Promulgated, Apr 14, 2021
MOHWV0050.00

# Method of Test for Veterinary Drug Residues in Foods - Test of Nitrovin (2)

## 1. Scope

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

## 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 and temperature control ≤ 10°C

2.1.6. High speed dispersing device: SPEX SamplePrep 2010 GenoGrinder®, ≥ 1000 rpm, or an other mechanical shaker.

2.1.7. Ultrasonicator: temperature control ≥ 50°C.

2.1.8. Nitrogen evaporator.

2.2. Chemicals

Acetonitrile, HPLC grade;
Methanol, HPLC grade;
Ethyl acetate, HPLC grade;
*n*-Hexane, HPLC grade;
Formic acid, reagent grade;
Dimethyl sulfoxide, DMSO, reagent grade;
Magnesium sulfate anhydrous, reagent grade;
Sodium chloride, reagent grade;
Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);
Nitrovin, reference standard.

2.3. Apparatus

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

2.3.2. Extraction powder(note): containing 6 g of magnesium sulfate anhydrous and 1.5 g of sodium chloride.

Note: commercial extraction kits can be used as needed.

2.3.3. Ceramic homogenizer: Bond Elut QuEChERS P/N 5982-9313, or an equivalent product.

2.3.4. Volumetric flask: 50 mL.

2.3.5. Membrane filter: 0.22 μm, PTFE.

2.4. Reagents

2.4.1. Acetonitrile containing 1% formic acid

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

2.4.2. Acetonitrile containing 1% formic acid: methanol (95:5, v/v)

Mix acetonitrile containing 1% formic acid and methanol at the ratio of 95:5 (v/v).

2.4.3. Acetonitrile: ethyl acetate (4:1, v/v)

Mix acetonitrile and ethyl acetate at the ratio of 4:1 (v/v).

2.4.4. 50% Acetonitrile

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

2.4.5. *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 nitrovin 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, mix appropriate volume of the standard stock solution, and dilute with methanol to 100 ng/mL as the standard solution.

2.7. Sample solution preparation

2.7.1. Muscle, viscera, eggs, honey and milk

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; transfer about 2 g of the well-mixed honey accurately weighed; accurately transfer 2 mL of the milk sample into a 50-mL centrifuge tube. Add one ceramic homogenizer and 10 mL of pre-cooled deionized water, and stand for 10 min. Add 5 mL of acetonitrile containing 1% formic acid: methanol (95:5, v/v), cap the centrifuge tube, and vortex-mix for 1 min. Shake at 1000 rpm for 1 min by the high speed dispersing device or shake vigorously by hands for 1 min. Add the extraction powder, cap the centrifuge tube, shake vigorously several times by hands to prevent coagulation of salts, and then shake at 1000 rpm for 1 min by the high speed dispersing device or shake vigorously by hands for 1 min. Centrifuge at 5000 ×g for 1 min at 10$^{o}$C, and collect the supernatant. Add 5 mL of acetonitrile containing 1% formic acid: methanol (95:5, v/v) to the residue, cap the centrifuge tube, vortex-mix for 1 min, and repeat the extract procedure described above. Combine the supernatants as the sample stock solution. Transfer 500 μL (a) of the sample stock solution into a 15-mL centrifuge tube, and then evaporate to near dryness by gently flushing with a stream of nitrogen at 40$^{o}$C in a water bath. Dissolve and dilute the residue with 50% acetonitrile to 1 mL (b). Add 1 mL of *n*-hexane saturated with acetonitrile, shake for 1 min, and centrifuge at 5000 ×g for 1 min. Take the lower layer, and filter with a membrane filter. Take the filtrate as the sample solution.

2.7.2. Fat

Transfer about 2 g of the fine-cut and homogenized fat sample accurately weighed into a 50-mL centrifuge tube. Add one ceramic homogenizer and 10 mL of acetonitrile: ethyl acetate (4:1, v/v), cap the centrifuge tube, vortex-mix for 1 min, and ultrasonicate for 15 min at 50°C. Centrifuge at 5000 ×g for 1 min at 10°C, and collect the supernatant as the sample stock solution. Transfer 500 μL (a) of the sample stock solution into a 15-mL 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% acetonitrile to 1 mL (b). Add 1 mL of *n*-hexane saturated with acetonitrile, shake for 1 min, and centrifuge at 5000 ×g for 1 min at 10°C. Take the lower layer, and filter with a membrane filter. Take the filtrate as the sample solution.

2.8. Matrix-matched calibration curve preparation

Take a blank sample, and follow the procedure described in section 2.7 to obtain the blank sample stock solution. Transfer 5 mL of the blank sample stock solution into a 15-mL 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% acetonitrile to 5 mL. Take 500 μL of the above solution, add 1-100 μL of the standard solution respectively, and dilute with 50% acetonitrile to 1000 μL. Add 1 mL of *n*-hexane saturated with acetonitrile, shake for 1 min, and centrifuge at 5000 ×g for 1 min at 10°C. Take the lower layer, filter with a membrane filter, and 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 nitrovin by the peak areas of nitrovin vs. the added concentrations in the range of 0.1-10 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 → 1.0 | 80 → 80 | 20 → 20 |
| 1.0 → 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.

Ion spray voltage: 5.5 kV.

Ionization mode: $ESI^+$.

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 potential (V) | Collision energy (eV) |
|---|---|---|---|
| | Precursor ion (*m/z*)> product ion (*m/z*) | | |
| Nitrovin | 361 > 222* | 200 | 27 |
| | 361 > 302 | | 29 |

*The quantitative ion.

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 matrix-matched standard solutions into LC-MS/MS separately.

Operate according to the conditions in section 2.8. Identify nitrovin based on the retention time and the relative ion intensities(note). Calculate the amount of nitrovin in the sample by the following formula:

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

Where,

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

V: the volume of acetonitrile containing 1% formic acid: methanol (95:5, v/v) or acetonitrile: ethyl acetate (4:1, v/v) 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 nitrovin is 0.001 ppm.
2. Further validation should be performed when interfering compounds appear in the samples.

**Reference**

Tao, Y., Yu, H., Chen, D., Liu, Z. Y., Yang, D., Pan, Y., Wang, Y., Huang, L. and Yuan, Z. 2010. Determination of sodium nifurstyrenate and nitrovin residues in edible food by liquid chromatography-tandem mass spectrometry after ultrasound-

assisted extraction. J. Chromatogr. B 878: 3415-3420.