Comparison of Different Methods for Extraction and Estimation of Aflatoxin B_1 in Groundnut

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Manuscript received 28 *November* 1983; *revised* 11 *September* 1984

Aflatoxin extraction methods namely Best Food (BF), Contaminant Branch (CB), Pons' and Romer's and analytical methods namely thin-layer chromatography, spectrophotometric and minicolumn techniques, in different combinations, were compared for their efficiency for determining aflatoxin B_1 occurring naturally in groundnuts and also for estimating aflatoxin B_1 levels in groundnut meal samples spiked with known quantity of the toxin. The BF and Pons' methods gave better efficiency than the other two methods. Spectrophotometric method was more effective than TLC and minicolumn techniques. BF method was found relatively less expensive and less time consuming as compared to other extraction methods. The Pons' method was found convenient while handling large numbers of samples especially in the absence of centrifuge facilities required for the BF method.

Several methods for extraction and estimation of aflatoxins from groundnuts, groundnut products and other agricultural commodities have been described by various workers. The commonly used methods for aflatoxin extraction are the BF[1], CB[2], Pons'[3], and Romer's[4]. The first two are the standard methods accepted by AOAC (Association of Official Analytical Chemists) for extraction and estimation of aflatoxins in groundnuts and groundnut butter. The Pons' method was developed for determination of aflatoxins in cottonseed products but has been used for the estimation of aflatoxins in many other agricultural commodities. The method of Romer has been used for extraction and estimation of aflatoxins in mixed feeds including groundnut meal. While analyzing over one hundred samples of groundnuts for aflatoxins using two methods of extraction namely Pons' and Romer's and two methods of quantitation, i.e., TLC and minicolumn techniques, we found marked differences. This prompted us to test the extraction efficiency of four different methods (Pons', Romer's, BF and CB methods) and the accuracy of three analytical methods (TLC, minicolumn and spectrophotometric methods) for determining naturally occurring aflatoxin B_1 in groundnuts and for estimating aflatoxin B_1 levels in groundnut meal samples spiked with known quantities of the toxin.

Materials and Methods

Groundnut seeds (cv. 'TMV2') were obtained from the 1981 rainy season crop raised at the ICRISAT farm. The seeds (6 kg) were finely ground and divided into two lots of 3 kg each. One lot was used for determining naturally occurring aflatoxins. The other lot was spiked with pure aflatoxin B_1 (obtained from Makor Chemicals Ltd., Jerusalem, Israel) to give a concentration of 20 μg/kg. This material was used for testing recovery of the toxin by different extraction and analytical methods. For spiking, a measured volume of aflatoxin B_1 standard solution in chloroform was added directly to the finely ground sample which was then mixed thoroughly. The same aflatoxin was used as the spotting standard for thin-layer chromatography (TLC) and for preparation of standard minicolumns.

Extraction and analytical methods: The methods of extraction used were those of (*i*) Pons' (*ii*) Romer's, (*iii*) CB and (*iv*) BF. The detection and estimation of aflatoxin levels were done by (*i*) thin layer chromatography (TLC) using silica gel G coated (250 μm thickness) plates, (*ii*) spectrophotometry described by Nabney and Nesbitt[5], and (*iii*) minicolumn method[4] making use of Velasco Fluorotoxin meter.

All twelve combinations of the above extraction and analytical methods were tested on both naturally contaminated and spiked samples of the groundnuts. For each test, four replicates each of 50 g sample were used. All reagents used were of BDH analytical grade.

For TLC, standard spots of different concentrations (2, 4, 6, 8, 10, 15 ng) of aflatoxin B_1 with an appropriate aliquot (usually 20μl) of sample extract were spotted. The plates were developed in chloroform: acetone (9:1,

ICRISAT Journal Article No. 375.

TABLE 1. AFLATOXIN B_1 (PPB) IN GROUNDNUTS EXTRACTED AND ANALYSED BY DIFFERENT METHODS

Extraction method	Naturally contaminated			Spiked samples		
	TLC	Spectro-photometric	Minicolumn	TLC	Spectro-photometric	Minicolumn
BF	4.7±0.47*	6.0±0.44	5.2±0.45	20.5±2.06	18.6±1.55	16.6±0.78
CB	4.0±0.37	4.5±0.18	5.2±0.21	16.0±1.41	20.0±0.82	15.2±0.28
Pons'	4.9±0.41	5.2±0.33	5.6±0.19	20.0±1.41	18.6±1.55	16.8±0.64
Romer's	2.1±0.19	2.6±0.18	2.3±0.08	7.7±0.62	9.4±0.60	10.6±0.70

*Mean±SE (based on 4 observations)

v/v) in an unlined and unequilibrated tank. Aflatoxin B_1 was determined quantitatively by visual comparison of the fluorescence intensities of the sample extract spots with those of the standard aflatoxin spots under UV light at 365 nm.

The following procedure was used for estimating an unknown quantity of aflatoxin B_1 occurring naturally in groundnuts and also for calculating the efficiencies of different combinations of extraction and analytical methods for extracting and estimating the toxin.

Let μ be the unknown quantity of aflatoxin B_1 occurring naturally in groundnuts. Consider the following two cases: Let (*i*) $x_1, x_2 \ldots\ldots\ldots\ldots x_p$ be the quantities of aflatoxin B_1 observed in p indepedent homogeneous samples of same size by using certain combinations of extraction and analytical methods for determination of naturally occurring toxin. (*ii*) $y_1, y_2 \ldots\ldots\ldots\ldots y_q$ be the quantitites of aflatoxin B_1 observed in q independent samples each sample spiked with α known quantity (20 ppb in present case) of the toxin.

The means and variances from the above two cases can be written as:

$$\bar{x} = \Sigma x_i / p, \quad \bar{y} = \Sigma y_i / q$$

$$s_x^2 = \Sigma(x_i - \bar{x})^2 / (p-1)$$

$$s_y^2 = \Sigma(y_i - \bar{y})^2 / (q-1)$$

The efficiency (e) of a method is defined as:

$$e = \frac{\text{amount of aflatoxin determined}}{\text{amount of aflatoxin present in the sample}} \times 100$$

It is reasonable to assume that the method has the same efficiency for extracting aflatoxin B_1 occurring naturally in groundnuts and for extracting aflatoxin B_1 from groundnut meal spiked with the known quantity of the toxin. This assumption leads to the following relations:

$$\frac{e}{100} = \frac{\bar{x}}{\mu} = \frac{\bar{y}}{\mu + a} \qquad (1)$$

$$\mu = a\bar{x} / (\bar{y} - \bar{x}) \qquad (2)$$

$$e = 100\ (\bar{y} - \bar{x}) / a \qquad (3)$$

The variances of the two means x and y may be estimated by s_x^2/p and s_y^2/q respectively. Hence, the standard error (SE) of μ will be estimated by

$$\mathrm{SE}(\mu) = a\bar{x}\ \{s_x^2/(p\bar{x}^2) + s_y^2/(qd^2) - 2s_x^2/(p\bar{x}d)\}^{\frac{1}{2}}/d \qquad (4)$$

where $d = \bar{y} - \bar{x}$

and that of e by

$$\mathrm{SE}(e) = 100 \times (s_x^2/p + s_y^2/q)^{\frac{1}{2}}/a \qquad (5)$$

Results and Discussion

The means and their standard errors of the determinations of aflatoxin B_1 from the naturally contaminated groundnuts and from the samples spiked with the 20 ppb of the toxin are presented in Table 1. The estimates of the unknown quantity of the toxin occurring naturally in groundnuts are given in Table 2 using equations (2) and (4) as described under materials and methods. Table 3 gives the per cent efficiency of the methods in determining the toxin using the equations (3) and (5).

TABLE 2. ESTIMATES OF NATURALLY OCCURRING AFLATOXIN B_1 (PPB) IN GROUNDNUTS BY DIFFERENT EXTRACTION AND ANALYTICAL METHODS

Extraction method	TLC	Spectro-photometric	Minicolumn
BF	5.9±0.88*	9.5±1.23	9.1±0.76
CB	6.5±0.87	5.7±0.34	10.0±0.36
Pons'	6.5±0.71	7.7±0.94	10.0±0.60
Romer's	7.4±0.93	7.7±0.76	5.6±0.50

*Estimate±SE

TABLE 3. EFFICIENCY (PER CENT) OF DIFFERENT EXTRACTION AND ANALYTICAL METHODS FOR RECOVERY OF AFLATOXIN B_1 IN GROUNDNUTS

Extraction method	TLC	Spectro-photometric	Mini-column	Weighted* average
BF	79±11[a]	63±8	57±4	60±3
CB	60±7	78±4	50±2	56±2
Pons'	76±7	67±8	56±3	60±3
Romer's	28±3	34±3	41±4	—
Weighted av.‡	70±4	73±3	53±2	—

[a]Efficiency of combination of extraction and analytical method ±SE

*Weighted average (over all analytical methods) efficiency of extraction method±SE

‡Weighted average (over BF, CB and Pons' methods only) efficiency of analytical method±SE

— = Not considered.

There were marked differences among the extraction and analytical methods in the determination of naturally occurring aflatoxin B_1 in groundnuts (Table 1). The BF and Pons' methods showed better extraction efficiency than the other two methods. The CB method was slightly less efficient than the BF and Pons' methods, while Romer's method extracted considerably lower amounts of aflatoxin B1 than did the other three methods (Table 3). The two alkali treatment steps using NaOH and KOH during clean-up procedures may possibly be responsible for the low extraction efficiency of Romer's method. In another experiment, when these two steps were omitted from the method, there was some improvement in the recovery of the toxin (Mehan, unpublished data). Spectrophotometric method was more effective than TLC and minicolumn techniques (Table 3). The BF and Pons' methods coupled with TLC and CB procedure coupled with spectrophotometric analysis showed more than 75 per cent efficiency in determining aflatoxin B_1. Pons *et al* also reported wide differences between several extraction methods while determining levels of aflatoxin B1 in groundnuts and groundnut meals employing TLC as an analytical procedure for visual estimations. Pons' method was reported to be better than the other four methods used. Romer's method recovered markedly lower levels of aflatoxin B_1 with all the three analytical methods (Table 1). However, none of the procedures could provide more than 80 per cent efficiency and accuracy.

Economy and speed of analysis are important factors in choosing methods for mycotoxin estimation. Although CB procedure is efficient[6], it is time consuming because of the lengthy clean-up procedure and expensive as well. Cost and time required per sample for extracting aflatoxins by each of the extraction methods were calculated and it was noted that BF method was superior to the other methods in both respects. This method costs about Rupees 15 per sample as compared to those of Romer (Rs. 20), Pons' (Rs. 23) or CB method (Rs. 70). The BF procedure also requires less time (1.35 hr) in comparison with 2.05 hr, 2.50 hr, and 5.30 hr taken by Romer's, Pons' and CB, respectively. Pons' method was the next best in respect of cost and time requirements. Although Pons' method was originally developed for extracting aflatoxins from cottonseeds, it has been effectively used for groundnuts[7]. It has been found to be very convenient when handling large numbers of samples and it is particularly suitable for laboratories that lack the centrifuge facilities required for the BF method. It is concluded that the Pons' extraction procedure coupled with TLC or spectrophotometric analytical method is reasonably an efficient and quicker one for estimation of aflatoxin B_1 in groundnuts under such conditions.

Acknowledgement

We thank Dr. Murari Singh for going through the manuscript critically and for statistical analysis.

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