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Application of dispersive liquid–liquid microextraction coupled with vortex-assisted hydrophobic magnetic nanoparticles based solid-phase extraction for determination of aflatoxin M1 in milk samples by sensitive micelle enhanced spectrofluorimetry

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ABSTRACT

An efficient, simple and fast low-density solvent based dispersive liquid–liquid microextraction (LDS-DLLME) followed by vortex-assisted dispersive solid phase extraction (VA-D-SPE) has been developed as a new approach for extraction and preconcentration of aflatoxin M1 in milk samples prior to its micelle enhanced spectrofluorimetic determination. In this LDS-DLLME coupled VA-D-SPE method, milk samples were first treated with methanol/water (80:20, v/v) after removing the fat layer. This solvent was directly used as the dispersing solvent in DLLME along with using 1-heptanol (as a low-density solvent with respect to water) as the extracting solvent. In VA-D-SPE approach, hydrophobic oleic acid modified Fe_3O_4 nanoparticles were used to retrieve the analyte from the DLLME step. It is considerably that the target of VA-D-SPE was 1-heptanol rather than the aflatoxin M1 directly. The main parameters affecting the efficiency of LDS-DLLME and VA-D-SPE procedures and signal enhancement of aflatoxin M1 were investigated and optimized. Under the optimum conditions, the method was linear in the range from 0.02 to 200 $\mu g\ L^{-1}$ with the correlation coefficient (R^2) of 0.9989 and detection limit of 13 $ng\ L^{-1}$. The intra-day precision was 2.9 and 4.3% and the inter-day precision was 2.1 and 3.3% for concentration of 2 and 50 $\mu g\ L^{-1}$ respectively. The developed method was applied for extraction and preconcentration of AFM_1 in three commercially available milk samples and the results were compared with the official AOAC method.

1. Introduction

Aflatoxins (AFs) are toxic secondary metabolites of various fungi growing on a wide range of food and animal feedstuffs [1] such as *Aspergillus flavus* and *Aspergillus parasiticus* and the rare *Aspergillus nomius* [2]. These compounds frequently contaminate cereal crops, such as corn, beans, peanuts, and dried fruit [3] and there are at least 20 different types of them occur naturally. 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]. Aflatoxin M_1 (AFM_1) is the main monohydroxylated derivative of AFB_1 which produces by means of cytochrome P450-associated enzymes in liver and also known as “milk toxin”. Mammals that ingest AFB_1 contaminated diets, excrete AFM_1 into milk in the range of 0.3–6.3% of consumed AFB_1 and subsequently it can be found in a large variety of dairy products [5]. In fact, AFB_1 quickly absorbs from gastro-intestinal track and it appears as AFM_1 in blood after just 15 min [6]. It can be detected in milk in 12–24 h after the first ingestion of AFB_1 [7]. Thus, AFM_1 concentration in milk and milk products depends on the level of exposure and the amount of AFB1 ingested.

The toxicity of AFM_1 was initially classified as a Group 2B agent, but it has now moved to Group 1 by the International Agency for Research on Cancer (IARC) [8]. European Community Legislation limits the concentration of AFM_1 in milk and processed milk

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products at 0.050 μg kg^{-1} for adults [9] and 0.025 μg kg^{-1} for infants or baby-food [10]. AFM_1 is relatively stable in raw and processed milk products and is not affected by pasteurization (even those using UHT techniques) or cheese processing performed in dairy industry [11]. Thus, the presence of AFM_1 in these products has become a worldwide concern due to the widespread consumption of them. Therefore, accurate evaluation of AFM_1 in milk is of great interest.

Determination of AFM_1 is generally performed by different techniques such as thin layer chromatography [12], ultra-high performance liquid chromatography with mass detection [13,14], electrochemical methods [15] and chemiluminescence [16]. However, the "gold standard" for aflatoxin determination is high performance liquid chromatography (HPLC) followed by fluorimetric (FL) or mass spectroscopic (MS) analysis [17,18] which is time-consuming and costly and mainly limited to laboratory uses. Thus, developing a fast and rugged method which produces reliable data and leads to precise and reproducible results is of imminent interest to researchers. Regular development characteristics which need to be met include linearity, limit of detection (LOD) and limit of quantification (LOQ), accuracy and repeatability [19]. On the other hand, in order to obtain low limits of detection and good selectivity of AFs in food matrices, a sample preconcentration step is usually required prior to the instrumental analysis.

Several extraction techniques have been employed to extract and preconcentrate AFs from different sample matrices, such as solid phase extraction (SPE) [20], liquid–liquid extraction [21], and solid-phase microextraction (SPME)[22]. However, most of these methods require considerable extraction time to obtain satisfactory extraction efficiency (for example at least 90 min for liquid–liquid extraction [21]).

Featuring rapid extraction and high efficiency, since its first introduction by Rezaee et al. [23], dispersive liquid–liquid microextraction (DLLME) has been applied to the extraction of mycotoxins [24,25]. However, the limitation of using extracting solvents with higher density than water (typically chlorinated solvents that are comparatively less environmentally friendly) for the convenient collection of analytes (as the sedimented phase after centrifugation) is the main drawback of this method. On the other hand, the method requires additional processing steps apart from the mandatory centrifugation, including refrigeration to freeze the organic solvent, manually retrieving it to let it thaw, and use of additional materials and apparatus such as surfactants or conical-bottom test tubes [26]. To overcome theses drawbacks, some modification techniques that resulted in DLLME improvement including the use of organic solvents with lower density than water and applying MNPs-based SPE in combination with DLLME were developed [27,28].

In this study, a novel and fast LDS-DLLME procedure using 1-heptanol as the extraction solvent was applied to extract AFM_1 analyte-containing methanol/water (80:20, v/v) dispersing solvent and then, a magnetic nanoparticles-based VA-D-SPE using hydrophobic oleic acid modified Fe_3O_4 nanoparticles as an efficient adsorbent was applied to retrieve the analyte-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 the solvent and the nano-adsorbent cause the analyte was 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 and thawing, manual collection of extractant or specialized apparatus. Then, a surfactant enhanced spectrofluorimetric determination using triton X-100 micelles was used for determination of AFM_1. The main experimental parameters affecting the two-step extraction procedure were investigated in details and the analytical characteristics of the method were evaluated. The method was successfully applied for determination of AFM_1 in commercial milk samples.

2. Experimental

2.1. Instrumentation

A Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA, USA) equipped with a xenon lamp was used for fluorescence spectra recording of AFM_1 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 365 and 460 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. A Labinco BV L46 Vortex mixer (Breda, Netherlands) was used to mix and accelerate the reactions between reagents.

2.2. Standards and materials

Standard of AFM_1 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 ($(C_2H_5)_2O$), and trichloromethane ($CHCl_3$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), Triton X-100, oleic acid and the other used chemicals were supplied by Merck (Darmstadt, Germany). Deionized water was used throughout the experiments. As safety notes, all used laboratory glassware were treated with an aqueous solution of sodium hypochlorite (5% w/v) before re-using to minimize interferences and health risks due to AFM_1 contamination.

Blank liquid milk sample (10 ± 0.1 mL) was accurately weighed and transferred into a 50 mL centrifuge tube. The sample was spiked with appropriate amounts of AFM_1 and centrifuged at 2150*g* for 15 min and the fat layer was removed. Then, the supernatant was diluted to 100 mL with methanol: water (80:20, v/v) in a capped container with intensive shaking. An aliquot of diluted aqueous phase (3 mL) was directly used as the dispersing solvent for DLLME process.

2.3. Synthesis of oleic acid modified MNPs

The Fe_3O_4 nanoparticles were prepared via a simple chemical co-precipitation method previously reported [29] with slight modifications. Briefly, 5.8 g of $FeCl_3 \cdot 6H_2O$ and 2.1 g of $FeCl_2 \cdot 4H_2O$ were dissolved in 100 mL deionized water under nitrogen atmosphere with vigorous stirring at 85 °C. Then, aqueous ammonia solution (20 mL, 25% w/w) was added to the solution. The color of bulk solution changed from orange to black immediately. The obtained Fe_3O_4 nanoparticles were separated from the solution using an external supermagnet. The supernatant was removed and the precipitate was washed sequentially with deionized water (100 mL, three times) and 0.02 mol L^{-1} of sodium chloride (100 mL, twice). Then, oleic acid (1.0 g) was introduced and the reaction was kept at 80 °C for 3 h. The suspension was cooled down to the room temperature and the resulting precipitate was washed sequentially with deionized water (2 × 100 mL), methanol (2 × 100 mL) and deionized water (3 × 100 mL) by magnetic decantation with the help of an external magnet. Finally, the oleic

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

2.4. Recommended extraction procedure

An aliquot of 320 μL of 1-heptanol was added to 3 mL of MeOH/water (80:20, v/v) containing analyte 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 about 1650 g for 60 s to equilibrate. After that, 500 μL of the adsorbent containing 40 mg of oleic acid modified MNPs was quickly added to the vial. Vortex was applied for 2 min to facilitate the interaction of organic solvent containing target analyte to the surface of magnetic adsorbent. Then, the adsorbent was collected in the bottom of the vial by applying an external magnet and supernatant was removed. The adsorbed analyte was desorbed from the adsorbent by addition of 2 mL of MeCN for 2 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 1 mM Triton X-100 in 12% (v/v) MeCN/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. 1, the mean diameter of oleic acid modified MNPs is 9 ± 1 nm and the particles have uniform size distribution and most of them are quasi-spherical in shape. FT-IR spectroscopy was used to characterize the chemical interaction between Fe_3O_4 nanoparticles and oleic acid. As can be seen from Fig. 2, 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 ν_{as} (COO–) and the symmetric as ν_s (COO–) stretch, instead [30,31]. 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.

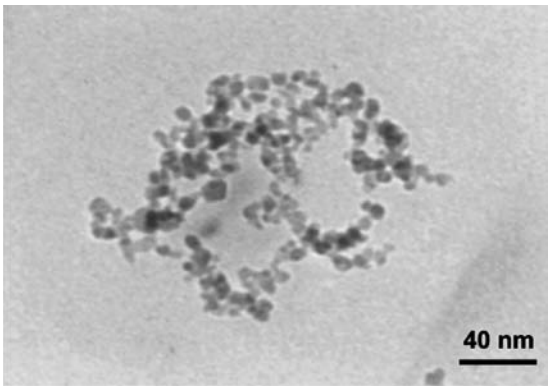

Fig. 1. TEM image of oleic acid modified MNPs.

3.2. Signal enhancement conditions

As a pentahetrocyclic and highly conjugated compound, AFM_1 exhibits native fluorescence. Thus, sensitive analytical techniques for its detection are based on either its native emission properties or enhanced fluorescence after chemical complexation/drevitization. Generally, the fluorescence of mycotoxins is quenched in water and increasing surfactant or some complexing agents like β-cyclodextrin enhances their fluorescence intensity [32,33]. This confirms the microenvironment around them in these situations is quite different from pure aqueous solutions. In this study, Triton X-100 was selected as signal enhancement agent for AFM_1 determination. This surfactant has a long tail length which forms large micelles around AFM_1 molecules, provide a better environment to encapsulate and restrict the intramolecular rotation of AFM_1 to boost emission. The effect of surfactant addition on the fluorescence intensity of AFM_1 was investigated by adding different amounts of Triton X-100 in the range of 0.1–2 mM to the desorbed AFM_1. As can be seen from Fig. 3, a significant fluorescence enhancement was observed with increasing Triton X-100 concentration and reached maximum in 1 mM which is above the critical micellar concentration (CMC) value of 0.2 mM for Triton X-100. The effect micelle formation time on the fluorescence signal of AFM_1 was also investigated in the range of 1–10 min and the results (see Fig. S1, S refers to the electronic supplementary materials) revealed that 5 min was enough for maximum signal enhancement and used for subsequent experiments.

3.3. Optimization of the LDS-DLLME procedure

3.3.1. Selection of dispersing solvent

The applicability of several organic 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 taking into account that it should has the capability of extracting AFM_1 from milk samples. The results (Fig. 4) revealed that the maximum extraction efficiency was achieved by MeOH/water (80:20 v/v) and therefore, it was selected to act as both the extraction solvent of AFM_1 from milk samples and as the disperser solvent in DLLME for subsequent experiments.

Furthermore, the effect of disperser solvent volume on the recovery of AFM_1 was investigated in the range of 1–5 mL. The obtained results (see Fig. S2) 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 AFM_1 in aqueous phase and decreasing the 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

Selection of extractant is of great importance in microextraction in order to obtain an efficient extraction performance. Choosing the extractant is influenced by several requirements. It should be immiscible with water, has the capability to extract analyte, good emulsification efficiency in the aqueous sample, low density and low vapor pressure to prevent loss during agitation. Furthermore, it should be relatively nonvolatile to prevent potential solvent loss during extraction. 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. The dispersion of the extraction solvent

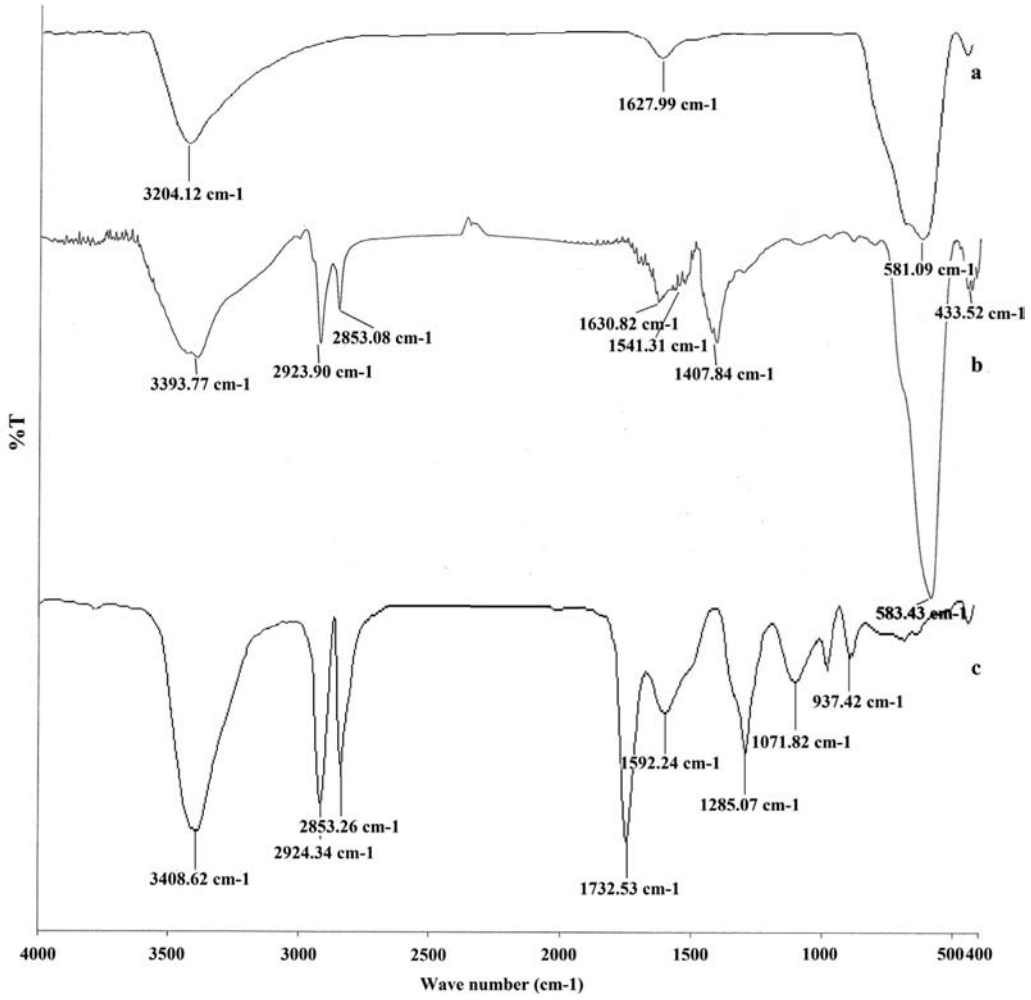

Fig. 2. FT-IR spectra of MNPs (a) oleic acid modified MNPs (b) and oleic acid (c).

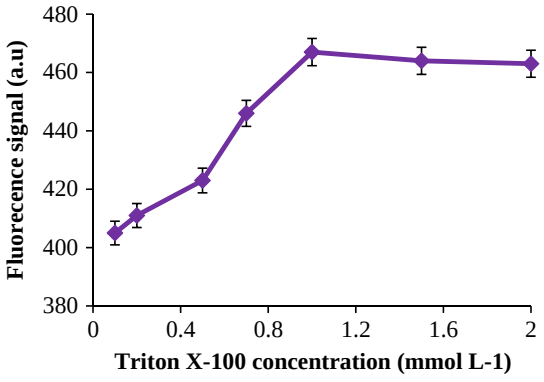

Fig. 3. Effect of Triton X-100 concentrations on the fluorescence signal of the AFM_1. The excitation and emission wavelengths were 365 and 460 respectively.

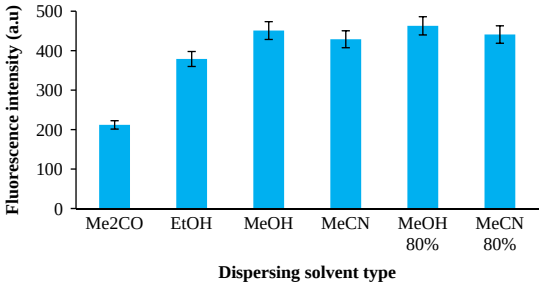

Fig. 4. Effect of dispersing solvent on the recovery of total AFM_1. Conditions: extraction solvent, 320 μL of 1-heptanol; water volume, 15 mL, equilibration time, 60 s, adsorbent amount, 40 mg; adsorption time, 2 min; desorption time, 2 min, and desorption solvent volume and type, 2 mL of MeCN.

determines enrichment efficiency for the analyte in DLLME step. The fine droplets of extraction solvent afford larger surface area to contact with the water sample and higher the extraction performance can be attained. As can be seen from Fig. 5, 1-heptanol gave the highest fluorescence signal for the analyte and was considered as the most suitable extraction solvent for subsequent experiments. The effect of extracting solvent volume, salt addition and water volume represent in Supplemental materials.

3.3.3. *Effect of equilibration time*

The equilibration time is defined as the interval time from the occurrence of 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 about 1650 g to maximize mass transfer and reduce mixing time. As can be seen in Fig. S6, the intensity of fluorescence signal was not affected remarkably in different extraction times showing that the mass transfer from sample solution to extracting solvent are very fast. Since the equilibrium state can be quickly achieved in DLLME, the required extraction time is short. In fact, short extraction time is one

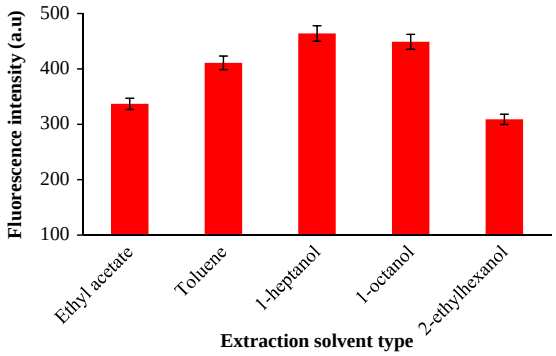

Fig. 5. Effect of extracting solvent on the recovery of total AFM_1. Conditions: dispersive solvent, 3 mL of MeOH/water (80:20 v/v); water volume, 15 mL, equilibration time, 60 s, adsorbent amount, 40 mg; adsorption time, 2 min; desorption time, 2 min, and desorption solvent volume and type, 2 mL of MeCN.

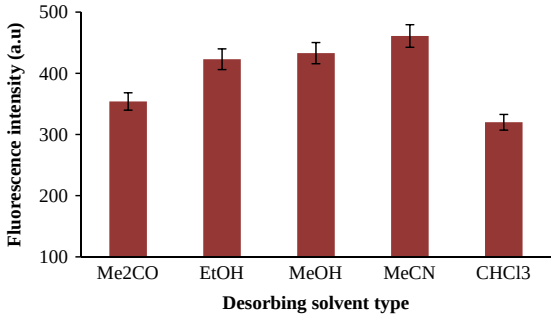

Fig. 6. Effect of desorption solvent type on the recovery of total AFM_1. Conditions: dispersing solvent volume and type, 3 mL of MeOH/water (80:20 v/v), extraction solvent, 320 μL of 1-heptanol, water volume, 15 mL, equilibration time, 60 s, adsorbent amount, 40 mg; adsorption time, 2 min; desorption time, 2 min, and desorption solvent volume, 2 mL.

of the remarkable advantages of the DLLME technique. Based on the results, 60 s was selected for subsequent experiments.

3.4. Optimization of MNPs-based VA-D-SPE procedure

3.4.1. Effect of MNPs amount and vortex time

Compared to conventional micron-sized adsorbents, MNPs offer high extraction capacity, rapid extraction dynamics and high extraction efficiency. Therefore, satisfactory results can be achieved by lower amounts of these adsorbents. The amount of hydrophobic adsorbent has direct effect on the extraction efficiency of the analyte because it guarantees the quantitative separation of extraction solvent-containing AFM_1 from DLLME step. Thus, different amounts of oleic acid modified Fe_3O_4 nanoparticles in the range of 10–100 mg were added to the sample solution. The results were summarized in Fig. S7 and revealed that the extraction efficiency increases with increasing in adsorbent amount up to 40 mg and then leveled off. High surface to volume ratio of nanoparticles causes the quantitative extraction could perform using very low amount of adsorbent. Therefore, 40 mg was selected for the next experiments.

It is well-known that vortex process is an effective way to enhance mass transfer from aqueous phase to the extraction phase. In order to realize the effect of adsorption time on the recovery of the analyte, the vortex time was investigated in the range of 1–7 min. The experimental results (see Fig. S8) indicate that 2 min is sufficient for achieving appropriate adsorption of the analyte and it was used for the next experiments.

3.4.2. Desorption conditions

To select the best eluent for desorbing analyte from the adsorbent, five common organic solvents including Me_2CO, EtOH, MeOH, MeCN and $CHCl_3$ were examined. As it is depicted in Fig. 6, the best elution of analyte was attained by using MeCN and it was selected as the elution solvent in the subsequent experiments. Furthermore, the eluent volume influences sensitivity of the method as it determines the maximum preconcentration factor that can be achieved for the target analyte. Ideally, it should be as low as possible but providing a quantitative and reproducible elution of the compounds. The effect of desorbing solvent volume on the recovery of AFM_1 was investigated in the range of 0.5–5 mL and the maximum recovery was obtained with volumes higher than 1 mL (Fig. S9). Therefore, 2 mL of acetonitrile was selected for the next experiments. In addition, the effect of desorption time on the recovery of AFM_1 was examined in the range of 1–10 min. As can be seen from Fig. S10, the duration time of 2 min was appeared to be sufficient for complete desorption of the analyte. Since, modified nanoparticles can be easily and rapidly collected from the sample solution using an external magnetic field, the analysis time greatly reduces compared to the conventional SPE methods.

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 3.4% for recovery results) reclaimed the capacity of these materials to be an alternative sorbent for immunoaffinity columns.

3.5. Analytical parameters

Under the optimum experimental conditions, the calibration curve was linear over the concentration range of 0.02–200 $\mu g\,L^{-1}$ with the correlation coefficient (R^2) of 0.9989. Solutions for the construction of calibration curve were prepared by spiking appropriate amounts of AFM_1 working solutions to the non-contaminated milk sample and subjected to the proposed LDS-DLLME coupled VA-D-SPE procedure following the enhanced fluorescence measurements. 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 13 $ng\,L^{-1}$. 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 50 $\mu g\,L^{-1}$ 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 1 indicate good precision of the proposed method. The adsorption capacity of oleic acid modified MNPs was determined by the static method. For this purpose, 40 mg of hydrophobic adsorbent was added to 18 mL of solution containing dispersed analyte after DLLME step, at different concentration levels. After 15 min, the mixture was filtered and the supernatant was analyzed. The results showed that the amount of analyte adsorbed per mass unit of the adsorbent was increased with increasing in concentration of AFM_1 and then was reached to a plateau value (adsorption capacity value), represents saturation of active surface of hydrophobic adsorbent. The maximum adsorption capacity of the adsorbent for AFM_1 was found to be 581 $\mu g\,g^{-1}$ which is comparable or even higher than many

Table 1
The characteristic data for determination of AFM_1 by the proposed method.

Parameters	Value
Dynamic range ($\mu g\ L^{-1}$)	0.02–200
Correlation coefficient (R^2)	0.9989
Intra-day precision (RSD%, $n=5$)	2.9[a]
	4.3[b]
Inter-day precision (RSD%, $n=5$)	2.1[a]
	3.3[b]
Limit of detection ($3.3S_b/m$[c], ng L^{-1})	13

[a] 2 $\mu g\ L^{-1}$ of AFM_1.
[b] 50 $\mu g\ L^{-1}$ of AFM_1
[c] S_b is the standard deviation for ten blank measurements and m is the slope of the calibration curve.

Table 2
Determination of AFM_1 in spiked milk samples ($n=3$, $\pm$ SD).

Sample	Spiked ($\mu g\ kg^{-1}$)	Found ($\mu g\ kg^{-1}$)	Recovery (%)
Sample 1	0	0.27 ± 0.05	–
	2	2.14 ± 0.05	94.4
	50	49.31 ± 0.05	98.1
	150	149.06 ± 0.05	99.2
Sample 2	0	0.33 ± 0.07	–
	2	2.12 ± 0.05	91.3
	50	47.96 ± 0.05	95.3
	150	149.58 ± 0.05	99.5
Sample 3	0	ND[a]	–
	2	1.92 ± 0.05	96.3
	50	48.03 ± 0.05	96.0
	150	147.75 ± 0.05	98.5

[a] Not detected.

Table 3
Comparison of AFM_1 analyses (mean $\pm$ SD, $n=3$) in contaminated milk samples by the proposed and standard IAC-HPLC-FD method.

Sample	Proposed method AFM_1 ($\mu g\ kg^{-1}$)	HPLC-FD-IAC[a] AFM_1 ($\mu g\ kg^{-1}$)
Sample 1	0.27 ± 0.05	0.31 ± 0.04
Sample 2	0.33 ± 0.07	0.27 ± 0.05

[a] HPLC analysis by the AOAC standard method [38].

reported adsorbents previously used for determination of aflatoxins [34–37].

3.6. Real sample analysis

To evaluate the applicability of the proposed method in real matrices, it was applied to the determination of AFM_1 in commercial milk samples. Recovery studies were carried out by spiking the samples with different amounts of AFM_1 and the obtained results were summarized in Table 2. The acceptable recoveries in the range of 91.3–99.5% demonstrate that the matrix of milk sample was not affected on extraction efficiency of the analyte. Further examination of accuracy was performed by comparison of the results obtained from the proposed method and the AOAC standard method (IAC-HPLC-FL) [38] for determination of AFM_1 in two contaminated milk samples. The results are summarized in Table 3. 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

Table 4
Comparison of the proposed method with some previously reported methods for the determination of total AFM_1.

Assay type	Linear range (ng mL^{-1})	Detection limit (ng mL^{-1})	Time needed for assay[a]	Reference
Impedimetric immunosensor	0.015–1	0.015	28 h	[39]
HPLC-FL	0.2–4	0.006	30 min	[20]
Immunochip	0.45–3.9	0.24	2.5 h	[40]
Flow-injection immunoassay	0.02–0.5	0.011	26 h	[41]
Competitive ELISA[b]	28–164	28	2.5 h	[42]
Indirect competitive ELISA	0.1–3.2	0.04	27.5 h	[6]
SPE-fluorimetry	0.04–8	0.015	50 min	[33]
DLLME-D-SPE-fluorimetry	0.02–200	0.013	20 min	This work

[a] Not mentioned exactly, at least.
[b] Enzyme-linked Immunosorbent Assay.

of the analytical characteristics obtained by the proposed method and some other reported methods for determination of AFM_1 is presented in Table 4. As can be seen, the proposed method has distinct advantages in terms of low detection limit, wide linear range and simplicity.

4. Conclusion

A two-step extraction technique namely, LDS-DLLME followed by hydrophobic MNPs-based VA-D-SPE coupled with Triton X-100 micelle enhanced spectrofluorimetric detection was developed for the extraction of the AFM_1 in milk samples. The proposed method demonstrates that an organic solvent with lower density than water can be used in DLLME without involving any additional handling procedure and apparatus by application of hydrophobic magnetic nanoparticles to retrieve the extracting solvent of DLLME and combination of MNPs-based SPE with LDS-DLLME eliminates many time-consuming centrifugation, refrigeration to freeze and then thawing or manual collection of extracting solvent usually accompany with DLLME procedure. The method has many advantages including simplicity, low solvent consumption, low cost, excellent enrichment in low time, good precision and high accuracy. The good extraction recovery and the inherent sensitivity of spectrofluorimetric method reveal the potential of present method for determination of AFM_1 in milk samples.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.11.007.

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