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MOHWV0036.06

Method of Test for Veterinary Drug Residues in Foods- Test of Tetracyclines

1. Scope

This method is applicable to the determination of 7 tetracyclines (tetracycline etc. listed in the attached table) in muscle, viscera, fat, eggs and milk of livestock, poultry and aquatic products, and honey.

2. Method

After extraction and purification, tetracyclines are determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer.

2.1.1.1. Ion source: positive ion electrospray ionization, ESI^+.

2.1.1.2. Column: ACQUITY CSH C18, 1.7 μm, 2.1 mm i.d. × 10 cm, or an equivalent product.

2.1.2. Centrifuge: centrifugal force ≥ 12000 ×g, temperature control ≤ 4°C.

2.1.3. Shaker.

2.1.4. Homogenizer.

2.1.5. Nitrogen evaporator.

2.1.6. Solid phase extraction vacuum manifolds.

2.1.7. Vortex mixer.

2.2. Chemicals

Formic acid, HPLC grade;
Methanol, HPLC grade;
Acetonitrile, HPLC grade;
n-Hexane, HPLC grade;
Trichloroacetic acid, reagent grade;
Disodium hydrogen phosphate, reagent grade;
Citric acid, reagent grade;
Hydrochloric acid, reagent grade;
Disodium ethylenediaminetetraacetate dihydrate, EDTA-Na_2●$2H_2O$, reagent grade;
Dimethylsulfoxide, DMSO, reagent grade;
Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);

Tetracycline hydrochloride, chlortetracycline hydrochloride, oxytetracycline hydrochloride, doxycycline, 4-epimer-tetracycline, 4-epimer-oxytetracycline and 4-epimer-chlortetracycline, reference standards.

2.3. Apparatus

2.3.1. Volumetric flask: 2 mL and 20 mL.

2.3.2. Centrifuge tube: 50 mL, PP.

2.3.3. Solid phase extraction cartridge: Oasis HLB, 6 mL, 500 mg, or an equivalent product.

2.3.4. Membrane filter: 0.22 μm, Nylon.

2.3.5. Ceramic homogenizer: Bond Elut QuEChERS P/N 5982-9313, or an equivalent product.

2.4. Reagents

2.4.1. 0.1 M citric acid

Dissolve and dilute 19 g of citric acid with deionized water to 1000 mL.

2.4.2. 0.2 M disodium hydrogen phosphate

Dissolve and dilute 28.4 g of disodium hydrogen phosphate with deionized water to 1000 mL.

2.4.3. McIlvaine buffer solution

Mix 615 mL of 0.1 M citric acid and 385 mL of 0.2 M disodium hydrogen phosphate, and adjust pH to 4.0 using 0.1 M citric acid or 0.2 M disodium hydrogen phosphate.

2.4.4. Extraction solution

Dissolve and dilute 3.72 g of disodium ethylenediaminetetraacetate dihydrate with the McIlvaine buffer solution to 1000 mL.

2.4.5. 20% methanol

Mix methanol and deionized water at the ratio of 2:8 (v/v).

2.4.6. 20% methanol containing 0.1% formic acid

Dilute 0.1 mL of formic acid with 20% methanol to 100 mL.

2.4.7. 5% methanol

Mix methanol and deionized water at the ratio of 5:95 (v/v).

2.4.8. 2.5% trichloroacetic acid

Dissolve and dilute 25 g of trichloroacetic acid with deionized

water to 1000 mL.

2.5. Mobile phase

2.5.1. Solvent A

Dilute 1 mL of formic acid with deionized water to 1000 mL, and filter with a membrane filter.

2.5.2. Solvent B

Dilute 1 mL of formic acid with acetonitrile to 1000 mL, and filter with a membrane filter.

2.6. Standard solution preparation

Transfer equivalent 10 mg of tetracycline, chlortetracycline and oxytetracycline reference standards accurately weighed; transfer about 10 mg of doxycycline, 4-epimer-tetracycline, 4-epimer-oxytetracycline and 4-epimer-chlortetracycline reference standards accurately weighed to each 10-mL volumetric flask, dissolve and dilute to volume with methanol as the standard stock solutions. Store under freezing. When to use, mix appropriate volume of each standard stock solution, and dilute with methanol to 1 μg/mL as the standard solution.

2.7. Sample solution preparation

2.7.1. Extraction

Transfer about 2 g of the fine-cut and homogenized muscle, visceral or fat sample accurately weighed; remove eggs's shells, and transfer about 2 g of the mixed egg white and yolk sample accurately weighed; accurately transfer 2 mL of the milk sample; transfer about 2 g of the well-mixed honey sample accurately weighed into a centrifuge tube. Add 1 granule of a ceramic homogenizer and 5 mL of 2.5% trichloroacetic acid, vortex-mix for 1 min, shake for 5 min, centrifuge at 3200 ×g for 5 min at 4°C, and collect the supernatant. Add 10 mL of the extraction solution to the residue, vortex-mix for 1 min, shake for 5 min, centrifuge at 3200 ×g for 5 min at 4°C, collect the supernatant, and repeat the above procedure twice. Combine the supernatants, and centrifuge at 12000 ×g for 5 min at 4°C. Take the supernatant for purification.

2.7.2. Purification

Transfer the solution for purification from section 2.7.1 into a solid phase extraction cartridge prerinsed with 6 mL of methanol and 6 mL of deionized water, and discard the eluent. Wash the cartridge with 6 mL of deionized water and 6 mL of 5% methanol in order, and discard the eluent. Add 6 mL of methanol to the cartridge, and collect the eluent. Add 50 μL of DMSO into the eluent, and evaporate to dryness by gently flushing with a stream of nitrogen in a water bath at 40°C. Dissolve and dilute the residue of the muscle, eggs, fat, milk or honey sample with 20% methanol containing 0.1% formic acid to 2 mL; dissolve and dilute the residue of the visceral sample with 20% methanol containing 0.1% formic acid to 20 mL. Take 1 mL of the above solution, centrifuge at 10000 ×g for 3 min, and collect the supernatant. Filter with a membrane filter, and take the filtrate as the sample solution.

2.8. Matrix-matched calibration curve (for the muscle, visceral, fat, milk and honey sample)

Take a blank sample, and follow the extraction, purification and evaporation to dryness procedures described in section 2.7 to obtain the residue. Dissolve the residue of the muscle, fat, milk or honey matrix with 20% methanol containing 0.1% formic acid to 1 mL; dissolve the residue of the visceral matrix with 20% methanol containing 0.1% formic acid to 10 mL as the blank sample solution. Take several 500 μL of the blank sample solution, add 5-200 μL of the standard solution separately and 20% methanol containing 0.1% formic acid to achieve a final volume of 1000 μL. Mix well, centrifuge at 10000 ×g for 3 min, collect the supernatant, and filter with a membrane filter. Take the filtrates as the matrix-matched standard solutions. Operate LC-MS/MS according to the following conditions. Establish the matrix-matched calibration curve of each tetracyclines by the peak areas of each tetracyclines vs. the added concentrations in the range of 0.005-0.2 μg/mL.

LC-MS/MS operating conditions(note):

Column: ACQUITY CSH C18, 1.7 μm, 2.1 mm i.d. × 10 cm.

Column temperature: 40°C.

Mobile phase: a gradient program of solvent A and solvent B is as follows:

Time (min)	A (%)	B (%)
0 → 1	95 → 95	5 → 5
1 → 6	95 → 85	5 → 15
6 → 9	85 → 70	15 → 30
9 → 9.5	70 → 2	30 → 98
9.5 → 14.5	2 → 2	98 → 98
14.5 → 15	2 → 95	98 → 5
15 → 18	95 → 95	5 → 5

Flow rate: 0.2 mL/min.

Injection volume: 5 μL.

Capillary voltage: 2.5 kV.

Ionization mode: ESI^+.

Ion source temperature: 150°C.

Desolvation temperature: 500°C.

Cone gas flow rate: 150 L/hr.

Desolvation flow rate: 1000 L/hr.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, cone voltage and collision energy are shown in the attached table.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.9. Calibration standard curve (for the egg sample)

Take a blank sample, add 10-400 μL of the standard solution separately, and follow the procedure described in section 2.7 to obtain the calibration standard solutions. Operate LC-MS/MS according to the conditions described in section 2.8. Establish the calibration standard curve of each tetracyclines by the peak areas of each tetracyclines vs. the added concentrations in the range of 0.005-0.2 μg/mL.

2.10. Identification and quantification

Accurately inject 5 μL of the sample solution and the matrix-matched standard solutions or the calibration standard solutions into LC-MS/MS separately, and operate according to the conditions described in section 2.8. Identify each tetracyclines based on the

retention time and the relative ion intensities(note). Calculate the amount of each tetracyclines in the sample by the following formula.

$$\text{The amount of each tetracyclines in the sample (ppm)} = \frac{C \times V}{M}$$

Where,

C: the concentration of each tetracyclines in the sample solution calculated by the matrix-matched calibration curve or the calibration standard curve (μg/mL)

V: the final make-up volume of the sample (mL)

M: the weight of the sample (g) or the volume of the sample (mL)

Note: Relative ion intensities are calculated by peak areas of qualitative ions divided by peak areas of quantitation ions. Maximum permitted tolerances of relative ion intensities by LC-MS/MS are as follows:

Relative ion intensity (% of base peak)	Tolerance (%)
> 50	± 20
> 20-50	± 25
> 10-20	± 30
≤ 10	± 50

Remark

1. The limits of quantification (LOQs) for 7 tertacyclines are 0.005 ppm in muscle, fat, eggs, milk and honey, and 0.05 ppm in viscera.
2. Further validation should be performed when interfering compounds appear in samples.

Reference

1. Cetinkaya, F., Yibar, A., Soyutemiz, G. E., Okutan, B., Ozcan, A. and Karaca, M. Y. 2012. Determination of tetracycline residues in chicken meat by liquid chromatography-tandem mass spectrometry. Food Addit. Contam. Part B 5: 45-49.
2. Giannetti, L., Longo, F., Buiarelli, F., Russo, M. V. and Neri, B. 2010. Tetracycline residues in royal jelly and honey by liquid chromatography tandem mass spectrometry: validation study according to Commission Decision 2002/657/EC. Anal. Bioanal. Chem. 398: 1017-1023.

Reference chromatogram

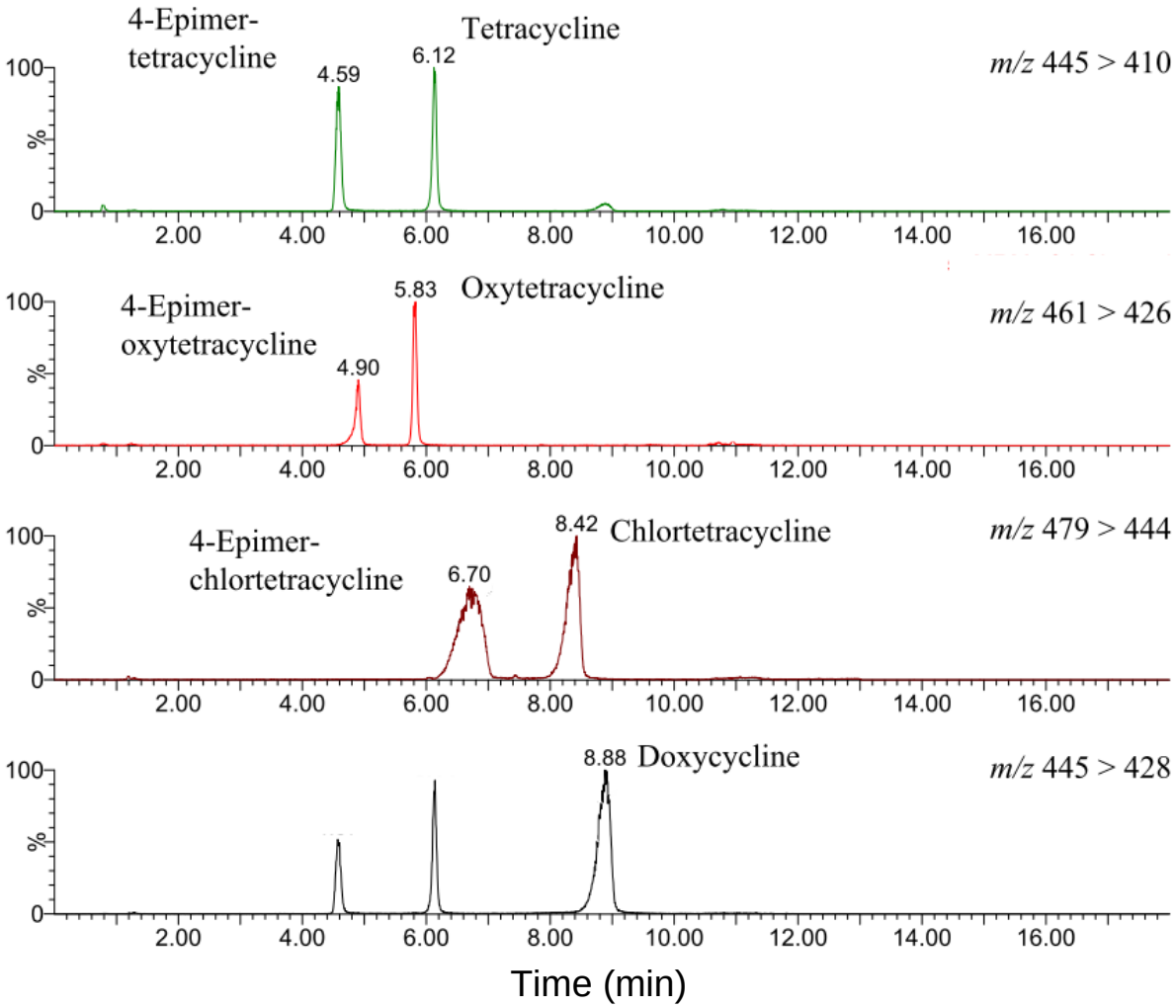

Figure. MRM chromatograms of 7 tetracycline standards analyzed by LC-MS/MS.

Table. The multiple reaction monitoring (MRM) parameters of 7 tetracyclines

Analyte	Ion pair: Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Tetracycline	445 > 410*	26	18
	445 > 427	26	12
	445 > 226	26	53
Oxytetracycline	461 > 426*	26	18
	461 > 443	26	12
	461 > 283	26	38
Chlortetracycline	479 > 444*	34	20
	479 > 462	34	18
	479 > 154	34	28
Doxycycline	445 > 428*	35	17
	445 > 154	35	28
4-Epimer-tetracycline	445 > 410*	24	20
	445 > 427	24	12
	445 > 392	24	25
4-Epimer-oxytetracycline	461 > 426*	28	20
	461 > 201	28	38
	461 > 444	28	15
4-Epimer-chlortetracycline	479 > 462*	34	20
	479 > 444	34	18

*The quantitative ion. The qualitative ion should be selected at least one ion depending on the matrix.