Determination of Tetracycline Residuals in Prepared Commercial Milk

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Purpose:

The purpose of this experiment is to introduce the students to a real-world application of high performance liquid chromatography (HPLC) in food safety. Oxytetracycline, a commonly used antibiotic by the dairy industry to treat illnesses in livestock, will be quantitatively determined in a prepared commercial milk sample. Solid phase extraction (SPE), an important sample preparation method routinely used in liquid chromatography will also be introduced.

Background:

Oxytetracycline (OTC, Figure 1) is a member of the tetracyclines (TCs), a group of broad-spectrum antibiotics. These compounds are not only used on humans but also in veterinary medicine as a feed additive or in drinking water to maintain animal health and food production[1]. TCs are amphoteric compounds that form crystalline hydrates and salts with acids and bases. Their UV spectra show strong absorption around 270 and 360 nm in neutral and acidic solutions. TCs are also soluble in alcohols and polar organic solvents such as n-butanol and ethyl acetate. There has been research reporting an estimated TC metabolic rate in dairy cows being in the range of 25-75%, leaving a significant percentage of the administered TCs to be excreted in bovine milk[2].
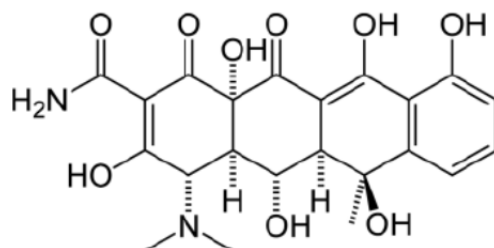

Figure 1

To prevent harmful effects of residual antibiotics in milk on the human health, various international health organizations have established the maximum residual limit (MRL) of OTC in all circulating milk in their countries. Some of these MRL are reported in Table 1. [(1-3)]

Table 1

Organization or Country	MRL for TCs in Milk (in μg/mL)
European Union (EU) World Health Organization (WHO) Chinese Ministry of Agriculture	0.1
U.S. Food and Drug Administration (USFDA)	0.3

Accurate monitoring of the contents of TCs in foodstuffs is important and is required by public health agencies. The most commonly used methods for measuring TCs in foods are microbiological assays although they are not the ideal method due to their varying precision and time-consuming nature. Liquid chromatographic methods can be equally time-consuming, but provide improved precision and accuracy.

A typical HPLC method of analyzing the TCs in milk samples involves a series of sample preparation steps including (1) extraction, (2) complexation, (3) deproteinization, (4) solid-phase extraction (SPE) and (5) evaporation[4]. A brief explanation for each of these steps is below:

- *Extraction* – Aqueous based extractions are primarily employed, with a McIlvaine/EDTA buffer system (pH 4) being the most prevalent. Aqueous systems provide greater solubility over most organics, excluding alcohols, and are compatible with most biological matrices of interest. One factor that may contribute to some degree of inaccuracy in the analysis is the fact that TCs are susceptible to acid degradation. It is generally believed that no major loss of TCs is expected over the course of at least 3 days when using the McIlvaine pH=4 buffer. This method has adopted a pH=2.9 McIlvaine buffer as the samples are analyzed immediately after the extraction.

- *Complexation* – TCs have a high tendency to form chelates with metals in their sample matrices. For instance, biological matrices that contain divalent metal ions (such as Ca^{2+} in milk) can interfere with the extraction of TCs. Disruption of these interactions is most commonly achieved through the addition of EDTA to the extraction buffer because of EDTA's higher affinity for most cations than TCs. EDTA is thus added to the McILvaine buffer to help recover chelated TCs in the original milk.

- *Deproteinization* – Chemical deproteinization within biological matrices is accomplished via acid, organics (i.e., acetonitrile), and heat. In this experiment, the acidic environment of the McIlvaine buffer also serves to deproteinize the milk matrix.

- *Solid phase extraction (SPE)* – SPE is widely used in chemical analysis for the purpose of sample cleanup and to concentrate the analyte of interest. Due to their carbon backbone, aromatic region, and varied functional groups, TCs are amenable to a wide range of SPEs. The non-polar regions of the ring system allow for cleanup using reversed phase - C18 or polymeric sorbents. The C18 SPE needs to be pretreated with the same McIlvaine/EDTA buffer to circumvent an overly strong attachment of the TCs to the silanol groups on the sorbent and to minimize possible metal contaminations.

- *Evaporation* - In some cases, due to the need to concentrate TCs collected form SPE extraction, solvents are evaporated from the final solutions before HPLC analysis.

Instrumentation:
An HPLC with UV detector will be used for this experiment. The basic operation steps of the HPLC are in the SOP for the instrument, available at chemlab.truman.edu.

HPLC conditions:
- Column: C18 (record the column-specific parameters printed on the column itself)
- Temperature: $40^{o}C$
- Mobile phase flow rate: optimized during experiment
- Detector: UV 269nm

Safety and Hazards information:
Tetracycline may be harmful if introduced by ingestion or inhalation. It may also cause skin irritation. Methanol can irritate the respiratory system if inhaled or cause skin irritation. Properly fitted nitrile or latex gloves should be worn to avoid skin contact with the irritants. Waste generated in the lab should not be poured down the drain; it should be collected in properly labeled waste containers placed in a lab fume hood.

Solution Preparation:

1. **McIlvaine buffer** – In a large beaker, dissolve 12.0 g sodium hydrogen phosphate (Na_2HPO_4), 3.72 g EDTA and 11.9 g anhydrous citric acid in approximately 1.0 L deionized water. Use concentrated phosphoric acid and/or 10M NaOH to adjust the pH to 2.90. Store in a clean reagent bottle. **(This will be prepared for you and will be stored in the refrigerator. You should test the pH prior to use.)**

2. **Oxytetracycline stock solution** – in a 50.0mL volumetric flask dissolve ~100mg oxytetracycline with HPLC grade methanol. This solution may be transferred to a plastic bottle and stored @ -10°C for up to four weeks. Calculate the concentration in μg/ml.

3. **Analytical mobile phase** – The mobile phase will be a mixture of 0.010 M aqueous oxalic acid, HPLC grade acetonitrile, and HPLC grade methanol. Prepare 500 mL of the oxalic solution. Prior to placing the mobile phase components into their respective reservoirs on the instrument, vacuum filter each solvent through a 0.45um nylon filter.

4. **2.0% methanol in water solution** (for eluting off sugar in the SPE step) – Mix 2.0mL methanol into 100mL di. water.

5. **Milk** – store in plastic container @ 4°C in the dark. (If spiking is desired, spike 2.5mg oxytetracycline into 50-mL milk)

6. **Concentrated Internal Standard** – Dissolve approximately 100 mg (weighed to the nearest 0.1 mg) in 50.0 mL methanol.

7. **Calibration Standards** – Using the ocytetracycline stock solution, prepare five standards in the range of ~1 to ~200 μg/mL by diluting the stock solution to 25.0 mLwith methanol. Prior to dilution, add 1.00 mL of the caffeine internal standard to each standard solution. Make no more than 25 mL of each standard.

Procedure:

Pre-treatment of the SPE cartridges:

1. Obtain three SPE cartridges with C18 packing and position them on the vacuum manifold as shown in the Figure 2. Be sure that the output liner of each valve is directed into a test tube. Ensure that the lid is properly seated on the manifold and that all valves on the top of the manifold are closed and secure before starting vacuum and introducing solvents.

2. Using vacuum tubing, attach the manifold to a trap and aspirator and turn water on to the aspirator. With all valves closed on the top of the manifold, use the white knurled valve next to the vacuum gauge on the manifold to adjust the pressure inside the chamber. To allow for slow flow (~1-2 mL/min), the needle on the gauge should be just outside the box labeled "ZERO" on the gauge (See Figure 3).

3. Add 2.0mL methanol to the cartridges, open the valve to each cartridge and elute the methanol at a flow rate of approximately 2-mL/min (~ 2 drops per 3 sec.). Close the valve to each cartridge
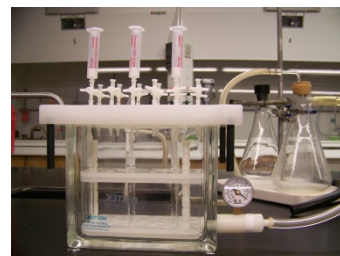
Figure 2. SPE Manifold

Figure 3. Pressure Gauge

4. Add 2.0mL McIlvaine buffer solution into each of the cartridges, open the valve to each cartridge and elute at 2 mL/min. Close the valve to each cartridge. Stop the aspirator and discard the drained solutions.

Preparation of the milk sample:

1. Commercial milk should be kept in plastic containers @4°C in the dark.

2. Obtain three 2 mL volumetric flasks and clean each with water followed by methanol.

3. Add 2.0-mL of the milk sample to each of three 15-mL plastic centrifuge tubes, and then add 6.0-mL of the McIlvaine buffer solution to each tube.

4. Agitate on the vortex mixer for 1 minute. Then centrifuge for 5 minutes to allow complete precipitation of the denatured protein.

5. Decant the supernatant from each tube and into a pretreated SPE column, and use vacuum to elute at 2 mL/min. Discard the eluent.

6. Add 1.0-mL of 2.0% methanol in H_2O solution to wash off residual sugars. Use vacuum to elute and discard eluent.

7. Place a cleaned 2 mL volumetric flask below each cartridge outlet and add 1 mL HPLC grade methanol to the cartridge to elute the tetracycline. Use vacuum to elute at no more than 2 mL/min (do not rush this step). If the volume exceeds the marked line, then let the extra solvent evaporate under a gentle stream of nitrogen gas to below the line.

8. To each sample solution, add enough caffeine internal standard to result in the same caffeine concentration as in the standard solutions (after dilution). After caffeine addition, dilute to the mark with methanol..

9. Analyze immediately on the HPLC or keep refrigerated in the dark. Shelf time should be kept to as short as possible.

HPLC Optimization:

Use one of your standards to identify the elution time of oxytetracycline and one of your milk samples to determine optimum separation conditions to resolve the analyte peak from the caffeine internal standard and any sample concomitants. A good place to start would be 10% methanol: 70% aqueous: 20% acetonitrile and a flow rate of ~1 mL/min. Use a 10 μL injection volume.

Once separation times have been optimized, run each of your standards and milk samples. Preform triplicate runs of one of your middle concentration standards. Replicate analyses for all solutions are desired if time allows. For each chromatogram, record the retention time, peak height and peak area for the oxytetracycline peak.

Calculations: Use both <u>peak areas</u> and <u>peak heights</u> for the following:

Determine the concentration of oxytetracycline in the milk sample and its associated uncertainty. If the actual concentration of the sample is known, calculate the percent recovery for your method. Using the precision the replicate runs of your standard as an estimate of the precision of the HPLC analysis, calculate the detection limit for the HPLC measurement. Is this also the detection limit for the method?

References:

1) "*Analysis of residual oxytetracycline in fresh milk using polymer reversed-phase column*" Kaale, E.; Chambuso, M; Kitwala, J. **Food Chem.**, 107 (2008) 1289-1293.
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3) "*Chromatographic analysis of tetracycline antibiotics in foods*" Oka, H.; Ito, Y.; Matsumoto, H. **J. Chrom. A**, 882 (2000) 109-133.
4) "*Complexities in tetracycline analysis – chemistry, matrix extraction, cleanup, and liquid chromatography*" Anderson, C.; Rupp, H.; Wu W. **J. Chrom. A**, 1075 (2005) 23-32.