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Development of a Simple Extraction Method for Tetracycline Analogues from Bovine Milk

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Abstract

Tetracycline and analogues are among the most used antibiotics in the dairy industry. Besides the therapeutic uses, tetracyclines are often incorporated into livestock feed as growth promoters. A considerable amount of antibiotics is released unaltered through milk from dairy animals. The presence of antibiotic residues in milk and their subsequent consumption can lead to potential health impacts, including cancer, hypersensitivity reactions, and the development of antibiotic resistance. Thus, it is important to monitor residual levels of tetracyclines in milk. The purpose of this study is to develop a quick and simple method for simultaneously extracting five tetracycline analogues from bovine milk. Specifically, five tetracycline analogues: Chlortetracycline (CTC), demeclocycline (DEM), doxycycline (DC), minocycline (MC), and tetracycline (TC) were simultaneously extracted from milk using trifluoroacetic acid. Subsequently, the extracted analogues were separated by reverse-phase high-performance liquid chromatography (RP-HPLC) and detected at 355 nm using UV/Vis. Calibration curves for all five tetracycline analogues show excellent linearity (r^2 value > 0.99). Percent recovery for MC, TC, DEM, CTC, and DC were: 31.88%, 96.91%, 151.29, 99.20%, and 85.58% respectively. The developed extraction method has good precision (RSD < 9.9% for 4 of the 5 analogues). The developed method with minimal sample preparation and pretreatment has the potential to serve as an initial screening test.

Keywords

Antibiotics, Tetracyclines, Extraction, Milk, HPLC

1. Introduction

Tetracyclines are one of the most commonly used antibiotics among farmed animals for prophylactic and therapeutic purposes [1] [2]. Tetracyclines are

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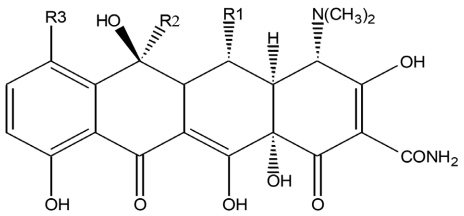
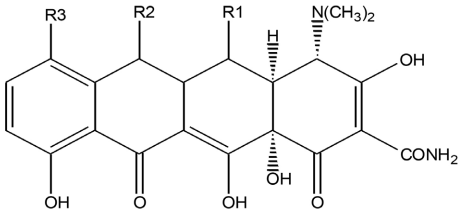
broad-spectrum antibiotics against a wide range of Gram-positive and Gram-negative bacteria. Besides the therapeutic uses, tetracyclines are often incorporated into livestock feed as growth promoters [3]. However, such practice promotes bacterial resistance and allergic reactions in animals and humans [4] [5]. There are about twenty tetracyclines available. The most commonly used tetracyclines for veterinary medicine are chlortetracycline (CTC), doxycycline (DC), oxytetracycline (OTC), and tetracycline (TC) [6]. In European countries and Switzerland, tetracyclines account for sixty-six percent of the total number of antibiotics used for animal farming [7]. The yearly consumption of tetracyclines for animal therapy in Europe is estimated to be more than 2500 tons [8]. Additionally, milk is widely consumed globally and has a significant value to human health [9]. However, a considerable amount of antibiotics is released unaltered through milk from dairy animals. The presence of antibiotic residues in milk and their subsequent consumption can lead to potential health effects, including cancer, hypersensitivity reactions, and the development of antibiotic resistance [10] [11]. Thus, it is important to monitor residual levels of tetracyclines in milk.

Various techniques such as chromatographic [12]-[17], immunological [18]-[21], microbiological [22]-[24], etc. have been applied to detect tetracyclines in milk. Immunological and microbiological techniques may offer faster results with reduced efficiency but can be applied at a cheaper rate. On the other hand, the chromatographic technique has higher sensitivity, higher specificity, and higher quantification capability. Thus, the chromatographic technique has become the most common detection method for tetracyclines in milk. Solid-phase extraction [13] [25] and liquid-liquid-phase extraction [26] [27] have been used for the extraction of tetracyclines from milk. Both extraction methods involve multiple steps and clean-up procedures. This study aims to develop a quick and simple extraction method for simultaneously extracting five tetracycline analogues: chlortetracycline (CTC), demeclocycline (DEM), doxycycline (DC), minocycline (MC), and tetracycline (TC) from milk. Results are compared with the solid-phase extraction method using Oasis HLB SPE cartridges. The simplicity of the developed method that required minimum sample cleanup and pretreatment has the potential to be used as the preliminary screening test. The structures of the five analogues are shown in **Figure 1**.





	R1	R2	R3
Chlortetracycline	H	CH_3	Cl
Demeclocycline	H	H	Cl
Tetracycline	H	CH_3	H

	R1	R2	R3
Doxycycline	OH	CH_3	H
Minocycline	H	H	$N(CH_3)_2$

Figure 1. Structures of tetracycline analogues. R1, R2, and R3 correspond to substitutions of the 5th, 6th, and 7th positions on the backbone of each tetracycline analogue, respectively [28].

2. Experimental

2.1. Instrumental

A Shimadzu HPLC system was used (Kyoto, Japan). It consisted of a photodiode array (Kyoto, Japan, SPD-M 10A VP), two pumps (LC-10AT), and a controller (SCL-10A VP). It was coupled to a C18 column (Phenomenex, 250 × 4.6 mm, 5-micron particles) through a high-pressure injection valve from Rheodyne Inc. (Cotati, CA, USA, model RH-7725i) with a 10 µL sample loop. The detection wavelength for tetracycline analogues was set at 355 nm.

The following equipment was used for the extraction procedures: a vortex mixer (American Scientific Products), a centrifuge (Fisher Scientific, Model: 225, 60 Hz), and an integrated SpeedVac system (Savant, model: ISS110). In addition, a 24-port solid phase vacuum manifold (Phenomenex) was used for the solid phase extraction comparison study.

2.2. Chemicals

All solvents were HPLC grade, and all chemicals were analytical grade. All reagents were used as received, except for degasification using ultrasonic agitation under vacuum prior to use. CTC, DEM, DC, MC, and TC as their hydrochlorides were obtained from Sigma Chemical (St. Louis, MO). Acetonitrile, methanol, oxalic acid, trifluoroacetic acid (TFA), sulfuric acid, hydrochloric acid, and aqueous ammonia were obtained from Fisher Scientific (Fairlawn, NJ). Bovine milk samples were obtained from commercial sources.

2.3. HPLC Conditions

The mobile phase consists of a mixture of 1:1.5:5 (v/v/v) mixture of methanol-acetonitrile-0.01 M aqueous oxalic acid solution (the pH of the oxalic acid was adjusted to 2.0 with ammonia solution). The flow rate was set at 1.0 mL/min. The retention time for tetracycline analogues was determined by injecting each compound individually into the HPLC system. Identification of each analogue from the extracted samples was determined by the corresponding retention time.

2.4. Calibration

Stock solutions of MC, TC, DEM, CTC, and DC were prepared by dissolving the corresponding chemicals in methanol to achieve concentrations of 5.11 mg/mL, 11.51 mg/mL, 9.99 mg/mL, 5.045 mg/mL, and 10.17 mg/mL, respectively. Next, a tetracycline standard mixture was made by transferring 1 mL of the MC stock solution, 1 mL of the TC stock solution, 2 mL of the DEM solution, 2 mL of the CTC stock solution, and 2 mL of the DC stock solution into a 10-mL volumetric flask. The mixture was then diluted to the mark with methanol. This standard mixture was subjected to subsequent serial dilutions for the preparation of five standard solutions with a concentration range of 0.03194 mg/mL - 0.511 mg/mL for MC, 0.07194 mg/mL - 1.151 mg/mL for TC, 0.1249 mg/mL - 1.998 mg/mL

for DEM, 0.06361 mg/mL - 1.009 mg/mL for CTC, and 0.1271 mg/mL - 2.034 mg/mL for DC. Each standard solution was injected three times. The calibration curve for each analogue was prepared by plotting the peak areas against the corresponding concentration of the standard solutions.

2.5. Determination of Limit of Detection

A standard stock solution was prepared, containing all five tetracycline analogues at the following concentrations: 1.81 mg/mL for MC, 1.177 mg/mL for TC, 1.132 mg/mL for DEM, 1.116 mg/mL for CTC, and 1.256 mg/mL for DC. Serial dilutions were made from this standard stock solution to determine the limit of detection.

2.6. Preparation of Samples

5 mL of dairy milk was spiked with 0.5 mL of a standard stock solution that was prepared as described in Section 2.5. The resulting concentrations of MC, TC, DEM, CTC, and DC in the spiked sample were 0.1081 mg/mL, 0.1177 mg/mL, 0.1132 mg/mL, 0.1116 mg/mL and 0.1256 mg/mL respectively. The spiked sample was vortexed for 10 min. A blank was prepared similarly with 5 mL of distilled water.

2.7. Selection of Acid Used for Extraction

Hydrochloric acid, sulfuric acid, and trifluoracetic acids were used for the extraction procedure of tetracycline analogues. It was determined that trifluoracetic acid was the best candidate for the procedure.

2.8. Simultaneous Extraction of Five Tetracycline Analogues

The spiked sample was vortexed for 10 minutes, followed by the addition of 0.5 mL of trifluoroacetic acid. Subsequently, it was centrifuged at the maximum speed of 4750 rpm for 15 minutes. The supernatant was collected and filtered with a 0.22 μm syringe filter. The filtered supernatant was dried using a SpeedVac. The dried sample was redissolved in 0.5 mL of methanol and injected into the HPLC system. Each dissolved sample was injected 3 times.

2.9. Percent Recovery Study

Percent recovery studies for the simultaneous extraction of five tetracycline analogues: MC, TC, DEM, CTC, and DC were performed by spiking milk samples and blanks as described above. Precision was evaluated by intra-day (3 samples) study.

2.10. Comparison Study on Solid Phase Extraction

8 mL of McIlvaine buffer was added to the spiked sample as described in Section 2.6. Subsequently, it was centrifuged at the maximum speed of 4750 rpm for 12 minutes. The supernatant was filtered using a Hirsch funnel. Extractions were

performed with the 3 mL Oasis HLB cartridges (Waters) and a 24-port vacuum manifold (Phenomenex). The cartridges were conditioned with 3.0 mL of methanol followed by 2.0 mL of distilled water. The extraction procedure was as follows: 1) 3.0 mL of the filtered supernatant was loaded onto the cartridge, 2) washed with 1.5 mL of 5% methanol, and 3) eluted with 2.0 mL of methanol. The eluted samples were dried using a SpeedVac. The dried sample was redissolved in 1.0 mL of methanol. Each dissolved sample was injected 3 times.

3. Results and Discussion

3.1. Calibration

Calibration curves for MC, TC, DEM, CTC, and DC are shown in Figures 2-6 respectively. As shown in these figures, all the calibration curves show excellent linearity with r^2 value greater than 0.99.

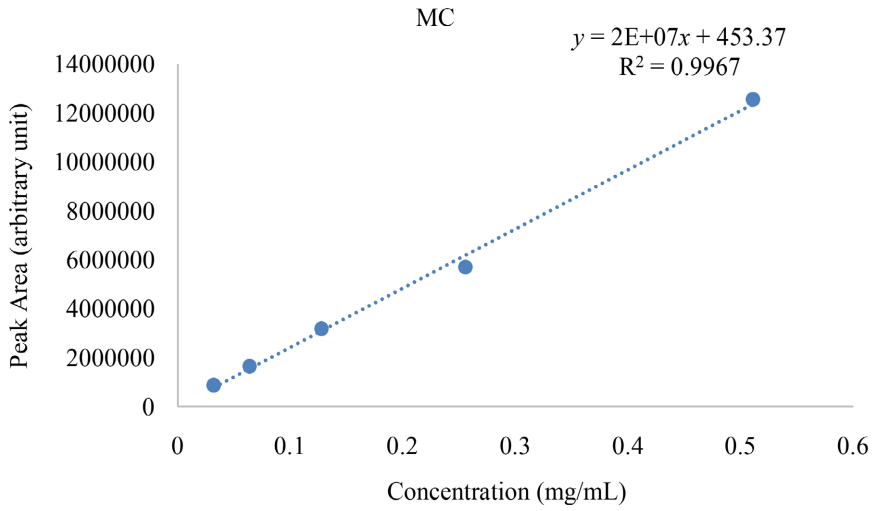


Figure 2. Calibration curve for minocycline (MC).

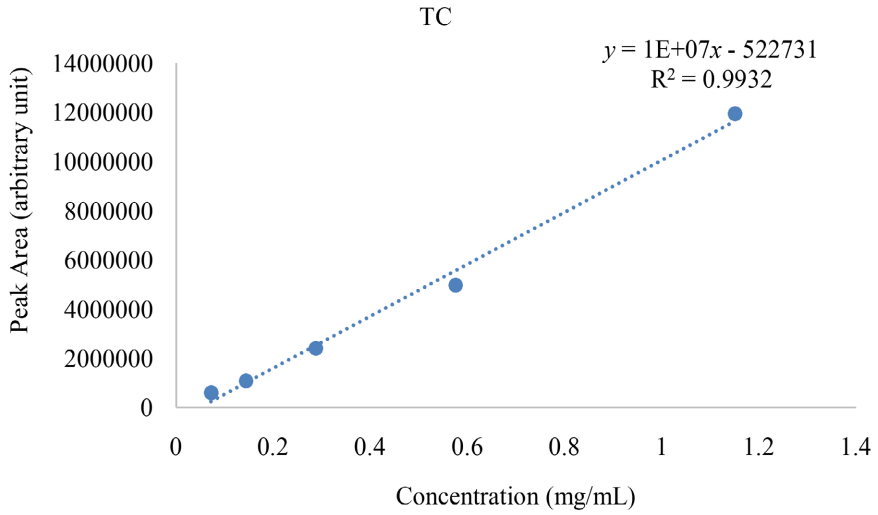


Figure 3. Calibration curve for tetracycline (TC).

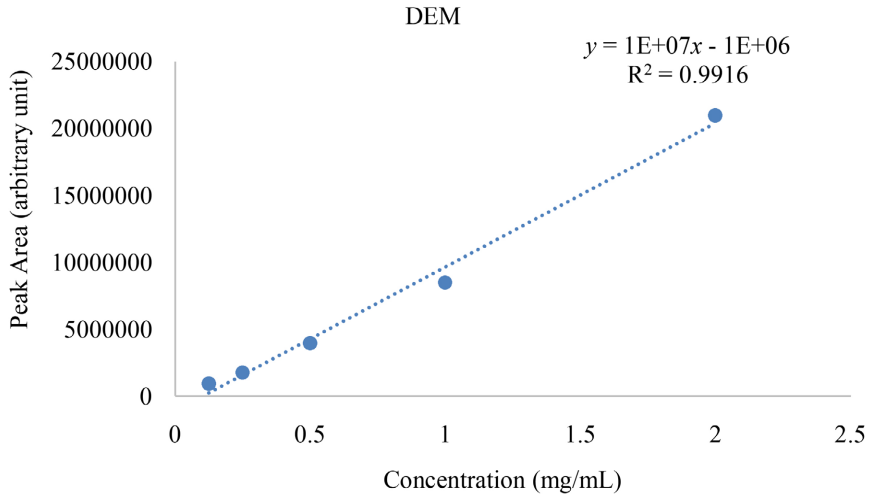


Figure 4. Calibration curve for demeclocycline (DEM).

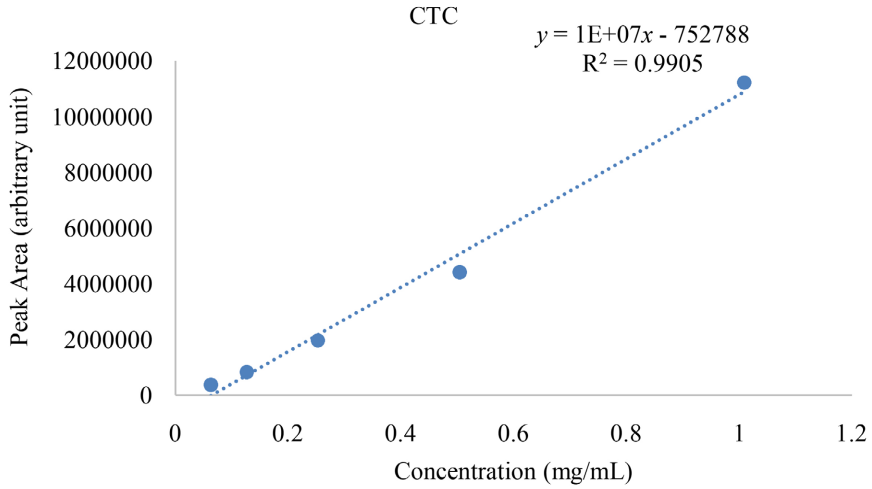


Figure 5. Calibration curve for chlortetracycline (CTC).

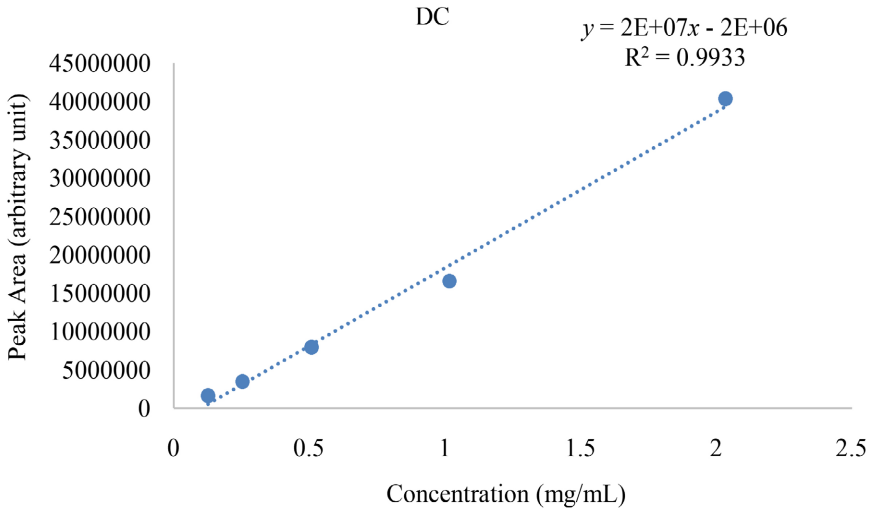


Figure 6. Calibration curve for doxycycline (DC).

3.2. Determination of Limit of Detection

The limit of detection for MC, TC, DEM, CTC, and DC are 0.0337 μg/mL, 0.367 μg/mL, 0.353 μg/mL, 0.348 μg/mL, and 0.393 μg/mL respectively.

3.3. Simultaneous Extraction of Five Tetracycline Analogues

A representative chromatogram of the milk sample extracted using trifluoroacetic acid is shown in Figure 7. Notably, all five tetracycline analogues were successfully separated, and no interference from the sample matrix was observed.

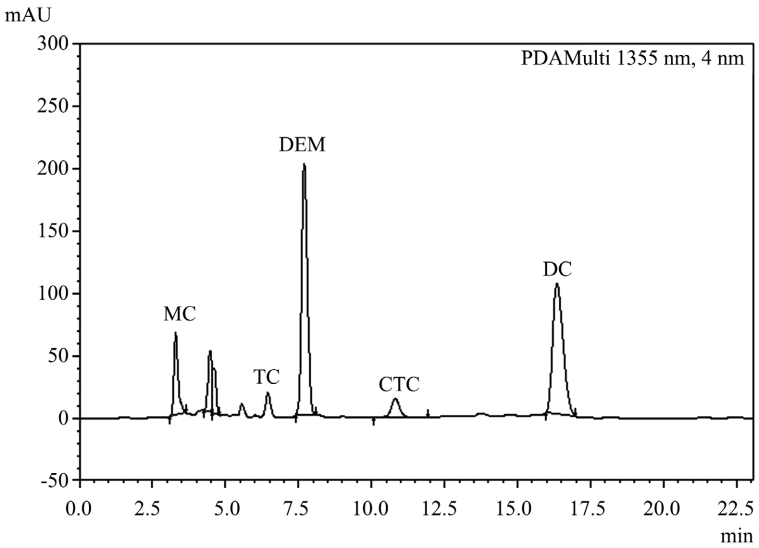


Figure 7. Chromatogram of milk sample extracted using trifluoroacetic acid.

3.4. Percent Recovery Study

Percent recovery was calculated as the ratio between the amount extracted from the spiked milk samples and the amount extracted from the blank. The results of the intra-day study are shown in Table 1. Percent recovery for TC, DEM, CTC, and DC was over 86%. The relative standard deviation (RSD) for each analogue is less than 9.9% for all 5 analogues except CTC. The high RSD value for the per cent recovery of CTC might be due to the relatively low signal of CTC. The results show that the developed extraction method has good precision.

Table 1. Percent recovery for simultaneous extraction of five tetracycline analogues by trifluoracetic acid.

	MC	TC	DEM	CTC	DC
Spiked Concentration	0.1081 mg/mL	0.1177 mg/mL	0.1132 mg/mL	0.1116 mg/mL	0.1256 mg/mL
Percent Recovery (n = 3)	31.68%	96.91%	151.29%	99.22%	85.58%
Relative Standard Deviation (RSD)	9.93%	2.84%	5.09%	23.63%	2.48%

3.5. Comparison Study with Solid Phase Extraction

Percent recovery was calculated as described in Section 3.4 and the results are shown in Table 2.

Table 2. Percent recovery for simultaneous extraction of five tetracycline analogues by solid phase extraction.

	MC	TC	DEM	CTC	DC
Spiked Concentration	0.1081 mg/mL	0.1177 mg/mL	0.1132 mg/mL	0.1116 mg/mL	0.1256 mg/mL
Percent Recovery (n = 1)	72.27%	63.56%	64.27%	66.76%	68.02%

4. Conclusion

A rapid and straightforward extraction method, using trifluoracetic acid, has been developed for simultaneously extracting five tetracycline analogues from milk. This method minimizes sample preparation and cleanup steps while achieving good precision (with a relative standard deviation of less than 9.9% for four out of the five studied analogues). The percent recovery using this method is comparable to solid-phase extraction. The high performance liquid chromatography (HPLC) conditions effectively separate all five tetracycline analogues. Additionally, the calibration curves for these analogues exhibit excellent linearity (with an r^2 value exceeding 0.99). The analysis was completed in under 20 minutes using an isocratic mode on a reversed-phase C18 column, employing a mobile phase composed of a 1:1.5:5 mixture of methanol, acetonitrile, and a 0.01M aqueous oxalic acid solution (the pH of the oxalic acid solution was adjusted to 2.0 with ammonia solution). The method's simplicity, requiring minimal sample cleanup and pretreatment, makes it a promising candidate for preliminary screening tests.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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