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Determination of Spinosyns A and D and Metabolites in Tree Nuts
(Almond and Pecan Nutmeats and Almond Hulls)
by High Performance Liquid Chromatography with Ultraviolet Detection

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A. Scope

This method is applicable for the quantitation of residues of spinosyns A and D and metabolites (spinosyns B, K, and *N*-demethyl spinosyn D) in almond and pecan nutmeats and almond hulls. The method was validated for each compound over the concentration range of 0.01 to 1.0 µg/g with a limit of quantitation of 0.01 µg/g. Chemical names and structures for spinosyns A, D, B, K, and *N*-demethyl spinosyn D are shown in Figure 1.

B. Principle

Residues of spinosyns A, D, B, K, and *N*-demethyl spinosyn D are extracted from almond and pecan nutmeats and almond hulls with an 8:2 solution of acetonitrile:water. The extracts are filtered and partitioned into 1-chlorobutane. Samples are purified by silica solid phase extraction (SPE), and cyclohexyl (CH) SPE. All five compounds are determined simultaneously in the purified extracts by reversed-phase high performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance detection at 250 nm. Confirmation of the residue identity in each matrix is accomplished by reanalyzing the purified extracts using liquid chromatography with mass spectrometric detection (LC/MS).

C. Safety Precautions

1. Each analyst must be acquainted with the potential hazards of the reagents, products, and solvents used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE: MATERIAL SAFETY DATA SHEETS (MSDS), LITERATURE, AND OTHER RELATED DATA. Safety information on non-DowElanco products should be obtained from the container label or from the supplier.

Disposal of reagents, reactants, and solvents must be in compliance with local, state, and federal laws and regulations.

2. Volatile or flammable organic solvents such as acetone, acetonitrile, 1-chlorobutane, dichloromethane, hexane, methanol, and triethylamine must be used in well-ventilated areas away from ignition sources.
3. Erlenmeyer flasks under vacuum are susceptible to implosion. Use polypropylene flasks or glass flasks covered with electrical tape. Evaporations under vacuum must be conducted behind appropriate shields while wearing eye protection.

D. Equipment (Notes L.1. and L.2.)

1. Balance, analytical, Model AE-100, Mettler Instrument Corporation, Hightstown, NJ 08520.
2. Balance, toploading, Model P-1200, Mettler Instrument Corporation.
3. Circulator heater for water bath, Model 1266-00, Cole-Parmer Instrument Co., Chicago, IL 60648.
4. Evaporator, Model TurboVap LV, Zymark Corporation, Hopkinton, MA 01748. Use a rotary vacuum evaporator (D.12.) if a TurboVap evaporator is not available. Do not use an N-Evap evaporator. Low recoveries have been seen with this equipment.
5. Filtration apparatus for HPLC solvents, catalog number 5-8061M, Supelco, Inc., Bellefonte, PA 16823.
6. Generator probe for homogenizer, 20 mm x 145 mm, catalog number 15401, Omni International, Waterbury, CT 06704.
7. Hammermill, Model 2001, equipped with a 3/16-inch screen, AGVISE Laboratories, Inc., Northwood, ND 58267.
8. High performance liquid chromatograph (for the mass spectrometer), Hewlett Packard Model 1050, Hewlett Packard, Wilmington, DE 19808.
9. High performance liquid chromatograph, Model 9012 solvent delivery system, Model 9100 autosampler, Model 9050 UV detector, and LC Star Workstation, Varian Associates Inc., Walnut Creek, CA 94598.
10. Homogenizer, Model 17105, Omni International.
11. Liquid chromatograph/mass spectrometer, Model LCQ, Finnigan MAT, San Jose, CA 95134.

12. Rotary vacuum evaporator, Model 1007-4 IN, Rinco Instrument Company, Inc., Greenville, IL 62246. If the rotating shaft evaporator is not available, the following evaporator may be substituted: Büchi rotary evaporator system, Model RE-121C, catalog number 09-548-105H, Fisher Scientific, Pittsburgh, PA 15219-4785.
13. Shaker, reciprocating, Model 6010, Eberbach Corporation, Ann Arbor, MI 48109.
14. Ultrasonic bath, Model FS14H, Fisher Scientific, Pittsburgh, PA 15219.
15. Vacuum manifold, SPE, cartridge, catalog number 5-7044, Supelco, Inc.
16. Vortex mixer, Model K-550-G, Scientific Industries, Inc., Bohemia, NY 11716.

E. Glassware and Materials (Notes L.1. and L.2.)

1. Bottles, 4-oz (118-mL), with PTFE-lined closures, catalog number 03-321-1A, Fisher Scientific.
2. Cartridge, cyclohexyl Bond Elut LRC, catalog number 1211-3032, Varian Sample Preparation Products, Harbor City, CA 90710.
3. Cartridge, Sep-Pak Plus silica, part number WAT20520, Waters, Milford, MA 01757.
4. Column, guard, ODS, 5- μm , 120 Å, 23 mm x 4.0 mm i.d., catalog number KGCQ-324, YMC, Inc., Wilmington, NC 28403.
5. Column, ODS-AM, 3- μm , 120 Å, 150 mm x 4.6 mm i.d., catalog number AM-302-3, YMC, Inc.
6. Filter paper, Whatman number 5, medium porosity, 7-cm diameter, catalog number 09-830K, Fisher Scientific.
7. Filter, glass fiber, 25-mm i.d., 1.0- μm pore size, catalog number 09-730-195, Fisher Scientific.
8. Filter, membrane, Nylon 66, 47-mm i.d., 0.45- μm pore size, catalog number 5-8067, Supelco, Inc.
9. Flask, side arm, 500-mL, catalog number 10-180E, Fisher Scientific.
10. Funnel, Büchner, 83-mm, catalog number 10-356C, Fisher Scientific.
11. Reservoir, SPE cartridge, 70-mL capacity with 20- μm polyethylene frits, catalog number 120-1027-F, Jones Chromatography, Lakewood, CO 80228.
12. Stopcock, SPE processing, PTFE, catalog number 121-0009, Jones Chromatography.

13. Vial, amber, 40-mL, catalog number 03-338-27A, Fisher Scientific.
14. Vial, clear, 40-mL, catalog number 03-339-5C, Fisher Scientific.
15. Vial, clear, 45-mL, catalog number 03-339-5D, Fisher Scientific.

F. Reagents and Prepared Solutions (Notes L.1. and L.2.)

1. Reagents

- a. Acetone, OmniSolv grade, catalog number AX0116-1, EM Science, Gibbstown, NJ 08027.
- b. Acetonitrile, ChromAR HPLC grade, catalog number 2856, Mallinkrodt Specialty Chemicals Company, Paris, KY 40361.
- c. Ammonium acetate, HPLC grade, catalog number A639-500, Fisher Scientific.
- d. 1-Chlorobutane, HPLC grade, catalog number B429-4, Fisher Scientific.
- e. Dichloromethane, HPLC grade, catalog number D150-4, Fisher Scientific.
- f. Hexane, OmniSolv grade, catalog number HX0296-4, EM Science.
- g. Magnesium sulfate (anhydrous), certified, catalog number M65-500, Fisher Scientific.
- h. Methanol, ChromAR HPLC grade, catalog number 3041, Mallinkrodt Specialty Chemicals Company.
- i. Nitrogen, gas, 99.99% purity, Airco Gas and Gear, Indianapolis, IN 46241.
- j. Nitrogen, refrigerated liquid, catalog number LQNI-230, Airco Gas and Gear.
- k. Sodium Chloride, Certified ACS grade, catalog number S-271-1, Fisher Scientific.
- l. Standards:
Obtain pure active ingredients or reference compounds of spinosyns A, D, B, K, and *N*-demethyl spinosyn D from Test Substance Coordinator, Building 304, DowElanco, Indianapolis, IN 46268-1053.
- m. Triethylamine, reagent grade, catalog number 04885-1, Fisher Scientific.
- n. Water, HPLC grade, catalog number WX001-4, EM Science.

2. Prepared Solutions

- a. 2% Ammonium acetate (w/v)
Prepare by dissolving 20.0 g of ammonium acetate per liter of HPLC grade water.
- b. 1:1 Solution of acetonitrile:methanol (v/v)
Prepare by mixing 1000 mL of each.
- c. 1:1:1 Solution of acetonitrile:methanol:2% ammonium acetate (v/v/v)
Prepare by mixing 300 mL of each.
- d. 42:42:16 Solution of acetonitrile:methanol:2% ammonium acetate (v/v/v)
Prepare by mixing 1680 mL of acetonitrile, 1680 mL of methanol, and 640 mL of 2% ammonium acetate and filtering through a 0.45- μ m membrane filter.
- e. 2:2:6 Solution of acetonitrile:methanol:water (v/v/v)
Prepare by mixing 500 mL of acetonitrile, 500 mL of methanol, and 1500 mL of HPLC grade water.
- f. 8:2 Solution of acetonitrile:water (v/v)
Prepare by mixing 1600 mL of acetonitrile and 400 mL of HPLC grade water.
- g. 1% Triethylamine in acetonitrile (v/v) (Note L.3.)
Prepare fresh daily by mixing 1.0 mL of triethylamine and 99 mL of acetonitrile.
- h. 2% Triethylamine in acetonitrile (v/v) (Note L.3.)
Prepare fresh daily by mixing 2.0 mL of triethylamine and 98 mL of acetonitrile.

G. Preparation of Standards

Prepare all solutions in clear glassware. Do not use amber glassware. Store prepared solutions at ambient temperature in the dark (Note L.4.). Do not store the solutions in a refrigerator.

1. Preparation of Spinosyns A, D, B, K, and N-Demethyl Spinosyn D Stock Solution

Separately weigh 10.0 mg of spinosyns A, D, K, B, and N-demethyl spinosyn D analytical standards or pure active ingredients into a series of 45-mL vials. Dissolve each standard in 10 mL of the 1:1 solution of acetonitrile:methanol (spinosyn D may require sonication to dissolve), and combine all solutions in the same 100-mL volumetric flask. Rinse all vials with 10 mL of the 1:1:1 solution of acetonitrile:methanol:2% ammonium acetate and add the rinsates to the volumetric flask. Dilute to

volume with the 1:1:1 solution of acetonitrile:methanol:2% ammonium acetate to obtain a stock solution containing 100 µg/mL of each compound.

2. Preparation of Spinosyns A, D, B, K, and N-Demethyl Spinosyn D Spiking and Calibration Solutions

Perform all dilutions with the 1:1:1 solution of acetonitrile:methanol:2% ammonium acetate.

- a. Combine separate 10.0-mL aliquots of the stock solution in 200-mL and 100-mL volumetric flasks, and dilute each flask to volume to obtain solutions containing 5.0 and 10.0 µg/mL of each compound, respectively. Using the appropriate aliquot of the 10.0-µg/mL solution, dilute to obtain solutions ranging in concentration from 0.015 to 1.25 µg/mL of each compound as follows:

Initial Conc. µg/mL	Initial Volume mL	Final Volume mL	Final Conc. µg/mL	Equivalent Sample Conc. ^a µg/g
10.0	0.30	200.0	0.015	0.003
10.0	0.50	200.0	0.025	0.005
10.0	1.0	200.0	0.050	0.010
10.0	2.0	200.0	0.100	0.020
10.0	5.0	200.0	0.250	0.050
10.0	25.0	200.0	1.25	0.250
5.00	--	--	5.00	1.00
10.0	--	--	10.0	2.00

^a The equivalent sample concentration is based on fortifying a 5.0-g sample with 1.0 mL of the respective standard solution.

- b. For fortifications, use standard solutions ranging in concentration from 0.050 to 5.00 µg/mL. To confirm the limit of detection, use the 0.015 µg/mL solution for fortification. For the calibration curve, use standard solutions ranging in concentration from 0.025 to 10.0 µg/mL.

H. High Performance Liquid Chromatography

1. Typical Operating Conditions

Instrumentation: Varian Model 9012 solvent delivery system
 Varian Model 9100 autosampler
 Varian Model 9050 UV detector
 Varian LC Star Workstation

Column: YMC ODS-AM, 3-µm, 120 Å,
 150 mm x 4.6 mm i.d.

Guard Column:	YMC ODS, 5- μ m, 120 Å 23 mm x 4.0 mm i.d.
Column Temperature:	Ambient
Mobile Phase:	Acetonitrile:methanol:2% ammonium acetate (42:42:16)
Flow Rate:	0.9 mL/min
Injection Volume:	250 μ L
Attenuation:	16
Chart Speed:	0.3 cm/min
UV Wavelength:	250 nm
Run Time:	25 min

2. Calibration Curves

Demonstrate that the calibration curves fit a least squares power regression equation (1) over the equivalent sample concentration range of 0.005 to 2.0 μ g/g for each compound. The least squares correlation coefficient (r^2) of each equation must be equal to or greater than 0.995. The exponent of each equation must fall in the range of 0.9 to 1.1. Representative calibration curves for spinosyns A, D, B, K, and *N*-demethyl spinosyn D are presented in Figure 2.

3. Typical Chromatograms

Typical chromatograms for almond nutmeat and hull samples obtained under the conditions cited in Section H.1. are presented in Figures 3 and 4, respectively. Both almond and pecan nutmeat samples exhibit similar chromatograms. A small background (<LOD) interference was observed at the retention time of spinosyn K in one analysis of the almond nutmeat control (shown in Figure 3). Background peaks were also observed to elute close to spinosyn B and *N*-demethyl spinosyn D, particularly in the almond hull samples (shown in Figure 4). These background peaks did not interfere with the performance of the method.

4. Liquid Chromatography/Mass Spectrometry (LC/MS) Conditions for Confirmation of Spinosyns A and D, and Metabolites

Instrumentation:	Finnigan Model LCQ LC/MS Hewlett-Packard Model 1050 high performance liquid chromatograph
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Column:	YMC ODS-AM, 3- μ m, 120 Å, 150 mm x 4.6 mm i.d.
Mobile Phase:	Acetonitrile:methanol:2% ammonium acetate (44:44:12)
Flow Rate:	1.0 mL/min
Injection Volume:	100 μ L
Selected Range Monitoring:	<i>m/z</i> 716-722 (spinosyns B and K) <i>m/z</i> 730-736 (spinosyn A and <i>N</i> -demethyl spinosyn D) <i>m/z</i> 744-750 (spinosyn D)
Scan Range:	<i>m/z</i> 700-770
Capillary Temperature:	150 °C
Sheath Gas:	75 psi N ₂
Auxiliary Gas:	40 psi N ₂
Ion Source Type:	Electrospray ionization
Electron Multiplier:	994 V

5. Typical Chromatograms

Typical chromatograms obtained under the conditions cited in Section H.4. are presented in Figures 5 and 6.

I. Determination of Recovery of Spinosyns A and D and Metabolites in Tree Nuts

1. Preparation of Recovery Samples for Tree Nuts (Almond and Pecan Nutmeats)

- a. Prepare tree nut samples for analysis by freezing with liquid nitrogen and grinding them through an AGVISE Model 2001 Hammermill with a 3/16-inch screen.
- b. Weigh 5.0 g of tree nut sample into a series of 4-oz bottles. For preparing fortified samples, use some of the samples as controls and fortify the remaining samples by adding 1.0 mL of the appropriate spiking solutions (Section G.2.) to obtain concentrations ranging from 0.01 to 1.0 μ g/g (Note L.4.). A reagent blank, containing no tree nut sample, is carried through the method with the samples.
- c. Add 70 mL of an 8:2 solution of acetonitrile:water and homogenize for at least 30 seconds using an Omni homogenizer at a speed control setting of 5.5. Rinse the generator probe with ~5 mL of acetonitrile, collecting the rinsate in the bottle. Cap

the bottle with a PTFE-lined closure and shake the sample for at least 10 minutes on a reciprocating shaker set at ~180 excursions/min.

- d. Place a Whatman number 5 filter paper in a Büchner funnel with a 500-mL side arm flask attached and filter the sample extract under vacuum. Rinse the bottle and the tree nut tissue with 15 mL of acetonitrile and add the rinsate to the Büchner funnel.
- e. Add 30 mL water to the filtrate and transfer the solution to a 250-mL separatory funnel. Add 75 mL of 1-chlorobutane to the separatory funnel and partition the analytes by vigorously shaking the solution for 20-30 seconds. Allow sufficient time for the layers to separate. Drain the aqueous (lower) layer into the 500-mL side arm flask and drain the remaining organic extract into a 500-mL evaporating flask.
- f. Return the aqueous layer to the separatory funnel and partition with a second 75 mL of 1-chlorobutane. Allow sufficient time for the layers to separate. Drain the aqueous (lower) layer to waste and combine the organic extracts in the 500-mL evaporating flask.
- g. Prior to evaporating the sample, rinse the rotary vacuum evaporators with methanol. Add 10 mL of methanol to the evaporating flask and evaporate the solvent using a rotary vacuum evaporator with the water bath heated between 40-45 °C (Note L.5.). If water is still present, add another 10 mL of methanol and evaporate to dryness.
- h. Dissolve the residue in 20 mL of hexane.
- i. Purify the sample on a silica SPE column utilizing the following procedures.

The vacuum and flow rate measurement for the silica SPE are summarized as follows:

Step	Solvent	Vacuum, (inches Hg)	Flow Rate, (mL/min)
I.1.i.(2)	Hexane	4	6
I.1.i.(3)	Dichloromethane	5	5
I.1.i.(4)	Acetonitrile	2.5	6
I.1.i.(5)	1% Triethylamine in Acetonitrile	2.5	3

These measurements are intended to be used as a guideline. The vacuum and flow rates may vary $\pm 15\%$ without affecting the final results. Do not let the column bed dry out in Steps I.1.i.(1) to I.1.i.(3).

- (1) Attach a 70-mL reservoir to a 1- μ m glass fiber filter, then a silica SPE column, followed by a stopcock, to an extraction vacuum manifold. Condition the column with 20 mL of hexane.

- (2) Add the sample solution from Step I.1.h. to the silica SPE column. After the hexane has eluted, rinse the sample flask with two separate 20-mL aliquots of hexane, and add both rinsates separately to the column.
- (3) Rinse the sample flask with 15 mL of dichloromethane. Add the rinsate to the cartridge when the level of hexane reaches the top of the frit in the reservoir.
- (4) Rinse the sample flask with 15 mL of acetonitrile. Add the rinsate to the cartridge when the level of dichloromethane reaches the top of the frit in the reservoir.
- (5) After the acetonitrile has completely eluted, turn off the vacuum and place a 40-mL amber vial in the vacuum manifold. Rinse the sample flask with 15 mL of 1% triethylamine in acetonitrile and add the rinsate to the cartridge. Using vacuum, collect the eluate in the 40-mL amber vial. (Note L.7.)
- (6) Transfer the eluate from the 40-mL vial to a 125-mL evaporating flask. Rinse the 40-mL vial two times with 5 mL of acetonitrile and add each rinsate to the evaporating flask.
- (7) Prior to evaporating the sample, rinse the rotary vacuum evaporators with methanol. Evaporate the solvent using a rotary vacuum evaporator with a water bath temperature maintained at 40-45 °C. (Note L.5.)
- (8) Dissolve the residue in 5 mL of a 2:2:6 solution of acetonitrile:methanol:water, and sonicate the solution for ~20 seconds.

j. Purify the extract on a CH SPE cartridge utilizing the following procedures.

The vacuum and flow rate measurement for the CH SPE are summarized as follows:

Step	Solvent	Vacuum, (inches Hg)	Flow Rate, (mL/min)
I.1.j.(2)	2:2:6 Soln. of Acetonitrile:Methanol:Water	12	2.5
I.1.j.(3)	Acetonitrile	4	3
I.1.j.(4)	Acetone	2.5	6
I.1.j.(5)	2% Triethylamine in Acetonitrile	1.5	3

The flow rate at Step I.1.j.(4) needs to be adjusted at ~6 mL/min or higher to avoid chromatographic elution of spinosyn K. The measurements for I.1.j.(2), I.1.j.(3), and I.1.j.(5) are intended to be used as a guideline. The vacuum and flow rates may vary $\pm 15\%$ without affecting the final results.

- (1) Attach a CH SPE cartridge to a stopcock, and attach the stopcock to a SPE vacuum manifold. Condition the CH cartridge under vacuum using the following sequence of solvents: 9 mL of methanol and then 18 mL of water. Do not let the column dry after it has been conditioned.

- (2) Add the sample from Step I.1.i.(8) to the CH cartridge. Rinse the sample flask with an additional 5 mL of the 2:2:6 solution of acetonitrile:methanol:water and add the rinsate to the column.
 - (3) Under full vacuum (~25 inches of Hg), dry the cartridge for ~2 minutes. Rinse the sample flask with 5 mL of acetonitrile, and add the rinsate to the cartridge.
 - (4) Under full vacuum (~25 inches of Hg), dry the cartridge for ~5 minutes. Reduce the vacuum to ~2.5 inches of Hg and add 3 mL of acetone directly to the cartridge with the vacuum open. Apply full vacuum when the acetone level is below the frit, and pull all the acetone through. (Note L.6.)
 - (5) Stop the vacuum and place a 40-mL amber vial in the vacuum manifold (Note L.7.). Turn off the hood light and add 6 mL of 2% triethylamine in acetonitrile directly to the cartridge. Using vacuum, collect the eluate in the 40-mL amber vial.
- k. Immediately place the vial in a TurboVap evaporator (60 °C, ~10 psi N₂) to evaporate the solvent to dryness.
 - l. Dissolve the residue in 1.0 mL of a 1:1:1 solution of acetonitrile:methanol: 2% ammonium acetate. Vortex the vial for 5 seconds to dissolve any residue on the side of the vial.
 - m. Transfer the solution to an HPLC sample vial.
 - n. Prepare the standard curves and analyze the sample solutions by HPLC as described in Section H.1.

Determine the suitability of the chromatographic system using the following performance criteria:

- (1) Standard curve linearity: Determine that the correlation coefficient (r^2) for the least squares equation which describes the detector response as a function of the concentration of calibration standards is equal to or greater than 0.995. Determine that the exponent of each equation falls in the range of 0.9 to 1.1.
 - (2) Appearance of chromatograms: Visually determine that the chromatograms resemble those in Figures 3 and 4 in terms of peak response, retention time, baseline, and background interference. Visually determine that the signal to noise ratio is at least 3:1 for the 0.025- μ g/mL calibration standard.
2. Preparation of Recovery Samples for Tree Nuts (Almond Hulls)
 - a. Proceed with Steps I.1.a. through I.1.d.
 - b. Rinse the bottle and almond hulls with a second 15 mL of acetonitrile and add the rinsate to the Büchner funnel.

- c. Transfer the extract to a 250-mL separatory funnel. Rinse the flask with 15 mL of acetonitrile and add the rinse to the separatory funnel. Add 30 mL of HPLC grade water and 75 mL of 1-chlorobutane to the separatory funnel.
- d. Partition the analytes by vigorously shaking the solution for 20-30 seconds. Allow sufficient time for the layers to separate. Drain the aqueous (lower) layer into a clean 4-oz bottle and drain the remaining organic extract into a 500-mL evaporating flask.
- e. Add ~12 g of sodium chloride and 15 mL of 1-chlorobutane to the 4-oz bottle. Seal the bottle with a PTFE-lined closure and shake vigorously for ~20 seconds or for ~5 minutes on a reciprocating shaker set at ~180 excursions/min.
- f. Centrifuge the sample for 5 minutes at ~2250 rpm. Using a disposable 10-mL pipete, transfer the organic (top) layer to the 500-mL evaporating flask in Step I.2.d. Add 20 mL of methanol to the evaporating flask.
- g. Prior to evaporating the sample, rinse the rotary vacuum evaporators with methanol. Evaporate the solvent using a rotary vacuum evaporator with the water bath heated between 40-45 °C. (Note L.5.)
- h. Dissolve the residue in 20 mL of hexane. Sonicate the sample for ~30 seconds.
- i. Purify the sample on a silica SPE column utilizing the following procedures.

The vacuum and flow rate measurement for the silica SPE are summarized as follows:

Step	Solvent	Vacuum, inches Hg	Flow Rate, mL/min
I.2.i.(2)	Hexane	4	6
I.2.i.(4)	2% Triethylamine in Acetonitrile	2.5	3

These measurements are intended to be used as a guideline. The vacuum and flow rates may vary $\pm 15\%$ without affecting the final results. Do not let the column bed dry out in Steps I.2.i.(1) to I.2.i.(2).

- (1) Attach a 70-mL reservoir to a 1- μ m glass fiber filter, then a silica SPE column, followed by a stopcock, to an extraction vacuum manifold. Add ~1 g of anhydrous magnesium sulfate to the reservoir. Condition the column with 20 mL of hexane.
- (2) Add the sample solution from Step I.2.h. to the silica SPE column. After the hexane has eluted, rinse the sample flask with two separate 20-mL aliquots of hexane, and add both rinsates separately to the column.
- (3) Proceed with Steps I.1.i.(3) through I.1.i.(4).

- (4) After the acetonitrile has completely eluted, turn off the vacuum and place a 40-mL amber vial (Note L.7.) in the vacuum manifold. Rinse the sample flask with 15 mL of 2% triethylamine in acetonitrile and add the rinsate to the cartridge. Collect the eluate in the 40-mL amber vial.
- (5) Proceed with Steps I.1.i.(6) through I.1.n.

3. Calculation of Percent Recovery

- a. Inject the calibration standards and determine the HPLC peak response for spinosyn A, D, B, K, and *N*-demethyl spinosyn D.
- b. Prepare standard curves for the five analytes by plotting the equivalent sample concentration on the abscissa (x-axis) and the resulting peak height response on the ordinate (y-axis) as shown in Figure 2. Determine the equation for the curve with respect to the abscissa using power regression analysis (1):

$$X = \left[\frac{Y}{\text{constant}} \right]^{1/\text{exponent}}$$

For example, using the spinosyn A data from Figure 2:

$$\text{Spinosyn A, Equivalent Sample Conc. } (\mu\text{g/g}) = \left[\frac{\text{Spinosyn A Peak Height}}{\text{constant}} \right]^{1/\text{exponent}}$$

$$\text{Spinosyn A, Equivalent Sample Conc. } (\mu\text{g/g}) = \left[\frac{\text{Spinosyn A Peak Height}}{22537.2} \right]^{1/0.996876}$$

- c. Determine the net residue concentration in each recovery sample by first subtracting the peak height response in the corresponding control sample from that of the recovery sample. Substitute the net peak height response obtained into the appropriate standard curve equation and solve for concentration.

For example, using the spinosyn A data from Figures 2 and 3, an almond nutmeat sample fortified with 0.010 $\mu\text{g/g}$ of spinosyn A had a peak height response of 195, and there was no interference in the corresponding control:

$$\text{Net Spinosyn A Residue Conc. } (\mu\text{g/g}) = \left[\frac{195 - 0.00}{22537.2} \right]^{1/0.996876}$$

$$\begin{array}{l} \text{Net Spinosyn A} \\ \text{Residue Conc.} \end{array} = 0.00852 \mu\text{g/g}$$

- d. Determine the percent recovery by dividing the net concentration ($\mu\text{g/g}$) by the theoretical concentration added:

$$\text{Percent Recovery} = \left[\frac{\mu\text{g/g Found}}{\mu\text{g/g Added}} \right] \times 100$$

For example, using the net concentration calculated in Step I.3.c. for spinosyn A:

$$\text{Percent Recovery} = \left[\frac{0.00852 \mu\text{g/g Found}}{0.010 \mu\text{g/g Added}} \right] \times 100$$

$$\text{Percent Recovery} = 85$$

4. Confirmation of Residue Identity

Confirm the identity of the analytes by analyzing the final solution from Step I.1.m. by the LC/MS conditions listed in Section H.4.

J. Determination of Spinosyns A and D and Metabolites in Tree Nuts

1. Prepare treated samples, a reagent blank, an untreated control sample (if available), and fortified recovery samples as described in Section I.1. for nutmeats and Section I.2. for almond hulls.
2. Prepare calibration curves for spinosyns A, D, B, K, and *N*-demethyl spinosyn D and determine the percent recovery of each analyte as described in Section I.3.
3. Determine the concentrations ($\mu\text{g/g}$) of the five analytes in the treated samples from their respective standard calibration curves, and calculate the uncorrected residue result as described in Step I.3.c.
4. If required, correct the residue result for the average of recoveries determined along with the sample as follows:

$$\text{Corrected Residue } (\mu\text{g/g}) = \text{Uncorrected Residue } (\mu\text{g/g}) \times \frac{100}{\text{PR}}$$

Where: PR = average percent recovery from fortified samples in the sample set

For example, using the net concentration calculated in Step I.3.c. for spinosyn A and the average percent recovery for spinosyn A recovery samples in the sample set from Table I:

$$\text{Corrected Residue } (\mu\text{g/g}) = 0.00852 \mu\text{g/g} \times \frac{100}{86} = 0.00991 \mu\text{g/g}$$

K. Results and Discussion

1. Method Validation

a. Recovery Levels and Precision

A method validation study was conducted to determine the percent recovery and the precision of the residue method. The results for each matrix are given in Tables I and II. The average recoveries for spinosyns A, D, B, K, and *N*-demethyl spinosyn D in nutmeats were 86%, 83%, 85%, 89%, and 82% with standard deviations of 3.8%, 3.9%, 5.9%, 9.2%, and 3.5%, respectively. The average recoveries for spinosyns A, D, B, K, and *N*-demethyl spinosyn D in almond hulls were 87%, 82%, 81%, 83%, and 79% with standard deviations of 4.0%, 4.8%, 3.1%, 3.4%, and 3.7%, respectively. Typical chromatograms of samples fortified at the validated limit of quantitation are illustrated in Figures 3 and 4.

b. Calculated Limits of Detection and Quantitation

The calculated limits of detection (LOD) and quantitation (LOQ) were determined using the standard deviation from the recovery results at the validated LOQ (0.01 $\mu\text{g/g}$). Following a published technique (2), the LOD was calculated as 3x the standard deviation, and the LOQ was calculated as 10x the standard deviation. The calculated results for nutmeats and almond hulls are presented in Tables I and II, respectively.

For all five analytes, the calculated LOD ranged from 0.0008 to 0.0035 $\mu\text{g/g}$ for all matrices. These calculated results support a LOD of 0.003 $\mu\text{g/g}$. The LOD was verified by fortifying samples at the 0.003 $\mu\text{g/g}$ level as shown in Tables I and II.

For all five analytes, the calculated LOQ ranged from 0.0027 to 0.012 $\mu\text{g/g}$ for all matrices. These calculated results support the validated LOQ of 0.010 $\mu\text{g/g}$, and representative chromatograms are shown in Figures 3 and 4.

c. Standard Curve Linearity

The average correlation coefficient (r^2) of the least squares power regression equations describing the detector response as a function of concentration was equal to or greater than 0.9997 for standard curves in each validation set for spinosyns A, D, B, K, and *N*-demethyl spinosyn D. The exponent of standard curves in each validation set ranged from 0.9892 to 1.002.

2. Confirmation of Residues of Spinosyns A and D, and Metabolites

Confirmation of the identity of spinosyns A, D, B, K, and *N*-demethyl spinosyn D can be achieved by reanalyzing representative final solutions from Step I.1.m. using LC/MS (Section H.4.). To confirm the residues, the resulting retention times of the analytes in the sample are compared with those of the standards by monitoring a selective range of ions from *m/z* 700-770. Typical chromatograms demonstrating the confirmation of spinosyns at 0.01- $\mu\text{g/g}$ (LOQ) in tree nuts are illustrated in Figures 5 and 6.

3. Assay Time

A set of 18 samples can be prepared for analysis during a typical workday, with overnight injection of the samples using an autosampler.

There are two acceptable stopping points in the method where sample preparation (Section I) may be suspended without deleterious effects on the sample analysis. These are indicated below.

- a. Steps I.1.h. and I.2.h. The assay may be stopped after the residues have been dissolved in hexane. Place the samples in the freezer to protect them from photolysis and degradation (Note L.4.). Sonicate the solution for at least 30 seconds before proceeding to Step I.1.i.(2) for nutmeats or Step I.2.i.(2) for almond hulls if the solution has been stored frozen.
- b. Step I.1.m. The samples are stable in the final solution for several days if protected from light (Note L.4.). Do not store the samples in the refrigerator.

4. Interference from Other Agricultural Chemicals

Commonly used agricultural chemicals have been tested for potential interference with spinosyns (3). At concentrations of 0.4 $\mu\text{g/mL}$ or higher, several pesticides were tested for interference by direct injection into the HPLC (Table III).

Chlorpyrifos, cypermethrin, ethion, fenvalerate, isopropanol, linuron, maneb, pendimethalin, pentachloronitrobenzene, propiconazole, and trifluralin produced interference peaks. However, none of these pesticides interfered when carried through the silica and CH SPE procedures and analyzed using the conditions described in Section H.1.

5. Standardization of Silica SPE Cartridge Elution Profile

Variation in the silica SPE cartridges can influence the elution profile of the spinosyns. It is necessary to obtain an elution profile for each lot of SPE cartridges used to ensure optimum recovery and clean-up efficiency. The following procedures may be used.

The vacuum and flow rate measurement for the silica SPE are summarized as follows:

Step	Solvent	Vacuum, (inches Hg)	Flow Rate, (mL/min)
K.5.c.	Hexane	4	6
K.5.d.	Dichloromethane	5	5
K.5.e.	Acetonitrile	2.5	6
K.5.f.	1% Triethylamine in Acetonitrile	2.5	3

These measurements are intended to be used as a guideline. The vacuum and flow rates may vary $\pm 15\%$ without affecting the final results.

- a. Prepare a silica SPE cartridge as described in Step I.1.i.(1).
- b. Pipet 1.0 mL of the 10.0- $\mu\text{g}/\text{mL}$ calibration solution into a 40-mL vial and evaporate the solutions to dryness using a TurboVap evaporator (60 °C, ~10 psi N₂). Dissolve the sample in 20 mL of hexane.
- c. Place three clean 40-mL vials in the vacuum manifold. Add the sample solution from Step K.5.b. to the conditioned SPE cartridge. Collect the eluate in one of the 40-mL vials. Rinse each sample vial with two separate 20-mL portions of hexane, and collect each eluate separately.
- d. Place two clean 40-mL vials in the vacuum manifold. Rinse the sample vial with 15 mL of dichloromethane, add the rinsate to the cartridge, and collect the eluate in one of the clean 40-mL vials.
- e. Rinse the sample vial with 15 mL of acetonitrile and add the rinsate to the SPE cartridge. Collect the eluate in the second clean 40-mL vial.
- f. Before eluting the spinosyns from the silica cartridge, make a 2-mL mark on ten 40-mL amber vials (Note L.7.). Place these vials in the vacuum manifold. Rinse the sample vial with 20 mL of the 1% triethylamine in acetonitrile and add the rinsate to the cartridge. Collect 2-mL fractions of the eluate in each vial.

To mark the vials, place 2 mL of acetonitrile into the vial, mark the meniscus, and pour out the solvent.

- g. Combine all of the hexane eluate from Step K.5.c. into a 125-mL evaporating flask and evaporate the solvent to dryness using a rotary vacuum evaporator. Place the vials of collected solutions from Steps K.5.d., K.5.e., and K.5.f. in a TurboVap evaporator (60 °C, ~10 psi N₂) to remove the solvent. Dissolve the residue in 10.0 mL of the 1:1:1 solution of acetonitrile:methanol:2% ammonium acetate. Vortex the flask and the vials so that the solvent dissolves any residue on the side. Transfer each solution to a sample vial and analyze by the HPLC conditions listed in Section H.1.

- h. Calculate separate percent recoveries of spinosyns A, D, B, K, and *N*-demethyl spinosyn D in each collected fraction using a standard curve.
- i. Typical elution profiles for a silica SPE cartridge are presented in Figure 7.
- j. If the elution profile for the spinosyns differ significantly from that shown in Figure 7, adjust the volume of acetonitrile or the 1% triethylamine in acetonitrile, respectively.

6. Standardization of CH SPE Cartridge Elution Profile

Variation in the CH SPE cartridges can influence the elution profile of the spinosyns. It is necessary to obtain an elution profile for each lot of CH SPE cartridges used to ensure optimum recovery and clean-up efficiency. The following procedures may be used.

The vacuum and flow rate measurement for the CH SPE are summarized as follows:

Step	Solvent	Vacuum, (inches Hg)	Flow Rate, (mL/min)
K.6.c.	2:2:6 Soln. of Acetonitrile:Methanol:Water	12	2.5
K.6.d.	Acetonitrile	4	3
K.6.e.	Acetone	2.5	6
K.6.f.	2% Triethylamine in Acetonitrile	1.5	3

The measurements for K.6.c., K.6.d., and K.6.f. are intended to be used as a guideline. The vacuum and flow rates may vary $\pm 15\%$ without affecting the final results.

- a. Prepare a CH SPE cartridge as described in Section I.1.j.(1).
- b. Pipet a 1.0-mL aliquot of the 10.0- $\mu\text{g}/\text{mL}$ calibration solution into a 40-mL vial and evaporate to dryness using a TurboVap evaporator (60 °C, ~10 psi N₂). Dissolve the sample in 5 mL of the 2:2:6 solution of acetonitrile:methanol:water.
- c. Place a clean 40-mL vial in the vacuum manifold. Add the sample solution from Step K.6.b. to the CH cartridge and collect the eluate in the clean 40-mL vial. Rinse the sample vial with an additional 5 mL of the 2:2:6 solution of acetonitrile:methanol:water and add the rinsate to the cartridge. Collect the eluate in the same vial.
- d. Dry the cartridge under vacuum for 2 minutes. Place a clean 40-mL vial in the vacuum manifold. Rinse the sample vial with 5 mL of acetonitrile, add the rinsate to the cartridge and collect the eluate. After the acetonitrile has eluted, dry the cartridge under full vacuum (~25 inch Hg) for 5 minutes.

- e. Place a clean 40-mL vial in the vacuum manifold and adjust the vacuum to ~2.5 inches of Hg. Add 3 mL of acetone directly to the cartridge under vacuum (Note L.6.) and collect the eluate. Apply full vacuum when the acetone level is below the frit and pull all acetone through.
- f. Turn off the vacuum and place eight 40-mL amber vials (Note L.7.) with 1-mL marks (as described in Step K.5.f. except use 1-mL of acetonitrile) into the vacuum manifold. Add 8 mL of 2% triethylamine/acetonitrile directly to the cartridge and collect 1-mL fractions of the eluate in separate vials.
- g. Place the vials collected in Steps K.6.d., K.6.e., and K.6.f. in a TurboVap evaporator (60 °C , ~10 psi N₂) to remove the solvent. Dissolve the residue in 10.0 mL of the 1:1:1 solution of acetonitrile:methanol:2% ammonium acetate. Vortex the vials to dissolve any residue on the side of the vial. Transfer the solutions to sample vials for HPLC analysis. Analyze the eluate from Step K.6.c. by HPLC directly without any concentration step due to its water content.
- h. Calculate separate percent recoveries of spinosyns A, D, B, K, and *N*-demethyl spinosyn D in each collected fraction using a standard curve.
- i. Typical elution profiles for a CH SPE cartridge are shown in Figure 8.
- j. If the elution profile for the spinosyns differ significantly from that shown in Figure 8, adjust the volume of acetone or the 2% triethylamine in acetonitrile, respectively.

L. Notes

1. Equipment, glassware, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are not listed here.
2. Because it is necessary to use a nonselective UV wavelength (250 nm) to obtain adequate sensitivity, certain precautions must be taken to avoid interferences that can result from the reagents or equipment. If interferences occur, individual reagents and chemicals must be tested for purity by treating them as they are used in the procedure and then analyzing the resulting solutions by HPLC to isolate the source(s) of the interferences. Those reagents or equipment found to be sources of interferences must be suitably purified or replaced with different sources of materials that do not produce interferences.

Certain equipment and reagents have been previously determined to cause interferences, and the following recommendations should be followed:

- a. Rinse dirty glassware with acetone before machine washing.
- b. Rinse the glassware with acetonitrile before use.

3. The elution profiles of spinosyn B and *N*-demethyl spinosyn D from the CH SPE changes when an aged triethylamine solution is used. Typically, lower recoveries of both compounds are seen. To avoid this problem, prepare the triethylamine solutions daily.
4. Avoid exposure of spinosyns to light whenever possible. These compounds degrade under normal laboratory light, which may adversely affect the shelf life of prepared standards and the results of sample recoveries.
5. Do not exceed a water bath temperature of 45 °C. At higher temperatures, spinosyn B and *N*-demethyl spinosyn D degrade.
6. Slow elution of acetone causes the chromatographic elution of spinosyn K at high amounts (>50 µg). To avoid this problem, add acetone to the cartridge with vacuum adjusted at ~2.5 inches of Hg.
7. The presence of triethylamine in the solution may promote photolysis of spinosyns. It is necessary to use amber vials whenever triethylamine is in the solution with spinosyns for extended periods of time.

M. References

1. Freund, J. E.; Williams, F. J. *Dictionary/Outline of Basic Statistics*; Dover Publications: Mineola, NY, 1991; p 170, eq I.3a.
2. Keith, L. H.; Crummett, W.; Deegan, J., Jr.; Libby, R. A.; Taylor, J. K.; Wentler, G. *Anal. Chem.* **1983**, *55*, 2210-2218.
3. Yeh, L. T.; Balcer, J. L., "Determination of Spinosad and Metabolites in Green and Cured Tobacco by High Performance Liquid Chromatography with Ultraviolet Detection", GRM 96.06, 1996, unpublished method of DowElanco.

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Table I. Recovery of Spinosyns from Nutmeats

Sample Number ^a	Date of Analysis	Fortification Level, µg/g	Spinosyn A		Spinosyn D		Spinosyn B		Spinosyn K		N-Demethyl Spinosyn D	
			Found µg/g	PR ^b	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR
17017802	17-Sept-1996	0.000	0.000		0.000		0.000		0.000		0.000	
18695301	17-Sept-1996	0.000	0.000		0.000		0.000		0.000		0.000	
17017802	23-Sept-1996	0.000	0.000		0.000		0.000		0.000		0.000	
18695301	23-Sept-1996	0.000	0.000		0.000		0.000		0.000		0.000	
17017802	01-Dec-1996	0.000	0.000		0.000		0.000		0.000		0.000	
17017802	01-Dec-1996	0.003	<0.010 ^c		<0.010		<0.010		<0.010		<0.010	
17017802	01-Dec-1996	0.003	<0.010		<0.010		<0.010		<0.010		<0.010	
17017802	17-Sept-1996	0.010	0.00852	85	0.00809	81	0.00842	84	0.00884	88	0.00822	82
17017802	17-Sept-1996	0.010	0.00857	86	0.00825	82	0.00825	82	0.00817	82	0.00828	83
18695301	17-Sept-1996	0.010	0.00936	94	0.00864	86	0.00863	86	0.00936	94	0.00825	82
18695301	17-Sept-1996	0.010	0.00870	87	0.00842	84	0.00804	80	0.00832	83	0.00796	80
17017802	24-Sept-1996	0.010	0.00857	86	0.00781	78	0.00781	78	0.00856	86	0.00791	79
17017802	24-Sept-1996	0.010	0.00853	85	0.00825	82	0.00833	83	0.00997	100	0.00810	81
18695301	24-Sept-1996	0.010	0.00813	81	0.00830	83	0.0106	106	0.0116	116	0.00733	73
18695301	24-Sept-1996	0.010	0.00857	86	0.00858	86	0.00821	82	0.0102	102	0.00767	77
			\bar{x} = 0.00862	86	0.00829	83	0.00854	85	0.00938	94	0.00797	80
			s = 0.00034	3.6	0.00027	2.7	0.00087	8.7	0.00117	12	0.00033	3.4
			RSD = 3.9	4.2	3.3	3.3	10	10	12	13	4.1	4.3
			LOD ^d = 0.0010		0.0008		0.0026		0.0035		0.0010	
			LOQ ^e = 0.0034		0.0027		0.0087		0.012		0.0033	

Table I. (Cont.) Recovery of Spinosyns from Nutmeats

Sample Number	Date of Analysis	Fortification Level, µg/g	Spinosyn A		Spinosyn D		Spinosyn B		Spinosyn K		N-Demethyl Spinosyn D	
			Found µg/g	PR	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR
17017802	17-Sept-1996	0.020	0.0158	79	0.0152	76	0.0171	86	0.0164	82	0.0166	83
18695301	17-Sept-1996	0.020	0.0173	87	0.0156	78	0.0164	82	0.0169	85	0.0163	82
17017802	24-Sept-1996	0.020	0.0170	85	0.0164	82	0.0168	84	0.0176	88	0.0162	81
			\bar{x} = 0.0167	84	0.0157	79	0.0168	84	0.0170	85	0.0164	82
			s = 0.00079	4.2	0.00061	3.1	0.00035	2.0	0.00060	3.0	0.00021	1.0
			RSD = 4.7	5.0	3.9	3.9	2.1	2.4	3.5	3.5	1.3	1.2
18695301	17-Sept-1996	0.050	0.0427	85	0.0413	83	0.0421	84	0.0431	86	0.0415	83
18695301	24-Sept-1996	0.050	0.0438	88	0.0431	86	0.0391	78	0.0431	86	0.0386	77
17017802	24-Sept-1996	0.050	0.0408	82	0.0407	81	0.0399	80	0.0357	71	0.0394	79
			\bar{x} = 0.0424	85	0.0417	83	0.0404	81	0.0406	81	0.0398	80
			s = 0.00152	3.0	0.00125	2.5	0.00155	3.1	0.00427	8.7	0.00150	3.1
			RSD = 3.6	3.5	3.0	3.0	3.8	3.8	11	11	3.8	3.9
17017802	17-Sept-1996	0.25	0.207	83	0.202	81	0.223	89	0.209	84	0.218	87
18695301	17-Sept-1996	0.25	0.219	88	0.213	85	0.215	86	0.217	87	0.211	84
18695301	24-Sept-1996	0.25	0.230	92	0.226	90	0.219	88	0.224	90	0.217	87
			\bar{x} = 0.219	88	0.214	85	0.219	88	0.217	87	0.215	86
			s = 0.0115	4.5	0.0120	4.5	0.00400	1.5	0.00751	3.0	0.00379	1.7
			RSD = 5.3	5.1	5.6	5.3	1.8	1.7	3.5	3.4	1.8	2.0

Table I. (Cont.) Recovery of Spinosyns from Nutmeats

Sample Number	Date of Analysis	Fortification Level, µg/g	Spinosyn A		Spinosyn D		Spinosyn B		Spinosyn K		N-Demethyl Spinosyn D	
			Found µg/g	PR	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR
17017802	18-Sept-1996	1.0	0.806	81	0.779	78	0.884	88	0.828	83	0.862	86
18695301	24-Sept-1996	1.0	0.906	91	0.897	90	0.841	84	0.876	88	0.832	83
17017802	24-Sept-1996	1.0	0.891	89	0.869	87	0.851	85	0.895	90	0.831	83
			\bar{x} = 0.868	87	0.848	85	0.859	86	0.866	87	0.842	84
			s = 0.0539	5.3	0.0617	6.2	0.0225	2.1	0.0345	3.6	0.0176	1.7
			RSD = 6.2	6.1	7.3	7.3	2.6	2.4	4.0	4.1	2.1	2.0
Total Average Percent Recovery:			\bar{x} =	86	83	85	85	85	89	82		
			s =	3.8	3.9	5.9	5.9	5.9	9.2	3.5		
			n =	20	20	20	20	20	20	20		
			RSD =	4.4	4.7	6.9	6.9	6.9	10	4.3		

^a Sample numbers 17017802 and 18695301 represent almond and pecan nutmeats, respectively.

^b PR = Percent recovery.

^c The residue was detected but was below the 0.01-µg/g limit of quantitation.

^d Calculated limit of detection.

^e Calculated limit of quantitation.

Table II. Recovery of Spinosyns from Almond Hulls

Sample Number	Date of Analysis	Fortification Level, µg/g	Spinosyn A		Spinosyn D		Spinosyn B		Spinosyn K		N-Demethyl Spinosyn D	
			Found µg/g	PR ^a	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR
18547701	07-Nov-1996	0.000	0.000		0.000		0.000		0.000		0.000	
18547701	01-Dec-1996	0.000	0.000		0.000		0.000		0.000		0.000	
18547701	01-Dec-1996	0.000	0.000		0.000		0.000		0.000		0.000	
18547701	01-Dec-1996	0.003	<0.010 ^b		<0.010		<0.010		<0.010		<0.010	
18547701	01-Dec-1996	0.003	<0.010		<0.010		<0.010		<0.010		<0.010	
18547701	07-Nov-1996	0.010	0.00775	78	0.00720	72	0.00767	77	0.00756	76	0.00726	73
18547701	07-Nov-1996	0.010	0.00841	84	0.00743	74	0.00843	84	0.00781	78	0.00735	74
18547701	07-Nov-1996	0.010	0.00784	78	0.00737	74	0.00828	83	0.00760	76	0.00761	76
18547701	01-Dec-1996	0.010	0.00849	85	0.00852	85	0.00761	76	0.00799	80	0.00758	76
18547701	01-Dec-1996	0.010	0.00869	87	0.00786	79	0.00767	77	0.00816	82	0.00785	79
18547701	01-Dec-1996	0.010	0.00914	91	0.00864	86	0.00776	78	0.00846	85	0.00778	78
18547701	01-Dec-1996	0.010	0.00899	90	0.00834	83	0.00812	81	0.00849	85	0.00768	77
18547701	01-Dec-1996	0.010	0.00938	94	0.00798	80	0.00827	83	0.00856	86	0.00884	88
			\bar{x} = 0.00859	86	0.00792	79	0.00798	80	0.00808	81	0.00774	78
			s = 0.00059	5.8	0.00055	5.4	0.00033	3.2	0.00040	4.1	0.00049	4.6
			RSD = 6.9	6.7	6.9	6.8	4.1	4.0	5.0	5.1	6.3	5.9
			LOD ^c = 0.0018		0.0017		0.0010		0.0012		0.0015	
			LOQ ^d = 0.0059		0.0055		0.0033		0.0040		0.0049	

Table II. (Cont.) Recovery of Spinosyns from Almond Hulls

Sample Number	Date of Analysis	Fortification Level, µg/g	Spinosyn A		Spinosyn D		Spinosyn B		Spinosyn K		N-Demethyl Spinosyn D	
			Found µg/g	PR	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR
18547701	07-Nov-1996	0.020	0.0166	83	0.0158	79	0.0153	77	0.0161	81	0.0151	76
18547701	01-Dec-1996	0.020	0.0174	87	0.0171	86	0.0157	79	0.0166	83	0.0168	84
18547701	01-Dec-1996	0.020	0.0176	88	0.0170	85	0.0160	80	0.0172	86	0.0162	81
			\bar{x} = 0.0172	86	0.0166	83	0.0157	79	0.0166	83	0.0160	80
			s = 0.00053	2.6	0.00072	3.8	0.00035	1.5	0.00055	2.5	0.00086	4.0
			RSD = 3.1	3.0	4.3	4.6	2.2	1.9	3.3	3.0	5.4	5.0
18547701	07-Nov-1996	0.050	0.0438	88	0.0428	86	0.0409	82	0.0426	85	0.0400	80
18547701	07-Nov-1996	0.050	0.0434	87	0.0426	85	0.0418	84	0.0418	84	0.0410	82
18547701	01-Dec-1996	0.050	0.0416	83	0.0401	80	0.0382	76	0.0411	82	0.0377	75
			\bar{x} = 0.0429	86	0.0418	84	0.0403	81	0.0418	84	0.0396	79
			s = 0.00117	2.6	0.00150	3.2	0.00187	4.2	0.00075	1.5	0.00169	3.6
			RSD = 2.7	3.0	3.6	3.8	4.6	5.2	1.8	1.8	4.3	4.6
18547701	07-Nov-1996	0.25	0.219	88	0.214	86	0.209	84	0.214	86	0.203	81
18547701	01-Dec-1996	0.25	0.217	87	0.210	84	0.200	80	0.212	85	0.195	78
18547701	01-Dec-1996	0.25	0.225	90	0.219	88	0.207	83	0.217	87	0.203	81
			\bar{x} = 0.220	88	0.214	86	0.205	82	0.214	86	0.200	80
			s = 0.00416	1.5	0.00451	2.0	0.00473	2.1	0.00252	1.0	0.00462	1.7
			RSD = 1.9	1.7	2.1	2.3	2.3	2.6	1.2	1.2	2.3	2.1

Table II. (Cont.) Recovery of Spinosyns from Almond Hulls

Sample Number	Date of Analysis	Fortification Level, µg/g	Spinosyn A		Spinosyn D		Spinosyn B		Spinosyn K		N-Demethyl Spinosyn D	
			Found µg/g	PR	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR	Found µg/g	PR
18547701	07-Nov-1996	1.0	0.853	85	0.838	84	0.794	79	0.836	84	0.768	77
18547701	07-Nov-1996	1.0	0.895	90	0.880	88	0.855	86	0.874	87	0.832	83
18547701	01-Dec-1996	1.0	0.875	88	0.845	85	0.817	82	0.858	86	0.792	79
			$\bar{x} = 0.874$	88	0.854	86	0.822	82	0.856	86	0.797	80
			$s = 0.0210$	2.5	0.0225	2.1	0.0308	3.5	0.0191	1.5	0.0323	3.1
			RSD = 2.4	2.8	2.6	2.4	3.7	4.3	2.2	1.7	4.1	3.9
Total Average Percent Recovery:			$\bar{x} =$	87	82	82	81	81	83	83	79	79
			$s =$	4.0	4.8	4.8	3.1	3.1	3.4	3.4	3.7	3.7
			$n =$	20	20	20	20	20	20	20	20	20
			RSD =	4.6	5.9	5.9	3.8	3.8	4.1	4.1	4.7	4.7

^a PR = Percent recovery.

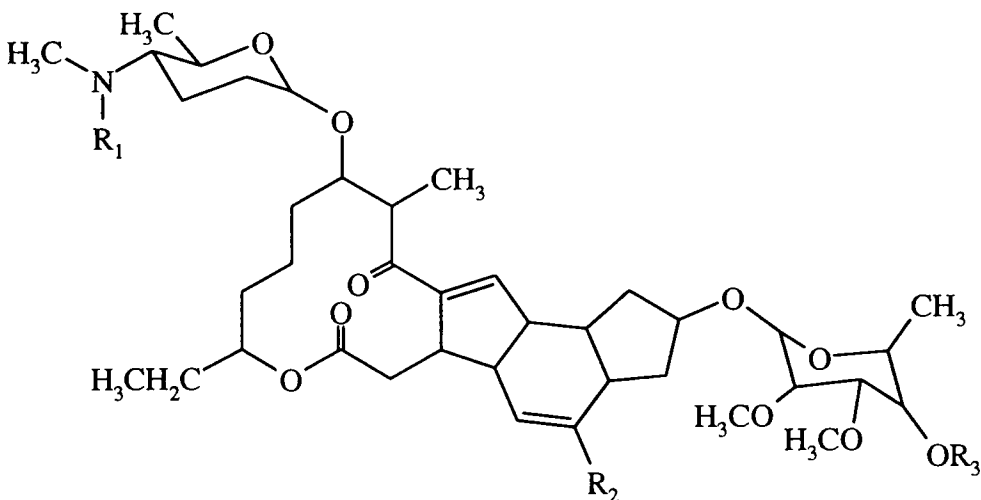
^b The residue was detected but was below the 0.01-µg/g limit of quantitation.

^c Calculated limit of detection.

^d Calculated limit of quantitation.

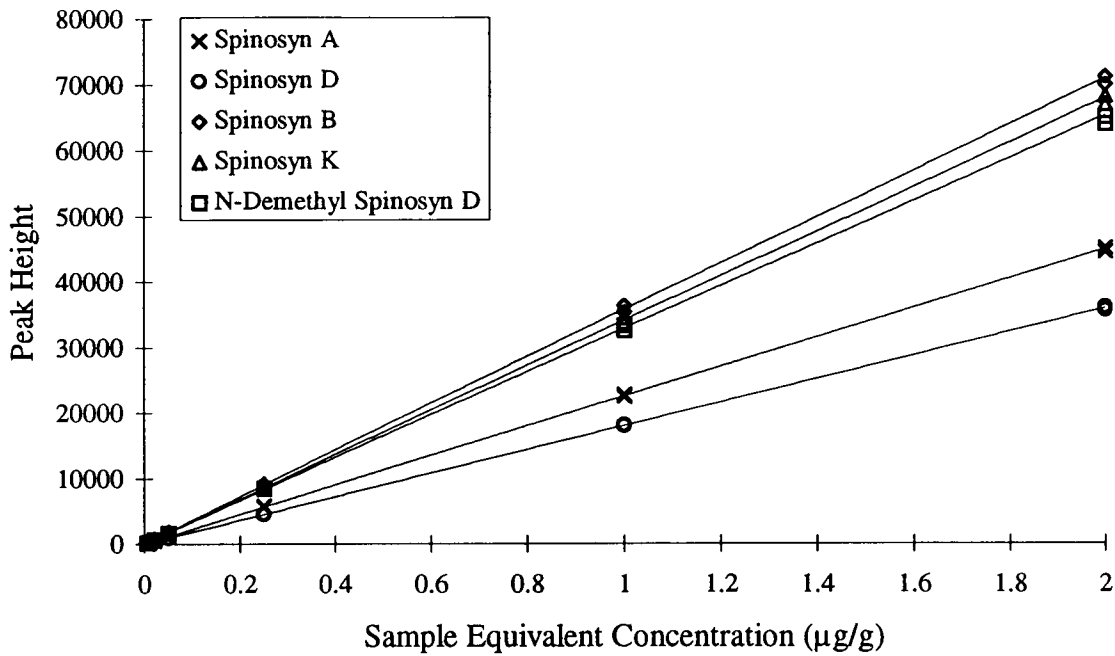
Table III. Chemicals Checked for Possible Interference with Spinosyns

Acephate	Ethoprop	Napropamide
Aldicarb	Fenamiphos	Norflurazon
Azinphos-methyl	Fenarimol	Oryzalin
Benfluralin	Fenvalerate	Oxamyl
Benomyl	Fluazifop-butyl	Oxydemeton-methyl
Bensulide	Fonofos	Oxyfluorfen
Carbaryl	Gibberallic acid	Paraquat dichloride
Carbofuran	Glyphosate	Pebulate
Chlorothalonil	Imidacloprid	Pendimethalin
Chlorpyrifos	Imidan	Pentachloronitrobenzene
Clofentazine	Iprodione	Permethrin
Cypermethrin	Isopropanol	Phorate
Cyromazine	Isoproturon	Phosphamidon
Cycloate	Linuron	Pronamide
DCNA	Malathion	Propargite
DCPA	Mancozeb	Propiconazole
Diazinon	Maneb	Sethoxydim
1,1-Dichloro-1-propene	Metalaxyl	Simazine
Dicofol	Methamidophos	Terbacil
Dimethoate	Methidathion	Thiophanate
Disulfoton	Methomyl	Triadimefon
Diuron	Metribuzin	Trifluralin
Endosulfon	Methyl parathion	Triforine
EPTC	Myclobutanil	Ziram
Ethion	Naled	



Factor	Chemical Name and Structures
Spinosyn A	Name: 2-[(6-deoxy-2,3,4-tri-O-methyl- α -L-mannopyranosyl)oxy]-13-[[5-(dimethylamino)tetrahydro-6-methyl-2H-pyran-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a,16b-tetradecahydro-14-methyl-1H-as-Indaceno[3,2-d]oxacyclododecin-7,15-dione (CAS Number 131929-60-7) Structure: $R_1 = \text{CH}_3$ $R_2 = \text{H}$ $R_3 = \text{CH}_3$
Spinosyn D	Name: 2-[(6-deoxy-2,3,4-tri-O-methyl- α -L-mannopyranosyl)oxy]-13-[[5-(dimethylamino)tetrahydro-6-methyl-2H-pyran-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a,16b-tetradecahydro-4,14-dimethyl-1H-as-Indaceno[3,2-d]oxacyclododecin-7,15-dione (CAS Number 131929-63-0) Structure: $R_1 = \text{CH}_3$ $R_2 = \text{CH}_3$ $R_3 = \text{CH}_3$
Spinosyn B	Name: 2-[(6-deoxy-2,3,4-tri-O-methyl- α -L-mannopyranosyl)oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a,16b-tetradecahydro-14-methyl-13-[(tetrahydro-6-methyl-5-(methylamino)-2H-pyran-2-yl)oxy]-1H-as-Indaceno[3,2-d]oxacyclododecin-7,15-dione (CAS Number 131929-61-8) Structure: $R_1 = \text{H}$ $R_2 = \text{H}$ $R_3 = \text{CH}_3$
Spinosyn K	Name: 2-[(6-deoxy-2,3-di-O-methyl- α -L-mannopyranosyl)oxy]-13-[[5-(dimethylamino)-tetrahydro-6-methyl-2H-pyran-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a,16b-tetradecahydro-14-methyl-1H-as-Indaceno[3,2-d]oxacyclododecin-7,15-dione (CAS Number 159195-00-3). Structure: $R_1 = \text{CH}_3$ $R_2 = \text{H}$ $R_3 = \text{H}$
<i>N</i> -Demethyl Spinosyn D	Name: 2-[(6-deoxy-2,3,4-tri-O-methyl- α -L-mannopyranosyl)oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a,16b-tetradecahydro-4,14-dimethyl-13-[(tetrahydro-6-methyl-5-(methylamino)-2H-pyran-2-yl)oxy]-1H-as-Indaceno[3,2-d]oxacyclododecin-7,15-dione (CAS Number 149439-70-3) Structure: $R_1 = \text{H}$ $R_2 = \text{CH}_3$ $R_3 = \text{CH}_3$

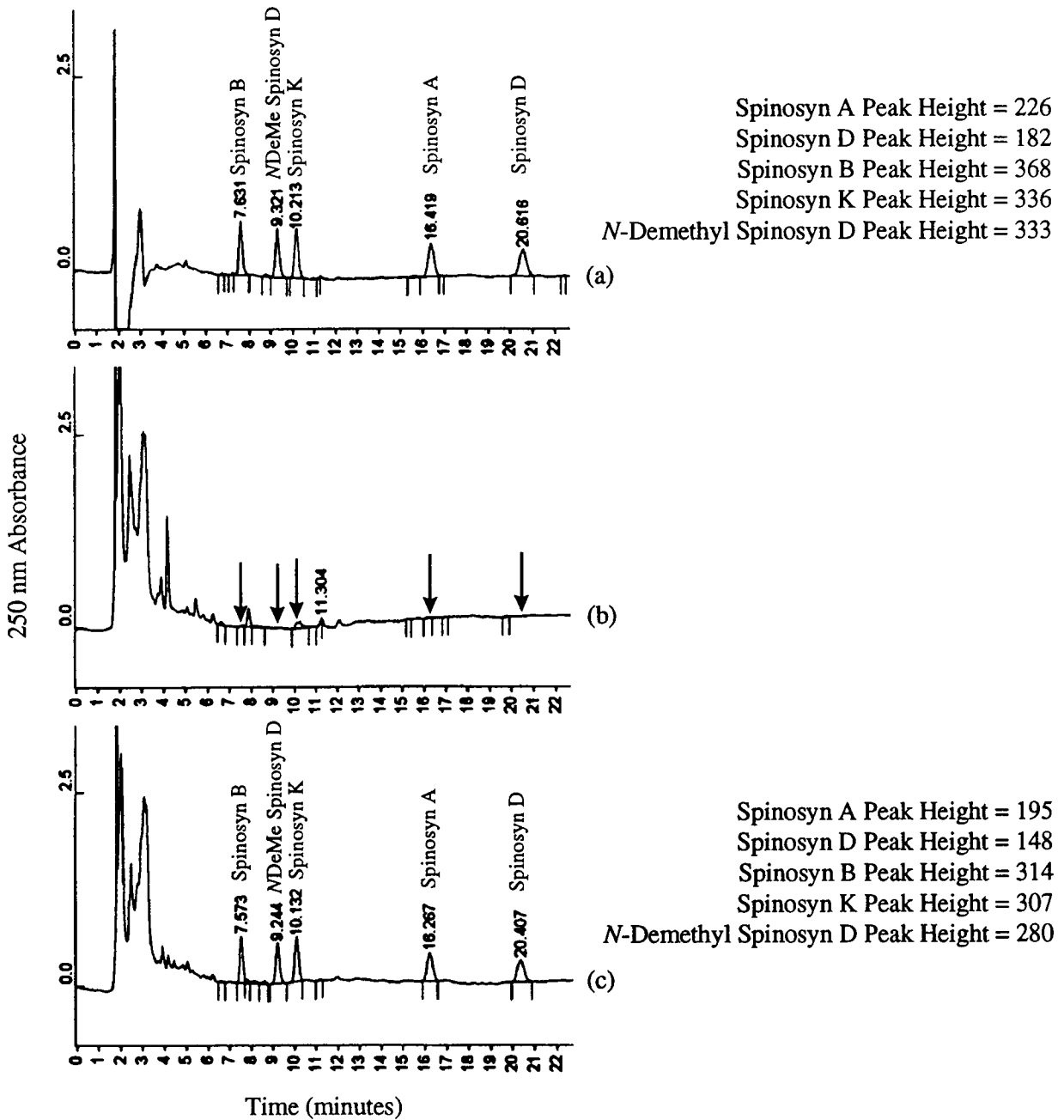
Figure 1. Chemical Names and Structures of Spinosyns A, D, B, K, and *N*-Demethyl Spinosyn D



Sample Equivalent Concentration µg/g	Peak Height				
	Spinosyn				N-Demethyl Spinosyn D
	A	D	B	K	
0.005	109	96	185	171	167
0.005	119	90	192	180	174
0.010	226	182	368	336	333
0.010	234	173	375	346	339
0.020	453	379	734	687	674
0.020	463	366	738	706	687
0.050	1119	900	1806	1699	1671
0.050	1138	901	1851	1754	1702
0.250	5682	4518	9112	8599	8388
0.250	5724	4567	9168	8684	8462
1.0	22408	17946	35300	33730	32498
1.0	22723	18168	36295	34421	33247
2.0	44440	35481	69965	66910	63885
2.0	45099	36065	71153	68172	64970
exponent:	0.996876	0.996570	0.990827	0.995856	0.992342
constant:	22537.2	17996.5	35712.4	34062.6	32820.3
r ² :	0.9999	0.9999	1.0000	0.9999	0.9999

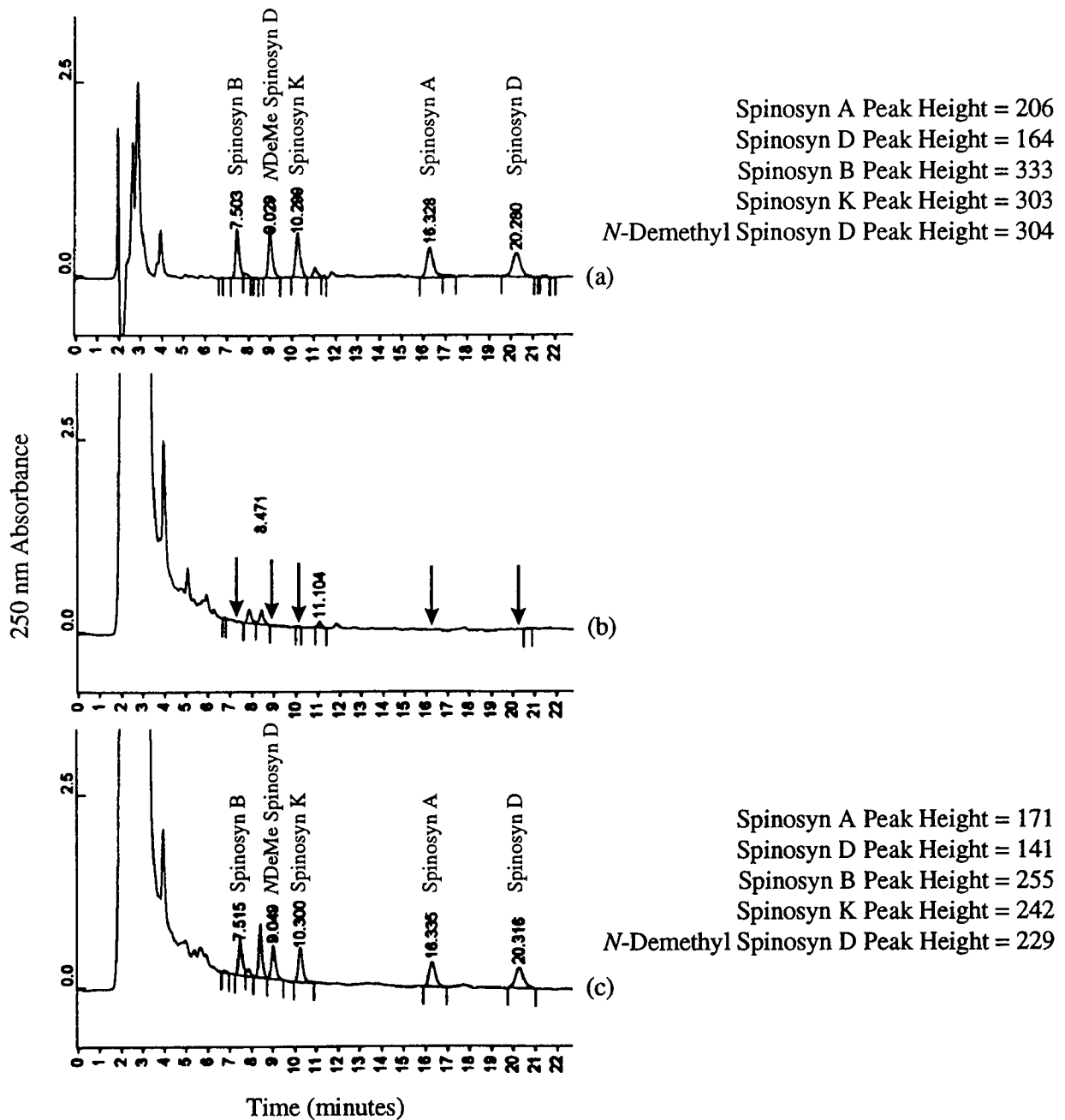
$$\text{Sample Equivalent Concentration } (\mu\text{g/g}) = \left[\frac{\text{Peak Height}}{\text{constant}} \right]^{1/\text{exponent}}$$

Figure 2. Typical Calibration Curves for the Determination of Spinosyns A, D, B, K, and N-Demethyl Spinosyn D in Tree Nuts



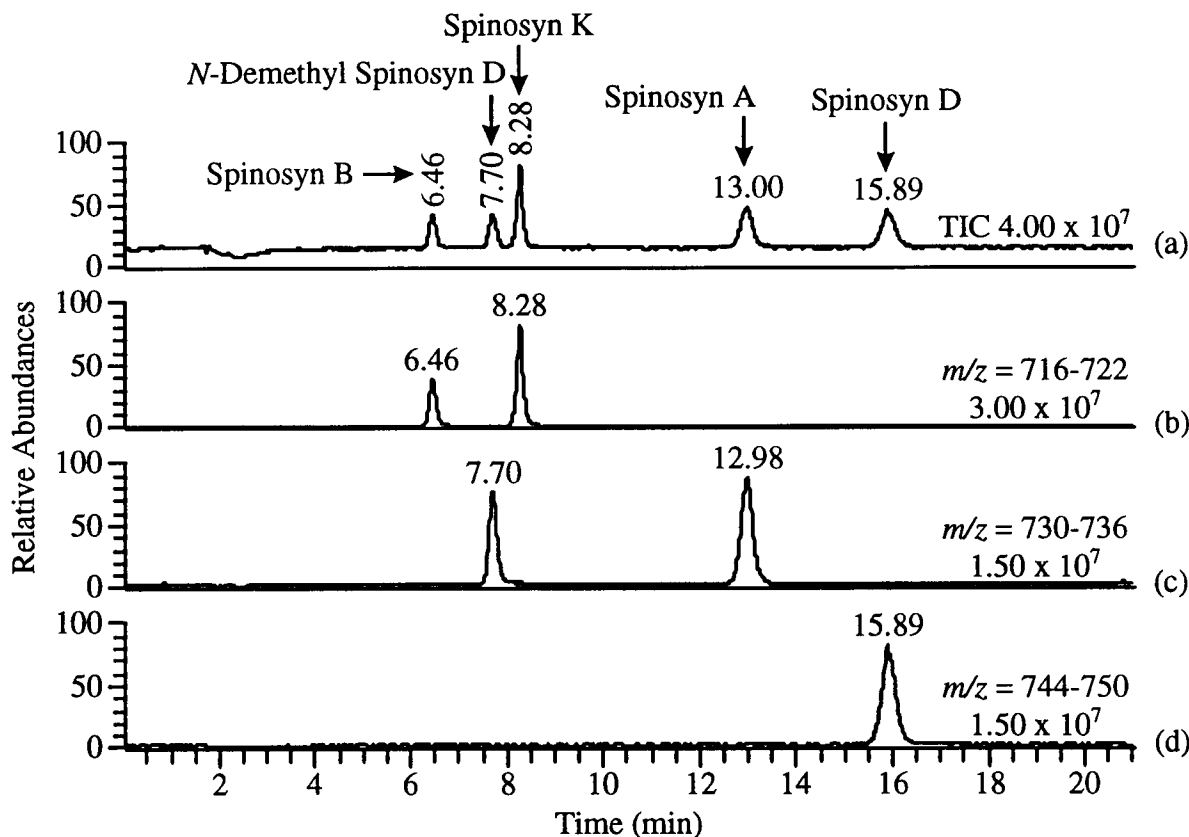
- (a) A 0.05- $\mu\text{g/mL}$ Calibration Standard, Equivalent to 0.010 $\mu\text{g/g}$ of Each Analyte in Tree Nuts
- (b) A Control Almond Nutmeat Sample
- (c) A Control Almond Nutmeat Sample Fortified at 0.010- $\mu\text{g/g}$ with Each Analyte, Equivalent to a Recovery of 85% Spinosyn A, 81% Spinosyn D, 84% Spinosyn B, 88% Spinosyn K, and 82% N-Demethyl (NDeMe) Spinosyn D

Figure 3. Typical Chromatograms from the Determination of Spinosyns in Tree Nuts (Nutmeats)



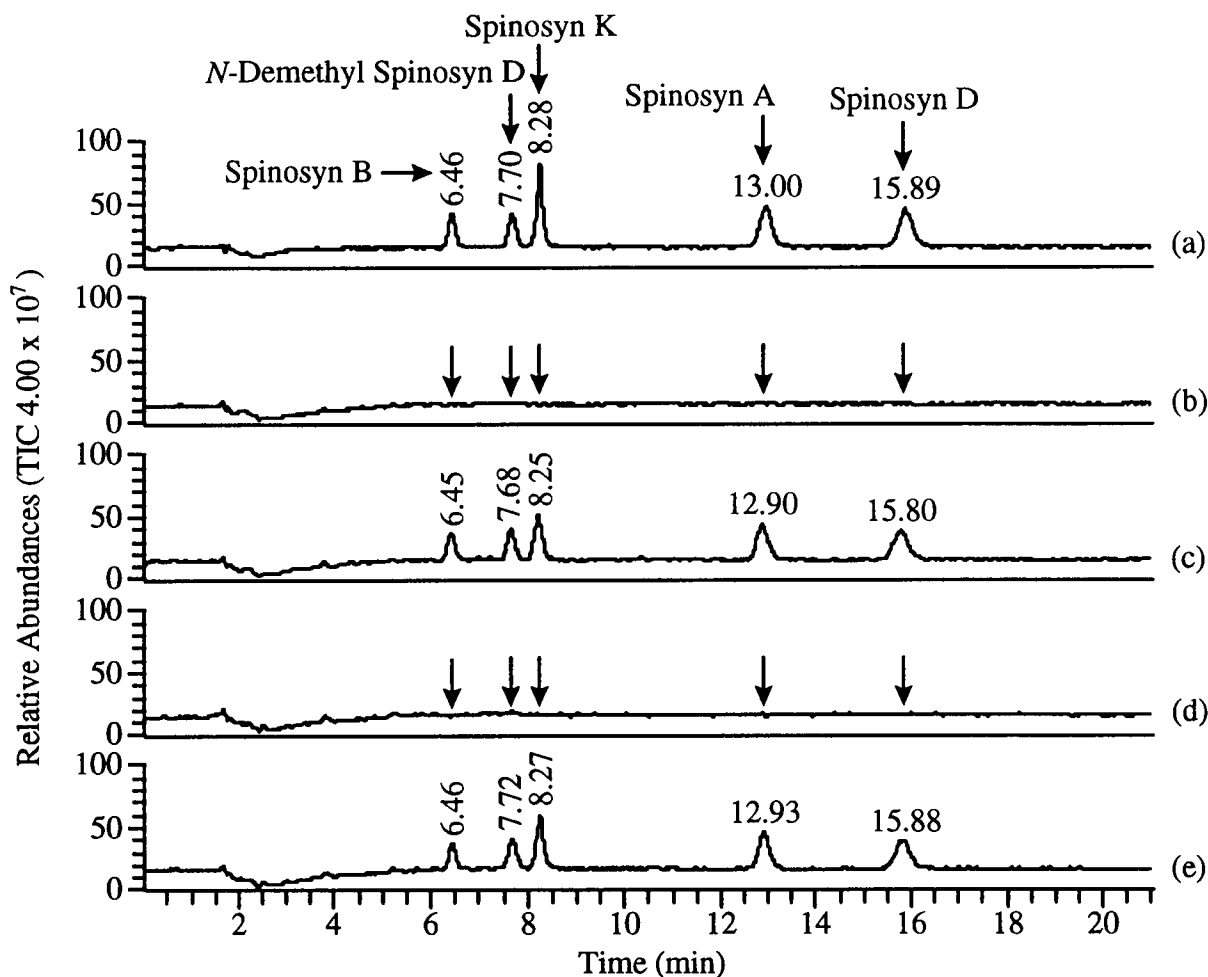
- (a) A 0.05- $\mu\text{g}/\text{mL}$ Calibration Standard, Equivalent to 0.010 $\mu\text{g}/\text{g}$ of Each Analyte in Tree Nuts
- (b) A Control Almond Hull Sample
- (c) A Control Almond Hull Sample Fortified at 0.010- $\mu\text{g}/\text{g}$ with Each Analyte, Equivalent to a Recovery of 85% Spinosyn A, 85% Spinosyn D, 76% Spinosyn B, 80% Spinosyn K, and 76% *N*-Demethyl (*NDeMe*) Spinosyn D

Figure 4. Typical Chromatograms from the Determination of Spinosyns in Tree Nuts (Hulls)



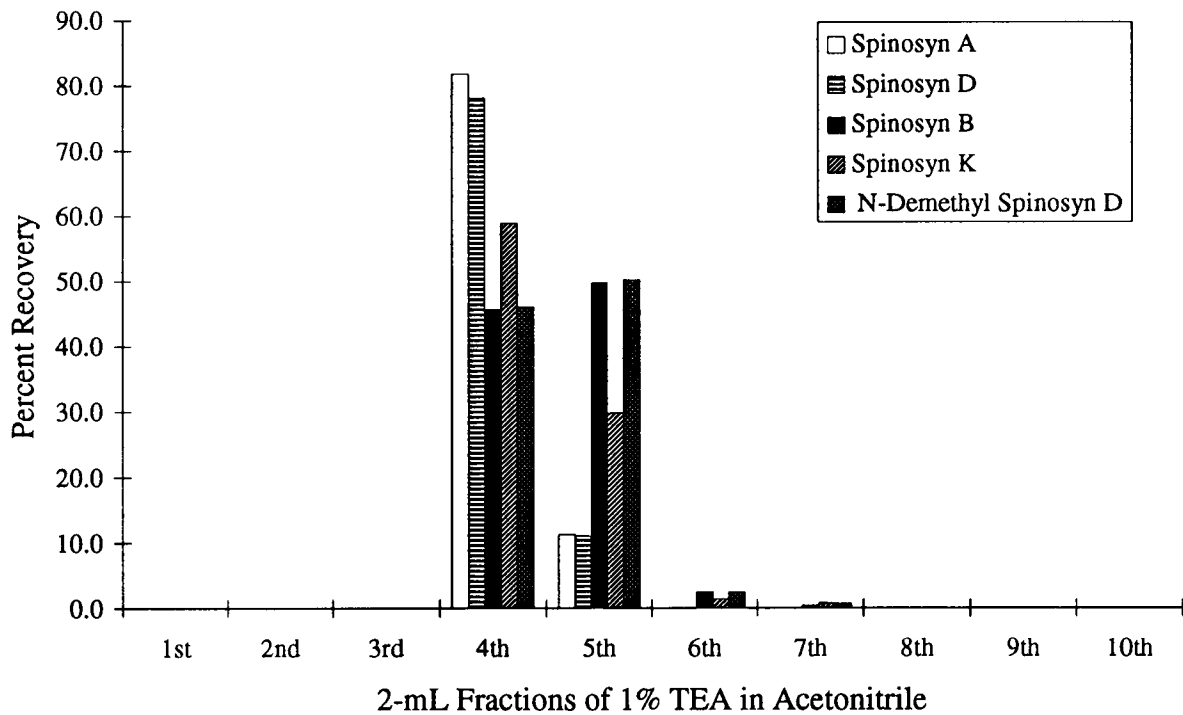
- (a) A Total Ion Chromatogram (TIC)
- (b) An Ion Chromatogram at $m/z = 716-722$, Specific to Spinosyns B and K
- (c) An Ion Chromatogram at $m/z = 730-736$, Specific to *N*-Demethyl Spinosyn D and Spinosyn A
- (d) An Ion Chromatogram at $m/z = 744-750$, Specific to Spinosyn D

Figure 5. Representative LC/MS Chromatograms for a 0.05- $\mu\text{g}/\text{mL}$ Calibration Standard, Equivalent to 0.010 $\mu\text{g}/\text{g}$ of Each Analyte in Tree Nuts



- (a) A 0.05- $\mu\text{g}/\text{mL}$ Calibration Standard, Equivalent to 0.010 $\mu\text{g}/\text{g}$ of Each Analyte in Tree Nuts
- (b) A Control Almond Nutmeat Sample
- (c) A Control Almond Nutmeat Sample Fortified at 0.010- $\mu\text{g}/\text{g}$ with Each Analyte
- (d) A Control Almond Hull Sample
- (e) A Control Almond Hull Sample Fortified at 0.010- $\mu\text{g}/\text{g}$ with Each Analyte

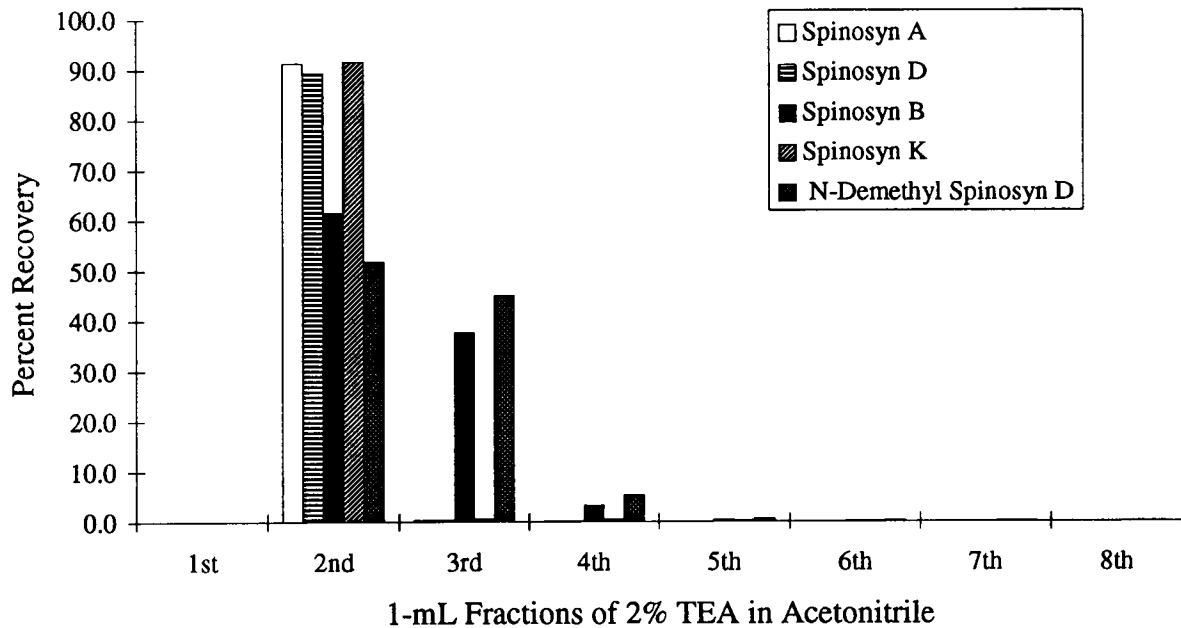
Figure 6. Representative Total Ion Chromatograms (TIC) for the Confirmation of Spinosyns in Tree Nuts by LC/MS



Solvent	Fraction	Percent Recovery				
		Spinosyn				N-Demethyl Spinosyn D
		A	D	B	K	
Hexane, 60 mL	Load	0.0	0.0	0.0	0.0	0.0
Dichloromethane, 15 mL	Rinse 1	0.0	0.0	0.0	0.0	0.0
Acetonitrile, 15 mL	Rinse 2	0.0	0.0	2.0	0.0	0.7
1% TEA in Acetonitrile, 2 mL	1	0.0	0.0	0.0	0.0	0.0
1% TEA in Acetonitrile, 2 mL	2	0.0	0.0	0.0	0.0	0.0
1% TEA in Acetonitrile, 2 mL	3	0.0	0.0	0.0	0.0	0.0
1% TEA in Acetonitrile, 2 mL	4	81.8	78.1	45.7	58.9	46.1
1% TEA in Acetonitrile, 2 mL	5	11.2	11.0	49.8	29.8	50.2
1% TEA in Acetonitrile, 2 mL	6	0.0	0.0	2.5	1.4	2.5
1% TEA in Acetonitrile, 2 mL	7	0.0	0.0	0.5	0.8	0.7
1% TEA in Acetonitrile, 2 mL	8	0.0	0.0	0.0	0.0	0.0
1% TEA in Acetonitrile, 2 mL	9	0.0	0.0	0.0	0.0	0.0
1% TEA in Acetonitrile, 2 mL	10	0.0	0.0	0.0	0.0	0.0
	Total ^a	93.1	89.1	98.5	90.9	99.5

^a Total does not include the breakthrough in the acetonitrile rinse.

Figure 7. Elution Profiles for 10.0 µg of Spinosyns on a Silica SPE Cartridge with 1% Triethylamine (TEA) in Acetonitrile



Solvent	Fraction	Percent Recovery				
		Spinosyn				N-Demethyl Spinosyn D
		A	D	B	K	
Loading solution, 10 mL	Load	0.0	0.0	0.0	0.0	0.0
Acetonitrile, 5 mL	Rinse 1	0.0	0.0	0.0	0.0	0.0
Acetone, 3 mL	Rinse 2	0.0	0.0	0.0	0.1	0.1
2% TEA in Acetonitrile, 1 mL	1	0.0	0.0	0.0	0.0	0.0
2% TEA in Acetonitrile, 1 mL	2	91.2	89.3	61.5	91.6	51.8
2% TEA in Acetonitrile, 1 mL	3	0.3	0.3	37.7	0.6	45.0
2% TEA in Acetonitrile, 1 mL	4	0.2	0.1	3.3	0.5	5.4
2% TEA in Acetonitrile, 1 mL	5	0.0	0.0	0.4	0.2	0.6
2% TEA in Acetonitrile, 1 mL	6	0.0	0.0	0.1	0.1	0.2
2% TEA in Acetonitrile, 1 mL	7	0.0	0.0	0.0	0.1	0.1
2% TEA in Acetonitrile, 1 mL	8	0.0	0.0	0.1	0.1	0.1
	Total^a	91.7	89.8	103.3	93.2	103.0

^a Total does not include the breakthrough in the acetone rinse.

Figure 8. Elution Profiles for 10.0 µg of Spinosyns on a CH SPE Cartridge with 2% Triethylamine (TEA) in Acetonitrile