irreversible adsorption of the aflatoxin B_1 onto the Florisil, onto the wall of the DEC, or onto the frits of the DEC. It has not so far been possible to increase the recovery of the analyte from the Florisil clean-up.

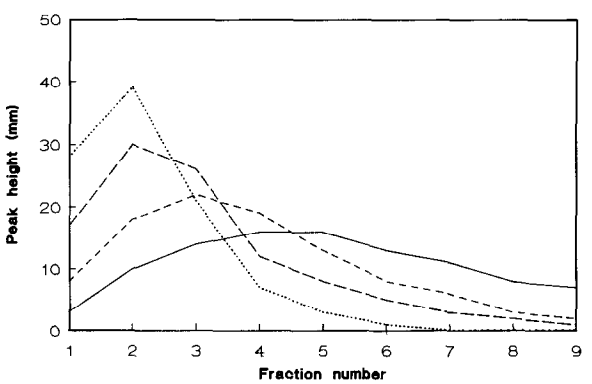

Fig. 2. Fraction number and peak height of aflatoxin B_1 chromatographed on the RP-C_{18} column with eluents of various water–acetone compositions: (—) 85:15; (- - -) 82.5:17.5; (– – –) 80:20; (. . . . .) 77.5:22.5. The effluent from the RP-C_{18} column was collected in 350-μl portions.

TABLE I

MEAN RECOVERIES AND CORRESPONDING COEFFICIENTS OF VARIATION FOR THE AFLATOXIN B_1 STANDARD ON THE SEPARATE FLORISIL AND RP-C_{18} COLUMNS AND FOR THE OVERALL PROCEDURE USING BOTH COLUMNS

Column	Mean recovery (%)	C.V. (%)	n
Florisil	50	9.2	10
RP-C_{18}	81	1.4	10
Overall procedure	44	12	20

The automated solvent evaporation may be another cause of the low recovery. Manual solvent evaporation and the subsequent redissolution of the residue did not cause losses of aflatoxin B_1. The automated solvent evaporation, however, is a time-based process which can easily be influenced by environmental conditions. Therefore it is a critical step in the analytical procedure.

Low recovery obviously results in low results of the analysis and affects the limit of determination (LOD). Nevertheless, because the recovery percentage is constant, the losses can be taken into account and the method can still be used for screening purposes, although a higher recovery is preferable.

The repeatability is comparable with that of the manual method, with a coefficient of variation (C.V.) of 12% for the overall procedure ($n=20$). Again, the Florisil clean-up shows the largest fluctuations (Table I), whereas the RP-C_{18} clean-up is very reproducible. The replicate analysis of a spiked sample (5 μg/kg aflatoxin B_1) showed a C.V. which was remarkably low compared with that obtained with standard aflatoxin solutions (C.V. = 3.0%; n = 5). Fig. 3 shows two chromatograms of samples to which aflatoxin B_1 had been added, corresponding to 5 and 10 μg/kg, respectively, prepared and analysed using the ASPEC–HPLC combination. The sample clean-up is good. The chromatograms are very similar to those obtained with the manual

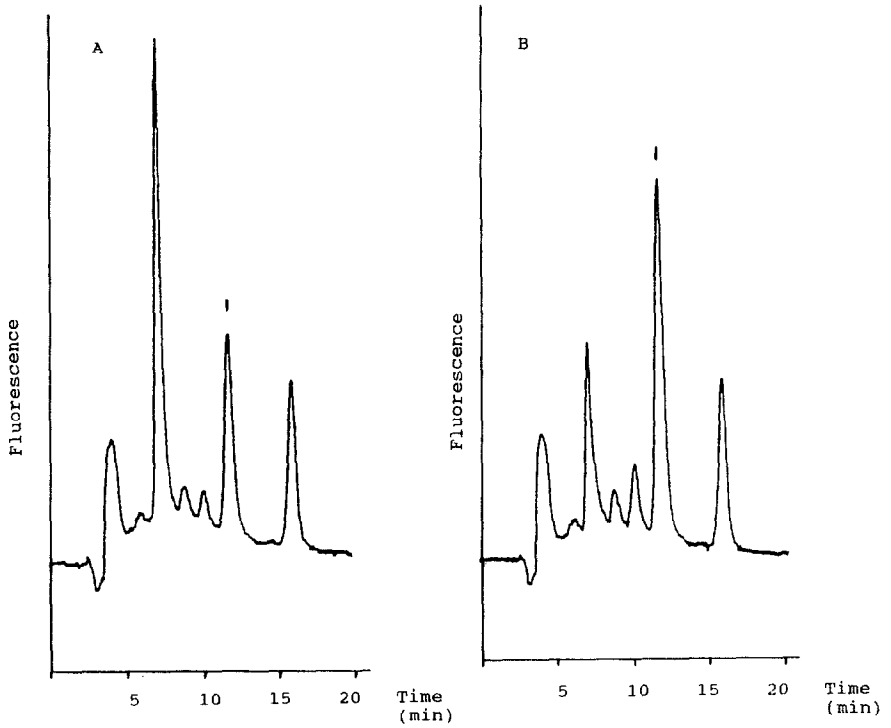

Fig. 3. Chromatograms of samples to which (A) 5 and (B) 10 μg/kg aflatoxin B_1 had been added. The last eluted peak is the internal standard aflatoxicol.

method. The LOD in spiked samples is approximately 2 μg/kg even when the recovery is as low as 40%.

The developed system is capable of calibrating the HPLC system with up to six calibrants. Up to 36 samples can be prepared and analysed in 24 h in unattended operation. Recalibration after ten samples is included in the automated protocol.

CONCLUSIONS

Automation of the method of analysis for aflatoxin B_1 which has been ring tested by the EC has been achieved. The recovery of the analyte is low but reproducible. This low recovery is probably due to losses during the Florisil clean-up procedure. With regard to repeatability and efficiency, the automated method is comparable with the manual method. Until there is an improvement in the percentage recovery, the developed method may only be used for screening purposes, with a LOD of approximately 2 μg/kg, taking the low but reproducible recovery into account. Its high sample turnover makes it useful in spite of the important drawback of incomplete recovery compared with the manual method.

The ASPEC software developed works well in the two-column clean-up with unattended intermediate solvent evaporation. With only minor adjustments this software can be used for other two-column clean-up procedures with or without solvent evaporation.

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REFERENCES

1 H. P. van Egmond, S. H. Heisterkamp and W. E. Paulsch, *Food Addit. Contam.*, 8 (1991) 17–29.
2 W. E. Paulsch, E. A. Sizoo and H. P. van Egmond, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 957–961.
3 W. A. Traag, J. M. P. van Trijp, L. G. M. Th. Tuinstra and W. Th. Kok, *J. Chromatogr.*, 396 (1987) 389–394.
4 W. Th. Kok, Th. C. H. van Neer, W. A. Traag and L. G. M. Th. Tuinstra, *J. Chromatogr.*, 367 (1986) 231–236.