Analytical Method for Captafol
(Targeted to Agricultural Products)

The target compound to be determined is Captafol.

1. Instrument

A gas chromatograph with an electron capture detector (GC-ECD) and a gas chromatograph/mass spectrometer (GC/MS or GC/MS/MS).

2. Reagents

In addition to the reagents and test solutions listed below, use those listed in Section C ***Reagents/Test Solutions, Etc., Part II Food additives***.

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

Acetonitrile: Use a rotary vacuum evaporator on 300 mL of acetonitrile to concentrate to the point of dryness. Dissolve the residue in 5 mL of n-hexane. For analysis, inject 5 μL of the solution into a GC-ECD. Peaks other than that of n-hexane in the resulting chromatogram should be as high as or lower than the peaks of γ-BHC at 2 x 10- 11g.

Acetone: Use a rotary vacuum evaporator on 300 mL of acetone to concentrate to the point of dryness. Dissolve the residue in 5 mL of n-hexane. For analysis, inject 5 μL of the solution into a GC-ECD. Peaks other than that of n-hexane in the resulting chromatogram should be as high as or lower than the peaks of γ-BHC at 2 x 10- 11g.

Sodium chloride: Sodium chloride (special grade). If the reagent is found to include any substance that may interfere with the analysis of substances to be analyzed from agricultural chemicals, wash with a solvent such as n-hexane before use.

Synthetic magnesium silicate (Florisil) for column chromatography: Heat florisil (150-250 μm in particle size) at 130°C for 12 hours or longer. Cool down to room temperature in a desiccator.

Diatomaceous earth: Diatomaceous earth for chemical analysis.

Ethyl acetate: Use a rotary vacuum evaporator on 300 mL of ethyl acetate to concentrate to the point of dryness. Dissolve the residue in 5 mL of n-hexane. For analysis, inject 5 μL of the solution into a GC-ECD. Peaks other than that of n-hexane in the resulting chromatogram should be as high as or lower than the peaks of γ-BHC at 2 x 10- 11g.

n-Hexane: Use a rotary vacuum evaporator on 300 mL of n-hexane to evaporate until

5 mL is left. For analysis, inject 5 μL of the solution into a GC-ECD. Peaks other than that of n-hexane in the resulting chromatogram should be as high as or lower than the peaks of γ-BHC at 2 x 10- 11g.

Water: Distilled water. If the distilled water is found to include any substance that may interfere with analysis of the target compositional substances from agricultural chemicals, wash with a solvent such as n-hexane before use.

Sodium sulfate (anhydrous): Sodium sulfate (anhydrous)(special grade). If the reagent is found to include any substance that may interfere with analysis of the target compositional substances from agricultural chemicals, wash with a solvent such as n-hexane before use.

3. Reference standard

Reference standard of Captafol: Contains not less than 98% of captafol, and its melting point is 159-161°C.

4. Procedure

a. Extraction

i. Cereal grains, legumes/pulses and seeds

Weigh 10.0 g of the test sample, previously ground so as to pass through a standard mesh sieve (420 μm).

Add 20 ml of 3% phosphoric acid solution to the obtained sample and leave it to stand for two hours.

Add 100 ml acetone and homogenize the mixture for three minutes.

Filter the homogenized sample by suction into a rotary vacuum evaporator through a filter paper covered with a 1-cm thick layer of diatomaceous earth.

Collect the residue on the surface of the filter paper and add 50 ml of acetone, and then homogenize the mixture for three minutes.

Repeat the above procedure and combine the filtrate into the rotary vacuum evaporator, and then concentrate the mixture to approximately 30 ml at 40°C or lower.

Transfer the concentrated solution to a 300-ml separating funnel already containing 100 ml of 10% sodium chloride solution.

Wash the eggplant-shaped flask of the above rotary vacuum evaporator with 100 ml of n-hexane,and add the washings to the separating funnel above.

Shake the mixture vigorously for five minutes using a shaker and leave it to stand, and then transfer the n-hexane layer to a 300-ml conical flask. Add 50 ml of n-hexane to the aqueous layer and repeat the above procedure, and add the n-hexane layer to the

conical flask above.

Add an adequate amount of sodium sulfate (anhydrous) to the flask and leave it to stand for 15 minutes with occasional shaking.

Filter the content of the flask into a rotary vacuum evaporator.

Wash the flask with 20 ml of n-hexane and wash twice the residue on the surface of the filter paper with the washings.

Add the washings into the rotary vacuum evaporator and remove the n-hexane at 40°C or lower.

Add 30 ml of n-hexane to the residue and transfer the mixture to a 100-ml separating funnel, and then add 30 ml of n-hexane-saturated acetonitril.

Shake the mixture vigorously for five minutes using a shaker and leave it to stand, and then transfer the acetonitrile layer into the rotary vacuum evaporator.

Add 30 ml of n-hexane-saturated acetonitrile to the n-hexane layer and repeat the above procedure twice, and then combine the acetonitrile layer into the rotary vacuum evaporator above.

Remove the acetonitrile at 40°C or lower and dissolve the residue in 5 ml of n-hexane.

ii. Fruit, vegetables, matcha and hops

In case of fruit and vegetables, weigh accurately 1 kg of the test sample and add 500 ml of 10% phosphoric acid solution.

Homogenize the mixture and mesure out a sample equivalent to 20.0 g.

In case of matcha, weigh 5.00 g of the tet sample and add 20 ml of 3% phosphoric acid solution, and then leave it to stand for two hours.

In case of hops, weigh 5.00 g of the test sample, previously ground, and add 20 ml of 3% phosphoric acid solution, and then leave it to stand for two hours.

Then, add 100 ml of acetone and homogenize the mixture for three minutes.

Filter the homogenized sample by suction into a rotary vacuum evaporator through a filter paper covered with a 1-cm thick layer of diatomaceous earth.

Collect the residue on the surface of the filter paper and add 50 ml of acetone, and then homogenize the mixture for three minutes. Repeat the above procedure and combine the filtrate into the rotary vacuum evaporator, and then concentrate the mixture to approximately 30 ml at 40°C or lower.

Transfer the concentrated solution to a 300-ml separating funnel already containing 100 ml of 10% sodium chloride solution.

Wash the eggplant-shaped flask of the above rotary vacuum evaporator with 100 ml

of n-hexane and add the washings to the separating funnel above.

Shake the mixture vigorously for five minutes using a shaker and leave it to stand, and then transfer the n-hexane layer to a 300-ml conical flask.

Add 50 ml of n-hexane to the aqueous layer and repeat the above procedure twice, and then combine the n-hexane layer to the conical flask above.

Add an adequate amount of sodium sulfate (anhydrous) to the flask and leave it to stand for 15 minutes with occasional shaking.

Filter the content of the flask into a rotary vacuum evaporator.

Wash the flask with 20 ml of n-hexane and wash twice the residue on the surface of the filter paper.

Add the washings into the rotary vacuum evaporator and remove the n-hexane at 40°C or lower.

Dissolve the residue in 5 ml of n-hexane.

iii. Teas except matcha

Weigh 9.00 g of the test sample and soak it in 540 ml of water at 100°C, and then leave it to stand at room temperature for five minutes.

Transfer 360 ml of the cooled filtrate into a 500-ml conical flask.

Add 30 ml of phosphoric acid, 100 ml of acetone and 2 ml of saturated lead acetate solution to the above conical flask, and then leave the mixture to stand for one hour at room temperature.

Filter the mixture by suction into a 1,000-ml separating funnel through a filter paper covered with a 1-cm thick layer of diatomaceous earth.

Wash the above conical flask with 50 ml of acetone and wash the residue on the surface of the filter paper with the washings.

Add the washings into the above separating funnel and also add 30 g of sodium chloride and 100 ml of n-hexane.

Shake the mixture vigorously for five minutes using a shaker and leave it to stand, and then transfer the n-hexane layer to a 300-ml conical flask.

Add 100ml of n-hexane to the aqueous layer and repeat the above procedure, and then add the n-hexane layer to the conical flask above.

Add an adequate amount of sodium sulfate (anhydrous) to the flask and leave it to stand for 15 minutes with occasional shaking.

Filter the content of the flask into a rotary vacuum evaporator.

Wash the flask with 20 ml of n-hexane and wash twice the residue on the surface of the filter paper with the washings.

Add the washings into the rotary vacuum evaporator and remove the n-hexane at 40°C or lower.

Dissolve the residue in 5 ml of n-hexane.

iv. Foods except those listed in i to iii above

Obtain the extracts according to the methods described in i or ii.

b. Clean-up

Add 5 g of florisil for column chromatography suspended in n-hexane into a chromatograph tube (15 mm in inner diameter, 300 mm in length) and add over approximately 5 g of sodium sulfate (anhydrous) into the column.

Spill out the n-hexane until only a small amount remains on the packing of the column and pour the solution obtained by the extraction described in 4-a into the column.

Then, pour 100 ml of n-hexane into the column and discard the effluent.

Pour 150 ml of ethyl acetate/n-hexane (1:9) into the column and collect the eluate into a rotary vacuum evaporator, and then romove the ethyl acetate and n-hexane at 40°C or lower.

Dissolve the residue in n-hexane to make exactly 5 ml of solution, which is used as the sample solution.

5. Measurement

a. Qualitative tests

Perform qualitative tests under the following conditions. Test results obtained must be the same as those obtained for the reference standard.

Testing conditions 1

Column: A silicate glass capillary column (0.25 mm inner diameter, 10-30 m in length) coated with methyl silicone for gas chromatography to a thickness of 1.5 μm.

Column temperature: Hold the column temperature at 50°C for one minute, followed by an increase of 25°C every minute until reaching 175°C, after which increase the temperature by 10°C every minute until reaching 300°C, and hold for five minutes.

Inlet temperature: 230°C

Detector: Operate at 300°C

Gas flow rate: Use helium as the carrier gas. Adjust the flow rate to the optimal condition.

Testing conditions 2

Column: A silicate glass capillary column (0.25 mm in inner diameter, 10-30 m in length) coated with 5% phenyl methyl silicone for gas chromatography to a thickness of 0.25 μm.

Column temperature: Hold the column temperature at 50°C for one minute, followed by an increase of 25°C every minute until reaching 125°C, after which increase the temperature by 10°C every minute until reaching 300°C, and hold for three minutes.

Inlet temperature: 230°C

Detector: Operate at 300°C

Gas flow rate: Use helium as the carrier gas. Adjust the flow rate to the optimal condition.

b. Quantitative tests

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

c. Confirmation tests

Perform gas chromatography/mass spectrometry under the same 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

0.01 mg/kg

Analytical Method for Captafol
(Targeted to animal and fishery products)

The target compound to be determined is captafol.

1. Instrument

Gas chromatograph equipped with electron capture detector (GC-ECD)

2. Reagents and test solutions

Use the reagent 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 compositional substances.

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

Ether: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

Sodium Chloride: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

Graphite Carbon Cartridge Column (250 mg): A polyethylene column of 8–9 mm in inner diameter packed with 250 mg of graphite carbon or a column equivalent to the specified one in separation capability.

Synthetic Magnesium Silicate Cartridge Column (910 mg): A polyethylene column of 8–9 mm in inner diameter packed with 910 mg of synthetic magnesium silicate or a column equivalent to the specified one in separation capability.

Ethyl Acetate: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

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

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

Anhydrous sodium sulfate: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

3. Reference standard

Reference standard of captafol : Contains not less than 97% of captafol.

4. Preparation of test solutions

a. Extraction

(i) Muscle, fat, liver, kidney, and fish/shellfish

For fat, weigh 5.00 g of the test sample, previously chopped and homogenized. For other products, weigh 10.0 g of the test sample, previously chopped and homogenized. For products that are very small in size, such as *shijimi* shellfish (bivalve), weigh the test sample accurately, grind and homogenize it with 10% (vol) phosphoric acid solution (a half amount of the sample by weight), then weigh an amount equivalent to 10.0 g of the ground sample.

Grind the weighed sample with 20 mL of 3% (vol) phosphoric acid solution (10 mL of water for *shijimi* shelfish) and 100 mL of acetone, filter it by suction, and collect the filtrate. Grind the residue on the filter paper with 50 mL of acetone and filter in the same manner. Combine the filtrates and evaporate to about 20 mL at a temperature not exceeding 40ºC. Add 100 mL of 10% (w/v) sodium chloride solution and extract twice by shaking with 100 mL of *n*-hexane and then with 50 mL of *n*-hexane. Combine the *n*-hexane extracts, dehydrate with anhydrous sodium sulfate, and remove the anhydrous sodium sulfate by filtration. Evaporate the filtrate at a temperature not exceeding 40ºC to remove the solvent. To the residue, add 30 mL of *n*-hexane and extract twice by shaking with 30 mL of acetonitrile saturated by *n*-hexane each time. Combine the acetonitrile extracts, and evaporate at a temperature not exceeding 40ºC to remove the solvent. Dissolve the residue in a 1:1 mixture of acetone and *n*-hexane and make exactly 20 mL. Transfer exactly 2 mL of the resulting solution (exactly 4 mL for fat samples) and evaporate it at a temperature not exceeding 40ºC to remove the solvent. Dissolve the residue by adding 5 mL of a 1:4 mixture of ether and *n*-hexane.

(ii) Milk, egg, and honey

Weigh 10.0 g of the test sample, previously homogenized, grind it with 20 mL of 3% (vol) phosphoric acid solution and 100 mL of acetone, centrifuge at 3,000 rpm for 5 minutes, and collect the supernatant. Grind again the residue with 50 mL of acetone (for honey, 20 mL of water and 50 mL of acetone) and centrifuge under the same conditions. Combine the supernatants and evaporate to about 20 mL (to about 50 mL for honey) at a temperature not exceeding 40ºC. To the concentrate, add 100 mL of 10% sodium chloride solution and extract twice by shaking with 100 mL of *n*-hexane and then with 50 mL of *n*-hexane. Combine the *n*-hexane extracts, dehydrate with

anhydrous sodium sulfate, remove the anhydrous sodium sulfate by filtration, and evaporate the filtrate at a temperature not exceeding 40ºC to remove the solvent. To the residue, add 30 mL of *n*-hexane and extract twice by shaking with 30 mL of acetonitrile saturated by *n*-hexane each time. Combine the acetonitrile extracts and evaporate at a temperature not exceeding 40ºC to remove the solvent. Dissolve the residue by adding a 1:1 mixture of acetone and *n*-hexane and make exactly 20 mL. Transfer exactly 2 mL of the resulting solution and evaporate it at a temperature not exceeding 40ºC to remove the solvent. Dissolve the residue by adding 5 mL of a 1:4 mixture of ether and *n*-hexane.

b. Clean-up

(i) Synthetic magnesium silicate column chromatography

Pour 5 mL of *n*-hexane into a synthetic magnesium silicate column (910 mg) and discard the effluent. Pour the solution obtained in section 4-a, pour 5 mL of a 1:4 mixture of ether and *n*-hexane and discard the effluent. Then pour 30 mL of a 1:9 mixture of ethyl acetate and *n*-hexane and evaporate the eluate at a temperature not exceeding 40ºC to remove the solvent. Dissolve the residue adding 5 mL of acetonitrile.

(ii) Graphite carbon column chromatography

Pour 5 mL of acetonitrile into a graphite carbon column (250 mg) and discard the effluent. Pour the acetonitrile solution obtained in section b-i, then pour 15 mL of acetonitrile, and evaporate the eluate at a temperature not exceeding 40ºC to remove the solvent. Dissolve the residue in *n*-hexane and make exactly 2 mL. Use this solution as the test solution.

5. Measurement

a. Calibration curve

Prepare captafol solutions (500 mg/L) by dissolving Captafol Reference Standard in acetone. Dilute 1 mL of the standard stock solution with acetone to exactly 25 mL (20 mg/L). Prepare several standard solutions with different concentrations by diluting the resulting solution with *n*-hexane. Inject them into a GC-ECD to prepare a calibration curve using the peak-height or peak-area method. When the test solution is prepared as directed in this method, the concentrate of captafol in the test solution that is equivalent to 0.01 ppm in the test sample is 0.005 mg/L.

b. Quantification

Inject the test solution in the GC-ECD and determine the content of captafol from the calibration curve prepared in section 5-a.

c. Confirmation tests

Conduct confirmation tests using the GC-ECD.

d. Measurement conditions

Column: 5% phenyl-methyl silicone (0.25 mm in inner diameter, 30 m in length, coated with a 0.25 µm thick layer)

Column temperature: Hold at 50°C for 1 minute, raise to 125°C at 15°C/min, then raise to 300°C at 10°C/min , and hold at 300°C for 5 minutes.

Injection port temperature: 230°C

Detector temperature: 300°C

Carrier gas: Helium

Injection volume: 1 µL

Retention time: About 18 minutes

6. Limit of Quantification

0.01mg/kg