Analytical Method for Diethylstilbestrol
(Targeted to Animal and Fishery Products)

The target compounds to be determined are diethylstilbestrol and diethylstilbestrol glucuronide.

1. Instrument

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

2. Reagents

Use the reagents listed in Section C ***Reagent/Test Solution, Etc.***, Part II ***Food Additives***, except the following.

Acetonitrile: Use a reagent not containing any substance that may interfere with the analysis of the target compounds.

Ethanol: Use a reagent not containing any substance that may interfere with the analysis of the target compounds.

Ethylenediamine-*N*-propylsilanized silica gel cartridge (1,000 mg): A polyethylene tube of 12-13 mm in inside diameter packed with 1,000 mg of ethylenediamine-*N*-propylsilanized silica gel, or a cartridge equivalent to the specified one in separation capability.

β-Glucuronidase solution: Contains 100,000 unit/mL of β-glucuronidase derived from *Helix pomatia.* One unit of this reagent is the amount of enzyme that liberates 1.0 μg of phenolphthalein per hour at pH 5.0 at 37-38ºC using phenolphthalein β-D-glucuronide as the substrate. Or use a reagent equivalent to the specified one in enzyme activity. The reagent should not contain any substance that may interfere with the analysis of the target compounds.

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

0.1 mol/L sodium acetate solution (pH 5.0)

Solution 1: Dissolve 0.82 g of sodium acetate in water to make exactly 100 mL.

Solution 2: Dissolve 0.60 g of Acetic acid in water to make exactly 100mL.

Add solution 2 to solution 1, mix and adjust pH to 5.0.

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

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

3. Reference standard

Reference standard of diethylstilbestrol: Contains not less than 98% of diethylstilbestrol.

4. Procedure

a. Extraction

Weigh 10.0 g of sample, add 50 mL of ethanol/water (9:1, v/v), homogenize, centrifuge at 3,000 rpm for 5 minutes, and collect the supernatant. Add 30 mL of ethanol/water (9:1, v/v) to the residue, homogenize, and centrifuge as described above. Collect the supernatant, combine the resulting supernatants, and add ethanol/water (9:1, v/v) to make exactly 100 mL. Take exactly 10 mL of the solution and concentrate to about 5 mL at below 40ºC. Add 10 mL of 0.1 mol/L sodium acetate solution (pH 5.0) and mix well.

b. Hydrolysis

Add 0.1 mL of β-glucuronidase solution to the solution obtained in "a Extraction", mix, let stand for 60 minutes at 37ºC with occasional shaking. Extract with shaking twice with 10 mL each of ethyl acetate/*n*-hexane (3:1, v/v). Combine the resulting extracts, concentrate at below 40ºC, and remove the solvent. Dissolve the residue in 2 mL of ethyl acetate.

c. Clean-up

Add 5 mL of ethyl acetate to an ethylenediamine-*N*-propylsilanized silica gel cartridge (1,000 mg), and discard the effluent. Transfer the solution obtained in "b. Hydrolysis", add 10 mL of ethyl acetate, and discard the effluent. Elute with 10 mL of ethanol/ethyl acetate (1:9, v/v), concentrate the eluate at below 40ºC, and remove the solvent. Dissolve the residue in acetonitrile/water (1:1, v/v) to make exactly 1 mL, and use this solution as the test solution.

5. Measurement

a. Calibration curve

Prepare diethylstilbestrol standard solution (acetonitrile/water (1:1, v/v)) of several concentrations. Inject each standard solution to LC-MS/MS, and make a calibration curve by peak-height or peak-area method. When the test solution is prepared following the above procedure, the sample containing 0.0005 mg/kg of diethylstilbestrol gives the test solution of 0.0005 mg/L in concentration.

b. Quantification

Inject the test solution to LC-MS/MS, and calculate the concentration of diethylstilbestrol from the calibration curve made in " a. Calibration curve".

c. Confirmation

Confirm using LC-MS/MS.

d. Measurement conditions

Column: Octadecylsilanized silica gel, 2.1 mm in inside diameter, 150 mm in length, 3 μm in particle diameter

Column temperature: 40°C

Mobile phase: Acetonitrile/2 mmol/L ammonium acetate (3:2, v/v)

Ionization mode: ESI (–)

Major monitoring ions (*m/z*): Precursor ion 267, product ion 237, 222

Injection volume: 5 μL

Expected retention time: 3 minutes

6. Limit of Quantification

0.0005 mg/kg