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Sensitive Procedure for Aflatoxin Detection in Peanuts, Peanut Butter, Peanut Meal, and Other Commodities

Charles E. Holaday* and Phillip C. Barnes, Jr.

The contamination of peanuts and many other commodities by aflatoxin continues to be a serious worldwide problem. A rapid and reliable screening method is urgently needed for this dangerous toxin. A technique is described which has a sensitivity of about 3–5 ppb and which can be completed in approximately 15 min. The procedure is based on millicolumn chromatography and can be used on a number of commodities. Proximate quantitation of the aflatoxin is also possible.

Aflatoxin contamination in peanuts and other commodities continues to be a serious problem for the food industry. Many crops are subject to contamination by fungal metabolites, such as aflatoxin, in this and other countries. A rapid and inexpensive method is needed for screening susceptible commodities for this dangerous toxin.

Holaday (1968) developed a rapid screening method for detecting aflatoxin in raw peanuts based on a technique called "millicolumn chromatography." Although this method was simple and had a sensitivity of *ca.* 5 ppb, other fluorescent compounds occasionally were extracted along with the aflatoxin, which considerably reduced the sensitivity of detection. Velasco (1972) proposed a method for detecting aflatoxin in cottonseed based on a technique similar to the millicolumn procedure. This method also had a sensitivity of about 10 ppb, but required about 20–30 min to complete. More recently, Cucullu *et al.* (1972) adapted Holaday's millicolumn method for detecting aflatoxin in cottonseed and other commodities and reported a sensitivity of about 10 ppb; the time required varies from 15 to 20 min, depending upon the commodity. Dickens and Welty (1969) proposed a visual method for detecting *A. flavus* spores on peanut kernels. This method was helpful in removing large quantities of contaminated peanuts from the marketing channels. Most of the present quantitative methods for aflatoxin are based on thin-layer chromatography. These procedures are time consuming and require trained technicians for making the analyses.

This paper describes an improved millicolumn procedure for detecting total aflatoxin content ($B_1 + B_2 + G_1 + G_2$), with a sensitivity of 3–5 ppb, that can be completed in about 15 min. This is faster than the original millicolumn method and the problem of interference from other fluorescent compounds has been eliminated. The millicolumns are similar to those described earlier by Holaday (1968) but are twice as long (9 cm) and twice the diameter (6 mm).

National Peanut Research Laboratory, United States Department of Agriculture, Agricultural Research Service, Southern Region, Peanut Quality Investigations, Dawson, Georgia 31742.

EXPERIMENTAL SECTION

Detection of Aflatoxin in Peanuts, Peanut Meal, Peanut Butter, and Corn. Equipment and reagents required for the test are as follows: chromatovue chamber—equipped with long-wave uv, Ultraviolet Products, Inc., San Gabriel, Calif.; millicolumns—packed with column-type silica gel 60–200 mesh, American Society of Testing Materials (MCB-Grade 950); plugs to hold silica gel inside glass tube are made from ashfree filter pulp (S & S No. 289). The silica gel column is 90 mm long. The glass tubing is 6 mm i.d. and 200 mm long. Prepared columns may be purchased from the Tudor Scientific Glass Co., Belvedere, S. C.; extracting solution—95 parts toluene, 5 parts acetonitrile (v/v); developing solution—97 parts chloroform–2 parts methanol–1 part acetone (v/v/v). All reagents are ACS grade.

Weigh a well-mixed 50-g sample into a 1-qt blender jar fitted with a screw cap, add *ca.* 10 g of filter aid and 100 ml of hexane, and blend at high speed for 1 min. Because of the highly flammable nature of hexane, it is recommended that the extraction be carried out under a well ventilated hood. Vacuum filter through a 90-mm glass fiber filter disk placed on the Buchner funnel into a 500-ml sideneck flask attached to an aspirator. Wash the jar twice with *ca.* 25 ml of hexane. Add these washings to the Buchner funnel. After the hexane has filtered through, place the homogenate, together with the filter disk, back in the blender jar, add 100 ml of the toluene–acetonitrile solution, and blend for 1 min. Acetonitrile is a poisonous substance and should not be inhaled. It is recommended that it be handled under a hood. Filter through a 90-mm glass fiber filter disk placed in the Buchner funnel into the 500-ml sideneck filter flask from which the hexane extract has been removed. Collect *ca.* 10–20 ml of the filtrate. Tap a millicolumn several times to pack and insert lower end into a one-hole rubber stopper placed in the neck of a 1000-ml filter flask. The sideneck of the filter flask is attached to an aspirator. Transfer 1.0 ml of the extract to the top of the millicolumn with a disposable pipet. The vacuum will pull the extract through the column rapidly; the aflatoxin, however, remains at the top of the column. Add *ca.* 1 ml of hexane to the top of the column and continue pulling the vacuum until all of the sol-

Table I. Proximate Quantitation of Aflatoxin in Peanuts and Pistachio Nuts

Peanuts[a]		Pistachio	
ml on column[b]	If no band, aflatoxin is less than (ppb)	ml on column[b]	If no band, aflatoxin is less than (ppb)
1.00	5	0.60	4
0.50	10	0.30	8
0.25	20	0.15	16
0.20	25	0.10	24
0.10	50	0.05	48
0.05	100	0.02	120
0.01	500	0.01	240

[a] Also applicable to peanut butter, peanut meal, and corn assays for aflatoxin. [b] Final sample extract.

vent is removed from the column. Remove column from vacuum and place in an upright position (a test tube rack is convenient for this purpose). Add *ca.* 0.75 ml of the developing solution and allow it to develop down through the column.

Expose the developed column for about 30 sec to the uv in the Chromatovue cabinet. Then examine the column on all sides for the presence of aflatoxin, which is indicated by a sharp blue fluorescent band *ca.* 1 cm below the top of the column. If no band is visible, there is less than 5 ppb of aflatoxin in the sample.

Detection of Aflatoxin in Pistachio Nuts. Pistachio nuts contain a large amount of chlorophyll, which is highly fluorescent and interferes with the detection of aflatoxin. Pons (1972) found that lead acetate precipitated chlorophyll. The procedure which we developed for detecting aflatoxin in pistachio nuts, therefore, is based on Pons' (1972) lead acetate cleanup procedure.

In addition to the reagents listed above, some additional solutions are required for pistachio nuts, as follows: acetonitrile–water solution—800 parts acetonitrile, 200 parts distilled water (v/v); saline solution—50 g of NaCl dissolved in 950 ml of distilled water; lead acetate solution—200 g of lead acetate trihydrate dissolved in 1000 ml of distilled water. Add 3 ml of glacial acetic acid to solution. All above reagents should be ACS grade.

Weigh a 50-g sample into a 1-qt blender jar equipped with a screw top, add 150 ml of the acetonitrile–water solution, and blend for 2 min. Vacuum filter extract through a 90-mm glass fiber filter disk placed on a Buchner funnel into a 500-ml sideneck filter flask. Pipette 5 ml of the extract into a 18 × 150 mm culture tube, and add 4 ml of the lead acetate solution and 11 ml of the 5% saline solution. Place a plastic cap on the culture tube and shake vigorously for 10 sec. Then filter contents of culture tube through a folded disk of the No. 541 filter paper placed in the plastic funnel into a second culture tube of the same size. After all the liquid has filtered through, remove funnel and add 2 ml of toluene to the culture tube. Place a plastic cap on the culture tube and shake vigorously for about 10 sec. Let phases separate and remove 0.6 ml of the upper phase and introduce into top of the millicolumn, the lower end of which is attached to a vacuum source as described in the peanut procedure. Pull the extract through the column. Add *ca.* 1 ml of hexane to top of column and continue pulling the vacuum until all the solvent is removed from millicolumn. Remove millicolumn from vacuum and place in an upright position. Add *ca.* 0.75 ml of the developing solution to top of column and let it develop down the column. Expose the column in the Chromatovue chamber for about 30 sec and then examine the column on all sides. A blue band *ca.* 1 cm from top of column indicates 4 ppb or more of aflatoxin. No band indicates less than 4 ppb.

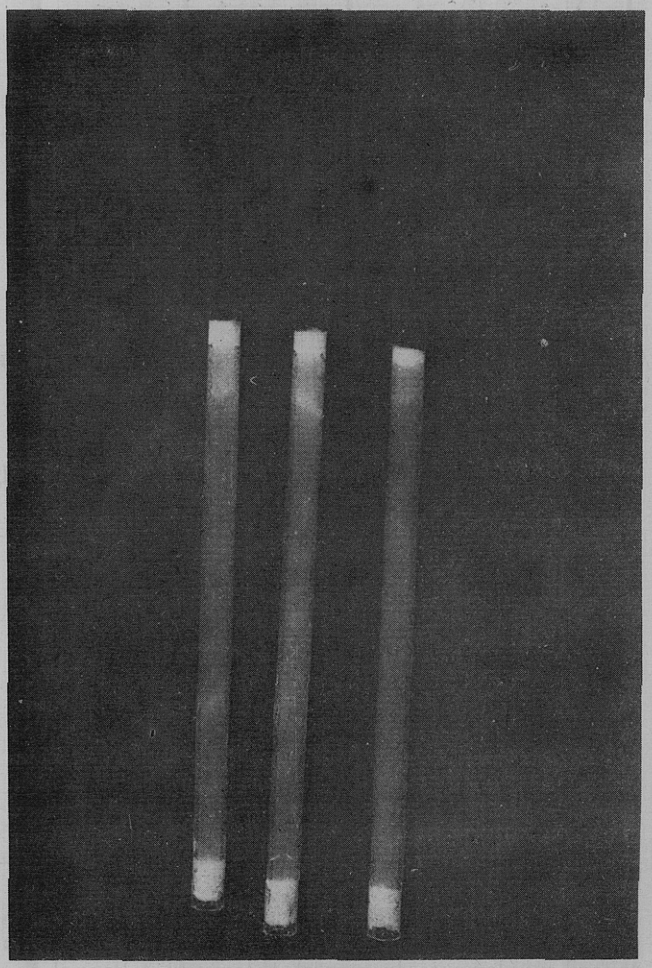

Figure 1. Three developed millicolumns representing 30, 20, and 5 ppb of aflatoxin, respectively, from left to right.

RESULTS AND DISCUSSION

The initial extraction with hexane removes most of the oil and interfering fluorescing materials from the sample. Aflatoxin is insoluble in hexane and remains with the sample. Hexane, however, does not remove chlorophyll from the pistachio nuts, and therefore a different extractant followed by a lead acetate cleanup is required. The millicolumns are not equilibrated at 80% relative humidity, as recommended by Holaday (1968), but are kept in an efficient desiccator until ready for use.

Time required for making a test is about 13–15 min, which is an improvement in time over the previous procedure of 15–25 min (Holaday, 1968). More sensitivity is possible if the Chromatovue is equipped with a transilluminator as well as overhead uv lamps.

Some degree of quantitation is possible by adding various amounts of the final sample extract to the millicolumns. Table I illustrates this technique. If no blue fluorescent band is visible after adding 0.6 ml to the millicolumn in the pistachio nut procedure, there is less than 4 ppb of aflatoxin in the sample. Adding 1.2 ml to the millicolumn would increase the sensitivity to *ca.* 2 ppb. In the procedure for the other commodities, 1.0 ml added to the top of the millicolumn would show no blue fluorescent band if less than 5 ppb of aflatoxin were present. If no band is visible after adding 2.0 ml to the top of column, there is less than 2–3 ppb.

Figure 1 shows three columns with 20, 10, and 5 ppb of the four aflatoxins (B_1 + B_2 + G_1 + G_2), respectively, from three samples of raw peanuts. All samples were contaminated by adding measured amounts of the four aflatoxins (B_1 + B_2 + G_1 + G_2) to them before extraction.

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Gel Electrophoretic Analysis of Peanut Proteins and Enzymes. I. Characterization of DEAE-Cellulose Separated Fractions

John P. Cherry,[1] Joseph M. Dechary, and Robert L. Ory*

The total proteins of Virginia 56R peanuts were solubilized by extracting the seeds with dilute phosphate buffer. The individual components were separated by DEAE-cellulose chromatography into eight major fractions which were then characterized by polyacrylamide disk gel electrophoresis. Using immunoelectrophoretic analysis, the peanut trypsin inhibitor was localized in the albumins fraction. Seven enzyme activities were examined by polyacrylamide disk and starch gel electrophoretic techniques. Zymograms of these enzymes, similar to those in the total cotyledonary extracts, seemed to be confined mostly to the fractions containing albumins and smaller molecular weight globulins. These studies provided information that may be potentially useful in the preparation of high-quality protein concentrates and in identification of specific changes in proteins caused by conditions used during processing of peanut meals into concentrates or isolates.

There have been many investigations on the reserve proteins of peanuts because of their potential usefulness as food supplements. In general, these studies have shown that most of the proteins are easily extractable (Altschul *et al.*, 1961, 1964a,b; Dawson, 1971; Dechary and Altschul, 1966; Dechary *et al.*, 1961) and can be separated into two major fractions, arachin and conarachin (Daussant *et al.*, 1969a,b; Dechary *et al.*, 1961; Evans *et al.*, 1962; Neucere, 1969; Neucere and Ory, 1970). More extensive purification of these two fractions (Dawson, 1971; Evans *et al.*, 1962; Tombs, 1965; Tombs and Lowe, 1967; Neucere, 1969) has revealed that they are composed of complex large molecular weight globulins (α-arachin, α-conarachin), plus some other closely related components, not completely separated by the usual techniques.

Gel electrophoresis has been used extensively to characterize and identify proteins and enzymes in biological systems (Cantagalli *et al.*, 1971; Cherry *et al.*, 1970, 1971a,b, 1972; Dawson, 1971; Haikerwal and Mathieson, 1971; Minetti *et al.*, 1971; Neucere and Ory, 1970; Sastry and Virupaksha, 1967; Tombs, 1963) and to detect experimentally induced modifications in these molecules (Jensen, 1959; Neucere, 1972; Neucere *et al.*, 1972).

Cherry *et al.* (1971b) and Cherry and Ory (1973a,b,c) have shown that the electrophoretic protein and enzyme profiles of individual peanuts are very complex, with much variation within the cultivars examined. The complexity of these electrophoretic patterns made it difficult to identify specific proteins that varied qualitatively and/or quantitatively. Identification of groups of proteins showing polymorphism could provide information that might be used during the fractionation of peanut proteins to produce concentrates or isolates of good amino acid balance. Also, standardization of these electrophoretic protein patterns could help in identifying changes in proteins caused by processing conditions.

For this investigation, peanut protein extracts were separated into eight fractions by DEAE-cellulose chromatography. The protein and enzyme components of each fraction were further characterized by polyacrylamide disk and starch gel electrophoretic techniques.

MATERIALS AND METHODS

Seeds. Virginia 56R certified peanut seeds were obtained "in shell" from a commercial supplier in Holland, Va. The seeds were shelled and hand-selected for uniform size and quality through the courtesy of W. K. Bailey.

Fractionation of Peanut Proteins. Most of the fractions were prepared by the method of Dechary *et al.* (1961). Fraction IV was separated into V and VI by chromatography over DEAE-cellulose in pH 8.0, I = 0.06, phosphate buffer, with a linear gradient of 0–0.5 *M* sodium chloride. Protein content of each fraction was determined by the method of Lowry *et al.* (1951). Arachin was purified by cryoprecipitation as described by Neucere (1969).

Gel Electrophoretic Techniques. Protein samples were dissolved in phosphate buffer, pH 7.9, I = 0.01, and examined by polyacrylamide (Cherry *et al.*, 1970) and starch (Brewbaker *et al.*, 1968) gel electrophoresis. Each fraction was examined at three protein concentrations (1.0, 0.6–0.8, and 0.4 mg/gel) to determine the major as well as all minor electrophoretic bands in each preparation. Methods for determining enzyme activities within each fraction were described by Cherry and Ory (1973a,b).

Immunochemical Techniques. Peanut trypsin inhibitor activity was analyzed according to Daussant *et al.* (1969a).

RESULTS AND DISCUSSION

Fractionation of Conarachin and Arachin. The complexity of the total proteins of peanut cotyledonary ex-

Oilseed and Food Laboratory, Southern Regional Research Laboratories, New Orleans, Louisiana 70179.
[1] Present address: Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843.