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

1. Scope

This method is applicable to the determination of chloramphenicol, thiamphenicol, florfenicol and florfenicol amine in honey, milk, eggs, muscle, viscera and fat of livestock, poultry and aquatic products.

2. Method

After extraction and purification, analytes 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: electrospray ionization, ESI.

2.1.1.2. Column: XSELECT HSS PFP, 2.5 μm, 2.1 mm i.d. × 10 cm, or an equivalent product.

2.1.2. Centrifuge: centrifugal force ≥ 3200 ×g.

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, reagent grade;
Ethyl acetate, reagent grade;
Ammonium hydroxide (30%), reagent grade;
Trichloroacetic acid, reagent grade;
Glacial acetic acid, reagent grade;
Sodium chloride, reagent grade;
Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);
Chloramphenicol, thiamphenicol, florfenicol and florfenicol amine, reference standards;
Chloramphenicol-d_5, thiamphenicol-d_3, florfenicol-d_3 and

florfenicol amine-d_3, isotope-labelled internal standards.

2.3. Apparatus

2.3.1. Volumetric flask: 100 mL.

2.3.2. Centrifuge tube: 50 mL, PP.

2.3.3. Solid phase extraction cartridge: Oasis MCX, 150 mg, 6 mL, or an equivalent product.

2.3.4. Membrane filter: 0.22 μm, PVDF.

2.4. Reagents

2.4.1. 50% methanol

Dilute 50 mL of methanol with deionized water to 100 mL.

2.4.2. 5% acetic acid

Dilute 5 mL of glacial acetic acid with deionized water to 100 mL.

2.4.3. 5% trichloroacetic acid

Dissolve and dilute 50 g of trichloroacetic acid with deionized water to 1000 mL.

2.4.4. Ethyl acetate containing 0.6% ammonium hydroxide

Dilute 20 mL of ammonium hydroxide with ethyl acetate to 1000 mL.

2.4.5. Methanol containing 3% ammonium hydroxide

Dilute 10 mL of ammonium hydroxide with methanol to 100 mL.

2.4.6. *n*-Hexane saturated with acetonitrile

Add 100 mL of acetonitrile to 1000 mL of *n*-hexane, shake to mix well, and then stand until complete layering. Take the *n*-hexane layer.

2.4.7. 25% methanol

Dilute 25 mL of methanol with deionized water to 100 mL.

2.5. Mobile phase

2.5.1. Mobile phase for analysis of chloramphenicol

2.5.1.1. Solvent A

Deionized water.

2.5.1.2. Solvent B

Methanol.

2.5.2. Mobile phase for analysis of thiamphenicol, florfenicol and florfenicol amine

2.5.2.1. Solvent A

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

2.5.2.2. Solvent B

Methanol.

2.6. Internal standard solution preparation

Transfer about 10 mg of chloramphenicol-d_5, thiamphenicol-d_3, florfenicol-d_3 and florfenicol amine-d_3 isotope-labelled internal standards accurately weighed to each 100-mL volumetric flask, dissolve and dilute with 50% methanol to volume as the internal standard stock solutions. Store under freezing. When to use, mix appropriate volume of each internal standard stock solution, and dilute with 50% methanol to 1 μg/mL for chloramphenicol-d_5, and 10 μg/mL for thiamphenicol-d_3, florfenicol-d_3 and florfenicol amine-d_3 as the internal standard solution.

2.7. Standard solution preparation

Transfer about 10 mg of chloramphenicol, thiamphenicol, florfenicol and florfenicol amine reference standards accurately weighed to each 100-mL volumetric flask, dissolve and dilute with 50% methanol to volume as the standard stock solutions. Store under freezing. When to use, mix appropriate volume of each standard stock solution, and dilute with 50% methanol to 0.0015-0.04 μg/mL for chloramphenicol, and 0.05-1 μg/mL for thiamphenicol, florfenicol and florfenicol amine as the standard solutions.

2.8. Sample solution preparation

2.8.1. Extraction

2.8.1.1. Muscle

Transfer about 5 g of the fine-cut and homogenized muscle sample accurately weighed into a centrifuge tube, and add 5 μL of the internal standard solution and 20 mL of ethyl acetate containing 0.6% ammonium hydroxide. Vortex-mix for 1 min, shake for 10 min, centrifuge at 3200 ×g for 10 min, and collect the supernatant. Add 2 mL of 5% acetic acid to the supernatant, vortex-mix for 1 min, and concentrate to about 2 mL by gently

flushing with a stream of nitrogen in a water bath at 45°C. Add 4 mL of 5% acetic acid, vortex-mix, and take the mixture for purification.

2.8.1.2. Viscera and eggs

Transfer about 5 g of the fine-cut and homogenized viscera sample accurately weighed; remove eggs' shells and transfer about 5 g of the mixed egg white and yolk sample accurately weighed into a centrifuge tube, and add 5 µL of the internal standard solution and 20 mL of 5% trichloroacetic acid. Vortex-mix for 1 min, shake for 10 min, centrifuge at 3200 ×g for 10 min, and collect the supernatant. Add 20 mL of *n*-hexane to the supernatant, vortex-mix for 1 min, centrifuge at 3200 ×g for 5 min, and take the lower layer for purification.

2.8.1.3. Milk

Accurately transfer 5 mL of the milk sample into a centrifuge tube, and add 5 µL of the internal standard solution and 20 mL of 5% trichloroacetic acid. Vortex-mix for 1 min, shake for 10 min, centrifuge at 3200 ×g for 10 min, and take the supernatant for purification.

2.8.1.4. Honey

Transfer about 5 g of the well-mixed honey sample accurately weighed into a centrifuge tube, and add 5 µL of the internal standard solution and 10 mL of 5% acetic acid. Vortex-mix for 1 min, shake for 10 min, and take the mixture for purification.

2.8.1.5. Fat

Transfer about 5 g of the fine-cut and homogenized fat sample accurately weighed into a centrifuge tube, and add 5 µL of the internal standard solution, 5 mL of deionized water, 1 g of sodium chloride and 20 mL of acetonitrile. Vortex-mix for 1 min, shake for 10 min, centrifuge at 3200 ×g for 10 min, and collect the supernatant. Add 20 mL of *n*-Hexane saturated with acetonitrile to the supernatant, vortex-mix for 1 min, centrifuge at 3200 ×g for 5 min, and take the lower layer. Evaporate to dryness by gently flushing with a stream of nitrogen in a water

bath at 45°C. Dissolve the residue with 5 mL of 5% acetic acid, vortex-mix, and take the mixture for purification.

2.8.2. Purification

Transfer the solution for purification from section 2.8.1 into a solid phase extraction cartridge prerinsed with 5 mL of methanol and 5 mL of deionized water, and discard the eluent. Wash the cartridge with 5 mL of 5% acetic acid, and discard the eluent. Add 5 mL of methanol containing 3% ammonium hydroxide to the cartridge, and collect the eluent. Evaporate the eluent to dryness by gently flushing with a stream of nitrogen in a water bath at 45°C. Dissolve the residue with 1 mL of 25% methanol, mix well, and filter with a membrane filter. Take the filtrate as the sample solution.

2.9. Matrix-matched calibration curve

Take a blank sample without adding the internal standard, and follow the extraction, purification and evaporation to dryness procedures described in section 2.8 to obtain the residue. Add 0.5 mL of the standard solution at different concentrations and 5 µL of the internal standard solution to each of the residue, dilute with deionized water to 1 mL, mix well, 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 analyte by the ratios of peak area of each analyte to that of the internal standard vs. the added concentrations of each analyte.

LC-MS/MS operating conditions(note 1):

Column: XSELECT HSS PFP, 2.5 µm, 2.1 mm i.d. × 10 cm.

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

Time (min)	A (%)	B (%)
0.0 → 1.0	95 → 95	5 → 5
1.0 → 2.0	95 → 85	5 → 15
2.0 → 5.0	85 → 30	15 → 70
5.0 → 10.0	30 → 20	70 → 80

10.0 → 10.1	20 → 95	80 → 5
10.1 → 15.0	95 → 95	5 → 5

Flow rate: 0.3 mL/min.

Injection volume: 10 µL.

Ion spray voltage: ESI^+, 5.5 kV; ESI^-, -4.5 kV.

Turbo heater temperature: 550°C.

Nebulizer gas (GS1): 50 psi.

Heated gas (GS2): 50 psi.

Curtain gas: 20 psi.

Collision gas: High.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, declustering potential and collision energy are as follows:

Analyte	Ionization mode	Ion pair	Declustering potential (V)	Collision energy (eV)
		Precursor ion (*m/z*)> product ion (*m/z*)		
Chloramphenicol	ESI^-	321 > 152*	65	22
		321 > 257	65	14
Thiamphenicol	ESI^-	354 > 185*	73	26
		354 > 290	73	16
Florfenicol	ESI^-	356 > 336*	73	12
		356 > 185	73	26
Florfenicol amine	ESI^+	248 > 230*	46	13
		248 > 130	46	33
Chloramphenicol-d_5 (I.S.)	ESI^-	326 > 157	65	22
Thiamphenicol-d_3 (I.S.)	ESI^-	357 > 188	73	26
Florfenicol-d_3 (I.S.)	ESI^-	359 > 339	73	12
Florfenicol amine-d_3 (I.S.)	ESI^+	251 > 233	46	17

*The quantitative ion.

Note 1: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.10. Identification and quantification

Accurately inject 10 µL of the sample solution and the matrix-matched standard solutions into LC-MS/MS separately, and operate according to the conditions described in section 2.9. Identify each analyte based on the retention time and the relative ion intensities(note 2). Calculate the amount of each analyte in the

sample by the following formula:

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

Where,

C: the concentration of each analyte in the sample solution calculated by the matrix-matched calibration 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 2: Relative ion intensities are calculated by peak areas of qualitative ions divided by peak areas of quantitative ions (≤ 100%). Maximum permitted tolerances of relative ion intensities are as follows:

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20～50	± 25
> 10～20	± 30
≤ 10	± 50

Remark

1. The limits of quantitation (LOQs) are 0.00015 ppm for chloramphenicol and 0.005 ppm for thiamphenicol, florfenicol and florfenicol amine in muscle, viscera, fat, eggs, milk and honey.
2. If the mobile phase A in section 2.5.1.1 used for the analysis of chloramphenicol is replaced with that in section 2.5.2.1, verification of the LOQs shall be performed to meet the requirements of the validation guideline.
3. Further validation shall be performed when interfering compounds appear in samples.

Reference

1. Zhang, S., Liu, Z., Guo, X., Cheng, L., Wang, Z. and Shen, J. 2008. Simultaneous determination and confirmation of chloramphenicol, thiamphenicol, florfenicol and florfenicol amine in chicken muscle by liquid chromatography–tandem mass spectrometry. J. Chromatogr. B 875: 399-404.
2. European Commission. 2019. Commission Regulation (EU) 2019/

1871 of 7 November 2019 on reference points for action for non-allowed pharmacologically active substances present in food of animal origin and repealing Decision 2005/34/EC. Off. J. Eur. Union L 289: 41-46.

Reference chromatogram

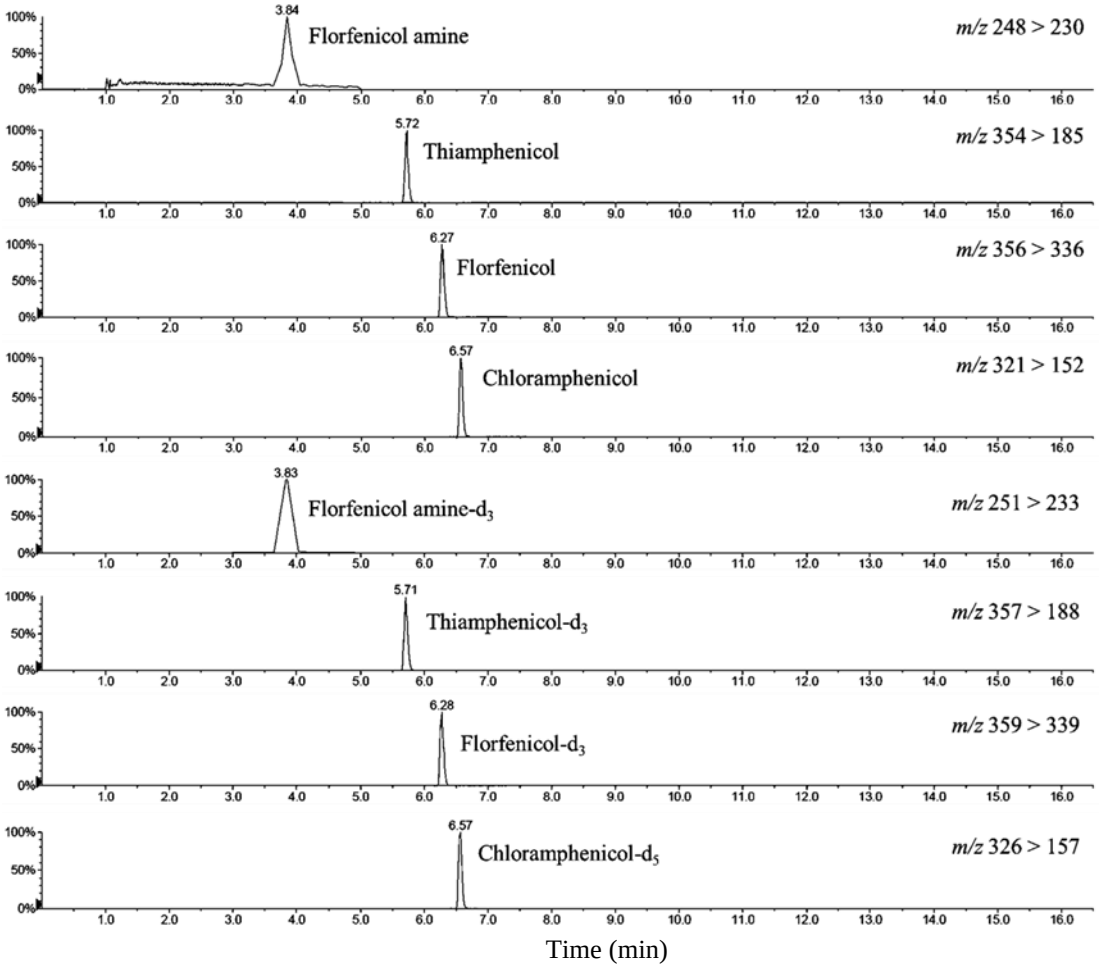

Figure. MRM chromatograms of chloramphenicols standards and their isotope-labelled internal standards analyzed by LC-MS/MS.