Cover Sheet for

ENVIRONMENTAL CHEMISTRY METHOD

Pestcide Name: Isoxaben

MRID #: 400595-08

Matrix: Soil

Analysis: HPLC/UV

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Agricultural Analytical Chemistry
Lilly Research Laboratories
Division of Eli Lilly and Company
Greenfield, Indiana 46140

Methods are routinely revised.
Interested parties may receive revisions
by request to the above address.

DETERMINATION OF EL-107[1] AND/OR ITS SOIL METABOLITE[2] IN SOIL

AM-AA-CA-R042-AC-755

PRINCIPLE

EL-107 and its soil metabolite are extracted from soil by refluxing with methanol-water. An aliquot of the extract is purified by liquid-liquid partitioning and alumina column chromatography. Detection and measurement require HPLC with UV detection. EL-107 and its metabolite are collected as separate samples from the alumina column and measured using different mobile phase solutions.

REAGENTS

1. Methanol, reagent-grade
2. Methanol/water, 80:20 (v/v)
3. Sodium chloride solution, 5-percent aqueous
4. Dichloromethane, reagent-grade
5. Sodium sulfate, anhydrous, methanol washed and dried
6. Alumina, Alcoa F-20 (deactivated with water, 4 percent)
7. Ethyl acetate, reagent-grade
8. Dichloromethane/ethyl acetate, 80:20 (v/v)
9. Dichloromethane/methanol, 99:1 (v/v), 98:2 (v/v), 97:3 (v/v)
10. HPLC mobile phase: methanol/water, 70:30 (v/v) for EL-107; methanol/water, 60:40 (v/v) for metabolite (Solvents must be HPLC quality, filtered, and degassed.)

APPARATUS

1. Sample grinding and blending equipment
2. Reflux apparatus with water-cooled condenser

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1 N-[3-(1-Ethyl-1-methylpropyl)-5-isoxazolyl]-2,6-dimethoxybenzamide, Serial No. 121607

2 N-[3-(1-Hydroxy-1-methylpropyl)-5-isoxazolyl]-2,6-dimethoxybenzamide, Serial No. 201469

3. Rotary vacuum evaporator with water bath set at 40-45°C

4. Chromatographic columns, 25 cm x 14 mm i.d., equipped with stopcocks

5. High-performance liquid chromatograph equipped with UV detector capable of operation at 0.01 AUFS at 254 nm.

STANDARD PREPARATION

Prepare separate standards for EL-107 and its soil metabolite.

1. Stock standard solution (50 mcg/ml) -- Accurately weigh about 10 mg of reference standard. Transfer it to a 200-ml volumetric flask and dilute to volume with methanol. Mix well.

2. Working standard solution (1.25 mcg/ml) -- Dilute the stock solution with methanol/water to obtain a solution containing 1.25 mcg/ml and mix well. (Use methanol/water in the same proportions as the mobile phase. See REAGENTS, Step 10.)

3. Standard curve -- Prepare standard solutions over the range of 1.25 to 0.156 mcg/ml by diluting aliquots of the working standard solution with methanol/water in the same proportions as the mobile phase.

Note: The stock standard solution is stable for at least two months if kept refrigerated. The 1.25 mcg/ml standard and standard curve solutions should be prepared fresh weekly.

PROCEDURE

A. Preparation of Soil Samples

Soil samples should be blended as necessary to yield homogeneous material. Dry silica sand (about equal weight) may be added if the soil is too moist to flow freely.

B. Extraction of EL-107 and Its Soil Metabolite From Soil

1. Weigh 50 g of soil into a 500-ml refluxing flask.

2. Add 200 ml of methanol/water, 80:20.

3. Attach a water-cooled condenser and heat to reflux for one hour.

4. Allow to cool to room temperature and allow solids to settle.

5. Transfer 100 ml of the supernatant solution to a 250-ml separatory funnel.

6. Add 50 ml of 5-percent aqueous sodium chloride solution.

7. Extract by shaking with three 70 ml portions of dichloromethane. Allow the phases to separate and drain the lower layer through a layer of anhydrous sodium sulfate (previously washed with 10-15 ml dichloromethane) into a 250-ml boiling flask. (Do not allow any water into the flask containing the dichloromethane extract.)

8. Rinse the sodium sulfate with additional dichloromethane.

9. Evaporate the sample solution on a rotary vacuum evaporator with water bath temperature at 40-45°C. If water droplets remain, add small portions of dichloromethane and repeat evaporation.

C. Purification

1. Prepare an alumina column for each sample as follows:
   a. Tamp a pledget of glass wool to the bottom of the column with a stirring rod.
   b. Add 13±0.5 ml of standardized alumina (See section E). Tap the sides of the column gently to settle alumina. Note: The alumina must be added to the columns in a reproducible manner to assure a consistent elution pattern for all samples within a set.
   c. Add about 1.5 cm of anyhydrous sodium sulfate, layering it carefully to avoid disturbance of the alumina surface.
   d. Wash the column with 30 ml dichloromethane, draining the solvent to the top of the sodium suflate layer.

2. Transfer the sample residue to the column using two 5-ml portions of dichloromethane, allowing each addition to pass into the adsorbent. Rinse the boiling flask with 25 ml dichloromethane and add it to the column, rinsing down the sides of the column. Allow the solvent to drain to the alumina surface. Discard the eluate.

3. Wash the column with 50 ml of 80:20 dichloromethane/ethyl acetate. Discard the eluate.

4. Wash with 25 ml of 99:1 dichloromethane/methanol. Discard the eluate.

5. Add 50 ml of 99:1 dichloromethane/methanol and collect the eluate in a 125-ml boiling flask. This fraction should contain EL-107.

6. Wash the column with 20 ml of 98:2 dichloromethane/methanol. Discard the eluate.

7. Add 75 ml of 97:3 dichloromethane/methanol and collect the eluate in a 125-ml boiling flask. This fraction should contain the soil metabolite.

Note: The solvent volumes used in steps 3 through 7 are dependent on the column profile and are suggested as a guideline. A column profile should be run when the procedure is introduced into the laboratory and when a new batch of alumina is used. See section E, Standardization of Alumina, for determination of the column profile.

8. Evaporate the eluate from step 5 and/or step 7 by rotary vacuum evaporation.

9. Dissolve the residue in 1.0 ml of methanol/water in the same proportions as the appropriate mobile phase and proceed with HPLC analysis.

D. Standard Recovery and Control Samples

A standard recovery sample of 0:025 ppm and a control sample are assayed with each set of experimental samples. The standard recovery sample is prepared by adding 1.0 ml of the appropriate 1.25 mcg/ml standard solution to 50 g of control soil. If control soil is unavailable, a system recovery is run which simulates the 0.025 ppm recovery level. Recovery and control samples are assayed exactly as experimental samples.

E. Standardization of Alumina

1. The alumina, as received, is deactivated prior to use by the addition of 4-percent (v/w) water, followed by tumbling in a closed container for 30 minutes. Allow the material to stand for two hours prior to use. Keep storage container tightly closed.

2. Prepare an alumina column as described in step C.1.

3. Evaporate to dryness 1.0 ml of the appropriate 1.25-mcg/ml working standard solution and transfer the residue to the column with two 5-ml portions of dichloromethane. Wash with 25 ml dichloromethane and discard washings.

4. Elute with two 25-ml portions of 80:20 dichloromethane/ethyl acetate, collecting each fraction in a separate boiling flask.

5. Elute with three 25-ml portions of 99:1 dichloromethane/ methanol, collecting each fraction in a separate boiling flask.

6. Elute with 20 ml of 98:2 dichloromethane/methanol; collect fraction in a boiling flask.

7. Elute with four 25-ml portions of 97:3 dichloromethane/ methanol, collecting each fraction in a separate boiling flask.

8. Evaporate the samples in steps 4, 5, 6 and 7 to dryness using rotary vacuum evaporation.

9. Dissolve the residues in 1.0 ml of methanol/water in the same proportions as the appropriate mobile phase and proceed with HPLC analysis.

10. From the resulting chromatograms, determine which column fractions contain EL-107 or its metabolite. Collect the corresponding fractions when assaying experimental samples.

F. High-Performance Liquid Chromatography

1. HPLC pump: Capable of delivering a constant flow rate with pulseless operation

2. UV detector: Capable of operation at 0.01 AUFS at 254 nm

3. Injector: Fixed loop or constant volume injector

4. Recorder: Compatible with detector

5. Analytical column: 25 cm x 4.6 mm i.d. Spherisorb ODS II, 5µm

6. Guard column: Co: Pell ODS, 30-38 µm, or equivalent packing or equivalent pellicular packing

7. Operating conditions:

Mobile phase:
a. methanol/water, 70:30 for EL-107
b. methanol/water, 60:40 for soil metabolite
Flow rate: 1 ml/min
Injection volume: 70 µl
Retention time of EL-107: approx. 7.0 minutes
Retention time of soil metabolite: approx. 5.5 minutes

Note: These parameters may require slight adjustments for optimum sensitivity.

G. Measurement

1. Assay standard curve solutions, control, standard recovery, and experimental samples using the HPLC conditions described for EL-107 or its metabolite.

2. Measure the peak height of the EL-107 or metabolite peak for each injection. If the peak height of any sample is not within the range of the standard curve, appropriate dilutions should be made for reassay by HPLC.

3. Plot the peak heights versus concentration for the standard curve on linear graph paper or perform linear regression analysis on a scientific calculator.

4. Using the peak heights of the experimental samples, standard recoveries, and control, determine the concentration in mcg/ml of each from the standard curve plot.

H. Calculation

1. Standard Recovery

$$\% \text{ Recovery} = \text{mcg/ml (from std curve)} \times \frac{200 \text{ ml}}{\text{mcg added}} \times \frac{1 \text{ ml}}{100 \text{ ml}} \times 100$$

2. Experimental Samples

$$\text{ppm EL-107 or metabolite} = \text{mcg/ml (from std curve)} \times \frac{200 \text{ ml}}{\text{g Sa}} \times \frac{1 \text{ ml}}{100 \text{ ml}} \times \frac{\text{dilution factor*}}{\text{percent std recovery}} \times \frac{100}{1}$$

*dilution factor only necessary if sample required further dilution.

I. Discussion

Method R042-AA was developed under study S-AAC-81-09. Validation data are contained in notebook 4E7. Recoveries from three different soil types fortified at 0.1 ppm ranged from 82 to 92%. Triplicate samples of one soil type fortified at 0.1 ppm and assayed on three separate days gave a relative standard deviation of 6.8%.

Revision R042-AB was validated by assaying duplicate soil recovery samples over a four day period. Fortification levels used were 0.05 ppm and 0.005 ppm.

	0.05 ppm	0.005 ppm
Day 1	94.7%	90.3%
1	98.9%	87.1%
2	101.0%	90.3%
2	94.7%	93.5%
3	94.2%	96.7%
3	94.2%	90.3%
4	94.2%	93.5%
4	94.2%	90.3%
Mean =	95.8%	91.5%
S.D. =	2.65	2.93
RSD =	2.77%	3.20%

Validation data are found in notebook HR8, under study No. I-BSR-84-9.

Revision R042-AC was written to include the determination of the soil metabolite, serial no. 201469. An aerobic soil metabolism

study (ABC-0224) and ^{14}C field soil study (AB-0097 and AB-0146) have shown this to be the major soil metabolite of EL-107. Validation data for the metabolite include recovery at 0.025 and 0.005 ppm in three soil types on one day and recovery at both levels in one soil type over a 2-week period.

	Soil A		Soil B		Soil C	
	.025 ppm	.005 ppm	.025 ppm	.005 ppm	.025 ppm	.005 ppm
	98.4	101.8	104.4	81.8	93.2	107.5
	100.5	91.4	101.3	74.1	95.3	97.1
	99.5	91.4	100.3	74.1	93.2	97.1
Mean =	99.5	94.9	102.0	76.7	93.9	100.6
S.D. =	1.05	6.00	2.14	4.45	1.21	6.00
RSD =	1.1	6.3	2.1	5.8	1.3	6.0

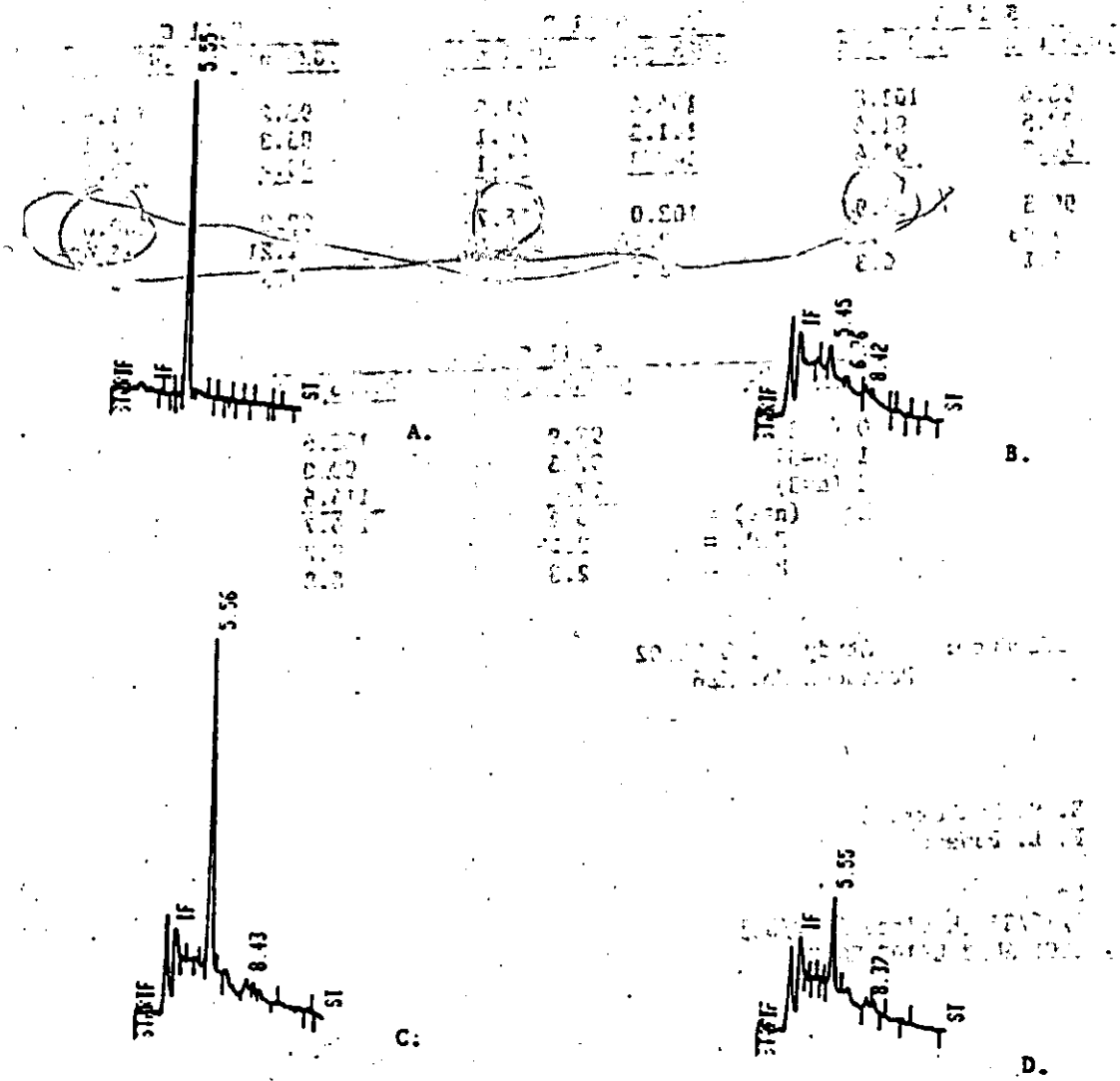
	Soil C	
Time, Wk.	0.025 ppm	0.005 ppm
0 (n=3)	93.9	100.6
1 (n=3)	97.3	99.0
2 (n=3)	97.4	114.6
Mean (n=9) =	96.2	104.7
S.D. =	2.23	9.22
RSD =	2.3	8.8

Reference: Study No. BSR8502
Notebook No. NS6

B. S. Rutherford
F. L. Powers

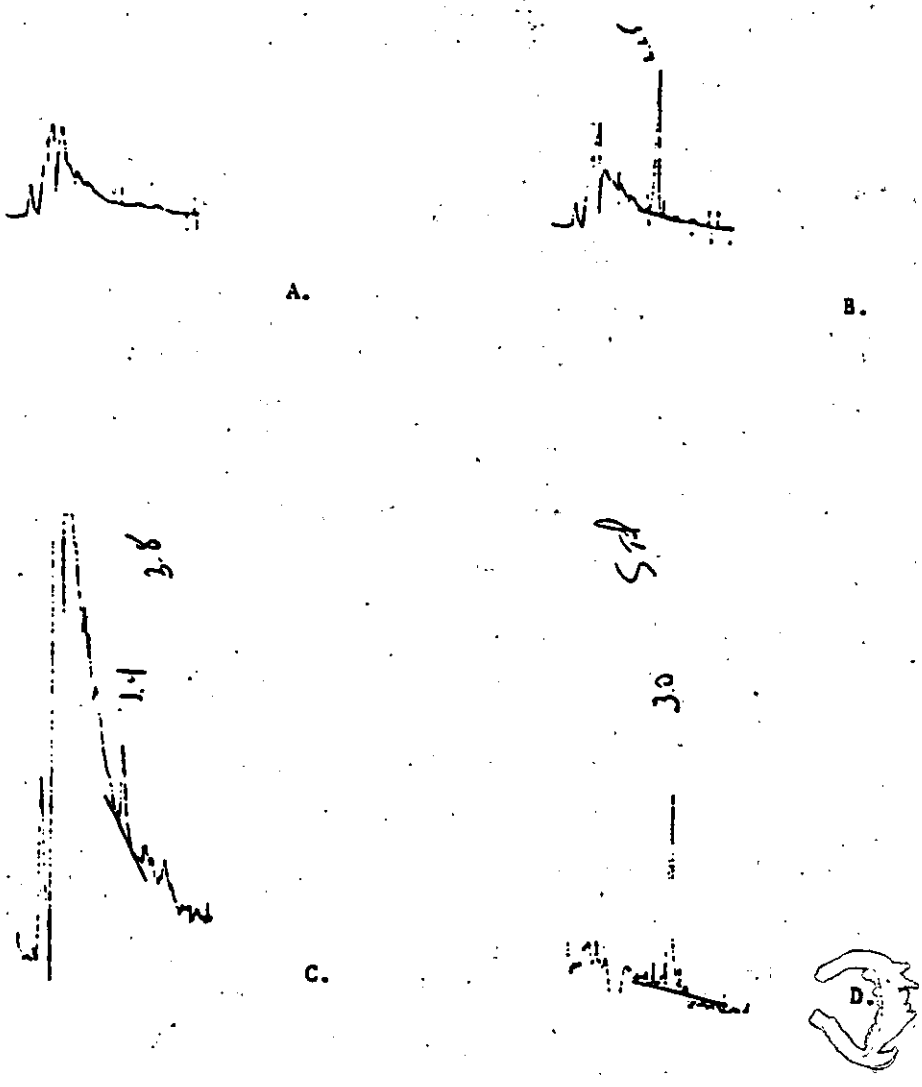
kg
1/16/85 (Revised 5/17/85)
GL01 AM33 EL107 R042

Representative Chromatograms of
EL-107 Soil Metabolite



A. 0.625 µg/ml STD
B. Control Soil
C. Control Soil plus 0.025 ppm metabolite
D. Control Soil plus 0.005 ppm metabolite
Mobile Phase: 60:40 MeoH/H_2O

Representative Chromatograms
of EL-107



A. Control Soil
B. Control Soil plus 0.05 ppm EL-107
C. Control Soil plus 0.005 ppm EL-107
D. 0. 25 µg/ml EL-107 Std

Mobile Phase: 70:30 MeoH/H_2O
A and B: Attenuation 2^3
C and D: Attenuation 2^0

APPENDIX F

RAW ASSAY DATA

1. Raw Data for Isoxaben Assays
2. Raw Data for Metabolite Assays

Each Residue Sample List contains all of the data on a given HPLC run. Each has been assigned a unique assay group number (AGN).

Some of the entries require further explanation:

Compound -- EL-107 is isoxaben; AA-045 is metabolite. (Some runs may list an m at the end of the AGN - this also designates an assay for the metabolite.)

Method No. -- R042-AC is an abbreviation of the procedure number of the method used on these samples.

Vial No. -- HPLC vial number in autosampler.

SN -- Sample number. The first five digits are the sample group number (SGN).

Sa Wt -- Weight of samples extracted in procedure in mg.

Lb/A Cf -- Pound/Acre conversion factor.

Bag Wt, Kg -- Weight of entire sample received.

Sa Vo -- Sample volume of final solution.

Af -- Aliquot factor which accounts for dilutions made or aliquots taken during assay procedure.

Pk A/Ht -- Peak heights of isoxaben or metabolite peak measured manually in cm; occasionally measured by computer in millivolts.

mcg/ml -- Concentration of final solution in mcg/mL as determined from the standard curve.

Rslt Type -- Units of result. Samples in lb/A; recoveries in ppm.

Description -- "Direct Standard" is a vial of standard isoxaben or metabolite which is injected periodically to monitor the performance of the HPLC system during a run. It is not used in the calculations. "System Recovery" is control plus standard. These results are used to correct the sample results and are listed below under standard recovery.

APPENDIX F (cont'd).

Instrument Conditions -- These were set up for GC runs, not HPLC runs; therefore:

GC Column = HPLC Column
Oven Temp ØC = Ambient Temp.
Attenuation N254 = Wavelength 254 nm.
GC Analyst = HPLC analyst

Other entries should be self-explanatory.