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Note

Sample clean-up and post-column derivatization for the determination of aflatoxin B1 in feedstuffs by liquid chromatography

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Contamination of cattle feed with aflatoxin B1 gives rise to a corresponding amount of aflatoxin M1 in milk. In Switzerland a limit for aflatoxin M1 was set at 0.05 μg/kg and a similar limit is presently under discussion in The Netherlands. Since the carry-over factor of aflatoxin B1 from feed to milk is 1–2%[1–3], the European Community (EC) has set a residue limit of 10 μg/kg for cattle feed.

It is desirable to have a limit of detection well below the tolerance, so that the limit of detection for cattle feed has to be 1–2 μg/kg. As cattle feed contains many different ingredients, the maximum allowable aflatoxin B1 content for these ingredients should also be set. In The Netherlands the tolerance for ingredients will be 200 μg/kg. So a limit of detection of about 20 μg/kg is necessary.

In a previous paper[4] we described a method for the determination of aflatoxin B1 in cattle feed, which has been in routine use in our laboratory since 1982. Cattle feed samples were purified by extraction with chloroform in the presence of water and Celite and by thin-layer chromatography (TLC). The determination of aflatoxin B1 was performed by high-performance liquid chromatography (HPLC) with fluorescence detection, and post-column derivatization with iodine at 70°C. This derivatization method was later optimized by Shepherd and Gilbert[5]. Although the method was satisfactory in respect of the limit of detection, there were several reasons for upgrading both the sample treatment and the derivatization procedure.

Concerning the sample treatment, the TLC clean-up procedure must be performed carefully in order to obtain reproducible results. In 1985 about 80 reference samples were analysed. From the results obtained it was concluded that the repeatability under routine circumstances had to be improved. At the Institute for Public Health and Environmental Hygiene (Bilthoven, The Netherlands) a clean-up procedure based on solid-phase extraction was developed[6]. After extraction with chloroform, a first clean-up is performed using a disposable mini-column filled with Florisil, and a further clean-up is achieved with a C_{18} mini-column. The final extract in aqueous acetone is analysed by HPLC.

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Concerning derivatization, the method had two drawbacks. First, the iodine reagent solution is not stable and has to be prepared freshly every day. Secondly, the post-column system requires an (expensive) pulse-less pump and a thermostating oven. Recently, a post-column derivatization method for the fluorescence detection of phenothiazines was developed, using on-line electrochemically generated bromine[7]. We have studied a similar system for the derivatization of aflatoxins[8]. It appeared that with bromine as the reagent the limits of detection were the same as with (added) iodine as the reagent. Only simple hardware was needed and unstable reagents were avoided.

In this paper we describe a study on the suitability of the new sample clean-up and derivatization method for the routine determination of aflatoxin B1 in feed stuffs. Since the demands on the detection limit for ingredients are less severe, the possibility of doing without the clean-up for these samples was studied.

EXPERIMENTAL

Apparatus

The HPLC equipment comprised two coupled cartridge columns (Chrompack, Middelburg, The Netherlands) packed with LiChrosorb RP-18 (particle size 5 μm), each 100 mm × 3 mm, an ACS 750/03 HPLC pump (Applied Chromatography Systems, Luton, U.K.) providing a flow-rate of 0.5 ml/min and a 420 fluorescence detector (excitation at 360 nm, emission at >420 nm) (Waters, Milford, MA, U.S.A.). Sample injection was done with a WISP (Waters).

For the iodine derivatization, a zero dead volume Tee (Valco, Houston, TX, U.S.A.) was used to combine the saturated aqueous iodine solution (flow-rate 0.4 ml/min) with the column effluent. The reaction coil with a hold-up time of about 40 s was kept at 70°C.

For the electrochemical bromine production a Kobra cell[9] was used, obtained from the Chemistry Department of the Free University (Amsterdam, The Netherlands). The current was delivered by a variable d.c. power supply with a 100 kΩ resistance in series with the cell. A reaction capillary, providing a reaction time of 4 s at a flow-rate of 0.5 ml/min, was used.

Materials

All reagents used were of analytical grade purity, so that under the given analytical conditions no interference with aflatoxin B1 occurred. The saturated iodine solution was prepared by adding 1 g of iodine to 200 ml water, mixing for at least 15 min and filtering through a Millipore filter (pore size 0.45 μm). The mobile phase was water–methanol–acetonitrile (13:7:4). For the bromine derivatization, 1 m*M* potassium bromide and 1 m*M* nitric acid were added to the mobile phase. Aflatoxin B1 standards for HPLC injection were prepared by evaporating the stock solution to dryness (1 μg/ml in chloroform) and redissolving in acetone–water (15:85).

Methods

Two different procedures were used for the extraction and purification of aflatoxin B1 from cattle feed. The first, based on clean-up by TLC, has been described[4].

In the second procedure, 25 g of the ground sample were extracted with 125

ml chloroform in the presence of 12.5 ml water and 12.5 g Celite (Johns-Manville, Denver, CO, U.S.A.). After filtration through a fluted filter-paper, 50 ml of the filtrate were passed through a Sep-Pak Florisil mini-column (Waters). The column was rinsed with 5 ml chloroform followed by 20 ml methanol. Aflatoxins were eluted with 15 ml of acetone–water (90:10). The acetone was removed using a rotary evaporator and 20 ml of water were added. This solution was passed through a Sep-Pak C_{18} mini-column (Waters). The column was eluted with 50 ml acetone–water (15:85). A 250-μl volume of the eluate was injected on the HPLC column.

For ingredients in cattle feed, 1.0 ml of the filtered chloroform extracts was evaporated to dryness, and the residue was dissolved in 5.0 ml aceton–water (15:85). A 250-μl volume was injected on the HPLC column.

RESULTS AND DISCUSSION

The proposed clean-up procedure based on solid-phase extraction[6] was tested using standard solutions of aflatoxin B1 in chloroform. On the Florisil mini-column, aflatoxin B1 is totally retained when chloroform and methanol are used as eluent. With 15 ml acetone–water (90:10), aflatoxin B1 is completely eluted.

Since the clean-up on reversed-phase material was an apparently critical step it was studied in greater detail. In Fig. 1 the elution pattern of aflatoxin B1 is presented. It is seen that aflatoxin B1 is completely eluted in the first 40 ml, so a volume of 50 ml acetone–water (15:85) will give quantitative results.

The long-term reproducibility of the solid-phase extraction was compared to that of the TLC method using spiked cattle feed samples. The results given in Table I demonstrate the greater reproducibility of the new method. With the TLC procedure a skilled technician obtained a lower coefficient of variation than an inexperienced one. Probably the whole manipulation involving the TLC clean-up is responsible for these problems. With the solid-phase extraction no such trend was noted; the method was also used successfully by an inexperienced technician.

As was shown before[8], the simplified derivatization with bromine gives the same detection limit as the iodine method. However, since the reaction of bromine

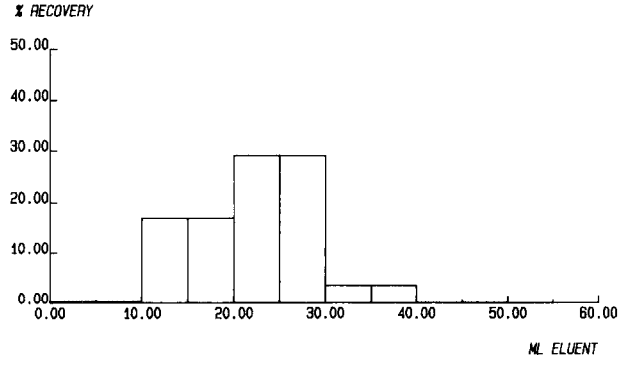

Fig. 1. Elution pattern (% recovery per 5 ml eluent) of aflatoxin B1 from C_{18} Sep-Pak. For other experimental conditions, see text.

TABLE I

RECOVERY OF AFLATOXIN B1 FROM CATTLE FEED SPIKED WITH 40 μg/kg ACCORDING TO TWO DIFFERENT CLEAN-UP METHODS

Clean-up method	*Recovery (%, mean ± S.D.)*	*Range (%)*	*Number of analyses*
TLC	96 ± 16	76–148	17
Solid-phase extraction	90 ± 7	77–103	25

TABLE II

INFLUENCE OF THE HARDWARE AND/OR EXTRACT COMPOSITION ON PEAK WIDTH

System	*Final extract composition*	*Injection volume (μl)*	*Peak width at half-height (s)*
Column directly coupled to detector	Methanol	20	5.6
Column coupled via iodine PCDS*	Methanol	20	7.0
Column coupled via Kobra	Methanol	20	6.0
Column coupled via Kobra	Acetone–water (15:85)	250	4.4

* PCDS = Post-column derivatization system.

TABLE III

COMPARISON OF AFLATOXIN B1 CONTENT (μg/kg) OF CATTLE FEED DETERMINED WITH IODINE OR BROMINE DERIVATIZATION

Recovery 1 = blank chemicals spiked with 40 μg/kg aflatoxin B1. Recovery 2 and 3: cattle feed spiked with 40 μg/kg aflatoxin B1.

Sample	*Iodine derivatization*	*Bromine derivatization*
Recovery 1	40.2	43.4
Recovery 2	44.4	42.1
Recovery 3	46.6	43.5
1	10.6	9.3
2	8.3	8.2
3	27.4	32.0
4	<1	1.9
5	<1	<1
6	<1	<1
7	<1	<1

TABLE IV

METHOD EVALUATION BY ANALYSIS OF REFERENCE SAMPLES

Method	*Ref. 4* *Sample A*	*This work* *Sample B*
Period (months)	12	9
Number of analyses	18	27
Aflatoxin B1 found (μg/kg) mean	11.2	8.9
Standard deviation (μg/kg)	3.9	1.5
Relative standard deviation (%)	35	17

is much faster, the reaction capillary could be shortened. The positive effect on band broadening is shown in Table II. A further reduction in peak width could be obtained with the new sample clean-up procedure. The final extract is dissolved in aqueous acetone, which causes a compression of the injection volume on the top of the column.

In order to compare the results obtained by HPLC and iodine derivatization with those obtained by HPLC and bromine derivatization, seven samples, two spiked samples and one spiked blank were extracted and subjected to clean-up as described[4]. The final extract in methanol was divided into two equal portions which were both analysed with the two derivatization methods (Table III). The agreement is satisfactory.

Also twelve ingredients, palm kernel, pollard, rape seed, niger-seed, cottonseed, citrus pulp, copra, tapioca, soya bean, groundnut, rice bran, corn gluten, were analysed with the two derivatization methods, but without any clean-up. It should be noted that these ingredients had already been defatted by the supplier. An aflatoxin B1 content above 50 μg/kg was not found in any of the samples. The limit of detection for both derivatization methods is about 10 μg/kg. Derivatization with bromine gave somewhat higher contents.

The differences between duplicate measurements both for the bromine derivatization and for the iodine derivatization are such that the method without clean-up can be used only for screening purposes. More detailed study of the individual ingredients will be necessary.

A final test of the suitability of the new method for routine analysis of cattle feed was made by repeated determination of reference samples. From a large batch of naturally contaminated cattle feeds, samples were drawn and given to a technician for analysis at regular intervals (without prior knowledge of the technician of the origin of the sample). The results are given in Table IV. It is clear that with the new method the relative standard deviation is halved.

CONCLUSIONS

A post-column procedure in which bromine is generated electrochemically in the column effluent is superior to post-column derivatization with iodine for the following reasons: (i) the equipment is less expensive; (ii) the installation, operation and maintenance is easier; (iii) in the case of citrus fruit the method is more selective; citrus peaks are absent or less intense[8].

Sample treatment based on solid-phase extraction is very suitable for routine analysis of aflatoxin B1 in cattle feed. Critical steps like evaporation to dryness are avoided. The repeatability in combination with bromine derivatization is excellent. A coefficient of variation half that obtained with the TLC method is feasible. The method is not time-consuming; 20–30 samples can be analyzed daily. For feed ingredients the method can be used without sample clean-up for screening purposes.

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