Analytical Method for α- trenbolone and β-trenbolone
(Targeted to Animal and Fishery Products)

The target compound to be α- trenbolone and β-trenbolone

1. Instrument

High-performance liquid chromatograph with an ultraviolet spectrophotometric detector (HPLC-UV)

Liquid chromatograph/mass spectrometer (LC/MS or LC/MS/MS)

2. Reagents

Use the reagent listed in Section C *Reagents/Test Solutions, Etc.,* Part II Food Additives, except the following.

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

Weakly basic anion exchanger cartridge column (500 mg): A polyethylene column of 8-9 mm in inner diameter packed with 500 mg of diethylaminopropyl weakly basic anion exchanger produced for column chromatography, or a column equivalent to the specified one in separation capability.

Hydroxypropyl dextran for column chromatography: Dextran (25-100 μm in particle size) chemically bonded with a hydroxypropyl group produced for column chromatography.

Dichloromethane: Dichloromethane (special grade).

3. Reference standard

Reference standard of α- trenbolone: Contains not less than 94% of α- trenbolone, and its melting point is 110°C.

Reference standard of β-trenbolone: Contains not less than 99% of β-trenbolone, and its melting point is 186°C.

4. Procedure

a. Extraction

Weigh 5.00 g out of test sample, previously ground. For muscle, remove the fat layer as much as possible before grinding. Add 20 ml of acetonitrile/methanol (4:1) and homogenize the mixture again. Centrifuge at 2,600 rpm for five minutes and collect the supernatant in a 100-ml separating funnel. Add 20 mL of acetonitrile/methanol (4:1) to

the precipitate and centrifuge the mixture under the above conditions, and then combine the acetonitrile and methanol layer to the separating funnel above. Add 20 mL of water-saturated n-hexane to the funnel and shake it vigorously for five minutes, and then leave it to stand. Transfer the acetonitrile and methanol layer to a 200-mL separating funnel. Add 40 mL each of 5% sodium sulfate solution and dichloromethane to the funnel and shake it vigorously for five minutes and leave it to stand, and then transfer the dichloromethane layer into a rotary vacuum evaporator. Add 15 mL of dichloromethane to the aqueous layer and repeat the above procedure and combine the dichloromethane layer into the rotary vacuum evaporator, and then remove the dichloromethane at 40°C or lower. Dissolve the residue in 1 mL of *n*-hexane/benzene (3:1).

b. Clean-up

i. Weakly basic anion exchanger column chromatography

Pour 6 ml of *n*-hexane/benzene (3:1) into a weakly basic anion exchanger cartridge column (500 mg) and discard the effluent. Pour the the solution obtained by the extraction described in 4-a into the column followed by 2 mL of *n*-hexane/benzene (3:1) and discard the effluent. Pour 3 mL of dichloromethane/methanol (9:1) into the column and collect the eluate into a rotary vacuum evaporator, and then evaporate the solvent to near dryness under nitrogen at 40°C or lower. Dissolve the residue in 0.5 mL of benzene/methanol (17:3).

ii. Hydroxypropyl dextran for column chromatography

Add hydroxypropyl dextran for column chromatography suspended in benzene/methanol (17:3), previously left to stand for 12 hours, into a polyethylene column (6 mm in inner diameter) so as to obtain a 120-mm long layer of dextran. Spill out the benzene/methanol (17:3) until only a small amount remains on the packing of the column. Pour the solution obtained by chromatography described in 4-b-i into this column followed by 10 mL of benzene/methanol (17:3). Discard 2.0 mL of the first effluent and collect the next eluate into a rotary vacuum evaporator, and then evaporate the solvent to near dryness under nitrogen at 40°C or lower. Dissolve the residue in 0.5 mL of acetonitrile/water (1:1), which is used as the sample solution.

5. Measurement

a. Calibration curve

Perfom qualitative tests under the following conditions. Test results obtained must be

the same as those obtained for the reference standard.

Testing conditions

Column packing: Octadecylsilane-bonded silica gel (5 μm in particle size).

Column: A stainless tube (4.0-6.0 mm in inner diameter, 150 mm in length).

Column temperature: 40°C

Detector: Operate with an absorption wavelength of 340 nm.

Mobile phase: Use acetonitrile/water (5:6). Adjust the flow rate so that α- trenbolone flows out in approximately 10 minutes.

b. Quantitative tests

Determine the quantity from the test results obtained under the conditions described in 5-a using either the peak height or peak area method.

c. Confirmation tests

Perform liquid chromatography/mass spectrometry under the conditions described in 5-a. Test results obtained must be the same as those obtained for the reference standard. Determine the quantity using either the peak height or peak area method, if required.

6. Limit of quantification

0.002 mg/kg