Development and Evaluation of a Minicolumn Assay for the Detection of Aflatoxin M₁ in Milk

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ABSTRACT

A practical field method for the chemiselective immobilization and detection of aflatoxin M₁ in milk has been developed and is being marketed. In this new method, aflatoxin M₁ is selectively adsorbed at the interface of a layer of neutral sand and a band of magnesium silicate (Florisil) packed in a glass minicolumn. Aflatoxin M₁ at ≥0.5 ppb in contaminated milk, can be easily visualized as a band of bright blue fluorescence. Briefly, raw or homogenized and pasteurized milk is diluted with water (1:1, vol/vol) and passed through a C₁₈ cartridge. Aflatoxin M₁ is then partitioned by polarity, eluted with acetone-methylene chloride, and added to the minicolumn. The minicolumn is washed and viewed under long wave UV light. The limit of detection for this assay was 0.2 ppb, which was similar to the 0.3 ppb obtained using an immunoaffinity column, followed by minicolumn detection. The assay was accurate, rapid, easy to perform, and stable.

(Key words: aflatoxin M₁, milk detection, minicolumn assay)

Abbreviation key: AF = aflatoxin (used with B₁, M₁, and Q₁), CSID-M₁ = chemiselective immobilization and detection of AFM₁, LD₅₀ = median lethal dose.

INTRODUCTION

Aflatoxins belong to a group of secondary fungal metabolites known as mycotoxins and are commonly produced by Aspergillus flavus or Aspergillus parasiticus as contaminants of a diverse variety of human foods and animal feeds (1). Following the ingestion of aflatoxin-contaminated feed by lactating dairy cows, aflatoxin M₁ (AFM₁) is secreted into the milk; AFM₁ is a major metabolite of parent AFB₁ that is formed by enzymatic hydroxylation at the 9α-position (Figure 1) and has an approximate overall conversion rate equal to 1 to 3% (1). Consequently, exposure to aflatoxins can occur directly through contaminated foods or indirectly by AFM₁-contaminated milk.

Early studies on the biological effects of aflatoxins in the bovine were directed toward the testing of acute toxicological response. Thus, little information is available on the effects of aflatoxins on physiological processes and metabolism (2). Studies (2) have indicated that bovine liver homogenates can produce significant amounts of AFM₁ and AFQ₁. Rumen metabolism of AFB₁ is reportedly associated with microbial biotransformation (2).

Toxicity to dairy cattle from ingestion of AFB₁-contaminated feed is characterized by anorexia, decreased milk production, lethargy, liver damage, and edema of the abdominal cavity (12). Also, AFB₁ elicits suppression of the immune system, resulting in illness and even death (6). In humans, high concentrations of AFB₁ have been associated with vomiting, abdominal pain, anorexia, and jaundice (13); centrilobular necrosis and fatty liver were also observed in these cases. In some laboratory animals, AFM₁ was equal in toxicity to AFB₁. In rats, the median lethal dose (LD₅₀) for AFM₁ was 1.5 mg/kg; the reported LD₅₀ for AFB₁ ranges from 1 to 18 mg/kg. Both mycotoxins produce significant liver damage (3, 4, 11, 14).

Another major concern with aflatoxins is their potent carcinogenicity. Recently, AFB₁ was categorized as a class 1 human carcino-
Figure 1. The structures of aflatoxin M₁ (AFM₁) and aflatoxin B₁. The only structural difference between the two compounds is the presence of the hydroxyl group at the 9α-position of AFM₁. Both molecules have the 8,9-double bond, which is the putative active site of the molecule.

gen; AFM₁ has been categorized as a class 2B (or probable) human carcinogen (15). Studies with laboratory animals have shown that AFB₁ and AFM₁ are carcinogenic in trout and rats and that AFB₁ is carcinogenic in nonhuman primates (7, 8, 16, 17, 18, 20). These findings suggest that food and feed contamination with aflatoxins is a potential problem. The AFM₁ is of particular concern because infants and children, who are uniquely vulnerable and are potentially more sensitive to aflatoxins than adults, are major consumers of milk.

Despite efforts to test animal feed for aflatoxins and to divert contaminated feed from dairy cattle, the problem of milk contaminated by AFM₁ still exists, partly because of the sampling error caused by aflatoxin hot spots. Hot spots are caused by variable growth of fungi and uneven distribution of aflatoxin in the sample. Feed can be sampled and tested and appear to be aflatoxin-free because only portions are contaminated. Corbett et al. (5) showed that feed was carefully sampled and tested to be negative for aflatoxins, but milk from dairy cows consuming the feed was positive for AFM₁. Because milk is homogeneous, sampling is not a problem. Therefore, testing of milk for AFM₁ can be used to monitor indirectly the aflatoxins in feed (5).

Occurrence of AFM₁ in milk in the US is common. Various methods for reduction of AFM₁ in contaminated milk have been reported (6); however, milk that has been treated to reduce AFM₁ is considered to be adulterated and is therefore not acceptable for sale. Therefore, AFM₁ must be prevented from entering the food chain via strategies for practical detection and diversion of contaminated milk supplies. Current FDA guidelines (6) allow .5 ppb of AFM₁ in fluid milk and 20.0 ppb of total aflatoxins in dairy cattle feed. Rapid, practical, and chemically stable techniques (capable of detecting .5 ppb of AFM₁) are required to test milk on site at individual dairy farms and dairy processing plants. In this paper, we report the development and evaluation of a minicolumn assay for chemiselective immobilization and detection of AFM₁ (CSID-M₁) that is accurate, does not require refrigerated storage, and is simple to use.

MATERIALS AND METHODS

Minicolumn

The CSID-M₁ minicolumns were packed with various adsorbent materials and neutral sand in borosilicate disposable blood bank dropper tubes (14.37 cm in length) that were screened for indigenous fluorescence. Only those pipets that did not fluoresce under long wave UV light (365 nm) were selected. Scoops were made from plastic pipet tips, sectioned diagonally with a razor, and were utilized to dispense the adsorbent materials. The cleanup layer consisted of alumina (activity grade I); 10% water was added to obtain activity grade IV. The aflatoxin detection band and control band consisted of magnesium silicate. For testing, minicolumns were packed according to the following configuration. The tip of the pipet was plugged with a polyethylene disc followed
MINICOLUMN ASSAY FOR AFLATOXIN M₁

Chemicals

Aflatoxin M₁, alumina (acid activity grade I), and Florisil (magnesium silicate at 100 to 200 mesh) were purchased from Sigma Chemical Co. (St. Louis, MO). Washed sea sand was obtained from Fisher Scientific (Fair Lawn, NJ). Polyethylene discs were obtained from Porex Technologies Corp. (Fairbourn, GA). Aflatest-10™ affinity columns were purchased from Vicam (Watertown, MA), and C₁₈ cartridges were purchased from Water’s Associates, (Milford, MA). Spectral grade solvents were used throughout this study, and all other reagents were of the highest purity commercially available.

CSID-M₁ Analysis

A mixture of 20 ml of raw or homogenized and pasteurized milk and 30 ml of water was loaded into a 60-ml syringe. A C₁₈ cartridge was preconditioned by elution with 10 ml of methanol followed by 10 ml of water. The cartridge was then attached to the syringe, and the sample was pushed through at a flow rate of approximately 1 drop per s. To remove the excess water, a 60-ml syringe was filled with air, which was forced through the cartridge twice. The cartridge was then washed with 10 ml of water and 20 ml of hexane. Next, air was forced through the cartridge again, and AFM₁ was eluted with 1 ml of 2.5% acetone in methylene chloride; .5 ml of eluate was collected in a test tube that was previously calibrated to .5 ml with a pipette. A minicolumn was prewetted by careful addition of 1 ml of 2.5% acetone in methylene chloride to prevent the formation of air bubbles. Next, the eluate was added to the minicolumn. The flow rate (1 drop/s) was enhanced by pressurization with a syringe. The minicolumn was washed with .5 ml of 2% methanol in methylene chloride and viewed under long wave UV light; positive samples were detected by the presence of a blue fluorescence at the target interface between sand and Florisil. Naturally contaminated milk (.3 ppb) was used in this experiment. The concentration of AM₁ was confirmed by a modified Takeda extraction (19) and reverse-phase HPLC (9). Milk was augmented or diluted to yield a range of concentrations (i.e., .5, .4, .2, and .1 ppb), and three replicate assays were performed at each concentration.

Immunaffinity Column

Vicam affinity columns were used in the initial cleanup phase of milk samples following a modified method of Hansen (9). Milk (25 ml) was passed through the affinity column followed by washing with two 10-ml portions of 10% methanol in water. The AFM₁ was eluted with 1 ml of 80% methanol. The eluate was dried under nitrogen and redissolved in .5 ml of 2.5% acetone in methylene chloride.

Figure 2. Schematic of the minicolumn assay for chemoselective immobilization and detection of aflatoxin M₁ in milk. The cleanup layer is alumina, activity IV. The detection and control bands are Florisil.
This extract was applied to a CSID-M$_1$ minicolumn that had been wetted with 2.5% acetone in methylene chloride. The minicolumn was rinsed with .5 ml of 2% methanol in methylene chloride and viewed under a long wave UV light. Positive samples were detected by a blue fluorescence at the target interface. The detection limit of this assay was determined and compared with the CSID-M$_1$ procedure. Milk samples used in this study were identical to those used in CSID-M$_1$ minicolumn analysis. Three replicates were performed at each concentration.

HPLC Analysis of AFM$_1$

Reverse-phase HPLC was used to determine the percentage of recovery of AFM$_1$ and to measure concentrations of AFM$_1$ in milk samples quantitatively (10). A solution of AFM$_1$ in water was standardized and used to establish a curve for the HPLC and to allow extrapolation of AFM$_1$ concentrations in milk samples. The analysis was conducted on a Water's HPLC system consisting of a pump (model 6000), an autosampler (model 710), and a radially compressed C$_{18}$ column (10 $\mu$m). The results were recorded on a Water's data module at 1 cm/min. Authentic AFM$_1$ was detected by fluorescence on a Water's fluorescence detector (model 420; excitation at 365 nm and emission at 425 nm). The mobile phase consisted of methanol:acetonitrile:water (15:15:70, vol/vol/vol) at a flow rate of 2 ml/min.

Milk was purchased at various locations in College Station, Texas or obtained for research purposes from the Texas Department of Health.

To verify the AFM$_1$ concentration, samples were extracted by a modified Takeda method (19) and analyzed by reverse-phase HPLC. Briefly, a C$_{18}$ cartridge was preconditioned with 10 ml of acetonitrile followed by 10 ml of deionized water. A mixture of 30 ml of water and 30 ml of milk was loaded onto the C$_{18}$ cartridge using a 60-ml disposable syringe. The diluted milk was passed through the cartridge with a flow rate $\leq$10 ml/min. The C$_{18}$ cartridge was washed with 10 ml of basic 10% acetonitrile (1:10:90, vol/vol/vol, ammonium hydroxide:acetonitrile:water), followed by a second wash of 10 ml of acidic 10% acetonitrile (1:10:90, vol/vol/vol, acetic acid:acetonitrile:water). These washes were discarded. The AFM$_1$ was eluted with 5 ml of acidic 30% acetonitrile (1:30:70, vol/vol/vol, acetic acid:acetonitrile:water). This eluate was collected in a test tube and extracted twice with five drops of saturated sodium chloride solution and 2 ml of methylene chloride. After centrifugation, the methylene chloride layer was transferred to a test tube and dried under nitrogen. The dried extracts were redissolved in 100 $\mu$l of acidic 30% acetonitrile solution. The tubes were vortexed, and the contents were transferred to a Water's limited insert for HPLC analysis. Seventy-five microliters were injected onto the HPLC.

Stability of the CSID-M$_1$ Minicolumn

Twenty minicolumns were prepared and divided into two groups. The first group was stored under ambient conditions, and the second group was stored in a desiccator containing SiO$_2$ (Sigma Chemical Co., Cherry Hill, NJ) to determine the effect of relative humidity on the performance of the minicolumn. After 2 wk, three minicolumns from each group were sampled and tested following the CSID-M$_1$ minicolumn assay. The study was repeated after 6 and 20 wk of storage. Before the CSID-M$_1$ minicolumn assay was performed, the AFM$_1$ concentration of milk was measured quantitatively using the modified Takeda method and reverse-phase HPLC. After the concentration of AFM$_1$ was established, milk was adjusted to approximately .5 ppb for testing.

Accuracy of the CSID-M$_1$ Minicolumn

The accuracy of the CSID-M$_1$ minicolumn was determined in a preliminary experiment by comparison with an established HPLC method of quantitative measurement of aflatoxin (10). Milk samples were extracted by the modified Takeda method (19) and analyzed by reverse-phase HPLC. The same milk samples were also analyzed by the CSID-M$_1$ minicolumn assay. The results of these two assays were compared to determine the accuracy of the CSID-M$_1$ minicolumn. The milk samples used in this experiment were naturally contaminated with AFM$_1$, except for one sample that was augmented to achieve .3 ppb.
RESULTS AND DISCUSSION

Detection Limits and Comparison of Cleanup Procedures

The CSID-M₁ minicolumns exhibited a relatively bright blue fluorescence at the interface of the detection band at .5 ppb of AFM₁. Results were positive (but less discernible) at .4, .3, and .2 ppb. Lower concentrations (i.e., .1 ppb) were not detected. Aliquots of these milk samples at concentrations of .5, .4, .3, .2, and .1 ppb were extracted with immunoaffinity columns and analyzed for AFM₁ using the CSID-M₁ minicolumn. Our results indicated that test concentrations of .5, .4, and .3 ppb of AFM₁ gave positive results but that lower concentrations were not discernible over the blank (Table 1). Increasing the volume of milk to 50 ml did not affect this limit. These findings indicate that the C₁₈ cartridge works as well as an immunoaffinity column for extraction and cleanup of whole milk samples in this analysis of AFM₁. In the antibody method, molecules containing a diketone moiety similar to that of AFM₁ may interfere with the assay. In milk, this interference would include an important component, diacetyl. Also, most assays that utilize immunoaffinity columns require that milk be defatted prior to analysis. In the CSID-M₁ assay (using a C₁₈ cartridge cleanup procedure), defatting of milk is not required.

The concentration of AFM₁ allowed in milk is currently set at .5 ppb by the FDA (6). Based on the results of this study, the CSID-M₁ minicolumn is capable of screening milk samples at diverse locations. The limit of detection of this assay possibly could be lowered even further by extraction and processing of a larger volume of milk. A positive sample detected using the CSID-M₁ assay must be confirmed and measured quantitatively by HPLC or TLC to delineate the extent of contamination and the problem. However, early detection and prevention of aflatoxin contamination may be possible by extensive screening of milk samples with rapid (20 to 30 min per sample) and cost-effective assays such as CSID-M₁.

Validation and Accuracy of the Minicolumn

The recovery of AFM₁ using the CSID-M₁ extraction procedure ranged from 58.7 to 76.0% at concentrations from .1 to 5 ppb of AFM₁. The variation in recovery may be due to the difficulty associated with the detection of very low concentrations. The fluorescence at the Florisil band was a function of the concentration of AFM₁. Increased concentrations of AFM₁ increase the intensity of fluorescence. However, at approximately ≥20 ppb of AFM₁, a steady state of fluorescence was observed; no further discernible increase in intensity occurred on the CSID-M₁ minicolumn. Figure 3 shows an example of CSID-M₁ minicolumns and the fluorescence associated with various concentrations of AFM₁.

Comparison of results from HPLC and CSID-M₁ assays showed that the CSID-M₁ assay was accurate. Milk samples that were contaminated at or above the detection limit of .2 ppb (based on quantitative measurement by HPLC) were positive when tested with the CSID-M₁ assay. Milk samples below the detection limit (based on quantitative measurement by HPLC) were negative in the CSID-M₁ minicolumn assay (Table 2). Based on these initial findings, CSID-M₁ is very accurate, resulting in no false positives or false negatives. The critical steps responsible for this

### Table 1. Study of the detection limits for aflatoxin M₁ (AFM₁) by the chemoselective immobilization and detection procedure¹ (CSID-M₁) and an immunoaffinity column procedure²

<table>
<thead>
<tr>
<th>AFM₁ Concentration (ppb)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>.50</td>
<td>+</td>
</tr>
<tr>
<td>.40</td>
<td>+</td>
</tr>
<tr>
<td>.30</td>
<td>+</td>
</tr>
<tr>
<td>.20</td>
<td>+</td>
</tr>
<tr>
<td>.10</td>
<td>+</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Milk samples at .5, .4, .3, .2, and .1 ppb were tested. Each sample was run in triplicate, and results of all three agreed. Positive samples were detected by a blue fluorescence on the detection band.

²Yicam Aflatest-IOTM columns were used for the extraction step. All samples were run in triplicate, and all three results agreed.

³Results are reported as positive (+) or negative (−) on the CSID-M₁ minicolumn.
Figure 3. Minicolumn assay for chemiselective immobilization and detection of aflatoxin M₁ (top); fluorescence of AFM₁ on the CSID-M₁ minicolumns (bottom). Standards of AFM₁ at 5, 3, 1, and 0 ppb (left to right) were added to the minicolumns.
TABLE 2. Determination of the accuracy of the chemiselective immobilization and detection (CSID-M1) minicolumn method for the detection of aflatoxin M1 in milk: comparison with an HPLC method.

<table>
<thead>
<tr>
<th>Sample</th>
<th>HPLC (ppb)</th>
<th>Minicolumn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>.80</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>.30</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>.35</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>&lt;.01</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>.20</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>&lt;.01</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>.06</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>.01</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>.03</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>.02</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>&lt;.01</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>.03</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>&lt;.01</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>.30</td>
<td>+</td>
</tr>
</tbody>
</table>

1 Results are expressed in parts per billion.
2 Results are reported as positive (+) or negative (-) for the CSID-M1 minicolumn.

Minicolumn accuracy are the elution of AFM1 from the C18 cartridge step and the final minicolumn wash. If a volume of >.5 to .6 ml is eluted from the cartridge, the recovery of potentially interfering compounds is enhanced. As shown in the HPLC chromatograph of an extract from the CSID-M1 procedure (Figure 4), other fluorescent compounds are recovered in the .5 ml of eluate from the C18 cartridge. These compounds are retained by the alumina cleanup layer at the top of the CSID-M1 minicolumn. If a large volume of 2% methanol in methylene chloride is added to the CSID-M1 minicolumn in the final washing step (> .8 ml), other interfering compounds may migrate to the Florisil band and result in a white fluorescence. Thus, the correct application of the final wash solution is very important in controlling the immobilization and movement of these interfering compounds on the CSID-M1 column.

Stability of CSID-M1 Minicolumns

After 2 and 6 wk, minicolumns that were stored in a desiccator in the laboratory worked as well as the columns stored under normal conditions prior to analysis of aflatoxin. The intensity of fluorescence on the Florisil band for AFM1 in milk was the same for both sets of columns. Columns were stable after 6 wk of unrefrigerated storage under both storage conditions. After 20 wk, the columns stored at ambient temperatures were as bright as con-
The reduced intensity of fluorescence on the CSID-MI minicolumns do not require refrigeration. The assay is rapid, cost-effective, and user friendly, which are attributes that make CSID-MI particularly useful for the field screening of milk. The CSID-MI assay was developed to screen samples of milk for aflatoxin contamination in the field as well as in the laboratory. The current concentration of AFM\textsubscript{1} allowed in milk in the US is .5 ppb, which can be easily attained with this method. The CSID-MI assay is accurate and chemically stable and does not require refrigeration. The assay is rapid, cost-effective, and user friendly, which are attributes that make CSID-MI particularly useful for the field screening of milk. The CSID-MI technology has been patented and licensed to TerraTek (Salt Lake City, UT). This assay should improve our ability to monitor milk for aflatoxin contamination and should expedite the diversion of contaminated dairy products from the food supply.

**TABLE 3. The effects of relative humidity on the performance of the CSID-MI minicolumns.**

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Ambient</th>
<th>Desiccator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage (wk)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>6</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1Chemiselective immobilization and detection of aflatoxin M\textsubscript{1}. Twenty minicolumns were prepared and divided into two groups. The first group was stored under ambient conditions, and the second was placed in a desiccator to determine the effect of relative humidity on their performance. After 2, 6, and 20 wk, three minicolumns were selected from each group and tested using contaminated milk with concentrations ranging from .5 to .7 ppb. The relative intensity of fluorescence on the CSID-MI minicolumn is indicated by number of plusses.

**ACKNOWLEDGMENTS**

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