Title: Recovery of Aflatoxin B_1 in Range of Food Commodities Utilizing a Matrix Resistant ELISA

By Thu P. Huynh and Christina Ly, Helica Biosystems Inc., Santa Ana, CA

INTRODUCTION:

Aflatoxin B_1 is a well-known potent human carcinogen produced by toxigenic fungi. Given its ubiquitous presence in a wide variety of foods and beverages, aflatoxin B_1 levels must be measured and monitored to prevent contaminated products from reaching the consumer. Enzyme linked immunosorbent assays (ELISAs) are frequently employed as a rapid and inexpensive method to screen samples that may contain aflatoxin B_1 concentrations above the legal permissible limit. To date, many available ELISAs are limited in the range of commodities that can be tested because they are subject to matrix interferences and require an additional clean-up step.

PURPOSE:

The aim of this study was to evaluate if a single ELISA kit can accurately detect aflatoxin B_1 in commodities that typically pose matrix interferences, including nuts, spices, and other common cooking ingredients.

MATERIALS AND METHODS:

Materials:

The Aflatoxin B_1 Low Matrix ELISA kit was prepared in-house (Helica Biosystems, Inc., Santa Ana, CA). The sodium chloride (NaCl), acetonitrile (ACN), hexane, methanol (MeOH), and aflatoxin B_1 (AFB_1) were purchased from Sigma (St. Louis, MO). The various comestibles (almonds, macadamia, peanuts, black pepper, chili, cinnamon, coriander, ginger, and coconut) were purchased from the local markets (Santa Ana, CA).

Methods:

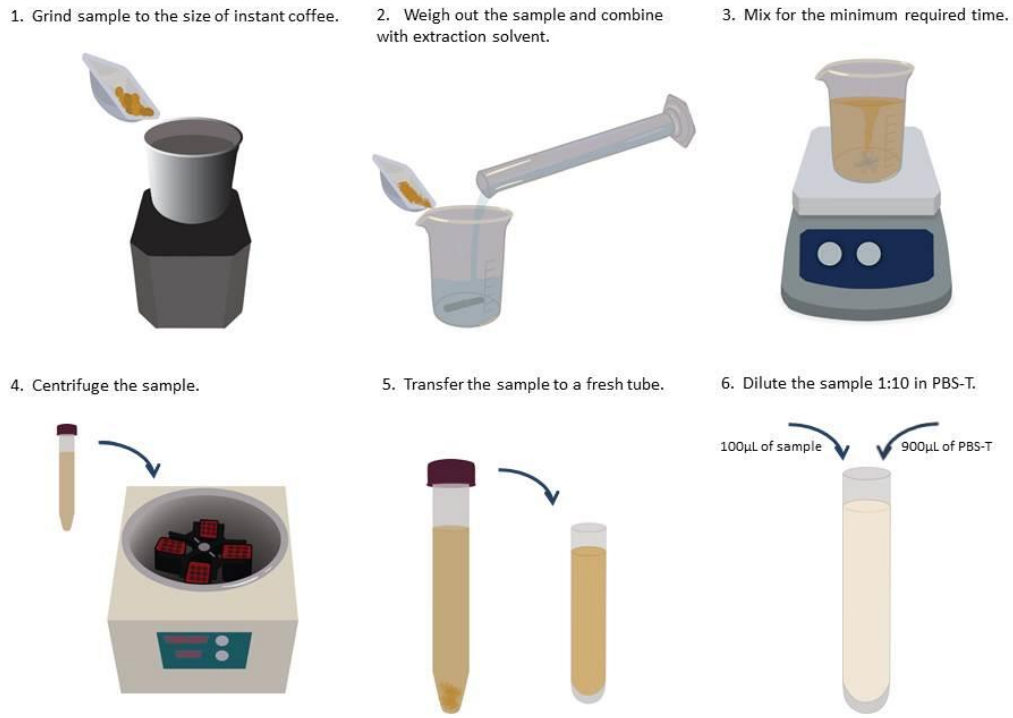
Sample Preparation

For the spike and recovery studies, all commodities were finely ground in a blender. Ingredients were weighed and spiked with a pure solution of AFB_1 at low, medium, and high concentrations. The commodity spiked AFB_1 was dried overnight at room temperature prior to extraction.

Extraction and Dilution

The samples were extracted according to the general scheme (Figure 1). A mass of each sample was transferred to a vessel and combined with 80% ACN. After a brief period of mixing, the samples were centrifuged and the supernatant was collected for analysis. Samples were diluted 1:10 into PBS-T prior to running the assay.

Figure 1 – General Extraction Procedure



Due to high oil content, the peanut was extracted according to the following method. 5g of peanut paste, 0.5g of NaCl, 30mL of 80% MeOH, and 10mL of hexane were combined and blended continuously for 3 minutes. A portion of the sample was filtered and the filtrate was diluted 1:10 into PBS-T prior to running the assay.

ELISA Method

The ELISA was performed according to the manufacturer's instructions (Helica Biosystems Inc, Santa Ana, CA). Briefly, all reagents were equilibrated to room temperature. 200µL of the assay diluent was transferred into each mixing well. 100µL of the standards or samples were pipetted into the appropriate mixing wells and mixed. 100µL of the mixture was transferred to the appropriate antibody-coated wells in duplicate and incubated at ambient temperature for 30 minutes. The wells were washed three times with PBS-T and tapped dry. 100µL of Aflatoxin HRP-conjugate was added to each antibody-coated well and incubated at room temperature for 30 minutes. The wells were washed three times with PBS-T and tapped dry. 100µL of the TMB

substrate was added to each microwell and the plate was incubated at ambient temperature for 10 minutes. 100µL of stop solution was added to each well. The optical density (OD) of each microwell was read at 450nm on an ELX800UV absorbance reader (Biotek, Winooski, VT) using a differential filter of 630nm.

Calculation and Analysis

The **%B/Bo** was calculated by dividing the OD of the sample by the OD for the 0ppb standard times 100 to obtain a percentage. The standard concentrations were plotted along the x-axis on a log scale. The corresponding %B/Bo values were plotted along the y-axis. Gen5 software (Biotek) was used to fit the standard curve (4-paramater logistics) and the concentration of the samples were interpolated from the standard curve. The original concentration was calculated after taking the final dilution factor into account.

The **limit of detection** (LOD) was calculated by taking the mean of twenty replicates of negative samples and subtracting three times the standard deviation of the negative samples to obtain a %B/Bo. The %B/Bo was used to interpolate the concentration from the standard curve to obtain the LOD concentration.

The **% recovery** was tabulated by taking the difference of the amount of AFB_1 spiked into the sample and the amount of AFB_1 recovered from the assay divided by the amount of AFB_1 spiked into the sample multiplied by 100 to obtain a percentage. In addition, the %CV was determined for each commodity at each spike level where each food sample was tested in triplicate at each spike level.

RESULTS AND DISCUSSION:

AFB_1 has long been recognized as a harmful contaminant present in food supplies throughout the economically developing world (1). Several commercial ELISA kits have been developed to detect AFB_1 in foods. However, a major challenge of using ELISAs for food analysis is the matrix effect, in which interfering substances from the commodity hinder enzyme activity or reduce the interaction between antibody and antigen (2). Previously, we have developed an ELISA kit for the quantitative detection of AFB_1 in corn, wheat, hay, snaplage, paprika, and pistachio with minimal interference from the various matrices. Due to the ubiquitous nature of AFB_1 in a wide array of dietary staples and agricultural products, we assessed the ability of our Aflatoxin B_1 Low Matrix ELISA kit to accurately quantitate the level of AFB_1 in additional food products (3).

Since food matrices can exert varying effects on an assay, we first determined the limit of detection (LOD) or sensitivity of the commodities under investigation. According to Table 1, the results demonstrate minimal interference by these particular

matrices. All LOD values were lower than 2.5ppb indicating that it would be possible to test these food matrices at levels above 2.5ppb.

Table 1 - Limit of Detection of Various Commodities

Matrix	LOD (μg/kg)
Almond	0.7
Macadamia	2.4
Peanut	2.1
Black Pepper	2.0
Chili	0.9
Cinnamon	0.3
Coriander	0.8
Ginger	1.2
Coconut	0.8

Since the commodities did not exhibit significant matrix interferences, we performed spike and recovery studies. The commodities under evaluation were spiked at three different levels of AFB_1 and the % recovery was determined. The overall recoveries were excellent for the nuts (Table 2).

Table 2 - Recovery of Aflatoxin B_1 from Nuts

Matrix	Spike level[a] (μg/kg)	Repeatability (%CV)	Recovery (%)
Almond	2.5	2.5	82.6
	5	17.6	100.5
	10	17.1	105.5
	Overall[b]	**12.4**	**96.2**
Macadamia	2.5	8.3	137.3
	5	14.7	132.2
	10	6.9	91.8
	Overall[b]	**10.0**	**120.4**
Peanut	10	4.7	85.9
	20	9.5	113.8
	Overall[b]	**7.1**	**99.8**

[a]Each spike level was tested in triplicate for each commodity.

[b]Overall recovery is the average recovery across all spike levels.

The recoveries were 96.2%, 120.4%, and 99.8% for almond, macadamia, and peanut, respectively. At the low 2.5ppb spike level for peanuts, recovery of $AFLB_1$ was not possible suggesting that the assay will not be useful for detecting low levels of $AFLB_1$ in peanuts. Excellent recoveries of AFB_1 in various spices were also obtained with the exception of cinnamon (Table 3).

Table 3 - Recovery of Aflatoxin B_1 from Spices

Matrix	Spike level[a] (μg/kg)	Repeatability (%CV)	Recovery (%)
Black Pepper	2.5	11.9	117.3
	5	22.0	101.7
	10	13.7	124.7
	Overall[b]	**17.9**	**113.2**
Chili	2.5	7.2	100.0
	5	3.6	96.0
	10	3.0	88.0
	Overall[b]	**3.3**	**92.0**
Cinnamon	2.5	31.8	56.9
	5	4.2	63.6
	10	15.1	50.6
	Overall[b]	**9.6**	**57.0**
Coriander	2.5	4	99
	5	5.5	100.3
	10	7.9	108
	Overall[b]	**6.7**	**104.2**
Ginger	2.5	8.7	92.7
	5	3.3	90.0
	10	4.2	97.5
	Overall[b]	**3.8**	**93.8**

[a]Each spike level was tested in triplicate for each commodity.

[b]Overall recovery is the average recovery across all spike levels.

The overall recoveries for black pepper, chili, cinnamon, coriander, and ginger were 113.2%, 92.0%, 57.0%, 104.2%, and 93.8%, respectively. The cause for low recovery in cinnamon spice is unclear, though it is possible that co-extractants in this spice may

bind to and mask AFB_1 detection. In addition, dried coconut was also tested and the overall recovery was excellent at 94.5% (Table 4).

Table 4 - Recovery of Aflatoxin B_1 from Coconut

Matrix	Spike level[a] (μg/kg)	Repeatability (%CV)	Recovery (%)
Coconut	5	14.2	94.3
	10	6.7	92.7
	20	7.1	96.3
	Overall[b]	**6.9**	**94.5**

[a]Each spike level was tested in triplicate for each commodity.

[b]Overall recovery is the average recovery across all spike levels.

In summary, all food items exhibited minimal matrix interferences. All commodities showed excellent overall recoveries of 92.0-120.4% with %CVs of less than 18%. The only exception was cinnamon, which had a recovery of 57%.

SIGNIFICANCE:

The data demonstrates that a single ELISA kit can be used to successfully quantify $AFLB_1$ in most commodities without the need for special extraction methods or clean-up procedures.

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