Rapid Determination of Vitamin D₃ in Aquatic Products by Polypyrrole-Coated Magnetic Nanoparticles Extraction Coupled with High-Performance Liquid Chromatography Detection

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1. Introduction

Vitamin D (VD), a group of fat-soluble secosteroids, plays important roles in the physiological activity of humans. The traditional role of VD is to maintain calcium and phosphorus homeostasis and normal bone function and structure [1]. Recently, more and more studies have reported that various chronic diseases, including insulin resistance, neurological disorders, diabetes, and cardiovascular disease are linked to a VD deficiency [2,3]. In nature, VD₃ comes mainly from plants and cholecalciferol (VD₂) comes from animals [4]. It is considered that fatty fish, fish liver, and fish oil are excellent supplementation sources for natural VD₃ [5,6]. Other foods, such as meat and egg yolk, also contain high amounts of VD₃ [5].

The complexity of food matrices means that measuring the content of VD₃ needs appropriate pretreatment before instrumental analysis. Appropriate extraction techniques can remove substances, including proteins, polysaccharides, and lipids that can interfere with VD₃ detection, and therefore, enhance the accuracy and detection limit of the method [7]. Popular extraction techniques are liquid-liquid extraction, solid phase extraction (SPE), dispersive liquid-liquid microextraction, magnetic solid phase extraction (MSPE), and enzyme linked immunosorbent assay [8–10]. Considering the amount of organic solvent
consumption, environmental friendliness, adsorbent usage, and time or cost [3,11], the MSPE technique has attracted great attention due to the rapid separation of the adsorbent from the sample matrix with a magnetic field [12]. Among the various magnetic nanoparticles, Fe$_3$O$_4$ nanoparticles have many unique properties (i.e., low toxicity, biocompatibility, and biodegradability) [13] and have been applied in various fields, such as heavy metal removal from industrial wastewater [14], trace component enrichment before detection [15,16], adsorption of target biomedical compounds [17], etc. However, Fe$_3$O$_4$ nanoparticles can be easily oxidized and can form aggregates [18]. Therefore, it is necessary to modify the surface of Fe$_3$O$_4$ nanoparticles to improve their stability.

Polypyrrole (PPy), the polymer of monomeric pyrrole, offers the advantages of low toxicity, low cost, and easy preparation [19]. PPy has been used on Fe$_3$O$_4$ nanoparticles to form an adhesive coat with more binding sites for target molecules by means of π-π stacking, hydrogen bonding, van der Waals forces, and charge interactions [20]. Fabricated Fe$_3$O$_4$@PPy composites have been successfully utilized for the removal of Ni (II) and Cr (VI) from industrial effluent [21] and rapid extraction of antiseptic ingredients or estrogens before HPLC-MS/MS determination [22,23]. In a recent study, Jiao, Zhang and Fan (2016) [10] described the effective extraction capacity of Fe$_3$O$_4$@PPy for vitamin D$_2$ and vitamin D$_3$ in the milk matrix. Although PPy coated Fe$_3$O$_4$ nanoparticles have been successfully prepared in the above literature, their structures and properties may be varied due to different synthesis methods. Moreover, the adsorption capacity of Fe$_3$O$_4$@PPy for different molecules, or even the same molecule, may vary in different food matrices. As far as we know, there are few reports on the application of Fe$_3$O$_4$@PPy for the extraction of VD$_3$ from aquatic products before detection.

The aim of this work was to prepare a suitable Fe$_3$O$_4$@PPy composite with a high adsorption efficiency for trace VD$_3$ in aquatic products before detection by high performance liquid chromatography (HPLC). The fabricated Fe$_3$O$_4$@PPy composites were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and thermogravimetric analysis (TGA) measurements. Furthermore, the main parameters affecting the efficiency of extraction, including sample pH, adsorbent dose, adsorption temperature, adsorption time, desorption solvent, and desorption time were optimized or determined. Finally, the method of Fe$_3$O$_4$@PPy extraction coupled with HPLC for detection of VD$_3$ was evaluated and employed for nine aquatic products. Our results provide an efficient, rapid, and environmentally friendly method for the detection of trace VD$_3$ in aquatic product matrices.

2. Materials and Methods
2.1. Materials

The aquatic products used in this study, including Penaeus sinensis (Solenocera crassicornis), Pacific white shrimp (Litopenaeus vannamei), cuttlefish (Sepia esculenta), squid (Loliolus japonica), clams (Cyclina sinensis and Paphia undulata), razor clam (Sinonovacula constricta), Chlamys farreri (Azunapectar furari), and silver pomfret (Pampus argenteus) were purchased from a local market in Zhoushan City, transported to the laboratory with ice bags within 1 h, and stored at −20°C for no more than one month. Vitamin D$_3$ standard (VD$_3$) (purity > 98%) and pyrrole were purchased from Aladdin Industrial Corporation (Shanghai, China). Chemicals used for HPLC analysis were HPLC grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other reagents were of analytical grade and obtained from commercial products.

2.2. Sample Saponification

All of the aquatic products used in this study were saponified to release VD$_3$ from bound protein using the method of Strobel, Buddhadasa, Adorno, Stockham and Greenfield (2013) [24] with further modifications. In brief, 4.0 g of minced sample was added to 10 mL of an 80% ethanol solution (containing 20% KOH), followed by the addition of 20.0 mg ascorbic acid. After 2 h of oscillation at room temperature, the mixture was centrifuged.
for 10 min at 6000 rpm. Finally, the supernatant was collected and used for the VD$_3$ adsorption experiments.

2.3. VD$_3$ Concentration Measurement

VD$_3$ concentration was determined by de Azevedo’s method [25] with slight modifications. Briefly, 10 µL of sample filtrate was loaded onto a 1260 Agilent HPLC system (Waldbronn, Germany) with a C$_{18}$ column (4.6 × 250 mm, 5 µm) (Elite, Dalian, China) at 40 °C. The mobile phase was 100% methanol with a flow rate of 0.6 mL/min and detection at 264 nm. To calculate sample VD$_3$ concentration, VD$_3$ standards were prepared in methanol with concentrations ranging from 0 to 10.0 µg/mL and assayed under the same conditions. VD$_3$ concentration was determined from the calibration curve for the VD$_3$ standards ($y = 15.38 \times -0.0583, R^2 = 0.9975$).

2.4. Preparation of Fe$_3$O$_4$@Polymerization of Pyrrole (Fe$_3$O$_4$@PPy)

2.4.1. Fe$_3$O$_4$ Nanoparticles (Fe$_3$O$_4$ NPs) Preparation

Fe$_3$O$_4$ NPs were prepared according to the method of Nalle, Wahid, Wulandari and Sabarudin (2019) [26] with slight modifications. In brief, 0.18 g of FeCl$_2$·4H$_2$O and 0.3 g of FeCl$_3$·6H$_2$O were dissolved in 15 mL of deionized water degassed by ultrasonic treatment, and stirred for 15 min at 55 °C. Then, 7.2 mL of 3 mol/L NaOH was rapidly added to the mixture and continuously stirred for 40 min. After 30 min of incubation at 90 °C in a water bath, the reaction solution was cooled to room temperature. The resulting black sediment was washed repeatedly with deionized water until a pH of 7.0 was achieved, then dried in a vacuum oven at 60 °C overnight. The generated Fe$_3$O$_4$ NPs were stored for further experiments.

2.4.2. Synthesis of Fe$_3$O$_4$@PPy Composites

Fe$_3$O$_4$ NPs were coated by polymerization of pyrrole (PPy) using the method of Zhang et al. (2020) [23] with few modifications. In brief, 0.028 g of sodium dodecyl sulfate and 0.2 g of Fe$_3$O$_4$ were added to 80 mL of deionized water and sonicated for 20 min to obtain a homogeneous dispersion. Subsequently, pyrrole monomer at a ratio of 1:1, 3:1, 5:1 ($v/w$) with respect to Fe$_3$O$_4$ NPs content was added and stirred for 10 min. Then, 10 mL of 1 mol/L FeCl$_3$·6H$_2$O was added slowly to the reaction and stirred for 12 h at room temperature. The generated Fe$_3$O$_4$@PPy particles coated with different PPy ratios were recovered using an external magnetic field, washed with deionized water three times, and finally dried in a vacuum oven at 60 °C overnight. The VD$_3$ adsorption rate and particle size of these freeze dried Fe$_3$O$_4$@PPy were then determined.

2.5. Adsorption Rate for VD$_3$

Fe$_3$O$_4$@PPy powder (50 mg) was added to a saponified solution (10 mL resulting from 4 g of Penaeus sinensis by-products). After 20 min of static adsorption at room temperature, the Fe$_3$O$_4$@PPy composites were separated from the mixture using an external magnet, washed with 2 mL of deionized water and 2 mL of ethanol. Then, the collected Fe$_3$O$_4$@PPy particles were added to 2 mL of methanol and desorbed for 10 min in a standing state, followed by separation of the Fe$_3$O$_4$@PPy particles using the action of a magnet. The remaining solution was evaporated at 45 °C to remove the methanol, and then redissolved in 500 µL of methanol. After filtration through a 0.22 µm filter, the concentration of VD$_3$ was determined as described in Section 2.3. The adsorption rate of VD$_3$ was calculated according to Equation (1) as follows:

$$\text{Adsorption rate} \% = \left( \frac{c \times v}{m_0} \right)$$

where $c$ represents the concentration of VD$_3$ (µg/mL), $v$ represents the total volume of the filtrate (mL), and $m_0$ (µg) represents the theoretical amount of VD$_3$ extracted with liquid-liquid extraction (LLE) using hexane as the solvent, calculated by multiplying the concentration of VD$_3$ in the LLE by the volume. The Fe$_3$O$_4$@PPy composites fabricated
with the ratio of PPy that showed the highest adsorption rate of VD₃ were used for further experiments.

2.6. Particle Size Measurement

The Fe₃O₄@PPy composites prepared with different ratios of PPy (1:1, 3:1, 5:1, v/w) were dispersed by sonication in pure water for 10 min. Then, 1.0–1.5 mL of the dispersion was dropped into the sample pool of a Zeta-sizer Nano-ZS90 (Malvern Instruments, Worcestershire, UK). The mean particle diameter (z-average) was determined in triplicate at 25 ºC.

2.7. Characterization of Fe₃O₄@PPy Composites

2.7.1. Scanning Electron Microscopy (SEM) and Transmission Electron Microscope (TEM)

Dried Fe₃O₄@PPy particles were characterized by SEM (JSM-7800F, JEOL, Japan) with an acceleration voltage of 10.00 kV, working distance of 6.1 mm, magnification at 50.00 KX, and signal A from the in-lens detector. A droplet of the Fe₃O₄@PPy composites suspension in distilled water was placed on a carbon coated copper mesh, and the excess liquid was removed with filter paper. Then, the Fe₃O₄@PPy particles were observed using TEM (Tecnai G2 F30, FEI, HILLSBORO, OR, USA) under an appropriate magnification. Corresponding SEM and TEM images of Fe₃O₄ NPs were used for comparison.

2.7.2. Fourier Transform Infrared (FTIR) Analysis

Dried Fe₃O₄@PPy composites or Fe₃O₄ NPs were ground with dried KBr at 1:100 (m/m), pressed into a thin slice, and then recorded with an FTIR spectrometer (IRAffinity, Shimadzu, Japan) from 400–4000 cm⁻¹.

2.7.3. X-ray Diffraction (XRD) Analysis

XRD patterns for the Fe₃O₄@PPy composites or Fe₃O₄ NPs were determined by an X-ray diffractometer (MiniFlex600, Rigaku, Japan) using Cu-Kα radiation in the region of 2θ from 10º to 70º at a scanning rate of 0.02º /s.

2.7.4. Thermogravimetric Analysis (TGA)

The thermogravimetric property of the Fe₃O₄@PPy composites was measured using a thermogravimetric analyzer (DTG-60, Shimadzu, Japan) under a N₂ atmosphere at temperatures ranging from room temperature to 600 ºC. Fe₃O₄ NPs were assayed in parallel for comparison.

2.8. Adsorbent Experiment

2.8.1. Adsorption Conditions

The adsorption experiments were carried out in a 100 mL conical flask containing 10 mL of the VD₃ saponification solution at various pHs (6.0–14.0) with a range of adsorbent doses (0.005–0.05 g Fe₃O₄@PPy composites), adsorption temperatures (25–55 ºC), and adsorption times (3–30 min). After adsorption, the Fe₃O₄@PPy composites were collected from the mixture with an external magnet. Subsequently, VD₃ desorption from Fe₃O₄@PPy composites was carried out using methanol as the desorption solvent according to the conditions described in Section 2.5. The concentration of VD₃ was determined by HPLC as described in Section 2.3. The amount of VD₃ adsorbed to the Fe₃O₄@PPy composites was calculated as qₑ (µg/g) using Equation (2) as follows:

$$qₑ = \frac{(c \times v)}{m_p} \quad (2)$$

where c represents the concentration of VD₃ (µg/mL), v represents the total volume of the filtrate (mL), and m_p is the mass of the Fe₃O₄@PPy composites used in the experiment (g).
2.8.2. Desorption Conditions

To develop a satisfactory desorption method, the desorption solvent and desorption time were investigated further. After VD₃ adsorption, the collected Fe₂O₄@PPy composites were added to 2.0 mL of desorption solvent (ethanol, acetonitrile, and methanol) and sonicated or allowed to stand for 1 to 15 min at room temperature. After magnetic separation of the Fe₂O₄@PPy composites, the resulting solution was evaporated and then redissolved in methanol as described in Section 2.4.2. Finally, 10 µL of filtrate was analyzed for VD₃ content using HPLC as described in Section 2.3. The desorption rate of VD₃ from Fe₂O₄@PPy composites was calculated according to Equation (3) as follows:

\[
\text{Desorption rate/\%} = \left( \frac{c \times v}{m_a} \right)
\]

where \( c \) represents the VD₃ concentration (µg/mL), \( v \) represents the total volume of the filtrate (mL), and \( m_a \) is the total amount of VD₃ adsorbed by the Fe₂O₄@PPy composites (µg).

2.9. Reusability of Fe₂O₄@PPy Composites

To investigate the recyclability of the Fe₂O₄@PPy composites as adsorbents for VD₃ in saponified aquatic products, one batch of Fe₂O₄@PPy composites was used as the adsorbent to conduct the adsorption and desorption experiments. The recovery of VD₃ from recycled Fe₂O₄@PPy composites was compared after repeated use. In addition, the characteristics of recycled Fe₂O₄@PPy composites were determined using SEM, TEM, XRD and FTIR measurements.

2.10. Method Evaluation

Quantitative parameters for the HPLC determination of VD₃ after Fe₂O₄@PPy composites extraction, including linearity, coefficient of determination (\( r^2 \)), limits of detection (LOD), limits of quantification (LOQ), accuracy and precision, were evaluated under optimal adsorption and desorption conditions. The sensitivity of the method was evaluated by the LOD and LOQ at a signal-to-noise ratio of 3 (S/N = 3) and 10 (S/N = 10), respectively. The accuracy of recovery was assessed by spiking saponified samples with a VD₃ standard (2 µg) and calculating recovery according to Equation (4). These samples were analyzed six times per day and the precision of the method was evaluated by intra-day relative standard deviation (RSD).

\[
\text{Recovery/\%} = \left( \frac{\text{Detected amount} - \text{sample amount}}{\text{Standard added amount}} \right) \times 100
\]

2.11. Application of Fe₂O₄@PPy Composites for VD₃ Detection in Aquatic Products

Aquatic products, mentioned in 2.1., were homogenized and saponified as described in Section 2.2. After the pH of the saponified sample solution was adjusted to 9.0, 25 mg of the Fe₂O₄@PPy composites were added for VD₃ extraction. After standing at room temperature (25 °C) for 11 min, the adsorbent was separated from the mixture using magnets. The adsorbent was rinsed with 2.0 mL of deionized water and then desorbed statically with 2.0 mL of methanol for 3 min. This methanol solution was magnetically separated from the Fe₂O₄@PPy particles and evaporated to dryness using a vacuum rotary evaporator, then redissolved in 500 µL of methanol. Finally, the methanol solution was filtered through a 0.22 µm organic filter, and 10 µL of the filtrate was analyzed by HPLC for VD₃ determination. The amount of VD₃ in aquatic products was expressed as µg per 100 g.

2.12. Statistic Analysis

All experimental results were expressed as the mean ± standard deviation. Analyses were performed with a one-way analysis of variance (ANOVA) and Tukey’s test using SPSS® software 19.0 (Chicago, IL, USA) to determine significant differences at \( p \leq 0.05 \).
3. Results and Discussion

3.1. Effect of PPy to Fe$_3$O$_4$ NPs Ratio on the Adsorption Rate of VD$_3$

As is shown in Figure 1a, the VD$_3$ adsorption rate for the Fe$_3$O$_4$@PPy composites increased with the dose of PPy. At ratio of 5:1 for PPy and Fe$_3$O$_4$ (v/m), the adsorption rate for VD$_3$ reached 100% ($p < 0.05$). The particle size of Fe$_3$O$_4$@PPy composites also increased with the amount of PPy added (Figure 1b). This indicated that an increase in pyrrole monomer led to a thickening of the coating on the surface of Fe$_3$O$_4$ NPs and provided more binding sites, thus improving the adsorption of VD$_3$. In this study, Fe$_3$O$_4$@PPy composites prepared at ratio of 5:1 (pyrrole/Fe$_3$O$_4$, v/w) had the highest adsorption rate for VD$_3$, and therefore, were selected for subsequent experiments.

![Figure 1.](image)

**Figure 1.** Effect of the ratio of pyrrole to Fe$_3$O$_4$ on the adsorption rate for VD$_3$. (a) Adsorption rate and (b) particle size of Fe$_3$O$_4$@PPy composites. Fe$_3$O$_4$ NPs were used for comparison (ratio of 0). Data is expressed as the mean ± standard deviation ($n = 3$). The different capital letters (A–D) indicate a significant difference ($p < 0.05$).

3.2. Characterization of Fe$_3$O$_4$@PPy Composites

3.2.1. SEM and TEM Observation

Both Fe$_3$O$_4$ NPs and Fe$_3$O$_4$@PPy composites comprised clusters of spherical particles when observed with SEM (Figure 2a,c). The co-ion effect of the Fe$^{3+}$ oxidant is considered the main reason for the aggregation of Fe$_3$O$_4$ NPs [18] and a similar phenomenon has also been reported for the complexation of Fe$^{3+}$ with PPy [23]. When viewed with TEM, the Fe$_3$O$_4$ NPs and Fe$_3$O$_4$@PPy composites had a different appearance. As is shown in Figure 2b, Fe$_3$O$_4$ NPs had a smooth and uniform surface morphology with diameters from 10 to 20 nm. By comparison, the image in Figure 2d shows that a PPy shell had been successfully coated onto the surface of the Fe$_3$O$_4$ NPs, where the clearly black regions are related to the Fe$_3$O$_4$ NPs at the core surrounded by light regions that have been formed by the polymerization of pyrrole monomers in the outer layer [27].

3.2.2. XRD Analysis

The Fe$_3$O$_4$ NPs prepared in this study showed typical peaks of XRD at 28 of 29.66°, 34.96°, 43.70°, 53.28°, 56.74°, and 62.22° (Figure 3a), which were accordance with previous reports on Fe$_3$O$_4$ NPs [28]. After coating with PPy, the XRD pattern of the Fe$_3$O$_4$@PPy composites had similar peaks to those detected in Fe$_3$O$_4$ NPs, indicating the presence of Fe$_3$O$_4$ NPs. Similar results were reported for the characteristics of iron oxides in Fe$_3$O$_4$@PPy [10]. However, the intensities of these peaks were all decreased to some extent. Furthermore, a broad peak was observed in the low range of 20–30°, which was ascribed to the typical amorphous structure of polypyrrole [21]. The findings in Figure 3a further provide further evidence of the existence of a PPy coating on the surface of the Fe$_3$O$_4$ NPs, consistent with TEM images (Figure 2d).
Figure 2. Microstructure of the Fe$_3$O$_4$@PPy composites under SEM and TEM. (a) SEM image of Fe$_3$O$_4$ NPs. (b) TEM image of Fe$_3$O$_4$ NPs. (c) SEM image of Fe$_3$O$_4$@PPy composites. (d) TEM image of Fe$_3$O$_4$@PPy composites.

3.2.3. FTIR Analysis

The FTIR spectra of the Fe$_3$O$_4$ NPs and Fe$_3$O$_4$@PPy composites are compared in Figure 3b. The typical band at 567 cm$^{-1}$ resulted from the stretching vibration of the Fe–O bond in Fe$_3$O$_4$ [29]. After interaction with PPy, the bands at 779 cm$^{-1}$ and 897 cm$^{-1}$ related to =C–H out-of-plane vibration of pyrrole rings [18] were observed in the Fe$_3$O$_4$@PPy composites. Furthermore, some of the typical bands associated with PPy, such as 1298 cm$^{-1}$ (C–H in-plane vibration), 1165 cm$^{-1}$ (=C–H in plane vibration), and 1062 cm$^{-1}$ (C–N stretching vibration) [30,31] were detected in the Fe$_3$O$_4$@PPy composites. The appearance of a band at 1635 cm$^{-1}$ might be ascribed to the red shift of basic C=C stretching of the Py ring due to slight over-oxidation [27,31]. Additionally, the band at 2744 cm$^{-1}$ related to C–H stretching vibration was dramatically increased in the Fe$_3$O$_4$@PPy composites. All the typical bands corresponding to PPy, as well as the missing band at 567 cm$^{-1}$, suggest that the Fe$_3$O$_4$ NPs were enveloped by a PPy coating.

3.2.4. TGA

Figure 3c shows the TGA curves for Fe$_3$O$_4$ NPs and Fe$_3$O$_4$@PPy composites. The weight loss from Fe$_3$O$_4$ NPs was 3.02% after heating from 25 °C to 100 °C, which was related to the evaporation of a small amount of water. The weight of Fe$_3$O$_4$ NPs (94.96%) was stable at temperatures above 100 °C. By comparison, three stages were detected in the TGA pattern for the Fe$_3$O$_4$@PPy composites. In the first stage, the evaporation of water and a slight degradation of PPy could be responsible for the observed weight loss of 6.58% when Fe$_3$O$_4$@PPy was heated from 100 °C to 200 °C. In the second stage, the Fe$_3$O$_4$@PPy composites decomposed dramatically at about 250 °C, which is consistent with the TGA results for a Fe$_3$O$_4$-PPy composite with a Fe$_3$O$_4$ content of 34% described by Chen et al. (2003) [30]. In the third stage, the TGA pattern for Fe$_3$O$_4$@PPy showed that
34.87% of the core content (Fe$_3$O$_4$) was left behind at 450 °C. The results in Figure 3c should be sufficient to prove that the fabricated Fe$_3$O$_4$@PPy composites have a core-shell structure.

![Figure 3](image-url)

**Figure 3.** Characteristics of the Fe$_3$O$_4$@PPy composites compared with Fe$_3$O$_4$ NPs. (a) XRD pattern, (b) FTIR spectra, and (c) TGA curves.
3.3. Conditions for VD₃ Adsorption to Fe₃O₄@PPy Composites

3.3.1. Effect of pH

The pH value of the adsorption environment is a critical factor in MSPE methods, since it can change the surface net charges for both the magnetic materials and the target compounds [23]. In this study, the initial pH of the sample solution (saponified Penaeus sinensis by-products) was 14.0. By decreasing pH, the adsorption (qₑ) of VD₃ increased from 5.91 µg/g at pH 14.0 to 31.64 µg/g at pH 9.0 for the Fe₃O₄@PPy composites (Figure 4a). However, when the pH was lowered further, the adsorption capacity decreased sharply and was reduced to zero at pH 6 and pH 5. These findings indicate that an acidic environment is not suitable for the adsorption of VD₃ to the Fe₃O₄@PPy composites. Under acidic conditions, some proteins with an isoelectric point of about 5.0 present in saponified aquatic products could precipitate, which might trap the originally released VD₃, thus resulting in poor adsorption of VD₃ to Fe₃O₄@PPy.

![Figure 4. Effects of factors on the adsorption of VD₃ by Fe₃O₄@PPy. (a) pH value of sample, (b) adsorbent dose, (c) adsorption temperature, and (d) adsorption time. Data are expressed as the mean ± standard deviation (n = 3).](image)

To reveal the role of pH on the adsorption capacity of the Fe₃O₄@PPy composites, the zeta potential was measured at different pH values. As shown in Figure 4a, the Fe₃O₄@PPy composites were negatively charged when pH conditions were greater than 9.0 (zeta potential < 0). In contrast, the Fe₃O₄@PPy composites were positively charged when the pH was below 9.0 (zeta potential > 0). It should be noted that the Fe₃O₄@PPy composites had a net charge close to neutral at pH 9.0 and demonstrated the highest adsorption capacity (qₑ) for VD₃. The results in Figure 4a indicate that the driving force for adsorption of VD₃ onto the Fe₃O₄@PPy composites does not rely on charge interactions, but rather, hydrophobic interactions and/or π–π stacking.

3.3.2. Effect of Adsorbent Dose

To achieve a good adsorption efficacy with a minimal dose of the Fe₃O₄@PPy composites, different amounts were applied to extract VD₃ from saponified Penaeus sinensis by-products. As the adsorbent dose increased from 5 mg to 50 mg, the amount of VD₃...
adsorbed by the Fe₃O₄@PPy composites increased with each increase in dosage up to 25 mg, when the maximum adsorption was achieved, and remained stable at higher doses (Figure 4b). Increasing the amount of adsorbent can provide more adsorption sites for the target ingredient, which helps improve the adsorption speed [23]. However, the efficiency of the adsorbent should also be considered because the amount of adsorbent comes at a cost. In this study, the adsorption capacity of the Fe₃O₄@PPy composites for VD₃ decreased gradually with each increase in dose, which could be ascribed to the existence of excess amounts of adsorbent. This could explain why the adsorption dose increased gradually, however the adsorption efficiency did not increase accordingly. Considering the high content of VD₃ adsorbed and a relatively higher qₑ compared to other doses, 25 mg was chosen as the optimum dose for the subsequent study.

3.3.3. Effect of Adsorption Temperature

VD is sensitive to heat and degrades easily under high temperatures [32]. The extraction efficiency of VD₃ from milk by Fe₃O₄@PPy decreased as the extraction temperature increased [10]. However, in this study, when the adsorption temperature ranged from 25 °C to 55 °C, the adsorption capacity of the Fe₃O₄@PPy composites for VD₃ remained stable (Figure 4c). A difference in the sample matrix between the saponified aquatic products used in this study and the unsaponifiable milk samples in the literature might explain the differential effect of temperature on VD₃ adsorption. Similar to our results, Zhang et al. (2020) [23] described that temperature had little effect on the extraction of 11 antiseptic ingredients with Fe₃O₄@PPy composites. Considering the energy saving and a convenient operation, 25 °C was selected as the adsorption temperature for subsequent experiments.

3.3.4. Effect of Adsorption Time

According to previous studies, compared to a conventional SPE method, a quicker equilibrium between the target component and Fe₃O₄@PPy nanoparticles can be reached as because of their high surface area and short diffusion route [33]. Figure 4d shows that the adsorption capacity of the Fe₃O₄@PPy composites for VD₃ increased rapidly from 0 to 11 min, then remained stable thereafter. This meant that 11 min was sufficient time to adsorb VD₃ molecules from the sample matrix. A similar adsorption time of 10 min was reported for VD extraction from milk with Fe₃O₄@PPy [10]. Therefore, we selected 11 min as the appropriate time for VD₃ adsorption to Fe₃O₄@PPy composites.

3.4. Desorption Conditions

Two desorption methods, namely ultrasonic and static treatment, were used to recover VD₃ from the Fe₃O₄@PPy composites (Figure 5a). No significant difference was found for the two desorption methods (measured as the VD₃ content in the desorbed solution) (p > 0.05). Acetonitrile, methanol, and ethanol were used as desorption solvents under static conditions to recover VD₃ from the Fe₃O₄@PPy composites (Figure 5b). Clearly, acetonitrile and methanol were more efficient than ethanol for desorbing VD₃ (p < 0.05). After a consideration of cost, applicability, and safety, we selected methanol as the desorption solvent. Subsequently, the desorption time with methanol was tested for the Fe₃O₄@PPy composites as shown in Figure 5c. The amount of VD₃ released was found to increase over time from 0.5 min to 3 min, and then remained stable after 3 min. Therefore, we chose 3 min as the desorption time for the follow-up study.

3.5. Regeneration of Fe₃O₄@PPy Composites

To determine if the Fe₃O₄@PPy composites prepared in this study could be reused after adsorption of VD₃ in aquatic products, the performance of the particles was evaluated after repeated adsorption and recovery of VD₃. After one cycle of adsorption and desorption of VD₃, the Fe₃O₄@PPy composites were dried in a vacuum oven and used for another round of adsorption and desorption. As is shown in Figure 6, the recovery of VD₃ decreased rapidly each time the Fe₃O₄@PPy composites were recycled. At the end of two cycles, the
recovery of VD₃ was 82.25%, but this dropped to 57.48% after three cycles. Our results suggested that the Fe₃O₄@PPy composites could be reused at least twice for a VD₃ recovery over 80%. The decrease in VD₃ recovery might be due to damage of the PPy shell with repeated use, thus leading to incomplete adsorption of VD₃.

![Figure 5](image_url)  

**Figure 5.** Effects of factors on VD₃ desorption from Fe₃O₄@PPy composites. (a) Desorption method, (b) desorption solvent, and (c) desorption time. Data are expressed as the mean ± standard deviation \((n = 3)\). The different capital letters (A,B) indicate a significant difference \((p < 0.05)\).

To investigate possible reasons for the decreased VD₃ recovery, the characteristics of recycled Fe₃O₄@PPy composites were compared using SEM and TEM, as well as XRD and FTIR analysis. No obvious microstructural changes were observed for the recycled Fe₃O₄@PPy composites with TEM (Figure 7c,d) when compared to its original condition (as shown in Figure 2d). In contrast, SEM identified some irregular areas wrapped in spherical particles after the composites that had been reused twice (see dotted outlines in Figure 7b), which were not present in unused composites (as shown in Figure 2c) or those that had been recycled once (Figure 7a). These changes might reduce the contact area for VD₃ adsorption, which might be responsible for the reduction in VD₃ adsorption.
Figure 6. Regeneration experiment using Fe$_3$O$_4$@PPy composites for VD$_3$ adsorption.

Figure 7. Characteristics of the Fe$_3$O$_4$@PPy composites after recycling once and twice. SEM images of Fe$_3$O$_4$@PPy composites reused (a) once and (b) twice. TEM images of Fe$_3$O$_4$@PPy composites reused (c) once and (d) twice. (e) XRD and (f) FTIR spectra of Fe$_3$O$_4$@PPy composites. The numbered spectra represent (1) Fe$_3$O$_4$@PPy, (2) Fe$_3$O$_4$@PPy after adsorption of VD$_3$, (3) Fe$_3$O$_4$@PPy after the first desorption, and (4) Fe$_3$O$_4$@PPy after the second desorption.

The specific peaks in the XRD spectrum of the Fe$_3$O$_4$@PPy composites related to the Fe$_3$O$_4$ NPs were not affected by adsorption of VD$_3$ (Figure 7e). A similar phenomenon was found for the reused magnetic composites. However, the broad peak in the range of 20–30$^\circ$ that was associated with the typically amorphous structure of PPy shifted obviously to smaller angles, suggesting structural changes in the PPy shell after the Fe$_3$O$_4$@PPy composites had been reused twice. Regarding the FTIR spectra, the band at 1635 cm$^{-1}$ associated with C=C stretching vibration of PPy disappeared from the Fe$_3$O$_4$@PPy com-
posites after adsorption of VD₃ (Figure 7f), which implied that the functional C=C group is involved in a hydrophobic interaction between Fe₃O₄@PPy and VD₃. However, the band representing C=C stretching vibration in reused Fe₃O₄@PPy composites was not restored after rinsing with methanol (the desorption solvent). This means this functional group might be irreversibly damaged by the desorption solvent. Furthermore, the reused Fe₃O₄@PPy composites showed increased intensities for the bands at 1062 cm⁻¹, related to C-N stretching vibration, and 1165 cm⁻¹, assigned to =C-H in plane vibration [30]. Similarly, the intensities of the bands at 897 cm⁻¹ and 779 cm⁻¹, attributed to the out-of-plane vibration of the =C-H in pyrrole rings [18], increased each time Fe₃O₄@PPy was recycled. In addition, the functional group at 2744 cm⁻¹ shifted towards blue after adsorption of VD₃ compared to a red shift that increased each time the Fe₃O₄@PPy composites were reused. These changes suggest that the band related to the C-H stretching vibration in PPy might be crucial for VD₃ adsorption and desorption. The increases in the intensities of the typical peaks related to the PPy ring indicate that the PPy coating might partially fall off the surface of the Fe₃O₄@PPy composites with VD₃ loading, which cannot be recovered by external magnetic adsorption, thus reducing VD₃ recovery.

3.6. Method Validation and Application

Good linearity for the VD₃ assay was achieved in the range of 0.1–10 µg/mL in saponified solutions of shrimp by-products with correlation coefficients (r²) reaching 0.9989. The limit of detection (LOD) (S/N = 3) and the limit of quantification (LOQ) (S/N = 10) were 10 ng/mL and 33 ng/mL, respectively. In the spiking test, the recovery of VD₃ was 97.72%, and the relative standard deviation (RSD) value was 1.78% (Table 1).

Table 1. Quality control parameters for the developed method for determining VD₃ content in saponified Penaeus sinensis by-products (n = 6).

| Linearity Range (µg/mL) | r²     | LOD (ng/mL) | LOQ (ng/mL) | Recovery (%) | RSD% |
|-------------------------|--------|-------------|-------------|--------------|------|
| 0.100–10.0              | 0.9989 | 10          | 33          | 97.72        | 1.78 |

The VD₃ content of Penaeus sinensis by-products and other aquatic products was determined using Fe₃O₄@PPy composites-based extraction coupled with HPLC detection. The results in Table 2 show significant variations in VD₃ content for the various species and parts tested in this study. Similarly, some authors have also reported significant differences in VD₃ content for fish, both between species and within species [6]. For example, the average content of VD₃ in mahi-mahi was only 1.11 µg/100 g, while it reached 45.3 µg/100 g in tilapia [34]. Baltic salmon had significantly higher VD₃ content (26.5 µg/100 g) than farmed Norwegian salmon (5.9 µg/100 g) [6]. In this study, low average VD₃ content was detected in squid (Lolium japonica) meat (2.86 µg/100g) and in silver pomfret (Pampus argenteus) (4.65 µg/100 g). By comparison, the tested clams Paphia undulata, Cyclina sinensis, and razor clam (Sinonovacula constricta) had high VD₃ content (>70 µg/100 g), suggesting these shellfish are a good source of dietary VD₃. Furthermore, it was noted that the average VD₃ content in the by-products of Penaeus sinensis (Solenocera crassicornis), Pacific white shrimp (Litopenaeus vannamei), and cuttlefish (Sepia esculenta) was higher than 10 µg/100 g, which exceeds the recommended daily intake of 5 µg/d [6]. In addition, the content of VD₃ was higher in the by-products than that the corresponding muscle tissue (p < 0.05). These findings also suggest that the by-product of Pacific white shrimp should be considered a good raw material for VD₃ extraction.
Table 2. Average content of VD₃ in the examined aquatic products.

| Species| VD₃ (µg/100 g) |
|--------|----------------|
|        | Mean ± SD      |
| Penaeus sinensis (Solenocera crassicornis) (by-products) | 12.12 ± 1.39 |
| Pacific white shrimp (Litopenaeus vannamei) (muscle tissue) | 10.77 ± 2.06 |
| Pacific white shrimp (Litopenaeus vannamei) (by-products) | 29.34 ± 0.36 |
| Cuttlefish (Sepia esculenta) (muscle tissue) | 10.56 ± 0.19 |
| Cuttlefish (Sepia esculenta) (by-products) | 10.94 ± 0.52 |
| Squid (Lolioius japonica) (muscle tissue) | 2.86 ± 0.63 |
| Squid (Lolioius japonica) (by-products) | 6.44 ± 0.20 |
| Clam (Cyclina sinensis) | 80.81 ± 10.39 |
| Clam (Paphia undulata) | 131.76 ± 3.72 |
| Razor clam (Sinonovacula constricta) | 73.77 ± 6.38 |
| Chlamys farre (Azunapeuten farre) | 23.10 ± 0.86 |
| Silver pomfret (Pampus argentaeus) | 4.65 ± 2.84 |

Note: *"* represents the VD₃ content, expressed as the mean ± standard deviation (SD) (n = 3). Different letters (a–i) against the mean content of VD₃ for each species suggest a significant difference (p < 0.05).

4. Conclusions

In the present work, we fabricated an effective adsorbent composed of Fe₃O₄ NPs functionalized with a PPy coating for VD₃ extraction. The results of SEM, TEM, XRD, FTIR and TGA prove that the Fe₃O₄@PPy composites have a core-shell structure. The adsorption of VD₃ from saponified Penaeus sinensis by-products to Fe₃O₄@PPy composites was optimal under the following conditions: pH 9.0 with a 25 mg dose at 25 °C and 11 min adsorption time. The adsorbed VD₃ could be effectively desorbed from the binding sites of the Fe₃O₄@PPy composites with methanol after static contact for 3 min. The accuracy and reproducibility of the developed method for VD₃ extraction and detection were quite satisfactory as evidenced by a high linear correlation coefficient and a low intra-day RSD. Compared to other conventional methods, the proposed method is more rapid since it does not require complicated extraction and concentration procedures. Instead, VD₃ can be separated from complex samples within minutes by quick and easy magnetic separation. Furthermore, this method is environmentally friendly since it requires less organic solvent. In addition, the results for VD₃ content in the tested aquatic products will provide important reference information for the rational selection of products for the development of VD₃-fortified foods.

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