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Selective enrichment of ochratoxin A using human serum albumin bound magnetic beads as the concentrating probes for capillary electrophoresis/electrospray ionization-mass spectrometric analysis

Chen-Yu Hong [a], Yu-Chie Chen [a,b,*]

[a] *Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan*
[b] *Institute of Molecular Science, National Chiao Tung University, Hsinchu 300, Taiwan*

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Abstract

Ochratoxin A (OTA) is a toxicant commonly present in many food products. Conventionally, immuno-affinity analysis is applied to rapidly screen the presence of OTA in food. However, antibodies are expensive. In this study, we present a new approach for selectively enriching OTA from aqueous samples using human serum albumin (HSA) bound magnetic beads as the affinity probes, followed by the analysis of CE/ESI-MS. In addition to demonstrating the feasibility of using the affinity probes to concentrate OTA, we also propose a rapid concentration and elution method for extraction, that is, OTA are extracted from aqueous samples by pipetting the samples in and out of a sample vial for 1 min followed by elution with pipetting for another minute. On the basis of the magnetic property, the affinity magnetic probe–target species could be rapidly isolated from the solution during the process of extraction and elution by magnetic separation. CE/ESI-MS, coupled by the electrodeless/sheathless interface, is used for the analysis of the samples. As this method features speed and cost-effectiveness, it is suitable for the purpose of rapid screening. In fact, the lowest detection limit for OTA is $\sim 4 \times 10^{-3}$ mg/L.

Keywords: CE–ESI-MS; Albumin; Magnetic beads; Ochratoxin A

1. Introduction

Ochratoxins generally contaminate food such as cereals, coffee, and wine. Ochratoxin A (OTA) and ochratoxin B (OTB) are secondary metabolites of several strains of *Aspergillus* and *Penicillium* [1–9]. OTA is significantly more toxic than OTB by about one order of magnitude [10]. Moreover, it has been demonstrated that OTA may result in several severe diseases [11–14]. However, no methods can remove OTA completely from food products. Thus, developing appropriate analysis methods to monitor the presence of OTA in food products is significant.

Conventionally, sample pre-treatment using immuno-affinity technique [9] and solid phase microextraction [13] to remove interferences are carried out prior to analysis by thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC) [15]. Though solid phase microextraction can concentrate analytes with similar polarities, it lacks selectivity. Meanwhile, immuno-affinity techniques have quite good selectivity for their corresponding target species; however, antibodies are expensive.

We herein propose an alternative approach by using serum albumin as the probe molecules to selectively concentrate OTA from sample solutions because it has been demonstrated that serum albumin can bind with OTA with a high affinity [14–17]. Furthermore, serum albumin can be obtained much easier to be obtained and is cheaper than antibodies. It was Chu [15] who first investigated the binding of OTA with bovine serum albumin using various spectroscopic methods. In addition to bovine serum albumin, several reports have also demonstrated the affinity interactions between human serum albumin (HSA) and OTA [16–18]. Using magnetic beads as affinity probes is quite suitable because it becomes very easy to isolate the

* Corresponding author at: Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan. Tel.: +886 3 5131527; fax: +886 3 5131527.
E-mail address: yuchie@mail.nctu.edu.tw (Y.-C. Chen).

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magnetic bead–target species conjugates from sample solutions after extraction by magnetic separation. We previously proposed several approaches using magnetic particles as the affinity probes for target species such as proteins [19,20], peptides [19,20], phosphopeptides [21,22], and pathogenic bacteria [23,24] followed by characterization of matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Capillary electrophoresis (CE) combined with electrospray ionization-mass spectrometry (ESI-MS) is an alternative analysis method to characterize the trapped species from magnetic affinity probes [25]. As a result, a straightforward electrodeless/sheathless interface combining CE with ESI-MS was demonstrated [25,26]. A pulled bare-capillary tip was used as the ESI emitter, which was not coated with any electrically conductive materials. A high external voltage was not applied on its outlet as well. This interface is very easy to operate. In this study, we employed HSA bound magnetic particles as the affinity probes to trap OTA from sample solutions followed by the analysis of CE/ESI-MS interfaced by the electrodeless/sheathless capillary emitter. A rapid enrichment approach that pipetted the sample solution for just 1 min to vigorously mix and to enrich target species from complex sample solutions could obtain OTA in sufficient quantities for CE/ESI-MS analysis. As a result, the combination of the use of magnetic affinity probes and rapid pipetting approach for OTA extraction followed by electrodeless/sheathless CE/ESI-MS was demonstrated.

2. Experimental

2.1. Reagents and materials

Acetic acid and iron(III) chloride 6-hydrate were obtained from Riedel de Haën (Seelze, Germany). Acetonitrile, hydrochloric acid, sodium hydroxide, and trifluororacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Human serum albumin, bradykinin, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES), and 3-aminopropyl triethoxysilane (APTES) were obtained from Sigma (Steinfein, Germany). 3-(2-Aminoethylamino)propyl trimethoxysilane (EDAS), and iron(II) chloride tetrahydrate were obtained from Aldrich (Milwaukee, WI). Ammonium hydroxide and 2-propanol were obtained from J.T. Baker (Phillipsburn, NJ). Ethanol and methanol were obtained from Tedia (Fairfield, OH), while ochratoxin A and tetraethoxysilane were obtained from Fluka (Buchs, Switzerland). The fused silica capillary (50-μm i.d. × 365-μm o.d.) was obtained from Polymicro Technologies (Phoenix, AZ). All wine was purchased from a local grocery store.

2.2. Preparation of functional magnetic beads

$FeCl_3$ (5.4 g) and $FeCl_2$ (2.0 g) were dissolved in an aqueous hydrochloric acid (2 M, 25 mL) at room temperature under sonication. After the salts were completely dissolved in solution, the mixture was degassed using a pump followed by filling with nitrogen. The mixture was slowly injected with ammonia (28%, 40 mL) while stirring under nitrogen protection. After stirring for 1 h, the generated particles were collected by aggregating the particles on the edge of the flask through applying the application of a magnet outside of the flask. The particles were rinsed with deionized water five times until the smell of ammonia totally disappeared, while the particles remaining in the flask were re-suspended in methanol (100 mL). A flask containing the iron oxide particle suspension (2.0 mg/mL, 100 mL) was injected with EDAS (2 mL) while stirring in a nitrogen-protected condition, and the mixture was refluxed in an oil bath at 110 °C. After 24 h, the supernatant was removed after aggregating the particles on the edge of the flask through an external magnet. The particles were rinsed three times with methanol and three times with deionized water followed by re-suspension in deionized water (10 mL), which gave the final concentration of this particle suspension ∼11.1 mg/mL.

The silanized magnetic beads obtained above were further covalently bound with HSA. The beads isolated from 2.8 mL of the suspension (11.1 mg/mL), HSA (10 mg), and EDC (100 mg) were mixed in MES buffer (100 mM, 10 mL, pH 6.8) through vortex mixing. After 24 h, the beads were isolated from the solution by using magnetic separation to remove the supernatant. The supernatant (1 mL) was measured by absorption spectroscopy. The absorption change of HSA at the wavelength of 280 nm before and after reaction was used to estimate the binding amount of HSA on the beads. The isolated beads (Fe_3O_4@HSA) were rinsed three times with deionized water, followed by re-suspension in deionized water (5 mL). The final concentration of the suspension was 6.9 mg/mL.

2.3. Fabrication of the capillary emitter

Either direct ESI-MS analysis or CE/ESI-MS was carried out using a pulled bare capillary as the emitter [25,26]. The inner surface of the capillary was modified by APTES to reverse the direction of electro-osmotic flow. The capillary was sequentially flushed with NaOH (0.1 M) and deionized water for 30 min using a pump. An APTES (10 mM) solution was flushed into the capillary for 10 min followed by successive flushes with deionized water (for 10 min) and air (for 10 min) using a pump (pressure: 18 mmHg). The modified capillary was placed in an oven at 110 °C for 90 min to strengthen the cross-linking of APTES on the capillary wall. The capillary was flushed with deionized water for 10 min using a pump. After letting it stand at room temperature overnight, the capillary was fabricated by applying a small weight (50 g) on the lower end of a vertical capillary. The lower part of the capillary was heated and then quickly drawn to form a narrow capillary tip. After cooling to room temperature, the capillary tip was immersed in HF solution (24%) for 10 min. The capillary was ready for the use in CE/ESI-MS after rinsed by methanol.

2.4. Enrichment of OTA by Fe_3O_4@HSA magnetic beads followed by CE/ESI-MS analysis

OTA is a photosensitive chemical, so it is required to wrap the sample vials with aluminum foil during the sample treatment

process. OTA sample solutions (2.02 mg/L) diluted in deionized water (1 mL) at different concentrations were mixed with Fe_3O_4@HSA magnetic beads (690 μg) by pipetting to vigorously mix for 1 min. The magnetic bead–target species were isolated by magnetic separation. The isolated beads were repeatedly rinsed with deionized water (1 mL × 2). The trapped species on the beads were eluted with acetonitrile/0.2% acetic acid (1/1, v/v) (20 μL) by pipetting, followed by addition of bradykinin (2 μL, 1 mg/mL), which was used as the internal standard for quantitative analysis. Standard addition method was employed to quantify OTA in wine samples using this approach. To simulate real samples, we first spiked a given amount of OTA into wine samples. Wine samples were sonicated for 30 min to remove gas prior to spiking with OTA. The wine samples containing OTA (12.11 mg/L) were then mixed with different amounts of OTA, followed by 100-fold dilution with deionized water before performing the extraction by the Fe_3O_4@HSA magnetic beads. A calibration curve was generated by plotting the relative intensity of the peak area at *m/z* 402 to that at *m/z* 1059 as a function of the concentration of OTA after extraction. The steps for enrichment of OTA by the Fe_3O_4@HSA magnetic beads are similar to those described above.

The samples obtained from enrichment by the Fe_3O_4@HSA beads was injected to a pulled bare capillary by applying a voltage of −10000 V for 4 s in the sample vial containing the inlet of the capillary and a platinum electrode. The inlet of the capillary was put back to a vial containing the running solution of acetonitrile/0.2% acetic acid (1/1, v/v) after sample injection. CE–ESI-MS analysis was carried out by applying a voltage of −10000 V on the vial containing the inlet of the capillary and the running solution.

2.5. Instrumentation

All the ESI mass spectra were obtained from an Esquire 2000 ion trap mass spectrometer (Bruker Daltonics, Leipzig, Germany). The negative ion mode was operated during ESI-MS analysis, while the operation mode was set on nano ESI on line. The gas flow rate was set at 3 L/min, while the temperature was set at 150 °C. Absorption spectra were obtained using a Varian Cary 50 spectrophotometer (Melbourne, Australia).

3. Results and discussion

Fig. 1 displays the absorption spectra of a HSA solution, before and after covalently binding with iron oxide magnetic beads. Apparently, the absorption band of the HSA solution decreased after reaction. The optical density (O.D.) of the absorption band of HSA solution (1 mg/mL) at 280 nm is ∼0.59, while the O.D. of the remaining HSA solution at 280 nm after reaction decreased to ∼0.14. The calibration curve of the HSA solution by plotting its absorption at 280 nm (*y*-axis) as a function of its concentration (*x*-axis) was acquired. The equation for this calibration curve was $y = 34362x + 0.0005$ and the correlation coefficient was equal to 1. On the basis of this calibration curve, we estimated the binding amount of HSA on 1 mg of beads was ∼4.2 nmol. We used the beads (Fe_3O_4@HSA)

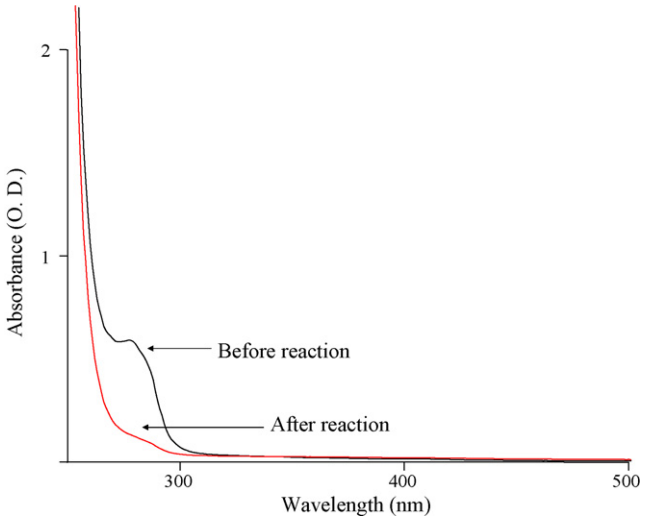

Fig. 1. Absorption spectra of a HSA solution (1 mg/mL, 10 mL), before and after covalently binding with iron oxide magnetic beads (31.1 mg). O.D. is the abbreviation of optical density.

as the affinity probes for OTA to carry out the study as follows.

Fig. 2a presents the direct ESI-MS spectrum of OTA (4×10^{-2} mg/L). The peak appearing at *m/z* 402 is the deprotonated pseudo-molecular ion of OTA ($[OTA\text{-}H]^-$). In addition to the peak at *m/z* 402, there are many peaks appearing in the same mass spectrum. Presumably, they are the impurities generated from either running solvents or ablated materials from the inner wall of the capillary. As the concentration of OTA was lowered down one order, the peak at *m/z* 402 could not be observed in the direct ESI mass spectrum. We employed our Fe_3O_4@HSA beads to enrich target species from a sample solution containing OTA (4×10^{-2} mg/L, 1 mL) by pipetting the mixture for 1 min followed by elution with acetonitrile/0.2% acetic acid (1/1, v/v) by pipetting for another 1 min. Fig. 2b displays the ESI-MS mass spectrum of the eluted solution from the beads after extraction, and as shown the intensity of $[OTA\text{-}H]^-$ pseudo-molecular ion at *m/z* 402 is dramatically raised. The results indicate that Fe_3O_4@HSA beads have the capability of being used as the affinity probes to selectively enrich trace of OTA from sample solutions. Furthermore, it only took 1 min to enrich OTA in sufficient quantities for CE–ESI-MS analysis.

One might suspect that the trapping capacity by pipetting for only 1 min might be worse than that of vortex mixing for a longer time. Thus, we also examined the results using these two different methods for enrichment of OTA. Fig. 3 presents the absorption spectra of OTA before and after extraction by the Fe_3O_4@HSA beads. The absorption band marked with dotted line (band (a)) is the absorption spectrum obtained from the original OTA solution before extraction. Two nearly overlapped absorption bands ((b) and (c)) indicated with arrows are the supernatants obtained using the Fe_3O_4@HSA beads to enrich OTA from sample solutions by pipetting 1 min and vortex mixing for 1 h, respectively. Apparently, although extraction by pipetting only took 1 min, the extraction efficiency was similar to that obtained by vortex mixing for 1 h.

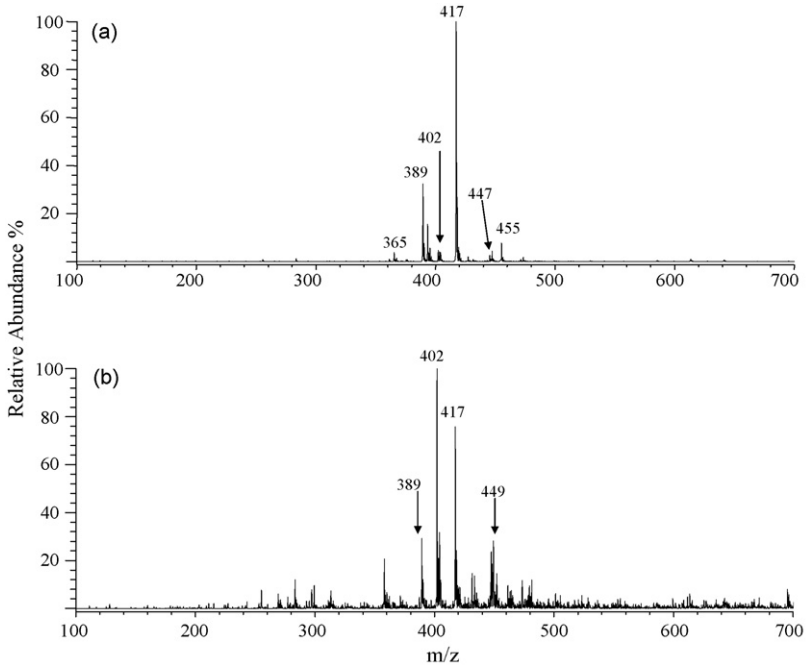

Fig. 2. (a) Direct ESI mass spectrum of OTA (4×10^{-2} mg/L), (b) ESI mass spectrum of the sample obtained using Fe_3O_4@HSA beads (690 μg) to selectively enrich their target species from a aqueous sample (1 mL) containing OTA (4×10^{-2} mg/L).

We further examined the trapping performance of Fe_3O_4@HSA for OTA. Fig. 4 displays the plotted trapping percentage (%) of Fe_3O_4@HSA for OTA as a function of the amount of Fe_3O_4@HSA beads, using absorption spectroscopy for investigation. OTA was totally trapped onto the beads as the amount of the beads was increased to more than 690 μg. There was about 14.5 nmol of OTA trapped by 1 mg of Fe_3O_4@HSA beads. We also examined the recovery of OTA eluted from the Fe_3O_4@HSA bead–OTA conjugates; that is, we examined the absorption change of the maximum absorption band of OTA at 333 nm after elution by pipetting. It was estimated that ~85% OTA was eluted from the beads (~690 μg). The results indicated that the recovery could achieve more than 80% even though the elution time was short.

The possibility of using this approach for quantitative analysis of OTA was also investigated. We used bradykinin ($[M\text{-}H]^-$ = 1059) as an internal standard because its migration time was near to that of OTA in CE–ESI-MS. OTA and bradykinin can be well separated in CE by using acetonitrile/0.2% acetic acid (1/1, v/v) as the running solvent. We used simulated samples as the real samples by first spiking a known amount of OTA into wine samples. Fig. 5a–c presents the total ion chromatogram (TIC) and the SIM chromatogram at *m/z* 402 and

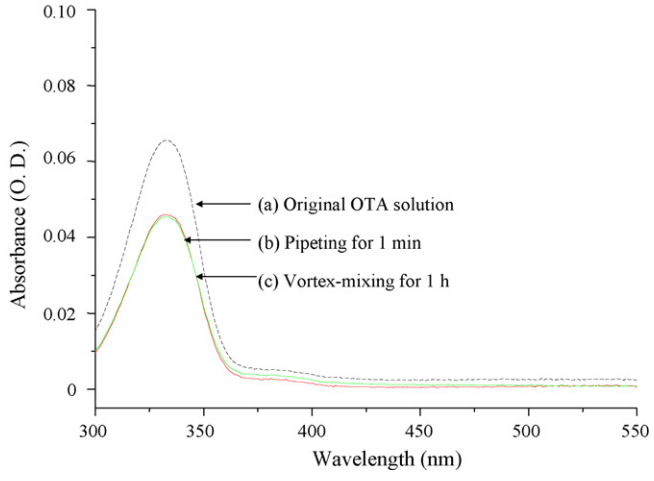

Fig. 3. Absorption spectra of OTA before and after extraction by the Fe_3O_4@HSA beads. (a) The dotted absorption band represents the absorption spectrum of the original OTA solution (10^{-5} M) before extraction. The absorption spectra of the supernatants obtained using the Fe_3O_4@HSA beads (690 μg) to enrich their target species from sample solutions (1 mL) containing OTA (10^{-5} M) (b) by pipetting 1 min and (c) by vortex mixing for 1 h.

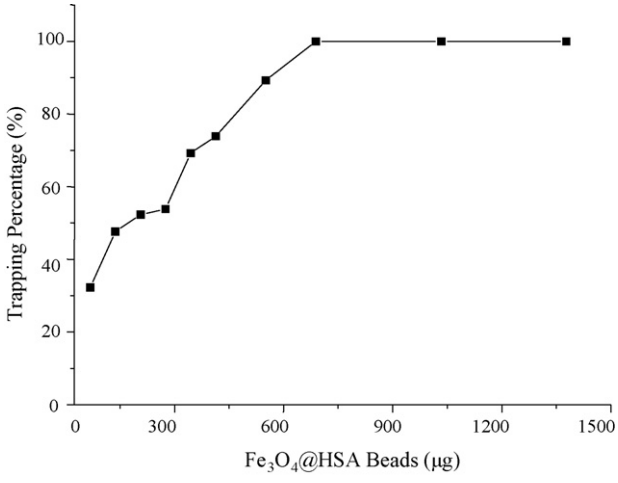

Fig. 4. Plot of the trapping percentage (%) of Fe_3O_4@HSA for OTA (total mole number = 10^{-5} M × 1 mL = 10 nmol) as a function of the amount of Fe_3O_4@HSA beads using absorption spectroscopy for examination.

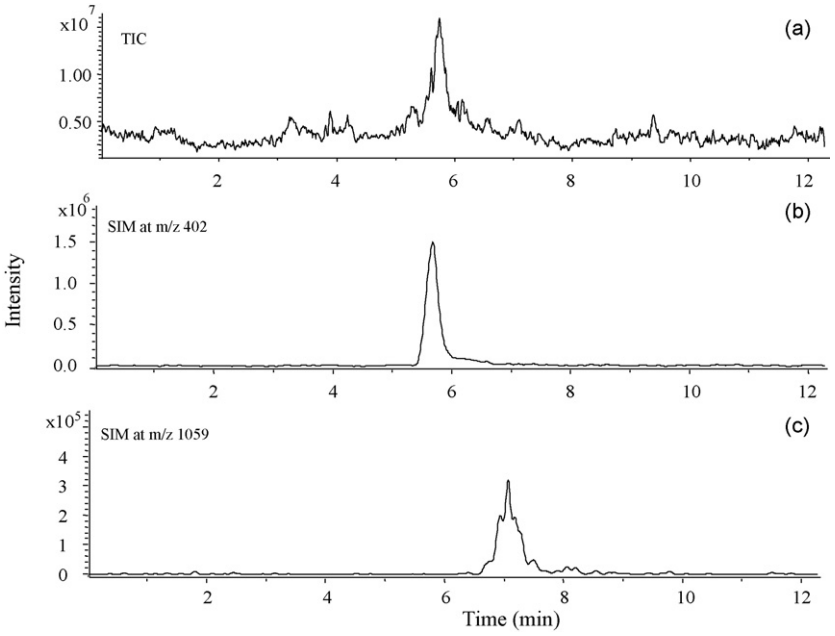

Fig. 5. (a) Total ion chromatogram (TIC) of CE–ESI-MS and SIM chromatograms at (b) *m/z* 402 (c) and 1059 obtained using Fe_3O_4@HSA beads (690 μg) to selectively enrich their target species from a diluted wine sample (1 mL) spiked with OTA (8×10^{-2} mg/L), followed by elution with acetonitrile/0.2% acetic acid (1/1 (v/v), 20 μL) solution. Bradykinin (1 mg/mL, 2 μL) was added into the eluted solution as the internal standard before CE–ESI-MS analysis.

1059, respectively, obtained using the Fe_3O_4@HSA magnetic beads to selectively enrich their target species from the wine sample. These two molecules can be well separated in the chromatogram. Fig. 6 shows the calibration curve obtained form the standard addition method by spiking different amounts of OTA into the wine sample to determine the original OTA concentration in the wine sample. The calibration curve was obtained by plotting the relative ratio of the peak area (*y*-axis) of the selected ion monitoring (SIM) peaks at *m/z* 402 and 1059 as a function of the concentration of OTA (*x*-axis). The equation for this calibration curve is $y = 2.64755 + 22.26738x$ and the correlation coefficient is 0.99942. The intercept of this curve is 0.1189, which indicates 0.1189 mg/L of OTA was present in the sample. Because the sample was diluted 100-fold with water before performing the extraction, the concentration of OTA in the wine sample was 11.89 mg/L. The experimental value shows only 1.82% difference from the true value (12.11 mg/L). The quantitative analysis result seems quite promising. However, the matrix interference in real samples may occasionally result in a higher error than that we report herein. When we employed this approach to quantify OTA from other types of wine samples, the analysis error may be up to 18% (results not shown). Thus, further studies may be required to improve this approach. We also found that the lowest detection limit for OTA is $\sim 4 \times 10^{-3}$ mg/L using this approach.

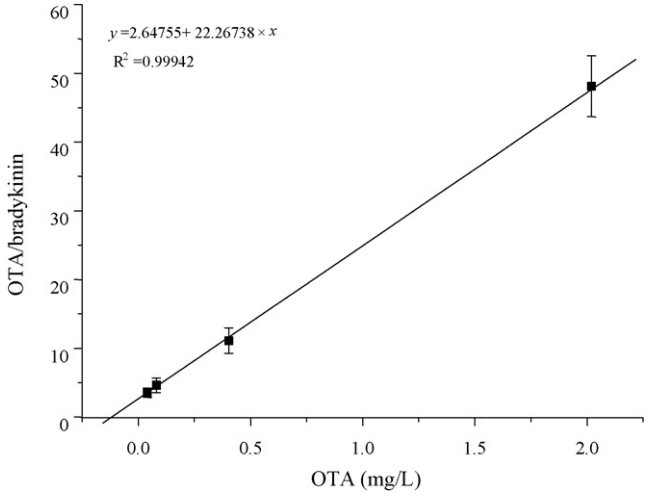

Fig. 6. Calibration curve obtained by plotting the relative intensity of the peak area of the SIM chromatogram at *m/z* 402 to that at 1059 as a function of the concentration of OTA spiked in the wine.

4. Conclusions

We have demonstrated that Fe_3O_4@HSA could selectively enrich traces of OTA from either aqueous solutions or wine samples. To the best our knowledge, this is the first time to apply this interaction in the method development for the selective enrichment of OTA. Since HSA is much cheaper and is easier to obtain than antibodies, this current approach is a more economic method than the immuno-affinity method. However, the selectivity of HSA for OTA from real samples might be worse than that using the antibody against OTA as the probe molecule. Nevertheless, because of its speed and cost-effectiveness, this approach is still suitable for rapid screening. Furthermore, HSA is not limited to binding with OTA as toxins other than OTA may interact with Fe_3O_4@HSA probes. Therefore, using these affinity probes for multi-targets may be applicable. Additionally, on-line extraction and elution directly carried out in capillary electrophoresis may be alternative means to improve the trap-

ping capacity and elution performance because the magnetic beads can be easily fixed on a specific area of the capillary by employing an external magnetic field. This approach is just the beginning of employing that feature of HSA capable of interacting with toxins for the development of analytical methods. Thus, the possibility of using HSA-immobilized magnetic beads as the affinity probes for multi-targets can be expected.

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