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

1. Scope

This method is applicable to the determination of nystatin residue in muscle, viscera, 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: Poroshell 120EC-C18, 2.7 μm, 3.0 mm × 10 cm, or an equivalent product.

2.1.2. Homogenizer.

2.1.3. Vortex mixer.

2.1.4. Centrifuge: centrifugal force ≥ 5000 ×g and temperature control ≤ 10°C.

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

2.2. Chemicals

Methanol, HPLC grade;
Formic acid, reagent grade;
Dimethyl sulfoxide, DMSO, reagent grade;
Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);
Nystatin, reference 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: 10 mL.

2.3.4. Membrane filter: 0.22 μm, PVDF.

2.4. Reagents

2.4.1. Methanol containing 1% formic acid

Mix methanol and formic acid at the ratio of 99:1 (v/v).

2.4.2. 1% Formic acid

Mix deionized water and formic acid at the ratio of 99:1 (v/v).

2.4.3. 0.1% Formic acid

Mix 1% formic acid and deionized water at the ratio of 1:9 (v/v).

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 nystatin reference standard accurately weighed to a 10-mL volumetric flask, dissolve and dilute with DMSO to volume as the standard stock solution. Store under freezeing. When to use, dilute appropriate volume of the standard stock solution with methanol to 100 μg/mL as the standard solution.

2.7. Sample solution preparation

Transfer about 2 g of the fine-cut and homogenized muscle or visceral sample accurately weighed; remove eggs' shells, and transfer about 2 g of the mixed egg white and yolk sample accurately weighed; accurately transfer 2 mL of the milk sample into a centrifuge tube. Add one ceramic homogenizer and 10 mL of methanol containing 1% formic acid, cap the centrifuge tube, and vortex-mix for 1 min. Shake at 1000 rpm for 10 min by the high speed dispersing device, and centrifuge at 5000 ×g for 5 min at 10°C. Dilute 250 μL (a) of the supernatant with 0.1% formic acid to 1000 μL (b), mix well, and filter with a membrane filter. Take the filtrate as the sample solution.

2.8. Calibration curve preparation

Take a blank sample, add 2-200 μL of the standard solutions, and follow the procedure described in section 2.7 to obtain the calibration standard solution. Operate LC-MS/MS according to the following conditions. Establish the calibration curve of nystatin by the peak areas vs. the added concentrations in the range of 5-500 ng/mL.

LC-MS/MS operating conditions(note)

Column: Poroshell 120EC-C18, 2.7 μm, 3.0 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 → 2.0	80 → 80	20 → 20
2.0 → 3.0	80 → 20	20 → 80
3.0 → 5.0	20 → 20	80 → 80
5.0 → 5.5	20 → 0	80 → 100
5.5 → 11.0	0 → 0	100 → 100
11.0 → 11.5	0 → 80	100 → 20
11.5 → 13.5	80 → 80	20 → 20

Flow rate: 0.3 mL/min.

Injection volume: 20 μL.

Ion sprary voltage: 5.5 kV.

Ionization mode: ESI^+.

Ion source temperature: 100°C.

Turbo heater temperature: 500°C.

Nebulizer gas (GS1): 50 psi.

Heated gas (GS2): 50 psi.

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	Ion pair	Declustering	Collision

	Precursor ion (*m/z*) > product ion (*m/z*)	potential (V)	energy (eV)
Nystatin	926.5 > 691.5*	50	28
	926.5 > 727.3		28
	926.5 > 673.4		24

*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.

2.9. Identification and quantification

Accurately inject 20 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 nystatin based on the retention time and the relative ion intensities(note). Calculate the amount of nystatin in the sample by the following formula:

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

Where,

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

V: the volume of the methanol containing 1% formic acid for sample extraction (10 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 ions (≤ 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 nystatin is 0.1 ppm.
2. Further validation should be performed when interfering compounds appear in the samples.

Reference

1. Scheuch, E., Gießmann, T. and Siegmund, W. 2006. Quantitative determination of nystatin in human plasma using LC-MS after inhalative administration in healthy subjects. J. Chromatogr. B 844: 84-88.
2. Huang, C. H., Shen, Y. U., Chen, H. M., Su, W. T., Ting, Y., Chang, S. H., Liao, C. D., Kao, Y. M., Tseng, S. H., Wang, D. Y. and Chen, H. F. 2016. Research for residual analysis of veterinary drugs in foods. The Research Project of the Food and Drug Administration, Ministry of Health and Welfare, Taiwan.

Reference chromatogram

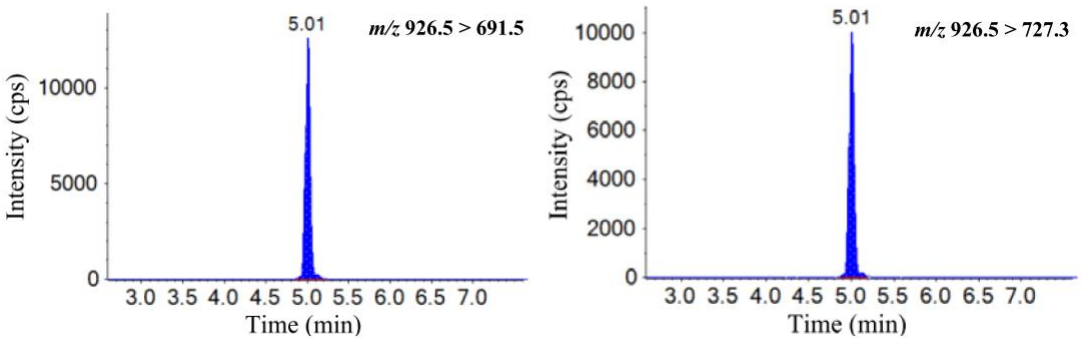

Figure. MRM chromatograms of nystatin analyzed by LC-MS/MS.