**Analytical Method for Daminozide**
**(Targeted to Agricultural, Animal and Fishery Products)**

The target compounds to be determined are daminozide and 1,1-dimethylhydrazine.

## 1. Instruments

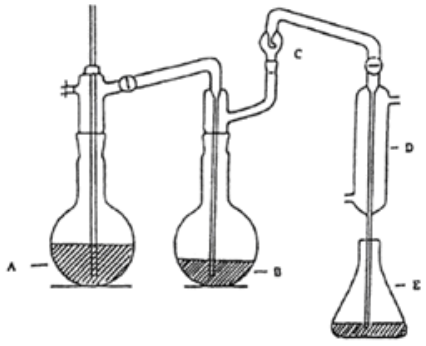
Gas chromatograph-flame thermionic detector (GC-FTD)

Gas chromatograph-nitrogen phosphorus detector (GC-NPD)

Gas chromatograph-mass spectrometer (GC-MS)

Steam distillation apparatus: Use the apparatus made of glass and roughly as the following figure.

A: 500-1,000 mL round-bottom flask
(for steam generation)
B: 500-1,000 mL round-bottom flask
(for distillation)
C: Distillation trap
D: Condenser
E: 100-mL conical flask




## 2. Reagents

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

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

Alumina (basic) cartridge (1,710 mg): A polyethylene tube of 8-9 mm in inside diameter packed with 1,710 mg of alumina (basic) or a cartridge equivalent to the specified one in separation capability.

Phase-separator filter paper: Use a siliconized filter paper for chemical analysis.

Defoaming silicone: Use a silicone produced for defoaming.

*o*-Nitrobenzaldehyde: *o*-Nitrobenzaldehyde (special grade).

1 w/v% *o*-Nitrobenzaldehyde-methanol solution: Dissolve 100 mg of *o*-nitrobenzaldehyde in 10 mL of methanol. Prepare each time before use.

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

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

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

Phosphate buffer solution (pH 5): Dissolve 13.15 g of potassium dihydrogen phosphate and 0.59 g of dipotassium hydrogen phosphate in water to make 100 mL.

## 3. Reference standard

Reference standard of 1,1-dimethylhydrazine: Contains not less than 97% of 1,1-dimethylhydrazine.

## 4. Procedure

a. Extraction

i. Agricultural products

For grains, legumes, nuts and seeds, grind sample to pass through a standard sieve (425 μm) and weigh 10.0 g of the sample. If it difficult to pass through the sieve, cut-up sample into about 2 mm square and weigh 10.0 g of the sample.

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 powdered tea, weigh 5.00 g of sample.

For tea leaves and hops, grind sample to pass through a standard sieve (425 μm) and weigh 5.00 g of the sample.

Add 80 mL of water, homogenize, and filter with suction using a glass fiber filter. Add 40 mL of water to the residue on the filter paper, homogenize, and filter as described above. Combine the resulting filtrates, and add water to make exactly 200 mL. For grains, legumes, nuts and seeds, fruits and vegetables, take exactly a 20 mL aliquot of the solution (exactly a 40 mL aliquot for tea and hops) to a round-bottom flask (for distillation), and add 80 mL of water.

ii. Animal and fishery products (except for milk, egg and honey)

Homogenize sample and weigh 10.0 g (5.00 g for fat) of the sample. Add 80 mL of water and 40 mL of *n*-hexane, homogenize, filter with suction using a glass fiber filter, and take aqueous and *n*-hexane layers. Add the *n*-hexane layer to the residue on

the filter, add 40 mL of water, homogenize, and filter as described above. Combine the obtained aqueous layers, and add water to make exactly 200 mL. Take exactly a 20 mL aliquot of the solution (exactly a 40 mL aliquot for fat) to a round-bottom flask (for distillation), and add 80 mL of water.

iii. Milk, egg and honey

Homogenize sample by thoroughly mixing, take 10.0 g of the sample to a round-bottom flask (for distillation), and add 80 mL of water.

b. Distillation

Add gradually and dissolve 60 g of sodium hydroxide cooling with water to the round-bottom flask used in " a. Extraction ". Add 1-2 drops of defoaming silicone and boiling stones, and attach the flask immediately to a steam distillation apparatus. Also attach a 100 mL conical flask containing 5 mL of phosphate buffer solution (pH 5) and 1 drop of phenolphthalein reagent. Heat a round-bottom flask (for steam generation) attached with the steam distillation apparatus. Distill the solution until the distillate becomes 45 mL, confirming that the distillate remains colorless. Adjust the heat so that the distillation finishes in about 15 minutes.

c. Derivatization

Add 1 mL of 1 w/v% *o*-nitrobenzaldehyde-methanol solution to the distillate obtained in " b. Distillation ", shake, and let stand at 40°C for 16 hours. Add 50 mL of *n*-hexane to the distillate, shake for 5 minutes, let stand, take the *n*-hexane layer, and filter using a phase-separator filter paper. Add 50 mL of *n*-hexane to the aqueous layer, treat as described above, and combine the obtained *n*-hexane layers. Wash the residue on the filter paper with 10 mL of *n*-hexane, transfer the washing to the *n*-hexane layer, concentrate at below 40°C and remove *n*-hexane. Dissolve the residue in 5 mL of acetone/*n*-hexane (1:19, v/v).

d. Clean-up

Add 10 mL of acetone/*n*-hexane (1:19, v/v) to a alumina (basic) cartridge (1,710 mg) and discard the effluent. Transfer the solution obtained in “c. Derivatization” to the cartridge, elute with 10 mL of acetone/*n*-hexane (1:19, v/v), collect the total eluate, concentrate at below 40°C and remove the solvent. Dissolve the residue in acetone to make exactly 1 mL for grains, legumes, nuts and seeds, tea, hops and animal and fishery products (except for milk, egg and honey), 2 mL for fruits and vegetables, 10 mL for milk, egg and honey, and use this solution as the test solution.

## 5. Measurement

a. Calibration curve

Prepare a 500 mg/L 1,1-dimethylhydrazine standard solution (water). Take a 1 mL aliquot of the solution, add 5 mL of phosphate buffer solution (pH 5) and 40 mL of water, and then add 1 mL of 1 w/v% *o*-nitrobenzaldehyde-methanol solution. Shake, and let stand at 40°C for 16 hours. Add 50 mL of *n*-hexane to the solution, shake for 5 minutes, let stand, take the *n*-hexane layer, and filter using a phase-separator filter paper. Add 50 mL of *n*-hexane to the aqueous layer, treat as described above, and combine the obtained *n*-hexane layers. Wash the residue on the filter paper with 10 mL of *n*-hexane, transfer the washing to the *n*-hexane layer, concentrate at below 40°C and remove *n*-hexane. Dissolve the residue and prepare standard solutions (acetone) of several concentrations, inject each standard solution to GC, 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 0.1 mg/kg of daminozide gives the test solution of 0.1 mg/L (as daminozide) in concentration.

b. Quantification

Inject the test solution to GC, and calculate the concentration of 1,1-dimethylhydrazine from the calibration curve made in “a. Calibration curve”. Use the following equation to calculate the concentration of daminozide.

Concentration of daminozide (ppm) = concentration of 1,1-dimethylhydrazine (ppm) × 2.655

c. Confirmation

Confirm using GC-MS.

d. Measurement conditions

(Example)

i. GC

Instruments: GC-FTD or GC-NPD

Column: Silicate glass capillary column 0.25 mm in inside diameter, 30 m in length coated with (trifluoropropyl) methylcyclotetrasiloxane for gas chromatography 0.25 μm in film thickness

Column temperature: The column temperature is held at 60°C for two minutes, followed by an increase of 10°C every minute until reaching 280°C, where it is held for 8 minutes.

Inlet temperature: 250°C

Detector: Should be operated at 280°C

Carrier gas: Helium

Injection volume: 2 μL

Expected retention time: 15 min

ii. GC-MS

Column: Silicate glass capillary column 0.25 mm in inside diameter, 30 m in length coated with (trifluoropropyl) methylcyclotetrasiloxane for gas chromatography 0.25 μm in film thickness

Column temperature: The column temperature is held at 80°C for 2 minutes, followed by an increase of 15°C every minute until reaching 200°C, after which the temperature is increased by 30°C every minute until reaching 250°C, where it is held for 3 minutes.

Inlet temperature: 250°C

Carrier gas: Helium

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

Major monitoring ions (*m/z*): 193, 77

Injection volume: 2 μL

Expected retention time: 9 min

**6. Limit of Quantification**

0.1 mg/kg