Analytical Method for Clenbuterol
(Targeted to Animal Products)

Target compounds to be determined is clenbuterol

1. Instrument

Liquid chromatograph-tandem mass spectrometer (LC-MS/MS)

2. Reagents

In addition to the reagents and test solutions listed below, use those listed in Section C ***Reagent/Test Solution, Etc.***, Part II ***Food Additives***,.

Reagents designated as “special grade” in this section must meet the requirements for “special grade” specified in the Japan Industrial Standards for the reagents.

Acetone: Use a reagent not containing any substance that may interfere with the analysis of the target compositional substances.

Acetonitril: Use a reagent not containing any substance that may interfere with the analysis of the target compositional substances.

Sodium chloride: Use a reagent not containing any substance that may interfere with the analysis of the target compositional substances.

Strongly acidic cation exchanger cartridge column (500 mg): A polyethylene column of 8–9 mm in inner diameter packed with 500 mg of benzenesulfony propylsilanized silica gel or a column equivalent to the specified one in separation capability.

Ethyl acetate: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

Silica gel cartridge column (1,000 mg): A polyethylene column of 8–9 mm in inner diameter packed with 1,000 mg of silica gel produced for column chromatography or a column equivalent to the specified one in separation capability.

Triethylamine: Triethylamine (special grade)

n-Hexane: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

Water: Use water suitable for chemical analysis, including distilled water, purified water, or pure water. If it contains any substance that may interfere with analysis of the target compositional substances, wash with a solvent such as *n*-hexane before use.

Sodium sulfate (anhydrous): Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

Methanol: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

3. Reference standard

Reference standard of clenbuterol : Contains not less than 98% of clenbuterol hydrochloric acid.

4. Procedure

a. Extraction

Weigh 10.0 g of the test sample for muscle, liver, kidney, fish/shellfish, milk, or egg, 5.00 g of the test sample for fat, and 10.0 g of the test sample for honey, respectively.

To the sample, add 20 mL of water to dissolve it. To this solution, add 100 mL of acetone, homogenize the mixture, and centrifuge at 3,000 rpm 5min.

Collect the supernatant.

Add 50 mL of acetone to the residue, homogenize the mixture, and centrifuge as directed above.

Combine the supernatants, and evaporate it at a temperature not more than 40°C to about 30 mL. Add 100 mL of 10% (w/v) sodium chloride and 5 mL of 2 mol/L sodium chloride, and perform shaking-extraction twice with ethyl acetate, (100 mL and then 50 mL).

Combine the extracts obtained, and add anhydrous sodium sulfate to dehydrate.

Remove the anhydrous sodium sulfate by filtration, and evaporate the filtrate at a temperature not more than 40°C to remove the solvent.

Add 30 mL of *n*-hexanes to the residue, and perform shaking-extraction twice with 30 mL of acetonitrile saturated with *n*-hexane each time.

Combine the extract obtained and evaporate it at a temperature not more than 40°C to remove the solvent.

Dissolve the residue by adding 5 mL of a mixture of acetone/triethylamine/*n*-hexane (30:1:170).

b. Clean-up

i. Silica gel column chromatography

Pour 10 mL each of acetone and *n*-hexane in series into a silica gel cartridge column (1,000 mg) and discard the effluent.

Into the column, pour the solution obtained in (1), pour 10 mL of a mixture of acetone/triethylamine/n-hexane (30:1:170), and discard the effluent.

Then pour 15 mL of acetone and evaporate the eluate at a temperature not more than 40°C to remove the solvent. Dissolve the residue by adding 5 mL of a mixture of water/methanol (1:1).

ii Strongly acidic cation exchanger cartridge column chromatography

Pour 5 mL each of methanol and water in series into a strongly acidic cation exchanger cartridge column (500 mg) and discard the effluent.

Into the column, pour the solution obtained in “i”, then pour 10 mL of methanol, and discard the effluent.

Pour 10 mL of a mixture of ammonia solution/methanol (1:49), evaporate the eluate at a temperature not more than 40°C to remove the solvent to remove the solvent.

Dissolve the residue in a mixture of acetonitrile/formic acid/water (300:1:700) to make exactly 2 mL (1 mL for a fat sample).

5. Measurement

a. Calibration curve

Prepare several solutions of clenbuterol Reference Standard in a mixture of acetonitrile/formic acid/water (300:1:700) with different concentrations.

Inject them into LC-MS/MS to prepare a calibration curve using the peak height or peak area method.

When the sample solution is prepared as directed in this method, the concentrate in the sample solution equivalent to 0.00005 mg/kg in the sample is 0.00025 mg/L.

b. Quantification

Inject the sample solutions in LC-MS/MS and determine the content of clenbuterol using the calibration curve prepared in “a”.

c. Confirmation

Conduct confirmation tests using LC-MS/MS.

d. Measurement conditions

Column: Octadecylsilanized silica gel (2.1 mm in inner diameter, 150 mm in length; 3.5 μm in particle size)

Column temperature: 40°C

Mobile phase: Maintain a mixture of 0.1% (vol) formic acid in acetonitrile/0.1% (vol) formic acid at 1:19 for 1 minute and then create a concentrate gradient of 1:19 to 3:7 in 13 minutes.

Ionized mode: ESI (+)

Main ions (m/z): Precursor ion 277; product ions 203, 132

Injection volume: 10 μL

Retention time: About 10 minutes

6. Limit of quantification

0.00005 mg/kg