Determination of Spinosad and Its Metabolites in Food and Environmental Matrices. 3. Immunoassay Methods

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Spinosad is an insect control agent that is derived from a naturally occurring soil bacterium and is effective on several classes of insects, especially Lepidopteran larvae. Spinosad is registered in many countries for use on a variety of crops, including cotton, corn, soybeans, fruits, and vegetables. Residue methods utilizing a magnetic particle-based immunoassay (IA) test kit have been developed and validated for determining spinosad in environmental and food matrices. These methods involve an extraction of the residues from the matrices with appropriate solvents. For some matrices, the sample extracts can be diluted and measured directly by IA without any cleanup. For other matrices, sample extracts are purified using liquid−liquid partitioning and/or solid phase extraction prior to measurement by IA. The methods determine the total residue of spinosad, which includes the active ingredients (spinosyns A and D) and several minor metabolites, including spinosyn B, spinosyn K, and *N*-demethylspinosyn D. The methods have validated limits of quantitation of 0.0001 μg/mL in water, 0.05 μg/g in sediment, and 0.010 μg/g in crops, crop processed commodities, and animal tissues. This paper briefly summarizes the residue methodology and method validation data for spinosad in 34 food, feed, and environmental matrices.

Keywords: *Spinosad; food; crops; soil; water; beef; milk; residue; analysis; immunoassay*

INTRODUCTION

Spinosad is a new insect control agent that is derived through the fermentation of a naturally occurring Actinomycetes bacterium, *Saccharopolyspora spinosa*. Spinosad comprises a mixture of spinosyns A and D and is the common name of the active ingredient that is present in Tracer Naturalyte, Success Naturalyte, Spin-Tor Naturalyte, and Conserve (all trademarks of Dow AgroSciences LLC) insect control products. These products are useful for the management of many insect pests, including caterpillars, thrips, flies, drywood termites, and some beetles.

Spinosad is registered in many countries on a variety of crops, including cotton, corn, soybeans, fruits, and vegetables (West et al., 2000). The efficacy, toxicity, and chemical and physical properties of the active ingredients have been presented (Sparks et al., 1995; Thompson et al., 1995).

Reliable analytical methods are an important aspect of monitoring pesticide residue levels to ensure human and environmental safety. Residue methods utilizing high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection have been previously reported for spinosad in cottonseed and cottonseed processed commodities (West, 1996), in soil, sediment, and water (West, 1997), in leafy vegetables, peppers, and tomatoes (Yeh et al., 1997), in meat, milk, cream, and eggs (West and Turner, 1998), in citrus crops and citrus processed commodities (West and Turner, 2000), and in numerous other crops and crop processed commodities (West et al., 2000). In addition, a method utilizing liquid chromatography with mass selective detection (LC-MS) has also been described for sample matrices that could not be analyzed by HPLC-UV (Schwedler et al., 2000).

Immunoassay (IA)-based kits are a relatively new development for pesticide residue analysis. Compared to traditional chromatographic methods, IA methods and test kits can provide highly sensitive and specific assays that are simple, rapid, and inexpensive. Thus, IA methods are ideal for instances in which large numbers of samples must be analyzed or the analysis by other techniques is difficult and/or expensive (Gabaldon et al., 1999). A potential disadvantage of IA methods is the difficulty of including multiple residue determinations. With spinosad, however, the immunoassay methods can be utilized as a screening technique to quickly determine the total residue of spinosad plus metabolites, whereas the HPLC methods can be used for confirmation of spinosad residues and/or for determining the residues of the individual analytes in selected samples. This approach effectively combines the strengths of both analytical techniques to obtain a maximum amount of information in the minimum amount of time.

Prior to developing residue methods for spinosad, studies were conducted using radiolabeled (^{14}C) material to determine the nature of the residue in crops, animal tissues, and environmental samples. These studies demonstrated that the two active ingredients in spinosad (spinosyns A and D) were not metabolized in cotton plants. However, the parent compounds were degraded or metabolized to spinosyn B and *N*-demethylspinosyn D in soil, water, and animal tissues. Spinosyns B and K and *N*-demethylspinosyn D were also identified as minor metabolites in crops (D. P. Rainey,

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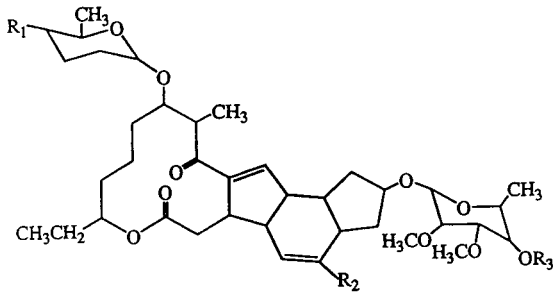


Spinosad and Metabolites

spinosyn A, $R_1 = N(CH_3)_2$, $R_2 = H$, and $R_3 = CH_3$
spinosyn D, $R_1 = N(CH_3)_2$, $R_2 = CH_3$, and $R_3 = CH_3$
spinosyn K, $R_1 = N(CH_3)_2$, $R_2 = H$, and $R_3 = H$
spinosyn B, $R_1 = NH(CH_3)$, $R_2 = H$, and $R_3 = CH_3$
N-demethyl spinosyn D, $R_1 = NH(CH_3)$, $R_2 = CH_3$, and $R_3 = CH_3$

Figure 1. Structures of spinosad and metabolites.

J. D. Magnussen, and D. F. Berard, Dow AgroSciences LLC, personal communication, 1994). The structures of spinosad and its metabolites are shown in Figure 1.

Following the nature of the residue studies with radiolabeled spinosad, several methods utilizing IA have been developed and validated for the determination of spinosad in 34 sample matrices. The methods involve an extraction of the analytes from the matrices with solvents or aqueous−organic solutions. The sample extracts for some sample matrices can be diluted and measured directly by IA without any sample purification. For other matrices, however, purification by liquid−liquid partitioning and/or solid phase extraction (SPE) is needed prior to immunochemical detection. The analytical methods for determining spinosad by IA determine the total residue, which includes the parent compounds and the metabolites.

This paper summarizes the residue methods and the method validation data for determining spinosad and its metabolites in the 34 different sample matrices by IA. A novel approach for removing chlorophyll-related interferences from leafy green crop extracts by the addition of bleach (sodium hypochlorite) is also reported. In addition, this paper presents correlation data for results obtained by IA and HPLC-UV.

EXPERIMENTAL PROCEDURES

Stabilization of Water Samples. Spinosad adsorbs from water samples onto the surface of plastic or glass containers. To prevent adsorption from decreasing the apparent concentration in water, samples were collected in glass containers, and Spinosad Water Stabilizer (Strategic Diagnostics, Inc., Newark, DE) was immediately added. The stabilizer was added at 0.5 mL/50 mL of water. However, because the stabilizer interferes with the HPLC-UV analysis of spinosad, a subsample was taken prior to the addition of the stabilizer in samples where both HPLC-UV and IA would be used.

Reagents. Triethylamine (TEA) was of reagent grade (Fisher Scientific, Pittsburgh, PA), and a new bottle of TEA was opened every two or three months to prevent the formation of contaminants. All other solvents and water were of HPLC grade. Nitrogen gas was 99.99% pure. The purified active ingredients used for analytical standards were obtained from the Test Substance Coordinator, Dow AgroSciences LLC, Indianapolis, IN. The immunoassay test kit was the Spinosad RaPID Assay 100 Tube Test Kit (Strategic Diagnostics, Inc.).

Standard Preparation. A standard stock solution was prepared at 100 μg/mL by weighing 10 mg of the spinosyn A purified reference material, quantitatively transferring to a 100-mL volumetric flask, dissolving in methanol, and diluting to volume. Fortification standard solutions for the determination of recovery were then prepared from the stock solution by performing appropriate dilutions with methanol.

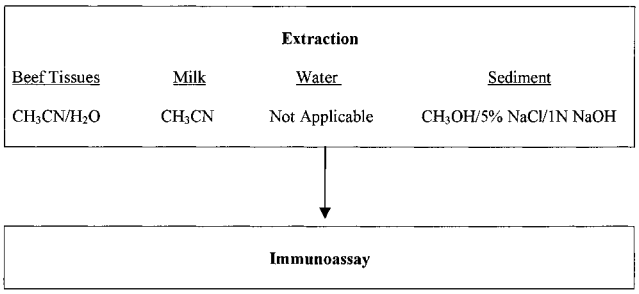


Figure 2. Flowchart showing the major steps for the immunochemical determination of spinosad in beef tissues, milk, water, and sediment.

The solutions for constructing the IA standard calibration curve were included in the Spinosad RaPID Assay Test Kit. The standard calibration curve solutions contained spinosyn A at concentrations of 0.05, 0.25, and 1.00 ng/mL.

Spinosyn A is the primary active ingredient in spinosad, typically comprising ~85% of the activity. Spinosyn A is also the major residue that occurs from the application of spinosad. In addition, spinosyn A is available as a highly pure reference compound. Because the composition of the commercial spinosad products varies with respect to the relative amounts of the other spinosyns present, it was not possible to obtain a standard containing a mixture of compounds that would always be representative of the commercial product or the residues present in various sample matrices. For these reasons, spinosyn A was used as the fortification and calibration standard. However, some control samples were also fortified with solutions containing technical grade spinosad, which contains both active ingredients (spinosyns A and D) and the minor metabolites and degradation products, to ensure that the IA method would determine the total residue.

Initial Sample Preparation and Storage. *(a) Sediment, Crops, and Animal Tissues.* Crop and animal tissue samples were diced with a knife or chopped with a cleaver. Sediment, crop, and animal tissue samples were frozen with liquid nitrogen and then ground through a hammer mill with a $^3/_{16}$-in. (48-mm) screen size (model 2001, Agvise Laboratories, Inc., Northwood, ND). After grinding, the samples were manually mixed in a plastic bag and then transferred to high-density polyethylene freezer cartons for storage at −15 to −20 °C.

(b) Water and Milk. Water and milk samples did not require sample preparation prior to being stored in a freezer or refrigerator.

The primary steps in the residue methods for determining spinosad and metabolites in the various sample matrices are summarized in the flowchart in Figures 2 and 3.

Sample Weighing and Fortification of Recovery Samples. *(a) Water.* Aliquots (50 mL) of the untreated control samples were transferred into a glass beaker, and fortified recovery samples were prepared by adding 1.0 mL of the appropriate fortification standard solution.

(b) Beef Tissues (Lean Meat, Liver, or Kidney). Untreated control beef tissue samples (20 g) were weighed into 250-mL boiling flasks, and fortified recovery samples were prepared by adding 1.0 mL of the appropriate fortification standard solution.

(c) Sediment, Milk, and Crops. Sediment, milk, or crop samples (5.0 g) were weighed into 40-mL clear glass vials (sediment or milk) or 250-mL clear glass bottles (crops). Fortified recovery samples were prepared by adding 0.5−1.0 mL of the appropriate fortification standard solution.

Sample Homogenization. *(a) Beef Tissues and Crops.* A 100-mL aliquot of 80% acetonitrile/20% water was added to each bottle, and the samples were blended for ~1−2 min using an Omni Mixer homogenizer (model 17105, Omni Interna-

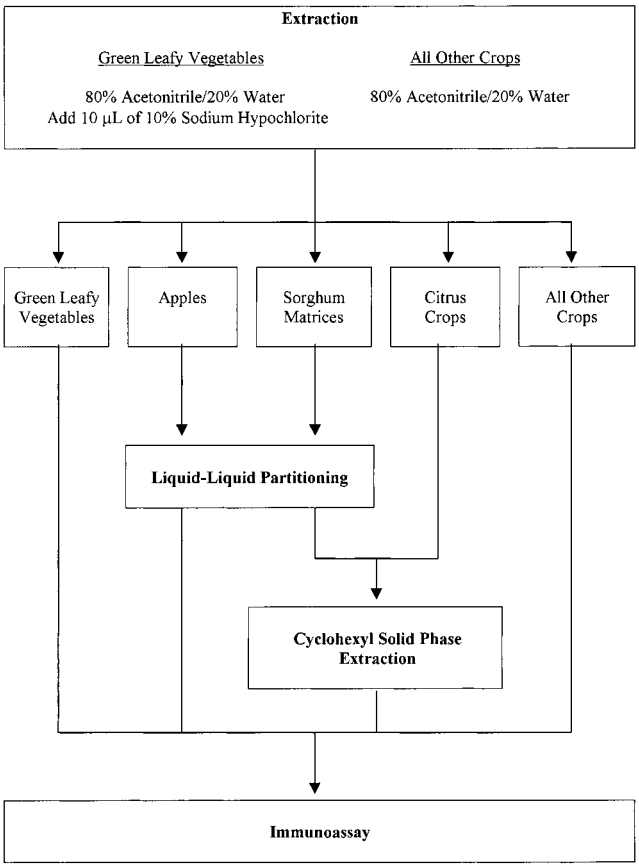


Figure 3. Flowchart showing the major steps for the immunochemical determination of spinosad in crops.

tional, Gainesville, VA). To prevent carry-over and cross-contamination of samples, the homogenizer probe was thoroughly cleaned between samples using a squirt bottle containing 80% acetonitrile/20% water. Additionally, the probe was immersed in a beaker containing 80% acetonitrile/20% water, and the homogenizer was run on medium speed for several seconds. Sample analysis was continued as described under Sample Extraction.

(b) Water, Milk, and Sediment. Water, milk, and sediment samples did not require homogenization.

Sample Extraction. *(a) Beef Tissues.* Approximately 2 g of boiling granules was added to the blended beef tissue samples, and the samples were refluxed for 1 h using heating mantles and chilled water reflux condensers. After refluxing, the flasks were capped and cooled to room temperature. The cooled supernatant extract was filtered through pleated filter paper (15-cm, Schleicher and Schuell, Keene, NH), and a 1.0-mL aliquot of the filtered extract was transferred to a 40-mL vial. Sample analysis for the beef tissues was then continued as described under Addition of Sample Diluent.

(b) Sediment. Sediment samples were extracted with an alkaline solution consisting of methanol/5% aqueous sodium chloride/1 N aqueous sodium hydroxide (65:27:8). The alkaline solution (15 mL) was added to the sample vial, which was then sealed with a PTFE-lined cap. The samples were mixed for 1 min with a vortex mixer (Vortex Genie, Fisher Scientific) and sonicated for at least 2 min in an ultrasonic bath (model FS14, Fisher Scientific). The samples were then shaken for at least 30 min on a reciprocating shaker (model 6000, Eberbach Corp., Ann Arbor, MI) at 180 excursions/min. Each vial was centrifuged for 1−2 min at 2500 rpm. The supernatant liquid was carefully decanted into a clean 40-mL glass vial. The soil extraction was then conducted a second time with another 15 mL of the alkaline solution, which was combined with the first 15-mL extract. Sample analysis for sediment samples was then continued as described under Addition of Sample Diluent.

(c) Apples and Sorghum. For apples and all of the sorghum matrices, the blended samples were extracted by sealing the sample bottle with a PTFE-lined lid and shaking on a reciprocating shaker at least 10 min at 180 excursions/min. The samples were centrifuged at 2000 rpm for 5 min, and 10.0-mL aliquots of the supernatant extracts were transferred to 125-mL separatory funnels for subsequent purification as described under Liquid−Liquid Partitioning.

(d) Citrus Crops. For citrus crops, the blended samples were extracted by sealing the sample bottle with a PTFE-lined lid and shaking on a reciprocating shaker for at least 10 min at 180 excursions/min. The samples were centrifuged at 2000 rpm for 5 min, and 5.0-mL aliquots of the supernatant extracts were transferred to a 40-mL vial. Water (5 mL) was added, and the solution was mixed with a vortex mixer prior to subsequent purification as described under Sample Purification by Cyclohexyl SPE.

(d) Other Crops. For all crops other than apples, sorghum, and citrus, the blended samples were extracted by sealing the sample bottle with a PTFE-lined lid and shaking on a reciprocating shaker for at least 10 min at 180 excursions/min. The samples were centrifuged at 2000 rpm for 5 min, and a 1.0-mL aliquot of the supernatant liquid was transferred to a 40-mL vial. For green leafy vegetables (mustard greens, celery, head lettuce, leaf lettuce, spinach, and tobacco), 10 μL of a 10% aqueous sodium hypochlorite solution (prepared fresh weekly) was added to the vial and mixed to bleach out interferences from chlorophyll. Sample analysis was then continued as described under Addition of Sample Diluent.

(e) Milk. Milk samples were extracted by adding 20 mL of acetonitrile, sealing the vial with a PTFE-lined cap, and shaking for at least 30 min at 180 excursions/min. A 1.0-mL aliquot of the supernatant acetonitrile extract was transferred to a 40-mL vial. (The protein portion of the milk was coagulated in the acetonitrile.) Analysis of the milk samples was then continued as described under Addition of Sample Diluent.

(f) Water. Water samples did not require extraction, and analysis was continued as described under Addition of Sample Diluent.

Sample Purification by Liquid—Liquid Partitioning. *(a) Sorghum Grain, Forage, Fodder, and Grain Dust.* Dichloromethane (10 mL) and water (5 mL) were added to the 125-mL separatory funnel containing the 10.0-mL aliquot of the sorghum grain, forage, fodder, and grain dust sample extracts (from Sample Extraction). The separatory funnels were shaken vigorously for 30 s. After the layers had separated, the organic (lower) layers were drained into 50-mL mixing graduated cylinders. The emulsified layers were retained in the separatory funnel along with the aqueous (upper) layer. The partitioning procedure was repeated with an additional 10 mL of dichloromethane, which was also drained into the same graduated cylinder. The volume in the graduated cylinders was then adjusted to 40 mL with dichloromethane. The graduated cylinders were capped, and the extract solutions were mixed. Analysis of the sorghum extracts was then continued as described under Sample Purification by Cyclohexyl SPE.

(b) Apples. Dichloromethane (10 mL) and water (5 mL) were added to the 125-mL separatory funnel containing the 10.0-mL aliquot of the apple sample extract (from Sample Extraction). The partitioning procedure was conducted as described above with sorghum samples, except that only one partitioning was conducted with dichloromethane, and the extract in the graduated cylinder was diluted to 20 mL instead of 40 mL. A 1.0-mL aliquot of the sample extract was transferred to a 40-mL vial. Analysis of the apple extracts was then continued as described under Sample Purification by Cyclohexyl SPE.

(c) Water, Milk, Sediment, Beef Tissues, and Other Crops. Water, milk, sediment, beef tissues, and crops other than apples or sorghum did not require cleanup by liquid−liquid partitioning.

Sample Purification by Cyclohexyl SPE. *(a) Citrus Crops.* For citrus crops, the diluted 10-mL extract described under Sample Extraction was transferred to a clean 40-mL

Table 1. Compounds Tested for the Potential to Interfere in the Spinosad RaPID Assay Test Kit

pesticides	pesticides	organic compounds	inorganic compounds
alachlor	malathion	*N*-acetylglucosamine	calcium chloride
aldicarb	metalaxyl	aflatoxin B1	copper chloride
azinphos-methyl	methamidophos	aflatoxin G1	hydrogen peroxide
carbaryl	methiocarb	humic acid	iron chloride
carbendazim	methomyl	β−lactose	magnesium chloride
carbofuran	metribuzin	methyl oleate	manganese chloride
chlorothalonil	parathion	Polyoxin D	mercuric chloride
chlorpyrifos	parathion-methyl	L-(+)-rhamnose	nickel sulfate
chlorpyrifos-methyl	phosmet		sodium chloride
cyanazine	picloram		sodium metasilicates
2,4-D	procymidone		sodium nitrate
dicamba	propachlor		sodium phosphate
dinoseb	thiabendazole		sodium sulfate
EPN	triclopyr		sodium sulfite
iprodine	vinclozolin		sodium thiosulfate
			zinc chloride

vial. The sample extract was then purified by cyclohexyl SPE using the following procedure:

A cyclohexyl (CH) Bond Elut LRC cartridge (Varian Sample Preparation Products, Harbor City, CA) was attached to a stopcock and an SPE vacuum manifold. Prior to using each new lot of CH cartridges, the elution profile was determined to ensure that the appropriate volumes of eluants were discarded and collected. The elution profile was standardized for each lot of CH cartridges by adding 0.5 mL of the 10.0 μg/mL fortification solution to 100 mL of 80% acetonitrile/20% water, diluting a 5.0-mL aliquot of the solution with 5 mL of water, and collecting fractions of the eluants for evaporation and analysis. The elution parameters for spinosad that are described below are typical for the CH columns used, but different lots might require different volumes of the eluants.

Before the sample extract was added, the CH SPE cartridge was conditioned under vacuum by successively rinsing with 9 mL of methanol and 18 mL of water. Without allowing the column to dry, the sample extract was added to the column, and the cartridge was dried under vacuum for 2 min after the solution had eluted. The sample vial was rinsed with 5 mL of acetonitrile, which was added to the column and eluted under vacuum. The sample vial was rinsed with 2 mL of acetone, which was added to the cartridge and eluted under vacuum. The cartridge was dried under vacuum for 5 min. Culture tubes (16 mm × 100 mm) were then placed in the vacuum manifold. The sample vial was rinsed with 4 mL of 2% TEA/98% acetonitrile (prepared fresh daily), which was added to the cartridge, and the eluate was collected in the culture tube. (Because TEA catalyzes the photodegradation of spinosad, exposure of the collected solutions to direct laboratory lighting was minimized.) Analysis of the citrus crop samples was continued as described under Sample Evaporation.

(b) Sorghum Grain, Forage, Fodder, and Grain Dust. For sorghum samples, a 10.0-mL aliquot of the purified extract (from Liquid−Liquid Partitioning) was transferred to a clean 40-mL vial. The sample aliquot was evaporated just to dryness using a TurboVap evaporator. The residue was dissolved in 1 mL of methanol with the aid of a vortex mixer, and 4 mL of 80% acetonitrile/20% water and 5 mL of water were added. After mixing, the sample extract was then purified by cyclohexyl SPE as described above for citrus crops, except that the volume of the eluting solvent 2% TEA/98% acetonitrile was 5 mL instead of 4 mL. Analysis of the sorghum samples was continued as described under Sample Evaporation.

(c) Water, Sediment, Milk, Beef Tissues, and All Other Crops. Other than citrus and sorghum samples, none of the other matrices required purification by CH SPE.

Sample Evaporation. *(a) Citrus Crops and Sorghum Matrices.* For all of the citrus crop and sorghum matrices, the purified sample extracts were immediately evaporated using a TurboVap evaporator at 60 °C with the nitrogen flow at 8 psi. The evaporation step required ∼10−20 min, and the samples were removed from the evaporator immediately upon evaporation of the solvent. Decreased recoveries were observed if the samples remained on the evaporator after solvent evaporation. In addition, care was taken to minimize the exposure to light of those sample extracts containing TEA from the CH SPE purification step to minimize the potential for photodegradation of spinosad.

Analysis of the citrus crop or sorghum samples was continued as described under Addition of Sample Diluent.

(b) All Other Crops, Beef Tissues, and Milk. For beef tissues, milk, and all crop matrices other than citrus and sorghum, the purified sample extracts were immediately evaporated using a TurboVap as described above for citrus and sorghum matrices, except that the temperature was set at 45 °C instead of 60 °C. For some crop samples, a small amount of water often remained in the flask upon evaporation of the organic solvents, but the volume was minimal (≤0.2 mL). Sample analysis was continued as described under Addition of Sample Diluent.

(c) Water and Sediment. Water and sediment samples did not require an evaporation step prior to the addition of sample diluent.

Addition of Sample Diluent. The sample extracts were initially dissolved in the following volumes of spinosad sample diluent (SSD) from the Spinosad RaPID Assay Test Kit. Further dilutions were made with the SSD as needed to bring the spinosad concentration within the range of the standard calibration curve. After addition of the SSD, the sample vials were capped with a PTFE-lined closure and were mixed with a vortex mixer for 20−30 s. The solution was allowed to equilibrate for at least 5 min to ensure homogeneity before proceeding as described under Immunoassay.

(a) Sediment. For sediment samples, a 1.0-mL aliquot of the sample extract solution from the sample extraction procedure was diluted with 5.0 mL of SSD.

(b) Sorghum Matrices. For all of the sorghum matrices, the residue from the sample evaporation was dissolved in 0.5 mL of methanol and then diluted with 4.5 mL of the SSD.

(c) All Other Crops, Beef Tissues, and Milk. For beef tissues, milk, and all crops other than sorghum, the residue was dissolved directly in the SSD. The volume of sample diluent was 3.0 mL for apples, 5.0 mL for citrus crops, and 10.0 mL for all of the other crop matrices, beef tissues, and milk.

During the analysis of hundreds of different crop samples using the dilution scheme described, some problems occurred with consistently determining spinosad concentrations at the LOQ at the bottom of the calibration curve. When this problem occurred, recoveries were low (50−70%) and the coefficient of variation was high (>20%) for replicate analyses. To solve this problem, the volume of SSD was reduced from 10.0 to 5.0 mL to move the analyte concentration closer to the midpoint of the calibration curve. This adjustment increased recoveries and lowered the assay variability.

(d) Water. Water samples did not require the addition of SSD prior to IA unless a dilution was needed to bring the spinosad concentration within the range of the standard calibration curve.

Immunoassay. The immunoassays were conducted using the Spinosad RaPID Assay Test Kit. The kit reagents were removed from refrigerated storage and allowed to warm to

room temperature for at least 30 min prior to use. Likewise, the analyzer was turned on for at least 30 min prior to use.

Samples were analyzed as described on the product information insert contained within the test kit. The average result from the duplicate test tubes constituted a single sample result.

Calculation of Results. Standard calibration curves were calculated using the preprogrammed data reduction capabilities of the RPA-1 RaPID Analyzer and the absorbances from the calibration standard solutions provided with the kit. The calibration curves were constructed by linear regression after performance of a ln/Logit data transformation of the concentration and absorbance values, respectively. Using the standard calibration curve, the RPA-1 RaPID Analyzer then calculated the concentration of spinosad in each sample tube and the mean concentration of duplicate tubes.

The concentration (μg/g) of the analytes in the samples was calculated from the concentration in the final solution (C), the final volume (V), the weight of the sample that was extracted (W), the aliquot factor (AF), and any additional sample dilution factor (DF) using the following equation:

$$\mu\text{g/g} = (C \times \text{AF} \times V \times \text{DF})/W \quad (1)$$

For those samples that did not require purification by liquid−liquid partitioning, the aliquot factor was calculated from the appropriate extraction and aliquot volumes, that is

$$\text{AF} = \text{extraction vol/aliquot vol} \quad (2)$$

For those sample types that required purification by liquid−liquid partitioning, the aliquot factor was calculated as follows:

$$\text{AF} = (\text{extraction vol/aliquot vol}) \times (\text{partitioning vol/aliquot vol}) \quad (3)$$

The percent recovery (R) was calculated from the concentration (μg/g) found in fortified recovery samples using the following equation:

$$R = [(\mu\text{g/g})/(\text{added } \mu\text{g/g})] \times 100\% \quad (4)$$

Calculated Limits of Detection and Quantitation. Using a technique described previously (Keith et al., 1983), the limits of detection (LOD) and quantitation (LOQ) for the residue methods were calculated from the standard deviation (s) of the μg/g results of fortified recovery samples. For water and sediment, the LOD and LOQ were calculated from the standard deviation of results from the 0.0001 μg/mL and 0.05 μg/g fortified recovery samples, respectively. For all other sample types, the LOD and LOQ were calculated from the results of the 0.010 μg/g fortified recovery samples. The LOD for each sample type was calculated as 3 times the standard deviation (3s), and the LOQ was calculated as 10 times the standard deviation (10s).

Interference Study. The potential interference with antibody binding in the assay was tested for 30 pesticides, 16 inorganic compounds, and 8 organic compounds. The compounds tested are listed in Table 1.

Specificity and Sensitivity. Several analogues, metabolites, and degradates of spinosad were tested to determine if the Spinosad RaPID Assay Test Kit would detect their presence in a water sample.

Confirmation and Comparison of Results. Residues that were detected in some of the sample solutions by IA were confirmed by also analyzing the samples by HPLC-UV (West et al., 2000) or by LC-MS (Schwedler et al., 2000). The results using the IA and chromatographic techniques were compared to determine the degree of correlation.

RESULTS AND DISCUSSION

Method Validation. Methods were validated over the concentration ranges that are listed in Tables 2−8, and the resulting recovery values are also summarized in Tables 2−8. For all 34 sample matrices, the average recoveries ranged from 77 to 112%.

Table 2. Recovery of Spinosad from Fortified Water and Sediment Samples

sample type	no. of recoveries	added, μg/g or μg/mL	% recovery[a] (mean ± SD)
water	31	0.0001−0.020	101 ± 7
sediment	39	0.05−0.35	77 ± 7

[a] No residues were detected in untreated control samples at a detection limit of 0.00005 μg/mL in water or 0.020 μg/g in sediment.

Table 3. Recovery of Spinosad from Fortified Beef and Whole Milk Samples

sample type	no. of recoveries	added, μg/g or μg/mL	% recovery[a] (mean ± SD)
beef kidney	14	0.010−0.50	78 ± 6
beef liver	19	0.010−5.0	77 ± 8
beef lean meat	22	0.010−0.50	77 ± 5
whole milk	32	0.01−0.50	84 ± 9

[a] No residues were detected in untreated control samples at a detection limit of 0.003 μg/g.

Table 4. Recovery of Spinosad from Fortified Vegetable Samples

sample type	no. of recoveries	added, μg/g or μg/mL	% recovery (mean ± SD)[a]
broccoli	17	0.010−1.0	105 ± 13
cabbage	18	0.010−1.0	110 ± 14
mustard greens	17	0.010−1.0	101 ± 7
tomatoes	18	0.010−1.0	106 ± 10
green peppers	17	0.010−1.0	102 ± 10
celery	19	0.010−1.0	105 ± 11
head lettuce	17	0.010−1.0	94 ± 6
leaf lettuce	19	0.010−1.0	102 ± 6
spinach	17	0.010−1.0	98 ± 10

[a] No residues were detected in untreated control samples at a detection limit of 0.003 μg/g.

Table 5. Recovery of Spinosad from Fortified Cucurbit, Legume, and Potato Samples

sample type	no. of recoveries	added, μg/g or μg/mL	% recovery[a] (mean ± SD)
cucumber	15	0.010−0.10	93 ± 15
muskmelon	18	0.010−0.25	94 ± 18
squash	14	0.010−0.10	89 ± 14
snow peas	15	0.010−0.50	94 ± 15
soybeans	15	0.010−0.50	112 ± 15
snap beans	12	0.010−0.50	109 ± 11
potatoes	16	0.010−1.0	103 ± 11

[a] No residues were detected in untreated control samples at a detection limit of 0.003 μg/g.

Linearity. The linearity of the IA standard calibration curve was determined using the calibration standards from the Spinosad RaPID Assay Test Kit. The standard concentrations ranged from 0.05 to 1.0 ng/mL. The correlation coefficient (r^2) for the linear regression equations describing the absorbance as a function of standard concentration ranged from 0.9900 to 1.0000 for al of the IA determinations.

LOD and LOQ. The calculated LOD (Keith et al., 1983) was 0.00004 μg/mL for water and 0.010 μg/g for sediment. For all other sample types, the calculated LOD ranged from 0.001 to 0.006 μg/g with an average of 0.003 μg/g.

Likewise, the calculated LOQ was 0.0001 μg/mL for water and 0.030 μg/g for sediment. For all other sample types, the calculated LOQ ranged from 0.006 to 0.020 μg/g with an average of 0.010 μg/g.

Table 6. Recovery of Spinosad from Fortified Corn and Wheat Samples

sample type	no. of recoveries	added, μg/g or μg/mL	% recovery[a] (mean ± SD)
sweet corn forage	14	0.010−1.0	100 ± 14
sweet corn grain	15	0.010−0.10	98 ± 15
sweet corn stover	13	0.010−1.0	94 ± 19
field corn grain	13	0.010−0.10	103 ± 13
wheat grain	15	0.010−0.20	102 ± 10
wheat forage	8	0.010	84 ± 12
wheat hay	4	0.010	97 ± 17
wheat straw	4	0.010	94 ± 20

[a] No residues were detected in untreated control samples at a detection limit of 0.003 μg/g.

Table 7. Recovery of Spinosad from Fortified Sorghum and Tobacco Samples

sample type	no. of recoveries	added, μg/g or μg/mL	% recovery[a] (mean ± SD)
sorghum grain	25	0.010−1.0	103 ± 12
sorghum grain dust	4	0.010−2.5	95 ± 12
sorghum forage	14	0.010−2.5	92 ± 14
sorghum fodder	17	0.01−1.0	98 ± 16
green tobacco	17	0.01−1.0	96 ± 11
cured tobacco	17	0.01−1.0	96 ± 12

[a] No residues were detected in untreated control samples at a detection limit of 0.003 μg/g.

Table 8. Recovery of Spinosad from Fortified Fruit Samples

sample type	no. of recoveries	added, μg/g or μg/mL	% recovery[a] (mean ± SD)
whole apples	18	0.010−1.0	102 ± 9
whole oranges	17	0.010−1.0	98 ± 15
whole grapefruit	17	0.010−1.0	96 ± 13
whole lemons	17	0.010−1.0	101 ± 11
orange peels	19	0.010−1.0	100 ± 15
edible oranges	17	0.010−1.0	97 ± 11

[a] No residues were detected in untreated control samples at a detection limit of 0.003 μg/g.

Method Ruggedness. During method development, the following critical factors for maintaining method ruggedness were discovered and incorporated into the analytical procedures described under Experimental Procedures: The addition of the stabilizing solution to water samples, the establishment of a three-month expiration date for TEA, the standardization of the elution profile for different lots of CH SPE cartridges, the use of low light levels to prevent photolysis of the analytes in the presence of TEA, the removal of the flasks from the evaporator immediately upon removal of the solvent, and the adjustment of the final volume to move the analyte concentrations closer to the midpoint of the calibration curve. To determine method ruggedness, the method validation data in Tables 2−8 were generated by multiple analysts on multiple days for all of the sample matrices, and adequate recoveries were obtained.

Interferences. The I_{50} for spinosyn A is ~0.0003 μg/mL, which is the concentration that results in 50% inhibition of conjugate binding to the available antibody. None of the compounds in Table 1 that were tested for potential interference with antibody binding in the immunoassay interfered at concentrations equivalent to 15000 times the I_{50} concentration for spinosyn A.

Specificity/Sensitivity. The results of the specificity and sensitivity testing are summarized in Table 9. The Spinosad RaPID Assay Test Kit was found to be sensitive (I_{50} < 2 ng/mL) to the active ingredients (spinosyns A and D) and all three of the metabolites (spinosyns B and K and *N*-demethylspinosyn D). Thus, the test kit determines the total residue of spinosad and metabolites in food, feed, or environmental matrices. Although the test kit sensitivity is generally similar for all of these compounds, results are not identical when the individual spinosyns are tested.

Table 9. Cross-Reactivity of Spinosad, Metabolites, and Degradation Products

I_{50} (ng/mL)				
spinosyn A	spinosyn D	spinosyn B	spinosyn K	*N*-demethyl-spinosyn D
0.29	1.88	0.61	0.92	1.80

To determine if the IA method could adequately detect and recover a mixture of spinosad-related compounds, fortified recovery samples were analyzed for some sample matrices that had been spiked with technical grade spinosad. Technical grade spinosad contains both active ingredients (spinosyns A and D) and trace amounts of other spinosad analogues, including the three degradation products. The resulting recoveries ranged from 66 to 81% for whole milk, from 68 to 73% for beef tissues, from 81 to 83% for apples, from 108 to 110% for broccoli, from 88 to 95% for head lettuce, from 80 to 89% for whole oranges, and from 71 to 75% for tobacco. Thus, the data indicated that adequate recoveries could be obtained for total spinosad. However, comparison with the corresponding recoveries in Tables 2−8 indicates that the apparent recoveries can be slightly lower when the metabolites are present due to the lower sensitivity of the metabolites in the immunoassay.

As a result, there is also a potential for the immunoassay to underestimate total residues of spinosad in samples in which the major residue is composed of the metabolites. However, the potential to underestimate residues is small because spinosyn A is the major residue and the metabolite residues are low in comparison. If results for individual components of the spinosad residue are desired, it is necessary to utilize analysis by HPLC-UV (West et al., 2000) or LC-MS (Schwedler et al., 2000).

False Positive/False Negative Rate. The false positive and false negative rates were determined for all 34 sample matrices. A false positive was defined as a measured concentration of spinosad at or above the LOD in a control sample known to be free of the analyte, and a false negative was defined as the failure to measure a concentration of spinosad in control samples fortified at the LOD. No false positives occurred in >150 control sample matrices, and no false negatives occurred in >100 control sample matrices that were fortified at the LOD.

Confirmation of Residues and Correlation of IA Results with HPLC Results. The detection and/or quantitation of spinosad in any of the sample matrices by IA can be confirmed with analysis by HPLC-UV or LC-MS. However, the IA and HPLC methods determine total spinosad residues differently. IA results are expressed as a sum of the total residue present, including the active ingredients (spinosyns A and D) and the three metabolites. Some other minor metabolites in addition to spinosyns B and K and *N*-demethylspinosyn D would also be expected to contribute a very small amount to the total residue detected by IA. In contrast, the HPLC

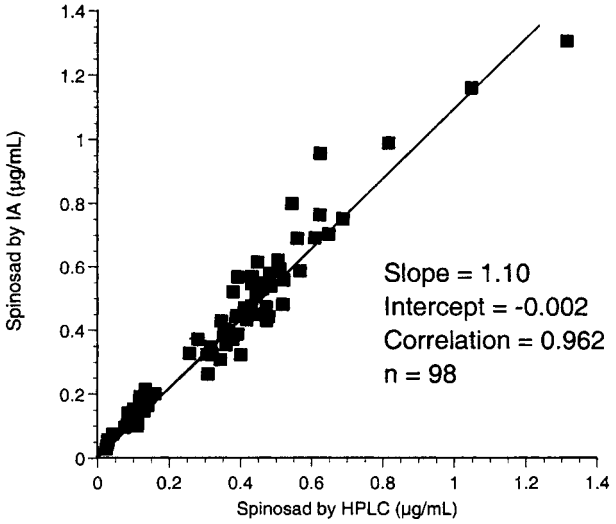


Figure 4. Correlation of total spinosad residues in whole milk determined by immunoassay with those obtained by HPLC-UV (expressed as the correlation coefficient, r^2).

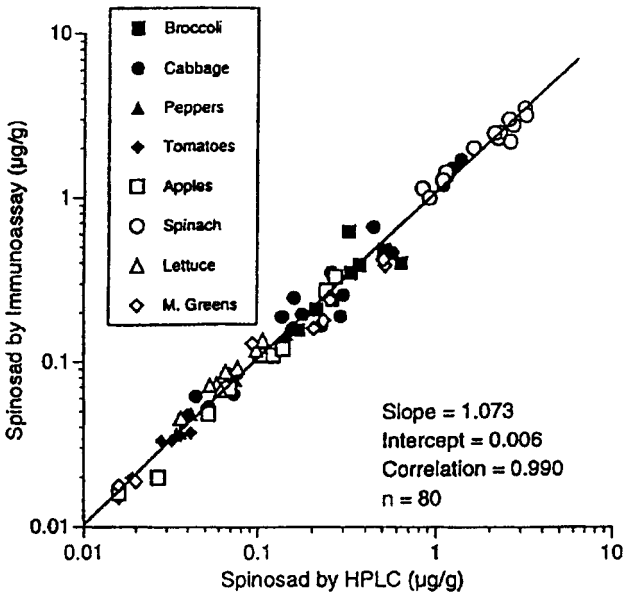


Figure 6. Correlation of total spinosad residues in crops determined by immunoassay with those obtained by HPLC-UV (expressed as the correlation coefficient, r^2).

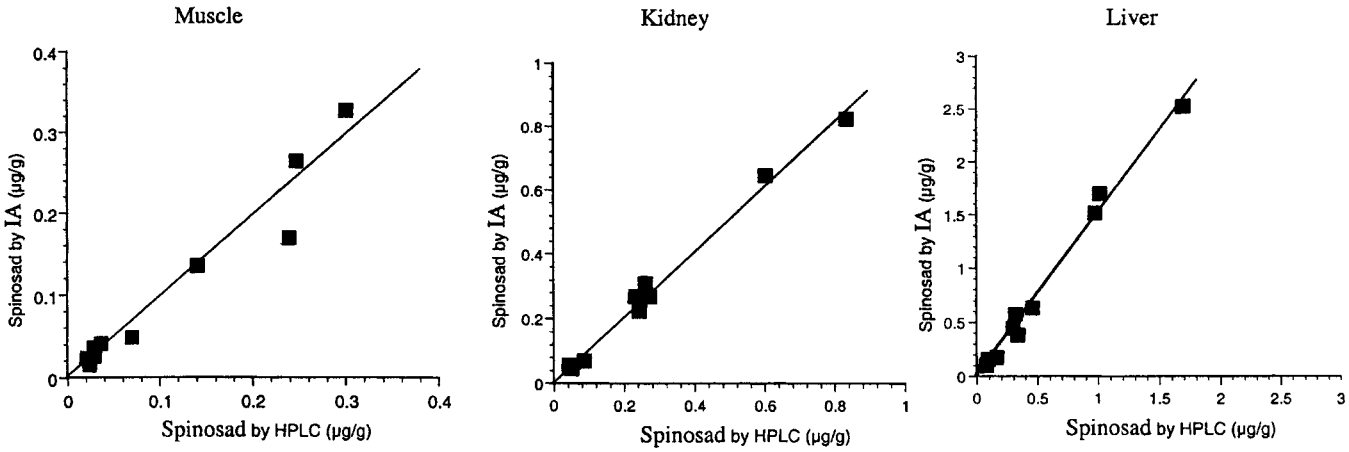
methods determine each of the five analytes separately, and the total residue consists of the sum of the individual concentrations. Thus, it was of interest to compare the total residue in various sample matrices as determined by both techniques.

Field samples treated with spinosad were used for the comparison of total residue results by the two approaches. Because the IA methods are much more rapid, IA was used as a screening technique to analyze all of the field samples from magnitude of residue studies. A subset of the samples was also analyzed by HPLC-UV, and the results were compared.

As shown in Figures 4−6, the total residue results for IA correlated well with those determined by HPLC-UV, with correlation coefficients (r^2) of 0.962 for milk, 0.945 for lean beef muscle, 0.995 for beef kidney, 0.993 for beef liver, and 0.990 for eight different crops. As a result of the excellent correlation with classical methods of analysis, spinosad IA methods were the first immunochemical methods accepted by the U.S. Environmental Protection Agency for tolerance enforcement (*Federal Register*, 1998).

Assay Time. The analysis of a typical set of 25 water samples by IA could be completed in ∼2 h. For other sample types requiring homogenization, extraction, and various degrees of sample purification, a typical set of 25 samples could be prepared in 4−8 h. These sample analysis times are considerably less than those required for HPLC-UV or LC-MS methods, in which 10−15 samples can typically be prepared for analysis in 8 h, followed by overnight injection into the LC using an autosampler and data analysis on the following day.

Conclusions. Immunoassay methodology has been developed and validated for determining residues of spinosad in 34 different sample matrices. The accuracy and precision of the methodology make it suitable for residue monitoring or tolerance enforcement. Factors affecting the successful performance of the method have been investigated, and precautions have been incorporated to enhance method ruggedness. Spinosad IA methods were the first immunochemical methods ac-



	Muscle	Kidney	Liver
Slope	0.990	1.02	1.55
Intercept	-0.003	-0.002	-0.004
Correlation	0.945	0.995	0.993

Figure 5. Correlation of total spinosad residues in beef tissues determined by immunoassay with those obtained by HPLC-UV (expressed as the correlation coefficient, r^2).

cepted by the U.S. EPA for tolerance enforcement. The IA methods provide reliable and cost-effective analytical methodology, and the data correlate well with those obtained by traditional LC techniques.

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