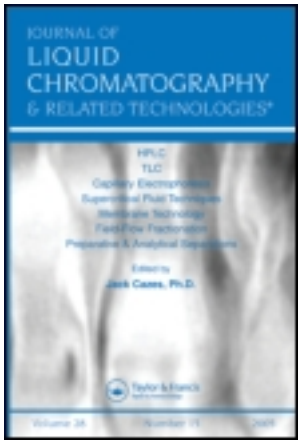
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QuEChERS-HPLC METHOD FOR AFLATOXIN DETECTION OF DOMESTIC AND IMPORTED FOOD IN JORDAN

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Ala' Yahya Sirhan,[1,2] Guan Huat Tan,[1] Ahmad Al-Shunnaq,[2] Lukman Abdulra'uf,[1] and Richard C. S. Wong[1]

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□ *A method for the analysis of aflatoxins in food samples was developed. The preliminary work focused on optimizing sample pretreatment conditions such as the extraction solvent; the type and amount of drying agents; the extraction time; and the solvent-sample ratio. A 60:40 methanol/acetonitrile (%, v/v) extraction mixture provided recoveries in the range of 71.8–107.7% at spike levels of 1.5–50.0 μg/kg with relative standard deviations (RSDs) lower than 15%. The separation of the four aflatoxin compounds with high selectivity and sensitivity, and within reasonable retention times, was performed by a C18 250 mm × 4.6 mm × 5 μm column (Nucleodur 250-5) at 45° C. The developed QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) procedure, high performance liquid chromatography (HPLC) method was then compared with the fluorometric determination method. It was found that the fluorometric determination method had a poor precision and positive bias as compared to QuEChERS-HPLC method. A total of 669 domestic and imported food samples in Jordan were analyzed for their aflatoxins content. Peanut and peanut butter samples showed the highest incidence of contamination (10 contaminations) followed by pistachio nut samples (6 contaminations) and sesame seed samples (2 contaminations).*

Keywords aflatoxin, design of experiment, extraction, HPLC, mycotoxin, QuEChERS

INTRODUCTION

Aflatoxins are a group of highly toxic and carcinogenic substances, which occur naturally, and can be found in food substances. Aflatoxins are secondary metabolites of certain strains of the fungi *Aspergillus flavus* and *A. parasiticus* and the less common *A. nomius*.[1] Aflatoxin B1, B2, G1, and G2 are the most important members, which can be categorized into two groups according to the chemical structure, namely, difurocoumarocyclopentenone

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series and ifurocoumarolactone. The aflatoxins fluoresce strongly under UV radiation (ca. 365 nm). Aflatoxins occur as colorless to pale-yellow crystals at room temperature.[2] The most common food commodities affected by aflatoxins are cereals (corn, wheat, barley, maize, oats, and rye), nuts (hazelnut, peanut, and pistachio nut), dried fruits (fig), and spices (chili powder).[3,1,4] Aflatoxins pose a potential threat to human and animal health through the consumption, contact or inhalation of foodstuffs and feedstuffs prepared from these commodities. As a result of the adverse health effects of mycotoxins, their levels have been strictly regulated especially in food and feed samples. While the European Union in general has set a much lower allowable residue level than the US Food and Drug Administration, they are generally in the μg/kg level for most of these compounds.[5] In order to ensure compliance with the international regulations, it is necessary to have a sensitive, reliable, and accurate method for the determination of mycotoxins in different food commodities.

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High performance liquid chromatography with fluorescence detection (HPLC-FLD) is considered as the most commonly used instrument of these methods for quantification of aflatoxins, because of its accuracy, high sensitivity, and ease in automation.[6] However, it requires one or more clean-up steps involving liquid–liquid extraction (LLE), solid-phase extraction (SPE), or immunoaffinity columns (IAC) clean-up and in addition, aflatoxin B1, and G1 suffer from fluorescence quenching in the HPLC mobile phase. Therefore, pre-column or post-column derivatization is necessary to enhance the fluorescence intensity of aflatoxin B1 and G1 which is needed to achieve the low limits of detection (low-ppb level).

Various derivatization methods including precolumn derivatization with trifluoroacetic acid (TFA),[7] postcolumn derivatization with iodine,[8] electrochemical derivatization with potassium bromide,[9] and electrochemical derivatization using the Kobra cell[6] have been developed. While, precolumn derivatization technique presents several significant drawbacks such as being laborious, time-consuming, and because of the high polarity and relative instability of these derivatives,[10] postcolumn derivatization with postcolumn photochemical reactor method for enhanced detection (PHRED) has been successfully used to overcome these drawbacks.[11]

The aim of this study is focused on optimizing the sample pretreatment conditions of the developed Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method and the optimization of the chromatographic conditions of an HPLC-FLD method with postcolumn photochemical derivatization for quantification of four(4) aflatoxins B1, B2, G1, and G2 in food. Sample pretreatment conditions such as the extraction solvent, the type and amount of drying agent, the extraction time, and the solvent-sample ratio were optimized. The developed QuEChERS-HPLC method was then validated and compared with the fluorometric determination method. The

methods were then used for the analysis of the selected aflatoxins in a total of 669 domestic and imported food samples in Jordan using a QuEChERS-HPLC method.

EXPERIMENTAL

Reagents and Materials

Certified mixed standard solutions consisting of aflatoxin B1, B2, G1, and G2 were obtained from Supelco (Bellafonte, PA, USA). Subsequently, daily working standard solutions were prepared by diluting the stock solutions in methanol. Water and acetonitrile (HPLC-grade) were purchased from VWR International (EC). Methanol (HPLC-grade) was purchased from Labscan (Dublin, Ireland). Sodium chloride was purchased from Acros Organics (New Jersey, USA). Anhydrous magnesium sulfate ($MgSO_4$) for QuEChERS was purchased from R&M Chemicals (Essex, UK). A non-sterile PTFE Syringe Filter with a disposable membrane filter (0.45 μm) was purchased from Whatman GmbH (Dassel, Germany).

QuEChERS-HPLC Method

Sample Preparation

Samples were prepared similar to the previous published aflatoxin QuEChERS method with some modification.[11]

Step I: A thoroughly homogenized food sample (1.0 g) was weighed in a polypropylene centrifuge tube (15 mL).

Sample recovery was performed with 1.0 g of the clean wheat, corn, rice, pistachio nut, peanut, and almond spiked with 5.0, 10.0, and 50.0 μg/kg of aflatoxin B1, G1, and 1.5, 3.0, and 15.0 μg/kg of aflatoxin B2, G2. The spiked samples were left overnight in the dark at room temperature to allow the solvent to evaporate and for aflatoxin absorption into the matrix. Then, they were extracted via the following steps (II to IV).

Step II: 3.0 mL of 60:40 (%, *v*/*v*) methanol/acetonitrile mixture was added and the centrifuge tube was shaken for 1 min to ensure that the solvent has mixed thoroughly with the entire sample, for the complete extraction of the analyte.

Step III: 1.32 g of anhydrous $MgSO_4$ and 0.25 g of NaCl were added into the mixture and the shaking procedure was repeated for 1 min to facilitate the extraction and partitioning of the four (4) aflatoxins into the organic layer.

Step IV: The extract was centrifuged for 5 min at 4000 rpm, and 0.5 mL of the upper organic layer was filtered through a 0.45-μm nylon syringe filter prior to HPLC analysis.

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HPLC Analysis

The HPLC analysis was performed using a Perkin Elmer Series 200 (Liantrisant, UK) system consisting of pump with a quaternary configuration, a vacuum degasser, a column oven, a fluorescence detector, and coupled with an autosampler equipped with a 200 µL sample loop. A post column PHRED from Aura Industries (NY, USA) with a low-pressure mercury lamp ($\lambda = 254$ nm) and a knitted reactor coil (25 m x 0.25 mm) was applied to enhance the detection of aflatoxin B1 and G1. The chromatographic separation was performed with a Nucleodur 250-5 C18 250 mm × 4.6 mm × 5 µm chromatographic column and was purchased from Macherey-Nagel (Duren, Germany). The sample extracts were analyzed isocratically using 65:25:10 water/methanol/acetonitrile mixture as the mobile phase. The column was kept in a column oven at 45°C at a flow rate of 1.0 mL/min to achieve the optimum resolution of the aflatoxins. The injection volume was maintained at 10 µL for both the sample and standard solutions.

Fluorometric Determination

Sample Preparation

For each sample, ten grams were extracted in a blender with 50 mL methanol: water (60:40, *v*/*v*) and 2.0 g of sodium chloride for 1 min in a Waring blender at high speed. After filtration through a fluted filter paper (24 cm, Vicam, Watertown, MA, USA), a 20 mL of the filtrate was diluted with 20 mL distilled water and was mixed vigorously. The mixture was then filtered a second time using a microfiber filter paper (11 cm, Vicam, Watertown, MA, USA) and 10 mL of filtrate (equivalent to 1.0 g sample) was applied to the immunoaffinity column (Aflatest, Vicam, Watertown, MA, USA). The column was washed twice with 5 mL of distilled water and the aflatoxins were eluted to the monoclonal antibodies by passing 1 mL of HPLC grade methanol through column at a rate of 1–2 drops/second.

Measurement

The fluorometric determination was performed by a VICAM Series 4 (G8002) Fluorometer optical system (MA, USA) equipped with a high intensity pulsed Xenon lamp with selected fluorescence excitation and emission filters. A linear calibration of the instrument was developed by subsequently reading three quinine sulfate dihydrate reference standards (Aflatest-MTM; VICAM). The red and the green mycotoxin calibration vials were used to set the higher and the lower calibration points and the last vial contains the yellow mycotoxin calibration standard, following the VICAM instruction manual. Exactly 1 mL of the eluate was mixed vigorously with 1 mL of the *AflaTest*

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Developer. The aflatoxin content was then obtained from the fluorometric measurements. The detection limit of the method was 1.0 μg/kg.

Food Samples

A total of 669 samples of domestic and imported samples were supplied to the Jordan food and drug administration in 2011 and analyzed. The samples consisted of 274 samples corresponding to cereals; eighty seven to peanuts and peanut butters; seventy eight to nuts (walnut, cashew, pine, hazelnut, and almond); forty six to sesame seeds; sixty one to pistachio nuts; fifty one to seeds (sunflower, water melon, etc.); fifty nine to green coffee, and the remaining samples were related to spices. Approximately 1–4 kg of each type of sample was obtained. The samples were stored in a dark place at room temperature (20–25°C). The samples were ground and mixed at room temperature till a fine and homogeneous powdered material was obtained. The powdered samples were then stored in plastic bags at 4°C in a refrigerator prior to analysis.

RESULTS AND DISCUSSION

QuEChERS-HPLC Method

Optimization of HPLC Conditions

Different combinations of water/methanol/acetonitrile such as 60:20:20, 65:25:10, 70:20:10, and 75:17:8 (%, *v*/*v*) were tested as the mobile phase in order to optimize the resolution of the aflatoxin standard mixture and to enhance the sensitivity. Aflatoxin G1 and B2 were not separated by the first mobile phase composition [60:20:20 (%, *v*/*v*)]. On the other hand, broad peaks and long retention times were produced using the third and fourth mobile phase systems, when the water composition exceeded 70%. In contrast, the second mobile phase offered an adequate separation between the aflatoxin G2, G1, B2, and B1 peaks with reasonable and acceptable retention times (Figure 1).

However, the bottleneck of a chromatographic system is the column, where the actual separation of the analyte mixture occurs. Column selection depends strongly on prior knowledge of the physicochemical properties of the analytes and of the matrix. The column type and its length were optimized by investigating various HPLC columns under the same chromatographic conditions in order to obtain the best chromatogram separation in the shortest analysis time. The investigated columns are:

1. ACE 5, 5 μm Phenyl 250 mm × 4.6 mm, from Hichrom (UK),
2. Nucleodur, 5 μm 250-5 C18 250 mm × 4.6 mm, from Macherey-Nagel (Duren, Germany),

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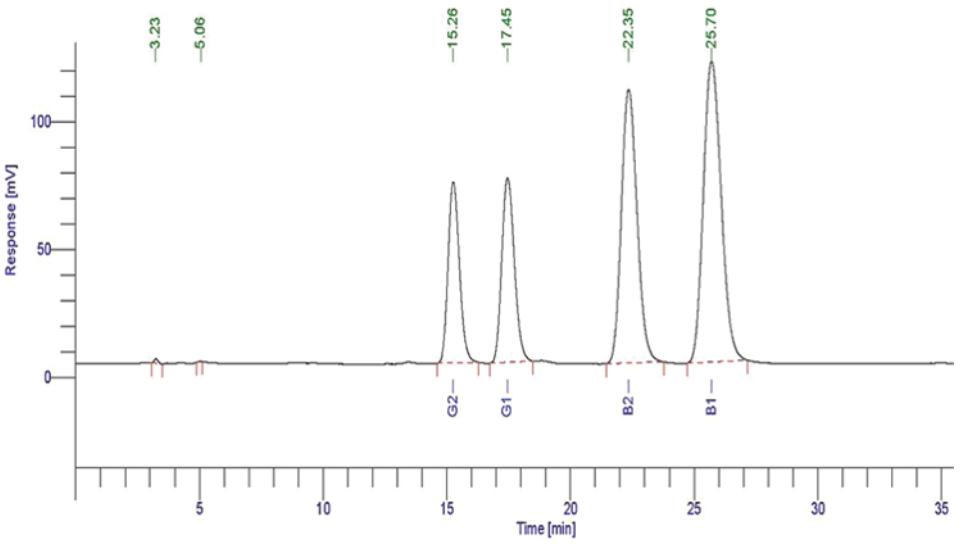


FIGURE 1 HPLC chromatogram of aflatoxin standard solutions containing 100 μg/L of aflatoxin B1 and G1 and 30 μg/L of aflatoxin B2 and G2. (Color figure available online.)

3. Brownlee Analytical, 5 μm C18 250 mm × 4.6 mm, from PerkinElmer (Shelton, USA),
4. Spherisorb 5, 5 μm ODS-1 C18 150 mm × 4.6 mm, from Phenomenex (USA),
5. Nucleodur 100-5, 5 μm C18 100 mm × 4.6 mm, from Macherey-Nagel.

Table 1 showed that the column has a significant effect on the retention time and sensitivity of aflatoxins. Aflatoxin G1 standard cannot be detected by the ACE 5, 5 μm Phenyl 250 mm × 4.6 mm, (column A). In the case of 100 mm column length (column E), their retention times were reduced to less than 20 min but was accompanied with an increasing risk of interferences and false positive results. While column D suffers from a significant decrease in the resolution between aflatoxin G2 and G1 ($Rs \approx 1.1$), column C has poor sensitivities for all analytes with a tailing peak for aflatoxin B2 (0.79 at 5% height). However, column B shows high efficiency (HETP = 2.3–3.9E-05 mm) and the best resolution ($Rs > 4.2$) but with longer retention times for the aflatoxins (≈50 min) (Table 1).

However, by increasing the column temperature to 45°C, the retention time was reduced to 30 min without affecting the resolution. The selectivity of the isocratic mixture of 65:25:10 (%, *v*/*v*) water/methanol/acetonitrile at 45°C column temperature are considered satisfactory as it enables aflatoxin quantification in the analyzed food commodities with higher selectivity and sensitivity within a reasonable run time. The repeatability and reproducibility

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TABLE 1 Retention Time, Area %, Tailing Factor, Resolution, and Theoretical Plate Number/Column Length (HETP) of Aflatoxins

Column	Aflatoxin	Retention Time (min)	Area %	Tailing Factor	Resolution	HETP (mm)
A ACE 5, 5 µm, Phenyl, 250 × 4.6 mm	G2	26.1	82.4	1.001	–	2.1E-05
	G1	–	–	–	–	–
	B2	36.8	100.0	0.800	–	1.8E-05
	B1	49.6	100.0	0.969	8.91	1.5E-05
B Nucleodur, 5 µm, C18, 250 × 4.6 mm	G2	21.1	100.0	1.080	–	3.9E-05
	G1	27.7	82.3	1.094	5.80	3.1E-05
	B2	33.2	99.0	1.007	4.16	3.2E-05
	B1	43.8	95.3	1.043	6.77	2.3E-05
C Brownlee Analytical 5 µm, C18, 250 × 4.6 mm	G2	13.6	81.6	1.157	–	6.3E-05
	G1	17.7	54.7	1.067	4.43	4.6E-05
	B2	21.1	81.6	0.788	3.22	3.6E-05
	B1	27.5	87.1	0.981	5.20	3.2E-05
D Spherisorb 5 µm, C18, 150 × 4.6 mm	G2	13.3	100.0	1.138	–	3.0E-05
	G1	14.2	100.0	1.023	1.09	2.8E-05
	B2	17.4	79.6	0.898	3.27	1.8E-05
	B1	22.0	89.7	1.098	4.20	2.2E-05
E Nucleodur 5 µm, C18, 100 × 4.6 mm	G2	8.1	97.8	1.095	–	5.5E-05
	G1	10.6	81.8	1.078	3.01	4.0E-05
	B2	12.9	74.8	0.824	2.44	3.4E-05
	B1	16.8	92.1	1.058	3.66	2.8E-05

of the retention times as measured by their relative standard deviation (RSD) ranged from 1.0% to 2.0% and 1.8% to 4.3%, respectively. In order to evaluate whether the aflatoxin mix standards could be distinguished and separated from interfering substances in the sample matrix, a naturally contaminated pistachio nut sample was pretreated using the QuEChERS method and separated using a Nucleodur 250-5. The chromatograms demonstrating the selectivity of the procedure are shown in Figure 2.

From the chromatograms of the naturally contaminated pistachio nut sample, it is evident that the peaks of the aflatoxin standard are well separated from the peaks of the interfering substances in the sample matrix and reasonable retention times were obtained.

Sample Pretreatment Optimization

For the mycotoxin determination, the sample pretreatment has a crucial impact on the accuracy of the results, especially when complex matrices such as cereals and nuts are analyzed for the very low levels of aflatoxins, which may be in the food. To attain this goal, a simple QuEChERS extraction procedure was adopted before the chromatographic determination. The extraction solvent, type and amount of drying agent, the extraction time and the solvent sample ratio affecting the efficiency of extraction, were all taken into consideration and optimized.

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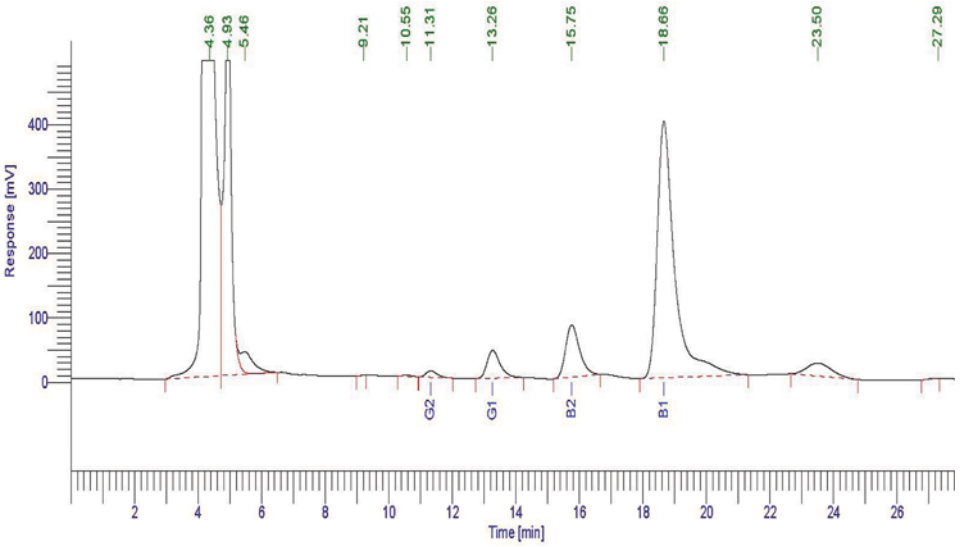


FIGURE 2 Representative HPLC chromatogram of a naturally contaminated pistachio nut sample. (Color figure available online.)

Determination of the Optimum Duration and Method of Extraction. After addition of the extraction solvents to the falcon tube, the tube was closed and manually shaken vigorously for 1 minute, according to the original QuEChERS procedure, to disperse the solvent and analytes. The effects of the duration and method of extraction in the aflatoxin recoveries were investigated by hand-shaking the falcon tube for 1 min in addition to vortexing the falcon tube between 1 min to 4 min. Manual shaking of the falcon tube for more than 1 min was not applied, as it was laborious. All experiments were carried out using the same procedure and were tested in a blank peanut sample that had been spiked with 10.0 μg/L of aflatoxin B1 and G1 and 3.0 μg/L of aflatoxin B2 and G2. After that, the recovery results were compared.

Figure 3 shows that the extraction times affect the recoveries of aflatoxins. For the first stage of extraction, the recoveries increased by increasing the vortex time with up to 15% enhancement for all analytes. However, the recoveries remained almost the same after 2 min of extraction. This is because there is a large concentration gradient for the solutes in the peanut sample and the extraction solvent interacts with the sample at the initial stage of extraction resulting in higher extraction rates. Additionally, the constituents located in the surface layers of the particles are more readily accessible than those in the deeper regions, which hardly diffuse out and hence slow down the rate of extraction.

Figure 3 indicates that manual shaking of the falcon tube for 1 min is more efficient than vortexing for the same period. This may be because

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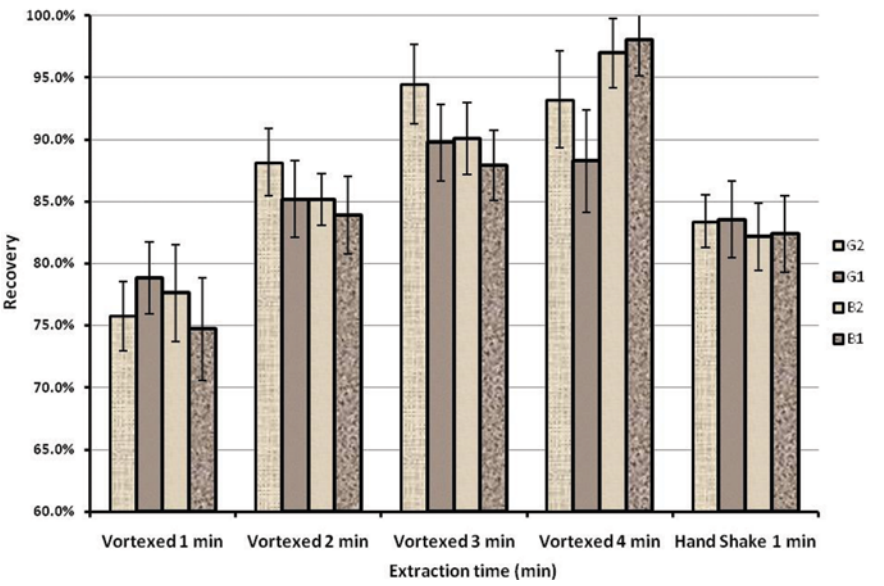


FIGURE 3 Effects of duration and method of extraction on the recovery of aflatoxin B1, B2, G2, and G1. (Color figure available online.)

hand shaking the falcon tube speeds up the extraction in addition to aiding in dispersing the solvent and analytes throughout the sample, thus ensuring that the solvent interacted well with the entire sample. However, manual shaking of the falcon tube for 1 min resulted in lower recoveries, which do not comply with the EU regulations. Therefore, a vortex mixer was employed, as it complies with the EU regulations in addition to being time and an effort-saving procedure.

Determination of the Appropriate Type and Amount of Drying Agent. A drying agent is an inorganic hygroscopic (readily absorbs water from the air) salt. Several salts are used routinely in the QuEChERS procedure. The most common is anhydrous $MgSO_4$. Other inorganic sulfate salts such as sodium sulfate (Na_2SO_4) may also be used in the same way. These salts are added to the extracts with a combination of sodium chloride (NaCl) to remove excess water and unwanted contaminants from the extracted samples as well as to provide a separate and distinct phase to facilitate the penetration of the mycotoxin into the organic layer.

In order to investigate the effects of adding different drying agents, blank peanut samples were spiked with 10.0 µg/L of aflatoxin B1 and G1 and 3.0 µg/L of aflatoxin B2 and G2, and analyzed after the addition of either anhydrous $MgSO_4$ or anhydrous Na_2SO_4 and the results from both analyses were compared. The results in Figure 4 show that anhydrous $MgSO_4$ is a far more effective drying agent than anhydrous Na_2SO_4 and

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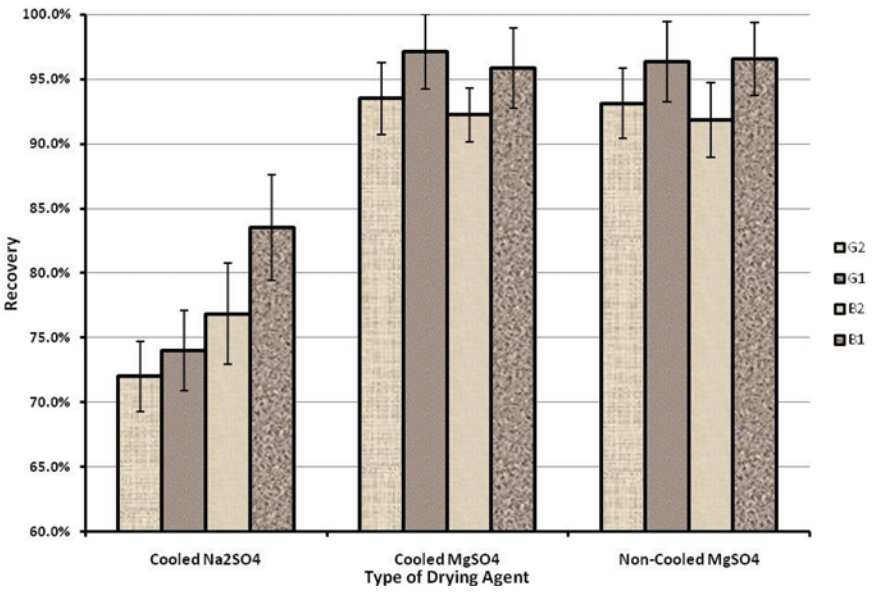


FIGURE 4 Comparison between drying agent type as well as comparison between a cooled falcon tube and non-cooled falcon tube effect on the recovery of aflatoxin B1, B2, G2, and G1. (Color figure available online.)

gave higher recoveries for both the targeted aflatoxins. An explanation for this observation is that anhydrous $MgSO_4$ is a fine powder while anhydrous Na_2SO_4 has a larger particle size, so that the drying power of $MgSO_4$ is greater together with its substantially higher capacity.

However, since anhydrous $MgSO_4$ is a highly exothermic compound, it is added to the extracts and not vice versa to avoid elevating the temperature in the falcon tube, which may lead to analyte decomposition and subsequently a reduction in the recoveries. For this reason, it is recommended to add ice into the falcon tube before adding the anhydrous $MgSO_4$ to absorb the released heat. This effect was investigated by comparing two blank peanut samples that were spiked with 10.0 μg/L of aflatoxin B1 and G1 and 3.0 μg/L of aflatoxin B2 and G2. The first sample was placed overnight in a freezer at −18°C and the second sample was left at room temperature. Both of them were analyzed and the results were compared (Figure 4).

Figure 4 indicates that recoveries for the selected aflatoxins were identical; this may be that the heat released after addition of anhydrous $MgSO_4$ was not sufficiently high enough to affect the analytes stability. Therefore, it is not necessary for an additional pre-cooling step of the falcon tubes.

The amount of anhydrous $MgSO_4$ required depends on the amount of water in the extraction solvents as well as in the samples. Therefore, the effect of the amount of anhydrous $MgSO_4$ in the recoveries was

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investigated by adding different amounts of anhydrous $MgSO_4$ from 0.5 to 4.0 g together with a constant amount of NaCl (Figure 5).

Figure 5 indicated that the recoveries were increased as the amount of anhydrous $MgSO_4$ was increased from 0.5 to 4.0 g. However, after addition of 2.0 g of anhydrous $MgSO_4$, it was found that the recoveries were not further increased for the majorities of the selected analyte. From these observations, it was determined that the addition of 2.0 g of anhydrous $MgSO_4$ is sufficient for the extracts without an additional pre-cooling of the falcon tube.

Effect of Dilution on Sample Extraction. Dilution of the sample extract is usually the method of choice to eliminate and reduce the matrix effects; dilution will also increase the extraction efficiency by decreasing the number of competing molecules per drop area to enhance the release of the analyte from the matrix. In addition, sample dilution shifts the equilibrium towards the free form of the analyte from the "bound" form with the matrix components. However, further dilution may adversely affect the sensitivity and lead to poorer detection limits. Therefore, it is necessary to look for an optimal dilution. In this study, the effect of dilution of the sample extracts was investigated by adding different amounts of extraction solvents ranging from a dilution ratio of 1:2 to 1:5 (Figure 6).

From Figure 6, it was found that for all the investigated dilution ratios, the recoveries for all target compounds fall within the acceptable range

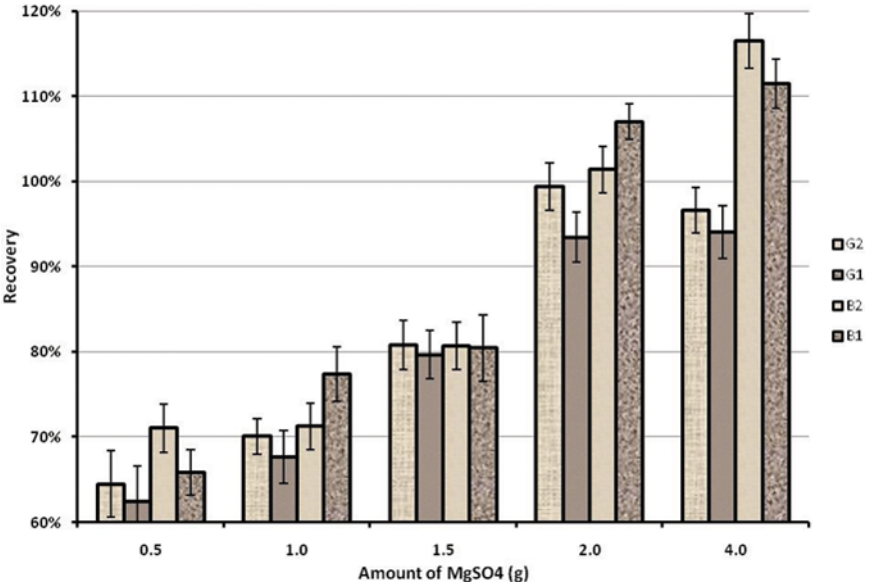


FIGURE 5 Effects of addition of various amounts of $MgSO_4$ on the recovery of aflatoxin B1, B2, G2, and G1. (Color figure available online.)

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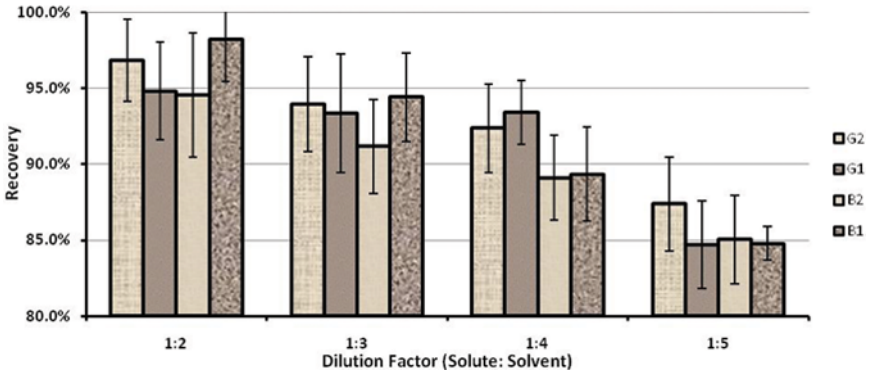


FIGURE 6 Effects of dilution of the sample extract on the recovery of aflatoxin B1, B2, G2, and G1. (Color figure available online.)

(between 85 to 98%) as well as complying with the EU regulations. It was found that increasing the amount of extraction solvents has a limited effect on the recoveries. Based on these data, dilution in the ratio of 1:3 was chosen as a good compromise between increased extraction efficiency and loss of sensitivity, and it was thus adopted for the subsequent experiments.

Optimization of the Extraction Solvent. Optimization of the effect of extraction solvent was investigated by using the design of the experiment (DOE) technique which configures the response surface methodologies (RSM) using mathematical and statistical techniques.[12] The basic assumption is that there is a possible interaction effect of factors which depend solely on the component proportions of the mixture and not on the component quantities; thus, the response of the property is entirely determined by the proportions of the total. Therefore, it is necessary to select the appropriate mixtures from which the response surface might be calculated; and having the response surface, a prediction of how such changes will affect the properties of the mixture can be obtained for any mixture by changing the mixture composition.

From a review of the literature, the use of different volume proportions of water, methanol and acetonitrile was frequently employed as the solvent mixture to extract aflatoxins.[4,13,3,14] Therefore, the DOE technique was applied to select the best combination of the three solvents (water, methanol and acetonitrile) to optimize the recovery of the aflatoxins in the food extracts by spiking a blank peanut sample with 10.0 μg/L of aflatoxin B1 and G1 and 3.0 μg/L of aflatoxin B2 and G2. ABCD designs with 21 experimental points were performed in duplicate, randomly at all points. The experimental data were then fitted to a quadratic polynomial model. An overlay contour tertiary plot (mixture profiler) of the aflatoxins recovery with 21 experimental points was then constructed as depicted in Figure 7.

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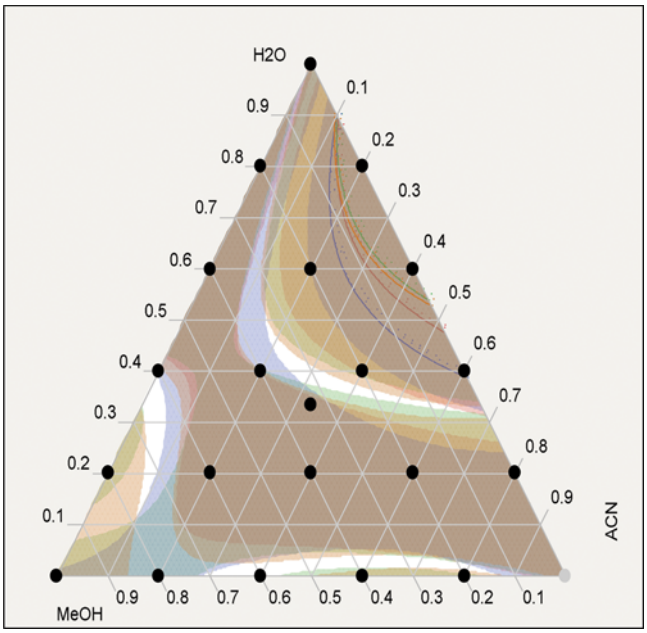


FIGURE 7 An overlay contour plot (mixture profiler) of aflatoxins recovery with 21 experimental points (the black dot point). The noncolored area as shown indicates the desirability of aflatoxins recovery (85–105%). (Color figure available online.)

The noncolored area in the plot in Figure 7 indicates the desirability of aflatoxins recovery (85–105%). To get the maximum desirability recoveries of the investigated analytes, the prediction profiler was applied, which shows the principal effects of the factors on the response. The diagram was constructed with assigned units to give immediate values for comparison (Figure 8 and Figure 9).

Figures 8 and 9 indicated that high extraction efficiency of the selected analyte was achieved when using 43:21:36 (%, *v*/*v*) water/ methanol/acetonitrile and 60:40 (%, *v*/*v*) methanol/acetonitrile. The second composition was selected; since this combination gave the desired responses as well as not containing water. In the presence of large amounts of water, anhydrous $MgSO_4$ tend to form lumps that can harden rapidly and reduce the supernatant layer. In addition, the water content cannot be evaporated easily and be reconstituted in a different solvent (mobile phase) compared to either methanol or acetonitrile. Therefore, mixtures of 60:40 (%, *v*/*v*) methanol/acetonitrile were applied in this study, as it gave recoveries fulfilling the EU Commission Directive 2006/401/EC for the analysis of mycotoxins in food samples.[15]

Method Validation

The in-house developed method was validated in terms of linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), intra-day

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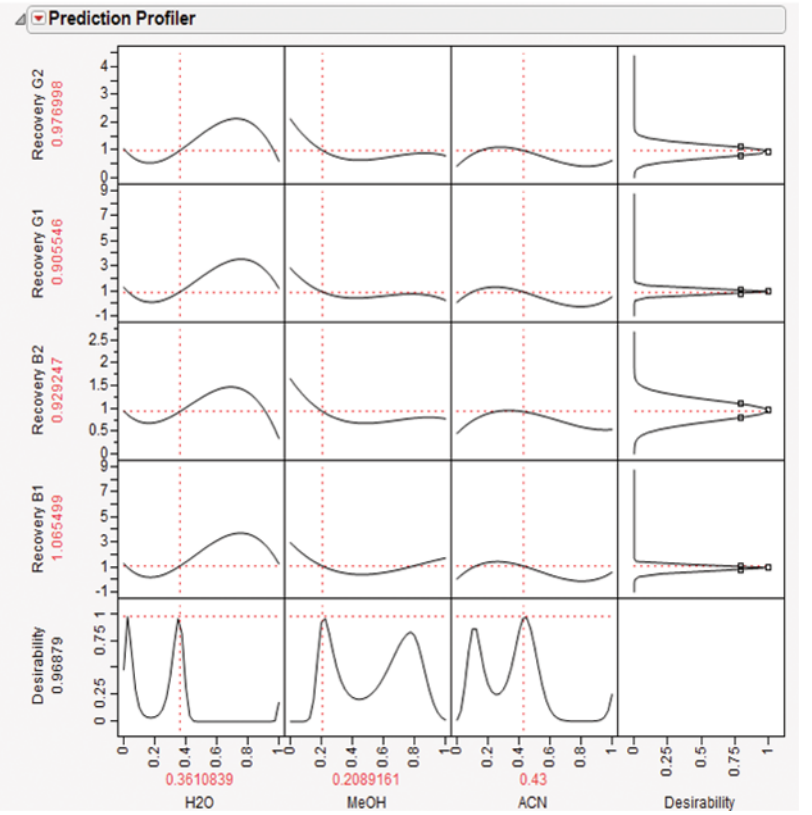


FIGURE 8 The profiler in "Maximum Desirability in Profiler for Mixture Analysis," displays optimal settings of 0.36 for water, 0.21 for methanol and 0.43 for acetonitrile, which give an estimated recovery between 91% and 101% of peanut samples spiked at 10.0 µg/L of aflatoxin B1 and G1 and 3.0 µg/L of aflatoxin B2 and G2. (Color figure available online.)

precision, and inter-day precision. The linearity was tested using a standard solution of the aflatoxins in the concentration range of 0.059 to 30.000 µg/kg for aflatoxin B2 and G2 and from 0.195 to 100.000 µg/kg for aflatoxin B1 and G1 using a least-squares regression analysis. Table 2 shows that good linear relationships with correlation coefficients greater than 0.993 for all the targeted analytes were obtained. Calibrations with standard solutions were used for quantification, because the ANOVA test did not show any significant difference ($P < 0.05$). Figure 10 shows the HPLC chromatogram of aflatoxin standard solutions containing 3.125 µg/L of aflatoxin B1 and G1 and 0.938 µg/L of aflatoxin B2 and G2.

The accuracy was tested by the determination of the recoveries of the aflatoxins in lean peanut samples spiked in triplicates at three-fortification levels (5, 10, and 50 µg/kg for aflatoxin B1 and G1, and 1.5, 3.0, and 5.0 µg/kg for aflatoxin B2 and G2) (Table 3).

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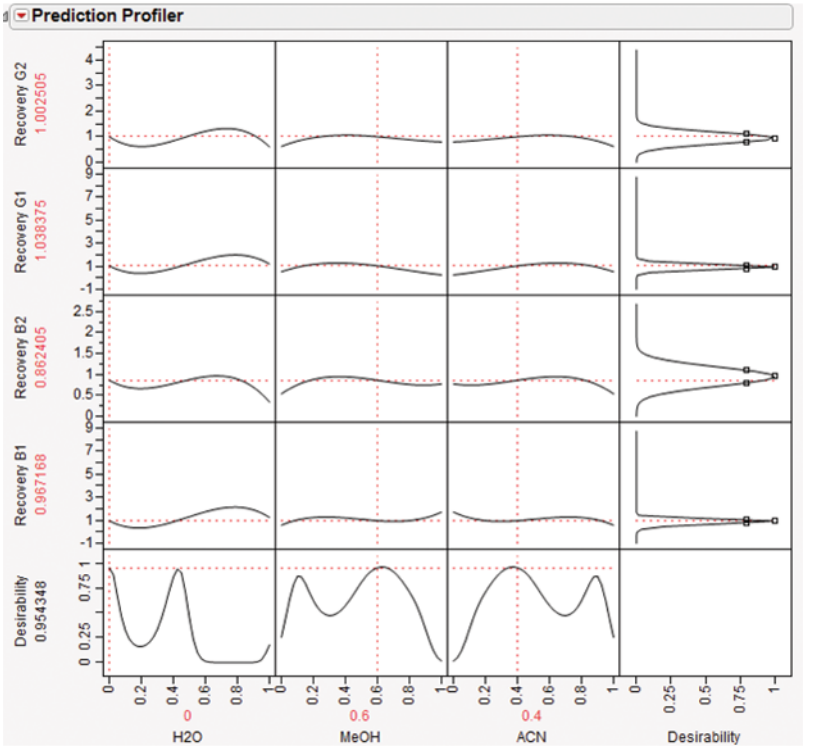


FIGURE 9 The profiler in "Maximum Desirability in Profiler for Mixture Analysis," displays optimal settings of 0.60 for methanol and 0.40 for acetonitrile, which give an estimated recovery between 86% and 104% of peanut samples spiked at 10.0 µg/L of aflatoxin B1 and G1 and 3.0 µg/L of aflatoxin B2 and G2. (Color figure available online.)

The recoveries obtained ranged between 76.3% and 98.0%, with RSD values of less than 10%. Alternatively, the method was tested for other matrices: corn, rice, pistachio nut, almond and sesame seed samples. The recovery results ranged between 71.8% and 107.7%, with RSD values of less than 15% at concentrations of 1.5 and 3.0 µg/kg for aflatoxin B2 and G2 as well as 5.0 and 10.0 µg/kg for aflatoxin B1 and G1. While the recoveries at the concentrations of 15.0 µg/kg for aflatoxin B2 and G2 and 50.0 µg/kg for aflatoxin B1 and G1 were from 81.0 to 107.5%, with RSD values of less than

TABLE 2 Linearity Range, Equation, r^2 Value, RSD, LOD, and LOQ of Aflatoxins

Aflatoxin	Linearity Range (µg/kg)	Equation	r^2	LOD (µg/kg)	LOQ (µg/kg)
B1	0.195–100.00	Y = (22078.573601) + (67517.549908) X	0.99396	0.17	0.57
B2	0.059–30.00	Y = (8866.164274) + (151599.561135) X	0.99648	0.05	0.18
G1	0.195–100.00	Y = (14087.825485) + (25425.320385) X	0.99446	0.35	1.17
G2	0.059–30.00	Y = (11493.592180) + (71338.219538) X	0.99536	0.06	0.20

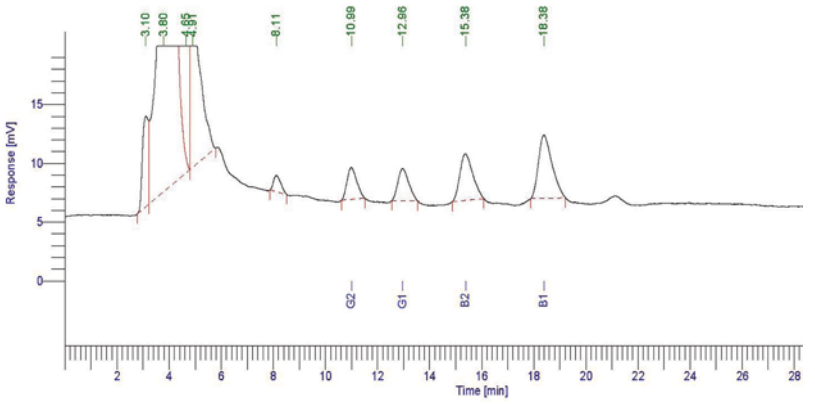


FIGURE 10 HPLC chromatogram of aflatoxin standard solutions containing 3.125 µg/L of aflatoxin B1 and G1 and 0.938 µg/L of aflatoxin B2 and G2. (Color figure available online.)

13%. These results fulfilled the requirements established by the European Union for the determination of aflatoxin contaminants in food samples.[15]

The sensitivity of the method was estimated by the LOD and LOQ. The LOD was verified as the lowest concentration giving a response of three-times the average of the base-line noise obtained from non-contaminated aflatoxin peanut samples spiked with a mixed standard stock solution containing the four (4) investigated aflatoxins. The LOQ was verified as the lowest concentration giving a response of six-times the average of the

TABLE 3 Mean of Recoveries and RSDs (n = 5) of Aflatoxins Spiked into Clean Wheat, Corn, Rice, Pistachio Nut, Peanut, Almond, and Sesame Seed Samples at Three Spiking Levels Using QuEChERS-HPLC Method

		Matrix						
Aflatoxin	Spiking Level (µg/kg)	Wheat	Corn	Rice	Pistachio Nut	Peanut	Almond	Sesame Seed
		Mean of Recovery (%) ± RSD (%)						
G2	1.5	75.7 ± 7.6	78.6 ± 5.8	96.3 ± 5.9	98.3 ± 8.2	80.5 ± 5.3	76.3 ± 3.4	106.7 ± 12.0
	3.0	74.5 ± 4.4	82.3 ± 6.7	104.4 ± 4.9	100.1 ± 8.1	95.3 ± 9.2	75.9 ± 6.2	102.5 ± 13.9
	15	85.4 ± 5.6	92.1 ± 4.9	94.4 ± 4.5	102.9 ± 7.6	97.5 ± 7.8	98.0 ± 9.9	107.5 ± 9.5
G1	5	73.1 ± 9.8	83.2 ± 2.7	102.8 ± 8.3	104.4 ± 6.1	95.5 ± 4.6	81.5 ± 4.5	86.0 ± 8.3
	10	87.7 ± 9.0	82.9 ± 7.2	91.9 ± 10.8	100.0 ± 10.4	92.2 ± 5.7	86.0 ± 6.2	102.6 ± 9.7
	50	81.6 ± 9.8	91.5 ± 6.9	105.7 ± 7.8	96.1 ± 9.8	98.0 ± 6.7	90.4 ± 6.1	103.4 ± 8.2
B2	1.5	73.8 ± 9.4	84.0 ± 8.5	90.0 ± 13.2	84.0 ± 7.7	77.8 ± 7.7	71.9 ± 5.6	102.7 ± 11.9
	3.0	82.1 ± 5.7	73.3 ± 8.4	101.9 ± 6.8	90.1 ± 8.1	82.4 ± 4.6	80.6 ± 6.2	103.4 ± 13.8
	15	87.5 ± 7.4	81.0 ± 6.2	103.6 ± 4.5	98.0 ± 4.5	93.5 ± 4.4	86.2 ± 6.4	106.7 ± 12.0
B1	5	75.4 ± 4.5	78.7 ± 7.2	97.4 ± 4.8	81.2 ± 5.0	76.3 ± 6.3	80.8 ± 9.8	86.1 ± 14.2
	10	71.8 ± 7.4	84.2 ± 8.4	104.9 ± 10.8	95.6 ± 8.1	89.4 ± 7.2	80.7 ± 6.2	107.7 ± 10.8
	50	84.1 ± 4.5	84.7 ± 5.9	97.0 ± 9.4	97.6 ± 5.1	97.0 ± 8.6	87.7 ± 9.5	97.0 ± 9.4

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base-line noise obtained from blank peanut samples spiked with a mixed standard stock solution containing the four (4) investigated aflatoxins.

The LOD values obtained (see Table 2) are between 0.16 μg/kg for aflatoxin B2 and 1.06 μg/kg for aflatoxin G1. While the LOQs for aflatoxin B1 are lower than 2.00 μg/kg, which is below the maximum permitted level as per the European regulations (2.00 μg/kg for aflatoxin B1 and 4.00 μg/kg for total aflatoxins in foodstuffs for direct human consumption with the exception of infant food).[16]

The intra-day precision and inter-day precision were evaluated by testing the RSD values of blank peanut samples spiked at 10 μg/kg with aflatoxin B1 and G1 and 3 μg/kg of aflatoxin B2 and G2. Five replicates for each concentration level were analyzed during each working day to test the intra-day precision and subsequently during five consecutive days to test for inter-day precision. The intra-day precision and inter-day precision results for all aflatoxins in the peanut samples showed acceptable precision. The RSD values for the intra-day precision and inter-day precision were below 12.0% and 18.0%, respectively (Table 4).

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Fluorometric Method

For the quantitative analysis of aflatoxins in food samples, the fluorometric method gave unsatisfactory results. This method has a poor precision since both positive and negative biases were detected (Table 5). In addition, it suffers from matrix effects as the results varied when the composition of the matrix was changed. However, the intra-day precision and inter-day precision were acceptable with RSD values for both the naturally contaminated food and the spiked sample of less than 9% and 8%, respectively.

A very significant factor in the validation of this method was the detection of matrix effects since both false positive and false negative results were noted (Table 5), with a positive and negative bias detected in the fluorometric method. The fluorometric method is a useful rapid screening technique capable of analyzing a large number of samples in a relatively short period. However, it would be necessary to confirm the positive results by other chromatographic methods.

TABLE 4 Intra-Day Precision and Inter-Day Precision of Aflatoxins Expressed as RSD (%) Values

Aflatoxin	Spiking Level (μg/kg)	Intra-Day Precision ($n=5$)[a]	Inter-Day Precision ($n=15$)[a]
B1	10	6.9	11.4
B2	3	11.6	17.1
G1	10	6.9	10.1
G2	3	10.9	15.0

TABLE 5 Mean of Recoveries and RSD Values ($n=5$) of Aflatoxins Spiked into Clean Wheat, Corn, Rice, Pistachio, Peanut, Almond, and Sesame Samples at Two Spiking Levels Using Fluorometric Method

		Matrix						
Aflatoxin	Spiking Level (μg/kg)	Wheat	Corn	Rice	Pistachio Nut	Peanut	Almond	Sesame Seed
		Mean of Recovery (%) ± RSD (%)						
Total Aflatoxins	5	118.8 ± 6.9	137.5 ± 8.5	140.2 ± 2.4	82.5 ± 5.2	123.8 ± 6.7	175.0 ± 4.1	118.8 ± 1.3
	10	83.1 ± 4.5	118.8 ± 5.5	190.8 ± 6.7	221.9 ± 2.1	96.9 ± 4.6	106.3 ± 6.7	336.3 ± 4.7

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Sample Analysis

The developed method was applied for the analysis of commercial products supplied from the Jordanian food and drug administration. Only 20 samples showed aflatoxin contamination. Most of these contaminations involved peanuts (8 contaminations) originating from Egypt. Aflatoxins are also frequently reported in pistachio nuts (6 contaminations) originating from Syria and two contaminations were detected in sesame seeds originating from India, as well as, two contaminations were detected in raw peanut butter originating from both China and India. Finally, one contamination was reported in almonds (in shell) originating from Syria as well as one in sunflower seeds originating from the United States. For cereals, coffee, and spices, none of the samples showed any detectable concentration of aflatoxins. The contaminated peanuts and pistachio nuts are all without shell. This may be because the micro-environment beneath the shell with the protection of the kernels is less susceptible to cross-contamination and mold growth, with consequently less toxin production.

The sharp increase of contamination by aflatoxins has caused concern to the authorities, since approximately 2.99% of 669 domestic samples and imported samples examined between April and November 2011 are contaminated by aflatoxins at levels higher than the levels permitted in Jordanian Regulations set by Jordan Institution for Standards & Metrology (JISM).[18]

These results have shown that contamination by aflatoxins are two times higher than what was reported in previous studies (1.38%) since 37 out of 2745 samples examined between January 2009 and March 2011 showed aflatoxin contamination (Table 6 and Figure 11), which used thin layer chromatography (TLC) as a screening method and fluorometric determination as the quantification method. TLC has been the most widely employed method for both qualitative and quantitative determination of aflatoxins since 1960s. It has been adopted as the official method for aflatoxins in food by the AOAC since 1990. Despite its advantages such as low cost, useful and an easy technique, it is time-consuming; has extremely low

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TABLE 6 Occurrence of Aflatoxins in Commercial Jordanian Foods from January 2009 to November 2011

Sample category	Total Sample of the Particular Category	Number of Positive Sample	Incidence (Positive/Total Sample of the Particular Category)	Incidence (Positive/ Total Sample)	Total Aflatoxins (μg/kg)
Fluorometric Method (January 2009–March 2011)					
Cereals[a]	792	4	0.5%	0.1%	23–110
Peanut and Peanut Butter	536	19	3.5%	0.7%	32–1200
Nuts[b]	412	0	0.0%	0.0%	–[c]
Sesame Seed	156	0	0.0%	0.0%	–[c]
Pistachio Nut	287	11	3.8%	0.4%	35–2065
Seeds[d]	290	4	1.4%	0.1%	23–150
Coffee	272	0	0.0%	0.0%	–[c]
Spices[e]	66	0	0.0%	0.0%	–[c]
Total	2745	38		1.4%	
QuEChERS HPLC Method (April 2011–November 2011)					
Cereals[a]	274	0	0.0%	0.0%	–[c]
Peanut and Peanut Butter	87	10	11.5%	2.6%	18–400
Nuts[b]	78	1	1.3%	0.3%	–[c]
Sesame Seed	46	2	4.3%	0.5%	100–1280
Pistachio Nut	61	6	9.8%	1.5%	21–1645
Seeds[d]	51	1	2.0%	0.3%	760
Coffee	59	0	0.0%	0.0%	–[c]
Spices[e]	13	0	0.0%	0.0%	–[c]
Total	669	20		3.0%	

[a]Rice, corn, corn and potato starch, wheat, semolina, flour, etc.
[b]Walnut, cashew nut, almond, pine nut, and hazelnut.
[c]Not detected or below LOD of the adopted method.
[d]Pumpkin seed, watermelon seed, and sunflower seed.
[e]Cardamom and chili.

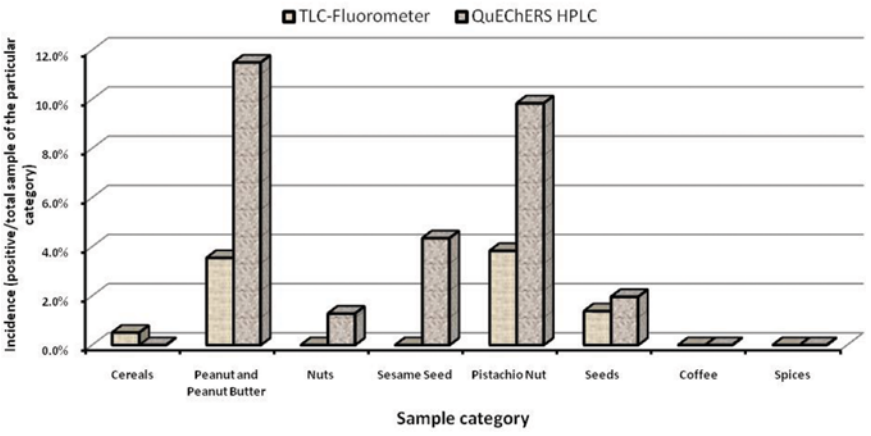


FIGURE 11 Comparison between TLC-Fluorometer method and QuEChERS-HPLC method on incidence of contamination for various sample categories. (Color figure available online.)

sensitivity for aflatoxins B1, B2, G1, and G2;[17] and gives unsatisfactory accuracy in quantification. Therefore, many researchers have used HPLC methods to achieve accurate quantification.[18,19] However, fluorometric determination, as mentioned earlier, suffers from matrix effects, so that it generally used as a rapid screening technique followed by more accurate chromatographic methods. Therefore, the differences in sample preparation and measurement methods may be responsible for these variations.

Figure 11 and Table 6 revealed a wide variation among sample categories in aflatoxin contamination across the 669 samples analyzed. There is a significant difference ($P > 0.05$) in aflatoxin contaminations between different types of sample categories. Approximately 11.5% of peanuts and peanut butters examined were contaminated with aflatoxins in the range of 18–400 μg/kg; 9.8% of pistachio nuts examined contained aflatoxins at concentrations of 21–1645 μg/kg; 4.3% of sesame seeds examined contained aflatoxins at concentrations of 100 and 1280 μg/kg; 2.0% of nuts examined contained aflatoxins at concentrations of 1680 μg/kg. However, samples of cereals, coffee and spices were found to be non-contaminated with aflatoxins. This may be because there were contaminated with other types of mycotoxins or the contaminations were below the LOD of the adopted method.

CONCLUSION

A simple, rapid, inexpensive, and effective sample preparation procedure using QuEChERS procedure has been developed. The developed sample preparation procedure is based on a single extraction step without employing a prior dSPE clean-up. Therefore, it can be recommended as an alternative to the time-consuming solid phase extraction or liquid–liquid extraction as well as to the more expensive immunoaffinity columns and multifunction column for aflatoxins determination in food. The extraction solvent was found to be the most important factor and it heavily affects the extraction efficiency. Other factors such as the type and amount of drying agents, the extraction time and the solvent-sample ratio have little effects. Excellent linearity, high recoveries, acceptable repeatability, and reproducibility with the LOQ values lower than the stipulated MRL were achieved indicating the suitability of the QuEChERS HPLC-FLD method for the determination of aflatoxins in various food commodities. On the other hand, the fluorometric method has a poor precision since both positive and negative biases were detected. Therefore, for the fluorometric method, it would be necessary to confirm the positive results by other chromatographic methods. Finally, it would be necessary to scrutinize the sharp increase of aflatoxins contaminations on the pistachio and peanut samples imported from Syria and Egypt, respectively, given the reported results from this and other studies.

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