

AMERICAN CYANAMID COMPANY  
AGRICULTURAL PRODUCTS RESEARCH DIVISION  
HUMAN AND ENVIRONMENTAL SAFETY  
P.O. BOX 400  
PRINCETON, NEW JERSEY 08543-0400

Recommended Method of Analysis - M 2332.01

Imazamox Herbicide (CL 299,263): HPLC Method for the Determination of Residues of CL 299,263 and CL 312,622 in Soil.

A. Principle

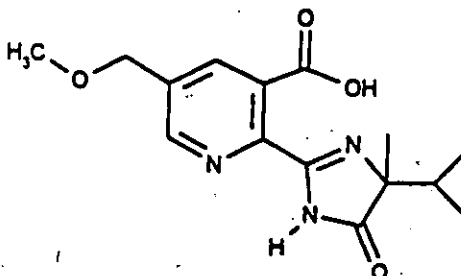
Residues of CL 299,263 and CL 312,622, a soil metabolite of CL 299,263, are extracted from soil with 0.5 N NaOH. The extract is initially cleaned-up by lead acetate precipitation and Celite filtration. The extract is then fractionated by differential elution of a C18 solid phase extraction (SPE) cartridge to separate the CL 299,263 from the CL 312,622 residues. Isolation of CL 299,263 is achieved by SCX-SPE and methylene chloride partitioning of the CL 299,263 fraction. The CL 312,622 fraction is cleaned-up using reverse-phase C18-SPE followed by QMA-SPE and SCX-SPE. Each fraction is analyzed by HPLC on a C8 reverse-phase column with detection by UV absorbance at 254 nm. Results are calculated by comparison of the peak response of the peak of interest to an external standard. The validated sensitivity (LOQ, limit of quantitation) of this method is 5 ppb for each compound.

B. Reagents

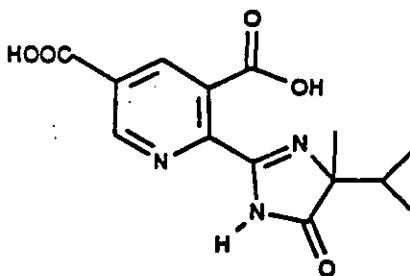
This is a list of suggested reagents. Items from other suppliers that have been shown to be functionally equivalent may be substituted.

1. Analytical Standards: Analytical grade of known purity. American Cyanamid Company, Agricultural Products Research Division, P.O. Box 400, Princeton, New Jersey, 08543.

- a. CL 299,263: Nicotinic acid, 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-(methoxymethyl)-.



- b. CL 312,622: 3,5-Pyridinedicarboxylic acid, 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-.



2. Solvents:

B & J Brand High Purity Solvent, Baxter, Burdick and Jackson, Muskegon, MI.

- Methanol, Catalog # 230-4
- Acetonitrile UV, Catalog # 015-4
- Methylene Chloride, Catalog # 300-4

Aldrich Chemical Company, Milwaukee, WI.

- Tetrahydrofuran, 99.9% HPLC Grade, Catalog # 27,038-5

3. Chemicals:

Baker "Analyzed" Reagents, J.T. Baker, Inc.

- Sodium hydroxide, J.T. Baker # 3722-01
- Hydrochloric acid, concentrated (36.5-38.0%), J.T. Baker # 9535-01
- Potassium phosphate, monobasic, J.T. Baker # 3246-01

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- d. Potassium phosphate, dibasic, J.T. Baker # 3252-01
- e. Potassium chloride, J.T. Baker # 3040-01
- f. Lead acetate trihydrate, J.T. Baker # 2271-01
- g. Phosphoric Acid, concentrated, J.T. Baker # 0260-01
- h. Glacial acetic acid, J.T. Baker # 9507-01

Other Suppliers

- f. Formic acid, 98%, Fluka Chemika # 06440.
- g. Celite 545 AW, Johns-Manville Corp.

- 4. Water, Purified: Milli-Q UV Plus water system fitted with ion exchange, carbon and Organex-Q cartridges, Millipore, Bedford, MA.
- 5. HPLC Mobile Phase: 14% Acetonitrile/83% water/2% tetrahydrofuran/1% formic acid. Mix 140 mL acetonitrile, 830 mL purified water, 20 mL tetrahydrofuran, and 10 mL formic acid. Filter the mixture through a 0.45 micron microporous HPLC solvent filter (Gelman FP-Vericel).

6. Reagent Solutions:

All reagent solutions should be stored at room temperature in borosilicate glass reagent bottles unless noted otherwise. Larger or smaller amounts of these solutions can be made by adjusting the volumes and amounts proportionately.

- a. 10 N NaOH. Carefully add and dissolve 400 grams of solid sodium hydroxide in about 600 mL of purified water using a magnetic stirrer. Caution, this solution becomes very hot. Let the solution cool and dilute to 1 liter with purified water. Store this solution in a high-density polyethylene (HDPE) bottle.
- b. 0.5 N NaOH. Dilute 50 mL of 10 N NaOH to 1 liter with purified water. Store this solution in a high-density polyethylene (HDPE) bottle.
- c. 6 N HCl. Slowly add 100 mL concentrated (36.5-38.0%) hydrochloric acid to 100 mL purified water that is being stirred in a glass beaker on a magnetic stirrer. Caution, this solution will become hot. Let cool and dilute to 200 mL.
- d. 0.24 N HCl. Mix 20 mL concentrated HCl with 980 mL purified water in a 1-L flask.
- e. 0.024 N HCl. Mix 2.0 mL concentrated HCl with 998 mL purified water in a 1-L flask.

f. 5% Methanol/0.024 N HCL. Mix 50 mL methanol with 950 mL 0.024 N HCL.

65/08 g. 1 M Potassium phosphate monobasic. Dissolve 27.2 g of potassium phosphate monobasic in about 150 mL purified water and dilute to 200 mL.

h. 1 M Potassium phosphate dibasic. Dissolve 34.8 g of potassium phosphate dibasic in about 150 mL purified water and dilute to 200 mL.

i. 100 mM Potassium phosphate monobasic. Dilute 50 mL 1 M potassium phosphate monobasic to 500 mL with purified water.

j. 100 mM Potassium phosphate dibasic. Dilute 50 mL 1 M potassium phosphate dibasic to 500 mL with purified water.

mM Phosphoric acid. Mix 3.4 mL concentrated phosphoric acid with 1 L purified water and dilute to 500 mL.

1 M Potassium phosphate, pH 5.0. Place 200 mL of 100 mM potassium phosphate monobasic in a beaker with magnetic stirring and titrate to pH 5.0 with 100 mM potassium phosphate dibasic using a pH meter to monitor the pH change.

m. 100 mM Potassium phosphate, pH 4.0. Place 200 mL of 100 mM potassium phosphate monobasic in a beaker with magnetic stirring and titrate to pH 4.0 with 100 mM phosphoric acid using a pH meter to monitor the pH change.

n. 20% Water/Methanol (v/v). Mix 100 mL purified water with 400 mL methanol.

o. 10% Methanol/Water (v/v). Mix 50 mL methanol with 450 mL purified water.

p. 25% Methanol/Water (v/v). Mix 125 mL methanol with 375 mL purified water.

5/8/91 q. 10% w/v Lead acetate trihydrate. Dissolve 50 g of lead acetate trihydrate in about 400 mL of purified water and dilute to 500 mL. Caution, lead acetate is highly toxic and should be handled with care and disposed of as hazardous waste.

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- r. 20% Methanol/0.5% acetic acid. Mix 100 mL methanol, 2.5 mL glacial acetic acid and 397.5 mL purified water.
- s. 2% Formic acid in methanol. Mix 2 mL of formic acid with 98 mL methanol. This reagent must be made fresh on the same day that it is used. Discard any unused reagent.
- t. Saturated potassium chloride in methanol. Mix about 20 g of potassium chloride with 400 mL of methanol in a glass reagent bottle, cap and shake by repeated inversion for several minutes. Leave the mixture in the capped bottle for at least 2 hours and shake occasionally. There should be excess undissolved salt in the bottle. The solution should be removed without disturbing the salt. As the solution is used, additional methanol and/or potassium chloride can be added and saturated by shaking.

C. Apparatus and Supplies

This is a list of suggested apparatus and supplies. Items, from other suppliers, that have been shown to be functionally equivalent may be substituted.

1. Liquid Chromatograph: Applied Biosystem Model 400 Pump and a Model 783A UV Absorbance Detector. Bodman Chemical Co., Aston, PA.
2. HPLC Column: Supelcosil LC-8-DB, 5 micron, 4.6 mm I.D. x 150 mm, Cat. No. 5-8344, Supelco, Inc.
3. Guard Column: Supelcosil LC-8-DB, 5 micron, 4.6 mm I.D. x 20 mm, Cat. No. 5-9553, Supelco, Inc.
4. Column Oven: Eppendorf Model CH-30 column heater with TC-50 Controller.
5. HPLC Injector: Alcott Model 738 autosampler or Reodyne Model 7125 manual sample injection valve fitted with a 100 mcL loop.
6. Integrator: Spectra-Physics, SP 4290 Recording Integrator.
7. Balance, Analytical: Mettler Model AT261, precision  $\pm 0.05$  mg.
8. Balance, Top-Loading: Sartorius Model L610D, precision  $\pm 0.01$  g.
9. Centrifuge: Sorvall RC-SC Plus centrifuge with GSA rotor for 250-mL bottles, DuPont. The centrifuge must be capable of applying approximately 5000 x G average to fully loaded 250-mL bottles.

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10. Reciprocating Shaker: Eberbach Model 6010, VWR Scientific. This shaker produces a 1-1/2 inch throw and 280 oscillations/minute.
11. Rotary Evaporator: Büchi, Model RE 121 fitted with dry-ice finger condenser and Model 461 Water Bath. Brinkmann Instruments.
12. SPE Vacuum Manifold: IST, International Sorbent Technologies Ltd. VacMaster 10, Cat. No. 121-1016, fitted with PTFE Stopcock/Needles, Cat. No. 121-0001. Distributor, Jones Chromatography, Lakewood, CO.
13. Magnetic Stirrer: Thermolyne, Model Nuova II.
14. Digital pH Meter: Orion Digital Ionalyzer, Model 501, fitted with a Beckman Futura Plus gel-filled combination electrode, Beckman part number 39846.
15. Laboratory Glassware: 1 liter, 250-mL and 100-mL graduated cylinders; 100-mL and 250-mL beakers; 100-mL volumetric flasks, 125-mL separatory funnels; 250-mL side-arm filtering flasks; 50-mL or 100-mL pear-shaped flasks with ground glass joints; 1-mL, 5-mL and 10-mL Class B or better pipets. All glassware should be made of borosilicate glass such as Corning Pyrex or Kimble Kimax glass.
16. Luer Stopcocks: Part number AI-121310-05, Varian Associates.
17. Silicone Tubing: 1/8 inch ID x 1/4 inch OD medical grade silicone tubing, Baxter.
18. HPLC Solvent Preparation and Delivery System: Mobile phase filter/degasser unit, Cat. No. 5-8093, Supelco, Inc.
19. Microporous Disk Filters: FP-Vericel membrane filter, 47 mm, 0.45 micron, HPLC Certified, Gelman Sciences, Inc.
20. Autoinjector Vials: Crimp seal type vials (1-mL nominal), teflon/silicone/teflon type septa (11 mm) sealed with hand type crimper, Supelco, Inc.
21. Centrifuge Bottles: 250-mL polypropylene centrifuge bottles, Nalgene.
22. Plastic Syringe, Disposable: Luer-Lok, 10- and 30-mL capacity, Becton Dickinson.
23. SPE Column Adapters: IST, International Sorbent Technologies Ltd. PTFE Adapters, Cat. No. 120-1100. Distributor, Jones Chromatography, Lakewood, CO.

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24. Solid Phase Extraction Cartridges:

- a. C18. Waters Sep-Pak Plus Environmental C18 Cartridge, Cat. No. 23635.
- b. QMA. Waters Sep-Pak Plus Accell QMA Cartridge. Cat. No. 20545
- c. SCX. IST, International Sorbent Technology, Isolute SCX Column, 1g/6mL, Cat. No. 530-0100-C, Distributor, Jones Chromatography, Lakewood, CO.

25. Reservoirs, Disposable: IST, International Sorbent Technology, 75-mL capacity, Cat. No. 120-1008F. Distributor, Jones Chromatography, Lakewood, CO.

26. HPLC Injection Syringe: Hamilton, Gastight Model 1000 Series, 100-mL capacity.

27. Filter Funnel: Whatman disposable 934-AH glass fiber filter funnel, Cat. No. 1920-1827, Whatman International Ltd.

28. Magnetic Stir Bars: Teflon Coated, 1.5 inch.

29. Pipettor: Pipetman P-1000 with blue plastic tips, Rainen RT-200 or equivalent, Rainen Instrument Company. Accuracy within 2% of nominal with precision within 1% repeatability.

30. Disposable Filter Unit: Vacuum filtration system, 150 mL capacity, Cat. No. 608N0115, Whatman International LTD.

31. Sonicator: Bransonic® Ultrasonic Cleaner, Branson Ultrasonic Cooperation.

D. Preparation of Standard Solutions

All of the standard solutions listed in this section should be stored in amber bottles at about 4 °C and are stable for one month past the preparation date of the stock solutions.

1. Stock Standard Solution:

- a. CL 299,263 Stock. Weigh accurately (to the nearest tenth of a mg) approximately 10 mg of CL 299,263 standard and place the known amount into a 100-mL volumetric flask. Add 10 mL of methanol and agitate by swirling to dissolve the solid. If the solid does not dissolve completely, sonicate the flask in a room temperature sonication bath and/or warm the flask slightly in warm tap water and agitate by swirling. When the solid is dissolved, dilute to the flask mark with purified water, stopper and invert.

the flask to mix the solution. Transfer the solution to a clean glass bottle. After correcting for standard purity, calculate, record and label the bottle with the exact concentration of CL 299,263 to three significant figures.

- b. CL 312,622 Stock. Weigh accurately (to the nearest tenth of a mg) approximately 10 mg of CL 312,622 standard and place the known amount into a 100-mL volumetric flask. Add 10 mL of methanol and agitate by swirling to dissolve the solid. If the solid does not dissolve completely, sonicate the flask in a room temperature sonication bath and/or warm the flask slightly in warm tap water and agitate by swirling. When the solid is dissolved, dilute to the flask mark with purified water, stopper and invert the flask to mix the solution. Transfer the solution to a clean glass bottle. After correcting for standard purity, calculate, record and label the bottle with the exact concentration of CL 312,622 to three significant figures.

2. Mixed Fortification Standard Solutions:

- a. Mixed Fortification Standard A. Pipet into a single 100-mL volumetric flask a volume of the CL 299,263 Stock Standard (D.1.a) and the CL 312,622 Stock Standard (D.1.b) to deliver 2500 mcg of each standard. Dilute to the flask mark with 10% Methanol/Water, stopper and mix by inversion. Transfer the solution to a small amber glass bottle and label with solution identification and concentration (25.0 mcg/mL each CL 299,263 and CL 312,622).
- b. Mixed Fortification Standard B. Pipet into a 100-mL volumetric flask 10 mL of the Mixed Fortification Standard A (D.2.a). Dilute to the flask mark with 10% Methanol/ Water, stopper and mix by inversion. Transfer the solution to a small amber glass bottle and label with solution identification and concentration (2.5 mcg/mL each CL 299,263 and CL 312,622).
- c. Mixed Fortification Standard C. Pipet into a 100-mL volumetric flask 10 mL of the Mixed Fortification Standard B (D.2.b). Dilute to the flask mark with 10% Methanol/ Water, stopper and mix by inversion. Transfer the solution to a small amber glass bottle and label with solution identification and concentration (0.25 mcg/mL each CL 299,263 and CL 312,622).

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3. Mixed Chromatography Standard Solutions:

- a. Mixed Chromatography Standard A. Pipet into a 100-mL volumetric flask 2 mL of the Mixed Fortification Standard B (D.2.b). Dilute to the flask mark with 10% Methanol/Water, stopper and mix by inversion. Transfer the solution to a small amber glass bottle and label with solution identification and concentration (0.05 mcg/mL each CL 299,263 and CL 312,622).
- b. Mixed Chromatography Standard B. Pipet into a 100-mL volumetric flask 4 mL of the Mixed Fortification Standard B (D.2.b). Dilute to the flask mark with 10% Methanol/Water, stopper and mix by inversion. Transfer the solution to a small amber glass bottle and label with solution identification and concentration (0.10 mcg/mL each CL 299,263 and CL 312,622).
- c. Mixed Chromatography Standard C. Pipet into a 100-mL volumetric flask 10 mL of the Mixed Fortification Standard B (D.2.b). Dilute to the flask mark with 10% Methanol/Water, stopper and mix by inversion. Transfer the solution to a small amber glass bottle and label with solution identification and concentration (0.25 mcg/mL each CL 299,263 and CL 312,622).
- d. Mixed Chromatography Standard D. Pipet into a 100-mL volumetric flask 2 mL of the Mixed Fortification Standard A (D.2.a). Dilute to the flask mark with 10% Methanol/Water, stopper and mix by inversion. Transfer the solution to a small amber glass bottle and label with solution identification and concentration (0.50 mcg/mL each CL 299,263 and CL 312,622).

E. HPLC Conditions

Operating conditions described below are provided for use as a guide in establishing actual operating conditions and should be adjusted as necessary to obtain peak shape and resolution from background peaks that is equivalent to or better than the chromatograms shown in Figure 2 and 3.

I. Instrument:

- a. HPLC Pump: Applied Biosystem Model 400 Pump.
- b. Detector: Applied Biosystem Model 783A UV Absorbance Detector.
- c. Integrator: Spectra-Physics Model SP4290.
- d. Autoinjector: Alcott Model 738.
- e. Column Oven: Eppendorf Model CH-30 with TC-50 Controller.

2. Column: Supelcosil LC-8-DB, 5 micron, 4.6 mm I.D. x 150 mm length and a Supelco guard column fitted with a Supelcosil LC-8-DB cartridge.

3. Instrument Conditions:

- |                                       |  |
|---------------------------------------|--|
| a. <u>Mobile Phase</u><br>(Isocratic) | Acetonitrile/Water/Tetrahydrofuran/Formic acid<br>14:83:2:1                |
| b. <u>Flow Rate</u>                   | 1 mL/minute  |
| c. <u>Column Temperature</u>          | 30°C controlled with column heater   |
| d. <u>Injection Volume</u>            | 50 microliter, autoinjector or manual loop<br>injector                     |
| e. <u>Detector Wavelength</u>         | 254 nm   |
| f. <u>Integration Parameters</u>      |  |
| Chart Speed                           | 0.5 cm/minutes   |
| Detector Output                       | 1 V/AU   |
| Attenuation                           | 4 mV Full Scale  |
| Peak Width                            | 30 seconds   |
| Peak Threshold                        | 1000   |
| Offset                                | 10% Full Scale   |
| End Run at                            | 10 minutes   |
| g. <u>Retention Times</u>             | Approximately 6.2 minutes for CL 312,622<br>and 8.4 minutes for CL 299,263 |

F. Linearity Check

The HPLC must be checked for linearity of response at least once for each related group of analyses. Linearity must also be confirmed following any change of column, modification of the instrument or significant alteration of chromatographic conditions. The linearity of response is checked by injection of at least three standards of different known concentrations. The response ratio for each standard injection is calculated and compared to the average response ratio.

1. Set up the HPLC conditions described above and obtain a stable chromatographic response for injection of 50 mL of the Mixed Chromatography Standard C (0.25 mcg/mL CL 299,263 and 0.25 mcg/mL CL 312,622).
2. Adjust the integrator to attain a peak height of approximately 40% full-scale deflection for the CL 312,622 peak after injection of 50 mL of the Mixed Chromatography Standard C, 0.25 mcg/mL, (12.5 ng CL 312,622 injected).
3. Inject and chromatograph 50 mL of each of the Mixed Chromatography Standards A, B, C, and D (0.05, 0.10, 0.25, and 0.50 mcg/mL of each compound).

4. Manually measure the peak heights with a ruler for the CL 299,263 and CL 312,622 peaks for each standard chromatogram or use the integrated peak area or integrated peak height provided by the data system or integrator. When integrated values are used, inspect each peak to insure that the baseline is properly defined and/or that the peak start and stop tick marks are placed properly. Calculate the response ratio for each standard injected by dividing the peak response (height or area) by the amount (nanograms) of standard injected. Calculate the average response ratio. Significant departure from linearity as indicated by deviation of any response ratio from the average response ratio of greater than 15% indicates instrument or experimental difficulties which must be corrected before proceeding.

#### G. Fortification Recovery Test

The validity and performance of the procedure must always be demonstrated by recovery tests. At least one fortified sample must be processed with every six samples analyzed. The fortification levels chosen for a study should include the level of sensitivity (LOQ, Limit of Quantitation, 5 ppb) of this method and should bracket the sample concentration range expected.

1. Weigh a 50 gram sample of a control soil into a 250-mL centrifuge bottle.
2. Add by pipet a volume of a Fortification Standard that is appropriate to the fortification level to be tested.
3. Process and analyze this fortified sample as described in sections H, I, J, K, L and M.

#### H. Extraction and Initial Sample Clean-Up

##### Extraction:

1. Weigh a 50 gram sample of soil into a 250-mL centrifuge bottle (Nalgene, polypropylene). Add 200 mL of 0.5 N NaOH to the bottle, cap and shake on a reciprocating shaker for 30 minutes (see Method Note H.1, Section N).
2. Centrifuge the bottle at about 5000 x G for 15 minutes. Decant the supernatant solution into a graduated cylinder and measure 100 mL of the sample solution into a clean beaker.

##### Lead Acetate Precipitation:

3. Titrate the sample solution to between pH 7 and pH 8 with 6 N HCl using a magnetic stirrer and a pH meter. If too much HCl is added and the pH goes below 7 then back-titrate the sample with 0.5 N NaOH to the proper pH range.

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4. Add 5 mL of 10% lead acetate trihydrate (w/v) and stir to mix [Caution, lead acetate is highly toxic and should be handled with care and disposed of as hazardous material.]

Filtration:

5. Add approximately 5 gram of Celite to each sample extract, stir to mix and filter the suspension through a Whatman disposable filter unit with a 934-AH glass fiber filter into a 250-mL vacuum flask using slight vacuum. The sample extract should be clear and light yellow in color.
6. Titrate the sample solution to between pH 1.7 and pH 1.8 with 6 N HCl using a magnetic stirrer and a pH meter. If a precipitate forms or the solution becomes cloudy, add 1 gram of Celite, stir to mix and filter again as described above. [Caution, the disposable glass fibre filter contains lead precipitates, which are highly toxic. Handle the filters and filtrates with care and dispose of as hazardous material.]

I. C18 SPE Fractionation

Vacuum Manifold Setup:

1. Set up the SPE vacuum manifold as shown in the attached Figure 1 and as described in Method Note I.1., Section N. The C18 cartridges will be attached to the SPE vacuum manifold by a length of silicone tubing to allow direct application of samples to the cartridges by vacuum.

C18 Preparation and Sample Loading:

2. Precondition one Sep-Pak C18 cartridge for each sample by washing it first with 10 mL of methanol followed by 10 mL of 0.024 N HCl using a 10-mL plastic syringe (see Method Note I.2., Section N).
3. Attach the C18 cartridge to the end of the vacuum manifold/tubing arrangement and place the cartridge into the sample in the vacuum flask (from step H.6.). Open the stopcock for the sample line and apply sufficient vacuum (about 100 to 200 mbar) to the manifold to achieve a flow of about two drops per second. The samples are drawn directly into the C18 cartridges. For details on the sample load procedure see Method Note I.3., Section N.

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C18 Cartridge Wash:

4. When the entire sample has been loaded and air has been drawn into the cartridge for approximately 30 seconds, rinse the sides of the sample flask down with 10 mL of 0.024 N HCl and allow the rinse to be drawn into the cartridge.
5. When the rinse is completely loaded, add an additional 30 mL of 0.024 N HCl to the flask and draw into the cartridge by vacuum until air enters the cartridge for approximately 30 seconds as in step I.4.
6. Add 40 mL of 5% methanol/0.024 N HCl to the flask and load onto the cartridge by vacuum until air enters the cartridge for approximately 30 seconds.
7. Remove the C18 cartridge from the vacuum manifold/tubing and wash with a total of 8 mL of 100 mM potassium phosphate pH 4.0, using a 10-mL plastic syringe and a plunger. [Caution, the C18 load that is collected in the side arm vacuum flask and rinse contains lead acetate, which is highly toxic. Handle this solution with care and dispose of properly.]

Elution of CL 312,622 Fraction:

8. Place a 50-mL clean beaker inside the vacuum manifold. Elute the C18 cartridge, using slight vacuum to achieve a flow rate of 2 drops per second, with a total of 30 mL of 100 mM potassium phosphate pH 5.0. This eluate contains the CL 312,622 residue and is processed further as described in section J.

Elution of CL 299,263 Fraction:

9. Using slight vacuum, wash the C18 cartridge from step I.8 above with 10 mL of 0.024 N HCl.
10. Place a new 50-mL beaker inside the vacuum manifold. Using slight vacuum to achieve a flow rate of 2 drops per second, elute the C18 cartridge into a beaker with a total of 30 mL of 25% Methanol/Water. This eluate contains the CL 299,263 residue and is processed further as described in section K.

J. CL 312,622 Clean-Up Track

C18 SPE Cartridge

1. Titrate the CL 312,622 fraction from step I.8 to between pH 1.7 and 1.8 with 6 N HCl using a magnetic stirrer and a pH meter to monitor pH change.

2. Using a 10-mL plastic syringe and a plunger, precondition the C18 cartridge by washing it first with 10 mL methanol, follow by 10 mL of 0.024 N HCl.
3. Connect the C18 cartridge to the vacuum manifold. Using slight vacuum to achieve a flow rate of 2 drops per second, load the CL 312,622 fraction from step J.1 onto the C18 cartridge.
4. Using slight vacuum, wash the C18 cartridge with 10 mL of 5% methanol/0.024 N HCl. This C18 cartridge will be attached and downloaded onto a QMA cartridge in the next step.

#### QMA-SPE Cartridge

5. Connect a 10-mL plastic syringe to one QMA cartridge and precondition the cartridge with 10 mL methanol, follow by 10 mL of 20% methanol/ 0.5% acetic acid.
6. Attach the QMA cartridge to a closed Luer stopcock on the vacuum manifold. Attach the C18 cartridge from step J.4 (loaded with the CL 312,622 fraction) to the top of the QMA cartridge and attach an empty 75-mL reservoir to the top of the C18 cartridge, using an adapter.
7. Add 40 mL of 20% methanol/0.5% acetic acid to the reservoir. Elute the C18 cartridge and download onto the QMA cartridge by opening the Luer stopcock and applying sufficient vacuum of about 100 to 200 mm Hg. When air enters both cartridges for approximately 30 seconds, close the stopcock, remove the reservoir, and discard the C18 cartridge.
8. Re-attach the reservoir to the top of the QMA cartridge.
9. Add 10 mL of 20% methanol/0.5% acetic acid to the reservoir and draw the wash through the QMA cartridge by vacuum until air enters the QMA cartridge.
10. Rinse the side of the reservoir with 10 mL of methanol and draw the methanol through the cartridge by vacuum until air enters the QMA cartridge.
11. Add the second 10 mL of methanol to the reservoir and draw through the cartridge until air enters the cartridge. This QMA and reservoir will be attached and downloaded onto an SCX column as described in the next step.

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### SCX-SPE Column

12. Attach one SCX-SPE column to a closed Luer stopcock on a vacuum manifold. Add 5 mL of methanol to the column and open the stopcock to apply vacuum and draw the methanol wash through the column. Repeat this methanol wash three times. Close the stopcock between each 5-mL wash before air enters the column.
13. Attach the QMA cartridge to the top of the SCX column using a SPE column adapter. (The reservoir should still be attached to the top of the QMA cartridge.)
14. Add 20 mL of freshly made 2% formic acid/methanol to the reservoir. Elute the QMA cartridge and download onto the SCX column by opening the Luer stopcock and applying the vacuum of about 100 to 200 mm Hg. After air enters the column, close the stopcock and remove the QMA cartridge and reservoir.
15. Attach the reservoir to the adapter on the SCX column and wash the SCX column twice by adding 10 mL of methanol to the reservoir and opening the stopcock to apply vacuum. Air should be allowed to enter the column after the wash to dry the column.
16. Open the vacuum manifold and place a 50-mL round-bottom flask (or other suitable container) in the vacuum manifold in a position to collect the eluate from the SCX column.
17. Close the vacuum manifold and elute the compound from the SCX column into the round-bottom flask with a total of 20 mL of 20% water in methanol by vacuum.
18. Remove the collected sample fraction from the vacuum manifold and evaporate the sample to dryness on a rotary evaporator (approximately 35°C water bath).
19. Dissolve the residue in 1 mL of 5% Methanol/0.024 N HCl and analyze by HPLC as described in section L.

### K. CL 299,263 Clean-Up Track

#### SCX-SPE Column

1. Add 3 mL of 0.24 N HCl to the CL 299,263 fraction from step I.10. to prepare it for loading on an SCX-SPE column.
2. Attach one SCX column to a closed Luer stopcock on a vacuum manifold.

3. Precondition the SCX column by washing 2 times with 5 mL of methanol followed by 2 times with 5 mL of 0.024 N HCl. Close the stopcock between each 5-mL wash before air enters the column.
4. Attach an adapter and a 75-mL reservoir to the top of the SCX column.
5. Pour the CL 299,263 fraction from step K.1 into the reservoir and open the stopcock to apply vacuum and load the fraction onto the column.
6. When air enters the column packing, rinse the reservoir with 10 mL of 20% methanol/0.5% acetic acid and draw the rinse through the column by vacuum.
7. Repeat this rinse two times with 10 mL of methanol and allow air to be drawn into the column for approximately 30 seconds to dry the column.
8. Open the vacuum manifold and place a 50-mL round-bottom flask (or other suitable container) in the vacuum manifold in a position to collect the eluate from the SCX column.
9. Close the vacuum manifold and elute the compound from the SCX column into the round-bottom flask with a total of 25 mL of saturated potassium chloride in methanol.
10. Remove the collected sample fraction from the vacuum manifold and evaporate the sample to dryness on a rotary evaporator (approximately 35°C water bath). This fraction is partitioned into methylene chloride as described in the next step.

#### Methylene Chloride Partition

11. Dissolve the dried residue in the flask from step K.10. in 10 mL of 0.024 N HCl and pour into a 125-mL separatory funnel.
12. Rinse the flask with an additional 10 mL of 0.024 N HCl and add to the separatory funnel. Add 20 mL of methylene chloride to the separatory funnel, stopper the funnel, shake briskly for 15 seconds and allow the phases to separate.
13. Draw off the methylene chloride phase (bottom phase) into a clean 100-mL pear-shaped flask taking care not to allow any of the aqueous phase to enter the flask.
14. Repeat this partition one more time with 20 mL of methylene chloride and draw off the methylene chloride phase (bottom phase) and add to the pear-shaped flask.

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15. Evaporate the combined methylene chloride phases with a rotary evaporator with the water bath set at approximately 35°C.
16. Dissolve the sample in 1.0-mL of 5% Methanol/0.024 N HCl and analyze by HPLC as described in section L.

L. HPLC Analysis

1. Set up the HPLC instrument according to the conditions listed in section E and obtain a satisfactory baseline and chromatographic response for a 50-microliter injection of the Mixed Chromatography Standard C (0.25 mcg/mL). Set the integrator attenuation to obtain about 40% full-scale deflection for the CL 312,622 peak of this standard. These conditions may be adjusted slightly to obtain equivalent or better peak shape and resolution as the chromatograms in Figure 2 and 3. Chromatograph 50 microliters of each of the Mixed Chromatography Standards A, B, C, and D (0.05, 0.10, 0.25, and 0.50 mcg/mL, respectively) for a linearity check as described in section F.
2. Inject and chromatograph 50 microliters of each processed soil sample and intersperse injections of Mixed Chromatography Standard C (0.25 mcg/mL, working standard) before the first sample injection and after a maximum of every two sample injections.
3. Identify the peak of interest (either CL 299,263 or CL 312,622) in each sample chromatogram and standard chromatogram and measure the peak response (peak height in millimeters or use the integrated peak area or integrated peak height provided by the data system or integrator). When integrated values are used, inspect each peak to insure that the baseline is properly defined and/or that the peak start and stop tick marks are placed properly. If the sample peak response is greater than the highest value from the linearity check, then dilute the sample to an appropriate volume with 5% Methanol/ 0.024 N HCl and rechromatograph the diluted sample.

M. Calculations

Calculate the apparent CL 299,263 and CL 312,622 residues in the injected samples (in ppb) from the sample peak response and the average peak response of the standard chromatograms obtained before and after the sample injection as follows:

$$\text{ppb} = \frac{R(\text{SAMP}) * V1 * V3 * C(\text{STD}) * V5 * D.F. * 1000}{R(\text{STD}) * W * V2 * V4}$$

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Where:

R(SAMP) = Sample Response (chromatographic response of the sample peak of interest).

R(STD) = Average Standard Response (average chromatographic response for the peak of interest in the working standard chromatograms before and after the sample chromatogram).

W = Weight of sample taken for analysis, in grams (50 g).

V1 = Volume of extraction solvent used, in milliliters (200 mL).

V2 = Aliquot of extract taken for analysis, in milliliters (100 mL).

V3 = Volume of solvent added to dissolve final residues for HPLC analysis, in milliliters (1.0 mL).

V4 = Volume of sample solution injected for HPLC analysis, in microliters (50 µL).

V5 = Volume of working standard solution injected for HPLC analysis, in microliters (50 µL).

C(STD) = Concentration of working standard solution injected in micrograms per milliliter (0.25 µg/mL)

D.F. = Dilution factor

1000 = Conversion Factor for ng/mcg.

Calculate the percent recovery for fortified samples as follows:

$$\text{Percent Recovery} = \frac{\text{ppb Found} * 100}{\text{FV} * \text{FC}/\text{W}} = \frac{\text{ppb Found}}{\text{ppb Added}} * 100$$

Where:

FV = Fortification Volume, in milliliters.

FC = Concentration of fortification solution in ng/mL.

W = Weight of sample taken for analysis, in grams (50 g).

Typical chromatograms for the determination of CL 299,263 and CL 312,622 in soil are shown in Figure 2 and 3, respectively.

N. Method Notes

This method supersedes M 2332 and is procedurally similar to M 2332 except for some minor procedural adjustments summarized below.

These method notes also provide additional detail and observations that may be useful to the analyst. They are numbered with the section and step to which they apply:

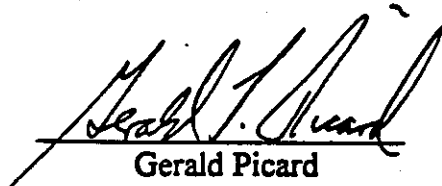
- H.1. The sample is shaken for 30 minutes instead of 15 minutes.
- H.2. Centrifuging is done at 5000 x G instead of 1000 x G.
- H.1 The Eberbach shaker described in Section C provides 280 reciprocations per minute with a linear reciprocation throw of 1.5 inches when set on high. To achieve the most thorough agitation of the soil, the bottle should be placed on the shaker in a horizontal position with the direction of reciprocation parallel to the long dimension of the bottle.
- I.1 Set up the SPE vacuum manifold as shown in the attached Figure 1 and described as follows. Connect a vacuum source to a large side arm vacuum flask. The flask must be large enough to contain the total volume of all the samples and washes that will be processed without spillover into the vacuum system. Connect a vacuum line from the flask to the vacuum controller of the SPE vacuum manifold. Attach Luer stopcocks to the ports of the manifold and close the stopcocks. Attach twelve inch lengths of silicone tubing to the Luer stopcocks. The C18 cartridges will be attached to the end of the silicone tubing to allow direct application of samples to the cartridges by vacuum.
- I.2 To pre-wash the cartridge, attach the cartridge to a 10-mL plastic syringe. Add 10 mL of methanol and push the methanol through the cartridge. Repeat this with 10 mL of the dilute HCl wash. Do not introduce air into the cartridge during this procedure.

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- I.3 To load the sample onto the cartridge, connect the male Luer outlet of a preconditioned C18 cartridge to the end of the vacuum manifold/tubing arrangement and place the cartridge (connected with the tubing to the vacuum manifold) into the sample in the vacuum flask. Tilt each sample flask slightly and place the open end of the cartridge into the bottom corner of the flask to facilitate the removal of the entire sample. Repeat for each sample to be processed. Open the stopcock for each sample line and apply sufficient vacuum (about 100 to 200 mbar) to the manifold to achieve a flow of about two drops per second. The samples are drawn directly into the C18 cartridges.

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