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Note

Determination of aflatoxins in cattle feed by liquid chromatography and post-column derivatization with electrochemically generated bromine

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Contamination of milk with aflatoxin (Af) M_1 can be caused by the contamination of cattle feed with AfB_1. The proposed tolerance for AfM_1 is 0.05 μg/kg in milk. Since the carry-over factor for AfB_1 from feed to milk is 1–2% (*e.g.*, ref. 1), the maximum amount of AfB_1 allowed in cattle feed in the European Community is set at 10 μg/kg. Therefore, a method for the determination of aflatoxins in cattle feed should be able to detect concentrations down to 1 μg/kg (1 ppb).

A method often used for the analysis of aflatoxins is high-performance liquid chromatography (HPLC) with fluorescence detection. Since the native fluorescence of AfB_1 is not high enough to reach the required detection limit, a derivatization step is often applied. Two derivatization reagents have been proposed. The first is applied in a pre-column procedure. AfB_1 is converted into the more highly fluorescent AfB_{2a} by acid hydrolysis (Fig. 1). With trifluoroacetic acid (TFA) the reaction takes 30 min at 50°C (ref. 2). In the second derivatization reaction iodine is the reagent. This

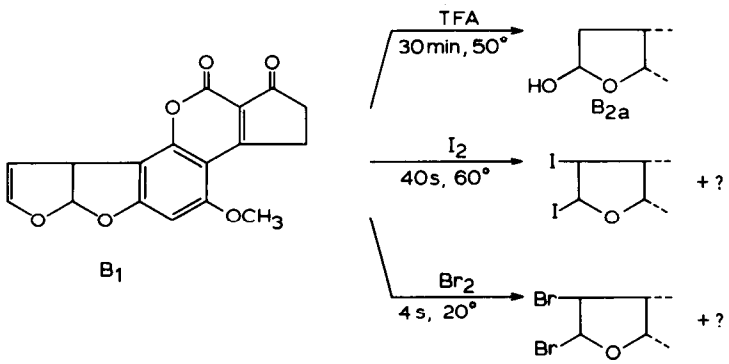

Fig. 1. Derivatization of aflatoxin B_1.

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reaction has been carried out before the separation (at 100°C), but more than one product is formed[3]. Therefore, a post-column reaction procedure is to be preferred. Tuinstra and Haasnoot[4] developed a method for the determination of AfB_1 in cattle feed based on the increase in fluorescence after the reaction with iodine. A saturated solution of iodine in water was mixed with the column effluent, and the reaction was carried out in a reaction capillary with a hold-up time of 40 s thermostatted at 60°C. The detection limit was 1 ppb.

Although the method was thus satisfactory in respect to sensitivity, there were two reasons to search for an alternative derivatization. (i) The iodine reagent solution is not stable and has to be prepared freshly every day. Day-to-day reproducibility is insufficient, so that frequent calibration is necessary. (ii) The post-column system requires an expensive pulseless pump and a thermostatting oven.

We have studied the fluorescence-signal enhancement of aflatoxins by derivatization with on-line, electrochemically generated bromine. Bromine can be produced from bromide present in the mobile phase, in a post-column electrochemical cell. The reagent concentration is controlled by the generating current. A similar system and the same set-up have been used in the determination of phenothiazines in human plasma[5]. The performance of this technique is compared with the iodine derivatization.

EXPERIMENTAL

Apparatus

The chromatographic equipment consisted of an ACS HPLC-pump, delivering a flow-rate of 0.5 ml/min, a Valco injection valve with 50-μl and 250-μl loops, a WISP sample processor, two coupled Chrompack Cartridge columns, each 100 × 3 mm I.D., packed with LiChrosorb RP-18 (5 μm), and a Waters 420-EC fluorescence detector with a 360-nm excitation filter and a >420-nm emission filter. For the iodine derivatization, 0.4 ml/min of the reagent solution was added with a second HPLC pump, through a zero-dead-volume T-piece. The reaction coil with a hold-up time of 40 s, was thermostatted at 60°C.

For the electrochemical bromine production a KOBRA-cell was used[6]. This cell was manufactured in the Chemistry Department Workshop of the Free University (Amsterdam, The Netherlands) and is available on request. Reaction coils providing reaction times of 4, 8 and 24 s at a flow-rate of 0.5 ml/min were used. The current was delivered by a variable DC-supply, with a 100-kΩ resistance in series with the KOBRA-cell.

Reagents and solutions

All chemicals used were of analytical grade purity. The mobile phase was a mixture of water, methanol and acetonitrile (13:7:4). For the bromine derivatization 1 m*M* potassium bromide and 1 m*M* nitric acid were added. The iodine reagent was prepared by adding 1 g of iodine to 100 ml of water, mixing for 15 min and filtering. Aflatoxin standard solutions were prepared by diluting stock solutions in chloroform with methanol or acetone–water (15:85).

For the extraction and purification of aflatoxins from cattle feed the procedure developed by Tuinstra and Haasnoot[4] was followed. The amount of the final extract

obtained which was injected on the column corresponded to typically 50 mg fresh weight of the cattle feed.

RESULTS AND DISCUSSION

Batch experiments showed that the fluorescence intensity of an AfB_1 standard solution increased after the addition of a bromine solution, with excitation and emission maxima at 365 and 435 nm, respectively. However, on standing the fluorescence decreased again. It may be concluded that when an excess of bromine is present, the first reaction step with bromine is followed by a second step, which yields a low-fluorescent product. The first step is probably the bromination of the 8,9-double bond (see Fig. 1), the second step may be the bromination of the phloroglucinol nucleus. It is clear that a post-column derivatization with bromine should be optimized carefully with respect to reaction time and amount of bromine produced (or generating current).

Optimization was carried out with standard injections of aflatoxins B_1, B_2, G_1 and G_2 (each 0.3 ng/injection). The aflatoxins eluted well separated from each other in the order G_2, G_1, B_2 and B_1. Fluorescence intensities were measured by peak heights with reaction coils of 4, 8 and 24 s reaction time and with various generating currents. The results with the 8-s capillary are given in Fig. 2. For AfB_1 and AfG_1 a steep rise in the fluorescence intensity is seen when bromine is produced, and a slower decline of the signals when the generating current is further increased. Some decrease of fluorescence intensity is also observed for AfB_2 and AfG_2. With the other reaction coils the same trend was found. Owing to increased band-broadening the signals are somewhat lower with the 24-s capillary. Further studies were done with the 4-s capillary and a generating current of 80–85 mÅ. This is the current observed with a voltage of 10 V over the cell and the 100-kΩ resistance.

Fig. 3 gives calibration plots for the four aflatoxins. Within the range studied (0.05–0.30 ng) deviations from linearity are minimal. It is interesting to note that for AfB_2 and AfG_2, which do not react with bromine owing to the absence of the double bond (see Fig. 1), the sensitivity with the bromine system is improved by a factor of *ca.* 2 compared with the iodine system. This is caused by the fact that the column effluent is now not diluted with the reagent solution. On the other hand, the sensitivity for AfB_1 is unchanged. Obviously, the product of the bromine reaction is less fluorescent than that of the iodine reaction, or side-reactions are more important.

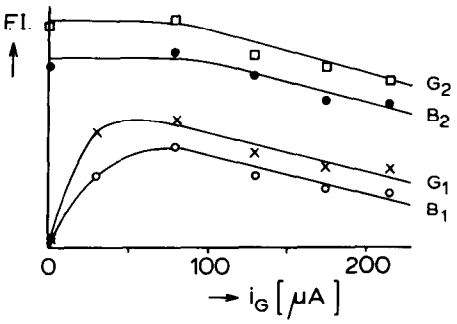

Fig. 2. Influence of the generating current on the fluorescence peak heights; reaction times, 8 s.

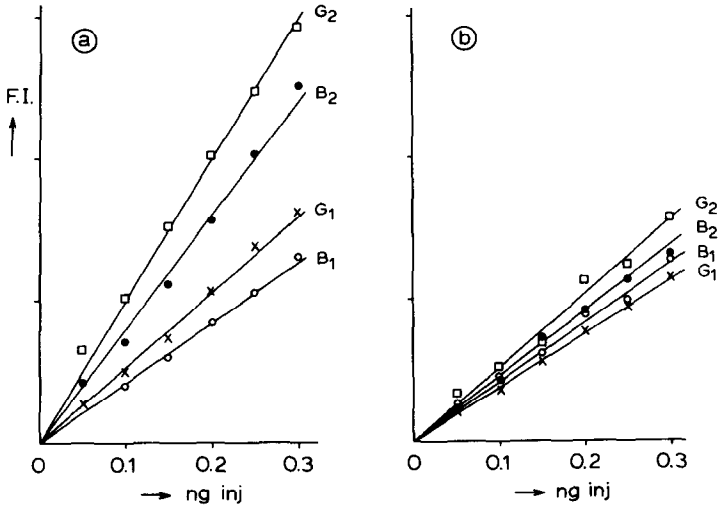

Fig. 3. Calibration plots. (a) Bromine derivatization; (b) iodine derivatization.

Table I lists the detection limits for standard solutions, without derivatization, with the iodine system and with the bromine system. The limits of detection observed correspond to 0.8 ppb of AfB_1 and AfG_1 and 0.4 ppb of AfB_2 and AfG_2 in cattle feed with the bromine derivatization.

The reproducibility was studied by repeated injection of a standard mixture of the four aflatoxins. Twenty consecutive injections were made, containing 0.25 ng of each aflatoxin, with a delay of 1 h between injections. The relative standard deviation of analysis was ± 1.3% for AfB_1 and AfG_1 and ± 1.2% for AfB_2 and AfG_2. During the 20-h measurement series the voltage drop over the reagent-production cell steadily increased from 1.7 to 3.6 V, and subsequently the current decreased from 83 to 64 μA. However, this had no significant influence on the sensitivity of detection. The original voltage drop was restored after flushing of the cell for a few minutes at zero current without injection of samples.

The replacement of the long reaction capillary, which is required for the iodine derivatization by a 4-s capillary on the one hand and the omission of the iodine

TABLE I

LIMITS OF DETECTION AFTER POST-COLUMN DERIVATIZATION

	Detection limit (ng)*			
	AfB_1	*AfG_1*	*AfB_2*	*AfG_2*
Underivatized	0.6	0.6	0.02	0.02
Iodine derivatization	0.04	0.04	0.04	0.03
Bromine derivatization	0.04	0.04	0.02	0.02

* Signal-to-noise ratio = 2.

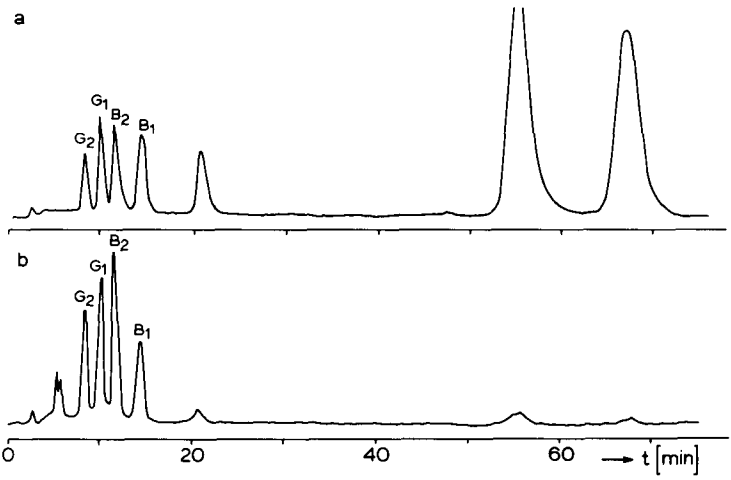

Fig. 4. Chromatograms of a (fortified) cattle-feed extract. (a) Iodine derivatization; (b) bromine derivatization.

solution flow on the other, resulted in an improvement of the resolution of the aflatoxin peaks. Peak widths were reduced by *ca.* 15%.

With the iodine derivatization procedure, in the chromatograms of cattle-feed samples, strong, late-eluting peaks are observed, especially when the cattle feed contained citrus pulp. Therefore, the total chromatographic run time is much longer than the retention time of AfB_1, which is *ca.* 15 min. By a lucky coincidence, these late-eluting peaks almost disappear when bromine is used as the reagent (see Fig. 4), and the run time can be reduced drastically.

For verification, we have compared the results obtained with the two post-column methods. Cattle-feed extracts were divided in two parts, and injected in both systems. As can be seen in Table II, the methods give comparable results.

The post-column procedure, in which bromine is generated electrochemically in the column effluent, is now being used in routine control of cattle-feed samples.

TABLE II

COMPARISON OF RESULTS OBTAINED WITH IODINE AND BROMINE DERIVATIZATION

Cattle-feed sample	*AfB_1 (ppb)*		*AfG_1 (ppb)*		*AfB_2 (ppb)*		*AfG_2 (ppb)*	
	Iodine	*Bromine*	*Iodine*	*Bromine*	*Iodine*	*Bromine*	*Iodine*	*Bromine*
A*	44	42	24	31	39	41	35	50
B*	47	44	25	35	40	42	38	48
C	11	9	N.d.**	1	1	3	N.d.**	2
D	8	8	5	6	2	2	1	1
E	27	32	2	1	8	8	7	7

* Fortified reference samples.

** N.d., below the detection limit.

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