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Preparation of titanium ion functionalized polydopamine coated ferroferric oxide core-shell magnetic particles for selective extraction of nucleotides from *Cordyceps* and *Lentinus edodes*

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**Highlights**

- Metal ions functionalized Fe₃O₄@PDA particles for the extraction of nucleotides.
- To extract nucleotides from complex matrix by Fe₃O₄@PDA@Ti⁴⁺ particles.
- Potential applications in phosphorylated small molecular compounds extraction.
Abstract

In this study, a titanium ion (Ti⁴⁺) functionalized polydopamine coated ferroferric oxide (Fe₃O₄@PDA@Ti⁴⁺) core-shell magnetic particle was prepared for the selective extraction of nucleotides. Firstly, different metal ions including Ti⁴⁺, Zr⁴⁺, Fe³⁺, Al³⁺, Cu²⁺, Zn²⁺, Ni²⁺ and Mg²⁺ were respectively immobilized onto Fe₃O₄@PDA particles and their extraction efficiency for five nucleotides [cytidine-5’-monophosphate (CMP), uridine-5’-monophosphate (UMP), guanosine-5’-monophosphate (GMP), thymidine-5’-monophosphate (TMP) and adenosine-5’-monophosphate (AMP)] were compared. Among these prepared materials, Fe₃O₄@PDA@Ti⁴⁺, which exhibited the highest extraction efficiency for nucleotides, was further characterized by Fourier transform infrared spectroscopy, scanning electron microscopy, transmission electron microscopy and energy dispersive X-ray spectroscopy. After being optimized of the extraction parameters including adsorbent amounts, extraction time, extraction temperature, type and concentration of the eluent, the prepared Fe₃O₄@PDA@Ti⁴⁺ magnetic particles were successfully applied for the selective extraction and determination of CMP, UMP, GMP, TMP and AMP in Cordyceps and Lentinus edodes. Good linearity (varying from 0.063-19.000 μg/mL, R²>0.999) and low limit of detection (LODs) (ranging between 0.0047 and 0.0141 μg/mL) for target analytes were achieved. These results demonstrated that the synthesized material in this study had potential for selective extraction of phosphorylated small molecular compounds in complicated matrix.

Key words: Magnetic solid phase extraction; Selective extraction; Nucleotides; Cordyceps; Lentinus edodes
1. Introduction

*Cordyceps* and *Lentinus edodes* are two typical medicinal and edible mushrooms in China, they have been recognized as an important source of bioactive compounds of high medical and nutritional values. In reality, *Cordyceps* has been frequently used for the treatment of various diseases [1]. And *L. edodes* is popularly consumed in Asian countries as a kind of healthy food. Until now, several studies have been reported for their chemical constituents and pharmacological activities [2,3]. Among them, nucleobases, nucleosides and nucleotides are of important bioactive ingredients, which possess many functions such as improving immunity, promoting blood circulation, regulating arrhythmia, inhibiting urinary tract infection, regulating the central nervous system and affecting fatty acids metabolism [4,5]. Owing to their important biological activities, the determination of nucleobase, nucleosides and nucleotides is of great interest. And sample preparation is an essential process for the analyses. So far, different sample pretreatment methods have been developed for the analysis of these analytes, such as boiling water extraction [6,7], perchloric acid extraction [8], pressurized liquid extraction [9]. However, those methods are of poor selectivity.

Selective extraction, as a new sample pretreatment technique, has attracted widespread attentions. Nowadays, several methods have been developed for the selective extraction and enrichment of target analytes. For example, molecularly imprinted polymers (MIP), which is based on the molecular recognition to achieve selective extraction of targets, has been widely used in areas including solid phase extraction (SPE) [10], magnetic solid phase extraction (MSPE) [11] and sensor [12,13]. However, the
selection of effective template molecules is a limitation of MIP. Metal-organic frameworks (MOFs), a type of crystalline porous hybrid materials, have strict pore size, active metal sites and large adsorption capacity, which make them potential in selective extraction of various targets such as natural products [14], environmental pollutants [15], and peptides [16]. However, their assembly mainly depends on the coordination between the metal ions and organic ligands, the stability of most MOFs materials are insufficient. On the other hand, covalent organic frameworks (COFs) are a new class of ordered crystalline organic polymers consist of organic monomers by covalent bond, due to their large specific surface area, great stability, permanent porosity by special design, they also be used as selective adsorbents in separation science [17,18]. The number of organic monomers constituting COFs is relatively small, and the development of COFs is still in the initial stage. Besides, immobilized metal ion affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC), which utilize the metal electrostatic interaction and/or chelation effect on phosphate-containing compounds, are also widely applied in the enrichment of phosphoproteins and phosphopeptides with low abundance [19-24]. These metal-based affinity materials may be further applied for the selective extraction of nucleotides, which are phosphate-containing compounds.

To date, in IMAC, several materials such as iminodiacetic acid (IDA) [25] nitrilotriacetic acid (NTA) [26] and chitosan [27] have been used as metal chelating ligands to immobilize metal ions and applied for the enrichment of phosphoproteins or phosphopeptides. However, these methods usually have some disadvantages, such as time-consuming, complex synthetic process and low yield. On the other hand, due to the
simple synthesis process, high hydrophilicity and good biocompatibility of polydopamine (PDA), the fabrications and broad applications of PDA-based materials have been rapidly developed in recent years. Especially, the applications of PDA-based adsorbents is a hot topic in separation science, in which PDA was used directly as a functional group for the sample preparations [28,29], or used as linking material for further functional modifications [30,31]. Furthermore, considering the large amount of catechol and amino groups on the surface, PDA might be used as convenient metal chelating agent in IMAC.

Therefore, in the present study, a MSPE method, which based on the immobilization of metal ions onto the Fe₃O₄ particles by using PDA as chelating agent, was developed for the selective extraction of nucleotides. A series of metal ions, including Cu²⁺, Mg²⁺, Ni²⁺, Zn²⁺, Al³⁺, Fe³⁺, Zr⁴⁺, Ti⁴⁺, were immobilized on the surface of Fe₃O₄@PDA core-shell composites and their extraction efficiency to five nucleotides [cytidine-5’-monophosphate (CMP), uridine-5’-monophosphate (UMP), guanosine-5’-monophosphate (GMP), thymidine-5’-monophosphate (TMP) and adenosine-5’-monophosphate (AMP)] were compared. The physicochemical properties of synthesized Fe₃O₄@PDA@Ti⁴⁺ magnetic particle were thoroughly characterized and some important parameters involved in extraction/elution process were carefully optimized. Finally, the developed method was applied for the selective extraction of nucleotides from three kinds of mushrooms, including natural *Cordyceps sinensis*, cultured *C. militaris* and dried *L. edodes*.

2. Experiment

2.1 Chemical and reagents

Cytidine, uridine, guanosine, thymidine, adenosine, GMP, TMP were all purchased from
Sigma (St Louis, MO, USA). CMP and UMP were obtained from Adamas Reagent Co., Ltd. AMP was from Huaimaike Biotechnology Co., Ltd. (Beijing, China). Methanol for liquid chromatography was purchased from InnoChem (Beijing InnoChem Science & Technology Co., Ltd., China). Titanium sulfate \([\text{Ti}(\text{SO}_4)_2]\) was from DiBai Biotechnology Co., Ltd. (Shanghai, China). Dopamine hydrochloride and zirconium oxychloride octahydrate \((\text{ZrOCl}_2\cdot8\text{H}_2\text{O})\) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Iron chloride hexahydrate \((\text{FeCl}_3\cdot6\text{H}_2\text{O})\), zinc sulfate heptahydrate \((\text{ZnSO}_4\cdot7\text{H}_2\text{O})\), nickel nitrate hexahydrate \([\text{Ni}(\text{NO}_3)_2\cdot6\text{H}_2\text{O}]\), aluminum nitrate nonahydrate \([\text{Al}(\text{NO}_3)_3\cdot9\text{H}_2\text{O}]\) and magnesium chloride hexahydrate \((\text{MgCl}_2\cdot6\text{H}_2\text{O})\) were of analytical reagent grade and purchased from KeLong Chemical Reagent Co., Ltd. (Chengdu, China). Cupric acetate monohydrate \([\text{Cu}(\text{CH}_3\text{COO})_2\cdot\text{H}_2\text{O}]\) was of analytical reagent grade and purchased from Ruijinte Chemical Co., Ltd. (Tianjin, China). Water used for all the experiments was purified by a water purification system (ATSelem 1820A, Antesheng Environmental Protection Equipment Co., LTD., Chongqing, China). All other chemicals and solvents, unless otherwise specified, were guaranteed reagent grade. Natural \(C. \text{sinensis}\) is from Langxian, Tibet province and cultured \(C. \text{militaris}\) is from Liaoning province. The dried \(L. \text{edodes}\) were purchased from the local supermarket and ground to powder (sieved by 65 mesh sieve). All the samples were deposited at the Pharmaceutical Engineering Laboratory in School of Chemistry and Chemical Engineering, Chongqing University, Chongqing, China.

2.2 Instruments

All the sample analysis were performed on an Agilent 1260 Infinity Series modular system
with quaternary pumps, a diode array detector (DAD), controlled by Agilent ChemStation software. An Agilent Zorbax SB-AQ column (250 × 4.6 mm, 5 μm) and a Zorbax SB-AQ guard column (12.5 × 4.6 mm, 5 μm) were used for the sample separation. The morphological and size evaluation of the magnetic particles were performed by scanning electron microscopy (SEM) (Quanta650, FEI, USA) and transmission electron microscopy (TEM) (JEM 2100F, JEOL, Japan). The elements of the magnetic particles were analyzed by energy dispersive X-ray spectroscopy (EDX) (Genesis Apollo XL, EDAX, USA). Fourier transform infrared spectroscopy (FT-IR) was carried out on a Nicolet 550 Fourier transform infrared spectroscopy (Thermo Scientific Inc., USA). The ultrasonic bath (KQL-100B, Kunshan Ultrasonic Apparatus Co. Ltd., China) was used for the preparation of samples. The samples were centrifuged on TGL-20M high speed refrigerated centrifuge (Shanghai Lu Xiangyi Centrifuge Instrument Co., Ltd). The SHZ-82 gas bath thermostat oscillator from Jintan Chengxi Experimental Instrument Factory (Changzhou, China) was used to oscillate samples.

2.3 Synthesis of the Fe₃O₄@PDA@M⁺⁺ magnetic particles

The schematic diagram of Fe₃O₄@PDA@M⁺⁺ synthesis procedure was shown in Fig. 1A. (1) The Fe₃O₄ magnetic particles were synthesized by solvothermal reaction according to the previous report with minor modifications [32]. Briefly, 2.740 g FeCl₃·6H₂O was dissolved in 80 mL ethylene glycol to form an orange transparent solution. Subsequently, 6.204 g sodium acetate anhydrous and 0.805 g sodium citrate dihydrate were added to the solution, a homogeneous black solution was formed under stirring at 150°C for 30 min. Then the mixture was transferred to the Teflon-lined stainless steel autoclave and heated
at 200°C for 8 h. Finally, the reaction products were cooled down to room temperature and collected by external magnetic field. The black products were rinsed with deionized water and ethanol for three times respectively and dried at 45°C in vacuum for 12 h. (2) The magnetic particles Fe₃O₄@PDA were synthesized according to the previous reported procedure with minor modifications [33] as follows: 80 mg Fe₃O₄ was firstly dispersed in 80 mL 10 mM Tris-HCl buffer (pH 8.5) with an addition of 160 mL ethanol under ultrasonic to form homogeneous solution, then under the continuous stirring, 120 mL aqueous containing 0.320 g dopamine hydrochloride was slowly added to the homogeneous solution and the mixture kept stirring mechanically for 12 h. The black products were collected by external magnetic field, washed with deionized water for three times and dried in vacuum at 45°C overnight. (3) The preparation of Fe₃O₄@PDA@M⁺ was achieved as follows: 50 mg Fe₃O₄@PDA were dispersed evenly in the 250 mL 50 mM metallic salt aqueous solutions and stirred for 12 h to make the metal ion immobilized onto the Fe₃O₄@PDA core-shell. The products were finally collected and rinsed with deionized water and ethanol respectively for three times and dried in vacuum at 45°C overnight.

2.4 Chromatographic conditions

For the chromatographic separation, the column temperature was set at 25°C, the detection wavelength was at 260 nm and the flow-rate of the mobile phase was maintained at 0.8 mL/min. Aliquots of 10 μL samples were injected into the HPLC for analysis. The mobile phase consisting of 1.0 mM ammonium acetate and 1.5 mM NaH₂PO₄-Na₂HPO₄ buffer solution and was adjusted to pH 4.9 using acetic acid (A) and methanol (B). The separations of five nucleotides (CMP, UMP, GMP, TMP and AMP) were accomplished
using isocratic elution: 98% (A) and 2% (B). And for the investigation of the selectivity of the synthesized materials, simultaneous separations of the ten nucleosides and nucleotides were performed using gradient elution: 0-20 min, 0%-2% B; 20-35 min, 2%-20% B; 35-55 min, 20% B; 55-60 min, 20%-0% B.

2.5 Preparation of the real sample solutions

According to our previous studies [6,34], boiling water extraction was adopted for the preparation of the samples with minor modifications. Briefly, powder of natural C. sinensis (0.1 g), cultured C. militaris (0.1 g) and dried L. edodes (1.0 g) were accurately weighed, 10, 10 and 15 mL boiling ultra-pure water (95-100°C) were added, respectively. After being ultrasonic treatment for 30 min at 75°C, the extracts were cooled to room temperature and centrifuged at 1737×g for 10 min. The supernatants were collected and stored at -20°C before analysis. The icy supernatants were melted and the desired solution were freshly prepared by dilution with ultra-pure water.

2.6 The procedure of magnetic solid phase extraction

For the typical MSPE (Fig. 1B), 30 mg the Fe₃O₄@PDA@Ti⁴⁺ microspheres were dispersed in 2 mL sample solution by ultrasonic treatment, the mixture was shaken under 120 rpm for 5 min at room temperature in a gas bath thermostat oscillator, then the adsorbents were rapidly isolated from the aqueous solution by external magnetic field. Next, 2 mL of 20 mM Na₃PO₄·12H₂O buffer was added to elute the target compounds with shaking for another 10 min. The adsorbents were separated from the eluent by a magnet and 2 mL eluent was taken out from the glass vial and filtered through the 0.22 μm nylon membrane (Shanghai Titan Scientific Co., Ltd., Shanghai, China) for HPLC analysis.
3. Results and discussion

3.1 Synthesis and characterization of synthesized materials

IMAC is an efficient strategy for the enrichment of phosphopeptides based on the strong affinity between the metal ions and phosphate groups. Considering the easy separation by magnet of magnetic particles and the feasibility of PDA as a good chelating agent, Fe₃O₄@PDA was prepared in this study by self-assembly polymerization of dopamine under alkaline condition and then eight kinds of metal ions, including Ti⁴⁺, Zr⁴⁺, Fe³⁺, Al³⁺, Cu²⁺, Zn²⁺, Ni²⁺ and Mg²⁺ derived from different metallic salt as the precursor, were fixed on the surface of Fe₃O₄@PDA core-shell materials by the catechol hydroxyl groups of PDA. The results of different metal ions immobilized Fe₃O₄@PDA core-shell magnetic particles for extraction of five nucleotides were shown in Fig. 2 and Table 1, the Fe₃O₄@PDA@Ti⁴⁺ and Fe₃O₄@PDA@Zr⁴⁺ magnetic particles had the highest extraction efficiency, which might be due to the strong affinity of Ti⁴⁺ and Zr⁴⁺ to the phosphorylated substances [35]. Meanwhile, the Fe₃O₄@PDA@Ti⁴⁺ was slightly better than that of Fe₃O₄@PDA@Zr⁴⁺. So the Fe₃O₄@PDA@Ti⁴⁺ was finally chosen for the following experiments.

The prepared magnetic particles were characterized by FT-IR, and the results were shown in Fig. 3. The strong adsorption peak at 590 cm⁻¹ was attributed to the Fe-O vibration. The peaks at 3400 cm⁻¹ and 1604 cm⁻¹ were corresponded to the vibration of O-H. Meanwhile, comparing to the Fe₃O₄, some other adsorption peaks appeared in the Fe₃O₄@PDA and Fe₃O₄@PDA@Ti⁴⁺. The adsorption peak at 1286 cm⁻¹ could be assigned to the C-O stretching of phenol compound. The characteristic peaks of 1440 cm⁻¹ and 1400 cm⁻¹ were corresponded to the benzene ring C-C vibration. The adsorption peak at 1130 cm⁻¹
and 3220 cm\(^{-1}\) could be attributed to the C-N and N-H stretching vibration [33]. Besides, through comparing the curve (b) and curve (c) in Fig. 3, the adsorption was weakened in the vicinity of 3400 cm\(^{-1}\) and 1600 cm\(^{-1}\), these may be due to the chelation of immobilized Ti\(^{4+}\) and hydroxyl, which weakened the vibration of hydroxyl.

To further verify the synthesized magnetic particles, the morphology and size of the products were observed by SEM and TEM. As shown in Fig. 4A, the Fe\(_3\)O\(_4\) magnetic particle was spherical and monodispersed with a diameter of about 400 nm. The Fe\(_3\)O\(_4\)@PDA and Fe\(_3\)O\(_4\)@PDA@Ti\(^{4+}\) were spherical but their monodispersity reduced, these might be due to the adhesion of PDA on the surface of Fe\(_3\)O\(_4\) (Fig. 4B and Fig. 4C). Meanwhile, there were some small and irregular particles on the Fe\(_3\)O\(_4\)@PDA and Fe\(_3\)O\(_4\)@PDA@Ti\(^{4+}\), which could be the dopamine monomer have the tendency to form free PDA particles [36]. Besides, from the image of TEM, the layer of PDA was modified on the edge of Fe\(_3\)O\(_4\) magnetic particles and the thickness of the PDA shell was about 50 nm (Fig. 4D). All of these results indicated that the PDA was successfully coated on the surface of the Fe\(_3\)O\(_4\). In addition, as shown in Fig. 5, the presence of Ti element by EDX, indicated that Ti\(^{4+}\) was successfully fixed on the surface of Fe\(_3\)O\(_4\)@PDA magnetic particles.

### 3.2 Optimization of the extraction conditions

The extraction performance of MSPE is influenced by several parameters, such as adsorbent amount, extraction time, extraction temperature, type and the concentration of the eluent. Therefore, effects of these parameters on the extraction of five nucleotides by Fe\(_3\)O\(_4\)@PDA@Ti\(^{4+}\) were systematically investigated in this study and each tests were performed for three times.
3.2.1 Effect of adsorbent amount

The amount of adsorbent plays an important role in the extraction efficiency of the analytes. The effect of different Fe₃O₄@PDA@Ti⁴⁺ amount on the extraction recovery of five nucleotides was compared in the range of 1 to 5 mg (the other conditions were: extraction time, 10 min; extraction temperature, 30°C). As shown in Fig. 6A, the recovery of target analytes increased when the adsorbent amount increased from 1 to 3 mg, and then basically remained constant with the further increase of absorbent amount. Therefore, 3 mg adsorbent was chosen for the subsequent experiments.

3.2.2 Effect of extraction time

The influence of the extraction time on the extraction efficiency was investigated from 0 to 6 min (the other condition were: adsorbent amount, 3 mg; extraction temperature, 30°C) and the results were shown in Fig. 6B. The extraction recovery increased with the extension of time within 5 min, and then it remained nearly constant. The result indicated that the extraction equilibrium could be achieved in a very short time, which was ascribed to the strong interaction between the Ti⁴⁺ and the phosphate group of the nucleotides. Overall, 5 min was chosen as the optimum extraction time.

3.2.3 Effect of the extraction temperature

The extraction temperature can affect the extraction efficiency by changing the distribution coefficient and diffusion coefficient between the target analytes and adsorbents to some extent. Hence, the effect of temperature to the extraction performance in the range of 25-35°C was investigated (the other conditions were: adsorbent amount, 3 mg; extraction time, 5 min). As shown in Fig. 6C, these results indicated that the temperature has little influence
on the extraction recovery, considering the economic and convenient principle, 25°C was chosen for the next experiments.

3.2.4 Effect of type and concentration of eluent

The desorption degree of the target analytes from the adsorbent depends on multi-factors, such as eluent type, eluent time and eluent concentration. In the process of enrichment of phosphopeptides, NH₃·H₂O was usually chosen as eluent due to the captured phosphopeptides were eluted at high pH [37]. In this study, different eluents including NH₄H₂PO₄ (pH 4.89), NH₃·H₂O (pH 10.67), Na₂HPO₄·12H₂O (pH 9.35) and Na₃PO₄·12H₂O (pH 12.06) with concentration of 40 mM were compared for the elution efficiency (the other conditions were: adsorbent amount, 3 mg; extraction time, 5 min; extraction temperature, 25°C). The results shown in Fig. 6D indicated that the highest efficiency can be obtained by using Na₃PO₄·12H₂O as eluent for eluting 10 min. The reason could probably attributed to two aspects: 1) the chelation interaction between the Ti⁴⁺ and phosphorylated group could be weaken under high pH; and 2) the free phosphate ions could bring the competition effect and displace the binding site of nucleotides. In addition, the influence of different concentrations (20, 40 and 60 mM) of eluent were also investigated. However, as the results shown in Fig. 6E, the eluent concentration hardly affected on the elution efficiency. Therefore, 20 mM Na₃PO₄·12H₂O buffer solution, eluted for 10 min as the final elution condition.

3.3 Evaluation of the selectivity, stability and magnetic response of the synthesized Fe₃O₄@PDA@Ti⁴⁺

For evaluating the performance of the synthesized materials, the selectivity, stability and
magnetic response were investigated. Mixed solution of CMP, UMP, GMP, TMP, AMP, cytidine, uridine, guanosine, thymidine and adenosine was prepared to study the selectivity of the synthesized absorbent. As shown in Fig. 7, the Fe₃O₄@PDA@Ti⁴⁺ magnetic adsorbent only extract five nucleotides with high efficiency, but almost had no effect on the five nucleosides. This property proved the possibility for selective enrichment of phosphorylated components in real samples and reduced the interference of non-target compounds by using Fe₃O₄@PDA@Ti⁴⁺ as adsorbent. Furthermore, the Fe₃O₄@PDA@Ti⁴⁺ was stored at room temperature for one month and its extraction efficiency was evaluated to study the stability of the prepared material. As the results shown in Table 2, the magnetic particles remained high extraction recovery for five nucleotides after one month’s storage, which indicated the good stability of the material. Finally, the magnetic property was tested by placing a permanent magnet close to the glass bottle containing the Fe₃O₄@PDA@Ti⁴⁺ particles solution, as shown in Fig. 8, the magnetic particles could be easily dispersed in water to form a dark-brown homogeneous solution by sonication and it could be isolated easily within ten seconds by applying a magnet.

3.4 Validation of the method and application in the analysis of real samples

The validation data of the method was given in Table 3. Under the optimized conditions, the linearity ranges were 0.063-16.000 μg/mL for UMP, 0.099-19.000 μg/mL for TMP, and 0.070-18.000 μg/mL for CMP, GMP and AMP, respectively. The limit of detection (LOD: S/N=3) and the limit of quantification (LOQ: S/N=10) were 0.0047-0.0141 μg/mL and 0.0148-0.0469 μg/mL for the five nucleotides, respectively. Besides, the linear correlation coefficient (R²) were all higher than 0.999. Then, to further evaluate the practical
application of the proposed method, the Fe₃O₄@PDA@Ti⁴⁺ core-shell particles extraction combined with HPLC analysis was used to simultaneously determination of the five nucleotides in real samples. All measurements were replicated three times (n=3) after pretreatment by Fe₃O₄@PDA@Ti⁴⁺. The results were shown in Table 4, and the HPLC chromatograms of the real samples were shown in Fig. 9. The identification of investigated compounds was carried out by comparison of their retention time and their UV spectra with those obtained injecting standards in the same conditions, as well as by spiking real samples with stock standard solutions. The content of five nucleotides were different in three samples, for natural C. sinensis, CMP, UMP, GMP, TMP and AMP were 282.36, 686.07, 1566.65, 126.84 and 1161.45 μg/g, respectively. However, TMP was not detected in cultured C. militaris and dried L. edodes, the content of GMP was much lower (345.94 μg/g) in cultured C. militaris. These can be affected by many factors, such as the growing environment, growing area and species differences. In addition, the recovery of GMP was low in cultured C. militaris, the possible reason for this result might be the incomplete separation of GMP. For dried L. edodes, CMP, UMP, GMP and AMP were 137.07, 381.72, 1097.49 and 733.85 μg/g, respectively. Overall, compared with previous studies [38], the five nucleotides determined by this method were basically on the same order of magnitude with direct determination. Furthermore, the selective extraction of Fe₃O₄@PDA@Ti⁴⁺ could reduce the interference of complex matrix to some extent. For example, GMP couldn’t be separated in cultured C. militaris, but the separation performance of GMP was improved after Fe₃O₄@PDA@Ti⁴⁺ treatment. Although the proposed method demonstrated the advantage of the selective extraction, there were still
some limitations, such as 1) the recovery is relative low in strong matrix interference, and 2) due to the phosphate ions occupy the adsorption site and the loss of metal ions to a certain extent in the eluent process, the reusability of the material will be deteriorated.

4. Conclusions

In this work, eight metal ions immobilized on Fe₃O₄@PDA core-shell magnetic particles were prepared, and Ti⁴⁺ was finally chosen after systematic comparison on their extraction efficiency to five nucleotides. The prepared Fe₃O₄@PDA@Ti⁴⁺ adsorbents were successfully applied for the selective extraction and determination of nucleotides from natural C. sinensis, C. militaris and dried L. edodes for the first time. The materials was stable and environmental friendly, meanwhile, the MSPE process was quick and facile. The developed method could be a promising tool for the extraction of phosphorylated compounds in complex natural products samples.

Conflict of interest

The authors all have read and approved present manuscript, and declared that they have not conflicts of interest.

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Figure Captions:

Fig. 1. The schematic diagrams of the Fe₃O₄@PDA@Mⁿ⁺ synthesis procedure (A) and the extraction procedure by Fe₃O₄@PDA@Ti⁴⁺ (B).

Fig. 2. The HPLC chromatograms of five nucleotides after extraction by Fe₃O₄@PDA@Mⁿ⁺. (a) Fe₃O₄@PDA@Ti⁴⁺, (b) Fe₃O₄@PDA@Zr⁴⁺, (c) Fe₃O₄@PDA@Fe³⁺, (d) Fe₃O₄@PDA@Al³⁺, (e) Fe₃O₄@PDA@Cu²⁺, (f) Fe₃O₄@PDA@Zn²⁺, (g) Fe₃O₄@PDA@Ni²⁺, (h) Fe₃O₄@PDA@Mg²⁺, (i) Fe₃O₄@PDA, (j) mixed reference compounds solution before extraction. Peaks: 1, CMP; 2, UMP; 3, GMP; 4, TMP; 5, AMP.
Fig. 3. The FT-IR spectra of (a) Fe₃O₄, (b) Fe₃O₄@PDA, and (c) Fe₃O₄@PDA@Ti⁺.
Fig. 4. The SEM image of (A) Fe₃O₄, (B) Fe₃O₄@PDA and (C) Fe₃O₄@PDA@Ti⁴⁺, and the TEM image of (D) Fe₃O₄@PDA@Ti⁴⁺.
Fig. 5. The EDX spectrum date of Fe₃O₄@PDA@Ti⁺.
Fig. 6. The optimized parameters of (A) adsorbent amount, (B) extraction time, (C) extraction temperature, (D) type of eluent, and (E) concentration of eluent. ( ) CMP, ( ) UMP, ( ) GMP, ( ) TMP, ( ) AMP.
Fig. 7. The HPLC chromatograms of ten nucleosides and nucleotides after treated with Fe₃O₄@PDA@Ti⁺⁺ for validating the selectivity of Fe₃O₄@PDA@Ti⁺⁺ magnetic particles. (a) mixed reference compounds solution before extraction. (b) supernatant after extraction. Peaks: 1, CMP; 2, UMP; 3, GMP; 4, cytidine; 5, TMP; 6, uridine; 7, AMP; 8, guanosine; 9, thymidine; 10, adenosine.
Fig. 8. The magnetic separation of the Fe₃O₄@PDA@Ti⁴⁺.

Zhou et al., Fig.7
Fig. 9. The HPLC chromatograms of different real samples, (A) natural C. sinensis, (B) C. militaris, (C) dried L. edodes, (a) sample solution before extraction (b) sample supernatant after extraction, (c) sample eluent after extraction, (d) sample eluent after extraction with spiked 800 ng/mL reference compounds, and (e) mixed reference compounds solution. Peaks: 1, CMP; 2, UMP; 3, GMP; 4, TMP; 5, AMP.
Zhou et al., Fig. 9
Table 1 Comparison on the extraction efficiency of eight different metal ions immobilized Fe₃O₄@PDA core-shell materials to five nucleotides (mean ± SD %, n=3)

<table>
<thead>
<tr>
<th>Absorbent</th>
<th>CMP</th>
<th>UMP</th>
<th>GMP</th>
<th>TMP</th>
<th>AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe₃O₄@PDA</td>
<td>0.59 ± 0.14</td>
<td>0.05 ± 0.19</td>
<td>2.71 ± 4.52</td>
<td>0.35 ± 0.39</td>
<td>0.09 ± 0.50</td>
</tr>
<tr>
<td>Fe₃O₄@PDA@Mg²⁺</td>
<td>1.33 ± 0.28</td>
<td>0.36 ± 0.67</td>
<td>2.94 ± 0.39</td>
<td>0.72 ± 0.40</td>
<td>2.41 ± 1.10</td>
</tr>
<tr>
<td>Fe₃O₄@PDA@Ni²⁺</td>
<td>2.42 ± 0.55</td>
<td>2.75 ± 0.58</td>
<td>2.52 ± 0.23</td>
<td>1.21 ± 0.88</td>
<td>3.11 ± 1.06</td>
</tr>
<tr>
<td>Fe₃O₄@PDA@Zn²⁺</td>
<td>1.29 ± 0.96</td>
<td>1.13 ± 0.55</td>
<td>2.16 ± 1.28</td>
<td>0.76 ± 0.64</td>
<td>3.01 ± 1.87</td>
</tr>
<tr>
<td>Fe₃O₄@PDA@Cu²⁺</td>
<td>36.15 ± 2.19</td>
<td>26.68 ± 1.88</td>
<td>87.09 ± 1.48</td>
<td>16.22 ± 2.14</td>
<td>68.63 ± 1.15</td>
</tr>
<tr>
<td>Fe₃O₄@PDA@Al³⁺</td>
<td>4.34 ± 0.31</td>
<td>2.75 ± 0.59</td>
<td>10.45 ± 1.58</td>
<td>3.25 ± 0.69</td>
<td>11.94 ± 1.73</td>
</tr>
<tr>
<td>Fe₃O₄@PDA@Fe³⁺</td>
<td>23.87 ± 5.51</td>
<td>7.17 ± 0.32</td>
<td>34.40 ± 2.33</td>
<td>5.45 ± 0.22</td>
<td>36.14 ± 2.32</td>
</tr>
<tr>
<td>Fe₃O₄@PDA@Zr⁴⁺</td>
<td>72.68 ± 2.51</td>
<td>79.37 ± 0.79</td>
<td>92.55 ± 0.36</td>
<td>75.35 ± 0.83</td>
<td>87.15 ± 0.71</td>
</tr>
<tr>
<td>Fe₃O₄@PDA@Ti⁴⁺</td>
<td>73.13 ± 6.23</td>
<td>90.73 ± 4.44</td>
<td>96.03 ± 1.20</td>
<td>83.83 ± 5.86</td>
<td>89.84 ± 2.60</td>
</tr>
</tbody>
</table>

Table 2 The extraction recovery (mean ± SD %, n=3) of five nucleotides by fresh prepared and one month storage Fe₃O₄@PDA@Ti⁴⁺

<table>
<thead>
<tr>
<th>Absorbent</th>
<th>CMP</th>
<th>UMP</th>
<th>GMP</th>
<th>TMP</th>
<th>AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>One month storage</td>
<td>82.43 ± 4.87</td>
<td>94.41 ± 1.83</td>
<td>96.55 ± 1.52</td>
<td>89.21 ± 3.15</td>
<td>91.36 ± 2.09</td>
</tr>
<tr>
<td>Fresh prepared</td>
<td>97.74 ± 1.89</td>
<td>95.46 ± 1.70</td>
<td>97.45 ± 1.32</td>
<td>89.31 ± 4.16</td>
<td>95.92 ± 0.24</td>
</tr>
</tbody>
</table>
### Table 3 Method validation for the determination of five nucleotides

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Calibration curves</th>
<th>Linear range (μg/mL)</th>
<th>R²</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>Y=46.074x-0.154</td>
<td>0.070-18.000</td>
<td>1.000</td>
<td>0.0113</td>
<td>0.0402</td>
</tr>
<tr>
<td>UMP</td>
<td>Y=84.882x+0.399</td>
<td>0.063-16.000</td>
<td>1.000</td>
<td>0.0076</td>
<td>0.0278</td>
</tr>
<tr>
<td>GMP</td>
<td>Y=83.790x-4.845</td>
<td>0.070-18.000</td>
<td>0.999</td>
<td>0.0056</td>
<td>0.0176</td>
</tr>
<tr>
<td>TMP</td>
<td>Y=74.570x+0.947</td>
<td>0.099-19.000</td>
<td>1.000</td>
<td>0.0047</td>
<td>0.0148</td>
</tr>
<tr>
<td>AMP</td>
<td>Y=134.800x-11.164</td>
<td>0.070-18.000</td>
<td>0.999</td>
<td>0.0141</td>
<td>0.0469</td>
</tr>
</tbody>
</table>

### Table 4 Determination of five nucleotides in three real samples (n=3).

<table>
<thead>
<tr>
<th>Analytes</th>
<th>C. sinensis</th>
<th>C. militaris</th>
<th>L. edodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contents (μg/g)</td>
<td>Recovery (%)</td>
<td>Contents (μg/g)</td>
</tr>
<tr>
<td>CMP</td>
<td>282.36 (8.93)a</td>
<td>100.79 (10.96)</td>
<td>84.92 (8.18)</td>
</tr>
<tr>
<td>UMP</td>
<td>686.07 (5.31)</td>
<td>108.33 (9.97)</td>
<td>719.25 (2.32)</td>
</tr>
<tr>
<td>GMP</td>
<td>1566.65 (1.19)</td>
<td>89.56 (14.32)</td>
<td>345.94 (5.31)</td>
</tr>
<tr>
<td>TMP</td>
<td>126.84 (6.90)</td>
<td>96.17 (6.01)</td>
<td>- b</td>
</tr>
<tr>
<td>AMP</td>
<td>1161.45 (1.46)</td>
<td>84.92 (8.18)</td>
<td>803.51 (4.59)</td>
</tr>
</tbody>
</table>

a, average of triplicates (RSD%);  
b, undetectable
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