Quantification of mycotoxins in vegetable oil by UPLC-MS/MS after magnetic solid-phase extraction

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ABSTRACT

The detection of mycotoxin contamination in foodstuffs is highly significant for public health. Herein we report an analytical method based on magnetic solid-phase extraction (MSPE) and UPLC-MS/MS for the simultaneous determination of mycotoxins, including fumonisins B₁ (FB₁), zearalenone (ZON) and ochratoxin A (OTA), in vegetable oil. Magnetic nanoparticles coated with double layers of silicon dioxide were synthesised and found to be an effective MSPE adsorbent for mycotoxins. The proposed MSPE procedure serves not only for sample clean-up but also for mycotoxin enrichment that enhances greatly the assay’s sensitivity. Under the selected MSPE conditions, linear matrix-matched calibration curves were obtained for mycotoxins in a concentration range from 0.178 to 625 μg kg⁻¹. The limits of detection were 0.210 μg kg⁻¹ for FB₁, 0.0800 μg kg⁻¹ for OTA and 1.03 μg kg⁻¹ for ZON. The proposed MSPE UPLC-MS/MS method was applied for the determination of mycotoxins in vegetable oil samples, including maize oil, rapeseed oil and soybean oil. ZON was detected in a maize oil at 101 μg kg⁻¹, which is below the European Union limit of 200 μg kg⁻¹ in foodstuffs.

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Introduction

Mycotoxins are toxic secondary metabolites mainly produced by different fungi such as *Aspergillus*, *Penicillium* and *Fusarium* species (da Rocha et al. 2014; Selvaraj et al. 2015), and they have significant negative impacts on human and animal health (Bryden 2012; da Rocha et al. 2014; Chen et al. 2015; Pinotti et al. 2016). Fumonisins (FBs) are a group of chemicals structurally similar to the sphingolipid intermediates sphinganine and sphingosine. They are produced by *F. verticillioides* and *F. proliferatum*, and are commonly found as corn contaminants worldwide. Among all the FBs, the most abundant naturally occurring analogue FB₁ has been classified as possible carcinogenic to humans by the IARC (Bryla et al. 2016; Wang et al. 2016). Zearalenone is one of the most common *Fusarium* mycotoxins in the temperate regions of America, Europe and Asia. It is most frequently encountered on corn, but also contaminates other cereals and plant products (Krska et al. 2003). Ochratoxin A (OTA) is produced by different species of fungi including *A. ochraceus*, *A. carbonarius*, *A. niger* and *P. verrucosum*, and it is an important food-borne mycotoxin that occurs in a wide variety of agricultural commodities ranging from cereal grains to dried fruits, to wine and coffee. Contamination generally occurs as a result of poor storage of commodities and suboptimal agricultural practices during the drying of foods (Bui-Klimke & Wu 2015; Marin & Taranu 2015). On the whole, mycotoxins contaminate a wide range of agricultural products, such as cereals, beans, oil seeds and so on. As one of the most important and widely consumed industrial products of those vegetables, vegetable oils may also be contaminated by mycotoxins.

Several analytical methods have been developed for mycotoxin determination in vegetable oils, including HPLC fluorescence (Papachristou & Markaki 2004; Bao et al. 2012; Gimenez et al. 2013), GC-MS/MS (Qian et al. 2015) and LC-MS/MS (Cavaliere et al. 2007; Gottschalk et al. 2009). Methods based on GC usually require a tedious derivatisation step. LC-MS/MS is an important tool for the analysis of various mycotoxins in agriculture products due to its high selectivity and specificity.
(Warth et al. 2012; Al-Taheer et al. 2013; Azaiez et al. 2014; Tsipalakou et al. 2014; Ediagbe et al. 2015; Jung et al. 2015; Fabregat-Cabello et al. 2016). However, a rapid and appropriate clean-up procedure is necessary to remove severe matrix effects in HPLC-MS analysis. In this sense, costly immunoaffinity columns have to be extensively used to extract mycotoxins from various sample matrices due to high specificity and sensitivity (Liao et al. 2011; Xue et al. 2014; Jung et al. 2015; Marley et al. 2015). Recently, sample extraction based on gel permeation chromatography (GPC) was applied for the analysis of mycotoxins in vegetable oil (Radova et al. 2001; Gottschalk et al. 2009; Qian et al. 2015). The major drawbacks of this method include an expensive solvent purification system and large consumption of toxic organic solvents.

In recent years, magnetic solid-phase extraction (MSPE) has been used to determine mycotoxins (Wu et al. 2011; Mashhadizadeh et al. 2013; Hashemi et al. 2014; McCullum et al. 2014). In MSPE, magnetic nanoparticles (MNPs) are dispersed into the sample solution to extract analytes and the adsorbent together with the analytes can be conveniently separated from the solution by applying an external magnetic field. The time-consuming column elution or filtration operations encountered in SPE can then be avoided. Therefore, MNPs can be widely used for the selective separation and pre-concentration procedure due to their high selectivity, simple operation procedures and low price. Up to date, the reported MSPE methods were designed to extract only one mycotoxin, or one group of mycotoxins with similar chemical structures, and there are no reports on the application of MSPE for multi-myco
toxin analysis.

In this paper, we developed a simple and cost-effective method using MNPs coated with double layers of silicon dioxide as a solid-phase adsorbent for UPLC-MS/MS determination of FB1, ZON and OTA in vegetable oils. Fe₃O₄@nSiO₂@mSiO₂ were synthesised and characterised with transmission electron microscopy (TEM) and Fourier-transform infrared spectra (FTIR). The experimental parameters affecting the extraction efficiency such as sorbent amount, adsorption time, eluting solvents and desorption time were investigated and analytical characteristics of the method were evaluated. To the best of our knowledge, this is the first time that MSPE is applied for the simultaneous separation and determination of three different kinds of mycotoxins. Finally, real vegetable oil samples were analysed to demonstrate the applicability of the proposed method.

Materials and methods

Reagents and chemicals

Acetonitrile (ACN), methanol (MeOH) and formic acid were HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Fumonisin B₁ (50 μg ml⁻¹) and ochratoxin A (OTA) (10 μg ml⁻¹) were purchased from Romer Laboratories (Tulln, Austria). Zearalenone (ZON) (50 μg ml⁻¹) (structures shown in Figure 1) was purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Other chemicals and solvents were of analytical grade. All toxin solutions were stored at -20°C. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA).

Preparation of Fe₃O₄@nSiO₂@mSiO₂

The MNPs were prepared following a previously reported process with a little modification (Liu et al. 2014). Briefly, magnetic Fe₃O₄ nanoparticles were prepared by a solvothermal reaction: 0.72 g sodium citrate, 4.8 g sodium acetate and 3.0 g FeCl₃·6H₂O were dissolved in ethylene glycol (100 ml) by vigorously stirring for 30 min. Then the solution was transferred into a Teflon-lined autoclave and reacted at 200°C for 12 h. The obtained black MNPs were rinsed with ethanol and water for several times before freeze drying at −50°C for 12 h. Then, 0.3 g of the obtained magnetic Fe₃O₄ nanoparticles were dispersed into 150 ml of HCl (0.1 M) solution by ultrasonication for 15 min. Subsequently, the treated Fe₃O₄ nanoparticles were washed with deionised water for several times, and 240 ml ethanol, 60 ml deionised water and 3.0 ml ammonia aqueous solution (28 wt%) were added. Then, 1.5 ml tetraethoxysilane (TEOS) was added drop wise into the mixture solution under stirring and reacted at RT for 24 h. The product was magnetically separated and washed with ethanol and water. Finally, 0.3 g of the obtained Fe₃O₄@nSiO₂ were dispersed in a mixture solution composed of
240 ml of H$_2$O, 180 ml of ethanol, 0.9 g of cetyltrimethyl ammonium bromid (CTAB) and 3.3 ml of aqueous ammonia (28 wt%) under ultrasonication for 15 min. Then 1.5 ml of TEOS were added drop wise to the solution under stirring. After reacting at RT for 24 h, the resultant magnetic Fe$_3$O$_4$@nSiO$_2$@mSiO$_2$ nanoparticles were washed with a mixture of HCl and C$_2$H$_5$OH (HCl: C$_2$H$_5$OH = 5:95, v/v) to remove the residual CTAB, and then were washed with ethanol and water several times followed by freeze drying at –50°C for 12 h.

**Characterisation of magnetic nanoparticles (MNP**s

TEM was performed with a JEOL JEM-2100 transmission electron microscope. Vibrating sample magnetometer (VSM) data were obtained by a SQUID magnetometer. FTIR were recorded in KBr with a Perkin-Elmer FTIR spectroscope.

**Mycotoxins standard solutions**

A mycotoxins standard mixture containing 1250 ng ml$^{-1}$ FB$_1$, 2500 ng ml$^{-1}$ ZON and 300 ng ml$^{-1}$ OTA was stored at –20°C until use. The work standard solutions were prepared daily by appropriate dilution with methanol/water (50:50, v/v) containing 1% formic acid.

**Sample preparation**

Aliquots (2 g) of vegetable oil samples were diluted to 10 ml with a methanol/water solution (80:20, v/v), vortex-mixed for 5 min and centrifuged at 10,000 rpm for 10 min. The supernatant, approximately 8 ml, was transferred to a new tube, and 24 ml of water were added. Subsequently, 10 ml of hexane were added to each tube. The tube was shaken vigorously for 30 s to wash the contents of the tube. The hexane layer was discarded and the wash was repeated once. Then the suspension of tube was centrifuged at 10,000 rpm for 10 min and filtered through a 0.22 μm membrane filter.

**Magnetic solid-phase extraction (MSPE) of mycotoxins**

A total of 20 ml of the sample solution was transferred to a 100 ml beaker and MNPs adsorbent (5 mg) was added. The solution was stirred for 10 min on an oscillator to facilitate the adsorption of mycotoxins onto the nanoparticles. Then the adsorbents were collected at the bottom of the
beaker by applying an external magnetic field via a magnet. The supernatant was discarded. After washing twice with 1 ml of water, 500 μl of acetonitrile/methanol (1:1) containing 1% formic acid were added as the desorbing solution and the suspension was stirred for 5 min. After desorption, the magnetic adsorbents were removed by the application of a magnet. A total of 400 μl of supernatant was transferred to a centrifuge vial and mixed with 400 μl of water. The mixed solution was filtered through a 0.22 μm membrane filter and injected into the UPLC-MS/MS system for analysis.

**UPLC-MS/MS analysis of mycotoxins**

Chromatographic separation was carried out using an Acquity UPLC system equipped with a 100 mm × 2.1 mm i.d., 1.7 μm, Acquity UPLC®BEH C18 column (Waters, Milford, MA, USA). A MeOH/water mixture (60/40, v/v) containing 2 mM ammonium acetate and 0.2% formic acid was used as the mobile phase at a flow rate of 0.2 ml min⁻¹. The column temperature was maintained at 35°C and the injection volume was 10 μl.

The Acquity UPLC system was coupled to a triple quadrupole tandem mass spectrometer (TQ Detector; Waters) with an electrospray ion source (ESI). The MS detector was operated in positive-ion mode with the following settings: capillary voltage, 3.5 kV; source temperature, 120°C; desolvation temperature, 350°C; desolvation gas flow, 650 l h⁻¹; and cone gas flow, 50 l h⁻¹. Argon was used as the collision gas, and the collision gas flow was 0.20 ml min⁻¹. Cone voltage and collision energy were optimised by infusion of each individual analyte. Detection was carried out in MRM mode. MRM parameters for MS detection of the three mycotoxins are summarised in Table 1.

| Mycotoxins | Precursor ion (m/z) | Product ion (m/z) | Cone voltage (V) | Collision energy (V) |
|------------|---------------------|------------------|------------------|---------------------|
| FB₁        | 722.5               | 352.3            | 55               | 42                  |
|            |                     | 324.3*           |                  |                     |
| ZON        | 319.0               | 301.0*           | 55               | 36                  |
|            |                     | 303.0           |                  |                     |
| OTA        | 404.0               | 239.0*           | 30               | 12                  |
|            |                     | 269.0           |                  |                     |
|            |                     | 252.0           |                  |                     |
|            |                     | 358.0           | 45               | 26                  |
|            |                     | 453.0           |                  |                     |

Note: *Means of the quantifier ion.

**Results and discussion**

**Characterisation of magnetic nanoparticles (MNPs)**

The FTIR spectrum (Figure 2(a)) showed strong absorptions at 571 cm⁻¹ arising from the Fe-O bond vibrations, and the one at 1080 cm⁻¹ was ascribable to the asymmetric linear vibration of the Si-O-Si bond. After coating silica shells, the apparent absorption intensity of the Fe-O bond vibrations decreased, while the relative absorption intensity of Si-O-Si bond increased. Figure 2(b) shows the magnified hysteresis loops of samples. The saturation magnetisation value of Fe₃O₄ was 63.8 emu g⁻¹. After being coated with a layer of silica, the saturation magnetisation of the material decreased to 42.9 emu g⁻¹ and then further decreased to 25.6 emu g⁻¹ after coating with the second layer of silica. The decrease in saturation magnetisation was attributed to the presence of the non-magnetic SiO₂. TEM images of Figure 2(c) showed that after coating with silica, the size of nanospheres increased from 200 to 260 nm, and then 430 nm, which indicated that two silica shells had been coated on the surface of Fe₃O₄ core.

**Sample preparation**

Mixtures of MeOH/H₂O, ACN/H₂O have often been used as extraction solutions for the determination of the three mycotoxins. To choose a suitable extraction solution, extraction efficiencies of four different extraction solutions (ACN/H₂O at ratios of 100:0, 80:20 and MeOH/H₂O at ratios of 100:0, 80:20) were investigated. The extraction recoveries of mycotoxins were best when MeOH/H₂O (80:20 v/v) was used as extraction solution. To reach good recovery, MeOH/H₂O (80:20 v/v) was chosen to extract mycotoxins.

Loss of up to 20% of FB₁ was observed in glass tubes during evaporation of the MSPE eluate. It was suggested that the amine group of the mycotoxins could bind to the free silanol groups on the glass surface of the used test tubes. The loss level was lower than the results reported previously, which was up to 75% (Nielsen et al. 2015). Therefore, we selected the method of dilution of the MSPE eluate instead of evaporation.

**MSPE for mycotoxins with Fe₃O₄@mSiO₂@mSiO₂ MNPs**

MSPE is an equilibrium-driven process and its efficiency depends on the partitioning of mycotoxins
between the aqueous phase and the adsorbents. Therefore, it is necessary to optimise different parameters affecting the efficiency of MSPE procedure, such as sorbent amount, adsorption time, eluting solvents and desorption time. All optimisations were carried out by analysing 20 ml of a standard solution containing 300 ng of FB, 600 ng of ZON and 75 ng of OTA.

The amount of MNPs varied in the range of 1–15 mg to optimise the adsorbent amount. It was found that the highest extraction efficiency was obtained when increasing the amount of adsorbents up to 5 mg while no additional enhancement of peak area was observed with adsorbents more than that amount. Thus, 5 mg of magnetic adsorbent were used in all experiments. To achieve a better extraction efficiency with shorter analysis time, it is necessary to select an adsorption time that provides the equilibrium between sample solution and adsorbent. Extraction times of 5, 10, 15 and 20 min were investigated. It was found that 10 min was sufficient to attain an adsorption equilibrium between target compounds and adsorbents. Desorption of the analyte from the surface of the adsorbent is a rather critical step. The selection of an eluting solvent is of major importance for the optimisation of the analytical procedure. The usefulness of several eluting solvents including H₂O, MeOH, ACN, MeOH/1% HCOOH, ACN/1% HCOOH, 50% MeOH/50% ACN/1% HCOOH and 50% MeOH/50% ACN/2% HCOOH was investigated in the preliminary experiments. The best desorption efficiency was obtained when using 50% MeOH/50% ACN/1% HCOOH as the eluting solvent (Figure 3). Desorption time (2, 5 and 10 min) was investigated to achieve high retrieval efficiency. The experimental results indicated

Figure 2. Characterisation of magnetic nanoparticles: (a) FTIR spectra of Fe₃O₄, Fe₃O₄@nSiO₂, and Fe₃O₄@nSiO₂@mSiO₂; (b) magnified hysteresis loops of materials: Fe₃O₄, Fe₃O₄@nSiO₂ and Fe₃O₄@nSiO₂@mSiO₂; and (c) TEM images of Fe₃O₄, Fe₃O₄@nSiO₂ and Fe₃O₄@nSiO₂@mSiO₂.
that 5 min of stirring of the mixture solution was sufficient and no significant effect was observed when the time was greater than 5 min. An equal amount of water was added to the elution solution prior to UPLC-MS/MS analysis in order to minimise the solvent effects on the separation and improve detection sensitivity of FB$_1$.

During MSPE method development, we found that addition of more than 3 ml water per ml of methanol could achieve a better extraction efficiency. Conversely, addition of less water decreased the recoveries of ZON and OTA significantly.

Under the MSPE conditions selected, two groups of standard solutions containing the three mycotoxins at concentrations ranging from 2.73 to 22.7 ng ml$^{-1}$ and from 27.3 to 227 ng ml$^{-1}$ were extracted to determine the extraction recovery. The mycotoxins extracted were quantified using a calibration curve prepared from standard water solutions of mycotoxins. The recoveries for the three mycotoxins ranged from 85.0% to 94.7% (Table 2).

### UPLC-MS/MS optimisation

For the chromatographic separation, an UPLC BEH C$_{18}$ column was selected with different mobile phases being tested, including MeOH/H$_2$O (60:40) containing 2 mM ammonium acetate, MeOH/H$_2$O (60:40) containing 0.1% formic acid, MeOH/H$_2$O (60:40) containing 0.2% formic acid, MeOH/H$_2$O (60:40) containing 2 mM ammonium acetate and 0.2% formic acid, and ACN/H$_2$O (60:40) containing 2 mM ammonium acetate and 0.2% formic acid. Finally, after testing different mobile phases, MeOH/H$_2$O (60:40) containing 2 mM ammonium acetate and 0.2% formic acid was selected due to the best separation efficiency and detection sensitivity.

### Quantification of mycotoxins in vegetable oil samples

Analytical characteristics of the recommended procedure were evaluated under optimised conditions. The behaviours of chromatographic retention and MRM chromatogram of the three mycotoxins are shown in Figure 4. Matrix-matched calibration curves were performed by spiking known amounts of analyte to blank vegetable oil samples. With the optimised MSPE procedure, calibration curves were obtained by least-squares linear regression analysis of the peak area ($n=5$) versus concentration of each analyte using six concentration levels. Linearity of calibration curve was observed in concentrations ranging from 0.178 to 625 μg kg$^{-1}$ with correlation coefficients > 0.997. Inter-day (5 days) precisions of the slope and intercept of the calibration curves were found to be in the range 3.1–5.3% (RSD, $n=5$). From the calibration curves, the LODs were estimated to be 0.210 μg kg$^{-1}$ for FB$_1$, 0.0800 μg kg$^{-1}$ for OTA and 1.03 μg kg$^{-1}$ for ZON (signal/noise = 3). These results indicated that the present method was

![Figure 3](image_url). Comparison of the effectiveness of retrieving mycotoxins retained on magnetic nanoparticles (MNP) with different eluting solvents.

![Table 2](image_url). Extraction efficiency of the proposed magnetic solid-phase extraction (MSPE) for mycotoxins from water.

| Sample          | Mycotoxins | Concentration added (ng ml$^{-1}$) | Concentration found (ng ml$^{-1}$)$^a$ | SD (n = 3) | Recovery (%) |
|-----------------|------------|------------------------------------|---------------------------------------|------------|--------------|
| Standard solution 1 | FB$_1$     | 11.4                                | 10.6                                  | 0.5        | 93.0         |
|                 | ZON        | 22.7                                | 19.3                                  | 0.8        | 85.0         |
|                 | OTA        | 2.73                                | 2.53                                  | 0.1        | 92.7         |
| Standard solution 2 | FB$_1$     | 114                                 | 108                                   | 2.1        | 94.7         |
|                 | ZON        | 227                                 | 198                                   | 9.9        | 87.2         |
|                 | OTA        | 27.3                                | 24.7                                  | 0.6        | 90.5         |

Note: $^a$Means of three replicates.
Figure 4. MRM chromatogram of (a) a blank vegetable oil sample and (b) a blank vegetable oil sample spiked with 2.77 μg kg⁻¹ of FB₁, 5.54 μg kg⁻¹ of ZON and 0.665 μg kg⁻¹ of OTA.
sufficiently sensitive for the analysis of mycotoxins in vegetable oil.

Accuracy and precision were assessed by spiking blank vegetable oil sample with the analytes at three levels, respectively. Concentrations were calculated by matrix-matched standard calibrations. Data in Table 3 show recoveries ranging from 89.4% to 97.1% with associated RSDs ≤5.7%. The acceptable recoveries suggest that the proposed MSPE method is sufficiently applicable for the determination of the three mycotoxins in vegetable oil samples. Comparing with other sample preparation approaches previously reported for oil analysis, all recoveries of the present MSPE UPLC-MS/MS method are good. As shown in Table 4, analytical figures of this method are compared for several HPLC-based quantitative methods reported for analysis of mycotoxins in oils (Ferracane et al. 2007; Majerus et al. 2009; Drzymala et al. 2015; Li et al. 2015).

To assess the applicability of the proposed method for the detection of the three mycotoxins in vegetable oil samples, three samples including maize oil, rapeseed oil and soybean oil were purchased from local markets and analysed to determine the three mycotoxins. Only ZON was found at concentration of 111 μg kg⁻¹ in the maize oil; FB₁ and OTA were not detected in the three samples tested. Based on the European Union limit of 200 μg kg⁻¹ for ZON in refined maize oil, the residue level found was acceptable.

### Conclusions

In conclusion, MNPs coated with double layers of silicon dioxide were successfully prepared and used as a MSPE sorbent. A selective extraction of three mycotoxins from vegetable oil with the novel MSPE sorbent was developed. Extracted mycotoxins were subsequently subjected to UPLC-MS/MS analysis. This is the first time that MSPE has been used successfully for extracting three kinds of mycotoxins from vegetable oil samples. As demonstrated, the proposed MSPE UPLC-MS/MS method is fast, very sensitive and well suited for the rapid quantification of the three mycotoxins in foodstuff samples.

### Disclosure statement

No potential conflict of interest was reported by the authors.

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