Published, Jun 10, 2020
Revised, Nov 6, 2020
TFDAV0018.01

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

1. Scope

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

2. Method

After extraction and purification, piperazine 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: XBridge® BEH HILIC, 2.5 μm, 2.1 mm i.d. × 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, temperature control ≤ 4°C.

2.1.6. High speed centrifuge: centrifugal force ≥ 12000 ×g.

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

2.1.8. Ultrasonicator.

2.1.9. Solid phase vacuum extraction manifolds.

2.1.10. Nitrogen evaporator.

2.2. Chemicals

Acetonitrile, HPLC grade;
Methanol, HPLC grade;
n-Hexane, HPLC grade;
Ammonium acetate, reagent grade;
Formic acid, reagent grade;
Dimethyl sulfoxide (DMSO), reagent grade;
Trichloroacetic acid, reagent grade;
Ammonium hydroxide (25%), reagent grade;
Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);
Piperazine, reference standard;
Piperazine-d_8, 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. Solid phase extraction cartridge: Bond Elute Plex PCX, 200 mg, 6 mL, or an equivalent product.

2.3.4. Volumetric flask: 5 mL and 10 mL.

2.3.5. Membrane filter: 0.22 μm, PTFE.

2.4. Reagents

2.4.1. 5% trichloroacetic acid

Dissolve and dilute 10 g of trichloroacetic acid with deionized water to 200 mL.

2.4.2. Ammonium hydroxide: acetonitrile (1:9, v/v) solution

Mix ammonium hydroxide and acetonitrile at the ratio of 1:9 (v/v). Prepare freshly before use.

2.4.3. 2% formic acid

Dilute 4 mL of formic acid with deionized water to 200 mL.

2.4.4. *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.

2.4.5. 2 M ammonium acetate

Dissolve and dilute 15.42 g of ammonium acetate with deionized water to 100 mL.

2.4.6. 50 mM ammonium acetate

Dilute 25 mL of 2 M ammonium acetate with deionized water to 1000 mL.

2.5. Mobile phase

2.5.1. Solvent A

Dilute 1 mL of formic acid with 50 mM ammonium acetate to 1000 mL, and filter with a membrane filter.

2.5.2. Solvent B

Dilute 1 mL of formic acid with acetonitrile to 1000 mL, and filter with a membrane filter.

2.6. Internal standard solution preparation

Transfer about 10 mg of piperazine-d_8 internal standard accurately weighed to a 10-mL volumetric flask, dissolve and dilute with methanol 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 1000 ng/mL as the internal standard solution.

2.7. Standard solution preparation

Transfer about 10 mg of piperazine reference standard accurately weighed to a 10-mL volumetric flask, dissolve and dilute with methanol to volume as the standard stock solution. Store under freezing. When to use, dilute appropriate volume of the standard stock solution with methanol to 100 ng/mL as the standard solution.

2.8. Sample solution preparation

2.8.1. Extraction

Transfer about 2 g of the fine-cut and homogenized muscle, viscera or fat 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 200 μL of the internal standard solution, one ceramic homogenizer, 6 mL of 5% trichloroacetic acid and 5 mL of acetonitrile, cap the centrifuge tube, and vortex-mix for 1 min. Sonicate for 10 min under 30°C, centrifuge at 5000 ×g for 10 min at 4°C, and collect the supernatant. Add 6 mL of 5% trichloroacetic acid and 2 mL of acetonitrile to the residue, cap the centrifuge tube, and vortex-mix for 1 min. Repeat the procedure described above once, and combine the supernatants. Add 10 mL of *n*-hexane saturated with acetonitrile, and shake for 1 min. Centrifuge at 5000 ×g for 5 min at 4°C, and collect the lower layer for purification.

2.8.2. Purification

Transfer the solution for purification from section 2.8.1 into the solid phase extraction cartridge pre-rinsed with 5 mL of methanol, 5 mL of deionized water and 5 mL of 2% formic acid, and discard the eluents. Wash the cartridge with 5 mL of 2% formic acid, 5 mL of deionized water and 5 mL of methanol in order, and discard the eluents. Add 10 mL of ammonium hydroxide: acetonitrile (1:9, v/v) solution to the cartridge, and collect the eluent as the stock sample solution. Add 50 μL of DMSO, and evaporate the eluent to slight dryness by gently flushing with a stream of nitrogen at 40°C in a water bath. Dissolve and dilute the residue with acetonitrile to 10 mL. Take 1 mL of the above solution, centrifuge at 12000 ×g for 3 min

at room temperature, and collect the supernatant. Filter with a membrane filter, and 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. Add 50 μL of DMSO, evaporate the eluent to slight dryness by gently flushing with a stream of nitrogen at 40°C in a water bath, dissolve and dilute the residue with acetonitrile to 5 mL. Take 500 μL of the above solution, add 20-250 μL of the standard solution respectively and 20 μL of the internal standard solution, and dilute with acetonitrile to 1000 μL. Mix well, centrifuge at 12000 ×g for 3 min at room temperature, and collect the supernatant. Filter with a membrane filter, and take the filtrate as the matrix-matched standard solutions. Operate LC-MS/MS according to the following conditions. Establish the matrix-matched calibration of piperazine by the ratios of peak area of piperazine to that of the internal standard vs. the added concentrations in the range of 2-25 ng/mL.

LC-MS/MS operating conditions(note):

Column: XBridge® BEH HILIC, 2.5 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 → 1.0	5 → 5	95 → 95
1.0 → 8.5	5 → 30	95 → 70
8.5 → 10.0	30 → 50	70 → 50
10.0 → 11.0	50 → 5	50 → 95
11.0 → 23.0	5 → 5	95 → 95

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 (DP) and collision energy (CE) are as follows:

Analyte	Ion pair	Declustering potential (V)	Collision energy (eV)
	Precursor ion (*m/z*) > product ion (*m/z*)		
Piperazine	87 > 44*	7	25
	87 > 70	12	21
Piperazine-d_8 (I.S.)	95 > 48	12	27

* The quantitative ion.

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

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

Where,

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

V: the final make-up volume of the sample (10 mL)

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

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 (%)	Tolerance (%)
> 50	± 20
> 20-50	± 25
> 10-20	± 30
≤ 10	± 50

Remark

1. Limit of quantitation (LOQ) for piperazine is 0.01 ppm in muscle, viscera, milk, eggs and fat.
2. Further validation should be performed when interfering compounds appear in the samples.

Reference

1. Xie, K., Liu, Y. N., Sun, L., Pang, M., Xie, X., Gao, Q., Wang, B., Zhang, Y., Wang, R., Zhang, G., Dai, G. and Wang, J. 2017. Quantification of piperazine in chicken muscle by ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry. Food Anal. Methods 10: 1736-1744.
2. Chung, S. W. C. and Lam, C. H. 2015. Development of a 15-class multiresidue method for analyzing 78 hydrophilic and hydrophobic veterinary drugs in milk, egg and meat by liquid chromatography-tandem mass spectrometry. Anal. Methods 7: 6764-6776.

Reference chromatogram

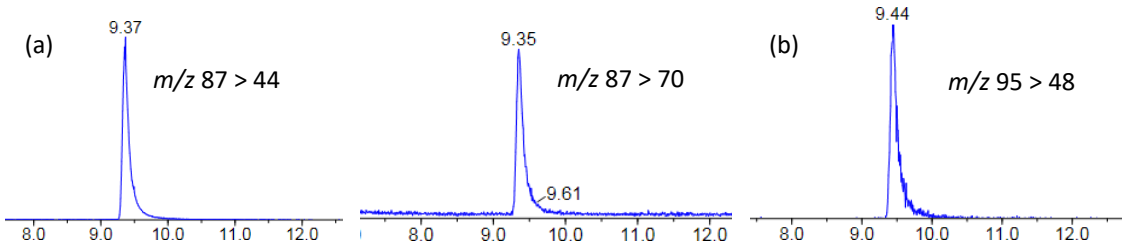

Figure. MRM chromatograms of piperazine standard (a) and piperazine-d_8 internal standard (b) analyzed by LC-MS/MS.