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Determination of aflatoxin M_1 in liquid milk using high performance liquid chromatography with fluorescence detection after magnetic solid phase extraction

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Abstract

A new and sensitive method based on the magnetic solid phase extraction (MSPE) with antibody free modified magnetic nanoparticles (MMNPs) followed by high performance liquid chromatography with post-column derivatization and fluorescence detection (HPLC-PCD-FD) has been developed for separation and determination of aflatoxin M_1 (AFM_1) in liquid milk. Magnetic nanoparticle coated with 3-(trimethoxysilyl)-1-propanthiol (TMSPT) and modified with ethylene glycol bis-mercaptoacetate (EGBMA) was used as adsorbent. Usefulness of MMNPs has been validated as antibody free clean-up 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 optimum conditions the calibration curve for AFM_1 determination showed good linearity in the range of 0.015–10.0 μg L^{-1} (R^2 =0.9998) and the limit of detection (S/N=3) was estimated to be 0.005 μg L^{-1}. The intra-day and inter-day precision (RSD %) of AFM_1 were in the range of 3.1–5.1 %. The good spiked recoveries ranging from 91.2 to 102.2 % were obtained. The main advantages of developed method are simple, rapid, inexpensive and accurate, also the results are compared with official method based on the conventional immunoaffinity columns (IAC).

Keywords: Aflatoxin M_1, Antibody free modified magnetic nanoparticles (MMNPs), Magnetic solid phase extraction (MSPE), HPLC-PCD-FD

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1. Introduction

Aflatoxins (AFs) are toxic compounds which are produced as secondary metabolites by the fungi *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius.* AFs frequently contaminate a wide range of foods and feedstuffs. Among different AFs, the most toxic and diffuse is the aflatoxin B_1 (AFB_1), which has been classified by International Agency of Research on Cancer as a group I human carcinogens.[1] When AFB_1 is ingested by the mammals through contaminated feed, it is converted into its monohydroxylated metabolites which have been designated as AFM_1.[2] The molecular structure of AFM_1 is showed in Fig. 1a. About 0.5-5% of AFB_1 in animal feeding is converted to AFM_1 in milk.[3] AFM_1 has exhibited toxic and carcinogenic effects [4] because of its possible accumulation and linkage to DNA. The toxicity of AFM_1 was initially classified as a Group 2B agent, but it has now moved to Group 1 by IARC.[1] European Community legislation limits the concentration of AFM_1 for milk and processed milk products intended for adults at 0.050 μg kg^{-1} and for milk intended for infants or baby-food production at 0.025 μg kg^{-1}.[5,6] AFM_1 is relatively stable during pasteurization, sterilization and storage of milk and milk-based products and AFM_1 intake, even at low concentrations, poses a significant threat to human health, especially to children who are the major consumers of milk.[7] Therefore, it is important to devise accurate, specific and sensitive methods for determining AFM_1 in milk. Various analytical methods were applied for AFM_1 analyses such as TLC,[8] ELISA,[9] LC-MS,[7,10] HPLC-FD,[11-15] UHPLC-MS-MS,[16] fluorimetriy,[17,18] and electrochemical methods.[19-21] Among different analytical methods, HPLC-FD technique is the most widespread analytical method for quantitative detection of AFM_1 due to its good sensitivity and suitable selectivity. Since the matrices of dairy product is complex and concentration of AFM_1 is very low for sensitive determination of AFM_1 in real samples a pretreatment step is necessary for sample enrichment and clean-up. Immunoaffinity column (IAC), C18, Carbograph-4 and multifunctional clean-up column

were reported to have preferable purification effect for AFM_1 clean-up in different dairy products.[7,11-15,22] Although SPE with antibody based IACs is the most common clean-up method for AFM_1 which allow a highly selective separation of analyte from a complex matrix. However, they have some important disadvantage such as time consuming, not recyclable, relatively expensive, tedious and limited storage time.[23,24] Therefore, the development of new, fast and less costly extraction and purification methods is necessary for the analysis of AFM_1 in real samples. A new SPE technique based on the use of magnetic nanoparticles, called magnetic solid phase extraction (MSPE) has been introduced for separation and preconcentration of organic and inorganic species from complex matrixes. In MSPE, the magnetic nano-sorbents are dispersed into the sample solution and phase separation can be conveniently carried out by applying an external magnetic field outside the sample solution. Therefore, the time-consuming column passing or filtration operations encountered in SPE are avoided. Among the magnetic nanosized materials, iron oxides have been extensively used as adsorbent in MSPE because of their super paramagnetism, high magnetic saturation, low toxicity, simple preparation process and low price. The stability and selectivity of the MNPs can significantly improved by the modification of the surface of adsorbent with special functional groups. The main aim of this study is to investigate the applicability of the MSPE by MNPs coated with 3-(trimethoxysilyl)-1-propanthiol (TMSPT) and modified ethylene glycol bis-mercaptoacetate (EGBMA) for extraction and determination of AFM_1 in liquid milk by HPLC–PC-FD. To the best of our knowledge, this is the first time that magnetic solid phase extraction with antibody free MMNPS followed by HPLC–PC-FD has been applied for the separation and determination of AFM_1 in liquid milk samples. All the experimental parameters affecting the extraction procedure were intensively investigated and analytical characteristics of the method were evaluated and compared with official method (IAC-HPLC-FD).[25] The results of this study show that MSFE-HPLC–FD

method can be considered as a suitable method for quantitative analysis of AFM_1 in liquid milk samples.

2. Experimental

2.1. Standards and materials

The standard solution of AFM_1 (500 µg L^{-1} in acetonitrile), 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). $FeCl_3.6H_2O$, $FeCl_2.4H_2O$, TMSPT, EGBMA and other used chemicals were supplied by Merck (Darmstadt, Germany). Immunoaffinity columns for clean-up of AFs by official standard method were used from R-Biopharm Rhone (Glasgow, Scotland). 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. 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 AFM_1 contamination.

2.2. Instrumentation

The HPLC system used for AFM_1 determination was a Waters HPLC system equipped with a Waters 600 pump/controller, Waters 717 autosampler, Waters temperature control module, Waters 474 fluorescence detector and a bromation cell for post column derivatisation. 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 selected at 360 nm and 440 nm for excitation and emission, respectively. The modified magnetic nanoparticles were characterized by an S-4160 scanning electron microscope (SEM) (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. An Eppendorf 5810 centrifuge was used for centrifugation. 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 modified magnetic nanoparticles

The magnetic nanoparticles (MNPs) were prepared via improved chemical co-precipitation method and then modified according to the procedure described in Ref.[26] $FeCl_3.6H_2O$ (11.68 g) and $FeCl_2.4H_2O$ (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 was added to the solution. The color of 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 (MSPT) was carried with 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, twice), and deionized water (200 mL, once). Then, the supernatant was removed and the TMSPT-MNPs suspension was homogeneously dispersed into 150 mL of 1.0 % aqueous solution of EGBMA. The mixture was transferred to a 400 mL beaker and sonicated for 2 h. The resulting solid phase (EGBMA-TMSPT-MNPs) was separated by magnetic decantation and washed with deionized water (250 mL, three times)

and methanol (200 mL, two times), before it was dried in vacuum oven at 45 °C for 24 h. Schematic structure of synthesized EGBMA-TMSPT-MNPs is shown in Fig. 1b.

2.4. Sample preparation and clean up step by MSPE procedure

Liquid milk was accurately weighed (10 ± 0.1 g) into 50 mL centrifuge tube and centrifuged (4000 rpm) for 15 min. After centrifugation, fat layer was isolated and supernatant aqueous phase diluted to 40 mL with PBS solution (pH=7.4) in a capped container and shacken intensively. Then, the diluted aqueous phase was transferred to 100 mL vial and 110 mg of EGBMA-TMSPT-MNPs were added to it. The solution was stirred for 5 min to facilitate adsorption of the AFM_1 on MMNPs. Then, the magnetic adsorbent was collected using an external magnet and the supernatant was decanted. The adsorbed AFM_1 were desorbed from surface of adsorbent by addition of 2 mL $Me_2CO/MeCN/CH_2Cl_2$ (1:2:2, v/v/v) mixture and stirring for 3 min. Finally, the magnet was used again to settle the nanoparticles. The desorbing solvent was transferred to 5 mL vial and evaporated to dryness under a gentle nitrogen flow. The residue was reconstituted in 300 µL of mobile phase and injected to HPLC for analysis.

2.5. Clean-up step by official standard method (IAC-HPLC-FD)

Clean-up of the final diluted extract by IAC was conducted in accordance with instruction of AOAC official standard method.[25] The diluted and defatted aqueous phase of milk passed through the IAC column that previously equilibrated with 10 mL of PBS solution, at flow rate of 2–3 drops per second. Then the column was washed with 15 mL of distilled water and subsequently AFM_1 was eluted with 2500 µL of acetonitrile and the eluate was evaporated to dryness 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. Bonding of special functional groups on the surface of MNPs can cause an increase in 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 silica coated MNPs), amino group (3-aminopropyltriethoxysilane modified silica coated MNPs) and thiol group (TMSPT modified silica coated MNPs and EGBMA modified TMSPT coated MNPs) were investigated in our preliminary studies (Fig. 2). Among them, the best adsorption efficiency was obtained with EGBMA-TMSPT-MNPs. As Fig. 1b shows EGBMA-TMSPT-MNPs have two thiol groups and carbonyl groups which could be have electrostatic interactions through S and O atoms of EGBMA and MSPT with carbonyl group of lactone ring and –OH group in furfuran ring of AFM_1. This type of interaction has also been reported for adsorption of AFB_1 which has same structure, on some clay sorbents such as montmorillonite and smectite.[27,28] Therefore, EGBMA-TMSPT-MNPs were selected as suitable adsorbent for the further studies.

3.1. Characterization of the adsorbent

To confirm that TMSPT and EGBMA are bonded to the Fe_3O_4 NPs, the characterization was performed by FT-IR spectroscopy. The FT-IR spectra of TMSPT-MNPs and EGBMA-MSPT-MNPs are shown in Fig. 3a and 3b. The characteristic peak of Fe_3O_4 nanoparticles can be seen in TMSPT-MNPs and EGBMA-TMSPT-MNPs spectra, as a strong absorption band at 584 cm^{-1} and 592 cm^{-1}, respectively that corresponds to the metal–oxygen bond in bulk magnetite. Grafting of a silica network to the surface of MNPs was confirmed by the strong absorption bands at 1125 and 1025 cm^{-1}, which are related to Si-O-H and Si-O-Si stretching

vibration. Moreover absorption peaks at 2939 and 2921 cm^{-1} correspond to the stretching vibrations of CH_2 and CH_3 groups of the alkyl chain. Eventually, a band at 2592 cm^{-1}, which correspond to -SH group confirmed that the surface of MNPs contained thiol groups due to the modification procedure.

Fig. 4a and 4b display the SEM images of TMSPT-MNPs and EGBMA-TMSPT-MNPs, which illustrate the uniform prepared modified nanoparticles have uniform size distribution and the most of the particles are quasi-spherical in shape. The size of EGBMA-TMSPT-MNPs adsorbent was estimated about 35 nm by SEM images.

X-ray diffraction patterns of EGBMA-TMSPT-MNPs was shown in Fig. 4c, representing the reflection patterns at peak position (2θ) of about 30.3, 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.[29] The average particle size of EGBMA-TMSPT-MNPs adsorbent, based on the Scherrer equation, was approximately 11 nm corresponding to line broadening of the 311-diffraction peak, which was observed at 2θ of 35.3. This discrepancy may be due to the presence of aggregates in SEM grain consisting of several crystallites and/or poor crystallinity.[30]

3.2. Optimization of experimental parameters

To evaluate the ability of the MMNPs for separation of AFM_1, the effect of experimental parameters on the performance of MSFE, 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. Concentration of 0.025 μg L^{-1} of AFM_1 was used for optimization studies. The peak area was selected as the extraction efficiency under different experimental conditions and all the results were average of three replicate measurements.

3.2.1 Effect of pH

In MSPE procedure, the pH value of the sample solution plays a critical role in target analyte extraction. The pH of sample can change the nature of the EGBMA-TMSPT-MNPs surface due to oxidation of -SH groups. Also, in strong acidic and alkaline media, the nature of AFM_1 may change due to rupture of the lactonic ring and/or hydrolysis reaction.[31,32] The effect of sample pH was investigated in the range 4.0-9.0 using 110 mg of MMNPs. As Fig. 5a shows, the adsorbent exhibit maximum extraction efficiency of AFM_1 over the pH range of 7.2-7.8. Whereas AFM_1 is a nearly neutral compound, a neutral environment is necessary to increase the extraction efficiency. Thus, the pH of 7.4 was selected for further experiments.

3.2.2. Effect of sample volume

In order to obtain a higher enrichment factor, a larger volume of sample solution is required. The effect of sample volume on the AFM_1 extraction was investigated using different sample volumes in the range of 5–100 mL, which were spiked, with 0.025 μg L^{-1} of AFM_1 (Fig. 5b). It was found that the quantitative recoveries were obtained when the sample volume was less than 50 mL. The extraction efficiency was decreased because at the sample volumes more than 50 mL the analyte loss from the adsorbent surface. Thus, the volume of 40 mL was selected for subsequent experiments.

3.2.3. The MMNPs amount

Compared to conventional micro-size sorbents, MNP*s* sorbents have higher surface areas and satisfactory results can be achieved with fewer amounts of MNP*s*. Thus, to study the effect of adsorbent amount on the extraction efficiency, different amounts of adsorbent in the range of 10-130 mg were added to the analyte solution. The results showed that the extraction efficiency increased with increasing amounts of adsorbent up to 110 mg and then leveled off (Fig .6a). Therefore, 110 mg of adsorbent was found to be the optimum.

3.2.4 Effect of adsorption time

Generally in most equilibrium processes, sufficient contact time is required to achieve the equilibrium between sample solution and adsorbents. For studying the effect of adsorption time on extraction efficiency, adsorption time was investigated in the range of 1-10 min (Fig. 6b). It was found that an adsorption time of 5 min was sufficient to attain adsorption equilibrium. In fact, MNPs provide a large surface area and a short diffusion rout which facilitate mass transfer of analytes under vigorous stirring. This is a superior advantage over the conventional SPE and other microextraction techniques, which usually need more than 30 min to reach the equilibrium.[33]

3.2.5. Desorption conditions

In most of MSPE producers, desorption process is a rather critical step and selection of desorption solvent is very important. A suitable desorption 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 types of desorption solvents was examined. Results are shown in Fig. 7. As can be seen the best result was found with the mixture of Me_2CO/MeCN/CH_2Cl_2 (1:2:2, v/v/v). Whereas surface of MNPs has hydrophilic properties, the use of Me_2CO and MeCN could improve the dispersion efficiency of MNPs in CH_2Cl_2 which acts as a hydrophobic solvent. Also the effect of desorption time was investigated in the range of 1–10 min (Fig. 8a). A duration time of 3 min appeared to be sufficient for complete desorption and no significant effect was observed when the time of desorption was greater than 3 min. The effect of eluent volume on AFM_1 recovery was further investigated in the range of 1-8 mL (Fig. 8b). The maximum sensitivity was obtained over the range 2-8 mL. Therefore, 2 mL of Me_2CO/MeCN/CH_2Cl_2 (1:2:2, v/v/v) was selected to ensure complete elution of analytes for further experiments.

3.2.6. Effect of reconstituting solvent volume

Based on the above results, a mixture of Me_2CO/MeCN/CH_2Cl_2 (1:2:2, v/v/v) was used for effective desorption of AFM_1 from MMNPs. But injection of this mixture solvent to the chromatography column caused an increased base line. In order to avoid this problem, the desorbing solvent was evaporated and the 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 (H_2O/MeCN/MeOH (6:2:2, v/v/v)) was selected as reconstituting solvent for subsequent investigations.

3.2.7. Reusability and stability of adsorbent

Reusability of an adsorbent is a very important key parameter in solid phase extraction procedures. In order to investigate the reusing ability of the adsorbents in several successive adsorption processes, the adsorbent was rinsed with 3 mL of Me_2CO/MeCN/CH_2Cl_2 (1:2:2, v/v) and then with 5 mL of water before application in the next time. The reusing ability of the adsorbent in several successive adsorption processes was investigated. 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.

3.3. Analytical parameters

Analytical characteristics of the presented method were evaluated under optimized conditions. The results were listed in Table 1. Calibration curve was obtained by least-squares linear regression analysis of the peak area (n=5) versus concentration of analyte using ten concentration levels. The calibration curve was linear over the range 0.015–10.00 μg L^{-1}

with regression equation $A=2\times10^{+6}$ C+18.7 (A, peak area and C μg L^{-1} of AFM_1) and correlation coefficient of 0.9998. Limit of detection (LOD) based on signal to noise ratio of 3 was found to be 0.005 μg L^{-1}. The precision of the method was evaluated through of the investigation intra-day precision and inter-day precision as relative standard deviation (RSD %). The intra-day precision was evaluated over five replicates spiked at two concentration levels (0.025 and 0.100 μg L^{-1} of AFM_1) 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). As Table 1 shows the obtained values of RSD for presented method in the range of 3.1–5.1 % are in agreement with the Commission Regulation (EC) No. 401/2006 [34] in foodstuffs. 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. The values of MM-LOD and MM-LOQ were obtained to be 0.006 μg kg^{-1} and 0.017 μg kg^{-1}, respectively. Solutions for matrix-matched calibration were prepared by spiking appropriate amounts of AFM_1 working solutions to the none-contaminated milk sample and following the clean-up and HPLC-FD procedure. The results indicated that sample matrix cannot significantly affect the AFM_1 determination. The obtained LODs were lower than the maximum levels (MLs) imposed by current EU regulation for liquid milk intended for direct human consumption (0.050 μg kg^{-1} of AFM_1).[5] Furthermore, enrichment factor (EF) was calculated by $EF= Vs/V_R \times R\%$ definition (where Vs is the sample volume, V_R is the reconstituting solvent volume, and R% is extraction yield). In this study by extracting 40 mL of sample solution in 300 μL of reconstituting solvent (R=97.2%), the enrichment factor of 129.6 was achieved for AFM_1 determination by the developed method.

3.4. Analysis of real samples

To evaluate the applicability of the proposed method in real samples, it was applied to the determination of AFM_1 in milk samples. The typical chromatograms of the spiked (0.025 μg kg^{-1} of AFM_1) and non-spiked milk 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 milk matrix, suggesting the good selectivity of the proposed procedure for determination of AFM_1 in milk sample. Recovery studies were carried out by spiking the blank milk samples with different amounts of AFM_1. Results (Table 2) showed the recovery values were in the range 91.2 to 102.2 %. Acceptable recoveries demonstrated that the matrix of liquid milk sample had no effects on the performance of the presented method. Accuracy of the developed method for the determination of AFM_1 in contaminated real samples was checked by the AOAC standard official method (IAC-HPLC-FD).[25] The results are presented in Table 3. The statistical analysis of the results using Student's t-test showed that there are no significant differences between results obtained by two methods at 95% confidence level. Also the obtained chromatograms of contaminated milk samples by proposed method are shown in Fig. 10. A comparison of the analytical feature achieved by the proposed method and other methods for AFM_1 determination is presented in Table 4. The presented method has distinct advantages in term of low detection limit, wide linear range, simplicity, good sensitivity and satisfactory recovery values.

4. Conclusions

This recent study describes a new and simple method for determination of AFM_1 in liquid milk using solid phase extraction of AFM_1 on MMNPs followed by HPLC-FD detection system. Magnetic Fe_3O_4 NPs modified with TMSPT and EGBMA was used as effective adsorbent for MSPE. The developed method has many advantages including simplicity, rapidity, low cost, good repeatability and reproducibility, high sensitivity and good recovery.

The solid phase extraction with MMNPs integrates sample clean up, extraction and pre-concentration steps. The clean-up step by presented MSPE requires a shorter time (about 9 min) than the IAC approach (about 35 min). Also, the used adsorbent has high stability, suitable reusability and MSPE with MMNPs offers obvious advantages such as high extraction efficiency, ease of operation, cost-effective and is free from sample carry over interference. The result of this study demonstrated that, the developed method for AFM_1 determination in liquid milk can be considered as a suitable alternative for conventional method based on the IAC clean-up step. Furthermore, matrix effects are not present and simple calibration can be carried out in all cases.

References

1 Internationa Agency for Research on Cancer (IARC), Monographs on the Evaluation of Carcinogenic Risks to Humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, World Health Organization (WHO), Lyon, France, 2002, **vol. 82**, pp. 171-174.

2 J.L. Richard, *Int. J. food microbial*, 2007, **119**, 3-10.

3 M. Carvajal, F. Rojo, I. Mendez and A. Bolanos, *Food Addit. Contam*., 2003, **20**, 1077-1086.

4 F. Galvano, V. Galofaro and G. Galvano*, J. Food Prot*., 1996, **59**, 1079-1090

5 European Commission regulation (EC). No. 466/2001, Setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins, *Off. J. Eur. Union*, 2001, **L 77**, pp. 1-13.

6 European Commission regulation (EC). No. 683/2004, Regards Aflatoxins and Ochratoxin A in Foods for Infants and Young Children., *Off. J. Eur. Union*, 2004, **L 106**, pp. 3-5.

7 Ch. Cavaliere, P. Foglia, E. Pastorini, R. Samperi and A. Lagan, *J. Chromatogr. A*., 2006, **1101**, 69-78.

8 O. Atanda, A. Oguntubo, O. Adejumo, J. Ikeorah and I. Akpan, *Chemospere*, 2007, **68,** 1455-1458.

9 Sh.Ch. Pei, Y.Y. Zhang, S.A. Eremin and W.J. Lee, *Food Control*, 2009, **20**, 1080-1085.

10 L. K. Sørensen and T. H. Elbæk, *J. Chromatogr. B*, 2005, **820**, 183-196.

11 A.C. Manetta, L. Di Giuseppe, M. Giammarco, I. Fusaro, A. Simonella, A. Gramenzi and A. Formigoni, *J. Chromatogr. A*, 2005,**1083**, 219-222.

12 A. Gurbay, S. Aydin, G. Girgin, A.B. Engin and G. Sahin, *Food Control*, 2006, **17**, 1-4.

13 Y. Wang, X. Liu, Ch. Xiao, Zh. Wang, J. Wang, H. Xiao, L. Cui, Q. Xiang and T. Yue, *Food Control*, 2012, **28**, 131-134.

14 V. Siddappa,, D. Kallenahalli Nanjegowda and P. Viswanath, *Food Chem. Toxicol*. 2012**,** **50**, 4158-4162.

15 B. El. Marnissi, R. Belkhou, D. P.Morgavi, L. Bennani and H. Boudra, *Food Chem. Toxicol.*, 2012, **50**, 2819-2821.

16 E. Beltrán, M. Ibáñez, J.V. Sancho, M.Á. Cortés, V. Yusà and F. Hernández*, Food Chem*., 2011, **126**, 737-744.

17 T.J. Hansen, *J. Food Prot*., 1990, **53**, 75-77.

18 C. Cucci, A.G Mignani, R. DalÍAsta, R. Pela and A. Dossena, Sens. Actuators, B, 2007, **126** ,467-472.

19 L. Micheli, R. Grecco, M. Badea, D. Moscone and G. Palleschi, *Biosens. Bioelectron*., 2005, **21**, 588-596.

20 S. Rameil, P. Schubert, P. Grundmann, R. Dietrich and E. Märtlbauer, *Anal. Chim. Acta*, 2010, **661**, 122–127.

21 E. Dinckaya, Ö. Kınık, M.K. Sezgintürk, C. Altuğ and A. Akkoca, *Biosens. Bioelectron*., 2011, **26**, 3806–3811.

22 C-Y. Chen, W-J. Li and K-Y. Peng, *J. Agric. Food. Chem*., 2005, **53**, 8474-8480.

23 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.

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

25 AOAC Official methods of analysis, Natural Toxins, Aflatoxin M_1 in liquid milk, immunoaffinity column by liquid chromatography, AOAC International, Gaithersburg, USA, Official Method 2000.08, 2005, pp.45-47.

26 M.H., Mashhadizadeh, M. Amoli-Diva, M.H Shapouri and H. Afruzi, *Food chem*., 2014, **151**, 300-305.

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

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

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

30 R.N. Panda, M.F. Hsieh, R.J. Chung and T.S. Chin, *J. Phys. Chem. Solids*, 2003, **64**, 193–199.

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

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

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

34 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.

Figure Captions:

Fig. 1. The molecular structure of AFM_1 (a) and schematic structure of synthesized EGBMA-TMSPT-MNPs (b)

Fig. 2. Effect of the different MMNPs on the extraction efficiency. A) 3-mercaptopropionic acid modified silica coated MNPs B) 3-aminopropyltriethoxysilane modified silica coated MNPs C) TMSPT modified silica coated MNPs D) EGBMA modified TMSPT coated MNPs. Conditions: concentration of AFM_1, 0.025 μg L^{-1}; 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 $Me_2CO/MeCN/CH_2Cl_2$ (1:2:2); 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. 3. FT-IR spectra of TMSPT-MNPs (a) and EGBMA-TMSPT-MNPs (b).

Fig. 4. SEM image of TMSPT-MNPs (a) and EGBMA-TMSPT-MNPs (b). X-ray diffraction pattern of EGBMA-TMSPT-MNPs (c).

Fig. 5. Effect of pH (a) and sample volume (b) on the extraction efficiency. Conditions: concentration of AFM_1, 0.025 μg L^{-1}; adsorbent amount, 110 mg; adsorption time, 10 min; desorption time, 10 min; desorption solvent type and volume, 3 ml of $Me_2CO/MeCN/CH_2Cl_2$ (1:2:2); 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. 6. Effect of MMNPs amount (a) and adsorption time (b) on the extraction efficiency. Conditions: concentration of AFM_1, 0.025 μg L^{-1}; pH, 7.4; sample volume, 40 mL; desorption time, 10 min; desorption solvent type and volume, 3 ml of $Me_2CO/MeCN/CH_2Cl_2$ (1:2:2); 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. 7. 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) $1Me_2CO+1MeOH+1CH_2Cl_2$, H) $1Me_2CO+1MeCN+1CH_2Cl_2$, I) $1Me_2CO+2MeCN+1CH_2Cl_2$, J) $1Me_2CO+2MeCN+2CH_2Cl_2$. Conditions: concentration of AFM_1, 0.025 μg L^{-1}; pH, 7.4; sample volume, 40 mL; adsorbent amount, 110 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. 8. Effect of desorption time (a) and desorption solvent volume (b) on the extraction efficiency. Conditions: concentration of AFM_1, 0.025 μg L^{-1}; pH, 7.4; sample volume, 40 mL; adsorbent amount, 110 mg; adsorption time, 5 min; desorption solvent type, $Me_2CO/MeCN/CH_2Cl_2$ (1:2:2); 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. 9. MSPE-HPLC--FD chromatograms of non-spiked (1) and spiked (2) liquid milk sample under optimized experimental conditions: concentration of AFM_1, 0.025 μg kg^{-1} pH, 7.4; sample volume, 40 mL; adsorbent amount, 110 mg; adsorption time, 5 min; desorption time, 3 min; desorption solvent type and volume, 2 ml of $Me_2CO/MeCN/CH_2Cl_2$ (1:2:2); reconstituting solvent volume (mobile phase), 300 μl; HPLC conditions as described in Section 2.2

Fig. 10. MSPE-HPLC--FD chromatograms of contaminated milk sample 1 (a) and sample 2 (b) under optimized experimental conditions. Conditions as described in Fig 9

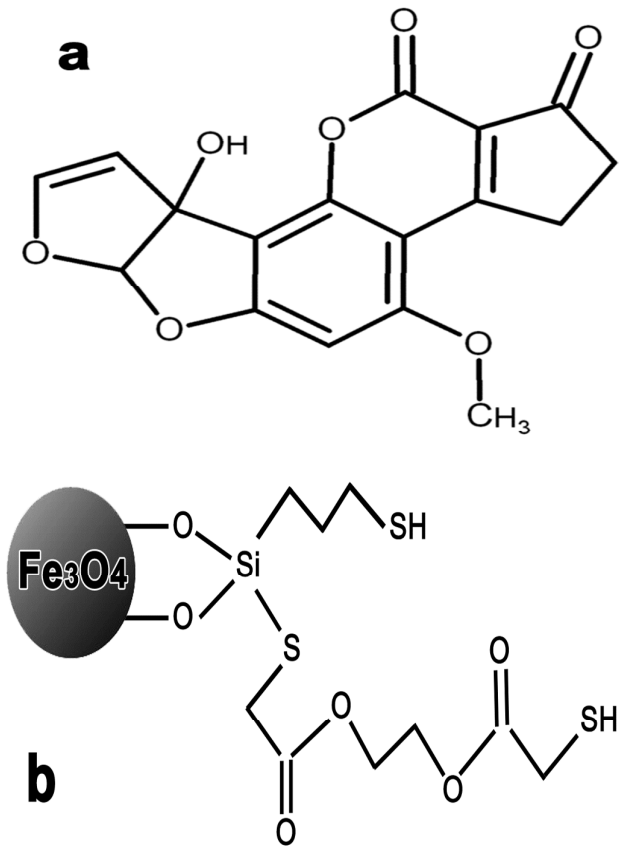

Fig.1

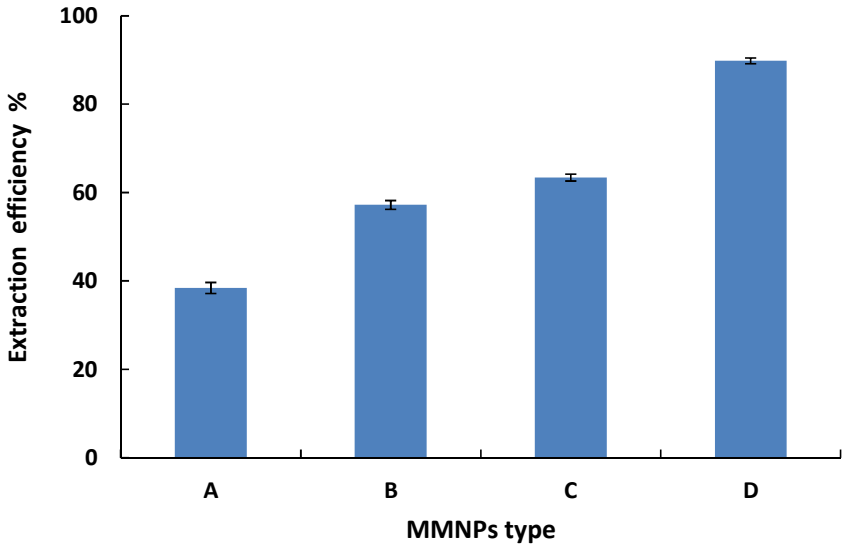

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Fig.2

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Fig.2

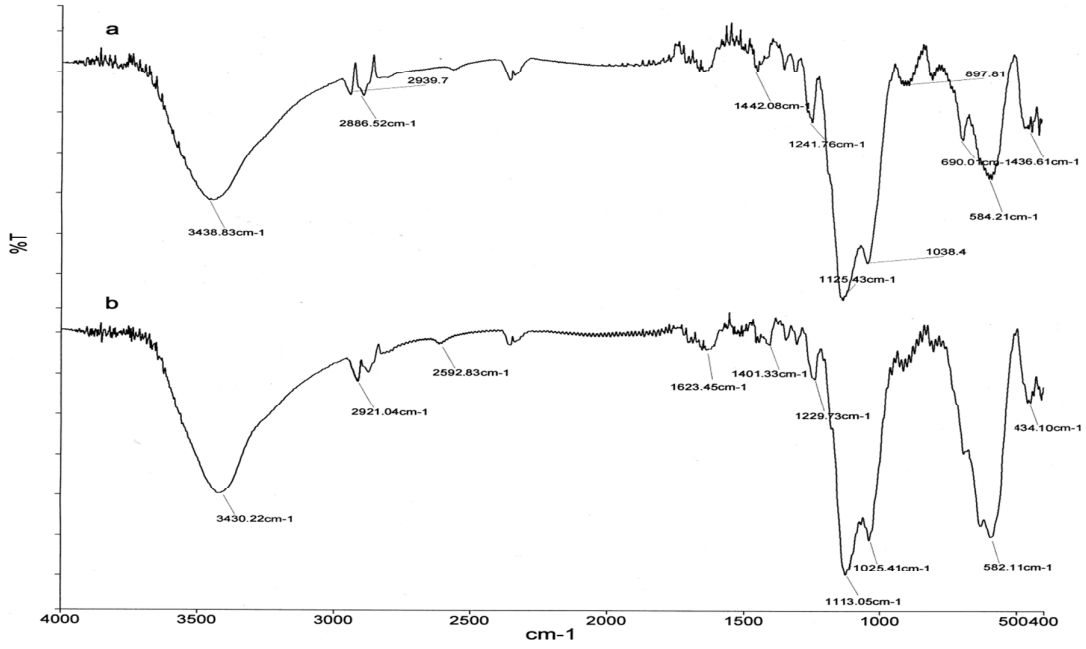


Fig.3

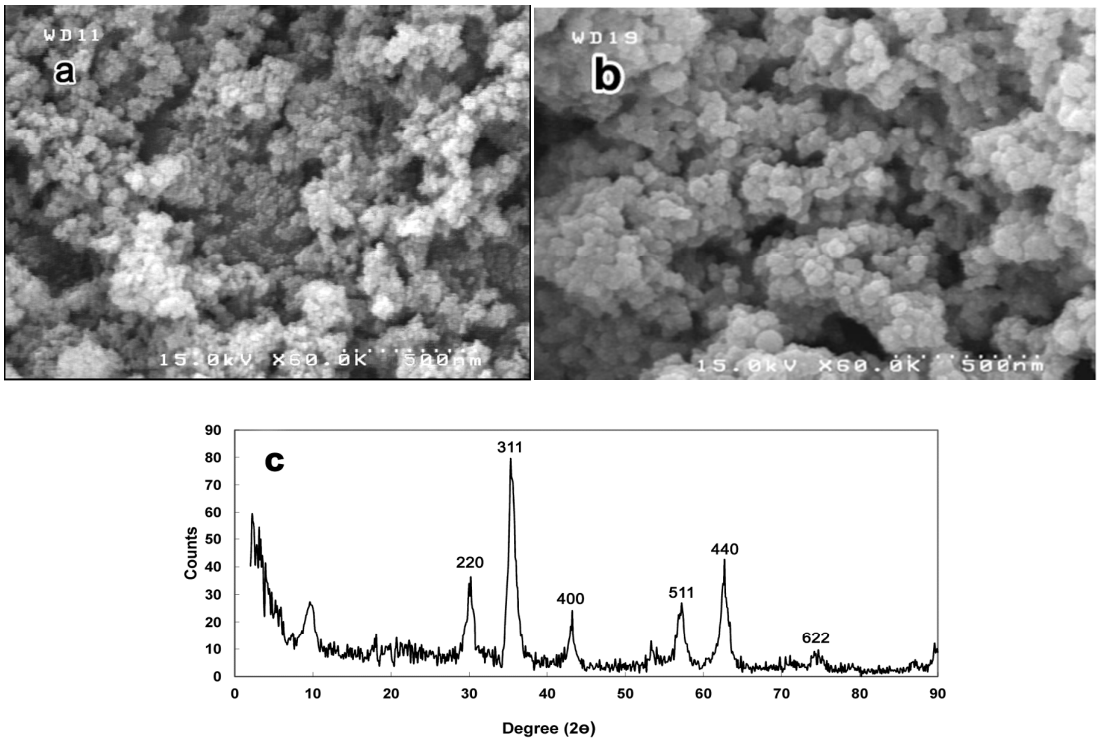


Fig.4

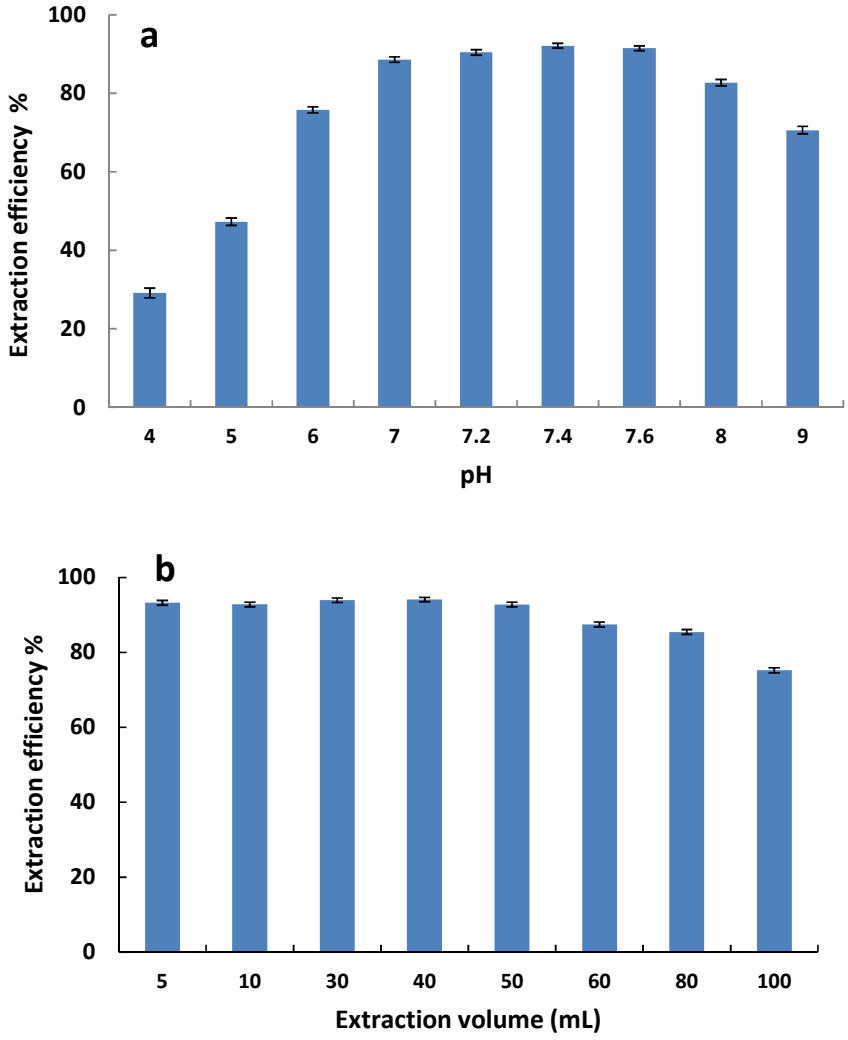
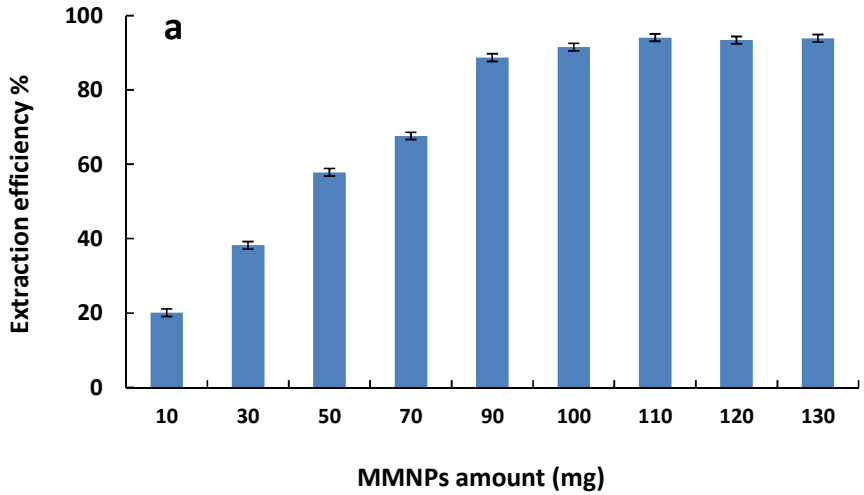


Fig.5



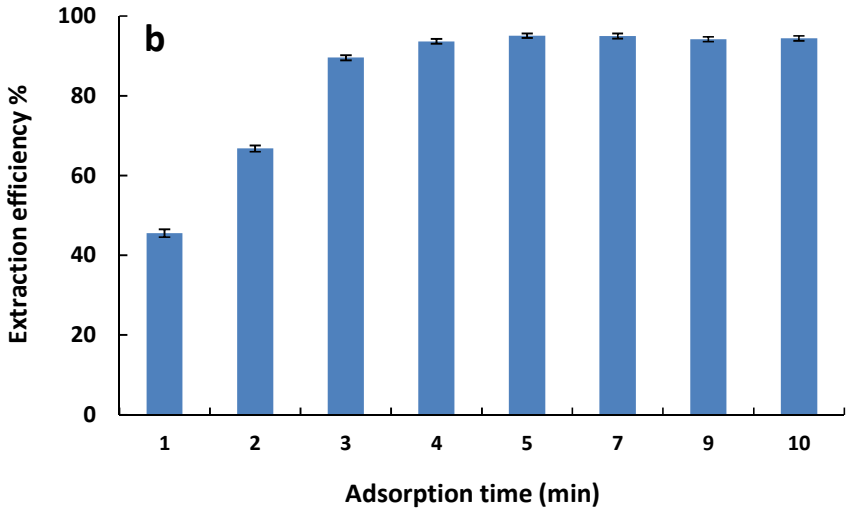


Fig.6

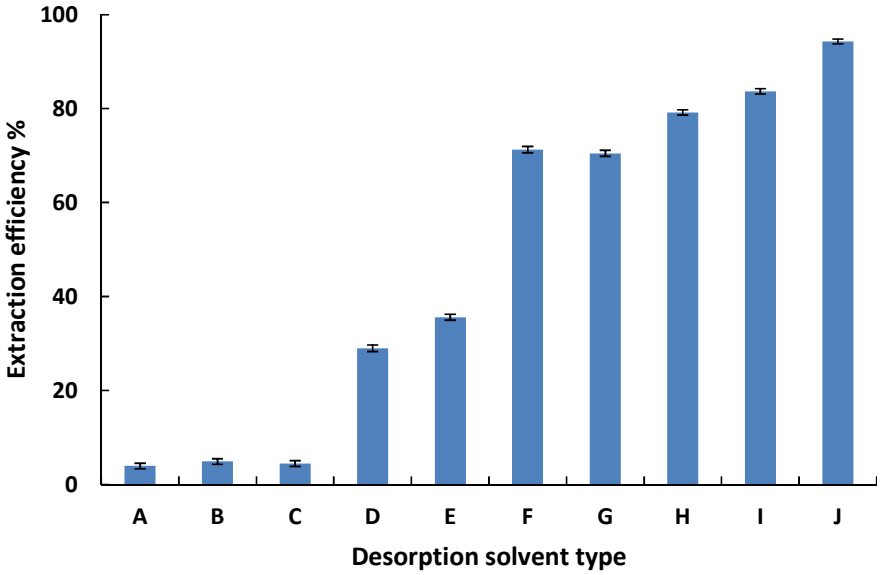


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Fig.7

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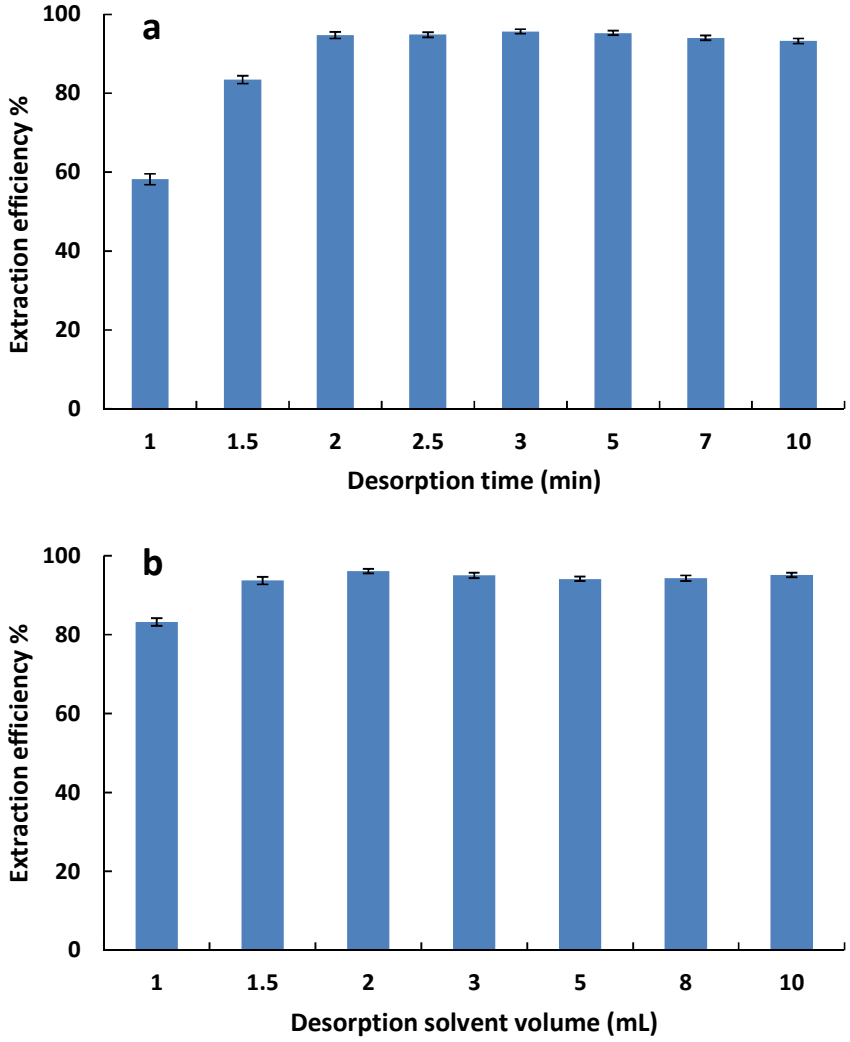

Fig.8

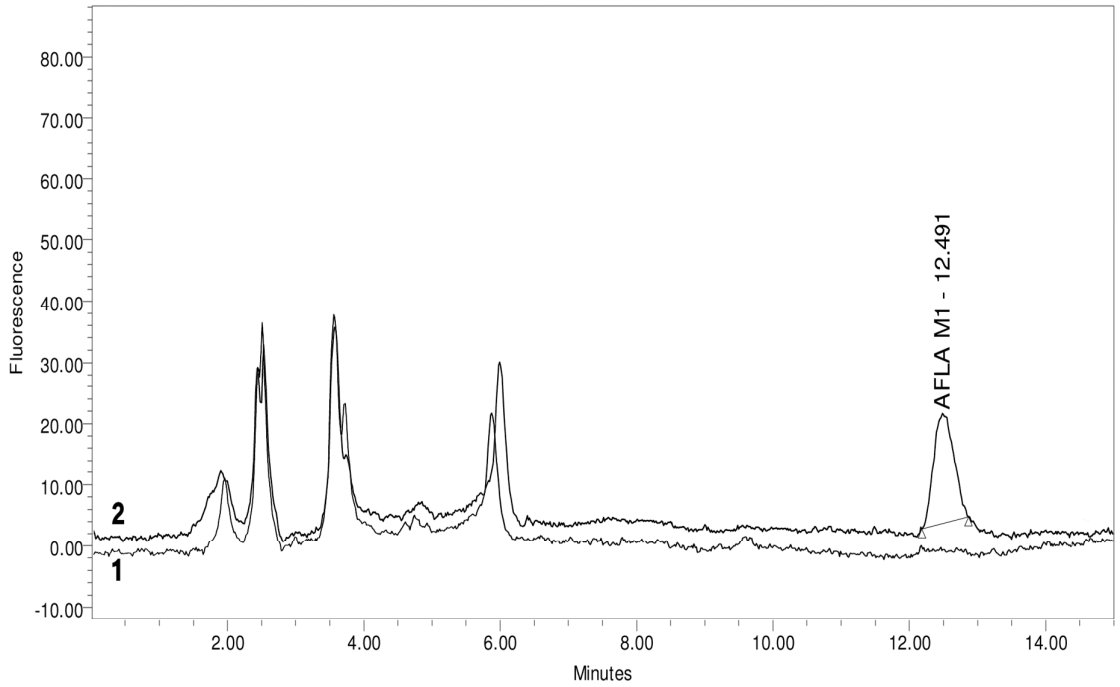

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Fig.9

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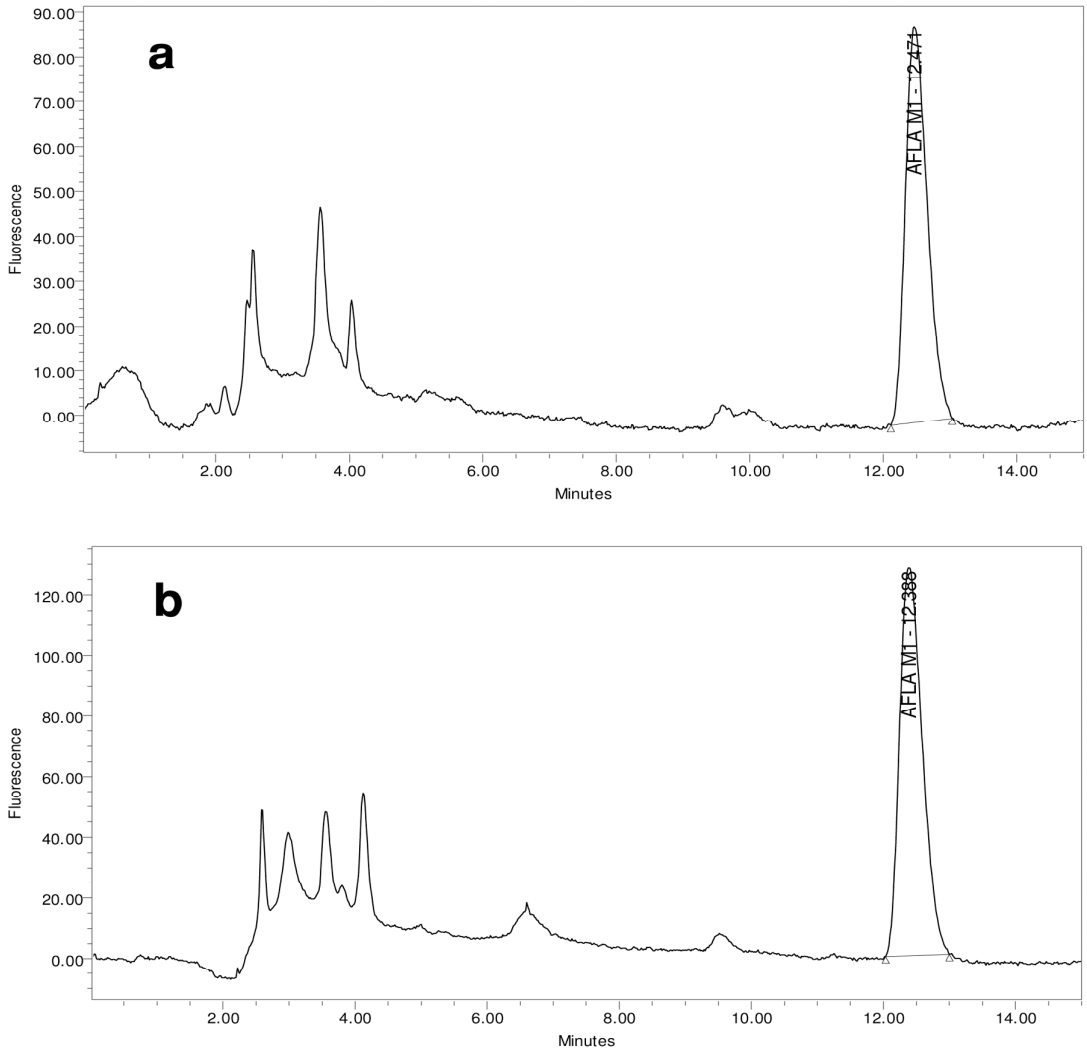


Fig.10

Table 1

The characteristic data of the proposed method.

Parameters	AFM_1
Calibration equation	$A = 2\times10^{+6} C + 18.7$
Dynamic range (μg L^{-1})	0.015 – 10.0
Correlation coefficient (R^2)	0.9998
Intra-day precision (RSD%, n=5)	4.8[a]
	3.1[b]
Inter-day precision (RSD%, n=15)	5.1[a]
	3.6[b]
Limit of detection ($3S_b^C$, μg L^{-1})	0.005

[a] For 0.025 μg L^{-1} of AFM_1

[b] For 0.100 μg L^{-1} of AFM_1

[c] $3S_b$ is defined as three times the standard deviation of the blank

Table 2

Results (mean ± SD based on three replicate analysis, n=3) of determination of AFM_1 by MSPE-HPLC-FD in spiked samples of liquid milk. HPLC conditions as described in Section 2.2

Milk sample	Added (μg kg^{-1})	Found (μg kg^{-1})	Recovery (%)
Sample 1	0.000	ND[a]	—
	0.050	0.051 ± 0.002	101.0
	0.500	0.456 ± 0.011	91.2
	0.750	0.733 ± 0.016	97.7
Sample 2	0.000	ND[a]	—
	0.050	0.046 ± 0.002	92.2
	0.500	0.511 ± 0.012	102.2
	0.750	0.724 ± 0.016	96.5
Sample 3	0.000	ND[a]	—
	0.050	0.047 ± 0.002	95.3
	0.500	0.467 ± 0.011	93.4
	0.750	0.751 ± 0.016	100.1
Sample 4	0.000	ND[a]	—
	0.050	0.048 ± 0.002	96.3
	0.500	0.472 ± 0.011	94.6
	0.750	0.718 ± 0.016	95.8

[a]Nd, not detected

Table 3

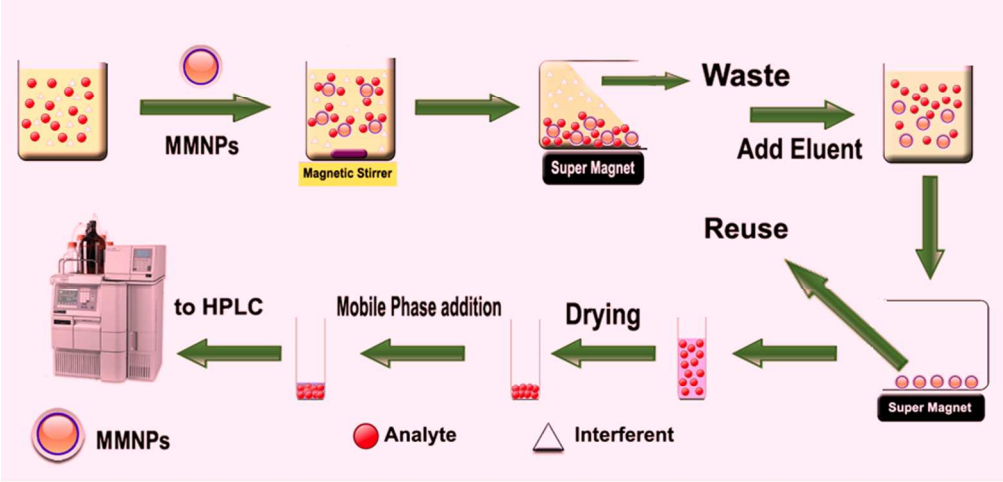
Comparison of AFM_1 analyses (mean ± SD, n=3) in contaminated liquid milk samples by MSPE-HPLC-FD and IAC-HPLC-FD methods. HPLC conditions as described in Section 2.2

Milk sample.	MSPE-HPLC-FD	IAC-HPLC-FD
	AFM_1 ($\mu g\ kg^{-1}$)	AFM_1 ($\mu g\ kg^{-1}$)
Sample 1	0.109 ± 0.003	0.102 ± 0.004
Sample 2	0.209 ± 0.005	0.211 ± 0.006

Table 4

Comparation of diverse methods for the determination of AFM_1.

Method	Linear range (μg L^{-1})	LOD (μg L^{-1})	Recovery (%)	Reference
IAC-Direct fluorimetry	—	0.050	97.0	17
Fluorimetric sensor	0.00-0.125	0.050	—	18
IAC-HPLC-FD	0.010-0.200	0.010	115.6-117.9	12
SPE-LC-MS	0.020-1	0.010	78-108	10
TLC	—	2	84.6-88.0	8
ELISA	0.040-5	0.040	87.9-128.3	9
Amperometric immunosensor	0.030-0.240	0.025	90-101	19
Impedimetric biosensor	1–14	1	107	21
Potentiometric immunosensor	0.125–2	0.040	74-136	20
MSFE-HPLC-FD	0.015-10.0	0.005	91.2-102.2	This work



Graphic Abstract
80x38mm (300 x 300 DPI)