An Improved Rapid Physicochemical Assay Method for Aflatoxin in Peanuts and Peanut Products[1]

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Abstract

A practical, short cut, sensitive method for more rapidly determining aflatoxin in peanuts and peanut products has been developed. This was in response to the need to reduce the time required for analyses of peanut products in process. Through reductions in solvent volumes, utilization of pressure filtration for clarification, and substitution of liquid:liquid extraction for a lengthy column clean up, equivalent results are possible in less than one half the time required for the current official procedures. Sensitivity, precision and accuracy are comparable to the current methods for raw nuts and peanut butter. It is now possible to analyze a given ground sample of peanuts within a period of less than 90 min and one analyst can assay more than 16 samples within an 8 hr working day.

Introduction

THE PROBLEM OF ANALYZING for aflatoxin has been with peanut shellers and processors since the early 1960's, when it was first realized that mold toxins might be present in peanuts (1–6). Many analytical methods were soon proposed (7–10) and currently the AOAC Official, First Action, Celite Method (11) is in general use in the laboratories approved by the Peanut Administrative Committee, (an organization directed by the Secretary of Agriculture to handle the business functions of the "Marketing Agreement Regulating the Quality of Domestically Produced Peanuts") hereafter referred to as the PAC Approved Laboratories. In addition, the CB Procedure, originated by the Food and Drug Administration and used in their laboratories, also has AOAC Official, First Action, status (12). These two methods, however, have one common limitation, namely, the long time (about three hours) required for completion of a given analysis.

The object in our studies was to develop a procedure giving values the same as those obtained by an official procedure but with a significant reduction in the time required for analysis.

Since the presence of aflatoxin contamination may frequently be due to only a small number of affected nuts distributed nonuniformly (13,14) throughout a carload shipment (containing 800 bags of raw nuts), it is almost impossible to assign a single value to represent the aflatoxin content of a carload of peanuts containing some contaminated nuts. In Table I there is shown, for illustrative purposes, the variability in results obtained in testing different lots of peanuts following repetitive samplings of each lot with the hope of obtaining an aliquot representative of the total lot. The variability in results obtained for each lot is not due to analytical variations since reasonably good checks are obtained in retesting the same ground aliquot of a given test sample. All analyses are reported in parts per billion ($\mu g/kg$).

The first sample for each car lot in Table I was a composite from a sampling by USDA, at the shelling plant, of 25% of the bags in the shipment. The next two samples were drawn by the purchaser, while the final two represent an additional independent sampling by USDA. Since there is no duplication of bag sampling during an individual sampling, the two final samples drawn by USDA represent 50% of the bags in the shipment. Peanut lots, found to contain such a high and variable degree of contamination as shown in Table I, are rejected by the processor. However, of major concern are the lots of peanuts which give a negative aflatoxin value but may still contain a few aflatoxin-positive peanuts within one or more of the bags. Not only must such contaminated nuts be completely removed (and electronic sorters are in wide use today for this purpose), but continuous monitoring of the finished product, for ex-

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TABLE I
Analyses of Peanuts by PAC Approved Laboratories

Car Lot	Sample: Identity	Sample: No. Bags Sampled	Analytical Laboratory	Aflatoxin,[a] ppb: B_1	B_2	G_1	G_2	Total
I	USDA-Official	200	Purchaser	48	37	0	0	85
	Purchaser-1	50	Purchaser	175	75	0	0	250
	Purchaser-2	100	Purchaser	0	0	0	0	0
	USDA-A	200	USDA					0
	USDA-B	200	USDA					28
II	USDA-Official	200	Purchaser	273	120	57	53	503
	Purchaser-1	50	Purchaser	0	0	0	0	0
	Purchaser-2	100	Purchaser	0	0	0	0	0
	USDA-A	200	USDA					75
	USDA-B	200	USDA					0
III	USDA-Official	200	Purchaser	9	6	0	0	15
	Purchaser-1	50	Purchaser	437	267	0	0	704
	Purchaser-2	100	Purchaser	25	10	0	0	35
	USDA-A	200	USDA					92
	USDA-B	200	USDA					158
IV	USDA-Official	200	Purchaser	75	38	0	0	113
	Purchaser-1	50	Purchaser	25	5	0	0	30
	Purchaser-2	100	Purchaser	0	0	0	0	0
	USDA-A	200	USDA					0
	USDA-B	200	USDA					6

[a] Only total values were reported by the USDA Laboratory. A value of "0" means less than 3 ppb ($\mu g/kg$) of aflatoxin.

TABLE II
Comparison of the Proposed Method for Aflatoxin With the Current Official Procedures

Steps	Proposed rapid procedure (A)	Celite Procedure (B)	CB Procedure (C)
Extraction	100 g peanuts or 50 g peanut butter Methanol-Water-Hexane Blender 3½ min + 5 min handling	Same as (A) Same as (A) Same as (A)	50 g peanuts or peanut butter Chloroform-Water Mechanical shaker 30 min + 5 min handling
Separation	Centrifuge 2000 rpm 5 min Seitz pressure filter 1 min + 5 min handling	Centrifuge 30 min + 5 min handling	Filter until 50 ml obtained 10—? min +5 min handling
Clean up	25 ml filtrate + 25 ml chloroform Liquid-liquid extraction in a separatory funnel 3 min	Mix with H_2O and pack into column Elute with hexane, then hexane-chloroform 20–60 min + 15 min handling	Silica-gel column Elute with hexane, ether and methanol-chloroform 45–90 min
Concentration	Evaporate 25 ml of chloroform-dilute to 250 μliter with benzene 10 min + 5 min handling	Evaporate 600 ml of chloroform-hexane 45 min + 5 min handling	Evaporate 150 ml of methanol-chloroform 20 min + 5 min handling
Minutes used to prepare extract	35–40	130+	120+
TLC	Silica gel plate developed using acetone-chloroform (1:9) 45 min + 5 min handling	Same as (A)	Same as (A)
Approximate total time	90 min[a]	180 + min	170+ min

[a] See footnote 2 in text.

ample the peanut butter, is required to insure that product shipped is free of aflatoxin. In order to handle the large number of samples required for a comprehensive testing of the product stream, a simpler, more rapid method of analysis, having at least equivalent sensitivity, accuracy and precision as the current official procedures, was urgently needed.

Using the official procedures as a starting point, individual steps of these methods were critically evaluated to determine their efficiency and necessity. From these studies, a procedure evolved which resembles the basic outline of the procedure reported by Campbell et al. (15).

Experimental Procedures

Analytical Method

The procedure utilized for initial extraction of aflatoxin is that used in the Celite Procedure (11) wherein a sample of 100 g peanuts is blended at high speed for 3.5 minutes with 500 ml of methanol-water (55:45 v/v) and 200 ml of hexane, or 50 g of peanut butter with 250 ml of methanol-water and 100 ml of hexane. For the sake of speed, it is preferable to weight the sample directly into a blending cup on a top loading balance. Immediately following blending, the slurry is transferred into 200 ml centrifuge bottles and centrifuged at 2000 rpm for 5 min.

At least 50 ml of the aqueous methanol layer is transferred into a Seitz filter, Model 6, (combination model with pressure lid but without check valve) containing a Whatman 42 filter paper (5.5 cm diameter). Application of about 2 psig of pressure from bottled nitrogen permits collection of about 30 ml of filtrate in less than 1 min in a graduated beaker.

Twenty-five milliliters of the filtrate are pipetted into a separatory funnel, 25 ml chloroform added, and the stoppered funnel shaken vigorously for 1 min. After separation of the solvent layers, the bottom ($CHCl_3$) layer is drawn off into a beaker and evaporated under nitrogen to about 1–2 ml. The extract is transferred to a four dram vial, with $CHCl_3$ rinsings, and evaporated to dryness under nitrogen. The material is taken up in 250 μliters of benzene and the mixture agitated vigorously to dissolve the aflatoxin. This provides the final test solution for TLC separation according to the procedure outlined in the Celite Method (11). In our laboratory the Chromato-Vue cabinet (obtained from Ultraviolet Products, Inc.) is regularly used when visually reading the TLC plates. However, for purposes of critical evaluation of the proposed rapid procedure, a densitometer was frequently employed in the present study.

In Table II there is a comparison of the steps in the proposed method with those of the official procedures; time advantages obtainable with the new rapid method are also shown. The times reported in connection with the individual steps of the proposed procedure as well as for the evaporation of the solvent in the Celite Method and in the CB Procedure are the average handling times in our laboratory. The range in time required for elution of the columns in the latter two procedures were calculated from the flow rates specified in the early published procedures (11,16). The latest revision of the CB Procedure (12) permits elution at maximum flow rate which generally requires 5 to 10 min less than the lower range limit shown on Table II. Therefore, while the approximate total time for the rapid method represents an average time, the total time recorded for each of the official procedures is roughly the minimum time required for one person to complete an analysis of one sample of ground raw peanuts for aflatoxin content.[2]

Recovery Studies

A series of recovery experiments were made by pre-weighing samples of both raw nuts and peanut butter previously shown to be free of aflatoxin. These

[2] Since this paper was submitted for publication, further refinements of a minor nature have been made and checked for reliability. These modifications have reduced the time required for the analysis from about 90 min to 60 min. These changes are: reduction of blending time in the initial extraction step to 1 min; omission of the Seitz filtration step by direct transfer of 25 ml of the aqueous methanol layer from the centrifuge bottle to the separatory funnel for subsequent extraction with chloroform; and the development of the TLC plate for only 20 min.

TABLE III

Recovery Experiments Using Proposed Method

Sample		Aflatoxin, ppb Added	Aflatoxin, ppb Found[c]	Recovery %
1) Ground raw nuts	B_1[a]	13.4	12.3	91.8
2) Ground raw nuts	B_1[a]	40.2	37.0	92.0
3) Peanut butter	B_1[a]	15.4	14.0	90.9
4) Peanut butter	B_1[a]	46.2	42.0	90.9
5) Ground raw nuts	B_1[b]	30	27.0	90.0
	B_2[b]	9	7.5	83.3
	G_1[b]	30	27.6	92.0
	G_2[b]	9	7.6	84.4

[a] Concentrations based upon spectrophotometric analyses of the pure aflatoxin B_1 solution used to provide the added B_1 to the aflatoxin-free substrate.
[b] Based upon concentrations established by the Southern Regional Laboratory of the USDA in solution provided by that laboratory; a dilution of this material in methanol was added to raw ground peanuts shown to be free of aflatoxin.
[c] Concentrations determined desitometrically.

preweighed samples were then purposely contaminated with known quantities of aflatoxin B_1 in chloroform and the solvent removed in a forced draft oven at 70 C. A Model 530 densitometer, manufactured by Photovolt Corporation, was employed in reading the plate for precise recovery studies. The developing solvent used and the techniques for instrument standardization were in accord with the published report by Pons et al. (17). Each sample was independently assayed on a separate TLC plate along with duplicate standards at three levels of concentration, included to provide a standard reference curve. All standards were from the same solution utilized for contaminating the samples. The recoveries of added aflatoxin averaged 91% as shown for samples 1 to 4 in Table III.

Also shown in Table III is another sample of the raw peanuts used for samples 1 and 2 which was spiked with a standard containing both the B and G aflatoxins. Again good recovery values were obtained for B_1 and G_1, however, recoveries for B_2 and G_2 were somewhat lower.

Discussion

In an attempt to find the cause of the apparent 9% loss of aflatoxin B_1 by the proposed procedure, the basic steps in this method were critically examined. These steps included the use of the pressure filter, the efficiency of the single chloroform liquid-liquid extraction, and the stability of the aflatoxin on the TLC plate.

To test the filtering step, the Seitz filter was included as an additional step in the Celite Procedure

TABLE IV

Analyses of Ground Raw Peanuts by the Proposed Procedure and the Official Celite Method Within One Laboratory

Sample No.	Method	Aflatoxin, ppb B_1	B_2	Total
V[a]	Improved Rapid	30.5	0	30.5
	Official Celite	30.5	0	30.5
VI	Improved Rapid	83	34	117
	Official Celite	75	30	105
VII	Improved Rapid	4	0	4
	Official Celite	4	0	4
VIII	Improved Rapid	90	25	115
	Official Celite	70	15	85
IX	Improved Rapid	8	0	8
	Official Celite	7	0	7
X	Improved Rapid	0	0	0
	Official Celite	0	0	0
XI	Improved Rapid	33	6	39
	Official Celite	32	10	42

[a] Concentrations of aflatoxin in this sample were determined densitometrically; all others were by visual comparison with standards on the TLC plates, when viewed under ultraviolet light in the Chromato-Vue cabinet.

TABLE V

Analyses in Different Laboratories of Samples of Raw Peanuts by the Proposed and the Celite Procedures

Sample No.	PAC Approved Laboratory	Method	Aflatoxin, ppb B_1	B_2	G_1	G_2	Total
XII	Alameda	Rapid	10	9	10	9	38
	Alameda	Celite	11	8	10	9	38
XIII	Alameda	Rapid	32	18	0	0	50
	Alameda	Celite	33	18	0	0	51
XIV	Alameda	Rapid	42	34	31	18	125
	Alameda	Celite	44	33	27	18	122
	Bayonne	Rapid	42	28	40	28	138
XV	Minneapolis	Rapid	13	0	8	0	21
	Minneapolis	Celite	13	0	8	0	21
	Bayonne	Rapid	7	0	6	3	16
XVI	Minneapolis	Rapid	42	16	0	0	58
	Minneapolis	Celite	38	13	0	0	51
XVII	Minneapolis	Rapid	38	25	31	13	107
	Minneapolis	Celite	38	19	38	13	108

immediately following the centrifugation step in this procedure. Comparison on a TLC plate of the final extract obtained in this manner with the corresponding extract from an identical sample, prepared using the unaltered Celite Procedure, showed no differences in the aflatoxin content of the two extracts; hence, the pressure filter was not the cause of losses found in the recovery studies of the proposed method.

The efficiency of extraction of aflatoxin from the aqueous methanol by chloroform was also evaluated. Similar liquid-liquid extraction with chloroform has been used by others in the analytical procedures presented (15,18–20) and by Parker and Melnick of our laboratory in studies showing the absence of aflatoxin from refined vegetable oils (21). In the present study samples of ground raw peanuts, known to contain aflatoxin, were extracted once with the specified volume of chloroform as outlined in the proposed procedure, and then were re-extracted three more times with 25 ml portions of chloroform. The latter three extracts were combined, evaporated and compared on the same TLC plates along with the first chloroform extracts. In this manner, it was shown that all of the aflatoxins were recovered by a single extraction within the limitations of the visual evaluations.

In a related study, the densitometer was employed for evaluation of extracts, obtained by the proposed method, from samples of aflatoxin-free peanuts to which known amounts of aflatoxin B_1 were added to the separatory funnels containing methanol-water extracts of the sample. In each case, about 98% to 99% of the added aflatoxin B_1 were found, which is in good agreement with similar studies made by Pons and Goldblatt (20). These data provide further evidence of the insignificant losses associated with the single chloroform extraction step.

TABLE VI

Collaborative Study of Aflatoxin B_1 in Simulated Peanut Butters Prepared With Reject Peanuts

Sample No.	Method	Aflatoxin, ppb found by PAC Approved Laboratory A	B	D	M	P	Average
1	Rapid	0	0	0	0	0	0
	Celite	3	0	0	0	0	0
2	Rapid	4	4	3	0	0	3
	Celite	5	4	3	0	3	3
3	Rapid	6	7	7	5	0	5
	Celite	9	6	5	5	7	6
4	Rapid	15	9	3	8	5	8
	Celite	21	8	3	8	6	9
5	Rapid	18	21	20	19	20	20
	Celite	25	18	20	15	11	18

TABLE VII

Analyses Within One Laboratory of Simulated Peanut Butters Prepared With Reject Peanuts Using Three Methods of Test

Sample No.	Method	Aflatoxin, ppb				
		B_1	B_2	G_1	G_2	Total
1	Rapid	0	0	0	0	0
	Celite	0	0	0	0	0
	CB	0	0	0	0	0
3	Rapid	7	0	0	0	7
	Celite	6	0	0	0	6
	CB	8	0	0	0	8
5	Rapid	21	6	0	0	27
	Celite	18	7	6	0	31
	CB	36	9	3	0	48
6	Rapid	45	16	0	0	61
	Celite	35	18	12	0	65
	CB	75	18	0	0	93
7	Rapid	70	20	10	0	100
	Celite	65	25	14	0	104
	CB	102	41	16	0	159

It was concluded from the results of these studies that there is no significant loss in the TLC or filtering step, but about 7% loss in the initial extraction or in our preparation of the samples for the recovery experiment. Since the Celite Method utilizes the same system of sample preparation up to the centrifuging of the initial extract, an equivalent 7% loss of aflatoxin would also occur in this procedure.

Results

The results of comparative studies of the proposed rapid method and the Celite Procedure are shown in Tables IV and V. In the case of the first sample in Table IV, the concentrations of aflatoxin were evaluated on the TLC plate using the densitometer. Identical values were obtained for the extracts prepared from sample V by each of these methods. The data in both tables indicate good agreement between the results obtained by the two methods on portions of the same ground sample. As in all previous cases, the raw nut samples were from rejected lots of raw peanuts.

A collaborative study using the proposed procedure and the Celite Procedure was conducted among five PAC Approved Laboratories. Summarized in Table VI are the values obtained for the aflatoxin B_1 present. The samples analyzed were simulated peanut butters specially prepared in the laboratory using pick-outs (that is, rejected nuts from roasted batches of peanuts). From 2 to 16 times as much rejected peanuts, as would be removed in plant processing, were blended with aflatoxin-free peanuts in the preparation of the contaminated peanut butters for this study. Agreement within each laboratory is considered to be good when comparing the two methods of test and the same is also true for the comparison of values for a given sample among the five different laboratories, with the possible exception of Laboratory A for Sample 4. Because pick-outs were used to provide the natural contaminant and such rejects also include over-roasted peanuts, the samples listed in Table VI showed extraordinarily high background interference on the TLC plates. For this reason, the values obtained for B_2 and G_1, also present in some of the samples, were more variable among the laboratories but this was noted to the same degree with the values obtained by either the proposed rapid method or by the Celite Method.

In another study in our laboratory which is summarized in Table VII, use was made of all three test methods, the proposed rapid method, the Celite Procedure and the CB Method. The results obtained in measuring aflatoxins other than B_1 are also reported. Two additional simulated, laboratory-made peanut butters, containing even larger amounts, 33% and 50%, of reject peanuts, were now included. The best agreement was found between the proposed rapid and the Celite Method. Ordinarily the CB Method gives plates with the least interfering background. However, in this series of simulated peanut butters prepared with pick-outs added in large amounts, the background interference noted with all three test methods was comparable.

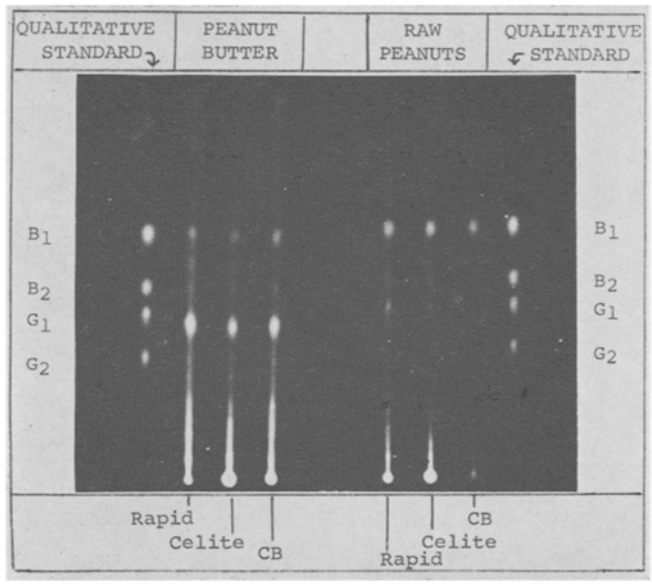

FIG. 1. A qualitative TLC plate illustrating the resolution of the aflatoxins in extracts of peanut products obtained by each of the three assay methods.

Figure 1 is a photograph of a qualitative TLC plate comparing the backgrounds noted using the proposed procedure and the official procedures in analyzing another sample of contaminated peanut butter and raw peanuts. The peanut butter was later found to contain, by the proposed method, about 8 ppb (μg/kg) of B_1 and 3 ppb of B_2 while the raw nut sample contained 19 ppb B_1, 10 ppb G_1 and traces of B_2 and G_2. The picture is illustrative of the excellent resolution of the four aflatoxins, B_1, B_2, G_1, G_2, from some of the background material generally found when analyzing peanut butter; the blue material between B_1 and B_2 and the blue-white peanut butter spot just below position for G_1. It is important that any TLC system employed be capable of comparable resolution or run the risk of errors in the estimation of aflatoxin if it is present in the peanut butter samples. We have never encountered a degree of interference which would prevent estimating low levels of aflatoxins (as low as 3 ppb) in raw peanuts and peanut butter by any one of these three methods when an adequate TLC system was used.

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