

**Analytical Method for 2, 4, 5-T**  
**(Targeted to Agricultural, Animal and Fishery Products)**

The target compound to be determined is 2, 4, 5-T.

**1. Instrument**

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

**2. Reagents**

Use the reagents listed in Section C *Reagents/Test Solutions, 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 compound.

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

Ether: Diethyl ether. Use a reagent not containing any substance that may interfere with the analysis of the target compound.

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

Graphitized carbon black/ethylenediamine-*N*-propylsilanized silica gel layered cartridge (500 mg/500 mg): A polyethylene tube of 12-13 mm in inside diameter packed with 500 mg of graphitized carbon black in the upper layer and 500 mg of ethylenediamine-*N*-propylsilanized silica gel in the lower layer, or a cartridge equivalent to the specified one in separation capability.

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

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

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

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 compound, wash with a solvent such as *n*-hexane before use.

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

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

### 3. Reference standard

Reference standard of 2,4,5-T : Contains not less than 98% of 2,4,5-T.

### 4. Procedure

#### a. Extraction

##### i. Grains, legumes, nuts and seeds

Add 20 mL of water to 10.0 g of sample, and let stand for 30 minutes. Add 5 mL of 4 mol/L hydrochloric acid and 100 mL of acetone, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and treat as described above. Combine the resulting filtrates, and add acetone to make exactly 200 mL.

Take exactly a 10 mL aliquot of the solution, add 100 mL of 10 w/v% sodium chloride solution, and extract with shaking twice with 100 mL and 50 mL of ethyl acetate/*n*-hexane (1:1, v/v). Combine the extracts, dehydrate with sodium sulfate (anhydrous), filter out the sodium sulfate (anhydrous), concentrate the filtrate at below 40°C, and remove the solvent.

Add 30 mL of *n*-hexane to the residue, and extract with shaking three times with 30 mL of acetonitrile/water (99:1, v/v). Combine the extracts, concentrate at below 40°C and remove the solvent.

##### ii. Fruits and vegetables

Add 5 mL of 4 mol/L hydrochloric acid and 100 mL of acetone to 20.0 g of sample, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and filter as described above. Combine the resulting filtrates, and add acetone to make exactly 200 mL.

Take exactly a 5 mL aliquot of the solution, add 100 mL of 10 w/v% sodium chloride solution, and extract with shaking twice with 100 mL and 50 mL of ethyl acetate/*n*-hexane (1:1, v/v). Combine the extracts, dehydrate with sodium sulfate (anhydrous), filter out the sodium sulfate (anhydrous), concentrate the filtrate at below 40°C, and remove the solvent.

##### iii. Tea and hops

Add 20 mL of water to 5.00 g of the sample, and let stand for 30 minutes. Add 5 mL of 4 mol/L hydrochloric acid and 100 mL of acetone, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and filter

as described above. Combine the resulting filtrates, and add acetone to make exactly 200 mL.

Take exactly a 10 mL aliquot of the solution, add 100 mL of 10 w/v% sodium chloride solution, and extract with shaking twice with 100 mL and 50 mL of ethyl acetate/*n*-hexane (1:1, v/v). Combine the extracts, dehydrate with sodium sulfate (anhydrous), filter out the sodium sulfate (anhydrous), concentrate the filtrate at below 40°C, and remove the solvent.

Add 30 mL of *n*-hexane to the residue, and extract with shaking three times with 30 mL of acetonitrile/water (99:1, v/v). Combine the extracts, concentrate at below 40°C, and remove the solvent.

iv. Muscle, liver, kidney, milk, egg and fish/shellfish

Add 5 mL of 4 mol/L hydrochloric acid and 100 mL of acetone to 10.0 g of sample, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and treat as described above. Combine the resulting filtrates, and add acetone to make exactly 200 mL.

Take exactly a 10 mL aliquot of the solution, add 100 mL of 10 w/v% sodium chloride solution, and extract with shaking twice with 100 mL and 50 mL of ethyl acetate/*n*-hexane (1:1, v/v). Combine the extracts, dehydrate with sodium sulfate (anhydrous), filter out the sodium sulfate (anhydrous), concentrate the filtrate at below 40°C, and remove the solvent.

Add 30 mL of *n*-hexane to the residue, and extract with shaking three times with 30 mL of acetonitrile/water (99:1, v/v). Combine the extracts, concentrate at below 40°C, and remove the solvent.

v. Fat

Add 5 mL of 4 mol/L hydrochloric acid and 100 mL of acetone to 5.00 g of sample, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and filter as described above. Combine the resulting filtrates, and add acetone to make exactly 200 mL.

Take exactly a 20 mL aliquot of the solution, add 100 mL of 10 w/v% sodium chloride solution, and extract with shaking twice with 100 mL and 50 mL of ethyl acetate/*n*-hexane (1:1, v/v). Combine the extracts, dehydrate with sodium sulfate (anhydrous), filter out the sodium sulfate (anhydrous), concentrate the filtrate at below 40°C, and remove the solvent.

Add 30 mL of *n*-hexane to the residue, and extract with shaking three times with 30 mL acetonitrile/water (99:1, v/v). Combine the extracts, concentrate at below 40°C, and remove the solvent.

vi. Honey

Dissolve 10.0 g of sample in 20 mL of water. Add 5 mL of 4 mol/L hydrochloric acid and 100 mL of acetone, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and filter as described above. Combine the resulting filtrates, and add acetone to make exactly 200 mL.

Take exactly a 10 mL aliquot of the solution, add 100 mL of 10 w/v% sodium chloride solution, extract with shaking twice with 100 mL and 50 mL of ethyl acetate/*n*-hexane (1:1, v/v). Combine the extracts, dehydrate with sodium sulfate (anhydrous), filter out the sodium sulfate (anhydrous), concentrate the filtrate at below 40°C, and remove the solvent.

b. Hydrolysis

Dissolve the residue obtained in “a. Extraction” in 2 mL of methanol, add 1 mL of a 1.5 mol/L sodium hydroxide solution. Attach a reflux condenser to the flask, heat at 80°C for 30 minutes in a water bath, and allowed to cool. Add 1.5 mol/L hydrochloric acid to adjust pH 7.5 - 8.0, then add 16 mL of 0.1 w/v% sodium hydrogen carbonate solution.

c. Clean-up

i. Octadecylsilanized silica gel column chromatography

Add 10 mL each of methanol and water to an octadecylsilanized silica gel cartridge (1,000 mg) sequentially, and discard the effluents. Transfer the solution obtained in “b. Hydrolysis” to the cartridge and discard the effluent. Elute with 20 mL of 0.1 w/v% sodium hydrogen carbonate/methanol (1:1, v/v), add 5 mL of 4 mol/L hydrochloric acid to the eluate, and adjust pH 1.0 or less. Add 100 mL of 10 w/v% sodium chloride solution to the resulting solution and extract with shaking twice with 50 mL ether. Combine the extracts, dehydrate with sodium sulfate (anhydrous), and filter out the sodium sulfate (anhydrous), concentrate the filtrate at below 40°C and remove the solvent. Dissolve the residue in 3 mL of acetonitrile/toluene (3:1, v/v).

ii. Graphitized carbon block/ethylenediamine-*N*-propylsilanized silica gel layered column chromatography

Add 10 mL of acetonitrile/toluene (3:1, v/v) to a graphitized carbon black/ethylenediamine-*N*-propylsilanized silica gel layered cartridge (500 mg/500 mg) and discard the effluent. Transfer the solution obtained in “i. Octadecylsilanized silica gel column chromatography”, add 7 mL of acetonitrile/toluene (3:1, v/v), and discard the effluent. Elute with 30 mL of acetonitrile/formic acid/toluene (75:1:25, v/v/v),

concentrated the eluate at below 40°C and remove the solvent. Dissolve the residue in methanol to make exactly 1 mL (exactly 0.5 mL for tea and hops) and use this solution as the test solution.

## 5. Measurement

### a. Calibration curve

Prepare 2, 4, 5-T standard solution (methanol) 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 0.01 mg/kg of 2, 4, 5-T gives the test solution of 0.005 mg/L in concentration.

### b. Quantification

Inject the test solution to LC-MS/MS and calculate the concentration of 2, 4, 5-T from the calibration curve made in “a. Calibration curve”.

### c. Confirmation

Confirm using LC-MS/MS.

### d. Measurement conditions

(Example)

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: Linear gradient from 5 mmol ammonium acetate solution/5 mmol/L ammonium acetate-methanol solution (7:3, v/v) to (1:9, v/v) in 20 min.

Ionization mode: ESI (-)

Major monitoring ions (*m/z*): Precursor ion 253, product ion 195

Precursor ion 255, product ion 197

Injection volume: 5 µL

Expected retention time: 12 min

## 6. Limit of Quantification

0.01 mg/kg