

Cover Sheet for

ENVIRONMENTAL CHEMISTRY METHOD

Pesticide Name: Spinosad (Tracer)

MRID #: 440451-02

Matrix: Sediment

Analysis: Immunoassay

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Determination of Residues of Spinosad in Sediment Using a Magnetic Particle-Based Immunoassay Test Kit

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A. Scope

This method is applicable for the determination of residues of spinosad in sediment samples over the concentration range of 50-350 ng/g with a validated limit of quantitation of 50 ng/g and a limit of detection of 20 ng/g. The range can be extended for analysis of samples containing higher concentrations of spinosad by dilution of a sample aliquot into the working range of the assay.

The antibody used to develop the spinosad immunoassay test kit is sensitive to several spinosyns as well as some metabolites and degradates (Table I, Figure 1). The method is not capable of differentiating individual spinosyns. Therefore, the concentrations measured using this method should be expressed without reference to specific factors.

The method is based upon use of the Ohmicron Spinosad RaPID Assay® test kit and RPA-1 RaPID Analyzer™.

B. Principle

Residues of spinosad are extracted from sediment with an alkaline methanol extraction solvent. The extract is diluted in Spinosad Sample Diluent and assayed for spinosad using the Ohmicron Spinosad RaPID Assay kit. The kit applies the principles of enzyme-linked immunosorbent assay (1,2) to the determination of residues of spinosad in sediment samples. An aliquot of the diluted sample is pipeted into a disposable test tube. Enzyme-conjugated spinosad and paramagnetic particles coated with antibodies specific to spinosad are then sequentially added to the tube and the mixture is incubated. During the incubation period, the spinosad and the enzyme-conjugated spinosad compete for antibody sites on the magnetic particles. At the end of the incubation period, a magnetic field is applied to the particles. The spinosad and enzyme-conjugated spinosad, which is bound to the antibodies on the particles, are held in the tube by the magnetic field while the unbound reagents are decanted. After decanting, the particles are washed to remove unbound enzyme conjugate. The presence of spinosad is detected by incubating the antibody-bound enzyme conjugate with an enzyme substrate (hydrogen peroxide) and a chromogen (3,3',5,5'-tetramethylbenzidine; TMB), generating a colored product. After this incubation period, the reaction is stopped and stabilized by the addition of acid.

Since the enzyme-labeled spinosad is in competition with the unlabeled (sample) spinosad for the antibody sites, the level of color development is inversely proportional to the concentration of spinosad in the sample (i.e., lower residue concentrations result in greater

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color development). The absorbance at 450 nm is measured in each tube using the RPA-1 RaPID Analyzer. Quantitation of residues of spinosad is accomplished through generation of a standard curve. The standard curve is constructed using linear regression after a ln/logit transformation of the concentration and absorbance values, respectively. The spinosad concentration in unknown samples is calculated from the regression equation using the preprogrammed software capabilities of the RPA-1 RaPID Analyzer.

C. Safety Precautions

1. Each analyst should be acquainted with the potential hazards of the reagents and products used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE: MATERIAL SAFETY DATA SHEETS, PRODUCT LITERATURE, AND OTHER RELATED DATA. Safety information on non-DowElanco products should be requested from the supplier. Disposal of reagents must be in compliance with local, state, and federal laws and regulations.
2. Avoid contact of Stopping Solution (0.5% sulfuric acid) with skin and mucous membranes. Wear protective clothing and safety glasses when working with this material. If this reagent comes in contact with skin, flush the exposed area with water.

D. Equipment (Note M.1.)

1. Balance, analytical, Mettler Model Number AE50, catalog number 01-909-402, Fisher Scientific, Pittsburgh, PA 15238.
2. Balance, portable, Ohaus Model Number CT600, catalog number 01-920-57, Fisher Scientific.
3. Centrifuge, Beckman Model Number GS-6 containing a GH 3.8 horizontal rotor, Beckman, Arlington Heights, IL 60004-1489.
4. Magnetic Separator Rack and Base, 60 position, catalog number A00004, Ohmicron Environmental Diagnostics Corp, Newtown, PA 18940.
5. Mixer, Vortex-Genie, catalog number 12-812, Fisher Scientific.
6. Photometer, fixed wavelength spectrophotometer RPA-1RaPID Analyzer, catalog number A00003, Ohmicron Environmental Diagnostics Corp.
7. Pipetter, Eppendorf, tri-volume, (100 μ L, 200 μ L, 250 μ L), catalog number 21-278-38, Fisher Scientific.
8. Pipetter, Eppendorf, 200-1000 μ L, catalog number 21-381-204, Fisher Scientific.
9. Pipetter, Eppendorf, repeater, catalog number 21-380-8, Fisher Scientific.
10. Shaker, variable-speed reciprocating, with box carrier, model 6000, Eberbach Corporation, Ann Arbor, MI 48106.
11. Timer, minutes and seconds with alarm, catalog number 14-649-14, Fisher Scientific.
12. Ultrasonic bath, Model FS14, catalog number 15-336-4, Fisher Scientific.
13. Water purification system, Milli-Q UV, Millipore Corporation, Bedford, MA 01730.

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E. Glassware and Materials (Note M.1.)

1. Caps, PTFE-lined, size 24-400, catalog number 02-883-3F, Fisher Scientific.
2. Combitips for repeater pipet, 12.5-mL, catalog number 21-380-8C, Fisher Scientific.
3. Culture tubes, disposable glass, 16 x 100 mm, catalog number 14-962-10B, Fisher Scientific.
4. Pipet tips, Eppendorf disposable, 0.1-1.0 mL catalog number 21-372-4, Fisher Scientific.
5. Pipets, 10-mL disposable, catalog number 13-678-31J, Fisher Scientific.
6. Pipets, 5-mL disposable, catalog number 13-678-25D, Fisher Scientific.
7. Pipets, 1-mL disposable, catalog number 13-678-25B, Fisher Scientific.
8. Vials, 11-dram glass, catalog number 03-339-5C, Fisher Scientific.

F. Reagents and Chemicals (Note M.1.)

1. Reagents

- a. Methanol, ChromAR HPLC grade, Mallinckrodt Specialty Chemicals, Paris, KY 40361.
- b. Sodium chloride, crystalline, catalog number 5640-500, Fisher Scientific.
- c. Sodium hydroxide, 1 N, catalog number SS266-1, Fisher Scientific.
- d. Spinosad RaPID Assay 100 Tube Kit, catalog number A00178, Ohmicron Environmental Diagnostics Corp. (Note M.2).

Kit contents include (Note M.3.):

- (1) Spinosad Antibody, coupled to paramagnetic particles
- (2) Enzyme Conjugate
- (3) Standards (i.e., calibration standards)
- (4) Quality Control Sample
- (5) Diluent/Zero Standard
- (6) Color Solution
- (7) Stopping Solution
- (8) Washing Solution
- (9) Test Tubes

- e. Spinosad Sample Diluent, catalog number A00180, Ohmicron Environmental Diagnostics Corp.

f. Standard

- (1) Spinosad technical grade active ingredient (TGAI) analytical standard. Obtain from Test Substance Coordinator, DowElanco, 9330 Zionsville Rd., Indianapolis, IN 46268.

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2. Prepared Solutions

a. 5% Sodium chloride (w/v):

Weigh 50 grams of NaCl, dispense into a 1-L flask. Add 1 L Milli-Q water using a graduated cylinder. Swirl flask to mix well.

b. Alkaline methanol extraction solution (methanol: 5% NaCl: 1 N NaOH solution at a ratio of 65:27:8):

For 1 L, add 650 mL of methanol, 270 mL of 5% NaCl from G.I., and 80 mL 1 N NaOH to a reagent bottle and mix well.

G. Instrument Settings

To obtain results from the Ohmicron Spinosad RAPID Assay use the following parameter settings on the RPA-1 RAPID Analyzer:

Parameter	RPA-1 Abbreviation	Setting
Protocol Name	Protocol Name	105PG/ML
Data Reduction	Data Reduct	Linear Regression
Transformation	Xformation	Ln/LgtB
Number of Calibrators	# of Calibrator	4
Number of Calibrator Replicates	# of Repts:	2
Calibrator #1 Concentration	Cal #1 Conc	0.00
Calibrator #2 Concentration	Cal #2 Conc	50.0
Calibrator #3 Concentration	Cal #3 Conc	250
Calibrator #4 Concentration	Cal #4 Conc	1000
Minimum Correlation	Correlation Flag	0.990
Maximum Concentration (pg/mL)	Normal Range Hi	1200
Minimum Concentration (pg/mL)	Normal Range Low	20.
Number of Controls	# of Controls	1
Number of Control Replicates	Ctrl Replicates	2
Number of Reagent Blanks	# Rgt Blk	0
Wavelength	Wavelength	450 nm
Read Mode	Read Mode	Absorbance
Units		Units pg/mL
Precision of Calibrators	Rep %CV Flag	100

H. Preparation of Fortification Stock Solutions

1. Dissolve 0.0500 g of spinosad TGA1 in methanol in a 100 mL volumetric flask. Dilute to volume with methanol to obtain a 500 µg/mL solution. Store in the dark when not in use.
2. Dilute 1.0 mL of the above 500 µg/mL spinosad solution with methanol in a 100-mL volumetric flask to obtain a 5.0 µg/mL stock solution. Store in the dark when not in use.

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I. Determination of Spinosad in Sediment

1. Preparation of Recovery Samples

- a. Prepare the following fortification stock solutions in methanol. Begin by using the 5.0 µg/mL spinosad solution described in H.2.

Stock Solution Concentration, µg/mL	Stock Volume, mL	Methanol Volume, mL	Final Volume, mL	Final Concentration, ng/mL
5.0	3.5	6.5	10.0	1750
5.0	2.0	8.0	10.0	1000
5.0	2.0	18.0	20.0	500 ^a
0.500	5.0	5.0	10.0	250
0.500	2.0	8.0	10.0	100

^a Use to prepare the 250 ng/mL and the 100 ng/mL fortification solutions.

- b. Use the fortification stock solutions from f.1.a. for preparation of the fortified sediment samples as follows.

Stock Solution Concentration, ng/mL	Stock Volume, mL	Sample Weight, g	Final Concentration, ng/g
100	1.0	5	20
250	1.0	5	50
500	1.0	5	100
1000	1.0	5	200
1750	1.0	5	350

Thoroughly mix the samples after fortification. The samples should be prepared on the day of use.

2. Sample Extraction Procedure

- Weigh a 5.00 ± 0.05 g sample into an 11-dram glass sample vial.
- Add 15 mL of the alkaline methanol extraction solution to each vial. Seal the vial with a PTFE-lined cap.
- Vortex the sample approximately 1 minute or until the extraction solvent and sediment are well mixed.
- Sonicate the sample vial for a minimum of 2 minutes.
- Shake the sample for a minimum of 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.
- Centrifuge each sample vial 1-2 minutes at approximately 2500 rpm.
- Decant the extract by gently pouring into another 11-dram sample vial. Avoid disturbing the packed sediment.

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- h. Repeat steps J.2.b.-J.2.f. an additional time. Combine the second extraction volume with the original decanted volume.
- i. **Critical step:** spinosad adheres to glass and plastic surfaces. Spinosad Sample Diluent described in F. 7. must be used to perform the following dilutions. Perform a 1:60 dilution of each combined extract as follows:
 - (1) Pipet 5 mL of sample diluent into a 16 x 100 culture tube.
 - (2) Add 1 mL of sample extract to the tube and vortex. This is a 1:6 dilution.
 - (3) Pipet 4.5 mL of sample diluent into a second 16 x 100 culture tube.
 - (4) Add 0.5 mL of the 1:6 diluted sample to the tube and vortex. This is a 1:10 dilution for a final sample dilution of 1:60. This is the sample to be assayed as described in I.4. below.
- j. If the sample contains greater than 350 ng/g of spinosad, perform an additional 1:10 dilution following steps I.2.I.3 and I.2.I.4, then reassay the diluted sample.

3. Reagent Preparation

Remove all samples and kit reagents from storage and allow them to equilibrate to room temperature for a minimum of 30 minutes.

4. Assay Procedure

Each test is conducted in an individual test tube. Duplicate analyses of a sample or standard constitutes two tests. A standard curve and the appropriate control and recovery samples must be included in each analytical batch. For further details consult the insert for the Ohmicron Spinosad RaPID Assay kit (Appendix A).

- a. Turn on the RPA-I Photoanalyzer at least 30 minutes prior to measuring absorbance in the completed assay.
- b. Label test tubes for standards, controls and samples and place the tubes in the proper rack position. Be sure that the rack is removed from the Magnetic Separator.
- c. **Critical step:** Accurate delivery of the correct sample volume and proper pipeting technique in this step are critical to obtaining accurate and precise data. Add 0.20 mL of the standard, quality control, or sample to each test tube using an Eppendorf pipetter. Pipet each sample or standard directly to the bottom of the tube, avoid liquid adhering to the sides of the test tube. Use a fresh pipet tip for each standard and sample.
- d. Using an Eppendorf repeater pipet equipped with a 12.5-mL combitip, add 0.25 mL (Dial Setting =1) of Enzyme Conjugate down the inside wall of each tube.
- e. Thoroughly mix the Antibody Coupled Paramagnetic Particles by swirling the bottle. Avoid vigorous shaking and foaming.
- f. Using a repeater pipet equipped with a 12.5-mL combitip, add 0.50 mL (Dial Setting =2) of the Antibody Coupled Paramagnetic Particles down the inside wall of each tube.
- g. When dispensing of the magnetic particles is completed, mix the samples by either gently vortexing (Vortex setting = 3-4) each tube for 1-2 seconds or by gently swirling the entire rack for a few seconds.

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- h. Incubate for 30 minutes at room temperature.
- i. After the incubation period, combine the rack and the magnetic base. Seat all tubes by pressing them into the base. Allow 2 minutes for the particles to separate.
- j. Do not separate the tube rack from the magnetic base. Using a smooth motion, invert the combined rack assembly over a collection container and pour out the tube contents. Keep the rack inverted and gently blot the test tube rims on several layers of paper towels.
- k. Using a repeater pipet equipped with a 12.5 mL combitip, add 1 mL (Dial Setting = 4) of Washing Solution to each tube. Wait 2 minutes. Decant the Washing Solution into an appropriate collection container using the technique described in Step I.4.j.
- l. Repeat Step I.4.k.
- m. Remove the tube rack from the magnetic separator then add 0.50 mL of Color Solution using a repeater pipet equipped with a 12.5 mL combitip (Dial Setting = 2).
- n. Gently vortex each tube for 1 to 2 seconds.
- o. Incubate for 20 minutes at room temperature. During this incubation, dispense approximately 1 mL of Washing Solution into a clean tube for use as an instrument blank.
- p. Add 0.5 mL of Stopping Solution to each tube using a repeater pipet (Dial Setting = 2) at the end of the incubation period.
- q. Analyze each tube using the RPA-1 RaPID Analyzer within 10 minutes after adding the Stopping Solution.

5. Operating Procedure for the RPA-1 RaPID Analyzer

The RPA-1 RaPID Analyzer is pre-programmed with the protocols for several RaPID Assay procedures. The following steps describe how to set up and run the analyzer to measure absorbance in the tubes for the Ohmicron Spinosad RaPID Assay.

- a. Switch on the instrument and allow it to warm up at least 30 minutes prior to use. The RPA-1 RaPID Analyzer will perform a self test. If all parameters are satisfactory, the "SELECT COMMAND" prompt will appear.
- b. At the "SELECT COMMAND" prompt press "RUN".
- c. At the "RUN PROTOCOL" prompt, scroll through the protocols using the arrow keys until "10SPG/ML" appears. Press "ENTER".
- d. At the "SPL. REPLICATES" (sample replicates) prompt, press "2" to indicate the number of replicates for each sample, then press "ENTER".
- e. At the "BLANK TUBE/INSERT TUBE" prompt, insert the tube with 1 mL of Washing Solution. The display will briefly read "EVALUATING TUBE" then "REMOVE TUBE" and the instrument will produce an audible beep indicating that the absorbance has been measured. After hearing the beep remove the tube.
- f. At the "CAL. #1 REP. #1/INSERT TUBE" prompt, insert the first replicate of the first standard/calibrator (0.00 µg/L). Remove the tube after the beep.
- g. Follow the prompts on the instrument display until all of the standards have been measured. The tube order is important. The RPA-1 RaPID Analyzer has been

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- programmed to evaluate the standards in ascending order, in duplicate, starting with 0.00 µg/L.
- h. After all the standards have been evaluated, the instrument will report the equation of the line, the transformed data and the standards data.
- i. Insert the control tubes, at the "CNTRL. #1 REP. #1" and "CNTRL. #1 REP. #2" prompts. The instrument will report the calculated values for each replicate of the control sample.
- j. Evaluate the results for the standard curve and the control samples. At the "EDIT CALIBRATORS YES/NO" prompt press "NO".
- k. At the "SPL. #1 REP #1/INSERT TUBE" prompt insert the first sample tube. Remove the tube after the beep.
- l. Continue sample analysis following the prompts on the instrument display. Press "STOP" after all the samples have been evaluated and the results have been reported by the RPA-1 RaPID Analyzer.

J. Calculations

1. Standard Curve

The RPA-1 RaPID Analyzer contains preprogrammed data reduction capabilities which calculates a standard curve for each analytical batch using the absorbances of the batch standards. The calibration curve is constructed by linear regression after performing a $\ln(C)/\text{Logit}(B/B_0)$ data transformation of the concentration and absorbance values respectively.

The regression equation is :

$$\text{Logit} \frac{B}{B_0} = \text{slope} * \ln(\text{Conc}) + Y \text{ intercept}$$

Where:

$$\text{Logit} \frac{B}{B_0} = \ln \frac{B/B_0}{1 - B/B_0}$$

- B = the absorbance measured at a specific spinosad concentration
- B₀ = the absorbance measured for the 0.00 ng/mL standard
- Conc = the spinosad concentration of the standard

Example: Calculation of the standard curve from Analytical Set 102894ADLY.

Concentration, µg/mL (Conc)	ln (Conc)	Absorbance, 450 nm	B/B ₀	Logit (B/B ₀)
0.00	NA*	1.256	NA	NA
50	3.91	1.004	0.7994	1.383
250	5.52	0.638	0.5080	-0.032
1000	6.91	0.338	0.2691	-0.999

*NA = Not Applicable.

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Using the $\ln(\text{conc})$ and $\text{Logit}(B/B_0)$ values calculated above in a linear regression, the results are:

Slope = -0.796
Y Intercept = 4.471

The results are presented graphically in Figure 2.

2. Calculation of Spinosad in Samples

- a. The RPA-1 RaPID Analyzer will calculate a predicted concentration of spinosad in each unknown sample using the preprogrammed data reduction parameters. It will report the absorbance value and calculated spinosad concentration for each replicate of each sample tube as well as the mean absorbance, the mean spinosad concentration and the percent coefficient of variation of the duplicate measurements for each sample. The mean values are the final result for each sample.

To calculate the concentration of spinosad in a sample use the following equation:

$$\text{Concentration} = e^a$$

where:

$$a = \left(\frac{\text{Logit} \frac{B}{B_0} - \text{Y intercept}}{\text{slope}} \right)$$

Example:

Sample fortified at 200ng/g, rack positions 33 and 34, from analytical set 102894ADLY:

Mean absorbance (450 nm) = 0.524

$$\frac{B}{B_0} = \frac{0.524}{1.256} \\ = 0.417$$

$$\text{Logit} \frac{B}{B_0} = \ln \frac{0.417}{1-0.417} \\ = \ln(0.715) \\ = -0.335$$

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$$\text{Concentration} = e^{\left(\frac{-0.335 - 4.471}{-0.796} \right)}$$

= 0.42 ng/mL (in diluted extract)

Final Concentration = Measured Concentration x Method Factor

Final Concentration (ng/g) = 0.42 ng/mL x 360 mL/g

Final concentration = 151.3 ng/g

b. The following information may appear as part of the data report:

"x.xx nd" (i.e., 0.04nd) indicates that the calculated concentration is below the LOD. The result should be reported "not detected" and the limit of detection should be noted.

"nd" indicates the absorbance measured is greater than or equal to the absorbance of the 0.00 ng/mL standard; therefore, a concentration cannot be calculated. The result should be reported as "not detected" and the limit of detection should be noted.

"x.xx HI" (i.e., 1.31 HI) indicates that the calculated value exceeded the upper range of the assay. The sample should be diluted 1:10 with Spinosad Sample Diluent then re-analyzed.

3. Calculation of the Method Factor of Diluted Samples

Method Factor = $\frac{\text{Total Volume of Extraction Solvent (mL)}}{\text{Weight of Sample (g)}} \times \text{Final Dilution Factor}$

Example: $\frac{30 \text{ mL}}{5 \text{ g}} \times 60 = 360 \text{ mL/g}$

4. Calculation of the Final Concentration in Diluted Samples

Final Concentration (ng/g) = Measured Concentration (ng/mL) x Method Factor (mL/g)

5. Calculation of Percent Recovery

The percent recovery for the quality control sample or for other fortified samples is calculated as follows:

$$\% \text{ Recovery} = \frac{\text{Calculated Concentration} - \text{Blank Concentration}}{\text{Fortified Concentration}} \times 100$$

K. Quality Control

1. Analytical Batch Definition

An analytical batch of samples is defined as a group of 60 tubes. The size of the batch is based on the capacity of the magnetic separator rack. An analytical batch of less than 60 tubes can be analyzed. The first 10 tubes (rack positions 1-10) are used for

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duplicate analysis of the four standards and a quality control solution. Following the quality control solution, up to 25 samples (recovery or study samples) may be analyzed in duplicate (two tubes). If more samples are to be analyzed than can be accommodated in one rack, the remaining samples should be analyzed as a distinct analytical batch with a new standard curve, control and recovery samples.

2. Quality Control Solution

A quality control solution containing 0.50 ng/mL of spinosad should be analyzed at the beginning of every batch of samples (rack positions 9 and 10). The quality control sample should be treated in the same manner as all other samples. Use the results obtained from analysis of the quality control solution to determine whether the assay was properly executed (see K.4.). Additional recovery samples should be analyzed to further ensure proper execution of the method.

3. Study Samples

All study samples should be analyzed in duplicate. If the concentration of spinosad exceeds the range of the assay, dilute the sample by a 1:10 dilution (e.g., 1.0 mL sample + 9.0 mL Spinosad Sample Diluent) then perform the analysis on the diluted sample aliquot. Multiply the result by the method factor to obtain the final result (see K.4.).

4. Criteria for Acceptance of an Analytical Batch

The correlation coefficient for the linear regression of the calibration curve should be greater than 0.990. The coefficient of variation should be less than 10% for each duplicate pair of standards and the quality control sample. The recovery value for the quality control sample should be within $\pm 20\%$ of the expected value (i.e., 0.4-0.6 ng/mL). If the data fail to meet these performance criteria, the analyst should evaluate the results, determine the potential source of the variation and, if necessary, repeat the analysis. Calibration and quality control data generated during validation of this method are tabulated and summarized in Table II.

5. Modifications and Potential Problems

No modifications to the assay procedure are recommended. This procedure is for use on sediment samples generated during an aquatic microcosm dissipation study. Validation of the method for analysis of other sediment or soil would be required prior to implementing this method for sample analysis.

6. Interferences

Thirty pesticides, sixteen inorganic compounds and eight additional organic compounds were tested for the potential to interfere with conjugate binding in the assay (3) (Table III). The only compound which exhibited an I_{50} concentration below 10 mg/mL was carbendazim ($I_{50} = 5.56$ mg/mL). The I_{50} concentration is the concentration which results in a 50% inhibition of conjugate binding to the available antibodies. In comparison, the I_{50} for Spinosyn A is approximately 0.0003 mg/mL(1).

7. Specificity/Sensitivity

Several factors, metabolites and degradates of spinosad have been tested to determine whether the Ohmicron Spinosad RaPID Assay test kit will detect their presence in a water sample. In general, the assay is sensitive ($I_{50} < 2$ ng/mL) to factors which have little or no modification to the trimethylpyranosyl ring. The assay is relatively insensitive ($I_{50} > 50$ ng/mL) to factors or degradates in which the trimethylpyranosyl

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ring portion of the molecule has been modified or is missing. One exception is that the assay is sensitive to spinosyn K (Table I, Figure 1). The level of sensitivity is not equivalent for all factors. Thus, when a mixture of spinosads and degradates are present in a sample, the assay response generated by the individual spinosyns is not additive.

L. Results and Discussion

1. Method Validation

a. Accuracy

Data used to calculate the mean recoveries for an estimate of method accuracy are presented in Table IV. The mean recoveries for sediment samples fortified with spinosad at several levels between 50 ng/g and 350 ng/g ranged from 74-78% (Table V).

b. Precision

Data used to calculate the recoveries for the estimation of method precision are presented in Table IV. The relative standard deviation of recoveries calculated for sediment samples fortified at several concentrations between 50 ng/g and 350 ng/g ranged from 1.4-10.4% (Table V).

c. Limits of Detection and Quantitation

Following published procedures (3), the calculated limit of quantitation (LOQ) for this method is 34 ng/g. This limit is based on 10 times the standard deviation (10s) of the results from analysis of 20 samples fortified at the proposed LOQ of 50 ng/g of spinosad TGAI (Table VI). The calculated LOQ supports the targeted LOQ of 50 ng/g.

In a similar fashion, the calculated limit of detection (LOD) for this method is 10 ng/g, based on 3 times the standard deviation (3s) from the analysis of samples fortified with 50 ng/g of spinosad TGAI (Table VI). In order to verify the target LOD (20 ng/g), five sediment samples were fortified with 20 ng/g of spinosad and analyzed using the procedure described above. Residues of spinosad were measured in all five samples.

d. Extraction Efficiency

To determine the extraction efficiency for aged residues of spinosad in pond sediment, a control sediment sample was fortified with 200 ng/g each of ¹⁴C spinosyn A, B, and D. The sample was aged for 34 days in a freezer and then analyzed by both HPLC-UV and radiochemical analysis. The two analytical techniques gave nearly identical recovery results, which ranged from 90-100% of theory for all three analytes. The results for spinosyn A were 180 ng/g for both the HPLC-UV and radiochemical analyses; for spinosyn B, 200 ng/g from both techniques; and for spinosyn D, 190 ng/g by HPLC-UV and 180 ng/g by radiochemical analyses.

2. False Positive / False Negative Rate

Fifteen control samples were analyzed during the validation study. The samples included analyses over several days and by two analysts. There were no false positives. (False positive is defined as a measured concentration of spinosad at or above the target LOD.) (Table IV) Spinosad was detected in each of five samples fortified at the target LOD of 20 ng/g.

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3. Ruggedness

The accuracy and precision estimates (Table V) calculated from the data in Table IV were within acceptable ranges at all fortification levels. These estimates include data generated by two analyses on multiple days.

4. Confirmatory Method

The detection and/or quantitation of spinosyns, Factors A, B, D, and *N*-demethyl spinosyn, Factor D in sediment can be confirmed by HPLC.

5. Assay Time

The time required to analyze a typical analytical set (25 samples or recoveries, four standards and the quality control sample in duplicate, including the sample extraction) is approximately five hours.

6. Correlation to a Non-Immunochemical Method

A direct correlation between this method and the existing HPLC (4) methodology has not been performed. However, data from an aquatic microcosm study indicate that both methods yield comparable results for freshly fortified control or unaged samples. When analyzing aged samples, the immunochemical method results in a measurement of higher residue levels than the HPLC method. Some of the photolytic and anaerobic degradates of spinosad cross-react with the antibody that was used to develop the Ohmicron Spinosad RaPID Assay kit (Table I). The HPLC method was designed to detect spinosyns A and D and their major metabolites. The higher residue concentrations measured in aged sediment samples using the immunochemical method may be due to the detection of degradates which were not analyzed in the HPLC method.

M. Notes

1. Equipment, glassware, materials, reagents and chemicals equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are assumed to be readily available and are not listed here.
2. Only the Ohmicron Spinosad RaPID Assay test kit may be used for performing this method. Other test kits should not be substituted.
3. Refrigerate all kit reagents and sample diluent at 2-8 °C. Do not freeze. Reagents may be used until the expiration date printed on the labels. The test tubes require no special storage conditions and may be stored separately from the kit reagents.

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N. References

1. Tijssen, T. *Practice and Theory of Enzyme Immunoassays*, Elsevier Science Publishers: Amsterdam, 1985.
2. Meulenbergh, E.P.; Mulder, W.H.; Stoks, P.G. "Immunoassays for Pesticides", *Environ. Sci. Technol.* 1995, 29, 553-561.
3. Keith, L. H.; Crummet, W.; Deegan, J.; Libby, R. A.; Taylor, J. T.; Wentler, G., "Principles of Environmental Analysis", *Anal. Chem.*, 1983, 55:2210-2218.
4. West, S. D., "Determination of XDE-T05 and Metabolites in Soil and Sediment by High Performance Liquid Chromatography with Ultraviolet Detection", GRM 94.20, 1994, unpublished method of DowElanco.

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Table I. Sensitivity of the Spinosad Immunoassay Test Kit to Individual Spinosyns, Metabolites and Degradates of Spinosad

High Sensitivity (ISO < 2.0 ng/mL)	Low Sensitivity (ISO > 50 ng/mL)
Spinosyn A	Spinosyn H
Spinosyn B	Spinosyn J
Spinosyn C	Spinosyn L
Spinosyn D	Spinosyn A Reverse pseudoaglycone
Factor B of D	Spinosyn A Aglycone
Spinosyn E	
Spinosyn F	
Spinosyn K	
Spinosyn A Pseudoaglycone	

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Table III. Compounds Tested For the Potential to Interfere in the Spinosad RaPID Assay Test Kit

Pesticides	Organic compounds	Inorganic compounds
Alachlor	N-Acetylglucosamine	Calcium (chloride dihydrate)
Aldicarb	Aflatoxin B1	Copper (chloride)
Azinphos methyl	Aflatoxin G1	Iron (chloride hexahydrate)
Carbaryl	Humic acid	Magnesium (chloride hexahydrate)
Carbendazim	β -Lactose	Manganese (chloride)
Carbofuran	Methyl oleate	Mercuric (chloride)
Chlorothalonil	Polyoxin D	Nickel (sulfate hexahydrate)
Chlorpyrifos	L(+)-Rhamnose	Nitrate (sodium)
Chlorpyrifos methyl		Peroxide (hydrogen)
Cyanazine		Phosphate (sodium, heptahydrate)
2,4-D		Silicates (sodium meta-)
Dicamba		Sodium chloride
Dinoseb		Sulfate (sodium)
EPN		Sulfite (sodium)
Iprodione		Thiosulfate (sodium, pentahydrate)
Malathion		Zinc (chloride)
Metaxyl		
Methamidophos		
Methiocarb		
Methomyl		
Metribuzin		
Parathion		
Parathion methyl		
Phosmet		
Picloram		
Procymidone		
Propachlor		
Thiabendazole		
Triclopyr		
Vinclozolin		

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Table IV. Recovery of Spinosad From Sediment

Sample Number	Date of Analysis	Analytical Set	Spinosad, ng/g		Percent Recovery
			Added	Found	
14909804	27-Oct-1994	102794ADLY	0	ND ^a	..
14909804	27-Oct-1994	102794ADLY	0	ND	..
14909804	27-Oct-1994	102794ADLY	0	ND	..
14909804	27-Oct-1994	102794ADLY	0	ND	..
14909804	27-Oct-1994	102794ADLY	0	ND	..
14909804	28-Oct-1994	102894ADLY	0	ND	..
14909804	28-Oct-1994	102894ADLY	0	ND	..
14909804	28-Oct-1994	102894ADLY	0	ND	..
14909804	28-Oct-1994	102894ADLY	0	ND	..
14909804	28-Oct-1994	102894ADLY	0	ND	..
14909804	04-Nov-1994	110494ACAM	0	ND	..
14909804	04-Nov-1994	110494ACAM	0	ND	..
14909804	04-Nov-1994	110494ACAM	0	ND	..
14909804	04-Nov-1994	110494ACAM	0	ND	..
14909804	04-Nov-1994	110494ACAM	0	ND	..
14909804	27-Oct-1994	102794BDLY	20	<LOQ ^b	NA ^c
14909804	27-Oct-1994	102794BDLY	20	<LOQ	NA
14909804	27-Oct-1994	102794BDLY	20	<LOQ	NA
14909804	27-Oct-1994	102794BDLY	20	<LOQ	NA
14909804	27-Oct-1994	102794BDLY	20	<LOQ	NA
14909804	27-Oct-1994	102794BDLY	50	36.9	74
14909804	27-Oct-1994	102794BDLY	50	41.8	84
14909804	27-Oct-1994	102794BDLY	50	39.3	79
14909804	27-Oct-1994	102794BDLY	50	40.8	82
14909804	27-Oct-1994	102794BDLY	50	40.5	81
14909804	27-Oct-1994	102794BDLY	50	35.8	72
14909804	27-Oct-1994	102794BDLY	50	43.6	87
14909804	27-Oct-1994	102794BDLY	50	39.4	79
14909804	27-Oct-1994	102794BDLY	50	41.6	83
14909804	27-Oct-1994	102794BDLY	50	40.6	81
14909804	28-Oct-1994	102894ADLY	50	33.4	67
14909804	28-Oct-1994	102894ADLY	50	37.9	76
14909804	28-Oct-1994	102894ADLY	50	34.5	69
14909804	28-Oct-1994	102894ADLY	50	44.3	89
14909804	28-Oct-1994	102894ADLY	50	36.3	73

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Table IV. (Cont.) Recovery of Spinosad From Sediment

Sample Number	Date of Analysis	Analytical Set	Spinosad, ng/g		Percent Recovery
			Added	Found	
14909804	04-Nov-1994	110494ACAM	50	36.4	73
14909804	04-Nov-1994	110494ACAM	50	34.7	69
14909804	04-Nov-1994	110494ACAM	50	35.4	71
14909804	04-Nov-1994	110494ACAM	50	32.8	66
14909804	04-Nov-1994	110494ACAM	50	34.7	69
14909804	27-Oct-1994	102794ADLY	100	70.6	71
14909804	27-Oct-1994	102794ADLY	100	73.9	74
14909804	27-Oct-1994	102794ADLY	100	77.2	77
14909804	27-Oct-1994	102794ADLY	200	147	73
14909804	27-Oct-1994	102794ADLY	200	168	84
14909804	27-Oct-1994	102794ADLY	200	157	78
14909804	28-Oct-1994	102894ADLY	200	158	79
14909804	28-Oct-1994	102894ADLY	200	151	76
14909804	28-Oct-1994	102894ADLY	200	197	98
14909804	28-Oct-1994	102894ADLY	200	163	81
14909804	28-Oct-1994	102894ADLY	200	173	87
14909804	04-Nov-1994	110494ACAM	200	155	77
14909804	04-Nov-1994	110494ACAM	200	138	69
14909804	04-Nov-1994	110494ACAM	200	152	76
14909804	04-Nov-1994	110494ACAM	200	142	71
14909804	04-Nov-1994	110494ACAM	200	137	68
14909804	27-Oct-1994	102794ADLY	350	273	78
14909804	27-Oct-1994	102794ADLY	350	273	78
14909804	27-Oct-1994	102794ADLY	350	267	76

* ND = Not Detected.

b <LOQ = Less than the limit of quantitation of 50 ng/g.

c NA = Not Applicable.

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Table V. Summary of the Accuracy and Precision Results Generated During the Method Validation Study

Spinosad, ng/g Added	Total Samples	Mean	Spinosad Found, ng/g Range		SD ^a	RSD ^b	Average Recovery, %
			Low	High			
50	20	38.0	32.8	44.3	3.4	8.9	76
100	3	73.9	70.6	77.2	3.3	4.5	74
200	13	157	137	197	16	10	78
350	3	271	267	273	3.5	1.3	77

^a SD = Standard Deviation

^b RSD = Relative Standard Deviation

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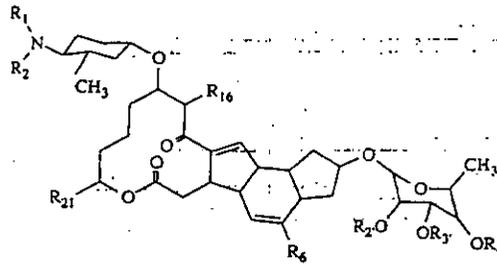
Table VI. Calculated Limits of Detection and Quantitation of Spinosad in Sediment

Sample Number	Analytical Set	Spinosad, ng/g	
		Added	Found
14909804	102794BDLY	50	36.9
14909804	102794BDLY	50	41.8
14909804	102794BDLY	50	39.3
14909804	102794BDLY	50	40.8
14909804	102794BDLY	50	40.5
14909804	102794BDLY	50	35.8
14909804	102794BDLY	50	43.6
14909804	102794BDLY	50	39.4
14909804	102794BDLY	50	41.6
14909804	102794BDLY	50	40.6
14909804	102894ADLY	50	33.4
14909804	102894ADLY	50	37.9
14909804	102894ADLY	50	34.5
14909804	102894ADLY	50	44.3
14909804	102894ADLY	50	36.3
14909804	110494ACAM	50	36.4
14909804	110494ACAM	50	34.7
14909804	110494ACAM	50	35.4
14909804	110494ACAM	50	32.8
14909804	110494ACAM	50	34.7
		\bar{x} =	38.0
		s =	3.4
		LOD ^a (3s) =	10.2
		LOQ ^b (10s) =	34.0
		n =	20

^aLOD = Calculated Limit of Detection.
^bLOQ = Calculated Limit of Quantitation.

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Spinosyn	R1	R2	R6	R16	R21	R2'	R3'	R4'
A	Me	Me	H	Me	Et	Me	Me	Me
B	H	Me	H	Me	Et	Me	Me	Me
C	H	H	H	Me	Et	Me	Me	Me
D	Me	Me	Me	Me	Et	Me	Me	Me
<i>N</i> -demethyl D	H	Me	Me	Me	Et	Me	Me	Me
E	Me	Me	H	Me	Me	Me	Me	Me
F	Me	Me	H	H	Et	Me	Me	Me
H	Me	Me	H	H	Et	H	Me	Me
J	Me	Me	H	H	Et	Me	H	Me
K	Me	Me	H	H	Et	Me	Me	H
L	Me	Me	Me	H	Et	Me	H	Me

Me = Methyl
 Et = Ethyl
 H = Hydrogen

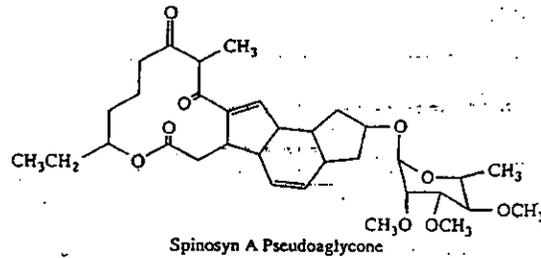
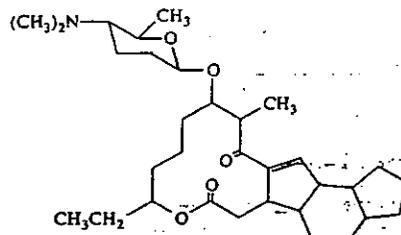


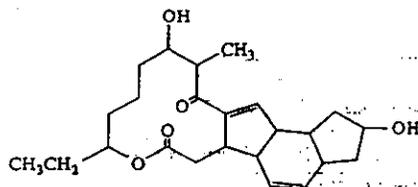
Figure 1. Structures of Some Spinosyns, Metabolites and Degradates

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Spinosyn A Reverse Pseudoaglycone



Spinosyn A Aglycone

Figure 1. (Cont.) Structures of Some Spinosyns, Metabolites and Degradates

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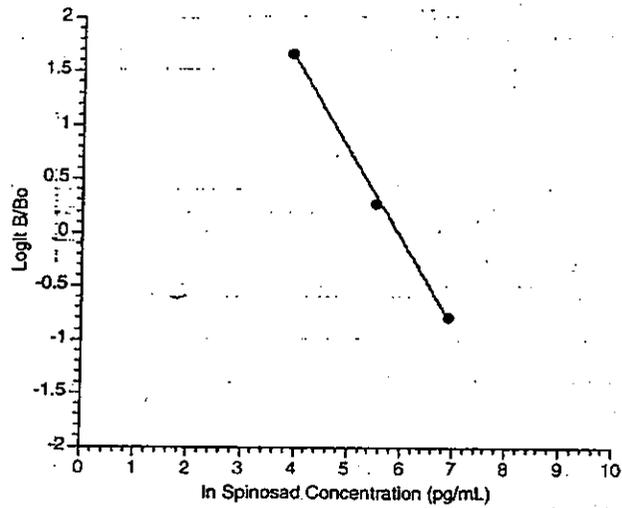


Figure 2. Standard Curve for Analytical Set 102894ADLY

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Appendix A
Spinosad RaPID Assay Kit-Insert

RaPID Assays®

Spinosad

• Intended Use

For detection of spinosad, a fluorescent labeled insect control agent composed primarily of spinosyn A and spinosyn D, in water (groundwater, surface water, well water), for soil and crop use refer to specific application labels.

• Principle

The Spinosad RaPID Assay® applies the principles of enzyme labeled immunosorbent assay (ELISA) to the determination of spinosad. The sample to be tested is added, along with an enzyme conjugate, to a disposable test tube, followed by paramagnetic particles with antibodies specific to spinosad attached. Both the spinosad (which may be in the sample) and the enzyme labeled spinosad (the enzyme conjugated complex for antibody binding sites on the paramagnetic particles). At the end of an incubation period, a magnetic field is applied to hold the paramagnetic particles (both spinosad and labeled spinosad) against the antibodies on the particles, in proportion to their original concentration in the tube and allow the unlabeled reagents to be discarded. After discarding, the particles are washed with Washing Buffer.

The presence of spinosad is detected by adding the enzyme substrate hydrogen peroxide and the chromogen 3,3',4,5'-tetramethylbenzidine. The enzyme-labeled spinosad reacting bound to the spinosad antibody catalyzes the conversion of the substrate chromogen mixture to a colored product. After an incubation period, the reaction is stopped and stabilized by the addition of acid. Since the labeled spinosad conjugated was in competition with the unlabeled spinosad (sample) for the antibody sites, the color developed is inversely proportional to the concentration of spinosad in the sample.

• Reagents

1. Spinosad Antibody-Coated Paramagnetic Particles
The spinosad antibody (both anti-spinosad) is covalently bound to paramagnetic particles, which are suspended in buffered saline with preservatives and stabilizers.

30 test kit: one 25 ml vial
100 test kit: one 85 ml vial

2. Spinosad Enzyme Conjugate
The horseradish peroxidase (HRP) labeled spinosad conjugate is diluted in buffered saline with preservatives and stabilizers.

30 test kit: one 10 ml vial
100 test kit: one 25 ml vial

3. Spinosad Standards
Three concentrations (0.05, 0.25 and 1.00 ng/ml) of spinosyn A standards in buffered saline with preservatives and stabilizers are supplied. Each vial contains 2.0 ml.

4. Control
A concentration (approximately 0.5 ng/ml) of spinosyn A in buffered saline with preservatives and stabilizers. A 2.0 ml volume is supplied in one vial.

5. DiacetZinc Standard
Buffered saline with preservatives and stabilizers without any detectable spinosad.

30 test kit: one 10 ml vial
100 test kit: one 25 ml vial

6. Color Solution
A solution of hydrogen peroxide and 3,3',4,5'-tetramethylbenzidine in an organic base.

30 test kit: one 20 ml vial
100 test kit: one 60 ml vial

7. Stopping Solution
A solution of sulfuric acid (0.5N).

30 test kit: one 20 ml vial
100 test kit: one 60 ml vial

8. Washing Buffer
Buffered saline containing preservatives and stabilizers.

30 test kit: one 70 ml vial
100 test kit: one 200 ml vial

9. Test Tubes
Polystyrene tubes (20) are packaged in a box.

30 test kit: one 30 tube box
100 test kit: three 30 tube boxes

• Reagent Storage and Stability

Store all reagents at 2-8°C. Do not freeze. Reagents may be used until the expiration date on the box. The test tubes require no special storage conditions and may be stored separately from the reagents to conserve refrigerator space.

Consult state, local and federal regulations for proper disposal of all reagents.

• Materials Required but Not Provided

In addition to the reagents provided, the following items are essential for the performance of the test:

Pipets* Precision pipet capable of delivering 200 and 1.0 ml, repeating pipet,
Water Meter* Thermally Stable Mix, Scientific Industries Water Meter, or equivalent

Magnetic Separation Rack* 96-Well Assay Rack or equivalent*
photometer capable of readings at 450 nm

* These items are available from Olinco.

• Sample Information

This procedure is recommended for use with water samples. Other samples may require modifications to the procedure and should be thoroughly validated.

Spinosad adheres to glass and plastic surfaces in aqueous solution. Water samples should be collected in glass vials with baffles cap break. Immediately upon collection, Water Stabilizer should be added at a 2:100 dilution (i.e., 1 ml per 100 ml sample), prior to splitting of samples, transfer to other containers or analysis to prevent adsorptive loss to the glass container.

Samples containing gross particulates matter should be centrifuged to remove particles.

Samples which have been preserved with monochloroacetic acid or other acids, should be neutralized with strong base e.g. 0N NaOH, prior to assay.

If the spinosad concentration of a sample exceeds 1 ng/ml, the sample is subject to repeat testing using a diluted sample. A ten-fold or greater dilution of the sample is recommended with an appropriate amount of DiacetZinc Standard. For example, in a separate test tube make a ten-fold dilution by adding 100 µl of the sample to 900 µl of DiacetZinc Standard. Mix thoroughly before assaying. Perform the assay according to the Assay Procedure and derive final results by multiplying the value obtained by the dilution factor e.g. 10.

The presence of the following substances up to 500 ppm were found to have no significant effect on Spinosad RaPID Assay results: copper, nickel, thiocarbates, sulfites, magnesium, calcium, mercury, neoprene and phosphates. Nitrate, iron, zinc, and peroxide were found to have no significant effect up to 100 ppm. In addition, basic acid concentrations up to 50 ppm, sodium chloride up to 1.0 M, silicate up to 1000 ppm and sulfate concentrations up to 10,000 ppm showed no effect on results.

• Reagent Preparation

All reagents must be allowed to come to room temperature and the antibody-coated paramagnetic particles should be mixed thoroughly before use.

• Procedural Notes and Precautions

As with all immunoassays, a consistent technique is the key to optimal performance. To obtain the greatest precision, be sure to treat each tube in an identical manner.

Add reagents directly to the bottom of the tube while avoiding contact between the reagents and the pipet tip. This will help assure consistent quantities of reagent in the test tubes.



