Analytical Method for Olaquindox and Carbadox (targeted to Animal and Fishery Products)

The target compound to be determined is 3-methyl-quinoxaline-2-carboxylic acid (MQCA) for Olaquindox and 2-quinoxalinecarboxylic acid (QCA) for Carbadox.

1. Instrument

A Liquid Chromatograph/Tandem Mass Spectrometer (LC/MS/MS)

2. Reagents

Use the reagents listed in Section C ***Reagent/Test Solution, Etc.***, Part II ***Food Additives***, except the following.Reagents designated as "special grade" in this section must meet the requirements for "special grade" specified in the Japan Industrial Standards for the reagents.

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

Ethylenediamine-*N*-propylsilanized silica gel cartridge column (500 mg): A polyethylene column of 8–9 mm in inner diameter packed with 500 mg of ethylenediamine-*N*-propylsilanized silica gel 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.

Weakly basic anion exchanger cartridge column (150 mg): A polyethylene column of 12–13 mm in inner diameter packed with 150 mg of weakly basic anion exchanger or a column equivalent to the specified one in separation capability.

Styrene-divinylbenzene co-polymer cartridge column (500 mg): A polyethylene column of 12–13 mm in inner diameter packed with 500 mg of styrene-divinylbenzene co-polymer or a column 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 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 a solvent such as *n*-hexane before use.

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

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

3. Reference standard

Reference standard of MQCA: Contains not less than 96% of MQCA.

Reference standard of QCA: Contains not less than 95% of QCA.

4. Procedure

A. Extraction

i. Muscle, liver, kidney, fish/shellfish, milk, egg, and fat

Weigh 10.0 g of the test sample for muscle, liver, kidney, fish/shellfish, milk, or egg. Weigh 5.00 g of the test sample for fat. To the sample, add 20 mL of 2 mol/L potassium hydroxide in methanol, heat the mixture in a water bath at 85°C for 2 hours under a reflux condenser, and allow to cool it. Add 60 mL of methanol, shake the mixture vigorously for 10 minutes, and filter it by suction. Wash the filter with 20 mL of methanol, combine the washings with the filtrate, and add methanol to make exactly 200 mL of a solution. Collect 20 mL of the resulting solution and add 2 mL of 3 mol/L hydrochloric acid.

ii. Honey

Add 20 mL of water to 10.0 g of the sample to dissolve it and add 0.5 mL of hydrochloric acid. Then add 100 mL of methanol, homogenize the mixture, and filter it by suction. Add 50 mL of methanol to the residue on the filter paper, homogenize the mixture, and filter it by suction. Combine the filtrates and add methanol to make exactly 200 mL of solution. Collect 20 mL of the resulting solution, add 100 mL of water, and perform shaking-extraction three times with 50 mL of ethyl acetate. Add anhydrous sodium sulfate to the extract obtained to dehydrate. Remove the sodium sulfate by filtration, and evaporate the filtrate at a temperature not more than 40°C to remove the solvent. Dissolve the residue by adding 5 mL of a mixture of formic acid/methanol (1:500).

B. Clean-up

i. Muscle, liver, kidney, fish/shellfish, milk, egg, and fat

a. Styrene-divinylbenzene co-polymer cartridge column chromatography

Pour 10 mL of a mixture of water/methanol (1:10) into a styrene-divinylbenzene co-polymer cartridge column (500 mg) and discard the effluent. Pour the solution obtained by the extraction into the column, then pour 6 mL of a mixture of water/methanol (1:10), transfer the combined eluate to a separating funnel, shake the eluate twice with 20 mL of *n*-hexane, and discard the hexane layer. Then add 100 mL of water, and perform shaking-extraction three times with 50 mL of ethyl acetate. Add anhydrous sodium sulfate to the extract obtained to dehydrate. Remove the sodium sulfate by filtration, and evaporate the filtrate at a temperature not more than 40°C to remove the solvent. Dissolve the residue by adding 5 mL of a mixture of formic acid/methanol (1:500).

b. Ethylenediamine-*N*-propylsilanized silica gel cartridge column chromatography

Pour 10 mL of a mixture of formic acid/methanol (1:500) into an ethylenediamine-*N*-propylsilanized silica gel cartridge column (500 mg) and discard the effluent. Into the column, pour the solution obtained in a, then pour 5 mL of a

mixture of formic acid/methanol (1:500), and discard the effluent. Pour 20 mL of a mixture of formic acid/methanol (3:97) and evaporate the eluate at a temperature not more than 40°C to remove the solvent. Dissolve the residue by adding 2 mL of a mixture of formic acid/methanol (3:97), add 3 mL of water, and mix well.

c. Weakly basic anion exchanger cartridge column chromatography

Pour 5 ml each of methanol and water in series to a weakly basic anion exchanger cartridge column (150 mg) and discard the effluent. Into the column, pour the solution obtained in b and discard the effluent. Pour 5 mL of water and discard the effluent. Then pour 10 mL of methanol, discard the effluent, pour 5 mL of a mixture of 25% ammonia solution/methanol (1:19), and evaporate the eluate at a temperature not more than 40°C to remove the solvent. Dissolve the residue in a mixture of water/methanol (1:1) to make exactly 1 mL of solution for a sample other than fat and to make exactly 0.5 mL for fat. Use the resulting solutions as the sample solutions.

ii. Honey

a. Ethylenediamine-*N*-propylsilanized silica gel cartridge column chromatography

Pour 10 mL of a mixture of formic acid/methanol (1:500) into an ethylenediamine-*N*-propylsilanized silica gel cartridge column (500 mg) and discard the effluent. Into the column, pour the solution obtained by extraction, then pour 5 mL of a mixture of formic acid/methanol (1:500), and discard the effluent. Pour 20 mL of a mixture of formic acid/methanol (3:97) and evaporate the eluate at a temperature not more than 40°C to remove the solvent. Dissolve the residue by adding 2 mL of a mixture of formic acid/methanol (3:97), add 3 mL of water, and mix well.

b. Weakly basic anion exchanger cartridge column chromatography

Pour 5 ml each of methanol and water in series to a weakly basic anion exchanger cartridge column (150 mg) and discard the effluent. Into the column, pour the solution obtained in (ii)a and discard the effluent. Pour 5 mL of water and discard the effluent. Then pour 10 mL of methanol, discard the effluent, pour 5 mL of a mixture of 25% ammonia solution/methanol (1:19), and evaporate the eluate at a temperature not more than 40°C to remove the solvent. Dissolve the residue in a mixture of water/methanol (1:1) to make exactly 1 mL. Use the resulting solution as the sample solution.

5. Calibration curve

Dissolve separately the reference standards of MQCA and QCA in methanol to prepare standard stock solutions and combine them. Prepare several standard solutions with different concentrations using a mixture of water/methanol (1:1). Inject them into LC-MS/MS to prepare a calibration curve using the peak height or peak area method. When the sample solution is prepared as directed in this method, the concentrate in the

sample solution equivalent to 0.001 mg/kg in the sample is 0.001 mg/L for each compound.

6. Quantitative tests

Inject the sample solutions in LC-MS/MS and determine the contents of MQCA and QCA using the calibration curve prepared in 5.

7. Confirmation tests

Conduct confirmation tests using LC-MS/MS.

8. Testing conditions

Column: Octadecylsilanized silica gel (2.1 mm in inner diameter, 150 mm in length; 3.5 μm in particle size)

Column temperature: 40°C

Mobile phase: Create a concentrate gradient of (1:19) to (1:1) acetonitrile/0.1% (vol) acetic acid in 15 minutes.

Ionized mode: ESI (+)

Main ions (*m*/z):

MQCA: Precursor ion 189; product ions 145,143

QCA: Precursor ion 175; product ions 129,102

Injection volume: 5 μL

Retention time: MQCA: About 11 minutes, QCA: About 11 minutes

9. Limit of quantification

0.001 mg/kg