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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'tetramethyl-benzidine; 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

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 µL, 200 µL, 250 µL), catalog number 21-278-38, Fisher Scientific.

8. Pipetter, Eppendorf, 200-1000 µL, 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.

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.

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.1., 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-1RaPID 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 Reps: | 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 TGAI 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.

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 I.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

a. Weigh a 5.00 ± 0.05g sample into an 11-dram glass sample vial.

b. Add 15 mL of the alkaline methanol extraction solution to each vial. Seal the vial with a PTFE-lined cap.

c. Vortex the sample approximately 1 minute or until the extraction solvent and sediment are well mixed.

d. Sonicate the sample vial for a minimum of 2 minutes.

e. Shake the sample for a minimum of 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

f. Centrifuge each sample vial 1-2 minutes at approximately 2500 rpm.

g. Decant the extract by gently pouring into another 11-dram sample vial. Avoid disturbing the packed sediment.

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.

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 "105PG/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

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)/Logit ($B/B_o$) data transformation of the concentration and absorbance values respectively.

The regression equation is :

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

Where:

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

B = the absorbance measured at a specific spinosad concentration
$B_o$ = 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, pg/mL (Conc) | ln (Conc) | Absorbance, 450 nm | $B/B_o$ | Logit ($B/B_o$) |
|---|---|---|---|---|
| 0.00 | NA[a] | 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 |

[a]NA = Not Applicable.

Using the ln(conc) and 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:

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

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

$$= 0.417$$

$$\text{Logit}\frac{B}{B_0} = \ln\frac{0.417}{1-0.417}$$

$$= \ln(0.715)$$

$$= -0.335$$

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

= 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

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

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:

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

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

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 ± 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

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.

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.

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.

N. References

1. Tijssen, T. *Practice and Theory of Enzyme Immunoassays*; Elsevier Science Publishers: Amsterdam, 1985.

2. Meulenberg, 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.

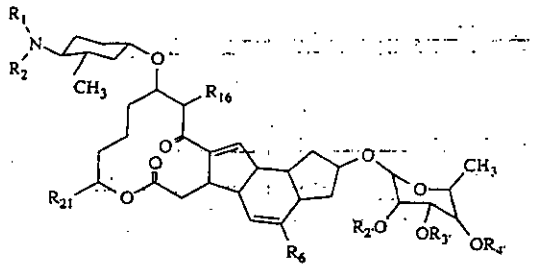
4. West, S. D., "Determination of XDE-105 and Metabolites in Soil and Sediment by High Performance Liquid Chromatography with Ultraviolet Detection", GRM 94.20, 1994, unpublished method of DowElanco.

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

| High Sensitivity ($I_{50}$ < 2.0 ng/mL) | Low Sensitivity ($I_{50}$ > 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 | |

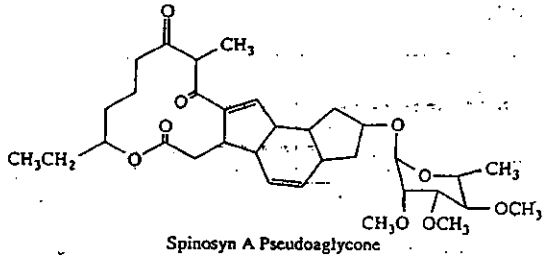
Table III. Compounds Tested For the Potential to Interfere in the Spinosad RaPID Assay Test Kit

| 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 | | Slufite (sodium) |
| Iprodine | | Thiosulfate (sodium, pentahydrate) |
| Malathion | | Zinc (chloride) |
| Metalaxyl | | |
| Methamidophos | | |
| Methiocarb | | |
| Methomyl | | |
| Metribuzin | | |
| Parathion | | |
| Parathion methyl | | |
| Phosmet | | |
| Picloram | | |
| Procymidone | | |
| Propachlor | | |
| Thiabendazole | | |
| Triclopyr | | |
| Vinclozolin | | |



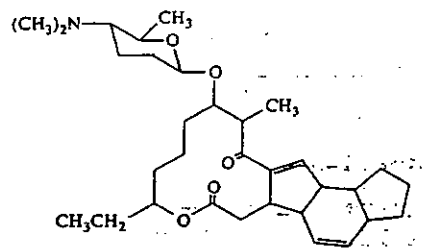
| Spinosyn | R1 | R2 | R6 | R16 | R21 | R2' | R3' | R4' |
|---|---|---|---|---|---|---|---|---|
| A | Me | Me | H | Me | Et | Me | Me | Me |
| B | H | Me | H | Me | Et | Me | Me | Me |
| C | H | H | H | Me | Et | Me | Me | Me |
| D | Me | Me | Me | Me | Et | Me | Me | Me |
| *N*-demethyl D | H | Me | 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

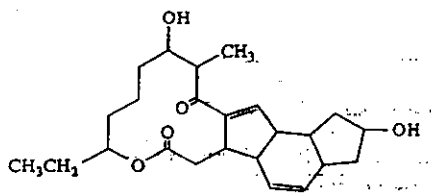


Spinosyn A Pseudoaglycone

Figure 1. Structures of Some Spinosyns, Metabolites and Degradates



Spinosyn A Reverse Pseudoaglycone



Spinosyn A Aglycone

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