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Method of Test for Veterinary Drug Residues in Foods - Test of Carbadox and its Metabolites

1. Scope

This method is applicable to the determination of carbadox and its metabolites in muscle and viscera of poultry and livestock 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: Symmetry C8, 3.5 μm, 2.1 mm i.d. × 10 cm, or an equivalent product.

2.1.2. Homogenizer.

2.1.3. Centrifuge: centrifugal force > 4000 ×g.

2.1.4. Rotary evaporator.

2.1.5. Shaker.

2.1.6. pH meter.

2.1.7. Solid phase extraction vacuum manifolds.

2.1.8. Vortex mixer.

2.1.9. Nitrogen evaporator.

2.2. Chemicals

Methanol, HPLC grade;

Acetonitrile, HPLC grade;

Acetic acid, reagent grade;

Phosphoric acid, reagent grade;

Metaphosphoric acid, reagent grade;

Deionized water, resistivity ≥ 18 MΩ·cm (at 25℃);

Carbadox (CBX), desoxycarbadox (DCBX) and quinoxaline-2-carboxylic acid (QCA), reference standards.

2.3. Apparatus

2.3.1. Centrifuge tube: 50 mL, PP.

2.3.2. Volumetric flask: 1 mL and 50 mL, amber.

2.3.3. Round bottom flask: 500 mL, amber.

2.3.4. Solid phase extraction cartridge: Oasis HLB cartridge, 6 mL, 200

mg, or an equivalent product.

2.3.5. Membrane filter: 0.22 μm, Nylon.

2.4. Reagents

2.4.1. 0.01% Acetic acid

Dilute 0.01 mL of acetic acid with deionized water to 100 mL.

2.4.2. 0.3% Metaphosphoric acid

Dissolve and dilute 1.5 g of metaphosphoric acid with deionized water to 500 mL.

2.4.3. Extraction solution

Mix 0.3% metaphosphoric acid and methanol at the ratio of 7:3 (v/v), prepare freshly before use.

2.4.4. 0.01% Acetic acid: acetonitrile (9:1, v/v)

Mix 0.01% acetic acid and acetonitrile at the ratio of 9:1 (v/v).

2.5. Mobile phase

2.5.1. Solvent A

Dilute 0.05 mL of acetic acid with deionized water to 500 mL, and filter with a membrane filter.

2.5.2. Solvent B

Dilute 0.05 mL of acetic acid with acetonitrile to 500 mL, and filter with a membrane filter.

2.6. Standard solution preparation

Transfer about 5 mg of carbadox, desoxycarbadox and quinoxaline-2-carboxylic acid reference standards accurately weighed into each 50-mL volumetric flask, dissolve and dilute with methanol to volume as the standard stock solutions. Store at -18℃ in the dark. When to use, mix appropriate volume of each standard stock solution, and dilute with 0.01% acetic acid: acetonitrile (9:1, v/v) to 1000 ng/mL as the standard solution.

2.7. Sample solution preparation

2.7.1. Extraction:

Transfer about 5 g of the homogenized sample accurately weighed into a homogenizer. Add 40 mL of extraction solution, homogenize for 2 min, and transfer into a 50-mL centrifuge tube. Centrifuge at 4000 ×g for 10 min, and collect the supernatant. Add 10 mL of extraction solution to the residue, vortex for 1 min,

and shake for 10 min. Centrifuge at 4000 ×g for 10 min, and combine the supernatant. Transfer the supernatant into a round bottom flask, and concentrate to about 10 mL under reduced pressure in a water bath at 40°C. Adjust pH to 4.0 ~ 4.5 with phosphoric acid, and centrifuge at 4000 ×g for 10 min. Collect the supernatant for further purification.

2.7.2. Purification:

Transfer the solution for purification from section 2.7.1. into an Oasis HLB cartridge prerinsed with 5 mL of methanol and 5 mL of deionized water, and discard the eluent. Wash the cartridge with 6 mL of deionized water, and discard the eluent. Dry the cartridge under vacuum for 1 min. Add 6 mL of methanol, and collect the eluent. Evaporate to near dryness with a stream nitrogen in a water bath at 40°C. Dissolve and dilute the residue with 0.01% acetic acid: acetonitrile (9:1, v/v) to 1 mL, mix well, and filter with a membrane filter. Take the filtrate as the sample solution.

2.8. Calibration curve

Take a blank sample, add 5 to 100 μL of the standard solutions, and follow the procedure described in section 2.7. to obtain the calibration standard solutions. Operate LC-MS/MS according to the following conditions. Establish the calibration standard curves of carbadox and its metabolites by peak areas vs. the added concentrations in the range of 5 ~ 100 ng/mL.

LC-MS/MS operating conditions(note)

Column: Symmetry C8, 3.5 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 (%)
0.0 → 6.0	100 → 0	0 → 100
6.0 → 6.5	0 → 100	100 → 0
6.5 → 10.0	100 → 100	0 → 0

Flow rate: 0.3 mL/min.

Injection volume: 10 μL.

Capillary voltage: ESI^+, 3.5 kV.

Ion source temperature: 100℃.

Desolvation temperature: 450℃.

Cone gas flow rate: 50 L/hr.

Desolvation flow rate: 800 L/hr.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, cone voltage and collision energy are shown as follows:

Analyte	Ion pair: Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
CBX	263 > 231*	25	15
	263 > 129	25	30
DCBX	231 > 143*	20	20
	231 > 199	20	15
QCA	175 > 129*	20	15
	175 > 102	20	25

*Quantitative ion pair.

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

2.9. Identification and quantification

Accurately inject 10 μL of the sample solution and the calibration standard solutions into LC-MS/MS separately. Operate according to the conditions in section 2.8. Identify carbadox and its metabolites based on the retention time and the relative ion intensities(note). Calculate the amount of carbadox and its metabolites in the sample by the following formula:

The amount of carbadox and its metabolites in the sample (ppm)

$$= \frac{C \times V}{M \times 1000}$$

Where,

C: the concentration of carbadox and its metabolites in the sample solution calculated by the calibration curves (ng/mL)

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

M: the weight of the sample (g)

Note: Relative ion intensities are calculated by peak areas of quantitative ions divided by peak areas of qualitative 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. Limits of quantification (LOQs) for carbadox and its metabolites are 0.001 ppm in muscle and 0.003 ppm in viscera.
2. Further validation should be performed when interfering compounds appear in samples.

Reference

Anna, M., George, K. and Georgios, T. 2012. Determination of carbadox and metabolites of carbadox and olaquindox in muscle tissue using high performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. B. 881-882: 90-95.