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アプリケーションノート

HPLC/UV Determination of Tetracyclines in Milk Using Mixed-Mode SPE and eXtended Performance [*XP*] 2.5 µm Columns

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Abstract

Tetracycline antibiotics are used in veterinary medicine for the treatment of animals bred for production of meat or milk. Worldwide maximum residue levels (MRL) for tetracycline antibiotics is 100 ppb (µg/L). This study presents an HPLC/UV method for reliable determination of these antibiotics in milk using a simple, selective SPE method and eXtended Performance [*XP*] 2.5 µm columns.

Benefits

- Improved productivity on HPLC using 2.5 µm particle columns
    - 35% faster than equivalent separation on 3.5 µm particle column
    - 60% faster than equivalent separation on 5 µm particle column
- Improved resolution compared to 3.5 and 5.0 µm particle columns at same flow rate and column dimension
- Simple and selective SPE for ppb level detection limits with UV detection
- LC Certified Vials for consistent cleanliness and inertness

Introduction

Tetracycline antibiotics are used in veterinary medicine for the treatment of animals bred for production of meat or milk. Of this class of antibiotics, oxytetracycline is the most commonly used for milk-producing cattle. Ingestion of antibiotic residues in milk can result in increased antibioticresistance as well as potential allergic reactions among the consuming population. Worldwide maximum residue levels (MRL) for tetracycline antibiotics is 100 ppb (µg/L). This study presents an HPLC/UV method for reliable determination of these antibiotics in milk using a simple, selective SPE method and eXtended Performance [*XP*] 2.5 µm columns.

Experimental

Sample and Buffer Preparation

1. EDTA/McIlvaine Buffer:
   In a 1-L volumetric flask, dissolve 28.41 g anhydrous dibasic sodium phosphate in approximately 900 mL water, dilute to volume, and mix. In a separate 1-L volumetric flask, dissolve 21.01 g citric acid

monohydrate in approximately 900 mL water, dilute to volume, and mix. Combine 1-L citric acid solution with 625 mL of phosphate solution. Add 60.5 g disodium EDTA, and mix well until dissolved. Prepare fresh weekly.

2. Initial Extraction/Precipitation:
   Transfer 1.5 mL milk to a 15-mL centrifuge tube. Add 6 mL of EDTA/McIlvaine buffer, and vortex for 30 s. Centrifuge at 4000 rpm for 5 min. Collect the supernantant, and adjust to pH 10 with 0.75 mL 1 M NaOH.

3. SPE Cleanup: SPE cleanup is performed using an Oasis MAX Cartridge (1 cc, 30 mg). Condition the cartridge with 2 mL methanol, followed by 2 mL water. Set flow rate to approximately 1 mL/min. Load pH adjusted supernatant obtained from the initial extraction. Wash with 0.5 mL 5% ammonium hydroxide, and then with 0.5 mL methanol. Elute with 0.5 mL 45:55 acetonitrile/75 mM aqueous oxalic acid. Dilute to 1.5 mL with reagent water prior to LC analysis.

LC conditions

System:	Waters Alliance e2690/5 HPLC with 2998 PDA Detector
Column:	XBridge BEH C_{18} XP 100 x 4.6 mm, 2.5 µm
Injection volume:	35 µL
Column temp.:	30 °C
Mobile phase A:	10 mM oxalic acid in water
Mobile phase B:	10 mM oxalic acid in acetonitrile
Flow rate:	1.20 mL/min
Gradient:	15% B initial, linear gradient to 50% B in 8.00 min, hold until 11.25 min, back to 15% B at 11.60 min. Hold and re-equilibrate until 12.85 min.
UV detection:	PDA (extracted 355 nm)
Vials:	Waters Certified Maximum Recovery

Results and Discussion

Method recovery was better than 80% for all tetracyclines.

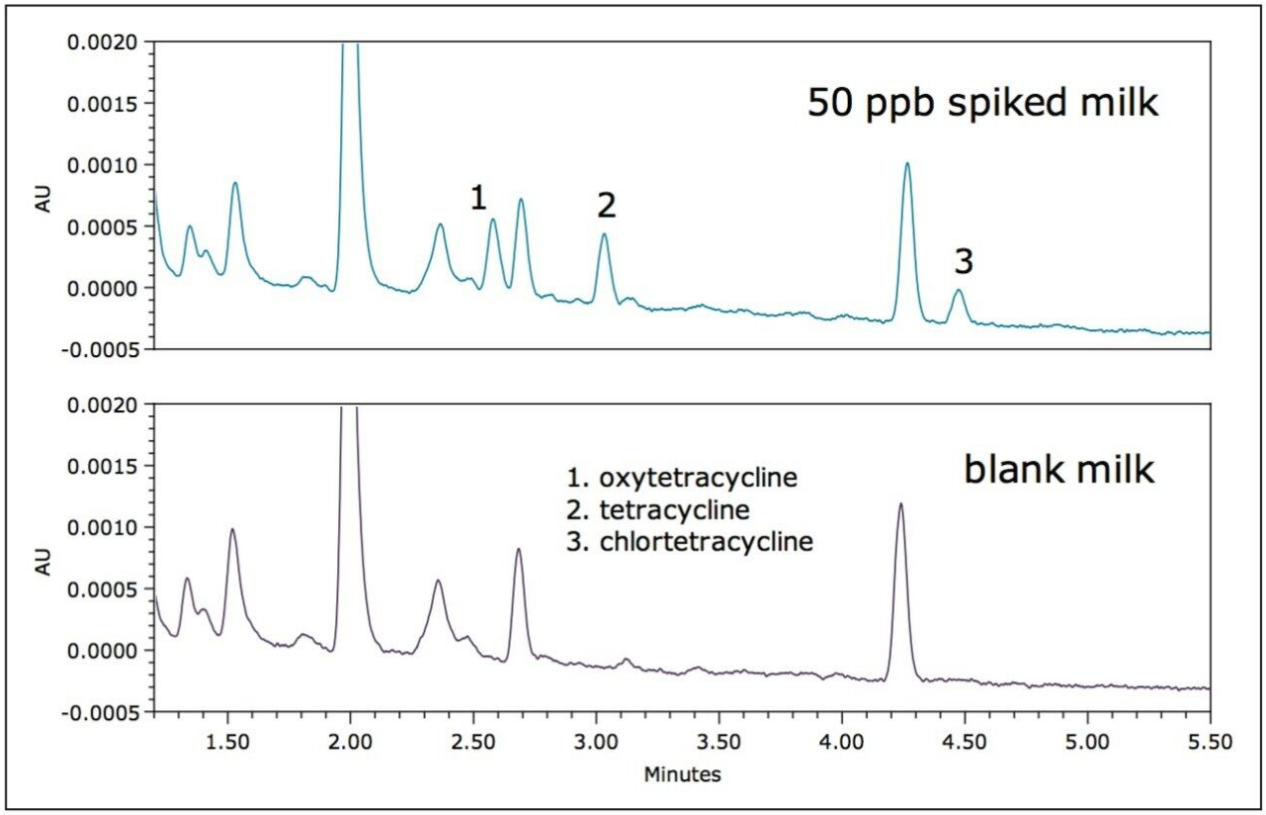

Figure 1. A typical HPLC/UV chromatogram (355 nm extracted wavelength, XP column) obtained from analysis of a sample spiked with 50 ppb (ng/g) of three tetracyclines.

Oxalic acid is utilized to acidify the SPE eluent and as a mobile phase modifier for the HPLC separation. The tetracyclines are strong chelators and can form complexes with metal ions such as calcium. For both SPE and HPLC, the added oxalic acid acts as an acidifying agent and also sequesters calcium. The result is better and more consistent performance.

The *XP* column used in this study provides better productivity compared with larger particle columns. The following table illustrates the savings in time and solvent using *XP* columns (1.2 mL/min flow rate, gradients scaled for similar peak capacity, injection volumes required for similar sensitivity).

Column dimension length (mm) x I.D. (mm) x dp (µm)	Injection volume	Analysis time
100 x 4.6 x 2.5 (***XP***)	35 µL	13 min
150 x 4.6 x 3.5	50 µL	20 min
250 x 4.6 x 5	90 µL	32 min

Conclusion

- The 2.5 µm *XP* column provides faster and more sensitive analysis using traditional HPLC instrumentation compared with larger particle columns.
- The EDTA/McIlvaine buffer is effective for protein precipitation and extraction of tetracycline antibiotics from milk.
- The Oasis MAX Cartridge provides effective cleanup prior to HPLC analysis.
- The methodology is rugged and straightforward; a single analysis can easily process 20 samples in an eight-hour day.

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Alliance HPLC <https://www.waters.com/514248>

2998 Photodiode Array (PDA) Detector <https://www.waters.com/1001362>

Available for purchase online

XBridge BEH C18 XP Column, 130Å, 2.5 µm, 4.6 mm X 100 mm, 1/pkg <https://www.waters.com/waters/partDetail.htm?partNumber=186006039>

Oasis MAX 1 cc Vac Cartridge, 30 mg Sorbent per Cartridge, 30 µm Particle Size, 100/pk <https://www.waters.com/waters/partDetail.htm?locale=en_US&partNumber=186000366>

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