Analytical Method for Milbemectin and Lepimectin (Agricultural Products)

1. Analytes

Compositional substances of Agricultural Chemicals	Analytes
Milbemectin	Milbemectin A3 (10*E*,14*E*,16*E*,22*Z*)-(1*R*,4*S*,5'*S*,6*R*,6'*R*,8*R*,13*R*,20*R*,21*R*,24*S*)-21,24-dihydroxy-5',6',11,13,22-pentamethyl-3,7,19-trioxatetracyclo[15.6.1.1^{4,8}.0^{20,24}]pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-2-one
	Milbemectin A4 (10*E*,14*E*,16*E*,22*Z*)-(1*R*,4*S*,5'*S*,6*R*,6'*R*,8*R*,13*R*,20*R*,21*R*,24*S*)-6'-ethyl-21,24-dihydroxy-5',11,13,22-tetramethyl-3,7,19-trioxatetracyclo[15.6.1.1^{4,8}.0^{20,24}]pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-2-one
Lepimectin	L.A3 (Lepimectin A3) (10*E*,14*E*,16*E*)-(1*R*,4*S*,5'*S*,6*R*,6'*R*,8*R*,12*R*,13*S*,20*R*,21*R*,24*S*)-21,24-dihydroxy-5',6',11,13,22-pentamethyl-2-oxo-3,7,19-trioxatetracyclo[15.6.1.1^{4,8}.0^{20,24}]pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-12-yl(*Z*)-2-methoxyimino-2-phenylacetate
	L.A4 (Lepimectin A4) (10*E*,14*E*,16*E*)-(1*R*,4*S*,5'*S*,6*R*,6'*R*,8*R*,12*R*,13*S*,20*R*,21*R*,24*S*)-6'-ethyl-21,24-dihydroxy-5',11,13,22-tetramethyl-2-oxo-3,7,19-trioxatetracyclo[15.6.1.1^{4,8}.0^{20,24}]pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-12-yl(*Z*)-2-methoxyimino-2 -phenylacetate

2. Instrument

High performance liquid chromatograph-fluorometric detector (HPLC-FL)

3. Reagents

Use the reagents listed in Section 3 of the General Rules, except the following.

Trifluoroacetic anhydride: Trifluoroacetic anhydride (Special grade)

Reference standard of milbemectin A3: Contains not less than 95% of milbemectin A3.

Reference standard of milbemectin A4: Contains not less than 95% of milbemectin A4.

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

4. Procedure

1) 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 100 mL of acetone to the sample, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and filter with suction. Combine the resulting filtrates, and add acetone to make exactly 200 mL. Take a 20 mL aliquot of the extract, concentrate at below 40°C and remove acetone. Add 100 mL of 10% sodium chloride solution, extract with shaking twice with 50 mL of ethyl acetate/*n*-hexane (1:4, v/v). Combine the extracts, and dehydrate with anhydrous sodium sulfate. Filter out anhydrous sodium sulfate, concentrate the filtrate below 40°C and remove the solvent. Add 30 mL of *n*-hexane to the residue, extract with shaking twice with 30 mL of acetonitrile saturated with *n*-hexane. Combine the extracts, concentrate at below 40°C and remove the solvent. Dissolve the residue in 5 mL of acetone/*n*-hexane (1:19, v/v).

ii) Fruits and vegetables

Add 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 with suction. Combine the filtrates, and add acetone to make exactly 200 mL. Take a 10 mL aliquot of the extract, concentrate at below 40°C and remove acetone. Add 100 mL of 10% sodium chloride solution to the residue, extract with shaking twice with 50 mL of ethyl acetate/*n*-hexane (1:4, v/v). Combine the extracts, and dehydrate with anhydrous sodium sulfate. Filter out anhydrous sodium sulfate, concentrate the filtrate at below 40°C and remove the solvent. Dissolve the residue in 5 mL of acetone/*n*-hexane (1:19, v/v).

iii) Tea leaves

Add 20 mL of water to 5.00 g of sample, and let stand for 30 minutes. Add 100 mL of acetone, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and filter with suction. Combine the filtrates, and add acetone to make exactly 200 mL. Take a 40 mL aliquot of the extract, concentrate at below 40°C and remove acetone. Add 100 mL of 10% sodium chloride solution to the residue, extract with shaking twice with 50 mL of ethyl acetate/*n*-hexane (1:4, v/v). Combine the extracts, and dehydrate with anhydrous sodium sulfate. Filter out anhydrous sodium sulfate, concentrate the filtrate at below 40°C and remove the solvent. Dissolve the residue in 5 mL of acetone/*n*-hexane (1:19, v/v).

2) Clean-up

Connect a silica gel cartridge (690 mg) at the bottom of a graphitized carbon black cartridge (500 mg), and add 10 mL each of acetone and *n*-hexane sequentially, and discard

the effluent. Transfer the extract obtained in 1) to the cartridges, add 15 mL of acetone/*n*-hexane (1:19, v/v), and discard the effluent. Then, elute with 30 mL of acetone/*n*-hexane (3:7, v/v), concentrate the eluate at below 40°C and remove the solvent. Dissolve the residue in 1 mL of toluene.

3) Fluorescence derivatization

Add 0.05 mL of triethylamine and 0.1 mL of trifluoroacetic anhydride to the solution obtained in 2). Seal tightly, and shake gently for 30 minutes at 40°C. Add 0.05 mL of triethylamine, and remove the solvent under a stream of nitrogen. Dissolve the residue in methanol to make exactly 5 mL, and use this solution as the test solution.

5. Calibration curve

1) Milbemectin

Prepare mixed standard solution of milbemectin A3 and milbemectin A4 (each 2 mg/L in acetonitrile). Take a 1 mL aliquot of the mixed standard solution, remove the solvent under a stream of nitrogen, and dissolve the residue in 1 mL of toluene. Prepare methanol solution of fluorescent derivatives for a calibration curve following the procedure described in **4** 3). Dilute with methanol to prepare solutions of several concentrations. Inject each solution to HPLC-FL, 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 milbemectin gives the test solution of 0.002 mg/L in concentration.

2) Lepimectin

Prepare standard solution of lepimectin (2 mg/L in acetonitrile). Take a 1 mL aliquot of the standard solution, remove the solvent under a stream of nitrogen, and dissolve the residue in 1 mL of toluene. Prepare methanol solution of fluorescent derivatives for a calibration curve following the procedure described in **4** 3). Dilute with methanol to prepare solutions of several concentrations. Inject each solution to HPLC-FL, and make a calibration curve by peak-height method or peak-area using the sum of peak heights or areas of lepimectin A3 and lepimectin A4. When the test solution is prepared following the above procedure, the sample containing 0.01 mg/kg of lepimectin gives the test solution of 0.002 mg/L in concentration.

6. Quantification

Inject the test solution to HPLC-FL and calculate the concentrations of milbemectin A3, milbemectin A4, and lepimectin from the calibration curve made in **5**.

7. Confirmation

Confirm using HPLC-FL.

8. Measurement conditions

Example

Detector: FL (excitation wavelength 368 nm, emission wavelength 460 nm)

Column: Octadecylsilanized silica gel, 4.6 mm in inside diameter, 150 mm in length and 5 μm in particle diameter

Column temperature: 40°C

Mobile phase: acetonitrile/water (9:1, v/v)

Injection volume: 20 μL

Expected retention time:

Milbemectin A3 14 min

Milbemectin A4 18 min

Lepimectin A3 13 min

Lepimectin A4 15 min

9. Limit of quantification

0.01 mg/kg

10. Explanatory note

1) Outline of analytical method

The method consists of extraction of milbemectin (milbemectin A3 and milbemectin A4) and lepimectin (lepimectin A3 and lepimectin A4) from sample with acetone, re-extraction with ethyl acetate/*n*-hexane (1:4, v/v), defatting with acetonitrile/hexane partitioning for grains, legumes, nuts and seeds, clean-up with a connected graphitized carbon black/silica gel cartridge, fluorescence derivatization, quantification and confirmation using HPLC-FL.

2) Notes

i) In the procedure of fluorescence derivatization, triethylamine is added to stop the reaction after shaking for 30 minutes. After removal of the solvent, 0.1–0.2 mL of residue remains. The fluorescent derivative is unstable, and thus, it is recommended to carry out the procedure after fluorescence derivatization quickly, and perform the measurement on the same day.

ii) Measurement conditions for confirmation

Detector: FL (excitation wavelength 368 nm, emission wavelength 460 nm)

Column: Phenylsilanized silica gel, 4.6 mm in inside diameter, 250 mm in length and 5 μm in particle diameter

Column temperature: 40°C

Mobile phase: acetonitrile and water (3:1, v/v)

Expected retention time:

Milbemectin A3 13 min

Milbemectin A4 15 min

Lepimectin A3 16 min

Lepimectin A4 18 min

Although the use of LC-MS/MS for confirmation was investigated when the analytical method for milbemectin and lepimectin was developed, the sensitivity of LC-MS/MS was insufficient.

iii) Because the reference standards of lepimectin A3 and lepimectin A4 were unavailable when the analytical method for lepimectin was developed, the mixed reference standard (containing lepimectin A3 less than 20% and lepimectin A4 more than 80%) was used for quantification. If each of the reference standards of lepimectin A3 and lepimectin A4 is available, quantify lepimectin A3 and lepimectin A4 individually, and regard the sum of the result as the analytical result of lepimectin.

11. References

Ministry of the Environment former Notification “Analytical Method for Milbemectin”

12. Type

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