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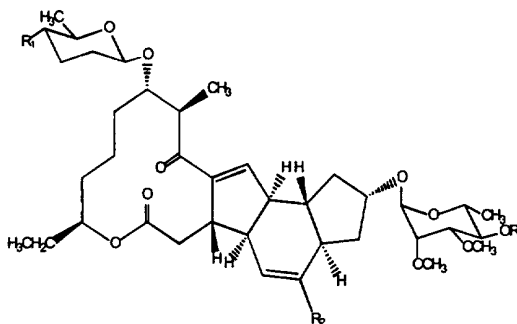
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Determination of Residues of Spinosad in Acidic Agricultural Crops by High Performance Liquid Chromatography with + Ion APCI Mass Spectrometry Detection

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1. SCOPE

This method is applicable to the quantitative determination of residues of spinosad (spinosyns A and D) and its metabolites (spinosyn K, spinosyn B and *N*-demethyl spinosyn D) in acidic agricultural crops over the concentration range 0.01 - 1.0 mg/kg. The method has been validated by the analysis of untreated and fortified samples for residues of spinosyns A, D, B, K and *N*-demethyl spinosyn D.



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

The chemical names and CAS numbers for these five compounds are presented in Table 1.

2. PRINCIPLE

Residues of spinosyns A, D, K, B and N-demethyl spinosyn D are extracted from the crop matrix by homogenising and shaking with a solution of acetonitrile/water. An aliquot of the extract is then diluted with acetonitrile and applied to a Strong Cation Exchange (SCX) solid phase extraction (SPE) cartridge. The spinosyns are eluted with a methanol:acetonitrile solution containing 0.1 M ammonium acetate. The eluate is evaporated to dryness and reconstituted in mobile phase.

Quantitation of the spinosyn residues is performed using High Performance Liquid Chromatography with + Ion APCI Mass Spectroscopy Detection.

3. SAFETY PRECAUTIONS

Each analyst should be acquainted with the potential hazards of the reagents, products and solvents before commencing laboratory work. SOURCES OF INFORMATION INCLUDE: SAFETY DATA SHEETS, LITERATURE AND OTHER INTERNALLY GENERATED DATA. Safety information on non-Dow AgroSciences products should be requested from the supplier. Disposal of reagents, reactants, and solvents must be in compliance with the appropriate government regulations.

4. EQUIPMENT*

The following list details the equipment that were used for the validation of this method. However equivalent equipment may be used when carrying out the procedure given in Section 6.6.

4.1 Laboratory Equipment

- 4.1.1 MSE Mistral 2000 centrifuge with adapters to take 8 dram vials.
 - 4.1.2 MSE model GF-8 centrifuge fitted with a six place rotor and 380 mL slotted cups with rubber cushions.
 - 4.1.3 Mettler PM460 2 figure balance with GA44 printer or equivalent.
 - 4.1.4 Mettler AT201 5 place balance with LCP45 printer or equivalent.
 - 4.1.5 Techne sample concentrator model SC3.
 - 4.1.6 Ultrasonic bath - Decon FS100.
- Items 4.1.1 - 4.1.6 available from Fisher Scientific UK.

- 4.1.7 Reciprocating shaker model LS 20 - Gerhardt UK Ltd.

*The full address of all suppliers named above is included in Appendix 1.

- 4.1.8 96 Well Extraction manifold for Isolute Array SPE cartridges – Jones Chromatography.

- 4.1.9 Gilson 'Microman' 100 and 200 fixed volume micropipettors. 250 and 1000 μ L variable volume micropipettors - Anachem Ltd.
- 4.1.10 Polytron Homogeniser – Philip Harris Scientific.
- 4.1.11 Finnpipette 8 channel multi stepper 1500 μ L pipette – Life Sciences International
- 4.1.12 Tecator homogeniser - Perstorp Analytical Ltd.

4.2 Mass Spectrometer and Chromatographic System

4.2.1 HPLC System consisting of:

Hewlett Packard HP-1100 Binary Pump.
Hewlett Packard HP-1100 Autosampler.
Hewlett Packard HP-1100 Solvent Degasser.
Hewlett Packard HP-1100 Thermostated Column Compartment.

All available from Agilent Technologies.

- 4.2.2 PE/SCIEX API2000 LC/MS/MS System. The HPLC and MS systems are centrally controlled by the PE/SCIEX Analyst software – PE Biosystems.
- 4.2.3 HPLC Column: Inertsil ODS3 5 μ m 10 cm x 4.6 mm id. - Hichrom Ltd.
- 4.2.4 Data Handling - Integration system capable of giving peak area or peak height information, e.g. PE/SCIEX Analyst Software.

4.3 Laboratory Glassware and Plasticware *

- 4.3.1 50, 100 and 500 mL measuring cylinders.
- 4.3.2 8 dram (30 mL) screw cap vials.
- 4.3.3 50 mL - 100 mL volumetric flasks.
- 4.3.4 Unicaps R3 plastic screw top to fit 8 dram vials.
- 4.3.5 2 mL plastic disposable transfer pipette.
- Items 4.3.1 - 4.3.5 available from Fisher Scientific UK.
- 4.3.6 2 mL gas chromatography vials and aluminium/red rubber vial caps - Owen Polyscience Ltd.
- 4.3.7 4oz (100 mL) glass jars and caps - Bristol Bottle Co. Ltd.

*The full address of all suppliers named above is included in Appendix 1.

5. REAGENTS AND MATERIALS*

5.1 Methanol - HPLC grade.

5.2 Water - HPLC grade.

5.3 Acetonitrile - HPLC grade.

5.4 Ammonium Acetate - HPLC grade.

Items 5.1 - 5.4 available from Fisher Scientific UK.

5.5 SCX SPE elution solvent : Methanol:acetonitrile (1:1 v/v) + 0.1 M ammonium acetate.

5.6 Isolute Array 100 mg (2mL reservoir volume) SCX SPE cartridges – Jones Chromatography.

5.7 Nitrogen for sample concentrator and LC/MS/MS System- Air Products Ltd.

5.8 Zero grade Air – Site compressor.

5.9 Analytical standard of Spinosad and it metabolites from The Sample Dispatch Coordinator, Dow AgroSciences, Crossbank Road, King's Lynn, Norfolk PE30 2JD, UK.

*The full address of all suppliers named above is included in Appendix 1.

6. PROCEDURE

6.1 Stock and Fortification Solutions

Dissolve 100 mg of analytical standard of each analyte (spinosyns A, D, K, B and N-demethyl spinosyn D) in separate 100 mL volumetric flasks with methanol/acetonitrile (50:50 v/v) to give 1000 µg/mL stock solution of each analyte. Combine 5.0 mL aliquots from each of the stock solutions in a 50 mL volumetric flask, and dilute to volume with methanol/acetonitrile (50:50 v/v) to give 100 µg/mL mixed standard stock solution. Dilute this mixed standard stock solution to give 10 and 1.0 µg/mL spiking solutions in methanol/acetonitrile (50:50 v/v) for recovery determinations. Make the dilutions according to the following table.

Mixed Parent Std (µg/mL)	Volume Taken (mL)	Final Volume (mL)	Fortification Solution (µg/mL)
100	5	50	10
10	5	50	1

6.2 Calibration Solutions.

Pipette 1.0 mL and 10.0 mL of the 100 µg/mL mixed standard solution (prepared from stock solution - Section 6.1) into separate 100 mL volumetric flasks and dilute to volume with methanol:acetonitrile:water (4:4:2 v/v) + 0.1 %w/v ammonium acetate. Prepare dilutions of this 1.0 and 10 µg/mL solutions in methanol:acetonitrile:water (4:4:2 v/v) + 0.1 %w/v ammonium acetate to give calibration standards over the range 0.0003 – 0.06 µg/mL and chromatograph using the conditions in Section 6.3. Plot peak area against concentration to establish detector linearity (note 9.1). Make the dilutions according to the following table.

Standard Concentration (µg/mL)	Volume Taken (mL)	Final Volume (mL)	Standard Concentration (µg/mL)
100.0	1.0	100	1.00
10.0	1.0	100	0.1
0.1	10.0	100	0.01
1.0	3.0	50	0.06
1.0	3.0	100	0.03
0.1	3.0	50	0.006
0.1	3.0	100	0.003
0.1	1.0	100	0.001
0.01	3.0	50	0.0006
0.01	3.0	100	0.0003

6.3 Chromatographic and Mass Spectrometer Conditions

6.3.1 Chromatographic Conditions

Column	: Inertsil ODS3 5 µm , 10 cm x 4.6 mm id
Mobile Phase	: A = 1:1 v/v Methanol/Acetonitrile + 0.1 % w/v Ammonium Acetate : B = Water + 0.1 % w/v Ammonium Acetate
Flow Rate	: 1.0 mL/min, Isocratic (95:5 A:B)
Injection Volume	: 100 µL

6.3.2 Mass Spectrometer Conditions

Ionisation Mode	: + ion, APCI
Vaporiser Temperature (TEM)	: 425°C
Interface Heater (ihe)	: On (1)
Nebulizer Current (NC)	: 2µA

Nebulizer Gas (Gas 1)	: 80 psi Nitrogen
Auxiliary Gas (Gas 2)	: 20 psi Nitrogen
Electron Multiplier	: 2100V
MS Scan Parameters	: 718.6amu over 0.5da scan width for 0.15 sec, 732.6amu over 0.5da scan width for 0.15 sec, 746.6amu over 0.5da scan width for 0.15 sec, cycled throughout run
Total analysis time	: 6 minutes
Quantitation	: Peak area (Note 9.1)

6.4 Sample Preparation

Samples were prepared using a Tecator homogeniser with or without dry ice. Prepared samples were stored deep frozen prior to analysis.

6.5 Method Validation

Validate the analytical procedure given in Section 6.6 by analysing the following:

At least one untreated sample for each matrix (each in duplicate).

At least one untreated sample after fortification at the lowest validation level (in duplicate). The lowest validation level is defined as "at least 4 times the average untreated value". At least one sample (in duplicate) fortified at an intermediate level and one sample at a level exceeding the expected maximum residue (in duplicate).

6.6 Procedure for Acidic Agricultural Crops

Include a reagent blank and procedural recovery in each analytical batch. Analyse all treated and untreated samples in duplicate.

- 6.6.1 Weigh duplicate 10g \pm 0.10g portions of sample into 4 oz jars. Add the required volume of the appropriate fortification solution to the recovery samples.
- 6.6.2 Add 50 mL of acetonitrile:water (80:20 v/v) to each jar and homogenise for one minute with a Polytron homogeniser (17000 rpm).
- 6.6.3 Cap the jar and shake for 30 mins at 150 rpm. Centrifuge the sample at 2000 rpm for 5 minutes.
- 6.6.4 Pipette a 0.5 mL aliquot of the extraction solution into a clean 8 dram, add 1.5 mL of acetonitrile and mix.
- 6.6.5 Condition a 100 mg SCX isolate array SPE cartridge with 1 mL acetonitrile followed by 1 mL water.

- 6.6.6 Apply the extract from step 6.6.4 to the cartridge and pull through at approximately 1 mL/min. Pull dry for 30 seconds. Wash the cartridges with 2 x 1.0 mL of methanol. Suck the cartridge dry for 1 minute under full vacuum. Wash the cartridge with 1 mL of water and pull the cartridge dry at full vacuum for 30 seconds. Wash the cartridge with 1 mL of a 0.1 M aqueous ammonium acetate solution and suck the cartridge dry for 5 mins at full vacuum.
- 6.6.7 Insert the collection rack into the SPE manifold and elute the spinosyns with two 750 μ L aliquots of 0.1 M ammonium acetate in acetonitrile:methanol (1:1 v/v). Pull approx 200 μ L of the elution solvent through the cartridge then stop the flow and allow to soak for 1 min before slowly eluting at approximately at 1 mL/min). Elute with a further 750 μ L of elution solvent. Suck the cartridge dry for a further 30 seconds once the final aliquot of elution solvent has passed through the cartridge.
- 6.6.8 Transfer the samples to clean 8 dram vials and evaporated to dryness at 45°C using a gentle stream of nitrogen. Reconstitute the samples in 1 mL of acetonitrile:methanol:water (4:4:2 v/v/v) and transfer to a 2 mL vial and chromatograph samples using the conditions given in Section 6.3 injecting the calibration standards throughout the run. For sample extracts which contain spinosad concentrations >0.06 μ g/mL, dilute with acetonitrile:methanol:water (4:4:2 v/v/v) to give a concentration <0.06 μ g/mL after re-injection.

7. CALCULATIONS

7.1 Calculation of Residues

Spinosad residues are calculated from the individual spinosyn calibration curve using the following equation:

$$\text{mg/kg} = \frac{\text{Sample Response} - \text{Intercept}}{\text{Slope}} \times A \times B \times C$$

The intercept and slope are calculated by linear regression

where:

$$A = \text{Method factor} = \frac{(50/0.5)}{10} \times 1 = 10$$

B = Additional dilution factor

C = Moisture correction factor

A correction factor is applied when moisture from the sample makes a significant contribution (where G is ≥ 1.08) to the total extraction volume. The moisture content can be determined by heating pre-weighed samples at 105°C for a minimum of 16 hours or by reference to a standard text.

The moisture contribution factor (G) is calculated as follows:

$$G = \frac{A + B}{A}$$

where A = Volume of extracting solvent added

$$B = \frac{\text{wt of sample} \times \% \text{ water}}{100}$$

The moisture correction factors used in this method were:

Substrate	Moisture Correction factor	Substrate	Moisture Correction factor
Orange pulp	1.18	Lemon pulp	1.18
Orange peel	1.16	Lemon peel	1.17
Orange juice	1.17	Lemon Juice	1.18
Orange whole fruit	1.17	Lemon whole fruit	1.18
Mandarin pulp	1.19		
Mandarin peel	1.15		
Mandarin Juice	1.17		
Mandarin whole fruit	1.17		

7.2 Calculation of % Recovery

$$\% \text{ Recovery} = \frac{[\text{mg/kg found} - \text{mg/kg control}]}{\text{fortification level, mg/kg}} \times 100$$

8. METHOD PARAMETER DEFINITIONS AND STATISTICS

The validation of this method was performed as Dow AgroSciences study 000313.

8.1 Control and recovery values

Using the procedure outlined in Section 6, control and recovery values for the determination of spinosad and its metabolites in the matrices are shown in Tables 2-3. A summary of spinosyn recoveries in the matrices is shown below:

Substrate	Average Recovery %				
	Spinosyn B	Spinosyn N-demethyl D	Spinosyn K	Spinosyn A	Spinosyn D
Acidic crops	95 (76-111)	93 (70-113)	95 (71-110)	95 (77-110)	95 (72-110)

Typical calibration plots are shown in Figures 1 – 5. Typical chromatography is presented in Figure 6.

8.2 Limits of detection and quantitation

The limit of detection of this method is defined as 20% of the lowest validated level (0.01 mg/kg). i.e. 0.002 mg/kg. Study samples results calculated as values <0.002 mg/kg or are classified as Not Detected (ND). The limit of quantitation of this method is defined as the lowest validated level i.e. 0.01 mg/kg. Study sample results calculated as greater than the limit of detection but less than 0.01 mg/kg are designated < 0.01 mg/kg.

8.3 Precision

The relative standard deviation (RSD) over the validated range (0.01 - 1.0 mg/kg) are shown below for each spinosyn. Note that the recovery experiments were performed in five batches by four different analysts.

Substrate	Relative Standard Deviation % (R.S.D)				
	Spinosyn B	Spinosyn N-demethyl D	Spinosyn K	Spinosyn A	Spinosyn D
Acidic Crops	9.7	8.9	8.0	8.5	8.7

8.4 SPECIFICITY

The method is specific for the determination of spinosyns A, D, B, K, and *N*-demethyl D by virtue of the chromatographic separation and selective detection system used. The presence of spinosyns A, D, B, K, and *N*-demethyl D is identified by comparison of peak retention time of a known standard with that for the sample.

8.5 CONFIRMATION

Confirmation of the residues can be achieved by re-injecting the samples using the Alltech Mixed mode C18/Cation exchange (4.6 mm x 15 cm) instead of Inertsil ODS3 column.

9. NOTES

- 9.1 Peak area was used for quantitation during the validation of this method, although peak height could also be used.
- 9.2 The extraction and clean up of a batch of 50 determinations can be completed within a normal working day with the final quantification being carried out either overnight or during the following day. Based on this experience a batch could be analysed within a 24 hour period with no break points being required. If break points are required, they could be introduced at steps 6.6.3, 6.6.4 and 6.6.8 (all extracts should be kept in a refrigerator).

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TABLE 1Chemical Names and CAS Numbers for Spinosyns A, D, K, B, N-demethyl D

Spinosyn A: 2-[6-deoxy-2,3,4-tri-O-methyl- α -L-mano-pyranosyl)oxy]-13-[[5-(dimethylamino)-tetrahydro-6-methyl-2H-pyran-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14, 16a,16b-tetradecahydro-14-methyl-1H-as-indeceno[3,2-d]oxacyclododecin-7,15-dione (common name: Factor A) - (CAS No. 131929-60-7).

Spinosyn D: 2-[6-deoxy-2,3,4-tri-O-methyl- α -L-mano-pyranosyl)oxy]-13-[[5-(dimethylamino)-tetrahydro-6-methyl-2H-pyran-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14, 16a,16b-tetradecahydro-4,14-dimethyl-1H-as-indeceno[3,2-d]oxacyclododecin-7,15-dione (common name: Factor D) - (CAS No. 131929-63-0).

Spinosyn B: 2-[6-deoxy-2,3,4-tri-O-methyl- α -L-mannopyranosyl)oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10, 11,12,13,14,16a,16b-tetradecahydro-14-methyl-13-[(tetrahydro-6-methyl-5-(methylamino)-2H-pyran-2-yl)oxy]-1H-as-Indaceno[3,2-d]oxacyclododecin-7,15-dione (common name: Factor B) - (CAS No. 131929-61-8).

N-demethyl Spinosyn D: 2-[6-deoxy-2,3,4-tri-O-methyl- α -L-mannopyranosyl)oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10, 11,12,13,14,16a,16b-tetradecahydro-4,14-dimethyl-13-[(tetrahydro-6-methyl-5-(methylamino)-2H-pyran-2-yl)oxy]-1H-as-Indaceno[3,2-d]oxacyclododecin-7,15-dione (common name: Factor B of D or N-Demethyl Spinosyn D) - (CAS No. 149439-70-3).

Spinosyn K: 2-[6-deoxy-2,3-di-O-methyl- α -L-mannopyranosyl)oxy]-13-[[5-(dimethylamino)-tetrahydro-6-methyl-2H-pyran-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a, 16b-tetradecahydro-14-methyl-1H-as-Indaceno[3,2-d]oxacyclododecin-7,15-dione (common name: Factor K) - (CAS No. 159195-00-3)

Table 2 - Control Values for Spinosad in Acidic Agricultural Crops

Sample No.	Batch No.	Residue Found (mg/kg) ^a				
		Spinosyn B	Spinosyn N-demethyl D	Spinosyn K	Spinosyn A	Spinosyn D
96/217 Whole oranges	B1	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
R99-999-073 Orange peel	B1	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
R99-999-074 Orange pulp	B1	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
R99-999-056 Whole mandarins	B1	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
96/250 Mandarin pulp	B1	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
R96-108-005 Mandarin peel	B1	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
96/213 Whole lemons	B1	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
96/225 Lemon peel	B1	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
R00-999-011 Lemon pulp	B1	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
R00-999-026 Orange juice	B3	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
R00-999-027 Mandarin juice	B3	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
R00-999-028 Lemon juice	B3	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000	0.0000 0.0000
	Ave Control	0.0000	0.0000	0.0000	0.0000	0.0000

^a The results quoted are only numerical values used to calculate the lowest fortification concentration and hence the determination limit of the method.

Table 3 - Recovery of Spinosad from Fortified Acidic Agricultural Crops

Sample No.	Batch No.	Fortification Rate (mg/kg)	% Recovery				
			Spinosyn B	Spinosyn N-demethyl D	Spinosyn K	Spinosyn A	Spinosyn D
96/217 Whole oranges	B1	0.01	83 92	80 88	86 96	81 84	88 84
R99-999-073 Orange peel	B1		87 80	93 88	95 93	90 90	95 92
R99-999-074 Orange pulp	B1		90 89	92 94	94 91	95 91	87 93
R99-999-056 Whole mandarins	B1		86 88	89 91	91 91	90 91	91 94
96/250 Mandarin pulp	B1		89 87	92 88	94 90	94 89	95 88
R96-108-005 Mandarin peel	B1		85 84	93 88	98 93	89 84	96 90
96/213 Whole lemons	B1		83 92	94 94	96 97	87 93	97 94
96/225 Lemon peel	B1		86 80	92 88	89 96	95 93	95 87
R00-999-011 Lemon pulp	B1		86 91	88 92	92 96	86 95	84 92
R00-999-026 Orange juice	B3		94 97	96 98	94 98	99 103	98 101
R00-999-027 Mandarin juice	B3 B4		84 77	80 86	84 75	86 78	78 78
R00-999-028 Lemon juice	B3		79 96	78 89	79 92	85 94	81 91
	Mean		87	90	92	90	90
	Range		77-97	78-98	75-98	78-103	78-101
	n		24	24	24	24	24
	S.D.		5.2	5.0	5.8	5.7	6.0
	R.S.D.		6.0	5.5	6.3	6.3	6.7

Table 3 Continued - Recovery of Spinosad from Fortified Acidic Agricultural Crops

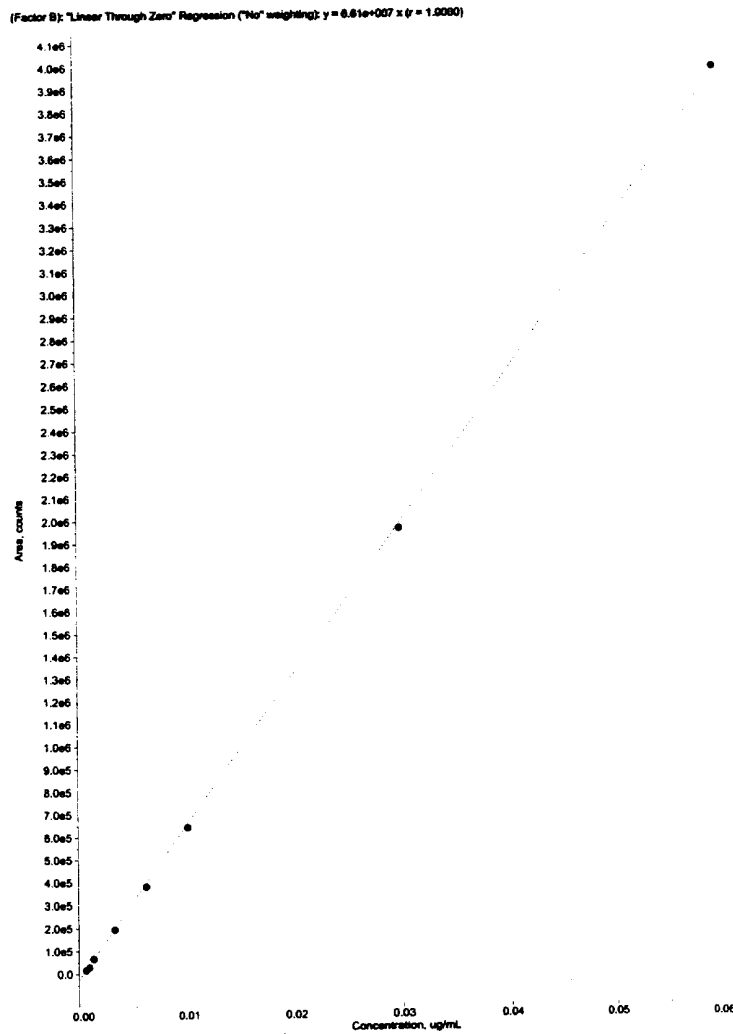
Sample No.	Batch No.	Fortification Rate (mg/kg)	% Recovery				
			Spinosyn B	Spinosyn N-demethyl D	Spinosyn K	Spinosyn A	Spinosyn D
96/217	B2	0.10	105	99	102	105	107
Whole oranges			105	105	103	104	106
R99-999-073	B2R		106	104	103	105	103
Orange peel	B4		87	82	81	85	85
R99-999-074	B2		103	96	102	105	104
Orange pulp			105	99	104	102	104
R99-999-056	B2		109	103	105	105	107
Whole mandarins			110	105	107	109	108
96/250	B2		106	104	103	103	103
Mandarin pulp			105	101	103	102	104
R96-108-005	B2R		99	93	95	101	100
Mandarin peel	B4		83	77	79	81	83
96/213	B2		101	101	98	98	96
Whole lemons			105	102	103	103	103
96/225	B2		97	94	97	97	97
Lemon peel			99	95	98	100	99
R00-999-011	B2R		100	92	98	99	92
Lemon pulp	B2		104	101	101	98	99
R00-999-026	B3		99	95	93	101	98
Orange juice			92	87	87	93	91
R00-999-027	B3	85	83	81	88	87	
Mandarin juice		94	91	92	97	96	
R00-999-028	B3	93	89	89	93	85	
Lemon juice		76	70	71	77	72	
		Mean	99	95	96	98	97
		Range	76-110	70-105	71-107	77-109	72-108
		n	24	24	24	24	24
		S.D.	8.9	9.3	9.6	8.1	9.1
		R.S.D.	9.0	9.9	10.0	8.3	9.4

Table 3 Continued - Recovery of Spinosad from Fortified Acidic Agricultural Crops

Sample No.	Batch No.	Fortification Rate (mg/kg)	% Recovery				
			Spinosyn B	Spinosyn N-demethyl D	Spinosyn K	Spinosyn A	Spinosyn D
96/217	B2	1.00	92	86	89	88	88
Whole oranges			92	89	91	88	92
R99-999-073	B5		106	106	102	106	109
Orange peel			111	113	108	110	110
R99-999-074	B2		92	88	93	88	89
Orange pulp			91	85	91	88	89
R99-999-056	B2		104	99	100	99	102
Whole mandarins			104	100	101	102	98
96/250	B2		99	95	98	96	95
Mandarin pulp			95	92	94	92	90
R96-108-005	B2		110	104	101	105	105
Mandarin peel			110	107	106	108	109
96/213	B2		92	87	91	89	90
Whole lemons			92	87	90	89	87
96/225	B2		92	87	93	87	88
Lemon peel			90	86	91	86	87
R00-999-011	B2		102	96	98	97	97
Lemon pulp			97	91	96	93	92
R00-999-026	B5		111	111	110	110	107
Orange juice			100	100	100	99	103
R00-999-027	B5		94	95	90	91	93
Mandarin juice			104	106	100	102	101
R00-999-028	B5		100	101	97	102	103
Lemon juice			104	102	96	99	102
	Mean	99	96	97	96	97	
	Range	90-111	85-113	88-110	86-110	87-110	
	n	24	24	24	24	24	
	S.D	7.2	8.7	6.0	8.0	7.9	
	R.S.D.	7.3	9.0	6.2	8.3	8.1	

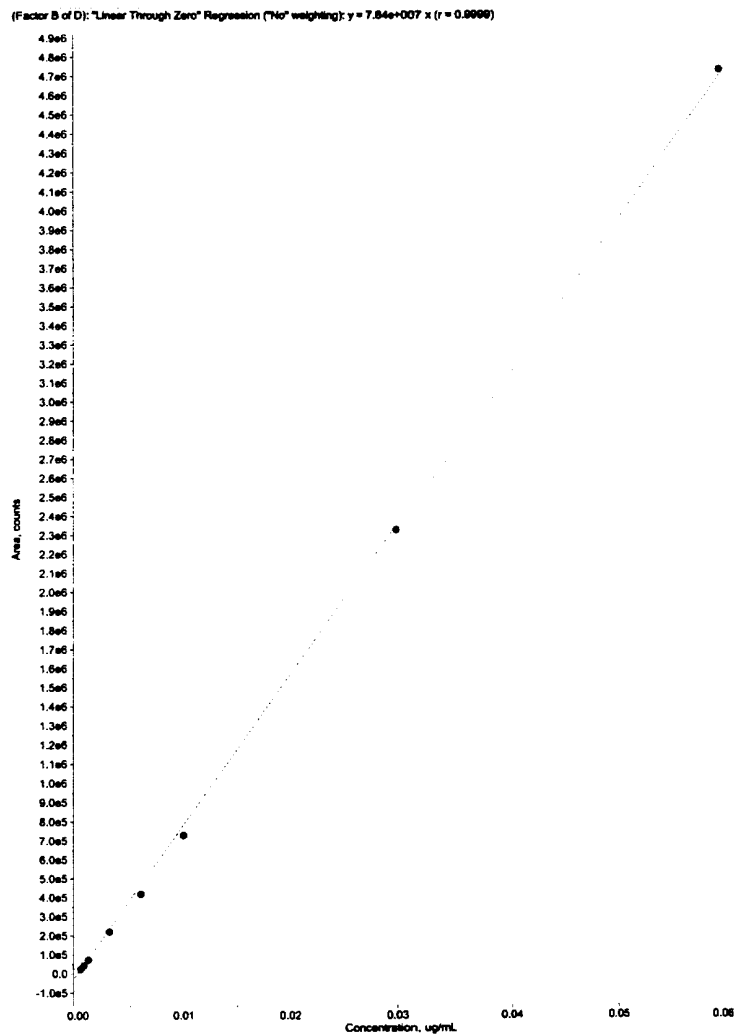
Mean	95	93	95	95	95
Range	76-111	70-113	71-110	77-110	72-110
n	72	72	72	72	72
S.D	9.2	8.3	7.6	8.0	8.3
R.S.D.	9.7	8.9	8.0	8.5	8.7

FIGURE 1 - TYPICAL CALIBRATION PLOT FOR SPINOSYN B



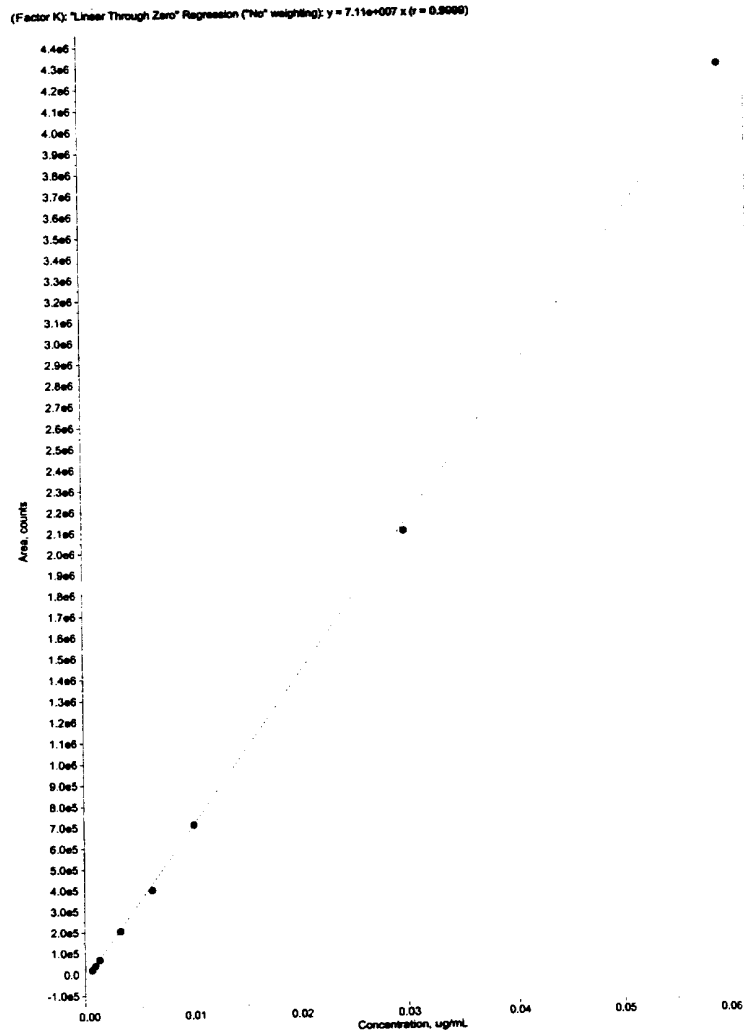
Standard Concentration (µg/mL)	MS Response Ion 718.6 amu (Peak Area)
0.0003	1.69e4
0.0006	2.97e4
0.001	6.65e4
0.003	1.93e5
0.006	3.80e5
0.01	6.41e5
0.03	1.96e6
0.06	3.99e6

FIGURE 2 - TYPICAL CALIBRATION PLOT FOR SPINOSYN N-DEMETHYL D



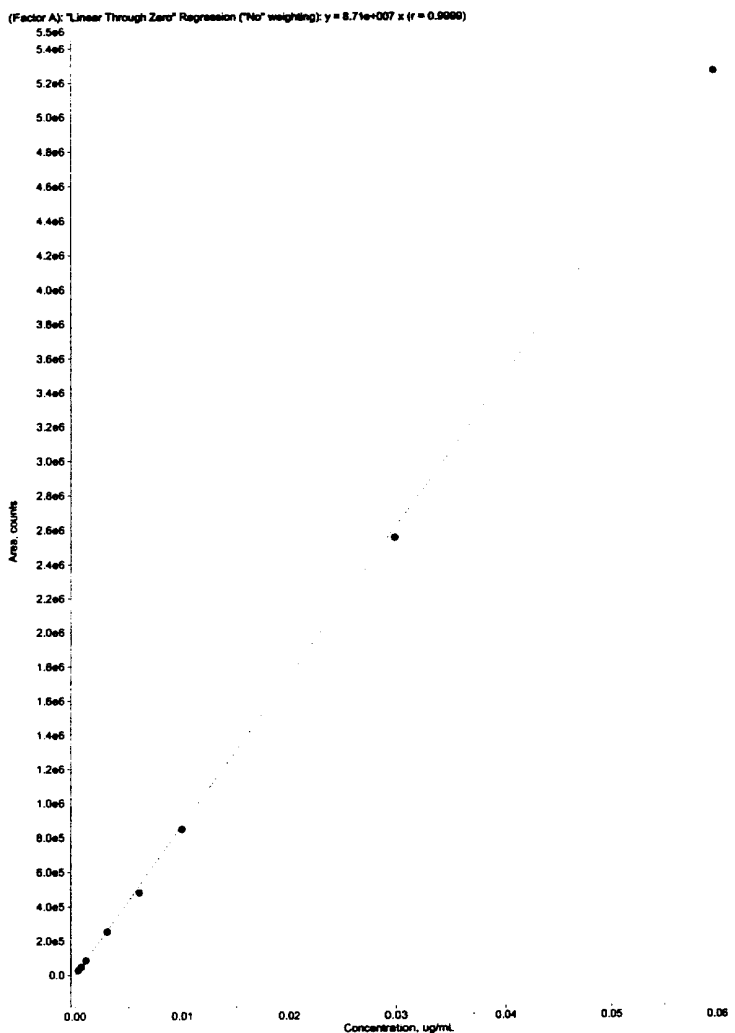
Standard Concentration (µg/mL)	MS Response Ion 732.6 amu (Peak Area)
0.0003	2.41e4
0.0006	4.22e4
0.001	7.18e4
0.003	2.20e5
0.006	4.18e5
0.01	7.29e5
0.03	2.33e6
0.06	4.73e6

FIGURE 3 - TYPICAL CALIBRATION PLOT FOR SPINOSYN K



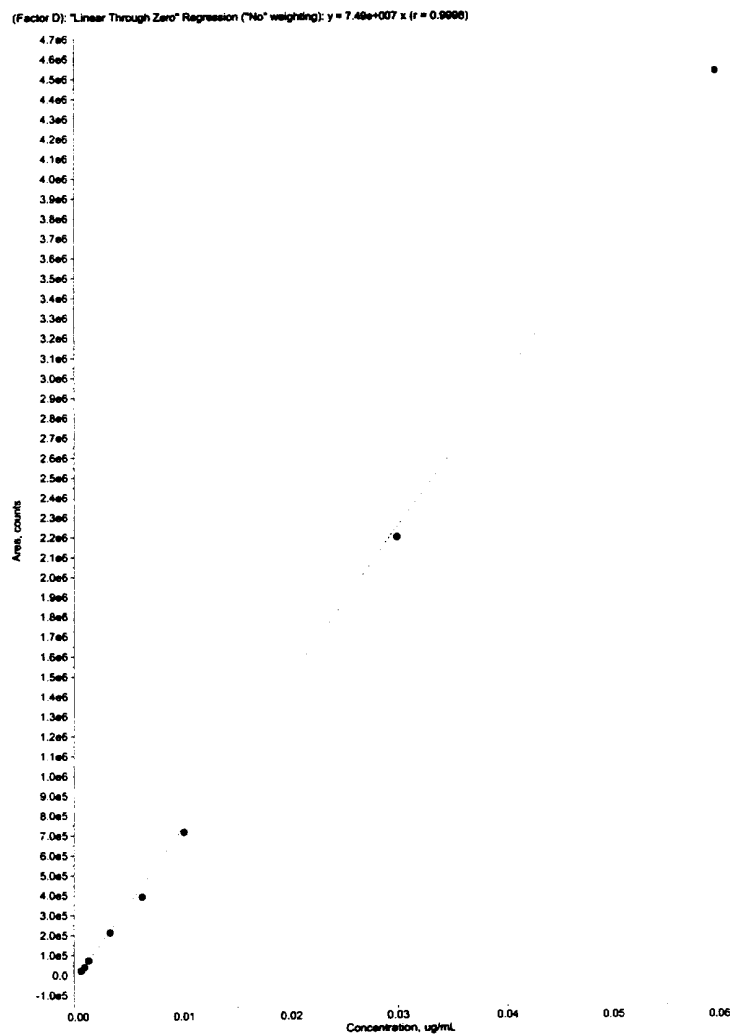
Standard Concentration (µg/mL)	MS Response Ion 718.6 amu (Peak Area)
0.0003	2.11e4
0.0006	4.00e4
0.001	6.89e4
0.003	2.05e5
0.006	3.99e5
0.01	7.09e5
0.03	2.10e6
0.06	4.29e6

FIGURE 4 - TYPICAL CALIBRATION PLOT FOR SPINOSYN A



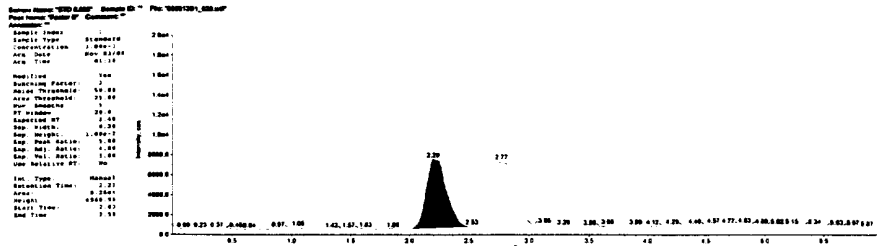
Standard Concentration (µg/mL)	MS Response Ion 732.6 amu (Peak Area)
0.0003	2.66e4
0.0006	4.71e4
0.001	8.42e4
0.003	2.54e5
0.006	4.80e5
0.01	8.48e5
0.03	2.55e6
0.06	5.26e6

FIGURE 5 - TYPICAL CALIBRATION PLOT FOR SPINOSYN D

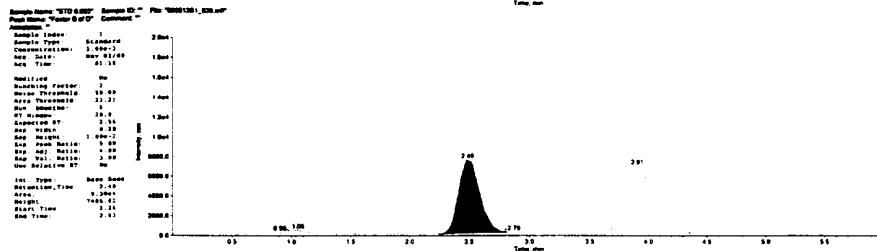


Standard Concentration (µg/mL)	MS Response Ion 746.6 amu (Peak Area)
0.0003	2.30e4
0.0006	3.97e4
0.001	7.30e4
0.003	2.14e5
0.006	3.92e5
0.01	7.16e5
0.03	2.19e6
0.06	4.53e6

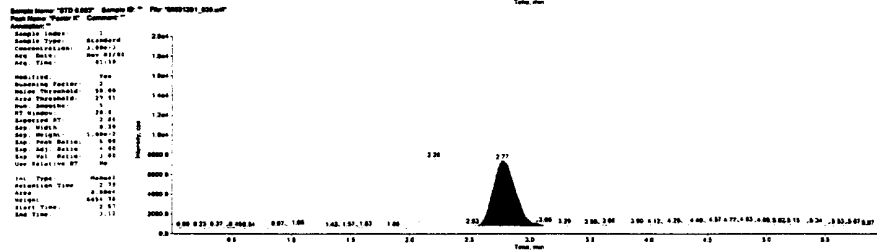
FIGURE 6 - TYPICAL CHROMATOGRAPHY FOR THE ANALYSIS OF SPINOSAD IN WHOLE ORANGES



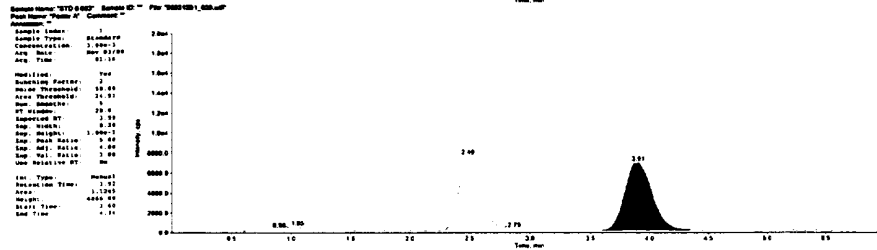
Spinosyn B



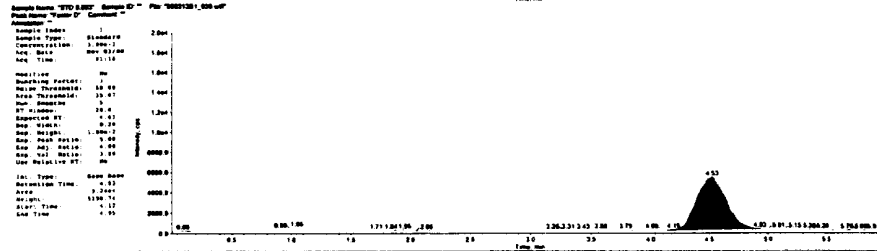
Spinosyn N-Demethyl D



Spinosyn K



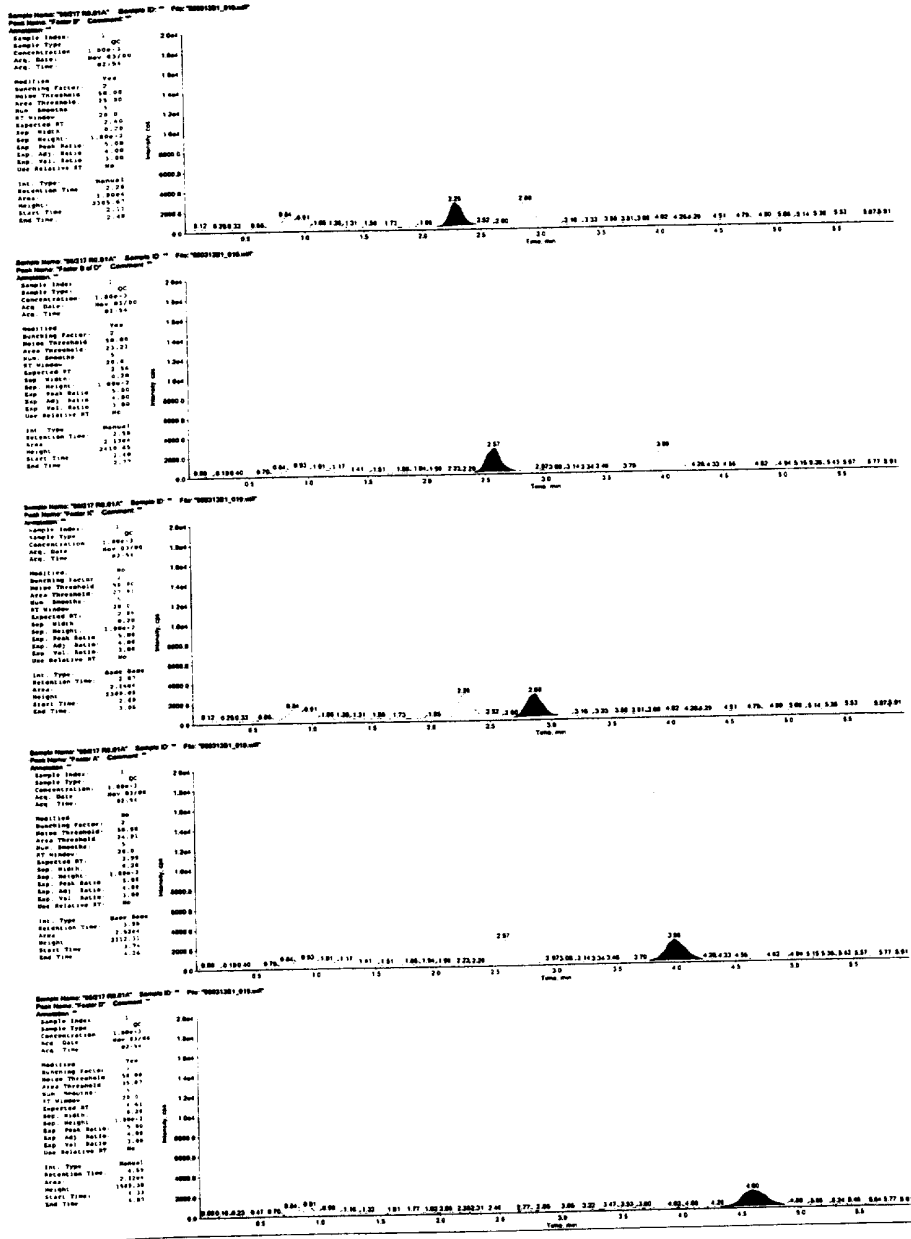
Spinosyn A



Spinosyn D

Mixed Spinosyn Standard
 0.003 µg/mL

FIGURE 6 CONTINUED - TYPICAL CHROMATOGRAPHY FOR THE ANALYSIS OF SPINOSAD IN WHOLE ORANGES



Spinosyn B

Spinosyn N-Demethyl D

Spinosyn K

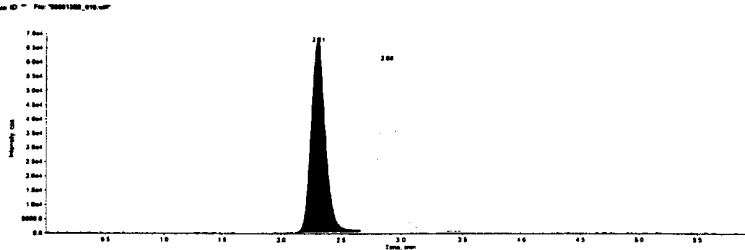
Spinosyn A

Spinosyn D

Control whole orange sample 96/217 A
 Fortified at 0.01 mg/kg
 83% (Spinosyn B), 80% (Spinosyn N-Demethyl D), 86% (Spinosyn K),
 81% (Spinosyn A), 88% (Spinosyn D)

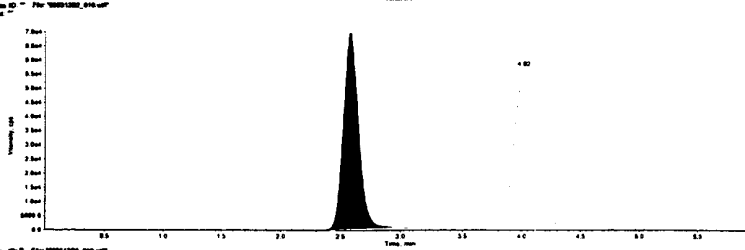
FIGURE 6 CONTINUED - TYPICAL CHROMATOGRAPHY FOR THE ANALYSIS SPINOSAD IN WHOLE ORANGES

Sample Name: "96/217 A1.A" Sample ID: "96091302_016.A" File: "96091302_016.A"
 Peak Name: "Spinosyn B" Comment: "
 Acquisition: "
 Sample Index: 1
 Sample Type: QC
 Concentration: 0.10
 Acq. Date: Nov. 02/99
 Acq. Time: 21:24
 Method: "
 Inlet Type: Manual
 Retention Time: 27.21
 Area: 4.0845
 Height: 7.0845
 Alert Time: 2.26
 End Time: 2.26



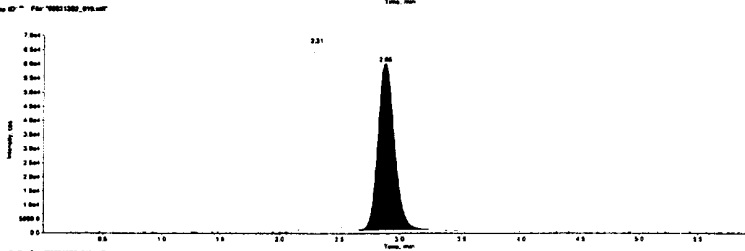
Spinosyn B

Sample Name: "96/217 A1.A" Sample ID: "96091302_016.A" File: "96091302_016.A"
 Peak Name: "Spinosyn N-Demethyl D" Comment: "
 Acquisition: "
 Sample Index: 1
 Sample Type: QC
 Concentration: 0.10
 Acq. Date: Nov. 02/99
 Acq. Time: 21:24
 Method: "
 Inlet Type: Manual
 Retention Time: 28.24
 Area: 4.3345
 Height: 7.0845
 Alert Time: 2.26
 End Time: 2.26



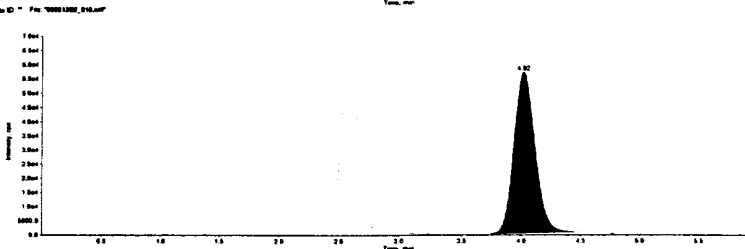
Spinosyn N-Demethyl D

Sample Name: "96/217 A1.A" Sample ID: "96091302_016.A" File: "96091302_016.A"
 Peak Name: "Spinosyn K" Comment: "
 Acquisition: "
 Sample Index: 1
 Sample Type: QC
 Concentration: 0.10
 Acq. Date: Nov. 02/99
 Acq. Time: 21:24
 Method: "
 Inlet Type: Manual
 Retention Time: 29.24
 Area: 5.3345
 Height: 7.0845
 Alert Time: 2.26
 End Time: 2.26



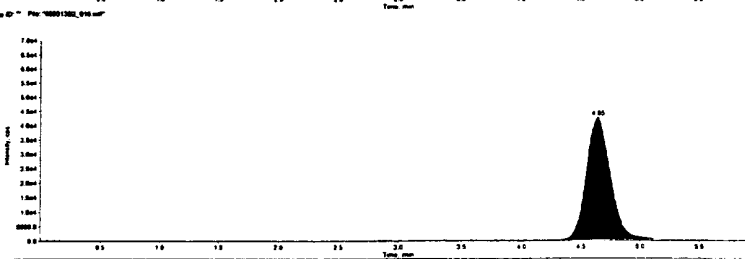
Spinosyn K

Sample Name: "96/217 A1.A" Sample ID: "96091302_016.A" File: "96091302_016.A"
 Peak Name: "Spinosyn A" Comment: "
 Acquisition: "
 Sample Index: 1
 Sample Type: QC
 Concentration: 0.10
 Acq. Date: Nov. 02/99
 Acq. Time: 21:24
 Method: "
 Inlet Type: Manual
 Retention Time: 30.24
 Area: 5.3345
 Height: 7.0845
 Alert Time: 2.26
 End Time: 2.26



Spinosyn A

Sample Name: "96/217 A1.A" Sample ID: "96091302_016.A" File: "96091302_016.A"
 Peak Name: "Spinosyn D" Comment: "
 Acquisition: "
 Sample Index: 1
 Sample Type: QC
 Concentration: 0.10
 Acq. Date: Nov. 02/99
 Acq. Time: 21:24
 Method: "
 Inlet Type: Manual
 Retention Time: 31.24
 Area: 4.3345
 Height: 7.0845
 Alert Time: 2.26
 End Time: 2.26



Spinosyn D

Control whole orange sample 96/217 A
 Fortified at 1.00 mg/kg
 92% (Spinosyn B), 86% (Spinosyn N-Demethyl D), 89% (Spinosyn K),
 88% (Spinosyn A), 88% (Spinosyn D)
 X 10 Dilution

Appendix 1Suppliers Addresses

Air Products (GB) Ltd., Hampshire International Business Park, Chineham, Basingstoke, Hampshire, UK

Agilent Technologies., King Street, Wokingham, Berkshire, UK.

Anachem Ltd, 20 Charles Street, Luton, Bedfordshire, UK.

Bristol Bottle Co. Ltd., Unit 1, Ashmead Trading Estate, Keynsham, UK.

Fisher Scientific UK, Ltd., Bishop Meadow Road, Loughborough Road, Leicestershire, UK.

Gerhardt UK Ltd., Underwood Lane, Crewe, Cheshire, UK.

Hichrom Ltd., 1 The Markam Centre, Station Road, Theale, Reading, Berkshire, UK.

Jones Chromatography Ltd., New Road, Hengoed, Mid Glamorgan, UK.

Life Sciences International Ltd, Unit 5, The Ringway Centre, Edison Rd, Basingstoke, Hampshire, UK.

Owen Polyscience Ltd., 34 Chester Road, Macclesfield, Cheshire, UK.

PE Biosystems, Kelvin Close, Birchwood Science Park North, Warrington, Cheshire, UK.

Perstorp Analytical Ltd., Highfield House, Foundation Park, Roxborough Way, Maidenhead, Berkshire, UK.

Philip Harris Scientific, 618 Western Avenue, Park Royal, London W3, UK.

Stephan Machinery (UK) Ltd., Unit 7, Felthambrook Industrial Estate, Felthambrook Way, Feltham, Middlesex, UK.

GLP COMPLIANCE STATEMENT

Study Title: VALIDATION OF ANALYTICAL METHOD GRM 00.27 -
DETERMINATION OF SPINOSAD RESIDUES IN ACIDIC
AGRICULTURAL CROPS BY HIGH PERFORMANCE LIQUID
CHROMATOGRAPHY WITH + APCI MASS SPECTROSCOPY
DETECTION

Method Number: GRM 00.27

Study Number: 000313

Study starting date: 20 Oct 00 **Experimental starting date:** 01 Nov 00

Experimental completion date: 10 Nov 00 **Study completion date:** 06 Dec 00

I confirm that I fulfilled the responsibilities of Study Director for the above analytical method validation study. The work was conducted in compliance with OECD Principles of Good Laboratory Practice [ENV/MC/CHEM(98)17] and UK regulations embodying these principles.

The method fully and accurately reflects the procedures adopted and the raw data generated during the study.



Name: M Hastings
Study Director



Date

ARCHIVING STATEMENT

The following study related documentation is lodged in the Archive of Dow AgroSciences, Letcombe Laboratory, Letcombe Regis, Wantage, Oxon, OX12 9JT, UK.

The Study Plan including any amendments or deviations
The original laboratory raw data
Relevant study-specific correspondence
A copy of the final report

QUALITY ASSURANCE STATEMENT

Study Title: VALIDATION OF ANALYTICAL METHOD GRM 00.27 -
**DETERMINATION OF SPINOSAD RESIDUES IN ACIDIC
 AGRICULTURAL CROPS BY HIGH PERFORMANCE LIQUID
 CHROMATOGRAPHY WITH + APCI MASS SPECTROSCOPY
 DETECTION**

Method Number: GRM 00-27

Study Number: 000313

The above non-clinical health and environmental safety (regulatory) study was examined for conformance with OECD Principles of Good Laboratory Practices [ENV/MC/CHEM(98)17] and UK regulations embodying these principles.

The report was determined to reflect the procedures adopted and the raw data generated.

The dates of Quality Assurance activities are given below:

Type of Inspection	Inspection Date	Date findings reported
Study Plan review (reported to Study Director only)	20 Oct 00	20 Oct 00
Study / Process-based Inspections - lab: Sample Analysis *	26 Oct 00	27 Oct 00
Reported data: Method	22 Nov 00	01 Dec 00

For this study type monitoring of technical procedures, processes, facilities and personnel are performed on a regular basis. Dates relevant to the experimental timing have been quoted. Inspections are reported to the Study Director and management.

* Inspections not directly related to this study and not reported to the Study Director or the Study Director's management

I Brownlee

I Brownlee
 European Quality Assurance Auditor
 Dow Agrosiences
 Letcombe Laboratory
 Letcombe Regis, Wantage
 Oxon OX12 9JT, UK

06 Dec 2000
 Date