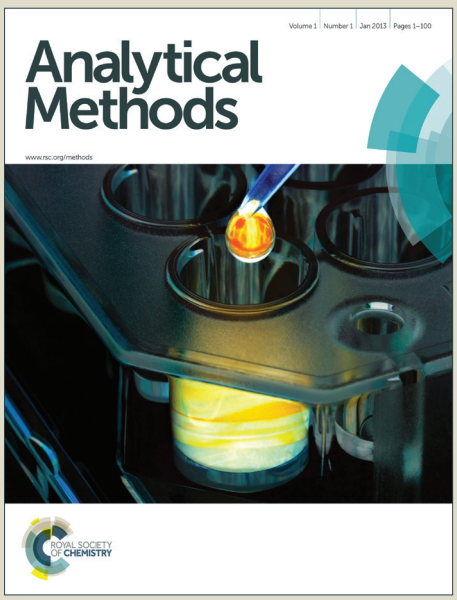
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Separation and determination of aflatoxins B_1, B_2, G_1 and G_2 in pistachio samples based on the magnetic solid phase extraction followed by high performance liquid chromatography with fluorescence detection

Mahdi Hashemi *, Zohreh Taherimaslak

Department of Analytical Chemistry, Faculty of Chemistry, Bu-Ali Sina University, Hamedan, Iran

Abstract

A simple and fast method based on the magnetic solid phase extraction (MSPE) followed by high performance liquid chromatography with post column derivatization-fluorescence detection system has been developed for simultaneous separation and determination of aflatoxins (AFs) B_1, B_2, G_1 and G_2 in pistachio. After primary extraction, purification of AFs was occurred by MSPE procedure. Magnetic nanoparticles coated by 3-(trimethoxysilyl)-1-propantiol and modified with 2-amino-5-mercapto-1,3,4-thiadiazole were used for extraction and purification of AFs. Efficiency of modified magnetic nanoparticles has been validated as an antibody-free adsorbent. The experimental parameters affecting the extraction efficiency such as pH, adsorption and desorption times, amount of adsorbent, type and volume of desorption solvent were investigated and optimized. Under the optimized conditions, the calibration curves were linear in the ranges of 0.10-15 μg L^{-1} for AFB_1 and AFG_1 and 0.04 - 3.00 μg L^{-1} for AFB_2 and AFG_2. Limits of detection (LODs) were in the range of 0.012- 0.035 μg L^{-1}. The intra-day and inter-day precision (RSD %) were in the range of 2.3–5.4 % and 2.8–5.9 %, respectively. The developed method has been successfully applied to the determination of AFs in pistachio samples with good recoveries (92.5-103.2%). Also, performance of the method was compared with the conventional method based on the immunoaffinity chromatography. The results demonstrated, the developed method is simple, rapid, inexpensive, accurate and selective.

Keywords: Aflatoxins; Magnetic solid phase extraction; Modified magnetic nanoparticles; High performance liquid chromatography; Pistachio.

* Corresponding author. Tel.: +98 81 38282807; Fax: +98 81 38257407

E-mail addresses: mhashemi@bacu.ac.ir (M. Hashemi)

1. Introduction

Aflatoxins (AFs) are a group of highly oxygenated heterocyclic compounds which are produced as secondary metabolites by the food spoilage fungi, particularly *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. Under favorable temperature and humidity conditions for these toxigenic fungi, AFs may be formed during any phase of the production, processing and transformation of food products.[1] Among 18 different types of AFs identified, major ones are AFB_1, AFB_2, AFG_1 and AG_2 (Fig. 1). These AFs show potency of toxicity, carcinogenicity and mutagenicity in the order of AFB_1>AFG_1>AFB_2>AFG_2. The International Agency of Research on Cancer has classified all four AFs as Group 1 carcinogens, primarily affecting liver.[2] The European commission has set the maximum levels (MLs) of AFs in cereals, peanuts, dried fruits, nuts, spices and processed products for human consumption as 4 μg kg^{-1} for total aflatoxins (AFB_1, AFG_1, AFB_2 and AFG_2) and 2 μg kg^{-1} for AFB_1 alone.[3] AFs frequently contaminate a wide variety of important agricultural products. The agricultural products with the highest risk of AFs contamination include corn, peanuts, pistachio nuts, cottonseed, figs, spices and copra.[4] Iran is the largest producer and exporter of pistachio with a production of more than 180,000 tons per year. It is also one of the important agricultural products, which is exported as well as being largely consumed in Iran. Pistachio nuts are a rich source of fat and contain fatty acids such as oleic, linoleic and linolenic acids, which are essential for the human diet.[5] They also contain minerals (Ca, Mg, K, P, Cu, etc.), vitamins A, B_1, B_2, B_6 and many bio-active constituents such as antioxidants, phytosterols and other phytochemicals.[5] AFs are founding in pistachio and are known to be toxic, mutagenic and immunosuppressive.[6] Due to high toxicity, frequent occurrence and low concentration of AFs, development of rapid, sensitive and reliable methods for screening and determination of AFs in a wide range of samples is of great interest. Various

analytical methods for the determination of AFs in the food samples include thin-layer chromatography,[7] enzyme-linked immunosorbent assay,[8] fluorescence polarization assay,[9] high-performance liquid chromatography with fluorescence detection,[10-14] high-performance liquid chromatography with diode-array detection,[15] high-performance liquid chromatography with tandem mass spectrometry,[16-21] and adsorptive voltammetry.[22] Among different analytical methods, high-performance liquid chromatography with fluorescence detection (HPLC-FD) is the most frequently used method due to its good sensitivity and suitable selectivity. Since the matrices of the food samples are often complex and concentration of AFs are relatively low, a pretreatment step is necessary for sample enrichment and clean-up. Usually pretreatment step involves a methanol–water (80:20, v/v) extraction followed by a purification step. Different clean-up methods have been reported for purification of AFs such as dispersive liquid–liquid microextraction,[10] solid-phase microextraction,[13,19] and solid-phase extraction with immunoaffinity columns (IACs),[11,12,16] or other solid phase sorbents.[15,17,18,20,23] Generally, IACs allow an efficient and highly selective separation of AFs. However, they have some important disadvantage such as relatively high cost, lake of reusability and short shelf-life time.[24] Moreover, the collection efficiency of IACs has failed to provide complete satisfaction for users.[25] In recent years, a new solid phase extraction technique, based on the use of magnetic or magnetically modified adsorbent called magnetic solid-phase extraction (MSPE), has been developed for separation and preconcentration of organic and inorganic species from complex matrices. Magnetic nanoparticles (MNPs) have been extensively used as adsorbent in MSPE because of their super paramagnetism, high magnetic saturation, low toxicity, simple preparation process and low price. Also, MNPs possess large surface areas which provide high interfacial area, fast mass transfer and rapid equilibrium. The stability and selectivity of the MNPs can

significantly improve by the modification of their surfaces by favorable functional groups.[26,27] Recently, the different modified nanoparticles and nanobeads with monoclonal antibodies have been applied for separation and determination of mycotoxins by immunoassay methods.[28-31] But synthesis of these nanoparticles and nanobeads often is so laborious, time consuming and expensive. Also the life time of these adsorbents is a serious problem. The aim of the present work is to investigate the applicability of modified magnetic nanoparticles (MMNPs) for the separation and determination of AFB_1, AFB_2, AFG_1 and AFG_2 in pistachio samples by HPLC-FD. All the experimental parameters affecting the separation procedure were intensively investigated and analytical characteristics of the proposed method were evaluated and compared with official method (IAC-HPLC-FD).[32] To the best of our knowledge, this is the first time that magnetic solid phase extraction with antibody-free adsorbent has been applied for the separation and determination of AFB_1, AFB_2, AFG_1 and AFG_2 in pistachio samples. The method was demonstrated to be applicable for the determination of AFs in real samples.

2. Experimental

2.1. Standards and materials

The standards solutions of AFs containing AFB_1 and AFG_1 at 1000.0 µg L^{-1} and AFB_2 and AFG_2 at 200.0 µg L^{-1} and all HPLC-grade solvents such as acetone (Me_2CO), acetonitrile (MeCN), dichloromethane (CH_2Cl_2), methanol (MeOH) and water (H_2O) were purchased from Sigma–Aldrich (St. Louis, MO, USA). $FeCl_3.6H_2O$, $FeCl_2.4H_2O$, 3-(trimethoxysilyl)-1-propantiol (TMSPT), 2-amino-5-mercapto-1,3,4-thiadiazole (AMT), 3-mercaptopropionic acid (MPA), 3-aminopropyltriethoxysilane (APTES), tetraethylorthosilicate (TEOS) and other used chemicals were supplied by Merck (Darmstadt, Germany). Phosphate buffered saline (PBS, pH=7.4) was prepared by dissolving 0.20 g KCl, 0.20 g KH_2PO_4, 1.16 g Na_2HPO_4 and 8.00 g

NaCl in 1L water. Immunoaffinity columns for purification of AFs by official standard method were used from R-Biopharm Rhone (Glasgow, Scotland). As safety notes, all used laboratory glassware were treated with an aqueous solution of sodium hypochlorite (5%) before the discarding to minimize health risks due to AFs contamination.

2.2. Instrumentation

The HPLC system used for AFs determination was a Waters HPLC system equipped with a Waters 600 pump/controller, Waters 717 autosampler, Waters 474 fluorescence detector and a bromination cell for post column derivatisation. AFB_1 and AFG_1 suffer a significant fluorescence quenching in the reversed phase solvent systems. Bromination of these AFs converts the weakly fluorescent AFB_1 and AFG_1 into their highly fluorescent bromine derivatives. Schematic illustration of this reaction is shown in Fig. S1 (Electronic Supplementary information; ESI). The chromatographic separation was performed on a Waters C18 column (150 × 4.6mm, 5 μm particle size) using a H_2O/MeCN/MeOH (6:2:2, v/v/v) mobile phase at a flow-rate of 1.0 mL min^{-1} (for each 1L mobile phase 120 mg of potassium bromide and 350 μL of 4 mol L^{-1} nitric acid were added). The detection wavelengths were fixed at 360 nm and 440 nm for the excitation and emission, respectively. The modified magnetic nanoparticles were characterized by an H-800 transmission electron microscope (TEM) (Hitachi, Japan), APD2000 x-ray diffractometer (XRD) (Italstructures, Italy) and FT-IR spectrometer (Perkin Elmer, spectrum version 10.01.00, USA). A permanent magnet of Nd-Fe-B (100 mm×50 mm×40 mm, Model N48, China) was used for magnetic separation. Ultrasonic bath (Uc-150 Sturdy Industrial CO LTD, Taiwan) was used in modification step. A pH-meter (Corning, Model 140, Switzerland) with a double junction glass electrode was used to check the pH of the solutions.

2.3. Synthesis of adsorbent

The magnetic nanoparticles (MNPs) were prepared via improved chemical co-precipitation method and then modified according to the procedure described in Ref.[33] A schematic illustration of the preparation procedure is shown in Fig. 2. $FeCl_3 \cdot 6H_2O$ (11.68 g) and $FeCl_2 \cdot 4H_2O$ (4.30 g) were dissolved in 200 mL deionized water under nitrogen atmosphere with vigorous stirring at 85 °C. Then, 20 mL of 30% aqueous ammonia solution was added to the solution. The color of the bulk solution changed from orange to black immediately. The magnetic precipitates were washed twice with deionized water and once with 0.02 mol L^{-1} sodium chloride solution. Then, 20 mL of prepared magnetic suspension was placed in a 250 mL round-bottom flask and allowed to settle. The supernatant was removed and coating of MNPs with 3-(trimethoxysilyl)-1-propanthiol (TMSPT) was carried with the addition of an aqueous solution of TMSPT (10%, v/v, 80 mL), followed by glycerol (60 mL). The mixture was then stirred and heated at 85 °C for 2 h under a nitrogen atmosphere. After cooling to room temperature, the suspension was washed sequentially with deionized water (200 mL, three time), methanol (100 mL, three time), and deionized water (200 mL, five time). In the next step, the supernatant was removed and the TMSPT-MNPs suspension was homogeneously dispersed into 150 mL of 1.0 % aqueous solution of AMT. The solution was transferred to a 500 mL beaker and ultrasonicated for 2 h. After that, the resulting modified nanoparticles (AMT-TMSPT-MNPs) were washed three times with deionized water and twice with methanol and then them were dried in vacuum oven at 45 °C for 2 h. The other investigated adsorbents were prepared according to previously published procedures.[34-36]

2.4. Sample pretreatment

Pistachio samples were purchased from a local market. All samples were stored at 4 °C until their analysis. Initially all pistachio samples were weighed. Then 50 g of thoroughly homogenized sample together with 5 g of NaCl were dissolved in 200 mL of methanol: PBS (80:20, v/v) solution 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 separating funnel and the lower aqueous phase was filtered by a filter paper (Whatman, 30 µm) and 20 ml of filtered extract was diluted by 130 ml of PBS solution to obtain 150 mL diluted extract. Finally, the diluted extract was passed through a glassy microfibers filter paper (Whatman, 16 µm) and filtrate was collected for subsequent MSPE.

2.5. Purification step by MSPE procedure

Initially, an aliquot of 50 mL of sample solution was mixed with 150 mg of AMT–TMSPT-MNPs in a 100 mL sample vial. The suspension was stirred for 5 min to facilitate adsorption of the AFs on the surface of MMNPs. Then, the magnetic adsorbent was collected using an external magnet and supernatant solution was decanted. The adsorbed AFs were desorbed from surface of the adsorbent by addition of 2 mL Me_2CO/MeCN/CH_2Cl_2 (1:1:2, v/v/v) mixture and stirred for 3 min. Finally, the magnet was used again to settle the nanoparticles, and the desorbed solution was evaporated under a gentle nitrogen flow. The residue was reconstituted in 300 µL of mobile phase and injected to HPLC for analysis.

3. Results and discussion

The choice of adsorbent is very important for the MSPE process. An ideal adsorbent must have several characteristics. It should have good stability, suitable affinity for compound of interest, high surface area for effective adsorption and can be easily separated from solution in a short

time. MNPs have certain advantages for MSPE, however their selectivity is relatively low, especially for complex matrices. Also, bare MNPs can easily form large aggregates, which may alter their magnetic properties, decrease their surface area and thus decrease their adsorption capacity. Bonding of special functional groups on the surface of MNPs can be used to avoid these disadvantages and cause an increase in selectivity and extraction efficiency for target analytes. On the basis of these considerations, the usefulness of MNPs modified with different functional groups including, carboxylic group (3-mercaptopropionic acid modified tetraethylorthosilicate coated MNPs), amino group (3-aminopropyltriethoxysilane coated MNPs), thiol group (3-(trimethoxysilyl)-1-propantiol coated MNPs) and both amino and thiol groups (2-amino-5-mercapto-1,3,4-thiadiazole modified 3-(trimethoxysilyl)-1-propantiol coated MNPs) were investigated in our preliminary studies (Fig. 3). The best adsorption efficiency was obtained with AMT–TMSPT-MNPs. This adsorbent has both amino and thiol groups which can act as reactive sites for effective electrostatic interactions with carbonyl group of lactone ring in AFs. This type of interaction has also been reported for adsorption of AFB_1 on some clay sorbents such as montmorillonite and smectite.[37,38] Therefore, AMT–TMSPT-MNPs were selected as suitable adsorbent for the further studies.

3.1. Characterization of the adsorbent

To confirm that TMSPT and AMT are bonded to the Fe_3O_4 NPs, the characterization was performed by FT-IR spectroscopy. The FT-IR spectra for MNPs, TMSPT-MNPs and AMT–TMSPT-MNPs are shown in Fig. 4a, 4b and 4c. The broad feature in the range 3441–3220 cm^{-1} is due to O–H stretching vibration, which corresponds to the hydroxyl groups attached by the hydrogen bonds to the iron oxide surface (Fig. 4a). After initial coating step, the characteristic peaks at 1125-1039 cm^{-1} are related to the O–Si stretching vibration, at 2931 and 2886 cm^{-1} are

attributed to –C–H stretching and at 1442 cm^{-1} to the $–CH_2$ bending. The transmittance wave band from 690 to 580 cm^{-1} corresponds to the metal–oxygen bonds (Fig. 4b). When the coated nanoparticles are modified with 2-amino-5-mercapto-1,3,4-thiadiazole (AMT), at the first an increase was observed for the broad feature (3441–3220 cm^{-1}) that related to $–NH_2$ stretching band and two new vibrational bands appear at 1404 cm^{-1} and 1634 cm^{-1} (Fig. 4c). The characteristic peak at 1404 cm^{-1} is attributed to C-N stretching vibration, at 1634 cm^{-1} is assigned to the heterocyclic ring resulted from immobilization of AMT on the surface of TMSPT-MNPs in modification step. Also, Fig. 5a and 5b display the TEM images of TMSPT-MNPs and AMT–TMSPT-MNPs, which illustrate the relatively uniform size distribution of these adsorbents with a mean diameter of approximately 11 $\pm$ 1.2 nm. X-ray diffraction patterns of AMT–TMSPT-MNPs was shown in Fig. 6, representing the reflection patterns at peak position (2θ) of about 30.2, 35.3, 43.2, 57.2, 62.7, and 74.2 which correspond to the reflection planes of 220, 311, 400, 511, 440, and 622, respectively. The position and relative intensity of all diffraction peaks are consistent with the standard pattern of Fe_3O_4 according to the JCPDS card.[39] The average particle size of AMT-TMSPT-MNPs adsorbent according to the Scherrer equation and based on the most intense XRD peak (311-diffraction peak at 2θ=35.3) was found to be 10 nm, which is in good agreement with that obtained by TEM image.

3.2. Optimization of experimental parameters on MSFE

To evaluation the ability of the MMNPs for separation of AFs, the effect of experimental parameters on the performance of microextraction, such as sample pH, amount of adsorbent, adsorption time, desorption time and the type of desorption solvent were investigated by HPLC-FD using one variable at a time. Concentrations of 1.2 µg L^{-1} of AFB_1 and AFG_1 and 0.24 µg L^{-1}

of AFB_2 and AFG_2 were used for optimization studies. The peak area was selected as the extraction efficiency under different experimental conditions.

3.2.1. Effect of pH

The pH of the sample solution plays an important role in the adsorption of AFs by affecting both the form of analytes and the stability of the adsorbent. In strong acidic and alkaline media, the nature of AFs may change due to rupture of the lactonic ring and/or hydrolysis reaction.[40,41] Also the pH of sample can change the favorable nature of the adsorbent surface due to porotonation of –NH and or oxidation of –SH groups. The effect of sample pH on the adsorption of AFs was investigated in the range of 4.0-9.0 (Fig. 7). As Fig. 7 shows, the highest extraction efficiency for AFs was obtained over the pH range 7.0-7.6. AFs are neutral compounds and therefore a neutral environment is necessary to increase the extraction efficiency. Hence, a pH of 7.4 was selected for further studies.

3.2.2. Effect of sample volume

In order to obtain a higher enrichment factor in MSPE procedure, a larger volume of sample solution is required. The effect of sample volume on the AFs extraction was investigated using different sample volumes in the range of 5–150 mL, which were spiked, with 1.2 μg L^{-1} of AFB_1 and AFG_1 and 0.24 μg L^{-1} of AFB_2 and AFG_2.(Fig. S2). The results showed that the extraction efficiency was constant in the range of 5-60 mL and then decreased at higher sample volumes due to the analyte loss from the adsorbent surface. Thus, the volume of 50 mL was selected for subsequent experiments.

3.2.3. The MMNPs amount

Compared to conventional adsorbents (micro-size particles), nanoparticles have higher surface area. Therefore, satisfactory results can be achieved with fewer amounts of nanoparticle adsorbent. To study the effect of adsorbent quantity on the extraction efficiency, different amounts of adsorbent in the range of 10-170 mg were added to the solution. The results showed that the extraction efficiency increased with increasing amounts of adsorbent up to 150 mg and then leveled off (Fig. S3). Therefore, 150 mg of adsorbent was selected for the further experiments.

3.2.4. Effect of adsorption time

To achieve better extraction efficiency with shorter analysis time, it is necessary to select an adsorption time that provides the equilibrium between sample solution and adsorbent. The effect of adsorption time was investigated in the range of 1-10 min (Fig. S4). It was found that an adsorption time of 5 min was sufficient to attain adsorption equilibrium. The high surface area of MNPs along with homogeneous distribution of the nano-sorbent throughout the sample could be the possible reasons for achieving such a fast extraction process.[42] Therefore, the equilibrium state is achieved quickly and adsorption time is very short. This is the most important advantage of this purification method as compared to purification method using IAC in official method, which has a typical extraction times higher than 35 min in purification step.

3.2.5. Desorption conditions

The selection of a suitable desorbing solvent is of great importance for optimization of MSPE process. A good desorbing solvent should effectively elute the adsorbed analytes with the minimum volume and less interfering impurities co-eluted. It also should not damage the nature of the adsorbent surface. On the basis of the above considerations, the usefulness of several

desorbing solvents, including single-solvent systems (MeCN, Me_2CO, MeOH), binary solvent systems (MeCN/CH_2Cl_2 (1:1,v/v), MeOH/CH_2Cl_2 (1:1,v/v) and Me_2CO/CH_2Cl_2 (1:1, v/v)) and ternary solvents systems (Me_2CO/MeOH/CH_2Cl_2 (1:1:1, v/v/v), Me_2CO/MeCN/CH_2Cl_2 (1:1:1,v/v/v) and Me_2CO/MeCN/CH_2Cl_2 (1:1:2,v/v/v)), was investigated in the preliminary experiments (see Fig. 8). Very low AFs desorption was observed in the experiments carried out with single solvent systems. With binary solvent systems, good desorption efficiency was observed for AFB_1 and AFB_2, but low desorption efficiencies were found for AFG_1 and AFG_2. Among the investigated ternary solvent systems, the best desorption efficiencies for all AFs were obtained by mixture of Me_2CO/MeCN/CH_2Cl_2 (1:1:2, v/v/v). Whereas, MNPs surfaces have hydrophilic properties, the use of acetone and acetonitrile could improve the dispersion efficiency of MNPs in dichloromethane, which acts as a hydrophobic solvent. The effect of eluent volume on AFs recovery was further investigated in the range of 1-10 mL (Fig. 5S). The maximum sensitivity was obtained over the range of 2-10 mL. Therefore, 2 mL of Me_2CO/MeCN/CH_2Cl_2 (1:1:2, v/v/v) mixture was selected to ensure complete elution of analytes for further experiments. The effect of desorption time was also studied over the range 1-10 min (Fig. 6S). The experimental results indicated that 3 min of stirring of the desorbing solvent and adsorbent mixture was sufficient and no significant effect was observed when the time of desorption was greater than 3 min.

3.2.6. Effect of reconstituting solvent volume

As mentioned above a mixture of Me2CO/MeCN/CH2Cl2 (1:1:2, v/v/v) was used for effective desorption of AFs from MMNPs. But injection of this mixture solvent to the chromatography column caused an increased base line. In order to avoid this problem, desorbing solvent was evaporated and residual was reconstituted in mobile phase as a suitable solvent for injection. In

order to obtain a higher enhancement factor, a fewer volume of mobile phase is required for reconstituting of the residues of target analytes. The effect of reconstituting solvent volume was studied in the range of 300-5000 µL. The experimental results showed that a volume of 300 µL is enough to obtain best enrichment of AFs. Therefore, 300 µL of mobile phase (H2O/MeCN/MeOH (6:2:2, v/v/v)) was selected as reconstituting solvent for subsequent investigations.

3.2.7. Reusability and capacity of adsorbent

In order to investigate the recycling of the adsorbent under optimized conditions, the adsorbent was rinsed with 3 mL of Me_2CO/MeCN/CH_2Cl_2 (1:1:2, v/v) and then with 5 mL of water before application in the next time. No obvious changes were observed in the recoveries after 10 times. The results of this study indicate that the adsorbent is reusable and stable with no analyte carryover during extraction procedure. Adsorption capacity of adsorbent is investigated by static desorption method. For this purpose 150.0 mg of adsorbent was equilibrated with 50.0 mL of each analyte solution containing various concentrations at optimum conditions. After 10 mine the mixture was filtered and supernatant were analyzed. The results showed that the amount of analyte adsorbed per unit mass of adsorbent was increased linearly with the initial concentration of AFs and then was reached to a plateau value (adsorption capacity value), which represent saturation of the active surface of adsorbent for each analyte. The maximum adsorption capacities of prepared adsorbent for AFB_1, AFG_1, AFB_2 and AFG_2 were found to be 0.172, 0.166, 0.158, and 0.156 mg g^{-1}, respectively.

3.3. Analytical performance of the proposed method

Analytical characteristics of the presented method were evaluated under optimized conditions. Calibration curves were obtained by least-squares linear regression analysis of the peak area

(n=3) versus concentration of each analyte using eight concentration levels. Good linearity was obtained with correlation coefficients ranging from 0.9990 to 0.9998. Limits of detection (LODs) based on signal to noise ratio of 3, ranged from 0.012 to 0.035 μg L^{-1}. The precision of the method was evaluated through investigation of the intra-day precision and inter-day precision. The intra-day precision was evaluated over five replicates spiked at two concentration levels (0.2 and 1.2 μg L^{-1} for AFB_1 and AFG_1 and 0.04, 0.24 μg L^{-1} for AFB_2 and AFG_2) within one day (n=5). The inter-day precision was evaluated over five daily replicates, spiked at same level per work day, over a period of three days (n=15). The intra-day and inter-day precision (RSD %) were in the range 2.3–5.4 % and 2.8–5.9 %, respectively. The obtained values of RSD for presented method are in agreement with the Commission Regulation (EC) No. 401/2006[43] in foodstuffs. The results were listed in Table 1. Also, to investigate the possible matrix effect on the AFs determination in real sample, the limits of matrix-matched detection (MM-LOD, S/N=3) and quantification (MM-LOQ, S/N=10) were evaluated from matrix-matched calibration. Solutions for matrix-matched calibrations were prepared by spiking appropriate amounts of AFs working solutions to the blank pistachio sample and following the clean-up and HPLC-FD procedure. The results (Table 2) indicated that sample matrix cannot significantly affect the AFB_1, AFB_2, AFG_1, and AFG_2 determination. The obtained LODs are lower than the MLs imposed by current EU regulation for foodstuff,[3] suggesting the suitability of the method for the determination of the target analytes in the pistachio. Furthermore, enrichment factor (EF) was calculated by $EF= V_S/V_R \times R\%$ definition (where V_S is the sample volume, V_R is the reconstituting solvent volume, and R% is extraction yield). In this study, by extracting 50 mL of sample solution in 300 μL of reconstituting solvent, enrichment factors of 161 (R=97.2%), 160

(R=96.0%), 158 (R=94.8%) and 157 (R=94.3%) were obtained for AFB_1, AFG_1, AFB_2 and AFG_2, respectively.

3.4. Real sample analysis

To test the applicability of the proposed method in real samples, it was applied to the determination of AFs in pistachio samples. The typical chromatograms of a blank and a spiked pistachio sample under optimized conditions are shown in Fig. 9. It can be seen, there are no interfering peaks in the elution area of the analytes for pistachio matrix which suggesting the good selectivity of the proposed procedure for determination of AFs in pistachio samples. Recovery studies were conducted by spiking AFs to the non-contaminated pistachio samples with different concentrations of AFs. Three replicate samples were studied at each concentration. Results (Table 3 and Table 4) showed that the recovery values were in the range of 92.5-103.2%. Acceptable recoveries demonstrated that the matrix of pistachio sample had no effects on the performance of the presented method. Accuracy of the presented method was checked by the official method based on the IAC-HPLC-FD procedure.[32] The results are presented in Table 5. The statistical analysis of the results using Student t-test showed that there are no significant differences between results by two methods at 95% confidence level. Also, the obtained chromatograms of natural contaminated pistachio samples by the proposed method are shown in Fig. 10. High selectivity, good sensitivity and suitable baseline separation for the four AFs demonstrated that the proposed method is sufficiently applicable for determination of trace amounts of AFs in pistachio samples. A comparison of the analytical feature achieved by the developed method and other methods for AFs (AFB_1, AFB_2, AFG_1 and AFG_2) determination is presented in Table 6. The proposed method has the advantages in term of simplicity, low detection limits, good sensitivity and satisfactory recovery values.

4. Conclusion

In this study, a MSPE procedure followed by HPLC-FD system has been successfully applied for simultaneous determination of AFs (AFB_1, AFB_2, AFG_1 and AFG_2) in pistachio sample. Magnetic Fe_3O_4 NPs modified with TMSPT and AMT was used as effective antibody-free adsorbent for purification of AFs. The magnetic solid phase extraction with MMNPs integrates sample clean up, extraction and pre-concentration at two fast steps (adsorption and desorption steps). Regarding analysis time, the purification by the presented MSPE method requires a shorter time (about 9 min) than the IAC approach (about 35 min). Also, compared to traditional purification method with IACs which are not recyclable the used MMNPs have high stability and suitable reusability. In addition, matrix effects are not present and simple calibration can be carried out in all cases. The result of this study demonstrated that, the new method for purification of AFs in pistachio sample can be considered as a suitable alternative for conventional purification method with IACs. This work is currently in progress to extend the application of the proposed approach to other matrices and mycotoxins such as ochratoxin A, zearalenone and deoxynivalenol.

References

1 N.W. Turner, S. Subrahmanyam and S.A. Piletsky, *Anal. Chim. Acta*. 2009, **632**, 168-180.

2 Internationa Agency for Research on Cancer (IARC), Monograph on the Evaluation of carcinogenic risks in humans, World Health Organization (WHO), Lyon, France, 1993, **vol. 56**, pp. 245-393.

3 European Commission regulation (EC). No.165/2010, Setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins, *Off. J. Eur. Union*, 2010, **L 50**, pp. 8-12.

4 Codex Committee on Food Additives and Contaminants (CX/FAC), 37th Session, Discussion paper on aflatoxins in Brazil nuts, the Hague, the Netherlands, 2005, pp. 25-29.

5 E. Kucukoner and B. Yurt, *Eur. Food Res. Technol*., 2003, **217**, 308-310.

6 M. Aghamohammadi, J. Hashemi, Gh. Asadi Kram and N. Alizadeh, *Anal. Chim. Acta*, 2007, **582**, 288–294.

7 I. Var, B. Kabak and F. Gök, *Food Control*, 2007, **18**, 59-62.

8 B.H. Liu, Y.T. Hsu, Ch.Ch. Lu and F.Y. Yu, *Food Control*, 2013, **30**, 184-189.

9 M.S. Nasir and M.E. Jolley, *J. Agric. Food Chem*, 2002, **50**, 3116-3121.

10 L. Campone, A.L. Piccinelli, R. Celano and L. Rastrelli, *J. Chromatogr. A*, 2011, **1218** 7648-7654.

11 D. Afzali, M. Ghanbarian, A. Mostafavi, T. Shamspur and S. Ghaseminezhad, *J. Chromatogr. A*, 2012, **1247**, 35-41.

12 F. Hepsaga, O. Golgea and B. Kabakb, *Food Control*, 2014, **38**, 75-81.

13 M. Quinto, G. Spadaccino, C. Palermo and D. Centonze, J. Chromatogr. A, 2009, 1216, 8636-8641.

14 M. Rezaee, F. Khalilian, H. A. Mashayekhi and N. Fattahi, Anal. Methods, 2014, DOI: 10.1039/C3AY42244E.

15 M. Vosougha, M. Bayat and A. Salemi, Anal. Chim. Acta, 2010, 663, 11-18.

16 R. Wei, F. Qiu, W. Kong, J. Wei, M. Yang, Z. Luo, J. Qin and X. Ma, *Food Control*. 2013, **32**, 216-221.

17 B. Huang., Zh. Han, Z. Cai, Y. Wu and Y. Ren, *Anal. Chim. Acta*, 2010, **662**, 62-68.

18 R. Romero-Gonzalez, J.L.M. Vidal, M.M. Aguilera-Luiz and A. Garrido Frenich, *J. Agric. Food Chem*. 2009, **57**, 9385-9392.

19 Y. Nonaka, K. Saito, N. Hanioka, S. Narimatsu and H. Kayaoka, *J. Chromatogr. A*. 2009, **1216**, 4416-4422.

20 M. Ventura, A. Gómez, I. Anaya, J. Díaz, F. Broto, M. Agut and L. Comellas, *J. Chromatogr. A*, 2004, **1048,** 25-29.

21 A. Santini, R. Ferracane, G. Meca and A. Ritieni, *Anal. Methods*, 2010, **2**, 884–889.

22 R. Hajian and A.A. Ensafi, *Food Chem*, 2009, **115**, 1034-1037.

23 V.S. Sobolev, *J. Agric. Food Chem*, 2007, **55**, 2136-2141.

24 E. González-Peñas, C. Leache, M. Viscarret, A. Pérez de Obanos, C. Araguás and A. López de Cerain, *J. Chromatogr. A*, 2004, **1025**, 163-168.

25 M. Castegnarol, M. Tozlovanul, Ch. Wild, A. Molini, A. Sylla and A. Pfohl-Leszkowicz, *Mol. Nutr. Food Res*.,2006, 50, 480-487.

26 Y.S. Lin, P.J. Tsai, M.F. Weng and Y.C. Chen, *Anal. Chem*. 2005, **77**, 1753-1760.

27 J.E. Smith, C.D. Medley, Z. Tang, D. Shangguan, C. Lofton and W. Tan, *Anal. Chem.*, 2007, **79**, 3075-3082.

28 S.H. Cha, J.K. Lee, H.S. Jung, S.H. Kim, H.J. Kim, Ch.U. Choi and H.G. Kang, *NSTI-Nanotech*.,2010, **3**, 569-572.

29 Sh. Wu, N. Duan, Ch. Zhu, X. Ma, M. Wang and Zh. Wang, *Biosens. Bioelectron*., 2011, **30**, 35-42.

30 T. Dianping, YU. Yongliang, N. Reinhard, M. Manuel and K. Dietmar, *Analyst*., 2010, **135**, 2661-2667.

31 A. Sharma, Z. Matharu, G. Sumana, P.R. Solanki, C.G. Kim and B.D. Malhotra, *Thin Solid Films*, 2010, **519**, 1213-1218.

32 AOAC Official methods of analysis, Natural Toxins, AOAC International, Gaithersburg, USA, Official Method 999.07, 18rd ed., 2005, pp.32-35.

33 M.H. Mashhadizadeh and Z. Karami, *J. Hazard. Mater*., 2011, **190**, 1023-1029.

34. M.H. Mashhadizadeh and M. Amoli-Diva, *J. Anal. At.Spectrom*., 2013, **23**, 251-258.

35. H. R Shaterian and M. Aghakhanizadeh, *Catal. Sci. Technol*., 2013, **3**, 425-428

36. A. Beiraghi, K. Pourghazi and M. Amoli-Diva, *Anal. Methods*, 2014, **6**, 1418-1428.

37 Q. Desheng, L. Fan, Y. Yanhu and Z. Niya, *Poult. Sci*., 2004, **84**, 959-961.

38 Y. Deng, A.L. Barrientons Velazquez, F. Billes and J.B. Dixon, *Appl. Clay Sci*., 2010, **50**, 92-98.

39 JCPDS card 19-0629, Joint Committee on Powder Diffraction Standards, Swarth-more, 1989.

40 F.K. saalia and R.D. Phillips, *Food Control*, 2010, **21**, 1066-1069.

41 I. Nicolas-Vazquez, A. Mendez-Albores, E. Moreno-Martınez, R. Miranda and M. Castro, *Arch. Environ. Contam. Toxicol*., 2010, **59**, 393-406.

42 H. Bagheri, O. Zandi and A. Aghakhani, *Anal. Chim. Acta*., 2011, **716**, 61-65.

43 European Commission regulation (EC). No. 401/2006, setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins, *Off. J. Eur. Union*, 2006, **L 70** pp. 12-34.

Table 1

Performance data for determination of AFs in standard solution by MSPE-HPLC-FD. HPLC conditions as described in section 2.2

AF	Calibration Equation	Linearity ($\mu g\ L^{-1}$)	R^2	LOD ($\mu g\ L^{-1}$)	intra-day precision (RSD%, n=5)	intra-day precision (RSD%, n=15)
AFB_1	y=186192 x+23.52	0.10-15.0	0.9990	0.032	2.3[a] 3.8[b]	2.8[a] 4.4[b]
AFG_1	y=142370 x+19.82	0.10-15.0	0.9997	0.035	2.4[a] 3.7[b]	2.9[a] 4.3[b]
AFB_2	y=270813 x+12.31	0.04-3.00	0.9998	0.012	3.7[a] 5.4[b]	3.9[a] 5.8[b]
AFG_2	y=176013 x+12.64	0.04-3.00	0.9997	0.013	3.8[a] 5.3[b]	4.1[a] 5.9[b]

[a] For 1.2 $\mu g\ L^{-1}$ of AFB_1 and $AFG_1$and 0.24 $\mu g\ L^{-1}$ of AFB_2 and AFG_2
[b] For 0.2 $\mu g\ L^{-1}$ of AFB_1 and $AFG_1$and 0.04 $\mu g\ L^{-1}$ of AFB_2 and AFG_2

Table 2

The obtained limits of matrix-matched detection (MM-LODs) and quantification (MM-LOQs) by the proposed method.

AF	MM-LOD (μg kg^{-1})	MM-LOQ (μg kg^{-1})
AFB_1	0.034	0.112
AFG_1	0.037	0.115
AFB_2	0.015	0.042
AFG_2	0.014	0.044

Table 3

Determination of AFB_1 and AFG_1 in spiked pistachio samples. HPLC conditions as described in Section 2.2.

Pistachio sample	Spiked ($\mu g\ kg^{-1}$)		Found ($\mu g\ kg^{-1}$) [a]		Recovery (%)		RSD (%)	
	AFB_1	AFG_1	AFB_1	AFG_1	AFB_1	AFG_1	AFB_1	AFG_1
Sample 1	0.000	0.000	ND[b]	ND	—	—	—	—
	1.200	1.200	1.131	1.165	94.2	97.1	2.4	2.2
	5.000	5.000	5.161	4.691	103.2	93.8	1.2	1.4
Sample 2	0.000	0.000	ND	ND	—	—	—	—
	1.200	1.200	1.134	1.123	94.5	93.6	2.4	2.5
	5.000	5.000	4.628	4.763	92.6	95.3	1.8	1.2
Sample 3	0.000	0.000	ND	ND	—	—	—	—
	1.200	1.200	1.161	1.213	96.7	101.2	2.3	2.2
	5.000	5.000	4.661	4.710	93.2	94.2	1.6	1.3
Sample 4	0.000	0.000	ND	ND	—	—	—	—
	1.200	1.200	1.162	1.143	96.8	95.2	2.1	2.3
	5.000	5.000	5.101	4.744	102.2	94.9	1.1	1.5

[a] Mean of three determinations.
[b] ND, not detected

Table. 4

Determination of AFB_2 and AFG_2 in spiked pistachio samples. HPLC conditions as described in Section 2.2.

Pistachio sample	Spiked ($\mu g\ kg^{-1}$)		Found ($\mu g\ kg^{-1}$) [a]		Recovery (%)		RSD (%)	
	AFB_2	AFG_2	AFB_2	AFG_2	AFB_2	AFG_2	AFB_2	AFG_2
Sample 1	0.000	0.000	ND[b]	ND	—	—	—	—
	0.240	0.240	0.225	0.243	93.7	101.2	3.3	3.9
	1.000	1.000	0.961	0.945	96.1	94.4	2.5	2.6
Sample 2	0.000	0.000	ND	ND	—	—	—	—
	0.240	0.240	0.217	0.228	92.5	94.4	3.7	3.5
	1.000	1.000	0.937	0.968	93.7	96.8	2.8	2.4
Sample 3	0.000	0.000	ND	ND	—	—	—	—
	0.240	0.240	0.233	0.231	97.4	96.3	4.2	3.4
	1.000	1.000	1.031	0.981	103.1	98.1	2.3	2.4
Sample 4	0.000	0.000	ND	ND	—	—	—	—
	0.240	0.240	0.229	0.225	95.4	93.8	3.6	3.8
	1.000	1.000	0.967	0.953	96.8	95.3	2.5	2.6

[a] Mean of three determinations.
[b] ND, not detected.

Table 5

Comparison of AFs analyses (mean ± SD, n=3) in contaminated pistachio samples by proposed method and IAC-HPLC-FD method. HPLC conditions as described in Section 2.2.

Sample	AF	MSPE-HPLC-FD	IAC- HPLC-FD
		Found ($\mu g\ kg^{-1}$)	Found ($\mu g\ kg^{-1}$)
Sample 1	AFB_1	1.23± 0.03	1.19± 0.04
	AFG_1	0.98± 0.03	1.01± 0.03
	AFB_2	0.18± 0.01	0.16± 0.01
	AFG_2	0.15± 0.01	0.14± 0.01
Sample 2	AFB_1	0.99± 0.03	1.04± 0.04
	AFG_1	ND [a]	ND
	AFB_2	0.073± 0.003	0.080± 0.004
	AFG_2	ND	ND

[a] ND, not detected.

Table 6

Comparation of diverse methods for the determination of AFB_1, AFB_2, AFG_1 and AFG_2 in real samples.

Method	Matrix	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	Recovery (%)	Reference
TLC	helva	1	—	66.6-86.6	[7]
DLLME-HPLC-FD	Maize flour	0.03-0.17	0.10-0.57	74-92	[10]
IAC-HPLC-FD	Pistachio	0.10-0.11	0.11-0.14	88.7-97.5	[12]
SPME-HPLC-FD	wheat flour	0.035-0.2	0.1-0.63	55-59	[13]
SPE-HPLC-DAD	Pistachio	0.2-3.0	—	73-115	[15]
SPE-UHPLC-MS	Peanuts	0.009-0.212	0.012-0.273	74.7-86.8	[17]
SPE-DLLME-HPLC-FD	Pistachio	0.02-0.04	—	85-93	[14]
MSPE-HPLC-FD	Pistachio	0.014-0.037	0.042-0.115	92.5-103.2	This work

Figure Captions:

Fig. 1. Structure of Aflatoxin B_1, Aflatoxin G_1, Aflatoxin B_2 and Aflatoxin G_2.

Fig. 2. Schematic illustration of the preparation procedure of the ATM-TMST-MNPs adsorbent.

Fig. 3. Effect of the different MMNPs on the extraction efficiency. A) 3-mercaptopropionic acid modified tetraethylorthosilicate coated MNPs, B) 3-aminopropyltriethoxysilane coated MNPs, C) 3-(trimethoxysilyl)-1-propantiol coated MNPs and D) 2-amino-5-mercapto-1,3,4-thiadiazole modified 3-(trimethoxysilyl)-1-propantiol coated MNPs. Conditions: Concentration of AFs, 1.2 μg L^{-1} of AFB_1 and AFG_1 and 0.24 μg L^{-1} of AFB_2 and AFG_2; pH, 7.; sample volume, 30 mL; adsorbent amount, 100 mg; adsorption time, 10 min; desorption time, 10 min; desorption solvent type and volume, 3 ml of $Me_2CO/MeCN/CH_2Cl_2$ (1:1:2); reconstituting solvent volume (mobile phase), 300 μl; HPLC conditions as described in Section 2.2. Error bars represent the standard deviation of the mean recovery for three replicates.

Fig. 4. FT-IR spectra of naked MNPs (a), TMSPT-MNPs (b) and AMT–TMSPT-MNPs (c).

Fig. 5. TEM image of TMSPT-MNPs (a) and AMT–TMSPT-MNPs (b).

Fig. 6 X-ray diffraction pattern of AMT–TMSPT-MNPs

Fig. 7. Effect of pH on the extraction efficiency. Conditions: Concentration of AFs, 1.2 μg L^{-1} of AFB_1 and AFG_1 and 0.24 μg L^{-1} of AFB_2 and AFG_2; sample volume, 30 mL; adsorbent amount, 130 mg; adsorption time, 10 min; desorption time, 10 min; desorption solvent type and volume, 3 ml of $Me_2CO/MeCN/CH_2Cl_2$ (1:1:2); reconstituting solvent volume (mobile phase), 300 μl; HPLC conditions as described in Section 2.2. Error bars represent the standard deviation of the mean recovery for three replicates.

Fig. 8. Effect of desorption solvent type on the extraction efficiency.

A) MeOH, B) MeCN, C) Me_2CO, D) $1MeOH+1CH_2Cl_2$, E) $1MeCN+1CH_2Cl_2$, F) $1Me_2CO+1CH_2Cl_2$, G) $1Me_2CO+1MeOH+2CH_2Cl_2$, H) $1Me_2CO+1MeCN+1CH_2Cl_2$, I) $1Me_2CO+1MeCN+2CH_2Cl_2$. Conditions: Concentration of AFs, 1.2 μg L^{-1} of AFB_1 and AFG_1 and 0.24 μg L^{-1} of AFB_2 and AFG_2; pH, 7.4; sample volume, 50 mL; adsorbent amount, 150 mg; adsorption time, 5 min; desorption time, 5 min; desorption solvent volume, 2 ml; reconstituting solvent volume (mobile phase), 300 μl; HPLC conditions as described in Section 2.2. Error bars represent the standard deviation of the mean recovery for three replicates.

Fig. 9. MSPE-HPLC--FD chromatograms of non-spiked pistachio sample (1) and spiked pistachio sample (2) under optimized experimental conditions. AFs added: 1.2 μg L^{-1} of AFB_1 and AFG_1 and 0.24 μg L^{-1} of AFB_2 and AFG_2. Conditions as described in Fig 8.

Fig. 10. MSPE-HPLC--FD chromatograms of contaminated pistachio samples. sample 1 (a) and sample 2 (b) under optimized experimental conditions. Conditions as described in Fig 8.

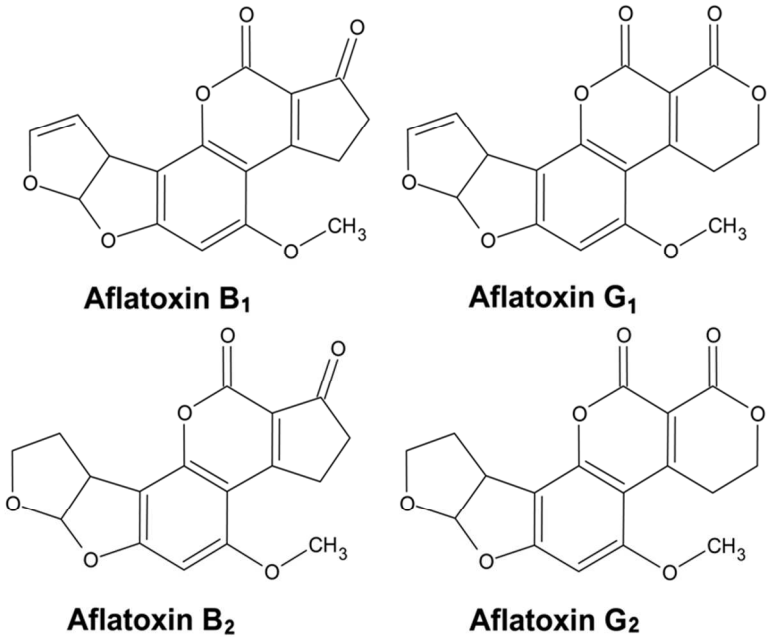


Fig. 1

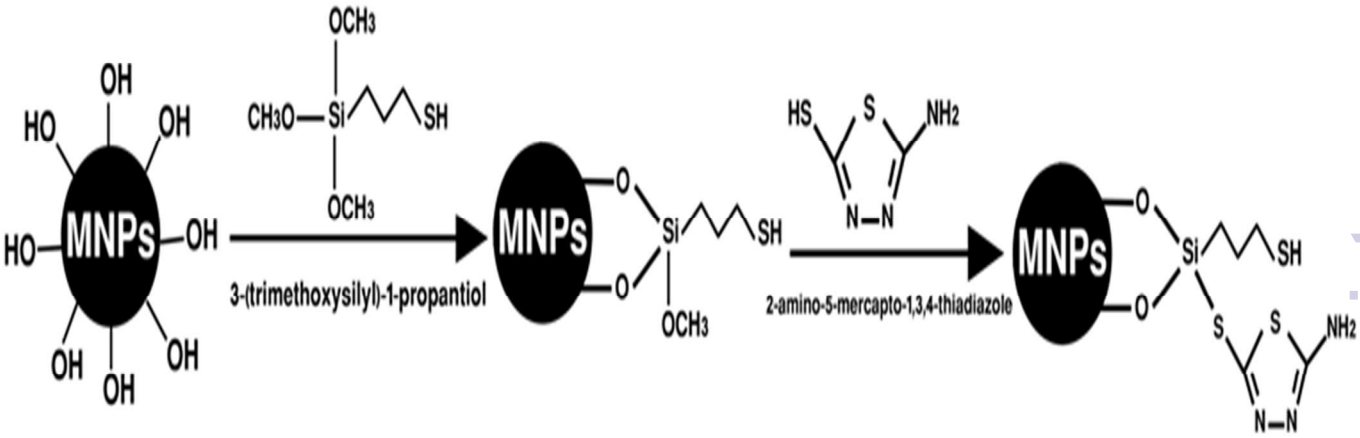


Fig. 2

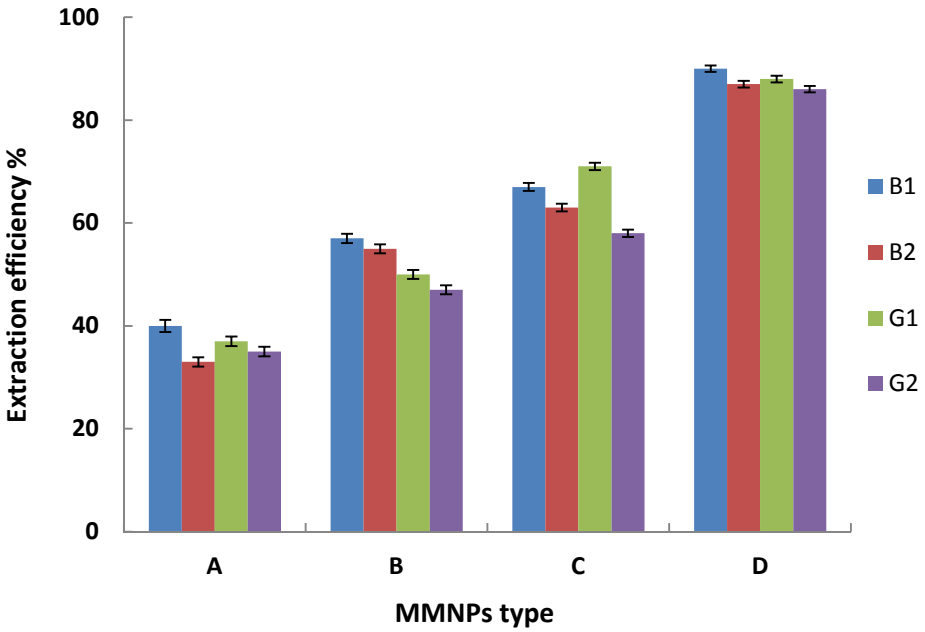


Fig. 3

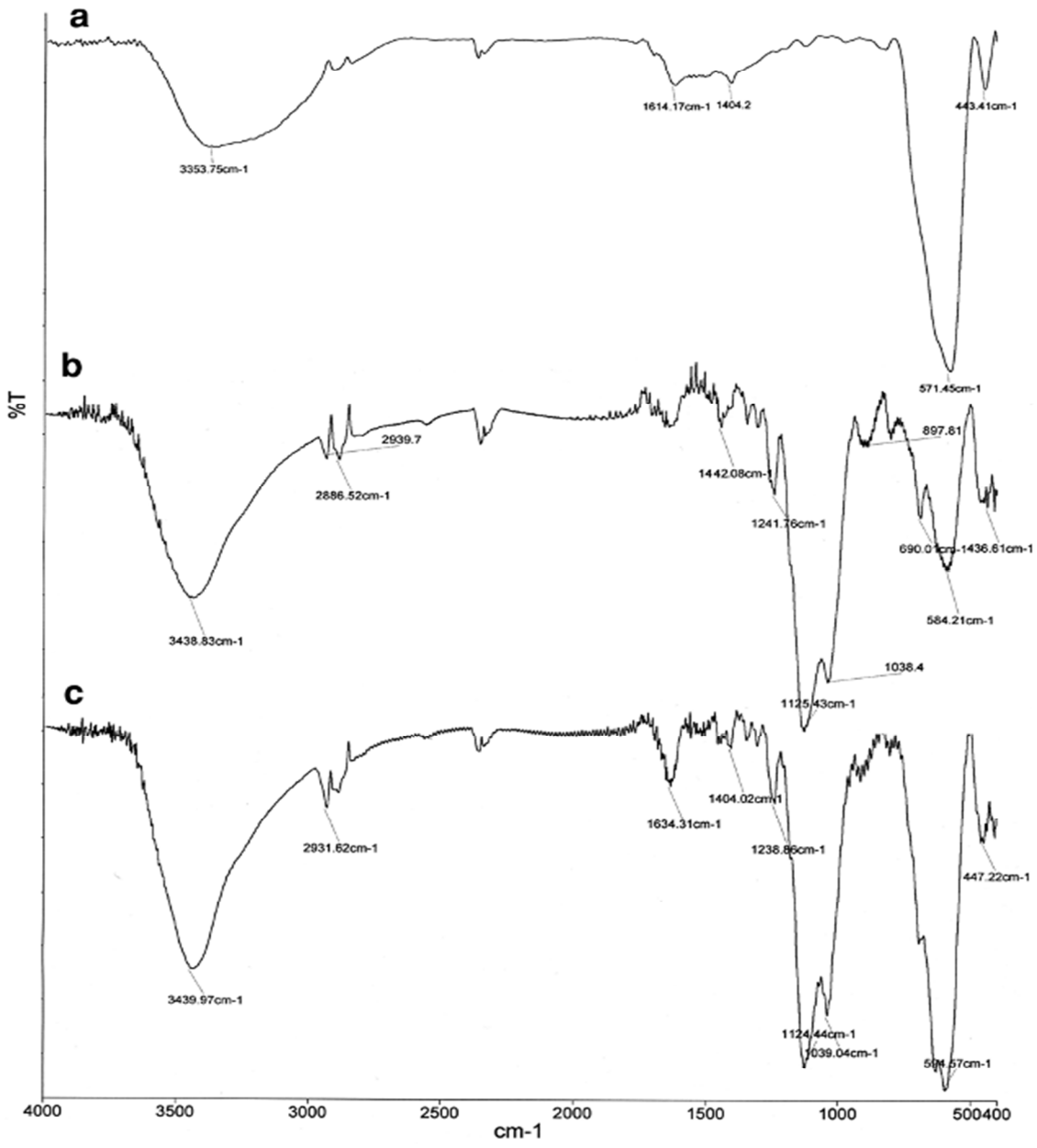


Fig.4

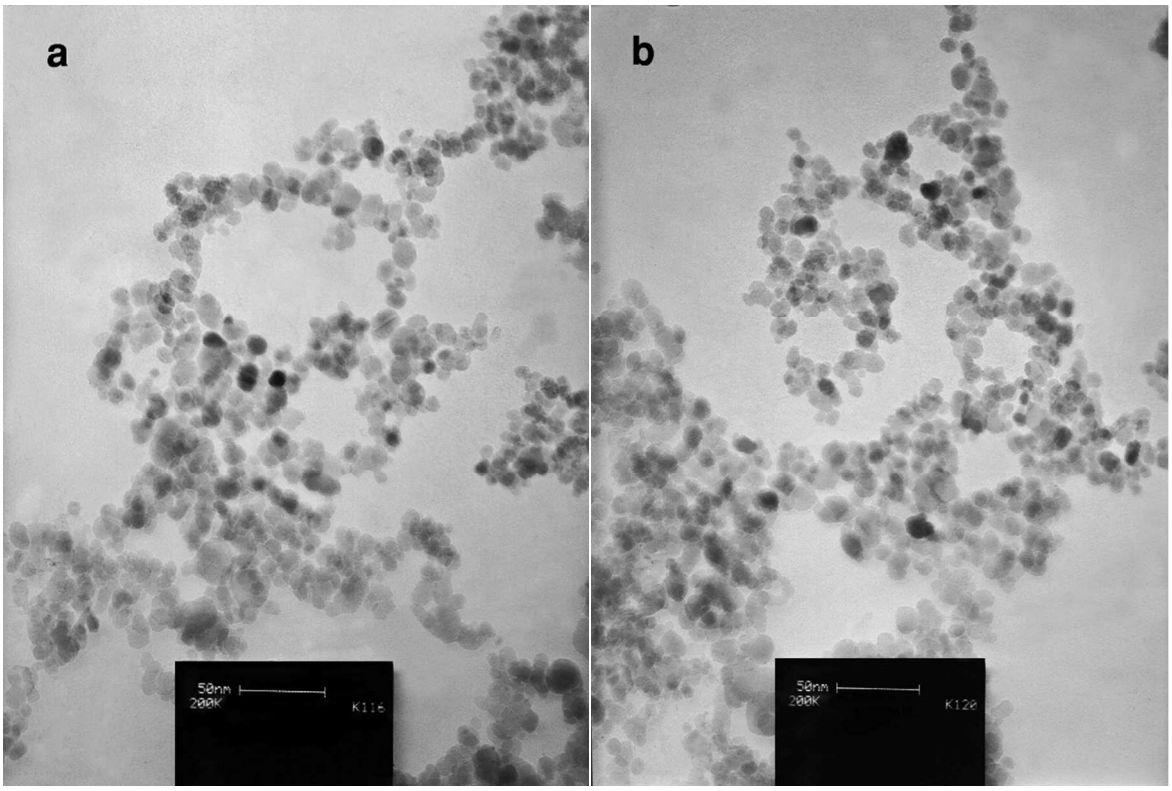


Fig. 5

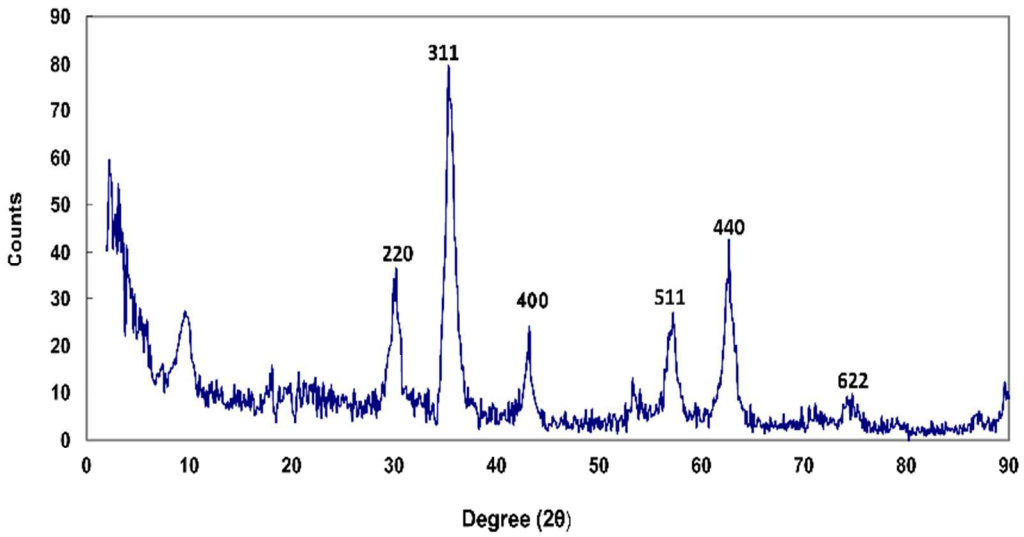


Fig. 6

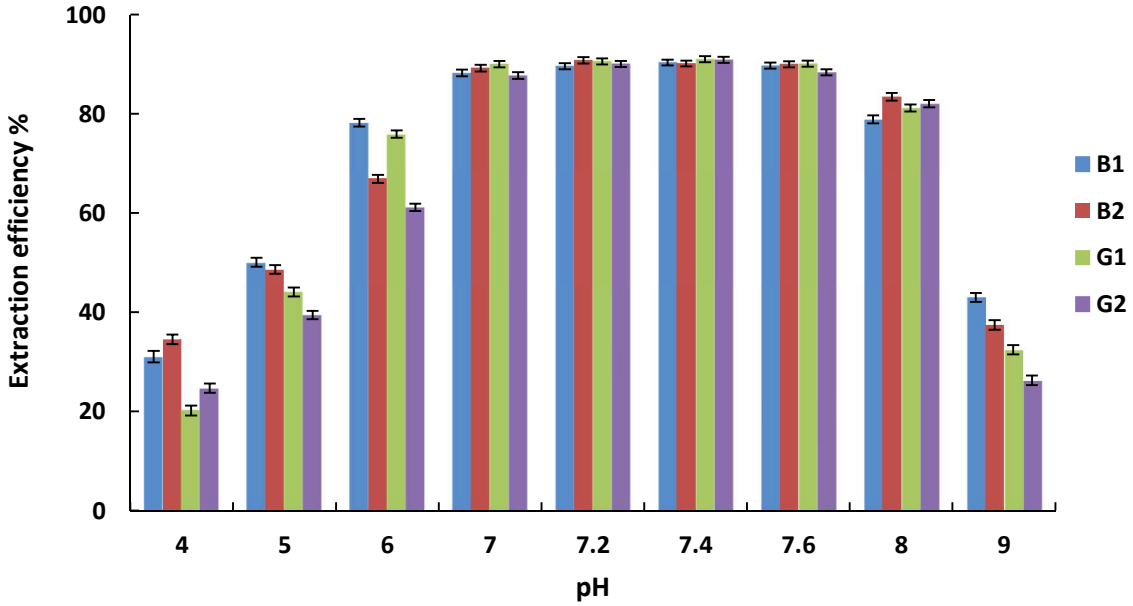


Fig.7

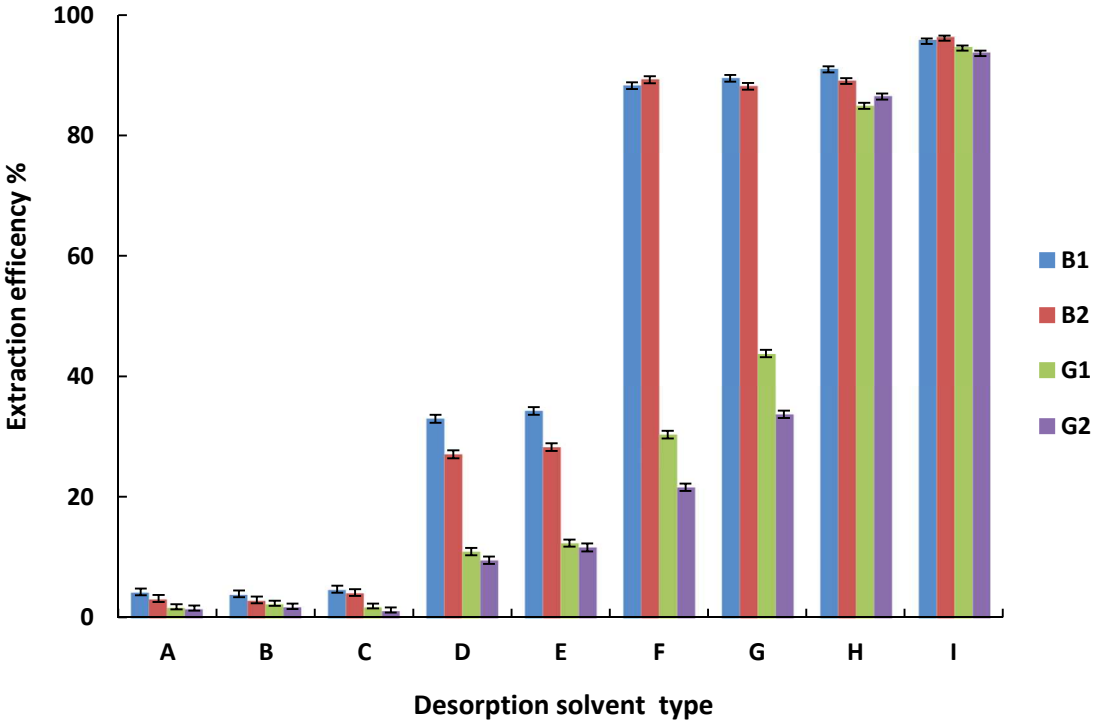


Fig. 8

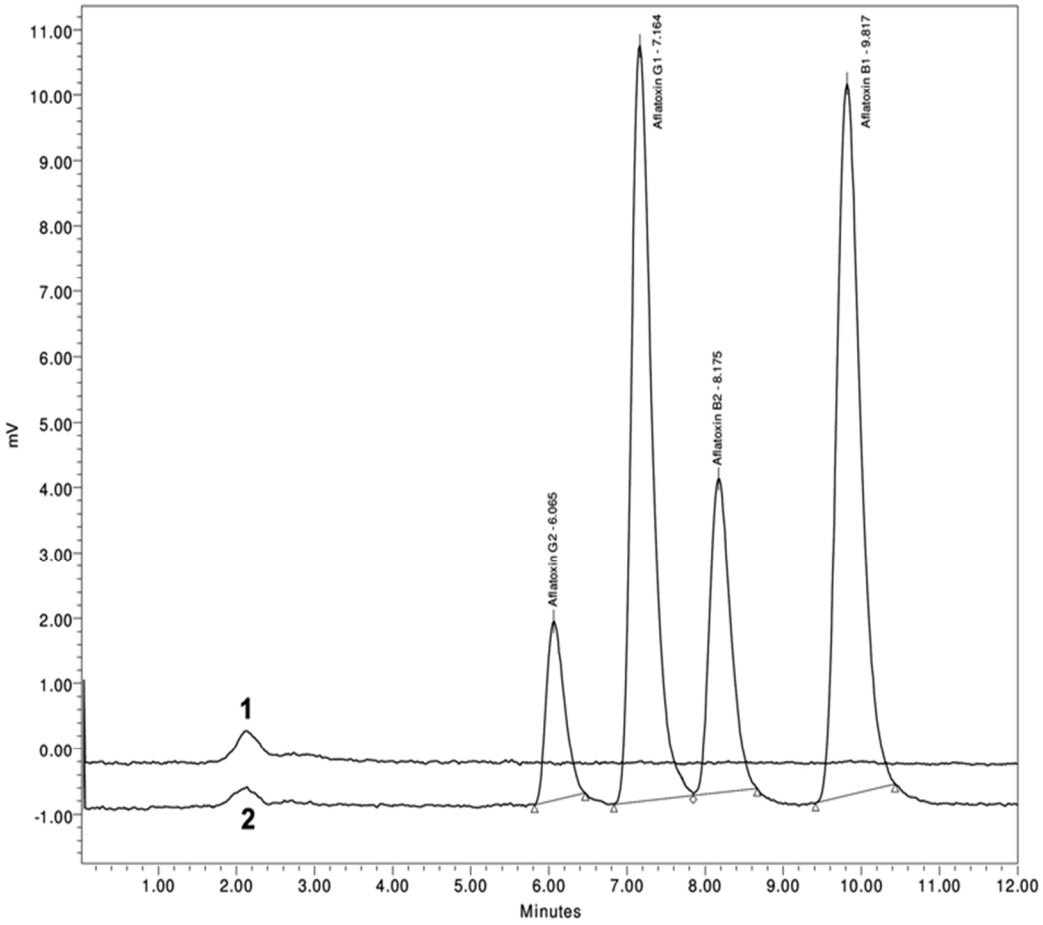


Fig. 9

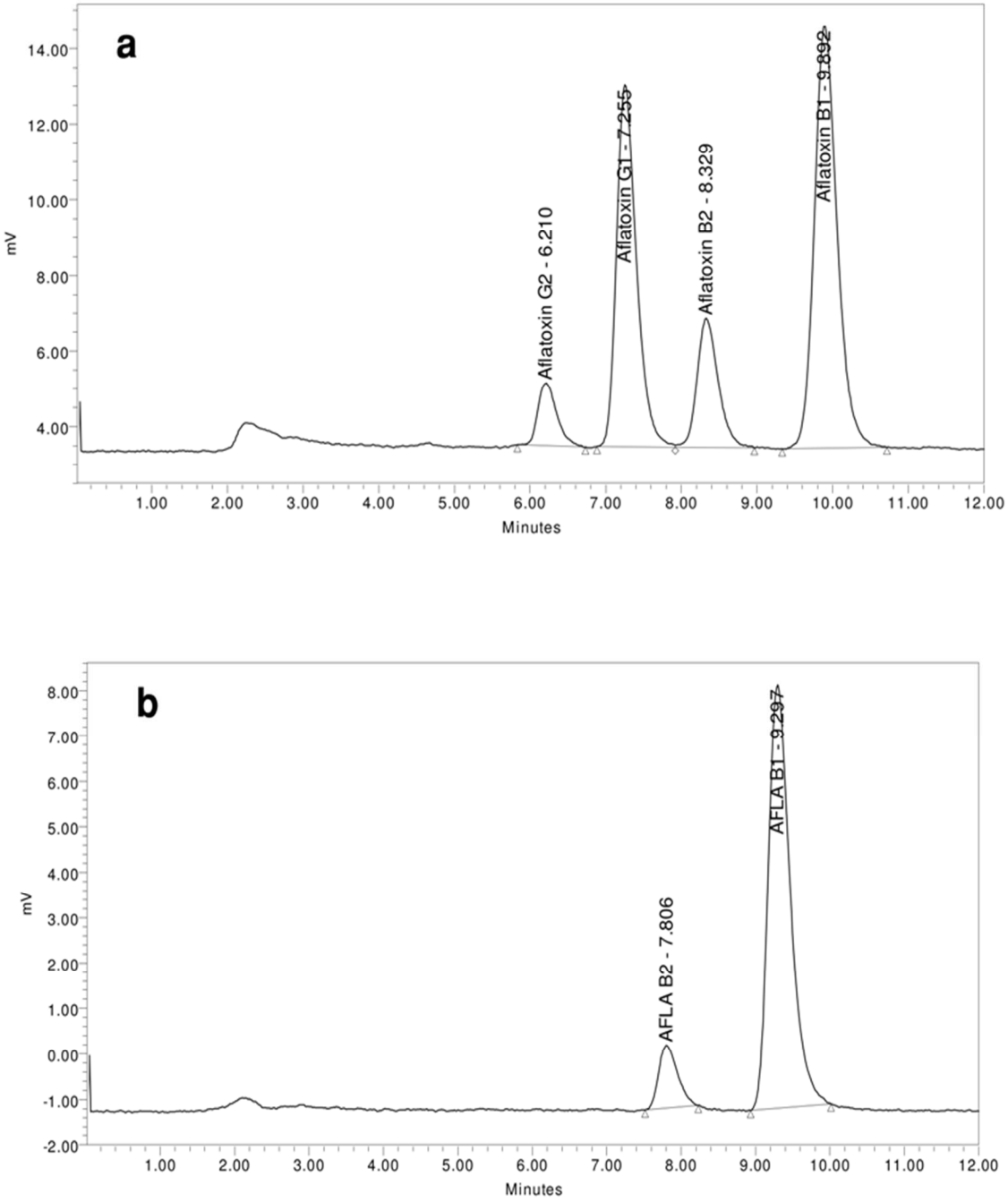


Fig. 10