Determination of Spinosad and Its Metabolites in Citrus Crops and Orange Processed Commodities by HPLC with UV Detection

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Spinosad is an insect control agent that is derived from a naturally occurring organism and is effective on a wide variety of crops, including citrus crops. A method is described for the determination of spinosad and its metabolites in citrus crops and orange processed commodities. The method determines residues of the active ingredients (spinosyns A and D) and three minor metabolites (spinosyn B, spinosyn K, and *N*-demethylspinosyn D). For dried orange pulp and orange oil, the method has a limit of quantitation (LOQ) of 0.02 μg/g and a limit of detection (LOD) of 0.006 μg/g. For all other sample matrices (whole fruit, edible fruit, juice, and peel), the method has an LOQ of 0.01 μg/g and an LOD of 0.003 μg/g. The analytes are extracted from the various sample types using appropriate solvents, and the extracts are purified by liquid−liquid partitioning and/or solid-phase extraction. All five analytes are determined simultaneously in the purified extracts by reversed-phase high-performance liquid chromatography with ultraviolet detection at 250 nm.

Keywords: *Spinosad; spinosyn A; spinosyn D; spinosyn B; spinosyn K; N-demethylspinosyn D; citrus; oranges; grapefruit; lemons; mandarins; quantitation; HPLC-UV*

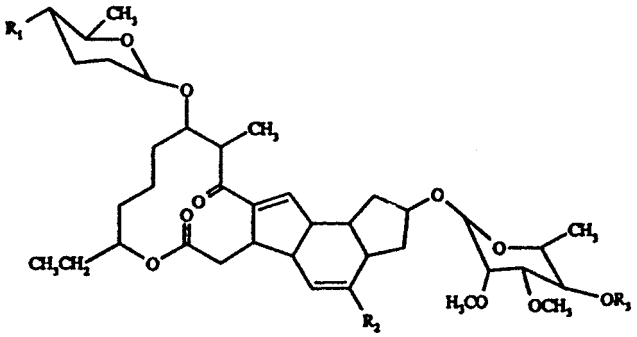
INTRODUCTION

The spinosyns are insect control agents that are derived from a naturally occurring Actinomycetes bacterium, *Saccharopolyspora spinosa*. Spinosad, which is composed of a mixture of spinosyns A and D, is the common name of the active material that is derived from a fermentation broth. Spinosad is used for the management of insect pests in citrus crops and a variety of other crops, including cotton (Sparks et al., 1995; Thompson et al., 1995). The structures of spinosad and its metabolites are shown in Figure 1, and the chemical names and CAS Registry No. are included in Table 1.

Analytical methods were needed to determine the magnitude of residues in the fruit and processed commodities of citrus crops treated with spinosad. Residue methods 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), and in meat, milk, cream, and eggs (West and Turner, 1998). Previous studies using radiolabeled (^{14}C) material demonstrated that spinosyns A and D were metabolized to spinosyn B, spinosyn K, and *N*-demethylspinosyn D in crops (D. P. Rainey, J. D. Magnussen, and D. F. Berard, Dow AgroSciences LLC, personal communication, 1994). Thus, the following methods are presented for the determination of all five analytes in citrus crops and orange processed commodities by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection.

EXPERIMENTAL PROCEDURES

Apparatus. *HPLC with a UV Detector.* A Hewlett-Packard model 1050 HPLC with a UV detector was used in combination with a Hewlett-Packard model 3396 series II recording integrator for the measurement of peak height responses. The primary HPLC column was an ODS-AM [3-μm particle size, 150 × 4.6 mm i.d. (YMC, Inc., Wilmington, NC)], maintained at 30 °C. (ODS-AM is a high-carbon load C_{18} packing that has been subjected to an endcapping step that improves peak shape with some analytes.) The mobile phase consisted of 44% reservoir A/44% reservoir B/12% reservoir C (isocratic), with reservoir A containing methanol, reservoir B containing acetonitrile, and reservoir C containing 2% aqueous ammonium acetate/acetonitrile (67:33). The flow rate was 0.4 mL/min. The injection volume was 175 μL, the integrator attenuation was 2^3, and the chart speed was 0.2 cm/min. The UV detector was operated at 250 nm. The five analytes eluted with retention times ranging from approximately 13 to 26 min.



Spinosad and Metabolites

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

Figure 1. Structures of spinosad and metabolites.

The confirmatory HPLC column was a C_{18}/cation mixed-mode [5-μm particle size, 150 mm × 4.6 mm i.d. (Alltech, Deerfield, IL)]. (The mixed-mode column is a multifunctional support for separating hydrophobic as well as ionic species.

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Table 1. Chemical Names and CAS Registry Numbers[a] for Spinosyns

name (CAS Registry No.)	chemical name
spinosyn A (131929-60-7)	2-[(6-deoxy-2,3,4-tri-*O*-methyl-α-L-mannopyranosyl)oxy]-13-[(5-(dimethylamino) tetrahydro-6-methyl-2*H*-pyran-2-yl)oxy]-9-ethyl-2,3,3*a*,5*a*,5b,6,9,10,11,12,13,14,16*a*,16*b*-tetradecahydro-14-methyl-1*H*-*as*-indaceno(3,2-*d*)oxacyclododecin-7,15-dione
spinosyn D (131929-63-0)	2-[(6-deoxy-2,3,4-tri-*O*-methyl-α-L-mannopyranosyl)oxy]-13-[(5-(dimethylamino) tetrahydro-6-methyl-2*H*-pyran-2-yl)oxy]-9-ethyl-2,3,3*a*,5*a*,5*b*,6,9,10,11,12,13,14,16*a*,16*b*-tetradecahydro-4,14-dimethyl-1*H*-*as*-indaceno(3,2-*d*)oxacyclododecin-7,15-dione
spinosyn K (159195-00-3)	2-[(6-deoxy-2,3-di-*O*-methyl-α-L-mannopyranosyl)oxy]-13-[(5-(dimethylamino) tetrahydro-6-methyl-2*H*-pyran-2-yl)oxy]-9-ethyl-2,3,3*a*,5*a*,5*b*,6,9,10,11,12,13,14,16*a*,16*b*-tetradecahydro-14-methyl-1*H*-*as*-indaceno(3,2-*d*)oxacyclododecin-7,15-dione
spinosyn B (131929-61-8)	2-[(6-deoxy-2,3,4-tri-*O*-methyl-α-L-mannopyranosyl)oxy]-9-ethyl-2,3,3*a*,5*a*,5*b*,6,9,10,11,12,13,14,16*a*,16*b*-tetradecahydro-14-methyl-13-[(tetrahydro-6-methyl-5-(methylamino)-2*H*-pyran-2-yl)oxy]-1*H*-*as*-indaceno(3,2-*d*)oxacyclododecin-7,15-dione
N-demethylspinosyn D (149439-70-3)	2-[(6-deoxy-2,3,4-tri-*O*-methyl-α-L-mannopyranosyl)oxy]-9-ethyl-2,3,3*a*,5*a*,5*b*,6,9,10,11,12,13,14,16*a*,16*b*-tetradecahydro-4,14-dimethyl-13-[(tetrahydro-6-methyl-5-(methylamino)-2*H*-pyran-2-yl)oxy]-1*H*-*as*-indaceno(3,2-*d*)oxacyclododecin-7,15-dione

[a] Supplied by the author.

The support consists of a high-purity, 100-Å, spherical silica substrate bonded with a cationic amine functionality and a conventional reversed-phase C_{18} functionality.) The mobile phase was 40% reservoir A/40% reservoir B/20% reservoir C (isocratic), with reservoir A containing methanol, reservoir B containing acetonitrile, and reservoir C containing 2% aqueous ammonium acetate/acetonitrile (67:33). The flow rate was 0.6 mL/min. All of the other parameters were the same as those listed above for the primary column. The five analytes eluted with retention times ranging from approximately 8 to 14 min.

Reagents. Water was purified using a Milli-Q UV Plus purification system (Millipore Corp., Milford, MA). The following reagents were of HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA): acetone, acetonitrile, dichloromethane, hexane, methanol, and ammonium acetate. Triethylamine (TEA) was of reagent grade (Fisher Scientific), and a new bottle of TEA was opened every two or three months to prevent the formation of impurities that produced interference peaks on the chromatogram. The sodium sulfate was of certified ACS grade, anhydrous, granular, 10−60 mesh, and tested for pesticide residue analysis (Fisher Scientific). The purified active ingredients used for analytical standards were obtained from the Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 306/A1, Indianapolis, IN 46268.

Standard Preparation. The purity of the analytical standards ranged from 95 to 97%. Individual stock solutions of the five analytes were prepared at 200 μg/mL by weighing 20 mg of each standard, quantitatively transferring to separate 100-mL volumetric flasks, dissolving in 50% methanol/50% acetonitrile, and diluting to volume. Aliquots (5.0 mL) of all five stock solutions were then combined in a 100-mL volumetric flask and diluted to volume with methanol/acetonitrile/2% aqueous ammonium acetate (1:1:1) to obtain a mixture containing 10.0 μg/mL of each analyte. Aliquots of this solution were further diluted with methanol/acetonitrile/2% aqueous ammonium acetate (1:1:1) to obtain HPLC calibration standards at concentrations of 0.0, 0.10, 0.50, 1.0, and 1.5 μg/mL.

Solutions for fortifying control citrus crop and processed commodity samples for the determination of recovery were prepared by combining 10.0-mL aliquots of the five 200 μg/mL stock solutions in a 50-mL volumetric flask and diluting to volume with 50% methanol/50% acetonitrile to obtain a mixture containing 40.0 μg/mL of the analytes. Aliquots of this solution were further diluted with 50% methanol/50% acetonitrile to obtain fortification standards at concentrations of 0.03, 0.06, 0.1, 0.2, 0.4, 0.5, 1.0, 2.0, and 10.0 μg/mL. All standard fortification solutions were prepared in clear glass volumetric flasks. The use of amber glass flasks was avoided because spinosyn B and *N*-demethylspinosyn D dissolved in 50% methanol/50% acetonitrile tend to gradually adsorb onto amber glassware. The fortification solutions containing analyte concentrations of <2.0 μg/mL were prepared fresh daily, and the more highly concentrated fortification solutions were prepared weekly.

Precautionary Protection from Light. During the sample extraction and purification steps, the extracts were protected from light to prevent photolysis. Protective measures included working under reduced lighting conditions (e.g., turning off the lights in fume hoods during liquid−liquid partitioning and SPE cleanup steps) and placing the samples in the dark during any interruptions during sample processing. Long interruptions were generally avoided during sample analysis, except that the analysis could be delayed overnight prior to the silica or CH SPE procedures by storing the sample extracts in a freezer. No delays in sample preparation occurred during the analysis of orange oil samples due to instability of the analytes in orange oil.

Initial Sample Preparation and Storage. *(a) Whole Fruit, Edible Fruit, Peel, and Dried Pulp.* Fruit, peel, and pulp samples were diced with a knife, frozen with liquid nitrogen, and then ground through a hammer mill with a $^3/_{16}$-in. 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) Orange Juice and Orange Oil. Juice and oil samples did not require sample preparation prior to being stored as above.

Sample Weighing and Fortification of Recovery Samples. *(a) Whole Fruit, Edible Fruit, Peel, and Dried Pulp.* Untreated control samples (10 g of dried pulp or 20 g of the other sample matrices) were weighed into 8-oz (237-mL) glass bottles (Qorpak, with PTFE-lined lids, Fisher Scientific). Fortified recovery samples were prepared by adding 1.0 mL of the appropriate fortification standard solution to the appropriate control samples, and sample analysis was continued as described under Sample Homogenization.

(b) Orange Juice. Juice samples were thawed (if frozen) and then thoroughly shaken to obtain homogeneity prior to analysis. Untreated control samples (20 g) were weighed into 8-oz (237-mL) glass bottles, and fortified recovery samples were prepared by adding 1.0 mL of the appropriate fortification standard solution. Purified water (40 mL) and 80 mL of 40% acetonitrile/60% dichloromethane were added to the sample extraction bottle, which was then sealed with a PTFE-lined lid. Sample analysis was continued as described under Purification by Liquid−Liquid Partitioning.

(c) Orange Oil. Fortified orange oil recovery samples were prepared by adding 1.0 mL of the appropriate fortification standard solution to a 125-mL boiling flask, evaporating the solvent just to dryness using a rotary vacuum evaporator (model 1007-4, Rinco Instrument Co., Greenville, IL) at 35−45 °C, and weighing 5.0 g of orange oil into the flask. The sample was then sonicated using an ultrasonic bath (model FS14H, Fisher Scientific) for 20 s to dissolve the residue in the oil. The oil was dissolved in 20 mL of hexane, and sample analysis was continued as described under Purification by Silica SPE.

The primary steps in the residue methods for determining spinosad and metabolites in the various sample matrices are

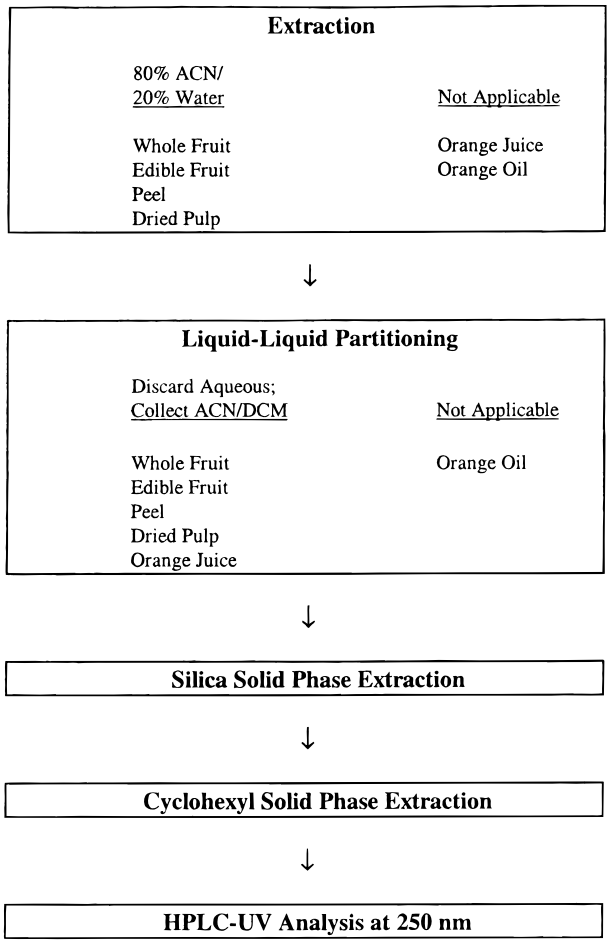

Figure 2. Flowchart for the determination of spinosad and metabolites in citrus crops and processed commodities by HPLC-UV (ACN, acetonitrile; DCM, dichloromethane).

summarized in the flowchart in Figure 2. The solvents and extraction techniques used for extracting the citrus crop samples were those shown during a radiolabeled (^{14}C) study to result in essentially complete extraction of the analytes from crop sample matrixes (D. P. Rainey et al., personal communication, 1994).

Sample Homogenization. *Whole Fruit, Edible Fruit, Peel, and Dried Pulp.* An 85-mL aliquot of 80% acetonitrile/20% water was added to each bottle, and the samples were blended for ~1 min using an Omni Mixer homogenizer (model 17105, Omni International, Gainesville, VA) with a 20-mm-diameter generator and sawtooth blades. The samples were blended on a speed setting of 5, which was equivalent to ~10000 rpm as measured by a tachometer. Using a 20-mL glass syringe, the homogenizer blades were rinsed with 15 mL of 80% acetonitrile/20% water, and the rinse solution was added to the sample bottle. The analyses were continued as described under Sample Extraction.

Sample Extraction. *Whole Fruit, Edible Fruit, Peel, and Dried Pulp.* Fruit, peel, and dried pulp samples were extracted by sealing the sample bottle with a PTFE-lined lid and shaking on an orbital shaker (New Brunswick model G-33, Fisher Scientific) at 250 rpm for 5 min. The samples were centrifuged at 2250 rpm for 5 min, and a 75-mL aliquot of the supernatant liquid (or 65 mL for dried pulp samples) was transferred to a clean 8-oz bottle. Purified water (40 mL) and dichloromethane (75 mL) were added, and the bottle was sealed with a PTFE-lined lid. The analyses were continued as described under Purification by Liquid−Liquid Partitioning.

Purification by Liquid−Liquid Partitioning. *Whole Fruit, Edible Fruit, Peel, Dried Pulp, and Juice.* The samples were shaken in an upright position on an orbital shaker at 250 rpm for 5 min and were then centrifuged at 2250 rpm for 5 min. Before remixing of the solvent layers could occur, the aqueous (upper) layer was completely aspirated off and discarded using a vacuum and a disposable 9-in. Pasteur transfer pipet. The organic (lower) layer was transferred to a 100-mL graduated cylinder, and the organic layer was partially aspirated off until the volume was reduced to 100 mL. (For juice samples, the volume was reduced to 40 mL.) The remaining 100-mL sample aliquots (or 40 mL for juice) were transferred to clean, 250-mL boiling flasks. The sample bottles were rinsed with 10 mL of dichloromethane, and the rinse was added to the boiling flasks. The sample extracts were evaporated using a rotary vacuum evaporator under a vacuum of −26 in. of Hg and a water bath temperature of 35−50 °C. The residue was then dissolved in 20 mL of hexane, and the analyses were continued as described under Purification by Silica SPE.

Purification by Silica SPE. All sample types were purified using the same silica SPE procedure. Prior to using each new lot of silica SPE columns, the elution profile was determined to ensure that the appropriate volumes of solvents were discarded and collected in the following procedure. The elution profile described below was determined using a standard solution containing all five analytes at 2.0 μg each in 20 mL of hexane.

A 25-mL column reservoir was attached to each silica SPE column. A ball-shaped plug of pesticide-grade glass wool was firmly pressed into the bottom of the SPE column reservoir, and ~4 mL (6 g) of pesticide-grade sodium sulfate was added to the reservoir. The glass wool plug was placed above the narrow neck of the column reservoir to prevent plugging of the neck with sodium sulfate so that the eluting solvents would adequately flow through the column. Prior to addition of the samples to the silica SPE column, the column was conditioned with 20 mL of hexane, which was eluted under a vacuum of −5 in.

The volumes of eluting solvents that are specified below were typical for the silica SPE procedure, but the volumes might require modification for different lots of silica SPE columns. The sample was added in 20 mL of hexane, which was eluted under vacuum. The evaporating flask was rinsed with 20 mL of hexane, which was added to the column reservoir and eluted under vacuum. The flask was rinsed with 20 mL of dichloromethane, which was added to the column and eluted. The column was then air-dried under vacuum for 2 min. The flask was rinsed with 20 mL of acetonitrile, which was added to the column and eluted under vacuum. All of the solvent that had eluted thus far was discarded.

A precleaned, 40-mL amber glass vial (Fisher Scientific) was then placed in the vacuum manifold for solvent collection, and the hood lights were turned off to minimize photolysis of the analytes in the presence of TEA. The evaporating flask was rinsed with 15 mL of 1% TEA/99% acetonitrile, which was added to the column and eluted into the vial under vacuum. (It was necessary to prepare the 1% TEA/99% acetonitrile solution immediately before use due to the instability of TEA in the solution.) The eluate from the 40-mL vial was transferred to the previously used 250-mL boiling flask, and the vial was rinsed twice with 2-mL aliquots of acetonitrile, which were transferred to a 250-mL boiling flask. To prevent reduced recoveries, the sample solution was immediately evaporated using a rotary vacuum evaporator at 35−45 °C, and the evaporation process was monitored closely so that the sample vials could be removed from the evaporator immediately upon evaporation of the solvent. The use of a TurboVap evaporator (Zymark Corp., Hopkinton, MA) was avoided at this step, because the TurboVap evaporator caused greatly reduced recoveries for some of the sample types, especially for orange oil. The residues were dissolved in 5 mL of 20% methanol/20% acetonitrile/60% water with the aid of an ultrasonic bath for 10−20 s. The flasks were carefully rotated and swirled to dissolve the residues on the side of the flasks. The analyses were continued as described under Purification by Cyclohexyl SPE.

Purification by Cyclohexyl SPE. All sample types were purified using the same cyclohexyl (CH) SPE procedure. Prior

to using each new lot of CH SPE columns, the elution profile was determined to ensure that the appropriate volumes of solvents were discarded and collected in the following procedure. The elution profile described below was determined using a standard solution containing all five analytes at 2.0 μg each in 5 mL of 20% methanol/20% acetonitrile/60% water. Prior to addition of the sample to the SPE column, the column was conditioned by adding the following sequence of eluants and eluting under a vacuum of −5 in.: 9 mL of methanol, 9 mL of acetonitrile, and then 18 mL of purified water.

The following volumes were typical for the CH SPE procedure, but the volumes might require modification for different lots of CH SPE columns. The samples were added in 5 mL of 20% methanol/20% acetonitrile/60% water, and the solvent was eluted under vacuum. The vials were rinsed with an additional 5 mL of 20% methanol/20% acetonitrile/60% water, which was added to the column reservoirs and eluted under vacuum. The columns were dried under vacuum for 2 min after the solution had eluted. The vials were rinsed with 5 mL of acetonitrile, which was added to the columns and eluted under vacuum. The columns were dried under vacuum for 5 min after the acetonitrile had eluted. The vials were rinsed with 5 mL of acetone, which was added to the columns and eluted under vacuum. All of the solvent that had eluted thus far was discarded.

The original sample vials were rinsed with 6 mL of 1% TEA/99% acetonitrile, which was added to the columns and eluted under vacuum into precleaned, 40-mL amber vials. (It was necessary to prepare the 1% TEA/99% acetonitrile solution immediately before use due to the instability of TEA in the solution.)

The sample solutions were immediately evaporated using a TurboVap evaporator set at 60 °C and a nitrogen flow of 8 psi. To prevent reduced recoveries, the evaporation process was monitored closely so that the sample vials could be removed from the evaporator immediately upon evaporation of the solvent. The residues were dissolved in 1.0 mL of methanol/acetonitrile/2% aqueous ammonium acetate (1:1:1). Because the analytes adsorb very strongly to glass, the vials were thoroughly swirled and rotated to ensure that the residues had dissolved. The final solutions were not filtered through a 0.45-μm filter, because some types of filters produced interference peaks in the chromatogram. However, because the solutions were highly purified at this point, the lack of filtration did not decrease column performance over a period of several weeks.

HPLC. Solutions were analyzed by HPLC using the conditions described previously under Apparatus. The suitability of the chromatographic system was determined using the following performance criteria: (a) It was determined that the correlation coefficient (r^2) exceeded 0.995 for the least-squares equation that described the detector response as a function of the concentration of the calibration standards. (b) It was visually determined that baseline resolution was achieved for all five analytes. (c) It was visually determined that a signal-to-noise ratio of approximately 10:1 was achievable for the 0.1 μg/mL calibration standard. If the peak response for any of the samples exceeded the range of the calibration curve, the samples were diluted with methanol/acetonitrile/2% aqueous ammonium acetate (1:1:1).

Calculation of Results. Separate calibration curves were prepared for all five analytes by plotting the concentration of the calibration standards on the abscissa (*x*-axis) and the resulting peak heights on the ordinate (*y*-axis). Using regression analysis, the equation for the calibration curve was determined with respect to the abscissa. The concentration (C) of the analyte in the final solution was calculated from the measured peak height response (PR) and the least-squares coefficients for the slope (m) and *y*-axis intercept (b) as follows:

$$C = (\mathrm{PR} - b)/m \quad (1)$$

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), and the aliquot factor (AF) using the following equation:

$$\mu\mathrm{g/g} = (C \times \mathrm{AF} \times V)/W \quad (2)$$

The aliquot factor was calculated from the appropriate extraction and aliquot volumes for each sample type, that is

$$\mathrm{AF} = (\text{total extraction volume/aliquot volume}) \quad (3)$$

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

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

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 method were calculated from the standard deviation (s) of the micrograms per gram results of fortified recovery samples. For all of the sample types except dried orange pulp and orange oil, the LOD and LOQ were calculated from the standard deviation of results from the 0.01 μg/g fortified recovery samples. For the dried orange pulp and orange oil samples, the LOD and LOQ were calculated from the results of the samples fortified at 0.02 μg/g. The LOD was calculated as 3 times the standard deviation ($3s$), and the LOQ was calculated as 10 times the standard deviation ($10s$).

Confirmation of Results. Residues that were detected in some of the sample solutions injected onto the primary HPLC column (ODS-AM) were confirmed by also injecting those solutions onto a different type of HPLC column (C_{18}/cation mixed mode). Confirmation required that the retention times of the analytes in the samples matched those in the standards on both columns and that the C_{18}/cation confirmatory column gave results that were within ±20% of those obtained on the primary column.

Interference Study. Seventy pesticides commonly used on cotton, fruit, and vegetables have been previously tested for interference by direct injection into the liquid chromatograph (West, 1996). Any compounds that produced peaks at the retention times of the analytes were carried through the entire analytical procedure and analyzed using the primary HPLC-UV conditions to determine if they would still interfere after going through the sample purification procedures.

RESULTS AND DISCUSSION

Method Validation. The method was validated over the following ranges of concentrations: 0.01−2.0 μg/g for whole citrus fruits (oranges, grapefruit, lemons, and mandarins), edible orange fruit, and orange juice; 0.01−10.0 μg/g for orange peel; and 0.02−10.0 μg/g for dried orange pulp and orange oil. The results of the validation study are summarized in Table 2. For all nine commodities, the average recoveries ranged from 88 to 113% for spinosyn A, from 87 to 110% for spinosyn D, from 88 to 110% for spinosyn K, from 76 to 99% for spinosyn B, and from 77 to 96% for *N*-demethylspinosyn D.

Chromatograms. Typical chromatograms obtained under the primary and confirmatory HPLC conditions for oranges are included in Figures 3 and 4, respectively. Chromatograms for the other sample matrices were similar. The alternative conditions used for the confirmation of residues (Figure 4) resulted in a different order of elution for the analytes compared to that obtained with the primary HPLC conditions (Figure 3).

Linearity. The linearity of the detector was determined using five calibration standards ranging in concentration from 0 to 1.5 μg/mL. The average correlation coefficient (r^2) for the least-squares equations describing the detector response as a function of concentration of the standard curve solutions was >0.9999

Table 2. Recovery of Spinosyns A, D, K, and B and *N*-Demethylspinosyn D (NDSD) from Citrus Crops and Processed Commodities

			% recovery[a] (mean ± SD)				
sample type	added, μg/g	*n*	A	D	K	B	NDSD
whole fruit[b]	0.010−2.0	20	104 ± 4	101 ± 4	99 ± 5	98 ± 7	95 ± 8
edible oranges	0.010−2.0	20	101 ± 4	98 ± 5	98 ± 6	92 ± 7	90 ± 7
orange peel	0.010−10.0	20	97 ± 9	95 ± 7	98 ± 5	90 ± 8	90 ± 7
dried orange pulp	0.020−10.0	20	113 ± 5	110 ± 4	110 ± 4	99 ± 8	96 ± 8
orange juice	0.010−2.0	20	102 ± 5	94 ± 5	104 ± 4	94 ± 6	90 ± 6
orange oil	0.02−10.0	20	88 ± 7	87 ± 6	88 ± 5	76 ± 7	77 ± 5

[a] No residues were detected in untreated control samples. Peaks were detected in samples fortified at the method LOD (0.003 μg/g or 0.006 μg/g), but the residues were below the limit of quantitation. [b] Whole fruit included oranges, grapefruit, lemons, and mandarins.

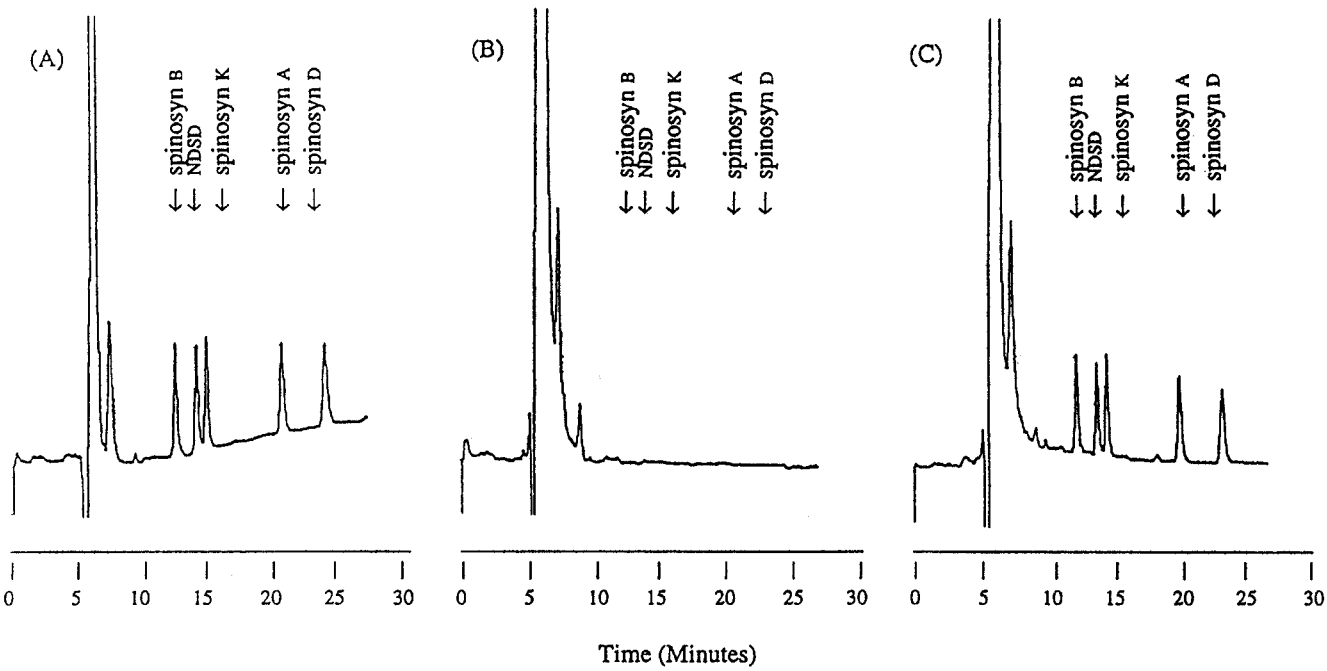

Figure 3. Typical chromatograms from the determination of spinosyns A, D, K, and B and *N*-demethylspinosyn D (NDSD) in oranges using the primary column (ODS-AM): (A) standard, 17.5 ng of each analyte; (B) control oranges containing no detectable residue; (C) control oranges fortified with 0.01 μg/g (LOQ), equivalent to recoveries of 89% (spinosyn B), 88% (*N*-demethylspinosyn D), 96% (spinosyn K), 101% (spinosyn A), and 97% (spinosyn D).

for all five analytes. Linearity at concentrations exceeding the range of the calibration curve (0.0−1.5 μg/mL) was not investigated.

LODs and LOQs. The calculated values for the LOD (3*s*) and LOQ (10*s*) are presented in Table 3. For all five analytes, the calculated LOD ranged from 0.001 to 0.005 μg/g in dried orange pulp and orange oil and from 0.001 to 0.003 μg/g in the other sample matrices. These calculated values support a method LOD of 0.006 μg/g for dried orange pulp and orange oil and 0.003 μg/g for the other sample types. The method LOD was further supported by the presence of detectable peaks in chromatograms resulting from the analysis of control samples fortified at 0.003 or 0.006 μg/g (Table 2).

Likewise, the calculated LOQ for all five analytes ranged from 0.003 to 0.015 μg/g in dried pulp and oil and from 0.002 to 0.009 μg/g in the other sample matrices (Table 3). The calculated values supported the validated method LOQs of 0.02 μg/g for dried pulp and oil and 0.01 μg/g for the other sample matrices. Chromatograms demonstrating the determination of the analytes in oranges at the validated LOQ are illustrated in Figure 3.

Critical Factors for Method Ruggedness. In addition to the critical factors noted during the analysis procedure, several factors were determined to have a potential effect on method ruggedness.

(a) Interferences. Because a nonselective wavelength (250 nm) was needed to obtain adequate sensitivity for the analytes, it was necessary to rinse glassware with acetone and methanol to remove interferences due to the detergent.

(b) Water in Extracts. It was necessary to remove traces of water from the sample solutions prior to purification by silica SPE to prevent a change in the elution profile. Water was removed by adding sodium sulfate to the column reservoirs.

(c) Analyte Instability. To prevent potential photolysis of the analytes, the samples were handled under low-light conditions during the purification steps. Photolysis was increased by the presence of TEA, so it was necessary to use amber glass containers when the sample solutions contained TEA. It was also necessary to remove samples from evaporators immediately upon evaporation of the solvents to prevent degradation, and the use of rotary vacuum evaporators instead of TurboVap evaporators in some method procedures was required to prevent loss of the analytes.

Specificity. Pesticides commonly used on cotton and vegetables were previously tested for potential interference with the analytes (West, 1996). Seventy pesticides

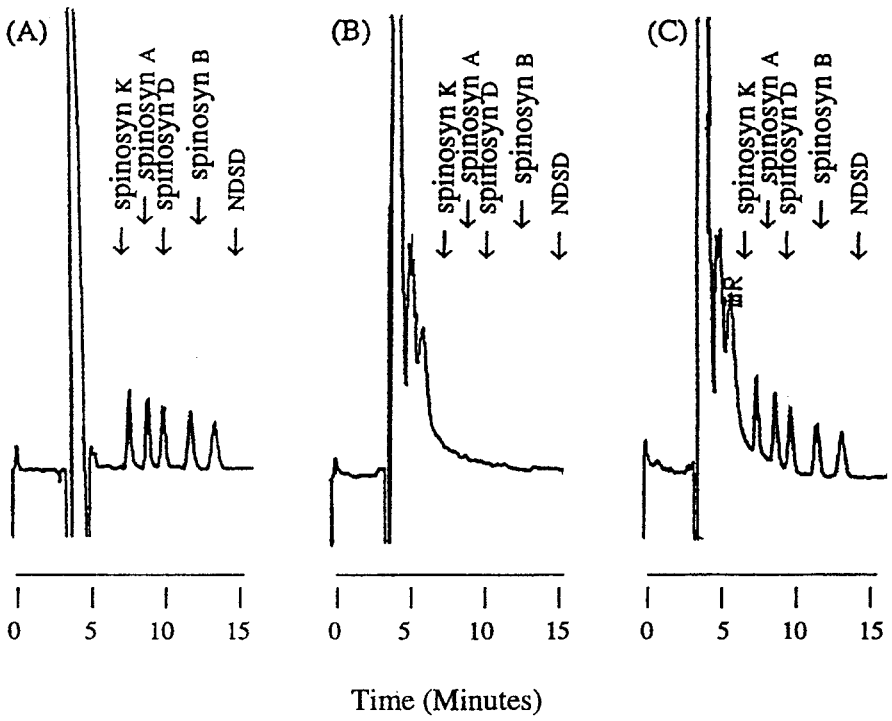

Figure 4. Typical chromatograms from the determination of spinosyns A, D, K, and B and *N*-demethylspinosyn D (NDSD) in oranges using the confirmatory column (C_{18}/cation mixed mode): (A) standard, 17.5 ng of each analyte; (B) control oranges containing no detectable residue; (C) control oranges fortified with 0.01 μg/g of all five analytes, equivalent to recoveries of 101% (spinosyn K), 104% (spinosyn A), 105% (spinosyn D), 93% (spinosyn B), and 97% (*N*-demethylspinosyn D).

Table 3. Calculated Limits of Detection and Quantitation for Spinosyns A, D, K, and B and *N*-Demethylspinosyn D (NDSD)

	LOD[a]					LOQ[b]				
sample matrix	A	D	K	B	NDSD	A	D	K	B	NDSD
whole fruit	0.001	0.001	0.002	0.001	0.001	0.002	0.002	0.006	0.004	0.004
edible oranges	0.002	0.001	0.002	0.002	0.002	0.005	0.004	0.005	0.005	0.007
orange peel	0.001	0.001	0.001	0.003	0.002	0.004	0.004	0.003	0.009	0.005
dried orange pulp	0.002	0.002	0.002	0.002	0.001	0.006	0.006	0.005	0.006	0.003
orange juice	0.001	0.001	0.001	0.002	0.002	0.004	0.004	0.003	0.006	0.006
orange oil	0.004	0.005	0.003	0.002	0.003	0.014	0.015	0.011	0.008	0.009

[a] Calculated LOD (μg/g), calculated as 3*s*. [b] Calculated LOQ (μg/g), calculated as 10*s*.

were tested for interference by direct injection into the liquid chromatograph. Most of the pesticides eluted with the solvent front, and only avermectin B_{1a}, dicofol, propargite, thiodicarb, and tralomethrin produced peaks that matched the retention times of the analytes. However, none of these five pesticides interfered when they were carried through the entire analytical procedure. Thus, the cleanup procedures described in the method effectively removed the potentially interfering compounds as well as the interfering coextractives from the samples.

Conclusions. Residue methodology has been validated for the determination of the active ingredients of spinosad (spinosyns A and D) and its three major metabolites (spinosyns B and K and *N*-demethylspinosyn D) in whole citrus fruits (oranges, grapefruit, lemons, and mandarins), edible oranges, orange peel, dried orange pulp, orange juice, and orange oil. 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. This method expands the list of sample matrices in which spinosad residues may be successfully determined.

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