Comparative Study of Three Different Methods for the Determination of Aflatoxins in Tahini

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Aflatoxins spiked at three different levels (6.5, 13.0, and 19.5 μg/kg) in tahini, a sesame butter, were analyzed by using three different methods: high-performance liquid chromatography (HPLC), fluorometry, and enzyme-linked immunosorbent assay (ELISA). An immunoaffinity column was used for cleanup and purification of extracts prior to detection by HPLC and fluorometry. All methods were statistically evaluated for accuracy, precision, and simple correlations. Additionally, 14 tahini samples randomly obtained from Turkish retail markets were analyzed using an immunoaffinity column cleanup procedure coupled with the HPLC detection method. The fluorometric determination method involving an immunoaffinity column cleanup step was found to be highly correlated with the HPLC method ($r = 0.978$). Both methods were found to be effective due to their high recoveries and low variance for the prediction of total aflatoxin contamination in tahini samples. The ELISA method, due to its high variation in replicates, was found to be applicable only as a screening method. The survey study demonstrated the need for control of aflatoxin contamination of foodstuffs involving sesame seeds as an ingredient.

KEYWORDS: Aflatoxin; analysis; sesame seeds; tahini; high-performance liquid chromatography (HPLC); fluorometry; enzyme-linked immunosorbent assay (ELISA); immunoaffinity column

INTRODUCTION

Aflatoxins B_1, G_1, B_2, and G_2, which are the secondary metabolites of the molds *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*, produce great risk by contaminating a wide variety of agricultural commodities and foodstuffs, especially those having high carbohydrate and/or fat contents, such as tree nuts and nut products, peanuts, corn, cereals, grains, oilseeds, dried figs and raisins, cottonseed, milk, feedstuffs, and dried spices (*1−8*). Tahini (sesame butter) produced by milling of dehulled and roasted sesame seeds is widely consumed as a mixture with sweet foods such as honey and grape molasses and also is an important ingredient of halva, which is a popular sweet food in Turkey and in some Middle East countries (*9, 10*). *A. flavus* and *A. parasiticus* have been reported to grow on sesame seed and produce aflatoxins (*11−13*). Several survey studies on sesame seeds (*14−17*) and tahini (*18*) have shown the extent of aflatoxin contamination.

In recent years, there has been an increase in demand for simple, quick, accurate, and specific methods for the determination of aflatoxins. The immunological methods that have been used since the 1990s are accepted as official methods for aflatoxin determination in some food commodities; however, it is still necessary to evaluate their efficiency in different foodstuffs and to correlate their results with other accepted analytical methods (*19*). High-performance liquid chromatog-

raphy (HPLC) and enzyme-linked immunosorbent assay (ELISA) methods were found to be highly correlated for the analysis of total aflatoxins in foodstuffs such as peanut, peanut butter, and corn and for aflatoxin B_1 in corn and mixed feeds, whereas in other foodstuffs, such as cereals, low correlations were observed (*20*). ELISA methods could be used only for screening purposes with the detection limit above the regulatory limits because they are not reliable when used as a quantitative method (*21, 22*). Analyses of aflatoxins B_1, B_2, and G_1 in corn and of total aflatoxin levels in peanut butter and aflatoxin B_1 in corn and roasted peanuts based on ELISA methods were adopted as AOAC Official Methods (*23*). Immunoaffinity column cleanup techniques coupled with HPLC detection or fluorometric detection are applied to various food matrices such as corn, peanuts, peanut butter, pistachios, Brazil nuts, almonds, nut confectionery products, maize gluten, soya expeller, beer, animal products, animal feedstuffs, and dried fruits such as figs, dates, and apricots (*22, 24−26*). The immunoaffinity column (Aflatest) procedure is an AOAC-approved method for the determination of aflatoxins in corn, raw peanuts, and peanut butter (*23*).

The objective of this study was to analyze aflatoxins in tahini, which is a traditional Turkish food, by HPLC, fluorometry, and ELISA methods. Correlations of three aflatoxin determination methods were investigated, and methods were compared in terms of accuracy and precision. A limited aflatoxin survey of tahini in 14 samples randomly collected from the market was also carried out.

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Table 1. Amount of Total Aflatoxins Added to the Samples and Aflatoxin B_1, B_2, G_1, and G_2 Concentrations after Spiking

level		amount of total aflatoxins spiked to 25 g of sample (ng)	volume spiked from stock solution (mL/ 25 g of sample)	individual aflatoxin concn (ppb)			
no.	aflatoxin (ppb)			B_1	B_2	G_1	G_2
1	6.5	162.5	1.25	2.5	0.75	2.5	0.75
2	13.0	325	2.5	5.0	1.5	5.0	1.5
3	19.5	487.5	3.75	7.5	2.25	7.5	2.25

MATERIALS AND METHODS

Apparatus. For HPLC analysis an HPLC system equipped with a Waters 501 solvent delivery system, a 7010 injection port, an HP 1046A fluorescence detector (excitation, 363 nm; emission, 440 nm), a 300 mm × 3.9 mm i.d. Millipore-Waters μ-Bondapak C18 LC column, and 810 Baseline software was used. A Vicam series 4 fluorometer, model Vicam V1.0 (Vicam, Watertown, MA), was used for the fluorometric determination of total aflatoxins. As an ELISA microstrip reader, a Statfax 303 Plus (Awareness Technology Inc., Palm City, FL) with a 650 nm filter was utilized.

Kits. Aflatest P columns (Vicam) and Veratox kits for aflatoxin (Neogen, Lansing, MI) were tested.

Samples. Tahini samples of 14 different trademarks were randomly selected from the Turkish retail market. One sample confirmed to contain a total aflatoxin level below the detection limit was used for the comparison of methods.

Due to the separation of oil–water phases during storage, samples were homogenized by mixing nearly for 30 min (Janke & Kunkel RW 20) prior to analysis. Twenty-five gram portions of samples were weighed into tightly capped small glass jars and wrapped with aluminum foil.

Aflatoxin Standard Solution. An aflatoxin B and G mixture purchased in dry concentrate form (Sigma, St. Louis, MO) was dissolved in 7.5 mL of benzene/acetonitrile (98:2, v/v). Five hundred microliters of a mixed aflatoxins stock standard solution, containing 5 μg/mL aflatoxin B_1, 1.5 μg/mL aflatoxin B_2, 5 μg/mL aflatoxin G_1, and 1.5 μg/mL aflatoxin G_2 (B_1/B_2/G_1/G_2 10:3:10:3), was transferred into an amber vial and evaporated just to dryness under a stream of nitrogen at room temperature. The total aflatoxin concentration was 130 ng/mL when the contents of the vial were dissolved in 50 mL of benzene/acetonitrile (98:2, v/v).

Spiking. Test samples were spiked with total aflatoxins B_1, B_2, G_1, and G_2 at 6.5, 13.0, and 19.5 μg/kg levels in triplicate. These spike levels represent values above and below the maximum allowable regulatory limits of the Turkish Food Codex, which is 10 μg/kg (27).

Each 25 ± 0.1 g of sample was spiked with the known amounts of 130 ppb stock aflatoxin solution given in **Table 1**. The spiked samples were kept in a fume hood to allow the benzene/acetonitrile solvent to evaporate and were kept at 4 °C until analysis.

Extraction. Aflatoxins were extracted from samples using AOAC Method 968.22 (28). Spiked samples were transferred from glass jars to the blender by rinsing the jar with 125 mL of methanol/water (70: 30, v/v). Five grams of NaCl was added and blended at high speed (23000 rpm) for 2 min. The sample extract was divided into three portions to be analyzed by three determination methods.

Immunoaffinity Column Cleanup. Affinity column cleanup was performed according to AOAC Method 968.22 (28). Filtered and diluted extracts were passed through the affinity columns followed by HPLC determination and fluorometric determination methods. The methanol eluate was collected in 2 mL Eppendorf tubes and evaporated under a stream of nitrogen gas. The dry residue was kept at 4 °C until analysis.

Derivatization. Aflatoxins were derivatized with trifluoroacetic acid (TFA) according to AOAC Method 990.33 with slight modifications prior to HPLC injections (23). The modification involved an increase in mixing time to 1 min and the use of 0.95 mL of a water/acetonitrile (9:1, v/v) solvent system, instead of 1.95 mL, to dissolve aflatoxins.

HPLC Determination. For liquid chromatographic determination a water/acetonitrile/methanol (70:17:17, v/v) mobile phase system

having a flow rate of 1 mL/min was used (23). The retention times of each aflatoxin component (G_1, B_1, G_2, and B_2) were 6.3, 7.5, 9.9, and 12.7 min, respectively, in a 20 min run time. Toxins were detected by fluorescence at an excitation wavelength of 363 nm and emission wavelength of 440 nm. The injection volume was 50 μL. Each sample was injected twice.

Fluorometric Determination. The dry sample was dissolved in 1 mL of HPLC grade methanol and mixed in a minishaker for 1 min at 1600 rpm. One milliliter of developer solution was added to the sample extract and mixed at 1600 rpm for 30 s. Following transfer of sample extract and developer solution into the fluorometry cuvette, the total aflatoxin content of the sample was read out in parts per billion. Each sample was measured twice (29).

ELISA Determination. ELISA analysis was performed according to the instructions of the Neogen Veratox aflatoxin procedure. Kits and extracts were brought to ambient temperature before analysis. A multichannel pipettor was used for ELISA analysis, and great attention was given to the incubation periods. Concentration of total aflatoxins in parts per billion was recorded from a 650 nm filter ELISA reader that was calibrated using aflatoxin standards (30).

Survey Study. Fourteen tahini samples randomly collected from various markets in Istanbul were analyzed by immunoaffinity cleanup and HPLC determination using the same conditions previously described.

Decontamination of Equipment. To avoid cross-contamination of samples, all glassware was soaked in a 10% solution of household bleach containing 5.25% NaOCl for a day, which was followed by washing with a mild detergent and rinsing with pure water (31). The container of the Waring blender was rinsed with methanol after washing with a mild detergent. The syringe barrel and the microsyringes (injector) were rinsed thoroughly with methanol (29).

Statistical Analysis. The performances of three different aflatoxin determination methods were compared by statistical analysis with an SPSS software program. At each spiking level, the recoveries (percent) and the coefficients of variation (percent) were calculated. The differences between the HPLC, fluorometry, and ELISA methods at total aflatoxin levels were analyzed by one-way ANOVA experimental design, where the variance component was accepted as the concentration of aflatoxins. Significant differences were analyzed by comparing the mean values using Duncan's new multiple-range test. A simple linear regression analysis was performed between the results of HPLC, fluorometry, and ELISA methods.

RESULTS AND DISCUSSION

The results obtained for the HPLC method for each aflatoxin component and total aflatoxin levels of tahini samples measured by HPLC, fluorometry, and ELISA are given in **Table 2**.

The coefficents of variation (CV%) of the HPLC analysis of aflatoxins B_1, G_1, B_2, and G_2 spiked at three levels range between 0.98 and 32.12%, between 7.81 and 31.82%, between 3.96 and 20.21%, and between 7.90 and 37.92%, respectively, and for total aflatoxins between 3.17 and 20.77%. In general, repeatability, as defined by the CV, of HPLC analysis of tahini samples for aflatoxin B_1 and total aflatoxins decreased as the spiking levels decreased. The analytical variations of aflatoxins B_1, G_1, B_2, and G_2 obtained through immunoaffinity cleanup followed by HPLC detection are presented in **Table 2**. For aflatoxins B_1, G_1, and G_2, values of CV% were at their lowest at the highest spike level, except for aflatoxin B_2. Although the spiking levels in this study are below the spiking levels of the studies conducted on oilseeds and peanut butter samples by Trucksess et al. (31), Carman et al. (32), and Patey et al. (33), using immunoaffinity column cleanup coupled with the HPLC detection method, the CV% for tahini samples analyzed with HPLC are nearly the same or even less.

Recovery values were calculated to determine the accuracy of the methods. For HPLC determination of aflatoxins in tahini, recovery values were in the range of 54.8−72.9% for aflatoxin

Table 2. HPLC, Fluorometry, and ELISA Results of Tahini Samples Spiked with Three Different Levels of Aflatoxins B_1, B_2, G_1, and G_2 (10:3:10:3) along with Standard Deviations for Repeatability (S_r), Recovery Values (Percent), and Coefficients of Variation (CV%)

	HPLC results[a]					fluorometry results[a]	ELISA results[a]
	aflatoxin (ppb)					total aflatoxin (ppb)	total aflatoxin (ppb)
sample	B_1	B_2	G_1	G_2	total	av	av
first control	ND[b]	ND	ND	ND	ND	2.43	4.3
second control	ND	ND	ND	ND	ND	1.30	4.8
mean						1.86	4.55
S_r						0.80	0.35
first level (6.5 ppb)	1.02	0.70	1.19	0.72	3.63	9.50	13.8
B_1, 2.5 ppb; B_2, 0.75 ppb	1.87	0.72	1.96	0.73	5.28	10.00	6.1
G_1, 2.5 ppb; G_2, 0.75 ppb	1.24	0.67	1.44	0.54	3.88	6.50	9.2
mean	1.37	0.69	1.53	0.66	4.26	8.67	9.70
S_r[c]	0.441	0.028	0.391	0.110	0.885	1.89	3.87
recovery %	54.8	92.0	61.2	88.0	65.4	133.3	149.23
CV%[d]	32.12	3.96	25.52	16.61	20.77	21.84	39.94
second level (13 ppb)	3.42	1.45	3.68	1.21	9.76	17.75	13.1
B_1, 5 ppb; B_2, 1.5 ppb	3.02	0.97	1.94	0.59	6.72	15.50	20.7
G_1, 5 ppb; G_2, 1.5 ppb	3.02	1.17	2.65	1.32	8.16	16.75	23.0
mean	3.15	1.20	2.76	1.04	8.21	16.67	18.93
S_r	0.230	0.242	0.877	0.393	1.522	1.13	5.18
recovery %	63.0	80.0	55.2	69.3	62.7	128.2	145.64
CV%	7.31	20.21	31.82	37.92	18.54	6.76	27.36
third level (19.5 ppb)	5.53	2.14	6.35	1.76	15.78	25.00	36.4
B_1, 7.5 ppb; B_2, 2.25 ppb	5.47	2.00	5.48	1.89	14.84	26.25	36.4
G_1, 7.5 ppb; G_2, 2.25 ppb	5.42	1.82	6.23	1.61	15.09	26.00	21.6
mean	5.47	1.99	6.02	1.75	15.24	25.75	31.47
S_r	0.054	0.159	0.470	0.138	0.483	0.66	8.54
recovery %	72.9	88.4	80.3	77.8	78.1	132.1	161.37
CV%	0.98	8.00	7.81	7.90	3.17	2.57	27.16

[a] The average of two injections or two readouts. [b] Not detected (detection limit for aflatoxin B_1, <0.3125 ppb; for aflatoxin B_2, <0.1875 ppb; for aflatoxin G_1, <0.3125 ppb; for aflatoxin G_2, <0.375 ppb; and for total aflatoxins, <1.1875 ppb). [c] S_r, the standard deviation for repeatability. [d] CV%, coefficient of variation percentage.

B_1, 55.2–80.3% for aflatoxin G_1, 80.0–92.0% for aflatoxin B_2, 69.3–88.0% for aflatoxin G_2, and 62.7–78.1% for total aflatoxins (**Table 2**). For each spiking level the highest recoveries were obtained for aflatoxin B_2. However, these recovery values are lower than those for corn, peanuts, and peanut butter presented by Trucksess et al. for total aflatoxins (*31*) and for aflatoxins B_1, B_2, and G_1 (*34*), based on the same analytical method. On the other hand, the recovery values were higher than the values obtained for peanut butters spiked at 28 and 47 μg/kg total aflatoxins by Patey et al. (*33*). The differences in recovery values may be related to the sample matrix that is analyzed and to the specificity of immobilized antibodies to these components (*24*). In addition, spike levels may also have an effect on the observed recovery values; when the recovery values are compared to those reported for aflatoxin B_1 (90%) and aflatoxin G_1 (99%) spiked at a 34 ppb level in sesame seeds by Carman et al. (*32*), the recovery values for tahini are observed to be lower, although it is a product of sesame seeds. Therefore, the results of each study may have a different range of recovery values depending on the sample matrix, the immunoaffinity column used during cleanup, and spike levels of aflatoxins.

Analysis of the spiked samples by the fluorometric method yielded CV% values in the range of 2.57–21.84% and recovery values in the range of 128.2–133.3% (**Table 2**). The decrease in CV% values by increase in spike level was similar to the results obtained by Trucksess et al. (*31*). High readouts from fluorometry are expected because the calibration of the fluorometer is made according to the losses during extract cleanup (*29*).

For the analysis of tahini sample by the ELISA method, the CV% values were found to be in the range of 27.16–39.94% and the recovery percentage values were between 145.6 and 161.4%. CV% values are found to be inversely proportional and recovery values to be directly proportional with the spiking levels. Whitaker et al. (*35*) related the high variation to nonfamiliarity of the analysts and the problem of standardized manufacturing of ELISA kits. In a study by Mühlemann et al. (*36*) the same ELISA kits gave recovery percentage values between 71 and 112% for different food commodities.

The analytical variations for total aflatoxins obtained from the three different methods of analysis are given in **Table 2**. CV% values decreased as the spiking level increased in all three determination methods except for the ELISA method, which had high values at all spiking levels. Results obtained by HPLC and ELISA methods were found to be statistically different, whereas there was no significant difference between HPLC–fluorometry and fluorometry–ELISA methods in tahini samples ($p \leq 0.05$). When the homogeneous subsets were evaluated, ELISA failed due to its high variability; however, the HPLC method was found to be superior due to its high accuracy and precision.

Simple linear regression analyses between the results of HPLC, fluorometry, and ELISA methods are presented in **Figure 1**. The variance analysis of the regression between the methods showed statistical significance for each of the combinations ($p \leq 0.05$). The highest correlation was observed between the results of HPLC and fluorometry methods ($r = 0.978$). However, correlation between results of HPLC and ELISA methods ($r = 0.816$) is lower than those of fluorometry and ELISA methods ($r = 0.840$).

Hongyo et al. (*20*) suggested using the same extraction solvent to increase the correlation between ELISA and HPLC methods. In addition, the same extraction procedure allows measuring only the effect of detection variances (*37*). The low regression
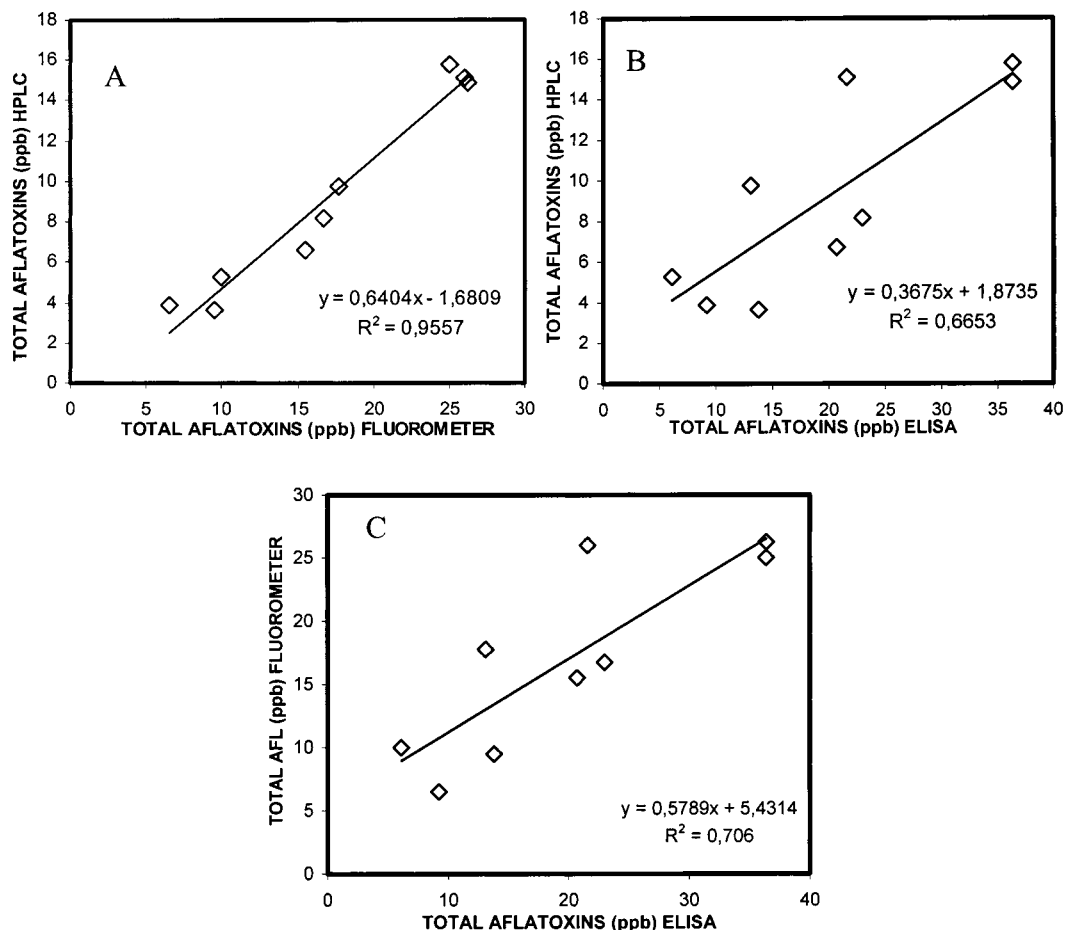
Figure 1. Regression line and regression equation for (A) HPLC versus fluorometry, (B) HPLC versus ELISA, and (C) fluorometry versus ELISA.

for HPLC−ELISA methods may be related to the absence of a cleanup step in the ELISA method. Similarly, the high regression obtained for HPLC and fluorometry methods may be due to the same cleanup procedure performed in both methods. Hongyo et al. (*20*) associated the high correlation coefficient values obtained for ELISA methods to the high specificity and reproducibility of the monoclonal antibody used in their study. The type of food matrix may also have an effect on correlation values between methods. Mühlemann et al. (*36*) reported low correlation coefficients for cereals and grains but high values for peanut and oilseeds.

Due to its high correlation with the HPLC method, the fluorometric determination method following an immunoaffinity column cleanup step was found to be efficient for the prediction of total aflatoxin contamination for tahini samples. The HPLC method can detect aflatoxins B_1, B_2, G_1, and G_2 individually with high accuracy; however, its cost and experienced analyst requirements may limit its application. Due to its high variation in replicates, the applicability of the ELISA method seems to be suitable only as a screening method.

To evaluate the method in practice, 14 tahini samples of different trademarks obtained from retail markets in Turkey were analyzed by immunoaffinity cleanup and HPLC detection method in order to understand the aflatoxin risk in tahini. As a result of this limited survey, 1 of 14 samples was found to be highly contaminated with aflatoxins (total aflatoxins > 176 ppb), whereas in the other tahini samples no aflatoxin was detected. It is obvious that there is a need for the control of aflatoxin contamination of foodstuffs having sesame seeds as an ingredient.

SAFETY

All used labware, pipet tips, and kit components should be soaked in a 10% solution of household bleach for 30 min before disposal (*31*). Aflatoxins are toxic and carcinogenic. A fume hood should be used, and gloves, protective clothing, and eyewear should be worn.

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