Analytical Method for Coumaphos
(Targeted to agricultural, animal and fishery products)

The target compound to be determined is coumaphos.

1. Instruments

Gas chromatograph-flame thermionic detector (GC-FTD)

Gas chromatograph-flame photometric detector (with interference filter for phosphorus, wavelength: 526 nm) (GC-FPD(P))

Gas chromatograph-nitrogen phosphorus detector (GC-NPD)

Gas chromatograph-mass spectrometer (GC-MS)

2. Reagents

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 compound.

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

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

Silica gel for column chromatography (particle size of 63 to 200 μm): Heat silica gel made for column chromatography (particle size of 63 to 200 μm) at 130°C for 12 hours or longer. Cool down to room temperature in a desiccator.

Diatomaceous earth: Use diatomaceous earth for chemical analysis.

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

Porous diatomaceous earth cartridge (to hold 20 mL of solution): A polyethylene tube of 20–30 mm in inside diameter packed with granular porous diatomaceous earth prepared for column chromatography (to hold 20 mL of solution), or a cartridge with equivalent to the specified one in separation capability.

Trimethylaminopropylsilanized silica gel/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 trimethylaminopropylsilanized silica gel 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.

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

3. Reference standard

Reference standard of coumaphos: Contains not less than 98% of coumaphos.

4. Procedure

a. Extraction

(i) Grains, legumes, nuts and seeds

Grind sample to pass through a standard sieve (425 μm), weigh 10.0 g of the sample, add 20 mL of water, and let stand for 30 minutes.

Add 100 mL of acetone to the sample, homogenize for 3 minutes, and filter through a filter paper, covered with a 1-cm-thick layer of diatomaceous earth, with suction into a vacuum rotary evaporator flask. Collect the residue on the filter paper, add 50 mL of acetone, homogenize for 3 minutes, treat as described above, combine the filtrate in the vacuum rotary evaporator flask, and remove acetone at below 40°C.

Transfer to a 300 mL separating funnel containing 100 mL of saturated sodium chloride solution. Wash the vacuum rotary evaporator flask with 100 mL of ethyl acetate/*n*-hexane (1:4, v/v), and transfer the washing to the separating funnel. Shake the separating funnel vigorously for 5 minutes with a shaker, let stand, and transfer the ethyl acetate/*n*-hexane layer to a 300 mL conical flask. Add 50 mL of ethyl acetate/*n*-hexane (1:4, v/v) to the aqueous layer, treat as described above, and combine the ethyl acetate/*n*-hexane layers in the conical flask. Add an appropriate quantity of anhydrous sodium sulfate to the ethyl acetate/*n*-hexane layer, let stand for 15 minutes with occasional shaking, and filter into a vacuum rotary evaporator flask. Wash the conical flask with 20 mL of *n*-hexane, and wash the residue on the filter paper with the washing. Repeat this step one more time. Combine the washings in the vacuum rotary evaporator flask, and remove ethyl acetate/*n*-hexane at below 40°C.

Add 30 mL of *n*-hexane to the residue, and transfer to a 100 mL separating funnel.

Add 30 mL of acetonitrile saturated with *n*-hexane to the separating funnel, shake vigorously for 5 minutes with a shaker, let stand, and transfer the acetonitrile layer to a vacuum rotary evaporator flask. Add 30 mL of acetonitrile saturated with *n*-hexane to the *n*-hexane layer, treat as described above twice, combine the acetonitrile layers in the vacuum rotary evaporator flask, and remove acetonitrile at below 40°C. Dissolve the residue in 5 mL of acetone/*n*-hexane (1:1, v/v).

(ii) Fruits, vegetables, tea leaves and hops

For fruits and vegetables, weigh about 1 kg of sample accurately, add an appropriate quantity of water (if necessary), homogenize, and then take the sample equivalent to 20.0 g.

For tea leaves and hops, grind the sample, weigh 5.00 g, add 20 mL of water, and let stand for 30 minutes.

Add 100 mL of acetone to the sample, homogenize for 3 minutes, and filter through a filter paper, covered with a 1-cm-thick layer of diatomaceous earth, with suction into a vacuum rotary evaporator flask. Collect the residue on the filter paper, add 50 mL of acetone, homogenize for 3 minutes, treat as described above, combine the filtrate in the vacuum rotary evaporator flask, and remove acetone at below 40°C.

Transfer to a 300 mL separating funnel containing 100 mL of saturated sodium chloride solution. Wash the vacuum rotary evaporator flask with 100 mL of ethyl acetate/*n*-hexane (1:4, v/v), and transfer the washing to the separating funnel. Shake the separating funnel vigorously for 5 minutes with a shaker, let stand, and transfer the ethyl acetate/*n*-hexane layer to a 300 mL conical flask. Add 50 mL of ethyl acetate/*n*-hexane (1:4, v/v) to the aqueous layer, treat as described above, and combine the ethyl acetate/*n*-hexane layers in the conical flask. Add an appropriate quantity of anhydrous sodium sulfate to the ethyl acetate/*n*-hexane layer, let stand for 15 minutes with occasional shaking, and filter into a vacuum rotary evaporator flask. Wash the conical flask with 20 mL of *n*-hexane, and wash the residue on the filter paper with the washing. Repeat this step one more time. Combine the washings in the vacuum rotary evaporator flask, and remove ethyl acetate/*n*-hexane at below 40°C. Dissolve the residue in 5 mL of acetone/*n*-hexane (1:1, v/v).

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

For muscle, liver, kidney and fish/shellfish, weigh 20.0 g of sample. For fat, weigh 5.00 g of sample.

Add 20 mL of 0.1 mol/L hydrochloric acid to the sample, homogenize, add 100 mL of acetone/*n*-hexane (1:2, v/v), homogenize again, centrifuge at 3,000 rpm for 5 minutes, and collect the organic layer. Add 50 mL of *n*-hexane to the residue, homogenize, and

centrifuge at 3,000 rpm for 5 minutes. Combine the resulting organic layers, dehydrate the extract with anhydrous sodium sulfate, and filter out the anhydrous sodium sulfate. Concentrate the filtrate at below 40ºC and remove the solvent. Dissolve the residue in *n*-hexane to make exactly 20 mL.

(iv) Milk, egg and honey

Weigh 10.0 g of sample.

Add 10 mL of 0.1 mol/L hydrochloric acid to the sample, homogenize, add 100 mL of acetone/*n*-hexane (1:2, v/v), homogenize again, centrifuge at 3,000 rpm for 5 minutes, and collect the organic layer. Add 50 mL of *n*-hexane to the residue, homogenize, and centrifuge at 3,000 rpm for 5 minutes. Combine the resulting organic layers, dehydrate the extract with anhydrous sodium sulfate, and filter out the anhydrous sodium sulfate. Concentrate the filtrate at below 40ºC and remove the solvent. For milk and egg, dissolve the residue in *n*-hexane to make exactly 10 mL. For honey, dissolve the residue in acetone/*n*-hexane (1:9, v/v) to make exactly 5 mL.

b. Clean-up

(i) Grains, legumes, nuts and seeds, fruits, vegetables, tea leaves and hops

Place 5 g of silica gel for column chromatography (particle size of 63 to 200 μm) suspended in acetone/*n*-hexane (1:1, v/v), and then about 5 g of anhydrous sodium sulfate in a chromatographic tube of 15 mm in inside diameter and 300 mm in length, and let flow out acetone/*n*-hexane (1:1, v/v) to the extent that only a small quantity of acetone/*n*-hexane (1:1, v/v) remains on the top of the column. Transfer the solution obtained in "a. Extraction" to the column, elute with 100 mL of acetone/*n*-hexane (1:1, v/v), collect the eluate in a vacuum rotary evaporator flask, and remove the acetone/*n*-hexane at below 40°C. Dissolve the residue in acetone to make exactly 5 mL, and use this solution as the test solution.

(ii) Muscle, fat, liver, kidney, fish/shellfish, milk and egg

① Porous diatomaceous earth column chromatography

For Muscle, fat, liver, kidney and fish/shellfish, transfer exactly 10 mL of the solution obtained in "a. Extraction (iii)" to a porous diatomaceous earth column (to hold 20 mL of solution). For milk and egg, transfer total solution obtained in "a. Extraction (iv)" to the column. Let stand the column for 10 minutes, and then aspirate the column for 10 minutes and remove most of the solvent. Elute with 90 mL of acetonitrile saturated with *n*-hexane, concentrate the eluate at below 40ºC and remove the solvent. Dissolve the residue in acetone/*n*-hexane (1:9, v/v) to make exactly 5 mL.

② Trimethylaminopropylsilanized silica gel/ethylenediamine-*N*-propylsilanized silica gel layered column chromatography

Add 10 mL of acetone/*n*-hexane (1:9, v/v) to a trimethylaminopropylsilanized silica gel/ethylenediamine-*N*-propylsilanized silica gel layered cartridge (500 mg/500 mg), and discard the effluent. Transfer exactly 2 mL of the solution obtained in "① Porous diatomaceous earth column chromatography" to the cartridge, elute with 10 mL of acetone/*n*-hexane (1:9, v/v), collect the total eluate, concentrate at below 40ºC and remove the solvent. Dissolve the residue in acetone/*n*-hexane (1:9, v/v) to make exactly 4 mL (1 mL for fat), and use this solution as the test solution.

(iii) Honey

Add 10 mL of acetone/*n*-hexane (1:9, v/v) to a trimethylaminopropylsilanized silica gel/ethylenediamine-*N*-propylsilanized silica gel layered cartridge (500 mg/500 mg) and discard the effluent. Transfer exactly 2 mL of the solution obtained in "a. Extraction (iv)" to the cartridge, elute with 10 mL of acetone/*n*-hexane (1:9, v/v), collect the total eluate, concentrate at below 40°C and remove the solvent. Dissolve the residue in acetone/*n*-hexane (1:9, v/v) to make exactly 4 mL, and use this solution as the test solution.

5. Measurement

a. Calibration curve

(i) Grains, legumes, nuts and seeds, fruits, vegetables, tea leaves and hops

Prepare coumaphos standard solutions (acetone) of several concentrations. Inject each standard solution to GC-FTD, GC-FPD(P) or GC-NPD, 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 coumaphos gives the test solution of 0.02 mg/L for grains, legumes, nuts and seeds, 0.04 mg/L for fruits and vegetables, and 0.01 mg/L for tea leaves and hops in concentration.

(ii) Muscle, fat, liver, kidney, fish/shellfish, milk, egg and honey

Prepare coumaphos standard solutions (acetone/*n*-hexane (1:9, v/v)) of several concentrations. Inject each standard solution to GC-FTD, GC-FPD(P) or GC-NPD, 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 coumaphos gives the test solution of 0.01 mg/L in concentration.

b. Quantification

Inject the test solution to GC-FTD, GC-FPD(P) or GC-NPD, and calculate the

concentration of coumaphos from the calibration curve made in “a. Calibration curve”.

c. Confirmation

Confirm using GC-MS.

d. Measurement conditions

(i) For quantification

Condition 1 (GC-FTD, GC-FPD(P) or GC-NPD)

Column: Silicate glass capillary column 0.53 mm in inside diameter, 10–30 m in length coated with methyl silicone for gas chromatography 1.5 μm in film thickness

Column temperature: The column temperature is held at 80°C for 1 minute, followed by an increase of 8°C every minute until reaching 250°C, where it is held for 5 minutes.

Inlet temperature: 230°C

Detector temperature: 280°C

Gas flow rate: Helium is used as the carrier gas. The flow rate should be adjusted to the optimal condition. The flows of air and hydrogen should also be adjusted to the optimal conditions.

Condition 2 (GC-FTD, GC-FPD(P) or GC-NPD)

Column: Silicate glass capillary column 0.32 mm in inside diameter, 10–30 m in length coated with 50% trifluoro propyl methyl silicone for gas chromatography 0.25 μm in film thickness

Column temperature: The column temperature is held at 70°C for 1 minute, followed by an increase of 25°C every minute until reaching 125°C, after which the temperature is increased by 10°C every minute until reaching 235°C, where it is held for 12 minutes.

Inlet temperature: 230°C

Detector temperature: 280°C

Gas flow rate: Helium is used as the carrier gas. The flow rate should be adjusted to the optimal condition. The flows of air and hydrogen should also be adjusted to the optimal conditions.

Condition 3 (GC-FPD(P))

Column: Silicate glass capillary column 0.32 mm in inside diameter, 30 m in length coated with 35% trifluoro propyl methyl silicone for gas chromatography 0.5 μm in film thickness

Column temperature: The column temperature is held at 60°C for 1 minute, followed by an increase of 25°C every minute until reaching 210°C, after which the temperature is increased by 10°C every minute until reaching 280°C, where it is held for 10 minutes.

Inlet temperature: 250°C

Detector temperature: 280°C

Gas flow rate: Helium is used as the carrier gas. The flow rate should be adjusted to the optimal condition. The flows of air and hydrogen should also be adjusted to the optimal conditions.

Injection volume: 2 µL

Expected retention time: 18 min

(ii) For confirmation

Condition (GC-MS)

Column: Silicate glass capillary column 0.25 mm in inside diameter, 30 m in length coated with 5% phenyl-methyl silicone for gas chromatography 0.25 µm in film thickness

Column temperature: The column temperature is held at 60°C for 1 minute, followed by an increase of 25°C every minute until reaching 210°C, after which the temperature is increased by 10°C every minute until reaching 300°C, where it is held for 8 minutes.

Inlet temperature: 250°C

Carrier gas: Helium is used as the carrier gas. The flow rate should be adjusted to the optimal condition.

Ionization mode (ionization energy): EI (70 eV)

Major monitoring ions (*m/z*): 364, 362, 226

Injection volume: 2 µL

Expected retention time: 15 min

6. Limit of quantification

0.01 mg/kg