A New Method for Determining Aflatoxins in Groundnut and Groundnut Cake Using Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance

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ABSTRACT: A new analytical method was developed for the determination of aflatoxins in groundnut and groundnut cakes by Fourier transform infrared (FTIR) spectroscopy using horizontal attenuated total reflectance technique. Groundnut and groundnut cake samples were used in this study. The wavelengths were selected for the four types of aflatoxins—B_1, B_2, G_1, and G_2—and the standards prepared for each by spiking some clean sample with the aflatoxins in concentrations of 0–1200 parts per billion. A partial least square regression was used to derive the calibration models for each toxin. The coefficients of determination (R^2) of the calibration model were computed for the FTIR spectroscopy predicted values vs. actual values of aflatoxins in parts per billion. The R^2 was found to be 0.9911, 0.9859, 0.9986, and 0.9789 for aflatoxins B_1, B_2, G_1, and G_2, respectively. Standard errors of calibration for groundnut samples were found to be 1.80, 2.03, 1.42, and 2.05 for aflatoxins B_1, B_2, G_1, and G_2, respectively. Calibration models were validated with an independent set of samples. The R^2 of validation models were computed. The SD of the difference for repeatability of the FTIR method was found to be better than that of the chemical method. Based on the results obtained, FTIR spectroscopy can be a useful instrumental method for determining aflatoxins in oilseeds and oilseed cakes. With its speed and ease of data manipulation by computer software, it is a possible alternative to the standard wet chemical methods for a rapid and accurate routine determination of aflatoxin levels in food and feed.

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KEY WORDS: Aflatoxin, ATR, FTIR, groundnut, PLS.

Some microorganisms (molds, yeast, and bacteria) produce toxins in their metabolites, for example, mycotoxins by molds. Mycotoxins have probably been with humans since the beginning of time, but have not been known as a health hazard until relatively recently. Interest in them was aroused in 1961 when an epidemic swept through the Christmas turkey population in England, killing a large number of them (1). Investigation into the death focused on the rancid groundnut meal from Brazil used in the poultry feed, eventually identifying aflatoxin—a then-unknown mycotoxin produced by *Aspergillus flavus.* Subsequently, it was found that *Aspergillus parasiticus* can also produce aflatoxin (2,3).

Aflatoxin is a very potent acute poison to animals, humans included (4,5), although there are some differences in sensitivity between different species (3). In sublethal doses, it is a carcinogen, causing liver cancer in 10–20 yr. Because of its great danger, the food and livestock feed industries are wary of it and go to great lengths to detect it and reject any contaminated foodstuff.

Among the oilseeds, aflatoxins pose the most serious problem in groundnut (6), but they can occur in all of them. Thus, at least 60 countries have proposed or established limits for the aflatoxin level in food/feed (7).

One of the first tests for aflatoxins was done on young ducklings, as they are particularly susceptible to the poison. The ducklings not only died but also produced characteristic liver lesions (1). The test was also used to quantify the toxin concentration by classical chemical procedures as the toxin emits a characteristic bright-blue fluorescence under ultraviolet (UV) light with the intensity related to the toxin level (1,2,8).

There were two peaks in the fluorescence—blue and green—resulting in the previously thought single poison being partitioned into aflatoxins B and G, respectively. But it was soon found that each of the two components also had two subcomponents differing in R_f on thin-layer chromatography (TLC). The four aflatoxins were therefore designated B_1, B_2, G_1, and G_2 in order of decreasing R_f values (9,10). More differentiation has since been made, and today more than 20 aflatoxins are recognized (6,11) in addition to their methoxy, ethoxy, and aceto derivatives. However, only a few of them, most importantly aflatoxin B_1, occur naturally.

Holaday (12) introduced the minicolumn chromatography method for rapid estimation of aflatoxins, and this was improved on by Velasco (13) and Holaday (14). Aflatoxins can also be quantified directly on TLC plates by fluorescence densitometry (15). Recently, there has been much interest in the use of high-pressure liquid chromatography as it gives much better reproducibility (2,16,17).

Most of the foregoing methods are rather expensive, time-consuming, labor-intensive, and/or use large quantities of toxic chemicals. Lopez *et al.* (18) suggested an easier method of visual estimation of aflatoxin production in groundnut with *Aspergillus* mutants that accumulate norosolorinic acid, an orange-pigmented intermediate in the synthetic pathway for aflatoxins.

The first tests for aflatoxins took nearly 3 d to do. The time has since been reduced, but it remains that there is no single

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quantitative analysis for the determination of aflatoxins in all commodities. A satisfactory method for groundnuts may not work well for cottonseed products or for mixed animal feeds.

This study aimed to develop and examine Fourier transform infrared (FTIR) spectroscopy as a rapid, easy, and convenient analytical method for determining aflatoxins in food/feed using mainly pure standard aflatoxins for calibration instead of preanalyzed groundnut and groundnut cake as in standard practice. The standard methods of analysis are recognized by official organizations such as the British Standards Institute (previously the Tropical Products Institute), the Association of Official Analytical Chemists, the American Oil Chemists' Society (AOCS), and the American Association of Cereal Chemists.

EXPERIMENTAL PROCEDURES

Samples and chemicals. All chemicals were of analytical grade. The chemicals and aflatoxin standards were purchased from Sigma Chemical Co. (St. Louis, MO). The groundnut samples were purchased from local retailers. Some aflatoxin-free samples were spiked with defined amounts of aflatoxins covering the range 0–1200 ppb. Groundnut cake was solvent-extracted after grinding up the nuts. Some samples of raw groundnut and cake were moistened and incubated (19,20) and exposed to air at room temperature for about 45 d. Pure aflatoxin standards B_1, B_2, G_1, and G_2 were prepared in different concentrations in benzene/acetonitrile (98:2) covering the range 0–1200 ppb. This is based on the AOCS Official Method Ab 6-68 (21).

Extraction and cleanup. A 100-g sample of groundnut or groundnut cakes was placed into a steel blender container (Waring Commercial, New Hartford, CT), and 500 mL methanol/water (55:45), 200 mL hexane, and about 4 g NaCl were added in order. The mixture was blended for 1 min at high speed and left to stand for 30 min to separate. Then 25 mL of the aqueous methyl alcohol phase was pipetted into a 250-mL separation funnel, and 25 mL chloroform was added. The funnel was stoppered, shaken for about 1 min, and then left for the layers to separate. The bottom chloroform layer was drained into a stainless steel beaker and evaporated over steam in an atmosphere of nitrogen just to dryness. The residue in the beaker was carefully flushed into a 25-mL glass-stoppered Erlenmeyer flask or a vial with three small chloroform rinses. The flask, or the vial, was placed over a hot-water bath and evaporated just to dryness. The extract was dissolved in 250 µL benzene/acetonitrile (98:2) for spotting on TLC plate. Duplicate tests were done for each sample.

TLC. The solutions from the 28 samples and reference standards were spotted on pre-coated 20 × 20 cm TLC plates (SIL G-25) of 0.25-mm silica gel layer (Macherey-Nagel GmbH, Düren, Germany). The plates were developed for about 25 to 35 min in a developing tank using chloroform/acetone (90:10) solution and then removed and allowed to dry before reading in UV light.

The plates were examined in a long-wavelength UV viewing chamber. Four clear spots were visible in the reference standards, and the R_f values of aflatoxins being identified should closely correspond to the respective standard spots. However, some interpolation may be necessary to identify sample spots between two standard spots.

Calculations. The concentration of the aflatoxin B_1 in µg/kg or (ppb) was derived by Equation 1:

$$\text{aflatoxin } B_1 \text{ (µg/kg or ppb)} = (S \times Y \times V)/(X \times W) \quad [1]$$

where S = µL aflatoxin B_1 standard equal to unknown; Y = concentration of aflatoxin B_1 standard, in µg/mL; V = µL of final dilution of sample extract; X = µL of sample extract spotted giving fluorescent intensity equal to S (B_1 standard); and W = grams of sample contained in aliquot transferred to separation funnel.

Calculate W as in the following example, using a sample size of 100 g and 25 mL aliquot to the separation funnel (Eqs. 2 and 3).

$$25/500 = 1/20 \quad [2]$$

$$1/20 \times 100 = 5 \text{ g} \quad [3]$$

The same procedure was followed in calculating the concentrations of the B_2, G_1, and G_2 spots. The total aflatoxins will be the total of the individual spots.

FTIR method. To quantify aflatoxin using FTIR spectroscopy, it is necessary first to obtain a calibration between the IR band intensity and aflatoxin content. With the calibration, the unknown content of the sample can be estimated provided that: (i) the spectra from the sample are recorded under the same conditions as for the calibration, and (ii) the standards used are representative of the unknown and cover the same range.

Although the sample extract could be applied to the FTIR before and/or after the cleanup step, the same standard-solution extracts (after cleanup) used in the TLC method were applied to the FTIR spectrometer for developing a calibration curve.

Spectra acquisition. The mid-band infrared attenuated total reflectance (ATR) spectra were obtained with a Fourier transform spectrometer (Series1725; PerkinElmer Ltd., Beaconsfield, Buckinghamshire, United Kingdom) equipped with a deuterated triglycine sulfate detector and a horizontal ATR accessory. Automatic dehumidifiers were used to protect against interference by water vapor. The samples were placed in contact with the ATR element (ZnSe crystal 45° ends) at room temperature. Spectra were collected from 32 scans from 4000 to 750 cm^{-1} wave numbers at 8 cm^{-1} resolution. A strong apodization was used. After each measurement, the crystal was cleaned three times with acetone and dried. The cleaned crystal was checked spectrally to ensure that no residue remained from the previous sample. Duplicate tests were done for each sample of the 28 prepared samples, and stored in JCAMP files on diskette for subsequent analysis.

Mathematical and statistical analysis. The Nicolet Turbo Quant IR calibration and prediction software package (Madison, WI), based on partial least squares (PLS) regression, was used to obtain the calibration. A validation of the model as

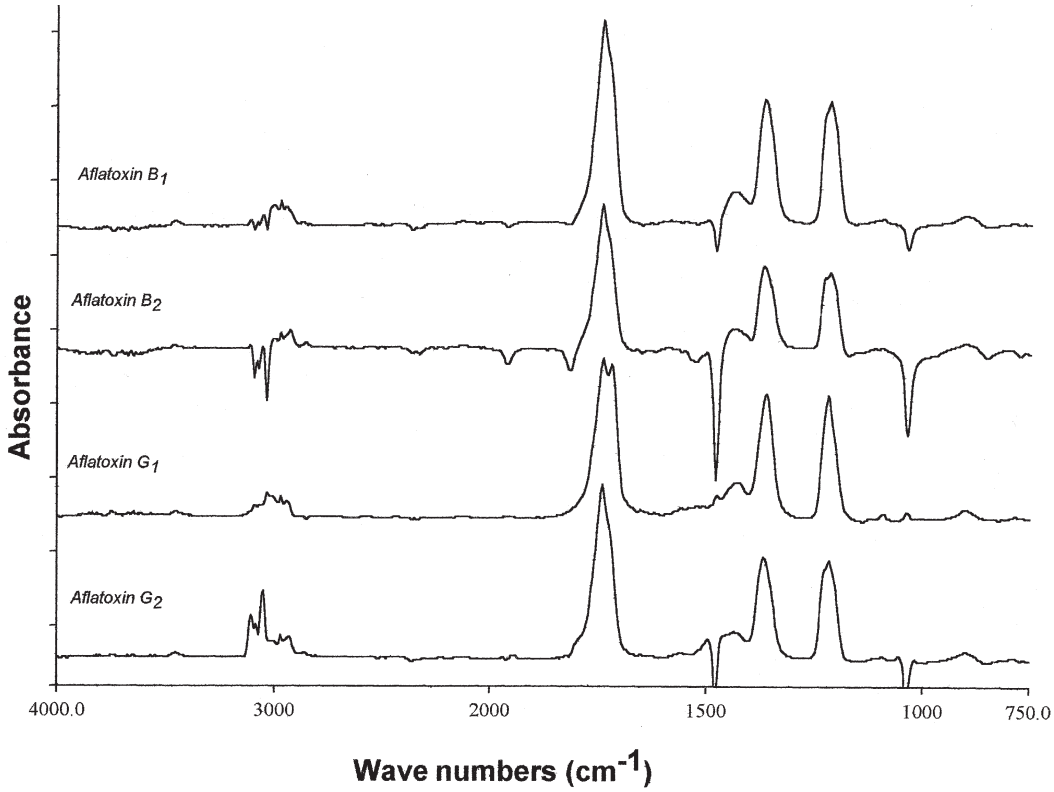


FIG. 1. Spectra of aflatoxins B_1, B_2, G_1, and G_2 after subtracting the solvent spectra.

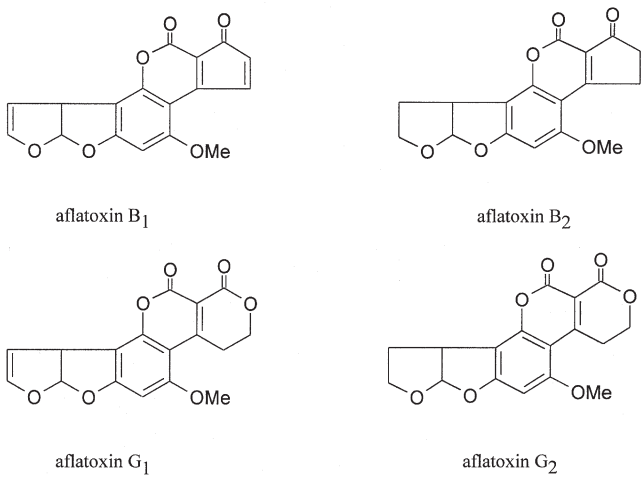
stated by Fuller *et al*. (22) was then carried out to assess the predictive capability of the model. Using the mean difference (MD) and SD of the difference (SDD_r) between the predicted and actual or chemical values, the calibration was further improved (23,24).

RESULTS AND DISCUSSION

One of the fundamental strengths of FTIR spectroscopy is that it can use spectral ratioing to discern small differences that would otherwise be missed in the raw spectrum (25). Ratio comparison of the spectrum of a sample contaminated with aflatoxins against that of aflatoxin-free sample or the spectrum of the solvent only permits the spectral features of aflatoxins to be seen. Figure 1 shows the spectra of aflatoxins B_1, B_2, G_1, and G_2 after subtracting the solvent spectrum. The aflatoxins exhibited characteristic absorption bands at wavelengths 3004–2969 cm^{-1} for CH_2, aromatic =CH, –C–H, C=C and phenyls, 1744–1720 cm^{-1} for C=O, 1364–1369 cm^{-1} for methyl adjacent to epoxy ring, 1217–1220 cm^{-1} for in-plane –CH bending of phenyl (26), 1035–1037 cm^{-1} for symmetric stretching of =C–O–C or symmetric bending of phenyl, and 900–902 cm^{-1} for possibly isolated H. Scheme 1 shows the chemical structure of the four aflatoxins.

Development of calibration models. The calibration standards were designed to obtain data for PLS regression, that is, as free as possible from interference by other components. Thus, only the data from regions that correlated with the features of interest were abstracted by the PLS software in order to obtain a calibration standard(s) spectrally representative of the samples to be analyzed (27). Table 1 shows the results (duplicate values) from the derived calibrations and SD analysis of data obtained from the AOCS reference and the FTIR methods.

The variance and correlation spectra were used to optimize the spectral data. The correlation spectrum (Fig. 2, line a) was used to select the best spectral regions for analysis, while the variance spectrum (Fig. 2, line b) displayed the regions with changed absorbance values over the calibration set. For the calibration set in Figure 2, the spectral regions with the highest correlation between concentration information and spectral response (27) were set to include all the data from 3000



SCHEME 1

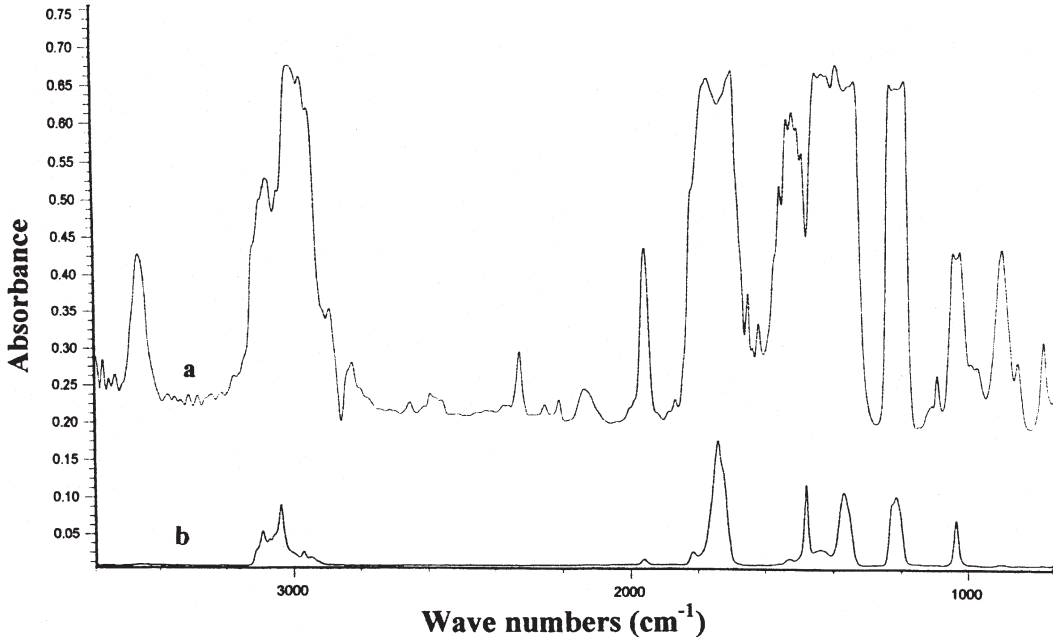


FIG. 2. Line a: The correlation spectrum obtained from the calibration standards of aflatoxin B_1; line b: the variance spectrum obtained from the calibration standards of aflatoxin B_1.

to 2932, 1832 to 1693, 1400 to 1329, and 1250 to 1187 cm^{-1} for aflatoxin B_1. A correlation plot was developed (Fig. 3) using the actual values and calibration set that gave a coefficient of determination (R^2) of 0.9911. The equation was

$$Y = 0.9758x + 7.8432 \quad [4]$$

and the intercept and slope were not significantly different ($P > 0.05$) from 0.0 and 1.0, respectively. The calibration was evaluated by a set of known spiked samples. The spectral regions used for the determination of aflatoxins B_2, G_1, and G_2 were selected in the same manner as for B_1. Figures 4 to 6 show the correlation and variance spectra used to select spectral regions for prediction. For the determination of aflatoxin B_2, the best correlation between concentration information and spectral response was obtained using all the data from 1800–1700, 1500–1452, and 1051–1015 cm^{-1}, which gave R^2 of 0.9859. For aflatoxin G_1, the best correlation ($R^2 = 0.9986$) was obtained using all the data from 3060–2924, 1852.5–1681.5, 1550–1451, 1265–1144.8, and 1073–1000 cm^{-1}. For aflatoxin G_2, the data were from 1825–1677, 1400–1320, and 1250–1167 cm^{-1} ($R^2 = 0.9789$). The standard error of calibration for the calibration models for aflatoxins B_1, B_2, G_1, and G_2 were 1.80, 2.03, 1.42, and 2.05, respectively (Table 2).

Figure 7 shows the results of validation of the predictive model for aflatoxin B_1, from comparing the data obtained by the chemical method and FTIR spectroscopy. This plot is linear with slope of 0.974 and R^2 of 0.9691. The standard error of prediction was 2.07, which indicates the accuracy for the FTIR method, as the values were so close to the chemical ones. Correlation plots were developed for aflatoxins B_2, G_1, and G_2 (Figs. 8–10, respectively).

The correlation between the FTIR spectrometry predictions and their chemical method values (Table 2) were all

TABLE 1
Calibration and Validation Statistics for Aflatoxins Determined by AOCS TLC and FTIR-ATR Spectroscopic Methods[a]

		AOCS TLC method		FTIR-ATR method	
Data set	Aflatoxins	Mean	SD	Mean	SD
Calibration	B_1	476.55	11.54	489.60	2.16
	B_2	461.26	12.81	475.38	3.04
	G_1	439.58	9.34	451.62	2.50
	G_2	430.92	10.63	442.32	2.28
Validation	B_1	362.35	13.78	380.93	2.79
	B_2	347.24	14.14	366.23	3.34
	G_1	318.09	12.59	334.57	3.66
	G_2	305.22	13.27	330.41	3.85

[a]AOCS, American Oil Chemists' Society; TLC, thin-layer chromatography; FTIR, Fourier transform infrared; ATR, attenuated total reflectance. Values reported as ppb.

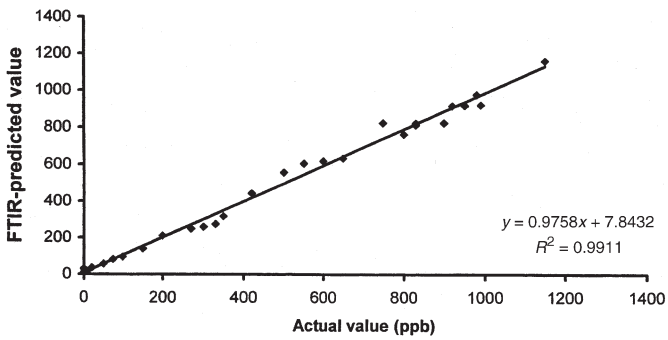


FIG. 3. A calibration plot of actual values vs. Fourier transform infrared (FTIR)-predicted values of aflatoxin B_1 for 28 samples, calculated with partial least squares (PLS) regression.

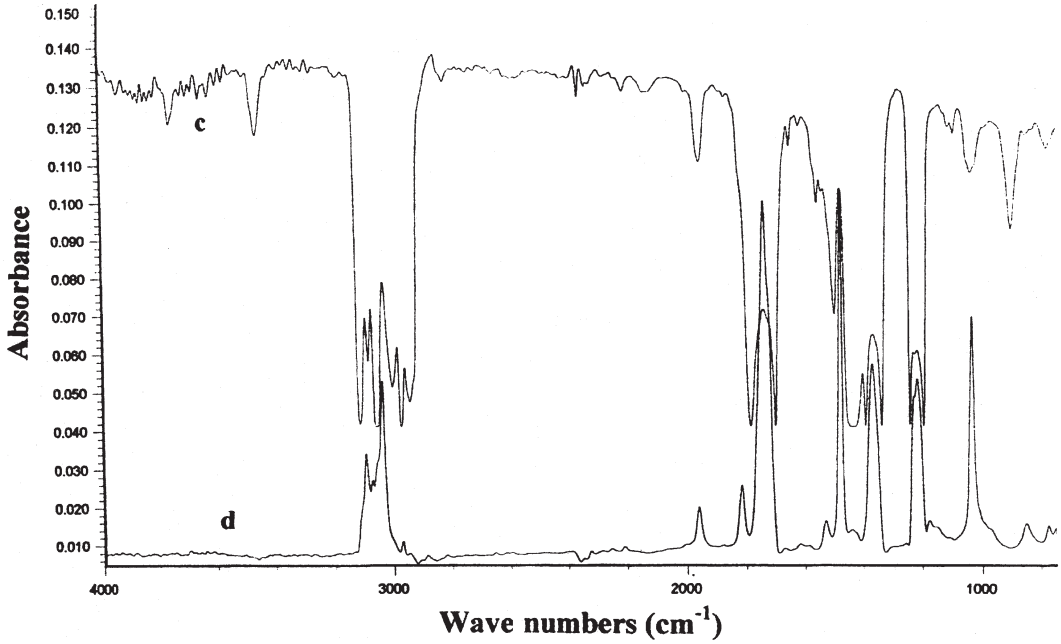


FIG. 4. Line c: The correlation spectrum obtained from the calibration standards of aflatoxin B_2; line d: the variance spectrum obtained from the calibration standards of aflatoxin B_2.

high as were the standard error of calibration and standard error of prediction. The MD_a and SDD_a were −13.05 and 4.17, respectively, for aflatoxin B_1. The CV was 0.035 to 6.50% for the FTIR method compared with 1.2 to 13.4% for duplicate chemical analysis. In terms of repeatability, the MD_r was 0.84 for the FTIR method and 7.22 for the duplicate chemical analysis. The SDD_r were 2.02 and 4.64 for FTIR and chemical TLC methods, respectively. Table 3 shows the results of calibration statistics for aflatoxin content in groundnut and groundnut cake samples obtained by AOCS TLC reference method and FTIR spectroscopic methods. The FTIR spectroscopic method was the more accurate as the TLC method was difficult for visually estimating small differences in intensity on the TLC plates.

This study has shown that FTIR-ATR spectroscopy can be used to determine aflatoxin contents in groundnut and groundnut cake. The analysis is rapid and requires only minimal sample size (>2 mL) and chemicals.

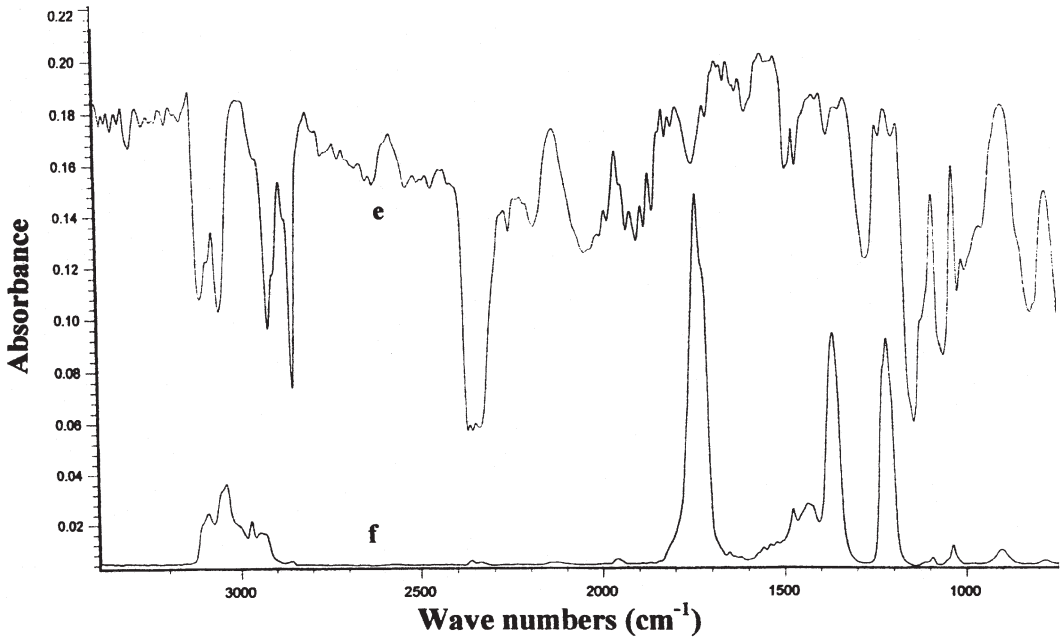


FIG. 5. Line e: The correlation spectrum obtained from the calibration standards of aflatoxin G_1; line f: the variance spectrum obtained from the calibration standards of aflatoxin G_1.

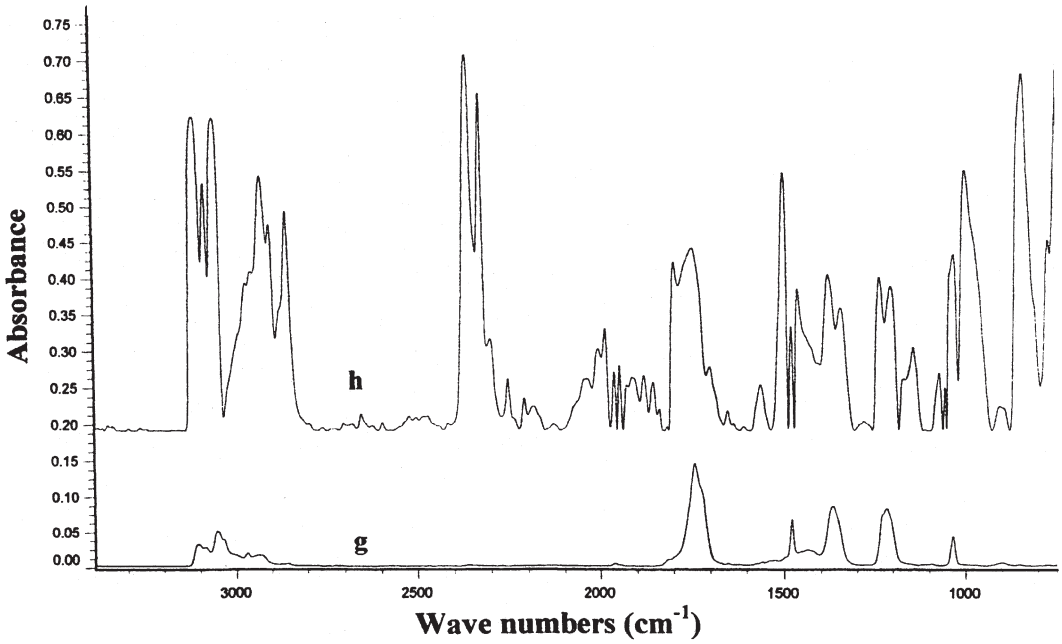


FIG. 6. Line h: The correlation spectrum obtained from the calibration standards of aflatoxin G_2; line g: the variance spectrum obtained from the calibration standards of aflatoxin G_2.

TABLE 2
Results of Calibration Models of AOCS TLC Reference Method for Aflatoxins in Reference Standards (groundnut and groundnut cake) and FTIR-ATR Methods[a]

		Regression equation		Error	
Samples	Aflatoxins	Calibration	Validation	SEC	SEP
Groundnut	B_1	$Y = 0.9758x + 7.8432$	$Y = 0.9740x + 12.271$	1.80	2.07
	B_2	$Y = 0.9770x + 13.960$	$Y = 0.8793x + 11.648$	2.03	2.18
	G_1	$Y = 1.0035x - 1.1941$	$Y = 0.8867x + 8.5750$	1.42	1.94
	G_2	$Y = 0.9690x + 7.7132$	$Y = 0.9726x + 8.3521$	2.05	2.21
Groundnut cake	B_1	$Y = 0.9332x + 8.9551$	$Y = 0.8950x + 9.0431$	1.62	1.85
	B_2	$Y = 0.8894x + 6.7830$	$Y = 0.9687x + 10.147$	1.87	1.99
	G_1	$Y = 0.8975x + 10.369$	$Y = 0.8884x + 9.5360$	2.11	2.06
	G_2	$Y = 1.0072x - 2.0103$	$Y = 0.9103x + 7.8994$	1.97	2.18

[a]SEC, standard error of calibration; SEP, standard error of prediction for validation; for other abbreviations, see Table 1.

TABLE 3
Calibration Statistics for Aflatoxin Content in Groundnut and Groundnut Cake Obtained by Classical TLC and the FTIR-ATR Methods[a]

	B_1		B_2		G_1		G_2	
Statistics	TLC	FTIR	TLC	FTIR	TLC	FTIR	TLC	FTIR
MD_r	7.22	0.84	9.35	1.27	7.94	0.97	10.05	2.00
SDD_r	4.60	2.02	5.13	2.54	3.82	1.98	5.06	1.88
MD_a	−13.05		−14.12		−12.04		−11.40	
SDD_a	4.17		5.02		3.69		4.58	

[a]MD, mean difference; SDD_r, standard deviation of difference; *r*, repeatability; *a*, accuracy; for other abbreviations, see Table 1.

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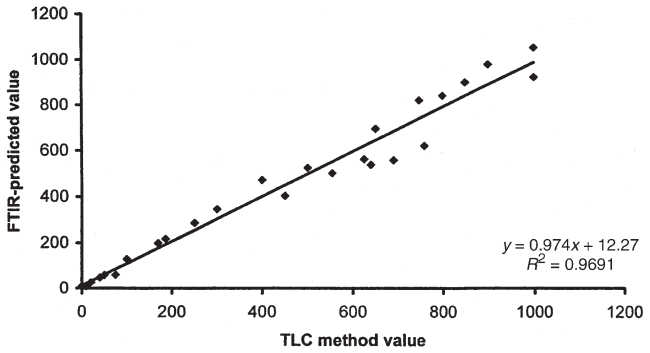

FIG. 7. A validation plot of FTIR-predicted values of aflatoxin B_1 vs. chemically determined aflatoxin B_1 for 28 groundnut samples, calculated with PLS regression. See Figure 3 for abbreviations.

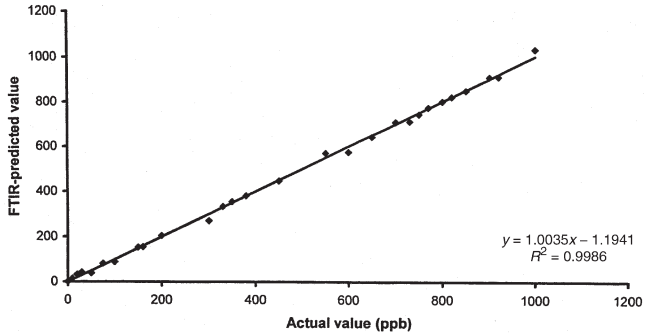

FIG. 9. A calibration plot of actual values vs. FTIR-predicted values of aflatoxin G_1 for 28 samples, calculated with PLS regression. See Figure 3 for abbreviations.

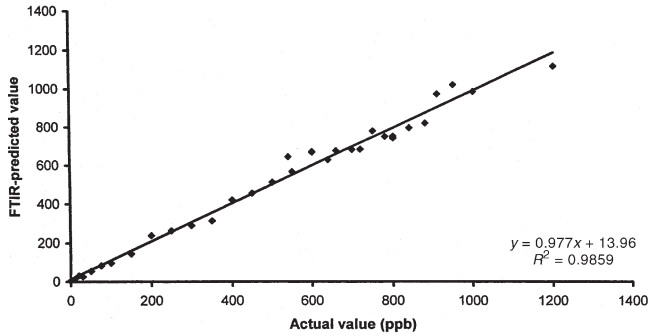

FIG. 8. A calibration plot of actual values vs. FTIR-predicted values of aflatoxin B_2 for 28 samples, calculated with PLS regression. See Figure 3 for abbreviations.

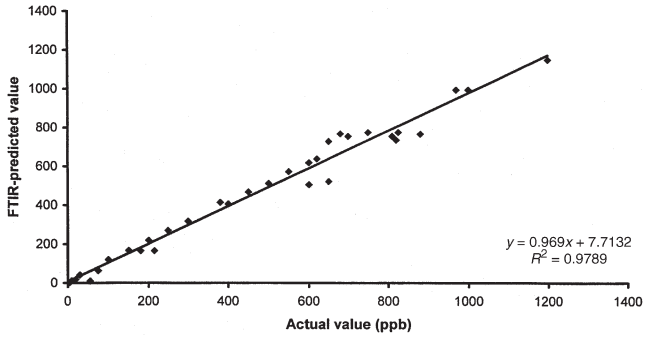

FIG. 10. A calibration plot of actual values vs. FTIR-predicted values of aflatoxin G_2 for 28 samples, calculated with PLS regression. See Figure 3 for abbreviations.

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