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

1. Scope

This method is applicable to the determination of bacitracin in eggs.

2. Method

After extraction and purification, bacitracin 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: SHIMADZU Shim-pack GIST C18, 2 μm, 2.1 mm i.d. × 10 cm, or an equivalent product.

2.1.2. Solid phase extraction vacuum manifolds.

2.1.3. Nitrogen evaporator.

2.1.4. Centrifuge: centrifugal force ≥ 10000 ×g, temperature control ≤ 0°C.

2.1.5. Ultrasonicator.

2.1.6. Vortex mixer.

2.2. Chemicals

Methanol, HPLC grade;

Acetonitrile, HPLC grade;

n-Hexane, HPLC grade;

Formic acid, reagent grade;

Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);

Bacitracin A, reference standard.

2.3. Apparatus

2.3.1. Volumetric flask: 1 mL and 10 mL.

2.3.2. Centrifuge tube: 15 mL and 50 mL, PP.

2.3.3. Membrane filter: 0.22 μm, PVDF

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

2.4. Reagents

2.4.1. 0.1% formic acid

Dilute 1 mL of formic acid with deionized water to 1000 mL.

2.4.2. 0.1% formic acid: acetonitrile (4:1, v/v)

Mix 0.1% formic acid solution and acetonitrile at the ratio of 4:1 (v/v).

2.4.3. Extraction solution

Mix 0.1% formic acid and methanol at the ratio of 5:2 (v/v). Prepare freshly before use.

2.5. Mobile phase

2.5.1. Solvent A

Dilute 2 mL of formic acid with deionized water to 1000 mL, and filter with a membrane filter.

2.5.2. Solvent B

Dilute 2 mL of formic acid with acetonitrile to 1000 mL, and filter with a membrane filter.

2.6. Standard solution preparation

Transfer about 10 mg of bacitracin A reference standard accurately weighed to a 10-mL volumetric flask, dissolve and dilute with 0.1% formic acid to volume as the standard stock solution. Store under refrigeration in the dark. When to use, dilute appropriate volume of the standard stock solution with 0.1% formic acid: acetonitrile (4:1, v/v) to 1000 ng/mL as the standard solution.

2.7. Sample solution preparation

2.7.1. Extraction

Remove eggs' shells, and transfer about 2.5 g of the mixed egg white and yolk sample accurately weighed into a 50-mL centrifuge tube. Add 25 mL of the extraction solution, sonicate for 10 min, centrifuge at 10000 ×g for 10 min at 0°C, and collect the supernatant. Add 10 mL of *n*-hexane to the residue, and vortex-mix for 1 min. Centrifuge at 10000 ×g for 10 min at 0°C, and collect the lower layer for purification.

2.7.2. Purification

Transfer the solution for purification from section 2.7.1 into the solid phase extraction cartridge prerinsed with 5 mL of methanol and 5 mL of deionized water, and discard the eluent. Wash the cartridge with 10 mL of deionized water, and discard the eluent. Add 5 mL of methanol to the cartridge, and collect the eluent. Evaporate the eluent to dryness by gently flushing with a stream of nitrogen at 40°C in a water bath. Dissolve and dilute the residue with 0.1% formic acid: acetonitrile (4:1, v/v) to 1 mL. Filter with a membrane filter, and take the filtrate as the sample solution.

2.8. Calibration standard curve preparation

Take a blank sample, add 25-150 μL of the standard, and follow the procedure

described in section 2.7 to obtain the calibration standard solutions. Establish the calibration standard curve of bacitracin A by the peak areas of bacitracin A vs. the added concentrations in the range of 25-150 ng/mL.

LC-MS/MS operating conditions(note1):

Column: SHIMADZU Shim-pack GIST C18, 2 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 → 3.0	95 → 0	5 → 100
3.0 → 5.0	0 → 0	100 → 100
5.0 → 6.0	0 → 95	100 → 5
6.0 → 8.0	95 → 95	5 → 5

Flow rate: 0.4 mL/min.

Injection volume: 5 μL.

Interface voltage: 1 kV.

Ionization mode: ESI^+.

Interface temperature: 295°C.

Nebulizing gas flow: 3.0 L/min.

Heating gas low: 15.0 L/min.

Desolvation line temperature: 185°C.

Heat block temperature: 300°C.

Drying gas flow: 5.0 L/min.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, Q1/Q3 Pre Bias and collision voltage are as follows:

Analyte	Ion pair	Q1/Q3 Pre Bias (V)	Collision energy (eV)
	Precursor ion (*m/z*) > product ion (*m/z*)		
Bacitracin A	475 > 199*	30/20	26
	475 > 669.8	30/24	15

*The quantitative ion.

Note 1: 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 5 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 bacitracin A based on the retention time and the relative ion intensities(note2). Calculate the amount of bacitracin in the sample by the following formula(note3):

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

C: the concentration of bacitracin A in the sample solution calculated by the calibration standard curve (ng/mL)

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

M: the weight of the sample (g)

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

Note 3: The amount of bacitracin in the sample is expressed as bacitracin A.

Remark

1. Limit of quantification (LOQ) for bacitracin is 0.01 ppm.
2. Further validation should be performed when interfering compounds appear in samples.

Reference

1. Lin, W. X., Sun, X. Q., Tian, M., Yu, L., Chen, X. and Li, Z. 2009. Determination of colistin, bacitracin and virginiamycin multiresidues in animal tissue by liquid chromatography - tandem mass spectrometry. J. Instrum. Anal. 28: 212 - 215.
2. Tao, Y., Xie, S., Zhu, Y., Chen, D., Pan, Y., Wang, X., Liu, Z., Huang, L., Peng, D. and Yuan, Z. 2018. Analysis of major components of bacitracin, colistin and virginiamycin in feed using matrix solid-phase dispersion extraction by liquid chromatography-electrospray ionization tandem mass spectrometry. J Chromatogr. Sci. 56: 285 - 291.

Reference chromatogram

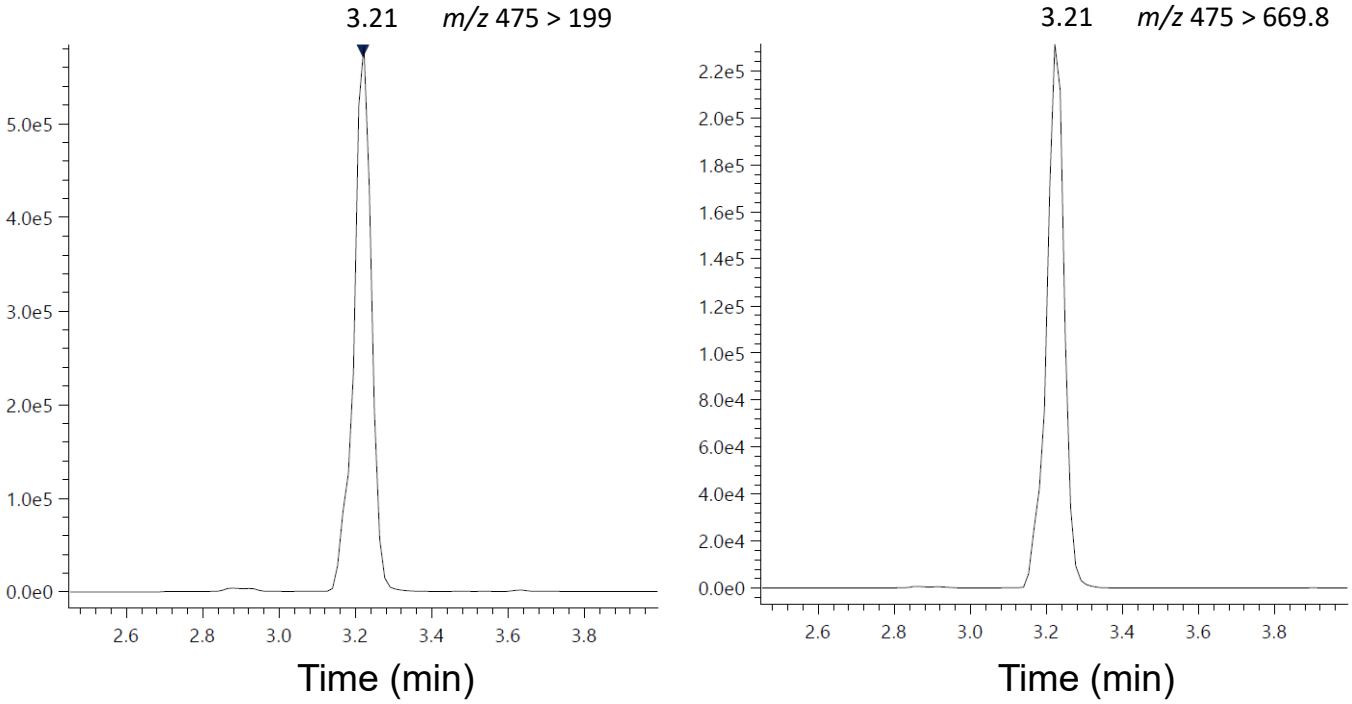

Figure. MRM chromatograms of bacitracin A standard analyzed by LC-MS/MS.