Helica Biosystems, Inc

1527 W. Alton Ave

Santa Ana, CA 92704

Phone: 714-578-7830

Fax: 714-578-7831

Email: info@helica.com

# Quantitation of Aflatoxin $B_1$ by ELISA in Commodities that Pose a Matrix Effect

**Thu Hyunh, Ph.D,, Chritina Ly, Peter Knight, Ph.D, and Wondu Wolde-Mariam**

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**ABSTRACT:**

Aflatoxin $B_1$ is a major fungal residue and potent carcinogen found in various food commodities and beverages. Enzyme linked immunosorbent assays (ELISAs) have long been utilized to quantitate mycotoxin levels because they enable the simple, rapid, and high-throughput detection of mycotoxins. Additionally, ELISAs are portable and allow for the detection of mycotoxins in the field where fast decisions are required. One drawback of ELISAs is the matrix effect, where components in the commodity may interfere with the assay and result in higher or lower than expected values. Previously, we developed an aflatoxin $B_1$ low matrix ELISA to quantitatively assess aflatoxin $B_1$ levels from wheat, snaplage, corn, hay, paprika, pistachio, and peanut. In this study, we augmented the array of commodities that could be tested with the aflatoxin $B_1$ low matrix ELISA kit. We investigated the use of this kit to accurately quantify aflatoxin $B_1$ in a wide array of food commodities that include common cooking spices, oils, sauces, and animal feed. With minor modifications in the extraction procedures for each commodity, the recoveries of aflatoxin $B_1$ from these commodities reached 80% or better. In brief, we have developed a kit to quantify aflatoxin $B_1$ with minimal matrix effects in a plethora of commodities. As a result, the aflatoxin $B_1$ low matrix ELISA kit is an inexpensive and highly valuable tool to monitor and ensure food safety worldwide.

## MATERIALS AND METHODS:

Materials:

The Aflatoxin $B_1$ Low Matrix ELISA kit was prepared in-house (Helica Biosystems Inc, Santa Ana, CA). The acetonitrile and aflatoxin $B_1$ ($AFB_1$) were procured from Sigma (St. Louis, MO). All spices, oils, sauces, and animal feed were obtained from the local market or food supply store (Santa Ana, CA).

Methods:

*Extraction and Dilution*

For the spike recovery studies, the animal feed and cilantro seed were finely ground. For the dumpling sauce, cooking oils, and the soy sauce, the samples were vortexed after spiking and immediately extracted. For all other commodities, the $AFB_1$ was dried onto the commodity overnight at room temperature prior to extraction.

The samples were extracted according to the general scheme (Figure 1). A mass or volume of sample was transferred to a vessel and combined with extraction solvent. For the solid samples, 2-20g of sample was used for the analysis. For the oils and sauces, 10-20mL of sample was assayed. After a brief period of mixing with 100-200mL of 80% acetonitrile, the sample was centrifuged and the supernatant was collected for analysis. Samples were diluted into PBS-T prior to running the assay.

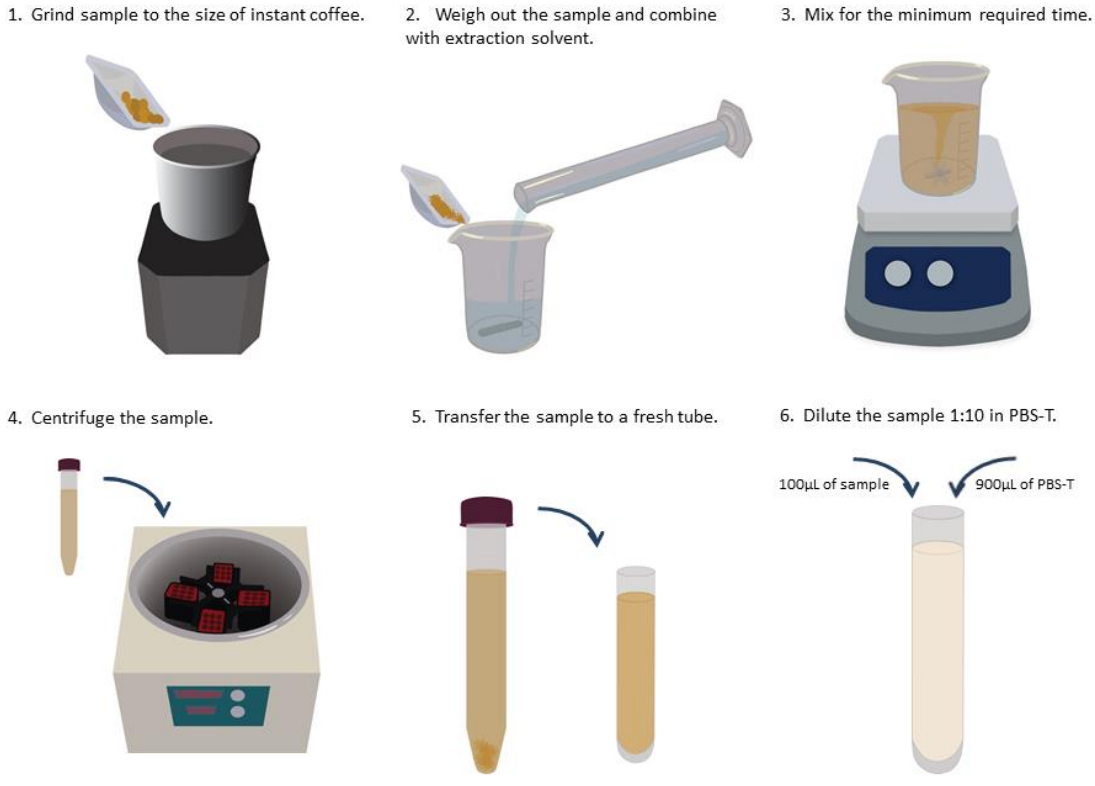


**Figure 1 – General Extraction Procedure**

*ELISA Method*

The ELISA was performed according to the manufacturer's instructions (Helica Biosystems Inc, Santa Ana, CA). Briefly, all reagents for the ELISA were equilibrated to room temperature before use. 200µL of the assay diluent was transferred into each mixing well. 100µL of the standards or samples were pipetted into the appropriate 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 a StatFax2100 (Awareness Technology Inc, Palm City, FL) using a differential filter of 630nm.

*Calculation and Analysis*

The %B/Bo was calculated by dividing the OD for 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. ReaderFit software (Hitachi Solutions America Ltd, San Francisco, CA) was used to fit the standard curve (4-paramater logistics) and the spiked samples were interpolated from the standard curve. The original concentration was calculated after taking the final dilution factor into account.

**To assess matrix interferences**, twenty replicates of blank, extracted commodity were assayed. A Student's *t*-test was performed using Microsoft Excel to determine if there is a statistically significant difference between the means of the extracted commodity and extraction solvent alone. All p-values less than 0.05 were considered significantly different.

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

**% 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%.

**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, pistachio, and peanuts 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 animal feed and food products common to many developing countries (3-4).

First, we determined if the commodities under investigation exhibit matrix interferences. According to Table 1, the mean signal of the commodities tested did not show a statistically significant difference compared to the mean signal of the extraction solvent as all p-values were greater than 0.05. The results demonstrate the lack of interference by these particular matrices.

**Table 1 - Assessment of matrix interferences and establishment of LOD**

| Matrix | p-value[a] | LOD (μg/kg) |
|---|---|---|
| Chili Powder | 0.279 | 0.0176 |
| Cilantro Seed | 0.959 | 0.0184 |
| Coriander Powder | 0.746 | 0.0163 |
| Corn Oil | 0.991 | 0.0102 |
| Peanut Oil | 0.942 | 0.0116 |
| Safflower Oil | 0.322 | 0.0172 |
| Sesame Oil | 0.301 | 0.0108 |
| Vegetable Oil | 0.976 | 0.0111 |
| BBQ Sauce | 0.864 | 0.0106 |
| Char Siu Sauce | 0.584 | 0.016 |
| Chili Sauce | 0.584 | 0.0079 |
| Dumpling Sauce | 0.219 | 0.0111 |
| Hoisin Sauce | 0.874 | 0.0135 |
| Hunan Sauce | 0.600 | 0.0126 |
| Oyster Sauce | 0.573 | 0.0154 |
| Plum Sauce | 0.121 | 0.0046 |
| Shrimp Paste | 0.382 | 0.0069 |
| Soy sauce | 0.130 | 0.0061 |
| Animal Feed | 0.963 | 0.009 |

[a]All p-values <0.05 were considered statistically significant.

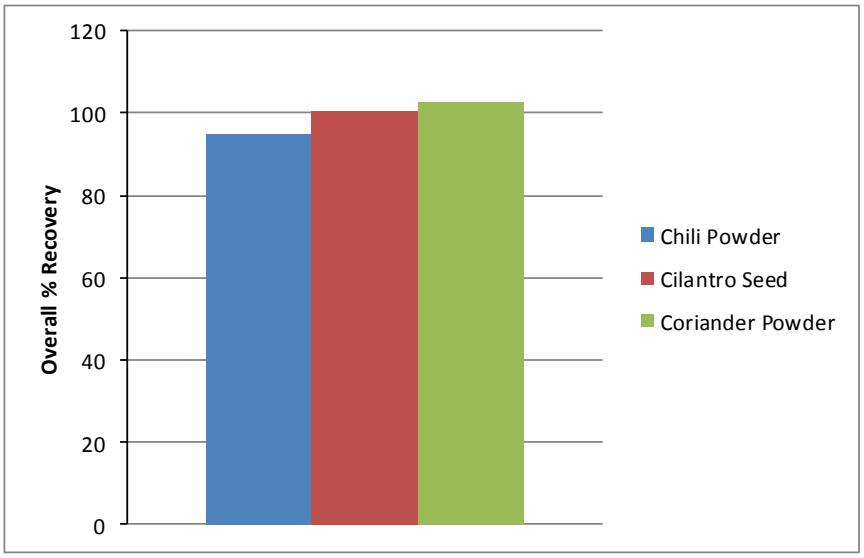
Since the commodities did not exhibit matrix interferences, we performed spike and recovery studies. The commodities under evaluation were spiked at three different levels of $AFB_1$ and the recovery was determined. Common cooking spices, which include chili powder, cilantro seed, and coriander powder all exhibited excellent recoveries of 88.3-110.1% at each spike level (Table 2). Furthermore, the overall % recoveries for chili powder, cilantro seed, and coriander powder were 95.0%, 100.6%, and 102.6%, respectively (Figure 2).

**Table 2 - Method precision and recovery of aflatoxin $B_1$ in common spices**

| Matrix | Spike level[a] (mg/kg) | Repeatability (%CV) | Recovery (%) |
|---|---|---|---|
| Chili Powder | 2.5 | 7.21 | 100.48 |
| | 5 | 3.57 | 96.20 |
| | 10 | 3.03 | 88.28 |
| Cilantro Seed | 2.5 | 5.24 | 95.48 |
| | 5 | 3.57 | 96.20 |
| | 10 | 2.76 | 110.08 |
| Coriander Powder | 2.5 | 3.96 | 99.44 |
| | 5 | 5.49 | 100.30 |
| | 10 | 7.92 | 108.02 |

[a]Five replicates at each level.

**Figure 2 - Overall % recovery of aflatoxin $B_1$ from spices**



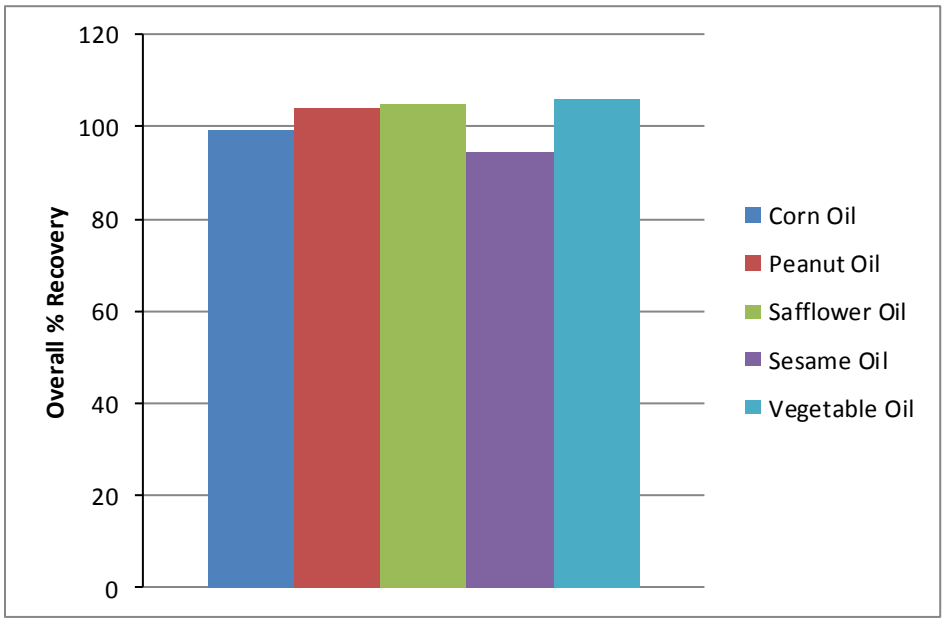

Furthermore, we investigated cooking oils frequently found in households, including corn oil, peanut oil, safflower oil, sesame oil, and vegetable oil. In initial studies, we extracted the spiked oils at the standard extraction protocol of 10 minutes. However, the % recoveries achieved ranged from 54-72% (data not shown). By extending the extraction time to 30 minutes, we were able to achieve better recoveries. The % recoveries at each level for each commodity ranged from 80.3-116.4% (Table 3). For corn oil, peanut oil, safflower oil, sesame oil, and vegetable oil, the overall % recoveries were 99.0, 103.7, 104.7, 94.4, and 105.9%, respectively (Figure 3). Augmenting the extraction time permitted the extraction solvent to mix thoroughly with the viscous sample and allowed for better recovery.

**Table 3 - Method precision and recovery of aflatoxin $B_1$ in cooking oils**

| Matrix | Spike level[a] (µg/kg) | Repeatability (%CV) | Recovery (%) |
|---|---|---|---|
| Corn Oil | 10 | 9.08 | 97.23 |
| | 20 | 7.26 | 87.53 |
| | 40 | 9.21 | 112.30 |
| Peanut Oil | 10 | 3.87 | 108.12 |
| | 20 | 9.80 | 89.58 |
| | 40 | 5.91 | 113.45 |
| Safflower Oil | 5 | 4.62 | 107.64 |
| | 10 | 6.33 | 93.00 |
| | 20 | 6.54 | 113.49 |
| Sesame Oil | 5 | 5.71 | 101.84 |
| | 10 | 6.26 | 80.30 |
| | 20 | 4.82 | 101.07 |
| Vegetable Oil | 5 | 4.85 | 107.88 |
| | 10 | 11.83 | 93.30 |
| | 20 | 4.70 | 116.44 |

[a]Five replicates at each level.

**Figure 3 - Overall % recovery of aflatoxin $B_1$ from cooking oils**




The 30 minute extraction time was not limited to cooking oils. Several common cooking sauces required longer exposure to the extraction solvent to achieve better recoveries. The recovery at each level for each cooking sauce is shown in Table 4. In particular, BBQ sauce, dumpling sauce, hoisin sauce, hunan sauce, plum sauce, shrimp paste, and soy sauce exhibited overall recoveries of 101.88, 129.28, 105.18, 100.7, 115.82, 103.28, and 112.92%, respectively (Figure 4). Other sauces, such as char siu, chili, and oyster required 10 minutes or less of extraction time to achieve overall

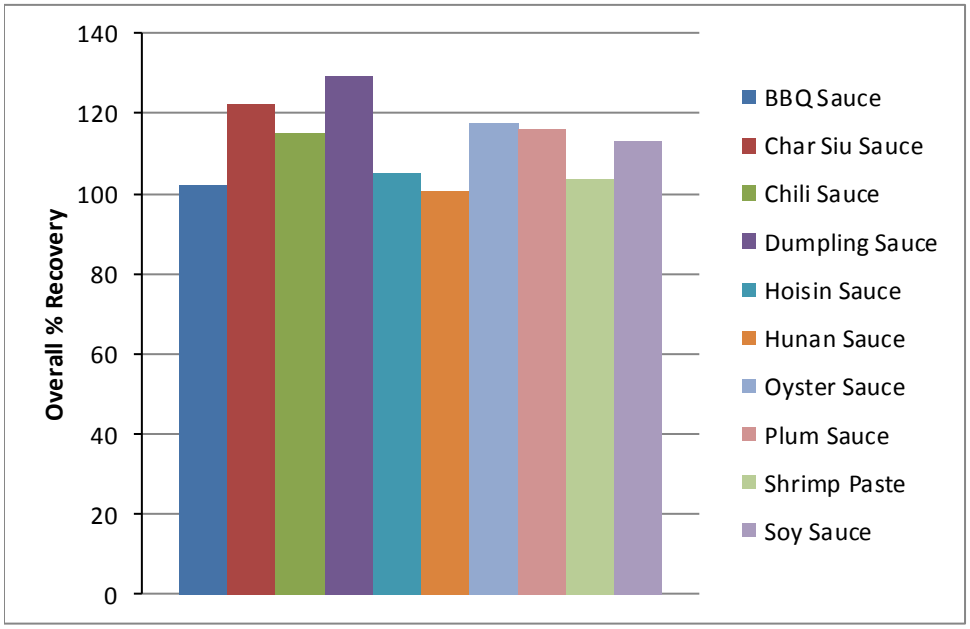
recoveries of 122.50, 115.0, and 117.63% (Figure 4). Overall, the extraction efficacy of cooking oils and sauces was enhanced by simply increasing the extraction time.

**Table 4 - Method precision and recovery of aflatoxin $B_1$ in common sauces**

| Matrix | Spike level[a] (µg/kg) | Repeatability (%CV) | Recovery (%) |
|---|---|---|---|
| BBQ Sauce | 2.5 | 9.18 | 105.03 |
| | 5 | 6.72 | 99.55 |
| | 10 | 7.81 | 101.06 |
| Char Siu Sauce | 2.5 | 9.87 | 120.10 |
| | 5 | 9.70 | 120.68 |
| | 10 | 8.29 | 126.72 |
| Chili Sauce | 2.5 | 6.55 | 115.96 |
| | 5 | 8.44 | 104.96 |
| | 10 | 3.88 | 124.27 |
| Dumpling Sauce | 2.5 | 7.73 | 157.76 |
| | 5 | 9.27 | 100.37 |
| | 10 | 7.05 | 129.70 |
| Hoisin Sauce | 2.5 | 6.98 | 99.57 |
| | 5 | 5.87 | 107.75 |
| | 10 | 6.15 | 108.22 |
| Hunan Sauce | 2.5 | 6.63 | 101.00 |
| | 5 | 2.86 | 105.75 |
| | 10 | 5.78 | 95.36 |
| Oyster Sauce | 2.5 | 9.09 | 116.05 |
| | 5 | 5.18 | 111.49 |
| | 10 | 6.95 | 125.37 |
| Plum Sauce | 2.5 | 9.55 | 107.06 |
| | 5 | 9.64 | 119.25 |
| | 10 | 6.03 | 121.15 |
| Shrimp Paste | 2.5 | 7.90 | 112.85 |
| | 5 | 8.47 | 93.23 |
| | 10 | 10.44 | 103.78 |
| Soy Sauce | 2.5 | 5.75 | 109.16 |
| | 5 | 7.44 | 103.63 |
| | 10 | 6.25 | 125.97 |

[a]Five replicates at each level.

**Figure 4 - Overall % recovery of aflatoxin $B_1$ from cooking sauces**




Finally, we evaluated the recovery of $AFB_1$ in animal feed spiked at 50µg/kg, 100µg/kg, and 200µg/kg. The % recoveries were 101.6, 104.9, and 113.6%, respectively (Table 5). The overall recovery of $AFB_1$ was 106.7% across the three concentrations. In short, the Aflatoxin $B_1$ Low Matrix ELISA can be used for routinely testing feed destined for animal consumption.

**Table 5 - Method precision and recovery of aflatoxin $B_1$ in animal feed**

| Matrix | Spike level[a] (mg/kg) | Repeatability (%CV) | Recovery (%) |
|---|---|---|---|
| Animal feed | 50 | 6.10 | 101.60 |
| | 100 | 4.84 | 104.88 |
| | 200 | 8.59 | 113.64 |
| | **Overall[b]** | **6.51** | **106.71** |

[a]Five replicates at each level.

[b]Overall results of 15 experiments at three levels of spiking.

## CONCLUSIONS:

We have successfully developed a single ELISA kit for the quantitative detection of $AFB_1$ in a wide selection of commodities. By modifying the existing standard protocol for each particular commodity, we have achieved good recoveries of $AFB_1$ in a wide selection of food products for human and animal consumption. In particular, the modification of extraction time was crucial to achieving better recoveries. The Aflatoxin $B_1$ Low Matrix ELISA kit is suitable for the routine screening of a large number of samples from a variety of commodities as there were no significant matrix interferences

in the commodities examined. The method performance was satisfactory with recoveries of 80% or better.

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