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DISPERSIVE MICRO-SOLID-PHASE EXTRACTION (D-µ-SPE) WITH POLYPYRROLE-GRAPHENE OXIDE (PPY-GO) NANOCOMPOSITE SORBENT FOR THE DETERMINATION OF TETRACYCLINE ANTIBIOTICS IN WATER SAMPLES

(Pengekstrakan Fasa-Pepejal-Mikro Secara Serakan (D-µ-SPE) menggunakan Polipirol-Grafin Oksida (Ppy-GO) Penjerap Nanokomposit bagi Penentuan Antibiotik Tetrasiklin dalam Sampel Air)

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Abstract

Dispersive micro-solid-phase extraction (D-µ-SPE) method using polypyrrole-graphene oxide (PPy-GO) nanocomposite sorbent has been developed for the extraction and pre-concentration of tetracycline antibiotics (TCs) residues, namely oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), demeclocycline (DMC) and doxycycline (DOC) in water samples prior to high-performance liquid chromatography-ultraviolet/diode array detector (HPLC-UV/DAD). The PPy-GO nanocomposite was prepared by *in situ* oxidative chemical polymerization. The effects of sample pH, a mass of sorbent, desorption solvent, extraction time and desorption time on the extraction of analytes were evaluated and optimized. Under the optimum conditions, the method demonstrated good linearity (R^2=0.9989-0.9995) over a concentration range of 10-1000 µg L^{-1}. The limit of detection (LOD) was in the range of 4.9-8.7 µg L^{-1} with satisfactory relative recoveries (80 – 105 %) and a good relative standard deviation (RSD) of ⩽ 2.3 % (n = 3) was obtained. The method was successfully applied to river water and tap water samples. Results obtained using the developed method were compared with results previously reported using other SPE-based methods.

Keywords: tetracycline antibiotics, PPy-GO, dispersive micro-solid-phase extraction, liquid chromatography, water samples

Abstrak

Kaedah pengekstrakan fasa-pepejal-mikro secara serakan (D-µ-SPE) menggunakan penjerap nanokomposit polipirol- grafin oksida (PPy-GO) telah dibangunkan untuk pengekstrakan dan pra-pemekatan sisa antibiotik tetrasiklin (TCs), iaitu oksitetrasiklin (OTC), tetrasiklin (TC), klortetrasiklin (CTC), demeklosiklin (DMC) dan doksisiklin (DOC) di dalam sampel air sebelum kromatografi cecair berprestasi tinggi-pengesan tatasusunan ultraungu/diod (HPLC-UV/DAD). Nanokomposit PPy-GO telah disediakan melalui pempolimeran oksidatif kimia *in situ*. Kesan pH sampel, jisim penjerap, pelarut nyahjerapan, masa pengekstrakan dan masa nyahjerapan pada pengekstrakan analit telah dinilai dan dioptimumkan. Di bawah keadaan optimum, kaedah menunjukkan kelinearan yang baik (R^2=0.9989-0.9995) dalam julat kepekatan 10-1000 µg L^{-1}. Had pengesanan (LOD) adalah dalam julat 4.9-8.7 µg L^{-1} dengan pemulihan relatif yang memuaskan (80-105%) dan sisihan piawai relatif (RSD) yang baik sebanyak ≤ 2.3% (n = 3) telah diperoleh. Kaedah tersebut berjaya diaplikasikan pada sampel air sungai dan air paip. Keputusan yang diperoleh menggunakan kaedah yang dibangunkan dibandingkan dengan keputusan yang dilaporkan sebelum ini menggunakan kaedah berasaskan SPE yang lain.

Kata kunci: antibiotik tetrasiklin, Ppy-GO, pengekstrakan fasa-pepejal-mikro secara dispersif, kromatografi cecair, sampel air

Introduction

The discovery of antibiotics in the 1940s has witnessed traditional medicine and herbal therapies being replaced by a more effective remedy for treating bacterial infections [1-2]. Since then, antibiotics have become essential in medicine and are critical components of the modern healthcare systems. Tetracycline, for example, is a group of broad-spectrum antibiotics active against several Gram-positive and Gram-negative bacteria [3]. Since their discovery, tetracycline antibiotics have played a prominent role in veterinary and human medicine, specifically for treating and preventing microbial infections and as additives in animal feed [4]. Although the role of antibiotics in fighting harmful bacteria cannot be neglected, the mishandling of antibiotics can adversely affect public health.

Tetracycline antibiotics (Figure 1) can be discharged from industrial production, animal foodstuff and human medicine into rivers and local groundwater [5]. The occurrence of these antibiotic residues in water samples in high concentrations will cause harmful effects on public health, including allergic reactions in humans, liver damage, accumulative toxicity, yellowing of teeth, the transfer of drug-resistant bacteria from food to humans, and gastrointestinal disturbance [6]. There is also growing concerned that antimicrobials are affecting water quality because they can accelerate the evolution of antimicrobial-resistant bacteria [7]. Hence, it is critical to develop reliable analytical methods for routine monitoring of tetracycline in the environment. The method should be rapid, precise, economical, less time-consuming, and environmentally friendly.

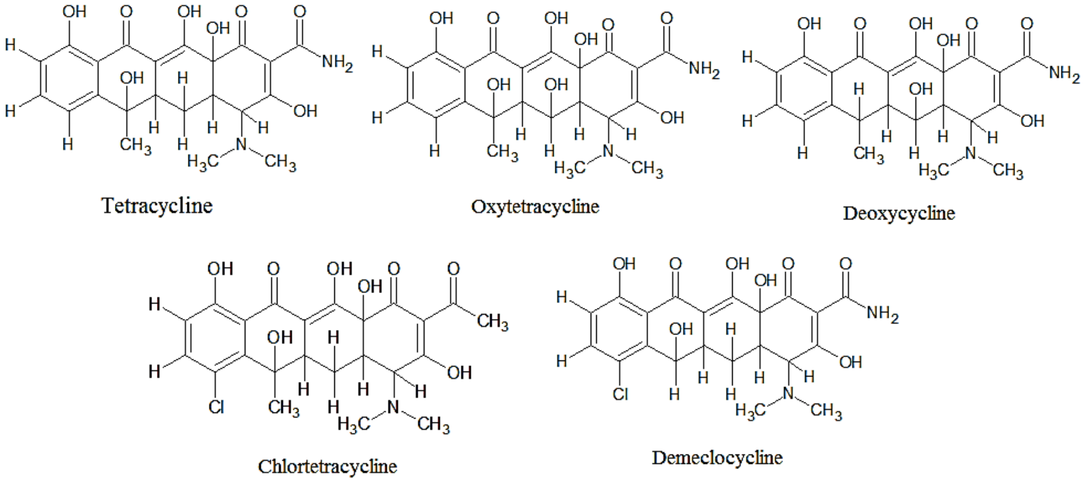

Tetracycline
Oxytetracycline
Deoxycycline
Chlortetracycline
Demeclocycline

Figure 1. Chemical structure of some commonly used tetracycline antibiotics

In recent years, numerous analytical methods have been developed for the detection of tetracycline in complex environmental matrices such as swine wastewater and manure [8, 9], livestock tissues [10], milk [11], honey [12], and soils [13]. Several extraction techniques have been reported mainly for the extraction and pre-concentration of tetracycline antibiotics in aqueous matrices. These include solid-phase extraction (SPE) [14], solid-phase microextraction (SPME) [15], liquid-liquid extraction [16] and pressurized liquid extraction [17].

SPE is the most common sample isolation and pre-concentration technique for the extraction of pharmaceuticals, including tetracycline in water samples [18]. Yang et al. [19] developed an analytical method for the determination of 11 tetracyclines using solid-phase extraction and liquid chromatography-electrospray ionization tandem mass spectrometry in the influent and effluent of domestic wastewater. Dzomba et al. [20] have reported a comparative study of ultrasonic coupling to the modern SPE method, such as tandem solid-phase extraction (TSPE), dispersive solid-phase extraction (DSPE) and matrix solid-phase dispersion (MSPD) for the extraction of tetracycline in river water.

Trapping media in SPE have aroused increasing interest in research on sample preparation as they have a key role in obtaining high clean-up and enrichment efficiency for trace analysis in complex matrices. In recent years, there have been many successful works on the use of varieties of commercialized sorbent materials for an efficient SPE technique [21]. Apart from commercially available sorbents, researchers are devoted and have been passionate on developing new sorbents to enhance the extraction process. In the past few decades, various type of solid sorbent has been developed for the determination of environmental decontamination, including carbon-based [22], molecularly imprinted polymers [23], nano-fibres [24] and carbon nanotubes [25].

Graphene has been extensively employed in a wide range of applications since its discovery by Novoselov et al. in 2004 [26]. Graphene oxide performs well as sorbent, with high porosity, chemical stability, unique surface structure, large specific surface area and hydrophilicity due to its richness in oxygenated groups [27]. However, because they tend to agglomerate easily due to the strong Van der Waals interaction between molecules and have low selectivity, their usage as extraction sorbent is restricted [28]. To address the aforementioned issues, varieties of graphene-based composite materials have recently been developed. Polypyrrole-graphene oxide (Ppy-GO) composites have been utilized as supercapacitor electrodes and biosensors due to their remarkable electrical and optical properties [29-30]. Nonetheless, the efficacy of PPy-GO as sorbent has not been explored much, especially in the detection of pharmaceutical residues in complex matrices.

Hence, this research aimed to investigate the use of PPy-GO as a promising material for the determination of tetracycline antibiotics in water samples with the aid of D-µ-SPE, which is believed to be a simple, rapid, selective and efficient alternative method compared to the traditional SPE.

Materials and Methods

Chemicals and materials

Five (5) tetracycline antibiotics, namely oxytetracycline (OTC), tetracycline (TC), demeclocycline (DMC), chlortetracycline (CTC), and doxycycline (DC) with purity assay in range of 98-101% were purchased from Sigma-Aldrich. Acetonitrile (ACN), methanol (MeOH), formic acid (of HPLC grade), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Pyrrole monomer and ferric chloride crystal ($FeCl_3.6H_2O$, 99.0%) were procured from Xian, China. Graphene oxide powder and methane sulfonic acid (MSA) were purchased from Sigma-Aldrich (St. Louis, USA). Ultrapure water was produced by Barnstead Nanopure (Thermo Scientific).

Preparation of standard and sample solutions

The individual stock solutions of OTC, TC, DMC, CTC, and DOC were prepared separately in HPLC grade methanol to a final concentration of 1000 mg L^{-1}. All standard solutions were stored in amber glass bottles at 4 °C when not in use. A series of working standard solutions were prepared in methanol by dilution before analysis to prevent the decomposition of analytes. Tap water samples were collected from a laboratory in Universiti Teknologi MARA (UiTM) Shah Alam, Selangor, Malaysia, while river water samples were collected from Hulu Langat, Selangor, Malaysia. The collected samples were filtered through 0.45 µm nylon filter paper to remove any suspended particles. Samples were kept in polyethene bottles pre-cleaned with acetone and stored at 4 ºC prior to the analysis process. For accuracy and precision studies, the river and tap water samples (10 mL, pH 3) were spiked with the standard mixture of five tetracycline antibiotics to give a final concentration of 1 mg L^{-1} for each analyte.

Preparation of PPy-GO nanocomposite material

The method of synthesis was adapted from literature [31]. The synthesis of PPy-GO nanocomposite material was prepared by in situ chemical polymerization of pyrrole. About 0.05 g GO was dispersed in distilled water and sonicated to form a 2 mg mL^{-1} homogeneous suspension. Then, 25 µL pyrrole monomer was slowly added to the yellowish-brown GO solution under ultrasonication for 40 min. Approximately 0.3 g $FeCl_3.6H_2O$ was dissolved in 10 mL distilled water, introduced into the mixture dropwise and followed by ultrasonication for another 0.5 h. After that, the black-colored PPy-GO composites were collected by centrifugation and washed with distilled water and ethanol several times, followed by vacuum drying at 45 ºC for 12 h.

Dispersive micro-solid-phase extraction procedure by using PPy-GO nanocomposite material

The extraction method of D-µ-SPE was adopted from Othman et al. [32]. About 50 mg of PPy-GO nanocomposite material was dispersed into the aqueous sample (10 mL, pH 3). The mixture was vigorously stirred with a magnetic stirrer to trap the analytes for 15 min. Subsequently, the PPy-GO nanocomposite material was isolated from the solution by centrifugation (4000 r min^{-1}, for 5 min). The supernatant was discarded. About 1 mL of desorption solvent was added, and then the mixture was sonicated for 10 min to desorb the analytes. The mixture was then centrifuged at 4000 rpm for 5 min. The solvent was collected and evaporated until dry under a soft stream of nitrogen gas. Finally, 10 µL of the extract was injected into the HPLC system for analysis. The workflow of this method is illustrated in Figure 2. The PPy-GO nanocomposite material can be reused several times after washing with 2 mL of methanol and water, respectively. Several optimization parameters for D-µ-SPE were optimized by changing one variable at a time.

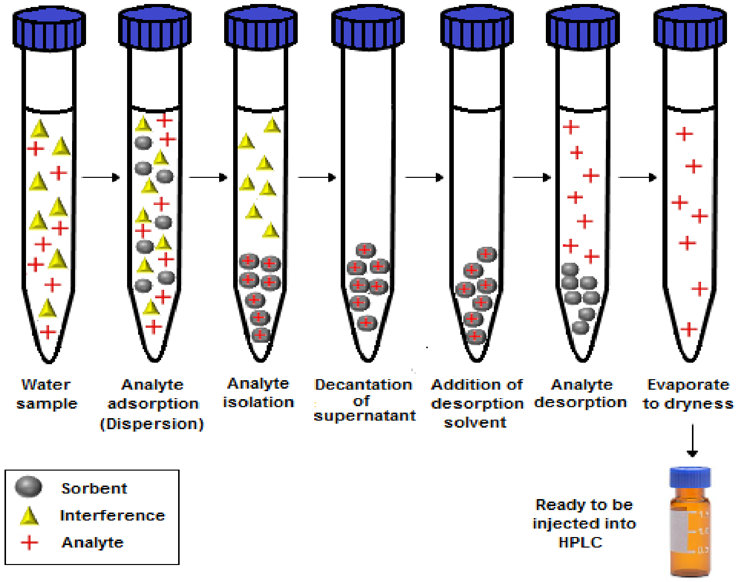


Figure 2. Dispersive micro-solid-phase extraction (D-µ-SPE) procedure by using Ppy-GO as sorbent

Chromatographic conditions

All analyses were performed using an automated high-performance liquid chromatography (HPLC) Dionex Ultimate 3000 (Sunnyvale, CA, USA) system, using an Acclaim Polar Advantage II (5 µm, 120 Å, 4.6×150 mm) (Dionex USA) as the analytical column. A gradient elution consisting of acetonitrile, methanol and formic acid was applied. The flow rate was programmed at 1 mL min^{-1}. For initialization of the LC analysis, the chromatographic system was cleaned and stabilized for 1 h. The quantification of TCs was performed by measuring the peak area of the chromatogram. TCs were detected using an ultraviolet/diode array detector (UV/DAD) at a selected wavelength of 270 nm.

Validation of the analytical method

The extraction method was assessed for linearity (R^2), the limit of detection (LOD), the limit of quantification (LOQ), precision (% RSD), and accuracy (% relative recovery) prior to sample analysis. Linearity was measured by plotting the calibration curve of five (5) different concentration levels for the standard mixture of TCs. The same calibration graph was used for the determination of LOD and LOQ. Method precision was measured using three (3) different extractions of the calibration standard at the lowest concentration.

Results and Discussion

Optimization of extraction parameter

The oxytetracycline (OTC), tetracycline (TC), demeclocycline (DMC), chlortetracycline (CTC), and doxycycline (DC) were selected as model analytes in this study as they are among the most commonly used TCs. To determine the optimum conditions for the extraction of TCs from aqueous samples, many parameters that can affect the extraction efficiency were investigated, namely the extraction time, desorption time, sample pH, extraction solvent and mass of sorbent. The optimization was carried out using one variable at a time while other parameters were kept constant. Optimization was carried out using deionized water samples spiked with each TC to give a final concentration of 1 mg L^{-1} in 10 mL of water samples. About 1 mL of acetonitrile was used as the desorption solvent. Triplicate extractions were carried out for each parameter optimized in this study.

Effect of sample pH on the extraction of TCs

The experiment was carried out by varying the pH value of the spiked sample solution from pH 3 to pH 11 to determine the effect of sample pH on the extraction of TCs. It was observed that the peak area slightly increased with increasing pH from pH 3 to pH 7 and highest at pH 7. This is shown in Figure 3(a). The peak area starts to decrease after pH 7.

The effect of sample pH on the adsorption of analytes can be explained based on its amphoteric characteristics under varied pH conditions [33]. Tetracycline is an amphoteric compound with various ionizable functional groups and will form different ionic species in an aqueous solution at different pHs. Thus, in the analysis of TCs, pH adjustment is a very crucial step [34]. TCs have three pKa values ($pKa_1 \approx 3$, $pKa_2 \approx 7$, $pKa_3 \approx 9$) [35].

Throughout the range of pH, tetracycline occurs as cationic in strong acid solution approximately at pH 3, zwitterionic at pH 4 to 7.7 and anionic at pH > 7.7 [36]. The adsorption of TCs onto Ppy-GO nanocomposite sorbent is strongly affected by either the acidity or basicity of the sorbent surface and the tetracycline speciation. At pH 4 - 7.7, TCs are mainly in their molecular form and are less protonated. Meanwhile, the sorbent surface had an increasing negative charge. Hence, the TCs exhibited increasing hydrophobicity with increasing pH, resulting in better adsorption on the sorbent. From the experiment carried out, the extraction of TCs was observed to be highest at pH 7. After pH 7, the adsorption became less efficient due to the electrostatic repulsion between anionic TCs and the negatively charged sorbent surface. Therefore, pH 7 was selected as the pH value used in subsequent experiments.

Effect of extraction time

The influence of extraction time on the peak area of analytes was studied in the range of 5 to 25 min. The effect of contact time between sorbent and analytes is shown in Figure 3(b). It was observed that the adsorption capacity of TCs rapidly increased from 5 to 15 min of extraction time. The highest extraction efficiency was obtained after 15 min of extraction time.

After 15 min, the adsorption of analytes became practically constant. This could be due to the long duration of extraction time, whereby all the active sites on the surface area of sorbent were fully saturated with the analyte as time progressed [37]. Therefore, 15 min was selected as the optimal extraction time and was used in further experiments.

Effect of desorption time

The effect of desorption time was studied in the range of 5-25 min of sonication. The sonication method was used for the desorption solvent to desorb TCs from the PPy-GO sorbent as this technique is suitable to be applied for reversible adsorption [38]. The maximum desorption time for the analytes was achieved within 10 min of sonication (Figure 3(c)). However, beyond 10 min of sonication, the peak area of analytes was constant. This was probably due to the analytes being fully desorbed from the sorbent into the desorption solvent. Therefore, a desorption time of 10 min of sonication was chosen for subsequent experiments.

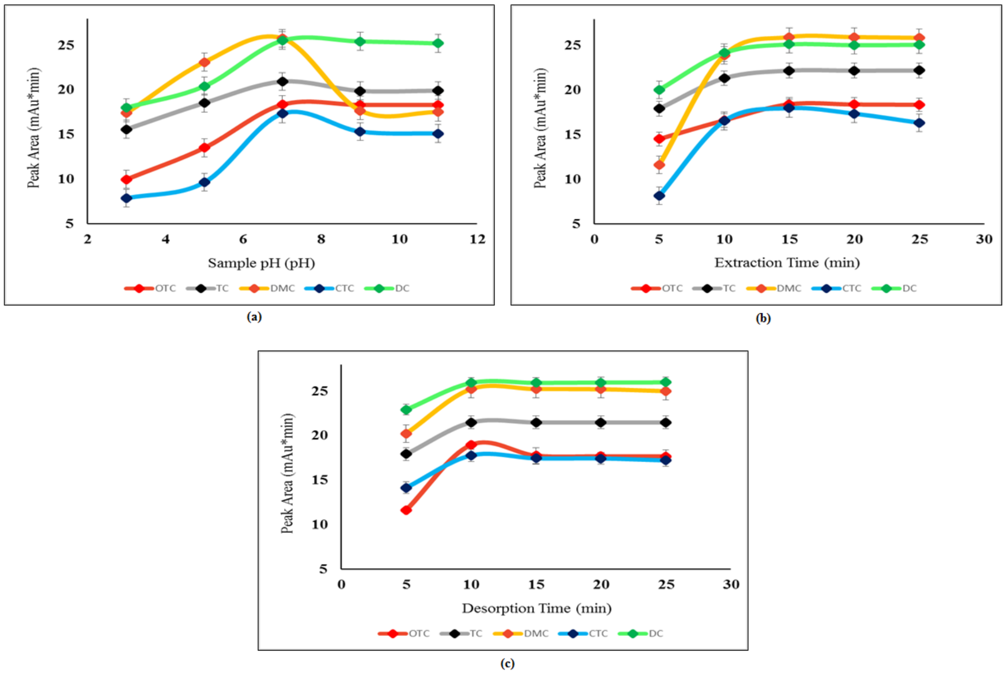


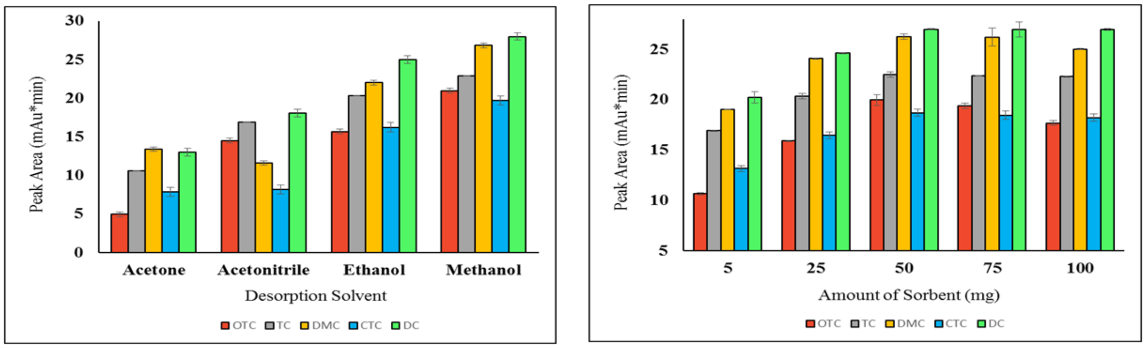
Figure 3. Optimization for the extraction performance of PPy-GO using D-µ-SPE. (a) Effect of sample pH, (b) extraction time and (c) desorption time on PPy-GO-D-µ-SPE of TCs in water samples. (Error bars represent standard deviations of results, n = 3)

Effect of desorption solvent

A complete back extraction process depends on the time of sonication and the strength of the desorption solvent [39]. The selection of a suitable organic solvent is crucial to ensure the complete elution of trapped analytes from the surface of the PPy-GO nanocomposite. In this study, the influence of different types of organic solvents was investigated to enhance the recovery of analytes. The solvents used in this study were acetone, acetonitrile, ethanol, and methanol, according to the increasing order of polarity. The experimental results depicted in Figure 4(a) demonstrate that the use of acetone as a desorption solvent yielded the lowest recovery and was therefore not sufficiently powerful for desorption of the targeted TCs. The data also showed that the best efficiency (expressed as peak area) was obtained when methanol was used as a desorption solvent. This can be related to its polar characteristic. Methanol has a stronger hydrogen bond and dipole-dipole intermolecular interactions with the targeted TCs, thus enabling effective desorption of TCs from the sorbent surface. Therefore, methanol was selected as the desorption solvent in all subsequent experiments.

Effect of mass of sorbent

For the optimization of the mass of sorbent, about 5 mg to 100 mg of PPy-GO powder was used. The result showed that the lowest proposed mass of sorbent (5 mg) produced the lowest peak area (Figure 4(b)). On the other hand, 50 mg of sorbent gave the highest peak area of analytes. No significant increase in the peak area was observed with a further increase in sorbent mass. This was probably due to all the analytes having been fully adsorbed by the sorbent while there were still a lot of active sites available for surface adsorption [40].



(a) (b)

Figure 4. Optimization for the extraction performance of PPy-GO using D-µ-SPE. (a) Desorption solvent and (b) sorbent amount on PPy-GO-D-µ-SPE of TCs in water samples. (Error bars represent standard deviations of results, n = 3).

Optimum extraction conditions

The final adopted PPy-GO-D-µ-SPE conditions were: sample pH, pH 7; extraction time, 15 min; desorption time, 10 min; desorption solvent, methanol; sorbent mass, 50 mg. Hence, these optimum extraction conditions were applied for subsequent studies.

Reusability of sorbent

The reusability of the sorbent was tested by rinsing the sorbent five times with 2 mL methanol with sonication after each extraction. Then, the sorbent was dried at 60 °C for 1 hour before further use in the next D-µ-SPE procedure. The result showed that the sorbent could be conveniently reused up to fifteen times without any significant loss in the extraction recovery (> 80%) for TCs before the recovery starts to decrease slowly. This was probably due to the loss of carboxyl groups from the surface of the sorbent after repeated use.

Method validation and analytical performance of D-µ-SPE

The optimization of D-µ-SPE was then validated for relative recoveries, sample calibration and LOD. From the linearity range of 10-1000 µg $L^{-1,}$ good linearity was obtained for the four analytes, where the coefficients of determination (R^2) were in the range of 0.9989-0.9995. The LODs and LOQs were calculated at a signal-to-noise ratio of 3, and the results were in the range of 4.9 - 8.7 µg L^{-1} for LODs. The precision of the method was measured by the relative standard deviation (RSD), and the results obtained were between 1.4-3.0 %. Table 1 shows the validation data for D-µ-SPE of TCs from water samples.

Table 1. Quantitative results of DSPE-LC of TCs in tap and river water samples

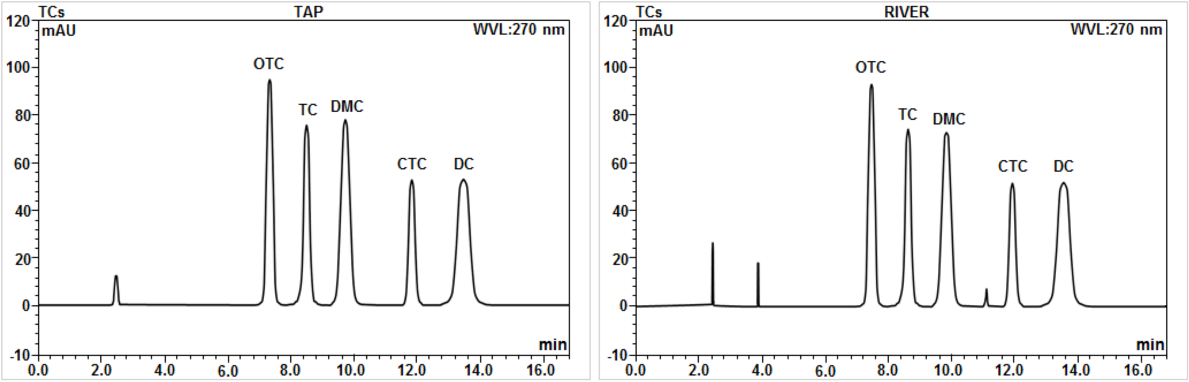
Sample	**Analyte**	**Linear Range (μg L^{-1})**	**Coefficient of Determination (R^2)**	**LOD (μg L^{-1})**	**RSD (%, *n* = 3)**
Tap water	OTC	10-1000	0.9991	8.6	2.5
	TC	10-1000	0.9994	5.6	1.4
	DMC	10-1000	0.9992	8.1	3.0
	CTC	10-1000	0.9995	7.4	2.0
	DC	10-1000	0.9990	5.9	1.9
River water	OTC	10-1000	0.9992	8.7	2.3
	TC	10-1000	0.9989	4.9	2.0
	DMC	10-1000	0.9993	8.6	2.1
	CTC	10-1000	0.9995	7.4	1.6
	DC	10-1000	0.9991	6.6	2.2

Application of D-µ-SPE on river and tap water samples

The developed PPy-GO-D-µ-SPE method was successfully applied to river and tap water samples. Relative recovery studies were conducted by spiking the water samples to give a final concentration of 50 and 500 µg L^{-1}. The results are presented in Table 2. It shows that good relative recoveries were obtained in the range of 80% to 105%. The results suggest that dispersive micro-solid-phase extraction is an efficient technique for extracting drugs in aqueous matrices. Figure 5 shows HPLC chromatograms of the OTC, TC, DMC, CTC and DC in river and tap water samples.

Table 2. Relative recovery studies of DSPE-LC of TCs from different spiked water samples (n = 3)

Analyte	**Spiked concentration (μg L^{-1})**	**Tap water**		**River water**	
		Relative Recovery (%)	**RSD (%)**	**Relative Recovery (%)**	**RSD (%)**
OTC	50	95	4.8	98	2.3
	500	103	3.9	98	0.8
TC	50	99	0.4	85	1.9
	500	100	0.9	88	1.1
DMC	50	97	1.2	102	1.8
	500	99	3.9	95	0.8
CTC	50	80	0.9	100	1.2
	500	105	1.0	98	0.2
DC	50	101	1.3	80	1.8
	500	99	0.1	94	2.0



(a) (b)

Figure 5. HPLC chromatogram of five tetracycline antibiotics spiked in (a) tap water samples and (b) river water samples

Comparison with other reported methods

The efficiency of the proposed D-μ-SPE method for TCs was compared with other reported methods in terms of analysis method, extraction sorbent, linear range and LODs. The comparative results are summarized in Table 3. As can be seen, the method developed in this study achieved a comparable LOD with other prior reported methods, which also used graphene-based nanocomposite. Furthermore, compared with other developed nanocomposite sorbents, Ppy-GO is easy to prepare and can be reused fifteen times. On the other hand, extraction of tetracycline using conventional SPE involves high consumption of organic solvents and sorbents, which is laborious, somewhat costly, and time-consuming [44]. On the other hand, D-μ-SPE only requires a small amount of organic solvent and sorbent for every single analysis. In addition, since D-μ-SPE requires simple devices such as a sonicator and centrifuge, it can be considered relatively inexpensive, simple, rapid and suitable for routine analyses.

Table 3. Comparison of Ppy-GO-D-μ-SPE with other published methods for the determination of TCs.

Analysis Method	**Sorbent**	**Type of Sample**	**Linear Range (μg L^{-1})**	**LOD (μg L^{-1})**	**Recoveries (%)**	**Ref.**
μ-SPE- HPLC-UV	PET-GO nano-fiber	Honey	10–5000	15.3	89–94	[24]
MSPE-DLLME	Fe_3O_4@SiO_2@GO-β-CD	Milk, Water	10–200	1.8–2.9	71-122	[41]
SPE-HPLC	Graphene	Milk	20–1000	10-20	82-104	[42]
MSPE- UPLC–TUV	Fe_3O_4@SiO_2@FeO	Tap, river	0.133–333	0.027-0.107	91-105	[43]
D-μ-SPE-HPLC-UV	Ppy-GO	Tap, river	10-1000	4.9-8.7	80-105	This work

μ-SPE- HPLC-UV: micro-solid-phase extraction high-performance liquid chromatography
MSPE-DLLME: magnetic solid-phase extraction dispersive liquid-liquid microextraction
SPE-HPLC: solid-phase extraction high-performance liquid chromatography
MSPE-UPLC-TUV: magnetic solid-phase extraction ultra-performance liquid chromatography tunable ultra-violet
D-μ-SPE-HPLC-UV: dispersive micro solid-phase extraction high-performance liquid chromatography ultra-violet

Conclusion

This paper reports the use of polypyrrole incorporated with graphene oxide for the extraction of tetracycline antibiotics using dispersive micro solid-phase extraction combined with HPLC-UV/DAD. Several parameters were optimized in the D-μ-SPE method. The optimum parameters were used to analyse real samples for successful extraction of oxytetracycline, tetracycline, demeclocycline, chlortetracycline, and doxycycline from river and water samples. Good linearities were achieved for the analytes with coefficients of determination R^2, in the range of 0.9989-0.9995. The method was successfully applied to the analysis of river and tap water samples, with good relative recoveries in the 80-105 % range. Compared with other previously reported methods that employed graphene-based nanocomposites, the D-µ-SPE method proved to be a simple, sensitive, selective and green extraction method that could potentially be used in the chemical laboratory for routine analysis of water samples.

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