ANALYTICAL METHOD FOR THE DETERMINATION OF PYRITHIOBAC SODIUM IN SOIL USING SUBCRITICAL WATER EXTRACTION, GRAPHITIZED CARBON CLEAN-UP, AND COLUMN-SWITCHING LC/UV ANALYSIS WITH CONFIRMATION BY LC/MS

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PURPOSE FOR REVISION

Revision No. 1 to AMR 2745-93 serves seven purposes:

1. The word cleaned-up in the title is changed to clean-up.
2. The number of ASE™ 200 extraction cycles is defined as one.
3. The typo in Step 1 of the analyte purification procedure is corrected: wash the ENVI-Carb tube with one 10-mL aliquot of **0.10** M formic acid in 90% dichloromethane (DCM)/10% methanol (MeOH).
4. Step 5 of the analyte purification procedure is clarified: cartridges are not allowed to air dry under vacuum after the wash solution passes through them.
5. A warning not to use a cyano guard column is added to the Equipment section and to the Modifications or Special Precautions section. Column-to-column reproducibility for pyrithiobac sodium has been horrible; the cyano guard column tends to increase the peak width and generate poor peak shape for pyrithiobac sodium.
6. The wording in the Setting the Time Window and Operating Conditions sections has been changed to clarify the intent of switching the **entire** pyrithiobac sodium peak from the CN column to the C18 column.
7. A new typical calibration plot for UV detection is added in Figure 4.

1.0 SUMMARY

Pyrithiobac sodium (pyrithiobac, DPX-PE350, KIH-2031, sodium 2-chloro-6-[(4,6-dimethoxypyrimidin-2-yl)thio]benzoate) is extracted from 10 g of soil by Milli-Q® water at subcritical conditions (100°C and 2000 psi) using a DIONEX ASE™ 200 Extractor. Pyrithiobac is separated from the resulting extract by passing it through a graphitized carbon column. Pyrithiobac is selectively eluted from the column from coextracts and then analyzed by column-switching liquid chromatography (LC) with ultraviolet (UV) absorption detection at 254 nm. The method detection limit (MDL) and limit of quantitation (LOQ) for the LC/UV method are 0.3 and 1.0 µg/kg (ppb), respectively.

This method meets U.S. EPA, Subdivision N, 164-5, Pesticide Assessment Guideline and EEC Directive 91/414/EEC: Annex II 4.2.2 criteria.

2.0 INTRODUCTION

This analytical method was developed to determine the levels of pyrithiobac sodium residues extracted from soil. Pyrithiobac sodium is the active ingredient in Staple® Herbicide which is used to control broad-leaf weeds in cotton. The structure and physicochemical data for pyrithiobac sodium (pyrithiobac, DPX-PE350, KIH-2031, sodium 2-chloro-6-[(4,6-dimethoxypyrimidin-2-yl)thio]benzoate) are found in Appendix 1.

Pyrithiobac is extracted from 10 g of soil by Milli-Q® water at subcritical conditions (100°C and 2000 psi) using a DIONEX ASE™ 200 Extractor. Extraction efficiency was demonstrated using standard ^{14}C methodology. After extraction, pyrithiobac is trapped on a graphitized carbon column. Pyrithiobac is selectively eluted from the column from coextracts and then analyzed by column-switching HPLC/UV (254 nm). The method detection limit (MDL) and limit of quantitation (LOQ) for the LC/UV method are 0.3 and 1.0 µg/kg (ppb), respectively.

Method ruggedness testing was performed. Three soil types, typical of soil where cotton is grown, of varying pH, % organic matter, % silt, and % clay were fortified, extracted, and analyzed using this method. Additionally, the extraction and clean-up steps of this method were performed by three analysts.

LC/MS methods were developed to confirm the results generated by LC/UV for selected samples.

3.0 MATERIALS

3.1 Equipment

Equivalent equipment may be substituted unless otherwise indicated. Note any specification in the following descriptions before making substitutions. Substitutions should be made only if equivalency/suitability has been verified with acceptable control and fortification recovery data.

ASE™ 200 Extraction Apparatus - extractor and the following parts: 22-mL stainless steel extraction cells, #49561; cellulose filters, #49458; 60-mL collection vials, #48784, septa for collection vial lids, #49464; O-rings, #049457; PEEK seals, #049455 DIONEX (Sunnyvale, Calif.). Silica gel 60, 0.040-0.063 mm particle size, #9385-3 EM Science (Gibbstown, N.J.).

LC/UV system - (Waters, Milford, Mass.)

- Pump control module, Waters;
- Three pumps, Waters, Model 510; Note: a three pump, high-pressure mixing HPLC system is not required for this method; a single pump, low-pressure mixing HPLC system will work too.
- Millennium 2010 v2.00 software run on a NEC 486/33 computer, Waters;
- Auto injector, Waters, Model 717 equipped with a 2.5-mL syringe;
- Temperature control module, Waters;
- Column heater module, Waters; and
- Six-port switching valve, (Valco Inst., Houston, Tex., Model E60, #EC6W)

HPLC Columns - Column I: Zorbax® SB-CN 4.6 x 150 mm, 5-µm particles, #883975-905; Column II: Zorbax® SB-C18 4.6 x 250 mm, 5-µm particles, #880975-902. **Do not substitute.** Do not use a cyano guard column. Column-to-column reproducibility for pyrithiobac sodium has been found to be unacceptable. A Zorbax® cyano guard column usually increases peak width and generates poor peak shape for pyrithiobac sodium.

Solid-Phase Extraction Apparatus - Solid-phase extraction manifold, #5-7044M, with disposable Teflon® solvent guides, #5-7059 (Supelco, Bellefonte, Pa.)

Solid-Phase Extraction Cartridges and Adapters - ENVI-Carb packing #5-7210 (Supelco, Bellefonte, Penn.), **do not substitute.** 25-mL reservoir with frits #1213-1017, and porous, polyethylene, 20-µm pore frits #1213-1023 (Varian Sample Preparation Products, San Fernando, Calif.).

Disposable Centrifuge Tubes - Blue Max centrifuge tubes with caps and rack, polypropylene, 50-mL volume, #21008-951 (VWR Scientific Co., Bridgeport, N.J.)

Evaporator - N-Evap® Model 111 laboratory sample evaporator/nitrogen manifold fitted with Teflon®-coated needles (Organomation Associates, South Berlin, Mass.). Unit is attached to a dry, clean nitrogen source.

Mobile Phase Filters and Vacuum Filter Apparatus - Use 0.45-µm pore, Cat. No. HATF 047 00, Type HA filters for the 0.1 M acetic acid. Use 0.5-µm pore, Cat. No. FHUP 047 00, Type FH filters for acetonitrile. The Millipore vacuum filter apparatus used to filter and degas mobile phases consists of a glass filter holder, #XX1004700, a ground glass base with stopper, # XX1004702, a funnel cover, #XX2504754, and a 1-L filter flask, #XX1004705 (Millipore, Inc., Bedford, Mass.).

Syringes - 2.5-mL disposable plastic syringe, Part No. Z11685-8 (Aldrich Chemical Co., Milwaukee, Wis.); Hamilton 100- and 500-µL syringes, #80600 and #80800, respectively (Hamilton, Reno, Nev.)

Syringe Filters - 4-mm nylon filters with 0.45-µm pore, #9001-10 (Chrom Tech, Inc., Apple Valley, Minn.)

pH Meter - Beckman Model PHI 11 (Beckman Instruments, Inc., Fullerton, Calif.)

Balances - Mettler A163 analytical and PM460 top-loading balances (Mettler Instrument Corp., Hightstown, N.J.)

Ultrasonic Bath - Branson Model 2200 ultrasonic bath (VWR Scientific Co., Bridgeport, N.J.)

Mixer - -Vortex Genie 2 (VWR Scientific Co., Bridgeport, N.J.)

Pipettes - Pipetman #P-1000 adjustable pipette and EDP-Plus pipette #EP-10ML (Rainin, Emeryville, Calif.)

Antistatic Gun - Zerostat antistatic gun, #Z3000 (Sigma, Chemical Co., St. Louis, Miss.)

3.2 *Reagents and Standards*

Equivalent reagents may be substituted for those listed below. To determine if substituted reagent impurities interfere with DPX-PE350, appropriate amounts of the solvents should be injected into the HPLC using the chromatographic conditions specified in this report for DPX-PE350.

Water - Deionized water passed through a Milli-Q® UV Plus water purification system #ZD60 115 UV (Millipore, Bedford, Mass.)

Dichloromethane (DCM) - EM Omni Solv®, residue grade dichloromethane, #DX0831-1 (EM Science, Gibbstown, N.J.). ***Warning*** - dichloromethane is a suspected carcinogen - use in a fume hood.

Methanol (MeOH) - EM Omni Solv®, HPLC-grade methanol, #MX0488-1 (EM Science)

Acetonitrile (ACN) - EM Omni Solv®, HPLC-grade acetonitrile, #AX0142-1 (EM Science)

Acetone - EM Omni Solv®, HPLC-grade acetone, #AX0116-1 (EM Science)

Ammonium Carbonate [$(NH_4)_2CO_3$] - Baker Analyzed® Reagent, reagent-grade ammonium carbonate #0642-01 (J. T. Baker, Inc., Phillipsburg, N.J.)

Hydrochloric Acid (HCl) - Reagent-grade 12 M hydrochloric acid, #9535-01 (J. T. Baker, Inc.)

Formic Acid - EM Suprapur® formic acid, #11670-1 (EM Science)

Acetic Acid - Baker Analyzed® glacial acetic acid, #9524-00 (J. T. Baker, Inc.)

Pyrithiobac Sodium (DPX-PE350, KIH-2031) - Reference substance used for HPLC analysis: analytical standard grade DPX-PE350, Lot #4, 98.7% pure (prepared by Kumiai/Ihara Chemical Co. for DuPont Agricultural Products, Global Technology Division, E. I. du Pont de Nemours and Company).

Radioactive pyrithiobac (DPX-PE350), NEN #2764-067, HOTC #370, 99.0% pure. Specific Activity: 70.210 µCi/mg. Radiolabel location: pyrimidine-2-^{14}C.

3.3 Safety and Health

No unusually hazardous materials are used in this method. All appropriate material safety data sheets should be read and followed, and proper personal protective equipment should be used.

Warning - dichloromethane is a suspected carcinogen - use in a fume hood.

Caution: extraction cells used for this method are extremely hot (100°C) after the extraction. Allow the cells to cool for at least 15 minutes before handling to avoid burns.

All material safety data sheets should be read and followed and proper protective equipment should be used.

4.0 METHODS

4.1 Principles of the Analytical Method

In this section is a brief discussion of procedures developed to extract pyrithiobac sodium from soil. This discussion is followed by a brief explanation of the analytical method using subcritical water extraction.

Pyrithiobac is stable in relatively extreme extraction conditions: acidic and basic conditions. Aged pyrithiobac residues may be efficiently extracted from soil by reflux in 20% 1 N sulfuric acid/80% acetone and by reflux in 1 N sodium hydroxide.

These acidic and basic extraction conditions sufficiently extract aged pyrithiobac residues from soil, but the clean-up steps that follow before analysis are extensive, requiring two to three days to complete. After acidic or basic extraction, and extensive clean-up, co-extracts still lead to interference peaks in chromatographic analysis.

Using single-column, reversed-phase LC/UV, the coextracts that remain after clean-up interfere with the quantitation of pyrithiobac at low levels (1 µg/kg). Column-switching LC/UV of these extracts may be performed to eliminate much of the interference, but spurious interference peaks still present problems for routine analysis.

Pyrithiobac may not be directly analyzed by GC, but must be derivatized. A reagent that works reasonably well is diazomethane, methylating the carboxylic acid on pyrithiobac. However, many analysts prefer not to work with diazomethane due to its potential hazards. Other reagents may be used to derivatize pyrithiobac, but the conditions required usually derivatize co-extracts that can lead to interference peaks in GC.

A method that would efficiently extract aged pyrithiobac sodium residues, but require little clean-up before its direct and routine analysis by LC/UV was desired. The analytical method described in this report accomplishes this objective.

Pyrithiobac sodium (pyrithiobac, DPX-PE350, KIH-2031, sodium 2-chloro-6-[(4,6-dimethoxypyrimidin-2-yl)thio]benzoate) is extracted from 10 g of soil by Milli-Q® water at subcritical conditions (100°C and 2000 psi) using a DIONEX ASE™ 200 Extractor. Pyrithiobac is separated from the resulting extract by passing it through a graphitized carbon column. Pyrithiobac is selectively eluted from the column and then analyzed by column switching HPLC/UV (254 nm). A flow diagram of the analytical method from extraction to analysis is shown in Figure 1.

4.2 *Analytical Procedure*

4.2.1 *Glassware and Equipment Cleaning*

Glassware and extraction cells should be scrubbed by brush with a soap solution, rinsed two to five times with water, and rinsed with acetone or other suitable solvents. Distilled or deionized water may be added to the rinse sequence. The glassware and extraction cells are air-dried.

4.2.2 *Preparation of Solutions*

The following solutions should be prepared weekly and stored at room temperature unless stated otherwise:

0.01 M Ammonium Carbonate - Dissolve 0.96 g of $(NH_4)_2CO_3$ in about 800-mL distilled water and dilute to 1.00 L in a volumetric flask.

0.1 M Hydrochloric Acid - Pipet 8.3 mL 12 M HCl into 1-L volumetric flask and bring to volume with Milli-Q® water.

90% DCM/10% MeOH - With 1000-mL graduated cylinder, measure 900 mL of dichloromethane and add to 1-L volumetric flask. With 100-mL graduated cylinder, measure 100 mL of methanol and add to the 1-L flask. Do not adjust the volume to 1-L mark.

0.1 M Formic Acid in 90/10 DCM/MeOH - Pipet 0.755 mL of formic acid into 200-mL volumetric flask. Bring to volume with 90 DCM/10 MeOH.

0.10 M Acetic Acid - Pipet 2.85 mL of glacial acetic acid into 500-mL volumetric flask and bring to volume with Milli-Q® water.

20% Acetonitrile/80% 0.10 M Acetic Acid - With 100-mL graduated cylinder, measure 100 mL of acetonitrile and add to a 500-mL volumetric flask. With a 500-mL graduated cylinder, measure 400 mL of 0.1 M acetic acid into the 500-mL volumetric flask. Do not adjust the volume to the 500-mL mark. Shake vigorously to mix.

HPLC Eluents - Eluent A: 100% acetonitrile; Eluent B: 100% 0.10 M acetic acid; Eluent C: 100% Milli-Q® water. Mobile phases should be thoroughly degassed daily. Solvents are degassed by filtering them through a Millipore® vacuum filtering apparatus while sonicating the apparatus. If a low-pressure mixing HPLC is used, mobile phases should be sparged at approximately 30 mL/min.

4.2.3 *Preparation and Stability of Stock Standard*

Use Class A volumetric flasks when preparing standard solutions.

Prepare a standard stock solution by accurately weighing 10 mg of pyrithiobac into a 100-mL volumetric flask on an analytical balance. *Record the weight of the standard used to make the stock solution.* Dissolve the standard in approximately 75 mL of HPLC-grade methanol. After dissolving, bring the solution to 100.00-mL volume using HPLC-grade methanol. This standard solution is stable for approximately 8 months when stored at approximately 4°C. The concentration of this solution is 100-µg/mL pyrithiobac in methanol.

4.2.4 *Preparation and Stability of Fortification Standard*

Use Class A volumetric flasks when preparing standard solutions.

Prepare a fortification standard solution by pipetting 1.00 mL of the 100-µg/mL pyrithiobac stock standard into a 100-mL volumetric flask. Bring to volume using HPLC-grade methanol. The concentration of this solution is 1-µg/mL pyrithiobac in methanol. This standard solution is stable for approximately 8 months stored at approximately 4°C.

4.2.5 *Preparation and Stability of Chromatographic Standards*

Use Class A volumetric flasks when preparing standard solutions.

The 1-µg/mL pyrithiobac in methanol fortification standard is used to prepare the chromatographic standards. Prepare the standards by pipetting volumes of the 1-µg/mL fortification standard solution of pyrithiobac into a 25-mL volumetric flask, as shown in the following table:

Desired Standard Concentration (µg/mL)	Volume of 1 µg/mL Standard Required (mL)
0.500	12.5
0.250	6.25
0.200	5.00
0.100	2.50
0.0500	1.25
0.0250	0.625
0.0100	0.250
0.00500	0.125
0.00100	0.0250

Evaporate the methanol (to dryness) in each of the 25-mL volumetric flasks using an N-Evap®. Add 20% acetonitrile/80% 0.10 M acetic acid to the volumetric flasks and dilute to 25.00 mL. These standard solutions are stable for approximately 6 months stored at 4°C.

Fortification Standard Solution - In most circumstances, the 1-µg/mL intermediate standard solution should be used for fortifications of samples analyzed by HPLC.

4.2.6 *Source of Samples*

Soil samples used to generate the recovery data in this report were from four states known for cotton production. These samples included soils from Madera, California; Bolivar County, Mississippi; Tarboro, North Carolina; and Donna, Texas. Soils from these areas were characterized for percent organic matter, sand, silt, and clay. The pH and texture of these soils were also determined. Typical physical properties are listed in the following table.

Origin	Tarboro, NC	Bolivar County, MS	Donna, TX	Madera, CA
Depth (feet)	0-0.5	0.5 - 1	—	0-0.5
pH	5.4-6.4	—	7.8	5.6
% Organic Matter	0.6-1.2	0.3-0.9	1.4	0.7
% Sand	88-92	48-72	47.2	76.0
% Silt	4-8	22-40	24	19.3
% Clay	4	6-12	28.8	4.7
Texture	Sand	Sandy Loam	Sandy Clay	Loamy Sand

4.2.7 *Storage and Preparation of Samples*

Soil samples should be received frozen, and should be sieved through a 1/4-inch screen to remove stones and plant debris. Samples may be composited and homogenized using a Hobart chopper or a ball mill. After homogenization, the soil samples are immediately returned to the freezer for storage until they are ready to be prepared for analysis.

4.2.8 *Sample Fortification Procedure*

Generally, fortified soil samples are prepared using the 1.0-µg/mL fortification standard solution. A syringe is used to add either 10, 20, or 50 µL of the intermediate standard solution to the soil and silica mixture, resulting in fortification levels of 1.0, 2.0, and 5.0-µg pyrithiobac sodium/kg soil (ppb), respectively. After fortification, the fortified soil should remain at room temperature for approximately 10 min.

Note: Soil should be fortified before mixing with silica for the extraction step described in the next section.

To test the linearity of this method over the range of pyrithiobac concentrations expected in field samples, the 100 µg/mL pyrithiobac stock standard was also used to fortify soils. From the stock standard, 5-, 10-, 50-, 100-, and 500-µg/mL volumes of

100-µg/mL pyrithiobac in methanol were added by syringe to 10 g of soil for 50-, 100-, 500-, 1000-, and 5000-µg/kg (ppb) fortification levels, respectively.

4.2.9 *Analyte Extraction Procedure*

Before extraction, weigh 10 g (±0.01 g) of soil into a 50-mL plastic centrifuge tube. Weigh 7 g of silica gel into the centrifuge tube and thoroughly mix the soil and silica by shaking. Use a clean spatula to break up soil clumps if necessary. The soil/silica matrix should be homogeneous.

Before an extraction, check the white O-rings installed in the exterior end of each extractor cell cap and in the ends of the rinse tubes. These O-rings should be pressed into place or replaced as needed.

Before loading an extraction cell, the PEEK seals for the cell should be checked to avoid leaks during an extraction. Worn PEEK seals are discolored and often have deep grooving on the surface. Replace worn PEEK seals before extraction.

Prepare to load the extraction cell by placing a new cellulose filter in the bottom of the cell on the stainless steel frit.

Transfer the sample to a 22-mL ASE™ extraction cell.

The loaded cell is extracted using the following conditions on the ASE™ extractor:

Heat Step:	5 min
Static Step:	10 min
Solvent Flush:	40%
Nitrogen Purge:	60 seconds
Extraction Temperature:	100°C
Extraction Pressure:	2000 psi
Extraction Solvent:	Milli-Q® water
Extraction Cycles:	1

A solvent rinse of the ASE™ extractor lines was performed between each extraction. The extract is collected in a capped, 60-mL vial. The extract is stable for at least three days at room temperature.

Although silica homogeneously mixed with soil should prevent cell plugging during subcritical water extraction, cell plugging may occur. Therefore, after a sample set has been extracted, each extraction cell should be opened and examined for evidence of plugging. If it is obvious after inspection that water covers the surface of the silica/soil matrix, the cell probably plugged during the extraction. (It is normal that a small amount of water remains adsorbed to the silica and soil after the nitrogen purge.) Extracts from plugged cells should not be cleaned-up and analyzed. Another ten grams of soil should be extracted for these samples using the above listed procedure with one modification: more silica should be added to the soil and mixed.

Caution: Extraction cells are extremely hot (100°C) after the extraction. Allow the cells to cool for at least 15 minutes before handling.

4.2.10 *Analyte Purification Procedure*

Each extract was subjected to purification using a disposable 2-g ENVI-Carb cartridge.

To prepare the ENVI-Carb extraction cartridge, weigh 2 g of ENVI-Carb packing into a 25-mL reservoir. Use the antistatic gun to prevent static charges during the weighing process. Leave two frits in the bottom of the reservoir. Add the packing on top of them. After adding the packing to the reservoir, add a 20-µm frit on top of the packing.

1. Wash the ENVI-Carb tube with one 10-mL aliquot of 0.10 M formic acid in 90% dichloromethane (DCM)/10% methanol (MeOH). Pull air through the tube for 15 minutes to dry. Wash the tube with 25 mL of 0.1 M HCl. Pull air through the tube for 2-3 seconds after the HCl has passed through the packing.
2. Add 10 mL of Milli-Q® water to the ENVI-Carb tube and pull through the packing until 1-2 mL of water remain above the top frit.
3. Add extract from the subcritical water extraction to the column. Pull the sample through the ENVI-Carb tube at a flow rate of 3-5 mL/min. Once all of the extract has been added to the column, rinse the collection vial with two 2-3 mL aliquots of Milli-Q® water and add to the column. Pull the final amount of sample through the packing until the first air bubble appears below the packing, then stop the flow.
4. Wash the ENVI-Carb tube packing with 15 mL of 0.01 M ammonium carbonate, pulling air through the packing for 2 minutes after the wash solution passes through the ENVI-Carb tube. Wash the ENVI-Carb tube with 2 mL of MeOH and pull air through the packing for 15 minutes to dry.
5. Wash the ENVI-Carb tube packing with 10 mL of 90% DCM/10% MeOH. Do not allow the ENVI-Carb cartridges to air dry under vacuum after adding the 10 mL of 90% DCM/10% MeOH. Just allow the solvent to pass through and stop the flow.
6. Elute pyrithiobac sodium from the ENVI-Carb tube with 25 mL of 0.10 M formic acid in 90% DCM/10% MeOH at a flow rate of 3-5 mL/minute, collecting the solution that passes through in a 50-mL plastic centrifuge tube.
7. Evaporate the DCM, MeOH, and formic acid solution to dryness using an N-Evap with the water bath at 40°C. The sample may be stored for at least two weeks if stored in a refrigerator at approximately 4°C.
8. Add 20% acetonitrile/80% 0.1 M formic acetic acid to a final volume of 1.0 mL. Vortex mix for approximately 10 seconds, making sure that the solution vortexes the lower one-third of the vial side. Sonicate the sample for 3 minutes, and vortex mix for 10 seconds. Filter the sample through a 4-mm diameter, 0.45-µm pore

syringe filter. Samples are stable for at least five weeks if stored in a refrigerator at approximately 4°C.

9. Analyze by column-switching LC/UV as described in the next section.

4.3 LC/UV Instrumentation

4.3.1 Description

Method validation data reported in this study were generated using the instrumentation described in Section 2.1 of this report. The high-pressure mixing HPLC system used for this work generated reproducible retention times for the column-switching routine that was used. However, low-pressure mixing systems using proportioning valves may require premixed solvents. If retention times shift or if the baseline fluctuates or is irregular during the gradient, solvent premixing may be required.

Isocratic, multi-dimensional HPLC was used with the columns listed in the Equipment section of this report. (For a review of multi-dimensional, column-switching HPLC, see References 1 and 2.) A diagram of the column switching valve arrangement is shown in Figure 2, where Column I and Column II are Zorbax® SB-CN and Zorbax® SB-C18 analytical columns, respectively. The column-switching routine used and a description of how the switching valve was connected to the HPLC and activated are described in Tables 1 and 2.

With the valve in Position 1, the effluent from Column I leaves the column through the valve, enters a bypass loop, flows back through the valve, and then flows to the detector. With the valve in Position 2, the effluent from Column I goes (via the valve) to Column II, back to the valve, and then to the detector. To obtain the data in this report, all tubing connecting the switching valve to the analytical columns and detector was 0.010-inch internal diameter tubing made as short as possible to minimize dead volume. *If smaller internal diameter tubing is used, the resulting back pressure developed when both columns are in series may be too great for the LC system.*

Before injection, the valve is put in Position 1, so that the HPLC flow bypasses Column II. Pump 28% ACN/72% 0.1 M acetic acid at 1.0 mL/min through Column I only. Just before pyrithiobac starts to elute from Column I, the valve is switched to Position 2 in order to trap the peak on Column II. After the pyrithiobac peak is collected at the head of Column II (after 1 min), the valve is switched back to Position 1.

Preparing for Analysis

If new analytical columns are used or if columns have not been used for a day or more and have been stored in ACN, MeOH, or a mixture of water with these organic solvents, they should be conditioned.

To condition the columns, position the switching-valve to join the columns in series. Pump 100% ACN through both columns at 1 mL/min. Monitor the baseline during this process. After achieving a stable baseline, set the columns in the mobile phases that are used for the analysis by doing the following. Pump 48% acetonitrile/52% 0.1 M acetic acid through both columns for 30 min at 1 mL/min. At the end of this step, position the switching valve to Position 1 and condition the SB-CN column with 28% acetonitrile/72% 0.1 M acetic acid for 5 min at 2 mL/min.

After conditioning the columns, the autosampler should be purged with 28% acetonitrile/72% 0.1 M acetic acid.

Setting the Time Window

The valve switching times (the "time window") are set at ± 0.50 minutes around the average retention time for three injections of pyrithiobac standards eluting from **Column I only**. (See the following section for the operating conditions.) The time window is determined immediately before the sample analysis run is started. The retention time (through Column I) percent relative standard deviation (% RSD = 100.* Std. Dev./Avg.) for the standards injected should be no greater than 0.4%.

The time for a significant baseline deflection after injection for the 15 cm SB-CN column used with 28% ACN/72% 0.1 M acetic acid at 1.0 mL/min at 40°C was typically two minutes. Note that this time is dependent on the dwell time of a specific HPLC. The HPLC system that generated the data for this report had a dwell time of 4.5 min (dwell time is defined in Reference 3).

Pyrithiobac typically eluted at approximately 11 min from the SB-CN column. Pyrithiobac peaks eluting from the SB-CN column were approximately one-minute wide at the base of the peak. The intent of the column switch is to transfer the entire pyrithiobac peak from Column I to Column II; the time window must accommodate this intent.

To assure the time window is adequate, the average retention time of pyrithiobac should be determined on the SB-CN column before starting the analysis of a sample set. Approximately 30 runs (including standards) can be made before reevaluating the average retention time of pyrithiobac on Column I. The retention time of pyrithiobac should be reevaluated because retention on the column may change slightly after injecting many soil samples.

The mobile phase used to determine the average retention time of the standards is 28% ACN/72% 0.1 M acetic acid at a flow rate of 1 mL/min. Using this mobile phase composition, typical pyrithiobac peak widths for standards injected are normally one minute, depending on the SB-CN column used and the pyrithiobac retention time on the SB-CN column. The pyrithiobac standards had a capacity factor of about 4.5 (k' ≈ 4.4) using the above stated conditions. ***Note that the column temperature must be maintained at 40°C throughout each chromatographic analysis.***

4.3.2 *Operating Conditions*

The following conditions are used to separate pyrithiobac from co-extracted compounds (see Figure 2 and Tables 1 and 2). A sample is injected into Column I. The initial mobile phase concentration is 28% ACN/72% 0.1 M acetic acid at a flow rate of 1 mL/min. At the beginning of the determined time window (the time window is typically about 10.5 to 11.5 min from the point of injection), the valve is switched from Position 1 to Position 2 and pyrithiobac is transferred to Column II. At the end of the time window, the valve is switched from Position 2 to Position 1. The intent of this column switch is to transfer the entire pyrithiobac peak from Column I to Column II.

After pyrithiobac is trapped on Column II and the valve is switched back to Position 1, the mobile phase is changed from 28% ACN/72% 0.1 M acetic acid to 80% ACN/20% 0.1 M acetic acid, and the flow rate is increased from 1 to 2.0 mL/min, to quickly clean off Column I (a 5 min wash). After cleaning Column I, the column is conditioned 10 min with 48% ACN/52% 0.1 M acetic acid at 2.0 mL/min (through Column I only). Column I is then reequilibrated at 1.0 mL/min for 1 min using this mobile phase composition. Following these steps, Column I is in the correct mobile phase to complete the analytical separation on Column II.

After setting Column I at Column II conditions, the valve is switched to Position 2 to elute pyrithiobac from Column II using the 48% ACN/52% 0.1 M acetic acid mobile phase. Pyrithiobac elutes from Column II at a retention time of about 32 min from the start of the run. After pyrithiobac elutes from Column II, the valve is switched to Position 1 and 28% ACN/72% 0.1 M acetic acid is passed through Column I only at 2 mL/min for 5 min. The flow rate is reduced to 1 mL/min and the system is allowed to run for another one minute. At this time, Column I and Column II are both ready for the next injection. A typical chromatogram of a 100-ng/mL pyrithiobac standard showing the events of the analysis from injection to the end of the separation is shown in Figure 3.

Common conditions for the LC/UV method are shown in the following table:

Wavelength	254 nm
Column Temp.	40.0°C
Injection Volume	0.100 mL
Mobile Phase A	100% ACN
Mobile Phase B	100% 0.1 M acetic acid
Mobile Phase C	100% Milli-Q® water

4.3.3 *Calibration Procedures*

For the data in this report, the external standard calibration technique was used to quantitate the amount of pyrithiobac sodium in soil samples. A calibration curve was generated by plotting the response of the UV detector (254 nm) in peak height versus the concentration of pyrithiobac sodium standards that were injected. A correlation

coefficient for each plot was determined. A typical calibration curve is shown in Figure 4.

4.3.4 *Sample Analysis*

Each set of samples analyzed for investigation purposes should include at least one unfortified sample (a sample which matches the investigation samples as closely as possible, preferably from an untreated plot). Soil, preferably from an untreated plot, should be fortified with the pyrithiobac at a known level, and carried through the procedure to verify recovery.

For the analysis, a standard should be injected at the beginning and end of an automated sequence, and after every two to three samples. Standards and fortifications should be injected in order of increasing concentration. If analysis is delayed, samples should be stored refrigerated or frozen until analysis. Extracted and cleaned-up samples should be stable for at least two weeks if kept refrigerated, and for at least five weeks if kept frozen.

Samples having detector responses for pyrithiobac sodium greater than the highest accompanying standard should be diluted to fall within the range of standards and reanalyzed.

Sample analysis should be done as outlined above. Selected samples may be analyzed by LC/MS to confirm the presence or absence of pyrithiobac in soil samples. Please see the discussion of the LC/MS confirmatory method in Section 4.4.3 of this report.

4.4 Calculations

4.4.1 *Methods*

Quantitation of the amount of pyrithiobac sodium found in extracted soils was done by using external standards. Known pyrithiobac concentrations (ng/mL) and responses (in peak height or area) from these standards were used to generate a linear least squares fit. The equation for the best fit is $y = mx + b$, where y is the peak height or area, x is the amount of pyrithiobac found in ng/mL, m is the slope of the line, and b is the y axis (ordinate) intercept. The solution to the equation for this line gives the concentration of pyrithiobac found in ng/mL as a function of the peak height or area:

$$\text{Concentration found, ng / mL} = x = (y - b) / m$$

The following calculation was used to determine the ppb pyrithiobac sodium found for each control and treated sample:

$$\text{ppb Found} = \frac{\text{(Concentration found, ng / mL)(Final volume, mL)(Dilution factor)}}{\text{Sample weight, g}}$$

The following equation was used to calculate the fortification level in ppb:

$$\text{Fortification level, ppb} = 1000\left(\frac{(\text{Volume of standard, mL})(\text{Concentration of standard, } \mu\text{g/mL})}{\text{Sample weight, g}}\right)$$

The following equation was used to calculate percent recovery for fortified samples:

$$\%\ \text{Recovery} = 100\left(\frac{\text{ppb Found}}{\text{Fortification level, ppb}}\right)$$

4.4.2 *Examples*

For a 1.0-ppb fortified soil sample (Spike 2 of Data Sheet Number 5 in Appendix III), the concentration found was 1.1×10^1 ng/mL (rounded to two significant figures). The ppb found was calculated as follows:

$$\text{ppb found} = \frac{(1.1 \times 10^1\ \text{ng/mL})(1.0\ \text{mL})(1)}{10.0\ \text{g}} = 1.1\ \text{ppb}$$

(ppb values are rounded to two significant figures in Table 3 of this report)

For this sample, the percent recovery found was calculated as follows:

$$\%\ \text{Recovery} = 100\left(\frac{1.1\ \text{ppb}}{1.0\ \text{ppb}}\right) = 111$$

(percent recoveries are rounded to the nearest whole number in Table 3 of this report, without rounding the concentration or ppb found)

5.3 *Modifications or Special Precautions*

Low pressure mixing LC instruments that use a proportioning valve to mix solvents may not be adequate for this method. Therefore, the mobile phases may need to be premixed. The need for premixing is determined by unstable retention times, or baseline fluctuations during the gradient. Mobile phases should be degassed, particularly when low pressure mixing systems are used.

Do not use a cyano guard column. Column-to-column reproducibility for pyrithiobac sodium has been found to be unacceptable. A Zorbax® cyano guard column usually increases peak width and generates poor peak shape for pyrithiobac sodium.

5.4 ***Method Ruggedness***

5.4.1 *Stability and Ruggedness Testing*

The stability of pyrithiobac sodium in standards and extracts has been stated in the respective sections of this report. The stability of reagents used in this method have also been stated.

Several variables were explored to establish the ruggedness of this method from sample extraction through column-switching LC/UV analysis. A variety of soil types were extracted and purified by multiple analysts.

Several soil textures were successfully extracted using the ASE™ 200: sand; sandy clay; sandy loam; silt loam; loamy sand; and loam soils. Soils having up to 78% silt were extracted without plugging by mixing the soil with silica gel. Soils having up to 21% clay were also successfully extracted using this method. All soils tested were successfully carried through this procedure.

Soils were mixed with silica gel as explained in this method and carried through the extraction. Addition of silica is important to prevent plugging of the extraction cell which would otherwise occur. The most likely cause of the plugging is the silt being compacted in the extractor.

Ionic strength in the extracts from different soils using subcritical water extraction varies. ENVI-Carb is an ion-exchange packing used in the clean-up step for this method that could be overloaded at specific ionic strengths causing the method to fail. Two grams of ENVI-Carb packing are more than adequate to accommodate this ionic strength variability.

The time window for the column-switching LC/UV analysis in this method is one minute. This window is wide enough to allow variability in pyrithiobac sodium's retention time. Approximately 30 samples, including standards, can be analyzed before a new time window should be established.

5.4.2 *Specificity/Potential Interference*

Due to the selective nature of the subcritical water extraction, ion-exchange clean-up using graphitized carbon, and column-switching liquid chromatography, interference in this method is less than the MDL at the retention time of pyrithiobac.

If interference in an unfortified control is suspect, the confirmatory LC/MS method discussed in the following section may be performed. The confirmatory method significantly reduces interference potential due to the mass selective nature of the detector.

5.4.3 *LC/MS Confirmatory Methods*

Liquid chromatography interfaced with mass spectrometry (LC/MS) employing both thermospray (TSP) and electrospray (ESI) modes of ionization on a single quadrupole instrument were successfully used for analysis of pyrithiobac sodium residues in soil. TSP-LC/MS was originally employed since it was an established technique for analysis of pyrithiobac in water (Reference 8). Conditions for analysis using ESI-LC/MS were developed due to the increased popularity and availability of instruments designed with electrospray ionization. Standard solutions and sample extracts are prepared as described for LC/UV analysis.

Details of the procedures for the analysis of pyrithiobac sodium in soil are contained in Appendix 4. For either approach, the instrument was operated using selected ion monitoring (SIM) for ions of mass/charge ratios (m/z) of 327 and 329 with a 0.6 amu window and the instrument in positive ion mode. Selection of these ions was based upon the mass spectrum generated during the method development process with the instrument in scanning mode. The spectrum generated by ESI-LC/MS for pyrithiobac is shown in Figure 8. TSP-LC/MS also yielded m/z 327 as the base peak. The spectrum generated by TSP-LC/MS is shown in Reference 8. The ions selected are those resulting from protonation of the acid of pyrithiobac sodium. The ratio of ion abundance for 329/327 is characteristic of a molecule containing one chlorine atom and can be used to confirm the identity of a peak eluting at the pyrithiobac retention time.

Chromatography and mass spectrometry conditions for TSP analysis are similar to those contained in Reference 8, are contained in Appendix 4 and summarized below.

TSP-LC/MS HPLC Conditions:

Column:	4.6 mm x 25 cm, Zorbax® SB-C18 analytical column with 5-µm diameter packing
Column Temperature:	50.0°C
Injection Volume:	0.050 mL
Flow Rate:	0.9 mL/min
Mobile Phase:	48% acetonitrile/52% 0.1 M acetic acid
Post-column Addition Flow: Composition:	 0.2 mL/min. 0.5 M ammonium acetate

Pyrithiobac has a retention time of approximately 9 minutes (t_0 Å 2.5 min). The total run time for one sample is 20 minutes. The HPLC column should be conditioned daily with 90% acetonitrile/10% Milli-Q® water to clean the column and reequilibrated with the mobile phase before analysis.

TSP-LC/MS Mass Spectrometer Conditions:

Ionization Mode:	filament off; discharge off
Ions Monitored:	m/z 326.9 ± 0.3 amu m/z 328.9 ± 0.3 amu
Scan Length	2 seconds
Electrospray Voltage:	3.9 kV
Electron Multiplier Voltage:	1400-3000 V, established daily
Temperatures:	probe: 85-100°C, established daily source: 200°C manifold: 70°C

Optimal chromatographic conditions for ESI-LC/MS differ from those for TSP analysis. HPLC and MS conditions for ESI-LC/MS are summarized below.

ESI-LC/MS HPLC Conditions:

Column:	3.0 mm i.d. x 25 cm, Zorbax® SB-C18 analytical column with 5-µm diameter packing
Column Temperature:	50.0°C
Injection Volume:	0.100 mL
Flow Rate:	0.4 mL/min
Mobile Phase:	48% acetonitrile/52% 0.1 M acetic acid

The retention time of pyrithiobac sodium is approximately 9.5 minutes; the total run time is 14 minutes (where the t_0 Å 2.5 minutes). The HPLC column should be conditioned daily with 90% acetonitrile/10% Milli-Q® water to clean the column and reequilibrated with the mobile phase before analysis.

ESI-LC/MS Mass Spectrometer Conditions:

Ions Monitored:	m/z 327.0 ± 0.3 amu m/z 329.0 ± 0.3 amu
Scan Length:	2 seconds
Electrospray Voltage:	3.9 kV
Electron Multiplier Voltage:	1840.V, established daily
Temperatures:	capillary heater: 200°C manifold: 70°C
Sheath Pressure:	60 psig

Since the electrospray interface is optimal at low flow rates, the HPLC flow is split post-column such that only 90 µL/min actually passes through the interface (~4.44:1 split); the remainder going to waste.

Quantitation for both LC/MS methods is from linear regression of peak areas for external standards. Calculations detailed for the column-switching LC/UV method apply (see Section 4.4). Typical calibration curves for thermospray and electrospray LC/MS methods are shown in Figures 9 and 10, respectively. Although the linear dynamic range for MS detection was not as great as for UV, adequate linearity was displayed over the range of 5 ng/mL to 100 ng/mL pyrithiobac sodium;

The MDL for pyrithiobac sodium in soil by LC/MS was estimated to be 0.4 ppb using the same evaluation technique as used for LC/UV data (see Section 5.1.5). Estimated MDL values should be determined by each lab using this method. In the case of LC/MS, the MDL might need to be routinely assessed if responses change significantly from day to day.

MS detection is inherently more difficult and less stable than UV detection. MS detection requires skilled operation of the mass spectrometer. Day-to-day and run-to-run variation in instrument performance can complicate instrument settings and create variable method detection limits. For these reasons, LC/MS analysis should be reserved for those cases where confirmation of LC/UV results is desired or matrix interference is present. The mass spectrometer is a very selective detector, and monitoring two ions of the analyte at pyrithiobac's retention time provides positive identification.

TABLE 1
TYPICAL COLUMN-SWITCHING TIMING SEQUENCE FOR SWITCHING VALVE

#	Time (min.)	Event	Function	Explanation
1	0.00	Event 3	On	Start run through Column I only
2	0.00	Event 4	Off	
3	10.49	Event 4	On	Start column switch; pyrithiobac is transferred
4	10.49	Event 3	Off	
5	11.49	Event 3	On	End column switch; Clean Column I
6	11.49	Event 4	Off	
7	23.00	Event 4	On	Start analytical separation on Column II
8	23.00	Event 3	Off	
9	35.90	Event 3	On	Set Column I to initial conditions
10	35.90	Event 4	Off	

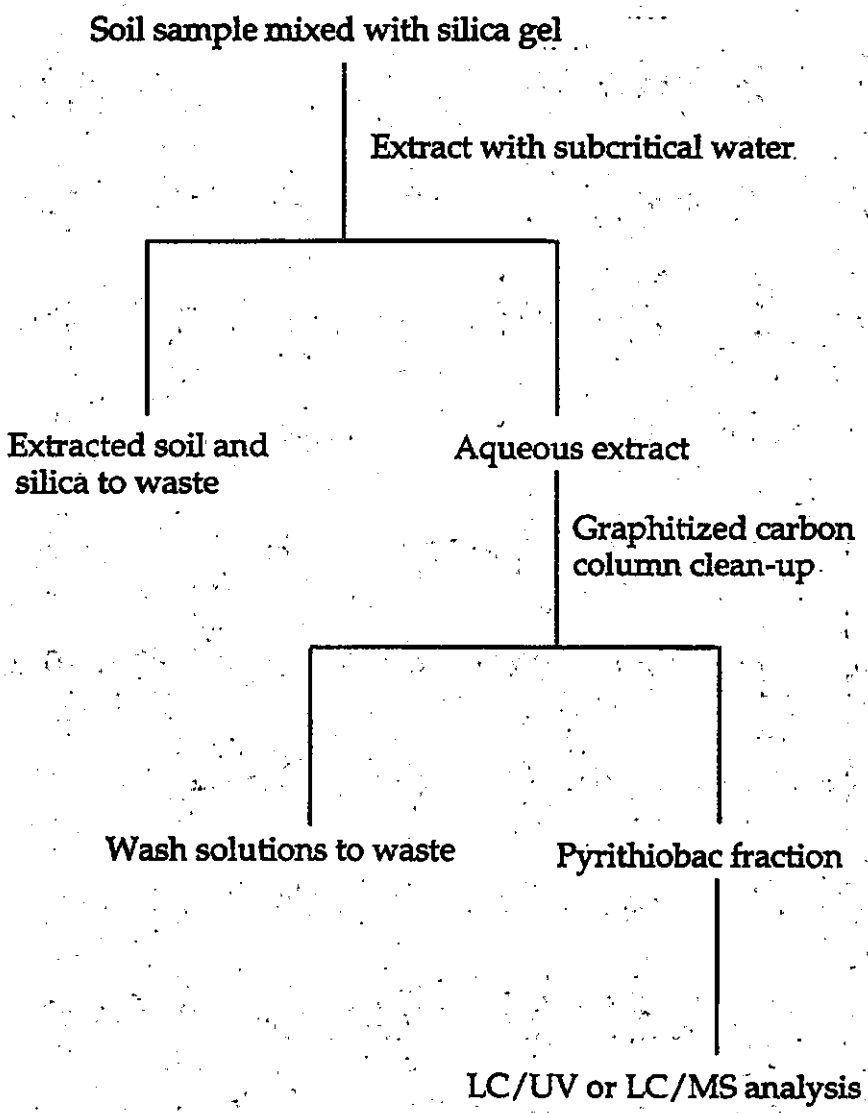
The Waters pump control module has four external contact closure (TTL to GND) events that are activated using the Millennium 2010 software. The values of Event 3 and Event 4 (on and off times) control the Valco column switching valve: Event 3 off, Event 4 on = valve in Position 1; Event 3 on, Event 4 off = valve in Position 2. The Valco valve wiring is hooked up in the following way to the pump control module: red coated wire to Event 3, black-coated wire to Event 4, and green-coated wire to a Waters 12 V power supply negative position. If both events are turned on at the same time, the valve continues to rotate; therefore, flow through the system stops.

TABLE 2
TYPICAL TIMES AND VALUES OF MOBILE PHASE MIXING AND FLOW RATE USING THE WATERS PUMP CONTROL MODULE

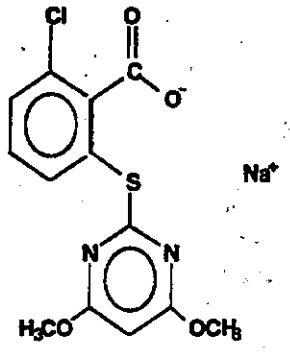
#	Time (min)	Flow (mL/min)	%A	%B	%C	Curve Type	Explanation
1	0.00	1.00	28.0	72.0	0.0	0	Start analysis on Column I only
2	13.00	2.00	80.0	20.0	0.0	11	Clean off Column I
3	17.00	2.00	48.0	52.0	0.0	11	Set Column I to Column II cond.
4	22.00	1.00	48.0	52.0	0.0	11	Set proper flow rate for analysis
5	36.00	2.00	28.0	72.0	0.0	11	Set Column I at initial cond.
6	45.00	1.00	28.0	72.0	0.0	11	Set at initial flow rate

Curve Type 0 on the Waters HPLC system is the starting condition for the analysis. Curve Type 11 on the Waters HPLC system is a step gradient that begins at the specified time. Mobile phases A, B, and C are 100% ACN and 100% 0.1 M acetic acid and Milli-Q® water, respectively.

FIGURE 1
FLOW DIAGRAM OF THE ANALYTICAL METHOD FOR THE DETERMINATION OF PYRITHIOBAC SODIUM EXTRACTED FROM SOIL



APPENDIX 1
STRUCTURE AND PHYSICOCHEMICAL PROPERTIES OF PYRITHIOBAC SODIUM



DPX-PE350

Bates (see Reference 4) has determined the following physico-chemical properties for DPX-PE350:

Melting Point:	233.8-234.2°C
Solubility:	
Water	728 g/L
Methanol	270 g/L
Acetone	812 mg/L
Acetonitrile:	347 mg/L
Partition Coefficient, n-octanol/pH 7 water:	0.14
Dissociation constant, pKa	2.34

APPENDIX 4
LC/MS CONFIRMATORY METHODS

TSP-LC/MS Method for Pyrithiobac Sodium in Soil
ESI-LC/MS Method for Pyrithiobac Sodium in Soil

TSP-LC/MS METHOD FOR PYRITHIOBAC SODIUM IN SOIL

1.0 INTRODUCTION

Liquid chromatography interfaced with mass spectrometry (LC/MS) employing thermospray (TSP) ionization on a single quadrupole instrument is described for the quantitative analysis of pyrithiobac sodium residues in soil at levels down to 1 ppb. This method was a natural extension of a previously established TSP-LC/MS method for analysis of the same active ingredient in water (Reference 8). Standard solutions and soil extracts are prepared as described for column-switching LC/UV analysis within the body of this report.

The instrument was operated using selected ion monitoring (SIM) for ions of mass/charge ratios (m/z) of 327 and 329 with a 0.6 amu window and the instrument in positive ion mode. The ion selection was based upon the mass spectrum generated during the method development process with the instrument in scanning mode. The spectrum generated by TSP-LC/MS yielded m/z 327 as the base peak with m/z 329 at approximately 30% abundance; pyrithiobac's spectrum is shown in Reference 8. The ions selected are those resulting from protonation of the acid of pyrithiobac sodium. The ratio of ion abundance for 329/327 is characteristic of a molecule containing one chlorine atom and can be used to confirm the identity of a peak eluting at the pyrithiobac retention time.

2.0 EQUIPMENT AND REAGENTS

2.1 Equipment

Equivalent equipment may be substituted unless otherwise indicated. Note any specification in the following descriptions before making substitutions. Substitutions should be made only if equivalency/suitability has been verified with acceptable control and fortification recovery data.

HPLC system - Minimum requirements for the HPLC system include an autosampler, column oven, a pumping system capable of mixing three solvents with a minimum of pulsing, a pulse-dampened pump for post-column addition, and a high-pressure switching valve to allow the HPLC effluent to be directed to the MS or to waste (the latter is included with the TSP interface accompanying the MS system below). Low-volume pump heads on low-pressure mixing systems with pulse-dampening or high-pressure mixing systems generally will produce the desired level of performance.

- Waters Model 616 HPLC pump module (Waters Corp., Milford, Mass.)
- Waters Model 717 autosampler equipped with a 250-µL syringe, temperature control module and column heater (Waters Corp.)

- Post column addition pump: Kratos/ABI Spectroflow model 400 HPLC pump (Bodman Industries, Aston, PA) with SSI model LP-21 pulse dampener #20-0218 (Rainin Instrument Co., Inc., Woburn, Mass.)
- Low dead-volume in-line solvent filters: 1.5 mm i.d., 0.5-µm filter, # 7315-010; 3.0 mm i.d., 0.5-µm filter, #7335-010 (Rainin Instrument Co., Inc.). Note that the low dead volume in-line solvent filter should be used to prevent post-column band broadening; a larger internal diameter pre-column filter was used immediately following the post-column addition pump.
- HPLC Column: 4.6 mm x 250 mm Zorbax® SB-C18, 5-µm particles, #880975-902 (Mac-Mod Analytical, Inc., Chadds Ford, Penn.). **Do not substitute.**

MS System - Minimum requirements are a single stage quadrupole instrument with a thermospray source/interface. Vendor software provides control of both the MS and the HPLC systems.

- Finnigan model SSQ7000 single-stage quadrupole MS with thermospray (TSP2) source/interface (Finnigan MAT, San Jose, Calif.)
- 104°C refrigerated vapor trap, #RVT4014, Cryocool liquid #SCC1 (Savant Instruments, Inc., Farmingdale, N.Y.) and 4-L glass vessel adapted for use with Finnigan TSP exhaust system and Savant vapor trap

Mobile Phase Filtration Apparatus - 0.45-µm pore, 47 mm diameter, Type HA filters, #HATF 047 00 with vacuum filter apparatus consisting of a glass filter holder, #XX1004700, a ground glass base with stopper, #XX1004702, a funnel cover, #XX2504754, and a 1-L filter flask, #XX1004705 (Millipore Corp.)

2.2 *Reagents*

Equivalent reagents may be substituted for those listed below. To determine if substituted reagent impurities interfere with pyrithiobac, appropriate amounts of the solvents should be injected into the HPLC using the chromatographic conditions specified in this appendix.

Water - Deionized water passed through a Milli-Q® UV Plus water purification system #ZD60 115 UV (Millipore Corp.)

Acetonitrile (ACN) - EM Omni Solv®, HPLC-grade acetonitrile, #AX0142-1 (EM Science, Gibbstown, N.J.)

Acetic Acid - Baker Analyzed glacial acetic acid, #9524-00 (J. T. Baker, Inc., Phillipsburg, N.J.)

Ammonium Acetate ($CH_3CO_2NH_4$) - Baker Analyzed Reagent®, reagent-grade ammonium acetate #0559-08 (J. T. Baker, Inc.)

Pyrithiobac Sodium (DPX-PE350, KIH-2031) - Reference substance used for HPLC analysis: analytical standard grade DPX-PE350, Lot #4, 98.7% pure (prepared by Kumiai/Ihara Chemical Co. for DuPont Agricultural Products, Global Technology Division, E. I. du Pont de Nemours and Company)

3.0 METHODS

3.1 *Glassware and Equipment Cleaning*

Glassware and extraction cells should be scrubbed by brush with a soap solution, rinsed two to five times with water, and rinsed with acetone or other suitable solvents. Distilled or deionized water may be added to the rinse sequence. Glassware is air-dried.

3.2 *Preparation of Solutions*

0.10 M Acetic Acid - Pipet 2.85 mL of glacial acetic acid into 1-L graduated cylinder and bring to 1-L final volume with Milli-Q® water. Prepare weekly.

HPLC Eluents - Eluent A: 100% acetonitrile; Eluent B: 100% 0.10 M acetic acid, Eluent C: 100% Milli-Q® water. Mobile phases should be thoroughly degassed daily; this is accomplished with the Waters system described here by sparging with helium. Components may be premixed at a ratio of 48% Eluent A and 52% Eluent B for use through a single pump channel, but then helium sparging should be minimized to avoid altering the mobile phase composition. Replace aqueous eluents weekly.

0.5 M ammonium acetate - Dissolve 19.27 g ammonium acetate in approximately 400 mL of Milli-Q® water. Use a 500-mL graduated cylinder and bring to 500-mL final volume with Milli-Q® water. Filter through a 0.45-µm type HV filter. Prepare weekly.

3.3 *Preparation and Stability of Standard Solutions*

Standard solutions are prepared as detailed in the body of this report. They are stored refrigerated if LC/MS analysis is to be delayed.

3.4 *Preparation of Sample Extracts*

Samples are extracted as for LC/UV analysis following the procedures detailed in the body of this report. Samples are stable for at least two weeks if stored refrigerated.

3.5 *Fortification of Samples*

Fortifications of soil with pyrithiobac sodium are performed following the procedures detailed in the body of this report.

3.6 *Chromatography*

Minimum requirements of the HPLC system are described in the Equipment section above. For thermospray ionization, the chromatographic system and the post-column addition pump used for ammonium acetate introduction should be designed to minimize pressure pulsing by the pumps, as pressure pulsing increases baseline noise in the mass spectrometer. Low dead-volume 0.5-µm filters are placed in-line following the LC and the post-column addition pump to reduce the chance of particulates (from pump seals, for example) entering the thermospray probe of the MS. Chromatography conditions for TSP-LC/MS analysis are the same as those

developed for analysis of pyrithiobac sodium in water (Reference 8); this is an isocratic reversed-phase analysis on a C18 column designed for use with low-pH mobile phases. Conditions used for analysis are summarized below.

HPLC Conditions:

Column:	4.6 mm x 25 cm, Zorbax® SB-C18 analytical column with 5-µm diameter packing
Column Temperature:	50.0°C
Injection Volume:	0.050 mL
Flow Rate:	0.9 mL/min
Mobile Phase :	48% acetonitrile/52% 0.1 M acetic acid
Post-column Addition Flow: Composition:	 0.2 mL/min. 0.5 M ammonium acetate

Pyrithiobac had a retention time of approximately 9 minutes (t_0 Å 2.5 min). The total run time for one sample was 20 minutes. The HPLC column should be conditioned daily with 90% acetonitrile/10% Milli-Q® water to clean the column and reequilibrated with the mobile phase before analysis. Use of a guard column is optional; if used, retention times will be slightly longer but will require no change in operating parameters.

A UV detector set at 254 nm may be included in the LC/MS system (either substituted for the MS detector or placed in-line preceding the MS) in order to monitor HPLC performance. The 0.0100-µg/mL pyrithiobac sodium standard specified in this method should produce a significant response (approximately 20:1 signal-to-noise), allowing evaluation of retention time and peak shape. If monitoring is desired, a variable-UV rather than diode array detector is suggested to provide adequate sensitivity, and a high pressure flow cell is desired if the detector is in-line with the MS.

3.7 Mass Spectrometry

The minimum specifications for the MS system are described in the Equipment section above. Effluent from a post-column addition pump is combined with that from the HPLC by way of a stainless steel low-dead-volume mixing tee. Ammonium acetate is added post-column to provide a proton source for ionization of the sample in the mass spectrometer without affecting the chromatographic separation. The mass spectrometer has a high-pressure switching valve which permits the effluent from the HPLC and post-column addition pump to be diverted from the mass spectrometer to waste. The flow is diverted for approximately the first five minutes of each chromatographic run to avoid introducing unnecessary sample material to the MS. This still allows adequate time for the TSP-LC/MS system to equilibrate before the pyrithiobac peak elutes.

The conditions outlined below are representative of those used for the particular instrument upon which this method was developed and evaluated.

Mass Spectrometer Conditions:

Ionization Mode:	positive ionization – filament off; discharge off
Ions Monitored:	m/z 326.9 ± 0.3 amu m/z 328.9 ± 0.3 amu
Scan Length	2 seconds
Electrospray Voltage:	3.9 kV
Electron Multiplier Voltage:	1400-3000 V, established daily
Temperatures:	probe: 85-100°C, established daily source: 200°C manifold: 70°C

Many of the mass spectrometer conditions were unique for the particular instrument used and varied daily. MS conditions were established and the instrument tuned while directly infusing a pyrithiobac sodium solution of approximately 0.5 µg/mL in 52% 0.1M acetic acid/48% acetonitrile at 0.9 mL/min (bypassing the HPLC column). Ammonium acetate was introduced by the post-column addition pump at 0.2 mL/min. The instrument was tuned to optimize stability and sensitivity of the signal for ions of m/z 327 and 329 by adjusting lens, repeller, quad offset voltages and TSP probe temperature. Calibration at m/z 327 and 329 was checked, and the instrument recalibrated using standard procedures as needed. The electron multiplier voltage was adjusted such that the signal intensity was approximately 10^6 abundance.

A 0.005- or 0.010-µg/mL chromatographic standard should be analyzed prior to the start of analyses to more closely establish the appropriate electron multiplier voltage setting for the desired limits of quantitation and detection. For the system used in this method, the electron multiplier voltage was adjusted such that injection of a 0.010-µg/mL pyrithiobac sodium standard solution yielded a detected peak with an area of approximately 80,000 to 100,000 abundance. Operating parameters must be tailored to the particular instrument used, particularly if it is to be an alternate vendor's instrument, and should be checked daily.

3.8 Sample Analysis

A standard should be injected at the beginning and end of an analysis sequence and after every two to three samples. If analysis is delayed, samples should be stored refrigerated or frozen until analysis. Sample extracts should be stable for at least two weeks if refrigerated, and for at least five weeks if frozen.

3.9 *Calculations*

Quantitation is from linear regression of peak areas for external standards. Calculations detailed for the column-switching LC/UV method apply (see Section 3.4 of the report). Adequate linearity over the range of 0.005 µg/mL to 0.1 µg/mL pyrithiobac sodium with correlation coefficient (R^2) values of 0.97 or greater should be achievable.

4.2 *Modifications or Special Precautions*

The MS detector is extremely sensitive to pressure fluctuations caused by the HPLC system. Although the chromatography may be adequate for UV detection as evidenced by a stable baseline, periodic baseline fluctuations may appear on chromatograms from the MS. In general, the cause can be traced back to poor check valve function due to pump seal wear or gasses in the mobile phase. Maintaining the pumping system of the HPLC is critical to the performance of the LC/MS system.

The TSP source/interface relies on an exhaust pump with a cold trap to remove the bulk of the HPLC effluent introduced into the mass spectrometer. The efficiency of the pump and trap greatly affects the response of the MS system. The instrument used for this method development and evaluation employed a -104°C 4-L capacity cold trap, which is able to effectively maintain a stable pressure over the course of 16 to 20 hours of continuous operation. Use of a less effective trapping system (such as liquid nitrogen or dry ice/acetone) causes the pressure to change over time, and thus the instrument response varies. If this is the case, calculations must be based on response factors from bracketing standards in order to account for the degradation in response.

* Response Factor = RF = peak area ÷ chromatographic standard concentration

ESI-LC/MS METHOD FOR PYRITHIOBAC SODIUM IN SOIL

1.0 INTRODUCTION

LC/MS employing electrospray ionization (ESI) on a single quadrupole instrument is described for the quantitative analysis of pyrithiobac sodium residues in soil at levels down to 1 ppb. This method was developed to accommodate the popularity of atmospheric pressure ionization (API) instruments and their greater availability at contract and enforcement laboratories. Chromatography is similar to the thermospray (TSP) LC/MS method previously described, with the same ions monitored by the mass spectrometer. Similar sensitivity has been demonstrated. Standard solutions and soil extracts are prepared as described for column-switching LC/UV analysis within the body of this report.

The instrument was operated using selected ion monitoring (SIM) for ions of mass/charge ratios (m/z) of 327 and 329 with a 0.6 amu window and the instrument in positive ion mode. The ion selection was based upon the mass spectrum generated during the method development process with the instrument in scanning mode. The spectrum generated by ESI-LC/MS yielded m/z 327 as the base peak with m/z 329 at approximately 30% abundance; pyrithiobac's spectrum is shown in Figure 6 of this report. The ions selected are those resulting from protonation of the acid of pyrithiobac sodium. The ratio of ion abundance for 329/327 is characteristic of a molecule containing one chlorine atom and can be used to confirm the identity of a peak eluting at the pyrithiobac retention time.

2.0 EQUIPMENT AND REAGENTS

2.1 ***Equipment*** Equivalent equipment may be substituted unless otherwise indicated. Note any specification in the following descriptions before making substitutions. Substitutions should be made only if equivalency/suitability has been verified with acceptable control and fortification recovery data.

HPLC system - Minimum requirements for the HPLC system include an autosampler, column oven, and a pumping system capable of mixing three solvents with a minimum of pulsing low-volume pump heads on low-pressure mixing systems with pulse-dampening or high-pressure mixing systems generally will produce the desired level of performance.

- Waters model 616 HPLC pump module (Waters Corp., Milford, Mass.)
- Waters model 717 autosampler equipped with a 250-µL syringe, temperature control module and column heater (Waters Corp.)
- Low dead-volume in-line solvent filter: 1.5 mm i.d., 0.5-µm filter, # 7315-010, (Rainin Instrument Co., Inc.)

- HPLC Column: 3.0 mm ID x 250 mm Zorbax® SB-C18, 5-µm particles, #880975-302 (Mac-Mod Analytical, Inc., Chadds Ford, Penn.). **Do not substitute.**

MS System - Minimum requirements are a single stage quadrupole instrument with an electrospray source/interface. Vendor software provides control of both the MS and the HPLC systems.

- Finnigan model SSQ7000 single-stage quadrupole MS with API source/interface configured for ESI operation (Finnigan MAT, San Jose, Calif.)

2.2 Reagents

Equivalent reagents may be substituted for those listed below. To determine if substituted reagent impurities interfere with pyrithiobac, appropriate amounts of the solvents should be injected into the HPLC using the chromatographic conditions specified in this appendix.

Water - Deionized water passed through a Milli-Q® UV Plus water purification system #ZD60 115 UV (Millipore Corp.)

Acetonitrile (ACN) - EM Omni Solv®, HPLC-grade acetonitrile, #AX0142-1 (EM Science, Gibbstown, N.J.)

Acetic Acid - Baker Analyzed glacial acetic acid, #9524-00 (J. T. Baker, Inc., Phillipsburg, N.J.)

Pyrithiobac Sodium (DPX-PE350, KIH-2031) - Reference substance used for HPLC analysis: analytical standard grade DPX-PE350, Lot #4, 98.7% pure (prepared by Kumiai/Ihara Chemical Co. for DuPont Agricultural Products, Global Technology Division, E. I. du Pont de Nemours and Company)

3.0 METHODS

3.1 Glassware and Equipment Cleaning

Glassware and extraction cells should be scrubbed by brush with a soap solution, rinsed two to five times with water, and rinsed with acetone or other suitable solvents. Distilled or deionized water may be added to the rinse sequence. Glassware is air-dried.

3.2 Preparation of Solutions

0.10 M Acetic Acid - Pipet 2.85 mL of glacial acetic acid into 1-L graduated cylinder and bring to 1-L final volume with Milli-Q® water. Prepare weekly.

HPLC Eluents - Eluent A: 100% acetonitrile; Eluent B: 100% 0.10 M acetic acid, Eluent C: 100% Milli-Q® water. Mobile phases should be thoroughly degassed daily; this is accomplished with the Waters system described here by sparging with helium. Components may be premixed at a ratio of 48% Eluent A and 52% Eluent B

for use through a single pump channel, but then helium sparging should be minimized to avoid altering the mobile phase composition. Replace aqueous eluents weekly.

3.3 *Preparation and Stability of Standard Solutions*

Standard solutions are prepared as detailed in the body of this report. They are stored refrigerated if LC/MS analysis is to be delayed.

3.4 *Preparation of Sample Extracts*

Samples are extracted as for LC/UV analysis following the procedures detailed in the body of this report. Samples are stable for at least two weeks if stored refrigerated.

3.5 *Fortification of Samples*

Fortifications of soil with pyrithiobac sodium are performed following the procedures detailed in the body of this report.

3.6 *Chromatography*

Minimum requirements of the HPLC system are described in the Equipment section above. This is an isocratic reversed phase analysis on a C18 column designed for use with low-pH mobile phases. Conditions used for analysis are summarized below.

HPLC Conditions:

Column:	3.0 mm i.d. x 25 cm, Zorbax® SB-C18 analytical column with 5 µm diameter packing
Column Temperature:	50.0°C
Injection Volume:	0.100 mL
Flow Rate:	0.4 mL/min
Mobile Phase:	48% acetonitrile/52% 0.1 M acetic acid

The retention time of pyrithiobac sodium is approximately 9.5 minutes; the total run time is 14 minutes (where the t_o Å 2.5 minutes). The HPLC column should be conditioned daily with 90% acetonitrile/10% Milli-Q® water to clean the column and reequilibrated with the mobile phase before analysis. Use of a guard column is optional; if used, retention times will be slightly longer but should require no change in operating parameters.

A UV detector set at 254 nm may be included in the LC/MS system in order to monitor HPLC performance. The 0.0020-µg/mL pyrithiobac sodium standard specified in this method should produce a significant response, allowing evaluation of retention time and peak shape. If monitoring is desired, a variable-UV rather than diode array detector is suggested to provide adequate sensitivity. Placing the UV detector in-line may produce unacceptable band-broadening for MS detection; it would be preferable to position the detector on the waste side of the effluent split,

taking its contribution to system back pressure into account when establishing the split ratio (see suggested split ratio below).

3.7 Mass Spectrometry

The minimum specifications for the MS system are described in the Equipment section above.

The conditions outlined below are representative of those used for the particular instrument upon which this method was developed and evaluated.

ESI-LC/MS Mass Spectrometer Conditions:

Ions Monitored:	m/z 327.0 ± 0.3 amu m/z 329.0 ± 0.3 amu
Scan Length	2 seconds
Electrospray Voltage:	3.9 kV
Electron Multiplier Voltage:	1840 V, established daily
Temperatures:	capillary heater: 200°C manifold: 70°C
Sheath Pressure:	60 psig

Since the electrospray interface is optimal at low flow rates, the HPLC flow is split post-column such that only 90 µL/min actually passes through the interface (~4.44:1 split), the remainder going to waste.

Many of the mass spectrometer conditions were unique for the particular instrument used and varied daily. MS conditions were established and the instrument tuned while directly infusing a pyrithiobac sodium solution of approximately 0.5 µg/mL in 52% 0.1M acetic acid/48% acetonitrile at 0.4 mL/min (bypassing the HPLC column). The instrument was tuned to optimize stability and sensitivity of the signal for ions of m/z 327 and 329 by adjusting lens, repeller, quad offset voltages, and TSP probe temperature. Calibration at m/z 327 and 329 was checked, and the instrument recalibrated as needed using standard procedures. The electron multiplier voltage was adjusted such that the signal intensity was approximately 10^6 abundance.

A 0.005- or 0.010-µg/mL chromatographic standard should be analyzed prior to the start of analyses to more closely establish the appropriate electron multiplier voltage setting for the desired limits of quantitation and detection. For the system used in this method, the electron multiplier voltage was adjusted such that injection of a 0.010-µg/mL pyrithiobac sodium standard solution yielded a detected peak with an area of approximately 80,000 to 100,000 abundance. Operating parameters must be tailored to the particular instrument used, particularly if it is to be an alternate vendor's instrument, and should be checked daily.

3.8 *Sample Analysis*

A standard should be injected at the beginning and end of an analysis sequence and after every two to three samples. If analysis is delayed, samples should be stored refrigerated or frozen until analysis. Sample extracts should be stable for at least two weeks if refrigerated, and for at least five weeks if frozen.

3.9 *Calculations*

Quantitation is from linear regression of peak areas for external standards. Calculations detailed for the column-switching LC/UV method apply (see Section 3.4 of the report). Adequate linearity over the range of 0.005 µg/mL to 0.1 µg/mL pyrithiobac sodium with correlation coefficient (R^2) values of 0.97 or greater should be achievable.

4.2 *Modifications or Special Precautions*

The MS detector is extremely sensitive to pressure fluctuations caused by the HPLC system. Although the chromatography may be adequate for UV detection as evidenced by a stable baseline, periodic baseline fluctuations may appear on chromatograms from the MS. In general, the cause can be traced back to poor check valve function due to pump seal wear or gasses in the mobile phase. Maintaining the pumping system of the HPLC is critical to the performance of the LC/MS system.