Determination of Spinosad and Its Metabolites in Meat, Milk, Cream, and Eggs by High-Performance Liquid Chromatography with Ultraviolet Detection

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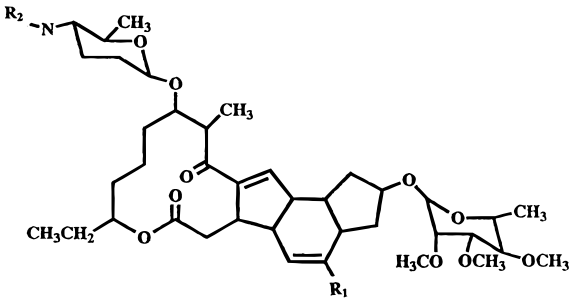
Spinosad is a naturally derived insect-control agent for use on cotton and a variety of other crops. A method is described for the determination of spinosad and its major metabolites in beef and poultry meat, milk, cream, and eggs. The method determines residues of the active ingredients (spinosyns A and D) and two metabolites (spinosyn B and *N*-demethylspinosyn D). For chicken fat, the method has a limit of quantitation (LOQ) of 0.02 μg/g and a limit of detection (LOD) of 0.006 μg/g. For all other chicken tissues, beef tissues, milk, cream, and eggs, the method has an LOQ of 0.01 μg/g and an LOD of 0.003 μg/g. The analytes are extracted from the various sample types using appropriate solvents, and the extracts are purified by liquid−liquid partitioning and solid-phase extraction. All four analytes are determined simultaneously in the purified extracts by reversed-phase high-performance liquid chromatography with ultraviolet detection at 250 nm.

Keywords: *Spinosad; spinosyn A; spinosyn D; spinosyn B; N-demethylspinosyn D; beef; poultry; chicken; meat; milk, cream; eggs; quantitation; HPLC-UV*

INTRODUCTION

The spinosyns are natural insect-control agents that are derived from an Actinomycetes bacterium, *Saccharopolyspora spinosa*. Spinosad, which is composed of a mixture of spinosyns A and D, is the common name of the active material that is derived from a fermentation broth. Spinosad is being developed for the management of insect pests in cotton and a variety of other crops (Sparks et al., 1995; Thompson et al., 1995).

Because of its anticipated uses, spinosad residues might occur in meat, milk, cream, or eggs if animals consume feed containing the residues. Consequently, analytical methods are needed to determine the magnitude of residues in feeding studies with spinosad in cows and chicken. Residue methods have been previously reported for spinosad in cottonseed and cottonseed processed commodities (West, 1996), soil, sediment, and water (West, 1997), and leafy vegetables, peppers, and tomatoes (Yeh et al., 1997). Previous studies using radiolabeled (^{14}C) material in goats and chickens demonstrated that spinosyns A and D were metabolized to spinosyn B and *N*-demethylspinosyn D, respectively (D. P. Rainey and J. D. Magnussen, Dow AgroSciences, personal communication, 1994). Thus, the following methods are presented for the determination of all four analytes in beef and poultry tissues, milk, cream, and eggs by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. The chemical names and CAS Registry Numbers for the analytes are included in Table 1.



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

EXPERIMENTAL PROCEDURES

Apparatus. *(a) HPLC with a UV Detector.* A Hewlett-Packard model 1050 HPLC with a UV detector was used in combination with a Hewlett-Packard model 3396 Series II recording integrator for the measurement of peak height responses. The primary HPLC column was an ODS-AQ [5-μm particle size, 150 × 4.6 mm i.d. (YMC, Inc., Wilmington, NC)], maintained at 30 °C. The mobile phase consisted of 44% reservoir A/44% reservoir B/12% reservoir C (isocratic), with reservoir A containing methanol, reservoir B containing acetonitrile, and reservoir C containing 2% aqueous ammonium acetate/acetonitrile (67:33). The flow rate was 0.5−0.6 mL/min for poultry tissues and 0.8−1.0 mL/min for beef tissues. The injection volume was 175 μL, the integrator attenuation was 2^3, and the chart speed was 0.2 cm/min. The four analytes eluted with retention times ranging from approximately 10 to 16 min for poultry tissues and from 8 to 14 min for beef tissues.

The confirmatory HPLC column was a C_{18}/cation mixed-mode column [5-μm particle size, 150 mm × 4.6 mm i.d.

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

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

[a] Supplied by the author.

(Alltech, Deerfield, IL)]. The mobile phase was 40% reservoir A/40% reservoir B/20% reservoir C (isocratic), with reservoir A containing methanol, reservoir B containing acetonitrile, and reservoir C containing 2% aqueous ammonium acetate/acetonitrile (67:33). All of the other parameters were the same as those listed above for the primary column. The four analytes eluted with retention times ranging from approximately 9 to 17 min.

Reagents. Water was purified using a Milli-Q UV Plus purification system. The following reagents were of HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA): acetone, acetonitrile, dichloromethane, hexane, methanol, and ammonium acetate. Triethylamine (TEA) was of reagent grade (Fisher Scientific), and a new bottle of TEA was opened every 2−3 months to prevent the formation of impurities that produced interference peaks on the chromatogram. The sodium sulfate was of certified ACS grade, anhydrous, granular, 10−60 mesh, and tested for pesticide residue analysis (Fisher Scientific). (Sodium sulfate from a different supplier resulted in reduced recoveries due to adsorption of the analytes.) The purified active ingredients used for analytical standards were obtained from the Test Substance Coordinator, Dow AgroSciences, Indianapolis, IN.

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

Solutions for fortifying control beef or poultry samples for the determination of recovery were prepared by combining 10.0-mL aliquots of the four 200 μg/mL stock solutions in a 100-mL volumetric flask and diluting to volume with 50% methanol/50% acetonitrile to obtain a mixture containing 20.0 μg/mL of the analytes. Aliquots of this solution were further diluted with 50% methanol/50% acetonitrile to obtain fortification standards at concentrations of 0.20, 0.50, 1.0, and 2.0 μg/mL. All standard fortification solutions were prepared in clear glass volumetric flasks. The use of amber glass flasks was avoided, because spinosyn B and *N*-demethylspinosyn D dissolved in 50% methanol/50% acetonitrile tend to gradually adsorb onto amber glassware. To prevent such losses, the fortification solutions containing analyte concentrations of <2.0 μg/mL were prepared fresh daily, and the more highly concentrated fortification solutions were prepared weekly.

Precautionary Protection from Light. During the sample extraction and purification steps, the extracts were protected from light to prevent photolysis. Protective measures included working under reduced lighting conditions [e.g., turning off the lights in fume hoods during liquid−liquid partitioning and solid-phase extraction (SPE) cleanup steps] and placing the samples in the dark during any interruptions during sample processing. Long interruptions were generally avoided during sample analysis, except that the analysis could be delayed overnight prior to the silica SPE procedure.

Initial Sample Preparation. *(a) Beef or Chicken Lean Meat, Liver, Kidney, Fat, and Chicken Meat with Skin and Associated Fat.* Tissue samples were by diced with a knife, frozen with liquid nitrogen, and then ground through a Hammermill with a $^3/_{16}$-in. screen size (model 2001, Agvise Laboratories, Inc., Northwood, ND). After grinding, the tissue samples were manually mixed in a plastic bag and then transferred to high-density polyethylene freezer cartons for storage at −15 to −20 °C.

(b) Eggs. Egg whites and yolks (minus the shells) were blended at high speed for ∼1 min with a Polytron homogenizer (model PT 10203500 with 10-mm generator and saw teeth, Brinkmann Instruments, Inc., Westbury, NY). The blended samples were stored as above.

(c) Milk and Cream. Milk and cream samples did not require sample preparation prior to being stored as above.

Sample Weighing and Fortification of Recovery Samples. *(a) Beef or Chicken Lean Meat, Liver, Kidney, Fat, Eggs, and Chicken Meat with Skin and Associated Fat.* Samples (10 g of chicken fat or 20 g of other tissues) were weighed into 8-oz (237-mL) glass bottles (Qorpak, with PTFE-lined lids, Fisher Scientific), and fortified recovery samples were prepared by adding 1.0 mL of the appropriate fortification standard solution to the appropriate control samples. (If the egg samples were thawed, the sample containers were thoroughly shaken prior to weighing to produce homogeneous samples.) Sample analysis was continued as described under Sample Homogenization.

(b) Milk and Cream. Milk and cream samples were thawed, and the sample containers were thoroughly shaken by hand to result in homogeneous samples. A 20-g aliquot was transferred into an 8-oz glass bottle. Fortified recovery samples were prepared as described above. The analysis was continued as described under Sample Extraction. (The primary steps in the residue methods for determining spinosad and metabolites in the various sample matrices are summarized in the flowchart in Figure 1.)

Sample Homogenization. *(a) Beef or Chicken Lean Meat, Liver, Kidney, and Chicken Meat with Skin and Associated Fat. A* 50-mL aliquot of 80% acetonitrile/20% water was added to each bottle, and the samples were blended at high speed for ∼1 min using a Polytron homogenizer with a 10-mm diameter generator and sawtooth blades. The homogenized sample solutions were transferred through filling funnels into 250-mL boiling flasks. The homogenizer blades were rinsed by blending with 50 mL of 80% acetonitrile/20% water in a graduated cylinder at high speed for ∼10 s. The rinse solution

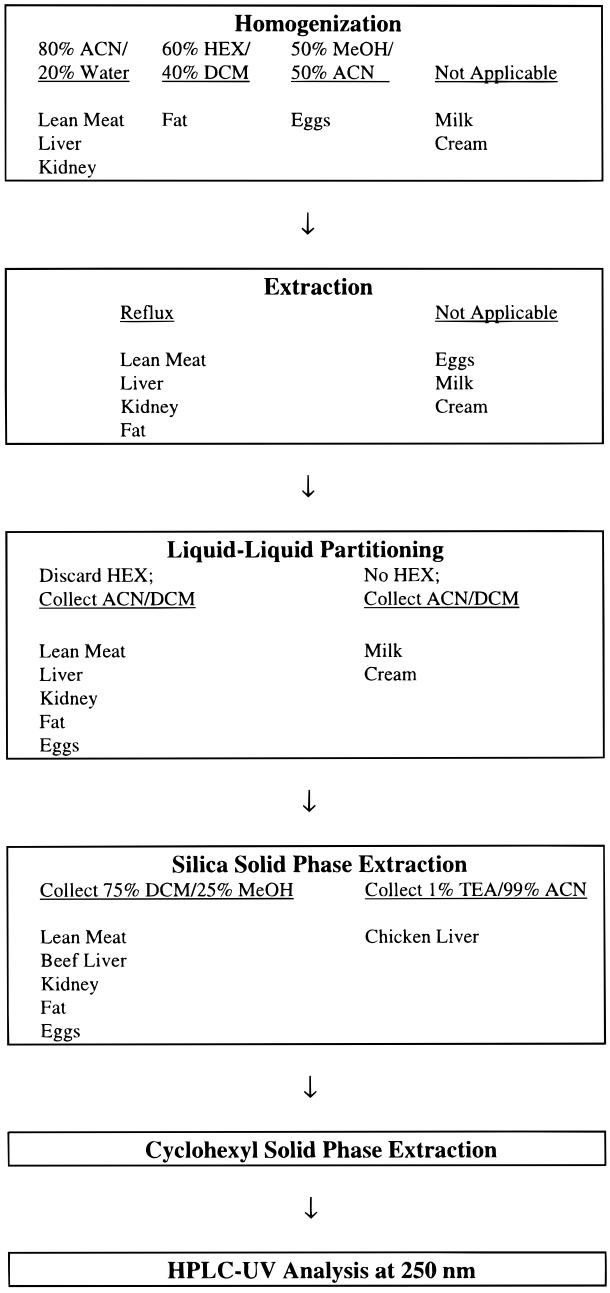

Figure 1. Flowchart for the determination of spinosad and metabolites in meat, milk, cream, and eggs by HPLC-UV (ACN, acetonitrile; HEX, hexane; DCM, dichloromethane; MeOH, methanol; TEA, triethylamine).

was combined with the blended sample solution in the 250-mL boiling flasks, and the analyses were continued as described under Sample Extraction.

(b) Beef or Chicken Fat. Fat samples were homogenized as just described, except that the extraction solution was 60% hexane/40% dichloromethane. Sample analysis was continued as described under Sample Extraction.

(c) Eggs. Egg samples were homogenized as just described, except that the extraction solution was 50% methanol/50% acetonitrile. The analyses were continued as described next under Sample Extraction.

(d) Milk or Cream. Milk or cream samples did not require any sample homogenization prior to sample extraction.

Sample Extraction. The solvents and reflux techniques used for extracting the various sample types were those shown during a radiolabeled (^{14}C) study to result in essentially complete extraction of the analytes from the sample matrices (Rainey and Magnussen, personal communication, 1994).

(a) Beef or Chicken Lean Meat, Liver, Kidney, Fat, and Chicken Meat with Skin and Associated Fat. Tissue samples were reflux-extracted using heating mantles (model 0-408, Glas-Col Apparatus Co., Terre Haute, IN), variable autotransformers (model 3PN1010, Staco Energy Products Co., Dayton, OH), and reflux condensers (300-mm sleeve length, 5-bulb, Fisher Scientific) cooled with chilled water (15 °C). To prevent excessive foaming, ~2 g of boiling granules (carborundum No. 12, Hengar Co., Philadelphia, PA) was added to the flasks. The heating mantle temperatures were approximately 150 ± 15 °C and caused the solutions to boil in ~10−15 min, and the heating was continued for 1 h. The cooled flasks were capped to prevent solvent evaporation, and the flasks were swirled to prevent clumping of the tissue. The sample extracts were cooled to room temperature, and 50-mL aliquots of the fat sample extracts (or 75-mL aliquots for the other sample types) were decanted through prepleated filter paper into 100-mL graduated cylinders. The 50-mL aliquots of the fat samples were transferred to 250-mL separatory funnels, whereas the 75-mL aliquots of the other sample types were transferred to clean, graduated, 8-oz bottles. The analyses were continued as described under Purification by Liquid−Liquid Partitioning.

(b) Eggs. Egg samples were shaken in an upright position on an orbital shaker (model G-33, New Brunswick, Fisher Scientific) at 250 rpm for 5 min. The samples were centrifuged at 2250 rpm for 5 min, and 75-mL aliquots of the supernatant solutions were decanted into graduated cylinders. The aliquots were then transferred to clean, graduated, 8-oz sample extraction bottles. The analyses were continued as described under Purification by Liquid−Liquid Partitioning.

(c) Milk and Cream. Acetonitrile (80 mL) was added to the samples, which were extracted and centrifuged as described for eggs except that the samples were shaken for 30 min. After the 75-mL aliquot had been transferred to an 8-oz bottle, the analyses were continued as described next under Purification by Liquid−Liquid Partitioning.

Purification by Liquid−Liquid Partitioning. *(a) Lean Meat, Liver, Kidney, Eggs, and Chicken Meat with Skin and Associated Fat.* For all sample types except fat, milk, and cream, 25 mL of hexane was added to the aliquots from the sample extraction. The bottles were sealed with a PTFE-lined lid, shaken in an upright position on an orbital shaker at 250 rpm for 5 min, and then centrifuged at 2250 rpm for 5 min. Before remixing of the solvent layers could occur, the hexane (upper) layer was carefully aspirated off and discarded using vacuum and a disposable 9-in. Pasteur pipet until only the acetonitrile (lower) layer and the narrow emulsion between the layers remained in the bottles. Dichloromethane (75 mL) was then added to the bottles. (For egg samples, 40 mL of ultrapure water was also added.) The bottles were sealed with a PTFE-lined lid, and the samples were shaken at 250 rpm for 5 min. The samples were centrifuged at 2250 rpm for 5 min. Before remixing of the solvent layers could occur, the aqueous (upper) layer was aspirated off and discarded using vacuum and a disposable 9-in. Pasteur pipet. After the aqueous layer was completely removed, the organic (lower) layer was partially aspirated off until the volume was reduced to the 100-mL graduation mark on the bottles. The remaining 100-mL sample aliquots were transferred to clean, 250-mL boiling flasks. The sample bottles were rinsed with 10 mL of dichloromethane, and the rinse was added to the boiling flasks. The sample extracts were evaporated using a rotary vacuum evaporator (model 1007-4, Rinco Instrument Co., Greenville, IL) under a vacuum of −26 in. of Hg and a water bath temperature of 35−50 °C. (For egg samples, it was necessary to monitor the evaporation closely for the beginning of excessive bubbling and foaming, which usually occurred when the volume was reduced to ~10 mL and solids began adhering to the side of the flask. When excessive bubbling began, the flask was immediately removed, 10 mL of methanol was added, and the evaporation was then completed using a reduced vacuum of −22 in. of Hg to prevent bumping and loss of the solution into the evaporator.) With most samples, oil remained in the flask upon evaporation of the solvents, and traces of water

were visible as droplets in the oil. If water remained in the flask of any of the sample types upon evaporation of the organic solvent, 10 mL of methanol was added, and the evaporation was repeated. The oily residue was then dissolved in 10 mL of hexane, and the analyses were continued as described under Purification by Silica SPE.

(b) Milk and Cream. For milk and cream samples, the hexane partitioning step was unnecessary. Thus, the liquid−liquid partitioning was conducted as just described in *(a)* for the other sample types, but beginning with the addition of 75 mL of dichloromethane. After rotary vacuum evaporation of the solvents and traces of water, the residue was dissolved in 10 mL of hexane, and analyses were continued as described under Purification by Silica SPE.

(c) Fat. For fat samples, 20 mL of hexane and 75 mL of acetonitrile were added to the separatory funnel containing the 50-mL sample aliquot. The separatory funnel was shaken vigorously for 30 s. After ∼2 min had been allowed for the layers to separate, the lower layer (dichloromethane/acetonitrile) was drained into a clean, 500-mL boiling flask. An additional 75 mL of acetonitrile and 10 mL of dichloromethane were added to the hexane remaining in the separatory funnel, and the partitioning was repeated. After separation, the lower layer was combined with that from the first partitioning in the 500-mL boiling flask. The upper (hexane) layer was discarded. After rotary vacuum evaporation of the solvents and traces of water, the residue was dissolved in 10 mL of hexane, and analyses were continued as described next under Purification by Silica SPE.

Purification by Silica SPE. Prior to using each new lot of silica SPE columns, the elution profile was determined to ensure that the appropriate volumes of solvents were discarded and collected in the following procedure. The elution profile described below was determined using a standard solution containing all four analytes at 2.0 μg each in 10 mL of hexane. A 25-mL column reservoir was attached to each silica SPE column. A ball-shaped plug of pesticide-grade glass wool was firmly pressed into the bottom of the SPE column reservoir, and ∼4 mL (6 g) of pesticide-grade sodium sulfate was added to the reservoir. (The glass wool plug was placed above the narrow neck of the column reservoir to prevent plugging of the neck with sodium sulfate so that the eluting solvents would adequately flow through the column.)

(a) Lean Meat, Kidney, Fat, Beef Liver, Eggs, Milk, Cream, and Chicken Meat with Skin and Associated Fat. The following procedure was used for all sample types except chicken liver. Prior to the addition of the samples to the silica SPE columns, the columns were conditioned with the following sequence of eluants, which were eluted under a vacuum of −5 in.: 10 mL of 75% dichloromethane/25% methanol, then 10 mL of acetonitrile, followed by 10 mL of dichloromethane, and 20 mL of hexane.

The volumes of eluting solvents that are specified below were typical for the silica SPE procedure, but the volumes might require modification for different lots of silica SPE columns. The samples were added in 10 mL of hexane. The evaporating flasks were rinsed with two 10-mL aliquots of hexane, which were added to the column reservoirs. The vacuum was turned on, and the hexane was eluted. The flasks were rinsed with 40 mL of hexane, which was added to the columns and eluted. The flasks were rinsed with 10 mL of dichloromethane, which was added to the columns and eluted. The flasks were rinsed with 8 mL of acetonitrile, which was added to the columns and eluted. All of the solvent that had eluted thus far was discarded, and 35-mL vials were then placed in the vacuum manifold for solvent collection. The evaporating flasks were rinsed with 12 mL of 75% dichloromethane/25% methanol, which was added to the columns and eluted into the vials. The sample solutions were immediately evaporated using a TurboVap evaporator set at 60 °C and a nitrogen flow of 8 psi. The evaporation process was monitored closely so that the sample vials could be removed from the evaporator immediately upon evaporation of the solvent (to prevent reduced recoveries), and the residues were dissolved in 5 mL of 20% methanol/20% acetonitrile/60% water with the aid of an ultrasonic bath for 10−20 s. The vials were carefully rotated so that the solvent could dissolve the residues on the sides of the vials. The analyses were continued as described under Purification by Cyclohexyl SPE.

(b) Chicken Liver. Chicken liver samples required a different eluting solvent to provide adequate cleanup with the silica SPE columns. The conditioning procedure that was described in *(a)* for the other sample types was followed except that the column was conditioned only with 20 mL of hexane. Also, after the addition of the chicken liver extracts, the elution scheme that was described for the other sample types was followed except that the 75% dichloromethane/25% methanol eluant was replaced with 25 mL of 1% TEA/99% acetonitrile, and the eluant was collected in 40-mL amber vials to protect the analytes from photodegradation in the presence of TEA. (It was necessary to prepare the 1% TEA/99% acetonitrile solution immediately before use due to the instability of TEA in the solution.) After evaporation of the eluting solvent and dissolving the residue in 5 mL of 20% methanol/20% acetonitrile/60% water, the analyses were continued as described next under Purification by Cyclohexyl SPE.

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

The following volumes were typical for the CH SPE procedure, but the volumes might require modification for different lots of CH SPE columns. The samples were added in 5 mL of 20% methanol/20% acetonitrile/60% water, and the solvent was eluted. The vials were rinsed with an additional 5 mL of 20% methanol/20% acetonitrile/60% water, which was added to the column reservoirs and eluted. The columns were dried under vacuum for 2 min after the solution had eluted. The vials were rinsed with 5 mL of acetonitrile, which was added to the columns and eluted. The columns were dried under vacuum for 5 min after the acetonitrile had eluted. The vials were rinsed with 5 mL of acetone, which was added to the columns and eluted. All of the solvent that had eluted thus far was discarded. The original sample vials were rinsed with 6 mL of 2% TEA/98% acetonitrile, which was added to the columns and eluted into precleaned amber vials (40 mL). (It was necessary to prepare the 2% TEA/98% acetonitrile solution immediately before use due to the instability of TEA in the solution.) The sample solutions were immediately evaporated using a TurboVap evaporator set at 60 °C and a nitrogen flow of 8 psi. The evaporation process was monitored closely so that the sample vials could be removed from the evaporator immediately upon evaporation of the solvent (to prevent reduced recoveries), and the residues were dissolved in 1.0 mL of methanol/acetonitrile/2% aqueous ammonium acetate (1:1:1). Because the analytes adsorb very strongly to glass, the vials were thoroughly swirled and rotated to ensure that the residues had dissolved. The final solutions were not filtered through a 0.45-μm filter, because the filters produced interference peaks in the chromatogram. The lack of filtration did not decrease column performance over a period of several weeks.

HPLC. Solutions were analyzed by HPLC using the previously described conditions. The suitability of the chromatographic system was determined using the following performance criteria: (a) It was determined that the correlation coefficient (r^2) exceeded 0.995 for the least-squares equation that described the detector response as a function of the concentration of the calibration standards. (b) It was visually determined that baseline resolution was achieved for all four analytes. (c) It was visually determined that a signal-to-noise

Table 2. Recovery of Spinosyns A, D, and B and *N*-Demethylspinosyn D (NDSD) from Beef and Poultry Tissues, Milk, Cream, and Eggs[a]

sample	added, μg/g	*n*	% recovery (mean ± SD)			
			A	D	B	NDSD
whole milk	0.003−1.0	20	104 ± 6	101 ± 6	101 ± 5	102 ± 5
cream	0.003−10.0	11	103 ± 6	98 ± 9	107 ± 7	106 ± 5
lean beef	0.003−1.0	11	95 ± 9	87 ± 4	101 ± 6	98 ± 5
beef liver	0.003−1.0	11	106 ± 9	92 ± 9	107 ± 12	94 ± 12
beef kidney	0.003−1.0	11	84 ± 8	84 ± 8	114 ± 15	97 ± 6
beef fat	0.003−10.0	11	98 ± 9	95 ± 7	93 ± 6	82 ± 11
eggs	0.003−1.0	20	88 ± 8	87 ± 7	102 ± 6	97 ± 7
lean chicken	0.003−1.0	11	92 ± 7	88 ± 4	100 ± 6	95 ± 5
chicken liver	0.003−1.0	11	94 ± 8	84 ± 6	93 ± 6	93 ± 4
chicken meat with skin and fat	0.003−1.0	11	86 ± 5	77 ± 4	96 ± 5	92 ± 7
chicken fat	0.006−2.0	11	114 ± 6	112 ± 6	102 ± 8	99 ± 5

[a] No detectable residue occurred in unfortified control samples.

ratio of approximately 5:1 to 10:1 was achievable for the 0.1 μg/mL calibration standard. If the peak response for any of the samples exceeded the range of the calibration curve, the samples were diluted with methanol/acetonitrile/2% aqueous ammonium acetate (1:1:1).

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

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

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

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

For all samples except fat, the aliquot factor was calculated from the extraction volumes and aliquot volumes as follows:

$$\mathrm{AF} = [(100\ \mathrm{mL}/75\ \mathrm{mL}) \times (150\ \mathrm{mL}/100\ \mathrm{mL})] = 2 \quad (3)$$

For fat samples, the aliquot factor was calculated as

$$\mathrm{AF} = (100\ \mathrm{mL}/50\ \mathrm{mL}) = 2 \quad (4)$$

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

$$R = [(\mu\mathrm{g/g})/(\text{added } \mu\mathrm{g/g})] \times 100\% \quad (5)$$

Calculated Limits of Detection and Quantitation. For all of the sample types except chicken fat, the limits of detection (LOD) and quantitation (LOQ) for the residue method were calculated using the standard deviation of the micrograms per gram results from the samples fortified at 0.01 μg/g. For the chicken fat method, the LOD and LOQ were calculated from the results of the samples fortified at 0.02 μg/g. Following a technique described previously (Keith et al., 1983), the LOD was calculated as 3 times the standard deviation (3*s*), and the LOQ was calculated as 10 times the standard deviation (10*s*).

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

Interference Study. Seventy pesticides commonly used on cotton, fruit, and vegetables have been previously tested for interference by direct injection into the liquid chromatograph (West, 1996). In addition, 10 therapeutic compounds commonly used for weight gain and/or disease control in animals were tested for interference. Any compounds that produced peaks at the retention times of the analytes were carried through the entire analytical procedure and analyzed using the primary HPLC-UV conditions to determine if they would still interfere after going through the sample purification procedures.

RESULTS AND DISCUSSION

Method Validation. The method was validated at concentrations ranging from 0.01 to 10 μg/g for beef fat and cream, from 0.02 to 2.0 μg/g for chicken fat, and from 0.01 to 1.0 μg/g for all other sample types. The results of the validation study are summarized in Table 2. For all 11 commodities, the average recoveries ranged from 84 to 114% for spinosyn A, from 77 to 112% for spinosyn D, from 93 to 114% for spinosyn B, and from 82 to 106% for *N*-demethylspinosyn D.

Chromatograms. Typical chromatograms obtained under the primary and confirmatory HPLC conditions are included in Figure 2 (eggs) and in Figure 3 (whole milk), respectively. Chromatograms for the other sample matrices were similar. The alternative conditions used for the confirmation of residues (Figure 3) resulted in a different order of elution for the analytes compared to that obtained with the primary HPLC conditions (Figure 2).

Linearity. The linearity of the detector was determined using five calibration standards ranging in concentration from 0 to 1.5 μg/mL. The average correlation coefficient (r^2) for the least-squares equations describing the detector response as a function of concentration of the standard curve solutions was >0.9999 for all four analytes. Linearity at concentrations exceeding the range of the calibration curve (0.0−1.5 μg/mL) was not investigated.

Limits of Detection and Quantitation. The calculated values for the LOD (3*s*) and LOQ (10*s*) are presented in Table 3. In chicken fat, the calculated LOD for all four analytes ranged from 0.004 to 0.006 μg/g, and the calculated LOQ ranged from 0.013 to 0.019 μg/g. These calculated values supported the experimentally validated method LOD and LOQ of 0.006 and 0.02 μg/g. The method LOD was further supported by the

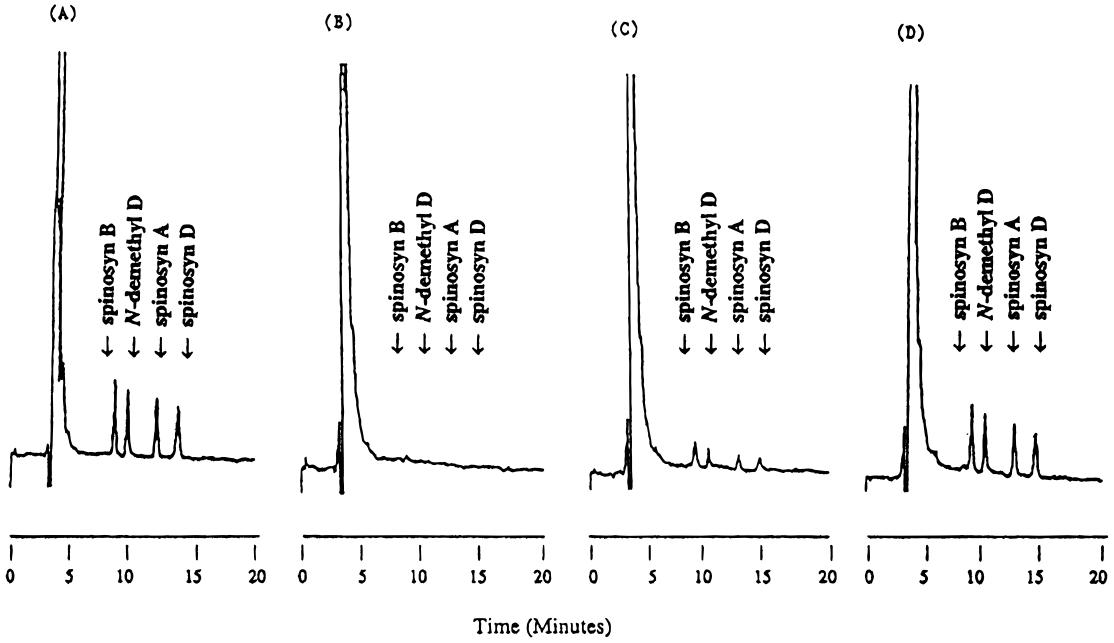

Figure 2. Typical chromatograms from the determination of spinosyns A, D, and B and *N*-demethylspinosyn D in eggs using the primary column (ODS-AQ): (A) standard, 17.5 ng of each analyte; (B) control eggs containing no detectable residue; (C) control eggs fortified with 0.003 μg/g of all four analytes (limit of detection); (D) control eggs fortified with 0.01 μg/g, equivalent to recoveries of 96% (spinosyn B), 93% (*N*-demethylspinosyn D), 93% (spinosyn A), and 88% (spinosyn D).

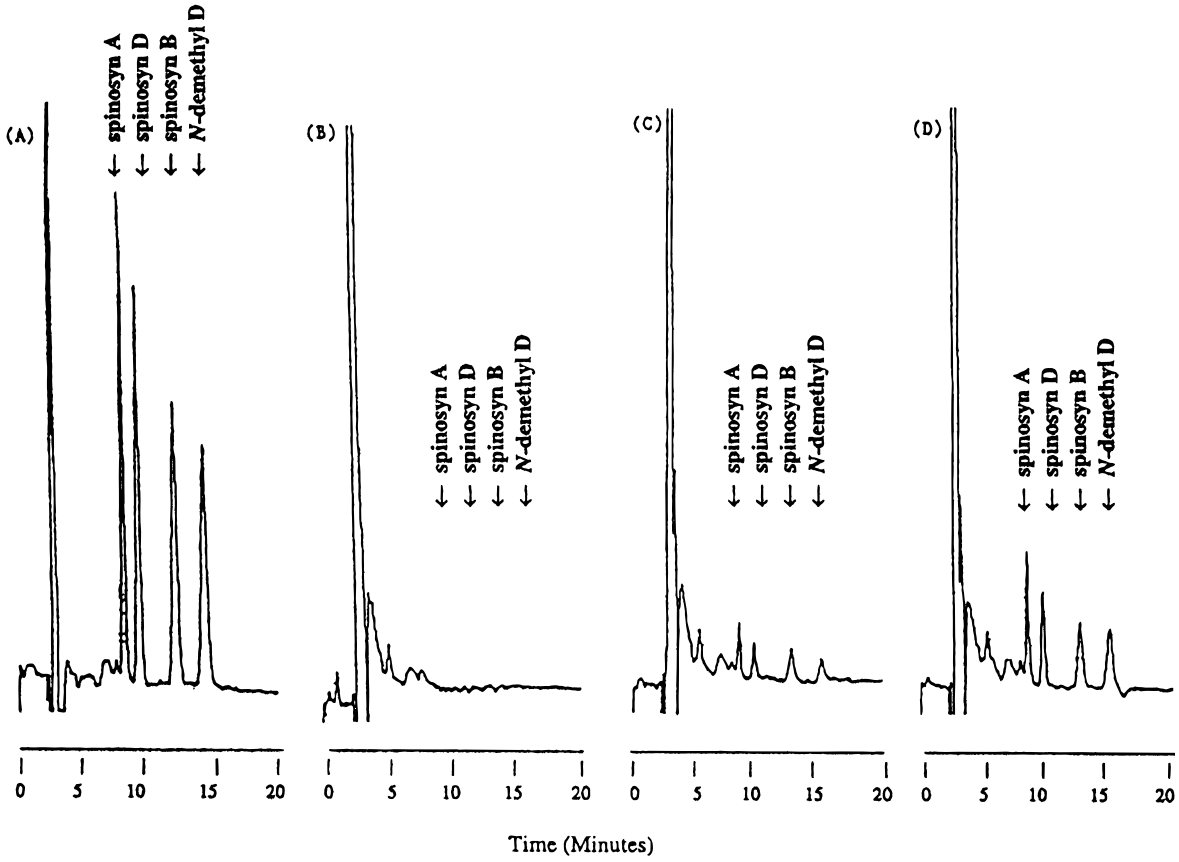

Figure 3. Typical chromatograms from the determination of spinosyns A, D, and B and *N*-demethylspinosyn D in whole milk using the confirmatory column (C_{18}/cation mixed mode): (A) standard, 175 ng of each analyte; (B) control milk containing no detectable residue; (C) control milk fortified with 0.01 μg/g of all four analytes, equivalent to recoveries of 103% (spinosyn A), 96% (spinosyn D), 106% (spinosyn B), and 93% (*N*-demethylspinosyn D); (D) control milk fortified with 0.025 μg/g of all four analytes, equivalent to recoveries of 99% (spinosyn A), 98% (spinosyn D); 102% (spinosyn B), and 102% (*N*-demethylspinosyn D).

presence of detectable peaks in chromatograms resulting from the analysis of control chicken fat samples fortified at 0.006 μg/g.

In all of the other commodities, the calculated LOD for all four analytes ranged from 0.001 to 0.004 μg/g, and the calculated LOQ ranged from 0.003 to 0.014 μg/

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

	LOD[a]				LOQ[b]			
sample	A	D	B	NDSD	A	D	B	NDSD
whole milk	0.002	0.002	0.002	0.002	0.006	0.007	0.005	0.005
cream	0.002	0.002	0.002	0.001	0.006	0.007	0.006	0.004
lean beef	0.002	0.001	0.002	0.001	0.006	0.004	0.006	0.004
beef liver	0.002	0.003	0.004	0.004	0.008	0.010	0.014	0.013
beef kidney	0.002	0.003	0.003	0.002	0.008	0.009	0.011	0.007
beef fat	0.003	0.002	0.002	0.003	0.009	0.008	0.007	0.010
eggs	0.002	0.002	0.001	0.001	0.005	0.005	0.004	0.004
lean chicken	0.001	0.001	0.002	0.001	0.004	0.004	0.005	0.003
chicken liver	0.002	0.002	0.002	0.001	0.007	0.007	0.006	0.003
chicken fat	0.004	0.004	0.006	0.004	0.013	0.013	0.019	0.013
meat/skin/fat	0.001	0.001	0.002	0.002	0.004	0.004	0.005	0.007

[a] Calculated limited of detection (μg/g), calculated as 3*s*. [b] Calculated limit of quantitation (μg/g), calculated as 10*s*.

g. The calculated values supported the validated method LOD and LOQ of 0.003 and 0.01 μg/g, respectively. The method LOD was further supported by the presence of detectable peaks in chromatograms resulting from the analysis of control samples fortified at 0.003 μg/g (Figures 2 and 3).

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

(a) Interferences. Because a nonselective wavelength (250 nm) was needed to obtain adequate sensitivity for the analytes, it was necessary to take precautions to avoid interferences from the reagents and equipment. After washing, it was necessary to rinse glassware with acetone and methanol to remove interferences due to the detergent. After each use, it was also necessary to rinse reflux condensers and rotary vacuum evaporators with methanol to prevent cross-contamination of samples.

(b) Water Temperature. The use of chilled water (15 °C) to cool the reflux condensers prevented the extraction volume from decreasing during sample extraction.

(c) Partitioning Time. Shaking the tissue or egg samples for >5 min during the liquid−liquid partitioning procedure resulted in increased emulsions that were more difficult to break, which sometimes increased interferences and decreased recoveries.

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

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

(f) Inadequate Cleanup. Some egg samples were found to be insufficiently purified when using the 75% dichloromethane/25% methanol eluant with the silica SPE columns. It was determined that these samples could be adequately purified using the 1% TEA/99% acetonitrile eluant that was specified for chicken liver samples.

(g) Aged Samples. Low recoveries of spinosyn B and *N*-demethylspinosyn D occurred with aged chicken fat samples, especially if the samples had been thawed and refrozen several times and the tissue had become spoiled. One sample that inadvertently thawed and became spoiled during a problem with the freezer produced recoveries of 30−50% for spinosyn B and *N*-demethylspinosyn D but essentially 100% for spinosyns A and D. Spoiled fat samples produced extracts that were very slow to filter after reflux extraction and caused very heavy emulsions that would not break adequately during liquid−liquid partitioning in the separatory funnels. Spinosyn B and *N*-demethylspinosyn D appeared to have an increased affinity for the spoiled chicken fat tissue compared to fresh tissue. To improve recoveries, it was thus necessary to increase the extractability of the analytes by changing from two 50-mL homogenization steps with 60% hexane/40% dichloromethane to one homogenization step with 100 mL of the more polar 80% acetonitrile/20% water. In addition, it was necessary to filter the chicken fat extract while it was still hot after refluxing to prevent losses on the filter paper. Also, to improve recovery of the two analytes from the liquid−liquid partitioning step, the hexane volume was increased from 20 to 95 mL, the number of partitionings with acetonitrile/dichloromethane was increased from two to three, and the emulsified solutions in the separatory funnel were centrifuged in 8-oz bottles to break the heavy emulsion and cause the layers to separate. The lower layer (acetonitrile/dichloromethane) was then removed with a pipet. These modifications resulted in quantitative recoveries of the analytes from spoiled chicken fat samples.

Specificity. Pesticides commonly used on cotton and vegetables were previously tested for potential interference with the analytes (West, 1996). Seventy pesticides were tested for interference by direct injection into the liquid chromatograph. Most of the pesticides eluted with the solvent front, and only avermectin B_{1a}, dicofol, propargite, thiodicarb, and tralomethrin produced peaks that matched the retention times of the analytes. However, none of these five pesticides interfered when they were carried through the entire analytical procedure. In addition, none of the following therapeutic compounds that are commonly used in commercial beef and poultry production produced interference peaks: bacitracin zinc, chlorotetracycline hydrochloride, monensin sodium, oxytetracycline hydrochloride, penicillin G potassium, propylene glycol, ractopamine hydrochloride, sulfathiazole, tilmicosin, and tylosin. Thus, the cleanup procedures described in the method effectively removed the potentially interfering compounds as well as the interfering coextractives from the samples.

Conclusions. A method has been developed and validated for the determination of the active ingredients of spinosad (spinosyns A and D) and its two major metabolites (spinosyn B and *N*-demethylspinosyn D) in beef and chicken tissues, milk, cream, and eggs. The accuracy and precision of the method make it suitable for residue monitoring or tolerance enforcement. Factors affecting the successful performance of the method have been investigated, and precautions have been incorporated to enhance method ruggedness. This

method expands the list of sample matrices in which spinosad residues may be successfully determined.

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