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Determination of Spinosad and Metabolites in Beef Tissues, Milk, and Cream by High Performance Liquid Chromatography With Ultraviolet Detection

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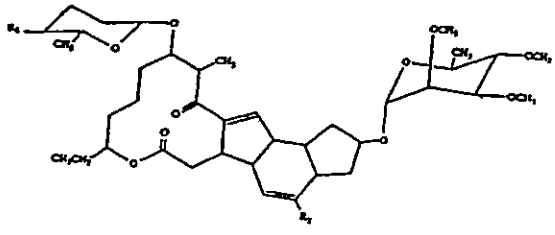
A. Scope

This method is applicable for the quantitative determination of residues of spinosad and its metabolites in milk, cream and beef tissues. The method has a validated limit of quantitation of 0.01 µg/g. It has been validated over the concentration range of 0.01-10.0 µg/g for beef fat and cream, and from 0.01-1.0 µg/g for milk, beef liver, lean, and kidney. The method simultaneously determines the parent compounds (spinosyns A and D) and two metabolites (spinosyn B and *N*-demethyl spinosyn D) in a single injection, with separation occurring on the HPLC column.



Spinosyn A, $R_1 = N(CH_3)_2$ and $R_2 = H$
Spinosyn D, $R_1 = N(CH_3)_2$ and $R_2 = CH_3$
Spinosyn B, $R_1 = NH(CH_3)$ and $R_2 = H$
N-Demethyl spinosyn D, $R_1 = NH(CH_3)$ and $R_2 = CH_3$

The chemical names for these four compounds are presented in Table I.

B. Principle

Residues of spinosyns A, D, B, and *N*-demethyl spinosyn D are extracted from milk and tissue samples using appropriate solvents. The extracting solvent is acetonitrile for milk and cream, 80% acetonitrile/20% water for beef lean, liver, and kidney, and 60% hexane/40% dichloromethane for fat. An aliquot of the extract is purified by liquid-liquid partitioning, followed by silica and cyclohexyl solid phase extraction (SPE). All four analytes are separated and determined simultaneously in the purified extracts by reversed-phase high performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 250 nm.

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, LITERATURE AND OTHER RELATED DATA. Safety information on non-DowElanco products may be obtained from the container label or from the supplier. Disposal of reagents and solvents must be in compliance with local, state, and federal laws and regulations.
2. Volatile and flammable organic solvents such as acetone, acetonitrile, 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 (Note L.1.)

1. Autotransformer, variable (for temperature control of heating mantles), Model 3PN1010, Staco Energy Products Co., Dayton, OH 45401.
2. Balance, analytical, Model AE-160, Mettler Instrument Corporation, Hightstown, NJ 08520.
3. Balance, toploading, Model P-1200 or BB2240, Mettler Instrument Corporation.
4. Bath, circulating, chilled water (for reflux condensers), Model 2095, Forma Scientific, Marietta, OH 45750.
5. Bath, circulating, heated water (for rotary vacuum evaporators), Model 1299-00, Cole-Parmer Instrument Co., Chicago, IL 60648.
6. Bath, ultrasonic, Model FS14H, Fisher Scientific, Pittsburgh, PA 15219.
7. Centrifuge, Model CU-5000, International Equipment Company, Needham Heights, MA 02194.
8. Condensers, reflux, water cooled, 300-mm sleeve length, 5 bulbs, catalog number 07-736B, Fisher Scientific.
9. Evaporator, TurboVap LV, Zymark Corporation, Hopkinton, MA 01748.
10. Evaporator, rotating shaft, catalog number 09-548-100, Fisher Scientific.
11. Filtration apparatus for HPLC solvents, catalog number 5-8061M, Supelco, Inc., Bellefonte, PA 16823.

12. Hammermill, Model 2001, equipped with a 3/16 inch screen, Agvise Laboratories, Inc., Northwood, ND 58267.
13. High performance liquid chromatograph with a UV detector, Model 1050, and a recording integrator, Model 3396 Series II, Hewlett-Packard, Wilmington, DE 19808.
14. Homogenizer, Polytron, catalog number PT 10 20 3500, with a 10-mm diameter generator with saw teeth, catalog number 27 11 330-3, Brinkmann Instruments, Inc., Westbury, NY 11590.
15. Mantles, heating (for reflux condensers), catalog number 0-408, Glas-Col Apparatus Co., Terre Haute, IN 47802.
16. Reservoirs (for SPE cartridges), catalog number WAT011390, Waters, Milford, MA 01757.
17. Shaker, gyratory, New Brunswick Model G-33, Fisher Scientific.
18. Vacuum manifold, solid phase extraction, catalog number 210016, Alltech Associates, Inc., Deerfield, IL 60015.
19. Water purifier, Milli-Q UV Plus, Millipore Corporation, Milford, MA 01730.

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

1. Bottles, glass, 8-oz (237-mL), graduated, with PTFE-lined lids, catalog number 03-320-11G, Fisher Scientific.
2. Cartridges, cyclohexyl (CH) Bond Elut LRC (500 mg), part number 1211-3032, Varian Sample Preparation Products, Harbor City, CA 90710.
3. Cartridges, Sep-Pak Plus, silica (690 mg), part number WAT20520, Waters.
4. Column, HPLC, C18/Cation, Mixed Mode, 5 µm, 150 mm x 4.6 mm i.d., catalog number 72575, Alltech Associates, Inc.
5. Column, HPLC, ODS-AQ, catalog number AQ-302-5, 5 µm, 150 mm x 4.6 mm i.d., YMC, Inc., Wilmington, NC 28403.
6. Filter paper, 15-cm, pre-pleated, Number 588 (0.19-mm thickness), catalog number 03710, Schleicher and Schuell, Keene, NH 03431.
7. Filters for HPLC solvents, membrane, Nylon-66, 47-mm i.d., 0.45 µm pore size, catalog number 5-8067M, Supelco, Inc.
8. Granules, boiling, carborundum Number 12, catalog number 133-B, Hengar Co., Philadelphia, PA 19144.
9. Pipets, Pasteur, 9-inch, catalog number 13-678-7C, Fisher Scientific.
10. Vials, amber glass, 40 mL, pre-cleaned, catalog number 03-338-27A, Fisher Scientific.
11. Vials, clear glass, 9.5 dram (35 mL), catalog number 03-339-21M, Fisher Scientific.

F. Reagents and Prepared Solutions (Note L.1.) (Critical Step--See Note L.2.)

1. Reagents

a. Acetone, OmniSolv, catalog number AX0116-1, EM Science, Gibbstown, NJ 08027.

b. Acetonitrile, ChromAR HPLC grade, catalog number 2856-09, Mallinkrodt Specialty Chemicals Company, Paris, KY 40361.

c. Ammonium acetate, HPLC grade, catalog number A639-500, Fisher Scientific.

d. Hexane, OmniSolv, catalog number HX0296-1, EM Science.

e. Methanol, ChromAR HPLC grade, catalog number 3041-09, Mallinkrodt Specialty Chemicals Company.

f. Methylene chloride (dichloromethane), Optima grade, catalog number DX0831-1, Fisher Scientific.

g. Nitrogen, refrigerated liquid, catalog number LQNI-230, Airco Gas and Gear, Indianapolis, IN 46241.

h. Standard:

Obtain pure active ingredients or reference compounds of spinosyns A, D, B, and *N*-demethyl spinosyn D from the Test Substance Coordinator, DowElanco, 9330 Zionsville Road, Indianapolis, IN 46268-1053.

i. Triethylamine, reagent grade, catalog number 04885-1, Fisher Scientific.

j. Water, ultra-pure, purified using Milli-Q UV Plus.

2. Prepared Solutions

a. 80% acetonitrile/20% water (v/v)

For each liter of solution, mix 800 mL of acetonitrile with 200 mL of ultra-pure water.

b. 2% aqueous ammonium acetate/acetonitrile (67:33) (v/v)

Dissolve 20.0 g of ammonium acetate per liter of ultra-pure water. For each liter of the 67:33 solution, mix 670 mL of 2% ammonium acetate and 330 mL of acetonitrile. Filter under vacuum through a 0.45-µm membrane filter using a filtration apparatus for HPLC solvents.

c. 60% hexane/40% dichloromethane (v/v):

Prepare by mixing 600 mL of hexane and 400 mL of dichloromethane per liter of solution.

d. 50% methanol/50% acetonitrile (v/v)

Prepare by mixing 500 mL of methanol and 500 mL of acetonitrile per liter of solution.

e. Methanol/acetonitrile/2% aqueous ammonium acetate (1:1:1) (v/v/v)

Prepare by mixing equal volumes of each.

f. 20% methanol/20% acetonitrile/60% water (v/v/v)

Prepare by mixing 200 mL of methanol, 200 mL of acetonitrile, and 600 mL of ultra-pure water per liter of solution.

g. 75% dichloromethane/25% methanol (v/v)

Prepare by mixing 750 mL of dichloromethane with 250 mL of methanol per liter of solution.

h. 2% triethylamine/98% acetonitrile (v/v)

Immediately before use, prepare by mixing 2 mL of triethylamine and 98 mL of acetonitrile per 100 mL of solution. (Note L.3.)

G. Preparation of Standards (Critical Step--See Note L.4.)

1. Preparation of XDE-105 Fortification Solutions

a. For each analyte (spinosyns A, D, B, and *N*-demethyl spinosyn D), weigh 20.0 mg of the pure active ingredient or reference compound (corrected for purity) using an analytical balance. Quantitatively transfer each one to separate 100-mL volumetric flasks and dissolve in 50% methanol/50% acetonitrile. Mix or shake until the solids completely dissolve. Dilute to volume to obtain stock solutions containing 200.0 µg/mL.

b. Combine 10.0-mL aliquots of each 200.0-µg/mL stock solution from Step G.1.a. in a 100-mL volumetric flask and dilute to volume with 50% methanol/ 50% acetonitrile to obtain a solution containing 20.0 µg/mL of each analyte.

c. Dilute appropriate aliquots of the above 20.0-µg/mL solution to volume with 50% methanol/50% acetonitrile in 100-mL volumetric flasks to obtain the desired concentrations for the fortification of recovery samples as shown in the table below.

Aliquot of Stock Soln.	Final Soln. Volume	Spiking Soln. Final Conc.	Equivalent Sample Conc.[a]
mL	mL	µg/mL	µg/g
1.0	100	0.2	0.010
2.5	100	0.5	0.025
5.0	100	1.0	0.050
10.0	100	2.0	0.100

[a] The equivalent sample concentration is based on fortifying a 20-g or 20-mL sample with 1.0 mL of spiking solution.

2. Preparation of HPLC Standard Calibration Solutions

a. Combine 5.0-mL aliquots of each 200.0-µg/mL stock solution from Step G.1.a. in a 100-mL volumetric flask and dilute to volume with methanol/acetonitrile/2% ammonium acetate (1:1:1) to obtain a solution containing 10.0 µg/mL of each analyte.

b. Dilute aliquots of the above 10.0-µg/mL solution to volume with methanol/acetonitrile/2% ammonium acetate (1:1:1) in 100-mL volumetric flasks to obtain the following calibration standards:

Aliquot of Stock Soln. mL	Final Solution Volume mL	Final Solution Concentration µg/mL
0.0	100	0.000
1.0	100	0.100
5.0	100	0.500
10.0	100	1.000
15.0	100	1.500

H. High Performance Liquid Chromatography

1. Typical Operating Conditions (Note L.5.)

Instrumentation:	Hewlett-Packard Model 1050 with a UV detector, an autosampler capable of injecting at least 175 µL, and a recording integrator or a computer
Column:	YMC ODS-AQ 5 µm 150 mm x 4.6 mm i.d.
Column (Oven) Temp.:	30 °C
Mobile Phase:	44% reservoir A/44% reservoir B/12% reservoir C reservoir A = methanol reservoir B = acetonitrile reservoir C = 2% aqueous ammonium acetate/acetonitrile (67:33)
Flow Rate:	0.8-1.1 mL/min (adjust to yield adequate retention times and baseline resolution)
Injection Volume:	175 µL
Detector:	Ultraviolet, 250 nm
Attenuation:	2^3 or 8 (adjust to yield a peak response of approximately 50% of full-scale deflection for spinosyn D in the 1.0 µg/mL standard)
Chart Speed:	0.2 cm/min
Run Time:	20 minutes per sample (longer time may be used if late-eluting peaks occur)

UV spectra are shown in Figure 1.

2. Calibration Curves

A typical calibration curve for spinosyn A is shown in Figure 2.

3. Column and Typical Conditions for Confirmation of Residues (Note L.5.)

Column:	C18/Cation Mixed Mode 5 µm 150 mm x 4.6 mm i.d.
Mobile Phase:	40% reservoir A/40% reservoir B/20% reservoir C Reservoir A = methanol Reservoir B = acetonitrile Reservoir C = 2% aqueous ammonium acetate/acetonitrile (67:33)
Flow Rate:	0.8-1.1 mL/min (adjust to yield adequate retention times and baseline resolution)
UV Wavelength:	250 nm, 235 nm, or 275 nm (Step I.3.)
Other Parameters:	Same as in Step H.1.

4. Typical Chromatograms

Typical chromatograms obtained under the conditions in Step H.1. are illustrated in Figures 3-8. Representative chromatograms are included for each level of fortification. The chromatograms for beef kidney (Figure 7) contained more extraneous peaks than those for the other commodities, although none of the extraneous peaks matched the retention times of the four analytes. (Note that slight differences in retention times for the analytes from various chromatograms within a given figure occurred because not all of the chromatograms in the figure were obtained on the same day.) Typical chromatograms for the confirmation of the analyte residues under the conditions in Step H.3. are shown in Figure 9.

I. Determination of Recovery of the Analytes

1. Preparation of Recovery Samples

a. Beef Lean, Liver, or Kidney

(1) Prepare the beef lean, liver, or kidney samples for analysis by chopping with a cleaver, then freezing with liquid nitrogen and grinding through an Agvise Model 2001 Hammermill with a screen size of approximately 3/16 inch. Composite and manually mix the tissue in a plastic bag to produce a homogeneous sample.

(2) Thaw the tissue samples, if necessary. Using a toploading balance, weigh duplicate 20-g samples of the untreated control tissue into 250-mL boiling flasks using a technique that prevents the tissue from sticking to the neck of the flask. A recommended approach is as follows: Tare the weight of the boiling flask on the balance. Place a wide-neck funnel on top of the boiling flask,

observe the weight of the funnel, and add slightly more than 20 g of tissue to the funnel. Using a narrow spatula, carefully transfer the tissue from the funnel down into the flask. Remove the funnel and record the weight of the tissue that was transferred to the flask. (Some of the tissue will stick to the funnel.)

(3) For laboratory recovery samples, add 1.0 mL of the appropriate fortification solution from Step G.1.c. Add 50 mL of 80% acetonitrile/20% water, and blend the sample on high speed for approximately 1 minute using a Polytron homogenizer with a 10-mm diameter generator and sawtooth blades.

(4) Add 50 mL of 80% acetonitrile/20% water to a 50-mL graduated cylinder, and rinse the blades by blending on high speed for approximately 10 seconds. Combine the rinse with the blended solution in the 250-mL boiling flask for a total extraction volume of 100 mL.

(5) Add approximately 2 g of boiling granules to the boiling flask, and place the flask on a heating mantle. Attach the flask to a chilled-water reflux condenser, and heat the samples to reflux for approximately 1 hour. (Note L.6.)

(6) After heating for 1 hour, turn off the heating mantle, but leave on the chilled water to continue cooling the condenser. Carefully raise up the hot flask and the condenser, and use a spatula or other suitable tool to prop up the flask above the mantle until the flask has cooled sufficiently to handle safely.

(7) Remove the flask from the condenser, and place a plastic cap or a glass stopper in the flask to prevent solvent loss. Transfer the flask to a hood until the sample has cooled approximately to room temperature. Clean the reflux condenser by rinsing with water and then methanol, and discard the rinse.

(8) Filter a 75-mL aliquot of the supernatant liquid through pleated filter paper into a 100-mL graduated cylinder. Transfer the 75-mL aliquot to a graduated, 8-oz bottle.

(9) Add 25 mL of hexane to the 75-mL sample aliquot in the 8-oz bottle, cap the bottle with a PTFE-lined lid, and shake the sample in an upright position on a gyratory shaker at 250 rpm for approximately 5 minutes. (Note L.7.)

(10) Centrifuge the sample at approximately 2250 rpm for about 5 minutes. Proceed immediately to Step (11). Do not allow the samples to stand for several minutes before proceeding, or some remixing will occur.

(11) Using any suitable technique, aspirate off the hexane (upper) layer. For example, use Nalgene tubing and attach a 9-inch disposable Pasteur pipet to a sidearm flask that is also attached to a vacuum line. Turn on the vacuum, and use the pipet to carefully draw off the hexane (upper) layer. Aspirate off all of the hexane, but leave the narrow band of emulsion on top of the 75 mL of acetonitrile. Do not aspirate off the acetonitrile (lower) layer. Discard the hexane that was collected in the sidearm flask.

(12) Add 75 mL of dichloromethane to the bottle, cap the bottle with a PTFE-lined lid, and shake the sample in an upright position on a gyratory shaker at 250 rpm for approximately 5 minutes.

(13) Centrifuge the sample at approximately 2250 rpm for about 5 minutes. Proceed immediately to Step (14). Do not allow the samples to stand for several minutes before proceeding, or some remixing will occur.

(14) Using any suitable technique (e.g., a 9-inch disposable Pasteur pipet attached to a sidearm flask under vacuum), carefully draw off the aqueous (upper) layer. After the aqueous layer has been completely removed, transfer the organic (lower) layer to a 100-mL graduated cylinder, and continue drawing off the organic layer down to 100 mL. (Alternatively, reduce the volume of the organic layer to the 100-mL mark in the graduated bottle.) Discard the solvents that were collected in the sidearm flask.

(15) Transfer the remaining 100-mL sample aliquot to a clean 250-mL boiling flask. Rinse the container with 10 mL of dichloromethane, and add the rinse to the boiling flask. Prior to concentrating the extract, turn on the vacuum and rinse the rotary vacuum evaporator with hexane, followed by methanol, to prevent sample contamination. Evaporate the organic solvents with the rotary vacuum evaporator and a water bath heated to approximately 35-50 °C. Add 10 mL of methanol, and evaporate to dryness to remove traces of water. (Critical step - See Note L.8.) Dissolve the residue in 10 mL of hexane.

(16) Before using each new lot of silica SPE cartridges, determine the elution profile as described in Section K.5.a. If the elution profile differs from that described below, modify the volumes of acetonitrile to be discarded in Step (16)(c) or the volume of 75% dichloromethane/25% methanol to be collected in Step (16)(d).

(a) Attach a reservoir and a stopcock to a silica SPE cartridge, and attach the stopcock to an SPE vacuum manifold. Prior to adding the sample solution, condition the cartridge under a vacuum of approximately -5 inches (-127 mm) of Hg using the following sequence of eluants: 10 mL of 75% dichloromethane/25% methanol, then 10 mL of acetonitrile, followed by 10 mL of dichloromethane, and 20 mL of hexane. Although it is not necessary to dry the silica cartridges between solvent additions, the cartridges may be allowed to dry without affecting the results.

(b) Add the sample from Step (15) in 10 mL of hexane. Rinse the evaporating flask with 10 mL of hexane, and add the hexane to the cartridge. Repeat with an additional 10 mL of hexane. Turn on the vacuum and elute the 30 mL of hexane. Rinse the flask with an additional 40 mL of hexane, add the solvent to the cartridge, and elute.

(c) Rinse the evaporating flask with 5 mL of dichloromethane, add the rinse to the SPE cartridge, and elute. Rinse the flask with two 4-mL aliquots of acetonitrile, add both rinses separately to the cartridge, and elute them separately. Discard all of the eluate collected thus far.

(d) After the second 4-mL acetonitrile rinse has eluted, place a 35-mL clear glass vial in the vacuum manifold. Rinse the evaporating flask with 12 mL of 75% dichloromethane/25% methanol and add the solvent to the cartridge. Collect the eluate in the 35-mL vial.

(e) Evaporate the sample solution to dryness using a TurboVap evaporator at 60 °C and nitrogen gas at 8 psi. Remove the samples from the TurboVap evaporator as soon as possible after the solvent has evaporated.

(f) Dissolve the residue in 5 mL of 20% methanol/20% acetonitrile/60% water with the aid of an ultrasonic bath for approximately 10-20 seconds. Carefully rotate the vial so that the solvent can dissolve the residue on the wall of the vial.

(17) Before using each new lot of CH Bond Elut LRC cartridges, determine the elution profile as described in Section K.5.b. If the elution profile differs from that described below, modify the volumes of acetone to be discarded in Step (17)(f) or the volume of 2% triethylamine/98% acetonitrile to be collected in Step (17)(g). Purify the extract on a CH SPE cartridge utilizing the following procedure.

(a) Attach a CH SPE cartridge to a stopcock and attach the stopcock to an SPE vacuum manifold. Condition the CH cartridge under a vacuum of approximately -5 inches (-127 mm) of Hg using the following sequence of eluants: 9 mL of methanol, followed by 18 mL of ultra-pure water. Do not completely dry the cartridge between solvent additions unless otherwise specified.

(b) Add the sample from Step (16)(f) to the CH cartridge. Rinse the vial with an additional 5 mL of 20% methanol/20% acetonitrile/60% water, and add the rinse to the column.

(c) Air dry the cartridge under vacuum for approximately 2 minutes after the solution has eluted.

(d) Rinse the vial with 5 mL of acetonitrile, and add the rinse to the cartridge.

(e) Air dry the cartridge under vacuum for approximately 5 minutes after the acetonitrile has eluted.

(f) Rinse the vial with 5 mL of acetone (Note L.9.), add the rinse to the cartridge, and discard all of the eluate collected thus far.

(g) Place a pre-cleaned, 40-mL amber collection vial in the vacuum manifold. Rinse the clear sample vial with 6 mL of 2% triethylamine/98% acetonitrile, turn off the hood lights, and transfer the rinse to the cartridge. Collect the eluant in the amber vial. (Note L.10.)

(18) Immediately evaporate the sample solutions to dryness using a TurboVap evaporator at 60 °C and nitrogen gas at 8 psi. Immediately remove the samples after the solvent has evaporated.

(19) Dissolve the residue in 1.0 mL of methanol/acetonitrile/2% ammonium acetate (1:1:1). Swirl the vial to dissolve the residue on the bottom of the container, then tilt the vial to nearly a horizontal position and slowly rotate it so that the solvent dissolves any residue on the side of the vial. Repeat the swirling and tilting/rotating one time to ensure that the residue is dissolved. (Note L.11.)

(20) Using a disposable Pasteur pipet, transfer the solution to an HPLC sample vial and seal the vial with a cap and crimper. Do not filter the final solution. (Note L.2.b.)

(21) Analyze the standard and sample solutions by HPLC as described in Steps H.1.-H.2. Determine the suitability of the chromatographic system using the following performance criteria:

(a) Standard curve linearity: Determine that the correlation coefficient (r^2) equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.

(b) Peak resolution: Visually determine that baseline resolution has been achieved for all four analytes.

(c) Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 3-8 in terms of peak response, baseline noise, and background interference. Visually determine that a signal-to-noise ratio of approximately 5:1 to 10:1 is attainable for the 0.1-µg/mL standard calibration solution.

(22) If the peak response for any of the samples exceeds the range of the standard calibration curve, dilute the sample with methanol/acetonitrile/2% ammonium acetate (1:1:1) to yield a response within the range of the calibration curve.

(23) Proceed to the calculations in Step I.2.

b. Milk or Cream

(1) Thaw the milk or cream sample, if necessary. Shake the sample container thoroughly to result in a homogeneous sample. Immediately collect a 20-mL aliquot of the sample using a pipet or a graduated cylinder, and transfer the aliquot to a graduated, 8-ounce glass bottle. For laboratory recovery samples, add 1.0 mL of the appropriate fortification solution(s) from Step G.1.c.

(2) Add 80 mL of acetonitrile, and shake the sample in an upright position on a gyratory shaker at 250 rpm for approximately 30 minutes. (Note L.7.)

(3) Centrifuge the sample at 2250 rpm for approximately 5 minutes. Proceed immediately to Step (4). Do not allow the samples to stand for several minutes before proceeding, or some remixing will occur.

(4) Decant a 75-mL aliquot of the supernatant solution into a graduated cylinder, and transfer the aliquot to a clean, 8-ounce graduated glass bottle.

(5) Proceed as described in Step I.1.a.(12) for beef lean, liver, or kidney.

c. Beef Fat

(1) Thaw the fat sample, if necessary. Using a toploading balance, weigh duplicate 20-g samples of the untreated control tissue into 250-mL boiling flasks using a technique that prevents the tissue from sticking to the neck of the flask. A recommended approach is as follows: Tare the weight of the boiling flask on the balance. Place a wide-neck funnel on top of the boiling flask, observe the weight of the funnel, and add slightly more than 20 g of tissue to the funnel. Using a narrow spatula, carefully transfer the tissue from the funnel down into the flask. Remove the funnel and record the weight of the tissue that was transferred to the flask. (Some of the tissue will stick to the funnel.) For laboratory recovery samples, add 1.0 mL of the appropriate fortification solution(s) from Step G.1.c.

(2) Add 50 mL of 60% hexane/40% dichloromethane, and blend the sample on high speed for approximately 1 minute using a Polytron homogenizer with a 10-mm diameter generator and sawtooth blades.

(3) Add 40 mL of 60% hexane/40% dichloromethane to a 50-mL graduated cylinder, and rinse the blades by blending on high speed for approximately 10 seconds. Combine the rinse with the blended solution in the 250-mL boiling flask. (The homogenization and melting of 20 g of tissue typically produces about 10 mL of solution, so that the total extraction volume is approximately 100 mL.)

(4) Add approximately 2 g of boiling granules to the boiling flask, and place the flask on a heating mantle. Attach the flask to a chilled-water reflux condenser, and heat the samples to reflux for approximately 1 hour. (Note L.6.) A setting of approximately 45% of full scale on the variable autotransformer has produced heating mantle temperatures of approximately 150 ± 15 °C and have brought the solution to boil in approximately 10 minutes. Adjust the percent of full-scale setting as needed to produce the desired temperature.

(5) After heating for 1 hour, turn off the heating mantles, but leave on the chilled water to continue cooling the condenser. Carefully raise up the hot flask and the condenser, and use a spatula or other suitable tool to prop up the flask above the mantle until the flask has cooled sufficiently to handle safely.

(6) Remove the flask from the condenser, and place a plastic cap or a glass stopper in the flask to prevent solvent loss. Transfer the flask to a hood until the sample has cooled to approximately room temperature. Decant a 50-mL aliquot of the supernatant liquid into a 50-mL graduated cylinder.

(7) Transfer the 50-mL aliquot to a 250-mL separatory funnel containing 20 mL of hexane and 75 mL of acetonitrile.

(8) Shake the separatory funnel vigorously for approximately 30 seconds. Wait for approximately 2 minutes for the layers to separate, and then drain the lower layer (acetonitrile/dichloromethane) into a clean 500-mL boiling flask.

(9) Add an additional 75 mL of acetonitrile to the separatory funnel. Repeat Step (8), combining the two acetonitrile extractions in the same boiling flask. Discard the upper hexane layer.

(10) Prior to concentrating the extract, turn on the vacuum and rinse the rotary vacuum evaporator with hexane, followed by methanol, to prevent sample contamination. Evaporate the organic solvents with the rotary vacuum evaporator and a water bath heated to approximately 35-50 °C. Add 10 mL of methanol, and evaporate to dryness to remove traces of water. (Critical step - See Note L.8.) Dissolve the residue in 10 mL of hexane.

(11) Proceed as described in Step L1.a.(16) for beef lean, liver, or kidney.

2. Calculation of Net Percent Recovery

a. Inject the calibration standards described in Section G.2.b. into the HPLC and determine the peak response for all four analytes.

b. Prepare separate standard curves for each analyte by plotting the concentrations on the abscissa (x-axis) and the resulting peak responses on the ordinate (y-axis) as shown in Figure 2. Using any suitable technique (e.g., regression analysis), determine the equation for the curve with respect to the abscissa.

For example, the calculation of a linear equation for the standard curve can be accomplished as follows:

$$PR = mC + b$$

where: PR = peak response
m = slope of the line
C = concentration
b = y-axis intercept

Rearranging the above equation, the concentration (C) of the analyte in the final solution can be calculated from the standard curve as:

$$C = \frac{(PR - b)}{m}$$

For example, the following equation results from using the values for b and m for the standard calibration curve in Figure 2:

$$C = \frac{[PR - (-0.01792)]}{3.69987}$$

c. Determine the net concentration in each recovery sample by first subtracting the response in the corresponding control sample from that of the recovery sample. Substitute the net peak response obtained into the above equation and solve for concentration.

For example, the milk sample fortified with 0.010 µg/mL of spinosyn A (Figure 3, chromatogram D) had a peak response of 0.37 x 10^4, and there was no interference in the corresponding control sample (Figure 3, chromatogram B). Thus, the net peak height was calculated as:

$$\text{net peak height } (\times 10^4) = 0.37 - 0.00 = 0.37$$

and the concentration in the final sample solution was calculated as:

$$C = \frac{[0.37 - (-0.01792)]}{3.69987} = 0.10484 \ \mu g/mL$$

d. Determine the residue concentration (µg/mL or µg/g) of the analyte in the fortified recovery sample as follows:

$$\mu g/mL \text{ or } \mu g/g = \frac{C \times AF \times V}{W}$$

where: W = sample weight or volume (normally 20 g or 20 mL)
C = concentration (µg/mL) as determined above
V = final volume (normally 1.0 mL unless further diluted)
AF = aliquot factor (normally 2), i.e., (100 mL/50 mL) for fat and [(100 mL/75 mL) x (150 mL/100 mL)] for all other sample types

For the example above, the µg/mL concentration of spinosyn A in the milk sample is calculated as:

$$\frac{0.10484\ \mu g/mL \times 2.0 \times 1.0\ mL}{20\ mL} = 0.010484\ \mu g/mL$$

e. Determine the net percent recovery (R) by dividing the net concentration (µg/mL) by the theoretical concentration added:

$$R = \frac{\mu g/mL\ Found}{\mu g/mL\ Added} \times 100\%$$

For the above example,

$$\frac{0.010484}{0.010} \times 100\% = 105\%$$

3. Confirmation of Residue Identity

a. If necessary, confirm the identity of the analytes in the residue sample by analyzing the same final solution from Step I.1.a.(20) under the different chromatographic conditions specified in Step H.3.

b. For maximum sensitivity, maintain the UV wavelength at 250 nm and utilize the alternative column and mobile phase conditions listed in Step H.3. Compare the resulting retention times of the analytes in the samples with those of the standards. Also, compare the resulting concentrations of the analytes in the samples with those obtained using the conditions in Step H.1. to determine if they are similar (i.e., within approximately ± 20% of the primary result.)

c. To utilize different wavelengths, inject the standard and sample solutions with the UV wavelength set at 235 nm. Repeat at 275 nm. Compare the resulting concentrations with those obtained at 250 nm (Step H.1.) to determine if they agree within approximately ± 20% of the primary result. However, the absorbance of the analytes will be decreased at 235 nm or 275 nm (Figure 1).

J. Determination of Spinosad and Metabolites in Milk, Cream, and Beef Tissues

1. Prepare treated samples, a system (reagent) blank, fortified recovery samples, and an untreated control (if available) as described in Section I.1. For the treated tissue samples and the unfortified controls, record the exact weight (within ± 0.1 g) of the samples to be extracted. For the fortified recovery samples, record the weight as 20 g, so that the concentration will accurately reflect the desired fortification levels and the resulting values may be used for the statistical calculation of the LOD and LOQ obtained during

the study. (The use of slightly different weights for the fortified recovery samples would skew the LOD and LOQ calculations that are based upon standard deviation by slightly changing the concentration in the fortified samples.)

2. Prepare separate standard calibration curves for the four analytes and determine the percentage recovery of each analyte as described in Section I.2.

3. Determine the concentrations (µg/mL) of each analyte from the respective standard calibration curve, and calculate the uncorrected residue result as follows:

$$\text{Uncorrected Result } (\mu g/mL \text{ or } \mu g/g) = \frac{\mu g/mL \text{ (from std. curve)} \times AF \times V}{W}$$

Where: AF = aliquot factor (normally 2.0)
V = final volume (normally 1.0 mL unless further diluted)
W = weight or volume of extracted sample (normally 20 g or 20 mL)

For example, using the same data from the fortified milk sample in Section I.2., the uncorrected residue (µg/mL) is calculated as:

$$\mu g/mL \text{ (uncorrected)} = \frac{0.10484\ \mu g/mL \times 2.0 \times 1.0\ mL}{20\ mL} = 0.01048$$

4. If desired, correct the residue result for the recovery obtained as follows:

$$\text{Corrected residue } (\mu g/mL) = \text{uncorrected residue } (\mu g/mL) \times \frac{100\%}{R_a}$$

where: R_a = average % recovery from fortified samples (Section I.2.)

For the sample above, the residue corrected for recovery is calculated as:

$$\text{Corrected residue } (\mu g/mL) = 0.01048\ \mu g/mL \times \frac{100\%}{105} = 0.01\ \mu g/mL$$

K. Results and Discussion

1. Method Validation

a. Recovery Levels and Precision

A method validation study was conducted to determine the recovery levels and the precision of the residue method for the four analytes in all of the commodities, and the results are summarized by sample type and fortification level in Tables II-VII. A statistical summary of the recovery values for all fortification levels combined is contained in Table VIII. For the six commodities, average recoveries ranged from 84-106% for spinosyn A, 84-101% for spinosyn D, 93-114% for spinosyn B, and 82-106% for *N*-demethyl spinosyn D. The corresponding relative standard deviations ranged from 6-10% for spinosyn A, 5-10% for spinosyn D, 5-13% for spinosyn B, and 5-13% for *N*-demethyl spinosyn D (Table VIII).

b. Standard Curve Linearity

The average correlation coefficient (r^2) for the least squares equations describing the detector response as a function of standard curve concentration was greater than 0.9999 for all four analytes from 20 standard curve determinations during the method validation.

c. Calculated Limits of Detection and Quantitation

Following a published technique (1), the limits of detection and quantitation were calculated using the standard deviation from the results of the fortified recovery samples at the targeted limit of quantitation, which was 0.01 ppm (0.01 µg/g or 0.01 µg/mL). The limit of detection (LOD) was calculated as 3X the standard deviation, and the limit of quantitation (LOQ) was calculated as 10X the standard deviation (1). For all four analytes in the six different commodities, the calculated LOD ranged from 0.001-0.004 ppm, and the calculated LOQ ranged from 0.004-0.014 ppm (Table IX). These calculated values supported the validated LOQ of 0.01 ppm and a claimed LOD of 0.003 ppm for all commodities. Recovery samples fortified at 0.003 ppm resulted in detectable peaks for all of the analytes in all of the sample types, thereby verifying the claimed LOD. Although the method has an LOD of 0.003 ppm, numerical values should be reported as less than the LOQ (<0.01 ppm) for residues that are above the LOD but below the validated LOQ, because these residue levels cannot be reliably quantitated.

2. Interference from Other Pesticides and Therapeutic Compounds

Several pesticides commonly used on cotton and vegetables have been tested for potential interference with the analytes. Using 10 µg/mL solutions, the following seventy pesticides were tested for interference by direct injection into the HPLC. Only avermectin, dicofol, propargite, thiodicarb, and tralomethrin produced interference peaks. However, none of these five pesticides interfered when carried through the entire analytical procedure and analyzed using the conditions described in Step H.1.

Acephate	Fenamiphos	Oryzalin
Aldicarb	Fenvalerate	Oxamyl
Avermectin	Fluazifop-butyl	Oxyfluorfen
Azinphos-methyl	Flumetsulam	Paraquat dichloride
Benomyl	Fluometuron	PCNB
Bensulide	Fonofos	Pendamethalin
Bifenthrin	Glyphosate	Permethrin
Bloc	Imidan	Profenofos
Botran	Iprodione	Prometryn
Butifos	Isoproturon	Pronamide
Carbaryl	Karmex (DCMU)	Propargite
Chlorothalonil	Kelthane	Sethoxydim
Chlorpyrifos	Malathion	Simazine
Cyanazine	Mancozeb	Sulprofos
Cyhalothrin	Metalyxil	Terbicil
Cypermethrin	Mepiquat chloride	Terbufos
DCPA	Methidathion	Thiodicarb
Diazinon	Methomyl	Tillam
Dicofol	Methyl parathion	Tralomethrin
Dimethoate	Metribuzin	Triadimefon

Disulfoton	MSMA	Trifluralin
Endosulfan	Napropamide	Triforine
EPTC	Norflurazon	Ziram
Ethephon		

In addition to the seventy pesticides, ten therapeutic compounds commonly used for weight gain and/or disease control in animals were tested for potential interference. None of the following compounds produced interference peaks for any of the analytes:

Bacitracin zinc	Propylene glycol
Chlorotetracycline hydrochloride	Ractopamine hydrochloride
Monensin sodium	Sulfathiazole
Oxytetracycline hydrochloride	Tilmicosin
Penicillin G potassium	Tylosin

3. Confirmation of Residue Identity

Confirmation of the analyte residues is described in Section L3. If the retention times of the analytes in the samples do not match those of the standard when using the different column and mobile phase, consider the residue to be due to compounds other than the analytes. If the retention times match, but significantly different concentrations are obtained using the primary and confirmatory conditions (i.e., a difference of more than ± 20% of the primary value), consider the detected residue to be due at least in part to interfering compounds and not to the analytes.

Typical chromatograms demonstrating the confirmation of the analyte residues in milk are illustrated in Figure 9. (Note that the alternative conditions utilized for confirmation result in a different order of elution for the four analytes compared to the primary conditions.) If additional confirmation is required beyond that discussed in this method, an alternative detection system such as HPLC-mass spectrometry or immunoassay might be required.

4. Assay Time

A set of 12-16 samples can be prepared for analysis during a typical workday, with overnight injection of the samples using an autosampler. If desired, completion of the sample analysis may be delayed after Step L1.a.(15), prior to the silica SPE cleanup, if the sample solutions are capped and protected from light.

5. Standardization of SPE Elution Profiles

a. Silica SPE Elution Profile

Variation in the silica SPE cartridges can influence the elution profile of the analytes. 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 can be used:

(1) Prepare a silica SPE cartridge as described in Step L1.a.(16)(a).

(2) Transfer 1.0 mL of the 2.0-µg/mL spiking standard solution (Step G.1.c.) to a 125-mL boiling flask and evaporate to dryness using a rotary vacuum evaporator. (Note L.12.) Dissolve the sample in 10 mL of hexane.

(3) Add the sample to the SPE cartridge. Rinse the evaporating flask with two separate 10-mL portions of hexane and add both hexane rinses to the SPE cartridge, and elute the hexane. Rinse the boiling flask with an additional 40 mL of hexane, add the solvent to the cartridge, and discard all of the solvent that has eluted thus far.

(4) Rinse the evaporating flask with 5 mL of dichloromethane, add the rinse to the cartridge, and discard the eluate.

(5) Rinse the evaporating flask with two separate 4-mL aliquots of acetonitrile, add them separately to the cartridge, and collect both of the eluates in separate 35-mL vials.

(6) Add at least four 4-mL volumes of 75% dichloromethane/25% methanol to the SPE cartridge and collect each 4-mL fraction in a separate 35-mL vial.

(7) Evaporate the sample solutions from Steps (5) and (6) to dryness using a TurboVap evaporator at 60 °C and nitrogen gas at 8 psi.

(8) Dissolve the residue in 2.0 mL of methanol/acetonitrile/2% ammonium acetate (1:1:1). Swirl the vial to dissolve the residue on the bottom of the container, then tilt the vial to nearly a horizontal position and slowly rotate it so that the solvent dissolves any residue on the side of the vial. Repeat the swirling and tilting/rotating one time to ensure that the residue is dissolved. (Note L.11.)

(9) Using a disposable Pasteur pipet, transfer the solution to an HPLC sample vial and seal the vial with a cap and crimper. Do not filter the final solution. (See Note L.2.b.)

(10) Analyze the standard and sample solutions by HPLC as described in Step H.1.

(11) Calculate separate percentage recoveries for all four analytes as described in Step I.2.

(12) If the elution pattern for the analytes differs from that shown in Figure 10, adjust the volume of acetonitrile rinse to be discarded or the volume of 75% dichloromethane/25% methanol to be collected.

b. Cyclohexyl SPE Profile

Variation in the cyclohexyl SPE cartridges can influence the elution profile of the analytes. 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 can be used:

(1) Prepare a cyclohexyl SPE cartridge as described in Step L.1.a.(17)(a).

(2) Transfer 0.5 mL of the 20.0-µg/mL spiking standard solution (Step G.1.b.) to a 125-mL boiling flask and evaporate to dryness using a rotary vacuum evaporator. (Note L.12.) Dissolve the residue in 5 mL of 20% methanol/ 20% acetonitrile/60% water.

(3) Add the standard solution to the SPE cartridge. Rinse the evaporating flask with an additional 5 mL of 20% methanol/20% acetonitrile/60% water, and add the rinse to the SPE cartridge.

(4) Rinse the evaporating flask with 5 mL of acetonitrile, and add the rinse to the cartridge. Air dry the cartridge under vacuum for approximately 5 minutes. Discard all of the eluate collected thus far.

(5) Rinse the evaporating flask with 3.0 mL of acetone, add the rinse to the cartridge, and collect the eluate in a clear, 35-mL vial. Repeat with an additional 2.0 mL of acetone, and collect the eluate in a separate 35-mL vial. ~~Air-dry the cartridge under vacuum for approximately 2 minutes.~~

(6) Add at least four 3-mL aliquots of 2% triethylamine/98% acetonitrile to the SPE cartridge, and collect each 3-mL fraction in separate 40-mL amber vials.

(7) Immediately evaporate the sample solutions to dryness using a TurboVap evaporator at 60 °C and nitrogen gas at 8 psi (Critical step—See Note L.10.).

(8) Dissolve the residue in 2.0 mL of methanol/acetonitrile/2% ammonium acetate (1:1:1) (for the acetone eluant) or 10.0 mL (for the 2% triethylamine/ 98% acetonitrile eluant). Swirl the vial to dissolve the residue on the bottom of the container, then tilt the vial to nearly a horizontal position and slowly rotate it so that the solvent dissolves any residue on the side of the vial. Repeat the swirling and tilting/rotating one time to ensure that the residue is dissolved. (Note L.11.)

(9) Using a disposable Pasteur pipet, transfer the solution to an HPLC sample vial and seal the vial with a cap and crimper. Do not filter the final solution. (See Note L.2.b.)

(10) Analyze the standard and sample solutions by HPLC as described in Step H.1.

(11) Calculate separate percentage recoveries for all four analytes as described in Step I.2.

(12) If the elution pattern for the analytes differs from that shown in Figure 11, adjust the volume of acetone rinse to be discarded or the volume of 2% triethylamine/98% acetonitrile to be collected.

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 assumed to be readily available and 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. When following the procedures as described, interferences in the chromatograms of system (reagent) blanks have not occurred. However, 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 a source 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. Rotary vacuum evaporators: Thoroughly rinse rotary vacuum evaporators as described in the method to prevent contamination of samples.

b. 0.45-µm filters: Do not remove particulates by filtering the final solutions through 0.45-µm filters prior to injection into the HPLC unless it has been demonstrated that the filters do not produce interferences under the HPLC conditions specified. (Failure to clarify the final solutions has not resulted in a noticeable chromatographic problem after several months of daily operation.)

c. Glassware: Glassware residues, especially detergent, are a common source of interference. If interferences in the chromatograms of reagent blank samples are found to be due to the glassware, rinse dirty glassware with deionized water before machine washing. After washing, rinse the glassware with acetone. If previously used glassware produces interferences, it might also be necessary to acid-wash the glassware before use.

3. Triethylamine is unstable in solution with acetonitrile, resulting in changes in the elution profile for the CH SPE cartridge and an increased solvent front on the chromatograms. Prepare an appropriate volume of the solution immediately before each use, and discard any unused portions.

4. If desired, prepare standard solutions at other concentrations by making appropriate dilutions. Store the solutions in a location that is protected from light, but avoid the use of amber glass containers for the fortification standards. Spinosyn B and *N*-demethyl spinosyn D dissolved in 50% methanol/50% acetonitrile tend to gradually adsorb onto amber glassware, so that only clear glass volumetric flasks (e.g., Kimax) should be used to prepare the fortification standards.

5. If necessary, modify the typical HPLC conditions to obtain optimum performance or to meet the criteria for system suitability in Step L1.a (21).

6. If chilled water is used to cool the reflux condensers, the volume of the extracting solution has not been observed to decrease during the refluxing procedure. However, if chilled water is not used, accurately mark the initial fluid level on the boiling flask, and add extracting solution to return the volume to the initial level after the refluxed solutions have cooled to approximately room temperature.

7. Shaking the samples in a horizontal position instead of a vertical position has resulted in a small interference peak on the chromatogram, presumably due to contact of the solvent with the PTFE-lined lid.

8. Water in the sample extract will alter the elution profile of the silica SPE cartridge. Add methanol to assist the evaporation of trace amounts of water.

9. At high sample concentrations of the analytes (approximately 10 µg/g), spinosyns A and D might partially elute in the 5-mL acetone wash with some lots of CH SPE cartridges. If breakthrough occurs, reduce the volume of the acetone rinse (e.g., to 3 mL).

10. The presence of triethylamine in the solution can result in the photodegradation of the analytes under normal laboratory lighting. It is necessary to use amber vials and reduced lighting whenever triethylamine is in solution with the analytes.

11. The analytes tend to adsorb very strongly to glass. Use the solvent and technique described to ensure that the residues are completely dissolved in the final solution. With a final volume of only 1.0 mL, the use of a vortex mixer has not adequately dissolved the residue from the side of the vial, so that it has been necessary to rotate and tilt the vials as described. For final volumes of at least 2.0 mL, a vortex mixer may be used, if desired, to assist the dissolving process.

12. Do not use a TurboVap evaporator for this step. Partial loss (approximately 20%) of spinosyns A and D has occurred when standard solutions have been evaporated with a TurboVap evaporator in the absence of the sample extract. No losses have occurred with a rotary vacuum evaporator, even in the absence of the sample extract.

M. References

1. Keith, L. H.; Crummett, W.; Deegan, J.; Libby, R. A.; Taylor, J. K.; Wentler, G., "Principles of Environmental Analysis", *Anal. Chem.*, 1983, 55, pp 2210-2218.

Table I. Chemical Names for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D)

Analyte	Chemical Name
A	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).
D	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).
B	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).
Met-D	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: not yet available).

Table II. Summary of Net Recovery Data[a] for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D) in Whole Milk

Added, µg/mL	N	Compound	Net Percent Recovery			
			Range	$\bar{x}$	s	RSD
0.0	4	A	ND[b]	ND	-	-
		D	ND	ND	-	-
		B	ND	ND	-	-
		Met-D	ND	ND	-	-
0.003	2	A	NA[c]	NA	-	-
		D	NA	NA	-	-
		B	NA	NA	-	-
		Met-D	NA	NA	-	-
0.010	8	A	99 - 116	108	6	6
		D	90 - 114	104	7	7
		B	95 - 109	102	5	5
		Met-D	96 - 112	104	5	5
0.025	3	A	97 - 104	101	4	4
		D	91 - 101	97	6	6
		B	94 - 100	97	3	3
		Met-D	92 - 101	96	5	5
0.050	3	A	95 - 106	99	6	6
		D	93 - 103	97	6	6
		B	94 - 107	100	7	7
		Met-D	92 - 105	98	7	7
0.10	3	A	100 - 108	104	4	4
		D	98 - 105	101	4	4
		B	98 - 106	103	5	5
		Met-D	97 - 105	102	5	5
1.0	3	A	103 - 105	104	1	1
		D	102 - 102	102	0	0
		B	102 - 104	103	1	1
		Met-D	101 - 103	102	1	1

[a] Net percent recovery is the gross (uncorrected) recovery minus the background interference in the corresponding control sample. (None of the control milk samples contained a background interference.)

[b] None detected at a detection limit of 0.003 µg/mL.

[c] Not applicable. (A peak response was detected, but it was below the 0.010-µg/mL limit of quantitation.)

Table III. Summary of Net Recovery Data[a] for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D) in Cream

Added, µg/mL	N	Compound	Net Percent Recovery Range	$\bar{x}$	s	RSD
0.0	3	A	ND[b]	ND	-	-
		D	ND	ND	-	-
		B	ND	ND	-	-
		Met-D	ND	ND	-	-
0.003	2	A	NA[c]	NA	-	-
		D	NA	NA	-	-
		B	NA	NA	-	-
		Met-D	NA	NA	-	-
0.010	8	A	96 - 113	102	6	6
		D	87 - 102	95	7	7
		B	96 - 116	105	6	6
		Met-D	97 - 110	104	4	4
10.0	3	A	100 - 114	106	7	7
		D	103 - 115	108	6	6
		B	108 - 115	112	4	4
		Met-D	105 - 113	110	5	5

[a] Net percent recovery is the gross (uncorrected) recovery minus the background interference in the corresponding control sample. (None of the control cream samples contained a background interference.)

[b] None detected at a detection limit of 0.003 µg/mL.

[c] Not applicable. (A peak response was detected, but it was below the 0.010-µg/mL limit of quantitation.)

Table IV. Summary of Net Recovery Data[a] for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D) in Lean Beef Tissue

Added, µg/g	N	Compound	Net Percent Recovery Range	$\bar{x}$	s	RSD
0.0	3	A	ND[b]	ND	-	-
		D	ND	ND	-	-
		B	ND	ND	-	-
		Met-D	ND	ND	-	-
0.003	2	A	NA[c]	NA	-	-
		D	NA	NA	-	-
		B	NA	NA	-	-
		Met-D	NA	NA	-	-
0.010	8	A	89 - 107	99	6	6
		D	83 - 93	89	4	4
		B	91 - 107	100	6	6
		Met-D	92 - 101	96	4	4
1.0	3	A	81 - 86	83	3	4
		D	82 - 86	83	2	2
		B	102 - 107	104	3	3
		Met-D	100 - 107	103	4	4

[a] Net percent recovery is the gross (uncorrected) recovery minus the background interference in the corresponding control sample. (None of the control samples of lean beef tissue contained a background interference.)

[b] None detected at a detection limit of 0.003 µg/g.

[c] Not applicable. (A peak response was detected, but it was below the 0.010-µg/mL limit of quantitation.)

Table V. Summary of Net Recovery Data[a] for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D) in Beef Liver

Added, µg/g	N	Compound	Net Percent Recovery			
			Range	$\bar{x}$	*s*	RSD
0.0	3	A	ND[b]	ND	-	-
		D	ND	ND	-	-
		B	ND	ND	-	-
		Met-D	ND	ND	-	-
0.003	2	A	NA[c]	NA	-	-
		D	NA	NA	-	-
		B	NA	NA	-	-
		Met-D	NA	NA	-	-
0.010	8	A	97 - 120	109	8	7
		D	83 - 110	90	10	11
		B	88 - 125	109	14	13
		Met-D	71 - 109	92	13	14
1.0	3	A	97 - 99	98	1	1
		D	94 - 98	96	2	2
		B	98 - 102	100	2	2
		Met-D	96 - 101	98	2	2

[a] Net percent recovery is the gross (uncorrected) recovery minus the background interference in the corresponding control sample. (None of the control beef liver samples contained a background interference.)

[b] None detected at a detection limit of 0.003 µg/g.

[c] Not applicable. (A peak response was detected, but it was below the 0.010-µg/mL limit of quantitation.)

Table VI. Summary of Net Recovery Data[a] for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D) in Beef Kidney

Added, µg/g	N	Compound	Net Percent Recovery Range	$\bar{x}$	s	RSD
0.0	3	A	ND[b]	ND	-	-
		D	ND	ND	-	-
		B	ND	ND	-	-
		Met-D	ND	ND	-	-
0.003	2	A	NA[c]	NA	-	-
		D	NA	NA	-	-
		B	NA	NA	-	-
		Met-D	NA	NA	-	-
0.010	8	A	76 - 97	82	8	10
		D	76 - 99	85	9	11
		B	98 - 136	120	11	9
		Met-D	86 - 107	98	7	7
1.0	3	A	83 - 93	88	6	7
		D	80 - 88	83	4	5
		B	94 - 102	97	5	5
		Met-D	92 - 99	95	4	4

[a] Net percent recovery is the gross (uncorrected) recovery minus the background interference in the corresponding control sample. (None of the control beef kidney samples contained a background interference.)

[b] None detected at a detection limit of 0.003 µg/g.

[c] Not applicable. (A peak response was detected, but it was below the 0.010-µg/mL limit of quantitation.)

Table VII. Summary of Net Recovery Data[a] for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D) in Beef Fat

Added, µg/g	N	Compound	Net Percent Recovery Range	$\bar{X}$	s	RSD
0.0	3	A	ND[b]	ND	-	-
		D	ND	ND	-	-
		B	ND	ND	-	-
		Met-D	ND	ND	-	-
0.003	2	A	NA[c]	NA	-	-
		D	NA	NA	-	-
		B	NA	NA	-	-
		Met-D	NA	NA	-	-
0.010	8	A	81 - 108	101	9	9
		D	84 - 107	97	8	8
		B	85 - 102	94	7	7
		Met-D	56 - 85	78	10	13
10.0	3	A	88 - 93	90	3	3
		D	89 - 92	90	2	2
		B	92 - 93	93	1	1
		Met-D	91 - 93	92	1	1

[a] Net percent recovery is the gross (uncorrected) recovery minus the background interference in the corresponding control sample. (None of the control beef fat samples contained a background interference.)

[b] None detected at a detection limit of 0.003 µg/g.

[c] Not applicable. (A peak response was detected, but it was below the 0.010-µg/mL limit of quantitation.)

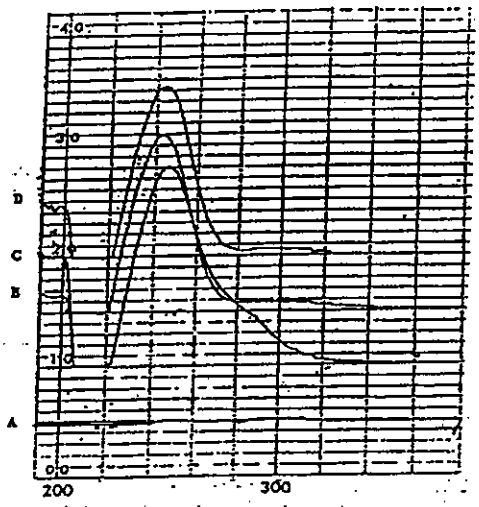
Table VIII. Statistical Summary of Overall Recovery Values for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D)

Sample Type	Added, µg/mL or µg/g	N	Analyte	Percent Recovery Range	$\bar{x}$	s	RSD
Whole Milk	0.010-1.0	20	A	95 - 116	104	6	6
			D	90 - 114	101	6	6
			B	94 - 109	101	5	5
			Met-D	92 - 112	102	5	5
Cream	0.010-10.0	11	A	96 - 114	103	6	6
			D	87 - 115	98	9	9
			B	96 - 116	107	7	7
			Met-D	97 - 113	106	5	5
Beef Lean	0.010-1.0	11	A	81 - 107	95	9	9
			D	82 - 93	87	4	5
			B	91 - 107	101	6	6
			Met-D	92 - 107	98	5	5
Beef Liver	0.010-1.0	11	A	97 - 120	106	9	8
			D	83 - 110	92	9	10
			B	88 - 125	107	12	11
			Met-D	71 - 109	94	12	13
Beef Kidney	0.010-1.0	11	A	76 - 97	84	8	10
			D	76 - 99	84	8	10
			B	94 - 136	114	15	13
			Met-D	86 - 107	97	6	6
Beef Fat	0.010-10.0	11	A	81 - 108	98	9	9
			D	84 - 107	95	7	7
			B	85 - 102	93	6	6
			Met-D	56 - 93	82	11	13

Table IX. Calculated Limits of Detection and Quantitation (µg/mL or µg/g) for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D) in Milk, Cream, and Beef Tissues

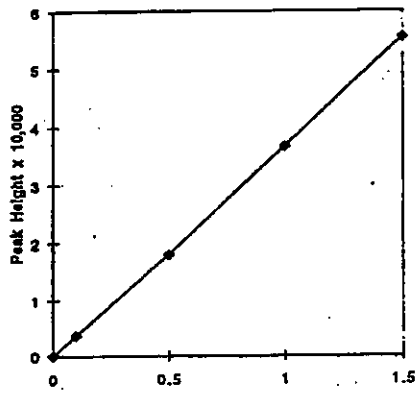
Matrix	Analyte	$\bar{x}$[a]	s[b]	LOD[c]	LOQ[d]
Whole Milk	A	0.0108	0.0006	0.002	0.006
	D	0.0104	0.0007	0.002	0.007
	B	0.0102	0.0005	0.002	0.005
	Met-D	0.0104	0.0005	0.002	0.005
Cream	A	0.0102	0.0006	0.002	0.006
	D	0.0095	0.0007	0.002	0.007
	B	0.0105	0.0006	0.002	0.006
	Met-D	0.0104	0.0004	0.001	0.004
Beef Lean	A	0.0099	0.0006	0.002	0.006
	D	0.0089	0.0004	0.001	0.004
	B	0.0100	0.0006	0.002	0.006
	Met-D	0.0096	0.0004	0.001	0.004
Beef Liver	A	0.0109	0.0008	0.002	0.008
	D	0.0090	0.0010	0.003	0.010
	B	0.0109	0.0014	0.004	0.014
	Met-D	0.0092	0.0013	0.004	0.013
Beef Kidney	A	0.0082	0.0008	0.002	0.008
	D	0.0085	0.0009	0.003	0.009
	B	0.0120	0.0011	0.003	0.011
	Met-D	0.0098	0.0007	0.002	0.007
Beef Fat	A	0.0101	0.0009	0.003	0.009
	D	0.0097	0.0008	0.002	0.008
	B	0.0094	0.0007	0.002	0.007
	Met-D	0.0078	0.0010	0.003	0.010

[a] Mean values of the net results for the 0.01-µg/mL or 0.01-µg/g recovery samples.
[b] Standard deviation of the net results for the 0.01-µg/mL or 0.01-µg/g recovery samples.
[c] Calculated limit of detection, calculated as 3*s*.
[d] Calculated limit of quantitation, calculated as 10*s*.



(A). Solvent blank
(B) Spinosyn D
(C) Spinosyn A
(D) Spinosyn B

Figure 1. UV Spectra for Spinosyns A, D, and B at 10 µg/mL in Methanol/Acetonitrile/ 2% Ammonium Acetate (44/44/12)

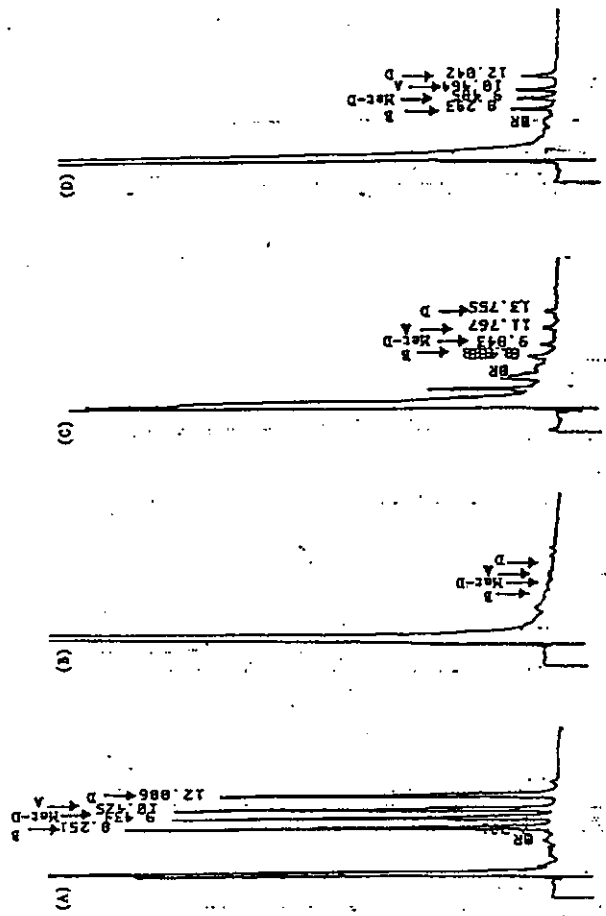


Spinosyn A Concentration, µg/mL

Concentration (µg/mL)	Peak Height (x10⁴)
0.0	0.0
0.1	0.36
0.5	1.80
1.0	3.67
1.5	5.55

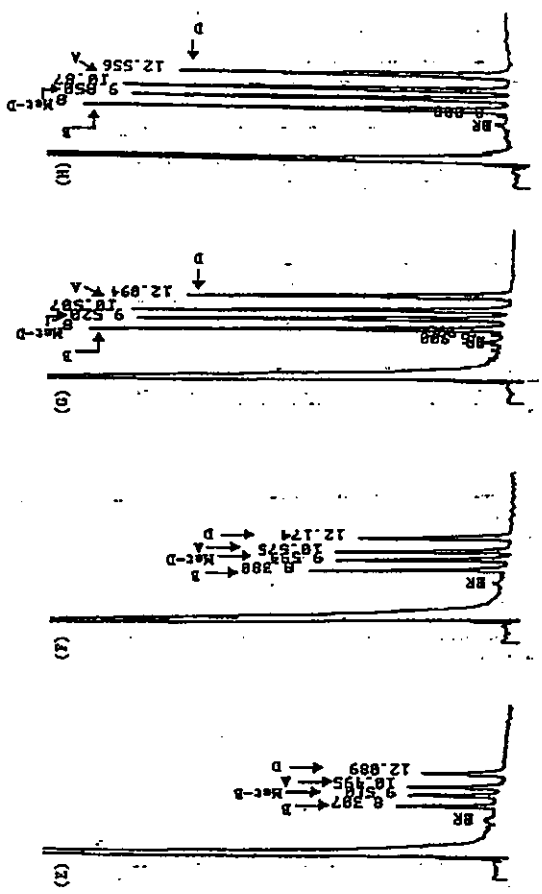
Slope (m) = 3.69987
Y-axis intercept (b) = -0.01792
Correlation coefficient (r^2) = 0.99996

Figure 2. Typical Calibration Curve for the Determination of Spinosyn A



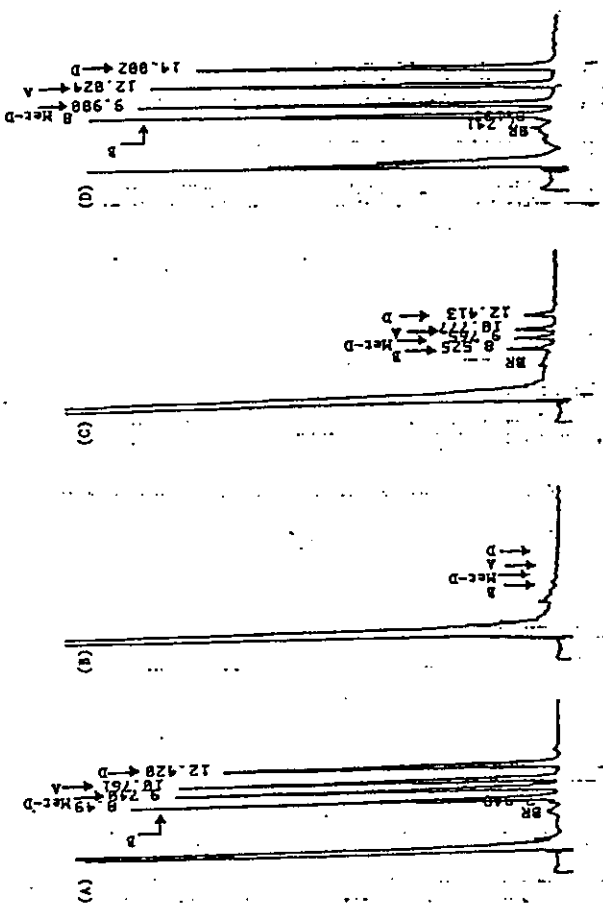
(A) Standard, 175 ng of each analyte injected (equivalent to a theoretical milk residue of 0.1 µg/mL of each).
(B) Untreated control milk containing no detectable residue of the analytes.
(C) Control milk fortified with 0.003 µg/mL of all four analytes (limit of detection).
(D) Untreated control milk fortified with 0.01 µg/mL (limit of quantitation), equivalent to recoveries of 102% (spinosyn B), 104% (Mel-D), 105% (spinosyn A), and 105% (spinosyn D).

Figure 3. Typical Chromatograms Demonstrating the Determination of Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Mel-D) in Whole Milk



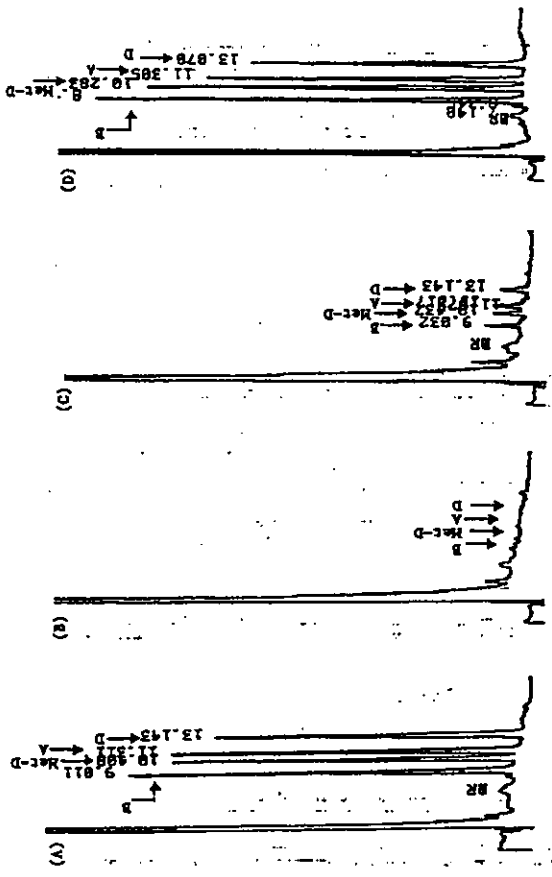
(E) Untreated control milk fortified with 0.025 µg/mL, equivalent to recoveries of 96% (spinosyn B), 96% (Mel-D), 101% (spinosyn A), and 100% (spinosyn D).
(F) Untreated control milk fortified with 0.05 µg/mL, equivalent to recoveries of 94% (spinosyn B), 92% (Mel-D), 95% (spinosyn A), and 93% (spinosyn D).
(G) Untreated control milk fortified with 0.1 µg/mL, equivalent to recoveries of 98% (spinosyn B), 97% (Mel-D), 100% (spinosyn A), and 98% (spinosyn D).
(H) Untreated control milk fortified with 1.0 µg/mL (diluted to 10 mL final volume), equivalent to recoveries of 102% (spinosyn B), 101% (Mel-D), 104% (spinosyn A), and 102% (spinosyn D).

Figure 3. (Cont.) Typical Chromatograms Demonstrating the Determination of Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Mel-D) in Whole Milk



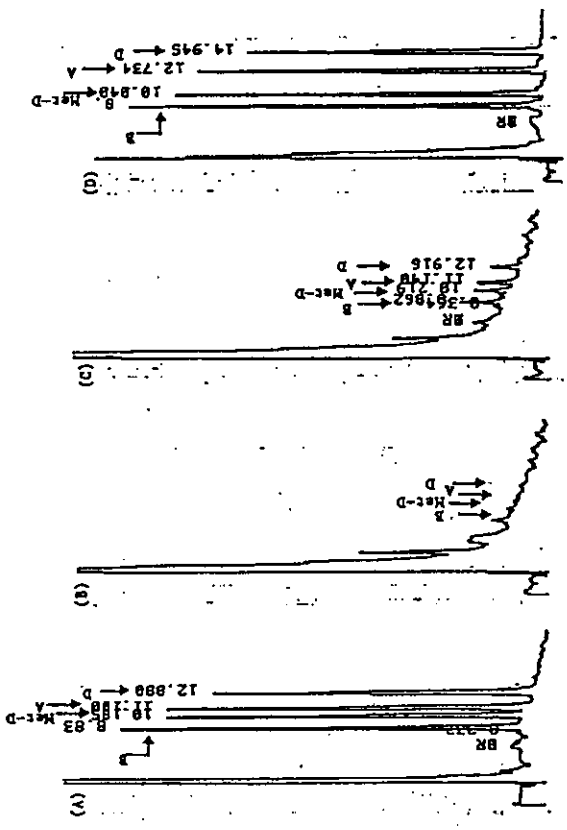
(A) Standard, 175 ng of each analyte injected (equivalent to a theoretical cream residue of 0.1 µg/mL of each).
(B) Untreated control cream containing no detectable residue of the analytes.
(C) Untreated control cream fortified with 0.01 µg/mL (limit of quantitation), equivalent to recoveries of 101% (spinosyn D), 99% (Met-D), 102% (spinosyn A), and 87% (spinosyn D).
(D) Untreated control cream fortified with 10.0 µg/mL (diluted to 100 mL final volume), equivalent to recoveries of 108% (spinosyn B), 105% (Met-D), 105% (spinosyn A), and 105% (spinosyn D).

Figure 4. Typical Chromatograms Demonstrating the Determination of Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D) in Cream



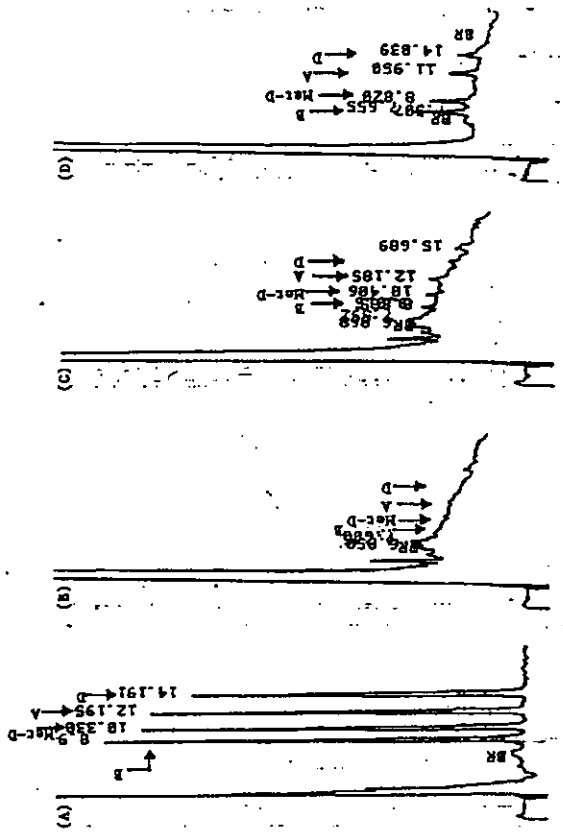
(A) Standard, 175 ng of each analyte injected (equivalent to a theoretical beef residue of 0.1 µg/g of each).
(B) Untreated control lean beef containing no detectable residue of the analytes.
(C) Untreated control lean beef fortified with 0.01 µg/g (limit of quantitation), equivalent to recoveries of 91% (spinosyn B), 92% (Mel-D), 89% (spinosyn A), and 93% (spinosyn D).
(D) Untreated control lean beef fortified with 1.0 µg/g (diluted to 10.0 mL final volume), equivalent to recoveries of 104% (spinosyn B), 103% (Mel-D), 86% (spinosyn A), and 86% (spinosyn D).

Figure 5. Typical Chromatograms Demonstrating the Determination of Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Mel-D) in Lean Beef Tissue



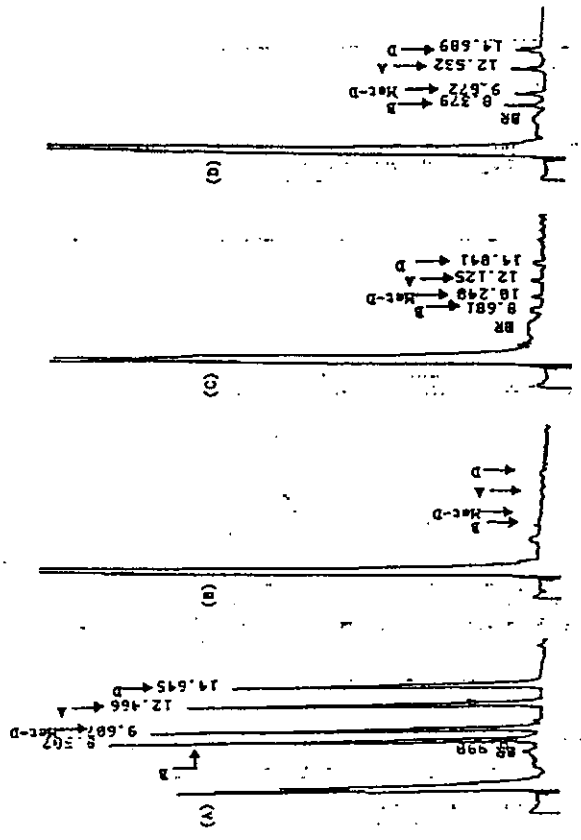
(A) Standard, 175 ng of each analyte injected (equivalent to a theoretical liver residue of 0.1 µg/g of each).
(B) Untreated control beef liver containing no detectable residue of the analytes.
(C) Untreated control beef liver fortified with 0.01 µg/g (limit of quantitation), equivalent to recoveries of 96% (spinosyn B), 86% (Mel-D), 97% (spinosyn A), and 86% (spinosyn D).
(D) Untreated control beef liver fortified with 1.0 µg/g (diluted to 10.0 mL final volume), equivalent to recoveries of 98% (spinosyn B), 96% (Mel-D), 97% (spinosyn A), and 94% (spinosyn D).

Figure 6. Typical Chromatograms Demonstrating the Determination of Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Mel-D) In Beef Liver



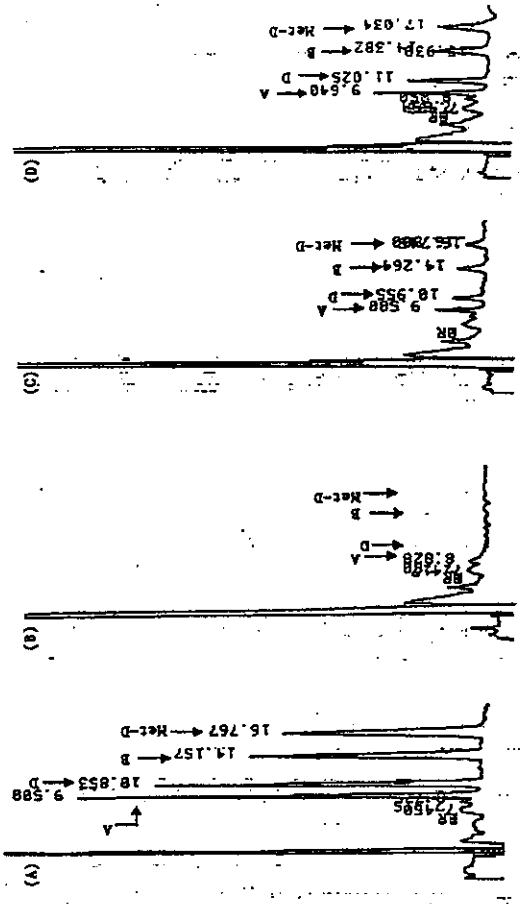
(A) Standard, 175 ng of each analyte injected (equivalent to a theoretical kidney residue of 0.1 µg/g of each).
(B) Untreated control beef kidney containing no detectable residue of the analytes.
(C) Untreated control beef kidney fortified with 0.003 µg/g (limit of detection).
(D) Untreated control beef kidney fortified with 0.01 µg/g (limit of quantitation), equivalent to recoveries of 124% (spinosyn B), 99% (Mel-D), 76% (spinosyn A), and 76% (spinosyn D).

Figure 7. Typical Chromatograms Demonstrating the Determination of Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Mel-D) in Beef Kidney



(A) Standard, 175 ng of each analyte injected (equivalent to a theoretical fat residue of 0.1 µg/g of each).
(B) Untreated control beef fat containing no detectable residue of the analytes.
(C) Untreated control beef fat fortified with 0.003 µg/g (limit of detection).
(D) Untreated control beef fat fortified with 0.01 µg/g (limit of quantitation), equivalent to recoveries of 90% (spinosyn B), 75% (Mel-D), 96% (spinosyn A), and 84% (spinosyn D).

Figure 8. Typical Chromatograms Demonstrating the Determination of Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Mel-D) in Beef Fat



(A) Standard, 175 ng of each analyte injected (equivalent to a theoretical milk residue of 0.1 µg/mL of each).
(B) Untreated control milk containing no detectable residue of the analytes.
(C) Untreated control milk fortified with 0.01 µg/mL (limit of quantitation), equivalent to recoveries of 103% (spinosyn A), 96% (spinosyn D), 126% (spinosyn B), and 93% (Met-D).
(D) Untreated control milk fortified with 0.025 µg/mL, equivalent to recoveries of 99% (spinosyn A), 98% (D), 102% (spinosyn B), and 102% (Met-D).

Figure 9. Typical Chromatograms Demonstrating the Confirmation of Spinosyns A, D, B, and *N*-Demethyl Spinosyn D (Met-D) in Whole Milk using the Confirmation Column (C18/Cation Mixed Mode)

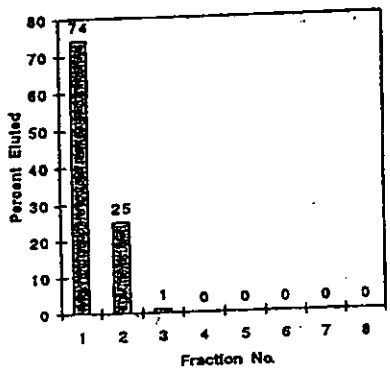


Figure 10. Typical Silica SPE Elution Profile for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D in 4-mL Fractions of 75% Methylene Chloride/25% Methanol (Fraction Number 1 = 0-4 mL, Fraction 2 = 4-8 mL, etc.)

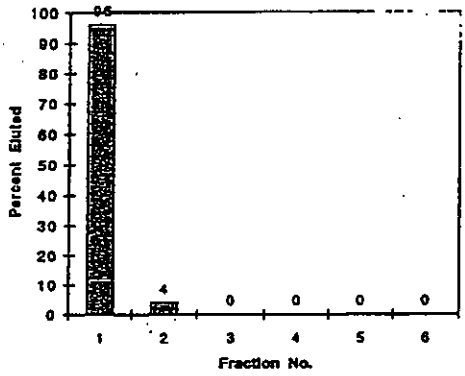


Figure 11. Typical Cyclohexyl SPE Elution Profile for Spinosyns A, D, B, and *N*-Demethyl Spinosyn D in 3-mL Fractions of 2% Triethylamine/98% Acetonitrile (Fraction Number 1 = 0-3 mL, Fraction 2 = 3-6 mL, etc.)

EPA ADDENDUM

PP#6F4761/6H5754

DowElanco Method GRM 95.03

Conditions and Substitutions used by ACLB

1. ACLB used a Hewlett-Packard 1090 Series HPLC equipped with a diode array detector. The YMC ODS-AQ analytical column used for analyses was thermostatted at 35 ^{o} C. The injection volume was 175 uL. The elution profiles of spinosads from the silica and cyclohexyl solid phase extraction columns were checked and adjusted as needed to obtain satisfactory recovery rates. Five hundred (500) mL flat-bottomed boiling flasks were used in place of the 250 mL boiling flasks to reflux the liver samples. Hot plates were used in place of heating mantles. A 13 mm X 100 mm (~ 10mL) disposable glass tube was used in place of the 35 and 40 mL vials for solid phase extraction (SPE) extraction. Sample eluates from the SPE cartridges were then evaporated in a Turbo-Vap using the same disposable tube. Aluminum foil was wrapped around the tubes used to collect the cyclohexyl eluates to protect the samples from light in place of amber bottles. Sample work-up was stopped after step I.1.a(16d) for both the milk and the liver extracts. Sample solutions were capped and placed in a refrigerator overnight until sample analyses resumed the next day. An HPLC flow rate of 1.0 mL/min was used for all analyses. Milk samples were injected every 20 minutes while liver samples were injected every 30 minutes to allow for adequate baseline equilibration. All other parameters were followed as written in the method.

2. The linearity of Spinosad A & D was established. Spinosad A and Spinosad D standard responses were found to be linear over a solution concentration range of 0.05 - 0.25 ug/mL injected. Sample analyses were conducted in this range.

3. The method suggests the use of calibration curves to determine sample concentrations. ACLB determined sample concentrations from a ratio of sample responses to the average of standard responses that bracketed the samples.

ACLB's Recommended Changes to the Method

4. ACLB used rotary evaporator traps and experienced no problems concentrating the milk samples during step I.1.a(15) using a 50 ^{o} C water bath temperature and the technique of very gradually increasing the vacuum to prevent bumping. Several of the liver samples however still bumped over into the traps at this step using this same technique. The liver extracts should be closely monitored during the rotary vacuum evaporation step. The use of rotary evaporator traps is strongly recommended. From ACLB's experience this is a critical step.