Original: Japanese
Provisional translation

Analytical Method for Ethofumesate
(Agricultural Products)

1. Analytes

Ethofumesate

Metabolite M2 [2,3-dihydro-3,3-dimethyl-2-oxo-benzofuran-5-yl methanesulfonate]

Metabolites converted to metabolite M2 by thermal acid treatment (including metabolite M3 [2-(2-hydroxy-5-methanesulfonyloxyphenyl)-2-methylpropionic acid] and conjugates of metabolite M3)

2. Applicable food

Vegetables

3. Instrument

Gas chromatograph-tandem mass spectrometer (GC-MS/MS)

4. Reagents

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

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

Reference standard of metabolite M2: Contains not less than 98% of metabolite M2.

Reference standard of metabolite M3 sodium salt: Contains not less than 95% of metabolite M3 sodium salt.

5. Procedure

1) Extraction

Add 100 mL of acetone and water (4:1, v/v) to 20.0 g of the sample, homogenize, centrifuge at 3,000 rpm for 5 min, and collect the supernatant. Add 50 mL of acetone and water (4:1, v/v) to the residue, homogenize, and centrifuge as described above. Combine the resulting supernatants and add acetone to make exactly 200 mL. Residues after centrifugation are used in 4) i) Hydrolysis.

2) Sodium hydroxide solution/hexane partitioning

Take exactly a 2 mL aliquot of the extract obtained in 1) and concentrate to less than 0.5 mL at below 40°C. Add 5 mL of 0.1 mol/L sodium hydroxide solution and 5 mL of *n*-hexane and shake. Collect the *n*-hexane layer, add 5 mL of *n*-hexane to the aqueous layer, and shake. Combine the resulting *n*-hexane layers, concentrate at below 40°C, and remove the solvent. Dissolve the residue in 1 mL of ethyl acetate and *n*-hexane (1:9, v/v). The aqueous layer after partitioning is used in 4) i) Hydrolysis.

3) Ethofumesate test solution

Inject 10 mL of ethyl acetate and *n*-hexane (1:9, v/v) into a silica gel cartridge (1,000 mg) and discard the effluent. Transfer the solution obtained in 2) to the cartridge, add 10 mL of

ethyl acetate and *n*-hexane (1:9, v/v), and discard the effluent. Then, add 10 mL of ethyl acetate and *n*-hexane (3:17, v/v), concentrate the eluate at below 40°C, and remove the solvent. Dissolve the residue in acetone and *n*-hexane (1:1, v/v) to make exactly 4 mL and use this solution as the test solution.

4) Test solution for metabolite M2 and metabolites converted to metabolite M2 by thermal acid treatment (including metabolite M3 and conjugates of metabolite M3).

i) Hydrolysis

Weigh accurately the residue obtained in 1) and immediately weigh out an amount equivalent to 1/100 of the residue. Combine the aqueous layer obtained in 2), add 5 mL of hydrochloric acid, seal tightly and heat at 80°C for 2.5 hrs. Allow it to cool, add 10 mL of water, and extract with shaking 3 times using 20 mL of diethyl ether. Combine the extracts, concentrate at below 40°C, and remove the solvent.

ii) Conversion to metabolite M2

Add 0.5 mL of acetic anhydride to the residue obtained in i) and heat at 80°C for 15 min. Allow it to cool and add 5 mL of ethyl acetate and *n*-hexane (1:9, v/v).

iii) Silica gel column chromatography

Inject 10 mL of ethyl acetate and *n*-hexane (1:9, v/v) into a silica gel cartridge (1,000 mg) and discard the effluent. Transfer the solution obtained in ii) to the cartridge, add 10 mL of ethyl acetate and *n*-hexane (1:9, v/v), and discard the effluent. Then, add 20 mL of ethyl acetate and *n*-hexane (1:4, v/v), concentrate the eluate at below 40°C, and remove the solvent. Dissolve the residue in acetone and *n*-hexane (1:1, v/v) to make exactly 4 mL and use this solution as the test solution.

6. Calibration curve

Prepare standard stock solutions using the reference standards of ethofumesate and metabolite M2, respectively. Mix each stock standard solution appropriately, dilute with acetone and *n*-hexane (1:1, v/v), and prepare standard solutions of several concentrations. Inject each standard solution into GC-MS/MS, and make calibration curves by peak-height or peak-area method. When the test solution is prepared following the above procedure, the concentration of ethofumesate and metabolite M2 in the test solution corresponding to 0.01 mg/kg (metabolite M2 and metabolite M3 are calculated as ethofumesate) in the sample results in 0.0005 mg/L (metabolite M2 and metabolite M3 are calculated as ethofumesate).

7. Quantification

Inject the test solution into GC-MS/MS, calculate the concentration of ethofumesate and metabolite M2* from the calibration curve obtained in 6., and calculate the concentration of ethofumesate including metabolite M2* using the following equation.

Concentration of ethofumesate (including metabolite M2*) (ppm) = A + B × 1.117

A: Concentration of ethofumesate (ppm)

B: Concentration of metabolite M2* (ppm)

* Including metabolites (including metabolite M3 and conjugates of metabolite M3) converted to metabolite M2 by thermal acid treatment.

8. Confirmation

Confirm using GC-MS/MS.

9. Measurement conditions

(Example)

Column: 5% phenyl-methyl silicone, 0.25 mm inside diameter, 30 m in length and 0.25 μm in film thickness

Column temperature: 50°C (1 min) → 25°C/min → 220°C → 5°C/min → 240°C → 30°C/min → 310°C (5 min)

Inlet temperature: 260°C

Carrier gas: Helium

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

Major monitoring ions (*m/z*)

Ethofumesate: Precursor ion 286, product ions 207, 161

Metabolite M2: Precursor ion 256, product ions 177, 149

Injection volume: 2 μL

Expected retention time

Ethofumesate: 10 min

Metabolite M2: 10 min

10. Limit of quantification

Ethofumesate: 0.01 mg/kg

Metabolite M2: 0.01 mg/kg (equivalent to ethofumesate)

Metabolite M3: 0.01 mg/kg (equivalent to ethofumesate)

11. Explanatory note

1) Outline of analytical method

The method consists of extraction of ethofumesate and its metabolites [metabolite M2 and metabolites converted to metabolite M2 by thermal acid treatment (including metabolite M3 and conjugates of metabolite M3)] from the sample with acetone and water (4:1, v/v), partitioning ethofumesate into the *n*-hexane layer and the metabolites into the aqueous layer by sodium hydroxide solution/hexane partitioning, for ethofumesate, clean-up the *n*-hexane layer using a silica gel cartridge, quantification and confirmation using GC-MS/MS, for the metabolites, adding hydrochloric acid to the aqueous layer after partitioning and to the residue during extraction, heating, hydrolysis of the conjugate of metabolite M3 to metabolite M3 (at this stage, part of metabolite M3 is converted to metabolite M2), transfer into diethyl ether for re-dissolution, converting metabolite M3 to metabolite M2 with acetic anhydride, clean-up using a silica gel cartridge, and quantification and confirmation metabolite M2 using GC-MS/MS. In the method, ethofumesate and metabolite M2 [including the metabolites converted to metabolite M2 (including metabolite M3 and

conjugates of metabolite M3) by thermal acid treatment] are quantified respectively. For the concentration of ethofumesate including the metabolites, the concentration of metabolite M2 is converted to the concentration of ethofumesate by multiplying by the conversion factor, and the sum of the concentration of ethofumesate and metabolite M2 is regarded as the analytical result of ethofumesate.

2) Notes

i) The supernatant should be filtered with filter paper if suspended materials are observed after centrifugation in the extraction process.

ii) When the residue is weighed after extraction, the solvent should be removed with a nitrogen stream if the weight varies due to the influence of the solvent in the residue.

iii) As a result of the hydrolysis operation, an equilibrium mixture of metabolite M2 and metabolite M3 is formed. Therefore, acetic anhydride is used to convert unconverted metabolite M3 into metabolite M2.

iv) Confirm that conversion to metabolite M2 is fully undergone by conducting a spike and recovery test using the reference standard of metabolite M3 sodium salt. The conversion factor for metabolite M3 sodium salt to ethofumesate is 0.9665.

v) When an emulsion is formed in the liquid-liquid extraction using diethyl ether after hydrolysis, leave the emulsion, collect the diethyl ether layer, and then perform the second and further operations on the remaining emulsion and water layer.

vi) If matrix effects or tailing are observed during the measurement, polyethylene glycol-300 should be added to both the test solution and the standard solution prepared for the calibration curve. These methods are described below.

Test solution: Take exactly a 1 mL aliquot of the test solution obtained in 5. 3) or 5. 4) iii) and add a 100 µL mixture of acetone containing 0.1 w/v% polyethylene glycol-300 and *n*-hexane (1:1, v/v) accurately.

The standard solution prepared for the calibration curve: Take exactly a 1 mL aliquot of the standard solution prepared for the calibration curve obtained in 6. and add a 100 µL mixture of acetone containing 0.1 w/v% polyethylene glycol-300 and *n*-hexane (1:1, v/v) accurately.

vii) When the analytical methods for ethofumesate and metabolite M2 using GC-MS/MS were developed, the following monitoring ions were used:

Ethofumesate

for quantitative ions (*m/z*): precursor ion 286, product ion 207

for qualitative ions (*m/z*): precursor ion 286, product ion 161

Metabolite M2

for quantitative ions (*m/z*): precursor ion 256, product ion 149

for qualitative ions (*m/z*): precursor ion 256, product ion 177

viii) Food items used to develop the analytical method: onion, sugar beet and garlic

12. References

Ethofumesate: Magnitude of the residue on onion (dry bulb), Appendix 4 Analytical summary report, Bayer Cropscience, 2004.

13. Type

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