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Method of Test for Veterinary Drug Residues in Foods – Test of Closantel

1. Scope

This method is applicable to the determination of closantel residue in muscle, viscera, fat, eggs and milk of poultry and livestock products.

2. Method

After extraction and purification, analyte is 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: CORTECS C18, 2.7 μm, 2.1 mm × 10 cm, or an equivalent product.

2.1.2. Homogenizer.

2.1.3. Vortex mixer.

2.1.4. Shaker.

2.1.5. Centrifuge: centrifugal force ≥ 5000 ×g.

2.1.6. High speed dispersing device: SPEX SamplePrep 2010 GenoGrinder®, ≥ 1000 rpm, or an equivalent product.

2.1.7. Nitrogen evaporator.

2.2. Chemicals

Acetonitrile, HPLC grade;
Methanol, HPLC grade;
Acetone, reagent grade;
n-Hexane, reagent grade;
Formic acid, reagent grade;
Dimethyl sulfoxide, DMSO, reagent grade;
Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);
Closantel, reference standard;
Closantel-$^{13}C_6$, isotope-labelled internal standard.

2.3. Apparatus

2.3.1. Centrifuge tube: 50 mL, PP.

2.3.2. Ceramic homogenizer: Bond Elut QuEChERS P/N 5982-

9313, or an equivalent product.

2.3.3. Volumetric flask: 50 mL.

2.3.2. Membrane filter: 0.22 μm, PTFE.

2.4. Reagents

2.4.1. Extraction solution

Mix acetonitrile and acetone at the ratio of 60:40 (v/v).

2.4.2. 50% methanol

Dilute 500 mL of methanol with deionized water to 1000 mL.

2.4.3. *n*-Hexane saturated with acetonitrile

Add 50 mL of acetonitrile to 500 mL of *n*-hexane. Shake to mix well, and then stand until complete layering. Take the *n*-hexane layer.

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 methanol to 1000 mL, and filter with a membrane filter.

2.6. Standard solution preparation

Transfer about 5 mg of closantel reference standard accurately weighed to a 50-mL volumetric flask, dissolve and dilute with DMSO to volume as the standard stock solution. Store under freezing. When to use, dilute appropriate volume of the standard stock solution with methanol to 1 μg/mL as the standard solution.

2.7. Internal standard solution preparation

Transfer about 5 mg of closantel-$^{13}C_6$ isotope-labelled internal standard accurately weighed to a 50-mL volumetric flask, dissolve and dilute with DMSO to volume as the internal standard stock solution. Store under freezing. When to use, dilute appropriate volume of the internal standard stock solution with methanol to 1 μg/mL as the internal standard solution.

2.8. Sample solution preparation

Transfer about 2 g of the fine-cut and homogenized sample accurately weighed; accurately transfer 2 mL of the milk sample into a centrifuge tube. Add 500 μL of the internal standard solution, one ceramic homogenizer and 10 mL of the extraction solution, cap the centrifuge tube, and vortex-mix for 1 min. Shake at 1000 rpm for 1 min by the high speed dispersing device, centrifuge at 5000 ×g for 1 min, and collect the supernatant. Add 10 mL of the extraction solution to the residue, and repeat the extraction procedure described above. Combine the supernatants, transfer 5 mL of the supernatant into a centrifuge tube, add 10 mL of *n*-hexane saturated with acetonitrile, and shake for 1 min by the high speed dispersing device. Centrifuge at 5000 ×g for 1 min, and collect the lower layer as the sample stock solution. Take 1 mL (a) of the the sample stock solution, and then evaporate to near dryness by gently flushing with a stream of nitrogen at 40°C in a water bath. Dissolve and dilute the residue with 50% methanol to 5 mL (b). Take 1 mL of the above solution, and filter with a membrane filter. Take the filtrate as the sample solution.

2.9. Matrix-matched calibration curve preparation

Take a blank sample without adding the internal standard, and follow the procedure described in section 2.8 to obtain the blank sample stock solution. Transfer 2 mL of the blank sample stock solution into a centrifuge tube, and then evaporate to near dryness by gently flushing with a stream of nitrogen at 40°C in a water bath. Dissolve and dilute the residue with 50% methanol to 5 mL. Take 500 μL of the above solution, add 0.5-20 μL of the standard solution respectively and 5 μL of the internal standard solution, and dilute with 50% methanol to 1000 μL as the matrix-matched standard solutions. Operate LC-MS/MS according to the following conditions. Establish the matrix-matched calibration of closantel by the ratios of peak area of closantel to that of the internal standard vs the added

concentrations in the range of 0.5-20 ng/mL.

LC-MS/MS operating conditions(note)

Column: CORTECS C18, 2.7 μm, 2.1 mm × 10 cm.

Column temperature: 40°C.

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

Time (min)	A (%)	B (%)
0.0 → 5.0	80 → 0	20 → 100
5.0 → 10.0	0 → 0	100 → 100
10.0 → 10.5	0 → 80	100 → 20
10.5 → 13.5	80 → 80	20 → 20

Flow rate: 0.3 mL/min.

Injection volume: 10 μL.

Capilliary voltage: -4.5 kV.

Ionization mode: ESI^-.

Ion source temperature: 100°C.

Turbo heater temperature: 500°C.

Nebulizer gas (GS1): 50 psi.

Heated gas (GS2): 50 psi.

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

Analyte	Ion pair: Precursor ion (*m/z*) > product ion (*m/z*)	Declustering potential (V)	Collision energy (eV)
Closantel	660.8 > 345*	-60	-50
	660.8 > 315		-48
	660.8 > 279		-52
Closantel-$^{13}C_6$	666.8 > 127	-20	-85

*The quantitative ion. The qualitation ion can be selected at least one ion depending on the matrix.

Note: 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. Operate according to the conditions in section 2.9. Identify closantel based on the retention time and the relative ion intensities(note). Calculate the amount of closantel in the sample by the following formula:

$$\text{The amount of closantel in the sample (ppm)} = \frac{C \times V \times F}{M \times 1000}$$

Where,

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

V: the volume of the extraction solution for sample extraction (20 mL)

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

F: the dilution factor, b/a

Note: Relative ion intensities are calculated by peak areas of qualitative ions divided by peak areas of quantitative ion (≤ 100%). Maximum permitted tolerances of relative ion intensities are as follows:

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

Remark

1. Limit of quantification (LOQ) for closantel is 0.025 ppm in muscle, viscera, fat, eggs and milk.
2. Further validation should be performed when interfering compounds appear in the samples.

Reference

1. Sun, H., Wang, F. and Ai, L. 2007. Validated method for determination of ultra-trace closantel residues in bovine tissues and milk by solid-phase extraction and liquid chromatography-electrospray ionization-tandem mass spectrometry. J. Chromatogr. A 1175: 227-233.
2. Lai, S. S., Yeung, H. S., Lee, W. O., Ho, C. and Wong, Y. T. 2011. Determination of closantel and rafoxanide in animal tissues by online anionic mixed-mode solid-phase extraction followed by isotope dilution liquid chromatography tandem mass spectrometry. J. Sep. Sci. 34: 1366-1374.

Reference chromatogram

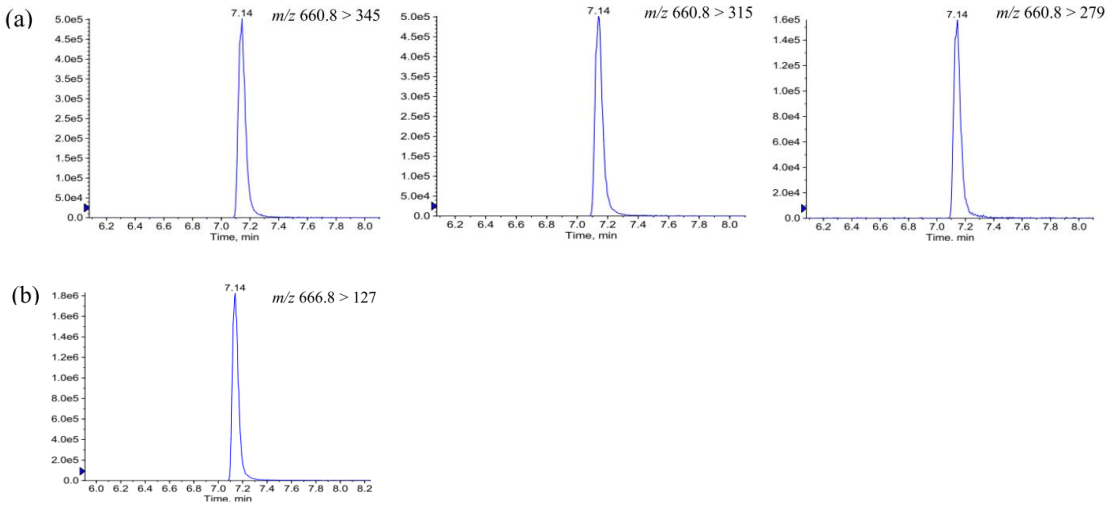

Figure. MRM chromatograms of closantel standard (a) and closantel-$^{13}C_6$ internal standard (b) analyzed by LC-MS/MS.