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Low-density solvent based dispersive liquid-liquid microextraction followed by vortex-assisted magnetic nanoparticles based solid-phase extraction and surfactant enhanced spectrofluorimetric detection for determination of aflatoxins in pistachio nuts

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Abstract:

A simple and efficient two-step extraction method, namely low-density solvent based dispersive liquid-liquid microextraction (DLLME) followed by vortex-assisted dispersive solid-phase extraction (VA-D-SPE) combined with analysis by surfactant enhanced spectrofluorimetry, was developed for the determination of total aflatoxins in pistachio samples. The analytes were firstly extracted with methanol/water (80:20, v/v) from solid pistachio matrices and this solution was directly used as the dispersing solvent in accompany with 1-heptanol as the low density extracting solvent in DLLME procedure. In VA-D-SPE approach, hydrophobic Fe_3O_4 nanoparticles (*i.e.* oleic acid modified magnetic nanoparticles) were used to retrieve the analytes from the DLLME step. It is noticeable that the target of hydrophobic nanoparticles was 1-heptanol rather than the aflatoxins directly. The main parameters affecting the efficiency in DLLME and VA-D-SPE and signal enhancement of the analytes were investigated and optimized. Under the optimum conditions, the calibration curve showed a good linearity in the range 0.05-500 µg L^{-1} (R^2=0.9984) with low detection limit of 21 ng L^{-1}. The repeatability and reproducibility of extraction (as RSD %) were in the range of 2.3-4.6 % and high recoveries ranging from 91.6 to 99.6 % were obtained. Finally, the proposed method was successfully applied to the determination of total aflatoxins in commercial pistachio samples. The obtained results revealed that the method is simple, inexpensive, accurate and remarkably free from interference effects. Furthermore, the proposed method reclaimed the versatility of DLLME because the selection of extraction solvent was not limited to high density solvents.

Keywords: Aflatoxins; Dispersive liquid-liquid microextraction; Vortex-assisted dispersive - solid phase extraction; Magnetic nanoparticles; Micelle enhanced spectrofluorimetry.

1. Introduction

Today more than 300 mycotoxins which are toxic metabolites of various fungi growing on a wide range of food and animal feedstuffs [1]. Among of them, aflatoxins (AFs, Fig. 1), produced by some *Aspergillus* moulds such as *Aspergillus flavus* and *Aspergillus parasiticus*, represent the main threat worldwide owing to their occurrence and toxicity [2]. AFs are potentially hazardous to humans and animals and display strong immunosuppressive, mutagenic, teratogenic and carcinogenic effects [3]. Among AFs compounds, aflatoxin B_1 (AFB_1) has been reported to be the most toxic ones and classified as a group Al human carcinogen [4]. Many countries and international organizations have set stringent regulations about the level of AFs permitted in food commodities. The European Commission has established the maximum levels for AFs in groundnuts, nuts, dried fruits and cereals as 2 ng g^{-1} for AFB1 and 4 ng g^{-1} for total AFs [5].

The simultaneous determination of multiple aflatoxins in a single test considerably reduces the time and costs of each analysis and is the most attractive approach practically [6]. Currently, many simultaneous methods, such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and immunoassay based analysis have been developed for detection and identification of mycotoxins in food and feedstuffs [7-11]. However, most of these methods are time-consuming, costly, laborious, and require expensive instruments [12]. On the other hand, these methods are not sufficiently sensitive for direct determination of these compounds in food samples. In this context, development of methods for pre-concentration of AFs is necessary. Thus, methods normally used to analyze aflatoxins in sample matrices are based on extraction/preconcentration/clean-up with solid phase extraction (SPE) or immunoaffinity columns, followed by concentration steps [7, 8, 13]. On the other hand, modern trends in analytical chemistry are towards the simplification and miniaturization of sample

preparation procedures. Liquid-phase microextraction (LPME) has emerged in these last years as a powerful tool for preconcentration and matrix separation prior to detection. A new mode of LPME, namely dispersive liquid-liquid microextraction (DLLME), has been developed by Assadi et al. in 2006 [14] and is based on ternary component solvent systems. Here, an appropriate mixture of extraction solvent and dispersive solvent is injected rapidly into an aqueous solution, resulting in a cloudy state consisting of fine droplets of the extraction solvent dispersed in the aqueous phase, which markedly increased the contact surface between phases and reduce extraction time with the increasing enrichment factors [15, 16]. The advantages of the DLLME method are simplicity of operation, rapidity, low cost, high recovery and enrichment factors [17]. But, in conventional DLLME, the density of extraction solvent should be higher than water [18], the applications of DLLME in most cases were limited to water samples and the volume of the sedimented phase in some cases was dependent on the surrounding temperature. These limitations caused some development on DLLME. Some modification techniques resulted in DLLME improvement are the use of organic solvents with lower density than water and applying SPE in combination with DLLME [19, 20].

In this study, a DLLME procedure using 1-heptanol as the extraction solvent was applied to extract AFs from pistachio samples and a vortex assisted dispersive solid phase extraction (VA-D-SPE) using hydrophobic oleic acid modified Fe_3O_4 nanoparticles as the adsorbent was applied to retrieve the AFs-containing extracting solvent from DLLME step. Since, 1- heptanol is a large alcohol with a non-polar hydrophobic chain, a hydrophobic interaction can occur between this solvent and the hydrophobic nanoparticles and the analytes were rapidly partitioned on the surface of magnetic nanoparticles (MNPs). Separation was quickly carried out by the application of an external magnetic field overcoming the need for centrifugation, refrigeration to freeze,

manual collection of extractant or specialized apparatus. Then, a surfactant enhanced spectrofluorimetric determination using triton X-100 micelle formation was applied for determination of AFs. All the experimental parameters affecting the two-step extraction procedures were investigated in details and the analytical characteristics of the method were evaluated. The method was successfully applied for determination of AFs in pistachio samples.

2 Experimental

2.1 Standards and materials

Standards of AFB_1, AFB_2, AFG_1, and AFG_2 and all HPLC-grade solvents including acetone (Me_2CO), acetonitrile (MeCN), dichloromethane (CH_2Cl_2), methanol (MeOH), ethanol (EtOH), ethyl acetate ($C_4H_8O_2$), toluene (C_6H_5-CH_3), 1-heptanol ($C_7H_{16}O$), 1-octanol ($C_8H_{18}O$), 2-ethylhexanol ($C_8H_{18}O$), diethyl ether ((C_2H_5)$_2$O), and trichloromethane ($CHCl_3$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$), Triton X-100, oleic acid and the other used chemicals were supplied by Merck (Darmstadt, Germany). Deionized water was used throughout the experiments.

After preparation of standard solutions of each aflatoxin, their concentration was determined by using an UV-Vis Spectrophotometer through AOAC Official method No. 971.22, chap. 49.2.03 [21]. These standards were used to prepare tertiary stock solution of mixed standards as total AFs 1000 µg mL^{-1} (AFB_1, AFG_1=400 µg mL^{-1}; AFB_2, AFG_2=100 µg mL^{-1}), and the working standard solution was prepared by diluting stock solution with methanol and water.

Since AFs are potential carcinogen compounds, extreme handling precautions must be warranted. Gloves and other protective clothing must be worn as safety precaution and it is

necessary to protect analytical works from sunlight because of degradation in light. All glassware should be soaked in 5% sodium hypochlorite solution to destroy AFs residue before re-use.

2.2 Instrumentation

A Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA, USA) equipped with a xenon lamp was used for fluorescence spectra recording of AFs with scan rate of 1200 nm min^{-1}. All measurements were performed using 10 mm quartz microcells at room temperature and spectra recording were carried out with slit widths of 5 nm. The excitation and emission wavelengths were 360 and 450 nm respectively. The modified magnetic nanoparticles were characterized by a Hitachi H-800 (Tokyo, Japan) transmission electron microscope (TEM). Chemical interactions were studied using a Perkin Elmer Spectrum one Bv5.3.0 FT-IR spectrometer (Waltham, Massachusetts, US) in the range of 400-4000 cm^{-1} with KBr pellets. UV-Vis spectra of AFs standards were carried out using UV-240 Shimadzu spectrophotometer (Tokyo, Japan). A Labinco BV L46 Vortex mixer (Breda, Netherlands) was used to mix and accelerate the reactions between reagents.

2.3 Synthesis of oleic acid modified MNPs

The Fe_3O_4 nanoparticles were prepared via a simple chemical co-precipitation method previously reported [22] with slight modifications. Briefly, $FeCl_3 \cdot 6H_2O$ (5.8 g) and $FeCl_2 \cdot 4H_2O$ (2.1 g) were dissolved in 100 mL deionized water under nitrogen atmosphere with vigorous stirring at 85 °C. Then, 20 mL of aqueous ammonia solution (25% w/w) was added to the solution. The color of bulk solution changed from orange to black immediately. The magnetic precipitate was washed with deionized water (2×100 mL) and 0.02 mol L^{-1} (1×100 mL) sodium chloride. Then, oleic

acid (1.0 g) was introduced and the reaction was kept at 80 ºC for 3 h. Finally, the suspension

was cooled to room temperature. The resulting nanoparticles were washed sequentially with

deionized water (2×100 mL), methanol (2×100 mL) and deionized water (3×100 mL) and

separated from the solution with the help of an external magnet. Finally, oleic acid modified

magnetite nanoparticles were stored in deionized water at a concentration of 80 mg mL^{-1}.

2.4. Real sample pretreatment

For preparation of pistachio samples (oily sample), 50 g of thoroughly homogenized nuts and 5 g

NaCl were dissolved in 200 mL of methanol: water (80:20, v/v) and then, the mixture was added

to 100 mL of n-hexane in a blender and mixed thoroughly for 3 min. The mixture was transferred

to the separating funnel and the lower aqueous phase was filtered and diluted to 150 mL with

methanol: water (80:20, v/v) and shacked intensively. The separated phase was then passed

through a glass microfiber filter paper and an appropriate aliquot of the filtrate was used for

DLLME step.

2.5. Analytical procedure

310 µL of 1-heptanol was added to 3 mL of sample solution (*i.e.* MeOH/H$_2$O (80:20, v/v)

containing analytes) and the mixture was rapidly injected into a conical-bottom vial containing

15 mL of deionized water. Then, the vial was sealed and swirled on a vortex agitator at 3500 rpm

for 1 min (equilibration time). After that, 750 µL of the adsorbent (containing 60 mg of modified

MNPs) were quickly added to the vial. The solution swirled again using the vortex agitator for 2

min to facilitate interaction of organic solvent containing AFs to the surface of oleic acid

modified MNPs. Then, the magnetic adsorbent was collected by applying an external magnet and

the supernatant was removed. The adsorbed AFs were desorbed from the adsorbent by addition

of 2 mL of MeCN for 3 min. After desorption, the eluent was separated by magnetic decantation

and evaporated to dryness under nitrogen gas flow at room temperature. The dry residue was

dissolved in 2 mL of 0.5 mM Triton X-100 in 15 % (v/v) acetonitrile/water and the solution was

stirred for 5 min. The final solution was evaporated to 500 μL under nitrogen flow and used for

taking fluorescence spectra.

3 Results and discussion

3.1 Characterization of the adsorbent

The size and morphology of oleic acid modified Fe_3O_4 nanoparticles were characterized by TEM

images. As can be seen from Fig. 2, the modified nanoparticles had a uniform size distribution

and most of them are quasi-spherical in shape with the mean diameter of 9±1.2 nm. FT-IR

spectroscopy was used to characterize the chemical interaction between Fe_3O_4 nanoparticles and

oleic acid. As can be seen from Fig. 3, the characteristic peak of Fe_3O_4 nanoparticles can observe

as a strong absorption band at 583 cm^{-1} corresponds to the Fe-O band of bulk magnetite. This

band can be observed in oleic acid modified MNPs spectrum too. The two sharp bands at 2923

and 2853 cm^{-1} are attributed to the asymmetric and symmetric CH_2 stretch, respectively. It is

worth to note that C=O stretch band of the carboxyl group, which generally appears at 1700-

1750 cm^{-1} was absent in the spectrum (b) belongs to the oleic acid modified MNPs and there

appeared two new bands at 1541 and 1630 cm^{-1} which were characteristic of the asymmetric v_{as}

(COO-) and the symmetric as v_s (COO-) stretch, instead [23, 24]. These results reveal that oleic acid

were chemisorbed onto the Fe_3O_4 nanoparticles as a carboxylate and its hydrocarbon tail is free

to interact with analyte containing 1-heptanol solvent.

3.2 Signal enhancement conditions

The fluorescence of mycotoxins is quenched in water and increasing surfactant or some complexing agents like β-cyclodextrin enhances the fluorescence intensity [25, 26]. This confirms that the microenvironment around AFs in these solutions is quite different from pure aqueous solutions. In this study, Triton X-100 was selected as signal enhancement agent. It possesses a long tail length which forms large micelles around AFs molecules, provide a better environment to encapsulate and restrict the intramolecular rotation of AFs to boost emission. The effect of surfactant addition on AFs fluorescence signal was investigated by adding different amounts of Triton X-100 (0.02-1 mM) to the desorbed AFs. As can be seen from Fig. 4, significant fluorescence enhancement was occurred with increasing Triton X-100 concentration and reached maximum in 0.5 mM which is above the critical micellar concentration (CMC) value of 0.2 mM for this surfactant. The effect micelle formation time on the fluorescence signal of AFs was also investigated in the range of 1-15 min. The results revealed that 5 min was enough for maximum signal enhancement and used for subsequent experiments.

3.3 Optimization of the DLLME procedure

3.3.1 Selection of dispersing solvent

Since the solvent used to extract AFs from solid pistachio matrix was applied directly as the disperser solvent in DLLME, its selection must take into account both the properties required for AFs extraction from solid sample matrix and DLLME dispersant [27]. Generally, an aqueous mixture of MeOH was applied for extraction of AFs and, Me_2CO, MeCN and MeOH are the commonly used disperser solvents in DLLME method. Thus, the applicability of several

solvents, including Me_2CO, MeOH, MeCN, EtOH, MeOH/water (80:20 v/v) and MeCN/water (80:20 v/v) was investigated in the preliminary experiments. The results (Fig. 5) revealed that the extraction efficiency achieved by MeOH/water (80:20 v/v) was higher than the other solvents and therefore, it was selected to act as both AFs extractant from pistachio samples and as the disperser solvent in DLLME for subsequent experiments.

Furthermore, the effect of disperser volume on the AFs recovery was investigated in the range of 1-5 mL. The obtained results (see Fig. S1, supplementary materials) revealed that the extraction efficiency increases with increasing the volume of MeOH/water (80:20 v/v) up to 3 mL and then, decreases due to the increase in solubility of AFs in aqueous phase and decrease in their distribution ratio. Based on the results, further studies were performed using 3 mL of MeOH/water (80:20 v/v) as the dispersing solvent.

3.3.2 Selection of extracting solvent

An appropriate extraction solvent has a crucial role in DLLME procedure. It should meet special conditions, and has several characteristics. For example, it must has good emulsification efficiency in the aqueous sample, high affinity for compounds of interest, low solubility in water, low density and low vapor pressure to prevent loss during agitation. Five organic solvents were evaluated as extraction solvent including ethyl acetate (density, $d=0.897$ g mL^{-1}), toluene ($d=0.865$ g mL^{-1}), 1-heptanol ($d=0.818$ g mL^{-1}), 1-octanol ($d=0.827$ g mL^{-1}), and 2-ethylhexanol ($d=0.833$ g mL^{-1}) in the preliminary experiments. As can be seen from Fig. 6, 1-heptanol gave the highest fluorescence signal for the analytes, followed by 1-octanol and toluene, and finally ethyl acetate and 2-ethylhexanol. Thus, 1-heptanol was considered as the most suitable extraction solvent for the subsequent experiments.

The volume of extracting solvent is another important parameter affecting the cloudy state formation and efficiency of extraction process. So, the effect of 1-hepanol volume on the extraction of AFs was investigated in the range of 250-350 µL. The obtained results (Fig. S2 supplementary materials) revealed that the fluorescence intensity of AFs increases with increasing 1-heptanol volume in the range of 290 to 330 µL. Decreasing in signal intensity above 330 µL is due to the dilution effects and in down to 290 µL corresponds to the dissolution of organic phase in aqueous media. Based on the results, 310 µL was selected as an optimum volume for further studies.

3.3.3 Effect of salt addition

Addition of salt to the sample may have several effects on the extraction efficiency of the analyte. Generally, salt addition can decrease solubility of target analyte in aqueous phase and promote analyte transfer toward the organic phase resulting to the improvement in the extraction efficiency and known as salting-out effect [28]. On the other hand, salt addition increases viscosity and density of sample solution and it can reduce the efficiency of emulsification phenomenon because of lower solubility of extracting solvent in aqueous phase. In this study, the effect of salt addition on the extraction efficiency of AFs was investigated by adding different amounts of NaCl in the range of 0-5 % (w/v) to the samples. The obtained results (see Fig. S3, supplementary materials) shown that the extraction efficiency of AFs was not affected by the presence of NaCl. Thus, the experiments were carried out without adding salt.

3.3.4 Effect of water volume

The recovery of AFs was also affected by the water volume used in DLLME because it can influence the solubility of them in the aqueous phase. The effect of water volume on the extraction efficiency of the analytes was investigated using different volumes in the range of 2.5-25 mL. The results (Fig. S4, supplementary materials) were shown that the extraction efficiency was constant in the range of 12.5-18 mL. Based on the results, 15 mL was selected for the subsequent experiments.

3.3.5 Effect of equilibration time

In this study, the equilibration time is defined as the interval time from the occurrence of the cloudy state and just before addition of hydrophobic magnetic nanoparticles. The equilibration time was investigated in the range of 0-200 s maintaining the rotational speed at 3500 rpm to maximize mass transfer and reduce mixing time. As can be seen in Fig. S5, supplementary materials, the intensity of fluorescence signal was not affected remarkably in different extraction times above 60 s, showing that the mass transfer from sample solution to the extracting solvent are very fast. Based on the results, 60 s was selected for the subsequent experiments.

3.4 Optimization of MNPs based VA-D-SPE procedure

3.4.1 Effect of MNPs amount and vortex time

The amount of hydrophobic MNPs is a key parameter to accomplish quantitative separation of the extraction solvent containing AFs in DLLME step. Thus, different amounts of oleic acid modified Fe_3O_4 were added in the range 10-100 mg to the sample solution. The results showed that the extraction efficiency increases with increasing in the amount of magnetic adsorbent up to 60 mg and then leveled off (Fig. S6, supplementary materials). MNPs have significantly higher

surface area and short diffusion route compared to ordinary sorbents. Thus, satisfactory results with lower adsorbent amount can be achieved with these sorbent materials. Based on the results, 60 mg was selected for the next experiments. In order to investigate the effect of adsorption time on the recovery of the analyte, the vortex time was varied in the range of 1-10 min. The experimental results (see Fig. S7, supplementary materials) indicate that 2 min is sufficient for achieving appropriate adsorption of AFs and it was used for the next experiments.

3.4.2 Desorption conditions

After extraction, the AFs containing extracting solvent were desorbed from the adsorbent into a suitable organic solvent. So, desorption capability of solvent was investigated using five commonly used organic solvents including Me_2CO, EtOH, MeOH, MeCN and $CHCl_3$. As can be seen from Fig. 7, the best result was found with MeCN as the desorbing solvent.

Furthermore, the effect of desorbing solvent volume on the recovery of AFs was investigated in the range of 0.5-5 mL and the maximum recovery was obtained with volumes higher than 2 mL (Fig. S8, supplementary materials). Thus, 2 mL of acetonitrile was selected as the optimum desorbing solvent for the subsequent experiments. In addition, the effect of desorption time on the recovery of the analytes was examined in the range of 1-10 min (Fig. S9, supplementary materials). As can be seen, the duration time of 3 min was appeared to be sufficient for complete desorption. Since, magnetic nanoparticles can be easily and rapidly collected from the solution using an external magnetic field, the analysis time greatly reduces compared to the conventional SPE methods and combination of MNPs based SPE with DLLME eliminates many time-consuming centrifugation, refrigeration to freeze and then thawing or manual collection of extracting solvent usually accompany with DLLME procedure.

3.4.3 Reusability of the adsorbent

In order to investigate the re-applicability of hydrophobic adsorbent, the oleic acid modified MNPs which was used in one VA-D-SPE procedure was further desorbed and analyzed under the same conditions and the reproducibility of recovery data was investigated. The experimental result show that oleic acid modified MNPs are capable of being used for up to 10 extractions without sacrificing the analytical results (obtained RSD% less than 3.1% for recovery results) reclaimed the capacity of these materials to be an alternative sorbent for immunoaffinity columns.

3.5 Effect of interferences

Selectivity and competitive extraction experiments were carried out using total AFs, zearalenone (ZEN), ochratoxin A (OTA) and deoxynivalenol (DON) which are other mycotoxins that may exist in pistachio. Therefore, the possible interference effects of ZEN, DON and OTA was studied by co-existing of them alone and in mixture. The obtained results (table 1) showed that the recoveries of total AFs in the presence of ZEN, OTA, DON and mixture of them were not significantly affected by the presence of the interferences, indicating good selectivity for determination of total AFs in pistachio. Two possible reasons are: the difference in excitation/emission wavelengths of these mycotoxins with the corresponding ones for total AFs and unsuitability of solvent medium (*i.e.* desorbing solvent in VA-D-SPE step) for both quantitative desorption from the adsorbent and taking analytes fluorescence.

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3.6 Analytical parameters

The proposed method was validated in terms of linearity, limit of detection, and intra-day and inter-day precisions. Samples for construction of the calibration curve were prepared by spiking appropriate amount of total AFs stock solution (with final concentration of 0.05, 0.1, 0.5, 5, 20, 50, 100, 250, 400 and 500 µg L^{-1}) to the non-contaminated pistachio samples and subjected to the proposed DLLME-µ-SPE procedure following the enhanced fluorescence measurements. Under optimum experimental conditions, the calibration curve was linear over the concentration range of 0.05-500 µg L^{-1} with calibration equation of Y=87.61C+4.71 and correlation coefficient (R^2) of 0.9984. Furthermore, sensitivity of the method for each individual aflatoxin was also measured by analyses of non-contaminated pistachio samples spiked with each aflatoxin with corresponding concentration in samples containing total AFs. Thus, samples containing 0.02, 0.04, 0.2, 2, 8, 20, 40, 100, 160, and 200 µg L^{-1} for AFB_1 or AFG_1 and samples containing 0.005, 0.01, 0.05, 0.5, 2, 5, 10, 25, 40, and 50 for AFB_2 or AFG_2 were analyzed. The calibration equations of Y= 38.93C+3.77 (R^2 of 0.9982), Y=279.80 C+8.49 (R² of 0.9992), Y=49.43 C-21.96 (R² of 0.9993) and Y=236.22 C-9.99 (R² of 0.9991) were obtained for AFB_1, AFB_2, AFG_1 or AFG_2 respectively. The limit of detection (LOD=3.3S_b/m, where S_b is the standard deviation of ten replicates measurements of blank solution and m is the slope of the calibration curve) was found to be 21 ng L^{-1}. The precision of the method was evaluated as RSD% through investigation of intra-day and inter-day variations. The intra-day precision was evaluated using five replicates measurements of two spiked samples with the concentration of 2 and 200 µg L^{-1} in the same day and the inter-day precision was evaluated using five replicates measurements of spiked samples at same concentration levels in five consecutive days. The results which were summarized in table 2 indicate good precision of the proposed method. The adsorption capacity

of adsorbent was also determined by the static method. For this purpose, 60 mg of hydrophobic adsorbent was equilibrated with 18 mL of solution containing dispersed analyte after DLLME step, at different concentration levels at the optimum conditions. After 10 min, the mixture was magnetically decanted and the supernatant was analyzed. The results showed that the amount of analytes adsorbed per mass unit of the adsorbent was increased with increasing in concentration of total AFs and then was reached to a plateau value (adsorption capacity value), represents the saturation of active surface of hydrophobic adsorbent. The maximum adsorption capacity of the adsorbent for total AFs was found to be 531 μg g^{-1}.

3.7 Real sample analysis

To evaluate the applicability of the proposed method in real matrices, it was applied to the determination of AFs in pistachio samples. Recovery studies were carried out by spiking the samples with different amounts of total AFs and the obtained results were summarized in Table 3. The acceptable recoveries in the range of 91.6 to 99.6 % demonstrate that the matrix of pistachio sample was not affected the extraction efficiency. Further examination of accuracy was performed by comparison of the results obtained from the proposed method and the AOAC standard method (IAC-HPLC-FL) No. 999.07:2000, chap. 49.2.29 [21] for determination of AFs in five contaminated pistachio samples. The results are summarized in Table 4. The statistical t-test analysis of the results showed that there are no significant differences between data obtained by the two methods at 95% confidence level. Furthermore a comparison of the analytical characteristics obtained by the proposed method and some other reported methods for determination of AFs is presented in Table 5. As can be seen, the proposed method has distinct advantages in terms of low detection limit, wide linear range, ease of operation and simplicity.

4. Conclusion

In this study, a two-step extraction technique namely, DLLME coupled with magnetic nanoparticles-based VA-D-SPE followed by surfactant enhanced spectrofluorimetric detection was developed for the extraction of total AFs in pistachio samples. The proposed method demonstrates that an organic solvent with lower density than water can be used in DLLME and it can be retrieved without involving any additional handling procedure and apparatus by application of hydrophobic magnetic nanoparticles. The method has many advantages including simplicity for extraction, low organic solvent consumption, excellent enrichment in a short period of time, good repeatability and reproducibility for determination of AFs, low cost and high accuracy. The good recoveries obtained for real samples and the inherent sensitivity and selectivity of spectrofluorimetric method showed that the present method was effectively applicable for determination of AFs in pistachio samples.

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Figure Captions:

Fig. 1 The molecular structure of aflatoxin B_1, B_2, G_1 and G_2.

Fig. 2 TEM image of oleic acid modified MNPs.

Fig. 3 FT-IR spectra of MNPs (a) and oleic acid modified MNPs (b).

Fig. 4 Effect of Triton X-100 concentration on the fluorescence intensity of the AFs. The excitation and emission wavelengths were 360 and 450 respectively.

Fig. 5 Effect of dispersing solvent on the recovery of total AFs. Conditions: extraction solvent, 310 µL of 1-heptanol; water volume, 15 mL, equilibration time, 60 s, adsorbent amount, 60 mg; adsorption time, 2 min; desorption time, 3 min, desorption solvent volume and type, 2 mL of MeCN.

Fig. 6 Effect of extracting solvent on the recovery of total AFs. Conditions: dispersive solvent, 3 mL of MeOH /water (80:20 v/v); water volume, 15 mL, equilibration time, 60 s, adsorbent amount, 60 mg; adsorption time, 2 min; desorption time, 3 min, desorption solvent volume and type, 2 mL of MeCN.
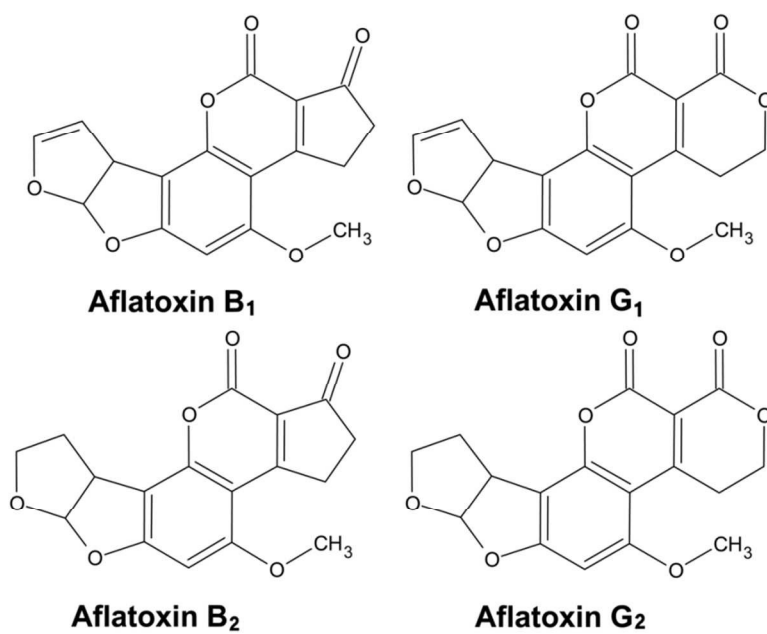
Fig. 7 Effect of desorption solvent type on the recovery of total AFs. Conditions: dispersing solvent volume and type, 3 mL of MeOH/water (80:20 v/v), extraction solvent, 310 µL of 1-heptanol, water volume, 15 mL, equilibration time, 60 s, adsorbent amount, 60 mg; adsorption time, 2 min; desorption time, 3 min, desorption solvent volume, 2 mL.
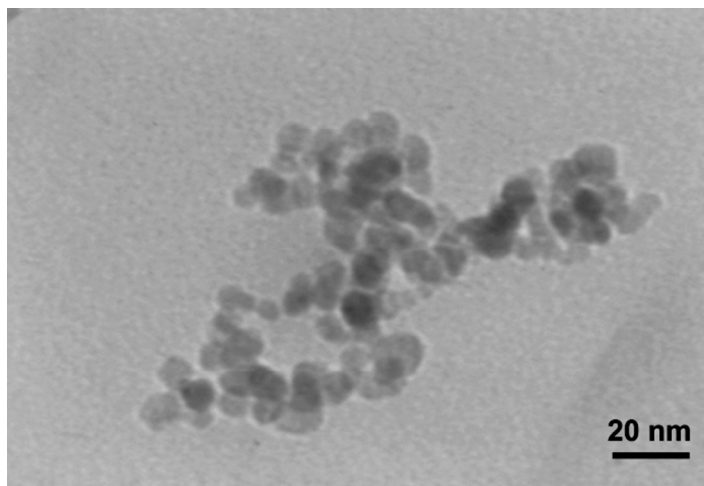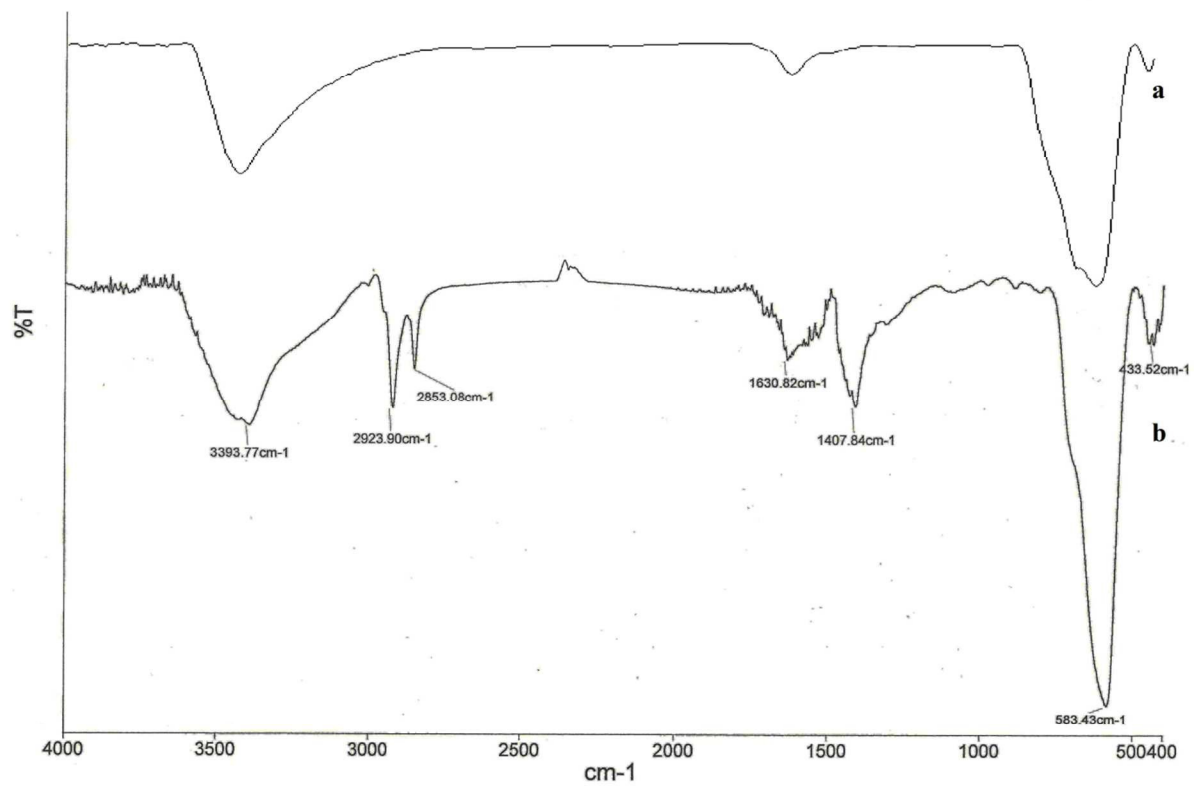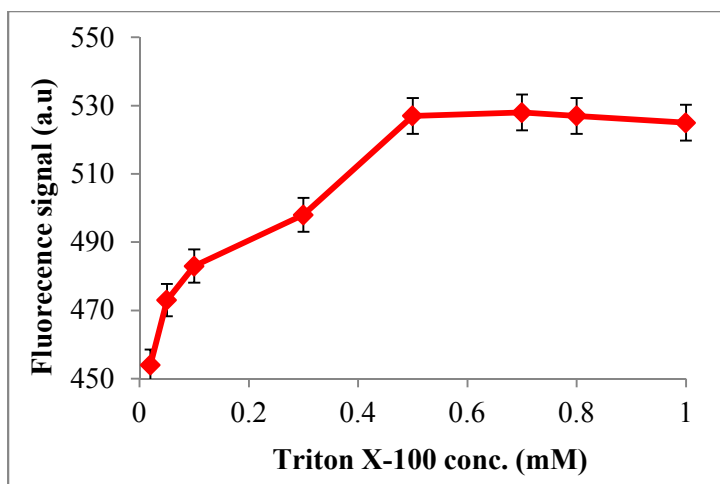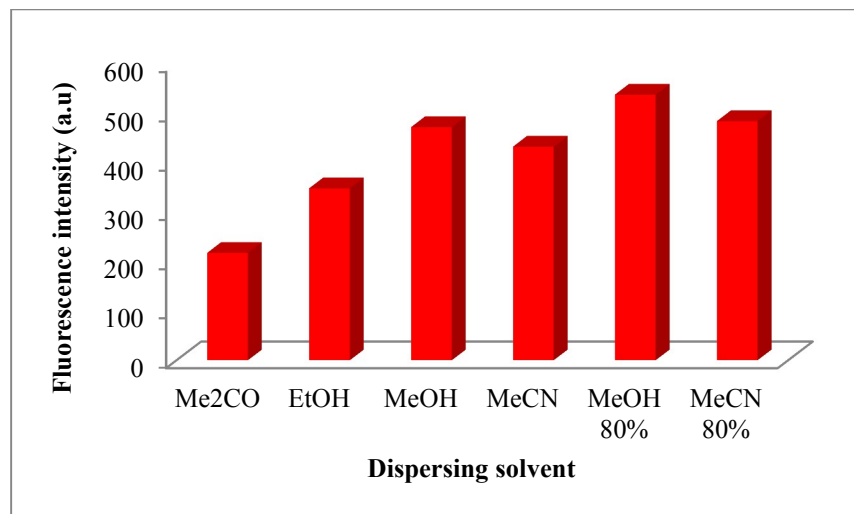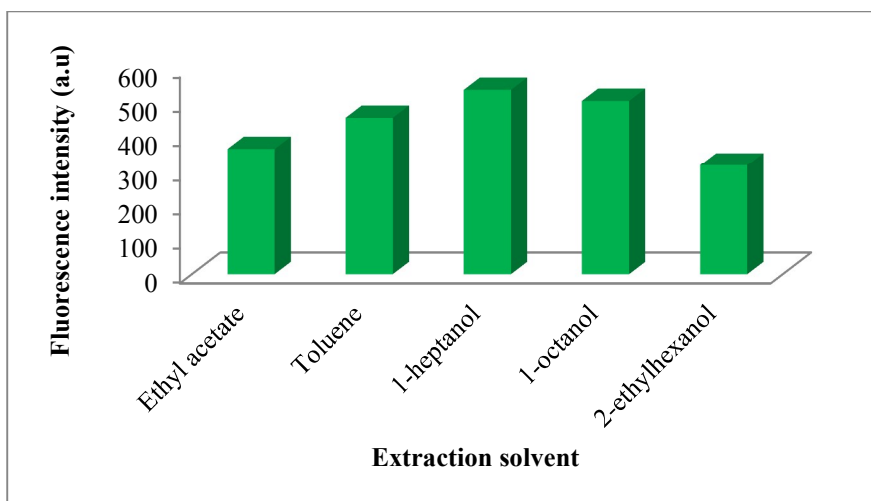
Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

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Fig. 6

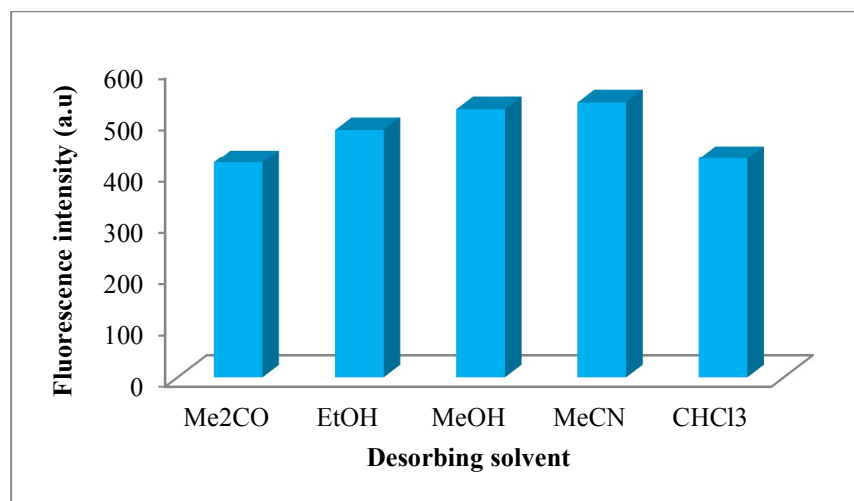**Fig. 7**

Table 1

Effect of mycotoxin interferences on the recovery of total AFs (5 µg kg^{-1}, n=3).

Interference	Concentration (µg kg^{-1})	Recovery (%)
ZEN	5	95.13
OTA	5	94.92
DON	5	98.73
Mixture	total	94.11

Table 2

The characteristic data for determination of total AFs by the proposed method.

Parameters	value
Dynamic range ($\mu g\ L^{-1}$)	0.05-500
Correlation coefficient (R^2)	0.9993
Intra-day precision (RSD%, n=5)	3.3[a]
	2.3[b]
Inter-day precision (RSD%, n=5)	4.6[a]
	3.4[b]
Limit of detection ($3.3S_b/m$ [c], ng L^{-1})	21

[a] For 2 $\mu g\ L^{-1}$ of total AFs

[b] For 200 $\mu g\ L^{-1}$ of total AFs

[c] S_b is the standard deviation for ten blank measurements and m is the slope of the calibration curve.

Table 3

Determination of total AFs in pistachio samples (n=3, ± SD).

Sample	Spiked (µg kg^{-1})	Found (µg kg^{-1})	Recovery (%)
Sample 1	0	2.21 ± 0.41	-
	10	11.44 ± 1.63	93.69
	50	50.74 ± 2.81	97.18
	300	300.12 ± 2.89	99.32
Sample 2	0	ND[a]	-
	10	9.16 ± 1.78	91.67
	50	47.66 ± 2.73	95.32
	300	298.81 ± 2.85	99.60
Sample 3	0	ND	-
	10	9.39 ± 2.19	93.91
	50	46.73 ± 1.97	93.46
	300	294.32 ± 2.63	98.11

[a]Not detected

Table 4

Comparison of AFs analyses (mean ± SD, n=3) in contaminated pistachio samples by the

proposed and standard IAC-HPLC-FD method.

Sample	Proposed method	HPLC-FD-IAC[a]
	AFs ($\mu g\ kg^{-1}$)	AFs ($\mu g\ kg^{-1}$)
Sample 1	2.21 ± 0.41	2.33 ± 0.13
Sample 2	2.78 ± 0.29	2.52 ± 0.30
Sample 3	1.98 ± 0.43	2.12 ± 0.37
Sample 4	3.55 ± 0.29	3.32 ± 0.41
Sample 5	2.41 ± 0.24	2.55 ± 0.22

[a] HPLC analysis by AOAC standard method.

Table 5

Comparison of the proposed method with some previously reported methods for the

determination of total AFs.

Method	Analyte	Matrix	Linear range $(\mu g\, kg^{-1})$	LOD $(\mu g\, kg^{-1})$	Ref.
HPLC-IAC	B_1, G_1	Pistachio	0.12-8	87.7	2
HPLC-IAC	B_2, G_2	Pistachio	0.06- 4	87.7	2
HPLC-MS[a]	B_1, B_2, G_1, G_2	Leaves	0.04-50	0.01-0.03	29
UHPLC-MS/MS[b]	B_1	Seeds	0.3-10	0.09	30
UHPLC-MS/MS	B_2, G_1, G_2	Seeds	0.6-20	0.11-0.22	30
DLLME-HPLC-FD	B_1, G_1	Cereals	0.1-20[c]	0.06-0.17	27
DLLME-HPLC-FD	B_2, G_2	Cereals	0.025-5[c]	0.01-0.04	27
DLLME-D-SPE-Fluorimetry	B_1, B_2, G_1, G_2	Pistachio	0.05-500	0.021	This work

[a] High performance liquid chromatography-mass spectrometry

[b] Ultra-high performance liquid chromatography-tandem mass spectrometry

[c] ng mL^{-1}