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Extraction of Aflatoxins from Liquid Foodstuff Samples with Polydopamine-Coated Superparamagnetic Nanoparticles for HPLC-MS/MS Analysis

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ABSTRACT: A facile magnetic solid phase extraction (MSPE) of aflatoxins (AFs) from liquid samples was developed using polydopamine-coated magnetic nanoparticles (PD-MNPs) as the adsorbent. PD-MNPs were prepared from amine-terminated MNPs and dopamine via an in situ oxidative self-polymerization approach. Under the selected MSPE conditions, extraction yields ranging from 59.3% for AF G_2 to 89.0% for AF B_1 were obtained with good repeatability. Coupled with HPLC-MS/MS quantification, the MSPE procedure serves not only for sample cleanup but also for AFs enrichment that is highly desired for trace analysis. The proposed MSPE-HPLC-MS/MS method had a linear calibration curve in the concentration range from 0.00600 to 3.00 ng/mL aflatoxin and limits of detection of 0.0012 ng/mL for AF B_1, AF B_2, and AF G_1, and 0.0031 ng/mL for AF G_2.

KEYWORDS: *aflatoxin quantification, HPLC-MS/MS, sample pretreatment, magnetic solid phase extraction, polydopamine coated magnetic nanoparticles, wine analysis*

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by organisms of the fungi kingdom, commonly known as molds.[1,2] Aflatoxins (AFs) are mycotoxins derived by *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus* (*A. parasiticus*) and are listed as Group I carcinogens by the International Agency for Research on Cancer (IARC), a body of the World Health Organization (WHO). There are four major natural aflatoxins (i.e., AF B_1, AF B_2, AF G_1, and AF G_2). AF B_1 and AF B_2 are produced by *A. flavus*, whereas AF G_1 and AF G_2 are produced by both *A. flavus* and *A. parasiticus*. AF B_1 is the molecule with the highest toxic significance. The hierarchy of toxicity, carcinogenicity, and mutagenicity of different aflatoxins is in the order AF B_1 > AF G_1 > AF B_2 > AF G_2.[3,4] The occurrence of aflatoxins is influenced by certain environmental factors. Hence, the extent of contamination will vary with geographic location, agricultural and agronomic practices, and the susceptibility of commodities to fungal invasion during preharvest, storage, and/or processing periods. On a worldwide scale, aflatoxins are found in stored food commodities and oil seeds such as corn, peanuts, and so forth.[5,6]

To achieve quantitative analysis of aflatoxins, analytical methods based on various instrumental techniques have been developed.[7,8] High-performance liquid chromatography−mass spectrometric (HPLC−MS) methods have drawn the most interest due to their excelling selectivity. However, HPLC−MS analysis is prone to matrix effects, which are dependent on analytes, sample matrices, LC−MS conditions, and sample preparation.[9,10] To some degree, use of internal standards, especially stable isotope dilution, alleviates the problems associated with matrix effects.[11,12] A major disadvantage of HPLC-MS stable isotope dilution analysis is the need for expensive isotope-labeled analogues of the analytes. In addition, when analyte concentrations in a sample solution are below the limit of quantitation (LOQ) of the method, enrichment of the analytes must be carried out prior to analysis. Many procedures for sample cleanup and/or analyte enrichment have been reported. Immunoaffinity columns have been used for long to extract aflatoxins from various sample matrices.[13,14] The advantages of these affinity extraction procedures are that they are selective and sensitive. However, the immunoaffinity columns are normally costly. To reduce the cost of analysis, chemical mini-columns, particularly florisil-packed columns were proposed and proven effective for sample cleanup in aflatoxin analysis.[15−17] Major drawbacks of these column-based procedures include a time-consuming elution of the retained analytes and use of significant quantities of toxic organic solvents. Solid-phase extraction (SPE) of aflatoxins with C_{18} cartridges has been successfully developed.[18−20]

Magnetic solid-phase extraction (MSPE) is a new version of SPE. It is based on the use of superparamagnetic nanoparticles as adsorbent.[21−23] In MSPE, the adsorbent needs not to be packed into a cartridge as in traditional SPE. Instead, a suspension of the nanometer-sized adsorbent is added and mixed well with the sample solution to extract analytes. After extraction, the adsorbent can be easily separated from the solution and collected through magnetic decantation by means of an external magnet. There is no need for tedious centrifugation or filtration in MSPE. Therefore, MSPE is

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quick, easy to perform, and importantly, well-suited to handle liter volumes of samples, which means a large enrichment factor can be conveniently obtained. Use of MSPE in quantitative analysis of estrogens[24] and isoflavones[25] in milk samples has been reported.

The aim of the present research was to develop a rapid, cost-effective, and efficient MSPE procedure for HPLC−MS/MS quantification of aflatoxins in liquid foodstuff samples. This procedure serves not only for sample cleanup, eliminating any potential matrix effects on the subsequent HPLC−MS/MS analysis, but also for AF enrichment, allowing the quantification at very low levels. To achieve high extraction efficiency, superparamagnetic Fe_3O_4 nanoparticles with different surface modifications were synthesized, characterized by using TEM and FTIR and evaluated as the MSPE adsorbent. MSPE parameters affecting the extraction efficiency, including extraction time, eluting solvent, and so forth were investigated. Finally, rapid analysis of low levels of aflatoxins in red wine samples by using the proposed MSPE−HPLC−MS/MS method was demonstrated.

MATERIALS AND METHODS

Reagents and Chemicals. Aflatoxin standards, dopamine (structures are shown in Figure 1), 1,6-hexamethylenediamine, anhydrous sodium acetate, iron(III) chloride hexahydrate ($FeCl_3·6H_2O$), ethylene glycol, HPLC grade acetonitrile, methanol, and formic acid were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Other chemicals and solvents were of analytical grade. Milli-Q water (Millipore, Bedford, MA) was used throughout the work.

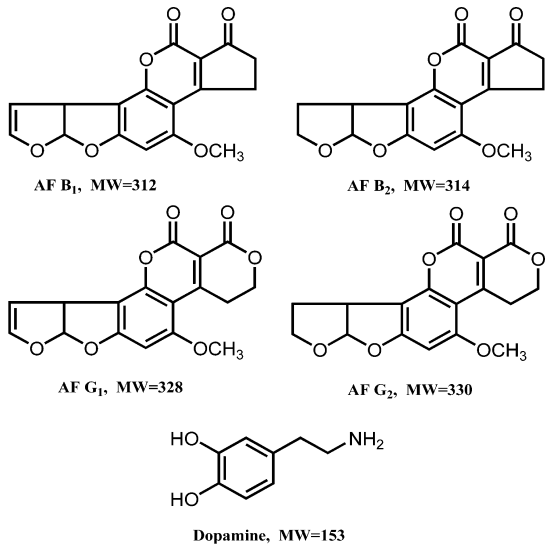

Figure 1. Chemical structures of the compounds involved in this work: AF B_1, AF B_2, AF G_1, AF G_2, and dopamine.

Preparation of Polydopamine-MNPs. Amine-terminated Fe_3O_4 magnetic nanoparticles (AMNPs) were prepared by a one-pot hydrothermal procedure previously reported (illustrated in Figure 2).[26] Briefly, 4.33 g of 1,6-hexanediamine, 1.33 g of anhydrous sodium acetate, and 0.66 g of $FeCl_3·6H_2O$ were dissolved in 25 mL of ethylene glycol by vigorously stirring at 50 °C to obtain a clear solution. It was then transferred to a Teflon-lined autoclave for 6 h at 198 °C to obtain AMNPs. The AMNPs were rinsed with water and ethanol twice. After each rinse step, the AMNPs were separated from the supernatant by using an external magnet. AMNPs were dried at 50 °C under N_2 overnight. To prepare polydopamine coated MNPs (PD-MNPs), an in situ oxidative self-polymerization procedure was used.[27−29] A suspension of AMNPs was prepared by dispersing 220 mg of AMNPs in 10 mL of 10 mM Tris-HCl buffer solution (pH 8.5) through sonication for 15 min. Dopamine HCl (2.5 mg/mL) was added to the AMNPs suspension with vigorous stirring, and the pH of the mixture was adjusted to 8.5 by addition of 10 mM sodium hydroxide. The solution was placed on a shaker for 12 h after which PD-MNPs were collected by magnetic decantation, washed three times with water, and finally redispersed in 5.0 mL water by sonication for 15 min.

Characterization of PD-MNPs. Transmission electron microscopic (TEM) images were acquired on a JEOL, JEM-1011 with a resolution of 0.2 nm lattice. FT-IR spectra were obtained by a Nexus 670 E.S.P. Fourier transform-infrared spectrometer.

Aflatoxin Standard Solutions. Aflatoxin standard mixture containing 1000 ng/mL AF B_1, AF G_1 and 300 ng/mL AF B_2, AF G_2 (Sigma-Aldrich, St. Louis, MO) was allotted at 100 uL per centrifuge tube and stored at −20 °C until use. The work standard solutions were prepared daily by appropriate dilution with methanol/water (50:50, v/v).

Magnetic Solid Phase Extraction of Aflatoxins. To an Erlenmeyer flask containing 50 mL of sample, 100 μL of the PD-MNPs suspension prepared above was added. The mixture was shaken for 10 min on an oscillator. The flask was placed on a magnet for 30 s to let PD-MNPs settle down. The supernatant was discarded. After washing twice with 500 μL of water, aflatoxins were eluted from the PD-MNPs with 250 μL of warm acetonitrile/methanol (1:1) at about 60 °C for 3 min. After magnetic separation, 100 μL of supernatant was transferred to a centrifuge vial and mixed with 100 μL of water. After being filtered through a 0.22 μm filtration membrane, portions (5 μL) were injected into the HPLC−ESI−MS/MS

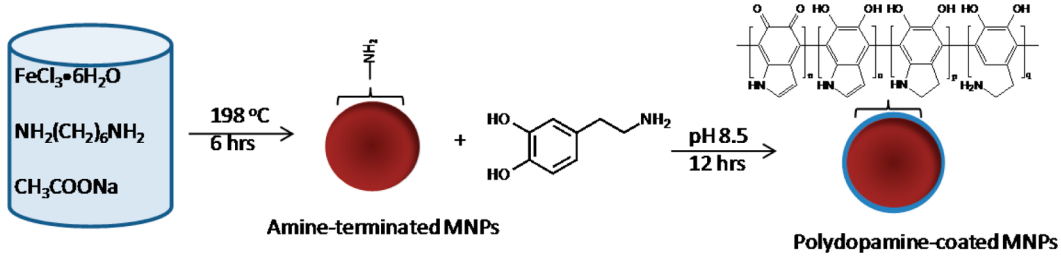

Figure 2. Illustration of PD-MNPs preparation involving two steps: (1) one-pot synthesis of amine-terminated magnetic nanoparticles (AMNPs) from $FeCl_3$ and hexanediamine and (2) coating AMNPs with polydopamine via in situ oxidative self-polymerization of dopamine.

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system for analysis without further purification. The MSPE procedure is illustrated in Figure 3.

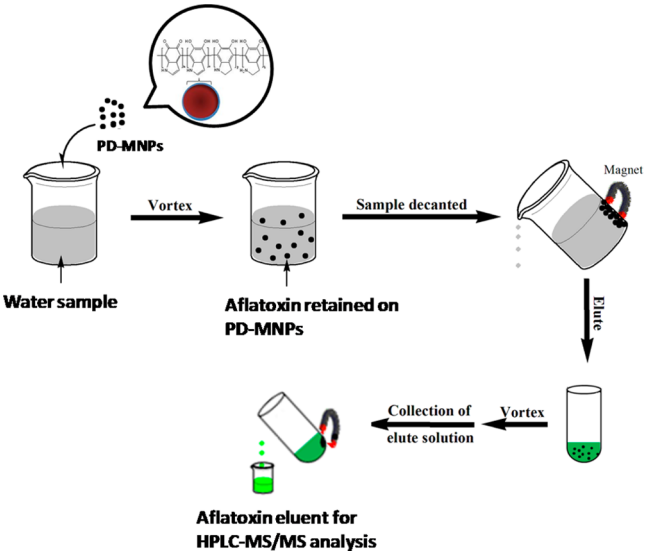

Figure 3. Illustration of the proposed MSPE procedure for facile extraction of aflatoxins followed by HPLC–MS/MS quantification.

HPLC–MS/MS Analysis of Aflatoxins. The system consisted of two pumps (LC-10ADvp, Shimadzu, Toyoto, Japan), an online degasser (DGU-12A, Shimadzu), and a triple quadrupole mass spectrometer equipped with a heated ESI source (TSQ Quantum, Thermo Scientific, San Jose, CA). Both the LC and mass spectrometer were controlled by Xcalibur software (Thermo Finnigan). A C_{18} reversed-phase column (Ascentis, 3 μm particle size, 10 cm × 2.1 mm, Sigma-Aldrich, St. Louis, MO) was used for separation. MeOH/water mixture (60/40, v/v) containing 5 mM ammonium acetate was used as the mobile phase at a flow rate of 0.150 mL/min. Sample injection volume was 5 μL. Data were acquired in full scan and SRM mode. The MS detector was operated in the positive ion mode with the following settings: spray voltage of 3 kV, vaporization temperature of 270 °C, capillary temperature of 300 °C, sheath gas pressure of 35 (arb), auxiliary gas pressure of 10 (arb), tube lens voltage of 150 V, and capillary voltage of 35 V. SRM parameters for MS detection of aflatoxins are summarized in Table 1.

Table 1. SRM Data Acquisition Parameters for Aflatoxins

aflatoxin	molecular weight	precursor ion (m/z)	product ion (m/z)	collision energy
AF B_1	312	313	285	28
AF B_2	314	315	259	28
AF G_1	328	329	243	28
AF G_2	330	331	245	28

RESULTS AND DISCUSSION

Synthesis and Characterization of PD-MNPs. MSPE is gaining attention because it is quick, easy to perform, inexpensive, and suitable for working with large volume samples.[21−23] The success of a MSPE procedure depends largely on the surface chemistry of the superparamagnetic nanometer-sized adsorbent particles. Polydopamine-coated nanoparticles have been widely used in biological and pharmaceutical technology arenas.[27−30] In this study, it was found that a highly stable polydopamine coating could be formed on the surface of amine-terminated Fe_3O_4 superparamagnetic nanoparticles via oxidative self-polymerization of dopamine. Because the polydopamine coating thus formed is composed of dihydroxyindole, indoledione, and dopamine units (as illustrated by Figure 2),[31,32] PD-MNPs are expected to have a high affinity for aflatoxins through a combination of charge transfer, π-stacking, and hydrogen-bonding interactions, and therefore, they are expected to serve as a highly effective MSPE adsorbent for extracting these compounds from solutions.

One-pot synthesis of amine-terminated MNPs (AMNPs) was achieved by following a procedure previously reported[26] using $FeCl_3{\cdot}6H_2O$ as the precursor of magnetic particles and hexamethylenediamine as an amino group source. From the TEM results, AMNPs prepared were ∼30 nm in diameter in average and had a circular shape. The FT-IR spectrum (Figure 4A) shows strong absorptions at 575 cm^{-1} arising from the vibration of the Fe−O bond and at 3133.5 and 3420.9 cm^{-1} from N−H $_{bond}$ in the amine group. PD-MNPs were easily prepared by incubating AMNPs with dopamine in a Tris buffer at pH 8.5 for 3 h. TEM analysis showed that PD-MNPs obtained had an average diameter of ∼40 nm. In the FT-IR spectrum of PD-MNPs (Figure 4B), a strong absorption band at 1400 cm^{-1} and a moderate band at 1600 cm^{-1} are observed, clearly indicating the presence of polymerized aromatic structures. In addition, an absorption band at 3100−3450 cm^{-1} is much stronger than that in AMNPs spectrum, which is due to the overlapping of hydroxyls and amines in polydopamine. These results suggest that PD coating of AMNPs was successful. It was also noted that the suspendability of PD-MNPs was much improved from that of the precursor AMNPs or bare Fe_3O_4 nanoparticles. A diluted suspension of PD-MNPs was stable for several days in terms of its appearance. UV−vis monitoring of the suspension for 1 week did not detect any leaching of polydopamine or its components from the nanoparticles.

MSPE of Aflatoxins with PD-MNPs. In the MSPE study, alflatoxin standard solutions prepared in water were used. PD-MNPs were added to 50 mL of a standard solution in each extraction test. Aflatoxins extracted from the solution were subsequently retrieved from the PD-MNPs and quantified by HPLC–MS/MS. Several HPLC–MS methods have been reported for aflatoxin quantification.[11,12,18,20,33] In the present study, several mobile phases, including MeOH/H_2O (60:40) containing 5 mM ammonium acetate, MeOH/H_2O (60:40) containing 0.1% formic acid, ACN/H_2O (60:40) containing 5 mM ammonium acetate, and MeOH/ACN/H_2O (60:20:20), were tested for the separation on a C_{18} column. It was found that isocratic elution with MeOH/H_2O (60:40) containing 5 mM ammonium acetate resulted in the best analytical results in terms of separation efficiency and detection sensitivity. The aflatoxins tested were separated within 4.5 min. Intriguingly, introducing ammonium acetate to the mobile phase significantly improved the detection sensitivity. However, no similar effects were observed with formic acid (0.1% v/v), a commonly used additive to enhance ionization efficiency in HPLC–MS analysis.

Effects of PD-MNPs amount on extraction efficiency were investigated. In a set of extraction tests, 10, 25, 50, 75, 100, 150, 200, and 500 μL of PD-MNPs suspension were added to different 50.0 mL portions of a standard solution containing 0.100 ng/mL AF B_1, respectively. On the basis of the peak areas

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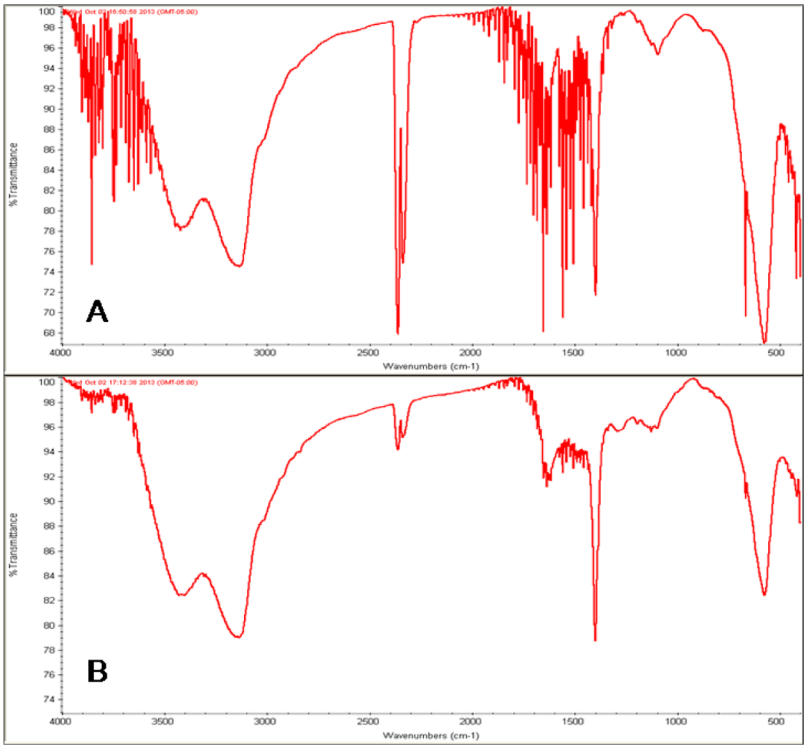

Figure 4. FT-IR spectra of AMNPs (A) and PD-AMNPs (B).

obtained from the HPLC–MS/MS analysis of the eluent from each test, the extraction efficiency of AF B1 was assessed. It was found that extraction efficiency increased [from 33.1 ± 7.2% to 89.2 ± 2.6% (n = 3)] with the increase in PD-MNPs amount and remained nearly constant when >75 μL of PD-MNPs suspension was used. For further studies, 100 μL of PD-MNPs suspension was added to 50 mL of sample to extract aflatoxins. Extraction times of 5, 10, and 20 min were tested. The extraction efficiency of AF B1 obtained were 86.7 ± 2.2%, 89.7 ± 1.7%, and 89.5 ± 0.3% (n = 3), respectively. Desorption of aflatoxins retained on PD-MNPs is also critical to the success of the MSPE development. Elution conditions, including eluting solvent, elution time (1, 2, 3, 5, and 10 min), and elution temperature (30, 45, 60, 80 °C) were investigated to achieve a high retrieval efficiency. Several eluting solvents were tested. Results of retrieval efficiency for AF B_1 are summarized in Figure 5. Elution with water produced an eluent solution containing nondetectable aflatoxins. Methanol/acetonitrile (1:1) was found most effective to retrieve aflatoxins from PD-MNPs. On the basis of these studies, methanol/acetonitrile (1:1) as the eluting solvent and an elution time of 3 min at 60 °C were selected for desorption of aflatoxin from PD-MNPs. An equal amount of water was added to the elution solution prior to HPLC–MS/MS analysis in order to minimize the solvent effects on the separation. Interestingly, when using AMNPs or bare Fe_3O_4 MNPs for MSPE of aflatoxin, the extraction efficiency was found to be significantly lower. This may be due to either a low affinity of aflatoxins to these adsorbents or poor desorption of aflatoxins from them under the selected elution conditions. PD-MNPs exhibit a very high affinity for aflatoxins because of combined interactions arising from charge transfer, π-stacking, and hydrogen bonding, most of which do not exist in the case of AMNPs or bare Fe_3O_4 MNPs.

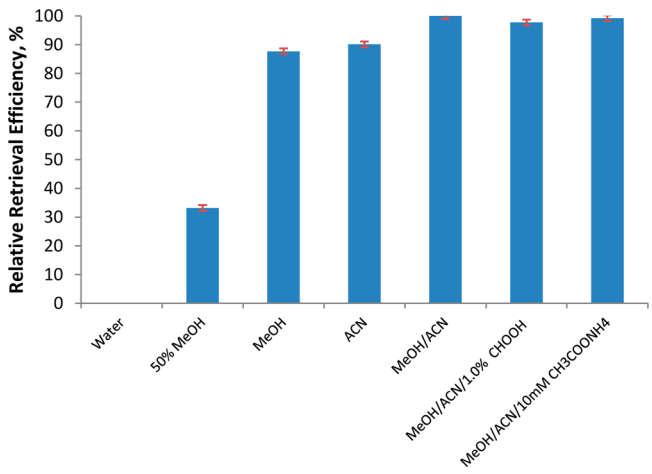

Figure 5. Comparing the effectiveness of retrieving AF B_1 retained on PD-MNPs with different eluting solvents.

Under the selected MSPE conditions, two standard solutions of aflatoxins at concentrations ranging from 0.0100–0.300 ng/mL were extracted to determine the extraction recovery for all the four aflatoxins tested. Aflatoxins extracted were quantified by using a calibration curve prepared from standard water solutions of aflatoxins. The recovery for the four aflatoxins ranged from 58.6 to 91.0% (Table 2). The difference in recovery is likely because of the fact that AF B_1 and AF B_2 are more hydrophobic than AF G_1 and AF G_2. These recovery values are significantly higher than many of those obtained from SPE procedures. It should be pointed out that the proposed MSPE offers an enrichment factor of 100, which is attractive for analysis of samples containing aflatoxins at very low levels.

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Table 2. Extraction Efficiency of the Proposed MSPE for Aflatoxins from Water

sample	aflatoxin	conc added (ng/mL)	conc found[a] (ng/mL)	SD (n = 3)	recovery (%)
std solution no. 1	AF B1	0.0300	0.0261	0.0012	87.0
	AF B2	0.0100	0.0074	0.0004	74.0
	AF G1	0.0300	0.0194	0.0012	64.6
	AF G2	0.0100	0.0060	0.0005	60.0
std solution no. 2	AF B1	0.300	0.2731	0.0119	91.0
	AF B2	0.100	0.0732	0.0056	73.2
	AF G1	0.300	0.1981	0.0077	66.0
	AF G2	0.100	0.0586	0.0021	58.6

[a]Means of three replicates.

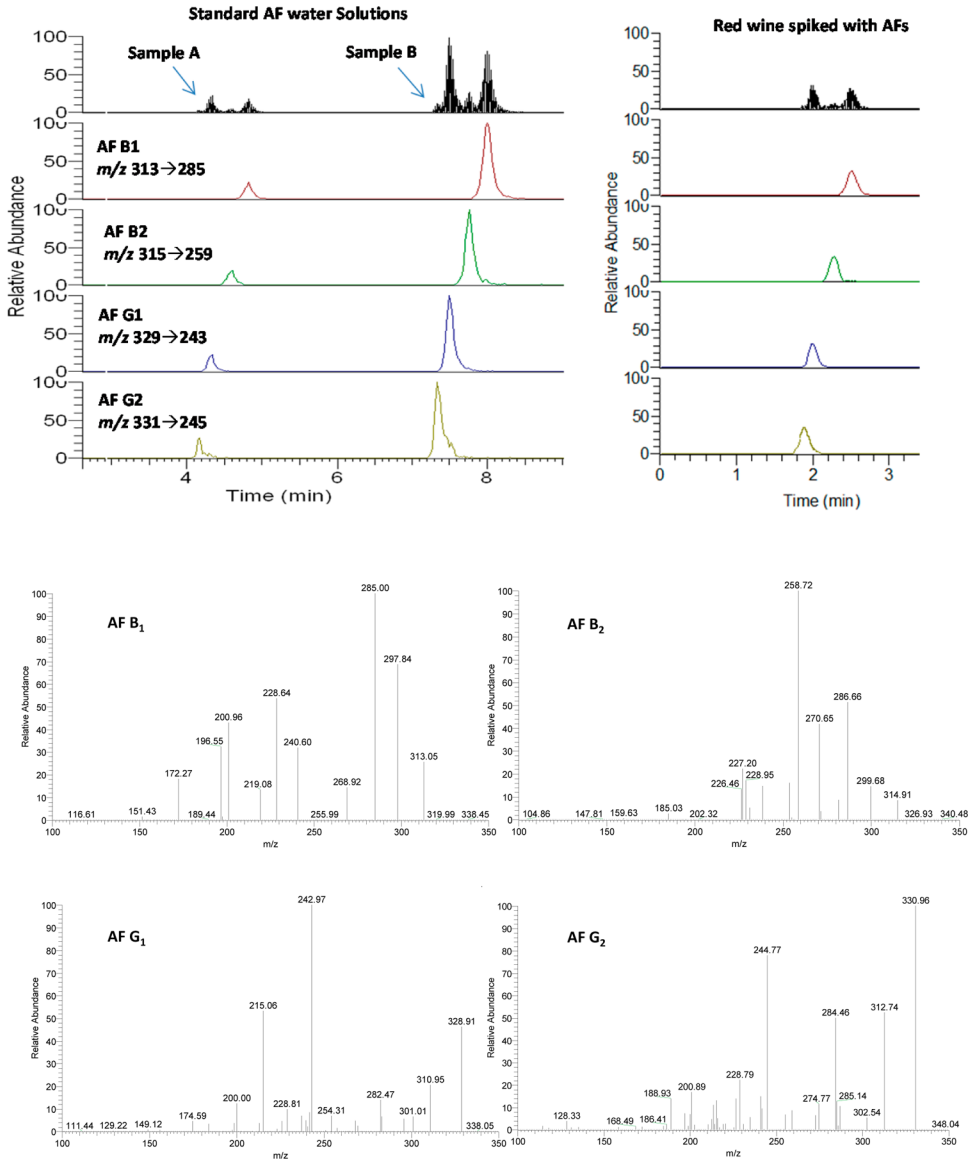

Figure 6. MSPE−HPLC−MS/MS determination of aflatoxins in water and red wine: chromatograms and MS^2 spectra for each aflatoxin involved. Sample A: water spiked with 0.0300 ng/mL of AF B_1 and G_1 and 0.0100 ng/mL of AF B_2 and G_2. Sample B: water spiked with 0.150 ng/mL of AF B_1 and G_1 and 0.0500 ng/mL of AF B_2 and G_2. Red wine: sample spiked with 0.0600 ng/mL of AF B_1 and G_1 and 0.0200 ng/mL of AF B_2 and G_2.

Quantification of Trace Aflatoxins in Red Wine Samples. Monitoring aflatoxin levels in foodstuff is highly important. In this work, quick, selective, and sensitive quantification of trace aflatoxins in red wines by coupling the proposed MSPE with HPLC−MS/MS analysis is demonstrated. To obtain the analytical figures of merit for the MSPE−HPLC−MS/MS method, simultaneous quantification of authentic AF B_1, AF B_2, AF G_1, and AF G_2 solutions was performed. The behaviors of chromatographic retention and product ion spectra of these four aflatoxins are shown in Figure 6. Under the selected HPLC−MS analytical conditions, the four aflatoxins were well-separated from each other. For each

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Table 3. Analytical Results of Red Wine Samples

	AF found (ng/mL)	AF added (ng/mL)	total AF found (ng/mL)	RSD (%, n = 3)	recovery (%)
sample no. 1					
AF B_1	ND	0.0100	0.0108	6.7	108.0
AF B_2	ND	0.0033	0.0030	7.1	90.9
AF G_1	ND	0.0100	0.0099	3.5	99.0
AF G_2	ND	0.0033	0.0033	5.2	100.0
sample no. 2					
AF B_1	ND	0.0600	0.0607	2.3	101.2
AF B_2	ND	0.0200	0.0194	1.8	97.0
AF G_1	ND	0.0600	0.0614	3.9	102.3
AF G_2	ND	0.0200	0.0197	3.6	98.5

ND: not detected.

aflatoxin tested, a characteristic product ion was obtained. Ion transitions (i.e., m/z 313 → 285 for AF B_1, m/z 315 → 259 for AF B_2, m/z 329 → 243 for AF G_1, and m/z 331 → 245 for AF G_2) were monitored using the SRM detection mode. Five-point calibration curves were prepared with authentic aflatoxin solutions at concentrations ranging from 0.00600 to 3.00 ng/mL in water. These solutions were submitted to the proposed MSPE procedure and injected into the HPLC−MS system for quantification. Peak areas were used for the calculation. Regression analysis of the results yielded linear calibration equations for all four aflatoxins tested with r^2 values >0.995. Interday (5 days) precisions of the slope and intercept of the calibration curves were found to be in the range between 3.5% and 5.6% (RSD, n = 5). From the calibration curves, the limits of detection were estimated to be in the range from 0.0012 ng/mL for AF B_1, AF B_2, and AF G_1 to 0.0031 ng/mL for AF G_2 (signal/noise = 3). These results indicate that the present method is very sensitive for the analysis of aflatoxins. It should be pointed out that these LODs are so low because the MSPE extraction provides a 100-fold enrichment factor (i.e., the sample is preconcentrated by a factor of 100 through the extraction), which is a significant gain of using the proposed MSPE procedure.

The proposed MSPE−HPLC−MS/MS method was applied to quantification of aflatoxins in liquid foodstuff samples, taking red wine as a model system. Two red wine samples were purchased from a local store and analyzed to determine the four aflatoxins. To verify the analytical results these samples were spiked with authentic aflatoxins and analyzed again. A typical chromatogram obtained from these analyses is shown in Figure 6. Peaks corresponding to AFs were well-identified. No unknown peaks appeared in the chromatogram, indicating that the method was specific for the determination of aflatoxins. The analytical results are summarized in Table 3. As can be seen, both the accuracy and the repeatability of the present MSPE−HPLC−MS method are good, because all recovery values are >90% and RSDs are <8%. These results suggest that the proposed method is useful for rapid quantification of aflatoxins in red wines. As shown in Table 3, aflatoxins were not detected in the wine samples tested. These results are in agreement with those reported previously.[12] Unlike some other mycotoxins such as ochratoxin A, aflatoxins in wine do not present a problem. Red wine samples are analyzed in this work as a model to demonstrate the usefulness of the proposed MSPE procedure. It is expected that the proposed method can be applied to analysis of other liquid foodstuff samples such as beer, vegetable fluids, among others. It is worth noting that because AF contents in these samples are normally below the LODs of HPLC−MS methods, enrichment of the analytes is needed prior to the analysis. The present MSPE procedure offers an enrichment factor of 100 for aflatoxins, which is attractive in analysis of these liquid foodstuff samples.

In conclusion, polydopamine-coated Fe_3O_4 core−shell superparamagnetic nanoparticles (PD-MNPs) can be easily prepared from amine-terminated Fe_3O_4 MNPs and dopamine via an in situ oxidative self-polymerization approach. The resultant PD-MNPs are of high stability and suspensibility. More importantly, they exhibit a high affinity to aflatoxins, thus enabling a facile and effective magnetic solid phase extraction (MSPE) of these toxins from large volume liquid samples. Using PD-MNPs as the adsorbent, MSPE of aflatoxins from liquid foodstuff samples proves effective for subsequent HPLC−MS/MS analysis and offers a considerable enrichment factor that is highly desired for quantification of trace aflatoxins. To the best of our knowledge, this is the first report on MSPE of this important group of toxins from foodstuff samples. As demonstrated in this work, the proposed MSPE−HPLC−MS/MS method is fast, very sensitive, and applicable to fast quantification of aflatoxins in water, red wine, and likely other liquid foodstuff samples.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AF, aflatoxin; AF B_1, aflatoxin B_1; AF B_2, aflatoxin B_2; AF G_1, aflatoxin G_1; AF G_2, aflatoxin G_2; MNPs, magnetic nanoparticles; AMNPS, amine-terminated magnetic nanoparticles; PD-MNPs, polydopamine coated magnetic nanoparticles; MSPE, magnetic solid phase extraction

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