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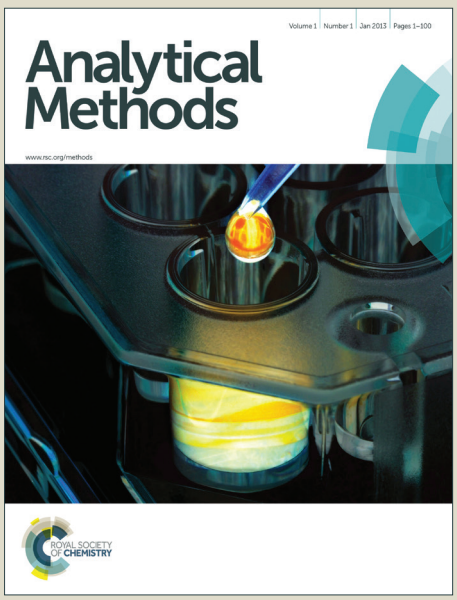

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Anionic surfactant coacervation extraction-magnetic solid phase microextraction for determination of malachite green

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Abstract

A novel, fast, and efficient two-step microextraction technique for preconcentration and extraction of trace amounts of malachite green in fishpond water, river water and flesh of fish was developed using spectrophotometry. MG with pH of 6.5 was extracted and mediated by the coacervation phase of anionic surfactant sodium dodecyl benzene sulfonate. The coacervation phase was then trapped by diatomite bonded Fe_3O_4 magnetic nanoparticles (DBMNPs) that can rapidly achieve two-phase separation in a magnetic field. The extracted surfactant-rich phase was diluted with ethanol and its absorbance was measured at 624 nm. A number of important parameters affecting extraction efficiency, such as volume of extraction solvent, amount of salt, pH, amount of DBMNPs, equilibration temperature and time, were investigated. The calibration graph was linear for MG ranging from 2 ng mL^{-1} to 180 ng mL^{-1} in the initial solution, with r^2 = 0.9994 (n = 10). The detection limit based on three times the standard deviation of the blank ($3S_b$) was 0.67 ng mL^{-1} and the relative standard deviation for 20 ng mL^{-1} of MG was 1.12% (n = 5). The method was applied to determine the trace amounts of MG from fishpond water, river water and flesh of fish.

1. Introduction

Malachite green (MG, Scheme 1) has functioned as an effective fungicide and antiseptic in aquaculture, and as antifungal, antimicrobial and anti-parasitic agents in the food industry since the 1930s. However, this chemical causes serious side effects.[1,2] High concentrations of MG severely damage the internal organs of fish and the growth of fish eggs.[3] MG also has toxic effects on human cells; it has mutagenic and carcinogenic properties. The use of MG in aquaculture has been banned in many countries because of its toxicity.[4,5] However, because of its high effectiveness and low cost, this harmful dye is still used and will probably continue to be used in aquaculture in some parts of the world. Developing a sensitive detection method for the presence of MG in various samples is therefore of importance.

Scheme 1

Given the low concentrations of MG in environmental samples and the difficulties in its extraction, preparing samples before determination is a necessity. Sample preparation is often the bottleneck that directly affects the accuracy, precision, and limits of detection, and is often the rate-determining step of the analytical process. The main direction in recent studies is towards the development of efficient, economical, simple, rapid, and clean sample preparation methods. To date, various methods have been reported for the preparation of aqueous MG samples. Liquid-liquid extraction is one of the oldest preconcentration methods in analytical chemistry. This technique is time consuming and requires large amounts of expensive and toxic organic solvents, which are subsequently evaporated.[6–9] Other methods, such as solid-phase extraction (SPE), use a limited amount of organic solvents but are relatively expensive. SPE techniques are also often non-specific and time-consuming.[10–14]

Various methods have also been reported for determining MG in aqueous samples, such as high-performance liquid chromatography (HPLC-UV),[11,12] spectrophotometry,[9,13–17] and liquid chromatography-mass spectrometry (LC-MS).[9,10] Although these methods presents certain advantages, they also have specific limitations and a number of methods, such as LC-MS, are

laborious and require sophisticated instruments.[9,10] While spectrophotometry is a simple and widely used analytical method for quantitative analysis, this method is not selective and requires the analytes present in a given sample to have different absorption spectra with low overlapping.[9,13–17]

Over the last decade, an increasing interest in the use of ionic surfactants has been observed in the field of separation science. Ionic surfactant solutions can facilitate coacervation and be used as extraction solvents.[18–20] However, these solutions are subject to certain limitations despite their versatility. For the majority of methods employing ionic surfactants, centrifugation is required for separating the donor phase (i.e., sample) from the acceptor phase (i.e., surfactant); this can be time-consuming when dealing with large sample volumes.[21–23]

The use of magnetic extractants has received considerable attention and has been reported in numerous articles. Cloud point extraction (CPE) uses non-ionic surfactant has reportedly been coupled with dispersive microsolid phase extraction for the purpose of sample preparation.[24] This method can be applied for the adsorption and separation of analytes from large volumes of environmental samples in a short period, and has been developed as a fast, simple, cost effective, and versatile extraction method based on the use of magnetic or magnetizable adsorbents.[24–26] The main advantage of this method is that phase separation can be conveniently performed by applying an external magnetic field. Overcoming specific steps associated with CPE, such as centrifugation to separate the surfactant-rich phase, refrigeration of the condensed micellar phase to reduce viscosity, and the use of appropriate apparatus to directly sample the surfactant-rich phase, significantly reduces the preparation time.

In the present study, a new, two-step method was developed to determine trace levels of MG residues in water and flesh of fish samples by using the anionic surfactant sodium dodecyl benzene sulfonate (SDBS) as the extraction solvent and DBMNPs as the trapping extractant. The possible factors affecting extraction efficiency, such as extraction solvent volume, amount of salt, pH, amount of DBMNPs, equilibration temperature and time were investigated and optimized.

2. Experimental

2.1. Reagents and materials

All chemicals used in this study were of analytical grade unless stated otherwise. Methanol and trichloroacetic acid (HPLC grade) were purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Double-distilled water (DDW) was used throughout the study. MG was obtained from Shanghai Chemical Reagent Company (Shanghai, China). A stock solution of 100 μg mL^{-1} of MG was prepared by dissolving 10 mg of the reagent in water and diluting to 100 mL in a volumetric flask. The desired concentrations were obtained by successive dilutions. SDBS was obtained from Acros Organics (New Jersey, America). 2% SDBS was prepared by dissolving 1 g SDBS in water and diluting to 50 mL in a volumetric flask. Britton Robinson (BR) buffer solution was prepared by adding NaOH to the BR buffer (phosphoric, acetic, boric; concentration was 0.04 mol L^{-1}) to adjust the pH using a pH meter. Water samples were obtained from the Fen River, and fishpond water was obtained from a local fishpond in Shanxi, China, which was filtered through a 0.45 μm nylon filter membrane (Jinteng Instrument Co., Ltd., Tianjin, China) before they were used. Sturgeon was purchased from local free market. Once in the laboratory, all of the fish were accuracy weighed, and their head, gills, skin, and thorns were removed. The muscle was separated, homogenised and stored at -18 ^{o}C until analysis. A whatman No. 2 filter paper (Whatman International Ltd., Brentford, Kent, United Kingdom) was used to filtered homogenates.

2.2. Apparatus

A CARY 300 Scan UV–visible spectrophotometer (Varian Ltd., Palo Alto, America) was used for recording absorption spectra and absorbance measurements using 0.7 ml quartz cell. A pH meter (Model pHS-3C, Shanghai Yidian Analytical Instruments, Ltd., shanghai, China) was used for pH adjustment. An Ultrasonic Cleaner (Model KH 2200DV, Kunshan Hechuang Ultrasonic Instrument Co., Ltd., kunshan, China) was used in the extraction. A centrifuge (MIKRO 22R, Hettich Zentrifugen, GmbH&Co., Tuttelingen, Germany) was used to prepare the

fish sample. An electric glass homogenizer was used to homogenize the flesh of fish (DY89-Ⅱ, Ningbo Xinzhi Biotechnology Co., Ltd., Zhejiang, Chain).

2.3. Synthesis of magnetic materials

2.3.1. Synthesis of pure maghemite nanoparticles

Pure maghemite nanoparticles (MNPs) were prepared according to the literature.[27,28] In a typical synthesis of monodisperse Fe_3O_4 MNPs with mesoporous structure, $FeCl_3 \cdot 6H_2O$ (0.8 g) was dissolved in ethylene glycol (EG) (16 mL) to form a homogeneous solution, followed by the addition of NaAc (2.4 g) and ethylenediamine (ETH) (8 mL). The mixture was stirred vigorously for half an hour and then sealed in a teflonlined stainless-steel autoclave (50 mL capacity). The autoclave was heated to and maintained at 200 °C for 8 h, and then allowed to cool to room temperature. The black products were washed several times with distilled water and ethanol and then dried at 60 °C in a vacuum for 8 h.

2.3.2. Synthesis of diatomite bonded Fe_3O_4 magnetic nanoparticles

The above process can be extended to the synthesis of diatomite bonded Fe_3O_4 magnetic nanoparticles using hydrothermal synthesis. In a typical synthesis, purified diatomite (0.3 g) was added to 24 mL of EG. Subsequently, 0.6 g of $FeCl_3 \cdot 6H_2O$ and 1.2 g of NaAc were dissolved in the EG solution at ambient temperature. After stirring for about 30 min, the solution was transferred to a 50 mL Teflon-lined stainless-steel autoclave kept at 200 °C for 8 h, and allowed to cool to room temperature. The black products were washed several times with distilled water and ethanol, and then dried at 60 °C in vacuum for 8 h.[29,30] The photography of the MNPs and the DBMNPs samples were examined by scanning electron microscope (SEM), as shown in Scheme 2.

Scheme 2

2.4. Extraction procedure

The first step, 0.1 ml of 10 μg mL^{-1} MG solution, 1.0 mL of BR buffer solution with pH 6.5,

0.9 mL of 2% of SDBS, and 1.2 g of $MgCl_2 \cdot 6H_2O$ were added to a 10 mL centrifuge tube and diluted to the target mark with water. The resultant solution was equilibrated at room temperature for 10 min. After this process, 35 mg of DBMNPs were added into the tube. The mixture was again vigorously shaken for 2 min. The SDBS phase was successfully trapped to the DBMNPs phase after the high-speed shaking process. A magnet was subsequently held around the test tube to collect the DBMNPs at the bottom of the test tube. The upper aqueous phase was removed and the surfactant-rich phase was diluted with 300 μL ethanol. The solution was then placed in an ultrasound for 2 min to desorb the SDBS from the DBMNPs. The DBMNPs were then separated from the solution by using a magnet, and the solution was measured at 624 nm. All experiments were performed in triplicate. The extraction procedure was show in Scheme 3. The DBMNPs were washed five times with ethanol under ultrasonic for 2 min. The ethanol was removed by magnetic decantation and DBMNPs were dried in a vacuum oven at 50 °C.

The flesh of fish was prepared according to Paleologos *et al* .[31] Fish samples used in shelf life experiments were cut in small pieces. 5 g of each sample were ground in a electric glass homogenizer for 3 min and thoroughly homogenized with 10 ml trichloroacetic acid (TCA) 6% (w/v). The homogenates were centrifuged (12 000 rpm, 20 min, 4 °C) to allow precipitation and filtered twice through Whatman No. 2 filter paper. The filtrates were transferred to 10 ml volumetric flasks and diluted with 6% (w/v) TCA to the mark. Extraction procedure was the same with water samples.

scheme 3

2.5. Calibration of the preconcentration factor and extraction recovery percentage

To evaluate the performance of the proposed method, enrichment factor (EF) and extraction recovery percentage (ER%) were determined using HPLC method[12] and calculation according to Eqs. (1) and (2):

$$EF = \frac{C_{des}}{C_0} \quad (1)$$

where C_{des} and C_0 are the concentrations of analytes in the desorbed phase and the initial concentration of analytes in the sample solution, respectively.

$$ER\% = \frac{C_{des} \times V_{des}}{C_0 \times V_0} \times 100\% = EF \times \frac{V_{des}}{V_0} \times 100\% \quad (2)$$

where V_{des} and V_0 are the volumes of the desorbed phase and sample solution, respectively.

3. Results and discussions

3.1. Effects of the volume of SDBS

A successful extraction procedure maximizes extraction efficiency by minimizing the phase volume ratio, thereby maximizing its enrichment factor. Thus, investigating the effects of surfactant volume on the performance of the extraction system is necessary. As shown in Fig. 1, the measured absorbance of the extracted solution increases as the surfactant amount increases, and then decreases when the amount of extracted surfactant has reached its maximum. This trend occurs because as the amount of SDBS increases, the final volume of analytical solution increases, which leads to the decrease in absorbance. The optimum surfactant volume of 0.9 mL SDBS was selected to achieve the optimal analytical signal in conjunction with the highest possible extraction efficiency.

Fig. 1

3.2. Effects of the amount of salt

Addition of salt can cause ionic surfactant solutions to separate into immiscible surfactant-rich and surfactant-poor phases. Several inorganic salts, including NaCl, Na_2SO_4, KCl, KBr, $CaCl_2$, $MgSO_4$, and $MgCl_2 \cdot 6H_2O$, were tested. $MgCl_2 \cdot 6H_2O$ is found to be the best among the selected salt. When the same amounts of the different types of salt were added, only $CaCl_2$, $MgSO_4$, and $MgCl_2 \cdot 6H_2O$ form the coacervation phase, whereas NaCl, Na_2SO_4, KCl, and KBr could not form the coacervation phase at room temperature even when the amount of salt was increased. Nevertheless, the coacervation phase formed by $CaCl_2$ could not undergo desorption using methanol. The solubility of $MgCl_2 \cdot 6H_2O$ is much better than that of $MgSO_4$. Therefore,

$MgCl_2 \cdot 6H_2O$ was added to induce production of coacervation phase and extraction of MG. To determine the effects of the amount of salt, experiments were conducted, wherein different amounts of $MgCl_2 \cdot 6H_2O$ were used. The other experimental conditions were kept constant. Fig. 2 shows with increase amounts of $MgCl_2 \cdot 6H_2O$ s from 0.6 g to 1.6 g, the absorbance first increases and then decreases because increasing the amount of $MgCl_2 \cdot 6H_2O$ can increase the volume of coacervation phase, which in turn decreases the absorbance. After a comprehensive consideration of the results, 1.2 g of salt was added in the subsequent experiments.

Fig. 2

3.3. Effect of pH

The pH of the working media is an important parameter considered in separation–preconcentration studies. Effect of pH values on sample solution was investigated given a pH range of 3.0 to 10.0 by adjusting pH of sample solution with hydrochloric acid and sodium hydroxide. Fig. 3 shows the effect of pH on the absorbance of the MG at 624 nm. The maximum absorbance is obtained at pH 6.5. Along with the pH values increase, MG becomes colorless and its absorbance is decreased. Based on the above result, pH 6.5 was selected for the subsequent experiments. Different buffer systems with pH of 6.5, such as acetic acid, sodium acetate, and BR buffer solution, were examined. The BR buffer solution was selected because its absorbance was higher than those of the others after extraction.

Fig. 3

Fig. 4

3.4. Effects of equilibration temperature and time

Using the shortest equilibration time and the lowest possible equilibration temperature is desirable as a compromise between completion of extraction and efficient separation of phases. Therefore, the effect of equilibration temperature ranging from 5 °C to 35 °C was examined as all other experimental conditions were kept constant. Room temperature was found to be adequate for the analysis. The dependence of extraction efficiency on equilibration time was also

investigated for a time interval of 5 min to 30 min. The results show that maximum extraction efficiency is achieved within 10 min extraction. No significant variation was observed when the extraction time exceeded 10 min. Therefore, 10 min was used as the optimum extraction time in all experiments.

3.5. Effect of the amount of DBMNPs

Compared with conventional MNPs adsorbents, we found that only DBMNPs could trap the coacervation phase. DBMNPs offer high extraction capacity, rapid extraction dynamics, and high extraction efficiency. The amount of DBMNPs has a direct effect on the extraction of coacervation phase. To determine the effect of DBMNPs on extraction recovery, various experiments were performed by adding 10 mg to 45 mg of DBMNPs. As shown in Fig. 4, the optimum amount of DBMNPs for trapping coacervates is 35 mg. Thus, 35 mg of DBMNPs was used in the subsequent experiments.

Fig. 5

3.6. Analytical performance

A linear calibration graph was obtained form 2 ng mL^{-1} to 180 ng mL^{-1} of MG in the initial solution under the optimized conditions. The equation for the line is $A = 3.4\times10^{-3}\ C + 5.9\times10^{-3}$ with a regression coefficient (r^2) of 0.9994 (n = 10), where A denotes the absorbance and C denotes the concentration of MG in ng mL^{-1}. The detection limit based on three times the standard deviation of the blank ($3S_b$) is 0.67 ng mL^{-1} and the relative standard deviation (RSD) of the developed method determined by analyzing the standard solution at 20 ng mL^{-1} of MG is 1.12% (n = 5). All samples were also measured by HPLC-UV to verify the results obtained by the developed method, in fact the values are consistent with UV measurement results. The ER% were 96.32%, and EF were 24.08 for MG.

The determination of MG using the two-step method was compared with other reported methods. The results are shown in Table 1. Unlike in the previously reported techniques, SDBS was used instead of a volatile and toxic organic solvent in the extraction phase. The results reveal

that the two-step method is a sensitive, environment friendly and reproducible technique that can be used for the preconcentration of MG from real samples.

Table 1

The adsorption-desorption cycles were performed and a change of -3% in the extraction efficiency was defined as tolerance limit. Results indicated that after eleven sorption–desorption cycles, as shown in Fig. 6, the extraction efficiency of DBMNPs for the drugs was remained within the tolerance limit but after twelve runs a 6% decrease in its performance was observed; therefore the reuse limit of the proposed sorbent was eleven cycles.

Fig. 6

3.7. Interference studies

Performing the procedure in the presence of interfering ions in the samples validated the selectivity of the coacervation phase for MG. Solutions containing 100 ng mL^{-1} of MG and various amounts of interfering ions were prepared following general procedure. The tolerance limit was defined as the amount of interfering ions causing less than ±5% change in the absorbance. Table 2 shows the results, which confirm good selectivity of the proposed method to the accurate determination of MG in real samples. Other organic coloring substances which may co-exist in the samples such as crystal violet and brilliant green did not interfere with the determination.

Table 2

3.8. Applications

The practical applicability of the proposed method was evaluated by extracting MG from samples of different sources, including fishpond water, river water and flesh of fish. The results show that MG residues in all samples are below the detectable level, indicating that these samples are practically free of MG. These samples were then spiked using standard amounts of MG at different levels to assess the matrix effect. The results are given in Table 3 that shows river water, fishpond water and flesh of fish spiked of MG. The relative recoveries (RRs) for the MG in river

water and fishpond water are in the ranges of 98.6%–101.1%, 96.1%–102.6% 97.6%–103%, 95.9%–102.3% and 88.7%–91.2%. The results of the proposed method were also compared with that of HPLC,[12] the results were shown (Table 4) the two methods had no significant differences after being evaluated by *t*-test and *F*-test method when the confidence level was 95%. The proposed method was satisfactorily applied to the determination of MG in real samples.

Table 3

Table 4

4. Conclusions

In the present study, a new, two-step microextraction technique based on SDBS coacervation phase extraction and DBMNPs trap was developed. The proposal has been optimized considering those variables, related to the extraction and adsorbent steps, which have a clear influence in its performance. Under the optimal extraction condition, the proposed method provides the best results in terms of sensitivity. Good linearity and repeatability were also achieved. Based on the results, the proposed method achieves greater simplification than conventional coacervation phase extraction procedures, thereby alleviating the need for specific sample handling treatments, such as centrifugation or freezing of the samples. It also shortens the overall analysis time. With the use of DBMNPs and SDBS in the extraction, the procedure can be described as environmentally friendly. The proposed method can successfully detect MG in river water, fishpond water and flesh of fish without matrix interferences.

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Table 1. Analytical parameter of different method

Method	Extraction solvent	The amount of extraction solvent	Limit of detection (ng mL^{-1})	Extraction time	Samples	Reference
DLLME-FO-LADS	$CHCl_3$	2ml	3.65	3 min	Water	[8]
MISPE-HPLC	MIP	-	0.1	24 h	Water	[12]
CPE-UV-Vis	TX-100	2 ml	1.2	20 min	Water	[15]
CPE-UV-Vis	TX-114	0.5 ml	2.9	15 min	Water	[16]
Two step method-UV-Vis	SDBS	300 µL	0.67	10 min	Water	Present work

FO-LADS fiber opticlinear array detection spectrophotometry HPLC high-performance liquid chromatographic

UV-Vis UltraViolet –visible MIP molecularly imprinted polymer (methacrylic acid-ethylene glycol dimethacrylate)

Table 2 The effect of interfering ions on the determination of 100 ng • mL^{-1} of MG

Tolerance ratio	Interfering ions
1000	Na^+, K^+, Ba^{2+}, Ca^{2+}, Mg^{2+}, Al^{3+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, Co^{2+}, Cr^{3+}, NH_4^+, Cl^-, NO_2^-, CrO_4^{2-}, $Cr_2O_7^{2-}$, F^-, Br^-, I^-, NO_3^-, SO_4^{2-}, $S_2O_3^{2-}$, $S_2O_8^{2-}$, BO_3^{3-}, $B_4O_7^{2-}$, CH_3COO^-, CO_3^{2-}, HCO_3^-, $C_2O_4^{2-}$, HPO_4^{2-}, $H_2PO_4^-$
500	Zn^{2+}, Sr^{2+},
100	Sn^{2+},
50	Pb^{2+}, Fe^{2+}, Fe^{3+},
8	MnO_4^-

Table 3 Determination of MG in aqueous samples (n=5)

Water Samples	Amount added (ng mL^{-1})	Amount found (ng mL^{-1})	Recovery (%)	RSD (%)
River water1	0	0	-	-
	5	4.93	98.6	1.93
	10	9.5	97.5	1.57
	15	15.07	100.5	1.04
	20	20.21	101.1	2.21
River water2	0	1.12	112	3.25
	5	5.13	102.6	1.22
	10	9.61	96.1	1.19
	15	14.94	99.6	2.33
	20	19.93	99.7	3.89
Fish pond water	0	0	-	-
	5	4.88	97.6	1.98
	10	10.30	103.0	2.53
	15	14.82	98.8	1.99
	20	19.89	99.5	1.65
Free market water	0	1.16	116	3.69
	5	4.96	99.2	1.68
	10	10.23	102.3	1.97
	15	14.92	99.5	1.99
	20	19.18	95.9	2.01
Sturge-on sample	0	0	-	-
	5	4.53	90.6	3.41
	10	8.87	88.7	4.13
	15	13.69	91.2	3.97
	20	17.25	86.2	3.85

Table 4 The determination results of the MG in real samples compared to literature (n=5)

samples	The proposed method		The No. 12 references method	
	Found (ng mL^{-1})	Equivalent nominal content (%) ±S.D.[a]	Found (ng mL^{-1})	Equivalent nominal content (%) ± S.D.[a]
River water1	98.39	98.39±1.41 (t, 0.76; F, 1.83)	98.15	98.15±1.96

The tabulate values of t and F at the 95% confidence limit are t=2.31 and F=6.39.

[a] Average of five determination

Fig.ure Captions

Scheme 1 The structure of malachite green.

Scheme 2 The photography of MNPs and DBMNPs was observed on a scanning electronic microscope (JSM-7500F, JEOL Ltd., Japan). A, MNPs, B, DBMNPs.

Scheme 3 The Scheme of extraction procedure.

Fig. 1 The effect of volume of SDBS. Extraction conditions: concentration of malachite green, 100 ng mL^{-1}; NaCl, 1.2 g; Extraction time, 10 min; DBMNPs, 35 mg; ultrasound, 2 min; desorption solvent, 300 μL ethanol; pH 6.5.

Fig. 2 The influence of amount of salt. Extraction conditions: concentration of malachite green, 100 ng mL^{-1}; SDBS, 0.9 mL; Extraction time, 10 min; DBMNPs, 35 mg; ultrasound, 2 min; desorption solvent, 300 μL ethanol; pH 6.5.

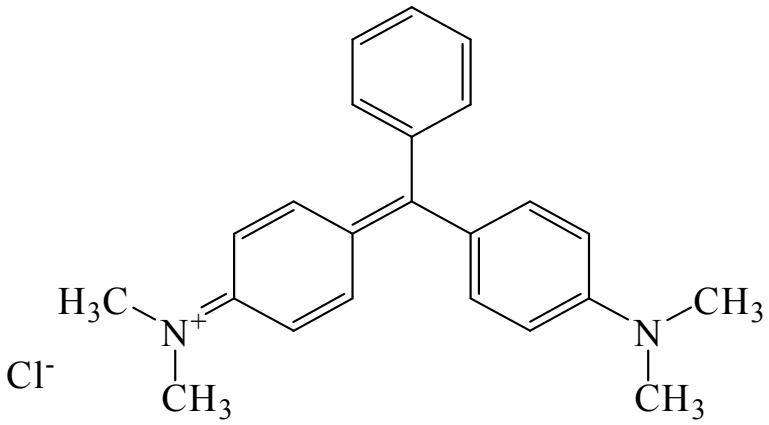
Fig. 3 The influence of pH. Extraction conditions: concentration of malachite green, 100 ng mL^{-1}; SDBS, 0.9 mL; Extraction time, 10 min; NaCl, 1.2 g; DBMNPs, 35 mg; ultrasound, 2 min; desorption solvent, 300 μL ethanol.

Fig. 4 The UV-vis spectrum in different pH media.

Fig. 5 The influence of amount of DBMNPs. Extraction conditions: concentration of malachite green, 100 ng mL^{-1}; SDBS, 0.9 mL; Extraction time, 10 min; NaCl, 1.2 g; ultrasound, 2 min;

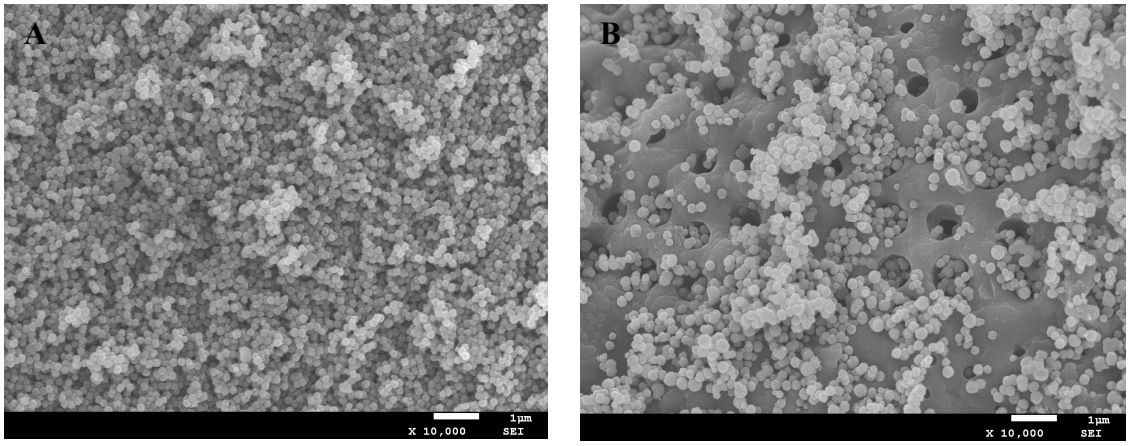
desorption solvent, 300 μL ethanol; pH 6.5.

Fig. 6 The effect of the reuse of the DBMNPs sorption–desorption cycle times. Extraction conditions: concentration of malachite green, 100 ng mL^{-1}; SDBS, 0.9 mL; Extraction time, 10 min; NaCl, 1.2 g; DBMNPs, 35 mg; ultrasound, 2 min; desorption solvent, 300 μL ethanol; pH 6.5.



Scheme 1

Scheme 2.



Scheme 3

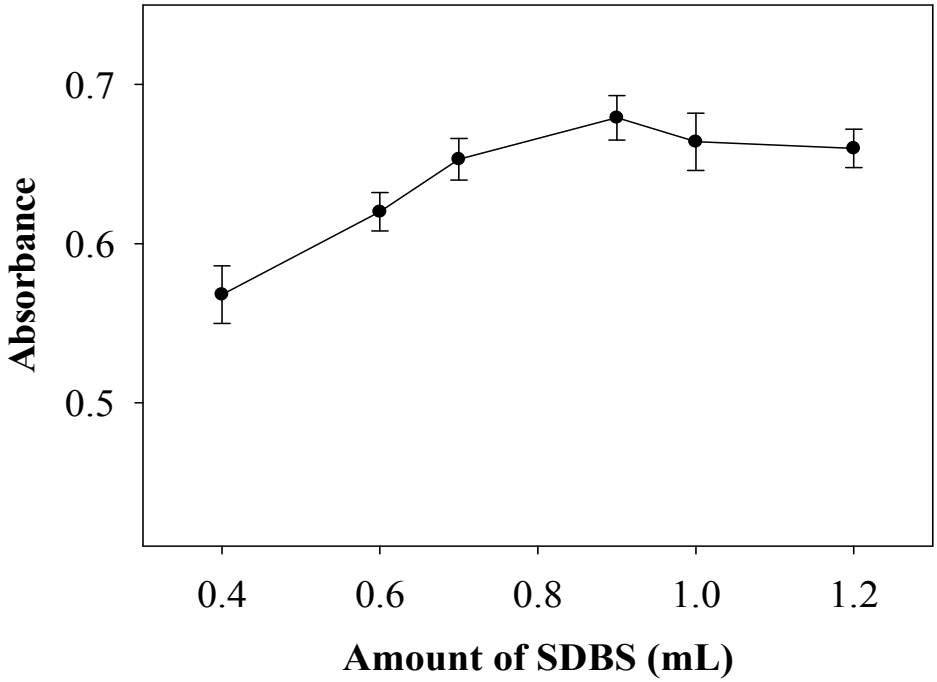


Fig. 1

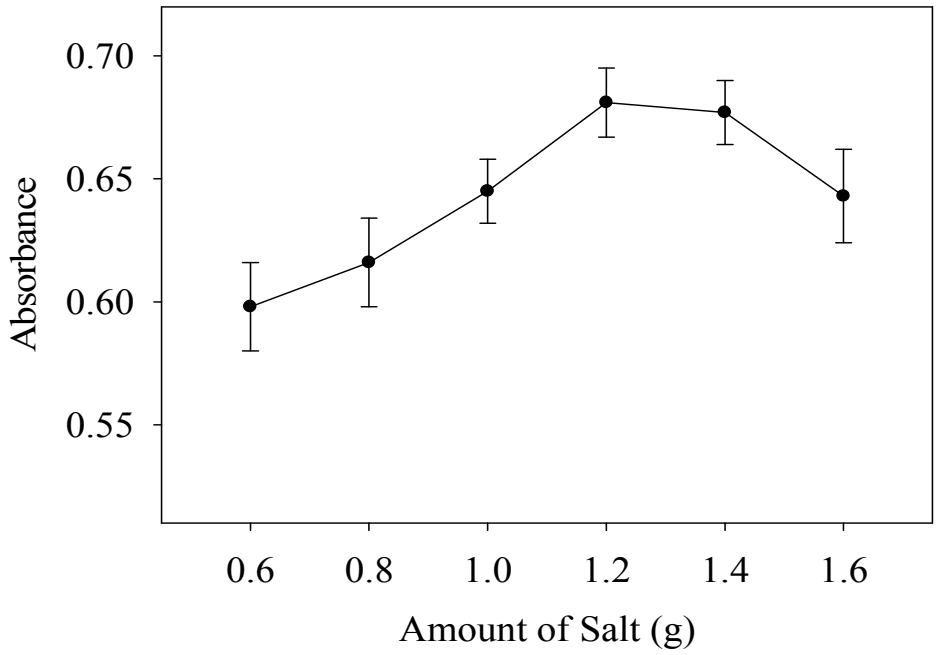


Fig. 2

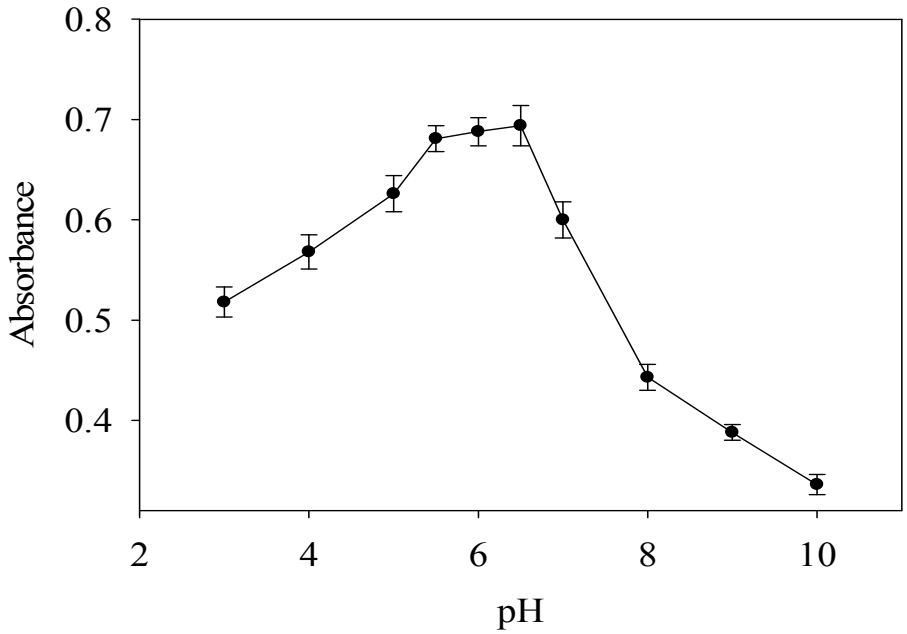


Fig. 3

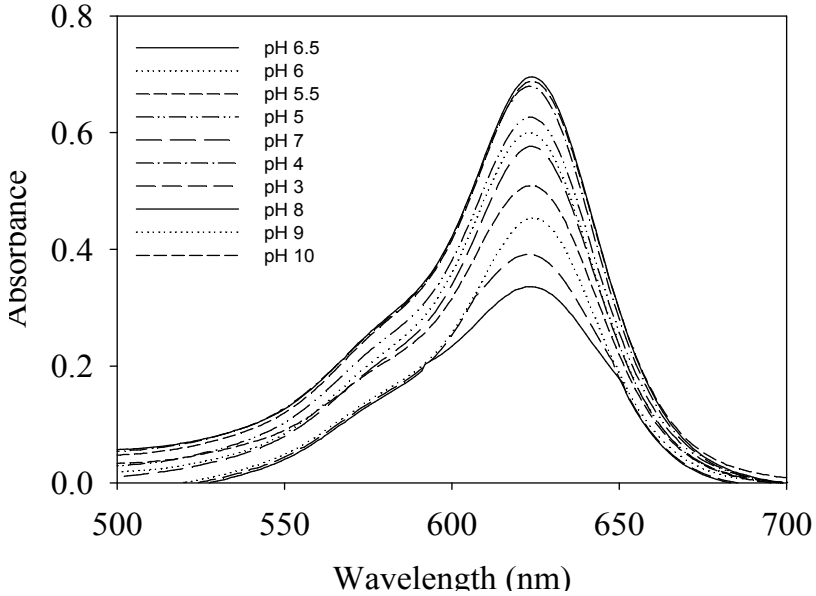


Fig. 4

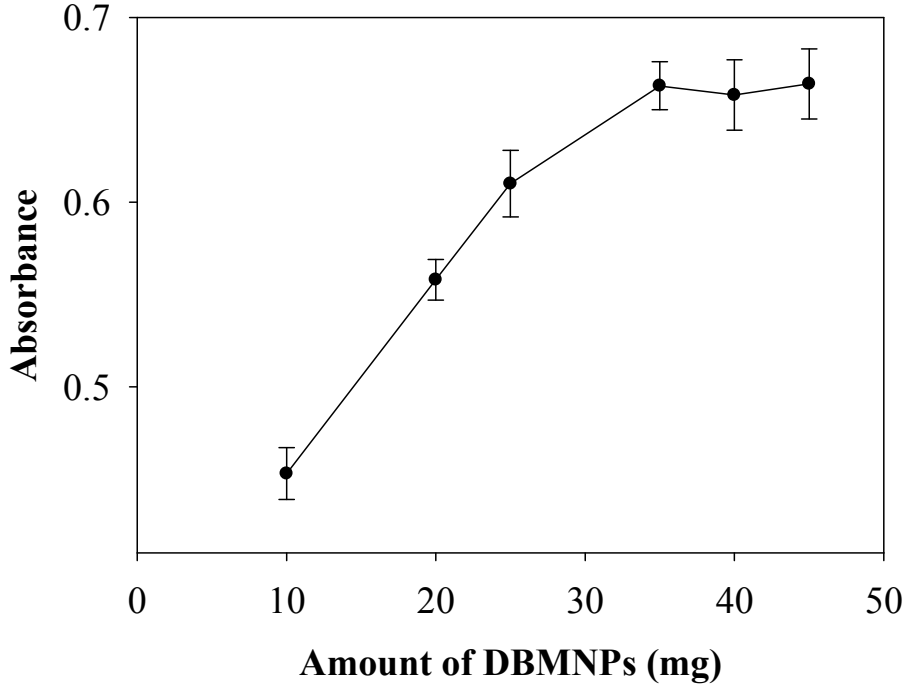


Fig. 5

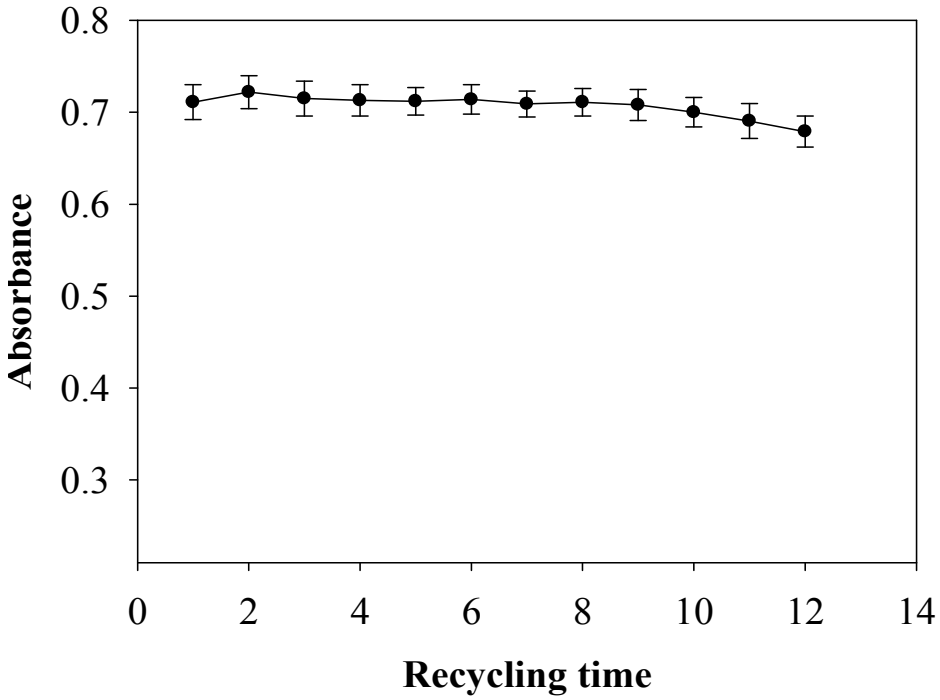


Fig. 6