Development of *in-situ* Dispersive Micro Solid Phase Extraction Assisted Cloud Point Extraction for the Determination of Tetracyclines by High Performance Liquid Chromatography

Kamolrat Phomai

A Thesis Submitted in Partial Fulfillment of Requirements for

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for Master of Science (Chemistry)

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The examining committee has unanimously approved this Thesis, submitted by Miss Kamolrat Phomai , as a partial fulfillment of the requirements for the Master of Science Chemistry at Mahasarakham University

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TITLE Development of *in-situ* Dispersive Micro Solid Phase Extraction Assisted Cloud Point Extraction for the Determination of Tetracyclines by High Performance Liquid Chromatography

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ABSTRACT

Under the principles of green analytical chemistry with the need for a small sample solution, little sorbent, low desorption solvent, and a proper extraction time, an *in-situ* dispersive micro solid phase extraction assisted cloud point extraction for the simultaneous analysis of tetracyclines (TCs) including oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC) by high performance liquid chromatography was developed. This proposed method, TCs were efficiently extracted by one step through the iron-mediated self-assembly between iron with tannic acid complexes (Fe-TA) under acetate buffer pH 5.0 and cloud point extraction. Under optimization of all experimental parameters, linear ranges were obtained in the ranges 20-1000 and 30-1000 μg L^{-1} for the determination of OTC, TC and CTC, DC, respectively, with the determination coefficients more than 0.993. Precisions represented in term relative standard deviations (RSDs) less than 4.75% were obtained. The applicability of the proposed method was successfully utilized to the quantification of TCs in wastewater, milk, egg, honey, chicken liver and chicken kidney samples with the recoveries ranging from 71.13-117.09% and this method gave good enrichment factors (EFs) of 33-90.

Keyword : in-situ Dispersive micro solid phase extraction, Cloud point extraction, High performance liquid chromatography

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CHAPTER I
INTRODUCTION

1.1 Problems and provenance

Antibiotics are organic molecules synthesized by various microorganisms to stop development of disease-causing small creatures. They are active biological molecules, both in the field of medicine and in veterinary medicine as the treatment and growth accelerator, feed additive in animals and prevent damage of products by bacteria. Tetracyclines (TCs) is one of the first antibiotics used worldwide to control bacterial infections in humans, animals and agricultural activities [1]. TCs have been extensively used for the treatment and prevention of infectious diseases in humans. Also, they have been utilized as additives in animal feed to promote a rapid growth and weight gain. Due to their broad spectrum activity and low cost, currently there are eight marketed tetracyclines, with four of them being most commonly used in the veterinary prescription of antimicrobials including tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) [2]. Because the antibiotics are poorly metabolized and absorbed by humans and animals, residues of tetracycline are frequently detected in environmental water and animal-derived foods such as milk and honey. The residues of antibiotics in food may lead to drug resistance and other adverse effects on humans. TCs can commonly cause gastrointestinal disturbances including abdominal discomfort, epigastric pain, nausea, vomiting, and anorexia. While taking TCs, discoloration of teeth, and inhibition of bone growth in children may occur [3]. Moreover, when the antibiotics enter the environment, all they may affect natural microbial communities. Thus, the determination of antibiotic residues in food and environmental is of importance [4]. The maximum residue limits (MRLs) for tetracyclines in milk, meat and other foods were established by European regulation 2377/90 and subsequent modifications. The MRLs for milk and muscle were set at 100 μg/kg for all species and the levels set for liver and kidney are 300 and 600 μg/kg, respectively [5]. In addition, MRL of 400 μg/kg is regulated in eggs by the Food Agriculture Organization (FAO).

Several analytical methods have been employed for the determination of TCs including electrochemical [6], spectrophotometric [7] and chromatographic methods

[8]. Although these analytical methods are powerful, an extra step as known as sample preparation involving extraction and preconcentration is usually required to enhance sensitivity of the analytical method for determination of TCs including hollow fiber liquid phase microextraction (HF-LPME) [9], solid phase extraction (SPE) [10], magnetic solid phase extraction (MSPE) in tandem with dispersive liquid-liquid microextraction (LLE) [11] and cloud point extraction (CPE) [12]. Among these method CPE is one of the most preferred methods to preconcentrate and separate organic molecules and metal ions complex owing to its high sensitivity, low cost, safety and environment-friendly. Furthermore, CPE according to green analytical chemistry (GAC) because these methods reduce the use of toxic organic solvents. Thus, an *in-situ* dispersive micro solid phase extraction assisted cloud point extraction for the determination of TCs by high performance liquid chromatography was developed which this method is high extraction efficiency, high enrichment factor, simple and rapid.

1.2 Objectives

1.2.1) To develop an *in-situ* dispersive micro solid phase extraction assisted cloud point extraction for preconcentration of four tetracyclines including Tetracycline (TC), Oxytetracycline (OTC), Chlortetracycline (CTC) and Doxycycline (DC) followed by high performance liquid chromatography.

1.2.2) To apply the developed method for trace analysis of TCs in real samples such as wastewater, milk, egg, honey, chicken liver and chicken kidney samples.

1.3 Scope of this work

1.3.1) Investigation on the optimization conditions of the *in-situ* dispersive micro solid phase extraction complexation reaction between tannic acid and iron(III) such as concentration of tannic acid, iron(III) and pH.

1.3.2) Investigation on the parameters that influence to the cloud point extraction efficiency such as mass of Triton X-114, vortex time, temperature and time of incubation, speed and time of centrifugation, concentration and volume of trifluoroacetic acid (TFA) in acetonitrile (ACN).

1.3.3) Investigation on the separation optimization conditions for the determination of four tetracyclines by high performance liquid chromatography such as ratio of mobile phase.

1.3.4) Investigation of analytical performance and method validation in order to evaluate the linearity, limit of detection (LOD), limit of quantitation (LOQ), precision (intra-day, inter-day), accuracy and enrichment factor (EF).

1.3.5) Study of the sample preparation for the determination of tetracyclines and application to real samples.

1.4 Benefit of research

High extraction efficiency, high enrichment factor, simple and rapid preconcentration method of four tetracyclines residues using *in-situ* of dispersive micro solid phase extraction assisted cloud point extraction can be obtained.

1.5 Venue of the study

1.5.1) Department of Chemistry, Faculty of Science, Mahasarakham University.

1.5.2) Scientific Instrument Science Unit, Mahasarakham University.

CHAPTER II
LITERATURES REVIEW

2.1 History and classification of tetracyclines

Tetracyclines were first discovered in 1945. Chlortetracycline and oxytetracycline were the first members of the tetracycline group [13]. They were produced by *Streptomyces aureofaciens* and *S. rimosus*, respectively. In the following years, other naturally occurring tetracycline molecules were discovered, e.g. dimethyl-chlortetracycline from *S. aureofaciens* and tetracycline from *S. viridofaciens*. Subsequently, a number of important semisynthetic tetracyclines were developed, e.g. methacycline, doxycycline, minocycline, rolitetracycline, lymecycline and the most recently produced glycylcyclines [14]. All these compounds above mentioned belong to the first class of tetracycline antibiotics, also referred to as “typical tetracyclines”. They exhibit bacteriostatic activity by means of interacting with bacterial ribosomes and blocking of the protein synthesis [15]. The second class, known as “typical tetracyclines” includes chelocardin, anhydrotetracycline, anhydrochlortetracycline and thiatetracycline and these exhibit bactericidal activity by targeting the cytoplasmic membrane [15-16]. These compounds are of no interest for the therapy due to their low-level inhibition of the protein synthesis and their cytotoxicity and they are not licensed to be used in the European Union and in the Czech Republic [18].

2.1.1) Applications of tetracyclines in veterinary medicine

Tetracyclines exhibit the activity against a broad spectrum of pathogenic microorganisms, they are well absorbed, exhibit low toxicity and are relatively inexpensive. These attributes led to the wide use of tetracycline antibiotics in the therapy of human and animal bacterial and non-bacterial infections as well as for the prophylaxis of infections in food animals and pets [19]. Tetracyclines are widely used in veterinary medicine mainly for the treatment of gastrointestinal, respiratory and skin bacterial infections, infectious diseases of locomotive organs and of genito-urinary tract as well as systemic infections and sepsis [20]. Target animal species for the application of these preparations are beef cattle, pig, sheep, goat, horse, dog, cat, poultry, rabbit and fish. Medication with tetracycline antibiotics is also

contraindicated in pregnant and young animals, because tetracyclines form chelates with calcium on the surface of teeth and bones, which results in the discoloration of teeth and in a retarded development of the skeleton [21]. Another adverse tetracycline drug reaction is increased photosensitivity, mainly in animals with low level of skin pigmentation but it is of particular importance. The above mentioned limitations are also valid for the medication of humans and tetracyclines cannot be used for the treatment of pregnant women and young children [22].

2.1.2) Chemical structures and maximum residue limits for tetracyclines

TCs and their degradation products may have harmful effects on consumers, such as possible allergic reactions, liver damage, yellowing of teeth, and gastrointestinal disturbance due to the selective pressure of antibiotics on human gut microflora. Figure 1 shows the chemical structure of tetracyclines [23].

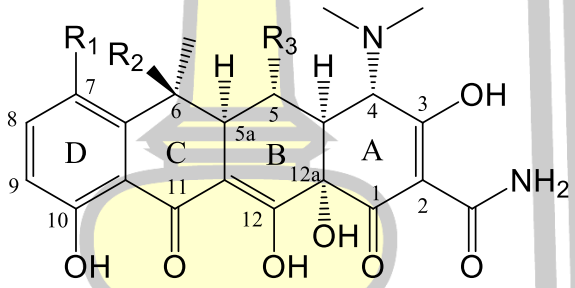

Figure 1 Chemical structure of tetracyclines.

Oxytetracycline: R_1=H, R_2=R_3=OH

Tetracycline: R_1=R_3=H, R_2=OH

Chlortetracycline: R_1=Cl, R_2=OH, R_3=H

Doxycycline: R_1=R_2=H, R_3=OH

Tetracyclines have three active pK_a sites. As shown in Figure 2, sites 1, 2, and 3 have different pK_a's. Site 1 is a conjugated trione system, which is acidic in nature, with a pK_a range from 2.8 to 3.4. The second site is a conjugated enone system which is slightly basic with a pK_a range of 7.2 to 7.8. Site 3 is a strong alkaline (diethyl amine group) with a pK_a range from 9.1 to 9.7 [24]. The combination of the three pK_a values of each antibiotic characterizes the specific antibiotic from the tetracycline

family. The different pK_a values are different from one tetracycline to another because of functional groups found at different locations.

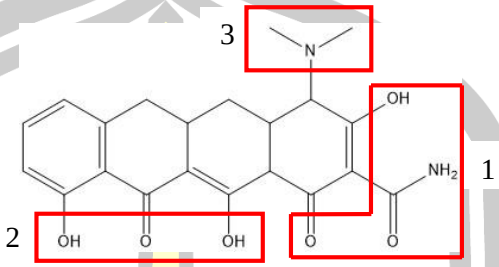

Figure 2 The three pK_a sites of tetracyclines.

The selected antibiotics including tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) was determined in this work. The three specific pK_a values, chemical and physical properties of each tetracyclines are listed in Table 1 [23].

Table 1 Chemical and physical properties of tetracycline antimicrobials.

Antimicrobial	**Systematic name**	**Acidity (pk_a)**	**Polarity (Log P)**	**Molecular mass (g/mol)**
Tetracycline (TC)	4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12apentahydroxy-6-methyl-1,11-dioxo-2-naphthacene carboxamide	pK_{a1} 3.3 pK_{a2} 7.7 pK_{a3} 9.7	-1.3	444.4
Oxytetracycline (OTC)	5-hydroxy-tetracycline	pK_{a1} 3.2 pK_{a2} 7.5 pK_{a3} 8.9	-3.6	460.4
Chlortetracycline (CTC)	7-chloro-tetracycline	pK_{a1} 4.5 pK_{a2} 7.8 pK_{a3} 9.8	-0.62	478.8

Antimicrobial	**Systematic name**	**Acidity (pk_a)**	**Polarity (Log P)**	**Molecular mass (g/mol)**
Doxycycline (DC)	6-deoxy-5-hydroxy-tetracycline	pK_{a1} 3.0 pK_{a2} 7.9 pK_{a3} 9.2	-1.9	444.4

To protect humans from the exposure to these drug residues, European Union (EU), Codex Alimentarius, Canadian, Brazilian (PNCRC) and the Chinese Ministry of Agriculture have established maximum residue limits (MRLs) for TCs. The MRLs of those TCs are presented in Table 2. .

Table 2 Maximum residue limits for tetracyclines in foods of animal origin.

Authority	Animal group	Target food	MRL (μg/kg)	Ref.
Codex Alimentarius	Poultry	Eggs	400	[25]
	Poultry	Muscle	200	
	Poultry	Liver	600	
	Cattle	Milk	100	
	Cattle and Swine	Muscle	200	
	Cattle and Swine	Liver	600	
	Fish	Muscle	200	
Canadian	Poultry	Eggs	400	[26]
	Poultry	Muscle	200	
	Poultry	Liver	600	
	Cattle	Milk	100	
	Cattle and Swine	Muscle	200	

Authority	Animal group	Target food	MRL (µg/kg)	Ref.
	Cattle and Swine	Liver	600	
European Union	Poultry	Eggs	200	[27]
	Poultry, Cattle and Swine	Muscle	100	
	Poultry, Cattle and Swine	Liver	300	
	Cattle	Milk	100	
Chinese	Poultry	Eggs	200	[28]
	All Animal Source Foods	Muscle	100	
	All Animal Source Foods	Liver	300	
	Cattle and Lamb	Milk	100	
Brazilian (PNCRC)	Poultry	Eggs	-	[29]
	Poultry, Cattle and Swine	Muscle	200	
	Poultry, Cattle and Swine	Liver	-	
	Cattle	Milk	100	

2.2 Green Analytical Chemistry

Green Chemistry is the use of chemistry techniques and methodologies that reduce or eliminate the use or generation of feedstocks, products, by-products, solvents, reagents, etc. that are hazardous to human health or the environment [30]. In short, it is the use of chemistry for pollution prevention. The same philosophy and ideas on Green Chemistry are those previously developed in analytical laboratories (GAC).

Green analytical chemistry (GAC) emerged from green chemistry in 2000 [31]. This relatively new area of activity within green chemistry concerns the role of analytical chemists in making laboratory practices more environmentally friendly, and it has gained a great deal of interest among chemists [31-32]. Aside from the development in instrumentation and methodologies, which are necessary for improvements in the quality of chemical analyses, efforts are being made to reduce the negative impact of chemical analyses on the environment and to enable implementation of sustainable development principles to analytical laboratories. In this context, GAC should be recognized as a stimulant to the progress of analytical chemistry. The most important challenge to the future of this discipline is to reach a compromise between the increasing quality of the results and the improving environmental friendliness of analytical methods. Guidelines that provide the framework for GAC are needed to meet this challenge.

In 1998 Anastas and Warner [34] formulated the 12 principles of green chemistry. Designed to meet the needs of synthetic chemistry, only some of these principles can directly be applied to analytical chemistry. The principles that find such application for both synthetic and analytical purposes are:

(i) Prevention of waste (principle number 1)

(ii) Safer solvents and auxiliaries (principle number 5)

(iii) Design for energy efficiency (principle number 6) and

(iv) Reduction of derivatization (principle number 8)

In our approach, the 12 principles of GAC are as follows [35]

1. Direct analytical techniques should be applied to avoid sample treatment.
2. Minimal sample size and minimal number of samples are goals.
3. *in situ* Measurements should be performed.
4. Integration of analytical processes and operations saves energy and reduces the use of reagents.
5. Automated and miniaturized methods should be selected.
6. Derivatization should be avoided.
7. Generation of a large volume of analytical waste should be avoided and proper management of analytical waste should be provided.
8. Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time.
9. The use of energy should be minimized.
10. Reagents obtained from renewable source should be preferred.
11. Toxic reagents should be eliminated or replaced.
12. The safety of the operator should be increased.

2.3 Extraction techniques

Over the years, the indiscriminate use of tetracycline drugs in animal feed has raised huge concerns regarding the quality of food, including honey, meat, fish, milk and eggs, available to consumers [35-37]. The contamination of these types of foods with tetracycline residues poses risk to human health, having the potential of causing harmful effects on the population. Sample preparation is usually the most critical step in the development of methods for residue analysis. The type of treatment adopted depends on the matrix type, and the chemical attributes of the analytes [39]. The typical steps involved in sample preparation include sampling, extraction, clean-up and preconcentration, followed by the final analysis. The sampling step is regarded the most important among them because an inadequate sampling can lead to erroneous results, regardless of whether the remaining steps in the analytical process have been flawless. Any portion or aliquot taken for analysis must be representative of the original bulk sample. For solid foodstuffs, proper crushing is recommendable to obtain smaller sample sizes. When conducting food composition analysis, smaller sample portions can be used. By contrast, for the determination of trace contaminants

in food, relatively larger portions of sample are often required in order to obtain lower limits of detection. Irrespective of the selected sampling mechanism, sample homogenization is essential for chemical analysis [40]. The extraction and clean-up methods for tetracycline residue analysis reported in the literature have been applied to a broad variety of matrices. Foodstuffs, such as milk and eggs, contain many interfering substances that need to be removed selectively. The difficulty encountered in the analysis of tetracycline antibiotics in this type of foods lies in the need to eliminate the main matrix components (e.g., lipids, proteins, vitamins, minerals, fats, and other nutritional and energy sources) that may interfere in the instrumental determination of the investigated compounds. This difficulty has led to the development of new strategies for the isolation and extraction of tetracycline residues from food matrices [39].

The most relevant analytical applications regarding the determination of tetracycline drugs in different foodstuffs was summarized in Table 3. The main techniques employed for the extraction and clean-up of tetracyclines from such matrices.

Table 3 Analytical applications for tetracycline residue determination in foodstuffs.

Author (year)	**Analytes / Samples**	**Sample preparation**	**Technique**	**LODs**
Capriotti *et al.* (2012) [41]	Antimicrobials and mycotoxins / Eggs	QuEChERS methodology	HPLC-ESI-MS/MS	11-27 μg kg^{-1}
Pena *et al.* (2007) [42]	OTC, TC and CTC/ Swine muscle and kidney	Extraction with McIlvaine-EDTA buffer, followed by SPE	HPLC-FL	-
Chen *et al.* (2009) [43]	TC, OTC, MC and CTC/ Chicken and eggs	MIPs based on magnetite	HPLC-ESI-MS/MS	0.07-0.19 μg kg^{-1}
Feng *et al.* (2016) [44]	CTC, TC, OTC and DC/ Eggs and swine tissue	MIP-SPE procedure	HPLC-DAD	20-40 μg kg^{-1}
Shalaby *et al.* (2011) [45]	CTC, TC, OTC and DC/ Chicken tissue	UAE and purification by SPE	HPLC-DAD	4.4-10 μg kg^{-1}
Mu *et al.* (2011) [46]	TC, OTC, and DC/ Milk	MSPD procedure	CE-UV	74.5-80.8 μg L^{-1}
Ibarra *et al.* (2011) [47]	CTC, TC, OTC and DC/ Milk	MSPE procedure	CE-UV	2-9 μg L^{-1}
Halvatzis *et al.* (1993) [48]	CTC, TC, OTC, DC and DMCC/ Honey	Dilution with water	CL-FIA	2.0-4.9 μg L^{-1}
Masawat *et al.* (2008) [49]	CTC, TC and DC/ Milk	Pretreatment McIlvaine buffer-EDTA solution and extraction using Florisil column	Spectrophotometry UV/VIS-FIA	0.38-1.55 μg mL^{-1}
Rodríguez *et al.* (2016) [50]	TC, OTC,CTC, and DC/ Egg supplements	US-DLLME procedure	Spectrophotometry UV/VIS-LWCC-FIA	6.4-11.1 μg L^{-1}

2.3.1) Cloud point extraction

Cloud point extraction (CPE), the role of extraction solvent is shown by a micellar (surfactant-rich) phase originating from a homogenous surfactant solution that is added to the sample. A surfactant aggregate (a micelle) orientates its hydrocarbon tails towards the center to create a nonpolar core. Isolated hydrophobic compounds (a large number of bioactive compounds) present in the aqueous solution are favorably partitioned in the hydrophobic core of micelles. In cloud point extraction, separation of two phases (i.e. the surfactant-rich phase and the aqueous supernatant phase concentrating the surfactant close to the critical micelle concentration (cmc)) requires appropriate experimental conditions depending on the nature of the surfactant. Thus, temperature change results in two-phase separation of non-ionic and zwitterionic surfactant solutions, while other parameters (e.g., pH, addition of ionic salt or organic solvent) are involved in two-phase separation process of ionic surfactants [40]. The extraction process of CPE technique is very simple and is shown in Figure 3. First, the surfactant or a concentrated surfactant solution is added to the aqueous solution containing the analytes to be extracted/preconcentrated. The final surfactant concentration must exceed its CMC in order to ensure formation of micelle aggregates. Analytes can remain referentially in the hydrophobic domain of the micelles in a surfactant-rich phase, thus being extracted and preconcentrated [51]. Next, the conditions are altered by raising or lowering the temperature and/or adding salt or other additives to obtain phase separation. After demixing of the biphasic system, either by gravity settling or centrifugation, the analytes are preconcentrated in a small volume of surfactant-rich phase. Depending on the density of the surfactant-rich phase, it can be either at the bottom or the top. The preconcentrated analytes in surfactant-rich phase is so viscous that it cannot be injected directly into instrument for analysis, thus, it needs to be diluted with aqueous or organic solvent [52]. The schematic representation of the cloud point extraction is presented in Figure 3 [12].

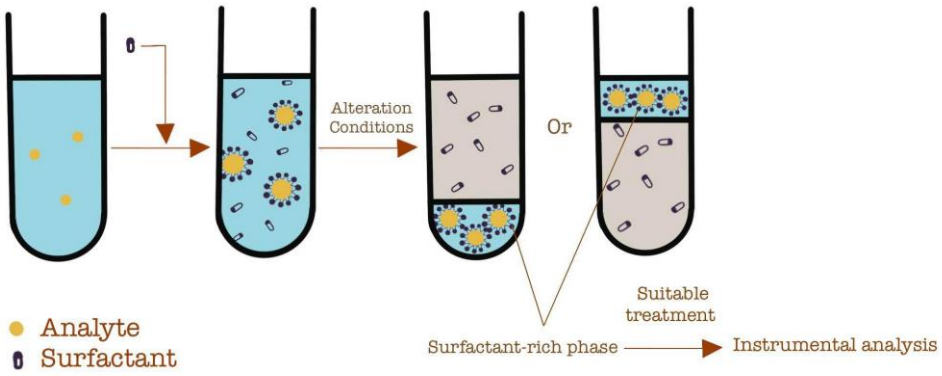

Figure 3 Schematic representation of the cloud point extraction.

2.3.2) Microextraction techniques

Microextraction techniques evolved from the classic sample preparation procedures and basically consist of the miniaturization of the latter. These techniques were developed to address the following concerns limited selectivity, incompatibility with instrumental techniques, the need to extract analyte traces from a large number of samples, the quest for the reduction of costs and time of analyses and for minimizing the consumption of toxic organic solvents that pose serious environmental and public health problem [53].

Dispersive micro-solid phase extraction (D-µSPE) is a miniaturized extraction method based on dispersion of micro- or nanosorbents in sample solution and isolation of solid sorbent by centrifugation, filtration or using an external magnetic field. D-µSPE is based on the SPE methodology, but a small amount of solid (µg or mg range) is dispersed in a sample solution containing the target analytes without conditioning. Dispersion phenomenon enables the sorbent to interact rapidly and uniformly with all the target analytes which lead to enhance the precision of method and reduce the extraction time. Ultrasounds and vortex (mechanical agitation) have been traditionally used to aid the dispersion of solids into liquid samples, although the terms ultrasound-assisted (UA) or vortex-assisted (VA) D-µSPE have been recently coined. The general procedure followed on both techniques shares similarities. The sample containing the sorbent is initially located on an extraction vessel where the ultrasounds or vortex agitation is applied for a defined period of time. After the extraction, the sorbent is usually recovered by centrifugation or filtration. In the case of using magnetic sorbents, the retrieval can be achieved by an external magnetic

field. The elution, which may involve re-dispersion, is finally done and the sorbent and eluate are separated (in the same way commented above) before the instrumental analysis [54]. The Schematic procedure of dispersive micro-solid phase extraction in demonstated in Figure 4 [55].

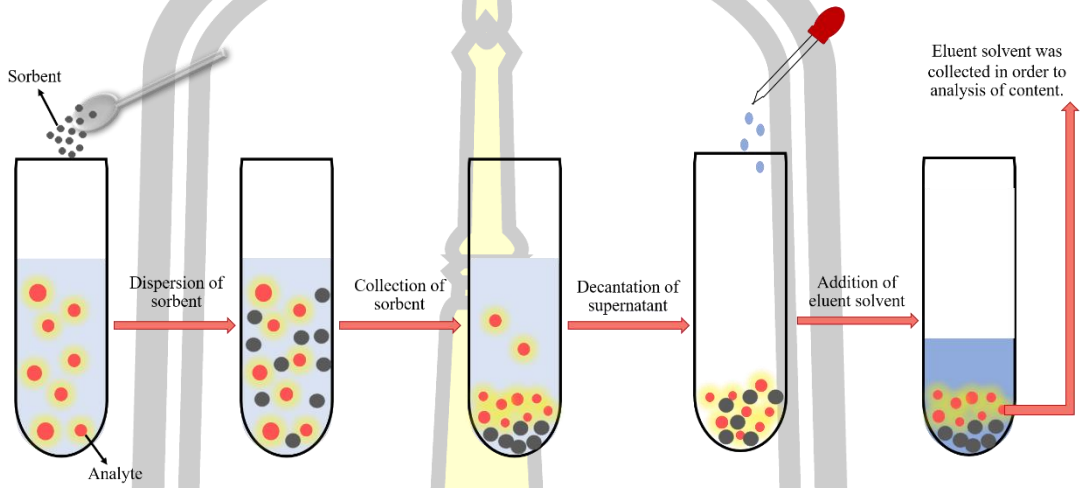

Figure 4 Schematic procedure of dispersive micro-solid phase extraction.

2.3.3) Nanostructured sorbents

Nanostructured sorbents have a high impact on microextraction techniques in the last decade. The reduction of the particle size, as it has been demonstrated by Witham et al. using a model based on perfect spherical particles, induces an exponential increase of the area-to-volume ratio. In this sense, Sangster and coworkers found a positive effect on the sorption capacity of aquatic sediments towards some hormones when the particle size was reduced. However, the efficient dispersion of the sorbent into the sample is also necessary to completely exploit the whole sorption capacity of a given nanomaterial [56].

Metal-organic frameworks (MOFs), these organic-inorganic hybrid solids consist of metal ions (or clusters) coordinated to organic linkers, with a large surface area and porosity showing high efficiency in adsorption. Consequently, these materials have been recommended as reliable and novel solid sorbents in sample preparation procedures. Rocio-Bautista et al. have recently published an article where evaluated the efficiency of five MOFs having different properties for the extraction of

six organic pollutants of different nature, concluding that is difficult to predict the adsorption of a target analyte in a specific MOF material [57]. The MOF's pore environment, pore size, and pore aperture widths, the presence of unsaturated metal sites, and the nature of the metal have a significant influence in their efficiency in D-μSPE.

2.3.3.1) Nanoparticles of iron(III)-tannic acid

Tannins are polyhydroxyphenols. They are soluble in water, alcohols and acetone and can coagulate proteins. They are yielded by extraction from wood substance, bark, leaves and fruits. Other components of the extraction solutions are sugars, pectins and other polymeric carbohydrates, amino acids and other substances. The content of non-tannins can reduce wood failure and water resistance of glued bonds. The polymeric carbohydrates especially increase the viscosity of the extracts [58]. The structure of tannins is shown in Figure 5 [59].

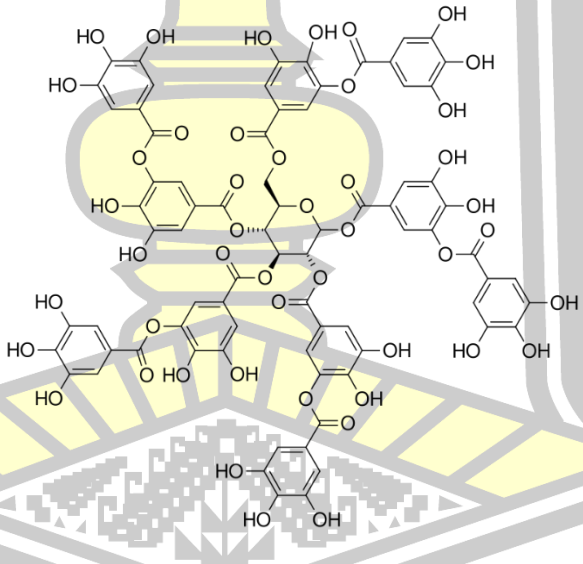

Figure 5 The structure of tannins.

Tannic acid (TA) is a typical kind of large natural polyphenol. It is considered as a polydentate ligand that can bind to various metal ions, especially ferric ion, to form high stable metal complexes. Additionally, unlike a small polyphenol, interaction of ferric ions and tannic acid has shown unique properties such as iron-mediated self-assembly of Fe–TA complexes, resulting in molecular nanoparticles of Fe–TA complexes. The large Fe–TA complexes are critical for enhancing the MRI

signal because large paramagnetic molecular nanoparticles have good capability of enhancing the rate of water–proton exchange by slowing down the rotational diffusion [60].

The Fe–TA NPs were easily obtained by mixing ferric chloride and tannic acid in phosphate buffered saline (PBS buffer) at room temperature for a few minutes in ambient air. Schematic illustration of the preparation of Fe–TA NPs is shown in Figure 6. Under this condition, Fe–TA complexes undergo an iron-mediated self-assembly process to form nanosized Fe–TA complexes [59]. It should be noted that PBS buffer was chosen as the reaction medium because this condition was suitable to form predominantly Tris-coordinated Fe–TA NPs (having a more stable structure) [50-51].

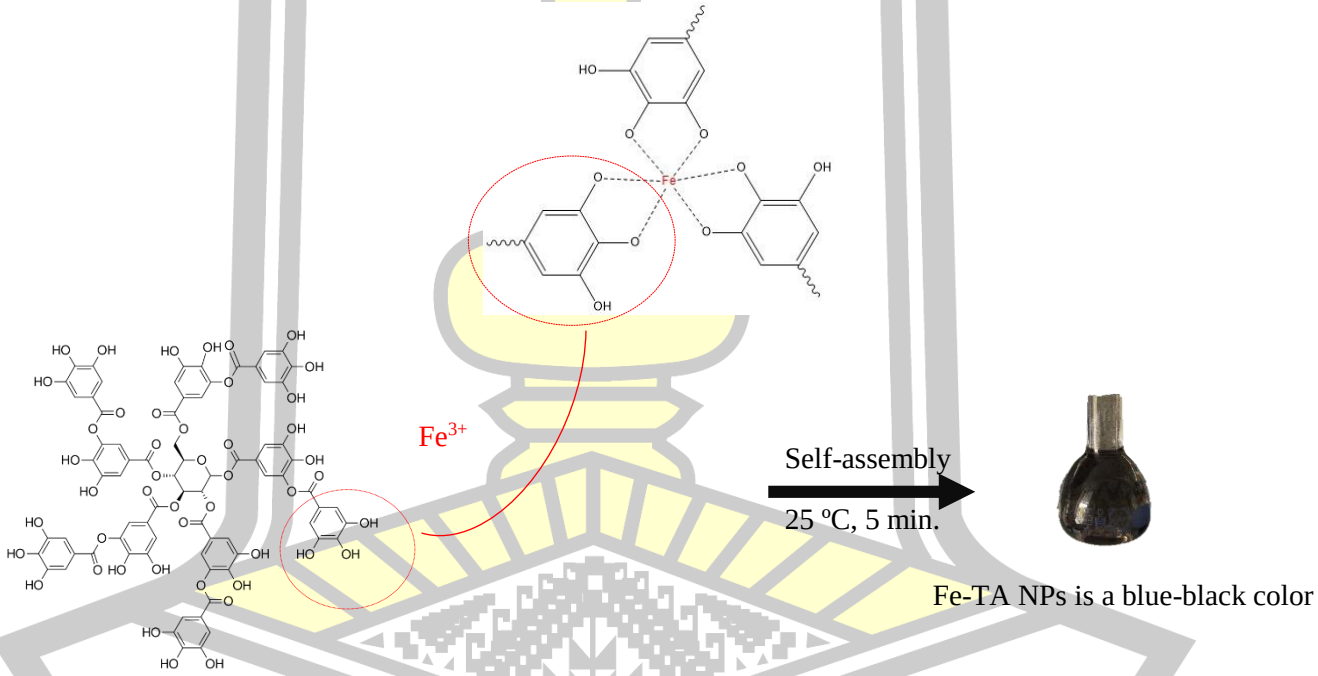

Figure 6 Schematic illustration of the preparation of Fe–TA NPs.

2.3.4) Application of microextraction for determination of tetracycline

Yi Wen et al, was developed and validated an in-tube SPME–HPLC method for the rapid, simple and simultaneous determination of four widely used tetracycline antibiotic (TC, CTC, OTC and DC) residues in fish muscle. A poly (methacrylic acid–ethylene glycol dimethacrylate) monolithic capillary column was selected as the extraction medium, which has successfully been used in the analysis of drugs in

biological fluids. Due to the biocompatibility of the extraction phase, no requirement of protein-precipitating and fat-removing steps were achieved [62]. Wen-Hsien Tsai et al, was studied the dispersive format, the reversible adsorption capabilities of different commercially available sorbents for preconcentration polar compounds such as TCs were evaluated. These sorbents include both silica-based sorbents and polymeric sorbents modified with various functionalized groups. The effects of various experimental parameters on the proposed dispersive SPME method were also investigated to develop a rapid and simple extraction method for four widely used tetracycline antibiotic residues from surface waters and milks. Moreover, from another point of view, for the purpose of obtaining higher final extract concentration for conventional LC-DAD, dispersive-SPME could also provide another method to further concentrate the MeCN extract from the extraction steps of QuEChERS method [63]. Ning Ma et al, the molecularly imprinted nano-polymer of minocycline was polymerized on the surface of a metal organic framework material to synthesize a novel composite. This composite was used as absorbent to develop a dispersive solid phase microextraction method for extraction of 7 tetracyclines in chicken muscle followed by determination with ultra performance liquid chromatography [64]. K. Cherkashina et al, was studied a procedure for separation and preconcentration of tetracyclines from human serum samples involving magnetic dispersive micro-solid phase extraction was proposed. The extraction efficiency of different tetracyclines was improved with the use of the surfactant coated Fe_3O_4 magnetic nanoparticles. Sorption mechanism was presented, and the potential use of magnetic Fe_3O_4 nanoparticles coated with different surfactants for tetracyclines adsorption was demonstrated for the first time. The procedure involved nanoparticle floating in a liquid sample phase for analyte extraction followed by elution and determination by high performance liquid chromatography with diode array detection [65].

2.4 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) functions by separating molecules and then detecting them. Liquid chromatography has two main components. The first component is the stationary phase. A column houses the stationary phase used in HPLC. There are many different types of columns; they come

in various lengths, material, and sizes. The stationary phase is responsible for separating the injected molecules as it interacts with the molecule at a molecular level. For example, the silica-based stationary phase octadecyl carbon chain (C18) can interact with a molecule through a series of chemical interactions such as ionic bonding, hydrogen bonding or hydrophobic interactions. Different sites on the molecule have different interactions with the stationary phase. Once separated, the analytes travel to the detector and read by a read-out device (i.e. monitor, printer, etc.). The C18 stationary phase is used in the column for reversed phase HPLC, where the stationary phase is less polar than the mobile phase [66].

The mobile phase usually consists of two solvents, aqueous and organic. The volume of mobile phase that is pumped through the system over an amount of time known as flow rate that is expressed in milliliters/microliters per minute. The percentage of aqueous and the organic solvents flowing through the system have two scenarios. If the percentage of aqueous and organic solvent are constant, then the method is known as an isocratic method. If the percentage of both phases are changing over time, then the method is known as a gradient method. Gradient methods are used to separate a wide range of molecules with different polarities. Gradient methods typically start at a low organic phase and then gradually increase the organic phase. For example, Oxytetracycline is released with smaller amounts of organic phase while Chlortetracycline requires higher organic phase content to be released. Figure 7 shows a diagram of the components of LC [66].

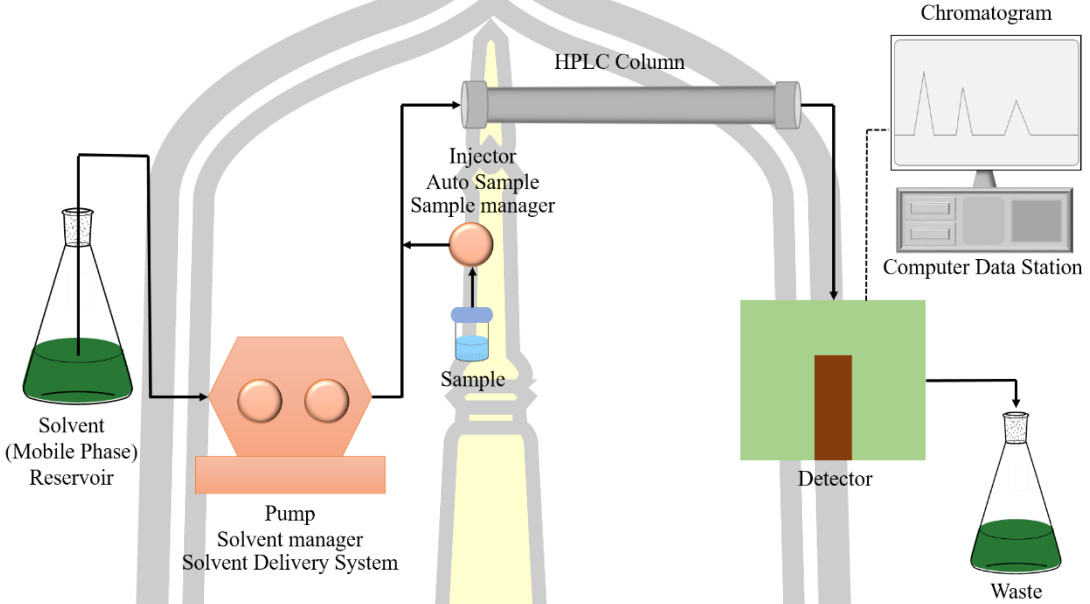

Figure 7 Liquid Chromatography System.

More detailed information about other analytical for determination of tetracyclines using chromatographic are summarized in Table 4.

Table 4 Selected methods recently published for TCs analysis.

	Reference			
	[67]	[68]	[69]	[70]
Matrix	Pig tissue	Turkey tissue	Beef and Pork Tissue	Salmon
Sample size (g)	1.5–5 (tissue dependant)	1	15	2
Analyte	TC, OTC, CTC, DC	OTC	TC, OTC, CTC	OTC
Extraction/deproteination	0.1 M succinic acid (pH 4)	McIlvaine (pH 4), MeOH	Water–ACN, 0.1 M phosphoric acid, hexane–DCM cleanup	Na_2EDTA–McIlvaine buffer (pH 4)
SPE/other	Oasis HLB	Metal chelate affinity chromatography	NA	Strata X, Waters aminopropyl
Evaporation	At 50 ºC under nitrogen	No	Partial evaporation at 40 ºC	No
Column	PLRP-S (250 mm × 4.6 mm I.D., 8 m)	LUNA C18 (150 mm × 4.6 mm I.D., 5m)	Prodigy ODS (4.6 mm × 150 mm, 5 m)	Varian Pursuit Diphenyl (300 mm × 4.6 mm ID, 5 m)
Mobile phase	1 mM oxalic acid–0.5% formic acid–THF	ACN–10 mM oxalic acid	4 mM oxalic acid–4 mM sodium decane sulfonate–ACN	$MgCl_2$ in tris(hydroxymethyl)-aminomethane (pH 7.5)–ACN
Detector	ESI MS	UV 355 nm	UV 370 nm	FLD
Spike range	50–1200 ng g^{-1}	50–1200 g kg^{-1}	100–1000 g kg^{-1}	100–1000 g kg^{-1}
Recovery (%)	>80	>75 (in muscle)	>90 for TC, OTC	≥70
Precision (%)	<14.7	<3.9	<6	<9

Table 4 (continued)

	Reference			
	[71]	[72]	[73]	[5]
Matrix	Milk and eggs	Honey	Salmon	Bovine milk and muscle
Sample size (g)	0.1 mL	3 g	5 g	5 g
Analyte	OTC	TC, OTC, CTC, DC, minocycline, methacycline	TC, OTC	TC, OTC, CTC, DC
Extraction/deproteination	Ultrasonic homogenization with 0.1 M succinic acid solution (pH 2.5); ultrafiltration	0.1 M Na2EDTA–McIlvaine buffer (pH 4)	Ultrasonication with Na_2EDTA–McIlvaine buffer (pH 4)–hexane (to remove fat)	Na_2EDTA–McIlvaine buffer
SPE	NA	Discovery DSC-phenyl	Oasis HLB	Oasis HLB
Evaporation	No	At 40 ºC, 240 bar	Partial evaporation at 30 ºC	Under nitrogen
Column	Mightysil® RP-4 GP (150 mm × 4.6 mm I.D., 5 m)	Discovery RP-Amide C16, 5m	Chromspher C8 (100 mm × 3 mm I.D., 5 m)	Hypersil C8 (250 mm × 4.6 mm I.D., 5m)
Mobile phase	2.1 mM succinic acid solution (pH 3.6)	10 mM oxalic acid (pH 3)–ACN	10 mM oxalic acid (pH 2)–ACN	10 mM oxalic acid–MeOH–ACN
Detector	UV 267 and 354 nm	DAD 270 and 355 nm	FLD	DAD 365
Spike range	Milk 0.05–0.2 g mL^{-1}; eggs 0.05–0.4 g mL^{-1}	500 ng g^{-1} (15 ng mL^{-1} LOD)	50–200 g kg^{-1}	50–150 g kg^{-1}
Recovery (%)	≥84	>92	>83	>81
Precision (%)	≤2.3	<5.5	<7.2	<5.3

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemicals and reagents used in this work listed in Table 5 were analytical regent grade and they were used without further purification.

Table 5 List of chemicals used in this work.

No.	Name	Formula	Grade	Company	Country
1.	Acetic acid	CH_3COOH	AR	Merck	Germany
2.	Acetonitrile	CH_3CN	HPLC	Merck	Germany
3.	Chlortetracycline hydrochloride	$C_{22}H_{23}ClN_2O_8 \cdot HCl$	HPLC	Sigma-Aldrich	Germany
4.	Deionized water	H_2O	-	Milli-Q, Millipore	United States
5.	Doxycycline hyclate	$C_{22}H_{24}N_2O_8 \cdot HCl \cdot 0.5H_2O \cdot 0.5C_2H_6O$	HPLC	Sigma-Aldrich	Germany
6.	Methanol	CH_3OH	HPLC	Merck	Germany
7.	Oxytetracycline hydrochloride	$C_{22}H_{24}N_2O_9 \cdot HCl$	HPLC	Sigma-Aldrich	Germany
8.	Sodium acetate 3- hydrate	$CH_3COONa.3H_2O$	AR	Ajax Finechem	New Zealand
9.	Stock solution of iron(III) 1000 mg L^{-1}	Fe^{3+}	AR	Merck	Germany
10.	Tannic acid	$C_{76}H_{52}O_{46}$	AR	Sigma-Aldrich	Germany
11.	Tetracycline hydrochloride	$C_{22}H_{24}N_2O_8 \cdot HCl$	HPLC	Sigma-Aldrich	Germany
12.	Trifluoroacetic Acid	$C_2HF_3O_2$	AR	Fisher Scientific	USA
13.	Triton X-114	$(C_2H_4O)n\ C_{14}H_{22}O$, n = 7 or 8	AR	Sigma-Aldrich	Germany

AR grade chemical means analytical reagent

HPLC grade chemical means high performance liquid chromatography

3.2 Instrumentation

The HPLC system consists of a Waters 1525 Binary HPLC pump (USA), and a Waters 2489 UV-Visible detector operated at 365 nm. A Purospher® STAR RP-18 endcapped column (4.6 × 150 mm, 5.0 µm) (Merck, Germany) was used as an analytical column carried out at room temperature. The injection volume is 20 µL. Breeze software was used for data processing. These TCs was separated using isocratic elution with 30% acetonitrile in water as the mobile phase at a flow rate of 0.8 mL/min. A vortex mixer (50 Hz, model ZX3, VELP Scientifica, Italy) and centrifuge (Model 1040series, labquip, United Kingdom) was used to mix the solution and accelerate the phase separation, respectively.

3.3 Experimental

3.3.1) Preparation of standard solution

3.3.1.1) Stock standard solution of TCs 1000 mg L^{-1}

Individual stock solution of TC, OTC, CTC and DC (1000 mg L^{-1}) was prepared by dissolving 0.0100 g of analytical standards in 10 mL of deionized water and stored in a dark glass bottle. Working TCs mixed solutions was prepared daily from the stock solution by a stepwise dilution with deionized water.

3.3.1.2) Stock standard solution of iron(III) 100 mg L^{-1}

A 100 mg L^{-1} iron(III) standard stock solutions was prepared by pipette 1 mL of 1000 mg L^{-1} of iron(III) into 10 mL volumetric flask. Then, the final volume was adjusted to 10 mL with deionized water.

3.3.1.3) Stock standard solution of tannic acid 1 mmol L^{-1}

A 1 mmol L^{-1} tannic acid standard stock solutions was prepared by dissolving 0.0425 g tannic acid in 25 mL of deionized water.

3.3.2) Preparation of 1 mol L^{-1} acetate buffer pH 5

Buffer solution was prepared by mixing appropriate amount of 54.36 g sodium acetate and 10.20 mL acetic acid. Then, the final volume was adjusted to 500 mL with deionized water. The required pH was performed by adjusting with 1 mol L^{-1} sodium hydroxide solution.

3.3.3) Preparation of mobile phase

The mobile phase 0.1% (v/v) TFA in acetonitrile (solvent A) and 0.1% TFA (solvent B) was prepared by pipette 1 mL of 99% TFA into 1000 mL volumetric flask. Then, the final volume was adjusted to 1000 mL with acetonitrile and deionized water for solvent A and solvent B, respectively.

3.3.4) Preparation of 9% TFA in acetonitrile

TFA 9% in acetonitrile for dissolving surfactant rich phase was prepared by pipette 0.9 mL of 99% TFA into 10 mL volumetric flask**.** Then, the final volume was adjusted to 10 mL with acetonitrile.

3.4 Sample preconcentration

An *in-situ* dispersive micro solid phase extraction assisted cloud point extraction procedure was carried out as follows, 0.05% w/v Triton X-114 was firstly added into a centrifuge tube (15 mL capacity). Then 1 mL standard or sample solution, 600 µL of 1 mmol L^{-1} tannic acid solution, 100 µL of 100 mg L^{-1} iron(III) solution and 5 mL of 0.5 M acetate buffer pH 5 was added, respectively. Then, the volume was adjusted to 10 mL with deionized water. After that, the solution was homogenized using a vortex mixer for 20 s and incubated at 40 ºC for 10 min. Then, the mixing solution was centrifuged at 4500 rpm for 20 min to completely isolate the precipitate of surfactant from the aqueous solution. And the collected Triton X-114 rich phase (lower phase) was withdrawn by syringe and diluted with 100 µL of 9%v/v TFA in ACN and filtered using a 0.45 µm membrane filter. Finally, 20 µL of the obtained solution was injected into the HPLC system for analysis. The Schematic representation of the *in-situ* dispersive micro solid phase extraction assisted cloud point extraction was presented in Figure 8. The diagram of *in-situ* dispersive micro solid phase extraction assisted cloud point extraction procedure was presented in Figure 9.

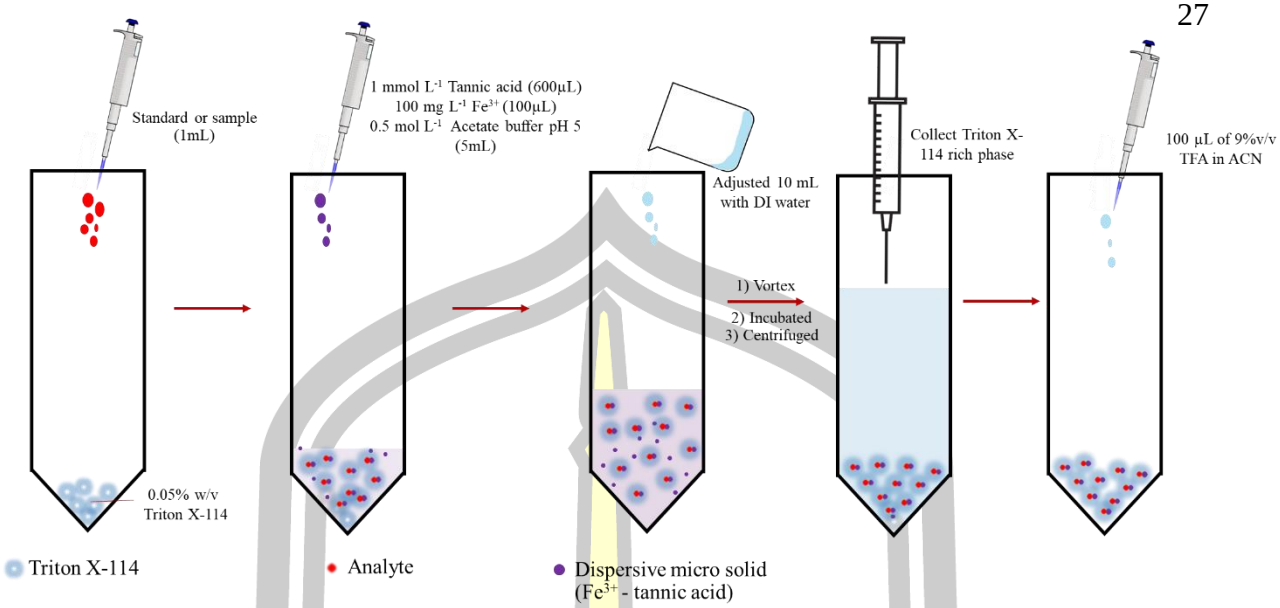

Figure 8 Schematic representation of the *in-situ* dispersive micro solid phase extraction assisted cloud point extraction

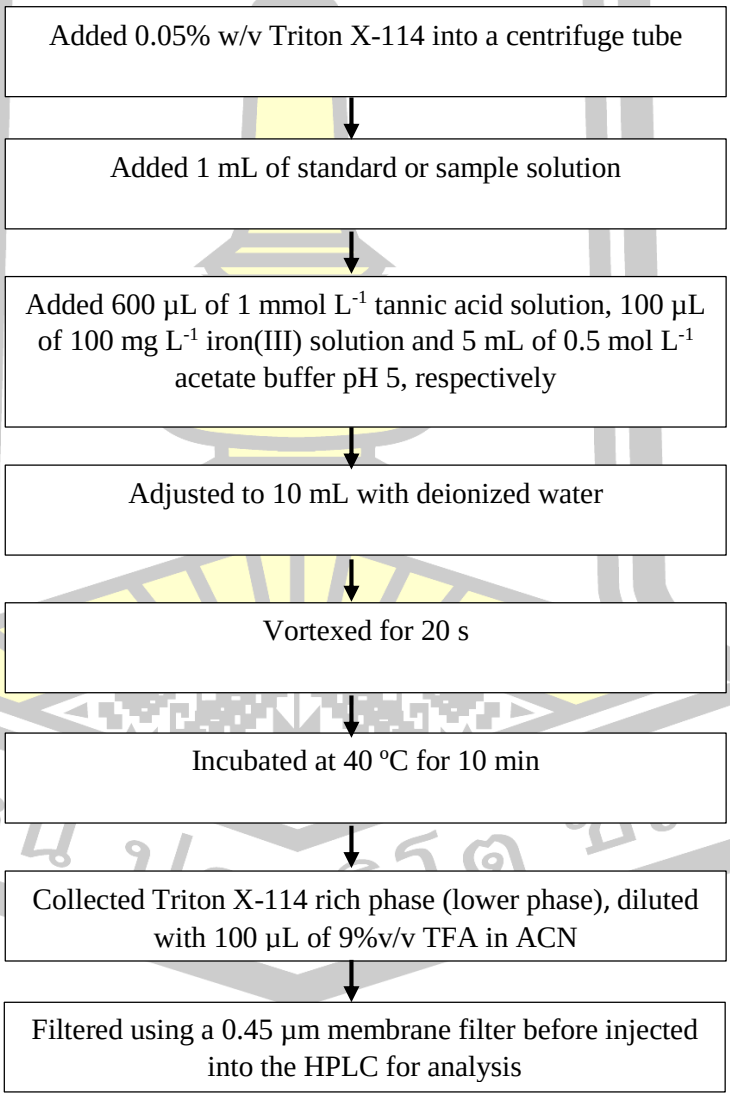

Figure 9 Diagram of an extraction process.

3.5 Optimization of experimental parameters

3.5.1) Effect of tannic acid concentration

Tannic acid concentration involve to iron-mediated self-assembly of Fe-TA complexes. The effect of tannic acid concentration was studied at 0.01, 0.02, 0.04, 0.06 and 0.08 mmol L^{-1}.

3.5.2) Effect of iron(III) concentration

Iron(III) concentration involve to iron-mediated self-assembly of Fe–TA complexes. The effect of iron(III) concentration was studied in the ranging of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}.

3.5.3) Effect of pH, concentration and volume

Due to the pKa value of tannic acid and the pH value effect to Fe-TA complex stability, the solution was chosen to adjust and control the pH value of the coordination reactions between tannic acid and iron(III). Therefore, the effect of pH was necessary investigated. The complex of tannic acid-iron(III) was studied in different acetate buffer at pH in the range 4-6, concentration of acetate buffer in ranging of 0.1-1.5 mol L^{-1} and acetate buffer volume was varied between 1-6 mL.

3.5.4) Effect of surfactants concentration

Surfactant concentration is one of the most important parameters affecting the efficiency of cloud point extraction. Because, the concentration of surfactant is low, the formation of micelle is not sufficiently realized and the extraction efficiency decreases. On the other hand, the concentration of surfactant is high, the pre-concentration factor was decreased as the volume of the surfactant rich phase increasing as expected [1]. The surfactant used for this purpose method was Triton X-114. In the preparation of the surfactant solutions, weight percentage (w/v) was preferred rather than volume (v/v) percentage, because it is very difficult to obtain repeatable volumes every time when prepared solutions in highly viscous surfactants. The effect of concentration of Triton X-114 was studied in the range of 0.001-0.10 %w/v.

3.5.5) Effect of vortex time

The vortex was used for agitation during the extraction step to provide the extraction efficiency the partition of sample and the extraction solvent into aqueous solution. The vortex time was studied at different intervals from 10-60 second.

3.5.6) Effect of incubation time and temperature

Another parameter needing optimization is the effect of incubation time and temperature. Temperature is an important parameter and required for micelle formation structure in cloud point extraction. In order to optimize the effect of incubation time on the cloud point extraction, it has been optimized between 1 to 25 min. And incubation temperature was studied in the range 35-55 ºC.

3.5.7) Effect of speed and time centrifugation

Centrifugation is another important parameter in procedure to achieve phase separation. In this extraction method, it was studied in the range of 4000-6000 rpm and 5-30 min for speed and time centrifugation, respectively.

3.5.8) Effect of concentration and volume of solvent for dilution surfactant rich phase

After obtained phase separation, surfactant rich phase (SRP) must be diluted with a suitable solvent prior to determination step. Because, high viscosity of SRP is not suitable for injection in this form. Volume of solvent used to dissolve the surfactant rich phase directly affects the enrichment factor, it is important to know that solvent volume was be taken. In order to obtain a high enrichment coefficient, the volume of the solvent must be the lowest. In this the proposed method concentration of TFA 0-10%v/v in ACN and volume of TFA in ACN to dilute surfactant rich phase in the range 50-500 µL was studied.

3.5.9) Morphology of tannic acid-iron(III) micro solid

The morphology of micro solid obtained from complex of tannic acid-iron(III) before and after utilized for extraction TCs was be investigated by TEM, SEM and FT-IR.

3.6 Method Validation

3.6.1) Linearity range, detection limits, precision and accuracy

The mixture of standard solutions including OTC, TC, CTC and DC was prepared and working solution was diluted with deionized water before injected into HPLC under the optimum conditions. A calibration curve for each analyte was constructed by plotting between the peak areas versus the concentration of mixed standard solution at eleven different concentrations for OTC, TC and nine different concentrations for CTC, DC. The linearity range was evaluated by the calibration curve ($y = mx+c$) and the correlation coefficient (r^2) value.

The sensitivity of the method was evaluated by limit of detection (LOD) calculated as three times the signal-to-noise ratio (3:1), and limit of quantitation (LOQ) calculated as ten times the signal-to-noise ratio (10:1). Precision of the method in terms of %RSD was determined by analyzing mixed standard solution at three different concentrations within a day and five difference days.

The percentage recoveries of *in-situ* dispersive micro solid phase extraction assisted cloud point extraction was studied by adding standard TCs at 30, 100 and 700 µg L^{-1}. The optimum conditions of analysis method was give the highest percentage recoveries, it was inferable that the proposed method can be used as an applicable and adequate method for analysis of tetracyclines residues in studied samples.

3.7 Real samples

The farm wastewater was collected from a pig farm, poultry farm and fish farm in Sisomdet District, Roi-Et Province, Thailand. All water samples was filtered through a 0.45 µm membrane filter, and were kept at 4 °C in refrigerator before analysis.

Food samples, including whole milk, UHT milk, egg, honey, chicken liver and chicken kidney were purchased from local supermarket in Kantharawichai District, Mahasarakham Province, Thailand. The milk and egg samples were deproteinized and defated before analysis followed the previously reported method [2]. Briefly, 1 mL of milk samples, 1 mL of ACN and 1 mL of 0.01 mol L^{-1} TFA was used for deproteinization and defatation. While, homogenized egg sample 1 g, 1.5 mL ACN

and 1.5 mL of 0.01 mol L^{-1} TFA was added to a 15 mL centrifuge tube, the solution was mixed using a vortex for 2 min and then centrifuge at 6000 rpm for 10 min. The supernatant was filtered through a 0.45 µm membrane filter and the final volume of the obtained solution was adjusted to 10 mL with deionized water, prior to extract by the proposed method.

For honey samples, 2 g of honey was diluted to 10 mL using water. The solution was mixed using a vortex for 2 min and then filtered using a 0.45 µm membrane filter. The diluted honey 1 mL was analysed by the proposed method.

Chicken liver and chicken kidney samples were prepared using the previously reported method [74]. One gram chicken meat or chicken liver samples, 2 mL of 0.1% formic acid in ethylenediaminetetraacetic acid (EDTA) 0.1% (w/v) were added to the samples and subsequently 2 mL of MeOH and 2 mL of ACN was used. After the addition of each solvent the tube was vortex-mixed for 30 s. The sample set was placed in an ultrasonic bath 20 min. Then, the samples were centrifuged at 4000 rpm for 10 min and the supernatant was filtered through a 0.45 µm membrane filter and the final volume of the obtained solution was adjusted to 10 mL with deionized water, prior to extract by the proposed method. The diagram of preparation real samples was presented in Figure 10.

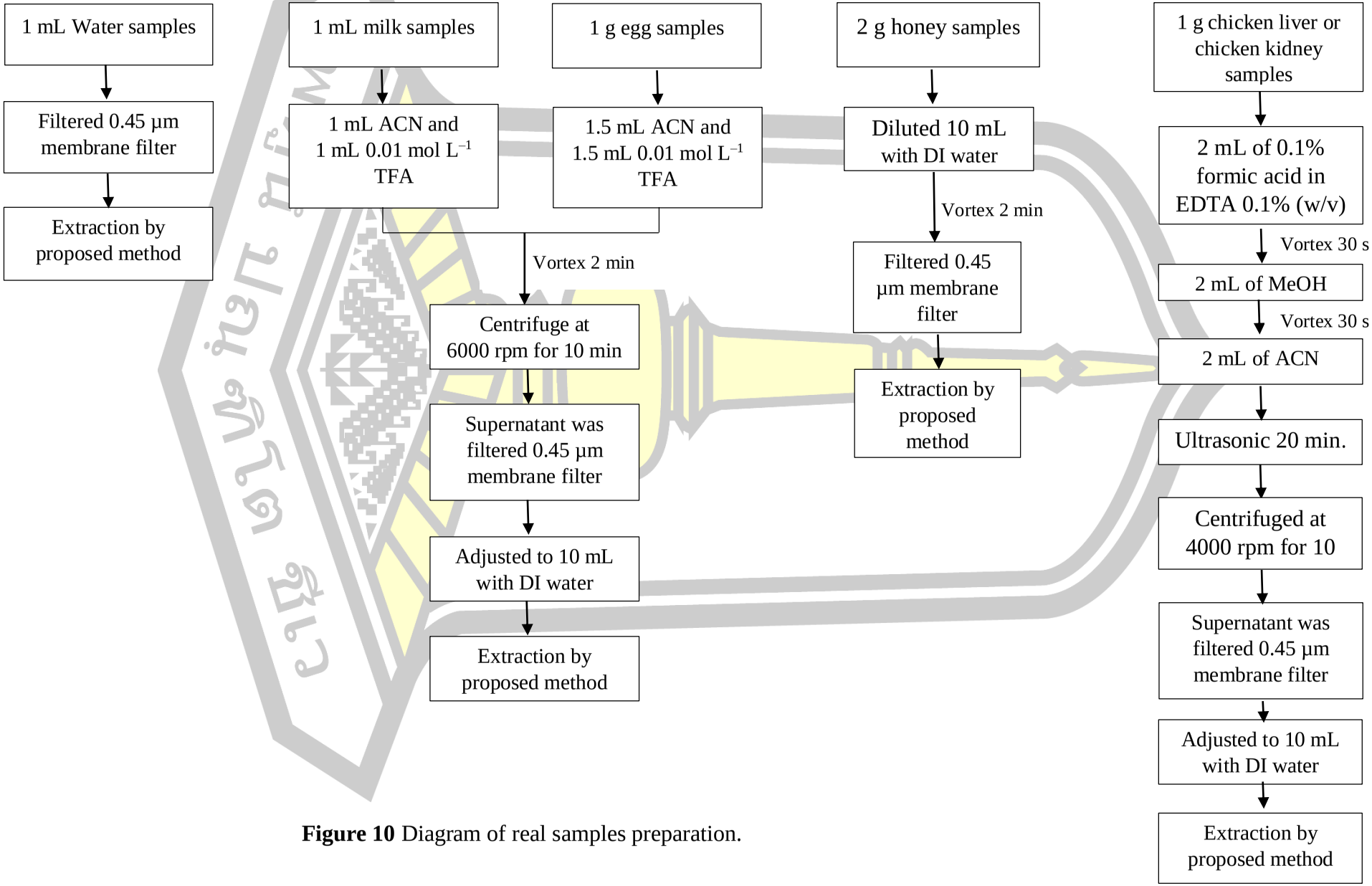

Figure 10 Diagram of real samples preparation.

3.8 Data analysis

The average result (mean) was calculated by summing the individual result and dividing by the number (n) of individual values:

$$\bar{X} = \frac{x_1 + x_2 + x_3 + \ldots}{n}$$

The standard deviation was measured of how precise the average is, that is, how well the individual number agree with each other. It is a measure of a type of error called random error. It is calculated as follows:

$$SD = \sqrt{\frac{(x_1-\bar{x})^2 + (x_2-\bar{x})^2 + (x_3-\bar{x})^2 + \ldots}{n-1}}$$

The percentage relative standard deviations (%RSD) are calculated from the standard deviation and mean using the equation:

$$\%RSD = \frac{SD}{\bar{x}} \times 100$$

The percentage recovery (%Recovery) was calculated by concentration of sample and spike sample using the equation:

$$\%Recovery = \frac{(spiked-unspiked)}{added} \times 100$$

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Ability of Fe-tannic acid micro solid extraction assisted cloud point extraction to extract and preconcentrate TCs

The ability of *in-situ* dispersive Fe-tannic acid micro solid extraction, cloud point extraction and *in-situ* dispersive Fe-tannic acid micro solid assisted cloud point extraction for extraction and preconcentration of TCs was investigated. Results of chromatogram are presented in Figure 11 (a) direct injection TCs, (b) preconcentration by cloud point extraction (CPE), (c) preconcentration by *in-situ* Fe-tannic acid micro solid extraction and (d) *in-situ* dispersive micro solid phase extraction assisted cloud point extraction. As can be seen that, CPE base on Triton X-114 (chromatogram b) can slightly extract of TCs while an *in-situ* dispersive Fe-TA micro solid extraction (chromatogram c) provided peak area higher direct injection method without preconcentration step (chromatogram a) but lower than an *in-situ* dispersive micro solid phase extraction assisted CPE (chromatogram d). Due to solid sorbents obtained from Fe-TA are nanoscale, it is difficult to complete phase separation by centrifugation. So, additional surfactant into the *in-situ* dispersive micro solid phase extraction can be accumulated all Fe-TA solid sorbents that interacted with TCs resulting to increase sensitivity. Therefore, it is clearly seen that, the proposed method was an effective method for the preconcentration of TCs.

4.2 Morphology of an *in-situ* dispersive micro solid phase

The hydroxyl groups in tannic acid can react with the Fe(III) to form of metal complexes. Complexes are formed between Fe(III) and phenolic groups with the presence of a third adjacent hydroxyl (pyrogallols) increase the stability of the complexes [75]. In this work, Fe-tannic acid micro solid can be utilized to extraction of TCs. To prove this hypothesis, SEM, FT-IR, TEM and Zeta potential were utilized to characterize of complex formation between Fe-tannic acid micro solid before and after extraction of TCs. The complexes were synthesized by adding 600 µL of 1 mmol L^{-1} tannic acid solution, 100 µL of 100 mg L^{-1} Fe(III) solution and 5 mL of 0.5 mol L^{-1} acetate buffer pH 5, respectively (used 1.0 µg mL^{-1} of each tetracyclines

The results SEM images are shown in Figure 12(a) and (b). SEM images reveals that Fe-tannic acid micro solids are spherical, and after the extraction of TCs micro solids agglomeration into large spherical shapes and the surface of micro solids was changed. While, TEM images reveals that after the extraction of TCs the morphology of micro solids has changed , as shown in Figure 12(c) and 12(d)

Figure 13 shows the FT-IR results of Fe-TA micro solid utilized as the sorbent to extraction of TCs. Obviously, polar functional group as hydroxyl (-OH) presented in the structure of TA may play an important role for the extraction of TCs interaction with polar functional groups on the TCs molecules as hydroxyl (-OH), carbonyl (-C=O) and amine ($-NH_2$) through dipole-dipole interaction and hydrogen bond. The results of IR spectra of Fe-TA before and after utilized for extraction TCs are shown in Figure 13. It was observed that, the stretching vibration peak of phenolic -OH group at 3425 cm^{-1} for Fe-TA micro solids is different from the stretching vibrations of -OH groups after extraction TCs (3415 cm^{-1}) which shifted to lower wavenumbers. The characteristics of another peaks in the Fe-TA micro solids by IR spectrum are as follows: the stretch of (C=C) of benzene ring band at 1687.00 cm^{-1}, the stretch vibration of (C-C) for benzene ring band at 1687.00, 1640.55 and 1553.72 cm^{-1}, the stretch (C-O) of phenolic band at 1424.82 and 1211.22 cm^{-1}, the stretch (C-O) of the carboxylic acid and ether band at 1051.57 and 1009.53 cm^{-1}. While, the peaks of phenolic (O-H) deformation with 1409.38 and 1245.29 cm^{-1} in the spectrum after the extraction of TCs by micro solids was slightly broadened than the Fe-TA micro solids (1424.82 and 1211.22 cm^{-1}). The absorption band of O-H bending at 1409.38, 645.63 and 621.93 cm^{-1} in the spectrum after the extraction of TCs which shifted to lower wavenumbers. These may be attributed to formation of hydrogen bond interaction between Fe-TA micro solids and TCs [65].

In order to confirm the stability of the micro solids, zeta potential measurements were investigated for the Fe-TA micro solids before and after extraction of TCs. The results, reveal that zeta potential values were found to be -21.8 ± 0.8 and -31.8 ± 1.1 mV for micro solid before and after extraction of TCs, respectively which, the zeta potential values was more than +25 or -25 mV indicating that the micro solids have high degree of stability [76]. Further characterizations using Dynamic Light Scattering, size distribution of TCs, Fe-TA micro solids and Fe-TA

micro solids-TCs were radius 3.2 ± 1.4, 101.7 ± 7.6 and 2780 ± 0.2 nm, respectively. The particle sizes of Fe-TA micro solids-TCs increased after the extraction process indicating the aggregation of Fe-TA micro solids and TCs.

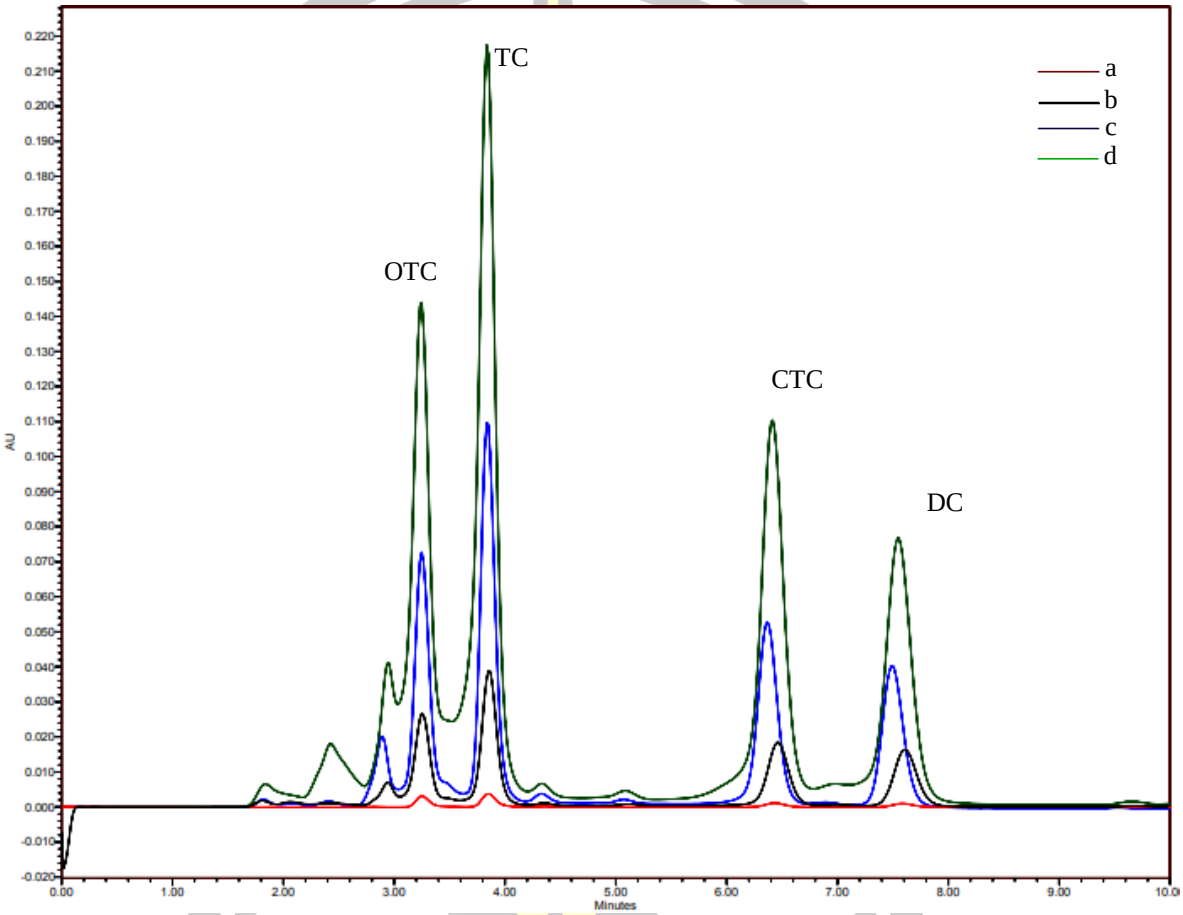

Figure 11 Chromatograms of TCs (a) direct injection method, (b) preconcentration by CPE, (c) preconcentration by *in-situ* dispersive Fe-TA micro solid extraction and (d) *in-situ* dispersive Fe-TA micro solid phase extraction assisted CPE.

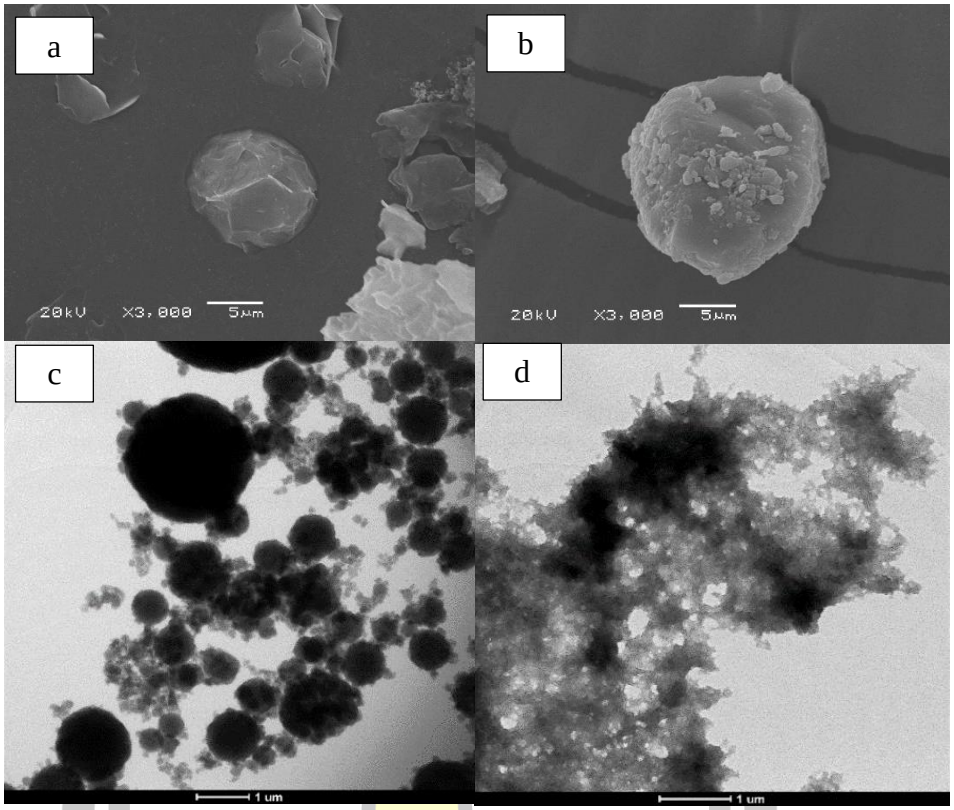

Figure 12 (a) SEM images of *in-situ* dispersive micro solids phase before and (b) after extraction TCs. and TEM images of *in-situ* dispersive micro solids phase before (c), after extraction TCs (d).

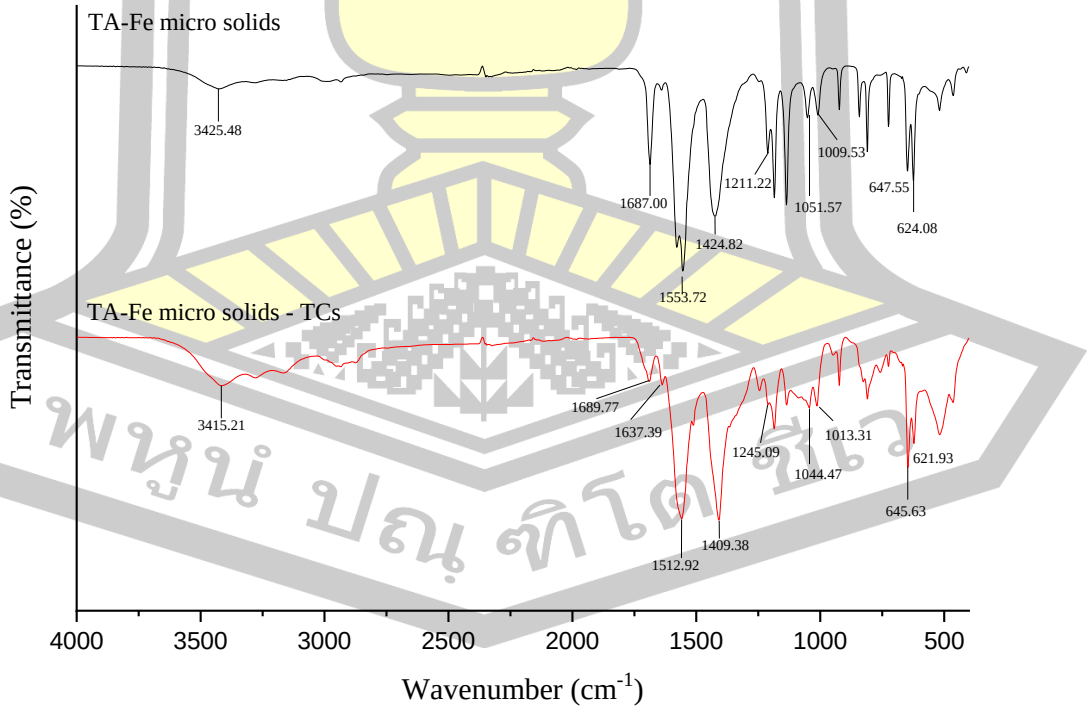

Figure 13 FTIR spectra of *in-situ* dispersive micro solid phase extraction (Fe-TA micro solids) and after extraction TCs (TA-Fe micro solids -TCs).

4.3 Optimization of an *in-situ* dispersive micro solid phase extraction assisted cloud point extraction

In order to achieve high extraction efficiency, the parameters that influence on the *in-situ* dispersive micro solid phase extraction assisted cloud point extraction procedure, such as concentration of tannic acid, concentration of Fe(III), pH, concentration of buffer, buffer volume, concentration of surfactants, vortex time, incubation time and temperature, centrifugation speed and time, concentration and volume of solvent for dilution surfactant rich phase were optimized and investigated through univariated method. The extraction efficiency was evaluated in term of the enrichment factor (EF), which was calculated using the ratio between the slope of the calibration plot established by the proposed method (S_{method}) and the slope of the standard curve obtained from the direct injection (S_{di}), $EF=S_{method}/S_{di}$. The optimization was carried out on the aqueous solution 10.00 mL containing 1.00 µg mL^{-1} of each tetracyclines. All the experiments were performed triplicated and the mean of the results were used to optimization.

4.3.1 Effect of tannic acid concentration

Tannic acid concentration is involved of Fe-mediated self-assembly of Fe-TA complexes. The effect of tannic acid concentration was studied at 0.01, 0.02, 0.04, 0.06 and 0.80 mmol L^{-1}. The obtained results (Figure 14) showed that the extraction efficiency was increased with increasing the concentration of tannic acid from 0.01 to 0.06 mmol L^{-1} and decreasing afterward. It may be due to the excessive forming of Fe-TA micro solids by increasing the tannic acid concentration more than 0.06 mmol L^{-1}, which leads to sufficient in extraction by sorbents. Therefore, tannic acid at 0.06 mmol L^{-1} was the optimum value for the formation of micro solids.

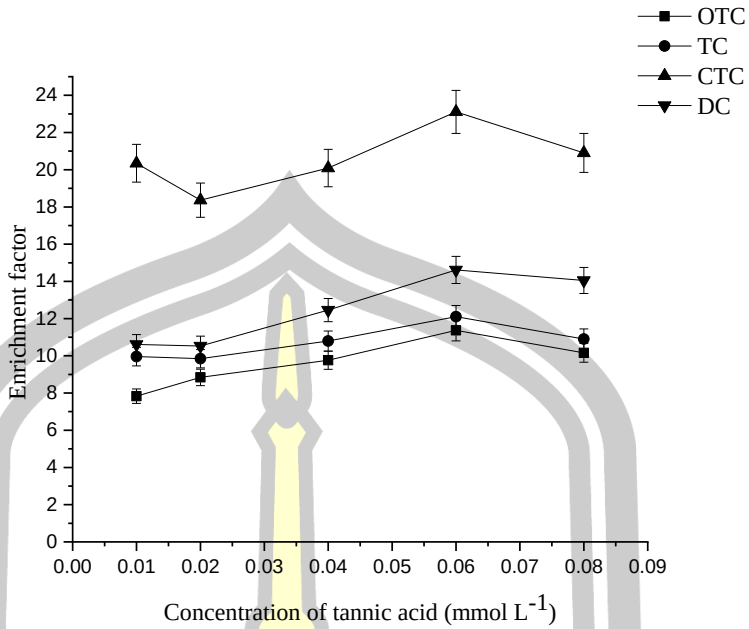

Figure 14 Effect of concentration of tannic acid. Conditions: 1.0% w/v Triton X-114, 1.0 mg L^{-1} Fe^{3+}, 1 mol L^{-1} acetate buffer pH 5.5, vortex time 20 sec, incubation at 45 ºC for 10 min, centrifugation at 5500 rpm for 10 min, 200 µL 9% v/v TFA in ACN for diluted surfactant rich phase, 1.0 µg mL^{-1} of each tetracyclines.

4.3.2 Effect of Fe(III) concentration

The effect of Fe(III) concentration on the self-assembly of Fe-TA complexes was investigated in the ranging of 0.5-3.0 mg L^{-1} of Fe(III). In Figure 15, the obtained results showed 1.0 mg L^{-1} value for the best signal as the optimum value.

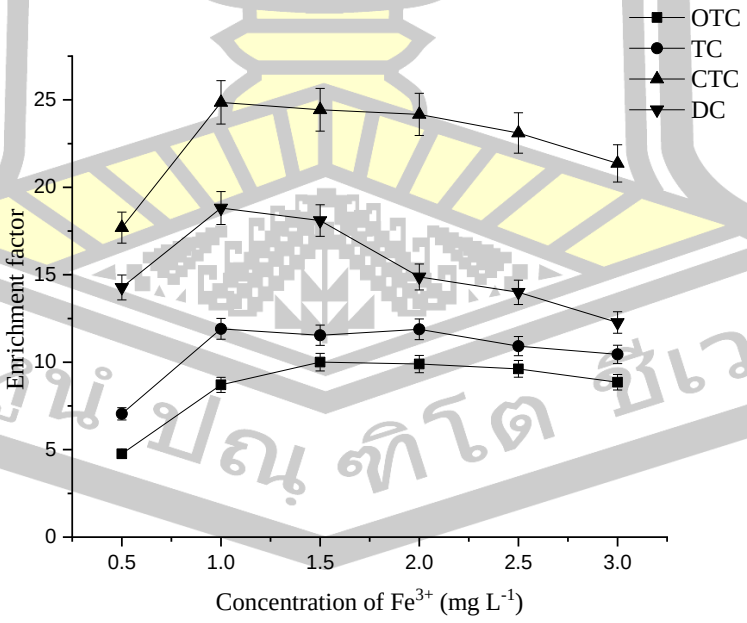

Figure 15 Effect of concentration of Fe^{3+}. Conditions: 1.0% w/v Triton X-114, 0.06 mmol L^{-1} tannic acid, 1 mol L^{-1} acetate buffer pH 5.5, vortex time 20 sec, incubation

at 45 ºC for 10 min, centrifugation at 5500 rpm for 10 min, 200 µL 9% v/v TFA in ACN for diluted surfactant rich phase, 1.0 µg mL^{-1} of each tetracyclines.

4.3.3 Effect of pH

It is widely known that the stability of the Fe-TA complexes are depended on pH. The coordination states of metal complexes are closely related to the electronic behavior. Thus, the pH was influenced to coordination state of Fe-TA micro solids, and also have an influence on the conformation of TCs. TCs can be presented in various forms; positive ($pH<pKa_1$), zwitterion ($pKa_1<pH<pKa_3$), or negative ($pH>pKa_3$) [1]. Therefore, in this study, the influence of pH on the stability of Fe-TA micro solids was investigated using 1 mol L^{-1} acetate buffer at pH in the range 4.0-6.0. The results (Figure 16) indicated that peak area increased as pH increased from 4.0 to 5.0 and then the signal reduced. Therefore, the optimum pH value and stability of the Fe-TA complexes for the enrichment process were selected pH 5.0

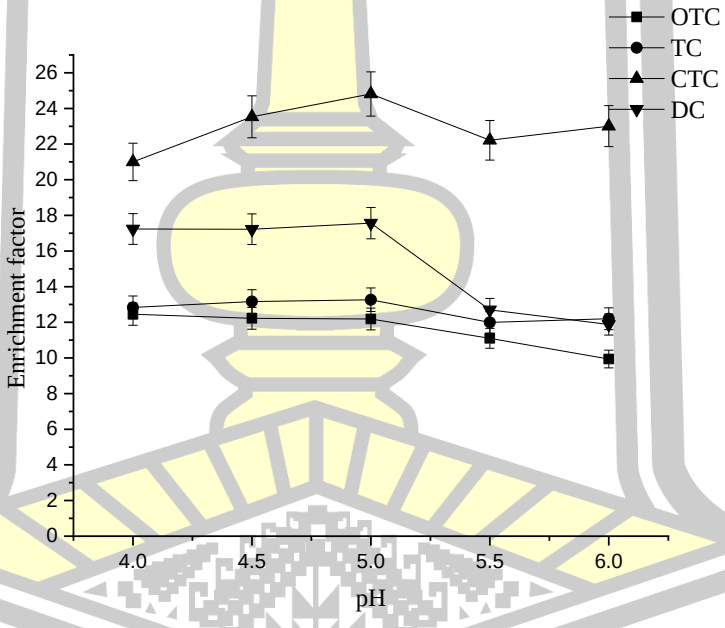

Figure 16 Effect of pH. Conditions: 1.0% w/v Triton X-114, 0.06 mmol L^{-1} tannic acid, 1.0 mg L^{-1} Fe^{3+}, 1 mol L^{-1} acetate buffer, vortex time 20 sec, incubation at 45 ºC for 10 min, centrifugation at 5500 rpm for 10 min, 200 µL 9% v/v TFA in ACN for diluted surfactant rich phase, 1.0 µg mL^{-1} of each tetracyclines.

4.3.4 Effect of buffer concentration

The concentration of acetate buffer, studied at the buffer concentration of 0.1-1.5 mol L^{-1}. The result shown in Figure 17 the signal was increased at 0.5 mol L^{-1} and then it was reduced. Therefore, the optimum concentration of acetate buffer was 0.5 mol L^{-1}.

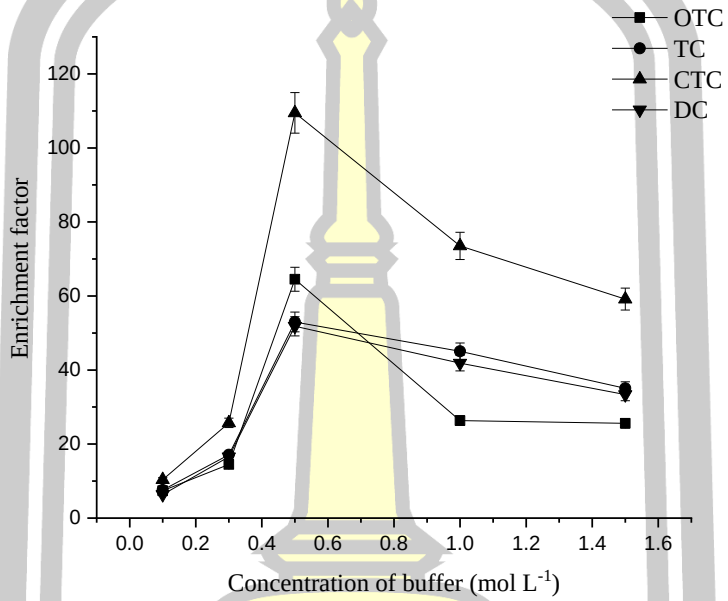

Figure 17 Effect of buffer concentration. Conditions: 0.05% w/v Triton X-114, 0.06 mmol L^{-1} tannic acid, 1.0 mg L^{-1} Fe^{3+}, acetate buffer pH 5.0, vortex time 20 sec, incubation at 45 ºC for 10 min, centrifugation at 5500 rpm for 10 min, 200 µL 9% v/v TFA in ACN for diluted surfactant rich phase, 1.0 µg mL^{-1} of each tetracyclines.

4.3.5 Effect of buffer volume

Volume of 0.5 mol L^{-1} acetate buffer pH 5.0 were studied different volume in the range 1-6 mL. The results shown in Figure 18. Enrichment factors increased as volume of acetate buffer increased from 1.0 to 5.0 mL, then it was slightly reduced. Thus, the optimum volume of 0.5 mol L^{-1} acetate buffer pH 5.0 was 5.0 mL.

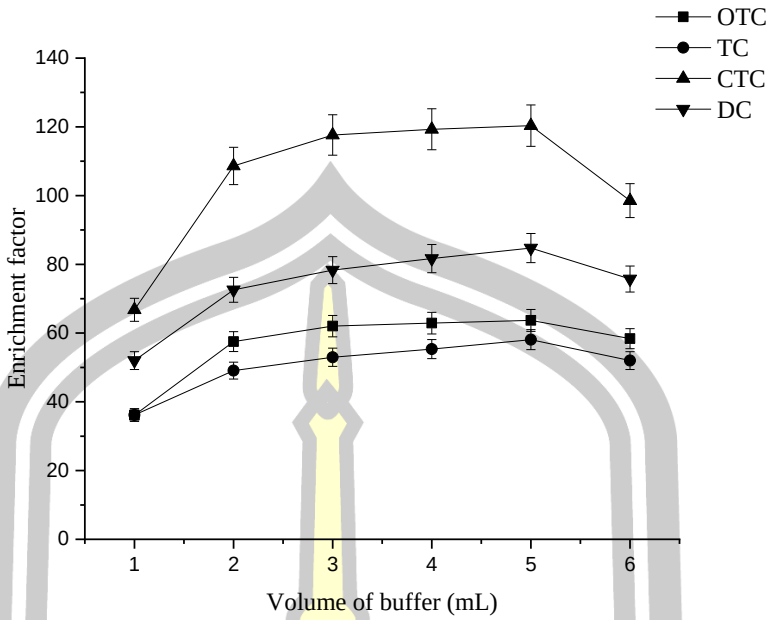

Figure 18 Effect of buffer volume. Conditions: 0.05% w/v Triton X-114, 0.06 mmol L^{-1} tannic acid, 1.0 mg L^{-1} Fe^{3+}, 0.5 mol L^{-1} acetate buffer pH 5.0, vortex time 20 sec, incubation at 45 ºC for 10 min, centrifugation at 5500 rpm for 10 min, 200 µL 9% v/v TFA in ACN for diluted surfactant rich phase, 1.0 µg mL^{-1} of each tetracyclines.

4.3.6 Effect of surfactants concentration

Surfactant concentration is one of the most important parameters affecting the extraction efficiency of cloud point extraction. The surfactant concentration affects both the extraction and preconcentration factor. Thus, the minimum concentration that produces quantitative extraction should be chosen in order to obtain the best aqueous phase volume/surfactant-rich phase volume ratio [77]. The effect of surfactant on cloud point extraction is considered to be very important because there is a narrow range for easy phase separation within maximum extraction efficiency and analytical signal detection. The solubilization/partition of non-polar organic molecules in the hydrophobic micellar core is an inherent property of all surfactant formulations. The efficiency of these procedures relies on the magnitude of analyte solubilization into the micelle (non-polar core and polar micelle-water interface). Non-ionic surfactants have always remained good vector for extraction of analyte from complex matrices [78]. Many nonionic surfactants such as polyethylene glycol 6000 (PEG-6000), Triton X-100 and Triton X-114 was used in cloud point extraction, in order to make the enrichment process under the best conditions. Nonionic surfactant used for this purpose was Triton X-114 due to low critical micelle concentration (cmc) and low cloud point at room temperature (25ºC) by avoiding degradation of antibiotic

molecules. In the preparation of the surfactant solutions, weight percentage (w/v) was preferred rather than volume (v/v) percentage, because it is very difficult to obtain repeatable volumes in every time when preparing solutions in highly viscous surfactants. The concentration of Triton X-114 was investigated in the ranging from 0.01-1.0% w/v. As can be seen in Figure 19 the extraction efficiency increased with increasing the concentration of Triton X-114 from 0.01 to 0.05% w/v and decreased afterwards. It may be due to increase surfactant concentration the surfactant rich phase also increasing, which resulting to decline of extraction efficiency by dilution effect. Thus, the best signal was obtained with 0.005 g of Triton X-114. This amount equals to 0.05% w/v Triton X-114 in final volume.

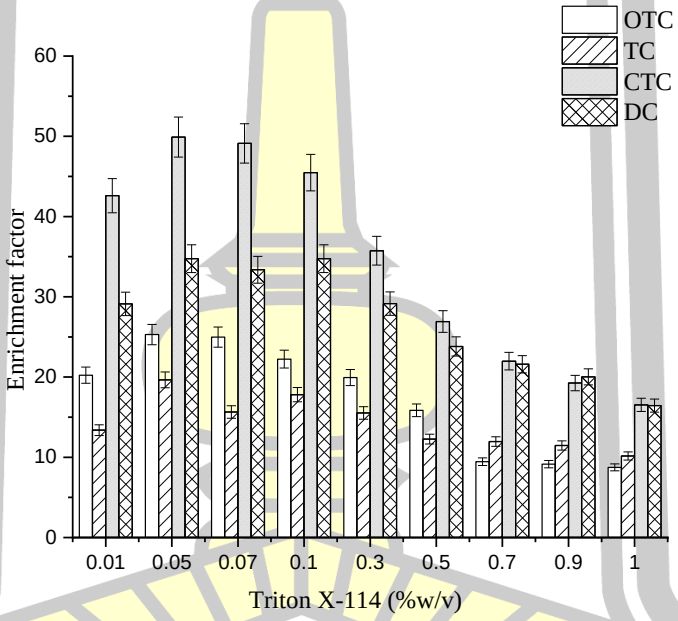

Figure 19 Effect of Triton X-114 concentration. Conditions: 0.06 mmol L^{-1} tannic acid, 1.0 mg L^{-1} Fe^{3+}, 0.5 mol L^{-1} acetate buffer pH 5, vortex time 20 sec, incubation at 45 ºC for 10 min, centrifugation at 5500 rpm for 10 min, 200 µL 9% v/v TFA in ACN for diluted surfactant rich phase, 1.0 µg mL^{-1} of each tetracyclines.

4.3.7 Effect of vortex time

The vortex was used for agitation of solution leading to ameliorate the contact between analytes with sorbent before incubation at water bath to provide the excellent extraction efficiency. The vortex time was studied at different intervals from 10-60 sec. According to experimental results shown in Figure 20, the homogenization of

mixer solution was effectively ensured at the 20 sec and it provided the best signal as the optimum value.

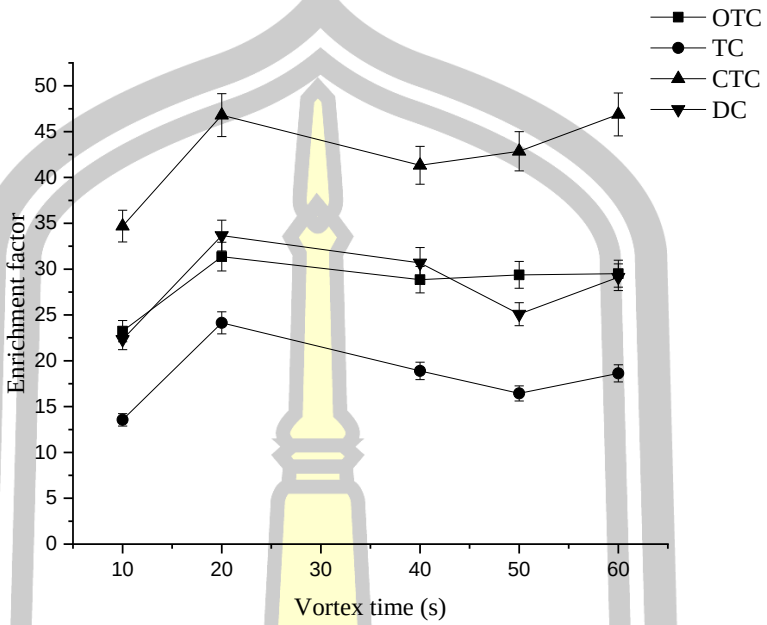

Figure 20 Effect of vortex time. Conditions: 0.05% w/v Triton X-114, 0.06 mmol L^{-1} tannic acid, 1.0 mg L^{-1} Fe^{3+}, 0.5 mol L^{-1} acetate buffer pH 5, vortex time 20 sec, incubation at 45 ºC for 10 min, centrifugation at 5500 rpm for 10 min, 200 µL 9% v/v TFA in ACN for diluted surfactant rich phase, 1.0 µg mL^{-1} of each tetracyclines.

4.3.8 Effect of incubation time and temperature

Another parameters needing to optimization are the effect of incubation time and temperature. Temperature is an important parameter and required for micelle formation structure in cloud point extraction. In order to optimize the effect of incubation temperature on the cloud point extraction was tested in the range 35-55 ºC and incubation time was examined between 1-25 min. The results (Figure 21a, b), the incubation temperature was observed at 40 ºC for the best signal as the optimum incubation temperature. And 10 min of incubation period was found to be appropriate, considering the total time spent in the experiment. Hence, the proposed method for TCs determination was incubated at 40 ºC for 10 min.

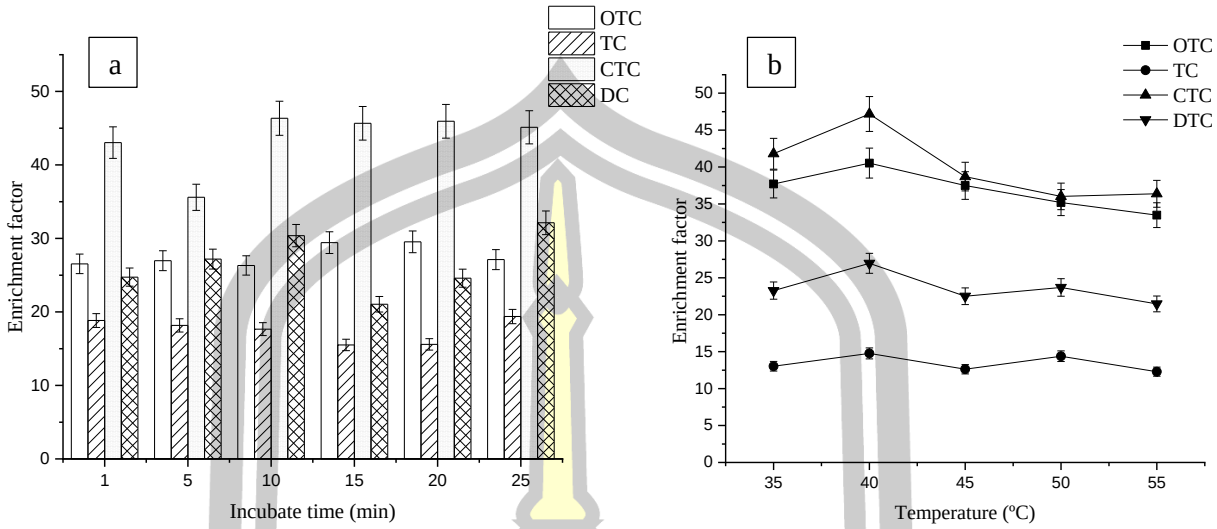

Figure 21 Effect of incubation time (a) and temperature (b). Conditions: 0.05% w/v Triton X-114, 0.06 mmol L^{-1} tannic acid, 1.0 mg L^{-1} Fe^{3+}, 0.5 mol L^{-1} acetate buffer pH 5, vortex time 20 sec, centrifugation at 5500 rpm for 10 min, 200 µL 9% v/v TFA in ACN for diluted surfactant rich phase, 1.0 µg mL^{-1} of each tetracyclines.

4.3.9 Effect of centrifugation speed and time

Centrifugation is another important parameter in extraction procedure to achieve completely phase separation from aqueous phase. In this extraction method, the centrifugation speed was studied in the range of 4000-6000 rpm and 5-30 min for time centrifugation. The effect of the speed and centrifugation time on the best signal of TCs is displayed in Figure 22. It can be seen that at 4500 rpm and 20 min were the appropriate speed and time centrifugation and these were used in further studies.

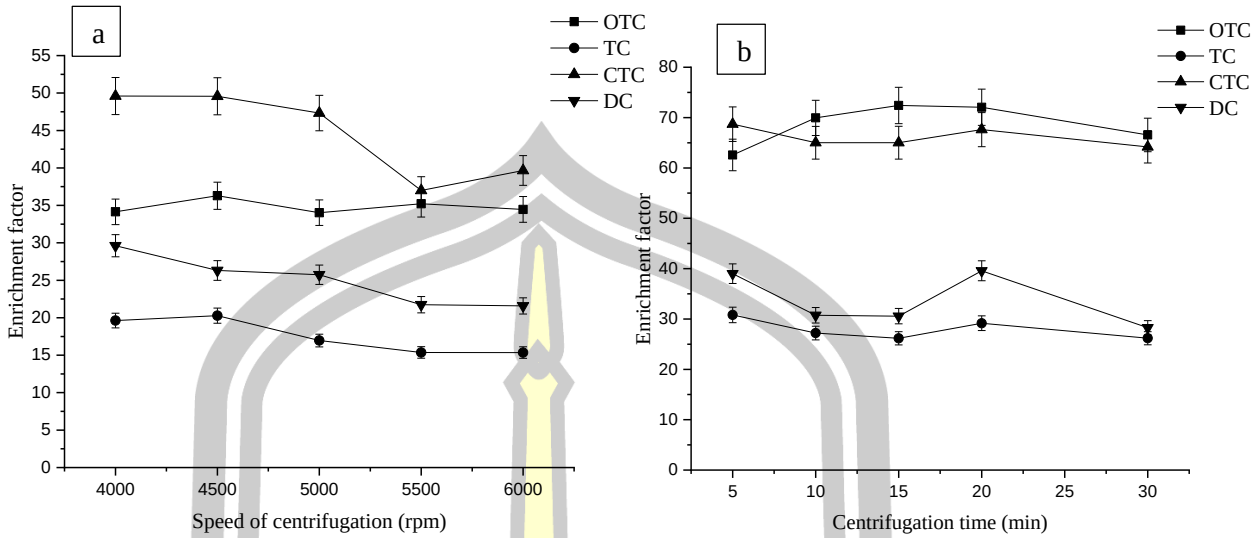

Figure 22 Effect of speed (a) and time centrifugation (b). Conditions: 0.05% w/v Triton X-114, 0.06 mmol L^{-1} tannic acid, 1.0 mg L^{-1} Fe^{3+}, 0.5 mol L^{-1} acetate buffer pH 5, vortex time 20 sec, incubation at 40 ºC for 10 min, 200 µL 9% v/v TFA in ACN for diluted surfactant rich phase, 1.0 µg mL^{-1} of each tetracyclines.

4.3.10 Effect of concentration and volume of solvent for dilution surfactant rich phase

After phase separation, surfactant rich phase (SRP) consist of micro solids must be desorbed and diluted with a suitable solvent prior to determination step. Because, TCs analyte can be adsorbed on Fe-TA micro solids and high viscosity of SRP is not suitable for injection in this form. Thus, TFA was utilized to dissolve the precipitates and release the enriched TCs by varying the concentration in the ranging of 0-10% v/v in ACN. As can be seen in Figure 23a, the highest signals were obtained with 9% TFA in ACN. Using TFA at low concentration than 9% v/v, it cannot be released TCs yielding to low sensitivity. Therefore, in the subsequent studies, the micro solids phase was dissolved with this solvent. Amount of solvent used to dissolve the phase directly affects the enrichment factor, it is important to know that solvent volume was taken. In order to obtain a high enrichment coefficient, the volume of the solvent must be the lowest and sufficient to inject to the HPLC. In this the proposed method volume of 9% v/v TFA in ACN to dilute SRP in the range 50-500 µL was employed. Volume of solvent for dilution SRP, the signals decreased with effect of dilution as expected Figure 23b. In order to obtain the best signals, sufficient to dissolve at

surfactant rich phase, easy filtration, enough to rinse micro syringe and repeat the injection 9% v/v TFA in ACN at 100 µL was selected in further studies.

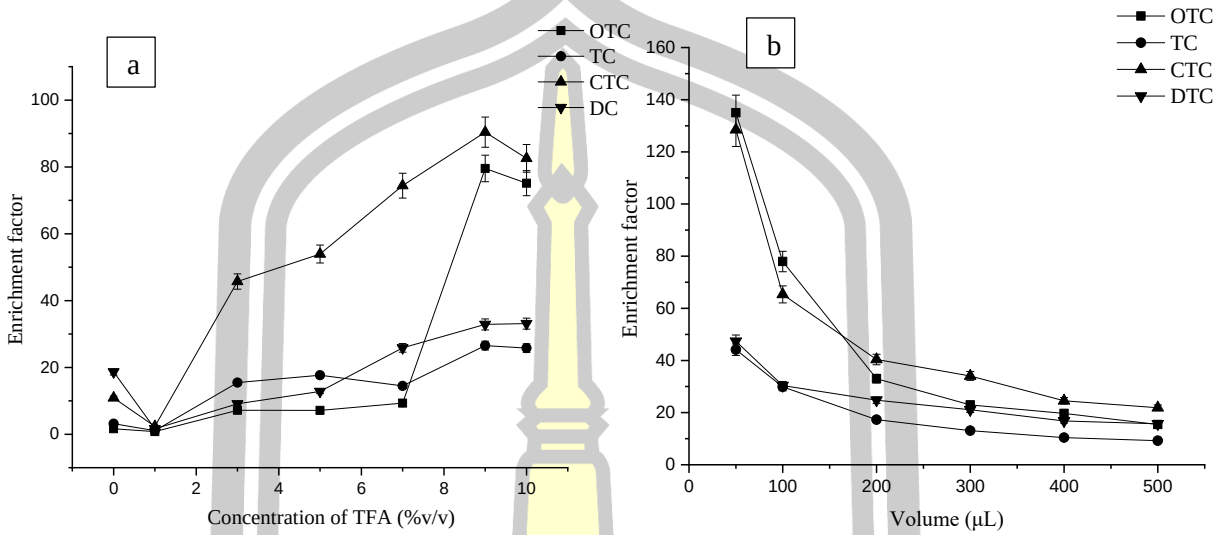

Figure 23 Effect of concentration of TFA (a) and volume of 9% TFA in ACN solvent for diluted SPR (b). Conditions: 0.05% w/v Triton X-114, 0.06 mmol L^{-1} tannic acid, 1.0 mg L^{-1} Fe^{3+}, 0.5 mol L^{-1} acetate buffer pH 5, vortex time 20 sec, incubation at 40 ºC for 10 min, centrifugation at 4500 rpm for 20 min, 1.0 µg mL^{-1} of each tetracyclines.

Table 6 The optimum conditions of *in-situ* dispersive micro solid phase extraction assisted cloud point extraction for the determination of tetracyclines by high performance liquid chromatography.

Parameters	Optimum conditions
Concentration of tannic acid	0.06 mmol L^{-1}
Concentration of Fe(III)	1.0 mg L^{-1}
pH of acetate buffer	0.5 mol L^{-1}, pH 5, 5 mL
Concentration of surfactants	0.05%w/v Triton X-114
Vortex time	20 sec
Incubation temperature and time	45 ºC, 10 min
Speed and time centrifugation	4500 rpm, 20 min
Concentration and volume of TFA	9%v/v TFA, 100 µL

4.4 Analytical performance and method validation

Under the selected and optimized conditions as summarized in Table 6, the analytical performance of the proposed method was evaluated in terms of linearity, limit of detection (LODs), limit of quantitation (LOQs), precision (intra-day, inter-day) and enrichment factor (EF). The chromatograms of TCs obtained from the proposed method and direct analysis are shown in Figure 24 while linearity range was summarized in Table 7. The linearity of TCs after preconcentration by the *in-situ* dispersive Fe-TA micro solids assisted cloud point extraction was observed in the range of 20-1000 μg L^{-1} for OTC, TC and 30-1000 μg L^{-1} for CTC, DC, with a coefficient of determination (r^2) ranging between 0.9939 and 0.9994. The obtained LODs were 5-7 μg L^{-1} and LOQs were 20-30 μg L^{-1}. EFs calculated as a slope ratio of the calibration curves with and without extraction were achieved between 33-90. The precisions expressed as a percentage of relative standard deviations (%RSDs, n=7) for the standard mixture at 30 μg L^{-1} in a day (intra-day) and three consecutive days (inter-day). The results indicated that proposed method had good precision with the RSDs less than 4.75% for peak area and 1.55% for retention time.

Table 7 Analytical performance of the proposed method for the determination of TCs.

Analyte	Linear range (μg L^{-1})	r^2	LOD (μg L^{-1})	LOQ (μg L^{-1})	EF	Precision (%RSD) 30 μg L^{-1}			
						Intra-day (n=7)		Inter-day (n = 5 × 3)	
						Peak area	t_R	Peak area	t_R
OTC	20-1000(150-1000) [a]	0.9994(0.9987)	5(50)	20(150)	71	2.54	0.35	1.19	0.81
TC	20-1000(100-1000)	0.9939(0.9981)	5(40)	20(100)	33	4.72	0.62	1.57	0.88
CTC	30-1000(200-1000)	0.9963(0.9986)	7(80)	30(200)	90	3.17	1.17	2.93	1.45
DC	30-1000(150-1000)	0.9956(0.9984)	7(60)	30(150)	48	4.75	1.31	1.30	1.55

( )[a] Value of standard without preconcentration (direct analysis)

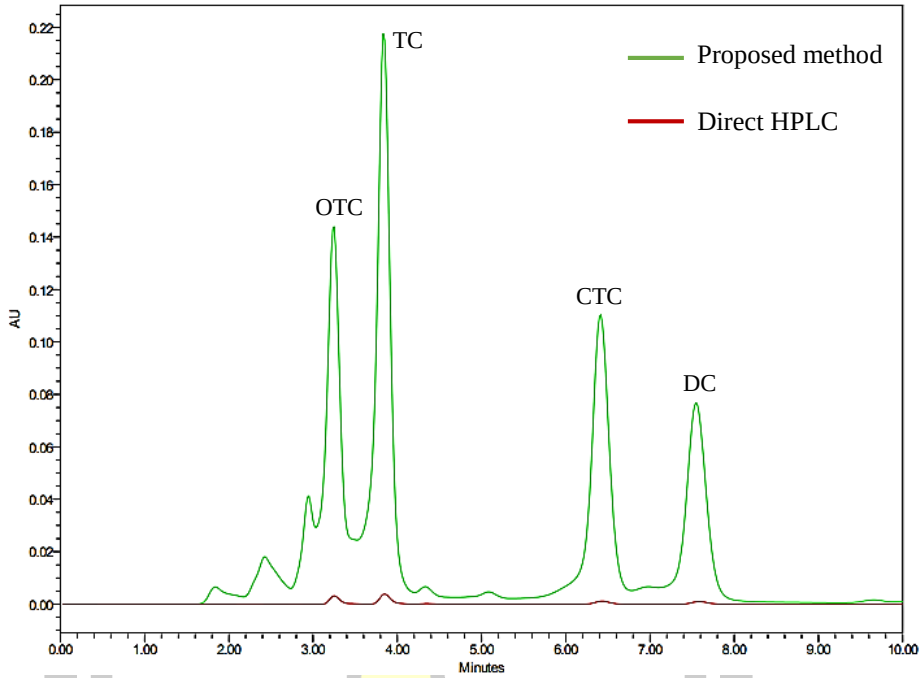

Figure 24 The overlaid chromatograms of the studied tetracyclines obtained from direct HPLC and preconcentrated by the proposed method (1.0 µg mL^{-1} of each tetracyclines).

4.5 Real samples analysis

The TCs residues were analyzed in various samples as the application of the developed method. The farm wastewater was collected from a pig farm, poultry farm and fish farm in Sisomdet District, Roi-Et Province, Thailand. Food samples, including whole milk, UHT milk, egg, honey, chicken liver and chicken kidney. All samples were purchased from supermarket in Kantharawichai District, Maha Sarakham Province, Thailand. Each sample was analyzed according to the sample preparation procedures described in Section 3.7 and analyzed by the developed method. In addition, the accuracy of the method, three different concentrations (30, 100 and 700 µg L^{-1}) of TCs were spiked to samples and calculated recovery values as shown in **Table 8**. Recovery were found in the range 79.91-108.51%, 75.25-112.50%, 71.53-115.87%, 76.32-115.59% and 71.13-117.09% for water, milk, egg, honey and chicken tissue respectively, that indicating this proposed method was provided good accuracy. Honey 1 sample was found to be contaminated with OTC at 530 µg L^{-1}. **Figure 25** shows typical chromatograms of the honey 1 sample (blank) and honey 1 sample spiked with TCs standard. These results indicated that the proposed method can detect TCs at concentrations of TCs less than MRLs in real samples.

Table 8 The determination of TCs and recovery in the studied water samples (n=3).

Analyte	Spiked (μg L^{-1})	Water 1			Water 2		
		Found (μg L^{-1})	Recovery (%)	%RSD	Found (μg L^{-1})	Recovery (%)	%RSD
OTC	0	ND	-	-	ND	-	-
	30	28.95	96.51	5.14	24.58	81.93	2.08
	100	92.76	92.76	0.13	81.81	81.81	1.84
	700	684.30	97.76	0.22	590.22	84.32	0.86
TC	0	ND	-	-	ND	-	-
	30	24.33	81.10	4.37	30.92	103.08	0.48
	100	92.76	92.76	0.13	98.45	98.45	4.33
	700	715.85	102.26	2.32	632.23	90.32	1.19
CTC	0	ND	-	-	ND	-	-
	30	24.25	80.85	3.38	24.06	80.20	0.64
	100	103.12	103.12	0.99	87.31	87.31	0.60
	700	693.12	99.02	1.60	665.34	95.05	0.71
DC	0	ND	-	-	ND	-	-
	30	28.56	88.68	1.79	30.46	101.55	6.32
	100	88.68	88.68	3.17	98.34	98.34	3.14
	700	699.03	99.86	0.48	642.68	91.81	1.21

ND: Not detected

Table 8 (continued)

Analyte	Spiked (μg L^{-1})	Water 3			Water 4		
		Found (μg L^{-1})	Recovery (%)	%RSD	Found (μg L^{-1})	Recovery (%)	%RSD
OTC	0	ND	-	-	ND	-	-
	30	27.09	90.32	2.25	23.97	79.91	1.11
	100	82.78	82.78	1.48	81.45	81.45	3.80
	700	591.16	84.45	1.24	585.58	83.65	0.72
TC	0	ND	-	-	ND	-	-
	30	27.95	93.16	1.72	29.21	97.38	2.28
	100	94.96	94.95	5.04	86.15	86.15	8.70
	700	753.42	107.63	0.61	590.49	84.36	2.86
CTC	0	ND	-	-	ND	-	-
	30	24.87	82.91	3.81	26.37	87.91	5.28
	100	86.61	86.61	1.24	90.00	90.00	0.74
	700	689.69	98.53	6.43	656.53	93.77	9.98
DC	0	ND	-	-	ND	-	-
	30	29.60	98.65	3.93	28.02	93.41	6.60
	100	108.51	108.51	1.69	88.66	88.66	1.35
	700	648.22	92.60	3.71	708.78	101.25	1.95

ND: Not detected

Table 8 (continued)

Analyte	Spiked (μg L^{-1})	Milk 1			Milk 2		
		Found (μg L^{-1})	Recovery (%)	%RSD	Found (μg L^{-1})	Recovery (%)	%RSD
OTC	0	ND	-	-	ND	-	-
	30	25.88	86.25	6.43	31.17	103.90	1.43
	100	80.86	80.86	1.66	83.37	83.37	0.71
	700	570.50	81.50	4.37	604.38	86.34	3.96
TC	0	ND	-	-	ND	-	-
	30	24.58	81.92	0.52	24.18	80.59	0.71
	100	101.80	101.80	4.50	94.80	94.80	0.76
	700	729.93	104.28	2.40	708.46	101.21	0.13
CTC	0	ND	-	-	ND	-	-
	30	22.58	75.25	1.48	23.80	79.32	1.78
	100	112.50	112.50	2.59	109.28	109.28	0.96
	700	588.41	84.06	3.47	633.15	90.45	0.53
DC	0	ND	-	-	ND	-	-
	30	29.69	98.95	2.16	32.06	106.88	2.02
	100	83.84	83.84	2.18	94.54	99.54	1.22
	700	592.56	84.65	1.64	647.44	92.49	1.32

ND: Not detected

Table 8 (continued)

Analyte	Spiked (μg L^{-1})	Egg 1			Egg 2		
		Found (μg L^{-1})	Recovery (%)	%RSD	Found (μg L^{-1})	Recovery (%)	%RSD
OTC	0	ND	-	-	ND	-	-
	30	25.86	86.20	4.61	26.50	88.33	2.68
	100	84.42	84.42	0.23	78.96	78.95	2.28
	700	711.11	101.59	0.97	579.93	82.84	0.45
TC	0	ND	-	-	ND	-	-
	30	30.10	100.34	6.89	30.97	103.22	1.22
	100	94.04	94.04	3.63	96.76	96.76	7.62
	700	640.94	91.56	4.70	679.22	97.03	2.63
CTC	0	ND	-	-	ND	-	-
	30	21.46	71.53	4.11	28.72	95.74	3.78
	100	86.94	86.94	2.25	86.94	86.94	1.10
	700	700.01	100.00	0.89	594.09	84.87	1.75
DC	0	ND	-	-	ND	-	-
	30	33.27	110.91	3.54	34.76	115.87	3.29
	100	105.17	105.17	0.65	100.91	100.91	1.72
	700	702.00	100.29	0.53	679.70	97.10	0.10

ND: Not detected

Table 8 (continued)

Analyte	Spiked (μg L^{-1})	Honey 1			Honey 2		
		Found (μg L^{-1})	Recovery (%)	%RSD	Found (μg L^{-1})	Recovery (%)	%RSD
OTC	0	10.60	-	6.43	ND	-	-
	30	35.29	82.28	5.17	22.90	76.32	6.14
	100	109.47	98.86	1.32	83.71	83.71	2.04
	700	643.64	90.43	1.62	573.52	81.93	0.92
TC	0	ND	-	-	ND	-	-
	30	23.38	77.93	1.94	27.73	92.42	2.02
	100	100.36	100.36	0.35	115.47	115.47	1.34
	700	756.81	108.12	2.07	648.70	92.67	7.63
CTC	0	ND	-	-	ND	-	-
	30	27.73	92.42	5.57	25.52	85.06	3.59
	100	91.24	91.24	8.28	104.85	104.85	1.83
	700	638.25	91.18	6.20	680.40	97.20	8.52
DC	0	ND	-	-	ND	-	-
	30	26.82	89.41	1.84	32.57	108.56	4.05
	100	92.54	92.54	6.68	115.59	115.59	3.37
	700	624.89	89.27	3.47	670.88	95.84	2.50

ND: Not detected

Table 8 (continued)

Analyte	Spiked (μg L^{-1})	Chicken liver			Chicken kidney		
		Found (μg L^{-1})	Recovery (%)	%RSD	Found (μg L^{-1})	Recovery (%)	%RSD
OTC	0	ND	-	-	ND	-	-
	30	21.34	71.13	9.32	24.72	82.41	1.62
	100	83.64	83.64	3.04	102.72	102.72	1.60
	700	582.91	83.27	3.46	737.37	105.34	0.33
TC	0	ND	-	-	ND	-	-
	30	30.63	102.30	4.04	31.58	105.26	2.76
	100	87.12	87.12	4.60	104.80	104.78	7.48
	700	565.38	80.77	3.20	568.95	81.28	3.71
CTC	0	ND	-	-	ND	-	-
	30	20.94	69.82	6.42	25.92	86.41	6.76
	100	77.57	77.57	2.33	117.09	117.09	1.11
	700	567.79	81.11	0.97	565.78	80.82	1.56
DC	0	ND	-	-	ND	-	-
	30	29.87	99.56	4.87	23.21	77.36	4.56
	100	110.93	110.93	3.22	88.50	88.50	0.44
	700	636.48	90.93	1.44	625.94	89.42	1.30

ND: Not detected

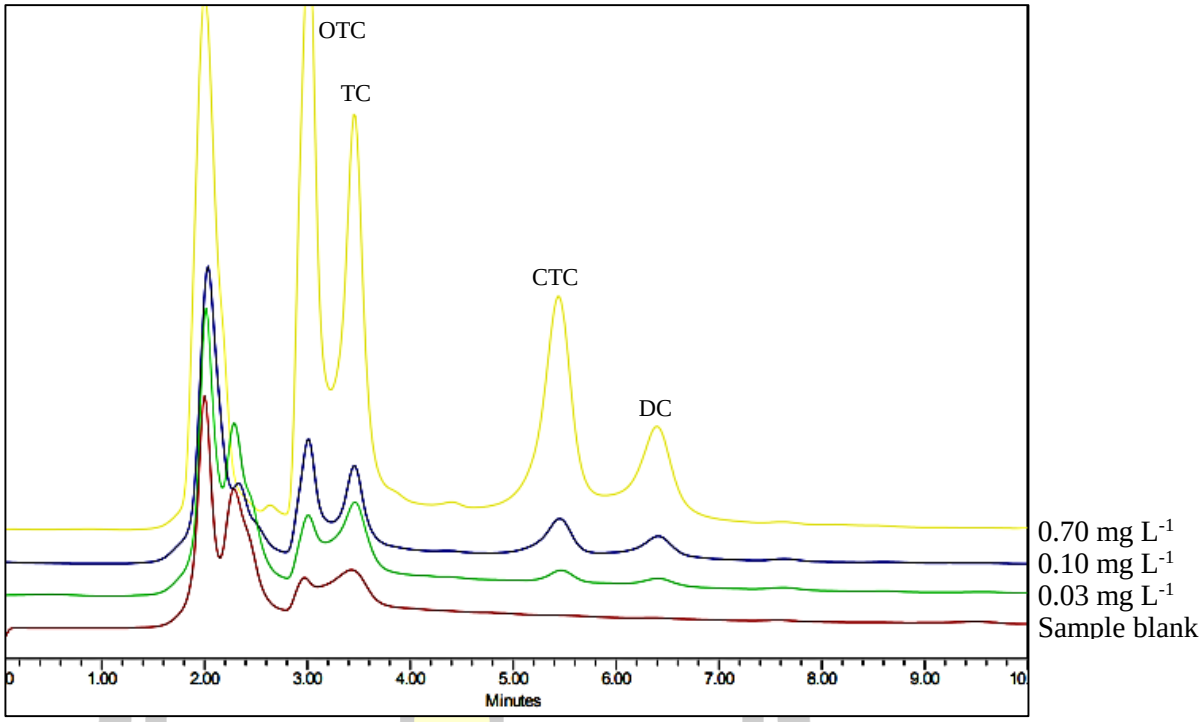

Figure 25 The overlaid chromatograms of sample blank and spiked honey 1 sample.

4.6 Comparison of proposed method with the other previous reports

The performance of the proposed method for the determination of TCs compared with the other reported methods shown in Table 9. As can be seen, The RSD% values of the proposed method are better than or comparable with those of the other methods. The LOD and LOQ for the proposed method are much better than other procedures. The enrichment factor in this study is better than with the previously published methods. Also, the proposed method was better than other methods in terms of sensitivity and simplicity since it avoided the synthesis step of solid sorbents, inevitably needed in conventional SPEs.

Table 9 Comparison of the proposed method with the other SPE extraction methods for the determination of TCs.

Extraction method	Sorbent material	Sample	LOD (μgL^{-1})	LOQ (μgL^{-1})	Recovery (%)	RSD (%)	EF	Analytical technique	Ref.
MSPE	GO-PET	Honey	15.30	-	89.00-94.00	4.90	-	HPLC	[79]
SPE	MIPs	Honey	16.10	-	62.60-82.30	4.50	-	HPLC-DAD	[80]
on-line SPE	zeolite imidazolate framework-8	Water, milk	1.50-8.00	5.00-26.70	70.30-107.40	≤3.60	35-61	HPLC	[81]
on-line SPE	Oasis HLB	Honey	5.00-12.00	17.00-40.00	84.20-120.60	≤8.90	-	HPLC-UV	[82]
in situ-DMSPE assisted CPE	Fe-TA micro solids	Wastewater, milk, egg, honey, chicken tissue	5.00-7.00	10.00	71.13-117.09	≤4.75	33-90	HPLC-UV	This work

CHAPTER V

CONCLUSION

In this work, a one-step *in-situ* dispersive micro solid phase extraction assisted cloud point extraction has been developed as an alternative preconcentration method for the analysis of TCs. Iron-mediated self-assembly of Fe-TA micro solids was utilized as sorbent and was created by *in-situ* processes in order to reduce time in preparation of sorbent. Furthermore, an *in-situ* dispersive micro solid phase extraction coupled with cloud point extraction was provided good extraction efficiency of TCs. For *in-situ* dispersive micro solid phase extraction assisted cloud point extraction, the optimal conditions were 0.05% w/v Triton X-114, 0.06 mmol L^{-1} tannic acid, 1.0 mg L^{-1} Fe^{3+}, 0.5 mol L^{-1} acetate buffer pH 5, vortex time 20 sec, incubation at 40 ºC for 10 min, centrifugation at 4500 rpm for 20 min. The extraction was then analyzed by Purospher® STAR RP-18 endcapped column (4.6 × 150 mm, 5.0 µm) (Merck, Germany) was used as an analytical column carried out at room temperature. The injection volume was 20 µL. Breeze software was utilized for data acquisition. These TCs was separated using isocratic elution using a mixture of 0.1%TFA in ACN and 0.1%TFA at ratio of 30/70 (v/v), at a flow rate of 0.8 mL min^{-1}. Under the optimum conditions, low limits of detection (LODs) were 5-7 µg L^{-1} for all target analytes which below the acceptable maximum residue limits (MRLs) for tetracyclines residues. The method was successfully applied for the determination of TCs residues in various samples including waste water, milk, egg, honey, chicken liver and chicken kidney. The proposed method was gave wide linear range, simple, rapid, good sensitivity, and high accuracy with good repeatability.

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