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Analysis of tetracyclines in shrimp samples based on a two-step extraction approach prior to high-performance liquid chromatography

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Abstract: This study presents a sensitive and reliable method for determining tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC) residues in shrimp samples. A two-step process involving liquid-liquid extraction (LLE) followed by solid-phase extraction (SPE) was developed prior to HPLC analysis. The target analytes were effectively extracted using EDTA/McIlvaine buffer (pH 4.0): methanol (80:20, %v/v), with subsequent clean-up using a C18 SPE cartridge. HPLC separation was conducted on a C18 column (250 mm × 4.6 mm i.d., 5 μm) at 30 °C, using 0.01 % trifluoroacetic acid (A) and acetonitrile (B) as the mobile phase. A gradient elution protocol was applied, transitioning from 85(A):15(B) %v/v to 70(A):30(B) %v/v at 7 min, with a 5 min hold, followed by adjustment to 85(A):15(B) %v/v for 13-14 min. The detection was performed using photodiode array (PDA) at 365 nm with a flow rate of 1.0 mL/min. The calibration curves exhibited good linearity within a concentration range of 0.4-6.0 μg/mL (R^2 > 0.995). The limits of detection (LOD) for TC, OTC, and CTC in shrimp were 0.034, 0.029, and 0.021 μg/mL, respectively. The limits of quantitation (LOQ) for TC, OTC, and CTC were found to be 0.114, 0.097, and 0.071 μg/mL, respectively. Recoveries of TC, OTC, and CTC from spiked shrimp samples ranged from 91.0 % to 95.5 %, 92.4 % to 97.2 %, and 93.3 % to 96.6 %, respectively. This method was successfully applied to the determination of TC, OTC, and CTC residues in shrimp samples sourced from various local markets.

Key words: tetracycline, oxytetracycline, chlortetracycline, solid-phase extraction, high-performance liquid chromatography

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1. Introduction

Antibiotics play a crucial role in animal husbandry, serving not only to treat diseases, but also to uphold overall health and stimulate growth.[1] Among these antibiotics, tetracyclines (TCs) are extensively used in veterinary practices in Thailand. This prevalence is attributed to their broad-spectrum antimicrobial activity, availability, and cost-effectiveness. However, the unauthorized administration of these antibiotics, deviations from the recommended label instructions, or inadequate withdrawal periods prior to animal slaughter can result in the persistence of residues in food samples, potentially posing adverse effects on human health.[2-5] In shrimp, residues of TC, OTC and CTC appear to be indispensable during the cultivation process because they may inhibit the protein synthesis of bacteria, thereby limiting their metabolic capacity and ultimately destroying them. The use of extremely high levels of these substances in the cultivation process leads to their contamination in shrimp.[3] Thus, the overuse or presence of TCs in animal production systems raises concerns about potential allergic reactions in sensitized individuals and their impact on the human gut microflora.[2,3] At the sub-therapeutic and therapeutic levels, these antibiotics may introduce resistant strains, alter the metabolic activity of the microflora, compromise the resistance barrier, and disrupt the ecological balance without immediate identifiable deleterious effects.[4-6] Recognizing the potential risks, regulatory authorities have established maximum residue limits (MRLs) for TCs, with a set threshold of 0.1 μg/g in muscle tissues.[7-8,22] Despite substantial research efforts dedicated to determining TC residues in shrimp samples in Thailand, some procedures often use a simple clean-up step or matrix dispersion before sensitive detection. Such a program is essential for the systematic detection of TC residues in shrimp to ensure the continued safety and integrity of the food supply chain.

Several methods have proven successful in monitoring TCs in shrimp tissue samples, employing various detection modes, such as UV-spectrophotometry, fluorescence, and mass spectrometry.[2,3,8] Generally, these methods incorporate a straightforward liquid-liquid extraction (LLE) coupled with a subsequent clean-up step using solid-phase extraction (SPE) or matrix dispersion.[5,9-13] Shrimp samples, characterized by variable matrices containing lipids, proteins, and other components, present a challenge because these co-extracted substances may disrupt the analysis.[14-19] Recognizing the need for a reliable extraction solution for this diverse group of compounds, McIlvaine buffer containing ethylenediaminetetraacetic acid (EDTA) has emerged as the most widely adopted method.[10,21,23] The effectiveness of this buffer lies in its ability to complex metal cations, ensuring a selective and efficient extraction process.[15-16,21] Although UV detectors have historically been employed in residue analysis, their limitations in sensitivity render them less desirable for accurate quantification.[18,20] However, mass spectrometry, which is highly sensitive, often incurs significant costs for operation.[17,19] In this context, high-performance liquid chromatography (HPLC) with photodiode array detection (PDA) is preferred.[26] This approach combines sensitivity with a wide scanning range, providing a robust and versatile platform for accurate detection and quantification of tetracyclines in shrimp samples. The utilization of HPLC-PDA not only ensures reliable results but also addresses the practical concerns associated with other detection methods, striking a balance between sensitivity and cost-effectiveness in residue analysis.

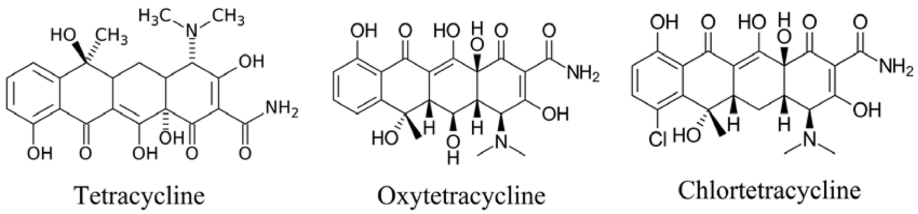

Fig. 1. Chemical structure of tetracyclines (TCs).

This study aimed to establish and validate an analytical method for the precise and sensitive determination of TC, OTC, and CTC, as shown in *Fig.* 1 for their chemical structure, in shrimp samples. The extraction protocol entails LLE utilizing EDTA/McIlvaine buffer (pH 4.0), followed by SPE employing both C18 and Oasis HLB cartridge. After extraction, the separation and detection of TCs were achieved using HPLC-PDA. The robustness and accuracy of the developed method were systematically validated to ensure reliability in the quantitative analysis.

2. Experimental

2.1. Materials and chemicals

HPLC-grade acetonitrile and methanol were obtained from RCI Labscan Limited (Thailand). Water used for HPLC purposes was purified via a Milli-Q Plus Water Purification system and further refined by filtration through a 0.45 μm filter before use. Analytical reagent (AR)-grade chemicals, including disodium hydrogen phosphate (Na_2HPO_4), formic acid, acetic acid, citric acid, and ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA), were procured from Merck (Germany). SPE cartridges, namely C18 and Oasis HLB (500 mg, 3 mL), were obtained from Waters (USA). For the preparation of standard solutions, high-purity standards of TC (95 %), OTC (96.2 %), and CTC (95 %) in hydrochloride form were purchased from Sigma-Aldrich Pvt. Ltd., USA. The use of these well-characterized standards ensured the accuracy and traceability of the analytical measurements in our study.

2.2. Methods

2.2.1. Stock solution and working solution preparation

Stock solutions were prepared by weighing 0.01 g of each standard and dissolving it in 10 mL of methanol, resulting in a concentration of 1000 μg/mL. To ensure stability, these stock solutions were carefully handled, protected from light, and stored at -20 °C. Working standard solutions with concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 4.0, 6.0, 8.0, and 10.0 μg/mL were prepared by diluting the respective stock solutions with methanol.[21,24,27] Owing to the inherent instability of the solutions at room temperature (30.0 ± 2 °C), all working solutions were prepared daily and promptly stored at 4 °C.[7,22]

2.2.2. Preparation of EDTA/McIlvaine buffer (pH 4.0): methanol

The EDTA/McIlvaine buffer (pH 4.0) was prepared through the dissolution of 14.20 g of anhydrous Na_2HPO_4, 33.60 g of Na_2EDTA and 21.0 g of citric acid monohydrate into 1.0 L of deionized water (DI water). The pH was meticulously adjusted to 4.0 using a 1.0 M citric acid solution or 1.0 M sodium hydroxide, followed by filtration through a 0.45 μm nylon filter. All buffer solutions were stored at 4 °C to maintain their stability. Additionally, various proportions of the washing solution (EDTA/McIlvaine buffer (pH 4.0): methanol) were explored, with the following ratios investigated: 60:40, 70:30, 80:20, and 90:10 (%v/v).

2.2.3. Sample collection and sample preparation

Shrimps were procured from three local markets (Ya Mo, Sa Khrok, and Pratu Phi) located in in Nakhon Ratchasima Province, Thailand. The sample bags were labeled, frozen, and brought to the laboratory under frozen conditions in a foam box containing shrimp packs. Nine shrimp samples were analyzed. The edible muscle tissues of the shrimp were chopped and immediately frozen at -20 °C until analysis. For the extraction and clean-up processes, each shrimp sample (1 kg) underwent trituration and homogenization in a blender. The homogenized material was sifted, and the resulting sample (1.0 g) was accurately weighed and placed into a glass tube. Subsequently, it was dissolved in 10 mL of EDTA/McIlvaine buffer (pH 4.0). The sample solution was centrifuged at 3,000 × g for 3 min. After filtration through a Buchner funnel, LLE was conducted using 10 mL of hexane in a separatory funnel. The aqueous phase was applied to a C18 SPE cartridge (500 mg/3 mL). Prior to use, the cartridge was conditioned with 3 mL of methanol and 3 mL of EDTA/McIlvaine buffer (pH 4.0).

Subsequently, the cartridge containing the sample was washed with 3 mL of EDTA/McIlvaine buffer (pH 4.0): methanol (80:20, v/v). The target compounds (TC, OTC, and CTC) were eluted with 3 mL of methanol. The eluate was concentrated to dryness under nitrogen at 40 °C and the resulting residue was dissolved in 1 mL of the mobile phase. The final eluate was filtered through a 0.45 μm membrane nylon filter and then injected into the HPLC system with a 10 μL of injection volume. This comprehensive sample preparation protocol ensured the extraction and concentration of TCs for subsequent accurate and reliable chromatographic analysis.

2.3. Instrumentation and HPLC procedure

The HPLC system utilized in this study was a Hitachi CM-5000 (Japan), equipped with a quaternary gradient pump, 20 μL loop injector, and PDA detector. The analytical column employed was a C18 (RP-C18) column with dimensions of 4.6 × 250 mm and a particle size of 5 μm from Cosmosil (Japan). The column temperature was maintained at 30 °C. To optimize the separation of the analyzed TCs and ensure a robust response within a reasonable runtime, various HPLC conditions were systematically adjusted. The mobile phase consisted of 0.01 % trifluoroacetic acid (TFA) (A) and acetonitrile (B), with a consistent flow rate of 0.8 ml/min. The detection was performed at a wavelength of 365 nm.[5,15,23] An absorbance maximum at 365 nm was established in this study due to the absence of matrix interference during the retention period,[23] as shown in *Fig.* S1. Gradient elution was investigated using three distinct systems, as outlined in *Table* 1. This thorough optimization process was crucial for achieving the desired separation efficiency and analytical performance for the subsequent analysis of TCs in shrimp samples.

Table 1. Systems of gradient elution for TCs analysis

Systems	Ratio of mobile phase (%v/v)		Time (min)
	0.01% TFA (A)	Acetonitrile (B)	
1	75	25	0–2
	80	20	4–6
	75	25	10
2	80	20	0–6
	75	25	8–12
	80	20	13
3	85	15	0–4
	70	30	7–12
	85	15	13–14

2.4. Method validation

2.4.1. Linearity

The method was validated based on the guidelines provided by the Association of Official Analytical Chemists (AOAC).[27] The linearity of method was assessed by constructing calibration curves using standard solutions and plotting the peak area against concentration. Calibration curves were established within the concentration range of 0.2–10.0 mg/mL.

2.4.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were calculated using the following equations: LOD = 3σ/*s* and LOQ = 10σ/*s*, where s is the standard deviation of the response and σ is the slope of TC, OTC, and CTC calibration curves. The values of σ were obtained from the peak areas of chromatograms of the spiked TCs standard at 0.4 μg/mL in shrimp sample after ten measurements.

2.4.3. Precision and accuracy

The accuracy and precision of the method were evaluated using % recovery and relative standard deviation (RSD). This was achieved by spiking three blank shrimp samples at three different concentrations (1, 6, and 10 μg/mL) and injecting them three times (n = 3) to obtain intraday results. This process was repeated over two consecutive days to assess inter-day variation (n = 3). The calculated values provided insights into the reliability and robustness of the method for the accurate determination of TCs in shrimp samples.

3. Results and Discussion

3.1. HPLC condition optimization

Various HPLC conditions were optimized to achieve

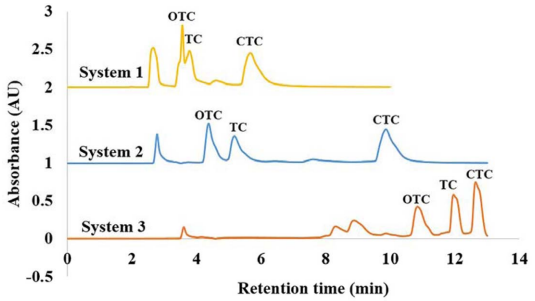

Fig. 2. Chromatogram of systems for separation of TCs at a flow rate of 0.8 mL/min.

effective separation of the analyzed TCs with the aim of achieving good resolution within a reasonable runtime. The results of the separation of TC, OTC, and CTC using gradient elution with three different systems are presented in *Fig.* 2. Suboptimal outcomes such as peak broadening and poor resolution were initially observed. However, the most favorable separation results were obtained using system 3 (*Fig.* 2). The optimized HPLC conditions involved a mobile phase composed of 0.01 % TFA (A) and acetonitrile (B). A gradient elution profile of 85(A): 15(B) %v/v was maintained from 0 to 4 min, transitioning to 70(A):30(B) %v/v at 7 min, with a subsequent hold time of 5 min. Following this, the composition was adjusted to 85(A):15(B) %v/v for the duration of 13–14 min. The flow rate was set at 0.8 mL/min, and detection was carried out using PDA at 365 nm. The obtained results demonstrated the excellent resolution of TCs, as depicted by symmetrical peaks and optimal runtime. Notably, *Fig.* 3 shows chromatograms with retention times for OTC, TC, and CTC

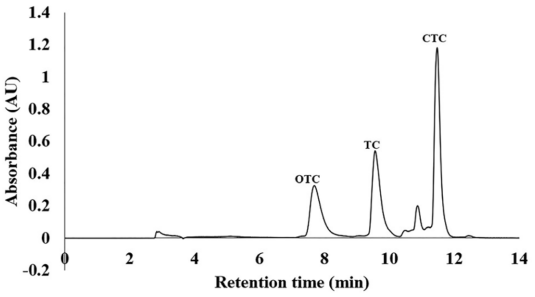

Fig. 3. HPLC chromatogram of TCs at a flow rate of 1.0 mL/min.

at 7.68, 9.56, and 11.47 min, respectively. These findings underscore the efficacy of the optimized HPLC conditions for achieving robust separation and accurate quantification of TCs in analyzed samples.

3.2. Extraction and clean-up procedure

The isolation of TCs from shrimp sample matrices presents challenges because of their affinity for sample lipids and proteins, and chelation with metal ions. Various studies have proposed the use of McIlvaine buffer solution with EDTA (pH 4.0) for LLE extraction, which enhances tetracycline recovery.[9,16-17] Additionally, different ratios of the SPE extraction solution (EDTA/McIlvaine buffer (pH 4.0): methanol) were investigated, including 60:40, 70:30, 80:20, and 90:10 (%v/v). As depicted in *Fig.* 4, optimal extraction efficacy for all three TCs was achieved at a ratio of EDTA/McIlvaine buffer (pH 4.0): methanol of 80:20 (%v/v). Thus, shrimp samples underwent LLE followed by SPE for cleanup. The analytes were extracted using EDTA/McIlvaine buffer (pH 4.0): methanol (80:20, %v/v), followed by C18 and Oasis HLB as a SPE cartridge clean-up procedure. To determine the most efficient clean-up method, two different commercial cartridges, C18 and Oasis HLB, were compared (*Fig.* 5). However, the results (*Fig.* 6) yielded very low recoveries, prompting the inclusion of LLE prior to SPE to eliminate nonpolar matrix compounds. Consequently, less interference was observed in the elution region of the analytes (*Fig.* 5(c)). Ultimately, the commercial C18 cartridge demonstrated superior recovery rates, exceeding 70 %. This cartridge was selected due to

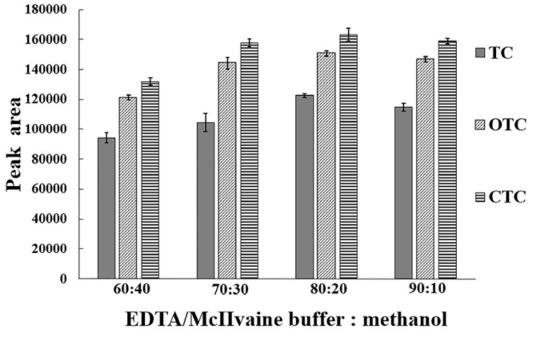

Fig. 4. Peak areas of TC, OTC, and CTC for extraction and clean-up procedure using a C18 cartridge.

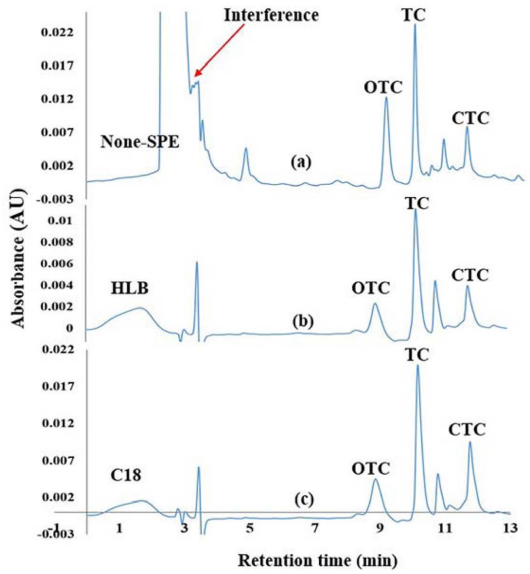

Fig. 5. Chromatograms of the extraction of homogeneous solution of shrimp sample spiked with 1 μg/mL of TCs: (a) none-SPE, (b) HLB, and (c) C18 cartridge.

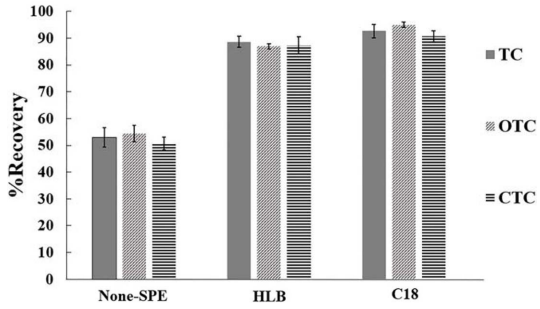

Fig. 6. Recovery of TC, OTC, and CTC for extraction and clean-up procedure using C18 and Oasis HLB cartridge.

its excellent recovery (> 90.0 %) as shown in *Fig.* 6. Moreover, a clean-up procedure was studied using both C18 and HLB cartridges, and it was clear that the two means were not significantly different at the $p > 0.05$, 95 % confidence level, as evaluated by SPSS analysis. These findings emphasize the significance of meticulous optimization and selection of extraction and clean-up methods to ensure the accurate and reliable quantification of TCs in shrimp samples.

3.3. Results of the method validation

The results of method validation are presented in *Tables* 2 and 3. Linearity was assessed based on six-point calibration curves with triple analysis.[21,27] The high correlation coefficients ($R^2 > 0.995$) indicated a strong correlation between analyte concentration and peak area within the range of 0.4–6.0 μg/mL for HPLC-PDA analyses as shown in *Fig.* S2. The LOD and LOQ values for tetracyclines in shrimp samples ranged from 0.021 to 0.034 μg/mL and from 0.071 to 0.114 μg/mL, respectively (*Table* 2). Recovery and precision were evaluated using spiked shrimp samples at levels of 1.0, 6.0, and 10.0 μg/mL. The relative recoveries for TC, OTC, and CTC for the HPLC-PDA method ranged from 91.0 % to 95.5 %, 92.4 % to 97.2 %, and 93.3 % to 96.6 %, respectively. The repeatability and within-laboratory reproducibility for TC, OTC, and CTC were less than 2.0 % at all spiked levels (*Table* 3). Precision, expressed as the relative standard deviation (RSD), indicated that the proposed methods can serve as reliable validation techniques for the analysis of TCs in shrimp samples. These results indicate the suitability and robustness of the proposed method for quantitative analysis of TC, OTC, and CTC in shrimp.

As shown in *Fig.* 7, the specificity of blank shrimp samples was examined using HPLC-PDA. The results obtained from the blank samples were compared with those of shrimp samples spiked with TC, OTC, and CTC, revealing no discernible interfering peaks. All TCs were identified based on their retention times and spectral characteristics, which were compared to

Table 2. Regression analysis data

TCs	Linearity range (μg/mL)	Regression equation	R^2	LOD (μg/mL)	LOQ (μg/mL)
TC	0.4-6.0	y=22541x–4406.1	0.9962	0.034	0.114
OTC	0.4-6.0	y=16869x+2273.1	0.9974	0.029	0.097
CTC	0.4-6.0	y=26373x–2132.5	0.9978	0.021	0.071

Table 3. Recovery and precision of TC, OTC, and CTC in spiked shrimp samples using a C18 cartridge

Compounds	Recovery (n=3)		Precision (n=3)	
	Spiked concentration (μg/mL)	Recovery (%)	Inter-day assay (RSD%)	Intra-day assay (RSD%)
TC	1.0	91.2 ± 0.53	0.98	0.58
	6.0	91.0 ± 0.12	0.47	0.13
	10.0	95.5 ± 0.77	0.17	0.81
OTC	1.0	92.4 ± 0.78	1.38	0.85
	6.0	94.1 ± 0.44	0.41	0.47
	10.0	97.2 ± 0.19	0.65	0.20
CTC	1.0	93.3 ± 0.69	0.42	0.74
	6.0	95.6 ± 0.73	0.86	0.77
	10.0	96.6 ± 1.08	1.22	1.12

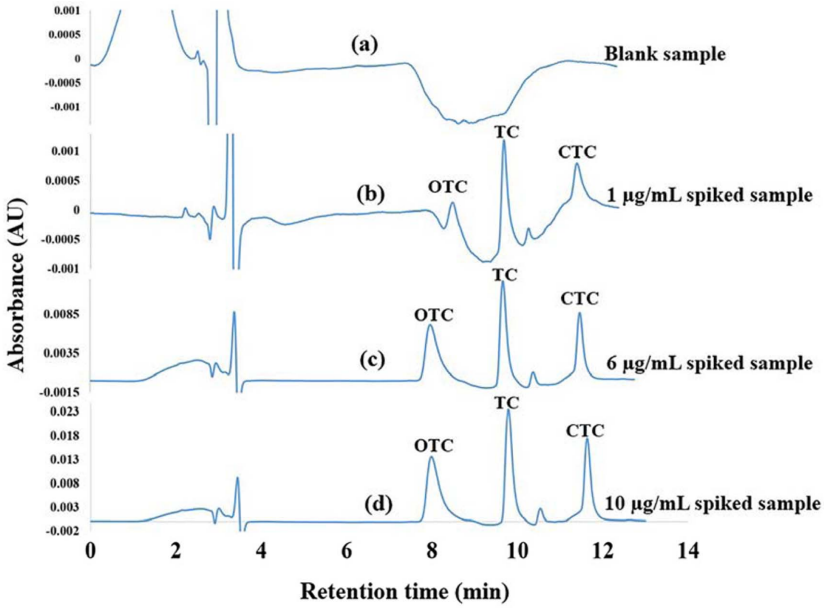

Fig. 7. Chromatograms of extraction with LLE-SPE(C18) in shrimp sample: (a) blank sample, and samples spiked with TCs at (a) 1 μg/mL, (c) 6 μg/mL, and (d) 10 μg/mL.

those of the standards. Standard mixture spikes were employed to confirm the identification of the analyte peaks. Furthermore, TCs in shrimp samples purchased from various local markets were determined. Nine samples were subjected to analysis; notably, none of these samples exhibited detectable levels of TCs contamination. These findings underscore the specificity and reliability of the analytical method for accurately discerning the presence or absence of TCs in shrimp samples obtained from diverse market sources. Moreover, the data show a comparison of the analytical performance of the proposed method with the other methods for the determination of TCs, as shown in *Table* 4. The results indicate the efficacy of the proposed method for detection of TCs in real samples.

4. Conclusions

The findings of this study confirm that the HPLC-PDA method for determining TC, OTC, and CTC in shrimp, when meticulously optimized and validated, meets all predetermined regulatory requirements.

Table 4. Comparison of the proposed method with the other method for the determination of TCs

Liquid-liquid extraction (LLE)	Solid-phase extraction (SPE)	Elution	Detection technique	LOD (μg/mL)	Samples	Recovery (%)	Ref.
EDTA/McIlvaine buffer	C18	Methanol	HPLC-FD	0.010	Tomatoes	97.9-101.9	[8]
McIlvane/EDTA	C18	Methanol	HPLC-PDA	-	Milk	71.5-91.5	[23]
McIlvaine buffer	Oasis HLB	Methanolic oxalic acid	HPLC-PDA	0.031	Buffalo meat	78.3-98.6	[24]
Sodium chloride and succinic acid	Oasis HLB	Methanol	LC-MS-MS	-	Shrimp and milk	77.0-93.0	[25]
EDTA and (methanol/ citrate; 80:20)	-	-	HPLC-PDA	0.015-0.062	Seafood products	95.0-105.0	[22]
EDTA/McIlvaine buffer (pH 4.0) and hexane	C18	Methanol	HPLC-PDA	0.021-0.034	Shrimp	91.0-97.2	This work

The method demonstrated satisfactory recovery, repeatability, and reproducibility, indicating its sensitivity and specificity. Recoveries ranging from 91.0 % to 97.2 % underscored high efficiency of the extraction procedure for tetracycline determination in shrimp samples. The optimized method exhibited good precision with a relative standard deviation (RSD) below 2 %. The synergistic use of LLE and SPE constitutes a robust approach for detecting TC, OTC, and CTC residues in shrimp. The developed method proved effective in detecting antibiotic concentrations below the maximum residue limits (MRLs) established by the Notification of the Ministry of Public Health (No. 303) B.E. 2550. These results validate the suitability and efficacy of the proposed method to ensure the safety and compliance of shrimp samples.

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