

METHOD NUMBER

KP-201R2

METHOD TITLE

LC/MS/MS ANALYTICAL METHOD FOR SIMULTANEOUS DETERMINATION OF
THIOPHANATE-METHYL AND MBC IN/ON CROPS

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The analytical method KP-201R0 was replaced by revised method No. KP-201R1.
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ABSTRACT

This Topsin®M analytical residue method was designed to obtain data on residues of thiophanate-methyl (TM) and methyl-2- benzimidazole carbamate (MBC) in or on crop matrices. The method consists of two sequential extractions with acidified methanol (MeOH) followed by centrifugation and vacuum filtration to separate solids from the extractable materials. A portion of combined methanol/aqueous extract is then neutralized with ammonium hydroxide to a pH range of 6.5 - 7.5. The neutralized extract is further cleaned-up in a reversed phase cartridge and the analytes are eluted with acetonitrile from the cartridge.

In preparation for instrumental analysis, the acetonitrile eluant is brought to a calibrated final volume with water and filtered through a syringe filter prior to instrumental analysis.

Instrumental analysis is accomplished using LC/MS/MS system and a reversed phase column. The molecular ions formed in positive ion mode are fragmented by collision with neutral gas. The fragment ions generated are filtered and only one product ions from each parent molecules are selected for quantitation. The method lower limit of quantitation is 0.01 ppm for both analytes.

Cautionary Notes: TM is unstable in the presence of heat, high pH, and is photosensitive. Experience has shown that it is imperative that extraction of the samples through the entire method should be completed in one day to obtain consistently good recoveries of TM; this also helps prevent conversion of TM to MBC. MBC is very stable under all conditions.

ANALYTICAL METHOD

I. Materials

A. Solvents and Reagents.

1. HPLC grade solvents or better should be utilized. Other brands of HPLC grade solvents may be substituted as long as they do not produce interferences with the chromatography.
 - a. Methanol (MeOH), HPLC grade, Fisher Scientific, Pittsburgh, PA
 - b. Acetonitrile, HPLC grade, Fisher Scientific, Pittsburgh, PA
2. Reagents should be ACS grade or better. Other brands of ACS grade reagents may be substituted as long as they do not produce interferences with the chromatography.
 - a. Phosphoric Acid (H₃PO₄), AR, Mallinckrodt, Paris, KY
 - b. Ammonium Hydroxide, concentrated, Fisher Scientific, Pittsburgh, PA 15238
 - c. L-(+)cysteine, J.T. Baker, Phillipsboro, NJ
 - d. Formic Acid(HCOOH), Mallinckrodt, Paris, KY
 - e. Celite 545, Fisher Scientific, Pittsburgh, PA 15238

B. Miscellaneous Materials

1. Oasis HLB cartridges, 6cc/200mg, Lot# WAT106202. Waters, Milford, MA.
2. Mixed Solvent Systems:
 - a. Extraction Solvent: Methanol : 3 N H₃PO₄ (1:1 v/v)
 - b. Transfer Solvent: MeOH: H₂O (25:75 v/v)

II. Equipment

A. LC/MS/MS System

TOPSIN M ANALYTICAL RESIDUE METHOD NO. KP-201R2

1. Waters Alliance HPLC system with cooled sample chamber and a column heater. Waters, Chromatograph Division of Millipore Corp., Milford, MA.
 2. Mass Spectrometer detector, PE Sciex 2000, Applied-Biosystem, Foster City, CA or Quattro Ultima, MicroMass, Beverly, MA or equivalent.
 3. A gradient pumps equivalent to Model 600 or 625 pump from Waters, Chromatograph Division of Millipore Corp., Milford, MA.
- B. SPE vacuum manifold, Supelco, Bellefonte, PA
- C. Adjustable pipettes, assorted sizes
- D. Orion pH Meter, model 920A, Orion Research Inc., Boston, MA, capable of accurately measuring 0-14 pH, with an accuracy of +/- 0.2 or equivalent.
- E. Hot plate magnetic stirrer
- F. Centrifuge, refrigerated, of a capacity capable of centrifuging 250 ml centrifuge containers at 2000 to 5,000 rpm.
- G. Omni Homogenizer mixer Model 17105, equipped with Omni Mier Stainless Steel 250 ml cup and blade assembly, OMNI international, Waterbury, CT or equivalent
- H. Wrist Action Shaker, Model 75, Burrell Corp, Pittsburgh, PA or equivalent
- I. Filters
1. #4 Qualitative filter paper 7.0 cm, VWR Scientific, Bridgeport, NJ
 2. 0.45 μ m 25 mm PTFE or nylon syringe filter, Corning Glass Works, Corning, NY.
- J. Glassware
1. Graduated test tubes 13 mm x 100 mm.
 2. Graduated cylinders, 250 ml and assorted.
 3. Beakers, various sizes.
 4. Buchner Funnel, porcelain, 90 mm opening.
 5. Class A volumetric flasks, assorted sizes
 6. Autosampler vials, Alltech Assoc., Inc., Deerfield, IL
 7. Centrifuge bottle, Nalgene, 250 ml

8. Glass filtration adaptor

K. Miscellaneous

1. Magnetic stir bars
2. Polyethylene 5-10 ml syringe with a non-lubricated polyethylene faced plunger assembly.

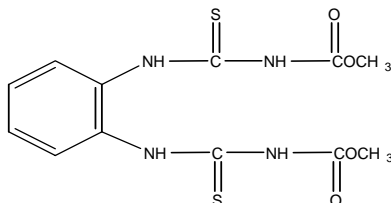
III. Test Substances

A. Parent Compound: Thiophanate Methyl; dimethyl[(1,2-phenylene) bis(imino-carbonothioyl)]bis[carbamate] (TM)

CAS No.: 23564-05-8

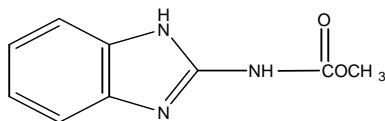
Trade Name: Topsin® M

MW 342.2



B. Metabolites: Methyl-2-benzimidazolyl carbamate (MBC)

MW 191



TM and MBC used as standards in their purest form should be considered a chemical hazard. All caution should be exercised when handling pure material or concentrated stock solutions. Avoid skin contact and inhalation. See Material Safety Data Sheet documentation accompanying standard shipment.

IV. Standard Preparation

A. Preparation of TM standards

1. Weigh 0.0100 g (with appropriate purity adjustment) of solid TM material in a glass weigh boat and transfer them to a 100 ml volumetric flasks using methanol. (Avoid use of plastics for the weighing of compounds and storing of reagents. The small amounts of plasticizer leaching into the sample will have a detrimental effect on the analysis.)
2. Half fill the volumetric flask with methanol and agitate gently until standards are completely dissolved.
3. Dilute to volume with methanol and mix by inverting several times. Final concentration = 100 F g/ml.
4. The concentrated standard solutions remain stable in a refrigerator at 0-7 °C for six months.

B. Preparation of MBC standard

MBC is most soluble in acidic aqueous solutions. Stock standard is, therefore, prepared in an acidic aqueous solvent.

1. Prepare a 0.05 N solution of phosphoric acid (H_3PO_4) to be used as the diluent.
2. Weigh 0.010 g of solid MBC material (with appropriate purity adjustment) into a glass weigh boat and transfer MBC into a 100 ml volumetric flask using 0.05 N H_3PO_4 . Rinse glass funnel thoroughly with 0.05 N H_3PO_4 into the flask.
3. Half fill the volumetric flask, sonicate the flask for a few minutes until all solid MBC is dissolved completely.
4. Bring to volume with 0.05 N H_3PO_4 and mix by inverting several times. Final stock solution concentration = 100 F g/ml.
5. The stock standard solution remains stable in a refrigerator at 0-7°C for six months.

C. Preparation of Fortification Standards

Fortification standards should be prepared on a monthly basis by dilution of the concentrated standard solution with methanol. Store in a refrigerator at 0-7°C.

D. Preparation of Calibration standards

Prepare calibration standards weekly by dilution of the concentrated stock standard solution with transfer solvent. Store in refrigerator at 0-7°C. A typical range of concentrations for the standards are 0.5, 1.0, 2.5, 5.0 and 10.0 ng/mL.

V. Sample Analysis Procedure

- A. Extraction – Rapid processing is essential to the success of this analysis due to the labile nature of thiophanate methyl. All sample extracts should be stored in refrigeration when not in use.
1. Weigh approximately 10-25 g (depending on the matrix) of randomly sampled crop to the nearest 0.01 g into a 350 ml mason jar for blender extraction, or a 250 ml HDPE, PTFE, or glass bottle for extraction by shaking.
 2. For the preparation of analytical recovery samples, fortify control samples in the extraction vessel by pipetting a known volume and concentration of the fortification standard onto the sample matrix.
 3. Add approximately 300 mg of L-(+)-cysteine (antioxidant) to each sample.
 4. Add 75 ml of extraction solvent: MeOH : 3N H₃PO₄ (1:1 v/v).
 5. If blending sample, blend the sample in the sealed extraction vessel for 3 minutes at moderate speed. If shaking the sample, place in a shaker and shake at moderate – high speed for approximately 10 minutes.
 6. Centrifuge sample in a centrifuge at 2,000 to 5,000 rpm depending on the sample matrix. If prolonged centrifugation is needed the centrifuge should have a refrigerated compartment.
If sample is not extracted in a container suitable for centrifugation, transfer to a centrifuge bottle (250 ml) with liberal rinses of extraction solvent.
 7. Separation of the extraction solvent from the solid sample matrix may be accomplished by filtration through a Buchner funnel or decantation.

In the case of filtration is used: seat a #4 Whatmen filter with a small amount of MeOH/water (25/75) into a Buchner funnel that has been secured onto a vacuum graduate cylinder while drawing a vacuum through the funnel and cylinder assembly. Top the filter paper with a thin layer of Celite 545 filter aid. Decant the supernatant extract to the Buchner funnel. Collect filtrate in a 250 ml graduate cylinder.

8. Re-extract the sample with 75 ml of extraction solvent once more and separation of extract from matrix as outlined in the steps V(A) 4-6. Centrifuge is not necessary if filtration is used. Combine the liquid phases from both extractions into the collection cylinder.

Note: When analyzing dry matrices such as hay, forage, etc., two additional extractions may be used with 50-75 ml of extraction solvent after the first extraction. In this case, the centrifuge step will be need in the second extraction step but not in the third extraction.

9. Rinse the extraction cap and bottle and include in the collection. Perform a final rinse of the filter cake in the Buchner funnel with a small amount of transfer solvent (25:75 MeOH:water) and allow for collection of the filtrate until the cake is nearly dry.
10. Dilute the total volume to 200 ml with transfer solvent and mix well.
11. Sample can be filtered through a 0.45 u syringe filter and directly analyzed on LC/MS/MS system.

The procedure B and C are designed as an optional further clean up and concentration steps depending on the sample matrices and the limit of the quantitation.

B. pH Adjustment

The control extract is used to go through pH adjustment on a pH meter in order to determine the amount of NH_4OH used to bring the pH of the sample to 6.5 - 7.5. Then same amount of NH_4OH will be added for the rest of sample. This test should be performed on each set and each type of the matrices.

1. Take 10 ml of the control extract into a 25 ml disposable plastic beaker. Add a stir bar and half full of a small spatula (approximately 30 mg) L-cysteine to the aqueous extract.
2. Place the sample beaker on a stir plate and stir rapidly.
3. Insert calibrated pH probe and measure pH continuously during the next step.
4. Using a adjustable pipettes, draw small amount (100 ul is suggested) of concentrated ammonium hydroxide and add dropwise to the

aqueous solution in the beaker until the pH meter reads steadily between 6.8 and 7.1. When the pH is close to 6.5, 25 or 50 ul of NH₄OH should be added each time. Record the total volume of ammonium hydroxide added.

5. Pipette 10 ml of each sample to a disposable test tube, add approximately 30 mg of L-cysteine and the same amount of NH₄OH determined from the step B.4 to each sample. Mix well.

C. Solid Phase Extraction Procedure

Waters Oasis HLB, 6cc/200mg cartridges is used in the procedure. Other reversed phase cartridges may be used as long as they are checked to insure good recoveries of TM and MBC. The flow rate should be controlled between 1.0-3.0 ml/minute. Volume of extract loaded on the cartridge and the final volume could be various depending on the instrument sensitivity and matrices.

- a. Pre-rinse the cartridge with 5 ml acetonitrile followed by 5 ml of acetonitrile:water 20:80 solution.
- b. Transfer the extract from section V.(C).(7) to the cartridge. Rinse the test tube with - 3 ml of acetonitrile:water 20:80 solution and add to the cartridge.
- c. Elute the cartridge with 5 ml acetonitrile and collect in a graduated test tube.
- d. Bring the volume of the test tube to 10.0 ml with water, mix the contents.
- e. Filter the extract through a 0.45 µm x 25 mm PTFE or Nylon syringe filter. Sample is ready for LC/MS/MS analysis.

VI. LC/MS/MS

Analysis of samples will be performed using LC/MS/MS system. A diverter switch valve may be utilized. In this case initially sample will be diverted to waste after passing through column. Just prior to elution of the analytes from column, the diverter is switched to the mass spectrometer. The diverter valve is returned to its initial position after the analytes is completely eluted from the column. A make-up mobile phase should be sprayed onto the mass spectrometer using an additional HPLC pump when the elution from the column is diverted into waste.

- A. Preparation of Mobile Phase:

A: Dilute 1 ml formic acid to 1 liter with water.

B: Methanol, HPLC grade or better.

B. HPLC Column Conditions:

Mobile and/or stationary phase conditions may be changed (depending on matrix conditions) in order to optimize chromatographic selectivity and/or efficiency.

Column: Betasil C8, 100 x 4.6 mm, 5F, ThermoHypersil-Keystone, Bellefonte, PA or equivalent.

Column Temperature: 28°C

Injection Volume: 25-100 ul

Run Time: 10 minutes

C. Mass spectrometer

APCI or electrode spray can be used for the analysis. Tuning and optimization on mass spectrometer should be performed by the operator to ensure good sensitivity. The following instrument parameters utilizing APCI interface on PE Sciex 2000 and a diverter should be used as a general guidance.

Ionization Mode: APCI

Scan Type: Positive MRM

Source Temperature: 400°C

Curtain Gas: 45 psi

Collision Gas: 4 psi

Nebulizer Gas: 15 psi

Auxiliary Gas: 60 psi

Nebulizer Current: 3.0 µA

Retention Time: MBC approximate 4.2, TM 6.3
 Make-up 65:35 0.1% HCOOH in water:MeOH
 Mobile Phase: 0.75 ml/min, isocratic (for Sciex only)

TM Q₁ mass 343, Q₃ mass 151

MBC Q₁ mass 192, Q₃ mass 160

Mobile phase gradient

Time min	Flow Rate ml/min	MeOH	0.1%HCOOH in water
0.0	0.75	20	80
4.0	0.75	100	0
7.0	0.75	100	0
7.5	0.75	20	80

VII. HPLC Quantitation Procedures

A. Standardization.

1. A linear regression response curve should be generated for every HPLC analytical run.
2. The curve should be generated by injections of at least four different concentrations of standards through the entire run. The standards should be injected prior to and immediately after a sequence of samples. If the sample set is >5 then an occasional standard should be analyzed between samples to demonstrate response stability.
3. A standard that represents a residue of 50% of the method's lower limit of quantitation (LOQ) for each analyte must be among those standards injected.
4. Any sample residue which exceeds the highest standard injected must be appropriately diluted and reinjected with a standard set such that it falls within the linear response range of the standards injected, or injected at a lower volume such that it falls within the linear response.

B. Calculations

Quantitation of thiophanate methyl and MBC are dependent on; sample size, the final sample volume, the final dilution volume. To standardize the

analysis, all samples of a given matrix should be analyzed using the same sample weight, aliquot (125 ml of 250 ml), brought to the same final sample volume (5 ml), injected same volume on HPLC, and analyzed on the HPLC with a standard injection volume. Dilution factors to achieve quantifiable concentrations for HPLC should be the only volume variables. With these standardizing assumptions, the following equation may be used to obtain parts per million values for TM and MBC:

$$ppm = \frac{0.2 \times C \times E \times \text{DilutionFactor}}{G \times F}$$

Where:

E = final sample volume for HPLC in milliliters.

C = concentration (ng /ml) as interpolated mathematically from a linear regression curve of the compound's HPLC response versus concentration of standard injected.

F = original sample weight in grams.

G = Aliquot of extract in milliliters taken for SPE procedure

The dilution factor is based on the ratio of the final dilution volume to the volume of any aliquot(s) removed from the final HPLC extract.

Example:

A 20 g sample was analyzed. After extraction, 10 ml is taken for SPE clean-up, it is brought to final volume of 10.0 ml for LC/MS/MS analysis.

$$ppm = [0.2 \times (C \text{ ng/ml}) \times (10.0 \text{ ml})] / (10 \text{ ml} \times 20 \text{ g})$$

Where:

C = The interpolated concentration value from a linear regression curve prepared from co-chromatographed standards and entered into the linear regression with the units of ng/ml.