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Title : **Thidiazuron: Method of Analysis for the Determination of Thidiazuron and its Metabolite Photo-Thidiazuron in Crops Using LC/MS/MS**

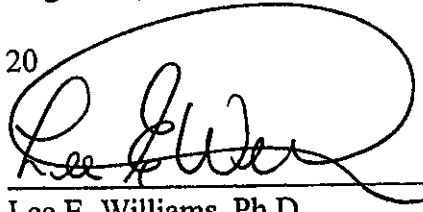
Analytes : Thidiazuron (TDZ) (AE B 049537) and Photo-Thidiazuron (p-TDZ) (AE F 132347).

Substrates : Crop Matrices

Date Issued : August 17, 2001

Total pages 20

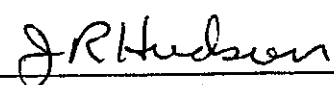
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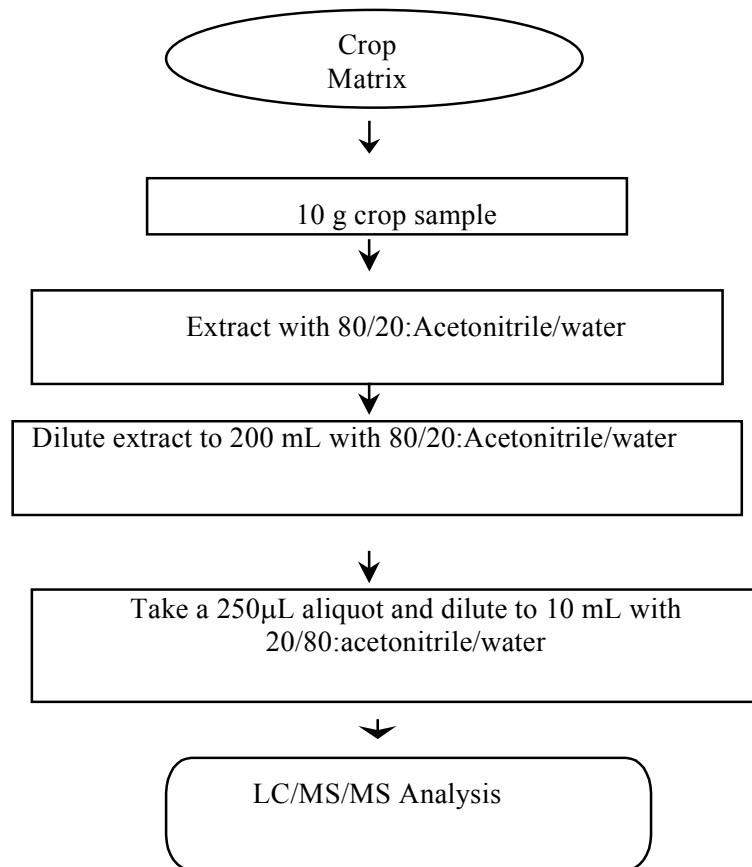
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August 17, 2001
Date

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Summary Flowchart of Analytical Method



Thidiazuron: Method of Analysis for the Determination of Thidiazuron and its Metabolite Photo-Thidiazuron in Crops Using LC/MS/MS

I. Introduction

This method has been developed for the analysis of Thidiazuron (AE B049537) and photo-thidiazuron (AE F132347) in crop matrices (mustard greens, radish roots and tops). This method has been verified during the method development stage at the spike level of fifty parts per billion (50 ppb).

A. Scope

This method sets forth the procedure for determining the residues of AE B049537 and AE F132347 in crop matrices. Recovery data collected during the method development stage are summarized at the end of this method.

B. Principle

Extractable residues of thidiazuron (AE B049537) and photo-thidiazuron (AE F132347) are removed from crop by blending with 80/20:acetonitrile/water. After blending the resulting extract is filtered, and diluted to a volume 200 mL. A 250 μ L aliquot of the forementioned extract is diluted to a final volume of 10 mL with 20/80 acetonitrile/ water. Following final dilution thidiazuron (AE B049537) and photo-thidiazuron (AE F132347) residues are quantified by High Performance Liquid Chromatography with MS/MS detection (LC/MS/MS)

C. Method Limits

The target level for the limit of quantitation (LOQ) is 50 ppb for thidiazuron (AE B049537) and photo-thidiazuron (AE F132347) in all crop matrices. Mean percent recoveries at the LOQ carried out during the method development were 91 and 89 percent for thidiazuron and photo-thidiazuron, respectively for all substrates.

D. Chemical Structure

Thidiazuron

C.A. Name: N-phenyl-N'-1,2,3-thiadiazol-5-ylurea

IUPAC Name: 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea

CAS Reg. No.: 51707-55-2

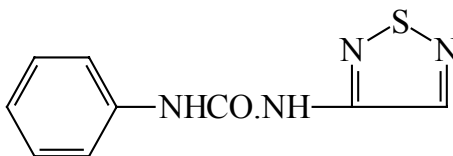


Photo-thidiazuron

C.A. Name: N-phenyl-N'-1,2,5-thiadiazol-3-ylurea

IUPAC Name: 1-phenyl-3-(1,2,5-thiadiazole-3-yl)urea

E. Safety

There are no particular safety hazards with this method. The method should be used by trained laboratory personnel. The user should review appropriate MSD sheets and observe normal laboratory safety procedures.

II. Materials

Reagents and solvents were used as received from supplier, unless otherwise noted.

Equivalent reagents, solvents and equipment may be substituted where appropriate.

A. Reagents

1. Acetic acid , glacial, 99.9%, JT Baker, Cat. No 9507-05

B. Solvents

1. Methanol, B&J ACS/HPLC, Burdick and Jackson, Cat. No BJAH230-4
2. Water, Milli-Q
3. Acetonitrile, Omnisolv, EM Science, Cat. No AX 0142-1

C. Solutions

1. 20/80: Acetonitrile/water solution
Add 200 mL of acetonitrile to a liter graduated cylinder. Add 800 mL of Milli-Q water and shake gently until it is mixed thoroughly.
2. 80/20: Acetonitrile/water solution
Add 800 mL of acetonitrile to a liter graduated cylinder. Add 200 mL of water and shake gently until it is mixed thoroughly.
3. 0.1% acetic acid in water
Add 1 mL of glacial acetic acid to 999 mL of Milli-Q water. Shake gently until it is mixed thoroughly.

D. Equipment

1. Analytical Balance, Mettler PM2000 or PG2002, VWR Cat. N° 11273-722
2. Professional Level Balance, Ohaus portable
3. Spoons, and/or spatula
4. Beakers, appropriate sizes
5. Blender, Sorvall Omni-Mixer, Omni International
6. Blender Blade Assembly, Omni International
7. Blending jars (Mason pint size canning)
8. Glass fiber filter paper, Whatman 934-AH (Cat. No 1827-90)
9. Kimax graduated mixing cylinders, 250 mL, Kimble, VWR Cat. No 24763-109
10. Volumetric flasks, and stoppers, appropriate sizes, class A, VWR, eg. Cat. No 29619-6xx
11. Volumetric Pipets, appropriate sizes, class A, VWR, eg. Cat. No 53046-xxx
12. Eppendorf pipettors and tips, appropriate sizes

13. Disposable Pasteur Pipets, sizes 5 ¾ inch and 9 inch, VWR, Cat. No 14673-010, 72050-900
14. Autosampler Vials, 1.5 mL, clear, Sun, Cat No. 200 250
15. Vial caps with split septa, Sun, Cat No. 500 061
16. Amber bottles, 4oz, VWR Cat. No 16153-135
17. Porcelain Büchner funnels, 83 mm, Coors USA, VWR Cat. N° 300310-109
18. Graduated cylinders, and stoppers appropriate sizes, VWR, eg. Cat. N° 34795-0xx
19. Optional : Vacuum adaptor, #27 stopper joint top and bottom, top female stopper joint is unground and measures 60 mm from the top of the joint to the top of the inner flair, (used for filtration directly into mixing cylinders), Chem. Glass Inc., VWR Part N°. VWR-9794-27H

E. Analytical Standards

Analytical Standard available from Aventis CropScience

CAS Name

Thidiazuron (AE B049537):	<u>N</u> -phenyl- <u>N'</u> -1,2,3-thiadiazol-5-ylurea
Photo-thidiazuron (AE F132347)	<u>N</u> -phenyl- <u>N'</u> -1,2,5-thiadiazol-3-ylurea

III. Fortification and Calibration Standard Solutions

A. Preparation

The stated concentrations of standard solutions should be adjusted to account for the purity of the neat solid standards. After preparation, standards should be transferred from the volumetric flasks into screw-capped amber bottles. Store standard solutions in the refrigerator at less than 5°C when not in use.

The following is provided as an example of how standard solutions may be prepared. Other concentrations may be used as appropriate. Number of digits advises for precision.

1. For the fortification standards:
 - 1.1 Weigh 50.0 ± 0.1 mg of each analytical standard into individual 50 mL volumetric flasks. Dilute in methanol and mix well. Dilute to final volume

with methanol. The concentration of this standard is 1 mg/mL or 1000µg/mL.

1.2 Withdraw a 1.0 mL aliquot from each 1.0 mg/mL standard and add to a 100 mL volumetric flask. Dilute to final volume with 20/80:acetonitrile/water and mix well. The concentration of this standard is 10.0 µg/mL of mixed standard.

1.3 Withdraw a 100µL aliquot from the 10.0 µg/mL standard and add to a 100 mL volumetric flask. Dilute to volume with 20/80:acetonitrile/water and mix well. The concentration of this standard is 10.0 ng/mL in 20/80:acetonitrile/water.

2. For the calibration standards:

2.1 Make serial dilution of the 10 ng/mL standard with 20/80:acetonitrile/water to obtain a standard curve of 0.02, 0.04, 0.10, 0.20, 0.40 and 0.80 ng/mL.

B. Stability

Calibration standard and fortification solutions of thidiazuron and its metabolite in 20/80 acetonitrile/water have been shown to be stable for a period of at least three months when stored below 0°C.

IV. Method of Analysis

A. Method Tips and Observations

1. For convenience, crop samples can be weighed out and stored in a freezer for future analyses.
2. Exchange micro pipet tips between samples when pipetting sample solutions for dilutions.

B. Analytical Method

1. Extraction

1.1 Weigh 10.0 ± 0.1 g of crop sample into a pint Mason® jar.

1.2 Known control samples may be fortified at this point for quality control purposes. For example, to obtain a 0.05 ppm spike, fortify a 10 g wheat forage with 0.50 mL of 1.0 µg/mL mixed standard of thidiazuron and photo-thidiazuron. Allow fortified samples to equilibrate for about 5 to 10 minutes.

1.3 Add 100 mL of 80/20 acetonitrile/water to each sample and blend for 8 to 10 minutes at medium speed.

- 1.4 Filter samples under suction through a Buchner funnel equipped with a pre-wetted glass fiber filter paper into a 250 mL graduated cylinder. Rinse the mason jar with 5 to 10 mL of 80/20:acetonitrile/water. Filter the rinse and combine with the extract.
- 1.5 Remove Buchner funnel and rinse the filter adaper with 2 to 5 mL of 80/20:acetonitrile/water. Dilute sample extract to 200 mL with 80/20:acetonitrile/water solution.
- 1.6 Transfer a 250 μ L aliquot to a 10 mL volumetric flask and dilute to the mark with 20/80:acetonitrile/water.
- 1.7 Vial samples for LC/MS/MS analysis.

2. Dilution

- 2.1 Sample may be diluted at the analyst's discretion.

3. Preparation of Standard Solutions

1. The liquid chromatographic calibration standards are prepared as needed in 20:80:acetonitrile/water (See section A2).

V. Liquid Chromatography

A. Instrumentation

LC/MS/MS System	Perkin Elmer Sciex API 3000 LC/MS/MS system with PE Sciex TurboIonSpray Electrospray Interface; Shimadzu LC-10AD VP HPLC pumps (2) with 250 μ L high pressure mixer and SCL-10A VP Pump Controller; Gilson Series 215 autosampler
Ionization and MS Mode:	Electrospray (TurboIonSpray) - positive ion mode
MS Mode:	MS/MS with multiple reaction monitoring (MRM)
Ion Spray:	5200 V
Nebulizer Setting:	5.0 (Air)

Curtain Gas Setting: 7.0 (Nitrogen)
 TurboIonSpray Settings: Heated air at ~8.5L/min, 375°C
 Collision Gas Setting: 7 (Nitrogen)
 Mass Transitions, Collision Energy, Declustering and Focusing Potential:
 Thidiazuron: 221/102 amu, 19V 41V 150V
 Photo-thidiazuron: 221/94 amu 21V 36V 140V

(Dwell Times 200ms)

Column: Supelco Discovery® RP Amide C16 10.0 cm
 x 2.1mm 5µm-particle size with an inline filter

Mobile Phase Flow Rate: 0.300 mL/minute, no split

Mobile Phase Composition: 20% Acetonitrile/80% Water (0.1% acetic acid)
 initial conditions

Gradient Profile:	Acetonitrile/Water (0.1% acetic acid)	Time
	20/80	2.0
	80/20	6.0
	80/20	9.0
	20/80	9.5
	20/80	12.5

Injection Volume: 75µL,

Retention times: 5.77 min for Thidiazuron and 6.57 photo-thidiazuron

Note the indicated LC-MS-MS parameters are guidelines and should be optimized for the instrument and column actually used. Instrument parameters and mobile phase compositions may be adjusted to improve separation from interfering peaks.

Critical Steps None

VI. Calculations

A. Calibration Curves

1. Linear regression should be used to generate calibration curves for the analyte. At least four different standard concentrations should be run with each set of samples. Extracts should be diluted such that the peak areas obtained are within the area range between the lowest and highest standards injected.
2. Linear regression coefficients should be calculated from 'peak area' (or 'peak height') versus 'ng / mL injected'. Data from the analytical standards should be fit to the linear equation : $y = a + bx$.
 where: y = peak area or height
 a = calibration line intercept
 b = calibration line slope
 x = concentration of analyte in injected solution

B. Quantification of Residues

1. Thidiazuron and photo-thidiazuron can be quantitated by comparison to their respective standard curves obtained from a linear regression analysis of the data.
2. Equations
 - 2.1 Concentration of analyte in sample in ppb (parts per billion=ng/g).

$$\text{ppb} = \frac{(y - a)}{b} \times \frac{c}{d \times e}$$

where : y = peak area, response of analyte of interest
 a = intercept of the linear regression curve (area)
 b = slope of the linear regression curve (response per ng/mL)
 c = final volume of sample (mL)
 d = sample weight (g)
 e = aliquot correction factor ($e=0.00125$)
 ppb = conc of analyte in sample (ppb)

- 2.2 Percent recovery

$$\text{Recovery (\%)} = \frac{\text{analyte found in sample (ppb)} - \text{amount found in control (ppb)}}{\text{fortification level (ppb)}} \times 100$$

VII. Recovery Data

The following tables represent recoveries obtained during method verification.

Recoveries are corrected for amounts found in untreated control (UTC).

ND means not detected.

A. Mustard Greens

Sample ID	Fortification (ppb)	Recovery (%) AE B049537	Recovery (%) AE F132347
UTC 25	ND	ND	ND
UTC 26 + 50	50 ppb	98	103
UTC 27 + 50	50 ppb	104	91
UTC 28 + 100	100 ppb	106	115

B. Radish roots

Sample ID	Fortification (ppb)	Recovery (%) AE B049537	Recovery (%) AE F132347
UTC 22	ND	ND	ND
UTC 23 + 50	50 ppb	89	85
UTC 24 + 50	50 ppb	86	81

C. Radish Tops

Sample ID	Fortification (ppb)	Recovery (%) AE B049537	Recovery (%) AE F132347
UTC 19	ND	ND	ND
UTC 20 + 50	50 ppb	79	73
UTC 21 + 50	50 ppb	78	77

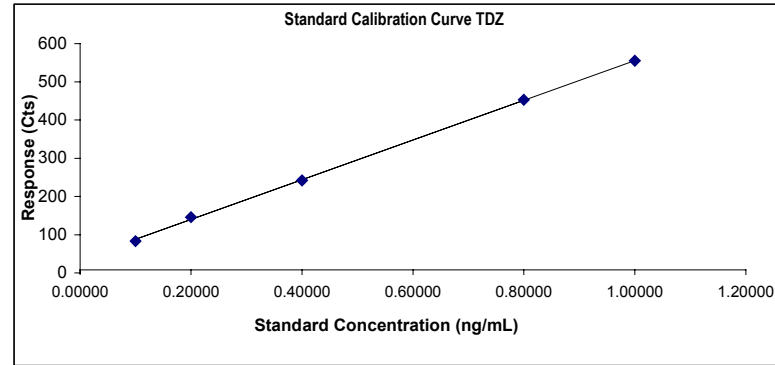
VIII APPENDIX I Typical Data and Example Chromatograms

Appendix I

Aventis CropScience

Residue Chemistry Calculation Spreadsheet

Study No.: TDZ/p-TDZ Set No.: Validation Set 2
 Analyst: Lee Williams Description: radish tops & roots
 Instrument: PE SCIEX API 3000 S/N 2899905 Room 2539
 Inj Date: 03-May-2001
 Completed: 04-May-2001



Standard Soln. Ref. #	Std. Conc. (ng/ml)	Response (Counts)	Calculated Line	Standard Calibration Curve		
				Slope (m)	Y-Int. (b)	Corr. Coeff.
	0.80000	70400.00	70428	87443.5	473.01075	0.9993
	0.10000	8660.00	9217			
	0.20000	19300.00	17962			
	0.40000	35000.00	35450			
	0.04000	3800.00	3971			
	0.02000	2090.00	2222			

Aventis Sample #	Sample Weight (g)	Amount Analyte Added (ng)	Fortification Level (ppm)	Initial Vol. (mLs) V ₁	Aliquot Vol. (mls) V ₂	Final Vol. (mLs) V ₃	Dilution Aliquot (mLs)	Dilution Volume (mLs)	Crop / Solv. Ratio (g/mL)	Response (Counts)	Concentration of p-TDZ (ng/mL) ^a	Determined Res. (ppm)	Determined Res. (ppb)	Percent Recovery
Section 1: Control Samples														
19 UTC radish tops	10.01	0.00	N/A	150 mL	0.250	10 mL	1 mL	1 mL	0.001668	0	N.D.	N.D.	N.D.	(BCR)
22 UTC radish roots	10.01	0.00	N/A	150 mL	0.250	10 mL	1 mL	1 mL	0.001668	0	N.D.	N.D.	N.D.	(BCR)
Section 2: Fortified Control Samples - Recoveries														
(20)UTC RT+ 0.05	10.01	500.00	50000.000	150 mL	0.250	10 mL	1 mL	1 mL	0.001668	6240.00	0.0660	39.5311	39531.0675	79.1%
(21)UTC RT+ 0.05	10.00	500.00	50000.000	150 mL	0.250	10 mL	1 mL	1 mL	0.001667	6160.00	0.0650	39.0217	39021.6730	78.0%
(23)UTC RR+ 0.05	10.03	500.00	50000.000	150 mL	0.250	10 mL	1 mL	1 mL	0.001672	6970.00	0.0743	44.4462	44446.2058	88.9%
(24)UTC RR+ 0.05	10.01	500.00	50000.000	150 mL	0.250	10 mL	1 mL	1 mL	0.001668	6750.00	0.0718	43.0270	43026.9721	86.1%
Section 3: Treated Samples														

crop/solvent ratio = (W / V₁) * (V₂ / V₃) * (dilution aliquot / dilution volume)

ug/ml Thiadiazuron = (peak response - b) / m

ppm = ug/ml / crop solvent ratio

^aCalculated value for concentration of Analyte (ug/mL) may not be zero due to linear fit not being forced through the origin.

N.D. = Not Detected (Zero Response) N/A = Not Applicable

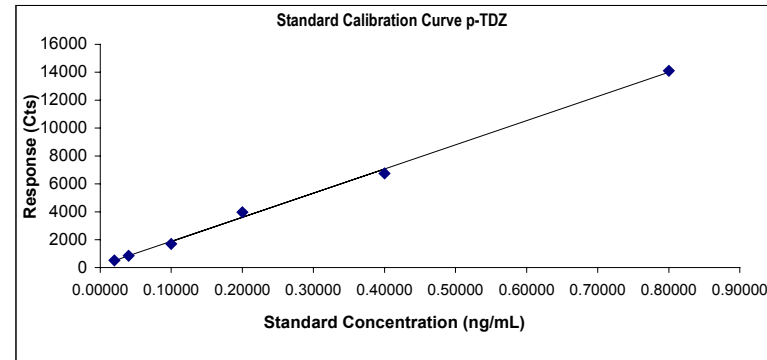
(BCR) Below Standard Calibration Curve

(OCR) Out of Standard Calibration Curve, Sample Requires Dilution

Aventis CropScience

Residue Chemistry Calculation Spreadsheet

Study No.: TDZ/p-TDZ Set No.: Validation Set 2
 Analyst: Lee Williams Description: Raddish Roots
 Instrument: PE SCIEX API 3000 S/N 2899905 Room 2539
 Inj Date: 03-May-2001
 Completed: 04-May-2001



Standard Soln. Ref. #	Std. Conc. (ng/ml)	Response (Counts)	Calculated Line	Standard Calibration Curve		
				Slope (m)	Y-int. (b)	Corr. Coeff.
	0.80000	14100.00	14007	17334.5	139.35215	0.9980
	0.10000	1700.00	1873			
	0.20000	3960.00	3606			
	0.40000	6750.00	7073			
	0.04000	853.00	853			
	0.02000	515.00	486			

Aventis Sample #	Sample Weight (g)	Amount Analyte Added (ng)	Fortification Level (ppm)	Initial Vol. (mLs) V ₁	Aliquot Vol. (mls) V ₂	Final Vol. (mLs) V ₃	Dilution Aliquot (mLs)	Dilution Volume (mLs)	Crop / Solv. Ratio (g/mL)	Response (Counts)	Concentration of p-TDZ (ng/mL) ^a	Determined Res. (ppm)	Determined Res. (ppb)	Percent Recovery
Section 1: Control Samples														
19 UTC raddish tops	10.01	0.00	N/A	150 mL	0.250	10 mL	1 mL	1 mL	0.001668	0	N.D.	N.D.	N.D.	(BCR)
22 UTC raddish roots	10.01	0.00	N/A	150 mL	0.250	10 mL	1 mL	1 mL	0.001668	0	N.D.	N.D.	N.D.	(BCR)
Section 2: Fortified Control Samples - Recoveries														
(20)UTC RT+ 0.05	10.01	500.00	50000.000	150 mL	0.250	10 mL	1 mL	1 mL	0.001668	1200.00	0.0612	36.6755	36675.4956	73.4%
(21)UTC RT+ 0.05	10.00	500.00	50000.000	150 mL	0.250	10 mL	1 mL	1 mL	0.001667	1250.00	0.0641	38.4428	38442.8196	76.9%
(23)UTC RR+ 0.05	10.03	500.00	50000.000	150 mL	0.250	10 mL	1 mL	1 mL	0.001672	1370.00	0.0710	42.4690	42468.9690	84.9%
(24)UTC RR+ 0.05	10.01	500.00	50000.000	150 mL	0.250	10 mL	1 mL	1 mL	0.001668	1310.00	0.0675	40.4791	40479.1186	81.0%
Section 3: Treated Samples														

crop/solvent ratio= (W / V₁) * (V₂/V₃) * (dilution aliquot / dilution volume)

ug/ml p-TDZ = (peak response - b) / m

ppm = ug/ml / crop solvent ratio

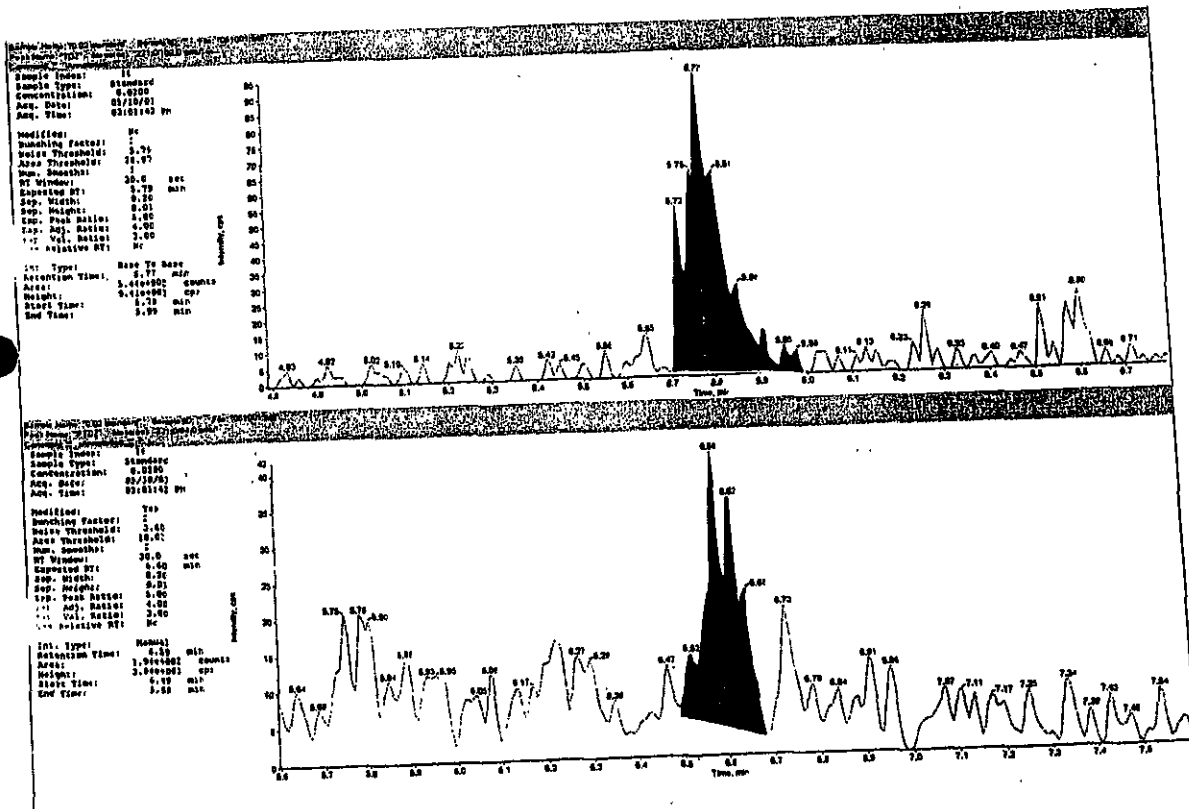
^aCalculated value for concentration of Analyte (ug/mL) may not be zero due to linear fit not being forced through the origin.

N.D. = Not Detected (Zero Response) N/A = Not Applicable

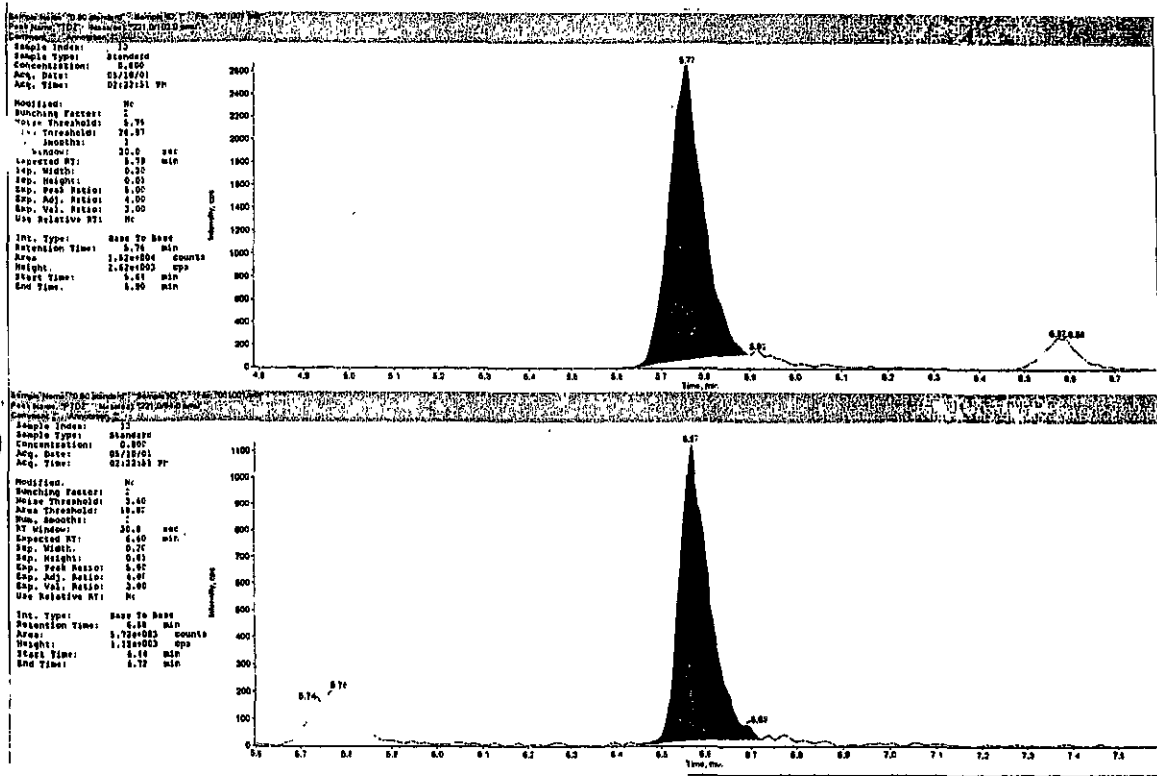
(BCR) Below Standard Calibration Curve

(OCR) Out of Standard Calibration Curve, Sample Requires Dilution

Chromatograms of 0.02 ng/mL Standard
Thidiazuron – top chromatogram
Photo-Thidiazuron – bottom chromatogram



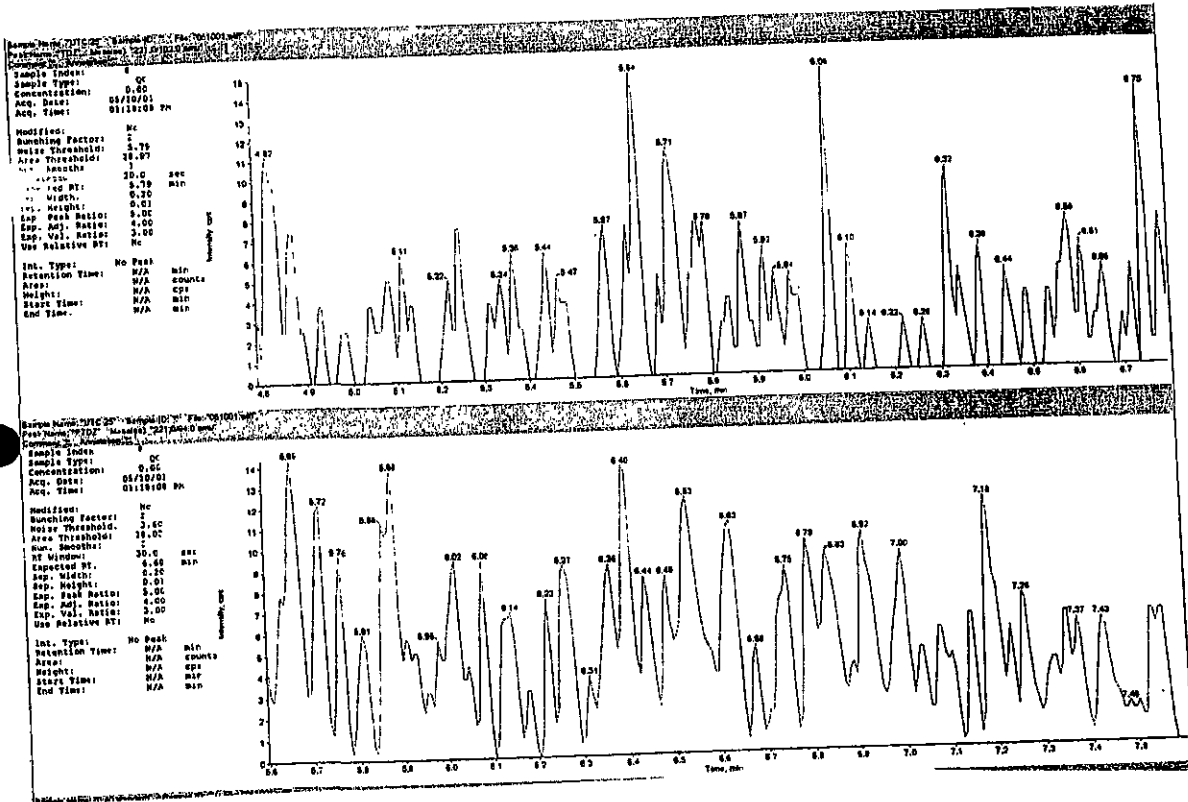
Chromatograms of 0.8 ng/mL Standard
Thidiazuron – top chromatogram
Photo-Thidiazuron – bottom chromatogram



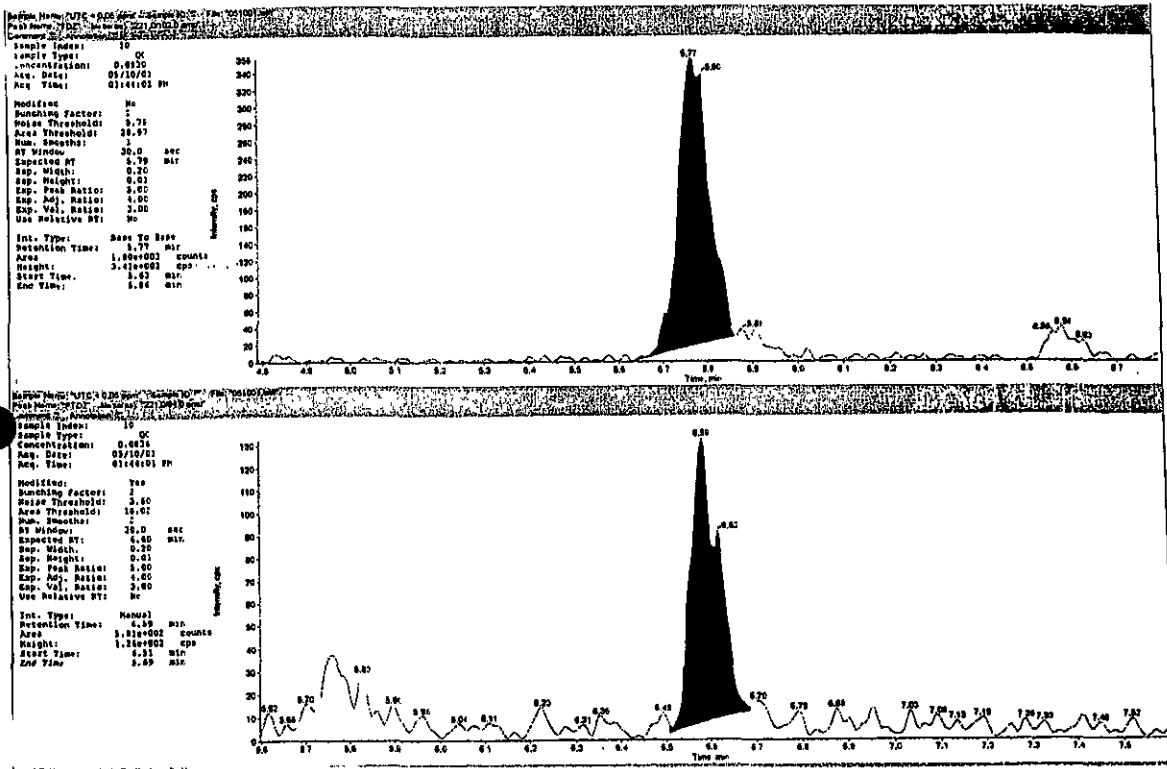
Chromatograms of Untreated Control Mustard Greens

Thidiazuron - top chromatogram

Photo-Thidiazuron - bottom chromatogram



Chromatograms of Mustard Greens Fortified
with 0.05 ppm, each, of Thidiazuron and Photo-Thidiazuron
Thidiazuron – top chromatogram
Photo-Thidiazuron – bottom chromatogram



Chromatograms of Mustard Greens Fortified with
0.1ppm, each, of Thidiazuron and Photo-Thidiazuron
Thidiazuron – top chromatogram
Photo-Thidiazuron – bottom chromatogram

