Determination of Tetracycline Antibiotics in Milk Using a Simple Strong Cation-Exchange SPE Cleanup Procedure and LC-MS/MS Analysis

UCT Part Numbers

SLDA100ID21-3UM
Selectra® DA HPLC column, 100 x 2.1 mm, 3 µm

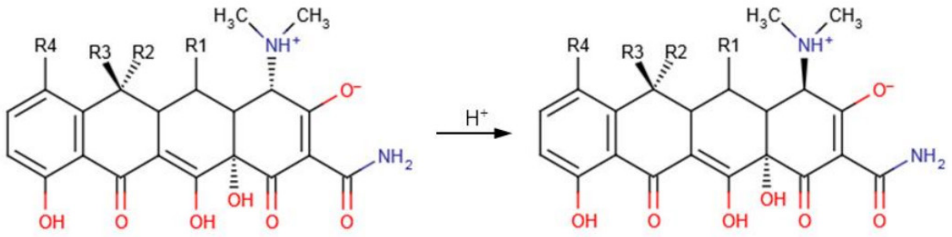
SLDAGDC21-3UM
Selectra® DA guard cartridge, 10 x 2.0 mm, 3 µm

CSDAU206
200 mg / 6 mL SPE cartridge

SLGRDHLDR
Guard cartridge holder

Introduction:

Tetracyclines (TC's) are broad spectrum antibiotics that are widely used in animal husbandry for the prevention, control, and treatment of bacterial infections [1]. They are amphoteric molecules that always carry a charge and only achieve a "neutral" state as zwitterions. As a result, they are highly polar molecules that are only soluble in polar organic solvents (e.g., alcohols) and acidic/basic solutions. TC's are prone to degradation under strongly acidic and alkaline conditions where they form anhydro-, iso- or epi-analogues [2]. Chlortetracycline (CTC) is vulnerable to alkaline decomposition and forms iso-CTC at high pH. Under mildly acidic aqueous conditions (pH 2–6) the TC's readily undergo epimerization at the C-4 position (amine). In addition to pH instability, TC's are also prone to degradation under certain light and redox conditions [2] (Figure 1.). All the degradation products can undergo additional epimerization or form alternative degradation products [3]. Under certain conditions TC's can undergo intramolecular H-bonding, while keto-enol tautomerization may also occur but appears to be temperature dependent [4].



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Compound	R_1	R_2	R_3	R_4
Tetracycline	H	CH_3	OH	H
Oxytetracycline	OH	CH_3	OH	H
Chlortetracycline	H	CH_3	OH	Cl
Demeclocycline	H	H	OH	Cl
Doxycycline	OH	CH_3	H	H
Minocycline	H	H	H	$N(CH_3)_2$

Figure 1. Structure of tetracycline antibiotics and the acid-catalyzed epimerization at C-4 position.

TCs are included in national chemical residue surveillance plans to ensure food safety and prevent unnecessary exposure of antibiotic drugs to consumers. The EU has established a maximum residue limit (MRL) for tetracycline, oxytetracycline, and chlortetracycline at 100 µg/kg in the muscle and milk of all food-producing species [5]. An MRL of 100 µg/kg has also been established for doxycycline in muscle, but it is not allowed for use in animals from which milk is produced for human consumption. The MRLs are based on the sum of the parent compound and its 4-epimer. In the US, tolerances are established for the sum of tetracycline residues (including chlortetracycline, oxytetracycline, and tetracycline) in muscle and milk at 2000 and 300 µg/kg, respectively [6].

TCs are challenging to analyze due to their instability, tendency to form chelation complexes with multivalent cations (i.e., metals), ability to bind with proteins, and the ability to interact with charged silanol groups on silica-based sorbents [7]. As such, it is essential to consider all these issues when developing a method or doing routine analysis of these compounds. Most reported methods for TC analysis use lengthy sample preparation procedures or do little to no sample cleanup. As a result, there is a need for a simple method for the analysis of TC antibiotics in foods of animal origin. Liquid/liquid extraction/partitioning is challenging due to TC's charge and low affinity for organic solvents. Therefore, solid-phase extraction (SPE) combined with LC–MS/MS analysis is the most widely used method for determining TC residues.

Aqueous-based extraction is the primary extraction mechanism. EDTA-McIlvaine's buffer (pH 4) is the most frequently used extraction solvent. At this pH, TCs exist as zwitterions and are in their most stable state. In addition, it is possible to sufficiently deproteinize biological samples at this pH prior to SPE cleanup. However, deproteinization can also be carried out under mildly acidic conditions using trichloroacetic acid (TCA), hydrochloric acid (HCl), or phosphoric acid [8]. Including EDTA in the extraction solvent minimizes the interaction of TCs with chelating complexes present in the sample [8] (Figure 2.). This addition is necessary when extracting milk which contains a large amount of calcium.

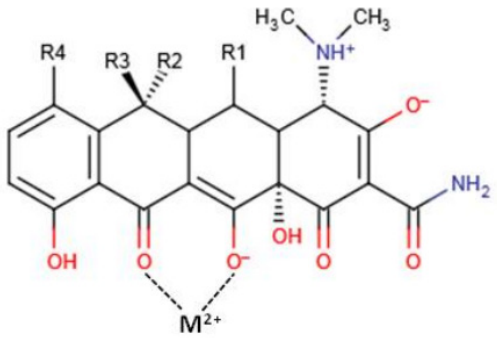

Figure 2. Chelation of divalent metal ions at the C-11 and C-12 position.

TC's chemical complexity and instability also cause problems during HPLC analysis, which often results in broad or tailing peaks and poor resolution. Additionally, the difficulty in controlling or preventing epimer formation (at the C-4 position) during the LC process can result in poor peak resolution between the parent TC and its 4-epimer, leading to peaks of varying intensity and affecting quantification. To overcome peak tailing, prewash an LC column with EDTA before use or use oxalic acid (a dicarboxylic acid with chelating properties) as a mobile phase additive [8].

This study aimed to develop a simple but efficient procedure for extracting, cleanup, and quantifying TC antibiotics in milk. Mixed-mode cartridges with a strong cation-exchange functionality were used to isolate the TCs from milk samples. HPLC separation was performed using a Selectra® DA HPLC column before detection by mass spectrometry. This simple method allows for the rapid analysis of TCs in milk while achieving good accuracy, precision, and sensitivity without an evaporation step.

Procedure:

1. Sample Extraction

a) Weigh 2 ± 0.1g of sample into a 15mL polypropylene centrifuge tube.
b) Add 10 mL of extraction buffer (50mM acetic acid + 10mM EDTA, pH 3.6) to each sample.
c) Shake or vortex samples for 15 minutes to deproteinize the sample and extract the tetracycline antibiotics.
   - For this work, a SPEX® SamplePrep® GenoGrinder® was used (operated at 1500 rpm).
d) Centrifuge the samples for 10 min at ≥3000 rcf and 4°C.

2. Condition Cartridge

a) Add 3 mL of methanol to cartridge **CSDAU206**.
b) Add 3 mL of buffer (50mM acetic acid + 10mM EDTA, pH 3.6).
Note: Do not let the cartridge go dry otherwise, repeat steps 2.a) and 2.b).

3. SPE Extraction

a) Load the supernatant from step 1d).
b) Adjust the vacuum pressure for a flow rate of 1–3 mL per minute.

4. Wash cartridge

a) Add 3 mL of ultrapure H_2O and slowly draw through.
b) Add 3 mL of MeOH and slowly draw through.
c) Dry under vacuum for ≈1 minute to remove excess solvent.

5. Elute Cartridge

a) Elute the tetracyclines from the SPE cartridge using 3mL elution solvent (1M oxalic acid + 2% TEA in MeOH).
b) Vortex the samples for 2 min and transfer a 1 mL aliquot to an autosampler vial for analysis.

LC-MS/MS Conditions:

HPLC Conditions	
HPLC column	UCT Selectra® DA, 100 x 2.1 mm, 3 µm **(p/n: SLDA100ID21-3UM)**
Guard Cartridge	UCT Selectra® DA, 10 x 2.0 mm, 3 µm **(p/n: SLDAGDC21-3UM)**
Guard Cartridge Holder	p/n: SLGRDHLDR
Column Temperature	40°C
Injection Volume	5 µL
Flow Rate	300 µL/min
Mobile Phase A	1mM oxalic acid in ultrapure H_2O
Mobile Phase B	1mM oxalic acid in MeOH

Gradient		
Time (min)	**A (%)**	**B (%)**
0.0	95	5
1.0	95	5
5.0	60	40
10.0	60	40
12.0	0	100
16.0	0	100
16.2	95	5
21.0	95	5

MS Conditions	
Instrumentation	Thermo Scientific™ TSQ Vantage™ tandem mass spectrometer
Ionization mode	ESI^+
Spray voltage	5000 V
Vaporizer temperature	350°C
Capillary temperature	350°C
Sheath gas pressure	60 arbitrary units
Auxiliary gas pressure	10 arbitrary units
Ion sweep gas	2 arbitrary units
Declustering potential	2 V
Q1 and Q3 peak width	0.2 and 0.7 Da
Collision gas	Argon
Collision gas pressure	2.0 mTorr
Cycle time	2 sec

Gradient							
Analyte	**t_R (min)**	**Precursor ion**	**Product ion 1**	**CE 1**	**Product ion 2**	**CE 2**	**S-lens (V)**
Thiabendazole$^{13}C_6$	8.6	208.00	137.05	31	181.03	25	43
Oxytetracycline	8.4	461.13	426.07	17	200.96	32	95
Tetracycline	9.0	445.10	410.04	18	153.91	24	79
Minocycline	10.0	458.10	441.09	16	282.98	41	136
Demeclocycline	10.3	465.10	448.04	16	430.02	19	99
Chlortetracycline	12.5	479.10	462.05	11	444.02	18	102
Doxycycline	13.2	445.08	428.08	16	321.01	28	90
Anhydrotetracycline	14.0	427.15	410.01	16	153.98	28	105

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Results and Discussion:

Polymeric SPE cartridges, particularly those containing polar-modified polymeric sorbent, have been used to extract TCs from food samples. A significant drawback of using these cartridges is the inability to rinse the sorbent with an organic solvent, which leads to "dirty" sample extracts containing matrix co-extractives. An alternative approach is to use ion-exchange SPE to retain the TC residues on the sorbent, which can then be rinsed with 100% organic solvent. An organic rinse effectively removes matrix interferences and produces a clean sample extract. Due to the instability and complex physico-chemical properties of TCs, the SPE procedure must be carefully optimized to achieve the best results.

Polymeric cation-exchange sorbents (strong and weak) were evaluated using a variety of different loading conditions (buffer type, pH, and ionic strength). Still, the TCs were found to be inadequately retained. A silica-based sorbent containing a strong cation exchange component was the best choice. Several different SPE elution solvents were evaluated but were not found to be effective at eluting the TCs from the SCX sorbent:

- 2% NH_4OH in MeOH is a commonly used elution solvent for eluting basic compounds from SCX sorbent. The high pH neutralizes amino functional groups on the analyte(s), allowing them to elute from the sorbent. However, it was not effective at eluting the strongly retained TCs. In addition, NH_4OH is not recommended for TCs due to potential alkaline degradation.
- 2% TEA in MeOH – also provides a high pH to neutralize amino functional groups but does not contain OH ions.
- Triethylammonium formate (1% TEA + 0.5% formic acid in MeOH) – At a low pH, the TEA is fully ionized and forms a protonated tertiary amine, which can function as a counter-ion to displace positively charged analytes from the SCX sorbent.

Accuracy & Precision Data for Tetracycline Antibiotics in Milk (100ppb, n=5)						
	Tetracycline	Oxytetracycline	Demeclocycline	Chlortetracycline	Doxycycline	Minocycline
Sample 1	74.7	53.4	86.7	87.6	88.8	129.2
Sample 2	70.8	52.0	87.7	85.4	91.4	128.9
Sample 3	75.5	51.9	88.7	85.7	86.4	126.6
Sample 4	87.4	58.1	80.8	87.1	99.7	136.3
Sample 5	84.4	66.9	74.6	83.8	91.5	153.4
Mean	**78.6**	**56.5**	**83.7**	**85.9**	**91.5**	**134.9**
RSD	**9.0**	**11.3**	**7.1**	**1.7**	**5.5**	**8.13**

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An alternative elution approach to pH manipulation is the use of a high salt concentration (≥ 1 M) to disrupt the ionic interaction between the analyte(s) and ion-exchange sorbent. It was determined that MeOH containing 1M oxalic acid was required to elute the TC's from the ion-exchange sorbent. Oxalic acid was used because it is a good metal chelating agent, which is necessary for the elution of TC's from silica-base sorbent. Lower concentrations of oxalic acid were not as effective, which is similar to the results reported by Pena et al. [9]. Minocycline, containing an additional amino functional group, did not elute with 1M oxalic acid. Therefore, 2% TEA was incorporated into the extraction solvent to function as a counter-ion and displace the minocycline from the sorbent. The eluted extracts can be analyzed direct- ly by LC-MS/MS. If lower sensitivity is required (low ppb range), an evaporation step could be included although great care should be taken (low temperature and N_2 flow).

Other problems faced during LC-MS/MS analysis of TCs include isobaric interference and the formation of epimers or degradation products. Isobaric interference is observed between tetracycline and doxycycline as they have similar m/z values and common fragment ions that a triple quadrupole mass spectrometer cannot distinguish. Therefore, sufficient LC separation (≈4 min) was obtained between the two compounds to overcome this problem (not evident in Figure 4 due to time-segmented acquisition). Isobaric interference and degradation products can also be generated from other TCs (e.g., tetracycline, anhydrotetracycline, CTC, and demeclocycline). As can be seen in Figure 4, chlortetracycline and demeclocycline have 2 peaks each. For CTC, there is a peak at 12.4 min corresponding to the analyte and a secondary peak at 10.36 min, which could correlate to demeclocycline (10.29 min), an epimer, or an alternative degradation product. For demeclocycline, there is a peak at 10.3 min corresponding to the analyte and a secondary peak at 8.9 min (epimer or degradation product). Epimerization can potentially create difficulties in accurate quantification as the epimer peaks can be equal to or larger than the parent TCs. Epimers have the same m/z values (parent and product ions), similar abundances, and usually elute close to the parent TCs.

It is often not possible to separate the epimers from the parent TCs, and some analysts will use a fast gradient so that the epimers co-elute with the TCs. The Selectra DA® column can separate the epimers. However, epimerization was not a significant problem in this work. Some minor peaks can be observed in the chromatograms but do not affect the quantification. Lastly, epimerization is readily observed if the final sample extracts (and calibration curve/QC standards) are prepared in an aqueous solution, particularly acidic solutions. However, if the final sample extracts are prepared in an organic solvent, the epimerization is drastically reduced, limiting the formation of large epimer peaks, and simplifying quantification.

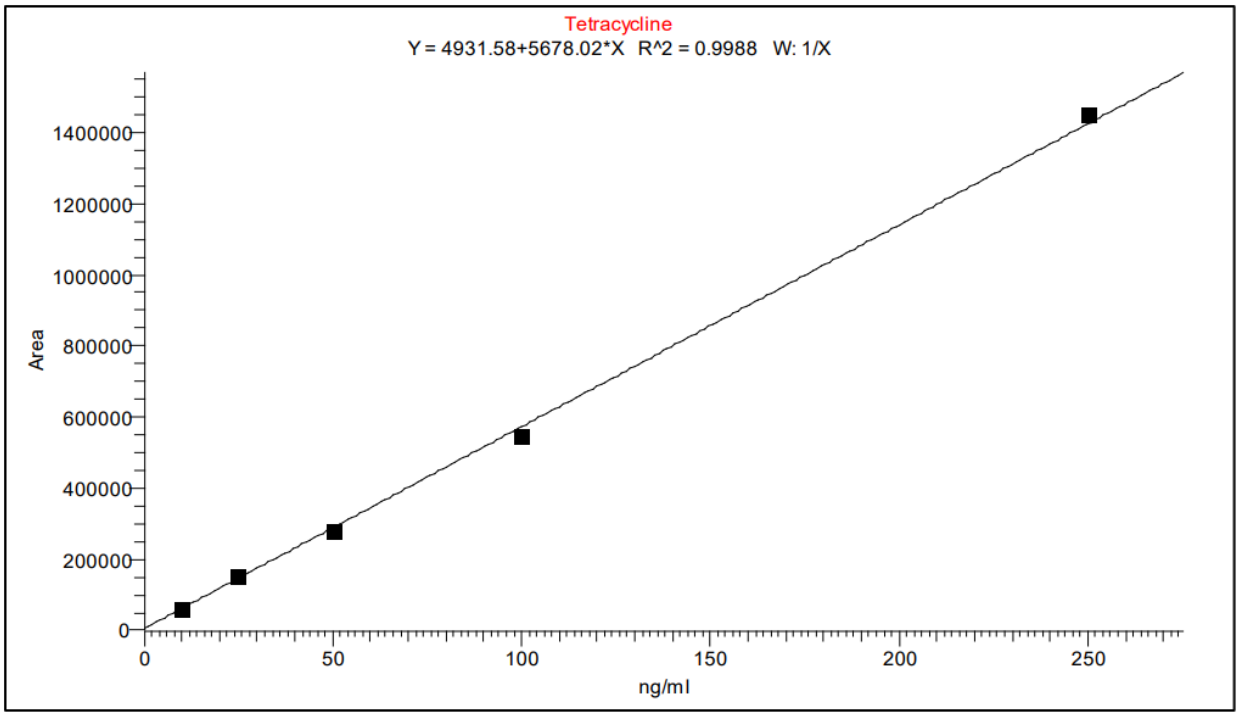

Figure 3. Calibration curve example (tetracycline).

Anhydrotetracycline was initially included in the method but was found to degrade rapidly on contact with the extraction buffer. The analyte is displayed in the chromatogram for informational purposes only and was not included in the final quantitative method. Thiabendazole $^{13}C_6$ was included as an internal standard in the method, but results were not improved when incorporated into the calculations. Therefore, it was excluded from the calculations and only used as a QC standard. If an isotopically labeled internal standard for one (or more) of the TCs is available, its inclusion in the method would be an obvious advantage. However, at the time of this research, appropriate isotopically labeled internal standards could not be commercially sourced.

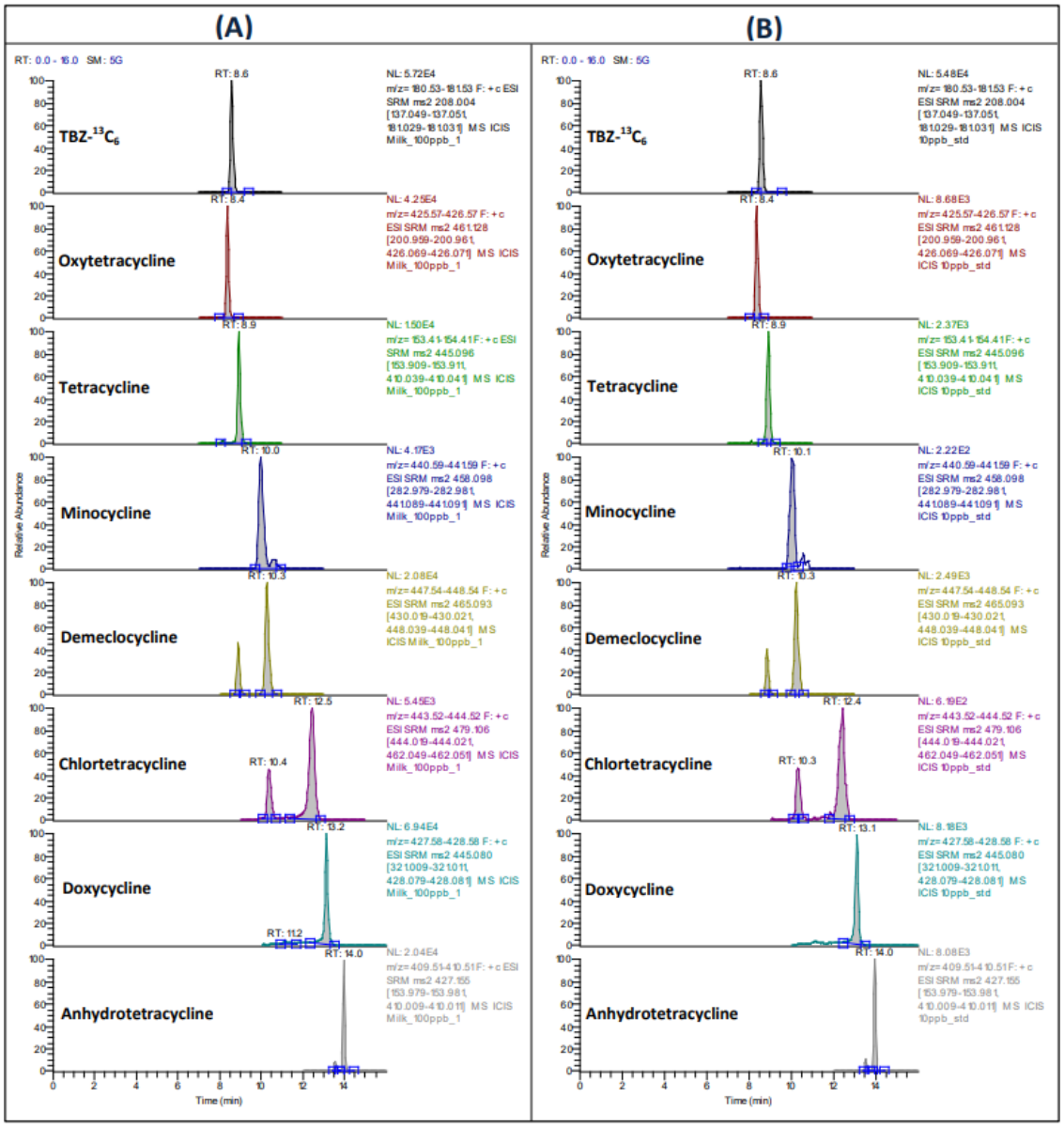

Figure 4. Chromatogram of (A) a milk sample fortified with tetracycline antibiotics at 100 ng/g (I.S. at 25 ng/g) and (B) a neat standard equivalent to 10 ng/g (I.S. at 25 ng/g).

Conclusions:

A simple, fast, cost-effective SPE and LC-MS/MS method was developed to detect and quantify six tetracycline antibiotics (TCs) in milk. Samples were extracted with pH 3.6 acetate buffer containing EDTA, and SPE cleanup was carried out using mixed-mode cartridges containing a strong cation exchange component. The TCs were separated within 16 min on a Selectra® DA HPLC column. The use of LC-MS/MS detection provided sufficient selectivity and sensitivity for identifying and quantifying the TCs. Good accuracy and precision were obtained for these problematic compounds. No evaporation step was included in the method, avoiding any potential loss during this step. Based on the lowest calibration point used, the method is capable of detecting TC residues at <10 ng/g. The accuracy and precision of the method could be further improved by incorporating an isotopically labeled internal standard for one of the TCs (if available). The method outlined here provides an attractive alternative to currently used methods for TC analysis.

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References:

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4103-01-02

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