

# Enzyme-Linked Immunosorbent Assay (ELISA) for Aflatoxin B<sub>1</sub> Estimation in Groundnuts

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## Abstract

*The commercially available hapten, afla B<sub>1</sub>-oxime-bovine serum albumin, was used to produce an antiserum in rabbits. The same hapten was coupled with alkaline phosphatase (hapten-BSA-ALP) and used in the competitive direct enzyme-linked immunosorbent assay (ELISA) for the detection of aflatoxin B<sub>1</sub>. Aflatoxin B<sub>1</sub> was extracted in methanol from naturally contaminated or 'spiked' groundnut seed samples.*

*Wells of a polystyrene microtitre plate were coated with the antiserum, the plates were washed in PBS-Tween, aflatoxin B<sub>1</sub> standards or groundnut sample extracts, and hapten-BSA-ALP conjugate were added and the plates incubated. The plates were again washed, and the amount of conjugate bound to the antibody was determined after addition of the substrate, p-nitrophenylphosphate.*

*The hapten-BSA-ALP conjugate has advantages in stability, simplicity of preparation, and high specificity, over the conventional toxin-enzyme conjugate in direct competitive ELISA. The assay method is more rapid and less expensive than the physico-chemical methods of aflatoxin analysis and it can detect levels of aflatoxin B<sub>1</sub> as low as 50 picograms.*

## Résumé

*Détermination de l'aflatoxine B<sub>1</sub> dans les arachides par la méthode ELISA : Le sérum albumine afla B<sub>1</sub>-oximebovine, commercialisé sous le nom de Hapten, a servi à l'élaboration d'un antiserum chez des lapins. Ce produit est associé à la phosphatase alcaline enzymatique (Hapten-ALP) et utilisé dans l'essai ELISA adéquat pour la détection de l'aflatoxine B<sub>1</sub>. Cette mycotoxine a été extraite avec le méthanol à partir des échantillons de graines d'arachide ayant subi une contamination naturelle ou celles artificiellement marquées.*

*Les parois d'une plaque de microtitrage en polystyrène sont enduites d'antisérum et les plaques sont ensuite lavées au produit PBS-Tween; l'aflatoxine B<sub>1</sub> étalon ou les extraits des échantillons d'arachide ainsi que le conjugué Hapten-ALP y sont ajoutés avant incubation. Les plaques sont à nouveau lavées et la quantité de conjugué liée à l'anticorps est déterminée en y ajoutant le substrat p-nitrophénylphosphate.*

*Les avantages de Hapten-ALP par rapport aux conjugués toxine-enzyme classiques pour le*

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*test ELISA sont sa stabilité, sa simplicité et sa forte spécificité. La méthode est plus rapide et moins coûteuse que les procédés physico-chimiques de dosage des aflatoxines et possède un seuil de détection de l'aflatoxine B<sub>1</sub> inférieur à 50 picograms.*

### **Resúmene**

**Determinación de la aflatoxina B<sub>1</sub> en el cacahuete por el método ELISA :** *Se utilizó albúmina de suero bovino-afla B<sub>1</sub>-oxime, conocido comercialmente con el nombre de "Hapten", para la producción de un antisuero en conejos. Este mismo "Hapten" fue ligado con fosfatasa alcalina (Hapten-BSA-ALP) y usado en el método directamente competitivo inmunoabsorbente vinculado a enzimas de cuantificación de anticuerpos, ELISA, para la detección de aflatoxina B<sub>1</sub>. La aflatoxina B<sub>1</sub> fue extraída con metanol de muestras de semilla de cacahuete contaminadas, ya sea en forma natural o artificialmente, con esa micotoxina.*

*Las depresiones de una placa de polistireno empleada para microtitulaciones se recubrieron con una capa delgada del antisuero; las placas se lavaron con el producto PBS-Tween; se agregaron soluciones calibradas de aflatoxinas B<sub>1</sub> o extractos de las muestras de cacahuates y, a continuación, el conjugado Hapten-BSA-ALP; luego se incubaron las placas. Se lavaron nuevamente las placas, y la cantidad de conjugado ligado al anticuerpo, se determinó después de la adición del sustrato, p-nitrofenilfosfato.*

*El conjugado Hapten-BSA-ALP tiene marcadas ventajas de estabilidad, simplicidad y alta especificidad, sobre el uso del conjugado toxina-enzima convencional, en el método competitivo directo ELISA. El método analítico primeramente citado es más rápido y menos costoso que los métodos físico-químicos de análisis de aflatoxinas y puede detectar niveles de aflatoxina B<sub>1</sub> hasta del orden de 50 picograms.*

## **Introduction**

The thin layer chromatography (TLC) systems developed in the 1960s and 1970s are still the most commonly used methods for detection and estimation of aflatoxins in groundnut and several other agricultural commodities. These methods are expensive and time consuming and so efforts have been made to develop more rapid and less expensive methods for aflatoxin analysis (Pestka et al. 1981, Morgan et al. 1986, and El-Nakib et al. 1981).

Several enzyme-linked immunosorbent assay (ELISA) procedures have been reported for the estimation of aflatoxin B<sub>1</sub> in groundnut and groundnut products (El-Nakib et al. 1981, Fan and Chu 1984, Morgan et al. 1986). These assays have advantages over conventional analytical procedures using TLC and high pressure liquid chromatography (HPLC) in terms of speed, ease of sample preparation and use, and are potentially cheaper for aflatoxin analysis. The major application of ELISA procedures at present is analysis of aflatoxin B<sub>1</sub> in such agricultural commodities as maize, groundnut, and groundnut products (El-Nakib et al. 1981, Fan and Chu 1984, Morgan et al. 1986). A few direct and indirect ELISA procedures have been developed for analysis of aflatoxin B<sub>1</sub> in groundnut, groundnut meal, and peanut butter. All direct competitive ELISA procedures necessitate the use of aflatoxin-horse radish peroxidase (HRP) conjugate. Two problems are encountered in preparation of the conjugate: (1) instability of the toxin-

enzyme conjugate, and (2) variations in the amounts of toxin conjugated to the enzyme (Fan and Chu 1984). Both these factors affect the sensitivity of ELISA. To overcome these factors, we have used alkaline phosphatase (ALP) enzyme in place of HRP and have coupled it directly with the commercially available afla B<sub>1</sub>-oxime-BSA. Using this afla B<sub>1</sub>-oxime-BSA-ALP conjugate, we have developed a simple, rapid, specific, and comparatively inexpensive direct competitive ELISA for analysis of aflatoxin B<sub>1</sub> in groundnuts.

## **Materials and Methods**

### **Chemicals and reagents**

Aflatoxin B<sub>1</sub>, bovine serum albumin (BSA, RIA grade), afla B<sub>1</sub>-oxime-BSA, alkaline phosphatase (Type VII-NT), p-nitrophenyl phosphate disodium, glutaraldehyde (RIA grade), and Tween 20 ® were purchased from Sigma Chemical Co., St. Louis, MO, USA. Complete and incomplete Freund's adjuvants were obtained from Difco Laboratories, Detroit, MI, USA.

Polystyrene microtitre plates were obtained from Dynatech Lab, Virginia, USA. All other organic solvents and inorganic chemicals used were of the highest analytical grade.

### **Production of antiserum against aflatoxin B<sub>1</sub>**

Antiserum against aflatoxin B<sub>1</sub> was produced by immunizing rabbits with afla B<sub>1</sub>-oxime-BSA, using the methods of Chu and Ueno (1977) and El-Nakib et al. (1981). Antiserum titre was determined by the indirect competitive ELISA procedure described by Morgan et al. (1986).

### **Preparation of afla B<sub>1</sub>-oxime-BSA-ALP conjugate**

Afla B<sub>1</sub>-oxime-BSA was conjugated to ALP through the glutaraldehyde bridge using the method of Avrameas et al. (1978). Several ratios of ALP and afla B<sub>1</sub>-oxime-BSA were tried in initial experiments and the most suitable ratio was 1 mg of afla B<sub>1</sub>-oxime-BSA to 4 mg of ALP. Afla B<sub>1</sub>-oxime-BSA was dissolved in phosphate buffered saline (PBS, pH 7.4, 1 mg mL<sup>-1</sup>) and mixed in proportions of 1:2, 1:4, 1:6, and 1:8 with ALP. The mixtures were dialyzed with 0.06% glutaraldehyde in PBS at room temperature for 2 h for conjugation. Excess glutaraldehyde was removed by dialyzing in PBS at 4°C for 3 h with three changes of PBS. The resultant afla B<sub>1</sub>-oxime-BSA-ALP conjugate was stored at 4°C.

### **Sample preparation and extraction**

Groundnut seeds (cv J 11) were obtained from the 1986 rainy-season crop grown at ICRISAT Center. Healthy, mature, finely ground seeds (500 g) were divided into several lots of 5- and 10-g samples and these were 'spiked' with aflatoxin B<sub>1</sub> standard to give concentrations of 10, 20, 40, and 50 µg kg<sup>-1</sup>. Samples were spiked by directly adding a measured volume of aflatoxin B<sub>1</sub> standard solution in methanol and then mixing thoroughly. These samples were used to test the recovery of the toxin by the ELISA procedure as described below. Some nonspiked samples were used to determine naturally occurring aflatoxin B<sub>1</sub>. Naturally contaminated seeds were also tested for aflatoxin B<sub>1</sub> levels using TLC and ELISA procedures. For ELISA, aflatoxin B<sub>1</sub> was

extracted from the spiked or naturally contaminated samples with 55% methanol ( $5 \text{ mL g}^{-1}$ ) in a Waring blender for 3 min. The extract was filtered through Whatman No.1 paper, the filtrate concentrated by flash drying, and then diluted in PBS to avoid methanol interference (to have below 11% methanol in each assay). The Pons' method of extraction (Pons et al. 1966) was used in the detection and estimation of aflatoxin  $B_1$  by TLC.

### **Aflatoxin $B_1$ standard**

Various concentrations of standard aflatoxin  $B_1$  ranging from 100 ng to 50 pg (in two-fold serial dilutions) in PBS containing 11% methanol were used in ELISA.

### **Direct Competitive ELISA Procedure**

In the first step, 200  $\mu\text{L}$  of crude antiserum (1:5000 in 0.05 M carbonate buffer, pH 9.6) were incubated in each well of polystyrene microtitre plates for 2 h at  $37^\circ\text{C}$ . The plates were then washed three times in PBS-Tween. Next, 100  $\mu\text{L}$  of various dilutions of aflatoxin  $B_1$  standard or sample extracts were added to each well, followed by 100  $\mu\text{L}$  of aflatoxin  $B_1$ -oxime-BSA-ALP conjugate (diluted in PBS-Tween containing 1% BSA, 1:4000). The plates were incubated  $37^\circ\text{C}$  for 2 h. The wells were then washed with PBS-Tween, 200  $\mu\text{L}$  of p-nitrophenyl phosphate (enzyme specific substrate) added, and the plates incubated at room temperature for 30 min. The reaction was stopped by the addition of 50  $\mu\text{L}$  of 3 mol sodium hydroxide, and the absorbance at 405 nm was measured using a micro-ELISA reader.

## **Results and Discussion**

The relationship between different concentrations of standard aflatoxin  $B_1$  and absorbance values in the ELISA procedure is shown in Figure 1. All points are means of two replicates. There was no strictly linear relationship across the different concentrations of aflatoxin  $B_1$  tested. This is probably due to reactions between different proportions of toxin and toxin-BSA-ALP conjugate that influence the amount of the toxin bound to the antibody adsorbed to the well surface. Only at optimal concentrations can a linear relationship be expected. A linear relationship can be obtained experimentally by utilizing a particular range of concentrations of the toxin and using a predetermined toxin-BSA-ALP conjugate dilution. The linear regression line (standard curve of aflatoxin  $B_1$ ) is shown in Figure 2. This linear regression equation was used to calculate the toxin concentrations from the sample extracts.

### **Recovery of Aflatoxin $B_1$ from Spiked Groundnut Samples**

The recovery of the toxin was 62–86% for 5-g samples and 70–107% for 10-g samples (Table 1). The recovery of the toxin was significantly lower when higher levels ( $50 \mu\text{g kg}^{-1}$ ) of the toxin added than when lower levels ( $10$  or  $20 \mu\text{g kg}^{-1}$ ) were added in the case of 5-g samples, while the recovery increased from 70 to 107% when 10-g samples were used. Similar results have been reported by other workers, particularly while using larger samples (Fremy and Chu 1984, Fan and Chu 1984, and El-Nakib et al. 1981). Dilution of the sample extracts would have avoided this problem. However, in both sample sizes, recovery of the toxin ranged from 70 to 96% from samples 'spiked' with concentrations of the toxin at  $10$  or  $20 \mu\text{g kg}^{-1}$ . These results are supported

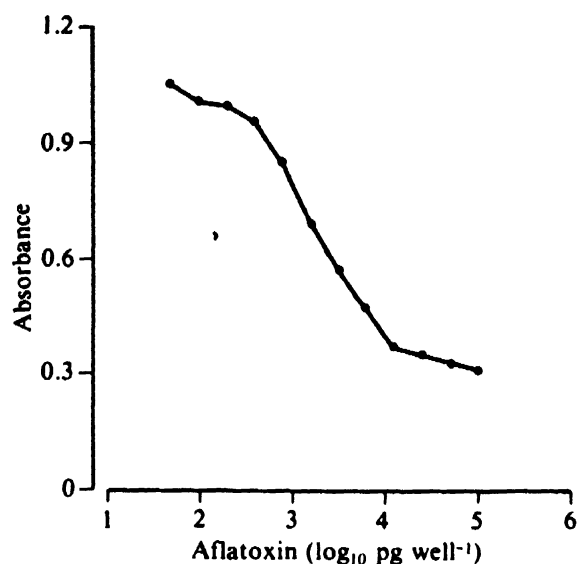


Figure 1. Relationship between aflatoxin  $B_1$  concentration and intensity of alkaline phosphatase (ALP) reaction measured as absorbance.

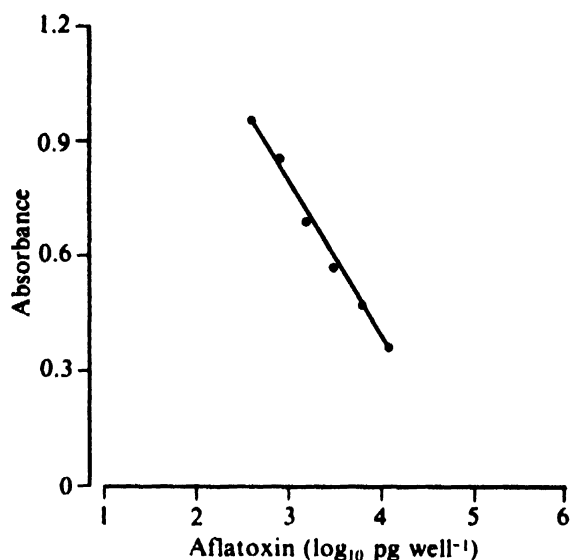


Figure 2. Linear regression line (standard curve) of aflatoxin  $B_1$ .  
 $y = 1.97 - 0.39x$  ( $r = 0.99$ ,  $P < 0.01$ )

**Table 1. Recovery of aflatoxin  $B_1$  by ELISA from 5-g and 10-g groundnut samples spiked with different concentrations of the toxin, ICRISAT Center, 1987.**

Toxin added ( $\mu\text{g kg}^{-1}$ )	Toxin recovered			
	( $\mu\text{g kg}^{-1}$ )	Recovery (%)	SE	CV (%)
<b>5-g samples</b>				
10	8.6	86	$\pm 3.9$	21
20	16.4	82	$\pm 2.9$	9
50	31.0	62	$\pm 3.9$	5
<b>10-g samples</b>				
10	7.0	70	$\pm 6.3$	20
20	19.2	96	$\pm 31.3$	30
40	43.0	107	$\pm 44.8$	18

by the findings of the other studies (Fan and Chu 1984, and Fremy and Chu 1984, and Hu et al. 1984). Aflatoxin was not detected in samples that had not been 'spiked'.

### Comparison of ELISA and TLC Procedures

The ELISA procedure estimated markedly more aflatoxin  $B_1$  in the samples than the TLC procedure (Table 2). The levels of toxin estimated by this ELISA procedure indicated that it is possible to detect high levels of toxin in naturally contaminated samples, provided appropriate

**Table 2. Comparison of TLC and ELISA procedures for estimation of aflatoxin B<sub>1</sub> in naturally contaminated groundnut samples, ICRISAT Center, 1987.**

Sample	Aflatoxin B <sub>1</sub> ( $\mu\text{g kg}^{-1}$ )			
	TLC method		ELISA	
	Mean	Range	Mean	Range
1	3.9	3.6-4.0	6.5	5.4-8.2
SE	$\pm 0.20$		$\pm 0.84$	
CV (%)	5		22	
2	3.9	3.6-4.0	6.0	4.9-7.0
SE	$\pm 0.20$		$\pm 0.61$	
CV (%)	5		18	

dilutions of the sample extracts are used. The procedure can detect aflatoxin at levels as low  $\mu\text{g kg}^{-1}$  with a simple extraction procedure. The ELISA procedure described in this paper can be used to screen large numbers of groundnut samples for aflatoxin B<sub>1</sub> content in either naturally or artificially contaminated seeds. Thus, it is possible to screen large numbers of genotypes simply and inexpensively for their ability to support aflatoxin production when seeds are artificially inoculated with aflatoxigenic isolates of *Aspergillus flavus*. This ELISA procedure has an advantage over the TLC procedure in terms of cost and time for aflatoxin analysis.

El-Nakib et al. (1981) described a direct competitive ELISA procedure for aflatoxin B<sub>1</sub> in groundnut and other agricultural commodities that is more sensitive (detecting down to 3  $\mu\text{g kg}^{-1}$ ) than the presently described procedure. However, they used the BF extraction for their ELISA procedure which also differed in its use of toxin-HRP conjugate prepared by the conversion of aflatoxin B<sub>1</sub> to aflatoxin B<sub>1</sub>-oxime and subsequently coupled with the HRP. In the presently described ELISA procedure, the commercially available aflatoxin B<sub>1</sub>-oxime-BSA has been used to produce antiserum as well as for conjugation with the enzyme-ALP. In this case, the preparation of aflatoxin B<sub>1</sub>-oxime-BSA-ALP conjugate is relatively simple and the conjugate is stable. No loss in activity of the toxin-enzyme conjugate was detected when the conjugate was stored at 4°C for 6 months. Other workers (Fan and Chu 1984, and Morgan et al. 1986) have described indirect competitive ELISA procedures for estimation of aflatoxin B<sub>1</sub>. These methods take longer than the direct competitive ELISA procedure because additional incubation steps are required. The ELISA procedure described in this paper is relatively simple and economic and only requires a simple extraction procedure, and low concentrations of antiserum and the toxin-enzyme conjugate. It can detect aflatoxin B<sub>1</sub> in samples at a level of 50 pg per assay.

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