Development of Dispersive Solid Phase Extraction Using Green Natural Sorbent for Enrichment of Antibiotics Prior to High Performance Liquid Chromatography Analysis

Nongnapas Nakhonchai

A Thesis Submitted in Partial Fulfillment of Requirements for
degree of Master of Science in Chemistry
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May 2024

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ABSTRACT

This study used chia seed mucilage as a biosorbent with modified the surface area by iron(III)-natural reagent particles for dispersive solid phase extraction of tetracycline prior to HPLV-UV. Four tetracyclines (TCs), including oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DC), were used with the optimal parameters for all experiments. The linear range was 2−500 µg L^{-1}, with a coefficient larger than 0.9957. The limits of detection (LODs) and quantification (LOQs) were 0.7−2.0 µg L^{-1} and 2.0−7.0 µg L^{-1}, respectively. Precisions were shown in the form of relative standard deviation (RSD%) values of less than 9.1% and 6.9% for intra-day and inter-day. For the first time, alternative biosorbents was successfully quantified TCs residues in milk samples, with recoveries ranging from 80.2 to 109.4%. The analytical performance of enrichment factors (EFs) was satisfactory in the 55-105 range. Furthermore, the selected biosorbent materials and the proposed method were praised for their natural mucilage and reagent, eco-friendly biopolymer, biodegradability, nontoxicity, cheap cost, lower solvent consumption, and reduced extraction procedure.

Keyword : Biosorbent, Chia seed, Tetracycline, Dispersive solid phase extraction, Food samples, Natural reagent

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Nongnapas Nakhonchai

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CHAPTER I
INTRODUCTION

1.1 Problems and provenance

Antibiotics treat infections in various human systems, including the respiratory, digestive, urinary, and skin. Antibiotics should be used effectively by consumers to gain high benefits for patients and avoid difficulties in the future such as drug use inappropriate for disease, drug resistance, antibiotic allergy, and adverse effects from obtaining antibiotics [1].

Tetracyclines (TCs) are one of the most commonly used antibiotics to treat bacterial infections in humans, animals, and agricultural settings [2]. The structure of TCs consists of four aromatic rings and an additional function group that can form chelate complexes with a variety of metal ions. Furthermore, the most commonly used antibiotics include tetracycline (TC), chlortetracycline (CTC), doxycycline (DC), and oxytetracycline (OTC) [3]. However, these antibiotics have created a concern among consumers about infections because they can be found in settings and foods such as animal milk and meat products, which can be directly poisonous and cause allergies in some sensitive patients. Furthermore, trace levels of antibiotics in food may influence microbial dispersion, which might lead to drug resistance if consumed for an extended period of time [4].

As a result, detecting residual antibiotics in animal-derived foods is important. Gas chromatography (GC) [5] and high performance liquid chromatography (HPLC) [6], and other analytical methods are commonly used to determine TCs. Nonetheless, antibiotic residues in animal-source foods have been detected at trace levels; thus, the sample preparation step is critical. The methods commonly utilized include liquid-liquid extraction (LLE) [7], solid phase extraction (SPE) [8], solid phase microextraction (SPME) [9], and dispersive solid phase extraction (DSPE) [10]. Among the methods stated earlier, DSPE can inject adsorbent directly into the sample solution, followed by sorbent dispersion to ensure interaction between the adsorbent and analyte. Biosorption is an attractive technology for problem solving, detoxification, and decontamination because it is both economical and environmentally beneficial. Adsorption, absorption, complexation, precipitation, and ion exchange are some of the processes involved in biosorption-based biosorbents. These processes occur spontaneously as a result of physical and chemical interactions between the analyte and the active surface area of the sorbent. The development of residual or natural materials to be employed as antibiotic adsorbents should be cost-effective and widely applicable [11].

Therefore, dispersive solid phase extraction using green natural sorbent was created for TC extraction and enrichment, followed by measurement via high performance liquid chromatography. Furthermore, we can enhance the extraction efficiency by appropriately modifying the natural adsorbent surface and/or synergizing with other extraction methods. The proposed approach is simple, low-cost, versatile, environmentally friendly, has a high extraction efficiency and enrichment factor, and is an alternative for determining TCs.

1.2 Objectives

1.2.1) To develop a dispersive solid phase extraction method-based natural adsorbent for the preconcentration of four tetracyclines, including tetracycline (TC), chlortetracycline (CTC), doxycycline (DC), and oxytetracycline (TC)

1.2.2) To apply the established approach to determining low TC concentrations in real samples

1.3 Scope of this work

1.3.1) Optimal chromatographic separation parameters for determining four tetracyclines using high performance liquid chromatography, including mobile phase composition and flow rate

1.3.2) Establish the optimum conditions for dispersive solid phase extraction to change the natural adsorbent between green natural reagent and iron (III), including iron (III) concentration, buffer concentration, pH, source, and amount of natural reagent.

1.3.3) Investigate parameters effecting dispersive solid phase extraction, including natural adsorbent amount, temperature, incubation time, vortex time, centrifugation speed, and concentration of trifluoroacetic acid (TFA) in acetonitrile (ACN)

1.3.4) Analytical performance and technique validation are investigated to determine linearity, limit of detection (LOD), limit of quantification (LOQ), precision (intraday, inter-day), accuracy, matrix effect, and enrichment factor (EF)

1.3.5) Investigate dispersive solid phase extraction with a natural adsorbent to detect tetracyclines and apply it to real samples

1.4 Benefit of research

The new approach, based on dispersive solid phase extraction with green natural sorbent, can detect trace levels of tetracycline residues in animal source food with excellent accuracy, precision, rapidity, simplicity, and low cost.

1.5 Venue of the study

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

1.5.2) Scientific Instrument Science Unit at Mahasarakham University

CHAPTER II
LITERATURES REVIEW

2.1 History of tetracyclines

Toby Hockett, a five-year-old kid, was treated with the yellow-colored substance known as "Aureomycin" delivered by the Lederle Laboratories Division of American Cyanamid in early 1948, and he was one of the first of many people to live safely as a result of aureomycin. Subsequently, the chemical technique of antibiotics was improved to produce a compound with superior performance, a better solubility profile, and good pharmacological activity, which was later dubbed "Tetracycline" [12]. Tetracyclines, natural compounds, were the most common products of *Streptomyces aureofaciens*, together with other tetracyclines such as oxytetracycline from *S. rimosus* and chlortetracycline from *S. aureofaciens* [13]. The most recently found tetracyclines are semisynthetic compounds known as glycylcyclines, which include doxycycline, lymecycline, methacycline, minocycline, and rolitetracycline [14]. The tetracycline generations are separated as follows: The first generation of antibiotics, including chlortetracycline, oxytetracycline, tetracycline, and demethylchlortetracycline, were recovered from a Missouri soil sample in the late 1940s. The second generation, which included rolitetracycline, lymecycline, doxycycline, and minocycline, was a modification of the oxytetracycline molecule. Tigecycline is a third-generation derivative example of minocycline [15]. Tetracyclines are utilized as antibiotics in animal husbandry and disease treatment because they have broad antibacterial activity, are active against both gram-positive and gram-negative bacteria, and have low toxicity [16]. Furthermore, tetracyclines are used to treat a variety of bacterial infections in the urinary and respiratory tracts [17].

2.1.1) Applications of tetracyclines in veterinary medicine

Veterinary medicine widely utilizes tetracyclines for therapeutic purposes due to their regular production, affordability, and widespread use worldwide. Tetracycline, oxytetracycline, chlortetracycline, and doxycycline are the most common tetracyclines used to treat food-producing animals. People used tetracyclines to treat infectious disorders like respiratory sickness in sheep, pigs, chickens, and cattle [18]. Tetracycline inhibits protein synthesis by a variety of mechanisms, including changes to the tetracycline ribosomal target, interference with the efflux pump, and tetracycline resistance in *Campylobacter* at the 30S subunit of ribosomes [19]. Overuse of tetracycline antibiotics leads to tetracycline resistance in bacteria in food and animal products, which spreads to human health. Consumer health risks include allergies, staining of the teeth, liver damage, and so on. Foods consumed orally in daily life, such as eggs, milk, meats, poultry, honey, and shellfish, may leave residues in the human body since animals excrete 17–90% of ingested antibiotics via urine and feces [20].

Therefore, we should control the presence of tetracycline antibiotics in the food sector to ensure consumer safety. Table 1 shows that the relevant government agency, Codex Alimentarius, determined maximum residue limits (MRLs) for veterinary medications, including those from the European Union (EU), Canada, China, and Brazil's Ministry of Agriculture.

Table 1 Maximum residue limits of tetracyclines for food stuffs in animal.

Agency	**Animal group**	**Food stuff**	**MRL (µg/kg)**	**Ref.**
Codex Alimentarius	Cattle	Muscle	200	[21]
		Liver	600	
		Milk (µg/L)	100	
	Fish	Muscle	200	
	Swine	Muscle	200	
		Liver	600	
	Poultry	Muscle	200	
		Liver	600	
		Eggs	400	
European Union	Cattle and Poultry	Muscle	100	[22]
		Liver	300	
		Milk	100	
		Eggs	200	
Canada	Cattle	Muscle	200	[23]
		Liver	600	
		Milk	100	
	Swine	Muscle	200	
		Liver	600	
	Poultry	Muscle	200	
		Liver	600	
		Eggs	400	

[a] Chlortetracycline, [b] Tetracycline, [c] Oxytetracycline and [d] Doxycycline

Table 1 (continued)

Agency	Animal group	Food stuff	MRL (µg/kg)	Ref.
China	Cattle	Muscle	200	[24]
		Liver	600	
		Milk	100	
	Fish	Muscle	200	
	Swine	Muscle	200	
		Liver	600	
	Poultry	Muscle	200	
		Liver	600	
		Eggs	400	
Brazil	Cattle, Swine and Poultry	Muscle	Sum equals 100 (for CTC[a], TC[b] and OTC[c]) and 100 for DC[d]	[25]
	Cattle	Milk	100	

[a] Chlortetracycline, [b] Tetracycline, [c] Oxytetracycline and [d] Doxycycline

2.1.2) Chemical structures of tetracyclines

Tetracyclines are composed of four linearly annulated six-membered rings, known as octahydronaphtacene or tetracene (naphthacene). The carbon 12a (sp^3) separates two unique chromophoric areas, the A ring and the BCD ring, which are partially saturated. The D ring is always aromatic, and when proper substitutions are made in carbon atom regions 4, 4a, 5, 5a, 6, and 12a, the ring becomes asymmetric [15]. The chemical fundamental structure of tetracyclines is given in Figure 1.

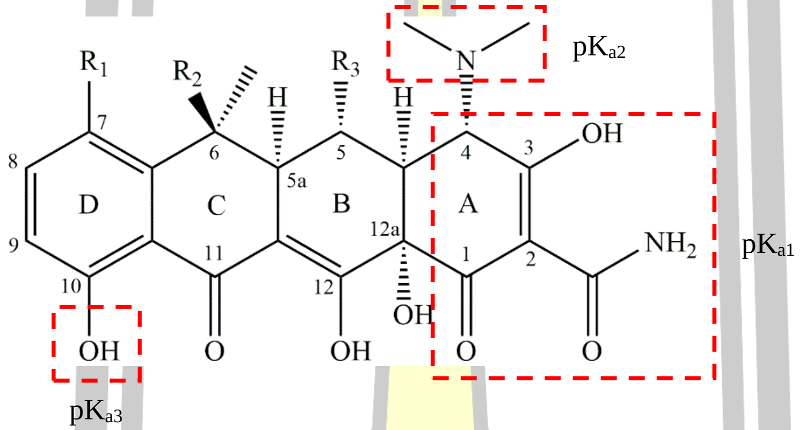

Figure 1 Chemical basic structure of tetracyclines [15].

Tetracyclines exhibit amphoteric activity via acid-base equilibria and function as chelating agents. Table 2 displays the three primary pK_a values of tetracyclines: a pH value less than 3 indicates a positive charge, a pH value between pK_{a1} and pK_{a2} indicates a neutral (zwitterionic state), and a pH value greater than 8 indicates a negative charge [26] as shown in Table 2. However, due to their widespread use in veterinary medicine, we selected antibiotics like oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DC) for analysis in this study.

Table 2 Chemical and physical properties of tetracyclines [26].

Compound	Systematic name	R_1	R_2	R_3	Molecular mass (g/mol)	Polarity (Log P)	Acidity (pK_a)
Oxytetracycline (OTC)	5-hydroxy-tetracycline	H	OH	OH	460.4	-3.6	pK_{a1} 3.2 pK_{a2} 7.5 pK_{a3} 8.9
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	H	OH	H	444.4	-1.3	pK_{a1} 3.3 pK_{a2} 7.8 pK_{a3} 9.6
Chlortetracycline (CTC)	7-chloro-tetracycline	Cl	OH	H	478.8	-0.62	pK_{a1} 3.3 pK_{a2} 7.6 pK_{a3} 9.3
Doxycycline (DC)	6-deoxy-5-hydroxy-tetracycline	H	H	OH	444.4	-0.19	pK_{a1} 3.0 pK_{a2} 7.9 pK_{a3} 9.2

2.2 Green analytical chemistry

Nowadays, all sciences, including chemistry, are developing novel approaches for determining lower or trace concentration levels of analytes, separating more complicated mixtures to achieve high precision and accuracy, and meeting the demands for smaller material volumes, simplicity, and speedy analysis. Analytical chemistry is currently sought to limit the negative impact of novel-generated methods and ensure environmental safety [27]. Paul Anastas, who was distinguished in his publications on green chemistry, proposed this concept known as "green chemistry (GC)" [28]. Anastas and Warner later published "Green Analytical Chemistry (GAC)".

The frequent use of this topic in both academic and applied laboratories has sparked interest [29]. GAC emerged at the end of the twentieth century, with Brazil, China, Spain, Poland, and the United States making the most contributions from 1994 to the present through journals such as Analytica Chimica Acta, Analytical Methods, Analytical and Bioanalytical Chemistry, Journal of Chromatography, Microchemical Journal, Talanta, and Trends in Analytical Chemistry. These journals, the most popular for publication, aim to replace toxic substances with non-toxic materials or less harmful products for analysis, thereby promoting method operator safety and minimizing environmental damage [30].

Finally, Anastas and Warner designed the "Twelve Principles of Green Chemistry" to guide sustainable chemistry. Gałuszka, Migaszewski, and Namieśnik's 2013 principles require real-time analysis to prevent pollution as they connect the GC and GAC concepts. Table 3 shows the principles of green chemistry and green analytical chemistry [31].

Table 3 Principles of green chemistry and green analytical chemistry [31].

Green chemistry	Green analytical chemistry
1. Prevent waste	**S**elect direct analytical techniques
2. Design safer chemicals and products	**I**ntegrate analytical processes and operations
3. Design less hazardous chemical syntheses	**G**enerate as little waste as possible and treat it properly
4. Use renewable feedstocks	**N**ever waste energy
5. Use catalyst not stoichiometric reagents	**I**mplement automation and miniaturization of methods
6. Avoid chemical derivatizations	**F**avour reagents obtained from renewable sources
7. Maximize atom economy	**I**ncrease safety of operator
8. Use safer solvents and reaction conditions	**C**arry out in situ measurements
9. Increase energy efficiency	**A**void derivatizations
10. Design chemicals and products to degrade after use	**N**ote that sample number and size should be minimal
11. Analyze in real time to prevent pollution	**C**hoose multi-analyte or multi-parameter methods
12. Minimize the potential for accidents	**E**liminate or replace toxic reagents

According to Table 3, GAC has a "SIGNIFICANCE" that affects sample preparation and extraction methods in analytical chemistry. This trend is still growing and presents a fresh research challenge. The present study employs the principles of gas chromatography and gas analytical chromatography (GC and GAC) to address the following topics: 1) waste prevention; 2) utilization of renewable feedstocks; 3) adoption of safer solvents and reaction conditions; 4) design of chemicals and 5) products to degrade post-use; real-time analysis to prevent pollution and minimize the risk of accidents for GC; integration of analytical processes and operations; preference for reagents obtained from renewable sources; enhancement of operator safety; and, finally, note that sample number and size should be kept to a minimum for GAC.

2.3 Natural phenolic compounds

Phenolic compounds are tiny molecular groups that occur abundantly as secondary metabolites in plants [32]. They are distinguished by their chemical structure, which includes an aromatic ring and at least one or more hydroxyl substituents, and can be classified as phenolic acids, coumarins, curcuminoids, flavonoids, lignans, quinones, stilbenes, and tannins. In the plant world, most phenolic chemicals exist in soluble or bound forms. The internal endoplasmic reticulum of plants produces them and stores them in vacuoles [33].

Tannin can be extracted from a variety of plant parts, including bark, leaves, pods, roots, wood, fruits, plant galls, and fruit, as well as from different plant species such as oak (*Quercus* sp.), eucalyptus (*Eucalyptus* sp.), and willow (*Salix caprea*). Tannins, or polyphenol compounds, are typically classified into two types: hydrolysable tannins (HTs) and condensed tannins (CTs). Hydrolysable tannins can be divided into gallotannins and ellagitannins, which are derived from gallic acid or hexahydroxydiphenic acid esters linked to a sugar group, respectively. Condensed tannins are polymers composed of three-ring flavanols linked by C-C linkages [34–36]. Figure 2 depicts the structures of hydrolysable and condensed tannins.

Tannins have an important function in daily life. People used them to treat diarrhea, skin burns, and rectal issues. Today, they serve as dyes and reagents in the production of rubber, inks, imitation horns, and tortoise shells. Furthermore, they were combined with beer and wine to clarify for photography, medicine preparation for pharmaceuticals, and chemical reagents in analytical laboratories [37–38].

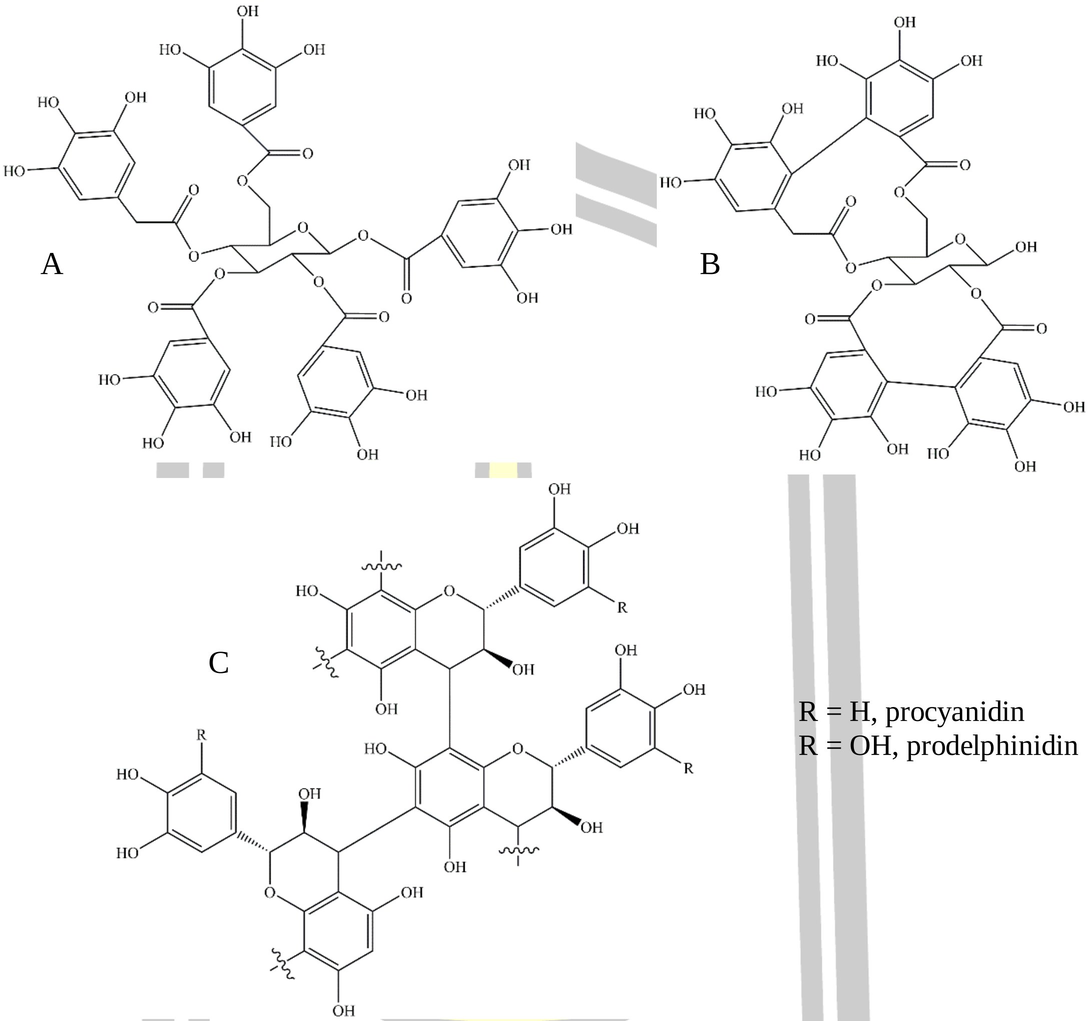

Figure 2 The structure of hydrolysable tannins with A) simple gallotannins, B) simple ellagitannins and C) condensed tannins trimer [35].

2.3.1) *Peltophorum pterocarpum* (DC.) Backer ex K.Heyne

Peltophorum pterocarpum is a well-known golden flamboyant, yellow poinciana, golden flamboyant, and copperpod tree that may be found and widely dispersed throughout the world, particularly in tropical Southeastern Asia, such as Indonesia, Malaysia, Thailand, Vietnam, and Sri Lanka. *Peltophorum pterocarpum* (PP) belongs to the native Fabaceae family, specifically the Caesalpinioideae subfamily. PP is a deciduous tree that can grow to be 15–25 meters tall and has a trunk diameter of up to 1 meter. PP is a pair of leaves 30–60 cm long with 16–20 petioles, each of which has 20–40 tiny leaves 8–25 mm long and 4–10 mm wide. The PP blooms are yellow, 2.5–4 cm in diameter, and grow in huge racemes up to 20 cm long. The PP fruit is a red pod 5–10 cm long and 2.5 cm wide that ripens to black and contains four seeds [39–40].

The most frequent type of PP is ornamental, and different sections of the PP tree are employed in a range of applications. The bark is used to treat diarrhea, tooth powders, muscle problems and sores, gargles, and eye lotion. The leaves are used to treat skin diseases through decoction. The flowers are used to make an astringent to treat or reduce intestinal diseases caused by childbirth pain, as well as lotion for eye problems, sprains, bruises, muscle pains, sores, and swellings. Furthermore, floral extract is utilized to produce good sleep and treat insomnia [41–42].

The PP tree is well-known for its abundance of biomolecules, which include antioxidants such as phenolic acids, flavonoids, ascorbic acid, quinines, and tannins [43]. Plant parts that contain antioxidants include bark, wood, stems, leaves, pods, and flowers [44]. Antioxidants can minimize and prevent oxidation in the human body, including the harmful consequences of free radicals, cancer, arteriosclerosis, and heart disease [45]. So, this study needs antioxidants from the PP tree to make biosorbent. It looks at different parts of the PP tree that are high in tannins, which are natural antioxidants. Tannins have a polydentate ligand that can bind to different metal ions for biosorption modification.

2.4 Adsorbents

An adsorbent is a solid substance used to bind atoms, ions, or molecules from a gas, liquid, or dissolved solid to a surface [46]. Adsorption is defined as the physical adherence of ions and molecules onto the surfaces of other molecular substances [47]. Adsorption procedures might be physical, chemical, or a combination of the two. Physical adsorption is exothermic and reversible, resulting from van der Waals and Coulomb interactions that cause attraction between the adsorbent and target molecules [48]. Chemical adsorption is endothermic and irreversible (thermodynamics), resulting from chemical bonds between the adsorbent surface and target molecules [49–50]. Adsorption is widely recognized as one of the most efficient methods for removing contaminants from wastewater or determining residues in food and drinking water, such as metal ions, azo dyes, pesticides, antibiotics, and organic substances. The ideal adsorbent should have a large surface area, a small quantity, high porosity, a low pore diameter, strong thermal and chemical strength, and great mechanical stability to result in high adsorption ability [51–53].

Adsorbents are typically utilized in the form of spherical pellets, rods, moldings, or monoliths, depending on the user's preference [51, 54]. Adsorbents are commonly categorized into five categories, as follows: 1) Natural materials. 2) Biosorbents. 3) Agricultural solid waste and manufacturing byproducts 4) Synthesized chemicals, and 5) Modified organic resources to improve their qualities and structure. Furthermore, adsorbents are classified as conventional or non-conventional adsorbents. Traditional adsorbents (ACs) include ion-exchange resins and inorganic compounds such as silica gel, alumina, and zeolite. Conventional adsorbents include ACs, zeolites, silica gel, and activated alumina, whereas non-conventional adsorbents originate from agricultural waste, organic resources, and manufacturing processes. Figure 3 depicts conventional and nonconventional adsorbents [54].

Nowadays, general adsorbents have negative environmental impacts and are expensive for material treatments [11]. These problems can be addressed using biological methods that are environmentally friendly, cost-effective, and have fewer negative consequences. The adsorbent preparation with a low usage cost while maintaining good performance is important in analysis [55]. Figure 4 presents a summary of adsorbent types, precursors, and processing procedures. In this study, biosorbents derived from nature are remarkable in terms of usage cost, environmental friendliness, and biodegradability [56].

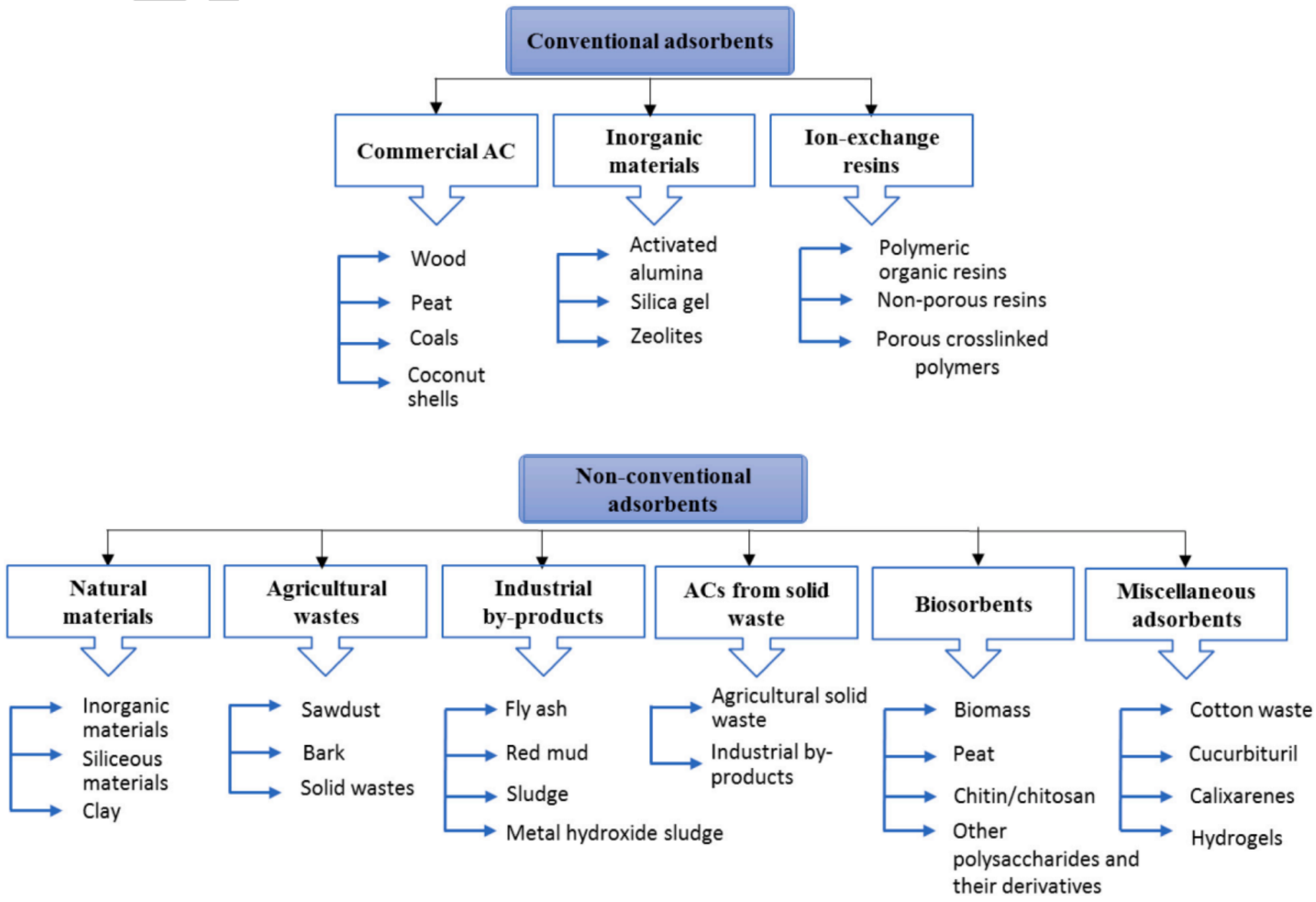

Figure 3 Conventional and non-conventional adsorbents [54].

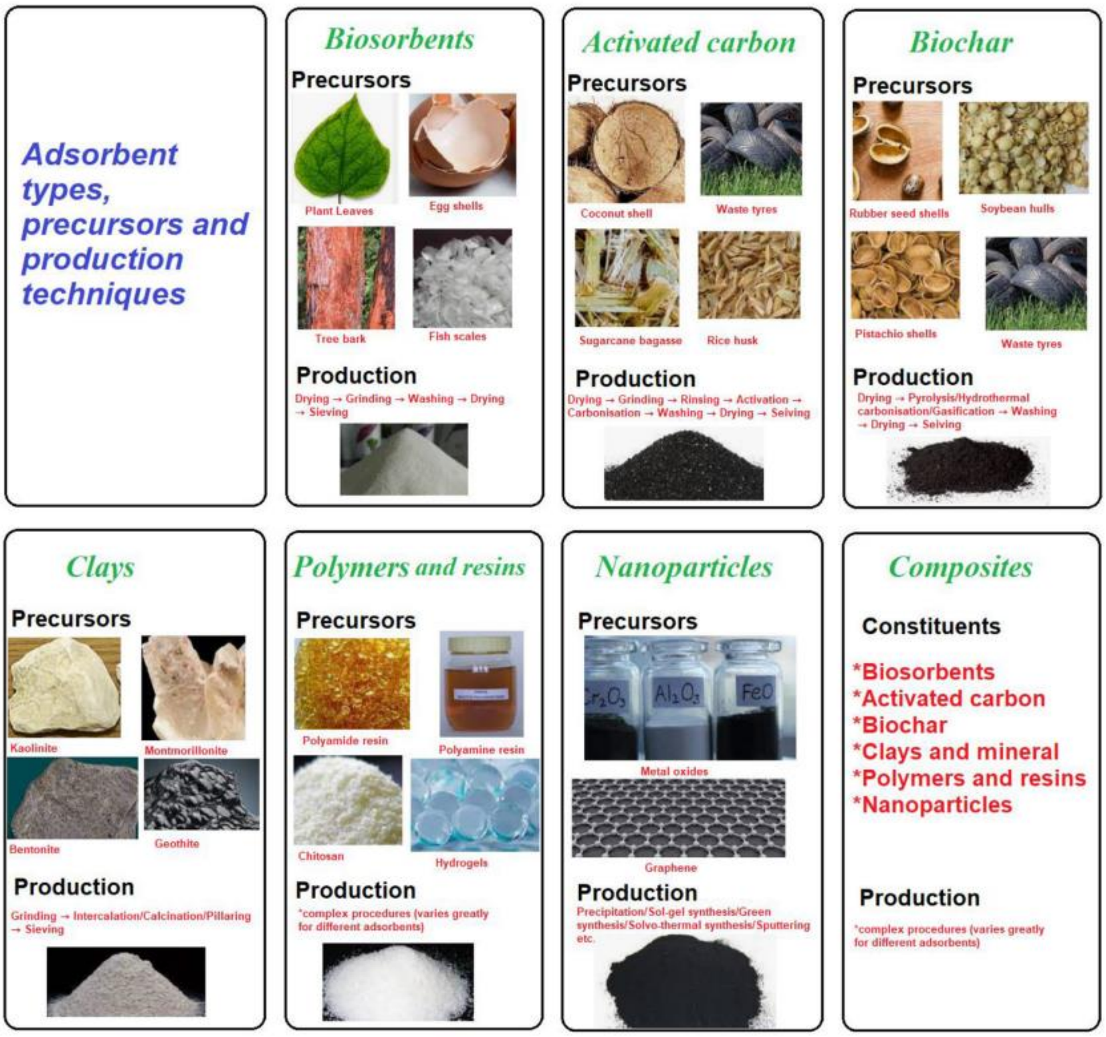

Figure 4 Summary of adsorbent types, precursors and processing techniques [56].

2.4.1) Biosorbents

Biosorbents are the most commonly used in green approach development because they are both environmentally benign and cost-effective [57–58]. Adsorption of a target molecule occurs as a result of physical and chemical interactions between the target molecule and the targeted biological material [59]. The development of low-cost waste materials is critical for selecting materials that have good affinity and can remove contaminants, antibiotics, or target compounds. The concepts of material selection as biosorbents are inexpensive, widely available, and have good adsorption [58].

Many categories classify biosorbents, such as natural biosorbents, microbial biomass, agricultural waste, industrial waste materials, microorganisms, and nanoparticles [11]. This research uses natural biosorbents and nanoparticles as sorbents.

2.4.1.1) Natural biosorbents

Natural materials include clays, chitosan, zeolites, industrial waste such as bagasse and ashes, as well as agricultural biomass such as seeds, barks, peels, and straw. These materials are utilized to adsorb pharmaceutical chemicals, heavy metals, industrial effluent dyes, and other organic contaminants [60–61]. Polymers derived from natural materials, such as polynucleotides, polysaccharides, and polypeptides, are extensively utilized as adsorbents for extraction and sample preparation in analytical chemistry [62]. Cellulose is one of the most widespread polysaccharides found in natural biopolymers and the main ingredient of plant cell walls. Cellulose is formed from β-d-glucopyranose units through β-1,4-glycosidic connections, resulting in a long-chain polysaccharide. These monomers consist mainly of methoxy and hydroxyl groups, which contribute to adsorption performance. On the outside of the polymer chain, there are several hydroxyl groups that work with the hydrophilic part of cellulose to change the surface by reacting with different chemical groups like amine, amidoxime, carboxyl, and nitrile [63]. However, natural materials are employed for analytical chemistry in the following ways: a sorbent for disk-SPE, cartridge SPE (c-SPE), dispersive SPE (d-SPE), Thin-Film Solid Phase Microextraction (TF-SPME), molecularly imprinted polymer SPE (MIP-SPE), and magnetic SPE [64–66].

2.4.1.2) Nanoparticles

Nanoparticles (NPs) are a type of adsorbent that is frequently studied, with diameters ranging from 1 to 100 nm. NPs have unique features and are widely used in a variety of commercial and domestic applications, including catalysis, medicine, agriculture, and engineering [66–69]. Clay, plants, metals, agricultural and industrial products, and chemicals are the raw materials used to manufacture nanomaterials [70–72]. NPs are now widely used in research, medicine, engineering, and agriculture due to their unique surface chemistry, electrical, and optical properties, which give high sensitivity, lower detection limits, and faster response times [73–75]. Green NP synthesis is interesting since it involves extracting stable NPs from plant components such as seed, bark, leaves, fungus, microbes, and others. Green methods using plant extracts offer numerous benefits, including cheaper costs, ease of preparation, environmental friendliness, and high purity and quality of the product obtained [76]. However, several other NPs are used as adsorbents based on extracted tannin from diverse plants since tannins are polyphenols with a large molecular weight, are natural polymers, and are inexpensive. Tannins are easily extracted from a variety of plant parts, including seeds, fruits, roots, and leaves. Polyphenolic compounds can be utilized as ligands in the adsorption process, as described in Section 2.3 [77].

Tannins are water-soluble and challenging to efficiently encapsulate in nanoparticles that inhibit tannin breakdown. Covalent bonding with poly (metacrylic acid) has been shown to boost antioxidant activity [78]. Tannins in their free form can precipitate proteins in plasma and serve as chelating agents, binding metal ions that are naturally present in the human body [79-80]. Furthermore, the ability of the chelating agent is exploited to produce complexes of more hydrophobic tannic acid-metal with desired properties [81-82]. Iron(III) is a favored metal ion for coordination with tannic acid due to its high linkability, robust contact with the ligand, and moderate toxicity when compared to other metal ions [82-83]. As a result, in this research, tannic acid-iron(III) nanoparticles are used as adsorbents to improve adsorption performance for target molecules. Figure 5 shows a possible TA-iron(III) complexation mechanism [84].

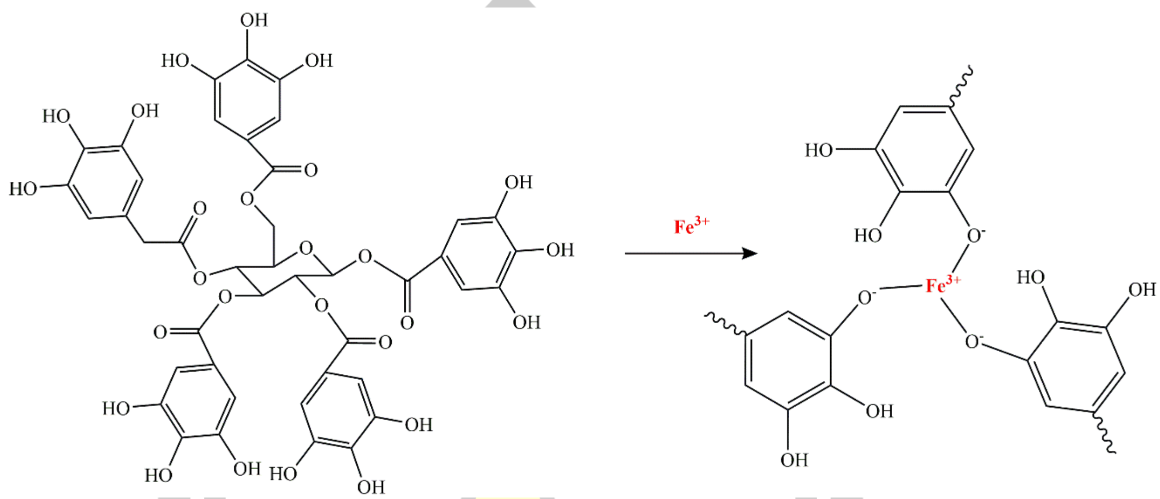

Figure 5 The possible complexation mechanism of TA with iron(III) [84].

2.4.2) *Salvia hispanica* L.

Salvia hispanica L., often known as Chia, is an annual herbaceous plant from the Lamiaceae family. It is native to southern Mexico and northern Guatemala. Nowadays, chia is grown in Mexico, Guatemala, Argentina, Australia, America, Bolivia, Brazil, Columbia, Peru, and Europe [85]. Several parts of this plant are commercially used around the world, particularly chia seeds, in food supplements and health food products to control weight loss for human consumption, such as whole grain in fruit juices, milk or drinking water, salads, an ingredient in bakery products, and the food industry [86]. Chia seeds contain a variety of nutritional properties and antioxidant, antimicrobial, and enzyme inhibitory activity, such as epilepsy, heart and cardiovascular protection, immune system, memory protective effect, dyslipidemia, diabetes, hypertension, and triglyceride control, among others [87–89]. The properties of chia seeds are known not only for these attributes but also for mucilage, which has interesting physical, chemical, and technological capabilities [89–90]. When chia seeds are immersed in water, mucilage (also known as hydrocolloid) is generated in response to hydration, with the ability to hold around 30 times their weight in water, indicating excellent water retention capacity [89, 92–93]. Figure 6 exhibits complete dry and hydrated chia seeds with mucilage surrounding the seed [89].

According to the chemical composition of mucilage, it is mostly composed of polymeric polysaccharides with a high molecular weight and organic acids [94]. The polymeric polysaccharides are heteropolysaccharides composed of D-xylose, D-mannose, L-arabinose, D-glucose, galacturonic acid, and glucuronic acid residues [95–97]. The structure of the polysaccharide in chia seeds has yet to be clearly described. Lin et al. (1994) and Yudianti et al. (2017) proposed the basic structure of mucilage as consisting of heteropolysaccharide repeating 1,4-β-D-xylopyranosyl-1,4-α-D-glucopyranosyl-1,4-β-D-xylopyranosyl units, with occasional 4-O-methyl-α-D-glucuronopyranosyl branches at the O-2 of some β-D-xylopyranoxyl moieties [89]. Furthermore, some research has indicated that pectin contains two separate polysaccharide components (mostly rhamnogalacturonan I, RG-I) and another fraction constituted of hemicellulose (largely arabinoxylans, AX). RG-I can covalently connect to hemicellulose via side chains, generating a super-macromolecule polymeric network [98-99]. Mucilage can interact with other cationic polymers via its anionic structure, forming a polyelectrolyte complex since the anionic structure of mucilage crosses bridges with the cationic structure of other polymers [100]. Mucilage has a high water-binding capacity, emulsifies, gels, acts as an encapsulating agent, is a is a texture modifier, and has a high consistency [97–101]. Figure 7 shows the chemical structures of known chia seed compositions based on repeating oligomers [102].

Mucilage from chia seeds is a green material because it is natural, eco-friendly, inexpensive, biodegradable, and nontoxic [92]. This research chose chia seed as the sorbent due to its diverse qualities and availability in nature. Table 4 shows the application of natural adsorbents for determining tetracyclines and other residues in the current publication.

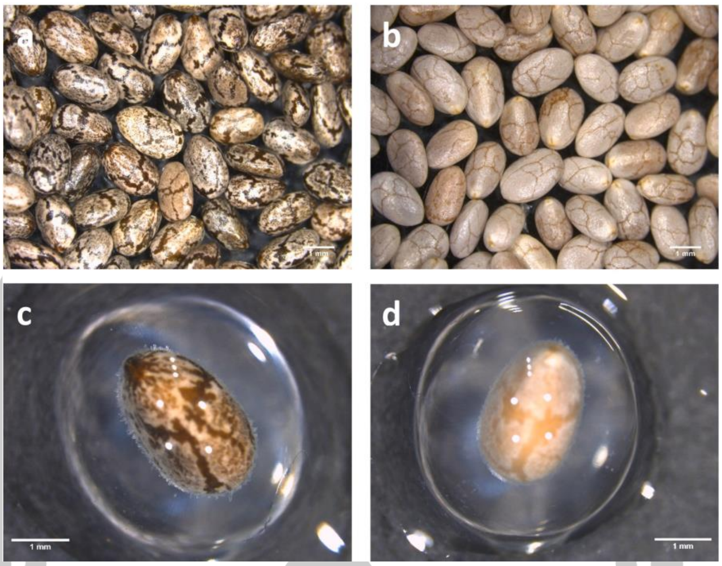

Figure 6 Whole dry a-b) and c-d) whole hydrated chia seed with the mucilage surrounding the seed [89].

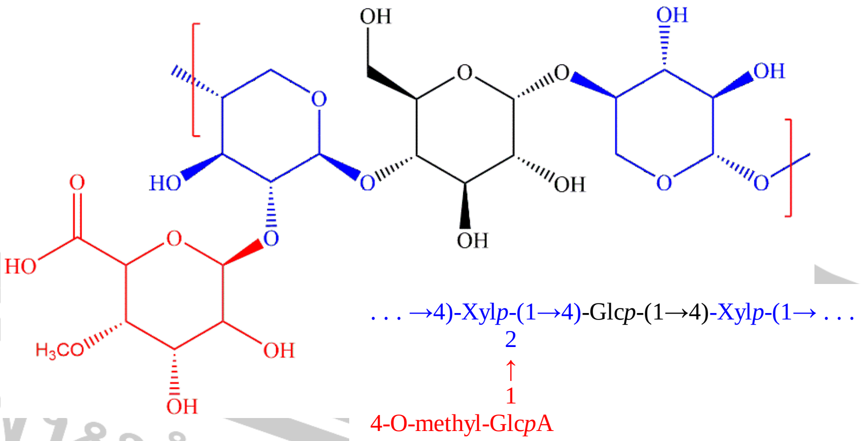

Figure 7 Chemical structures of reported compositions chia seed by repeating oligomer [102].

Table 4 Application of natural adsorbents for determination of tetracyclines and other residues in current publication.

Raw-material	**Adsorbent**	**Analyte**	**Sample**	**Author / Ref.**
Peanut shell	Sulfonated peanut shells (PNS-SO3H) biomass	Methylene blue and Tetracycline	Water	Islam *et al.* (2021) / [103]
Corn stover	Aminated graphitic carbon derived from corn stover biomass	Tetracycline	Aqueous solutions	Haghighat *et al.* (2020) / [104]
Date palm leaves	Date palm-based biochar upgraded with vitamin B6 onto biochar surface	Tetracycline	Aqueous solutions	Saremi *et al.* (2020) / [105]
Shrimp Shell Waste	Shrimp Shell Waste treated with HCl and NaOH solutions	Tetracycline	Aqueous Solutions	Chang *et al.* (2020) / [106]
Mucilage of basil seed	Sodium salt of *Ocimum basilicum* L. seed mucilage (Na-OBMS)	Cd(II)	Ground water	Lodhi *et al.* (2019) / [107]
Sawdust-based biochar	Improved sawdust-based using okra (*Abelmoschus esculentus* L.) mucilage modified biochar	Methylene blue	Water	Nath *et al.* (2021) / [108]
Mucilage of Quince seed	Modified silica aerogel using Quince seed mucilage	Pb(II)	Water and Wastewater	Ghahremani *et al.* (2021) / [109]

2.5 Extraction techniques

Extraction techniques offer a distinctive role in analytical chemistry and the laboratory. "Sample preparation" is the process of separating analytes from interfering sample components or matrix [110]. Sample preparation consists of removing matrix components to generate a more purified sample, which includes sampling, extraction, clean-up, and preconcentration, followed by analysis [111]. As a result, the words extraction and clean-up are sometimes used simultaneously [112]. The kind of treatment sample is determined by the matrix type, chemical parameters of the analyte, and procedure [113–114]. One of the most important concerns for analytical chemists is the development of methods to evaluate the transfer of pharmaceuticals from animals into the food chain and into the human body [115]. This paper proposes particular extraction and clean-up methods for tetracycline residue analysis. Tetracyclines are widely employed in the treatment and prevention of animal diseases, and they may be found in animal tissue that is generated as food for many clients, such as honey, meat, fish, milk, and eggs. The presence of tetracycline residues in above-limit tolerance levels or maximum residue limits (MRLs) was associated with a variety of health problems in the human body, as discussed in Section 2.1.1 [116]. The matrix of a food sample is complex, with many interfering compounds. To limit interference in instrumental determination, the primary matrix components (e.g., lipids, proteins, vitamins, minerals, fats, and other nutritional and energy sources) must be removed [113].

According to the outcome, analytical chemists have a challenge in developing sensitive and precise methods for quantifying tetracycline from various food product matrices. Sample preparation can be optimized to save time, reduce errors, and improve sensitivity. Analytical procedures have been developed to be more ecologically friendly, use fewer harmful solvents, and require smaller sample sizes than older approaches [116]. Table 5 summarizes the sample preparation used to determine tetracycline residues in foodstuffs.

Typically, the extraction techniques of analytical chemistry for isolation, clean-up, and preconcentration of residues of veterinary drugs were solid phase extraction (SPE) [117], liquid-liquid extraction (LLE) [118], solid phase microextraction (SPME) [119], magnetic solid-phase extraction (MSPE) [120], dispersive solid phase extraction (DSPE) [121], dispersive solid phase microextraction (DSPME) [122], molecularly imprinted polymer solid-phase extraction (MIP-SPE) [123] and QuEChERS [124].

2.5.1) Dispersive solid phase extraction

Dispersive solid phase extraction (DSPE) has been frequently employed and found since approximately 2000 [125]. DSPE has been effectively obtained for extraction, isolation, and clean-up methods in the analysis of various veterinary medications for the cattle business. The DSPE principle is followed by adding adsorbents, such as silica solids or polymers, to direct solution [139–141]. The dispersive approach was used to generate information on the contact surface area between adsorbents and analytes. Adsorbents for antibiotic residue identification are chemically changed to promote high performance adsorption by adding substances that interact with analytes while limiting interferences in the analytical matrix [129]. After dispersing, the adsorbents are centrifuged or filtered to separate adsorbents and analytes. The adsorbent formed by binding analytes might be easily eluted by adding suitable organic solvents. Figure 8 presents a scheme for the dispersion approach using dispersive solid phase extraction [10].

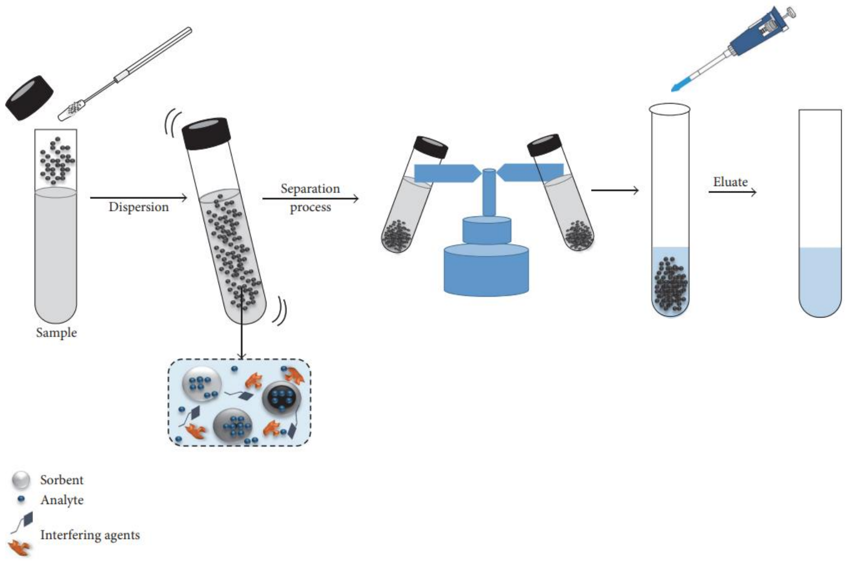

Figure 8 Scheme of dispersion methodology by dispersive solid phase extraction [10].

Table 5 The sample preparation applied for determination of tetracycline residues in foodstuffs.

Sample	**Deproteination**	**Extraction**	**Analyte**	**Recovery (%)**	**Precision (%)**	**Author / Ref.**
Milk, Egg, Chicken and Liver	EDTA- McIlvaine buffer	Solid phase extraction (SPE)	OTC, TC, CTC and DC	81.3–98.7	≤7.7	Zhao *et al.* (2019) / [130]
Milk, Water, and Honey	15% trichloroacetic acid (For Milk)	Vortex-assisted microextraction	TC	95.8–103.4	–	Lanjwani *et al.* (2023) / [131]
Milk, Skyr, Yoghurt, Buttermilk, Kefir, Quark, and Cream cheese	ACN	QuEChERS	OTC, MC, TC, DMC, CTC and DC	60.1–90.8	≤5.0	Zergiebel *et al.* (2023) / [132]
Milk-based infant formulas	0.01 M HCl	Vortex-assisted emulsification microextraction	OTC, CTC and DC	68.9–102.0	≤9.0	Sereshti *et al.* (2022) / [133]
Milk	1% Trichloroacetic acid	–	OTC, TC, CTC and DC	83.93–126.22	≤5.8	Chen *et al.* (2022) / [134]
Water	Adjusted McIlvaine buffer pH 5	Liquid-liquid microextraction	OTC, TC and DC	74–113	–	Sereshti *et al.* (2021) / [135]

Table 5 (continued)

Sample	**Deproteination**	**Extraction**	**Analyte**	**Recovery (%)**	**Precision (%)**	**Author / Ref.**
Milk, Meat, Yogurt and Dairy products (For Infant)	EDTA-McIlvaine buffer pH 5, ACN and Ammonium sulfate	Salting-out assisted liquid–liquid extraction	OTC, TC, CTC, DC and MTC	89.2–96.8	≤7.3	Moreno-González *et al.* (2017) / [136]
Milk and Honey	–	Solid-phase extraction (SPE)	OTC, TC, CTC, DC and MTC	81.5–101.4	≤7.7	Xu *et al.* (2016) / [137]
Milk, Egg and Pork	ACN and 0.01 M Trifluoroacetic acid pH 3.0	Solid-phase extraction (SPE)	OTC, TC, CTC and DC	84.0–97.0	≤8.1	Feng *et al.* (2016) / [138]
Egg, Fish and Shrimp	–	Pressurized liquid extraction (PLE)	OTC, TC, CTC, DC, DEMC, MINO and MTC	75.6–103.5	≤10	Liu *et al.* (2013) / [139]
Water, Chicken and Fish	Na_2EDTA-McIlvaine	Solid-phase extraction (SPE)	OTC and TC	80.4–106.0	≤5.7	He *et al.* (2020) / [140]
Milk	ACN and Evaporated under nitrogen	Magnetic solid phase extraction (MSPE)	TC	94.6–105.4	≤5.9	Vuran *et al.* (2021) / [141]
Chicken and Pork	ACN	Magnetic dispersive solid-phase extraction	OTC, TC and CTC	61.5–102.6	–	Castillo-García *et al.* (2015) / [142]

Furthermore, DSPE is a micro- and macroscale technology that is simple to understand and can reduce extraction and clean-up time. This DSPE adsorbent is not included in cartridges because it only contacts direct analytes and does not require treatment [143].

This study uses dispersive solid phase extraction (DSPE) as an extraction technique to determine tetracycline residues in food. DSPE is used to extract, isolate, clean up, and preconcentrate residues in complicated matrix samples. When compared to established procedures such as SPE and LLE, DSPE has the following advantages: lower solvent use, fewer complex matrixes, lower cost, and faster results [10].

2.5.2) Application of dispersive solid phase extraction for determination of tetracyclines

Yue-Hong Pang et al. developed metal-organic frameworks (MOFs) as adsorbents consisting of MIL-101 (Cr), MIL-100 (Fe), and MIL-53 in a 7:1:2 ratio for use in dispersive solid phase extraction (DSPE) for the determination of tetracyclines (OTC, TC, CTC, and DC) in honey using HPLC-MS/MS. The MOF powder was blended in a centrifuge tube overnight to ensure uniformity. 0.5 g of honey samples were combined with 5 mL of Na_2EDTA-Mcllvaine buffer solution, then 20 mg of compounded MOFs were added, centrifuged at 5.36 g for 3 minutes, and the supernatant was removed. The elute, 3 mL of methanol, was poured into the adsorbent and sonicated for 10 minutes before being centrifuged at 10.31 g for 6 minutes. The supernatant was transferred to a clean centrifuge tube and dried under nitrogen at 30 ºC in a water bath. Finally, the sample was diluted with 1 mL of water and filtered using a 0.22-μm nylon filter. The three compounded MOFs that were used as d-SPE adsorbents had different ligands, crystal structures, and pore sizes, all of which affected how well they could adsorb. However, the size was greater than the window length and the mesoporous pores of tetracyclines. Tetracyclines may easily adsorb inside the three compounded MOFs, resulting in high-performance adsorption by π-π interaction, aperture effect, and electrostatic attraction [144]. Vergara-Luis et al. studied the clean-up by SPE (solid phase extraction) or dispersive SPE after QuECHERS extraction with UHPLC-MS/MS for tetracycline (OTC, TC, CTC, and DC) detection in fresh vegetables. First, the SPE was mixed with 500 mg of Oasis

HLB cartridges. To isolate the sample volume, 1 mL of the supernatant was diluted with 20 mL of 0.05 mol L^{-1} citrate buffer (pH 4) and loaded to the working conditions. 5 mL of water was added to the wash, dried under vacuum, eluted with 9 mL of ACN, and evaporated to 1 mL. Aliquots of 125 µL were reconstituted in 250 µL using ACN:oxalic acid. Second, the DSPE was mixed with GCB 2.5 mg and 150 mg anhydrous Na2SO4, and 1 mL of the extractant was transferred to a centrifuge tube, vortexed, and centrifuged. Aliquots of 500 µL were reconstituted in 1 mL with ACN:Oxalic acid. In conclusion, SPE was the best clean-up for reducing the matrix effect and detecting tetracyclines, although DSPE required fewer steps and produced higher accuracy. As a result, it can provide guidance for optimizing appropriate clean-up for tetracycline detection in the future. Mahboob Nemati et al, was developed a new floating dispersive solid phase extraction, home-made device, based on deep eutectic solvents for determination of tetracycline antibiotics (OTC, TC, CTC and DC) from milk samples. 10 mL of milk sample was added to glass test tube and added 150 mg of trichloroacetic acid. Then, solution was vortexed for 1 min and centrifugation at 5000 rpm for 5 min. The supernatant was transferred into 500 mg lauryl betaine (as a surfactant). The mixture was transferred into the home-made device for extraction and added 100 mg of activated carbon into the aqueous phase that air stream allows passing the solution (flow rate of 0.4 mL/min) for 30 s. The sorbent particles were moved up-through the solution and collected on the top of the sample solution with the aid of air bubbles. After that the floated sorbent was collected by a spatula eluted with 150 µL of tetrabutyl ammonium chloride–propionic acid deep eutectic solvent under sonication for 1.5 min and centrifuged at 5000 rpm for 5 min. Finally, the supernatant injected into HPLC–DAD system. In this method, there was no need of organic dispersive, extraction solvents and without centrifugation [145]. Table 6 shows the application of dispersive solid phase extraction for tetracycline determination.

Table 6 Application of dispersive solid phase extraction for determination of tetracyclines.

Sample	Extraction / Clean-up	Analyte	Analysis	LOQs	LODs	Author / Ref.
Honey	Dispersive solid-phase extraction	OTC, TC, CTC and DC	HPLC–MS/MS	0.239–1.449 ng g^{-1}	0.073–0.435 ng g^{-1}	Pang *et al.* (2021) / [144]
Fresh vegetables	QuEChERS and Dispersive solid phase extraction	OTC, TC, CTC and DC	UHPLC-MS/MS	4.4–11.3 µg kg^{-1}	–	Vergara-Luis *et al.* (2023) / [121]
Swine wastewater	QuEChERS and Dispersive solid phase extraction	OTC, TC, CTC and DC	UPLC–MS/MS	0.050–0.100 ng mL^{-1}	0.020 –0.030 ng mL^{-1}	Wang *et al.* (2019) / [146]
Milk	Surfactant-assisted dispersive solid phase extraction	OTC, TC, CTC and DC	HPLC–DAD	0.6–1.0 µg kg^{-1}	0.1–0.3 µg kg^{-1}	Nemati *et al.* (2021) / [145]

2.6 High performance liquid chromatography

High performance liquid chromatography (HPLC) is very prevalent of separation technique in the analytical chemistry and many fields because it could determine the property of various compound in target molecules (analytes) by the low up to very high molecular mass of analytes. The principle of chromatography separation is involved on different adsorption/desorption abilities of components in between two phases that namely the stationary phase and the mobile phase. The mobile phase contained analytes, would pass into the stationary phase. The components of analytes were separated under pressure of the mobile phase by the retention times of components in the stationary phase were discovered different based on the nature of chemical structure and molecular weight of the analytes. Typically, the components of HPLC are consist of a solvent supply system (solvent container and degasser), pumping system (high-pressure pump and gradient device), sampling system (autosampler or manual syringes), separation system (chromatographic column), detection system (different types of detectors), and data processing system [147–148]. Figure 9 shows a schematic diagram of the components used in high-performance liquid chromatography [149].

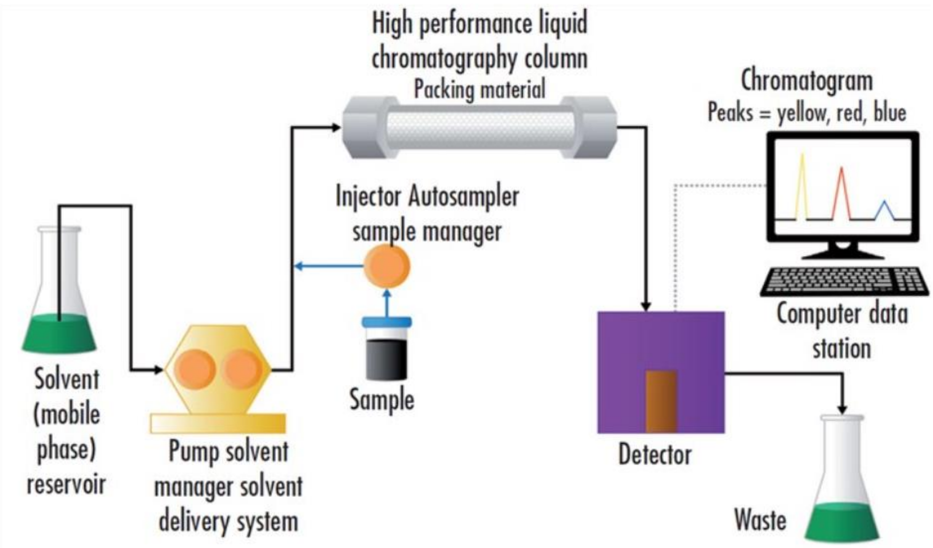

Figure 9 Schematic diagram of the high performance liquid chromatography components [149].

The three main components mentioned are the column, stationary phase, and mobile phase. A column for HPLC is substantially small, with an inner diameter of 2.0 to 5.0 mm and a length of 50.0 to 250 mm. Different materials are contained in the column that interact with the molecules of analytes as known in the stationary phase and are commonly unbranched alkanes, such as the silica-based stationary phase octadecyl carbon chain (C18), coupled to spherical particles. C18 can attach to chemical interactions such as hydrogen bonding, hydrophobic interactions, and ionic bonding. Analyte retention time is affected by their non-polarity, polarity, and characteristics. C18 is non-polar, which slows analyte migration. Consequentially, analytes with non-polar characteristics elute more slowly than polar ones. The mobile phase of HPLC should be sufficiently dissolved for analytes but not totally dissolved to identify analytes after passing through the column. Normally, the mobile phase of HPLC consists of two solvents: water and an organic liquid. Acetonitrile and methanol are two commonly used organic solvents. Furthermore, an acid, such as 0.1% formic acid or trifluoroacetic acid (TFA), was added to the mobile phase. It can aid in the demining of analytes at low pH. Organic molecules are more stable when they completely protonated. [150]. Distribution chromatography can distinguish between two types based on the polarity of the mobile and stationary phases. First, normal-phase partition chromatography consists of a non-polar mobile phase and a polar stationary phase. Second, reversed-phase partition chromatography consists of a polar mobile phase and a nonpolar stationary phase. Solvent delivery systems can distinguish between two types: isocratic and gradient elution. From start to finish, the isocratic mode uses a constant solvent ratio for elution, but it cannot effectively isolate mixtures of components with similar polarity to the mobile phase. On the other hand, gradient mode is adjusted throughout the run-in elution and may employ two or three different solvents with an inconstant solvent ratio. As a result, it can isolate a mixture of components with polarities similar to those of the mobile phase [147].

In this research, high performance liquid chromatography (HPLC) is recommended for the measurement of tetracycline residues in foodstuffs due to its high separation capacity, accuracy, and precision. Furthermore, they can analyze both qualitative and quantitative data using a single device. Table 7 summarizes how high performance liquid chromatography was used to determine tetracyclines.

Table 7 Application of high performance liquid chromatography for determination of tetracyclines in foodstuff.

	Author / Reference				
	Sereshti *et al*. (2021) / [133]	Xu *et al*. (2016) / [137]	Vuran *et al*. (2021) / [141]	Pang *et al*. (2021) / [144]	Sereshti *et al*. (2020) / [151]
Sample	Milk (For Infant)	Honey and Milk	Milk	Honey	Milk
Extraction	Vortex-assisted emulsification liquid–liquid microextraction (VA-ELLME)	Solid-phase extraction (SPE)	Magnetic solid phase extraction (MSPE)	Dispersive solid-phase extraction (DSPE)	Dispersive liquid-liquid microextraction (DLLME)
Deproteination	0.01 M HCl	–	ACN	–	ACN
Evaporation	–	–	Under nitrogen	Under nitrogen at 30 ºC	–
Analyte	OTC, TC and DC	OTC, TC, CTC, DC and MTC	TC	OTC, TC, CTC and DC	OTC, TC and DC
Column	C18 (250 mm × 4.6 mm, 5 µm)	RP SB-C18 (50 mm × 4.6 mm, 5 µm)	LUNA® Phenyl-Hexyl 100 Å (250 mm × 4.6 mm , 5 µm)	ACQUITY UPLC® HSS T3 (1.0 mm × 150 mm, 1.8 µm)	C18 (250 mm × 4.6 mm, 5 µm)
Mobile phase	ACN and MeOH–0.01 M oxalic acid aqueous solution (pH 4)	0.1% formic acid–MeOH	50 mM phosphate buffer (pH 4)–MeOH–ACN	0.4% formic acid–ACN	ACN and MeOH–0.01 M oxalic acid solution
Detector	UV 360 nm	Q-TOF/MS	DAD	MS/MS	UV 360 nm
Spiked range	100–200 µg kg^{-1}	0.65–34.25 µg kg^{-1}	100–300 ng mL^{-1}	10–50 ng g^{-1}	150–300 µg L^{-1}
Recovery (%)	68.9–102	81.5–101.4	94.6–105.4	88.1–126.2	70–113
Precision (%)	–	≤7.71	≤5.9	–	≤8.4

CHAPTER III
MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemicals and reagents used in this work listed in Table 8 were analytical grade, and they were used without further purification.

Table 8 List of chemicals used in this work.

No.	Chemicals	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.	Hydrochloric acid	HCl	AR	ANaPURE®	New Zealand
7.	Methanol	CH_3OH	HPLC	Merck	Germany
8.	Oxytetracycline hydrochloride	$C_{22}H_{24}N_2O_9 \cdot HCl$	HPLC	Sigma-Aldrich	Germany
9.	Sodium acetate 3- hydrate	$CH_3COONa \cdot 3H_2O$	AR	Ajax Finechem	New Zealand
10.	Sodium hydroxide	$NaOH$	AR	Ajax Finechem	New Zealand
11.	Stock standard solution of iron(III) 1000 mg L^{-1}	[$Fe(NO_3)_3 \cdot 9H_2O$ in HNO_3 0.5 N]	AAS	ITW Reagents	Germany
12.	Tetracycline hydrochloride	$C_{22}H_{24}N_2O_8 \cdot HCl$	HPLC	Sigma-Aldrich	Germany
13.	Trifluoroacetic Acid	$C_2HF_3O_2$	AR	Fisher Scientific	USA

AR grade chemical means analytical reagent

HPLC grade chemical means high performance liquid chromatography

AAS grade chemical means atomic absorption spectroscopy

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 was 20 µL. Breeze software would be used for data processing. A vortex mixer (50 Hz, model ZX3, VELP Scientifica, Italy) and centrifuge (Model 1000series, labquip, United Kingdom) were used to mix the solution and accelerate the phase separation, respectively. Ultrasonic bath (Model D-78224 singen, Elma, Germany) was used to extract and increase and mass transfer.

3.3 Biosorbent and natural reagent

3.3.1) Chia seed sorbent

Chia seed was purchased from a supermarket or local market in Kantharawichai District, Maha Sarakham Province, Thailand.

3.3.2) *Peltophorum pterocarpum* natural reagent

The suitable ingredient, *Peltophorum pterocarpum* natural reagent, was purchased on an online shopping application.

3.4 Experimental

3.4.1) Preparation of standard solution

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

Individual stock solutions (TC, OTC, CTC and DC, 1000 mg L^{-1}) were prepared by dissolving 0.0100 g of each TCs in 10 mL of methanol and stored in a dark glass bottle at 4 ºC in the refrigerator. Working TCs mixed solutions were prepared daily from the stock solution by a stepwise dilution with deionized water.

3.4.1.2) Stock standard solution of 10 mg L^{-1} iron(III)

A 10 mg L^{-1} of iron(III) standard stock solution was prepared by pipetting 0.5 mL from 1000 mg L^{-1} of iron(III) into 50 mL volumetric flask. Then, the final volume was adjusted to 50 mL with deionized water.

3.4.2) Preparation of 0.3 mol L^{-1} hydrochloric acid

A 0.3 mol L^{-1} of hydrochloric acid solution was prepared by pipetting 0.627 mL from 37%w/w of hydrochloric acid into 25 mL of volumetric flask. Then, the final volume was adjusted to 25 mL with deionized water.

3.4.3) Preparation of 0.05 mol L^{-1} acetate buffer pH 4.0

Buffer solution was prepared by mixing an appropriate amount of 0.4763 g sodium acetate and 1.250 mL acetic acid. Then, the final volume was adjusted to 500 mL with deionized water. Lastly, the required pH was adjusted with 1 mol L^{-1} sodium hydroxide solution.

3.4.4) Preparation of trifluoroacetic acid (TFA, v/v) in acetonitrile

3.4.4.1) Preparation of 5% TFA in acetonitrile

5% TFA in acetonitrile for the clean-up of the biosorbent was prepared by pipetting 1.250 mL of 99% TFA into a 25 mL volumetric flask. Then, the final volume was adjusted to 25 mL with acetonitrile.

3.4.4.2) Preparation of 9% TFA in acetonitrile

9% TFA in acetonitrile for the desorption process was prepared by pipetting 0.900 mL of 99% TFA into a 10 mL volumetric flask. Then, the final volume was adjusted to 10 mL with acetonitrile.

3.4.5) Preparation of mobile phase

The mobile phase was prepared by mixing 0.2% (v/v) TFA in acetonitrile (solvent A) and 0.2% (v/v) TFA in deionized water (solvent B) by pipetting 1 mL of 99% TFA into a 500 mL volumetric flask. Then, the final volume was adjusted to 500 mL with acetonitrile and deionized water for solvent A and solvent B, respectively.

3.4.6) Preparation of natural reagent

The *Peltophorum pterocarpum* including the bark, leaf and pod, was prepared by being cut into pieces prior to an oven at 60 ºC for 24 h, and then crushed with a blender. Subsequently, the crushed powder was filtered through a strainer and collected into a sealed container.

The natural reagent solution was prepared by heating and stirring 1.000 g of natural reagent powder of *Peltophorum pterocarpum* (bark, leaves and pod) in

100 mL of deionized water on a hot plate for 15 min. Then, the solutions were centrifuged at 6000 rpm for 10 min. The solutions were filtered (Whatman grade 1 filter paper) into a 100 mL volumetric flask and adjusted to 100 mL with deionized water, respectively.

3.5 Dispersive solid phase extraction of Chia seed mucilage modified by iron(III)–natural phenolic nanoparticles as biosorbent for preconcentration of TCs

The dispersive solid phase extraction using chia seed sorbent was carried out in 15 mL centrifuge tubes. Chia seed biosorbent 1.0 mg was cleaned up and activated by 1 mL of 0.3 mol L^{-1} hydrochloric acid, followed by vortexing for 1 min, and solutions were withdrawn by syringe. 1 mL of 5% v/v TFA in acetonitrile was carried out in the same way. Then, the sorbent was rinsed with 1 mL of deionized water (three replicates). The solution was withdrawn by syringe. The extraction procedures were performed by adding 1 mL of 10 mg L^{-1} iron(III) solution into centrifuge tubes that contain chia seed mucilage as a biosorbent. 200 µL of natural reagent, TCs standard or sample solution, and 3 mL of 0.05 mol L^{-1} of acetate buffer pH 4 were added, respectively. The final volume was adjusted to 10 mL with deionized water. Then, the solutions were incubated for 5 min at 30 °C in an ultrasonic bath, followed by vortexing for 30 s to mix the solutions. After that, the mixed solutions were centrifuged at 6000 rpm for 15 min to complete phase separation between the precipitate and the aqueous solution. In this step, the aqueous solutions were carefully withdrawn by syringe, and the lower phase of the complexed sorbent was collected. 50 µL of 5% v/v TFA in acetonitrile was added to desorb with vortex for 120 s and filtered through a 0.22 µm nylon membrane filter. Lastly, the final volume (20 µL) was injected into the HPLC system for analysis. The schematic representation of dispersive solid phase extraction using chia seed mucilage modified by iron(III)-natural phenolic nanoparticles as a biosorbent is presented in Figure 10.

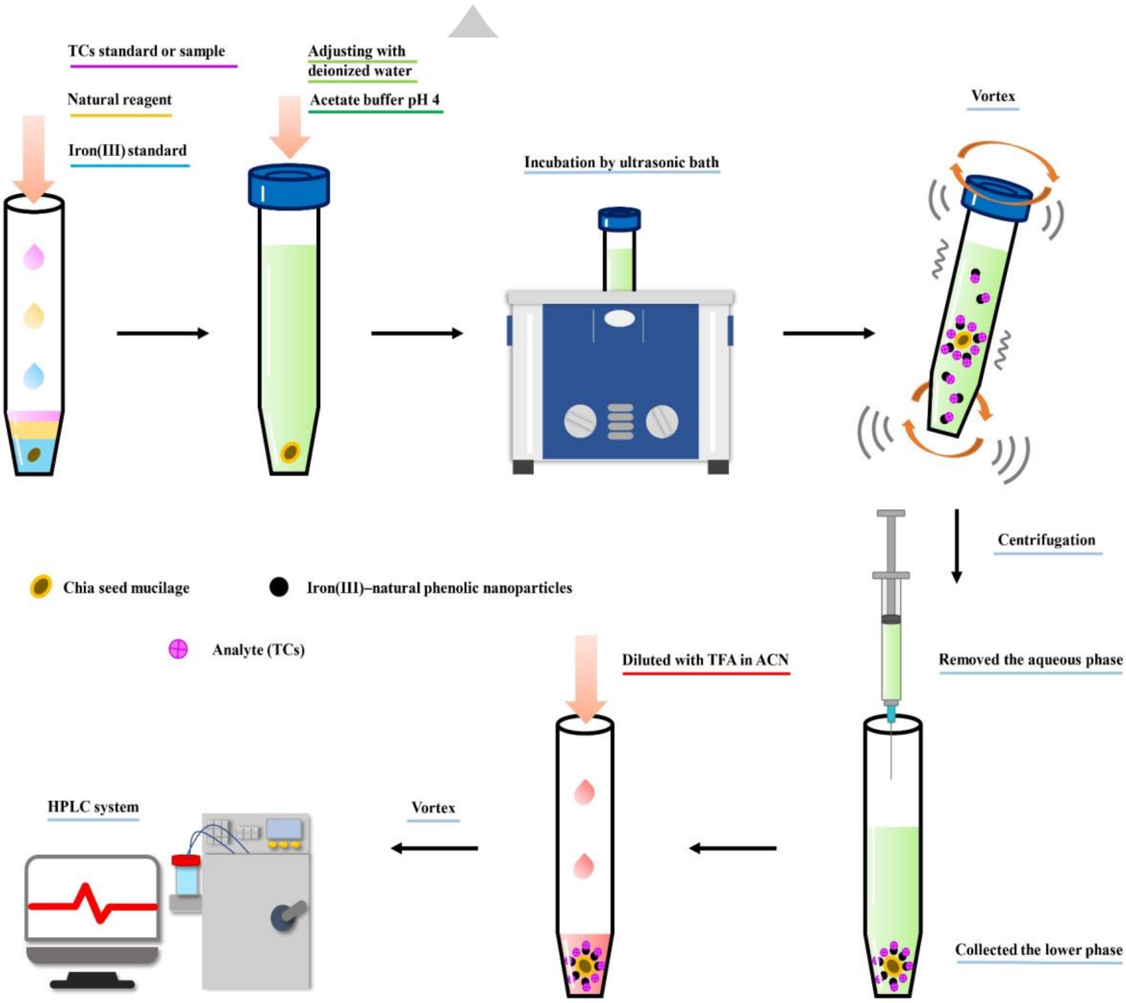

Figure 10 Schematic representation of the dispersive solid phase extraction using green natural biosorbent.

3.6 Optimization of experimental parameters

3.6.1) Natural phenolic reagent

3.6.1.1) Effect of suitable source of natural phenolic reagent

The effect of suitable sources of natural phenolic reagents was studied by using the bark, leaves and pod of *Peltophorum pterocarpum*.

3.6.1.2) Effect of amount of natural reagent powder

The effect of the amount of natural reagent powder was investigated in the range of 0.50, 1.00, 1.50, and 2.00 g.

3.6.1.3) Effect of natural reagent volume

The effect of natural reagent volume was tested in the range of 0, 100, 200, 300, and 400 µL.

3.6.2) Effect of amount of chia seed as natural adsorbent

The effect of the amount of chia seed adsorbent was studied in the range of 0, 1.0, 2.0, 3.0, and 5.0 mg.

3.6.3) Effect of iron(III) concentration

The effect of iron(III) concentration was examined in the range of 0, 0.5, 1.0, 1.5, and 2.0 mg L^{-1}.

3.6.4) Effect of pH

The effect of pH on extraction TCs was studied of 3.0, 4.0, 5.0, 6.0, and 7.0.

3.6.5) Effect of buffer concentration

The effect of acetate buffer concentration was studied in the range of 0, 0.005, 0.015, 0.025, and 0.035 mol L^{-1}.

3.6.6) Effect of temperature and incubation time

The temperature for incubation was studied in the range of 30 to 60 ºC under an ultrasonic bath. The incubation time was studied in the range of 1–15 min.

3.6.7) Effect of vortex time during extraction

The vortex time was used to mix the solution and enhance extraction efficiency. Vortex time was investigated in the range of 0 to 240 s.

3.6.8) Effect of speed and time centrifugation

Centrifugation is one of the important parameters for the separation phase. In this extraction method, it was studied in the range of 3000–6000 rpm and 5–20 min for speed and time centrifugation, respectively.

3.6.9) Effect of concentration and volume of desorption solvent

After the complete separation phase, the TCs on the adsorbent and complex of iron(III)-natural reagent particles were desorbed before injection into the HPLC system for quantitative analysis. In order to obtain a high enrichment coefficient, the concentration and volume of the desorption solvent were examined. For this extraction method, the concentration of desorption solvent was studied in the range 0–9% v/v of TFA in acetonitrile, and the desorption solvent volume was tested in the range 30–150 µL.

3.6.10) Effect of vortex time after extraction

The effect of vortex time after adding desorption solvent was studied in the range of 30 to 240 s.

3.6.11) Characteristic of natural biosorbent and complex of iron(III)-natural reagent particles

Natural biosorbent and iron(III)-natural reagent particles were characterized by FTIR, TEM, SEM, XRD, BET and zeta-potential in order to evaluate the functional group, morphology, crystalline, porous and surface charge of adsorbents, respectively.

3.7 Method validation

3.7.1) Linearity range

A mixture of standard solutions, including OTC, TC, CTC and DC, was prepared and the working solutions were diluted in deionization water before being injected into HPLC under the optimum conditions. The calibration curve for each analyte was constructed by plotting between the peak areas and the concentration of the mixed standard solution at the five concentration levels. The linearity was evaluated through the calibrations by the correlation coefficient (r^2) value of determination.

3.7.2) Detection limits

The sensitivity of the proposed method was evaluated by gradually reducing the concentration of the mixture standard solution. The limit of detection (LOD) was calculated by three times the signal-to-noise ratio (3:1), and the limit of quantitation (LOQ) was calculated by ten times the signal-to-noise ratio (10:1).

3.7.3) Accuracy and precision

The accuracy of the proposed method was evaluated by adding a mixed standard solution at three concentration levels to the sample solution, followed by a percentage recovery calculation.

The precision of peak area and retention time (t_R) were expressed intra-day and inter-day as the percentage relative standard deviation (%RSD). Precision was calculated by analyzing a mixed standard solution at three concentration levels within the same day and five different days, respectively.

3.7.4) Matrix effect

The matrix effect, also known as the standard addition method, was used to investigate all other components of the sample in the absence of the target molecule by analyzing the signal intensity, which might be higher or lower than the typical signal intensity. The matrix effect was reported as ME (%), and the study for each analyte was generated by comparing the slopes of the standard addition and calibration curve methods.

3.8 Real samples

Milk samples were purchased from a supermarket in Kantharawichai District, Maha Sarakham Province, Thailand. The milk samples were prepared for analysis by using protein precipitation before analysis. Briefly, 1 mL of milk samples and 4 mL of acetonitrile were added to a 15 mL centrifuge tube. The solutions were vortexed for 1 min. The milk sample solutions were centrifuged at 3000 rpm for 10 min, and then the supernatant was filtered through a 0.45 µm nylon membrane filter and evaporated under nitrogen gas to a final volume of 0.30 mL. The final volume was centrifuged at 6000 rpm for 10 min. The diagram of the preparation real samples is presented in Figure 11.

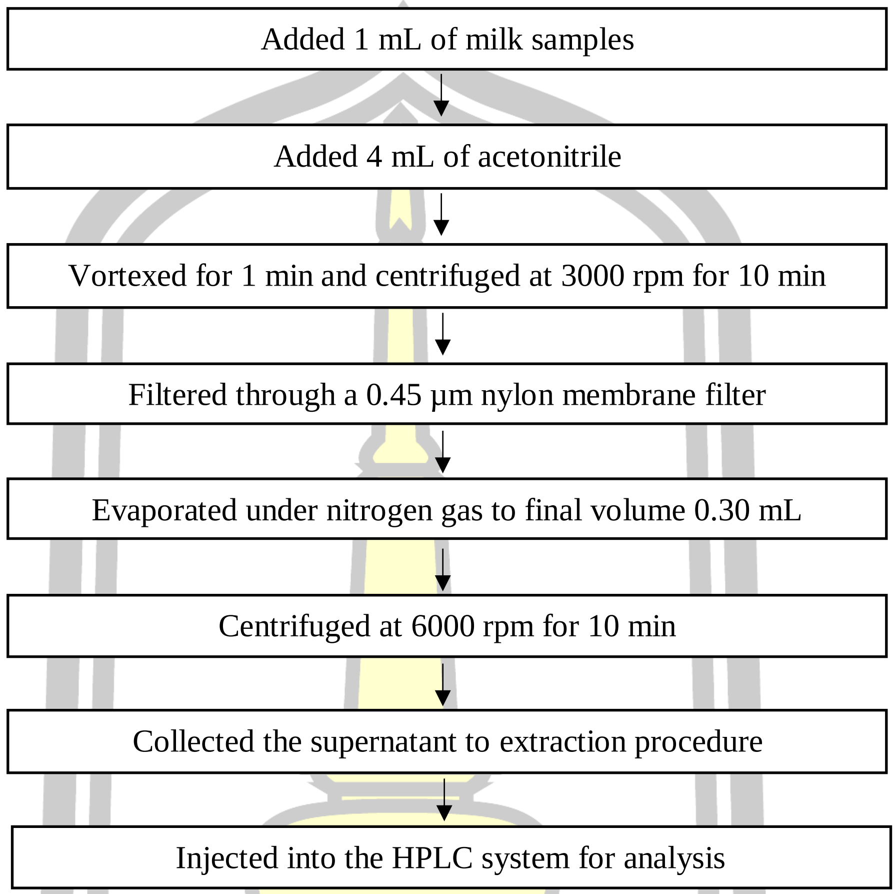

Figure 11 Diagram of preparation real samples.

3.9 Data analysis

The average results of all analyses, expressed by the mean ($\bar{x}$), were calculated by summing the individual results and divided by the number (n) of individual values. It was calculated as follows:

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

<u>Description</u>

x_n = Any data set consisting of the values x_1, x_2, x_3, …., x_n

n = The number of individual values

The standard deviation (SD) of all analyses was a measure of the amount of variation or dispersion in the in the data set. In order to indicate that the values tend to be close to the mean (a low standard deviation means that the values tend to be close to the mean), It was calculated as follows:

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

<u>Description</u>

$\bar{x}$ = Average values of each analysis

x_n = Any data set consisting of the values x_1, x_2, x_3, …., x_n

n = The number of individual values

The accuracy of all samples, expressed by the percentage recoveries (% recoveries), was a measure of the concentration of analyte that the values were close to the concentration of analyte in real samples. It was calculated as follows:

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

<u>Description</u>

C_{spiked} = Determined concentration in the sample solution that added the standard solution

$C_{unspiked}$= Determined concentration in the sample solution without added the standard solution

C_{added} = Added concentration in the sample solution

The precision of all analyses, expressed by the relative standard deviation (%RSD), was a measure of the repetitive analysis performance of the experimenter. It was calculated as follows:

$$\%RSD = \left(\frac{SD}{\bar{x}}\right) \times 100$$

Description

SD = The standard deviation of all analysis

$\bar{x}$ = Average values of each analysis

The matrix effect was expressed by ME (%) and obtained from the slope of the standard addition method and the standard solution. It was calculated as follows:

$$\%ME = \left(\frac{\text{slope of spiked real sample}}{\text{slope of standard solution}}\right) \times 100$$

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Characterization of chia seeds mucilage and iron(III)-natural reagent particles

4.1.1) IR spectroscopy

The FTIR technique was used to identify the functional groups of all adsorbents. Chia seed mucilage (CSM) showed the characteristic bands for polysaccharide, which are presented to the component of mucilage in Figure 12. The O−H stretching of hydrogen bonds, C−OH stretching of pyranose groups, C−H stretching, and COO− groups of uronic acid for asymmetric and symmetric stretching appear at 3287.85, 2924.70, 2854.02, 1458.73, and 1416.22 cm^{-1}. Moreover, O−H groups only appear in the chia seed mucilage at 1031.98 cm^{-1}, which is obtained from carbohydrate molecules. The peak at 3010.61 cm^{-1} was found to be the cis-type unsaturated C=C−H groups, and 1600.15 cm^{-1} was located in the stretching vibration for the carboxyl groups (−COOH), while 1743.55 cm^{-1} is indicated as a strong band for COOH stretching. These functional groups of CSM have been reported in other previous literature [152,153]. The reagent for the synthesis of the iron(III)-natural reagent particles was predicted to be tannin (one of the phenolic compounds in nature) that can coordinate with iron(III) to form the solid particles. To confirm the prediction, the obtained particles were compared with standard tannic acid and the other previous literature on the Fe-tannic acid complex [154–156]. The broad peak at 3388.92 cm^{-1} represents the O−H stretching of the hydrogen bond. The prominent peaks of tannic acid appear in this work, followed by 1693.50 cm^{-1} for C=C stretching of benzene ring, C−C groups for the stretch vibration of benzene ring at 1612.76, 1519.66, and 1446.96 cm^{-1}, the C−H plane of benzene ring at 1612.76 and 1519.66 cm^{-1}, the C−H deformation out of the plane at 867.09 cm^{-1}, the C−O stretching of phenolic groups at 1519.66 and 1446.96 cm^{-1}, and the last C−H groups for the torsion vibration of benzene ring at 765.77 cm^{-1}.

The combination of CSM and iron (III)-natural reagent particles is shown in Figure 12. The FTIR spectra obtained remain to present the position that corresponds with the characteristic band of both adsorbents. The comparison of FTIR spectra for TCs before and after preconcentration of all adsorbents found that the signals of O−H

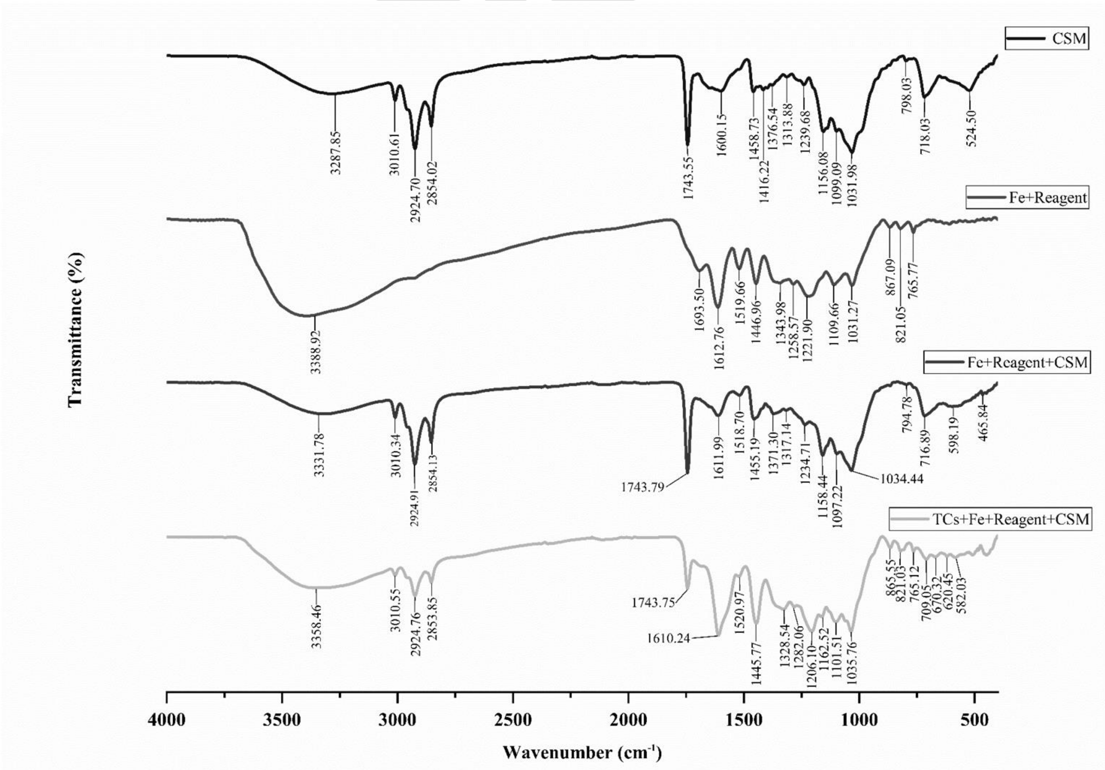

Figure 12 FTIR of CSM and iron(III)-natural reagent particles before combination of biosorbents and TCs extraction.

stretching from the hydrogen bond changed at 3358.46 cm^{-1} and C−C stretching of benzene rings and methylene, including C−O stretching of phenolics, broadened at 1445.77 cm^{-1}. The region of C−O stretching and O−H deformation of phenolics, including C−C stretching and C−H deformation in the plane of the benzene ring, was slightly shifted at 1328.54 cm^{-1}. C−OH bending (C−O and C−C) of uronic acid from carbohydrate molecules appeared at a lower wavenumber of 1206.10 cm^{-1}. Deformation in the plane of phenolic and carboxylic acid for O−H including stretching for C−C and deformation in the plane of benzene ring for C−H were shifted to higher wavenumbers at 1162.52 cm^{-1}.

4.1.2) XRD and BET analysis

The XRD patterns of all adsorbents are given in Figure 13. The CSM patterns provided an amorphous structure with two characteristic peaks. As shown in the 2θ scale at 15.32 (111) and 30.22 (222), it can be assigned that Magnesium Hydrogen Phosphate Hydrate (Mg $(H_2PO_2)_2(H_2O)_6$) from the data of the powder diffraction file PDF # 75-0029 [153]. Reagents of iron(III)-natural reagent particles synthesized were an amorphous structure. After the collaboration of all adsorbents is presented (without TCs and with TCs, respectively), that is not different for these lines.

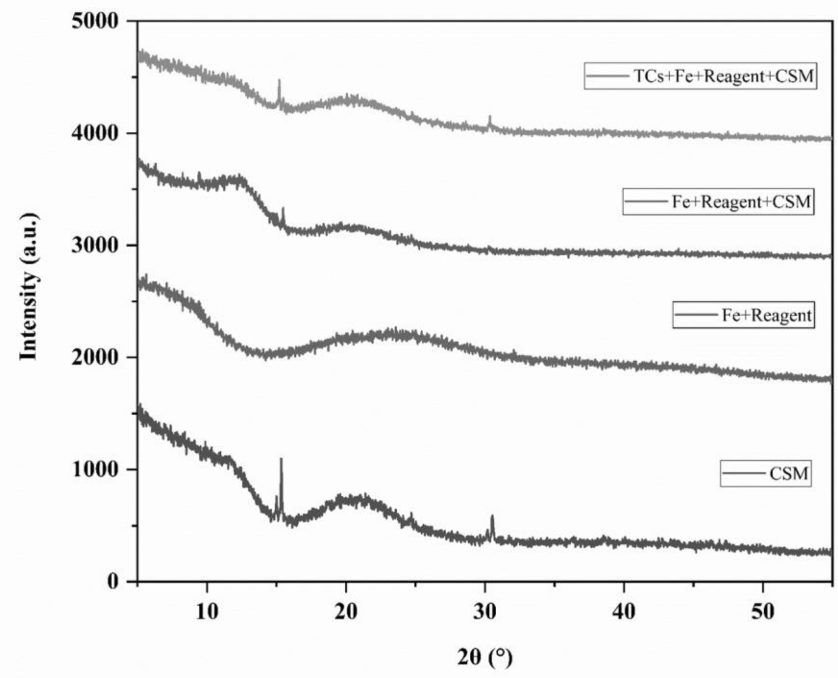

Figure 13 The XRD pattern of CSM, iron(III)-natural reagent particles (reagent) and the combination of biosorbents before and after TCs extraction.

According to the BET analysis for the iron(III)-natural reagent particles synthesized, it was revealed that the surface area and BJH adsorption average pore width (4V/A) (Aº) of the iron(III)-natural reagent particles were 5.932 m^2 g^{-1} and 70.993 Aº, respectively. On the other hand, the surface area decreased to 0.753 m^2 g^{-1} when CSM was combined with iron(III)-natural reagent particles because the particles synthesized were coated with CSM, which produced a larger size and a lower surface area than the pure particles synthesized. For BJH adsorption average pore width (4V/A) (Aº) was increased to 96.413 Aº because the dispersion of the particles synthesized was greater during coating on CSM. Moreover, both BJH adsorption average pore widths indicated the pore type of the adsorbent as mesoporous.

4.1.3) TEM and SEM analysis

The iron(III)-natural reagent particles synthesized were investigated for morphological adsorbent and particle size by TEM-EDX. As shown in Figure 14, the particles were quite spheres in the range of 14.83 to 24.88 nm, and the average particle size was 20.26±0.14 nm in Figure 14A. The particle size was changed after the coating of TCs, resulting in larger particles in the range of 40.41 to 62.12 nm (mean 51.91±0.10 nm) in Figures 14B and 14C. Moreover, the mapping results discovered nitrogen element (N), which may be gotten from TCs structure and presented in Figure 14D, and the EDX results in Figure 14F.

The SEM analysis of CSM and the iron(III)-natural reagent particles synthesized was performed to explore the morphology in Figure 15. The results revealed that the formation of a thick network of mucilage on the seed surface [157] in Figure 15A filled the CSM with small fibers, also known as fibrils, around the seeds. The average particle size of CSM was 1.90±0.49 mm (ranging from 1.37 to 2.75 mm). The iron(III)-natural reagent particles were quite a sphere and intensely overlayed in Figure 15B. When all adsorbents synergized together, CSM fibril was covered with particles that unified on fibril like plaster, as shown in Figure 15E. After TCs existence, the adsorbents were presented to differentiation, where the CSM fibril was characterized by a small dot and roughness, while the particles were covered and had a larger size of 2.14±0.59 mm (ranging from 1.54 to 2.88 mm), as shown in Figure 15F.

4.1.4) Zeta potential analysis

To confirm the charge of the adsorbent for interaction with TCs, the zeta potential was selected to be evaluated. The CSM and the iron(III)-natural reagent particles synthesized were examined by each adsorbent and showed negative charges at −17.73±1.36 and −17.77±0.12 mV, respectively [100,154]. After modification of these adsorbents, the negative charge was enhanced to −20.33 ±0.25 mV and decreased by the display of positive charge excess to −6.507±0.16 mV because of the interaction with TCs, which were the zwitterionic condition at pH 4 [158].

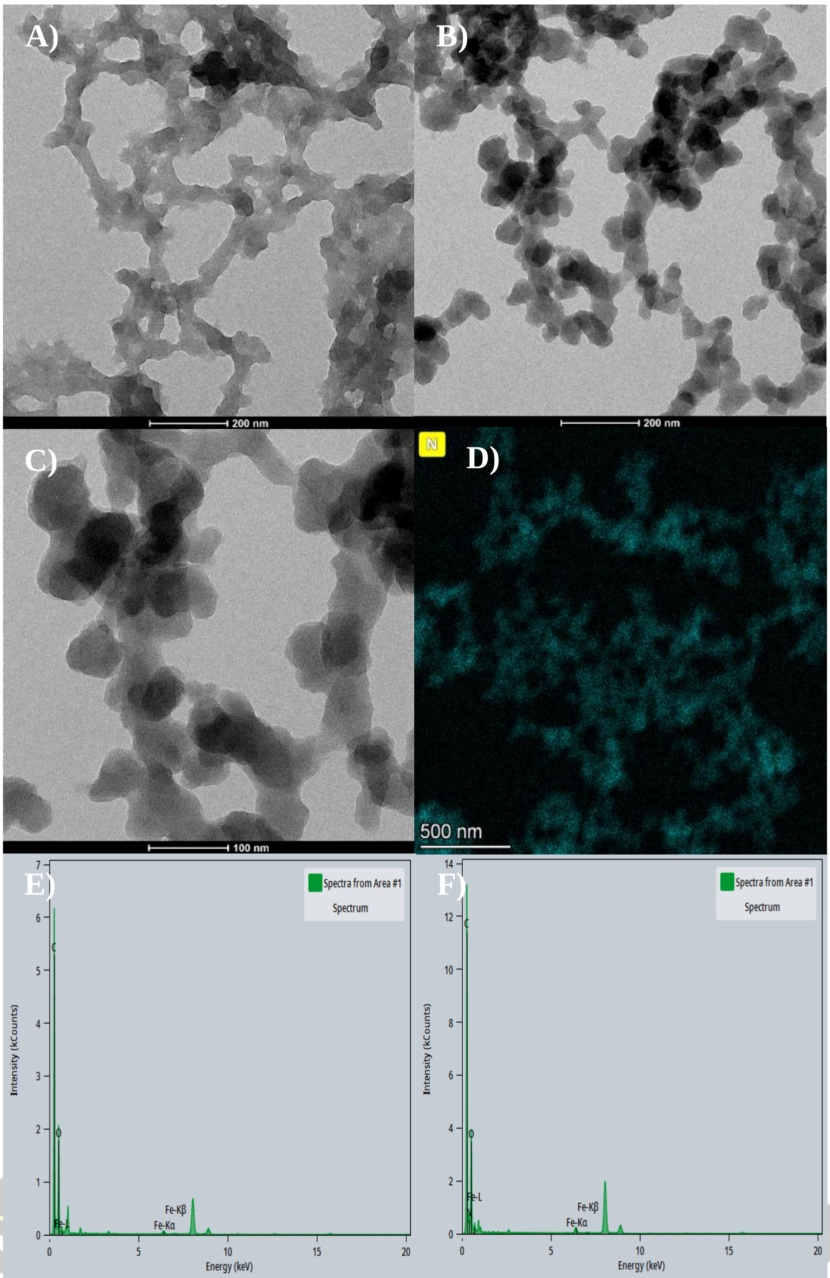

Figure 14 TEM images of iron(III)-natural reagent particles before and after TCs extraction; A) Before TCs extraction, B) and C) After TCs extraction, D) Mapping mode after TCs extraction, E) EDX before TCs extraction and F) EDX after TCs extraction.

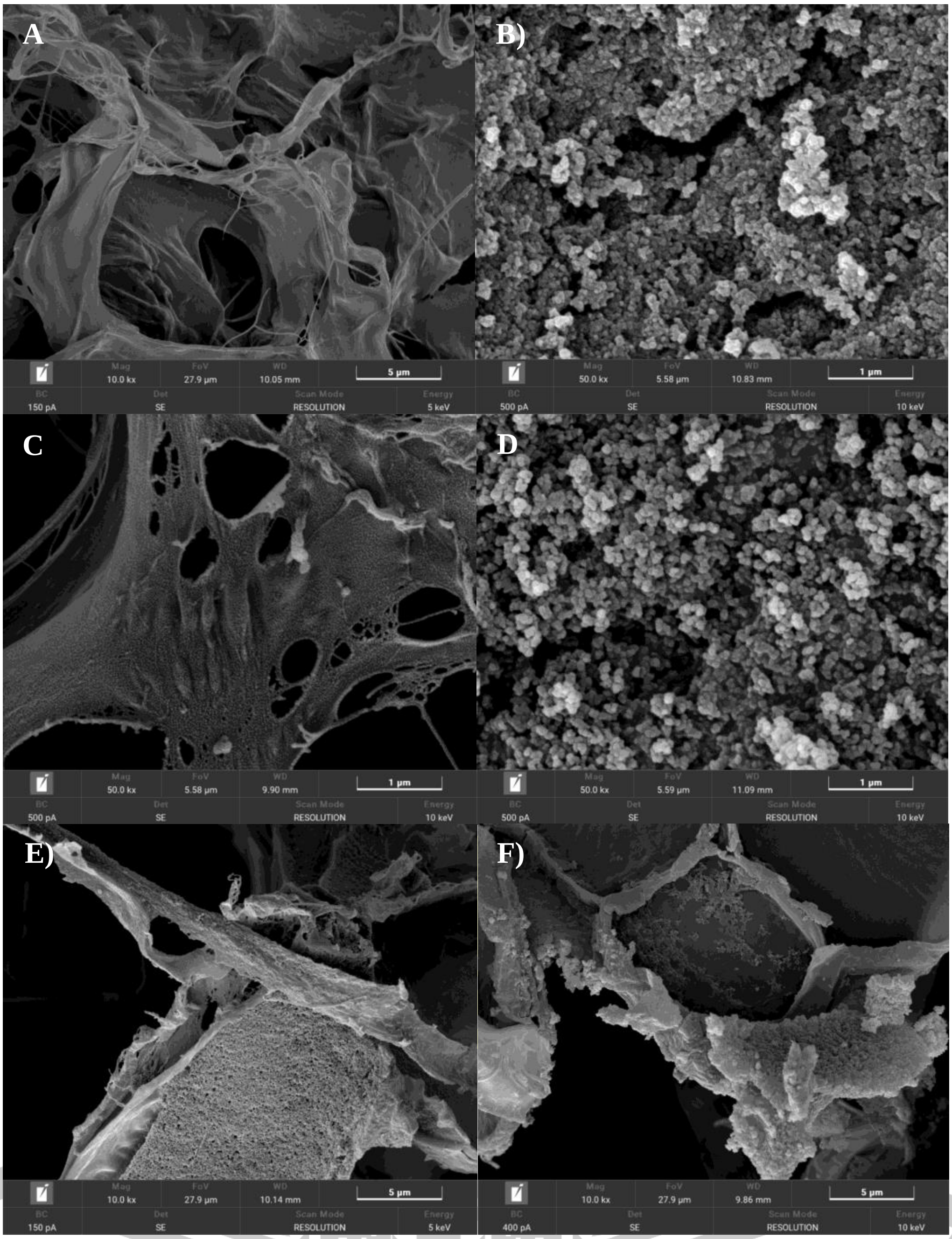

Figure 15 SEM images of CSM and iron(III)-natural reagent particles before and after TCs extraction; A) CSM surface area, B) Surface of area iron(III)-natural reagent particles, C) and D) After TCs extraction of CSM and iron(III)-natural reagent particles and E) and F) Combination of CSM modified by iron(III)-natural reagent particles after TCs extraction.

All characterizations indicated the successful modification of two adsorbents and the interaction between adsorbents with the target molecule of TCs by FTIR, XRD, TEM, SEM and Zeta potential was presented in Figure 16. The possible interaction was a hydrogen bond because the FTIR spectra of the adsorbent medicated were changed by the hydroxyl groups of CSM, the particles synthesized and TCs. The π–π interaction between the TCs and the particles synthesized structure from the aromatic ring. Lastly, the electrostatic interaction was caused by the anionic structure of CSM and the particles synthesized, while the TC structure was cationic (zwitterionic form). Figure 16 provides a graphitic overview of the extraction mechanism involving biosorbent modification and TCs.

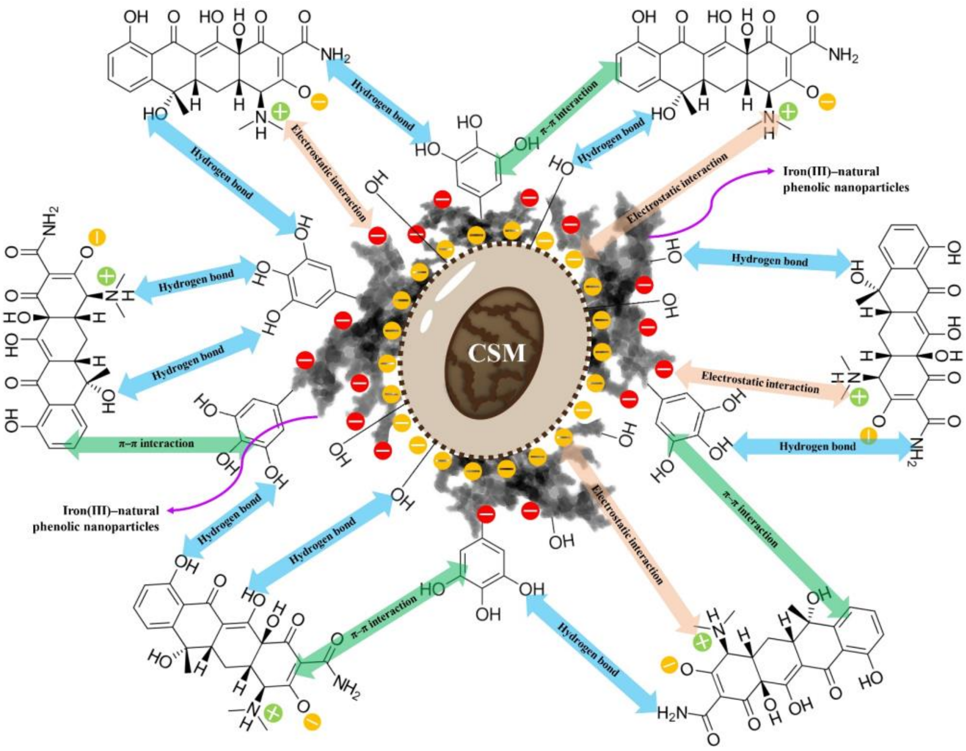

Figure 16 The illustration of interaction mechanism of CSM modified by iron(III)-natural reagent particles with TCs after extraction.

4.2 Optimization of DSPE using biosorbent

4.2.1) Ability of using chia seed mucilage synergistic with iron(III)-natural phenolic particles as sorbent

To explore the best adsorption efficiency, biosorbents were investigated under three conditions. In the first condition, only the chia seed mucilage was used in the extraction, and it was found that there was a little bit of adsorption for TCs when compared with the second condition. Iron(III)-natural reagent particles (second condition) provided higher adsorption efficiency because they were smaller, had a higher surface area, and were porous adsorbents than chia seed mucilage, which was not porous. Consequently, chia seed mucilage was synergized with iron(III)-natural reagent particles via an *in-situ* formation for DSPE procedure to obtain the best adsorption efficiency, and the results showed more impressive performance of TCs extraction in the last condition than before TCs extraction (STD), as shown in Figure 17.

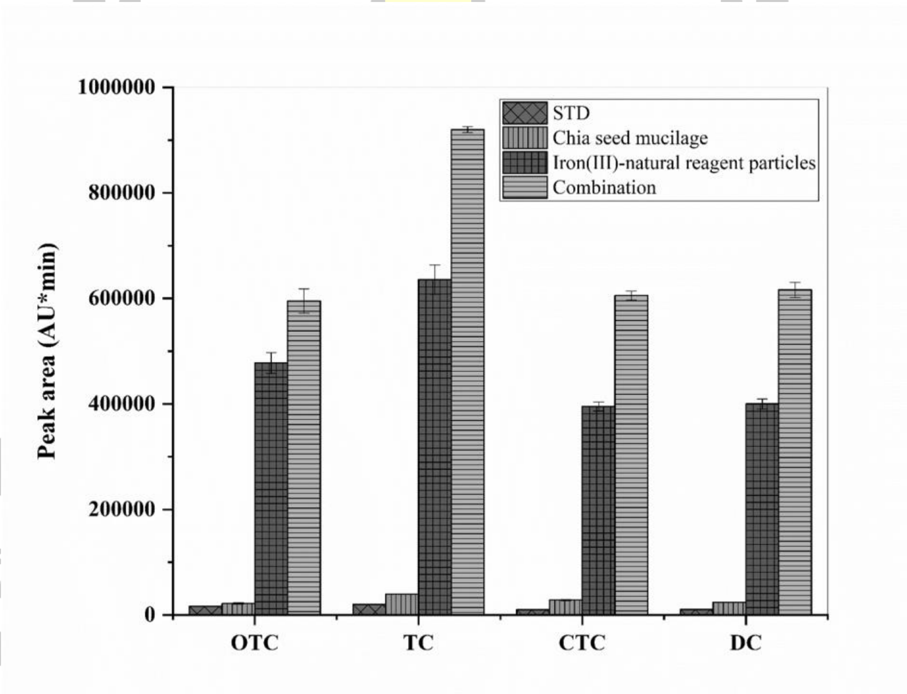

Figure 17 Selection of adsorbent type for the efficiency extraction to TCs analysis.

4.2.2) Natural phenolic reagent

4.2.2.1) Effect of suitable source of natural phenolic reagent

The effect of suitable sources of natural phenolic reagents was studied by using bark, leaves and pods of PP. It is well known that phenolic compounds have a high molecular weight and many hydroxyl groups [32]. Consequently, enhancing interaction with various antibiotics, the results showed that natural reagents from bark provided the best performance of adsorption and obtained the highest peak area and enrichment ratio in Figure 18.

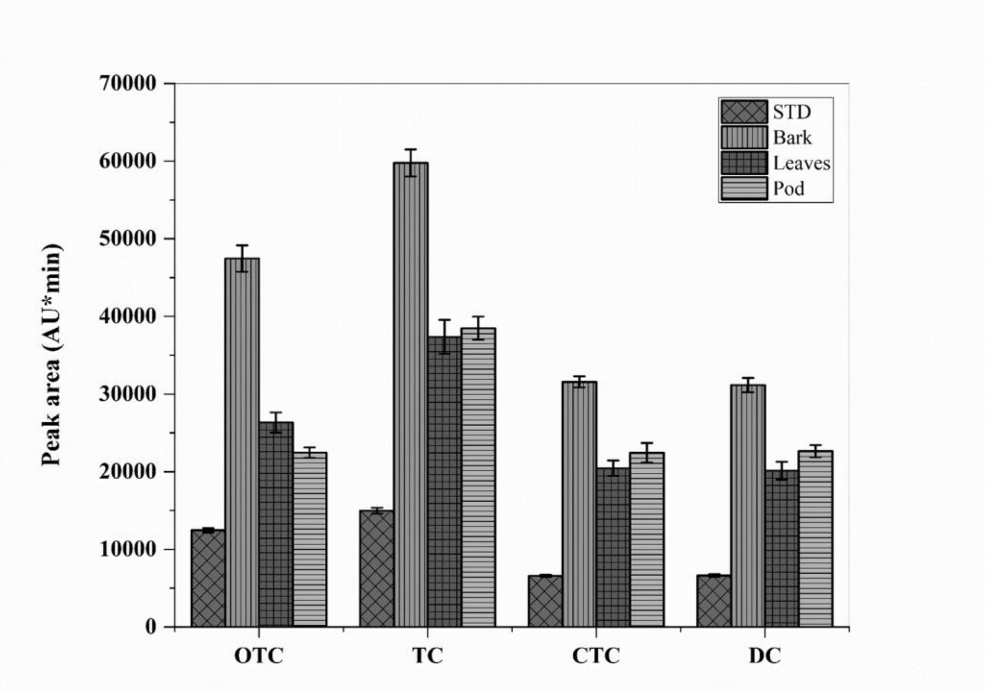

Figure 18 Natural phenolic reagent of for the optimized condition in DSPE extraction.

4.2.2.2) Effect of amount of natural reagent powder

The amount of natural reagent was investigated at 0.5−2.0 g (in 100 mL). As it can be seen in Figure 19, the best ratio was presented for 1.0 g after that, the efficiency of extraction was depreciated, and the tendencies of bark powder were constant because the ratio of particles formed between the phenolic compound and iron(III) was not appropriate. Therefore, we selected 1.0 g of PP bark powder for preconcentration in the extraction process.

4.2.2.3) Effect of natural reagent volume

Bark reagent, rich phenolic compounds such as tannin, volume was optimized as compositions for iron(III)-natural reagent particles and investigated in the range of 0−400 µL in Figure 20. The extraction efficiency increased with increasing natural reagent volume from 100−300 µL and then decreased. The volumes of 200 and 300 µL of bark reagent were considered for preconcentration in extraction because they provided good extraction efficiency, and both volumes showed no significant difference. Hence, 200 µL was chosen as the appropriate volume for DSPE.

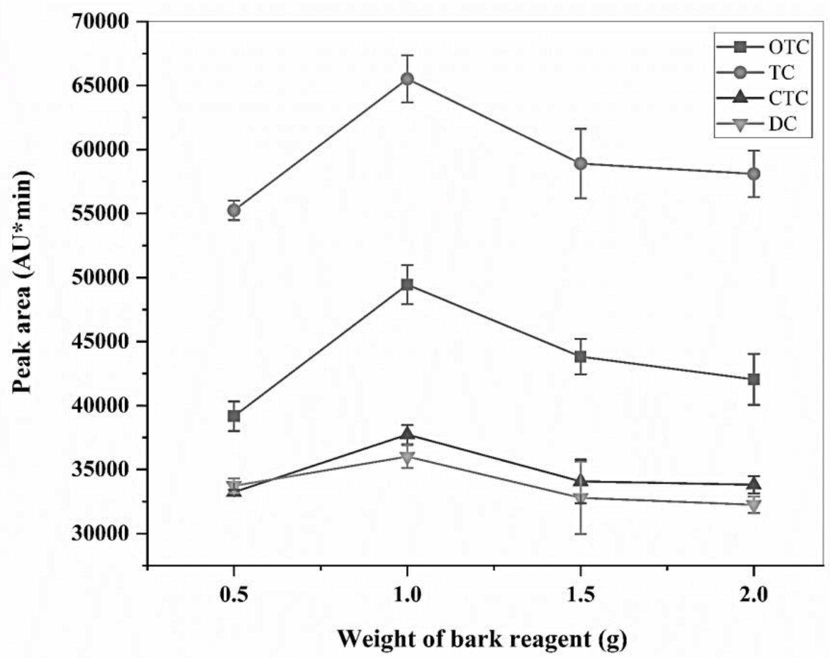

Figure 19 Amount of bark reagent for the optimized condition in DSPE extraction.

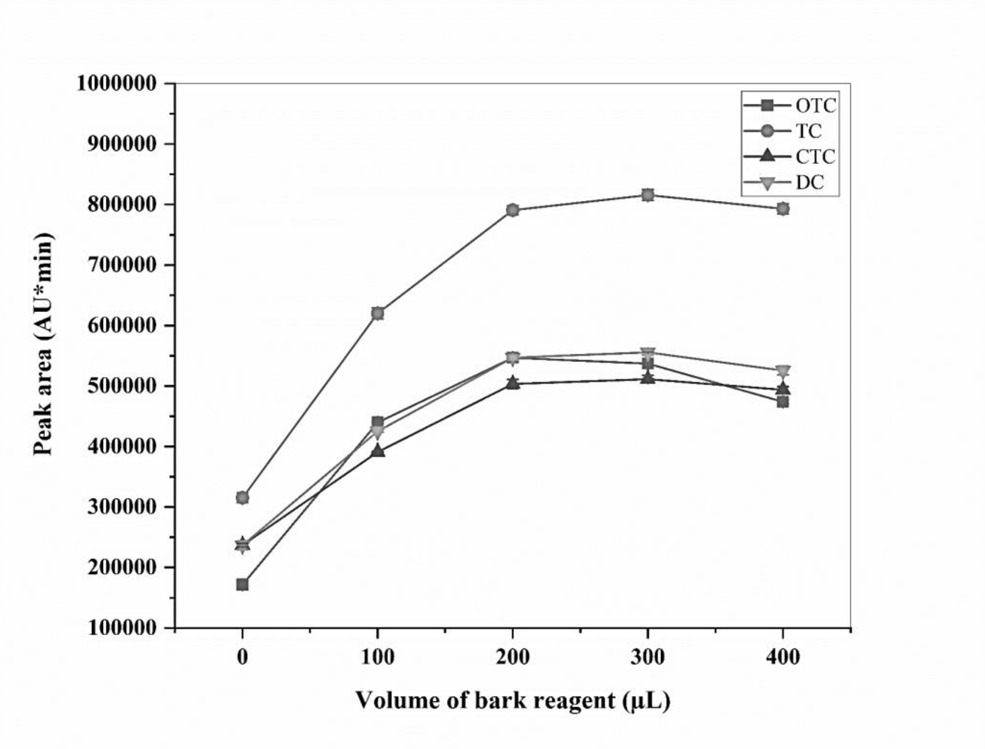

Figure 20 Volume of bark reagent for the optimized condition in DSPE extraction.

4.2.3) Effect of amount of chia seed as biosorbent

One of the important parameters for preconcentration in extraction is the amount of chia seed biosorbent that interacts with the sample solution containing the analytes. In this work, the amount of chia seed biosorbent was studied in the range of 0−5.0 mg, and the results showed that the addition of biosorbent 1.0 mg assisted in increasing the surface area for interaction with analytes in an appropriate amount of mucilage from the biosorbent. After that, the extraction efficiency decreased because excessive mucilage affected the eluted analytes due to the limited amount of desorption solvent. Therefore, 1.0 mg of biosorbent was selected because it provided the highest extraction efficiency, as shown in Figure 21.

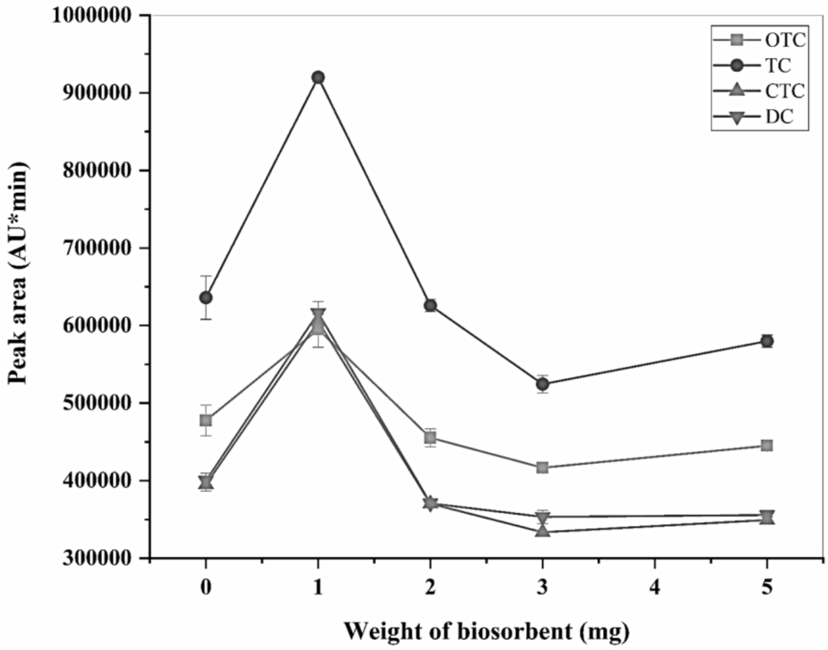

Figure 21 Amount of chia seed as biosorbent for the optimized condition in DSPE extraction.

4.2.4) Effect of iron(III) concentration

The construction of iron(III)-natural reagent particles is very prominent for preconcentration in extraction. Iron(III), one of the compositions, was assayed at a at a concentration in the range of 0.0–2.0 mg L^{-1}. As it can be seen in Figure 22, extraction efficiency was more expanded at 1.0 mg L^{-1} after that, reducing and approaching a stable condition. Consequently, iron(III) concentration for extraction and adsorption efficiency was obtained at 1.0 mg L^{-1} because the amount of particles was suitable for the desorption solvent volume.

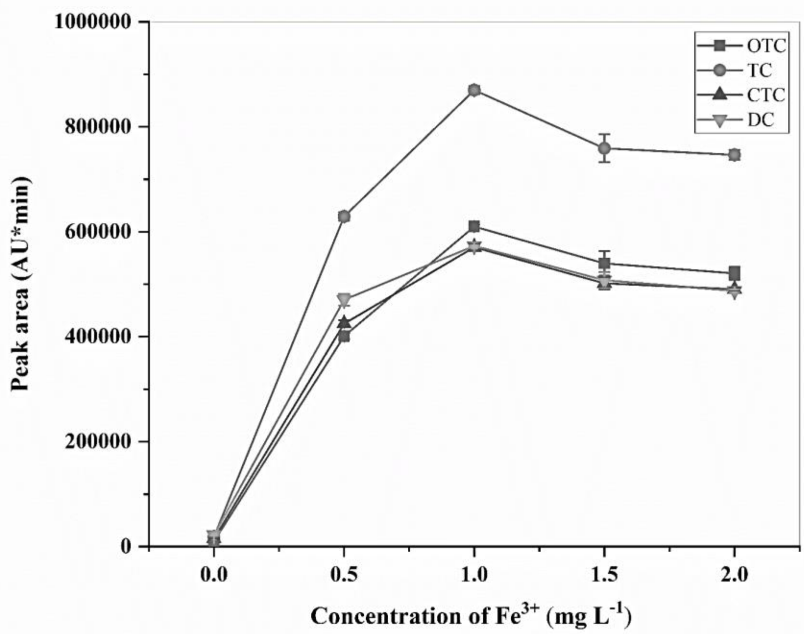

Figure 22 Iron(III) concentration for the optimized condition in DSPE extraction.

4.2.5) Effect of pH

The effect of pH is a very important parameter for the extraction efficiency of the proposed method. The pH of different sample solutions affects the coordination state of iron(III)-natural reagent particles, analyte forms (ionic or neutral), and the surface charge of the biosorbent for the physical and chemical interaction. This parameter was examined in the range of 3.0−7.0 using a 0.05 mol L^{-1} acetate buffer solution. According to the obtained results, pH 4.0 had the highest extraction efficiency, then decreased until pH 6.0 and approached a stable condition at pH 7.0 because the precipitate of $Fe(OH)_3$ can be formed at a pH greater than 5.5 [159] in Figure 23. Furthermore, iron(III)-natural reagent complexes could be formed +1, −1 and −3 at pH<2, pH 3 to 6, and pH>7.0, respectively [156], and TCs at pH between 3.3 and 9.0 are presented in zwitterion (neutral) because the pKa1 are 3.0−3.3 (3.2 for OTC, 3.3 for TC and CTC, and 3.0 for DC) [26]. Therefore, pH 4.0 was selected for the construction of iron(III)-natural reagent complexes because it provided the maximum extraction efficiency for TCs.

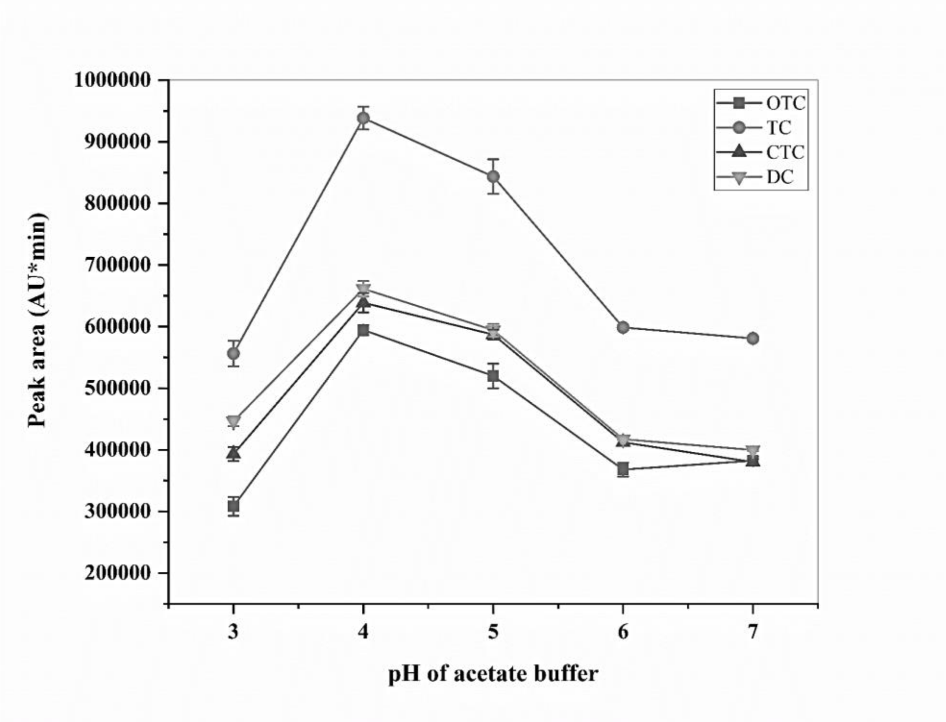

Figure 23 Effect of pH for the optimized condition in DSPE extraction.

4.2.6) Effect of buffer concentration

The pH 4.0 of the acetate buffer solution was studied at concentrations in the range of 0–0.035 mol L^{-1} to find the optimized condition for the formation of iron(III)-natural reagent particles as the results presented in Figure 24. The highest extraction efficiency was achieved at 0.015 mol L^{-1} after that, the signal was decreased because the high concentration was effected to equilibrium with the interaction between iron(III) and bark reagent solutions. The step of phase separation was difficult for the excessively formed particles. Therefore, 0.015 mol L^{-1} acetate buffer (pH 4.0) was selected as the appropriate condition for the formation of iron(III)-natural reagent particles to extract TCs.

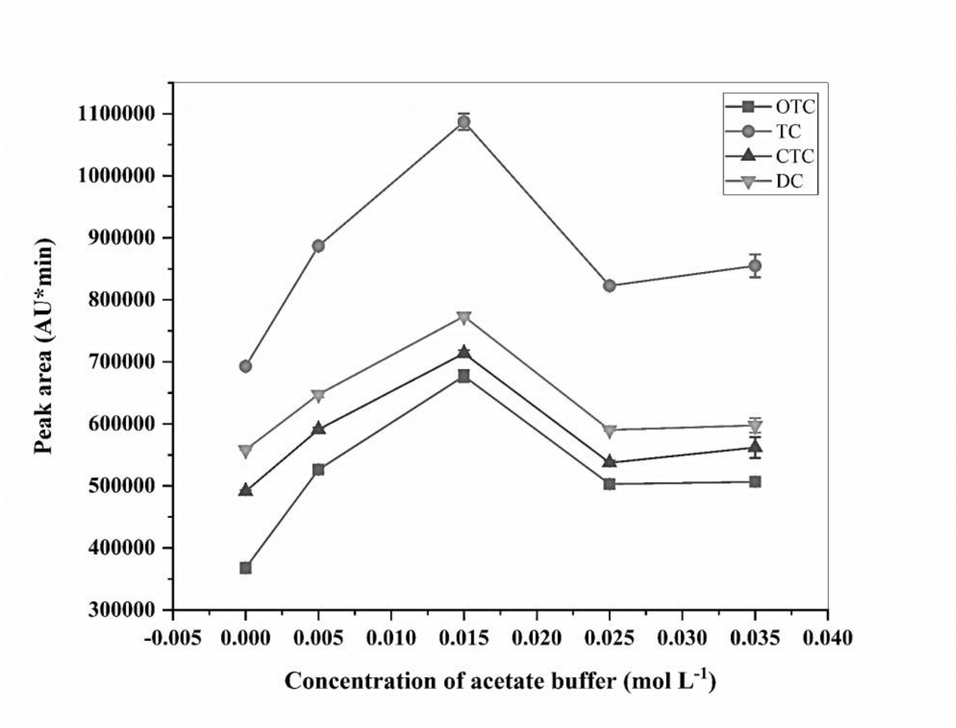

Figure 24 Buffer concentration for the optimized condition in DSPE extraction.

4.2.7) Effect of temperature and incubation time

Chia seeds mucilage-secreting cells can cover the whole seed. These cells are part of the seed fruit that get wet with a solvent or deionized water [157]. The mucilage swelling duration is higher at higher temperatures [160]. In this study, incubation temperature under ultrasound waves was considered to reduce the swelling duration of mucilage, and the optimum swelling duration time was examined. The temperature of ultrasonic incubations was investigated from 30 to 60 ºC, as shown in Figure 25. At 30 ºC provided the most extraction enrichment and continually decreased to 60 ºC because TCs target molecule was degenerate to other forms at high temperature, and mucilage, which was abundant, may affect the dilution effect of extraction. While incubation time was investigated at the durations of 1−15 min and 5 min showed higher extraction efficiency because ultrasound waves were speeding up the reaction of mass transfer. On the other hand, the complex polysaccharides are soluble in water and mucilage may dissolve in the aqueous phase after incubation for a long time [153]. Consequently, ultrasonic incubation at room temperature for 5 min was selected as the optimum swelling duration.

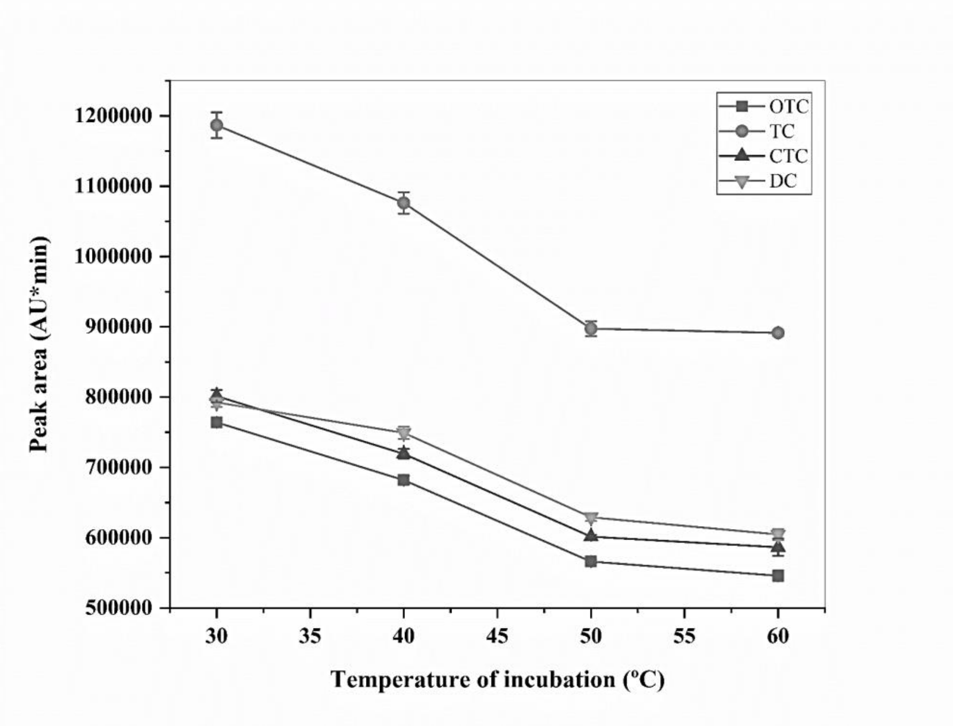

Figure 25 Effect of temperature of incubation for the optimized condition in DSPE extraction.

4.2.8) Effect of vortex time for DSPE

To enrich extraction efficiency and mix-well sample solution after adding all chemical orders, the effect of vortex time was studied at 0−240 s, and the results found that extraction efficiency had no significant difference in the range of 0−120 s. Then, the extraction efficiency dropped to 240 s. Vortex time was chosen at 30 s because the signal at 0 s (without vortex) obtained noise in the baseline of chromatogram which may interfere with the signal of TCs target molecule, while at 60 s, the noise of the baseline decreased.

4.2.9) Effect of speed and time centrifugation

After the extraction procedure, one of the essential parameters that helps to separate the biosorbents from the aqueous phase is centrifugation. Centrifugation speed was varied in the range of 3000−6000 rpm. The extraction efficiency was increased to 4000 rpm, approached stability at 5000 rpm for OTC, TC and CTC, and then slightly enriched again at 6000 rpm for all TCs. The centrifugation speed of DC since 4000−6000 rpm was increased continuously. The best choice of DSPE at

6000 rpm was considered the appropriate condition to make sure complete separation of biosorbent from solution in Figure 26. Moreover, the centrifugation time was tested at 5−20 min to support the separation phase. The results of TC, CTC and DC were presented as extraction efficiency between 15 and 20 min that provided the highest peak area, while OTC was a stable peak area. Therefore, 15 min was selected for the centrifugation time because there is no significant difference from 20 min and it can save the extraction time.

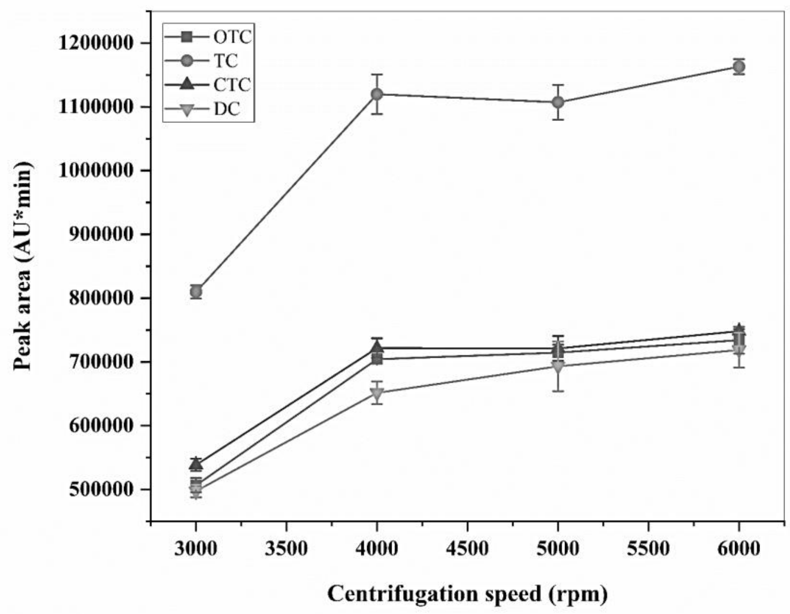

Figure 26 Effect of centrifugation speed for the optimized condition in DSPE extraction.

4.2.10) Effect of concentration and volume of desorption solvent

Normally, the type and volume of desorption solvent are the most important parameters because the sensitivity of determination and the elution efficiency are directly affected [161]. In this work, the type of desorption solvent was not studied, and TFA in ACN was chosen to elute TCs target molecule because the mobile phases were mixed by TFA. The desorption solvents were carried out by 0−9% TFA in ACN in Figure 27. The results indicated that the existence of TFA increased extraction efficiency better from 0−5% and then decreased at 9% TFA in ACN. Hence, 5% TFA in ACN was achieved as a suitable concentration for extraction because it obtained the highest extraction efficiency of TCs from desorption. To consume the minimized desorption solvent, the volume of 5% TFA in ACN was investigated for a duration of 30−150 μL. Results indicated that volume of solvent affected the extraction efficiency since 30−150 μL by dilution effect [162] in Figure 28. On the other hand, the appropriate desorption solvent volume should be enough for TCs elution. Consequently, 50 μL of 5% TFA in ACN was selected because it was provided high peak areas and high extraction efficiency for TCs analysis.

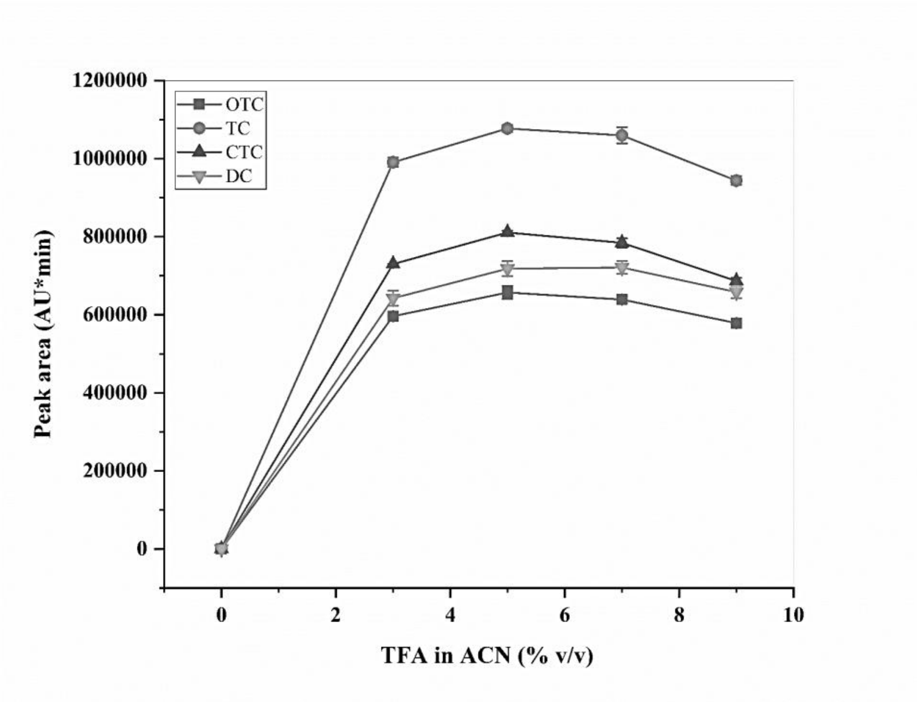

Figure 27 Concentration of desorption solvent for the optimized condition in DSPE extraction.

4.2.11) Effect of vortex time for desorption TCs analytes for solid sorbent

Desorption time was tested at 30 to 240 s, and it was found that the desorption time ranges at 30 to 120 s of extraction efficiency were increased for all TCs. After that, extraction efficiency was deceased until 240 s; the signal showed no significant difference for all TCs over 240 s. Hence, the vortex time of desorption was optimized at 120 s because it provided good extraction efficiency and short-time extraction.

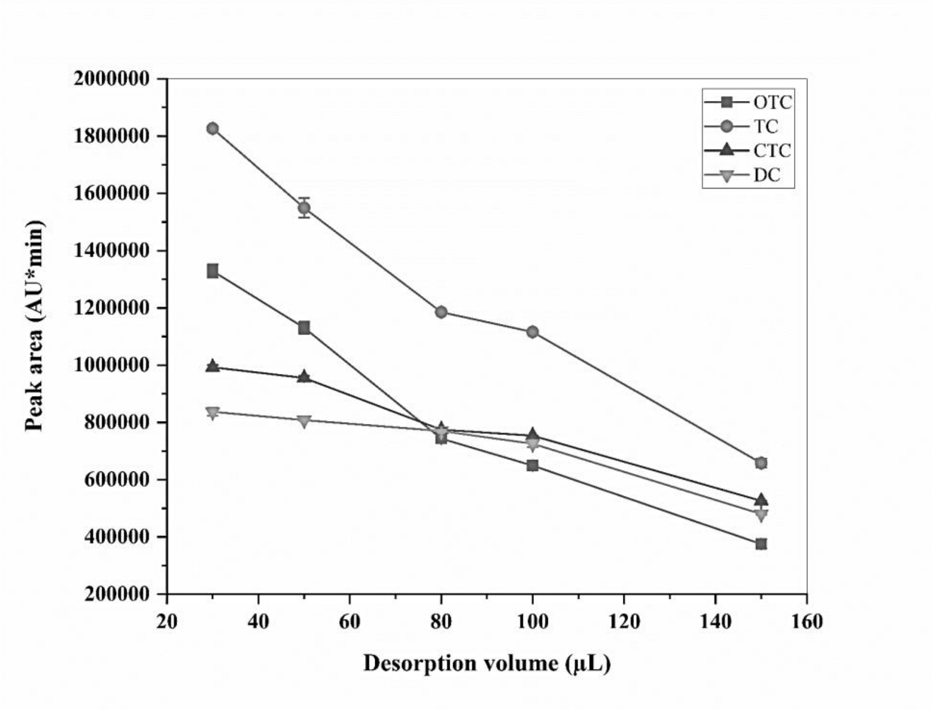

Figure 28 Desorption volume for the optimized condition in DSPE extraction.

Therefore, the optimun conditions for DSPE based on natural biosorbent synergistic with iron(III)-natural reagent particles for TCs enrichment are presented in Table 9.

Table 9 The optimized conditions of DSPE using biosorbent for the determination of tetracyclines.

Parameters	Optimum conditions
1. Natural phenolic reagent from *Peltophorum pterocarpum* (Suitable source, Amount and Volume)	Bark, 1.00 g and 200 µL
2. Amount of chia seed	0.0010 g
3. Iron(III) concentration	1.0 mg L^{-1}
4. pH	4.0
5. Buffer concentration	0.015 mol L^{-1}
6. Temperature and incubation time	30 ºC and 5 min
7. Vortex time during extraction	30 s
8. Speed and time centrifugation	6000 rpm and 15 min
9. Concentration and volume of desorption solvent	5%v/v of TFA in ACN and 50 µL
10. Desorption time	120 s

4.3 Method Validation

The analytical characteristics under the optimization of the method were evaluated by linear range, the limits of detections (LODs), the limits of quantification (LOQs), enrichment factors (EFs), and precision (intra-day and inter-day) based on the relative standard deviation (RSD%). The LODs and LOQs were calculated as 3S/N and 10S/N, where S and N are defined as the signal of the analytes and the noise of the blank, respectively. EFs were calculated from the ratio of calibration curve slopes before and after preconcentration by the development method. The matrix-matched calibration of mixed standard TCs at the ranges 2.0−500.0 µg L^{-1} presented good linearity with a coefficient of determination (r^2) greater than 0.9957. The results revealed that LODs and LOQs values were found to be 0.7−2.0 µg L^{-1} and 2.0−7.0 µg L^{-1}, respectively. The EFs of the development method were quite satisfactory in the ranges 55−105. The intra-day and inter-day precision values at three concentration levels (10.0, 50.0, and 100.0 µg L^{-1}) were lower than 9.1% and 6.9%, respectively.

The matrix effect (ME%) was evaluated in the range of 70–79%, indicating that matrix was quite influent on TCs extraction. So, the matrix-matched calibration method was applied to detect quantitative TCs in real samples. The summary of the obtained data and chromatogram are shown in Table 10 and Figure 29, respectively.

4.4 Real samples analysis

The accuracy and application of the developed DSPE-CSM-HPLC-UV method were confirmed by evaluating ten different milk samples including four pasteurized milks, one sterilized milk and five UHT milk samples. We prepared each milk sample according to the sample preparation procedures described in Section 3.8 and analyzed it using the developed method in Section 3.5. Three different concentrations were spiked into the sample (20.0, 50.0, and 100.0 µg L^{-1} for each of the TCs). The results found that TC residues did not exist for all milk samples, as shown in Table 11. The recoveries of TC residues in the analyzed samples were found in the range of 80.2−109.4%, proving that the developed method showed good accuracy and was applied to TC residues in milk samples. The chromatogram of milk samples spiked with standard TCs is presented in Figure 30.

4.5 Comparison the proposed method with the previous research

The proposed method was compared with the others published to assess its potential and analytical performance. The other published reports analyzes TCs residue in food samples by using HPLC, as presented in Table 12. According to the results, the linearity range, LOD and LOQ were lower than those of the MIP adsorbent with SPE. Moreover, we found that the LOD and LOQ (each of the analytes) in our method are better than the previous research. However, the temperature-sensitive combined ABS extraction by PPG_{1000}-based magnetic IL as an adsorbent showed better method validation than our method. However, considering the simplicity, type and volume of the solvent and its low cost, it is indicated that our method approach to eco-friendly and green adsorbents has a higher sensitivity than the others published.

Table 10 Analytical performance for the proposed method.

Analyte	Linear range ($\mu g\ L^{-1}$)	[a]LOD ($\mu g\ L^{-1}$)	[b]LOQ ($\mu g\ L^{-1}$)	[c]EF	Precision (%[d]RSD) 10.0 $\mu g\ L^{-1}$ Intra-day (n=5)		Inter-day (n=5×3)		Precision (%[d]RSD) 50.0 $\mu g\ L^{-1}$ Intra-day (n=5)		Inter-day (n=5×3)		Precision (%[d]RSD) 100.0 $\mu g\ L^{-1}$ Intra-day (n=5)		Inter-day (n=5×3)	
					Peak area	t_R	Peak area	t_R	Peak area	t_R	Peak area	t_R	Peak area	t_R	Peak area	t_R
OTC	2.0-500.0	0.7	2.0	55	9.1	0.1	6.0	0.3	3.9	0.2	6.6	0.5	5.8	0.2	2.3	0.3
TC	7.0-500.0	2.0	7.0	63	6.3	0.1	6.3	0.4	2.2	0.4	6.2	0.6	4.0	0.2	3.4	0.5
CTC	2.0-500.0	0.7	2.0	105	8.2	0.2	3.2	0.3	2.6	1.5	3.0	0.2	5.6	0.3	1.3	0.5
DC	2.0-500.0	0.7	2.0	80	8.0	0.2	6.9	0.4	6.6	1.3	3.0	0.3	3.5	0.3	3.0	0.7

[a] LOD: Limits of detection

[b] LOQ: Limits of quantification

[c] EF: Enrichment factors

[d] RSD: Relative standard deviations

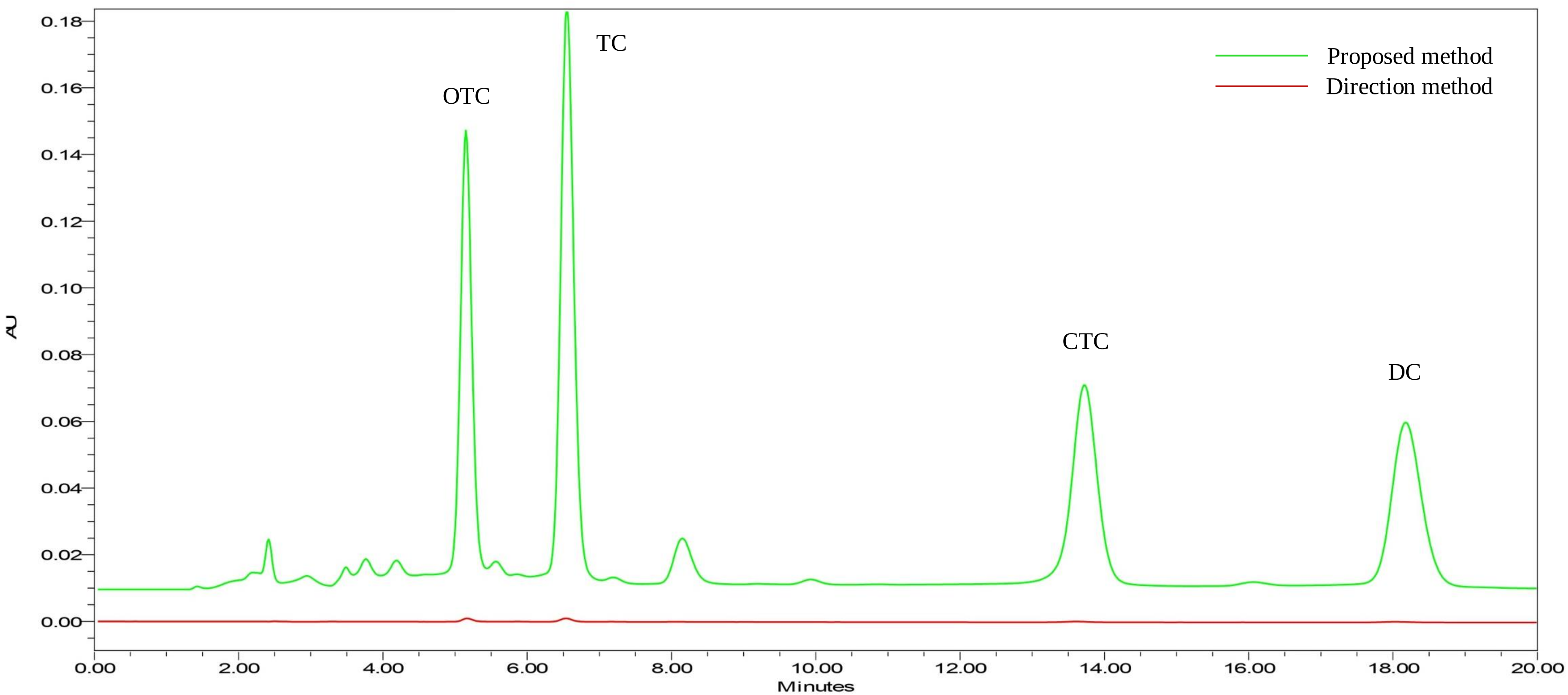

Figure 29 Chromatogram of comparison between the direct method and the proposed method with standard TCs at 500 μg L^{-1} under the optimum conditions of DSPE.

Table 11 Recovery of the proposed method for determination of TCs in milk samples.

Sample	Spiked (µg L^{-1})	OTC			TC			CTC			DC		
		Found (µg L^{-1})	Recovery (%)	%RSD	Found (µg L^{-1})	Recovery (%)	%RSD	Found (µg L^{-1})	Recovery (%)	%RSD	Found (µg L^{-1})	Recovery (%)	%RSD
Pasteurized milk 1	0	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
	20.0	19.28	96.4	0.7	21.08	105.4	3.2	20.29	101.5	2.3	21.85	109.3	1.9
	50.0	43.67	87.3	4.0	62.62	93.2	4.1	43.87	87.7	2.3	54.09	108.2	4.4
	100.0	81.70	81.7	1.3	85.35	85.4	0.1	82.17	82.2	0.7	104.51	104.5	2.7
Pasteurized milk 2	0	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
	20.0	20.50	102.5	7.2	20.97	104.8	3.3	20.56	102.8	11.8	21.59	107.9	2.0
	50.0	45.50	91.0	8.1	47.56	95.1	8.2	42.59	85.2	9.8	53.12	106.2	0.3
	100.0	83.62	83.6	1.3	85.49	85.5	1.4	80.22	80.2	1.5	102.74	102.7	4.6
Pasteurized milk 3	0	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
	20.0	21.13	105.6	3.4	21.61	108.0	2.6	19.61	98.0	1.9	20.89	104.4	1.8
	50.0	49.34	98.7	1.2	50.25	100.5	0.8	48.00	96.0	1.1	53.42	106.8	1.4
	100.0	91.85	91.9	2.1	100.51	100.5	0.6	91.34	91.3	1.2	106.41	106.4	0.4
Pasteurized milk 4	0	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
	20.0	17.70	88.5	8.1	20.41	102.1	4.0	18.48	92.4	1.0	21.13	105.6	6.5
	50.0	41.68	83.3	1.7	45.98	92.0	3.2	41.30	82.6	2.7	47.26	94.9	2.9
	100.0	93.04	93.0	0.7	102.40	102.4	1.9	89.14	89.1	2.7	104.07	104.1	0.7
Sterilized milk 1	0	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
	20.0	19.84	99.2	5.4	20.90	104.5	6.9	20.14	100.7	3.7	21.13	105.6	3.7
	50.0	49.26	98.5	0.9	49.25	98.5	0.9	48.14	96.3	1.2	51.47	102.9	1.6
	100.0	92.00	92.0	1.4	99.38	99.4	1.2	94.88	94.9	1.6	106.16	106.2	1.3

N.D. = Not detected

Table 11 (continued)

Sample	Spiked (µg L^{-1})	OTC			TC			CTC			DC		
		Found (µg L^{-1})	Recovery (%)	%RSD	Found (µg L^{-1})	Recovery (%)	%RSD	Found (µg L^{-1})	Recovery (%)	%RSD	Found (µg L^{-1})	Recovery (%)	%RSD
UHT milk 1	0	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
	20.0	20.97	104.8	2.3	21.44	107.2	3.3	20.80	104.0	9.1	21.88	109.4	1.1
	50.0	45.16	90.4	1.0	48.95	97.9	1.1.	43.05	86.1	1.3	53.39	106.8	1.1
	100.0	82.19	82.2	5.9	86.56	86.6	0.4	83.97	84.0	1.4	105.92	105.9	1.7
UHT milk 2	0	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
	20.0	19.72	98.6	3.0	20.33	101.6	6.8	19.80	99.0	7.5	21.13	105.9	4.0
	50.0	44.30	88.6	3.7	48.63	97.3	0.3	46.89	93.8	1.1	53.52	107.0	1.7
	100.0	91.80	91.8	1.0	94.23	94.2	0.5	93.00	93.0	1.2	100.23	100.2	0.4
UHT milk 3	0	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
	20.0	18.65	93.2	4.2	20.21	101.1	2.0	18.41	92.1	2.8	18.45	92.2	0.4
	50.0	41.53	83.1	3.0	45.20	90.4	1.8	46.90	93.8	2.0	49.25	98.2	1.3
	100.0	83.79	83.8	1.4	86.14	86.1	1.4	92.70	92.7	2.4	103.82	103.8	0.4
UHT milk 4	0	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
	20.0	17.70	88.5	1.7	19.55	97.7	5.0	18.77	93.8	1.6	21.13	105.6	3.5
	50.0	42.77	85.5	2.6	47.83	95.7	2.6	41.41	82.8	1.1	46.24	92.5	1.6
	100.0	81.87	81.9	0.8	90.54	90.5	0.8	81.78	81.8	1.3	105.40	105.4	1.6
UHT milk 5	0	N.D.	-	-	N.D.	-	-	N.D.	-	-	N.D.	-	-
	20.0	16.82	84.1	6.2	20.95	104.8	9.5	18.73	93.7	3.8	16.98	84.9	4.8
	50.0	42.60	85.2	2.1	48.27	96.5	1.5	42.18	84.4	0.8	47.82	95.6	1.1
	100.0	81.60	81.6	0.7	81.62	81.6	2.1	86.05	86.1	1.2	101.02	101.0	1.7

N.D. = Not detected

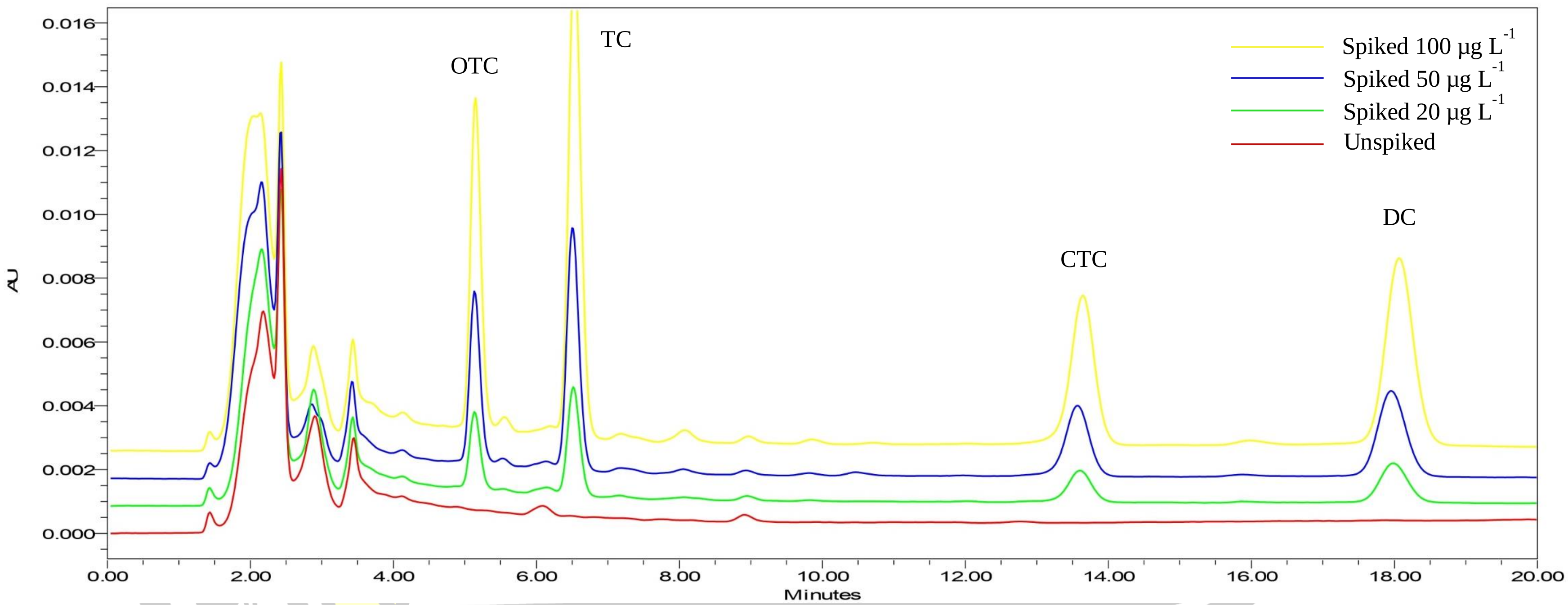

Figure 30 Chromatogram of milk sample spiked with standard TCs at 20, 50 and 100 µg L^{-1} under the optimum conditions of DSPE.

Table 12 Comparison of the proposed method and the previously reported research for determination of TCs in real samples by HPLC.

Adsorbent	Sample	Extraction method	Analytical technique	Linear range	[a] LOD	[b] LOQ	Recovery	[c] RSD	Ref
[d] MIP	Milk, egg and pork	[e] SPE	OTC	100–1000 ng mL^{-1}	20–40 ng mL^{-1}	50–80 ng mL^{-1}	74.00–93.00%	<4.20%	[138]
			TC	100–1000 ng mL^{-1}	20–40 ng mL^{-1}	50–80 ng mL^{-1}			
			CTC	100–1000 ng mL^{-1}	20–40 ng mL^{-1}	50–80 ng mL^{-1}			
			DC	100–1000 ng mL^{-1}	20–40 ng mL^{-1}	50–80 ng mL^{-1}			
[f] mNi@N-GrT	Milk	[g] MSPE	OTC	5.00–250 ng mL^{-1}	2.31 ng mL^{-1}	7.62 ng mL^{-1}	91.60–109.70%	<7.25%	[163]
			TC	5.00–250 ng mL^{-1}	1.29 ng mL^{-1}	4.26 ng mL^{-1}			
			CTC	5.00–250 ng mL^{-1}	1.31 ng mL^{-1}	4.32 ng mL^{-1}			
[h] PAN@COF-SCU1	Grass carp and duck	[i] PT-SPE	OTC	4.00–70 ng mL^{-1}	0.60 ng mL^{-1}	2.00 ng mL^{-1}	84.02–113.5%	<8.46%	[164]
			TC	4.00–70 ng mL^{-1}	0.60 ng mL^{-1}	2.00 ng mL^{-1}			
			CTC	4.00–70 ng mL^{-1}	3.00 ng mL^{-1}	10.00 ng mL^{-1}			
[j] PPG_{1000}-based magnetic IL	Milk	[k] Temperature-sensitive combined ABS	OTC	0.25–300 ng mL^{-1}	0.031–0.033 ng mL^{-1}	0.103–0.110 ng mL^{-1}	94.28–99.76%	<3.97%	[165]
			TC	0.25–300 ng mL^{-1}	0.035–0.036 ng mL^{-1}	0.117–0.120 ng mL^{-1}			
			CTC	0.25–300 ng mL^{-1}	0.065–0.067 ng mL^{-1}	0.217–0.223 ng mL^{-1}			
			DC	0.25–300 ng mL^{-1}	0.058–0.059 ng mL^{-1}	0.193–0.197 ng mL^{-1}			
[l] $Fe_3O_4@SiO_2$ MNG	Milk, egg, and honey	[m] DES-UA-DμSPE	OTC	2.50–750 μg L^{-1}	0.85 μg L^{-1}	2.74 μg L^{-1}	95.20–99.30%	<3.50%	[166]
			TC	1.50–500 μg L^{-1}	0.47 μg L^{-1}	1.61 μg L^{-1}			
			CTC	2.00–750 μg L^{-1}	0.66 μg L^{-1}	2.23 μg L^{-1}			
			DC	2.50–750 μg L^{-1}	0.81 μg L^{-1}	2.66 μg L^{-1}			
[n] CSM-Fe-NP	Milk	[o] DSPE	OTC	2.0-500.0 μg L^{-1}	0.7 μg L^{-1}	2.0 μg L^{-1}	80.2–109.4%	<9.1%	This work
			TC	7.0-500.0 μg L^{-1}	2.0 μg L^{-1}	7.0 μg L^{-1}			
			CTC	2.0-500.0 μg L^{-1}	0.7 μg L^{-1}	2.0 μg L^{-1}			
			DC	2.0-500.0 μg L^{-1}	0.7 μg L^{-1}	2.0 μg L^{-1}			

[a] LOD: Limits of detection

[b] LOQ: Limits of quantification

[c] RSD: Relative standard deviations

[d] MIP: Molecularly imprinted polymer

[e] SPE: Solid phase extraction

[f] mNi@N-GrT: Magnetic nitrogen doped graphene tube

[g] MSPE: Magnetic solid-phase extraction

[h] PAN@COF-SCU1: Polyacrylonitrile@Covalent organic framework-SCU stands for Sichuan University

[i] PT-SPE: Pipette tip solid-phase extraction

[j] PPG_{1000}-based magnetic IL: PPG_{1000} based magnetic ionic liquid

[k] Temperature-sensitive combined ABS: Temperature-sensitive combined aqueous biphasic system

[l] $Fe_3O_4@SiO_2$ MNG: $Fe_3O_4@SiO_2$ magnetic nano gel

[m] DES-UA-DμSPE: Deep eutectic solvent and ultrasound-assisted dispersive micro-solid phase extraction magnetic

[n] CSM-Fe-NP : Chia seed mucilage combined with iron(III)-natural phenolic nanoparticles

[o] DSPE : Dispersive solid phase extraction

CHAPTER V
CONCLUSION

In this research work, chia seed mucilage combined with iron(III)-natural reagent particles as a biosorbent was developed for the determination of tetracyclines by dispersive solid phase extraction and HPLC-UV analysis. Chia seed and reagents from the *Peltophorum pterocarpum* tree were available as natural materials and operated as biosorbents by in-situ processes. These biosorbents was the ability to adsorb the target analytes because of the overabundance of functional groups such as hydroxyl for the hydrogen bond and conjugation of electrons for π–π interaction. Also, electrostatic interaction of biosorbents (negative) and tetracycline (zwitterion). The optimized conditions were 1.0 mg of chia seed mucilage, 1 mL of 10.0 mg L^{-1} iron(III) solution, 200 µL of natural reagent, 3 mL of 0.05 mol L^{-1} of acetate buffer pH 4, incubation for 5 min about 30 ºC, vortex for 30 s, centrifugation at 6000 rpm for 15 min, desorption by 50 µL of 5% v/v TFA in acetonitrile, and vortex for 120 s. The final volume (20 µL) was analyzed by a Purospher® STAR RP-18 endcapped column as an analytical column carried out at room temperature. Breeze software would be used for data processing. Tetracyclines would be separated using isocratic elution with 25% acetonitrile in water as the mobile phase at a flow rate of 0.7 mL/min. Sensitivity was low at 0.7−2.0 µg L^{-1} and 2.0−7.0 µg L^{-1} for limits of detections (LODs) and limits of quantification (LOQs), respectively, by still the acceptable maximum residue limits (MRLs) of tetracyclines in milk.

However, the proposed method provided good linear range, good precision, accuracy, ease of search and preparation for biosorbents, eco-friendly, inexpensive and less negative effects. Finally, this method was successfully applied for tetracycline analysis in milk samples.

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