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A Research Note

Application of ELISA to Retail Survey of Aflatoxin B_1 in Peanut Butter

BHANU P. RAM[1], L. PATRICK HART[2], RICHARD J. COLE[3] and JAMES J. PESTKA[1,4*]

Department of Food Science and Human Nutrition and Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824 and National Peanut Research Laboratory, United States Department of Agriculture, Agricultural Research Service, Dawson, Georgia 31742

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ABSTRACT

A simple procedure was devised for the routine screening of aflatoxin B_1 (AFB_1) in peanut butter using enzyme-linked immunosorbent assay (ELISA). Peanut butter samples (5 g) were artificially contaminated with AFB_1 and extracted by blending with 25 ml of 55% methanol and 10 ml of hexane. The extract was filtered and aqueous filtrate analyzed by a direct competitive ELISA. Recovery of AFB_1 added to peanut butter samples ranged from 85 to 112%, with an average inter-well coefficient of variation of 18.4%. The inter-assay coefficient of variation was 22.7%. Using this procedure, only 3 of 63 commercial samples of peanut butter had detectable levels (>5.0 μg/kg) of AFB_1.

Aflatoxin B_1 (AFB_1) is monitored in peanuts, peanut products and other agricultural commodities because of its potential carcinogenic effects (*11,17,21*). AFB_1 is commonly determined by the chloroform-benzene (CB) and Best Foods (BF) methods (*19*) that detect aflatoxin levels of 5 μg/kg or greater (*18*). These methods and recently reported high performance liquid chromatography (HPLC) procedures (*1,20*) require lengthy procedures for sample extraction and clean up before analysis. Immunochemical detection procedures are potential alternatives for the routine screening of AFB_1 and other mycotoxins (*4,6,7,13-16*). This report describes a simplified protocol for screening AFB_1 in peanut butter. Essential features of the protocol include: (a) blending the sample with 55% methanol and hexane according to the BF method (*19*), (b) filtering the blended sample, and (c) direct competitive enzyme-linked immunosorbent assay (ELISA) of the filtrate for AFB_1.

[1]*Department of Food Science and Human Nutrition.*
[2]*Department of Botany and Plant Pathology.*
[3]*National Peanut Research Laboratory.*

MATERIALS AND METHODS

Chemicals

Tween-20, bovine serum albumin (BSA), 2,2'-azino di-(3-ethylbenzthiazoline sulfonic acid (ABTS), N-hydroxysuccinimide, horseradish peroxidase type VI (HRP), and AFB_1 were purchased from Sigma Chemical Co., St. Louis, MO. Complete and incomplete Freund's adjuvant were obtained from Difco Laboratories, Detroit, MI. 1,3-Dicyclohexycarbodiimide (DCCD) was from Aldrich Chemical Co., Milwaukee, WI. Other chemicals used were analytical grade or better.

AFB_1 conjugate preparation

AFB_1 was converted to AFB_1-oxime by the method of Chu et al. (*3*). AFB_1-oxime (1 mg) was conjugated to 10 mg BSA or HRP in the presence of N-hydroxysuccinimide and DCCD by the procedure described by Kitagawa et al. (*12*). Conjugates were divided into appropriate volumes and stored frozen at -20°C.

Antiserum production

New Zealand white female rabbits (Bailey Rabbitry, Alto, MI) were immunized with AFB_1-BSA conjugate as described previously (*4*). Titers of sera were determined by ELISA (*7*). Positive antisera were pooled and antibodies purified by ammonium sulfate precipitation (*13*). Antibodies were lyophilized and stored at -20°C.

Sample extraction for ELISA

AFB_1-free peanut butter was prepared and absence of detectable AFB_1 was verified by HPLC (see below). Commercial peanut butter samples were purchased from local East Lansing supermarkets.

For analysis, a 5-g sample of peanut butter was blended with 25 ml of 55% methanol (vol/vol) and 10 ml of hexane (extractant) for 3 min at high speed in an Omni-Mixer (Ivan Sorvall, Norwalk, CT). The extract, representing a 1:5 dilution of peanut butter, was filtered through three layers of Whatman No. 1 paper and 0.5 to 1 ml of the aqueous filtrate was collected for ELISA.

Direct competitive ELISA of the sample filtrate

Antiserum (1:500) was dried onto wells of a high binding capacity NUNC microtiter plate (Vangard International, Neptune, NJ) under forced air (40°C). Each well was washed three times with 300 μl of washing buffer [0.01 M phosphate-buffered saline solution (PBS, pH 7.2)], in 0.2% Tween 20 (PBS-Tween). Next, 300 μl of blocking solution (1% BSA in PBS; wt/vol) was added to each well and the plate incubated for 30 min at 37°C. The wells were washed with PBS-Tween three more times. AFB_1 standards (diluted in a 1:5 dilution of AFB_1-free peanut butter prepared as described above) or sample extract (prepared as above without further dilution) were mixed in equal volumes with AFB_1-HRP (peroxidase conjugate) previously diluted 200-fold in blocking solution containing 1% N,N dimethylformamide (vol/vol). Hence, both final sample:conjugate and standard:conjugate mixtures contained 0.1 g peanut butter/ml just before addition to the ELISA plate. Fifty μl of the mixture was added to wells and the plate incubated for 1 h at 37°C. After the wells were washed five times as above, 100 μl of 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) substrate solution was added to wells and the plate incubated at 37°C for 15 min. The reaction was terminated by the addition of 100 μl of stopping reagent [0.1% (wt/vol) sodium azide in 0.3 M citric acid] and the absorbance at 405 nm was determined on an EIA reader (Bio-Tek Instruments, Burlington, VT). A standard curve was prepared by plotting $\log_{10}$ AFB_1 concentration vs. absorbance.

HPLC determination of AFB_1 in commercial peanut butter

Aflatoxin analyses were performed on samples by means of the minicolumn method of Holaday and Lansden (*10*) followed by high-pressure liquid chromatography. High-pressure liquid chromatography was performed with a Waters Associates chromatography system equipped with two M-6000A pumps, a WISP 710 B autoinjector, a data module and a systems controller. Alfatoxins were separated with a Waters radial compression module containing a Radial-Pak Silica Gel column and a solvent system consisting of water-saturated chloroform supplemented with 0.6% methanol. A flow rate of approximately 2.0 ml/min or a flow rate sufficient to elute all four aflatoxins within 7 min was used. The aflatoxins were detected with a Varian Flurochrom fluorescence detector. Standard aflatoxin mixtures were obtained from Alltech Associates (Deefield, IL) for qualitative and quantitative analyses.

RESULTS

Effect of peanut butter extract on ELISA standard curve

When competitive ELISA curves were prepared in the presence of the filtrate of extracted AFB_1-free peanut butter and in extractant alone, the resulting competition curves differed (Fig. 1). The extractant curve showed a more pronounced decrease in absorbance with increasing AFB_1 concentration than did the extract curve, suggesting that the peanut butter extract interfered with the competitive ELISA curve. The linear reponse range of both curves was between 0.5 and 25 ng AFB_1/ml extract, with a correlation coefficient of 0.984 and 0.989 for extractant and extract curves, respectively. Because of the potential for interference by the peanut butter extract, standard curves prepared in AFB_1-free peanut butter extracts were used to calculate AFB_1 contamination in spiked and commercial peanut butter samples.

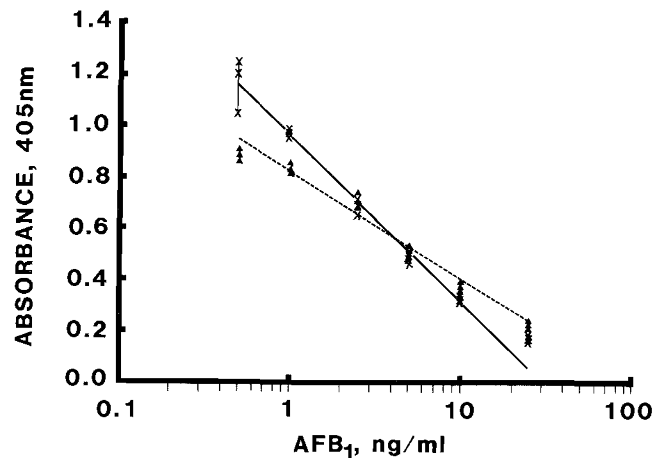

Figure 1. *Effect of peanut butter extract on the standard curve for AFB_1 determination by ELISA. Standard curves were prepared in both filtrates of extractant (▲) and AFB_1-free peanut butter extract (X). Power function best-fit linear regressions were constructed with Omicron Plotrax 2 software (Engineering-Science, Atlanta, GA).*

Recovery of AFB_1 in artificially contaminated peanut butter

AFB_1 was added to nine AFB_1-free peanut butter samples in concentrations ranging between 0.5 to 250 μg/kg of peanut butter. Spiked samples were extracted with extraction solvent and AFB_1 was determined in the filtrate by ELISA (Table 1). Aflatoxin in samples containing 0.5 and 1 μg AFB_1/kg was not detected by the ELISA, whereas the percent recovery for the other seven samples with AFB_1 levels at 2.5 ng/g peanut butter or greater ranged between 85 to 112%, with a mean recovery of 97%. Results presented in Table 2 reveal that the average inter-assay coefficient of variation was 22.7%.

TABLE 1. *Recovery of AFB_1 from artificially contaminated peanut butter by ELISA*[a].

AFB_1 added (ng/g)	AFB_1 recovery: Mean ± SD (μg/kg)	%[b]	CV[c] (%)
0.5	ND[d]	—	—
1.0	ND	—	—
2.5	2.8 ± 1.3	112	46.4
5	5 ± 1.9	100	38.0
10	10 ± 2	100	20.0
25	23 ± 0.9	92	4.0
50	47 ± 1.6	94	3.4
125	124 ± 5	99	4.0
250	213 ± 24	85	11.3

[a]AFB_1 was added to 5 g of peanut butter and the sample extracted with extraction solvent. Each sample was assayed in triplicate.

[b,c]Mean recovery and CV (inter-well variability) were 97 and 18.4%, respectively.

[d]ND, not detectable. Based on standard curve, theoretical detection limit was 5 μg/kg.

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TABLE 2. *Inter-assay variability of ELISA procedure used to detect AFB_1 in peanut butter.*

	AFB_1 in peanut butter extract, ng/ml[a]					
	0.5	1.0	2.5	5	10	25
Mean	0.7	1.4	2.0	5	10.4	21
SD	0.3	0.4	0.6	0.7	0.9	2.7
CV%[b]	42.8	28.5	30.0	14	8.6	12.8

[a]Aqueous extract containing 1.0 ng/ml before mixing with peroxidase conjugate would be equivalent to sample containing 5 μg AFB_1/kg.

[b]Determined by running the assay in triplicate on each of six different days. Average inter-assay variation (%CV) was 22.7 (n=6).

ELISA analysis of AFB_1 in commercial peanut butter

Sixty-three commercial samples of peanut butter representing 17 major and "generic" brand names were screened for AFB_1 by ELISA to validate the assay in a field situation. Three out of the 63 samples were positive for AFB_1 by ELISA, with all three being generic brands. These three samples along with seven other negative samples were also analyzed by HPLC to further establish the validity of the procedure (Table 3). HPLC similarily indicated the presence of AFB_1 in the samples found positive by ELISA, whereas sample Nos. 1 through 7 were negative for AFB_1 by ELISA but showed trace amounts of AFB_1 (≤5 ng/g) by HPLC.

DISCUSSION

Both conventional analytical methods and previously reported ELISA procedures for detection of AFB_1 in peanut butter require extensive extraction, sample clean up, evaporation, and redissolution steps (*1,2,5-7,18-20*) thereby increasing total analysis time. This constitutes a major disadvantage to laboratories involved in the routine screening of peanuts and peanut products for AFB_1. The ELISA reported herein required only the aqueous portion of the sample extract. The extraction procedure was based on the BF method (*19*) but did not employ centrifugation and phase separation steps. The aqueous fraction is collected by simply filtering the crude sample extract; extracted AFB_1 passes through the filter leaving the solids and hexane fraction in the filter. No sample evaporation or redissolution was required. The combined blending and filtration steps took only 5 min to carry out.

TABLE 3. *Analysis of AFB_1 in commercial peanut butter by ELISA and HPLC.*

Sample No.	AFB_1 (μg/kg) ELISA	HPLC
1	ND[a]	2
2	ND	3
3	ND	4
4	ND	4
5	ND	4
6	ND	4
7	ND	5
8	16	10
9	18	10
10	13	20

[a]ND, not detected; based on standard curve, the theoretical detection limit for AFB_1 was 5 μg/kg.

The direct ELISA described here is based on the competition of AFB_1 in the sample extract and AFB_1-peroxidase (enzyme conjugate) for the specific binding site on AFB_1 antibody absorbed to the microtiter plate well. To avoid interference in sample analysis, we prepared a standard curve using an AFB_1-free peanut butter extract so that samples and blanks both contained 0.1 g of peanut butter per ml upon final mixing with peroxidase conjugate. For AFB_1-free extracts we used commercial, high quality creamy peanut butter brands. Although the minimum statistically significant detection limit of the assay in the sample extract was 5 μg AFB_1/kg (equivalent to 1.0 ng AFB_1/ml after 5-fold sample dilution), an estimate could still be made at the 2.5 μg/kg level (Table 1).

ELISA sensitivity was comparable to that found previously for the CB or BF methods (*18,19*) and for two previously reported ELISA procedures (*6,7*). Coefficients of variation at 2.5 and 5 μg/kg exceeded 20%. Similar increases in variability have been observed with aflatoxin M_1 (*14*) and T-2 toxin (*15*) as the limits of detection were approached. Decreased precision might be expected at these levels because standard curves obtained by ELISA procedures are typically sigmoidal and begin to flatten as the analyte concentration approaches the detection limit. The inter-assay coefficient of variation for the ELISA procedure used to detect AFB_1 in peanut butter might be attributed to inter-well variability as was reported earlier (*13-15*), and similar coefficients of variation have been reported in previous ELISA procedures for AFB_1 in peanut butter. Nevertheless, overall precision of the assay was within an acceptable range when one considers that previously reported intra-laboratory coefficients of variation (repeatabilities) for two liquid chromatographic procedures ranged between 17 and 38% (*1*).

We have recently described the rapid extraction and ELISA of AFB_1 in naturally contaminated corn and cottonseed (*16*). The results presented herein suggest that the ELISA is also suitable for the rapid semiquantitative screening of AFB_1 in peanut butter. It should be noted that because of incomplete cross reactivity with AFB_2, AFG_1 and AFG_2 (*13*), the polyclonal AFB_1 antibody can be used to detect but not quantitate these aflatoxins. In the market survey described here, the ELISA detected AFB_1 in three samples out of 63 samples of commercial peanut butter; all three were generic brands. Similar results were obtained by HPLC on the three positive samples. The positive samples had AFB_1 levels of less than 20 μg/kg peanut butter and hence was still within the limits of FDA action level. Gilbert and Shepherd (*9*) have also recently determined that 31 of 32 samples of major brand-name peanut butters in the United Kingdom contained less than 10 μg AFB_1/kg. The extraction procedure and

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attendant ELISA described herein should find use as a quality control diagnostic in peanut processing facilities due to its simplicity, rapidity, sensitivity, safe reagents and ease of sample extraction. Using a 96-well microtiter plate, 36 sample extracts can be screened for AFB_1 in less than 2 h, allowing a high sample throughput in the routine determination of AFB_1 in peanut butter.

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