Selective magnetic nanographene oxide solid phase extraction with high performance liquid chromatography-fluorescence detection for determination of zearalenone in corn samples

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Abstract

A magnetic nanographene oxide sorbent as a selective sorbent for the magnetic solid phase extraction combined with high performance liquid chromatography-fluorescence detection

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was developed and proved to be a robust method for zearalenone determination in corn samples. Optimum extraction of zearalenone (20 mg magnetic nanographene oxide sorbent, extraction for 15 min, desorption time of 15 min using 1 mL of 0.5% formic acid in methanol) resulted in low limits of detection (0.05 mg/L) and quantitation (0.13 mg/L) and good linearity range of 0.13–1.25 mg/L with the correlation coefficient of 0.9957. The acceptable recoveries (79.3–80.6%) with relative standard deviations below 4.0% and satisfactory intra- and inter-day precisions (2.0–7.4%) were achieved. Additionally, the proposed method has been proved to be good in several aspects: easily prepared sorbent with high affinity to zearalenone, convenient and fast procedure and high extraction efficiency.

KEYWORDS

corn, zearalenone, graphene oxide, high performance liquid chromatography, magnetic solid phase extraction

Abbreviations: ZEA, zearalenone; MNGO, magnetic nanographene oxide; GO, graphine oxide; MNPs, Fe_3O_4 particles; MSPE, magnetic solid phase extraction; FLD, fluorescence detection

1 INTRODUCTION

Zearalenone are naturally occurring mycotoxins produced by several *Fusarium* species that contaminates commonly in maize and other crops *e.g.*, wheat, barley and rye [1–4]. Zearalenone is not classified as carcinogen by International Agency for Research on Cancer [3] but it and its metabolites induce adverse hormonal effects in animals such as reproductive disorders and abnormal fetal development due to mimicking the action of 17β-estradiol [1].

Additionally, zearalenone causes mycotoxicosis in farm animals including hepatocarcinogenesis, nephropathy and hematotoxicity in rodents and the reduction of milk production in cows [1, 5]. Therefore, for minimization of zearalenone risk to animal and human healths, the Panel on Contaminants in the Food Chain established a tolerable daily intake for zearalenone of 0.25 μg/kg [4] and the European Commission recommended the limit range of 0.1–3.0 mg/kg for zearalenone in feed materials, complementary and complete feedingstuffs [6].

Quantitative analysis of zearalenone in maize and other crop productions is still challenging due to the complex matrix of samples and requirement of pretreatment and extraction before analysis. Available extraction methods such as liquid-liquid extraction [1, 7–8] and SPE [9–10] have been employed for determination of zearalenone. The liquid-liquid extraction is limited because of time consuming and laborious. Compared to traditional SPE, magnetic solid phase extraction has attracted increasing interests since it avoids time-consuming and tedious on-column SPE step. The magnetic solid phase extraction leads to rapid and simple separation in the presence of an external magnet, without the need of centrifugation and filtration [11]. Recently, the nanomagnetic composite of graphene as a sorbent has been developed for determination of drug, pesticides and polysaccharides due to its high adsorptive properties and specific surface area with the separation convenience of magnetic materials [12–15]. Up to date, the determination of zearalenone by magnetic solid phase extraction based on magnetic nanographene oxide has not been reported.

In this study, the magnetic nanographene oxide was synthesized by a simple and rapid one-step method. Subsequently, we have applied it as a novel adsorbent for magnetic solid phase extraction in combination with HPLC-fluorescence detection for determining zearalenone in corn samples. To obtain the best performance extraction efficiency of

zearalenone, various parameters *i.e.*, amount of magnetic nanographene oxide, extraction time, ionic strength, desorption volume, concentration of formic acid in methanol and desorption time were thoroughly optimized and the analytical performance of the developed method was evaluated. As a result, not only the selectivity of zearalenone detection was much improved but also it demonstrated the successful application of method combined with HPLC-fluorescence detection for effective extraction and pre-concentration of zearalenone in corn samples with satisfactory analytical results. Furthermore, the synthesized magnetic nanographene oxide per gram (about 0.1 $) was 34 fold cheaper than commercial C_{18} cartridge (3.4 $).

2 MATERIALS AND METHODS

2.1 Reagents

All reagents were of analytical grade. Zearalenone (ZEA) was purchased from Sigma-Aldrich (USA). Graphene oxide solution (10 mg/mL, 7 μm) and iron (II) sulfate heptahydrate were purchased from TCI (Japan). Acetonitrile, methanol, sodium chloride and HPLC grade of acetonitrile and methanol were purchased from LabScan (Thailand). Iron (III) chloride hexahydrate was obtained from Lobachemie (India). Formic acid and ammonia solution were obtained from Merck (Germany). Ultrapure water was prepared by ELGA purification system (England).

A stock standard solution of ZEA was prepared by dissolving 10 mg solid standard in 10 mL methanol to obtain a concentration of 1000 mg/L and aliquots of standards were transferred to 1.0 mL amber vials for single use. Working solutions were further diluted with appropriate volume of methanol. These standard solutions were stored in the freezer at –20°C when not in use.

2.2 Synthesis of magnetic nanographene oxide

Magnetic nanographene oxide (MNGO) synthesis was based on the *in situ* chemical co-precipitation method according to a previous report [12] with modifications. The 150 µL of 10 mg/mL graphene oxide (GO) dispersion was added with 120 mL of a mixture of $FeCl_3.6H_2O$ (1.62 g) and $FeSO_4.7H_2O$ (0.84 g) and the dispersion was then vigorously stirred at room temperature. Ammonia solution was added dropwise to the solution until the pH of solution was in the range of 10–11.

Subsequently, the temperature of reaction was increased to 60°C under stirring for 2 h to ensure the complete synthesis of MNGO. After cooling down to room temperature, the MNGO were obtained and separated from the solution by external magnet. These MNGO sorbents were further washed repeatedly for 3 times with ultrapure water to remove residues such as sulfate and chloride and isolated by external magnet. Finally, the MNGO sorbents were dried at 70°C for 4 h and kept in desiccator. By this synthesis method, about 2 g MNGO sorbent was obtained. The surface morphology of synthesized MNGO was also compared which Fe_3O_4 particles (MNPs) homemade. The synthesis method of MNPs is similar to that of MNGO but 150 µL of 10 mg/mL GO was not added to a mixture of $FeCl_3.6H_2O$ (1.62 g) and $FeSO_4.7H_2O$ (0.84 g).

2.3 Sample preparation

Corn samples were supplied by the National Corn and Sorghum Research Center (Bangkok, Thailand) and collected from vendors in Songkhla province. The sample preparation was performed according to a previous report [1] with slight modification. Each sample was weighed to the nearest of 2.000 g into a 50 mL centrifuge tube and 10 mL of a mixture of acetonitrile/water (85/15, v/v) was added. After soaking the sample for 5 min, the solution was further extracted by ultrasonication for 30 min followed by centrifugation at 6000 rpm

for 20 min. An aliquot (8 mL) of resulting supernatant was transferred into a clean vial and dried under a stream of nitrogen gas at 50°C, and the dried sample was stored at 4°C. Before direct clean-up and pre-concentration of ZEA by magnetic solid phase extraction (MSPE), the dried sample was re-dissolved with 2 mL of a mixture of acetonitrile and water (5/95, v/v).

2.4 Magnetic solid phase extraction procedure

Magnetic solid phase extraction (MSPE) procedure is shown in Fig. 1. A 20 mg of MNGO was dispersed into a 2 mL of above sample solution and the mixture was shaken for 15 min to reach the adsorption equilibrium. Next, the adsorbent was isolated from the solution by external magnet that was deposited at outside of the sample vial. The target ZEA was desorbed from the isolated MNGO with 1 mL of methanol containing 0.5% formic acid by ultrasonication for 15 min. The magnet was used to isolate MNGO from the analyte solution. Finally, the resulting desorption solution was further filtered through 0.22 μm Nylon filter and 20 μL of sample solution was injected into the HPLC-fluorescence detection system for analysis.

2.5 HPLC-fluorescence detection system

The HPLC-fluorescence detection (FLD) analysis was performed on an Agilent 1200 series (USA) system. The separation was conducted on an Eclipse XDB C_{18} column (4.6 × 150 mm id, 5 μm) (Agilent, USA) at 40°C. The HPLC system was slightly modified from a previous report [16]. The isocratic mobile phase consisting of water/acetonitrile/methanol at the ratio of 35/10/55 (by volume) was used at a flow rate of 1.0 mL/min. The injection volume was 20 μL, and the detector was set for an excitation wavelength of 275 nm and emission wavelength of 450 nm.

3 RESULTS AND DISCUSSION

3.1 Characterization of magnetic nanographene oxide

The surface morphology of synthesized magnetic nanographene oxide (MNGO) was examined by SEM method and compared with Fe_3O_4 particles (MNPs) and graphene oxide (GO) as provided in Fig. 2. The MNPs consist of a large quantity of nearly uniform monodispersed spheres (Fig. 2A) whereas the GO appears to be sheet-like structure with smooth surface (Fig. 2B). The surface of MNGO (Fig. 2C) was clearly covered with monodispersed spheres of MNPs, resulting in a rougher surface than GO.

Furthermore, the successfully synthesized MNGO was confirmed by FTIR analysis in the range of 400-3900 cm^{-1} and compared with MNPs and GO (Fig. 3). The broad peak at 3400 cm^{-1} corresponds to the O–H stretching vibration. The band around 1625 cm^{-1} is appointed to the aromatic C-C stretching vibration peak. Furthermore, for GO and MNGO, the peak at 1063 cm^{-1} represents the C-O stretching. GO possesses peak at 1715 cm^{-1} that corresponds to the C=O stretching, however, this peak of MNGO was weakened. The distinct IR peak of MNGO and MNPs at 636 cm^{-1} is assigned to the formation of Fe–O, confirming the presence of Fe_3O_4 on the structure of MNGO. Results of SEM and FTIR indicated the successful synthesis of MNGO and were in good agreement with previous work [12].

Based on the structure of MNGO and ZEA (Fig. 1), the possible interaction between MNGO and ZEA might be due to (i) the hydrogen bond between hydroxyl groups on benzene ring of ZEA and active groups such as hydroxyl, carboxyl, and epoxy polar groups of MNGO and (ii) π-π stacking between aromatic rings of ZEA and MNGO. All these interactions can facilitate the adsorption of ZEA on the surface of MNGO which then enhance the extraction efficiency. It is possible that other compounds composed of aromatic or O/N groups can be adsorbed onto MNGO. However, the extraction parameters were optimized for ZEA,

resulting in high selectivity of MNGO. Furthermore, MNGO solid phase extraction combined with HPLC-fluorescence detector are sensitive and selective for ZEA in terms of efficient separation from other compounds. Thus, MNGO sorbent is ready to use for extracting ZEA in real samples.

3.2 Optimization of magnetic solid phase extraction conditions

To develop the method for the clean-up and preconcentration of ZEA using MNGO-MSPE method and achieve highest extraction efficiency, the considerable experimental parameters were evaluated (Fig. 4). A series of aqueous solution spiked with 0.25 mg/L of ZEA was used to study the extraction performance. Each parameter was studied while other parameters were kept constant. Three parallel experiments were performed for each parameter and HPLC peak area was presented as the evaluation of extraction efficiency. Statistical analyses were evaluated using the Microsoft Excel sofware 2007 and p value of <0.05 was considered significant.

3.2.1 Effect of magnetic nanographene oxide amount

The effect of different amount of MNGO was investigated in the range of 10–50 mg (Fig. 4A). The peak area response increased up to 20 mg which provided the sufficient surface for ZEA adsorption and then decreased, probably due to MNGO aggregation, resulting in a decrease of the effective adsorption surface area [12]. Based on the result, 20 mg of MNGO was selected for subsequent study.

3.2.2 Effect of extraction time

Different extraction times (3–20 min) with shaking were examined to maximize adsorption equilibrium process of ZEA between sorbent and sample solution (Fig. 4B). The highest peak area was achieved at 15 min, indicating sufficient contact time for ZEA and MNGO to reach

the equilibrium state. A decrease in response at 20 min was attributed to the longer extraction time that may result in the separation of MNGO particles from each other which can lower the interaction of ZEA and sorbent. Thus, 15 min was considered as the optimal extraction time.

3.2.3 Effect of ionic strength

Salt can increase the ionic strength of an aqueous sample and subsequently reduce the solubility of analyte due to the salting-out effect [13]. Thus, to examine whether ionic strength could affect the adsorption of ZEA onto MNGO, effect of ionic strength was studied in the range of 0.0–0.6% (w/v) NaCl. As can be seen in Fig. 4C, the peak area remained steady with the concentration of NaCl between 0 and 0.3% (w/v). This can be clearly proved that salting-out effect was not involved in the adsorption of ZEA. By increasing the concentration of NaCl higher than 0.3% (w/v), the response went down due to increased viscosity of sample solution. At higher solution viscosity, the diffusion coefficient of analyte will be low, and thus the mass transfer rate of ZEA in solution into MNGO may be slow. Based on these results, no NaCl was added for further study.

3.2.4 Effect of desorption volume

The desorption step is another important factor that affects the MSPE performance. Good desorption efficiency is based on desorption solvent that can elute the adsorbed analyte completely from the sorbent without destruction. Three desorption solvents with different polarity *i. e.*, methanol, acetonitrile and acetone were investigated with HPLC peak area values of 33.31±0.82, 17.59±1.16, 15.83±0.90, respectively. As a result, methanol was the most suitable desorption solvent. Further study involved with different volume of methanol ranging from 1.0–3.0 mL as illustrated in Fig. 4D. The extraction efficiency was highest at 1.0 mL, indicating the sufficient volume to elute ZEA completely. With further increase of

the volume (1.5–3.0 mL), the response dramatically decreased due to dilution effect. Thus, 1.0 mL of methanol was chosen for efficient desorption.

3.2.5 Effect of concentration of formic acid in methanol

It was reported that desorption efficiency of ZEA and its derivatives was enhanced by adding formic acid into the desorption solvent [1]. To ensure desorption of the target ZEA from the MNGO, the effect of concentration of formic acid (%) in methanol was investigated in the range of 0.0 to 5.0%. As can be seen in Fig. 4E, the response significantly increased from 0.0 to 0.5%. This phenomenon is consistent with a previous report based on multi-walled carbon nanotube magnetic nanoparticles [1]. The response then remained constant between 0.5 and 5.0%. Consequently, methanol containing 0.5% formic acid was chosen for next experiment.

3.2.6 Effect of desorption time

Different desorption times (5–25 min) were investigated to make sure that ZEA was completely desorbed (Fig. 4F). The response was enhanced with desorption time from 5–15 min and then decreased after 15 min possibly due to MNGO aggregation. Consequently, optimum desorption time was set to15 min.

Based on the above results, the optimized MSPE conditions for ZEA were as follows: 20 mg MNGO sorbent, extraction for 15 min, desorption time of 15 min using 1 mL of 0.5% formic acid in methanol as desorption solvent.

3.3 Method validation

To evaluate linearity of the method, a calibration curve was plotted using five concentrations of ZEA from 0.13–1.25 mg/L. Very good linear curve for ZEA ($y = 14.4920 (\pm 0.5500) x + 1.4189 (\pm 0.4182)$) was obtained with the coefficient of determination (r^2) of 0.9957.

The limit of detection (LOD) was calculated based on the determination of the slope of the calibration curve and the standard deviation (SD) of blank response using the equation [17]: $\mathrm{LOD} = 3.3\frac{\mathrm{SD}}{\mathrm{Slope}}$ In this work, the slope and SD of blank response were 14.8750 and 0.21 (n = 20), respectively, thus the LOD was 0.05 mg/L. According to US FDA guideline [18], the limit of quantitation (LOQ) is the lowest concentration of the calibration curve which can be measured with acceptable accuracy (80–120%) and precision of 20%. The LOQ was calculated to be 0.13 mg/L, with recovery of 82.9% and precision (RSD) of 5.1%.

The accuracy of the method was assessed by recovery test which was described as follows: 1 mL of appropriate concentration of ZEA was spiked to a blank corn sample separately to obtain final concentrations of 0.13, 0.25 and 0.61 mg/kg, respectively. These spiked samples were left at room temperature for 5 min to evaporate solvent and were prepared by a method mentioned in Section 2.3, followed by MSPE extraction and HPLC-FLD analysis. The recovery can be calculated by the equation: $\text{Recovery}\ (\%) = \frac{(C_S - C_U)}{C_{Std}} \times 100$ where C_S, C_U and C_{Std} are the analyte concentration in the spiked sample, the analyte concentration in the un-spiked sample and the concentration of spiked standard, respectively.

As presented in Table S1, satisfactory recoveries ranged from 79.3 to 80.6% with RSDs less than 4.0% that were acceptable within ranges of 70–120% set by the standard Commission Regulation (EC) No. 401/2006 [19]. This indicated the suitability of the method for determining ZEA by this work.

Precisions expressed as %RSD were validated by intra-and inter-day tests at two final concentration levels of 0.13 and 0.50 mg/kg which were spiked to corn samples. The intra-day precision (repeatability) was performed on the same day with three replicates whereas the

inter-day precision (reproducibility) was measured on two different days and each day was performed three replicates. The %RSD for intra-and inter-day precisions were 2.0–3.9% and 2.5–7.4%, respectively, which agreed well with AOAC values [20].

The preconcentration factor, defined as the ratio of the analyte concentration in the final extract to the initial analyte concentration in the sample solution before extraction [21], was calculated using the three final concentration levels (0.25, 0.50 and 1.00 mg/L). Under the optimum conditions, the average preconcentration factor was found to be 39. For above validation data, it was clearly presented that the MSPE based on MNGO followed by a HPLC-FLD technique was highly accurate, reliable and sensitive.

3.4 Matrix effect

The matrix effect can cause a positive or negative response of analyte [22], thus it was evaluated by comparing the slope of standard addition curve or matrix-matched calibration curve performed by addition of standards into the extracted samples with the slope of standard curve for the ZEA concentration ranges of 0.1–5.0 mg/L. The study of matrix effect was fully described in the supporting Information (Fig. S1). The slope of standard addition curve (11.0320) did not significantly differ from that of standard curve (11.1510), meaning that the determination of ZEA from corn samples was not influenced by the matrix interference. Thus, the standard addition curve was unnecessary and the standard curve constructed in methanol was applied for quantifying ZEA.

3.5 Comparison with other methods

The performance of the developed MNGO-based MSPE with HPLC-FLD method was compared with other previously reported methods in Table S2, including extraction method,

extraction time, LOD, recovery and precision (SD). The developed method has many advantages compared to previous methods. It consumes shorter extraction time which is of advantage to extract large numbers of samples. The liquid-liquid extraction method is time consuming, with attendant generation of chemical wastes, while method based on immune-affinity is limited in being time consuming and cost ineffective.

A comparable recoveries of other methods (71.9–119.9%) and our present work (79.3–80.6%) are within same range. For the precision (SD), our method was found to be a very precise method (1.5–3.2) when compared to other reports (0.3–14.6). The LOD for ZEA by ELISA (0.35 ng/mL) was lower than that reported in the present study (47.7 ng/mL), but ELISA is more expensive and time consuming (~150 min).

From several advantages of the developed method as mentioned earlier, it can be asserted that our method is very efficient and can be applied in the future as a routine method for monitoring of ZEA in corn samples.

3.6 Application in real samples

The developed method was applied to analyze ZEA in six corn samples as shown in Table S3. In this study, ZEA was not found in any sample. The comparison of ZEA contents in this study and in different countries was presented in Table S4. The mean concentration from different regions was found to be higher than our findings. This is probably due to many factors including weather on plant infection and toxin formation and different storage management [23].

Fig. S2 presents the HPLC chromatograms of ZEA standard solution, corn sample and spiked corn sample. As can be seen, the peak shape of ZEA standard is good with retention time of 6.7159±0.0602 min. In addition, the MNGO-MSPE degree of clean-up is clearly

shown in the absence of matrix interference peaks close to ZEA peak in samples, resulting in clean chromatogram of sample solution and spiked sample solution. It was reported that α-zearalenol, β-zearalenol, zearalanone, α-zearalanol and β-zearalanol are ZEA's derivatives. Thus, ZEA and its derivatives standards were investigated under current chromatographic conditions (Fig. S3A), which ZEA can be separated completely from its derivatives. Furthermore, the selectivity of MNGO towards ZEA together with its derivatives was proved by adding 0.25 mg/L of ZEA and its derivatives to corn samples, followed by extraction under optimized conditions. As seen in Fig. S3B, MNGO selectively absorbs ZEA but not its derivatives. These results demonstrate the selectivity of established method is high and allows ZEA to be quantified precisely.

4 CONCLUDING REMARKS

A new method based on the MSPE using MNGO with HPLC-FLD for determination of ZEA in corn samples was established for the first time. Our method has several advantanges. The MNGO has a high affinity to ZEA and the MSPE process is convenient and exhibited satisfactory performance including good linerity, low detection limit and high recovery with high precision. The determination of ZEA in samples can be performed using a calibration curve in methanol. Nevertheless, the established method was appropriate for separation and quantitative analysis of ZEA from real corn samples. The extraction method was not only effective but also faster than the traditional SPE, thus it can be an alternative method for routine work.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure captions in Electronic Supplementary Material

FIGURE S1 The comparison of standard curve and standard addition curve for zearalenone

FIGURE S2 HPLC chromatograms of (A) zearalenone standard solution of 0.5 mg/L, (B) corn sample and (C) corn sample spiked with 0.5 mg/L zearalenone

FIGURE S3 HPLC chromatograms of (A) standard mixture of 0.25 mg/L zearalenone (6) and its derivatives (β-zearalenol (1), α-zearalanol (2), β-zearalanol (3), zearalanone (4), α-zearalenol (5)) and (B) corn sample spiked with 0.25 mg/L zearalenone and its derivatives

Figure captions

FIGURE 1 The magnetic solid phase extraction procedure

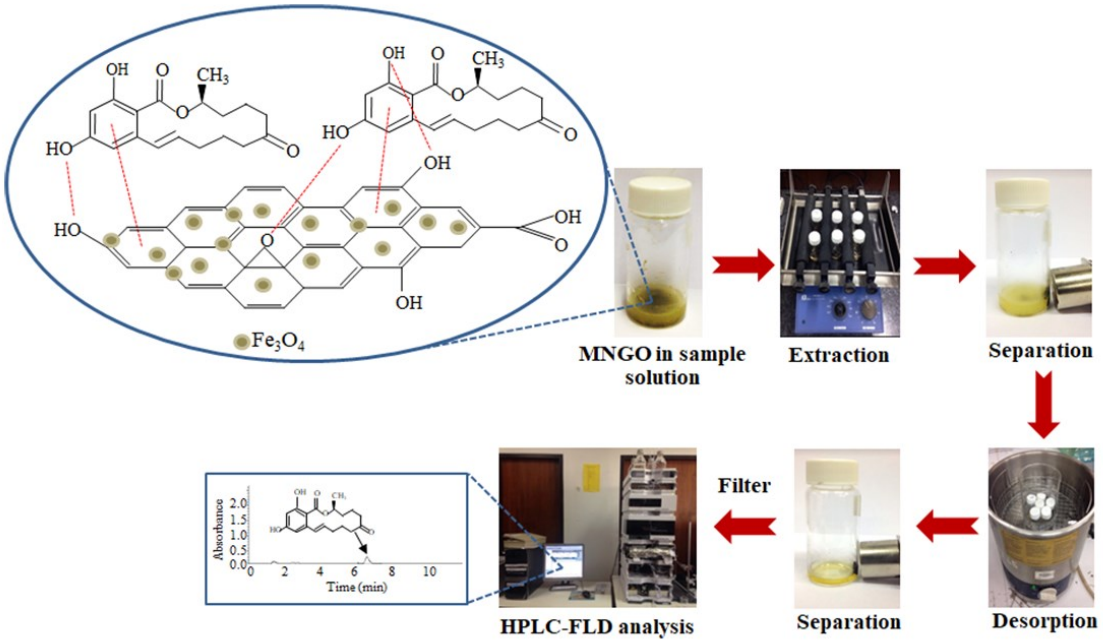

FIGURE 2 SEM images of (A) magnetic nanoparticles, (B) graphene oxide and (C) magnetic nanographene oxide

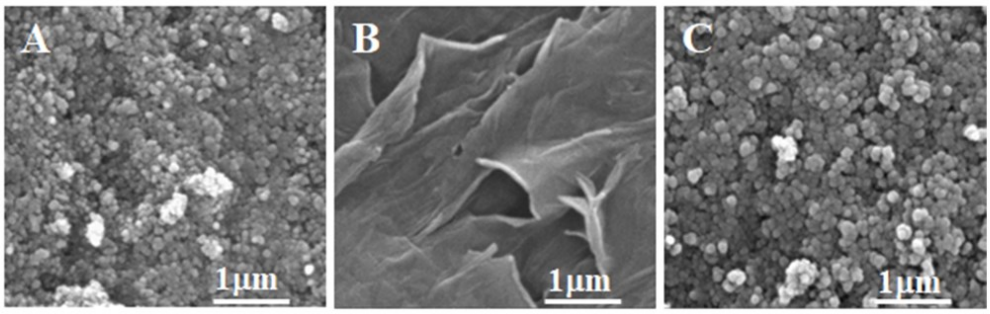

FIGURE 3 FTIR spectra of (A) magnetic nanoparticles, (B) graphene oxide and (C) magnetic nanographene oxide

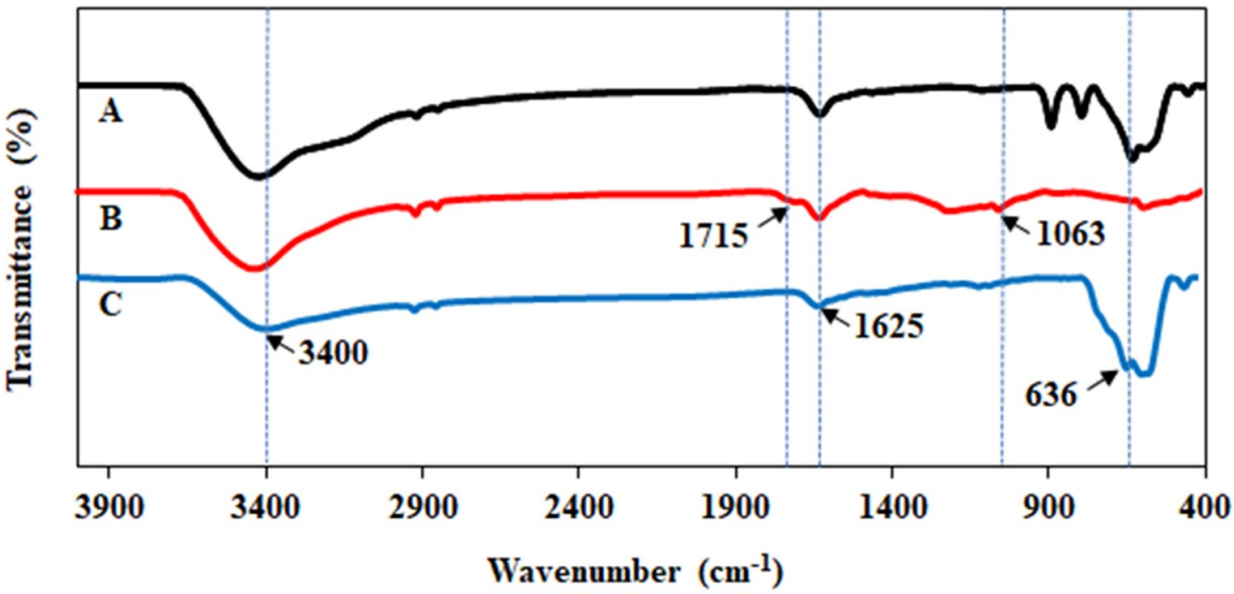

FIGURE 4 Effect of extraction conditions on the magnetic nanographene oxide-magnetic solid phase extraction efficiency of zearalenone. (A) Effect of magnetic nanographene oxide amount, (B) effect of extraction time, (C) effect of ionic strength, (D) effect of desorption volume, (E) effect of concentration of formic acid in methanol and (F) effect of desorption time