Minicolumn Chromatography: State of the Art

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ABSTRACT

It was recognized in the early 1960s that a rapid screening method for aflatoxin was needed. Holaday first proposed the minicolumn chromatography method as a rapid screening method in 1968. Since that time, many improvements have been made in this method. The latest minicolumn method has a limit of detection of 5 μg/kg and can be completed in 5-7 min. The minicolumn technique has been expanded to include screening commodities other than peanuts for aflatoxin, as well as for other mycotoxins including ochratoxin, zearalenone and aflatoxin M_1.

INTRODUCTION

During the early 1960s, the methodologies developed for detecting and quantitating the aflatoxins required several hours (1,2). Because of this, several researchers recognized the need for a reliable and rapid screening method for aflatoxin in peanuts, particularly at buying points and other peanut handling locations where little or no laboratory facilities were available. One of the early screening methods was based on minicolumn chromatography (3). Since this original development, several improvements have been made and the application has been broadened to include screening of other commodities for aflatoxins and other mycotoxins. The purpose of this report is to review the state of the art of the minicolumn technique.

Development of the Minicolumn Method for Aflatoxin

Holaday proposed the original minicolumn (MC) method for aflatoxin in peanuts in 1968 (3). The column used is shown in Figure 1. The MC was stored at 78-80% relative humidity for 24 hr before use. The procedure consisted of extracting a peanut sample with chloroform/acetone (97:3, v/v) for 1 min, filtering the blended material and placing the lower end of the minicolumn in the filtrate. The filtrate was allowed to develop to the top of the silica gel. The column was removed from the filtrate and warmed under a UV lamp for several minutes to dissipate any interfering fluorescence. Aflatoxin in the sample was indicated by a blue band about 10 mm from the lower end of the column. The limit of detection was estimated to be 10 μg/kg. Occasionally, some of the fluorescent material on the column did not completely dissipate, which made it difficult to detect the aflatoxin band at the lower levels of contamination.

In 1972, Velasco proposed a clean-up procedure that employed a ferric chloride gel obtained by adding sodium hydroxide to a ferric chloride solution until a pH of 4.6 was obtained (4). The extracting solution consisted of acetone/water (85:15). The MC is shown in Figure 2. After clean-up, the extract was dissolved in chloroform/acetone. One mL of the chloroform/acetone extract was pipetted into the top of the MC followed by 1 mL of chloroform/acetone. This was allowed to elute down through the column until it reached the Florisil-silica gel interface where the aflatoxin appeared as a blue fluorescent band. The limit of detection was reported to be 5 μg/kg. Although this procedure provided an excellent extract clean-up, it required an excessive amount of time for a rapid screening method which should require not more than 10 or 15 min.

In 1973, Pons et al. adapted Holaday's MC design for detecting aflatoxin in cottonseed and other commodities (5). In this procedure, a lead acetate solution cleaned up the extract of gossypol, chlorophyll and other fluorescing contaminants. A limit of detection of 10 μg/kg was reported and the time required varied from 15 to 20 min. Holaday and Barnes proposed an improved MC procedure in 1973 (6). The sample was first extracted with hexane to remove the oil and other interfering materials, followed by an extraction of the sample homogenate with toluene/acetonitrile to remove the aflatoxin. One mL of the filtered extract was pipetted into the MC and pulled through with a vacuum. The MC was then removed from the vacuum and ca. 0.75 mL of the developing solution consisting of chloroform and methanol (97:2, v/v) was added to the top of the column and allowed to develop down through the column. Aflatoxin was indicated by a blue fluorescent band ca. 1 cm below the top of the column. The MC was similar to the original Holaday MC, except that the glass was 6 mm id and 90 mm long. Limit of detection was reported to be ca. 4 μg/kg. Although this MC worked reasonably well, background fluorescence occasionally caused problems.

In 1974, Barabolak et al. (7) developed an MC procedure for aflatoxin in corn (CPC method). They used the same MC design as that reported by Velasco (4). The extractant used was acetone/water (85:15) at a ratio of 1 g sample/3 mL solvent. Celite filter aid was blended with the sample to expedite the filtration. The filtered extract was treated with a 40% solution of ammonium sulfate to remove the interfering fluorescent materials. The extract was partitioned against benzene and, after separation of the 2 layers, 1 mL of the benzene layer was pipetted onto the MC and allowed to drain down the column. Three mL of a chloroform/acetone solution (9:1) was added to the MC and allowed to drain. The column was placed under long-wave UV. Aflatoxin was indicated by a blue fluorescent band at the interface of Florisil and silica gel. A limit of detection of ca. 5 μg/kg was claimed.

Another MC procedure was proposed by Shannon et al. in 1975 (8). The sample was extracted with acetone/water (85:15, v/v). The extract was treated with aqueous ammonium sulfate to precipitate any interfering fluorescent material, filtered and then partitioned against benzene. The MC was placed in a portion of the benzene and allowed to wick up the column until the solvent front reached ca. 1 cm above the interface of the 2 adsorbents. The MC was then

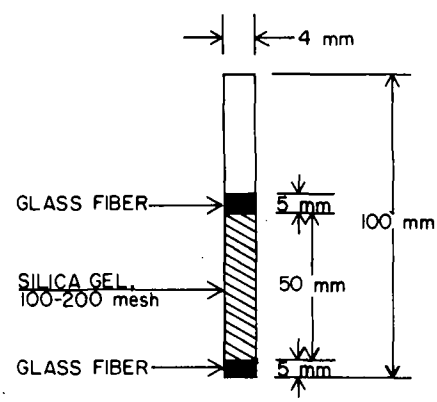

FIG. 1. Holaday's original minicolumn.

inserted into a solution consisting of chloroform/acetonitrile/isopropanol (93:5:2, v/v/v) and allowed to develop 5 min. The column used in this procedure is illustrated in Figure 3. A sharp blue fluorescent band ca. 1 cm above the interface of the aluminum oxide and the silica gel indicated aflatoxin. A limit of detection of 10 μg/kg was claimed. Occasionally, a white or yellowish band was present at the same location as the aflatoxin band which made it difficult to determine low levels of aflatoxins.

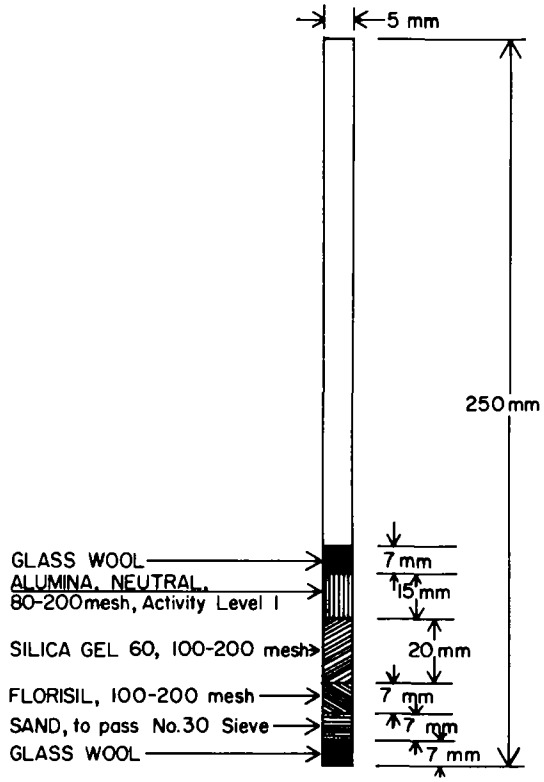

FIG. 2. Velasco's minicolumn.

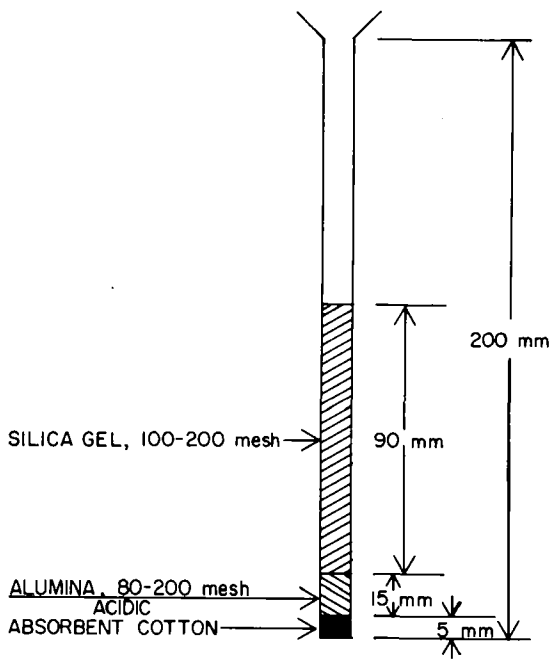

FIG. 3. Shannon's minicolumn.

In 1975, Holaday and Lansden reported an MC method that was both sensitive and rapid, and could be used with little or no laboratory facilities (9). The method used a 2-min extraction of the sample using methanol/water (80:20, v/v). The extract was filtered and to 15 mL of the filtrate was added 15 mL of a concentrated solution of zinc acetate/sodium chloride acidified with acetic acid. The salt solution precipitated most of the interfering fluorescing contaminants. The extract/salt mixture was filtered and 3 mL of benzene (subsequently replaced by toluene, private communication) was partitioned against 15 mL of the filtered extract/salt mixture and gently mixed. After separation of the 2 phases, 1 mL of the toluene (upper) phase was pipetted into the top of the MC, the lower end of which was attached to a vacuum. The toluene was pulled through the column and 3 mL of the eluting solution was added. Holaday and Lansden originally proposed hexane/acetone (80:20, v/v) as the eluant, but they later recommended methylene chloride/acetone (85:15, v/v) and finally, toluene/acetone (80:20, v/v) as changes in the activity level of the alumina adsorbent were made (private communication). The Holaday and Lansden method employed a simple MC design, as shown in Figure 4. The limit of detection was estimated to be ca. 4 μg/kg. Shannon and Shotwell conducted a collaborative study on 3 versions of the MC method in 1978 using both spiked and naturally contaminated samples of corn (10). The 3 versions included the Holaday/Lansden method (9), the CPC method which used a Velasco MC (7) and a combination of the Holaday/Lansden-CPC method. Results of the 20 collaborators indicated that a combination of the Holaday-Lansden extraction and clean-up procedure and the Velasco MC worked best. The study showed that the MC technique is effective for screening representative corn samples for aflatoxin. It should be pointed out that proper sampling methods are essential to get reliable aflatoxin results (8).

In 1979, a field study of the Holaday aflatoxin MC method was undertaken at the request of the peanut industry to accomplish the following: (a) determine if the MC method can be used under field conditions; (b) determine if the MC method can keep up with sample grading; (c) compare the MC method with the current method used at buying points. This method is based on the identification of *A. flavus* mold spores on the surface of a peanut kernel and is called the visual method; and (d) determine the economics of the MC method.

MC equipment was installed at 3 buying points in Alabama and at one in Georgia. Four inexperienced people were trained to run the tests. During the harvest season, 2705 samples were analyzed by the MC method. We found that the MC test could be done under primitive conditions and that it kept up with the grading. A comparison of the MC results with those of the visual method was made on the samples. Only 52 of the 86 samples that were positive by the visual method (segregation III) were positive by the MC method. (Segregation III peanuts are those that have visible *A. flavus* spores on the kernel surface. Segregation I peanuts have no *A. flavus* spores or other visible damage.) On the other hand, the MC found 60 samples to be positive that the visual method classified as negative. The economics of the MC method was difficult to assess due to the fluctuation of chemical and suppy prices; however, the approximate cost for the chemicals and supplies was $1.50, and $0.30 for labor to analyze one sample. These costs would be higher now.

Also in 1979, Shotwell and Holaday did a collaborative study on the MC technique with ground peanut samples which had been spiked with aflatoxin. Both the Holaday/Lansden and the Velasco MC, together with the Holaday/Lansden clean-up procedure, were used. The results will be

published in the *Journal of the Association of Official Analytical Chemists*.

The MC method for detecting aflatoxin in roasted peanuts and peanut butter requires a slightly different clean-up because of the increased amount of fluorescent materials in the extracts. Zinc sulfate is substituted for zinc acetate and no acetic acid is added. Zinc sulfate does a better job of removing the interfering fluorescent materials because of the gel formation, but because of the gel, the mixture filters slower and for that reason is not used with raw peanuts as time is often critical when analyzing this commodity.

The latest Holaday MC is shown in Figure 5. The sodium sulfate was added as the center layer to improve the interface with the Florisil, which increases sensitivity. To maintain the alumina at an activity level of III and the Florisil as dry as possible, the columns were kept in a desiccator. A test using the latest MC design can be completed in 5-8 min, depending on the operator's skill. Limit of detection is ca. 5 μg/kg. This latest MC design was used in a comprehensive test involving the MC, TLC, HPLC and the visual methods, the results of which will be published later.

Development of a Combined MC Procedure for Aflatoxin and Ochratoxin A

In 1976, Holaday proposed a rapid screening method for aflatoxins and ochratoxin A using a single extract after one clean-up procedure (11). The clean-up procedure consisted of treating a methanol/water (80:20) extract of the sample with an aqueous solution composed of 15% zinc sulfate and 5% phosphotungistic acid. After treatment, 15 mL of the filtered extract/salt mixture was partitioned against 3 mL of benzene. The subsequent steps to detect aflatoxin were the same as in the method proposed by Holaday and Lansden in 1975 (9). The procedure for detecting ochratoxin A involved adding 1 mL of the benzene layer to an MC. The MC is shown in Figure 6. The lower end of the column was attached to a vacuum. After the solvent was pulled through, 3 mL of methanol was added to the column and pulled through. The column was removed from the vacuum and 0.3 mL of a 0.25 N sulfuric acid solution was added to the top of the column and allowed to migrate down the column. Ochratoxin A produces a discrete blue fluorescent band ca. 1 cm from the top of the column when viewed under long-wave UV. Limit of detection of the aflatoxin was 4 μg/kg and for ochratoxin A, 8 μg/kg for some commodities and 16 μg/kg for others. The method was tested on peanuts, corn, rice, barley, rye, wheat and grain sorghum. Although the method worked satisfactorily in most cases, the last 4 commodities occasionally had background fluorescence which reduced the sensitivity.

Development of an MC Method for Zearalenone

While conducting research on zearalenone, Holaday found that its fluorescence under long-wave UV on alumina, was several times greater than when spotted on silica gel. This finding was instrumental in the development of an MC method for zearalenone (12). The extraction, clean-up and toluene partition steps were the same as was used on the latest aflatoxin method. The elution solvent consisted of 90% hexane and 10% acetone (v/v). The MC is shown in Figure 7. Other designs in which either basic or acetic alumina and 60-100 mesh Florisil were used did not work as well. A blue band at the interface of the alumina and the Florisil indicated at least 35 ppb of zearalenone. A dilution technique may be used to determine the approximate level of zearalenone. Time for a test is ca. 10 min for corn and grain sorghum; however, wheat causes some minor filtering problems. A wheat sample can also cause problems in the toluene partition step, because of an emulsion forma-

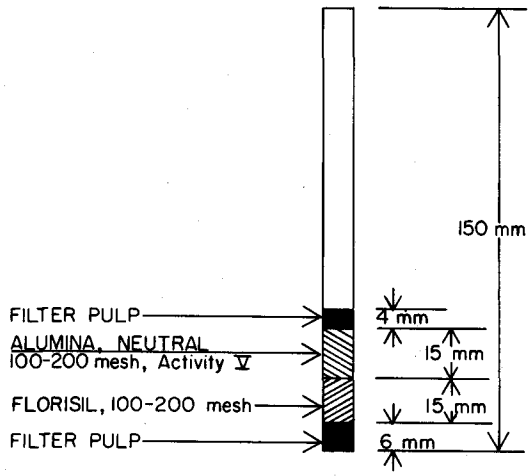

FIG. 4. Holaday and Lansden minicolumn.

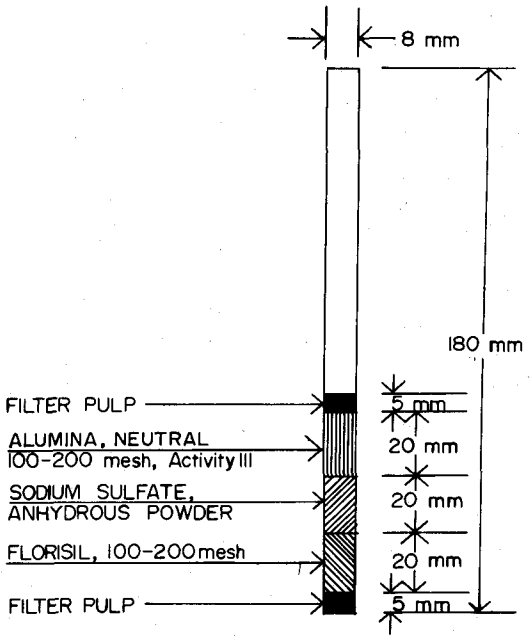

FIG. 5. Most recent Holaday minicolumn.

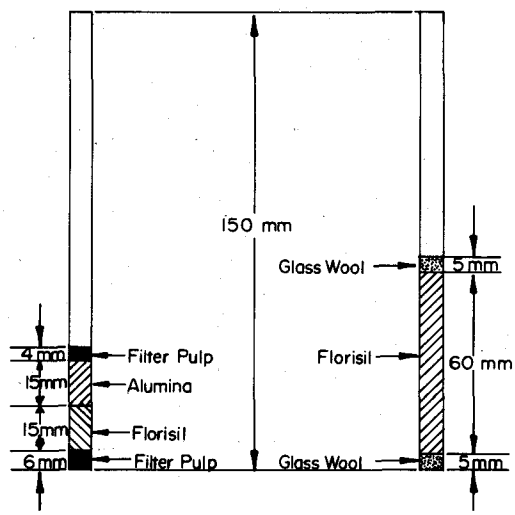

FIG. 6. Ochratoxin A minicolumn (*right*) and aflatoxin minicolumn (*left*).

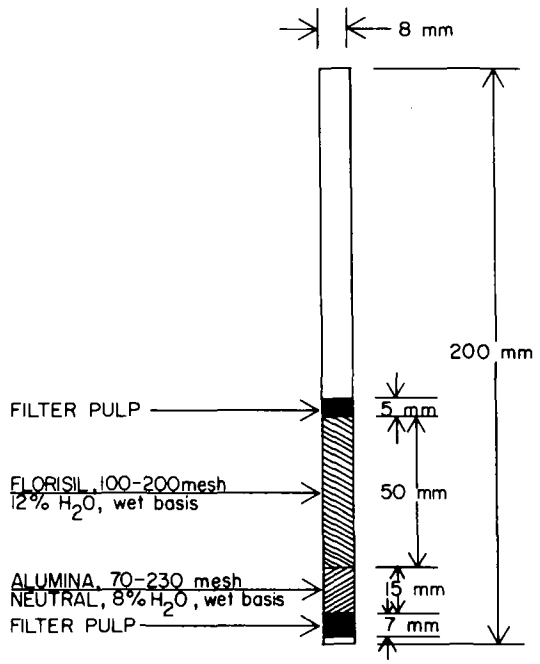

FIG. 7. Holaday's zearalenone minicolumn.

tion, unless the toluene is mixed very gently with the other ingredients. Although tests were only of corn, grain sorghum and wheat, other commodities could probably be analyzed for zearalenone with the MC without serious problems. The zearalenone MC method is basically the same as the aflatoxin technique and can be used at field locations. It should be very useful in screening for zearalenone in affected commodities.

Development of an MC Method for Aflatoxin M_1

Work was completed on an MC method for aflatoxin M_1 in raw and homogenized milk. Preliminary tests were also made with powdered milk with satisfactory results. The procedure consists of treating 30 mL of milk with 30 mL of a saturated solution of zinc sulfate and sodium chloride. To this mixture is added 3-4 g each of diatomaceous earth and absorptive magnesia. After vigorous mixing for a few sec, the mixture is filtered on a Buchner funnel. Forty mL of the filtrate are added to a separatory funnel together with 5 mL of toluene. The contents are thoroughly but gently mixed by up-ending the separatory funnel 4 or 5 times. After the 2 layers have separated, the lower layer is drained and discarded while the toluene (upper) layer is decanted into a test tube. An MC, the design of which is shown in Figure 8, is placed on a vacuum with the alumina side down and washed with 2 mL of toluene/methanol (80:20, v/v). After this has been pulled through, the MC is reversed on the vacuum (alumina side up) and 3 mL of the toluene extract is added and pulled through, followed by 3 mL of the toluene/methanol solution which is pulled through. The MC is viewed under long-wave UV. A blue band at the interface of the Florisil and the sodium sulfate indicates presence of at least 0.2 μg/kg of M_1. The limit of detection was determined by comparing the MC results with the TLC results on several samples with 0.2 μg/kg of M_1. Some quantitation is possible by using small increments of the 3 mL of toluene extract until a band becomes visible. That manuscript has been submitted to the *J. Assoc. Off. Anal. Chem.* for publication.

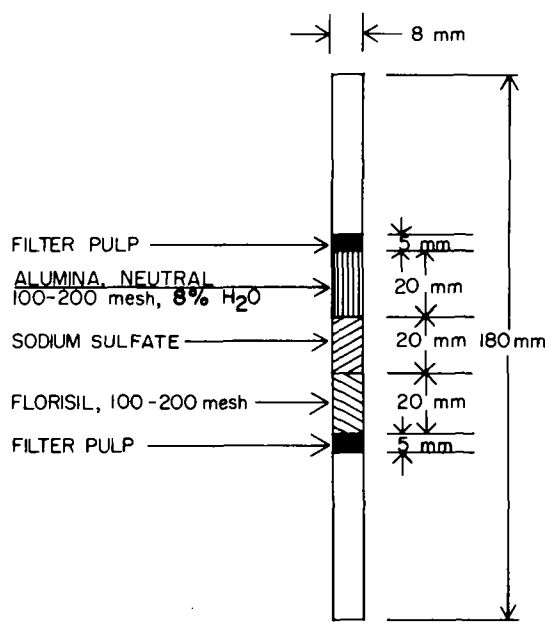

FIG. 8. Aflatoxin M_1 minicolumn.

Potential of the MC Chromatography Technique

The results of the recent field tests and collaborative studies have established the utility of the MC technique for detecting and approximating the aflatoxin in peanuts and corn. The technique is especially valuable at field installations and in plants for checking process lines. It is economical, costing only about one-tenth of an official TLC analysis. The application which we believe will have the greatest impact on the peanut industry is the use of the MC technique at peanut buying points; it should help reduce aflatoxin contamination in the food chain.

The MC technique developed for detecting zearalenone in corn, grain sorghum and wheat should also have excellent utility for identifying those lots which have dangerous levels of zearalenone. Although zearalenone is not as serious a problem as aflatoxin, its estrogenic effects can present problems. Because the technique is both rapid and simple, it can be used at elevators and feed lots to check feed before it is used.

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