Original: Japanese

Provisional Translation

Analytical Method for Oxytetracycline (Agricultural Products)

1. Analyte

Oxytetracycline

2. Instruments

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

Liquid chromatograph-mass spectrometer (LC-MS)

3. Reagents

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

Imidazole: Imidazole (G.R.)

Imidazole buffer: Dissolve 68.08 g of imidazole, 0.37 g of ethylenediaminetetraacetic acid disodium salt 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 containing ethylenediaminetetraacetic acid: Solution 1: Dissolve 21.0 g of citric acid in water to make exactly 1,000 mL. Solution 2: Dissolve 71.6 g of disodium hydrogen phosphate in water to make exactly 1,000 mL. Dissolve 1.86 g of ethylenediaminetetraacetic acid disodium salt in the mixture of 307 mL of solution 1 and 193 mL of solution 2.

Weakly acidic cation-exchange resin bonding carboxymethyl group cartridge (250 mg): Polypropylene tube of 12–13 mm in inside diameter packed with 250 mg of weakly acidic cation-exchange resin bonding carboxymethyl group, or other cartridge with equal separation characteristics.

Reference standard of oxytetracycline hydrochloride: 1.000 mg of reference standard of oxytetracycline hydrochloride contains not less than 0.850 mg potency of oxytetracycline.

4. Procedure

1) Extraction

i) Grains, legumes, nuts and seeds

Weigh 10.0 g of sample, add 20 mL of water, and let stand for 2 hours. Add 50 mL of citrate buffer containing ethylenediaminetetraacetic acid, homogenize for 3 minutes, centrifuge at 3,000 rpm for 10 minutes, and collect the aqueous layer. Add 20 mL of citrate buffer containing ethylenediaminetetraacetic acid to the residue, homogenize for 1 minute, and centrifuge as described above. Combine the aqueous layers and filter with suction. Add citrate buffer containing ethylenediaminetetraacetic acid to the filtrate to make exactly 100 mL.

ii) Fruits and vegetables

Weigh 20.0 g of sample, add 50 mL of citrate buffer containing ethylenediaminetetraacetic acid, homogenize for 3 minutes, centrifuge at 3,000 rpm for 10 minutes, and collect the aqueous layer. Add 20 mL of citrate buffer containing ethylenediaminetetraacetic acid to the

residue, homogenize for 1 minute, and centrifuge as described above. Combine the aqueous layers and filter with suction. Add citrate buffer containing ethylenediaminetetraacetic acid to the filtrate to make exactly 100 mL.

2) Clean-up

Add 10 mL of methanol, 10 mL of water and 5 mL of saturated ethylenediaminetetraacetic acid disodium salt solution to a styrene-divinylbenzene copolymer cartridge (500 mg) sequentially, and discard the effluent. Add 10 mL of methanol, 10 mL of 2% formic acid, 20 mL of water and 5 mL of methanol to a weakly acidic cation-exchange resin bonding carboxymethyl group cartridge (250 mg) sequentially, and discard the effluent. Transfer 20 mL of the extract obtained in 1) to the styrene-divinylbenzene copolymer cartridge, and discard the effluent. Add 30 mL of water and discard the effluent. Attach the weakly acidic cation-exchange resin bonding carboxymethyl group cartridge under the styrene-divinylbenzene copolymer cartridge, add 5 mL of methanol, and discard the effluent. Remove the styrene-divinylbenzene copolymer cartridge, elute with 5 mL of formic acid/methanol (1:1, v/v) from the weakly acidic cation-exchange resin bonding carboxymethyl group cartridge, concentrate the eluate under nitrogen stream and remove the solvent. Dissolve the residue in 1.36% potassium dihydrogen phosphate to make 1.0 mL, and use this solution as the test solution.

5. Calibration curve

Dissolve reference standard corresponding to 10.0 mg (potency) of oxytetracycline in methanol to make 10 mL and use this solution as reference standard solution. Dilute the reference standard solution with 1.36% potassium dihydrogen phosphate and prepare 0.02–1 mg/L standard solutions. Inject 10 µL of each standard solution to HPLC and make a calibration curve by peak-height or peak-area method.

6. Quantification

Inject 10 µL of the test solution to HPLC and calculate the concentration of oxytetracycline from the calibration curve made in **5**.

7. Confirmation

Confirm using LC-MS.

8. Measurement conditions

1) HPLC

Detector: FL (Excitation wavelength 380 nm, emission wavelength 520 nm)

Column: Octadecylsilanized silica gel, 4.6 mm in inside diameter, 150–250 mm in length and 5 µm in particle diameter

Column temperature: 30°C

Mobile phase: Imidazole buffer/methanol (17:3, v/v)

Expected retention time: 5 min

2) LC-MS

Column: Octadecylsilanized silica gel, 2.0–4.6 mm in inside diameter, 50–250 mm in length and 3-5 µm in particle diameter

Column temperature: 40°C

Mobile phase: Acetonitrile/0.1% formic acid/water (4:15:1, v/v/v)

Ionization mode: ESI (+)

Major monitoring ion (*m/z*): 461, 443

Expected retention time: 2.5 min

9. Limit of quantification

0.01 mg/kg

10. Explanatory note

1) Outline of analytical method

The method consists of extraction of oxytetracycline from sample with citrate buffer containing ethylenediaminetetraacetic acid, clean-up with a styrene-divinylbenzene copolymer cartridge and a weakly acidic cation-exchange resin bonding carboxymethyl group cartridge, quantification using HPLC-FL, and confirmation using LC-MS.

2) Notes

i) Centrifugation at 4°C is recommended.

ii) For a glutinous sample such as kiwi, the addition of about 5 g of Celite before extraction may improve the recovery.

iii) With the measurement conditions described in **8** 2), the peak may not be identified in the total ion chromatogram in low concentration samples. In such a case, concentrate the test solution or use alternative methods for confirmation.

11. References

1) MHLW Director Notice (Syoku-An No.0124001, January 24, 2005), Analytical Methods for Residual Compositional Substances of Agricultural Chemicals, Feed Additives and Veterinary Drugs in Food: Analytical Method for oxytetracycline, chlortetracycline and tetracycline (Animal and Fishery Products)

2) Yoshida K. and Uemori H., *Chromatography*, **26**, 67–69 (2005)

12. Type

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