STUDY TITLE

Residue Method Validation Report for the Determination of Residues of Spinosad in Bovine Tissues and Milk by Immunoassay

DATA REQUIREMENT

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PERFORMING LABORATORY

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RES95144

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Spinosad

Title: Residue Method Validation Report for the Determination of Residues of Spinosad in Bovine Tissues and Milk by Immunoassay

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d)(1)(A), (B), or (C).*

Company: DowElanco

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Date: 5/29/96

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Residue Method Validation Report for the Determination of Residues of Spinosad in Bovine Tissues and Milk by Immunoassay

Study Initiation Date: 28-July-1995 Study Completion Date: 10-June-1996
Experimental Start Date: 1-August-1995 Experiment Termination Date: 5-December-1995

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
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All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160, with the exception of the information provided in Table VII; the information provided in Appendix A; the interference study summarized in Table I of Appendix B; and verification of the preparation of spinosyn A calibrators included in the kit (GRM 95.14, Section F.1.d.(3) and GRM 95.14 Appendix A) and used to generate the standard curves in each data set. Extraction efficiency data was not generated under a GLP protocol. In addition, the determination of reactivity of spinosyn A metabolites in the immunoassay test kit was performed as a research study to obtain supporting data for the hydroxylated metabolites. Extraction efficiency and hydroxylated metabolite studies were performed in the same time period as the validation study. The interference study was performed as part of the development of the spinosad immunoassay test kit, prior to the study initiation date, by Ohmicron Corporation, Newtown, Pennsylvania.

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DowElanco Quality Assurance Unit
Good Laboratory Practice Statement Page

Compound: SPINOSAD **Study:** RES95144

Title: RESIDUE METHOD VALIDATION REPORT FOR THE DETERMINATION OF RESIDUES OF SPINOSAD IN BOVINE TISSUES AND MILK BY IMMUNOASSAY

Study Initiation Date: 7/28/95 **Study Completion Date:** 6/10/96

GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
7/28/95	7/28/95	PROTOCOL
8/3/95	8/7/95	FORTIFICATIONS, EXTRACTIONS
5/29,30/96, 6/3/96	6/3/96	REPORT AND RAW DATA

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined the report reflects the raw data generated during the conduct of this study.

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TABLE OF CONTENTS

Page

ABSTRACT 8

INTRODUCTION 9

EXPERIMENTAL 9

Sample Numbering, Preparation, and Storage 9

Fortification of Recovery Samples 10

Sample Extraction and Analysis 11

Extraction Procedure for Bovine Tissues 11

Extraction Procedure for Whole Milk 11

Immunoassay Procedure 11

Calculations 12

Statistical Treatment of Data 12

Study Personnel 12

RESULTS AND DISCUSSION 12

Calibration Curve Linearity 12

Analytical Recovery Data 13

Calculated Limits of Detection and Quantitation 13

False Positive and False Negative Rates 13

Specificity/Sensitivity 14

Radiolabeled Extraction Efficiency Study 14

Correlation to HPLC Method 15

CONCLUSIONS 15

ARCHIVING 15

ACKNOWLEDGEMENTS 16

REFERENCES 17

TABLE OF CONTENTS (CONT.)

Page

TABLES

Table I. Recovery of Spinosad from Bovine Kidney Tissues 18

Table II. Recovery of Spinosad from Bovine Liver Tissues 19

Table III. Recovery of Spinosad from Bovine Muscle Tissues 20

Table IV. Recovery of Spinosad from Bovine Whole Milk 21

Table V. Recovery of Spinosad TGAI from Bovine Tissues 23

Table VI. Recovery of Spinosad TGAI from Bovine Whole Milk 24

Table VII. Immunochemical Analysis of Radiochemical Samples in Goat Tissues from the Nature of Residue Study 25

APPENDIX

Appendix A Determination of the Reactivity of Spinosyn A Goat Metabolites in the Spinosad RaPID Assay 26

Appendix B DowElanco Method GRM 95.14 39

Residue Method Validation Report for the Determination of Residues of Spinosad in Bovine Tissues and Milk by Immunoassay

ABSTRACT

This report contains validation data for DowElanco Method GRM 95.14. Control samples of bovine tissues (kidney, liver, and muscle) and whole milk were analyzed for determination of residues of spinosad using a magnetic particle-based immunoassay test kit. The validated concentration range was 0.010-0.5 µg/g for each of the matrices. The range was validated up to 5.0 µg/g in liver. Over the range of fortification, the following results were obtained: for kidney, average recoveries ranged from 78 to 81% with relative standard deviations varying from 3 to 7%; for liver, average recoveries ranged from 76 to 82% with relative standard deviations varying from 3 to 9%; for muscle, average recoveries ranged from 71 to 78% with relative standard deviations varying from 2 to 8%; for whole milk, average recoveries ranged from 73 to 89% with relative standard deviations varying from 2 to 9%. The average recoveries were all within the acceptable range of 70 to 120% and relative standard deviations at each fortification level were less than 20%. The validated LOQ was 0.010 µg/g or µg/mL for each of the matrices. LOQs calculated from the data generated in this study support the targeted LOQ of 0.01 µg/g and were 0.006 µg/g for kidney, 0.007 µg/g for liver, 0.006 µg/g for muscle, and 0.008 µg/mL for milk.

INTRODUCTION

Spinosad is a fermentation derived insect control agent under development at DowElanco for the management of a wide range of insect pests on a variety of crops. The major active ingredients in spinosad are spinosyns A and D. Hydroxylated metabolites have also been identified in goat liver during a previous radiolabeled nature of residue study (1), however, quantities of these metabolites are not available for preparation of a pure analytical standard (Appendix A). The two major degradation products of spinosyns A and D are spinosyn B and *N*-demethyl spinosyn D, respectively. The chemical structures of these spinosyns are given in DowElanco Method GRM 95.14 (Appendix B).

The purpose of this study was to provide validation data for the determination of residues of spinosad in bovine tissues and whole milk using an immunochemical-based analytical method. Additionally, this study was designed to define the method precision, accuracy, linearity, specificity, and ruggedness, as well as to support the stated limits of detection (LODs) and quantitation (LOQs). A copy of the complete method is presented in Appendix B.

This report contains the analytical results and data for the validation of DowElanco Method GRM 95.14, "Determination of Residues of Spinosad in Bovine Tissues and Milk Using a Magnetic Particle-Based Immunoassay Test Kit". This method measures total spinosad and is not capable of differentiating individual spinosyns: thus, the concentration measured should be expressed without reference to specific spinosyns. The method is applicable for the quantitative determination of residues of spinosad over the concentration range of 0.010-0.5 µg/g or µg/mL, with a validated LOQ of 0.010 µg/g or µg/mL.

EXPERIMENTAL

Sample Numbering, Preparation, and Storage

Control whole milk samples from cows housed at the dairy barn at Eli Lilly and Company in Greenfield, Indiana, were used for the method validation study. The control bovine tissues (kidney, liver, and muscle) were obtained from Knightstown Locker in Knightstown, Indiana.

Upon receipt at the Global Environmental Chemistry Laboratory—Indianapolis Lab, the following unique sample numbers (SN) were assigned to the control samples: SN 16868801 (bovine kidney), SN 16869601 (bovine liver), SN 16866101 (bovine muscle), and SN 16865301 (whole milk). Data from additional bovine tissue control and fortified recovery samples collected during a dairy feeding study were also included in this report (2). The following unique sample numbers were assigned to the control samples from the feeding study: SN 16927701-03 (bovine kidney), SN 16926901-03 (bovine liver), and SN 16925001-03 (bovine muscle). These numbers were used to track the samples during storage and analysis.

Bovine tissue samples were prepared 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. Whole milk samples did not require any sample preparation prior to analysis. All of the bovine tissue and milk samples were transferred to high density polyethylene containers and were stored frozen until removed for fortification and analysis.

Fortification of Recovery Samples

Control samples were fortified with the appropriate fortification solution for the generation of recovery data. The analytical standards used in the validation study were the pure active ingredients or reference compounds listed in the following table:

Standard	Lot No.	Purity, %	Analysis Date	Reference
Spinosyn A	TSN100221	97	01-Mar-1995	FA&PC 950019
Spinosad, Technical Grade of Active Ingredient (TGAI)	AGR293707	76.1 Spinosyn A 11.9 Spinosyn D	16-May-1995	FA&PC 950151

The standards were obtained from the Test Substance Coordinator, DowElanco, 9330 Zionsville Road, Building 306/A1, Indianapolis, IN 46268-1053.

Recovery samples were fortified as described in DowElanco Method GRM 95.14 by adding 1.0 mL of the appropriate fortification solution to 20 g of the tissue control sample or by adding 1.0 mL of the appropriate fortification solution to 20 mL of the milk control sample. Milk results can be reported as either µg/g or µg/mL based on an average specific gravity of 1.03 (3).

Recovery samples of each matrix were fortified with spinosyn A at levels of 0.003 µg/g (the targeted LOD), 0.010 µg/g (the targeted LOQ), 0.10 µg/g and 0.50 µg/g. Liver was also fortified at 1.0 µg/g and 5.0 µg/g to verify that acceptable recoveries could be obtained from samples with higher residues. Untreated control samples were also prepared for analysis along with the recovery samples to check for background interferences.

The samples fortified with spinosyn A (described above) comprised the full validation set for each of the matrices. Additional recovery samples were fortified with TGAI (at 0.10 µg/g or µg/mL) following the same procedure as described for fortification with spinosyn A in DowElanco Method GRM 95.14. No corrections were made based on the reported purity of the TGAI fortification solution.

Sample Extraction and Analysis

The samples were extracted and analyzed as described in DowElanco Method GRM 95.14. Brief descriptions of the procedures are given below:

Extraction Procedure for Bovine Tissues A 20.0-g sample was extracted with a solution of acetonitrile/water (80/20) by homogenizing followed by refluxing. The extract was filtered. An aliquot of the filtrate was evaporated to dryness and re-dissolved in 10.0 mL of Spinosad Sample Diluent.

Extraction Procedure for Whole Milk A 5.0-mL sample was extracted by adding acetonitrile, vortexing and shaking. An aliquot of the extract was evaporated to dryness and re-dissolved in 10.0 mL of Spinosad Sample Diluent.

Immunoassay Procedure A 0.2-mL portion of the diluted extract was mixed with an aliquot of enzyme-conjugated spinosad in a disposable test tube. Magnetic particles coated with antibodies specific to spinosad were then added to the tube. After a 30-minute room temperature incubation period, the rack containing the tubes was placed into a magnetic base. Following separation of the magnetic particles, the solution was decanted and the particles were washed twice with a wash solution. The presence of spinosad was detected by adding an enzyme substrate (hydrogen peroxide) and a chromogen (3,3',5,5'-tetramethylbenzidine) to generate a colored product. After

an incubation period, the reaction was stopped by the addition of acid. The absorbance of the solution in each tube was then measured at 450 nm with the RPA-1 RaPID Analyzer.

Calculations

Example calculations of the standard curve, spinosad sample concentration, method factors, and percent recovery, are contained in Appendix B, DowElanco Method GRM 95.14, Section K.

Statistical Treatment of Data

Statistical treatment of data included the calculation of means, standard deviations, relative standard deviations and correlation coefficients.

Study Personnel

D. L. Young served as the study director and principal analyst for Study Number RES95144. C. A. Mihaliak, C. M. Knapp, and J. O'Neill served as additional analysts during the study.

RESULTS AND DISCUSSION

Calibration Curve Linearity

Calibration curves generated over the range of 0.00050 to 0.001 µg/mL were used for the quantitation of spinosad. A representative calibration curve is calculated and plotted in DowElanco Method GRM 95.14 (Appendix B, Figure 2). Correlation coefficient (r^2) values ranged from 0.9983 to 1.0000 for all analytical sets run during the method validation.

Analytical Recovery Data

A method validation study was conducted to determine the recovery levels and the precision of the method for spinosad in bovine tissues and whole milk. Data are summarized in Tables I-IV of this report and net recoveries in each matrix are summarized in DowElanco Method GRM 95.14 (Appendix B, Tables III-V). The validation was conducted using spinosyn A to fortify the recovery samples. Spinosyn A, which is the major component of spinosad and comprises approximately 76% of technical grade spinosad (4), was deemed the best choice for fortification of the validation recovery samples because technical grade spinosad can exhibit considerable lot-to-lot variability in the percentage of the individual spinosyns (5).

Additional control samples of each matrix were fortified with spinosad TGAI to verify detection of total spinosad in TGAI by the immunoassay method when extracted from bovine matrices. Data are summarized in Tables V-VI.

Calculated Limits of Detection and Quantitation

The LOD and LOQ for spinosad in bovine kidney, liver, muscle, and whole milk were calculated using the standard deviations from 0.010 µg/g (the targeted LOQ) recovery results. The LOD and LOQ were calculated as $3s$ and $10s$, respectively, following a published technique (6). The results are summarized in Table VII of DowElanco Method GRM 95.14.

The calculated LOD ranged from 0.0018 to 0.0024 µg/g , and the calculated LOQ ranged from 0.006 to 0.008 µg/g over all matrices. Recovery samples fortified at 0.003 µg/g (the targeted LOD) were detectable in all of the sample types. Recovery values for residues found at levels below the targeted LOQ (0.010 µg/g) were not calculated or reported.

False Positive and False Negative Rates

Unfortified control samples (matrix blanks) and samples fortified at the LOD (0.003 µg/g) were analyzed during the study to determine the false positive and false negative rate (7). A false positive result occurs when residue at or above the established LOD is measured in a control sample known to be free of analyte. A false negative result occurs when no residue is detected in

a sample fortified at the LOD. There were no false positives reported from the unfortified control samples and no false negatives from the LOD fortified samples analyzed during this study.

Specificity/Sensitivity

Several pesticides, inorganic compounds and organic compounds were tested for the potential to interfere with conjugate binding in the assay (8). The only compound which exhibited an I_{50} concentration below 10 µg/mL was carbendazim (I_{50} = 5.56 µg/mL). The I_{50} concentration is the concentration which results in a 50% inhibition of conjugate binding to the available antibodies, and is a commonly used reference value for expressing cross reactivity and determining the extent of interference. In comparison, the I_{50} for spinosyn A is approximately 0.0003 µg/mL.

Several analogs, metabolites and degradates of spinosad have been tested to determine whether the Spinosad RaPID Assay test kit will detect their presence in an aqueous sample (8). In general, the assay is sensitive (I_{50} < 0.002 µg/mL) to the major spinosad factors (spinosyns A, B, D, K, and *N*-demethyl spinosyn D), although the level of sensitivity is not identical for all spinosyns.

In addition, hydroxylated metabolites isolated from goat liver samples collected during a metabolism study were analyzed by radioassay and immunoassay (Appendix A). Metabolite concentration was determined and percent reactivity of the metabolites calculated. The hydroxylated metabolites were reactive in the immunoassay and quantifiable based on a spinosyn A calibration curve. The hydroxylated metabolites were formed from spinosyn A by hydroxylation on the tetracyclic macrolide combined, in some cases, with *N*-demthylation of the forosamine moiety.

Radiolabeled Extraction Efficiency Study

An extraction efficiency study was conducted using goat tissues and whole milk samples from a previous radiolabeled nature of residue study (1). Method GRM 95.14 was used to extract these samples for determination of total spinosad concentrations by immunoassay. Samples were reanalyzed radiochemically to ensure accurate ^{14}C results for comparison with the immunoassay

results. The radiochemical analysis was conducted using procedures described in the nature of residue report. Total residue was calculated from the radiochemical procedure and compared to the total residue determined from Method GRM 95.14. Data are summarized in Table VII of this report.

Correlation to HPLC Method

A direct correlation study between the immunoassay method and the existing HPLC methodology has been performed (9). Data from a dairy feeding study (2) indicate that both methods yield comparable results of total spinosad residues. Statistical calculations from analysis of whole milk, kidney, and lean bovine tissue samples resulted in a correlation coefficient of approximately 0.95. Statistical calculations from analysis of liver shows a positive bias in the immunoassay results which is believed to represent the hydroxylated metabolites of spinosad (Appendix A).

CONCLUSIONS

DowElanco Analytical Method GRM 95.14, "Determination of Residues of Spinosad in Bovine Tissues and Milk Using a Magnetic Particle-Based Immunoassay Test Kit" has been demonstrated to be suitable for use in the determination of residues of total spinosad in the matrices studied. The targeted LODs (0.003 µg/g) and LOQs (0.010 µg/g) were verified. Analysis of samples by different analysts and on different days demonstrated the ruggedness of the method. The average recoveries obtained for samples fortified with spinosyn A were within the acceptable range of 70-120%. Results obtained with this method compare favorably with those obtained by HPLC.

ARCHIVING

All raw data and the original of the final report are filed in the DowElanco testing facility archives, Indianapolis, Indiana.

ACKNOWLEDGEMENTS

Radiochemical analysis and the calculation of radiochemical results were accomplished by R. A. Collins, J. D. O'Neill, and D. P. Rainey. Initial sample documentation, handling, and preparation (grinding and blending) were conducted by R. D. Griggs, B. H. Arnold, T. L. Barnes, R. F. Elsharaiha, and M. W. Russell.

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Table I. Recovery of Spinosad from Bovine Kidney Tissues

Control Sample Number	Date of Analysis	Spinosyn A, µg/g Added	Spinosyn A, µg/g Found	Percent Recovery	Statistics[a]
16868801	08-Aug-1995	0	ND[b]	–	
16868801	08-Aug-1995	0	ND	–	
16927701	28-Sep-1995	0	ND	–	
16927702	28-Sep-1995	0	ND	–	
16927703	28-Sep-1995	0	ND	–	
16927701	05-Oct-1995	0	ND	–	
16927702	16-Nov-1995	0	ND	–	
16927703	05-Dec-1995	0	ND	–	
16927702	16-Nov-1995	0.003	NA[c]	–	
16927702	16-Nov-1995	0.003	NA	–	
16927703	05-Dec-1995	0.003	NA	–	
16868801	08-Aug-1995	0.010	0.0079	79	
16868801	08-Aug-1995	0.010	0.0082	82	
16868801	08-Aug-1995	0.010	0.0084	84	
16927701	28-Sep-1995	0.010	0.0083	83	
16927701	28-Sep-1995	0.010	0.0081	81	
16927701	28-Sep-1995	0.010	0.0070	70	
16927701	28-Sep-1995	0.010	0.0068	68	
16927701	05-Oct-1995	0.010	0.0077	77	$\bar{x}$ = 78
16927701	05-Oct-1995	0.010	0.0081	81	s = 5.7
16927703	05-Dec-1995	0.010	0.0072	72	RSD = 7.3
16927703	05-Dec-1995	0.100	0.065	65	
16868801	08-Aug-1995	0.500	0.389	78	$\bar{x}$ = 81
16868801	08-Aug-1995	0.500	0.416	83	s = 2.6
16868801	08-Aug-1995	0.500	0.410	82	RSD = 3.2
				$\bar{x}$ = 78	
				s = 6.2	
				n = 14	

[a] Statistical calculation for each fortification level.
[b] None detected at a detection limit of 0.003 µg/g.
[c] Not applicable (analyte was detected, but it was below the 0.010 µg/g LOQ).

Table II. Recovery of Spinosad from Bovine Liver Tissues

Control Sample Number	Date of Analysis	Spinosad, µg/g Added	Spinosad, µg/g Found	Percent Recovery	Statistics[a]
16869601	04-Aug-1995	0	ND[b]	–	
16869601	04-Aug-1995	0	ND	–	
16926901	03-Oct-1995	0	ND	–	
16926902	03-Oct-1995	0	ND	–	
16926903	03-Oct-1995	0	ND	–	
16926901	05-Oct-1995	0	ND	–	
16926902	25-Oct-1995	0	ND	–	
16926901	26-Oct-1995	0	ND	–	
16926902	16-Nov-1995	0	ND	–	
16926903	05-Dec-1995	0	ND	–	
16926901	03-Oct-1995	0.003	NA[c]	–	
16926903	05-Dec-1995	0.003	NA	–	
16869601	04-Aug-1995	0.010	0.0078	78	
16869601	04-Aug-1995	0.010	0.0075	75	
16869601	04-Aug-1995	0.010	0.0064	64	
16926901	03-Oct-1995	0.010	0.0081	81	
16926901	03-Oct-1995	0.010	0.0071	71	
16926901	05-Oct-1995	0.010	0.0085	85	
16926901	05-Oct-1995	0.010	0.0073	73	
16926902	25-Oct-1995	0.010	0.0081	81	$\bar{x}$ = 76
16926901	26-Oct-1995	0.010	0.0086	86	s = 7.1
16926903	05-Dec-1995	0.010	0.0070	70	RSD = 9.3
16926903	05-Dec-1995	0.100	0.065	65	
16869601	04-Aug-1995	0.500	0.425	85	
16869601	04-Aug-1995	0.500	0.410	82	$\bar{x}$ = 82
16869601	04-Aug-1995	0.500	0.401	80	s = 2.6
16926902	25-Oct-1995	0.500	0.397	79	RSD = 3.2
16926901	03-Oct-1995	1.00	0.91	91	
16926901	03-Oct-1995	1.00	0.80	80	
16926901	16-Nov-1995	5.00	3.91	78	
16926901	16-Nov-1995	5.00	3.37	67	
				$\bar{x}$ = 77	
				s = 7.5	
				n = 19	

[a] Statistical calculation for each fortification level.
[b] None detected at a detection limit of 0.003 µg/g.
[c] Not applicable (analyte was detected, but it was below the 0.010 µg/g LOQ).

Table III. Recovery of Spinosad from Bovine Muscle Tissues

Sample Number	Date of Analysis	Spinosyn A, µg/g Added	Spinosyn A, µg/g Found	Percent Recovery	Statistics[a]
16866101	03-Aug-1995	0	ND[b]	–	
16866101	03-Aug-1995	0	ND	–	
16925001	26-Sep-1995	0	ND	–	
16925002	26-Sep-1995	0	ND	–	
16925003	26-Sep-1995	0	ND	–	
16925001	27-Sep-1995	0	ND	–	
16925002	27-Sep-1995	0	ND	–	
16925003	27-Sep-1995	0	ND	–	
16925001	05-Oct-1995	0	ND	–	
16925002	16-Nov-1995	0	ND	–	
16925003	05-Dec-1995	0	ND	–	
16925001	26-Sep-1995	0.003	NA[c]	–	
16925001	27-Sep-1995	0.003	NA	–	
16925003	05-Dec-1995	0.003	NA	–	
16866101	03-Aug-1995	0.010	0.0086	86	
16866101	03-Aug-1995	0.010	0.0085	85	
16866101	03-Aug-1995	0.010	0.0082	82	
16925001	26-Sep-1995	0.010	0.0077	77	
16925001	26-Sep-1995	0.010	0.0079	79	
16925001	27-Sep-1995	0.010	0.0082	82	
16925001	27-Sep-1995	0.010	0.0069	69	
16925001	27-Sep-1995	0.010	0.0082	82	
16925001	27-Sep-1995	0.010	0.0071	71	
16925001	05-Oct-1995	0.010	0.0078	78	
16925002	16-Nov-1995	0.010	0.0085	85	$\bar{x}$ = 78
16925002	16-Nov-1995	0.010	0.0073	73	s = 6.1
16925003	05-Dec-1995	0.010	0.0069	69	RSD = 7.8
16925001	05-Oct-1995	0.100	0.073	73	$\bar{x}$ = 71
16925002	16-Nov-1995	0.100	0.071	71	s = 2.5
16925003	05-Dec-1995	0.100	0.068	68	RSD = 3.5
16866101	03-Aug-1995	0.500	0.376	75	
16866101	03-Aug-1995	0.500	0.385	77	
16866101	03-Aug-1995	0.500	0.386	77	
16925001	26-Sep-1995	0.500	0.384	77	$\bar{x}$ = 77
16925001	27-Sep-1995	0.500	0.393	79	s = 1.5
16925001	27-Sep-1995	0.500	0.377	75	RSD = 1.9
				$\bar{x}$ = 77	
				s = 5.4	
				n = 22	

[a] Statistical calculation for each fortification level.
[b] None detected at a detection limit of 0.003 µg/g.
[c] Not applicable (analyte was detected, but it was below the 0.010 µg/g LOQ).

Table IV. Recovery of Spinosad from Bovine Whole Milk

Sample Number	Date of Analysis	Spinosyn A, µg/g Added	Spinosyn A, µg/g Found	Percent Recovery	Statistics[a]
16865301	03-Aug-1995	0	ND[b]	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0	ND	--	
16865301	03-Aug-1995	0.003	NA[c]	--	
16865301	03-Aug-1995	0.003	NA	--	
16865301	03-Aug-1995	0.003	NA	--	
16865301	03-Aug-1995	0.003	NA	--	
16865301	03-Aug-1995	0.003	NA	--	
16865301	03-Aug-1995	0.010	0.0098	98	
16865301	03-Aug-1995	0.010	0.0093	93	
16865301	03-Aug-1995	0.010	0.0099	99	
16865301	03-Aug-1995	0.010	0.0098	98	
16865301	03-Aug-1995	0.010	0.0094	94	
16865301	03-Aug-1995	0.010	0.0081	81	
16865301	03-Aug-1995	0.010	0.0087	87	
16865301	03-Aug-1995	0.010	0.0085	85	
16865301	03-Aug-1995	0.010	0.0096	96	
16865301	03-Aug-1995	0.010	0.0088	88	
16865301	03-Aug-1995	0.010	0.0090	90	
16865301	03-Aug-1995	0.010	0.0100	100	
16865301	03-Aug-1995	0.010	0.0095	95	
16865301	03-Aug-1995	0.010	0.0076	76	
16865301	03-Aug-1995	0.010	0.0097	97	
16865301	03-Aug-1995	.0.010	0.0080	80	
16865301	03-Aug-1995	0.010	0.0084	84	
16865301	03-Aug-1995	0.010	0.0085	85	$\bar{x}$ = 89
16865301	03-Aug-1995	0.010	0.0084	84	s = 7.9
16865301	03-Aug-1995	0.010	0.0074	74	RSD = 8.9

Table IV. (Cont.) Recovery of Spinosad from Bovine Whole Milk

Sample Number	Date of Analysis	Spinosyn A, µg/g Added	Spinosyn A, µg/g Found	Percent Recovery	Statistics[a]
16865301	03-Aug-1995	0.100	0.073	73	$\bar{x}$ = 73
16865301	03-Aug-1995	0.100	0.067	67	s = 4.5
16865301	03-Aug-1995	0.100	0.077	77	RSD = 6.2
16865301	03-Aug-1995	0.500	0.397	79	
16865301	03-Aug-1995	0.500	0.380	76	
16865301	03-Aug-1995	0.500	0.388	78	
16865301	03-Aug-1995	0.500	0.371	74	
16865301	03-Aug-1995	0.500	0.394	79	
16865301	03-Aug-1995	0.500	0.379	76	
16865301	03-Aug-1995	0.500	0.384	77	$\bar{x}$ = 77
16865301	03-Aug-1995	0.500	0.384	77	s = 2.1
16865301	03-Aug-1995	0.500	0.403	81	RSD = 2.7
				$\bar{x}$ = 84	
				s = 9.2	
				n = 32	

[a] Statistical calculation for each fortification level.
[b] None detected at a detection limit of 0.003 µg/g.
[c] Not applicable (analyte was detected, but it was below the 0.010 µg/g LOQ).

Table V. Recovery of Spinosad TGAI[a] from Bovine Tissues

Matrix	Sample Number	Date of Analysis	Spinosad TGAI, µg/g Added	Spinosad TGAI, µg/g Found	Percent Recovery[b]
Kidney	16927702	16-Nov-1995	0.100	0.068	68
Liver	16926902	16-Nov-1995	0.100	0.061	61
Muscle	16925002	16-Nov-1995	0.100	0.073	73

[a] TGAI = Technical Grade Active Ingredient.
[b] Uncorrected for purity of 88% Spinosyn A + D

Table VI. Recovery of Spinosad TGAI[a] from Bovine Whole Milk

Sample Number	Date of Analysis	Spinosad TGAI, µg/mL Added	Spinosad TGAI, µg/mL Found	Percent Recovery	Statistics[b]
16865301	03-Aug-1995	0.100	0.072	72	
16865301	03-Aug-1995	0.100	0.070	70	
16865301	03-Aug-1995	0.100	0.067	67	
16865301	08-Aug-1995	0.100	0.066	66	$\bar{x}$ = 71
16865301	08-Aug-1995	0.100	0.070	70	s = 5.4
16865301	08-Aug-1995	0.100	0.081	81	RSD = 7.6

[a] TGAI = Technical Grade Active Ingredient.
[b] Statistical calculation for TGAI fortification.

Table VII. Immunochemical Analysis of Radiochemical Samples in Goat Tissues from the Nature of Residue Study

Matrix	dpm/g[a]	Theoretical Sample dpm	Total Extracted dpm[b]	% ^{14}C Extracted[c]	Extraction Volume (mL)	Spinosad Related Residues (µg/g or µg/mL) Immunoassay[d]	^{14}C[e]	% Recovery[f]
Kidney	7,290	72,900	68,928	94.6	64	0.92	0.91	101.1
Liver	11,900	119,000	107,331	90.2	57	1.19	1 42	83.8
Muscle	2,240	22,400	23,664	105.6	58	0.24	0.31	77.4
Milk	5,079	50,790	50,512	99.5	44	0.62	0.67	92.5

[a] Determined by combustion.

[b] For each tissue sample, a 10-g sample was extracted (dpm/g x 10g).

[c] Determined by dividing the total dpm extracted by the theoretical dpm in the sample. (Total Extracted dpm/Theoretical Sample dpm.)

[d] Calculated by multiplying the volume of each sample extract by the immunoassay spinosad residue result and dividing by the sample weight. (e.g., in liver [(57 mL x 0.208 µg/mL (from raw data))/10g = 1.19 µg/g]).

[e] Calculated by dividing the total dpm in the sample extract by 7548 dpm/µg (specific activity of the ^{14}C spinosyn A) to determine the total µg equivalent of spinosyn A in the extract and dividing that value by the sample weight, (e.g., in liver [(107331dpm/ 7548 dpm/µg)/10 g = 1.42 µg/g spinosyn A equivalents]).

[f] Determined by dividing the immunoassay residue by the radiochemical residue and multiplying the result by 100%.

Appendix A

Determination of the Reactivity of Spinosyn A Goat Metabolites in the Spinosad RaPID Assay

Determination of the Reactivity of Spinosyn A Goat Metabolites
in the Spinosad RaPID Assay

D. P. Rainey, J. D. O'Neill and R. A. Collins

ABSTRACT

Spinosyn A hydroxylated metabolites characterized in the goat metabolism study were reisolated from the original liver tissue sample using solvent partitioning, silica column chromatography and thin-layer chromatography. Each metabolite was then tested using the Ohmicron Spinosad RaPID Assay test kit to determine the reactivity of the metabolites in this assay procedure.

The results showed that hydroxylated metabolites, which were formed from spinosyn A by hydroxylation on the tetracyclic macrolide ring combined in some cases with *N*-demethylation of the forosamine moiety, were reactive in the immunoassay based on a spinosyn A standard curve. The responses from the hydroxylated metabolites were 60-80% of the calculated concentration from the radioassay determination. The data supports using the Ohmicron Spinosad RaPID Assay test kit to detect the hydroxylated metabolites of spinosyn A.

INTRODUCTION

Spinosad is a fermentation-derived tetracyclic macrolide produced by the actinomycete, Saccharopolyspora spinosa. Spinosad is active against a variety of lepidopterous and other insect pests present on cotton, tree crops and a variety of vegetable crops. The active ingredients in spinosad consists of spinosyns A and D which are present in a ratio of about 85:15.

Some of the proposed uses of spinosad include crops from which livestock feeds can be derived. A ruminant magnitude of residue study was conducted to determine the level of spinosad related residues in edible tissues and milk from lactating dairy cows fed various levels of spinosad for 28 days. The results from a ^{14}C spinosyn A and spinosyn D goat metabolism study (1) conducted prior to the ruminant magnitude of residue study showed that the predominant residue in tissues and milk was unmetabolized spinosyns A or D. Other major residues (greater than 10% of the total) were the metabolites spinosyn B (*N*-demethyl spinosyn A) and *N*-demethyl spinosyn D.

Additional metabolites present at levels between 5 and 10% of the total radioactive residue were also detected in the goat study. These metabolites were shown to be hydroxylated on the tetracyclic macrolide and, in several instances, also *N*-demethylated at the forosamine moiety. Structures could not be conclusively assigned to several of these metabolites, therefore, reference standards were not available or could not be prepared. As a result, the hydroxylated compounds were not included as analytes in the HPLC tissue and milk residue method which quantitatively determines spinosyns A, D, B, and *N*-demethyl spinosyn D.

The purpose of the work reported here was to determine the reactivity of the macrolide hydroxylated/*N*-demethylated metabolites in the Spinosad RaPID Assay. If the metabolites were highly reactive in this immunoassay procedure, it should be possible to use this procedure to determine the total spinosad related residue in tissues and milk from the animals in the magnitude of residue study. The metabolite level in these samples could then be estimated by determining the difference between the total residue by immunoassay and the residue attributed to the four analytes measured by the HPLC method.

MATERIALS AND METHODS

The metabolites used for the immunoassay reactivity determinations were isolated from goat liver. The liver tissue used was obtained from the goat metabolism study (1) which was completed in October, 1994. In that study, all major radiolabeled components detected in the liver were isolated and identified/characterized using mass spectroscopy.

The same metabolite isolation procedures described in the goat metabolism report were used to obtain liver metabolites for the reactivity determinations. For this report, only the specific techniques used to isolate metabolites will be discussed. Other information such as the dosing regimen, quantitation of metabolites and spinosyn A in liver, fat, lean, kidney, and milk, and a discussion of the results from the mass spectral characterization of liver metabolites can be found in the goat metabolism report (1).

Liquid Scintillation Counting (LSC)

Samples were counted in a Packard Tri-Carb scintillation counter (Model 2500TR). Performance was checked daily using a set of quenched standards. Counting efficiency was determined by use of an external standard and quench curve. Commercial scintillation cocktails such as Packard Ultima Gold and Beckman Ready Solve were used. Samples were generally counted for 5 or 10 minutes.

Combustion Analysis

Total radioactivity in extracted tissue samples was determined by combusting aliquots to CO_2 and water in a Harvey sample oxidizer (Model OX500 or OX300) and trapping the CO_2 in Carbon 14 Cocktail (R. J. Harvey, Hillsdale, New Jersey). The $^{14}CO_2$ trapped in the cocktail was then quantified by LSC. Oxidizer performance was checked each day by combusting samples containing a known amount of ^{14}C.

Thin Layer Chromatography (TLC)

TLC separations were carried out on Merck Silica Gel 60 plates (0.25 mm thickness with fluorescent indicator). Plates were developed in a solvent composed of toluene/isopropanol/diethylamine (84:7:7 by volume). Reference standards were visualized by UV light. Radioactive components were located by exposing plates to X-OMAT X-ray film or by phosphor screen autoradiography using the Phosphoimager SF (Molecular Dynamics, Sunnyvale, California).

Silica Gel Column Chromatography

Radioactivity in the liver extract was characterized by chromatography on silica gel columns. This procedure provided a profile of the extracted radioactivity based on differences in polarity of the components and also served as a sample clean-up procedure.

The extract concentrate was dried onto 7-10 mL of dry column silica gel 60 (E. Merck) and placed at the top of a 120-cm x 0.8-cm I.D. glass column previously dry packed with 50 mL of the same silica gel. The column was eluted at a flow rate of 20 mL/minute using a non-linear gradient developed by pumping solvent from a 190-mL stirred reservoir filled with one solvent into which a more polar solvent was being introduced as the elution progressed. The solvent sequence for elution is shown below:

Tube No.	Volume	Solvent*
1-10	200 mL	Hexane
11-30	400 mL	Hexane/Toluene (90/10)
31-50	400 mL	Hexane/Toluene (50/50)
51-70	400 mL	Toluene
71-90	400 mL	Toluene/EtOAc (90/10)
91-110	400 mL	Toluene/EtOAc (50/50)
111-130	400 mL	EtOAc
131-150	400 mL	EtOAc/MeOH/H_2O (80/20/2)
151-170	400 mL	MeOH/Water (95/5)
171-190	400 mL	MeOH/Acetic Acid (95/5)

*All solvents except tubes 171-190 contained 0.5% diethylamine.

Fractions of 20 mL each were collected and aliquots assayed for total radioactivity by LSC. Fractions constituting the same radioactivity peak or representing peaks of similar polarity were pooled and concentrated under vacuum.

Extraction of Radioactive Residues from Liver

Liver was extracted by refluxing duplicate 75-g samples with 375 mL of acetonitrile/H_2O (80:20 v/v) for one hour. Following the reflux period the samples were cooled and filtered. The filtrates were combined and then partitioned with an equal volume of dichloromethane. The volume of the spent aqueous fraction from this procedure was determined and duplicate 1.0-mL aliquots were radioassayed. The CH_2Cl_2 fraction was evaporated to dryness under vacuum, redissolved in 20 mL of CH_2Cl_2 and duplicate 0.5-mL aliquots were radioassayed. The extracted tissue fraction was air-dried, weighed, and residual ^{14}C activity determined by combustion analysis. The CH_2Cl_2 fraction was then subjected to silica gel column chromatography.

Metabolite Reactivity Determinations

Each of the radioactive components isolated from the liver extract which included spinosyn A and spinosyn B was appropriately diluted and then assayed in the Spinosad RaPID Assay kit. The specific assay procedures used were those supplied with the immunoassay kit. The concentration of each radioactive component as determined by immunoassay was compared to the concentration determined by radioassay to estimate the reactivity of each metabolite in the immunoassay test kit.

RESULTS AND DISCUSSION

Isolation of Liver Metabolites

The results from the initial extraction of liver showed that the dichloromethane fraction contained 90.4% of the sample radioactivity; the spent aqueous fraction 5.2% and the extracted tissue 4.4%. This distribution of radioactivity was essentially identical to that obtained from the original liver extraction in the goat metabolism study (1).

The results from the silica gel column separation of the dichloromethane fraction (90.4% of the liver radioactivity) are shown in Figure 1. The ^{14}C elution profile contains five radioactive peaks. The individual tubes comprising each of the five peaks were pooled and concentrated prior to TLC characterization. The amount of radioactivity and the µg equivalents of spinosyn A in each of the pooled fractions is also indicated in Figure 1.

TLC Characterization of Liver Metabolites

In order to further characterize the metabolite profile, the pooled fractions from the silica column separation of the liver extract were subjected to TLC with detection of the radiolabeled components by autoradiography.

The TLC autoradiograph of Fractions 1 and 2 from the silica column is shown in Figure 2 and Fractions 3 and 4 in Figure 3. Fraction 5 from the column contained only a small amount of radioactivity and was not subjected to TLC characterization.

Based on metabolite identification work carried out as part of the original goat metabolism study, the following assignments were made to the radiolabeled components present in Fractions 1, 2, 3, and 4 from the silica column:

Fraction 1. The single component in this fraction is spinosyn A.

Fraction 2. The two components in this fraction are spinosyn B and MET-1 which is identical to the metabolite designated as A-Li-2 in the goat metabolism report. Mass spectral data was obtained on this metabolite; however, a structure could not be assigned.

Fraction 3. The two metabolites in this fraction, MET-2 and MET-3, are identical to metabolites A-Li-3a and A-Li-3b from the goat metabolism report. These two metabolites were shown to be isomers which were hydroxylated on the tetracyclic macrolide.

Fraction 4. This fraction appears to contain a single component, MET-4. However, in the goat metabolism study this component was shown to be a complex of three metabolites which appeared to be isomers formed from spinosyn B by hydroxylation of the tetracyclic macrolide at three different locations. For purposes of determining the reactivity of these metabolites in the immunoassay, the complex was isolated and assayed as a single sample rather than as individual metabolites.

Reactivity of Metabolites in the Spinosad RaPID Assay

The metabolites used in the reactivity determinations were isolated from TLC plates by scraping the appropriate radioactive zone from the plate. The metabolite was then eluted from the silica gel with methanol; the solvent evaporated; the sample redissolved in 1.0 mL of methanol; and an aliquot radioassayed to determine the metabolite concentration.

Aliquots of each metabolite solution and appropriate dilutions were then assayed using the Spinosad RaPID Assay. The results of these assays are shown in Table I. The summarized data includes the metabolite concentrations as determined by radioassay and immunoassay and the percent reactivity. The percent reactivity of each analyte was calculated by determining the ratio of the immunoassay concentration to the radioassay concentration and multiplying by 100%. Thus, a metabolite which has reactivity of 100% would exhibit an equivalent concentration in the immunoassay and radioassay. This also means that the antibody utilized in the immunoassay kit binds the metabolite to the same degree as spinosyn A. If the reactivity of a metabolite was 50%, the concentration measured using the immunoassay would be 50% of the radioassay concentration.

The reactivity of the four metabolites (Table I) ranged from 60% (MET-4) to 79% (MET-1) with an average reactivity for the four metabolites of 68%. The reactivity of the isolated ^{14}C spinosyn A and spinosyn B are in the range which would be expected for these compounds. These results indicate that the Spinosad RaPID Assay test kit can be used to provide a measurement of the total concentration of the metabolites in animal tissues, including those which are not measured by the standard HPLC tissue residue method.

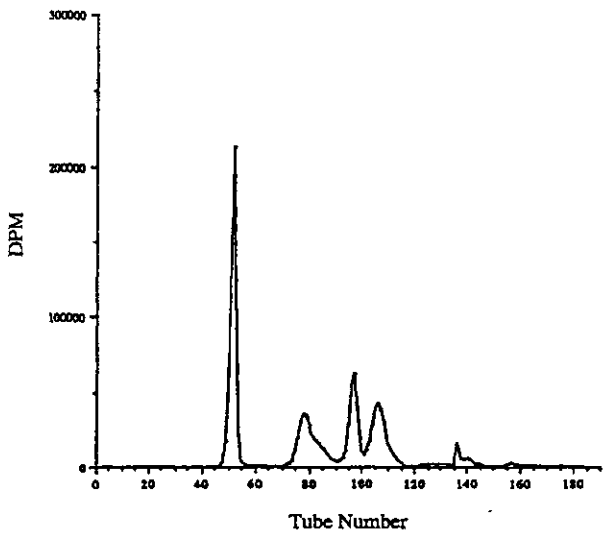
REFERENCES

1. Rainey, D. P. "^{14}C XDE-105 (Factor A and D) Goat Metabolism Study: Tissues, Milk, Excreta", GH-C 3396, MRID 43727406, 1994, unpublished report of DowElanco.

Table I. Reactivity of Isolated Spinosyn A Goat Liver Metabolites in the XDE-105 RaPID Assay

Compound	ng/mL		% Reactivity[a]
	Radioassay	Immunoassay	
spinosyn A	0.57	0.51	89
spinosyn B	0.15	0.12	80
MET-1	0.19	0.15	79
MET-2	0.44	0.27	61
MET-3	0.29	0.21	72
MET-4	0.25	0.15	60

[a] % reactivity = (immunoassay conc.÷ radioassay conc.) x 100.



Fraction #	Tube #	dpm	µg equivalents of spinosyn A[a]
1	48-55	581813	77.1
2	73-90	284697	37.7
3	91-101	251318	33.3
4	102-115	276961	36.7
5	136-144	53713	7.1

[a] Determined by dividing the dpm in fraction by 7548 dpm/µg (specific activity of spinosyn A used in the goat metabolism study.

Figure 1. Silica Column Separation of ^{14}C Residue in Dichloromethane Fraction of Spinosyn A Liver

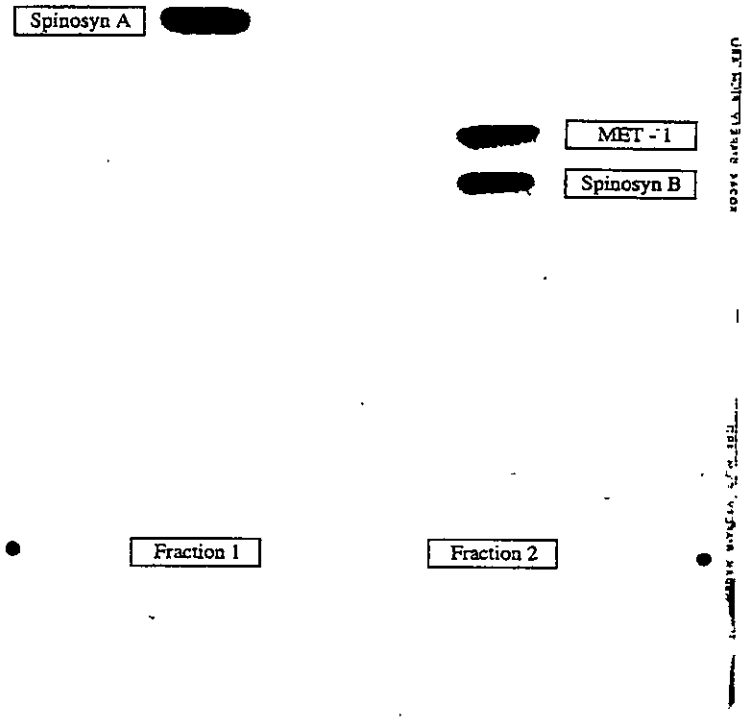


Figure 2. TLC Characterization of Silica Column Fractions 1 and 2 from Dichloromethane Fraction of Spinosyn A Liver

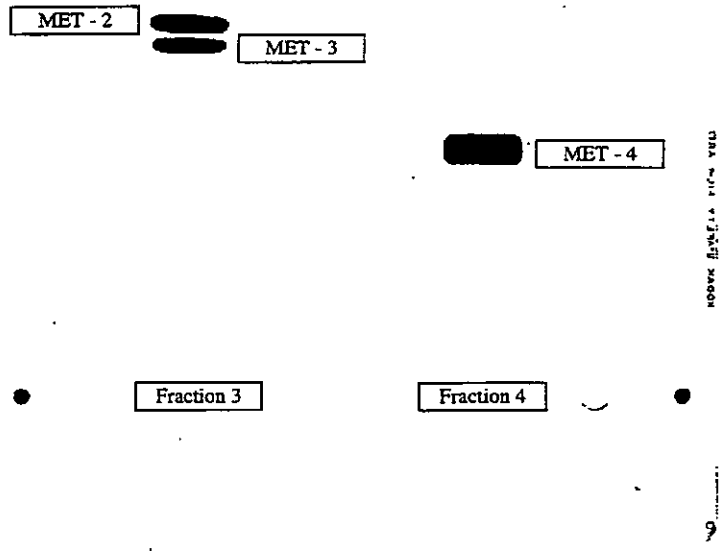


Figure 3. TLC Characterization of Silica column Fraction 3 and 4 from Dichloromethane Fraction of Spinosyn A Liver

Appendix B

DowElanco Method GRM 95.14

GRM.: 95.14
EFFECTIVE: May 22, 1996
SUPERSEDES: New

Determination of Residues of Spinosad in Bovine Tissues and Milk Using a Magnetic Particle-Based Immunoassay Test Kit

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A. Scope

This method is applicable for the quantitative determination of residues of spinosad in bovine tissues (kidney, liver, and muscle) and whole milk samples at a limit of quantitation (LOQ) of 0.010 µg/g and limit of detection (LOD) of 0.003 µg/g. The quantitative range is from 0.010 µg/g to 0.50 µg/g for bovine tissues and milk. The range can be extended for analysis of samples containing higher concentrations of spinosad by further dilution of the sample extract into the working range of the immunoassay kit.

B. Principle

The method is based upon use of the Ohmicron Spinosad RaPID Assay test kit and the RPA-1 RaPID Analyzer. The antibody used to develop the spinosad immunoassay test kit is sensitive to several spinosyns including the major metabolites and degradates (Figure 1). The kit uses spinosyn A, the major component of spinosad, for generation of the calibration curve and subsequent quantitation of spinosad. The method is not designed to differentiate individual spinosyns, but instead measures the total residue of the spinosyns.

Residues of spinosad and its degradation products are extracted from bovine tissues with an acetonitrile/water extraction solution and from milk with acetonitrile. An aliquot of the extract is evaporated to dryness and reconstituted with Spinosad Sample Diluent. The diluted sample is then assayed for spinosad residues using the Ohmicron Spinosad RaPID Assay kit, which applies the principles of enzyme-linked immunosorbent assay (1, 2) to the determination of residues of spinosad. An aliquot of the diluted sample is pipeted into a disposable test tube. Enzyme-conjugated spinosad and paramagnetic particles coated with antibodies specific to spinosad are then sequentially added to the tube and the mixture is incubated. During the incubation period, the spinosad and enzyme-conjugated spinosad compete for antibody sites on the magnetic particles. At the end of the incubation period, a magnetic field is applied to the particles. The spinosad and enzyme-conjugated spinosad, which is bound to the antibodies on the particles, are held in the tube by the magnetic field while the unbound reagents are decanted. After decanting, the particles are washed to remove unbound enzyme conjugate. The presence of spinosad is detected by incubating the antibody-bound enzyme conjugate with an enzyme substrate (hydrogen peroxide) and a chromogen (3,3',5,5' -tetramethyl-benzidine; TMB), generating a colored product. After this incubation period, the reaction is stopped and stabilized by the addition of acid.

Since the enzyme-labeled spinosad is in competition with free (sample) spinosad for the antibody sites, the level of color development is inversely proportional to the concentration of spinosad in the sample (i.e., lower residue concentrations result in greater color development). The absorbance at 450 nm is measured in each tube using the RPA-1 RaPID Analyzer. Quantitation of residues of spinosad is accomplished through generation of a calibration curve. The calibration curve is constructed using linear regression after a ln/Logit transformation of the concentration and absorbance values, respectively. The spinosad concentration in unknown samples is calculated from the regression equation using the preprogrammed software capabilities of the RPA-1 RaPID Analyzer. (Note N.1.)

C. Safety Precautions

1. Each analyst must be acquainted with the potential hazards of the reagents and products used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE: MATERIAL SAFETY DATA SHEETS, PRODUCT LITERATURE, AND OTHER RELATED DATA. Safety information on non-DowElanco products should be requested from the supplier. Disposal of reagents must be in compliance with local, state, and federal laws and regulations.

2. Avoid contact of Stopping Solution (0.5% sulfuric acid) with skin and mucous membranes. Wear protective clothing and proper eye protection when working with this material. If this reagent comes in contact with skin, flush the exposed area with water.

3. Volatile and flammable organic solvents such as acetonitrile and methanol must be used in well-ventilated areas away from ignition sources.

D. Equipment (Note N.2.)

1. Autotransformer, variable (for temperature control of heating mantles), Model 3PN1010, Staco Energy Products Co., Dayton, OH 45401.

2. Balance, analytical, Mettler, Model AE50, Fisher Scientific, Pittsburgh, PA 15238.

3. Balance, portable, Ohaus, Model CT600, Fisher Scientific.

4. Evaporator, TurboVap LV Evaporator, Zymark Corporation, Hopkinton, MA 01748.

5. Hammermill, Model 2001, equipped with 3/16 inch screen, Agvise Laboratories, Inc., Northwood, ND 58267.

6. Homogenizer, Polytron, catalog number PT 10 20 3500, with a 10-mm diameter generator with saw teeth, catalog number 27 11 330-3, Brinkman Instruments, Inc., Westbury, NY 11590.

7. Magnetic Separator Rack and Base, 60 position, catalog number A00004, Ohmicron Environmental Diagnostics Corp., Newtown, PA 18940.

8. Mantles, heating (for reflux condensers), catalog number 0-408, Glas-Col Apparatus Co., Terre Haute, IN 47802.

9. Mixer, Vortex-Genie, catalog number 12-812, Fisher Scientific.

10. Photometer, fixed wavelength spectrophotometer RPA-1RaPID Analyzer, catalog number A00003, Ohmicron Environmental Diagnostics Corp.

11. Pipetter, Eppendorf, tri-volume, (100 µL, 200 µL, 250 µL), catalog number 21-278-38, Fisher Scientific.

12. Pipetter, Eppendorf, 200-1000 µL, catalog number 21-381-204, Fisher Scientific.

13. Pipetter, Eppendorf, repeater, catalog number 21-380-8, Fisher Scientific.

14. Shaker, variable-speed reciprocating, with box carrier, Model 6000, Eberbach Corporation, Ann Arbor, MI 48106.

15. Timer, minutes and seconds with alarm, catalog number 14-649-14, Fisher Scientific.

E. Glassware and Materials (Note N.2.)

1. Boiling flasks, 250-mL, catalog number 09-552B, Fisher Scientific.

2. Bottles, glass, 8-oz (237-mL), graduated, with PTFE-lined lids, catalog number 03-320-11G, Fisher Scientific.

3. Combitips for repeater pipet, 12.5-mL, catalog number 21-380-8C, Fisher Scientific.

4. Condensers, reflux, water cooled, 300-mm sleeve length, 5 bulbs, catalog number 07-736B, Fisher Scientific.

5. Culture tubes, disposable glass, 16 x 100 mm, catalog number 14-962-10B, Fisher Scientific.

6. Filter paper, 15-cm, pre-pleated, catalog number 03170, Schleicher and Schuell, Keene, NH 03431.

7. Filtering funnels, glass, 65-mm diameter, catalog number 10-329B, Fisher Scientific.

8. Granules, boiling, Boileezers Alumina Granules, catalog number B365-250, Fisher Scientific.

9. Pipet tips, Eppendorf disposable, 0.1 mL-1.0 mL catalog number 21-372-4, Fisher Scientific.

10. Pipets, 10-mL disposable, catalog number 13-678-31J, Fisher Scientific.

11. Pipets, 5-mL disposable, catalog number 13-678-25D, Fisher Scientific.

12. Pipets, 1-mL disposable, catalog number 13-678-25B, Fisher Scientific.

13. Vials, 40-mL (11-dram) clear glass, catalog number 03-339-5C, Fisher Scientific.

F. Reagents and Prepared Solutions (Note N.2.)

1. Reagents

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

b. Methanol, ChromAR HPLC grade, catalog number 3041-09, Mallinckrodt Specialty Chemicals Company.

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

d. Spinosad RaPID Assay 100 Tube Kit, catalog number A00178, Ohmicron Environmental Diagnostics Corp. (Note N.3.).

Kit contents include (Note N.4.):

(1) Spinosad Antibody, coupled to paramagnetic particles
(2) Enzyme Conjugate
(3) Standards (i.e., calibration standards)
(4) Quality Control Sample
(5) Diluent/Zero Standard
(6) Color Solution
(7) Stopping Solution
(8) Washing Solution
(9) Test Tubes

e. Spinosad Sample Diluent, catalog number A00180, Ohmicron Environmental Diagnostics Corp. (Note N.4.).

f. Standard:

Obtain spinosyn A analytical standard from Test Substance Coordinator, DowElanco, 9330 Zionsville Rd., Building 306/A1, Indianapolis, IN 46268-1053.

g. Water, OmniSolv HPLC grade, catalog number WX001-4, EM Science, Gibbstown, NJ 08027.

2. Prepared Solutions

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

For each liter of solution, mix 800 mL of acetonitrile with 200 mL of HPLC grade water. Allow the solution to equilibrate.

G. Preparation of Fortification Stock Solutions and Recovery Samples (Note N.5.)

1. Preparation of Fortification Stock Solutions

a. Weigh 0.010 g of spinosyn A and quantitatively transfer to a 100-mL volumetric flask. Dilute to volume with methanol to obtain a 100-µg/mL stock solution. Store in the dark when not in use.

b. Pipet 10.0 mL of the 100-µg/mL spinosyn A solution from Section G.1.a. to a 100-mL volumetric flask and dilute to volume with methanol to obtain a 10-µg/mL stock solution. Store in the dark when not in use.

c. Pipet 2.0 mL of the 100-µg/mL spinosyn A solution from Section G.1.a. to a 100-mL volumetric flask and dilute to volume with methanol to obtain a 2.0-µg/mL stock solution. Store in the dark when not in use.

d. Pipet 10.0 mL of the 2.0-µg/mL spinosyn A solution from Section G.1.c. to a 100-mL volumetric flask and dilute to volume with methanol to obtain a 0.200-µg/mL stock solution. Store in the dark when not in use.

e. Pipet 3.0 mL of the 2.0-µg/mL spinosyn A solution from Section G.1.c. to a 100-mL volumetric flask and dilute to volume with methanol to obtain a 0.060-µg/mL stock solution. Store in the dark when not in use.

2. Preparation of Fortification Solutions

a. Use the stock solutions from G.1.b. and d. for fortification of bovine tissue samples in Section I.1.a. as follows:

Stock Solution Concentration µg/mL	Stock Volume mL	Sample Weight g	Final Concentration µg/g
10.0	1.0	20	0.500
0.200	1.0	20	0.010

b. Use the stock solutions from G.1.b., c., d., and e. for fortification of milk samples in Section I.1.b. as follows, thoroughly mixing the samples after fortification:

Stock Solution Concentration µg/mL	Stock Volume mL	Final Sample Volume mL	Final Concentration µg/g
10.0	1.0	20	0.500
2.0	1.0	20	0.100
0.200	1.0	20	0.010
0.060	1.0	20	0.003

H. Instrument Settings

To obtain results from the Spinosad RaPID Assay, use the following parameter settings on the RPA-1 RaPID Analyzer:

Parameter	RPA-1 Abbreviation	Setting
Protocol Name	Protocol Name	Spinosad
Data Reduction	Data Reduct	Linear Regression
Transformation	Xformation	Ln/LgtB
Number of Calibrators	# of Calibrator	4
Number of Calibrator Replicates	# of Reps:	2
Calibrator #1 Concentration	Cal #1 Conc	0.00
Calibrator #2 Concentration	Cal #2 Conc	50.0
Calibrator #3 Concentration	Cal #3 Conc	250
Calibrator #4 Concentration	Cal #4 Conc	1000
Minimum Correlation	Correlation Flag	0.990
Maximum Concentration (pg/mL)	Normal Range Hi	1200
Minimum Concentration (pg/mL)	Normal Range Low	20
Number of Controls	# of Controls	1
Number of Control Replicates	Ctrl Replicates	2
Number of Reagent Blanks	# Rgt Blk	0
Wavelength	Wavelength	450 nm
Read Mode	Read Mode	Absorbance
Units	Units	pg/mL
Precision of Calibrators	Rep %CV Flag	100

I. Determination of Spinosad in Bovine Tissues and Milk Recovery Samples

1. Preparation of Recovery Samples

a. Bovine tissues:

(1) Prepare the bovine tissue (kidney, lean, or liver) for analysis by chopping with a cleaver. Then freeze with liquid nitrogen and grind 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. Samples should be stored frozen.

(2) Thaw the sample, if necessary. Weigh 20-g samples 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. Some of the tissue will stick to the funnel. Remove the funnel and observe the weight of the flask and tissue. If necessary, add more tissue using the same technique.

(3) For recovery samples, add the appropriate volume of the fortification solution as described above in Section G.2.a.

(4) Add 50 mL of 80% acetonitrile/20% water and blend the sample on high speed for 1-2 minutes using a Polytron homogenizer with a 10-mm diameter generator and sawtooth blades. Add 50 mL of 80% acetonitrile/20% water to a 100-mL graduated cylinder and rinse the blades by blending on high speed for 5-10 seconds. Combine the rinse with the blended tissue 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 flask and place on a heating mantle. Attach the flask to a chilled-water reflux condenser and heat the samples to reflux for approximately 1 hour. A setting of 40 ±5% of full scale on the variable autotransformer has produced sufficient heat to bring the solution to boil within 15 minutes. Adjust the setting as needed. (Note N.6.)

(6) After heating for 1 hour, turn the mantle off, but allow the chilled water to continue cooling the condenser. Carefully raise the hot flask and condenser, and place a spatula or other suitable tool under the flask so that it will be propped up above the heating mantle to facilitate cooling of the flask. Allow the flask to cool until it can be safely handled.

(7) Remove the flask from the condenser and cap the flask to prevent solvent loss. Transfer the warm flask to a hood until the sample has cooled to approximately room temperature.

(8) Filter the supernatant extract through pleated filter paper into an 8-oz graduated glass bottle.

(9) Transfer 1.0 mL of the extract into a 40-mL (11-dram) clear glass sample vial.

(10) Evaporate to dryness using a TurboVap evaporator (45 °C, 8 psi N_2).

(11) Add 10.0 mL of Spinosad Sample Diluent to each vial, cap the vial with the PTFE-lined closure, and vortex for 20-30 seconds.

(12) Dilute fortified sample extracts containing greater than 0.050 µg/g (i.e., a 1:10 dilution by adding 0.5 mL of diluted sample extract to 4.5 mL of sample diluent into a new vial).

(13) Assay each sample following the procedure in Section I.2. If a sample contains more than 0.050 µg/g of spinosad, perform an additional 1:10 dilution of the sample from I.1.a (11) and reassay.

b. Milk:

(1) Thaw the milk and shake thoroughly to yield a homogenous sample. Transfer 5.0 mL into an 11-dram clear glass sample vial.

(2) For recovery samples, add the appropriate volume of the fortification solution as described above in Section G.2.b.

(3) Add 20 mL of acetonitrile to each sample.

(4) Cap each vial with a PTFE-lined closure and shake the sample for a minimum of 30 minutes on a reciprocating shaker at approximately 180 excursions/minute.

(5) Transfer 1.0 mL of the acetonitrile extract from Step I.1.b. (4) to an 11-dram glass sample vial. The protein portion of the milk will be coagulated in the acetonitrile.

(6) Evaporate to dryness using a TurboVap evaporator (45 °C, 8 psi N_2).

(7) Add 10.0 mL of Spinosad Sample Diluent to each vial, cap the vial with the PTFE-lined closure, and vortex for 20-30 seconds.

(8) Dilute fortified sample extracts containing greater than 0.050 µg/g (i.e., a 1:10 dilution by adding 0.5 mL of diluted sample extract to 4.5 mL of sample diluent into a second vial).

(9) Assay each sample following the procedure in Section I.2. If a sample contains more than 0.050 µg/mL of spinosad, perform an additional 1:10 dilution of the sample from I.1.b. (7) and reassay.

2. Assay Procedure

Conduct each test in an individual test tube. Duplicate tests of a sample or standard constitute a single analysis. A standard curve and the appropriate control and recovery samples must be included in each analytical batch. For further details, consult the Spinosad RaPID Assay Kit Insert (Appendix A).

Remove all kit reagents from refrigerated storage and allow them to equilibrate to room temperature. A minimum of 30 minutes is recommended for warming.

a. Turn on the RPA-I Photoanalyzer at least 30 minutes prior to measuring absorbance in the completed assay. See Section I.3. for operation instructions.

b. Label test tubes for standards, controls, and samples. Place the tubes in the proper rack position. Be sure that the rack is removed from the Magnetic Separator.

c. **Critical step: Accurate delivery of the correct sample volume and proper pipeting technique in this step are critical to obtaining accurate and precise data.** Add 0.20 mL of the standard, quality control, or sample to each test tube using an Eppendorf pipetter. Pipet each sample or standard directly to the bottom of the tube; avoid liquid adhering to the sides of the test tube. Use a fresh pipet tip for each standard and sample.

d. Using an Eppendorf repeater pipet equipped with a 12.5-mL Combitip, add 0.25 mL (Dial Setting = 1) of Enzyme Conjugate down the inside wall of each tube.

e. Thoroughly mix the Antibody Coupled Paramagnetic Particles by swirling the bottle. Avoid vigorous shaking and foaming.

f. Using a repeater pipet equipped with a 12.5-mL Combitip, add 0.50 mL (Dial Setting = 2) of the Antibody Coupled Paramagnetic Particles down the inside wall of each tube.

g. When dispensing of the magnetic particles is completed, mix the samples by either gently vortexing (Vortex setting = 3-4) each tube for 1-2 seconds or by gently swirling the entire rack for a few seconds.

h. Incubate for 30 minutes at room temperature.

i. After the incubation period, combine the rack and the magnetic base. Seat all tubes by pressing them into the base. Allow 2 minutes for the particles to separate.

j. Do not separate the tube rack from the magnetic base. Using a smooth motion, invert the combined rack assembly over a collection container and pour out the tube contents. Keep the rack inverted and gently blot the test tube rims on several layers of paper towels.

k. Using a repeater pipet equipped with a 12.5-mL Combitip, add 1 mL (Dial Setting = 4) of Washing Solution to each tube. Wait 2 minutes. Decant the Washing Solution into an appropriate collection container using the technique described in Step L2.j.

l. Repeat Step L2.k.

m. Remove the tube rack from the magnetic separator and then add 0.50 mL of Color Solution using a repeater pipet equipped with a 12.5-mL Combitip (Dial Setting = 2).

n. Gently vortex each tube for 1 to 2 seconds.

o. Incubate for 20 minutes at room temperature. During this incubation, dispense approximately 1 mL of Washing Solution into a clean tube for use as an instrument blank.

p. At the end of the incubation period, add 0.5 mL of Stopping Solution to each tube using a repeater pipet equipped with a 12.5-mL Combitip (Dial Setting = 2).

q. Analyze each tube using the RPA-1 RaPID Analyzer within 10 minutes after adding the Stopping Solution.

3. Operating Procedure for the RPA-1 RaPID Analyzer

The RPA-1 RaPID Photometric Analyzer is pre-programmed with the protocols for several RaPID Assay procedures. The following steps describe how to set up and run the analyzer to measure absorbance in the tubes for the Spinosad RaPID Assay.

a. Switch on the instrument and allow it to warm up at least 30 minutes prior to use. The RPA-1 RaPID Analyzer will perform a self test. If all parameters are satisfactory, the "SELECT COMMAND" prompt will appear.

b. At the "SELECT COMMAND" prompt, press "RUN".

c. At the "RUN PROTOCOL" prompt, scroll through the protocols using the arrow keys until "SPINOSAD" appears. Press "ENTER".

d. At the "SPL. REPLICATES" (sample replicates) prompt, press "2" to indicate the number of replicates for each sample, then press "ENTER".

e. At the "BLANK TUBE/INSERT TUBE" prompt, insert the tube with 1 mL of Washing Solution. The display will briefly read "EVALUATING TUBE" then

"REMOVE TUBE" and the instrument will produce an audible beep indicating that the absorbance has been measured. After hearing the beep, remove the tube.

f. At the "CAL. #1 REP. #1/INSERT TUBE" prompt, insert the first replicate of the first standard/calibrator (0.00 ng/mL). Remove the tube after the beep.

g. Follow the prompts on the instrument display until all of the standards have been measured. The tube order is important. The RPA-1 RaPID Analyzer has been programmed to evaluate the standards in ascending order, in duplicate, starting with 0.00 ng/mL.

h. After all the standards have been evaluated, the instrument will report the equation of the line, the transformed data and the standards data.

i. Insert the control tubes at the "CNTRL. #1 REP. #1" and "CNTRL. #1 REP. #2" prompts. The instrument will report the calculated values for each replicate of the control sample.

j. Evaluate the results for the standard curve and the control samples. At the "EDIT CALIBRATORS YES/NO" prompt, press "NO".

k. At the "SPL. #1 REP #1/INSERT TUBE" prompt, insert the first sample tube. Remove the tube after the beep.

l. Continue sample analysis following the prompts on the instrument display. Press "STOP" after all the samples have been evaluated and the results have been reported by the RPA-1 RaPID Analyzer.

4. Confirmation of Residue Identity

If confirmation of the residue identity is required, samples can be reanalyzed using method GRM 95.03, "Determination of Spinosad and Metabolites in Beef Tissues, Milk and Cream by High Performance Liquid Chromatography with Ultraviolet Detection" (3).

J. Determination of Spinosad in Bovine Tissues and Whole Milk

1. Prepare treated samples, a system (reagent) blank, fortified recovery samples, and an untreated control as described in Section L1. Record the weight of the treated tissue samples and the unfortified controls. 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. Use the RPA-1 RaPID Analyzer to calculate the uncorrected residue result from the calculated standard curve as discussed below in Section K.

3. If desired, correct the residue result for the analytical batch recovery as follows:

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

where: R_a = the average % recovery from fortified samples.

K. Calculations

1. Standard Curve

The RPA-1 RaPID Analyzer contains preprogrammed data reduction capabilities which calculate a standard curve for each analytical batch using the absorbances of the batch standards. The calibration curve is constructed by linear regression after performing a ln/Logit data transformation of the concentration and absorbance values, respectively (Figure 2).

The regression equation is :

$$\text{Logit}\,\frac{B}{B_0} = [\text{slope} \times \ln(\text{Conc})] + \text{Y intercept}$$

Where:

$$\text{Logit}\,\frac{B}{B_0} = \ln\frac{B/B_0}{1 - B/B_0}$$

B = the absorbance measured at a specific spinosad concentration
B_0 = the absorbance measured for the 0.00 ng/mL standard
Conc = the spinosad concentration of the standard

2. Calculation of Spinosad in Samples

The RPA-1 RaPID Analyzer will calculate the concentration of spinosad in each unknown sample using the preprogrammed data reduction parameters (Note N.7.). It will report the absorbance value and calculated spinosad concentration for each replicate of each sample tube as well as the mean absorbance, the mean spinosad concentration and the percent coefficient of variation (%CV) of the duplicate measurements for each sample. The mean values are the final result for each sample.

To calculate the concentration of spinosad in a sample, use the following equation:

Concentration = e^a

Where:

$$a = \left(\frac{\text{Logit}\,\frac{B}{B_0} - \text{Y intercept}}{\text{slope}} \right)$$

Example:

Milk sample fortified at 500 ng/mL, rack positions 31 and 32, from analytical set 080395CDLY (Figure 3):

Mean absorbance (450 nm) = 0.373

$$\frac{B}{B_0} = \frac{0.373}{1.194}$$

$$= 0.312$$

Therefore:

$$\text{Logit } \frac{B}{B_0} = \ln \frac{0.312}{1-0.312}$$

$$= \ln (0.453)$$

$$= -0.791$$

To calculate sample concentration:

$$\text{Concentration} = e^{\left(\frac{-0.791 - 4.576}{-0.808}\right)}$$

= 766.9 pg/mL (in diluted extract)
= 0.767 ng/mL (in diluted extract)

3. Calculation of the Method Factor (MF) of Bovine Tissue Samples

$$MF = \frac{\text{Volume of Extraction Solvent (mL)}}{\text{Weight of Sample (g)}} \times \text{Final Dilution Factor}$$

Therefore:

$$MF = \frac{100 \text{ mL}}{20 \text{ g}} \times 10$$

$$= 50$$

4. Calculation of the Method Factor (MF) of Milk Samples

$$MF = \frac{\text{Extraction Solvent Volume + Sample Volume (mL)}}{\text{Sample Volume (mL)}} \times \text{Final Dilution Factor}$$

Therefore:

$$MF = \frac{20 \text{ mL} + 5 \text{ mL}}{5 \text{ mL}} \times 100$$

$$= 500$$

5. Calculation of the Final Concentration in Diluted Samples

Final Concentration = Measured Concentration x Method Factor

From K.2. and K.4.:

Final Concentration (ng/mL) = 0.767 ng/mL x 500
Final Concentration = 383.5 ng/mL

6. Calculation of Percent Recovery

The percent recovery for the quality control sample or fortified samples is calculated as follows (Note M.8.):

$$\% \text{ Recovery} = \frac{\text{Calculated Concentration - Blank Concentration}}{\text{Fortified Concentration}} \times 100$$

From K.5.:

$$\% \text{ Recovery} = \frac{383.5 \text{ ng/mL} - 0 \text{ ng/mL}}{500 \text{ ng/mL}} \times 100$$

$$= 76.7\%$$

L. Quality Control

1. Analytical Batch Definition

An analytical batch of samples is defined as a group of 60 tubes. The size of the batch is based on the capacity of the magnetic separator rack. An analytical batch of less than 60 tubes can be analyzed. The first 10 tubes (rack positions 1-10) are used for duplicate analysis of the four standards and a quality control solution. Following the quality control solution, up to 25 samples (recovery or study samples) may be analyzed in duplicate (2 tubes). If more samples are to be analyzed than can be accommodated in one rack, the remaining samples should be analyzed as a distinct analytical batch with a new standard curve, control and recovery samples.

2. Quality Control Solution

A quality control solution containing 0.50 ng/mL of spinosyn A (supplied with the kit; Section F.1.d. (4)) should be analyzed at the beginning of every batch of samples (rack positions 9 and 10). The quality control solution should be assayed in the same manner as all other samples. Use the results obtained from analysis of the quality control solution to determine whether the assay was properly executed (see Section L.4.). Additional matrix fortified recovery samples should be analyzed to further ensure proper execution of the method.

3. Study Samples

All study samples should be analyzed in duplicate. If the concentration of spinosad exceeds the range of the assay, prepare a 10-fold dilution of the sample (e.g., 0.5 mL of sample + 4.5 mL of Spinosad Sample Diluent), and then assay the diluted sample aliquot. Multiply the result by the appropriate method factor to obtain the final result.

4. Criteria for Acceptance of an Analytical Batch

The correlation coefficient for the linear regression of the calibration curve should be greater than 0.990. The %CV should be less than 10% for each duplicate pair of standards. The %CV should be less than 20% for the quality control sample, and the recovery value should be within ±20% of the expected value (i.e., 0.4-0.6 ng/mL). If the data fail to meet these performance criteria, the analyst should evaluate the results, determine the potential source of the variation, and repeat the analysis if necessary. Calibration and quality control data generated during validation of this method are graphed and summarized in Figure 2.

5. Specificity/Sensitivity

Several analogs, metabolites and degradates of spinosad have been tested to determine whether the Spinosad RaPID Assay test kit will detect their presence in a water sample (4). In general, the assay is sensitive (I_{50} < 2 ng/mL) to factors which have little or no modification to the trimethylpyranosyl ring. The assay is relatively insensitive (I_{50} > 50 ng/mL) to factors or degradates in which the trimethylpyranosyl ring portion of the molecule has been modified or is missing (Figure 1). The level of sensitivity is not equivalent for all factors. Thus, when a mixture of spinosyns and degradates are present in a sample, the assay response generated by the individual spinosyns is not additive.

6. Interferences

Thirty pesticides, sixteen inorganic compounds and eight additional organic compounds were tested for the potential to interfere with conjugate binding in the assay (4) (Table I). The only compound which exhibited an I_{50} concentration below 10 mg/mL was carbendazim (I_{50} = 5.56 mg/mL). The I_{50} concentration is the concentration which results in a 50% inhibition of conjugate binding to the available antibodies. In comparison, the I_{50} for Spinosyn A is approximately 0.0003 mg/mL.

7. Modifications and Potential Problems

No modifications to the assay procedure are recommended. This procedure is for use on bovine tissue and milk samples generated during a dairy feeding study. Validation of the method for analysis of other sample matrices would be required prior to implementing this method for sample analysis.

M. 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 spinosad in bovine tissues (kidney, liver, and muscle) and milk. The results are summarized by sample type and fortification level in Tables II-V. Average recoveries of spinosad for all matrices over all fortification levels ranged from 77 to 84% and the corresponding relative standard deviations ranged from 7 to 11% (Table VI).

b. Standard Curve Linearity

The correlation coefficient (r^2) values for the linear regression equations describing the absorbance as a function of standard concentration ranged from 0.9983 to 1.0000 for 20 analytical batches analyzed during the method validation.

c. Limits of Detection and Quantitation

Following a published technique (5), the LODs and LOQs were calculated using the standard deviation from the results of the fortified recovery samples at the targeted LOQ, which was 0.010 µg/g. The LOD was calculated as three times the standard deviation ($3s$), and the LOQ was calculated as ten times the standard deviation ($10s$). The calculated LOD ranged from 0.0018 to 0.0024 µg/g, and the calculated LOQ ranged from 0.006 to 0.008 µg/g over all matrices (Table VII). Recovery samples fortified at 0.003 µg/g were detectable in all of the sample types, thereby verifying the targeted LOD of 0.003 µg/g. Although the method has an LOD of 0.003 µg/g, numerical values should be reported as less than the LOQ (< 0.010 µg/g) for residues that are above the LOD but below the validated LOQ, because these residue levels cannot be reliably quantitated.

2. False Positive/False Negative Rate

Unfortified control samples (matrix blanks) and samples fortified at the LOD (0.003 µg/g) were analyzed during the study to determine the false positive and false negative rate. A false positive result occurs when residue at or above the established LOD is found in a control sample known to be free of analyte (6). A false negative occurs when no residue is detected in a sample fortified at the LOD. There were no false positives from the unfortified control samples and no false negatives reported from the LOD fortified samples analyzed during this study.

3. Ruggedness

The average accuracy and precision values presented from the data in Tables III-VI are within the acceptable range of 70-120% for all matrices at all fortification levels. These estimates include data generated by multiple analysts on multiple days (7).

4. Confirmatory Method

The detection and/or quantitation of spinosyns A, B, D, and *N*-demethyl spinosyn D in bovine tissues and milk can be confirmed by HPLC (3).

A direct correlation between this method and the existing HPLC methodology has been performed (8). Data from a dairy feeding study indicate that both methods yield comparable results of total spinosad residues. Statistical calculations from analysis of milk, kidney, and lean bovine tissue samples resulted in a correlation coefficient of approximately 0.95.

5. Assay Time

The time required to analyze a typical analytical batch (25 samples or recoveries, four standards and the quality control sample in duplicate) in bovine tissues or milk is from 5 to 7 hours.

N. Notes

1. The method validation was performed using spinosyn A for the fortification of the recovery samples. Spinosyn A is the major component of spinosad, comprising approximately 76% of the technical grade spinosad. It is available as an analytical standard with consistently high purity and was therefore deemed the best choice for fortification of the recovery samples. Additional recovery samples fortified with technical grade spinosad were also assayed. Recoveries found in fortified milk samples ranged from 66 to 81% and from 68 to 73% in bovine tissues. Technical grade spinosad more closely represents the test substance administered in the feeding study; however, it exhibits lot-to-lot variability in its percentage of the individual spinosyns. This can effect the recovery calculations when analyzing total spinosad residue.

2. Equipment, glassware, materials, reagents, and chemicals 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.

3. Only the Ohmicron Spinosad RaPID Assay test kit may be used for performing this method. Other test kits should not be substituted.

4. Refrigerate all kit reagents and sample diluent at 2-8 °C. Do not freeze. Reagents may be used until the expiration date printed on the labels. The test tubes require no special storage conditions and may be stored separately from the kit reagents.

5. Standard solutions can be prepared 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. Some spinosyns have been found to gradually adsorb onto amber glassware. Only clear glass volumetric flasks should be used to prepare fortification stock solutions.

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

7. The following information may appear as part of the raw data report:

   "x.xx nd" (i.e., 0.04nd) indicates that the calculated concentration is below the LOD. The result should be reported "not detected" and the LOD should be noted.

   "nd " indicates the absorbance measured is greater than or equal to the absorbance of the 0.00 ng/mL standard; therefore, a concentration cannot be calculated. The result should be reported as "not detected" and the LOD should be noted.

   "x.xx HI " (i.e., 1.31 HI) indicates that the calculated value exceeded the upper range of the assay. The sample should be diluted (e.g., 1:10) with Spinosad Sample Diluent and then re-analyzed.

8. Measured concentrations in blank samples which are between the LOD and LOQ may be subtracted from the sample results. The analyst should recognize, however, that a concentration measured between the LOD and LOQ may be inaccurate.

O. References

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

Pesticides	Organic compounds	Inorganic compounds
Alachlor	N-Acetylglucosamine	Calcium (chloride dihydrate)
Aldicarb	Aflatoxin B1	Copper (chloride)
Azinphos methyl	Aflatoxin G1	Iron (chloride hexahydrate)
Carbaryl	Humic acid	Magnesium (chloride hexahydrate)
Carbendazim	β-Lactose	Manganese (chloride)
Carbofuran	Methyl oleate	Mercuric (chloride)
Chlorothalonil	Polyoxin D	Nickel (sulfate hexahydrate)
Chlorpyrifos	L(+) Rhamnose	Nitrate (sodium)
Chlorpyrifos methyl		Peroxide (hydrogen)
Cyanazine		Phosphate (sodium, heptahydrate)
2,4-D		Silicates (sodium meta-)
Dicamba		Sodium chloride
Dinoseb		Sulfate (sodium)
EPN		Sulfite (sodium)
Iprodine		Thiosulfate (sodium, pentahydrate)
Malathion		Zinc (chloride)
Metalaxyl		
Methamidophos		
Methiocarb		
Methomyl		
Metribuzin		
Parathion		
Parathion methyl		
Phosmet		
Picloram		
Procymidone		
Propachlor		
Thiabendazole		
Triclopyr		
Vinclozolin		

Table II. Summary of Net Recovery Data[a] for Spinosad in Bovine Kidney

Spinosyn A Added, µg/g	n	Net Percent Recovery			
		Range	$\bar{X}$	s	RSD
0.0	7	ND[b]	ND	--	--
0.003	3	NA[c]	NA	--	--
0.010	10	68-84	78	5.7	7.3
0.500	3	78-83	81	2.6	3.2

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

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

[c] NA = Not applicable. Analyte was detected, but it was below the 0.010-µg/g LOQ.

Table III. Summary of Net Recovery Data[a] for Spinosad in Bovine Liver

Spinosyn A Added, µg/g	n	Net Percent Recovery			
		Range	$\bar{Y}$	s	RSD
0.0	10	ND[b]	ND	--	--
0.003	2	NA[c]	NA	--	--
0.010	10	64-86	76	7.1	9.3
0.500	4	79-85	82	2.6	3.2

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

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

[c] NA = Not applicable. Analyte was detected, but it was below the 0.010-µg/g LOQ.

Table IV. Summary of Net Recovery Data[a] for Spinosad in Bovine Muscle

Spinosyn A Added, µg/g	n	Net Percent Recovery			
		Range	$\bar{X}$	s	RSD
0.0	11	ND[b]	ND	--	--
0.003	3	NA[c]	NA	--	--
0.010	13	69-86	78	6.1	7.8
0.100	3	68-73	71	2.5	3.5
0.500	6	75-79	77	1.5	1.9

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

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

[c] NA = Not applicable. Analyte was detected, but it was below the 0.010-µg/g LOQ.

Table V. Summary of Net Recovery Data[a] for Spinosad in Bovine Whole Milk

Spinosyn A Added, µg/mL	n	Net Percent Recovery			
		Range	$\bar{x}$	s	RSD
0.0	15	ND[b]	ND	--	--
0.003	5	NA[c]	NA	--	--
0.010	20	74-100	89	7.9	8.9
0.100	3	67-77	73	4.5	6.2
0.100 (TGAI)[d]	6	66-81	71	5.4	7.6
0.500	9	74-81	77	2.1	2.7

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

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

[c] NA = Not applicable. Analyte was detected, but it was below the 0.010-µg/mL LOQ.

[d] TGAI = Technical grade of active ingredient. Uncorrected for purity of 88% Spinosyn A + D.

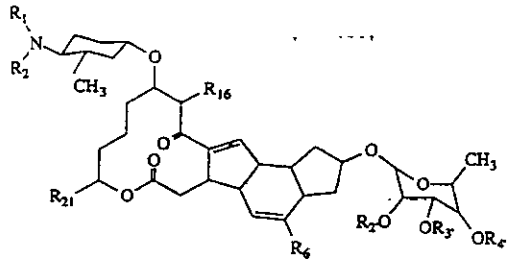
Table VI. Statistical Summary of Overall Recovery Data for Spinosyn A in Bovine Kidney, Liver, Muscle, and Whole Milk

Matrix	Added, µg/mL or µg/g	n	Percent Recovery			
			Range	$\bar{x}$	s	RSD
Kidney	0.010-0.500	14	68-84	78	6.2	7.9
Liver	0.010-5 00	19	64-86	77	7.5	9.7
Muscle	0.010-0.500	22	68-86	77	5.4	7.0
Milk	0.010-0.500	32	67-100	84	9.2	11.0

Table VII. Calculated Limits of Detection and Quantitation (µg/g) for Spinosad in Bovine Kidney, Liver, Muscle, and Whole Milk

Matrix	n[a]	$\bar{x}$[b]	s[c]	LOD[d]	LOQ[e]
Kidney	10	0.0078	0.0006	0.0018	0.006
Liver	10	0.0076	0.0007	0.0021	0.007
Muscle	13	0.0078	0 0006	0.0018	0.006
Milk	20	0.0089	0.0008	0.0024	0.008

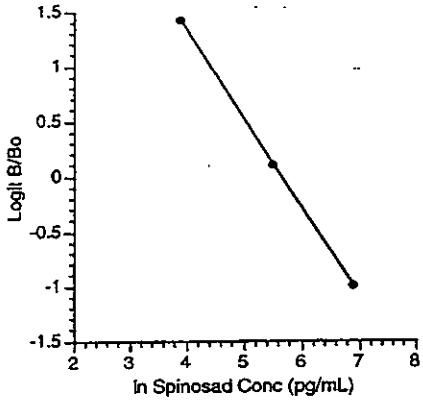
[a] Number of samples fortified with spinosyn A at 0.010 µg/g or 0.010 µg/mL.
[b] Mean values of the net results for the 0.010-µg/g or 0.010- µg/mL recovery samples.
[c] Standard deviation of the net results for the 0.010-µg/g or 0.010-µg/mL recovery samples.
[d] Calculated LOD (3s).
[e] Calculated LOQ (10s).



Spinosyn	R_1	R_2	R_6	R_{16}	R_{21}	$R_{2'}$	$R_{3'}$	$R_{4'}$
A	Me	Me	H	Me	Et	Me	Me	Me
B	H	Me	H	Me	Et	Me	Me	Me
D	Me	Me	Me	Me	Et	Me	Me	Me
N-Demethyl D	H	Me	Me	Me	Et	Me	Me	Me

Me = Methyl
Et = Ethyl
H = Hydrogen

Figure 1. Structures of Selected Spinosyns



Concentration, pg/mL	ln (Conc)	Absorbance, 450 nm	B/B_0	Logit (B/B_0)
0.00	NA[a]	1.194	NA	NA
50	3.91	0.962	0.8057	1.422
250	5.52	0.629	0.5268	0.107
1000	6.91	0.322	0.2697	-0.996

[a] NA = Not Applicable.

Slope = -0.808
Y-intercept = 4.576
Correlation coefficient (r^2) = 1.0000
Quality Control Solution = 0.573 ng/mL

Figure 2. Standard Curve for Analytical Set 080395CDLY

Effective Date: May 22, 1996 GRM 95.14

DowElanco Protocol Number		RES95144	Matrix	MILK
Analyst		DEBRA YOUNG	Analyte	XDE-105
Analysis	Date	Time	Method	105
Start	8/3/95	4:40 PM		
End	8/3/95	4:43 PM		

Batch information:

Batch ID #	080395CDLY
Data Filename	080395C.DLY

	Conc	Lot number	Exp. date
Kit		950638	09/95
Zero	0	950634	09/95
Standard 1	50	950630	09/95
Standard 2	250	950631	09/95
Standard 3	1000	950632	09/95
QC sample	500	950633	09/95

Instrument Settings:

Instrument protocol	105PG/ML
Data Reduction	Lin.Regression
Transformation	Ln/Lgt8
Read Mode	Absorbance
Wavelength	450 nm
Units	PG/ML
Quantitative range (pg/mL)	50 - 1000

Calibrator Data:

Concentration (pg/mL)		Absorbance	Difference	Observed concentration	% difference	Absorbance % CV
	0	1.164				
	0	1.224				
Mean	0	1.194				3.5
	50	0.994	-10.107	39.89	-25.30	
	50	0.930	10.707	60.71	17.60	
Mean	50	0.962	-0.250	49.75	-0.50	4.6
	250	0.623	9.444	259.44	3.60	
	250	0.636	-3.835	246.17	-1.60	
Mean	250	0.629	2.717	252.72	1.10	1.4
	1000	0.320	3.138	1003.10	0.30	
	1000	0.323	-14.505	985.50	-1.50	
Mean	1000	0.322	-5.733	994.27	-0.60	0.7

Transformed data:

Concentration	Transformed concentration	Transformed absorbance
50	3.91	1.421
250	5.52	0.108
1000	6.91	-0.998

Equation of line:

Slope	-0.808
Intercept	4.576
Correlation (r)	1.0000

Quality control samples:

Rack position	Absorbance	Concentration	Mean Absorbance	Mean Concentration	% Recovery	%CV
9	0.438	568.62				
10	0.434	577.25	0.436	572.93	115	1.1

Comments:
SECOND ANALYSIS. ANALYST 1. TGAI FORTIFIED SAMPLE REASSAYED.

Figure 3. Analytical Batch 080395CDLY

Effective Date: May 22, 1996

GRM 95.14

DowElanco Protocol# RES95144 Matrix MILK
Analyst DEBRA YOUNG Analyte XDE-105
Batch ID # 080395CDLY
Data Filename 080395C.DLY

Analysis Date 8/3/95

Sample ID	Rack position	Rep #	Absorbance	Conc. (pg/mL)	Mean absorbance	Mean conc. (pg/mL)	Conc. %CV	Corr Fact	Final conc. (pg/mL)
0-1	11	1	1.304	nd					
	12	2	1.251	nd					
		mean						50.0	
0-2	13	1	1.325	nd					
	14	2	1.300	nd					
		mean						50.0	
0-3	15	1	1.330	nd					
	16	2	1.289	nd					
		mean						50.0	
0-4	17	1	1.275	nd					
	18	2	1.352	nd					
		mean						50.0	
0-5	19	1	1.308	nd					
	20	2	1.305	nd					
		mean						50.0	
10-1	21	1	0.734	161.97					
	22	2	0.743	156.09					
		mean			0.738	159.03	2.6	50.0	7951.5
10-2	23	1	0.717	174.92					
	24	2	0.734	161.97					
		mean			0.725	168.44	5.4	50.0	8422.0
10-3	25	1	0.736	160.78					
	26	2	0.710	180.07					
		mean			0.723	170.42	8.0	50.0	8521.0
10-4	27	1	0.764	142.13					
	28	2	0.695	192.13					
		mean			0.729	167.13	21.2	50.0	8356.5
10-5	29	1	0.759	144.84					
	30	2	0.752	149.82					
		mean			0.756	147.33	2.4	50.0	7366.5
500-1	31	1	0.375	761.54					
	32	2	0.371	774.02					
		mean			0.373	767.78	1.2	500.0383890.0	
500-2	33	1	0.366	793.25					
	34	2	0.380	743.30					
		mean			0.373	768.27	4.6	500.0384135.0	
500-3	35	1	0.381	737.35					
	36	2	0.347	873.08					
		mean			0.364	805.22	11.9	500.0402610.0	

Figure 3. (Con't.) Analytical Batch 080395CDLY

Effective Date: May 22, 1996 GRM 95.14

DowElanco Protocol#			RES95144		Matrix	MILK			
Analyst			DEBRA YOUNG		Analyte	XDE-105			
Batch ID #			080395CDLY						
Data Filename			080395C DLY						
Analysis Date			8/3/95						
Sample ID	Rack position	Rep #	Absorbance	Conc. (pg/mL)	Mean absorbance	Mean conc. (pg/mL)	Conc. %CV	Corr Fact	Final conc. (pg/mL)
100TGAI-1	37	1	0 791	125 69					
	38	2	0.735	161.38					
		mean			0.763	143.53	17.6	500.0	71765.0
100TGAI-2	39	1	0 771	137.35					
	40	2	0.762	143.21					
		mean			0.767	140.28	3.0	500.0	70140.0
100TGAI-3	41	1	0.791	125.20					
	42	2	0.765	141.59					
		mean			0.778	133.40	8.7	500.0	66700.0
0	43	1	Empty	Empty					
	44	2	Empty	Empty					
		mean			Empty	Empty	Empty	1.0	Empty
0	45	1	Empty	Empty					
	46	2	Empty	Empty					
		mean			Empty	Empty	Empty	1.0	Empty
0	47	1	Empty	Empty					
	48	2	Empty	Empty					
		mean			Empty	Empty	Empty	1.0	Empty
0	49	1	Empty	Empty					
	50	2	Empty	Empty					
		mean			Empty	Empty	Empty	1.0	Empty
0	51	1	Empty	Empty					
	52	2	Empty	Empty					
		mean			Empty	Empty	Empty	1.0	Empty
0	53	1	Empty	Empty					
	54	2	Empty	Empty					
		mean			Empty	Empty	Empty	1.0	Empty
0	55	1	Empty	Empty					
	56	2	Empty	Empty					
		mean			Empty	Empty	Empty	1.0	Empty
0	57	1	Empty	Empty					
	58	2	Empty	Empty					
		mean			Empty	Empty	Empty	1.0	Empty
0	59	1	Empty	Empty					
	60	2	Empty	Empty					
		mean			Empty	Empty	Empty	1.0	Empty

Figure 3. (Con't.) Analytical Batch 080395CDLY