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Determination of aflatoxins in food by use of an automatic work station

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ABSTRACT

An automated HPLC method with postcolumn derivatization is described for the determination of aflatoxins B_1, B_2, G_1 and G_2 after an immunoaffinity column clean-up. It can be used for the determination of aflatoxins in a variety of foodstuffs such as nuts, nut-like products (pistachios, almonds, etc.) and dried fruit. The aflatoxins are extracted with methanol–water, followed by a filtration step. Dilution of the extract, mixing, immunoaffinity column clean-up, elution of the aflatoxins and optional on-line HPLC are performed by an automatic work station (Zymark BenchMate). The subsequent HPLC analysis includes a postcolumn derivatization step with iodine solution and fluorimetric detection. The method compared well with manual techniques and another automated method.

INTRODUCTION

Aflatoxins are extremely toxic secondary metabolites of the mould fungi *Aspergillus flavus* and *A. parasiticus*. The amount of aflatoxins represents a quality criterion for food and feedstuffs. As they belong to the most carcinogenic substances, there are very low legal limits for their occurrence in food. In Germany these limits are 4 μg/kg for the sum of aflatoxins B_1, B_2, G_1 and G_2 and 2 μg/kg for B_1 as a single component.

A variety of methods for the determination of aflatoxins have been described. A survey of the literature and a comparison between thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) were given by Werner [1].

In our laboratory, until 1991 aflatoxin analyses were performed by TLC, following published procedures [2,3]. Another TLC method has also been represented [4].

Since 1989 we have used HPLC with iodine postcolumn derivatization and immunoaffinity columns for sample clean-up. These columns contain a gel suspension of monoclonal antibodies covalently attached to a solid support. The antibodies are specific for aflatoxins B_1, B_2, G_1 and G_2. Various Clean-up [1,5,6] and HPLC [1,5–8] techniques have been published. The method used in our laboratory resembles most closely that the Trucksess *et al.* [5], which has been adopted as official first action by the AOAC as an AOAC–IUPAC method.

With the aim of rationalizing the immunoaffinity column clean-up for HPLC, we looked for possibilities of automation. Zymark provided

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an automatic work station, called BenchMate, which offers the options of pipetting, adding various reagents, weighing with an analytical balance, mixing solutions, performance membrane filtration, solid-phase extraction (SPE) (1- and 3-ml SPE columns can be used) and HPLC injection [9]. BenchMate procedures for sample preparation can be easily created and modified by the user. Another commercially available automated sample preparation system, called ASPEC, can also be used for aflatoxin analysis [10] and was compared with BenchMate.

An aflatoxin application for BenchMate had already been developed [11]. The procedure was adjusted to the requirements of our laboratory. Further research led to a simplification of our originally used procedure.

This paper describes automated HPLC analysis with postcolumn derivatization for the aflatoxins B_1, B_2, G_1 and G_2 after an immunoaffinity column clean-up. It can be used for the determination of aflatoxins in a variety of foodstuffs such as nuts, nut-like products (pistachios, almonds, etc.) and dried fruit (*e.g.*, figs).

EXPERIMENTAL

Field of application

The automated method can be applied to nuts and nut-like samples (pistachios, almonds, apricot kernels, peanuts, peanut butter, etc.), with the exception of walnuts, and to dried fruit (*e.g.*, figs). For other foods and for feedstuffs, manual techniques are performed because the sample clean-up is more complicated.

Principle

The aflatoxins, which are readily soluble in polar organic solvents, are extracted with methanol–water in a laboratory mixer, followed by a filtration sep. Dilution of the extract, mixing, immunoaffinity column clean-up, elution of the aflatoxins and optional on-line HPLC are performed by the BenchMate automatic work station (Zymark). The subsequent HPLC analysis includes a postcolumn derivatization step with iodine solution. Detection is carried out by spectrofluorimetry.

Reagents

Solvents were methanol (pure; *e.g.*, Riedel-de Haën Cat. No. 24228), LC-grade methanol and distilled water. The extraction solvent was pure methanol–distilled water (7:3, v/v). The LC mobile phase was LC-grade methanol–distilled water (1:1, v/v).

Postcolumn reagent. A 500-mg amount of iodine was dissolved in 10 ml of methanol, 1 l of water was added, the mixture was stirred for at least 10 min and filtered through a 0.45-μm filter. Fresh reagent was prepared every 2 days.

Aflatoxin standards. (1) Mixed aflatoxin stock standard solution (Sigma), containing 5 μg/ml each of aflatoxin B_1 and G_1 and 1.5 μg/ml each of aflatoxin B_2 and G_2. The flask contents [5 ml in benzene–acetonitrile (98:2)] were diluted with methanol (LC grade) to 50.0 ml giving 500 ng/ml of aflatoxins B_1 and G_1 and 150 ng/ml of B_2 and G_2). The solution was stored at −18°C.

(2) Mixed aflatoxin standard for standard addition: 40.0 ml of stock standard solution were diluted with methanol to 100.0 ml, giving 200 ng/ml of aflatoxins B_1 and G_1 and 60 ng/ml of B_2 and G_2. The solution was stored at −18°C.

(3) HPLC standard: 1.0 ml of stock standard solution was diluted with LC mobile phase to 100.0 ml, giving 5 ng/ml of aflatoxins B_1 and G_1 and 1.5 ng/ml of B_2 and G_2). This standard was kept in the dark at room temperature and prepared fresh weekly.

Apparatus

A blender with a 500-ml blender jar and cover, 18.5-cm filter-papers, prefolded, 0.45-μm membrane filters, diameter 50 and 30 nm, and borosilicate test-tubes (100 × 16 mm I.D.) were used.

The immunoaffinity columns were 1-ml AFLAPREP columns (Rhône-Poulenc) or 3-ml EasiExtract columns (Biocode). The specifications given by the manufacturers are as follows. AFLAPREP: when 4.0 ng of total aflatoxins are applied, a minimum of 80% of aflatoxin B_1 and G_1 and a minimum of 70% of aflatoxin B_2 and G_2 are recovered. The capacity of a column is 200 ng total aflatoxin, *i.e.*, larger amounts loaded result in decreased recoveries. EasiExtract: a minimum of 5.0 μg of aflatoxin B_1 is bound

when 10.0 μg are applied in 50 ml of 5% (v/v) methanol in phosphate-buffered saline. Greater than 80% of each aflatoxin is recovered when 3.5 ng of aflatoxins B_1, B_2, G_1 and G_2 are applied in 175 ml of diluted peanut butter extract (equivalent to 1 ppb of each aflatoxin).

A BenchMate automatic work station was provided by Zymark. A Baker SPE 10 system was used for manual immunoaffinity column handling. An HPLC system with a fluorescence detector and a data acquisition system was used. The LC column was Spherisorb S5 ODS-2 (250 mm × 4 mm I.D.). The postcolumn derivatization system consisted of a second LC pump, a zero-dead-volume T-piece, and a 320 cm × 0.5 mm I.D. PTFE reaction coil. Two column ovens were used with a temperature control module, heated to 35 and 70°C.

Sampling

Examples are as follows. For pistachios, a laboratory sample of about 50 kg was taken by sampling 10% of the bags following the random principle. The whole sampling material was mixed and divided into five subsamples of 10 kg. Three 10-kg subsamples of pistachios were ground in a 50-l cutter and a portion of each was analysed as described below. For figs, *ca.* 30 kg of sample were mixed and homogenized in a cutter after addition of 20% of water. Test portions were taken from various locations in the fig paste.

Extraction

The manual sample preparation prior to the BenchMate procedure was as follows. A 50-g homogenized sample was weighed into the blender jar and 100 ml of methanol–water (7:3, v/v) were added. The mixture was blended at high speed for 2 min, then filtered through a prefolded filter-paper. Approximately 12 ml of the extract were filled into a BenchMate test-tube.

Immunoaffinity column clean-up

The sample tubes, each provided with an immunoaffinity column on their caps, were placed in rack 1 of the BenchMate. Racks 2 and 3 were provided with empty "process" tubes and "final" tubes. In the BenchMate procedure, an aliquot of sample extract was pipetted, diluted with phosphate-buffered saline, mixed by cycling and then passed through the immunoaffinity column at a flow-rate of 4.2 ml/min. After washing the column with 20 ml of water, the aflatoxins were eluted with 1.5 ml of methanol at a flow-rate of 4.2 ml/min. Finally, 1.5 ml of water were added and the test solution was vortex mixed.

HPLC determination

The injections for HPLC can be performed on-line by the BenchMate directly after every sample clean-up. For an accelerated BenchMate clean-up and greater flexibility, an autosampler can be used. The test solutions in the BenchMate final tubes then have to be transferred into autosampler vials.

The HPLC conditions were as follows: column, Spherisorb S5 ODS-2 (250 mm × 4 mm I.D.), tempered at 35°C; precolumn RP-18 (4 mm × 4 mm I.D.); eluent, methanol–water (1:1, v/v); flow-rate, 0.85 ml/min; injection volume, 200 μl; detection, fluorescence with excitation at 360 nm and emission at 450 nm; derivatization, postcolumn derivatization with saturated iodine solution in water, flow-rate 0.2 ml/min, reaction coil 3.2 m × 0.5 mm I.D., temperature 70°C.

Fig. 1 shows a typical chromatogram of a

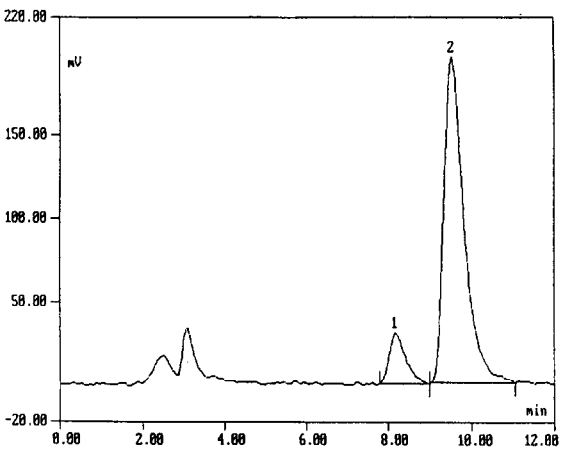

Fig. 1. Chromatogram of a naturally contaminated pistachio sample containing 8 μg/kg of aflatoxin B_1 and 0.5 μg/kg of aflatoxin B_2. Peaks: 1 = aflatoxin B_2; 2 = B_1.

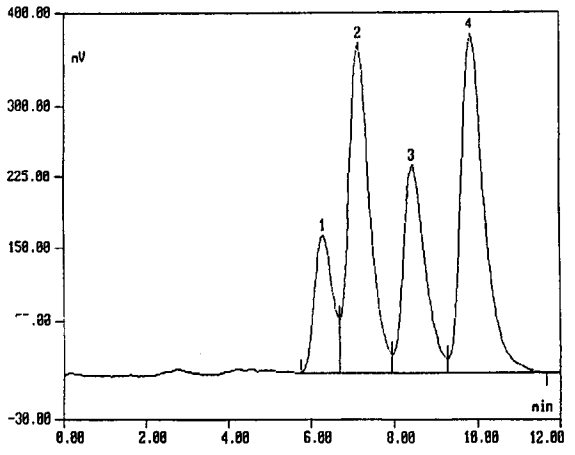

Fig. 2. Chromatogram of a standard solution containing 5 ng/ml each of aflatoxins B_1 and G_1 and 1.5 ng/ml each of aflatoxins B_2 and G_2. Peaks: 1 = aflatoxin G_2; 2 = G_1; 3 = B_2; 4 = B_1.

naturally contaminated pistachio sample with a content of 8 μg/kg of aflatoxin B_1 and 0.5 μg/kg of aflatoxin B_2, and Fig. 2 is a standard chromatogram for 5 ng/ml of aflatoxin B_1 and G_1 and 1.5 ng/ml of B_2 and G_2.

RESULTS AND DISCUSSION

For each sample matrix some recovery tests were carried out first by adding a mixed standard solution to the sample at the beginning of the preparation and processing the sample with and without standard addition. The amount of added standard was equivalent to 4 μg/kg of aflatoxin B_1 and G_1 and 1.2 μg/kg of aflatoxin B_2 and G_2. From the recovery results for spiked samples with low or no natural contamination, the repeatability of the method was calculated.

In a series of experiments, the same sample matrices as used for the BenchMate procedure were also processed manually in order to obtain comparative data about recoveries and repeatability. Because the volumes for the manual column clean-up are not as restricted as in the BenchMate procedure, the extracts to be treated manually were always diluted to a methanol concentration of 14% prior to immunoaffinity clean-up.

The following data (Table I) are based on the examination of 38 manually and 37 automatically

TABLE I

RECOVERIES AND RELATIVE STANDARD DEVIATIONS OF AFLATOXINS FOR NUT AND NUT-LIKE SAMPLES AFTER MANUAL (n = 38) AND AUTOMATIC (n = 37) CLEAN-UP WITH IMMUNOAFFINITY COLUMNS (AFLAPREP)

Clean-up	Parameter	Aflatoxin			
		B_1	B_2	G_1	G_2
Manual	Recovery (%)	73.6	55.7	78.2	32.1
	R.S.D. (%)	17.5	17.8	15.9	39.8
Automatic	Recovery (%)	74.5	64.2	83.7	34.7
	R.S.D. (%)	15.0	13.3	12.1	31.9

processed samples of pistachios, hazelnuts, brazil nuts, almonds, peanuts and apricot kernels by use of AFLAPREP columns. The recoveries obtained with the automated method are slightly better than those with the manual clean-up, although the methanol content of the automatically processed diluted extract (30%) is far higher than that of the diluted extract used for the manual method (14%).

The reason for better repeatability and higher recoveries is presumed to be the ability of the automatic work station to maintain slow and constant flow-rates for loading samples on to the immunoaffinity column and for the elution of the aflatoxins. Statistical data obtained in a collaborative trial of ten laboratories using HPLC with postcolumn derivatization have been reported [5]. The R.S.D. values for peanuts (and peanut butter in parentheses) were 16.4% (14.9%) for aflatoxin B_1, 23.1% (32.7%) for B_2, 6.2% (23.5%) for G_1 and 42.0% (79.1%) for G_2. These figures demonstrate the range of variation of results, which is normal for aflatoxin analysis. The repeatability obtained in our laboratory fits in the pattern of the published values.

We also carried out a study to compare AFLAPREP (Rhône-Poulenc) and EasiExtract (Biocode) aflatoxin immunoaffinity columns. Both types of column appear to be equally suitable for the clean-up of diluted pistachio sample extracts with a remaining methanol content of up to 15%. Higher methanol concen-

trations result is very low recoveries of aflatoxin B_2 and especially G_2 with the AFLAPREP column and in decreased recoveries of all aflatoxins with the EasiExtract column.

Comparison of BenchMate and ASPEC

The method developed for aflatoxin analysis using BenchMate was compared with that reported [10] using an ASPEC system.

The manual preparation of the test sample following our method is simpler and less time consuming than in the other method [10]; the latter involves mixing twice with an Ultra Turrax and has two manual dilution steps, whereas the dilution of the extract is automated in the BenchMate procedure, guaranteeing very precise dispensing volumes. The ASPEC procedure [10] includes a preconditioning step for the immunoaffinity columns. We omitted such a step, finding no differences in the results after clean-up with and without preconditioning of the columns.

The time needed per sample (double injection of sample, standard injections included) using the BenchMate procedure is 39 min, which is equivalent to 37 samples per day. If HPLC injection is carried out off-line, the preparation takes 20 min per sample, which means a throughput of 70 samples per day. Up to 50 samples can be put in a BenchMate rack and be processed unattended within 32 h. The ASPEC, on the other hand, has a sample carousel with which only 20 samples can be processed in an unattended operation time of 11 h. The time per sample (single injection, standard injections included) is 33 min, only slightly shorter than with BenchMate and double injection.

An obvious advantage of the ASPEC system is the greater working volume of the sample tubes of 50 ml in comparison with 12 ml for the BenchMate. The BenchMate can partly compensate for this restriction by the possibility of diluting concentrated extracts, also repeatedly by means of chained procedures. Hence higher volumes, *e.g.*, 50 ml of diluted extract, could be passed through the immunoaffinity column, but the time needed per sample would increase greatly. Owing to the higher sample tube volume, the ASPEC system allows the clean-up of samples which have been extracted with acetonitrile–water mixtures because immunoaffinity columns withstand only very low concentrations of acetonitrile, so that acetonitrile extracts must be more diluted than methanolic extracts. The BenchMate, in contrast, to achieve sufficient sensitivity in aflatoxin analysis, is suitable only for the clean-up of methanolic extracts.

The sensitivities of both methods are expected to be in the same range, calculated from the amount of sample per injection, although with the other method no detection limits were published [10]. With the equipment used, the BenchMate procedure gives detection limits equivalent to about 0.1 μg/kg for aflatoxins B_1 and G_1 and about 0.03 μg/kg for aflatoxins B_2 and G_2.

Advantages of automatic sample preparation over the manual technique

The automatic sample preparation gives better repeatability (see Table I) and reproducibility because of the precise control of flow-rates (column loading, rinsing, eluting) and volumes. Unattended sample preparation and HPLC injection (*e.g.*, overnight), including injection of calibration standards, can be performed, so that up to 4 h of manpower per day are saved in comparison with the manual technique. The BenchMate performs a gravimetric control of every preparation step which can be viewed afterwards.

Problems and limitations of automatic sample preparation

Restricted volumes of the sample and process tubes result in limitations on the construction of individual sample clean-up methods. It is very time consuming to pass high volumes of diluted extract through the column by means of chained procedures. Only sample matrices from which the aflatoxins can be extracted by methanol–water mixtures are suitable for the automatic procedure. Other extraction solvents (containing acetone or acetonitrile) have to be further diluted with water in order not to disturb the immunoaffinity process.

With BenchMate, only one sample at a time is processed, whereas manually up to six samples can be loaded on columns simultaneously by means of a Baker SPE system.

If any error occurs, the robot stops until it is attended to, which causes time losses. In on-line HPLC, this also means energy and material losses because the HPLC system is not switched off.

During a run nothing can be changed. Unnecessary injections cannot be skipped as would be possible with an autosampler. With versions I and II of the BenchMate work station there is no possibility of viewing and controlling the running procedure. Owing to matrix effects not all types of samples can be processed automatically, *e.g.*, nutmegs.

CONCLUSIONS

Sample preparation by use of immunoaffinity columns and a BenchMate automatic work station followed by HPLC is a suitable method for the determination of aflatoxins B_1, B_2, G_1 and G_2 in certain foodstuffs (nuts and nut-like products and dried fruit). It offers a numbers of advantages over other methods: lower detection limits than TLC and ELISA; greater confidence in the results than with all other methods because of the higher repeatability; very efficient clean-up; unattended sample preparation and optional on-line HPLC injection; economy of manpower in comparison with the manual technique; no need for large volumes of hazardous solvents; determination of all four aflatoxins simultaneously and over a wide range of concentrations (in contrast to the ELISA method).

In conclusion, the proposed method for the determination of aflatoxins by use of an automatic work station can be recommended for laboratories performing routine aflatoxin analyses. If necessary it can be combined with the manual immunoaffinity clean-up for certain sample matrices.

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