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Effect of Matrix Clean-Up for Aflatoxin Analysis in Corn and Dried Distillers Grains

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ABSTRACT

Aflatoxins are a group of highly carcinogenic mycotoxins that contaminate a wide variety of agricultural crops and have a detrimental economic impact on industries, such as corn and ethanol production. They are regulated by the FDA, and therefore, rapid, reliable cleanup techniques with low detection limits are needed for aflatoxins in a wide array of matrices. In this study the effect of using an immunoaffinity column versus simple filtering as a cleanup was tested for aflatoxins extracted from corn and Dried Distillers Grains (*DDG*). *The aflatoxins were analyzed by liquid chromatography tandem mass spectrometry* (*LC-MS/MS*). *The use of an immunoaffinity column resulted in greater signal-to-noise ratios* (*S/N*), *S/N of* 70 *vs S/N of* 5 *for corn, as well as fewer non-target peaks in the analysis. Recoveries of aflatoxin using immunoaffinity ranged from* 40% *to* 104.5% (*spiked substrate*) *and* 49% *to* 120% (*spiked extract*) *while percent recoveries of filtered samples ranged from* 84% *to* 119% (*spiked substrate*) *and* 88% *to* 119% (*spiked extract*). *This comparison study showed that filtering is acceptable for small sample sets or where rapid throughput is needed. However, for larger sample sets a more stringent cleanup method is necessary to ensure instrument performance.*

***Keywords*:** *Aflatoxin, LC-MS/MS, Immunoaffinity, SPE*

1. Introduction

Aflatoxins are a group of mycotoxins that are produced by several fungal species including the genus *Aspergillus*, most notably *A. flavus* and *A. parasiticus* [1-2]. The main aflatoxins are B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2) [2]. *A. flavus* is a ubiquitous fungus that has been found worldwide. It is a host pathogen known to infect such crops as corn, peanuts, and cotton [2]. This creates a regulatory issue with selling contaminated food products as aflatoxin B_1 has been found to be a potent carcinogen [3]. The FDA has set action levels for aflatoxins at 20 ppb (total aflatoxins) for foods designated for human consumption [4].

Aflatoxins are found worldwide [5-7], and contamination has a significant economic impact on corn crops within the United States. Southern states are especially impacted with losses due to aflatoxin contamination of corn each year [8] due to conditions that favor *A. flavus* growth. These conditions include drought stress, high temperatures during growing season, and insect damage that allows an entrance for the fungi [9].

Another economic sector impacted by aflatoxins is the ethanol industry, specifically the selling of dried distillers grains (DDG), which are a co-product of ethanol produced by fermentation of corn [10]. Ethanol production plants sell DDG as feed additives to increase profit margin. However, if contaminated corn is used as the feedstock, aflatoxins can be retained within the DDG [11]. This causes the DDG to be unsalable if addition to feedstuffs causes the combined product to exceed FDA limits, leading to a profit loss for the ethanol distillery [12].

Traditionally, aflatoxins have been detected using classical analytical methods such as thin layer chromatography [13]. Recently, the detection of aflatoxins has been moving towards analytical methods that can provide a higher throughput of samples such as enzyme-linked immunosorbent assay (ELISA) [14] and high-performance liquid chromatography (HPLC) coupled to fluorescence detection [15] or mass spectrometry (MS) [16]. With the advent of new column technology for HPLC systems, ultra high-pressure liquid chromatography (UHPLC) results can be achieved on a regular HPLC system, resulting in

faster analysis time. The use of these analytical methods, however, often requires sample cleanup. Immunoaffinity solid phase extraction (SPE) columns have become popular [17], as these columns are capable of greatly minimizing background detector noise while also reducing the chance of damaging a HPLC column. When coupled to LC-MS/MS, a sensitive and reliable detection method of aflatoxins is possible. The objective of this investigation was to compare the matrix removal capabilities of immunoaffinity SPE columns versus standard filter paper and determine when the use of each cleanup technique is appropriate.

2. Materials

2.1. Standards, Solvents, and Materials

Aflatoxin standards (AFB_1, AFB_2, AFG_1, AFG_2 and aflatoxin M_1 (AFM_1); >98% purity) were purchased from Sigma-Aldrich (Saint Louis, MO). Aflatoxin-free corn was obtained from the Mississippi State Chemical Laboratory. AflaCLEAN Immunoaffinity SPE columns and PBS buffer were obtained from Pickering Laboratories (Mountain View, CA). Optima grade methanol, acetonitrile, and water were purchased from Fisher Scientific (Fair Lawn, NJ). DDG were purchased from Sigma Aldrich (St. Louis, MO). Formic acid was purchased from Sigma Aldrich (St. Louis, MO). Whatman filter paper no. 1 was purchased from Fisher Scientific (Fair Lawn, NJ). BD 3 mL Luer-Lok Tip Syringes were purchased from Fisher Scientific (Fair Lawn, NJ). PTFE filters (0.45 μm) were purchased from Fischer Scientific (Fair Lawn, NJ).

2.2. LC-MS

An Agilent 1100 Liquid Chromatograph system (Santa Clara, CA) with a Phenomenex Kinetex Column (C18 150 × 4.6 mm i.d. with a particle size of 2.6 μm and a pore size of 100 Å) was used. Additionally, a HPLC Krud-Katcher Ultra Column In-Line Filter (0.5 μm Porosity × 0.004 in. ID) purchased from Phenomenex (Torrance, CA) was installed for added system protection. The mass spectrometer used was a Bruker Esquire (Billerica, MA) with an electrospray ionization (ESI) interface and ion trap.

3. Methods

3.1. Stock Solution

A stock solution of aflatoxins AFB_1, AFB_2, AFG_1, and AFG_2 was prepared at a concentration of 5 ppm and stored at 4˚C. A spiking solution was made from this stock solution by diluting an aliquot of the stock solution to 1 ppm. Spiked substrates were achieved by adding 1 mL of the spiking solution onto 5 g of corn/DDG and adjusting the extraction solvent to a final volume of 25 mL. Spiked extract samples were prepared by adding 0.2 mL spiking solution to 4.8 mL extract. A stock internal standard solution of AFM_1 was made by diluting an aliquot of the purchased standard (10 ppm) to 1 ppm and stored at 4˚C.

3.2. Cleanup Techniques

Spiked substrate samples (corn or DDG) were extracted using a modified method provided by Pickering Laboratories, Inc. Ground corn provided by the Mississippi State Chemical Laboratory or DDG were weighed out (5 g) into a 50-mL Falcon centrifuge tube and mixed with 25 mL of the extraction solvent, 80:20 methanol:water (v:v). This mixture was shaken for 15 min before being centrifuged at 3000 RPMs for 10 minutes. Samples were cleaned by either AflaCLEAN SPE or Whatman filter paper. For the AflaCLEAN samples, the extracted supernatant (1.4 mL) was mixed with 8.6 mL of PBS Buffer and passed through the SPE column on a vacuum manifold at a flow rate of 1 - 2 drops per second. After column loading, the immunoaffinity SPE column was washed with 10 mL of water before being eluted with 2 × 1 mL of methanol.

Spiked extract samples were obtained by spiking AFB_1, AFB_2, AFG_1, and AFG_2 into the extract of aflatoxin-free corn or DDG. The extracts were obtained according to the method described above, and collected after centrifugation. The spiked extract (1.4 mL) was mixed with 8.6 mL of PBS Buffer and passed through the AflaCLEAN column as described above.

Whatman spiked extract and spiked substrate samples, were gravity filtered using Whatman filter paper # 1. All samples, whether cleaned via SPE or Whatman, were filtered with a 0.45 μm PTFE filter prior to LC-MS/MS analysis. PTFE filtering showed no effective aflatoxin loss (data not shown). AFM_1 was used as an internal standard at a concentration of 50 ppb. AFM_1 was chosen as the internal standard as it is the metabolite of AFB_1 found in milk thus, there should be no AFM_1 found in these matrices [18].

3.3. LC-MS/MS Protocol

A solvent gradient program was used to maximize the signal-to-noise ratio (sensitivity). The solvents used were as follows: Solvent A-water, Solvent B-acetonitrile. Both solvents contained 0.1% formic acid by volume. The gradient used was: 0 min - 0.5 min—90% A, 0.51 min —50% A, 2.0 min - 9.0 min—20% A, 9.01 min -10 min —90% A. Mass Spectrometer settings were adjusted so that sensitivity was maximized. The conditions were as follows: Capillary: –4000 V, End Plate Offset: –500 V, Nebulizer: 30 psi, Dry Gas: 12 L/min, Dry Temp: 300˚C, scan range: 200 - 360 *m/z*, averages: 3. MS/MS was used

for absolute identification of aflatoxins and to further increase sensitivity. This can be seen in **Table 1**.

3.4. Standard Curves

In-matrix standard curves (corn Whatman, DDG Whatman, corn SPE, and DDG SPE) were produced with concentrations at 5, 25, 75, 250, and 500 ppb (3 replicates at each level). Each standard curve had AFM_1 added to each point for a final concentration of 50 ppb. Before AFM_1 was added, each point in each standard curve was filtered through a 0.45-μm PTFE filter.

3.5. Data Analysis

Limit of Detection (LOD) was determined by following the guidelines outlined in *Code of Federal Regulations*, Part 136, Appendix B [19]. LOD calculations were determined from a replicate set of n = 7 at a concentration of 5 ppb for each standard curve. Percent recoveries were calculated by dividing the amount of aflatoxin in each sample by aflatoxin amount calculated from spiked matrix. Signal-to-noise ratios were calculated by dividing the analyte signal by the background noise signal. Statistics were calculated for ANOVA Table (α = 0.05) and statistical difference was determined using *least squares means analysis* in Statistical Analysis Software (SAS) 9.2.

4. Results

Figure 1 shows the chromatographic separation and analysis of AFB_1, AFB_2, AFG_1, and AFG_2, and AFM_1 (internal standard) at a concentration of 75 ppb (50 ppb AFM_1) by LC-MS/MS. While baseline separation was not achieved for the aflatoxins, this was not a concern since this method was designed to be a rapid detection method and each had unique precursor and daughter ions. The elution order for the aflatoxins is (**Table 1**): AFB_1 (3.5 min), AFB_2 (3.4 min), AFG_1 (3.4 min), and AFG_2 (3.2 min), and AFM_1 (3.2 min). Limit of Detection (LOD) studies were performed for each of the four aflatoxins. These are instrument LODs and not method LODs. This can be seen in **Table 2**. LODs for Afla-CLEAN SPE columns (0.53 - 6.47 ppb for corn and 4.37 - 14.36 ppb for DDG) were generally lower than Whatman LODs (5.00 - 21.84 ppb for corn and 4.81 - 20.90 ppb for DDG). The pH was checked for each matrix extract (corn and DDG) and both were found to be in a range from 6.5 to 7.5. This is important because pH can play a role in ion enhancement or suppression in mass spectrometry. Since the pH was essentially neutral, no ion enhancement or suppression was thought to have occurred.

Method efficiencies (spiked substrate) were calculated from an n = 3 for each aflatoxin (AFB_1, AFB_2, AFG_1, and AFG_2) and can be seen in **Table 2**. These are a measure of how effective the method is from beginning to end of extracting aflatoxins from a particular matrix. They were from 84% to 119% (Whatman) and 40% to 104.5% (AflaCLEAN). Column efficiencies (spiked extract) were calculated from an n = 3 for each aflatoxin and can be seen in **Table 2**. These are a measure of how effective the column is releasing the aflatoxins during the elution step. They were from 88% to 119% (Whatman)

Table 1. MS/MS results for aflatoxins B_1, B_2, G_1, G_2, and M_1 (internal standard).

Aflatoxin	Time (min)	Precursor *m/z*	Product *m/z*
B_1	3.5	313	285.0, 298.0
B_2	3.4	315	259.0, 287.0, 297.0
G_1	3.4	329	243.0, 283.0, 301.0, 311.0
G_2	3.2	331	285.1, 303.1, 313.1
M_1	3.2	329	259.1, 273.1

Table 2. Percent Recoveries for Aflatoxins B_1, B_2, G_1, and G_2.

	B_1	B_2	G_1	G_2
		Corn Whatman		
spiked extract[a]	102 ± 15.49	94 ± 0.71	91 ± 7.07	101 ± 2.83
spiked substrate[b]	110 ± 0.07	93 ± 18.38	99 ± 18.68	101 ± 2.4
LOD[c]	8.08	21.84	10.56	5.00
		DDG Whatman		
spiked extract[a]	119 ± 8.49	105 ± 18.38	88 ± 43.72	108 ± 23.33
spiked substrate[b]	90 ± 1.41	118 ± 14.14	84 ± 1.41	119 ± 10.61
LOD[c]	10.62	20.90	4.81	16.19
		Corn Afla-CLEAN		
spiked extract[a]	120 ± 21.78	74 ± 9.07	108 ± 24.58	54 ± 6.11
spiked substrate[b]	104.5 ± 7.78	67 ± 9.9	88.5 ± 2.12	63 ± 5.66
LOD[c]	4.81	3.11	0.53	6.47
		DDG Afla-CLEAN		
spiked extract[a]	75 ± 23.97	49 ± 11.06	72 ± 27.07	54 ± 31.66
spiked substrate[b]	74 ± 10.07	40 ± 5.86	57 ± 3.00	59 ± 20.22
LOD[c]	7.98	14.36	9.93	4.37

[a]Column efficiency, [b]Method efficiency, [c]Instrument Limit of Detection in ppb [19].

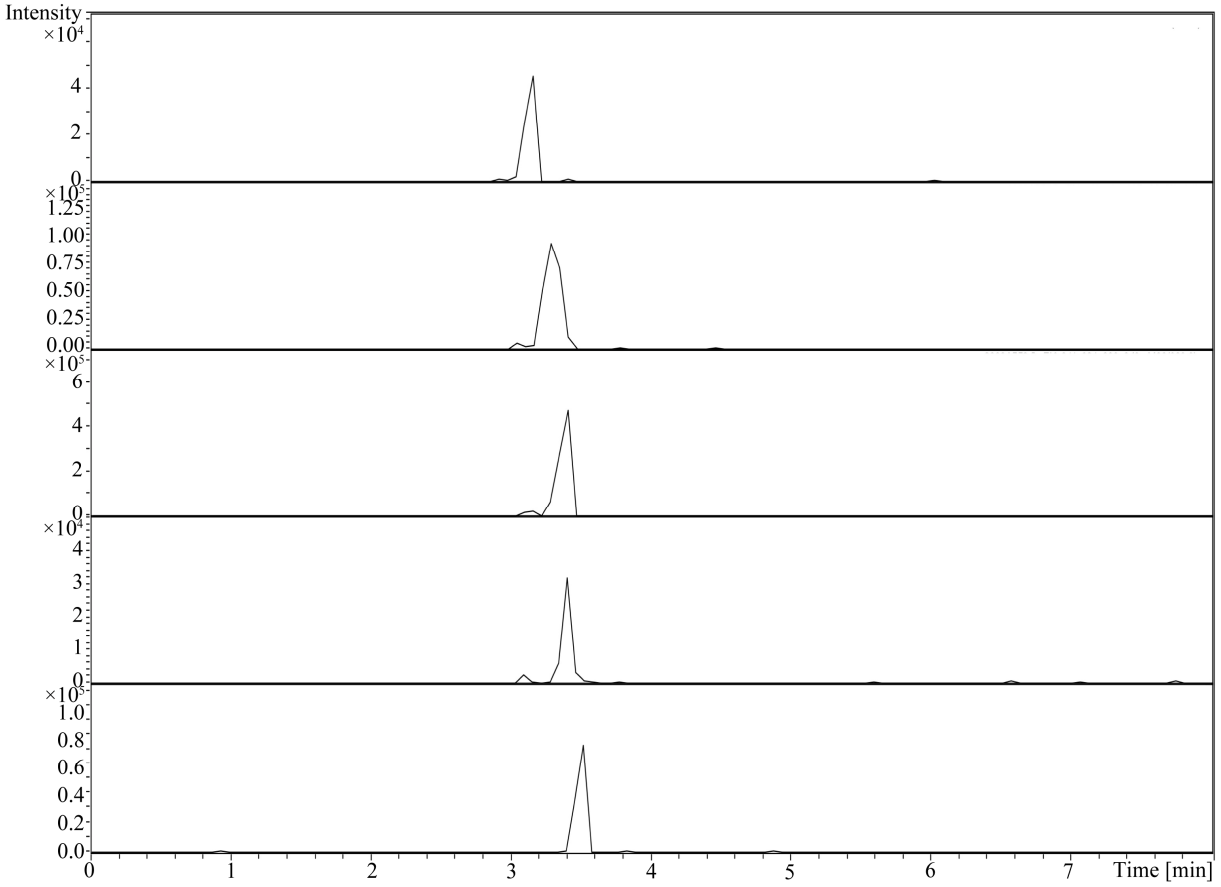

Figure 1. LC-MS/MS analysis of aflatoxin standard. Each aflatoxin (top to bottom: M_1, G_2, G_1, B_2, and B_1) is at a concentration of 75 ppb (M_1 is at 50 ppb). The elution is as follows: M_1-3.2 min, G_2-3.2 min, G_1-3.4 min, B_2-3.4 min, B_1-3.5 min Scales are different due to each aflatoxin has a different response factor.

and 49% to 120% (AflaCLEAN). From the results, it can be seen that corn SPE (spiked extract and spiked substrate) have higher percent recoveries (54% to 120%) when compared to DDG SPE spiked extract and spiked substrate (40% to 75%). From the statistical analysis, it was shown that there was a significant difference (α = 0.05) in the percent recoveries (corn and DDG SPE) for AFB_1 and AFB_2 but not AFG_1 and AFG_2 for spiked extract and spiked substrate. Statistical analysis for Whatman samples (spiked extract and substrate) showed no significant difference for AFG_1 and AFG_2 and no clear significant difference for AFB_1 and AFB_2. AFB_2 and AFG_2 Whatman samples were all significantly different (α = 0.05) from the SPE samples.

This comparative study was performed between the capabilities of Whatman filter paper and the AflaCLEAN immunoaffinity SPE column for cleanup of aflatoxins extracted from corn and DDG. Data for this can be seen in **Figures 2** and **3**. The corn matrix showed higher background noise (approximately 14-fold increase for AFB_2) for the Whatman cleanup when compared to the immunoaffinity SPE cleanup. Additionally, for the DDG matrix, higher background noise (approximately 35-fold increase for AFG_2) was seen for DDG cleaned with Whatman filter paper versus the immunoaffinity SPE column. Overall, samples cleaned with the immunoaffinity SPE column had a higher signal-to-noise ratio (S/N) over Whatman filter paper (S/N of 70 versus S/N of 5 for corn and S/N of 70 versus S/N of 2 for DDG, respectively).

5. Discussion

While baseline separation of the aflatoxins was not achieved, coelution of compounds is acceptable when using tandem mass spectrometry as this technique allows for definitive identification [20,21] and has been used in such fields as proteomics [22], pesticide analysis [23], and forensics [24]. Compounds are detected after being ionized and forming a specific mass-to-charge ratio (*m/z*). The mass spectrometer is capable of detecting multiple ions simultaneously, which are unique to each analyte. LOD were calculated for how low the instrument can

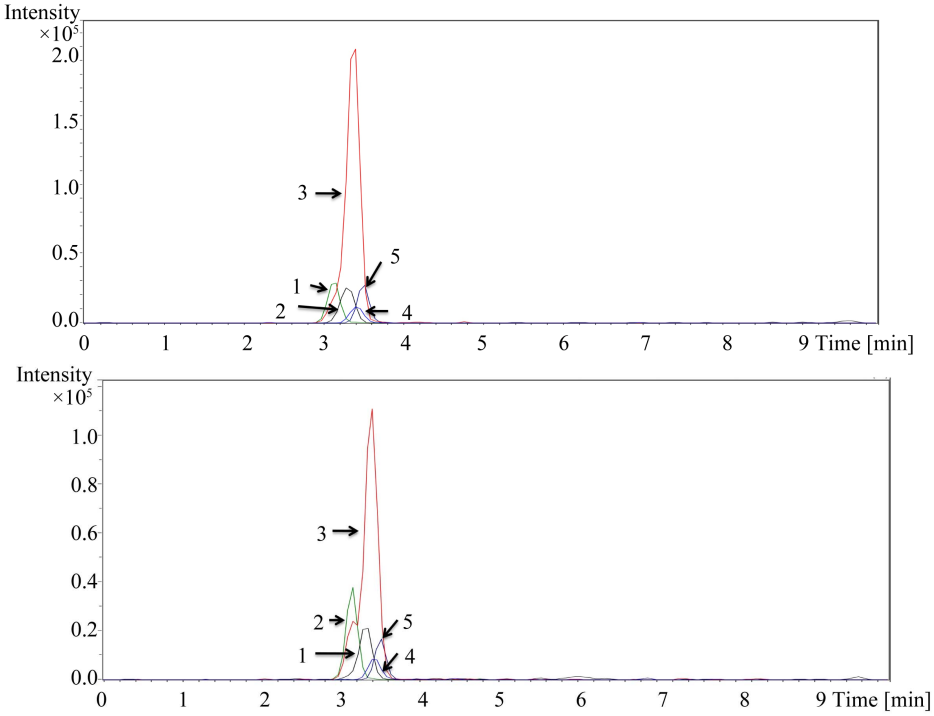

Figure 2. Differential analysis for immunoaffinity column cleanup for aflatoxins B_1-peak 5, B_2-peak 4, G_1-peak 3, G_2-peak 2, and M_1-peak 1 in corn (top) and Dried Distillers Grains (bottom). Both corn and DDG had a Signal-to-Noise ratio of 70.

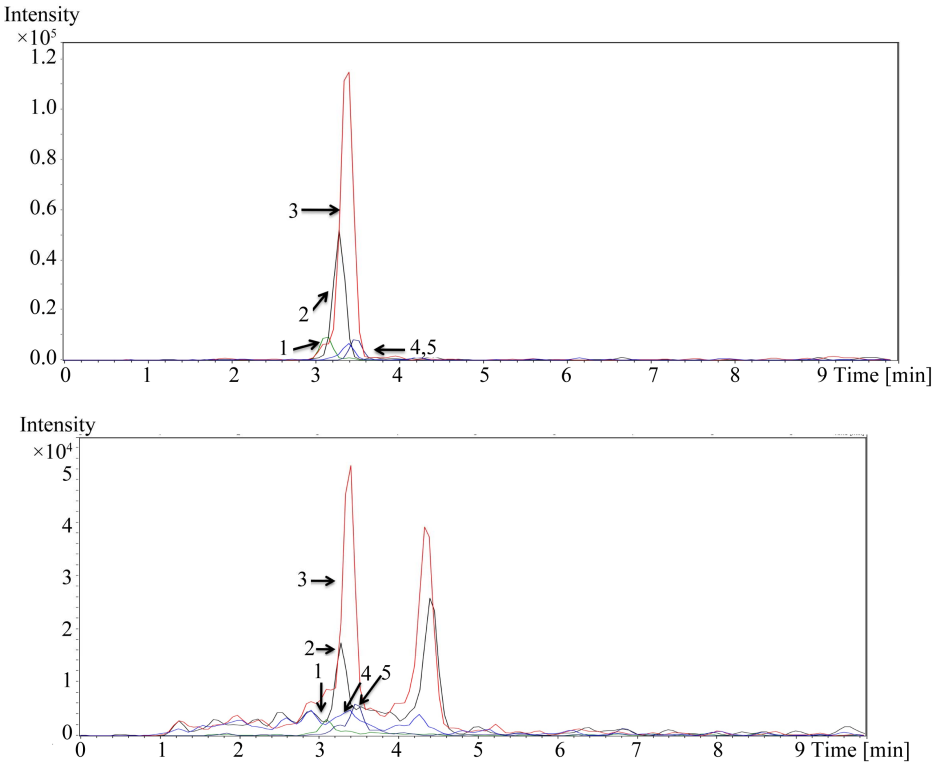

Figure 3. Contrast for whatman paper cleanup for aflatoxins B_1-peak 5, B_2-peak 4, G_1-peak 3, G_2-peak 2, and M_1-peak 1 in corn (top) and Dried Distillers Grains (bottom). Corn had a Signal-to-Noise ration of 5 versus a Signal-to-Noise ratio of 2 for DDG.

detect, but not the method limits of detection, which quantifies how low the method can detect. LOD for the aflatoxins were comparable to other detection methods designed for aflatoxins (LOD averaged among all four aflatoxins): LC-MS: 0.467 ppb [25] and ELISA: 2.5 ppb [26].

Spiked corn extract efficiencies (column efficiency) for AflaCLEAN SPE were calculated for all aflatoxins and shown to be greater than 74% (except AFG_2) indicating that the immunoaffinty column is not retaining the aflatoxins beyond the final elution step. It should be noted that column efficiencies have to be calculated from spiked matrix and not pure standards (made in methanol, the eluting organic solvent) as pure standards will not be retained on column. Column effiencies achieved in this study are comparable to other immunoaffinity SPE columns such as AFLASCAN, AFLA-RHONE, AflaTest, and AFLAPREP. For example, AflaTest column efficiencies for corn were calculated through the use of spiked sample extract and were: AFB_1, $AFG_1 \geq 90\%$, $AFB_2 \geq 85\%$, and $AFG_2 \geq 80\%$ [27]. However, for the DDG SPE spiked extract efficiencies, they were lower than 75% suggesting that the aflatoxins are not being released from the column. AFG_2 spiked extract efficiency was significantly lower than the other aflatoxins. This is because the binding affinity of AFG_2 to the antibodies within the immunoaffinity column seems to be lower than the other aflatoxins. This trend is seen in other immunoaffinity columns, not just the one produced by Pickering Laboratories: 17.7% (pH dependent) and 53.7% [28,29]. Spiked substrate efficiencies (method efficiency) for DDG were lower than those seen in corn, 57% vs 88.5% for aflatoxin AFG_1, respectively. Again, this could be due to aflatoxins not being released by the column due to the DDG matrix.

Before the inclusion of AFM_1 as the internal standard, percent recoveries for Whatman filter paper samples were lower than 60%. An internal standard was used to correct for ion suppression effects of the matrix. After the inclusion of AFM_1 as the internal standard, percent recoveries for corn samples (spiked extract and spiked substrate) rose to around 100%. However, for the DDG samples, percent recoveries ranged from 100% to 450% (data not shown for these recoveries). The theorized reason for this is that ion suppression is occurring for the aflatoxins in the DDG matrix and the internal standard is correcting for this. When this is coupled to using a standard curve without ion suppression (standards made up in pure methanol), very large percent recoveries were seen. To correct for this, the standard curves were switched from being made in pure methanol to matrix-match standards (standards made up with the matrix extract being used as the diluent). This solved the high percent recoveries seen for the Whatman filter paper samples. Due to this, AflaCLEAN SPE match standard curves were made up as well.

The use of AflaCLEAN SPE for matrix removal has been reported over a broad range of matrices: peanut butter (HPLC/Fluorescence detection) [30], wheat bran (HPLC/Fluorescence detection) [31], and sake and wheat beer (LC-MS/MS) [32]. However, this is the first study showing the effectiveness for DDG cleanup. Additionally, this comparison study shows that the immunoaffinity SPE column cleanup is superior in eliminating background noise versus Whatman filter paper. While the difference between the two techniques was not as profound for corn samples, there was a large amount of background noise for the DDG not removed by the Whatman filter paper. The immunoaffinity SPE column was superior for DDG cleanup versus the Whatman filter paper cleanup. This is most likely due to the removal of matrix effects that the Whatman filter paper is unable to capture. However, as can be seen from **Table 2**, percent recoveries for the two methods are comparable. Therefore, it was concluded that even though Whatman filter paper had higher background versus the immunoaffinity column, it was acceptable for cleanup of small sets of samples. Another area that Whatman filter paper cleanup would be useful is quick screening of samples for aflatoxins as this is a faster cleanup method than immunoaffinity SPE. For larger sets of samples or for sensitive instruments, it was concluded that using SPE was a better choice. This is due to the continued analysis of samples using Whatman filter paper as cleanup led to the instrument becoming extremely dirty, both on the LC column (pressure increase was seen which returned to normal after continued flushing of column) and the front of the mass spectrometer. If larger sample sets using Whatman filter paper for cleanup are to be analyzed, it is encouraged that frequent cleaning and flushing of the system is to be performed. From the results, both cleanup methods are useful for sample cleanup for aflatoxin analysis with the choice of which one to use depending on several factors (sample set size, sensitivity of instrument, time).

6. Safety

Aflatoxins are carcinogenic compounds that should be handled carefully at all times. Any handling of aflatoxins should be done using gloves and a lab coat. Any spills should be neutralized with a 10% bleach solution.

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