Original: Japanese

Provisional Translation

Analytical Method for Oxytetracycline, Chlortetracycline and Tetracycline (Animal and Fishery Products)

1. Analytes

Oxytetracycline

Chlortetracycline

Tetracycline

2. Instrument

High performance liquid chromatograph-fluorometric detector (HPLC-FL)

3. Reagents

Use the reagents listed in Section 3 of the General Rules, except the following.

Imidazole: Imidazole (GR)

Imidazole buffer solution: Dissolve 68.08 g of imidazole, 0.37 g of disodium ethylenediaminetetraacetate and 10.72 g of magnesium acetate in water to make 800 mL. Adjust pH to 7.2 with acetic acid, and add water to make exactly 1,000 mL.

Citrate buffer solution containing ethylenediaminetetraacetic acid (EDTA): Dissolve 21.0 g of citric acid in water to make exactly 1,000 mL (Solution I). Dissolve 71.6 g of disodium hydrogen phosphate in water to make exactly 1,000 mL (Solution II). Mix 307 mL of Solution I and 193 mL of Solution II, and dissolve 1.86 g of disodium ethylenediaminetetraacetate.

Styrene-divinylbenzene copolymer cartridge (265 mg): Polyethylene tube of 8-9 mm in inside diameter packed with 265 mg of styrene-divinylbenzene copolymer for column chromatography, or other cartridge with equal separation characteristics.

4. Reference standards

Reference standard of oxytetracycline hydrochloride: 1.000 mg of reference standard of oxytetracycline hydrochloride contains not less than 0.850 mg (titer) of oxytetracycline. Decomposition point of the standard is 190-194°C

Reference standard of chlortetracycline hydrochloride: 1.000 mg of reference standard of chlortetracycline hydrochloride contains not less than 0.900 mg (titer) of chlortetracycline hydrochloride. Decomposition point of the standard is above 210°C.

Reference standard of tetracycline hydrochloride: 1.000 mg of reference standard of tetracycline hydrochloride contains not less than 0.900 mg (titer) of tetracycline hydrochloride. Decomposition point of the standard is above 214°C.

5. Procedure

1) Extraction

i) Muscle, liver and kidney

For muscle, remove a fat part as much as possible, cut into small piece and mix uniformly, and weigh 5.00 g.

For liver and kidney, cut into small piece and mix uniformly, and weigh 5.00 g.

Add 30 mL of citrate buffer solution containing EDTA to the sample, homogenize for 1 minute, centrifuge at 3,000 rpm for 10 minutes, and transfer the aqueous layer to a 100 mL separating funnel. Add 20 mL of citrate buffer solution containing EDTA to the residue in the centrifuge tube, shake vigorously for 1 minute with a shaker, centrifuge as described above, and combine the aqueous layer in the separating funnel. Add 20 mL of *n*-hexane, shake vigorously for 5 minutes with a shaker, centrifuge at 3,000 rpm for 10 minutes, and take the aqueous layer.

ii) Fat

Remove a muscle part as much as possible, cut into small piece and mix uniformly, and weigh 20.0 g.

Add 200 mL of *n*-hexane, homogenize for 1 minute, add 40 mL of citrate buffer solution containing EDTA, homogenize for 1 minute again, centrifuge at 3,000 rpm for 10 minutes, and take an aqueous layer. Add 20 mL of citrate buffer solution containing EDTA to the *n*-hexane layer, shake vigorously for 1 minute with a shaker, centrifuge as described above, take the aqueous layer and combine with the first aqueous layer.

iii) Milk

Weigh 5.00 g of sample, add 30 mL of citrate buffer solution containing EDTA and 20 mL of *n*-hexane, and shake vigorously for 5 minutes with a shaker. Centrifuge at 3,000 rpm for 10 minutes, and take an aqueous layer.

iv) Egg

Remove eggshells, homogenize sufficiently, and weigh 5.00 g. Add 30 mL of citrate buffer solution containing EDTA to the sample, homogenize, add 100 mL of *n*-hexane, homogenize for 1 minute, centrifuge at 3,000 rpm for 10 minutes, and transfer the aqueous layer to a 100 mL separating funnel. Add 20 mL of citrate buffer solution containing EDTA to the residue in the centrifuge tube, shake vigorously for 1 minute with a shaker, centrifuge as described above, and combine the aqueous layer in the separating funnel. Add 20 mL of *n*-hexane, shake vigorously for 5 minutes with a shaker, centrifuge at 3,000 rpm for 10 minutes, and take the aqueous layer.

v) Fish/shellfish

For shellfish with shells, remove shells, cut into small piece and mix uniformly, and weigh 5.00 g.

For others, cut into small piece and mix uniformly, and weigh 5.00 g.

Add 30 mL of citrate buffer solution containing EDTA to the sample, homogenize for 1 minute, centrifuge at 3,000 rpm for 10 minutes, and transfer the aqueous layer to a 100 mL separating funnel. Add 20 mL of citrate buffer solution containing EDTA to the residue in

the centrifuge tube, shake vigorously for 1 minute with a shaker, centrifuge as described above, and combine the aqueous layer in the separating funnel. Add 20 mL of *n*-hexane, shake vigorously for 5 minutes with a shaker, centrifuge at 3,000 rpm for 10 minutes, and take the aqueous layer.

2) Clean-up

Add 10 mL of methanol, 10 mL of water and 5 mL of saturated disodium ethylenediaminetetraacetate solution to a styrene-divinylbenzene copolymer cartridge (265 mg) sequentially, and discard the effluent. Transfer the solution obtained in 1) to the cartridge, add 10 mL of water, and discard the effluent. Add 10 mL of methanol, collect the eluate to a vacuum rotary evaporator flask, and remove the solvent at below 40°C. Dissolve the residue in 1.0 mL of 1.36% potassium dihydrogen phosphate solution, and use this solution as the test solution.

6. Measurement

1) Qualification

Perform the test under the measurement conditions described below. The results shall agree with those obtained using the reference standards.

Measurement conditions

Column packing material: Octadecylsilanized silica gel (5 μm in particle diameter)

Column tube: Stainless tube 4.0-6.0 mm in inside diameter and 150 mm in length

Column temperature: 40°C

Detector: Perform with excitation wavelength 380 nm and emission wavelength 520 nm.

Mobile phase: For test of oxytetracycline and tetracycline, use imidazole buffer solution/methanol (17:3, v/v). Adjust the flow rate to elute oxytetracycline at about 5 minutes. For test of chlortetracycline, use imidazole buffer solution/methanol (3:1, v/v). Adjust the flow rate to elute chlortetracycline at about 7 minutes.

2) Quantification

Quantify using peak-height or peak-area method, on the basis of the results obtained using the measurement conditions described in 1).

7. Limits of quantification

1) Muscle, liver, kidney, milk, egg and fish/shellfish

Oxytetracycline: 0.02 mg/kg

Chlortetracycline: 0.03 mg/kg

Tetracycline: 0.02 mg/kg

2) Fat

Oxytetracycline: 0.005mg/kg

8. Explanatory note

1) Procedure

i) Centrifugation should be performed at room temperature.

ii) If the filtrate after filtration with suction is cloudy, centrifuge the filtrate.

iii) After transferring the extract to a styrene-divinylbenzene copolymer column, wash away EDTA from the column sufficiently with 30 mL of water divided into several aliquots.

2) Preparation of standard solutions

i) Dissolve a reference standard which corresponds to 10.0 mg of oxytetracycline in 10 mL of methanol, and use this solution as a stock standard solution of oxytetracycline (1,000 mg (titer) of oxytetracycline/L, 1,000 mg/L). This stock standard solution is stable for 1 year at -20°C.

ii) Dissolve a reference standard which corresponds to 10.0 mg of chlortetracycline in 10 mL of methanol, and use this solution as a stock standard solution of chlortetracycline (1,076 mg (titer) of chlortetracycline /L, 1,000 mg/L). This stock standard solution is stable for 1 year at -20°C.

iii) Dissolve a reference standard which corresponds to 10.0 mg of tetracycline in 10 mL of methanol, and use this solution as a stock standard solution of tetracycline (1,082 mg (titer) of tetracycline /L, 1,000 mg/L). This stock standard solution is stable for 1 year at -20°C.

iv) Dilute each stock standard solution of oxytetracycline, chlortetracycline and tetracycline with 1.36% of potassium dihydrogen phosphate solution gradually, and use these solutions as standard solutions for the calibration curves.

3) Others

i) If it is efficient to use a screening method which can test many samples simultaneously, test using “Screening Method for Oxytetracycline, Chlortetracycline and Tetracycline”, and then apply this analytical method to positive samples.

ii) When oxytetracycline, chlortetracycline and tetracycline are detected by this analytical method, it is recommended to use liquid chromatograph-mass spectrometer for confirmation.

9. References

None

10. Type

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