

Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

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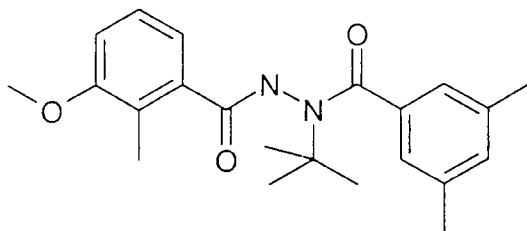


Determination of Residues of Methoxyfenozide in High Moisture Crops by Liquid Chromatography with Tandem Mass Spectrometry Detection

S. L. Byrne and M. J. Hastings

1. SCOPE

This method is applicable for the quantitative determination of residues of methoxyfenozide (3-methoxy-2-methyl benzoic acid 2-(3,5-dimethylbenzoyl)-2-(1,1-dimethylethyl) hydrazide). The method was validated in high moisture crops (carrots, sugar beets, radishes, onions) over the concentration range of 0.02-1.00 $\mu\text{g/g}$ with a validated limit of quantitation of 0.02 $\mu\text{g/g}$. For the purposes of this method, "high moisture" crops include all root and tuber vegetables, leaves of root and tuber vegetables, bulb vegetables, leafy vegetables, and brassica (cole) vegetables.



Methoxyfenozide
CAS No. 161050-58-4

The molecular formula and nominal mass for the above structure are provided in Table 1.

2. PRINCIPLE

Residues of methoxyfenozide are extracted from a high moisture crop sample by homogenizing with a 90% methanol/10% 0.1 N hydrochloric acid solution. The sample is shaken, centrifuged, and an aliquot of the extract is diluted with water and purified using a Phenomenex Strata 96-well solid phase extraction (SPE) plate. The SPE plate is washed with a 60% water/40% methanol/0.1% formic acid solution and eluted with acetonitrile. The eluate is evaporated to dryness and the residues reconstituted in a 70% water/30% acetonitrile mobile phase containing 0.1% formic acid. The purified extract is then analyzed by high performance liquid chromatography with tandem mass spectrometry (LC/MS/MS).

A calibration curve resulting from the injection of eight standard concentrations demonstrated linearity with a correlation coefficient of at least 0.9998. LC/MS/MS affords a highly specific method for both quantitation and confirmation of residue identity by retention time matching in conjunction with monitoring for both parent and product fragment ions, *m/z* 369.2 and 313.0, respectively.

3. SAFETY PRECAUTIONS

- 3.1. Each analyst must be acquainted with the potential hazards of the reagents, products, and solvents used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE: MATERIAL SAFETY DATA SHEETS, LITERATURE, AND OTHER RELATED DATA. Safety information on non Dow AgroSciences LLC products should be obtained from the container label or from the supplier. Disposal of reagents, reactants, and solvents must be in compliance with local, state, and federal laws and regulations.
- 3.2. Acetonitrile and methanol are flammable and volatile and should be used in well-ventilated areas away from ignition sources.
- 3.3. Formic acid and hydrochloric acid are corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be used when handling all chemicals.

4. EQUIPMENT (Note 12.1.)

4.1. Laboratory Equipment

- 4.1.1. Balance, analytical, Model AE200, Mettler-Toledo, Inc., Hightstown, NJ 08520.
- 4.1.2. Balance, pan, Model BB2440, Mettler-Toledo, Inc.
- 4.1.3. Centrifuge, with rotor to accommodate 8-oz jars, Model Centra-GP8, International Equipment Company, Needham Heights, MA 02194.
- 4.1.4. Evaporator, SPE-DualDry-96, Jones Chromatography, Lakewood, CO 80228.
- 4.1.5. Hammer mill, with 3/16-inch screen, Model 2001, AGVISE Laboratories, Inc., Northwood, ND 58267.
- 4.1.6. Homogenizer, Omni-mixer, Model ES, Omni International, Inc., Warrenton, VA 20187.
- 4.1.7. Homogenizer generator, Omni-mixer, 20-mm, catalog number 15010W, Omni International, Inc.
- 4.1.8. Pipetter, adjustable, Eppendorf, 10-100 μ L, catalog number 15010W, Omni International, Inc.

- 4.1.9. Pipetter, adjustable, Eppendorf, 50-1000 μ L, catalog number 21-378-83, Fisher Scientific Company, Pittsburgh, PA 15275.
- 4.1.10. Pipetter, adjustable, Eppendorf, 1000 to 5000 μ L, catalog number 22-46-134-6, Fisher Scientific.
- 4.1.11. Pipetter, positive displacement, 100-1000 μ L, model M1000, Gilson, Inc., Middleton, WI 53562.
- 4.1.12. Shaker, variable speed reciprocating with box carrier, Model 6000, Eberbach Corporation, Ann Arbor, MI 48106.
- 4.1.13. Ultrasonic cleaner, Model 1200, Branson Cleaning Equipment Company, Shelton, CT 06484.
- 4.1.14. Vacuum manifold, 96-well, catalog number 121-9601, International Sorbent Technology Ltd., Hengoed, Mid Glamorgan UK and distributed by Jones Chromatography USA, Inc.
- 4.1.15. Vortex mixer, Model G-560, Scientific Industries, Inc., Bohemia, NY 11716.
- 4.2. Chromatographic System
- 4.2.1. Column, analytical, Zorbax SB-C8, 4.6 x 75 mm, 3.5- μ m, catalog number 866953-906, Agilent Technology, Wilmington, DE 19808.
- 4.2.2. Liquid chromatograph autosampler, Model Series 1100, Agilent Technology.
- 4.2.3. Liquid chromatograph binary pump, Model Series 1100, Agilent Technology.
- 4.2.4. Liquid chromatograph degasser, Model Series 1100, Agilent Technology.
- 4.2.5. Mass spectrometer, Model API 3000, Applied Biosystems, Foster City, CA 94404.
- 4.2.6. Mass spectrometer data system, Analyst 1.1, Applied Biosystems.
- 4.2.7. Mass spectrometer data system, Model G1701CA, Agilent Technologies.
- 5. GLASSWARE AND MATERIALS (Note 12.1.)
- 5.1. Bottle, 8-oz (237-mL), round, wide-mouth, with PTFE-lined screw cap, catalog number B7768, National Scientific Company, Duluth, GA 30097.
- 5.2. Collection plate, 2-mL, catalog number 121-5203, International Sorbent Technology Ltd.

- 5.3. Collection plate sealing cap, catalog number 121-5205, International Sorbent Technology Ltd.
- 5.4. Culture tube, 12-mL (16 x 100 mm), with screw cap, catalog number 99449-16, Corning Products, Corning, NY 14831.
- 5.5. Cylinder, graduated, 100-mL, catalog number C7000-100, National Scientific Company.
- 5.6. Cylinder, graduated, 1000-mL, catalog number C7000-1L, National Scientific Company.
- 5.7. Cylinder, graduated, 2000-mL, catalog number C7000-2L, National Scientific Company.
- 5.8. Flask, volumetric, 100-mL, catalog number 161-8987, National Scientific Company.
- 5.9. Flask, Erlenmeyer, 250-mL, catalog number 617000-0424, Fisher Scientific.
- 5.10. Pipets, disposable Pasteur pipets (for transferring), catalog number 13-678-20A, Fisher Scientific Company.
- 5.11. Pipet, volumetric, 0.5-mL, catalog number 261-6010, National Scientific Company.
- 5.12. Pipet, volumetric, 1.0-mL, catalog number 261-6011, National Scientific Company.
- 5.13. Pipet, volumetric, 2.0-mL, catalog number 261-6012, National Scientific Company.
- 5.14. Pipet, volumetric, 3.0-mL, catalog number 261-6013, National Scientific Company.
- 5.15. Pipet, volumetric, 5.0-mL, catalog number 261-6015, National Scientific Company.
- 5.16. Pipet, volumetric, 10.0-mL, catalog number 261-6020, National Scientific Company.
- 5.17. Pipetter tips, Brinkmann Eppendorf, 1000 μ L tip, catalog number 22350901, Brinkmann Instruments, Inc., Westbury, NY 11590.
- 5.18. Pipetter tips, Brinkmann Eppendorf, 5-mL tip, catalog number 22350811, Brinkmann Instruments, Inc.
- 5.19. Pipetter tips, positive displacement, 1-mL tips, catalog number CP-1000, Gilson Inc.
- 5.20. SPE plate, Phenomenex Strata X 33 μ m, 30-mg packing, catalog number 8E-S100-TGB, Phenomenex, Torrance, CA 90501.

6. REAGENTS, STANDARDS, AND PREPARED SOLUTIONS (Note 12.1.)

6.1. Reagents

- 6.1.1. Acetonitrile, HPLC grade, catalog number 2856, Mallinckrodt Baker, Inc., Paris, KY 40361.
- 6.1.2. Formic Acid, 98% GR grade, catalog number FX0440-7, EM Science, Gibbstown, NJ 08027.
- 6.1.3. Hydrochloric acid, 0.1 N, certified concentration, catalog number SA54-1, Fisher Scientific, Pittsburgh, PA 15219.
- 6.1.4. Methanol, HPLC grade, catalog number 3041, Mallinckrodt Baker, Inc.
- 6.1.5. Nitrogen, refrigerated liquid, catalog number LQNI, BOC Gases, New Providence, NJ 07974.
- 6.1.6. Water, HPLC grade, catalog number WX0004-1, Fisher Scientific.

6.2. Standards

Methoxyfenozide (RH-2485): 3-methoxy-2-methyl benzoic acid 2-(3,5-dimethylbenzoyl)-2-(1,1-dimethylethyl) hydrazide

Obtain from Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268.

6.3. Prepared Solutions

- 6.3.1. 90% methanol/10% 0.1 N hydrochloric acid

Pour 1800 mL of methanol into a 2-L graduated mixing cylinder. Add 200 mL of 0.1 N hydrochloric acid. Place a glass stopper on the flask and invert several times to mix.

- 6.3.2. Water with 0.1% formic acid

Add 1.0 mL of formic acid to water in a 1-L graduated mixing cylinder and dilute to 1-L. Place a glass stopper on the cylinder and invert several times to mix.

6.3.3. Acetonitrile with 0.1% formic acid

Add 1.0 mL of formic acid to acetonitrile in a 1-L graduated mixing cylinder and dilute with acetonitrile to 1 L. Place a glass stopper on the cylinder and invert several times to mix.

6.3.4. 70% water/30% acetonitrile with 0.1% formic acid

Combine 700 mL of water containing 0.1% formic acid (6.3.2.) with 300 mL of acetonitrile containing 0.1% formic acid (6.3.3.) in a 1-L mixing cylinder, and mix well.

6.3.5. 60% water/40% methanol with 0.1% formic acid

Combine 120 mL of water and 80 mL of methanol in a stoppered flask. Using an electronic pipette, add 200 μ L of formic acid to the flask. Stopper and invert several times to mix.

7. PREPARATION OF STANDARDS

7.1. Preparation of Methoxyfenozide Fortification Solutions

7.1.1. Weigh 0.1000 g of methoxyfenozide analytical standard (RH-2485) and quantitatively transfer to a 100-mL volumetric flask. Dilute to volume with acetonitrile to obtain a 1000- μ g/mL stock solution.

7.1.2. Pipet 10.0 mL of the 1000- μ g/mL solution in Section 7.1.1 into a 100-mL volumetric flask and adjust to volume with acetonitrile to obtain a 100.0- μ g/mL stock solution.

7.1.3. Pipet 10.0 mL of the 100.0- μ g/mL solution in Section 7.1.2 into a 100-mL volumetric flask and adjust to volume with acetonitrile to obtain a 10.0- μ g/mL spiking solution.

7.1.4. Pipet 10.0 mL of the 10.0- μ g/mL mixed stock solution in Section 7.1.3 into a 100-mL volumetric flask and adjust to volume with acetonitrile to obtain a 1.0- μ g/mL spiking solution.

7.1.5. Pipet 10.0 mL of the 1.0- μ g/mL mixed stock solution in Section 7.1.4 into a 100-mL volumetric flask and adjust to volume with acetonitrile to obtain a 0.10- μ g/mL spiking solution.

7.2. Preparation of Methoxyfenozide Calibration Solutions

Prepare calibration solutions by diluting the 10.0- μ g/mL methoxyfenozide standard solution from Section 7.1.3 with the 70% water/30% acetonitrile containing 0.1% formic acid solution as described in the table below:

Original Standard Concentration	Aliquot of Original Standard	Final Solution Volume	Calib. Soln. Final Concentration	Equivalent Sample Concentration
$\mu\text{g/mL}$	mL	mL	$\mu\text{g/mL}$	$\mu\text{g/g}$
1.0	10.0	100.0	0.10	NA
1.0	5.0	100.0	0.05	2.0
1.0	3.5	100.0	0.035	1.4
1.0	2.0	100.0	0.02	0.8
1.0	1.0	100.0	0.01	0.4
0.10	5.0	100.0	0.005	0.2
0.10	1.0	100.0	0.001	0.04
0.01	5.0	100.0	0.0005	0.02
0.01	1.0	100.0	0.0001	0.004

8. LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS)

8.1. Typical Liquid Chromatography Operating Conditions (Note 12.2)

Instrumentation: Agilent Model 1100 autosampler
 Agilent Model 1100 binary pump
 Agilent Model 1100 degasser
 PE SCIEX API 3000 LC/MS/MS System
 PE SCIEX Analyst 1.1 data system

Column: Zorbax SB-C8
 4.6 x 75 mm, 3.5- μm

Column Temperature: 35 °C

Injection Volume: 25 μL

Run Time: 8.0 minutes

Mobile Phase: A –Water with 0.1% formic acid
 B –Acetonitrile with 0.1% formic acid

Flow Rate: 900 $\mu\text{L}/\text{min}$

Gradient:	Time, min	A, %	B, %
	0.0	70	30
	1.0	70	30
	8.0	10	90

8.2. Typical Mass Spectrometry Operating Conditions

API 3000:

Interface: TurbolonSpray
Polarity: Positive
Scan Type: MRM
Resolution: Q1 – unit, Q3 – low
Curtain Gas (CUR): 14
Collision Gas (CAD): 4.0
Temperature (TEM): 350 °C
Ion Source Gas 1 (GS1): 8
Ion Source Gas 2 (GS2): 7000
Period 1

Time: 8.0 minutes
Polarity: Positive
IonSpray Voltage (IS) 5500
Compound:

	<u>Ion, m/z</u>		<u>Time, ms</u>	<u>Collision Energy, V</u>
	<u>Q1</u>	<u>Q3</u>		
Methoxyfenozide	369.2	313.0	150	13

8.3. Mass Spectra

Typical mass spectrum of methoxyfenozide (RH-2485) and the product ion mass spectrum are presented in Figure 1.

8.4. Typical Calibration Curve

A typical calibration curve for the determination of methoxyfenozide is presented in Figure 2.

8.5. Typical Chromatograms

A typical chromatogram of a standard equivalent to the LOQ is shown in Figure 3. Typical chromatograms of a control sample, a 0.02- $\mu\text{g/g}$ (LOQ) recovery sample, and a 1.0- $\mu\text{g/g}$ recovery sample for the determination of methoxyfenozide in high moisture crops (sugar beet tops) are also illustrated in Figure 3.

9. DETERMINATION OF RECOVERY OF METHOXYFENOZIDE FROM HIGH MOISTURE CROPS

9.1. Method Validation

Validate the analytical procedure given in Section 9.3 by analyzing the following with each sample set:

At least one reagent blank.

At least one unfortified control.

At least one control fortified at the proposed limit of detection.

At least two controls fortified at the limit of quantitation.

At least two controls fortified at a level exceeding the expected residue concentration in the samples.

9.2. Sample Preparation

Prepare the samples for analysis by freezing with liquid nitrogen or dry ice and then grinding or chopping using a hammer mill with a 1/8-3/16 inch screen. Prepared samples are stored frozen at approximately -10 to -20 °C prior to analysis.

9.3. Sample Analysis

9.3.1. Weigh $5.0 \text{ g} \pm 0.05 \text{ g}$ of the sample into an 8-oz jar with a Teflon-lined lid. For recovery samples, add appropriate aliquots of the spiking solutions (Section 7.1) to obtain concentrations ranging from 0.02 $\mu\text{g/g}$ to 1.0 $\mu\text{g/g}$.

9.3.2. Add 100 mL of the 90% methanol/10% 0.1N hydrochloric acid extraction solution to the sample jar. Allow the sample to stand for approximately 30 minutes.

9.3.3. Homogenize the sample for approximately 1 minute, at approximately 10,000 rpm, with an Omni homogenizer fitted with a 20-mm probe.

9.3.4. Shake the sample for approximately 30 minutes at approximately 180 excursions per minute on a reciprocal shaker.

9.3.5. Centrifuge the sample jar for approximately 5 minutes at approximately 2000 rpm.

9.3.6. Using a positive-displacement pipet, transfer 1.0 mL of the sample solution into a 16-mm culture tube.

9.3.7. Dilute the sample from Step 9.3.6 with 2.0 mL of water and vortex mix for approximately 5 seconds.

- 9.3.8. Purify samples using the following SPE procedure (see Section 11.4.):
- a. Condition a Phenomenex Strata 96-well plate (30-mg) with 1.0 mL of methanol followed by 1.0 mL of water. Dry the plate under full vacuum for 10 seconds between solvents.
 - b. Transfer 1.5 mL of the sample solution from Step 9.3.7 to the 96-well plate. Pull the sample through the plate at approximately 1 mL/min, discarding the eluate. Dry the plate under full vacuum for 10 seconds after sample has eluted.
 - c. Wash the plate with 1 mL of a 60% water/40% methanol/0.1% formic acid solution, discarding the eluate. Dry the cartridge under full vacuum for 5 minutes.
 - d. Elute the methoxyfenozide from the plate at approximately 1 mL/min with two 750- μ L aliquots of acetonitrile, collecting the eluate in a 2-mL deep-well rack.
- 9.3.9. Evaporate the acetonitrile to dryness at approximately 50 °C using nitrogen (1 L/min) on a 96-well evaporator.
- 9.3.10. Reconstitute the samples in 1.0 mL of 70% water/30% acetonitrile containing 0.1% formic acid.
- 9.3.11. Transfer the calibration standards to unused wells and cap the deep-well collection plate. Gently vortex mix for 10-20 seconds and sonicate for approximately 20 seconds.
- 9.3.12. Analyze the samples along with the calibration standards using the LC/MS/MS conditions listed in Section 8. Determine the suitability of the chromatographic system using the following criteria:
- a. Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration.
 - b. Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figure 3 with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for the 0.0005- μ g/mL calibration standard (equivalent to 0.02 μ g/g of methoxyfenozide in the crop sample).
- 9.3.13. Dilute the samples exceeding the range of the standard curve with 70% water/30% acetonitrile containing 0.1% formic acid until the sample concentrations are within the range of the standard curve.

10. CALCULATIONS

10.1. Calculation of Standard Calibration Curve

- 10.1.1. Inject the series of calibration standards described in Section 7.2 using the conditions listed in Section 8, and determine the peak areas for methoxyfenozide as indicated below.

Methoxyfenozide (RH-2485) *m/z* Q1/Q3 369/313

- 10.1.2. Prepare a standard curve by plotting the equivalent analyte concentration on the abscissa (x-axis) and the respective peak area on the ordinate (y-axis) as shown in Figure 2. Using linear regression analysis (13.1), forced through zero, determine the equation for the curve with respect to the abscissa. Another option for calculations is to use a linear regression with a 1/x weighting rather than forcing through zero.

For example, using linear regression with the methoxyfenozide data from Figure 2:

$$X = \left(\frac{Y - \text{intercept}}{\text{slope}} \right)$$

$$\text{Methoxyfenozide} \quad = \quad \left(\frac{\text{Methoxyfenozide} - \text{intercept}}{2.82e7} \right)$$

($\mu\text{g/g}$)

$$\text{Methoxyfenozide} \quad = \quad \left(\frac{\text{Methoxyfenozide Peak Area} - 0}{2.82e7} \right)$$

($\mu\text{g/g}$)

10.2. Calculation of Percent Recovery

- 10.2.1. Determine the gross concentration in each recovery sample by substituting the peak area into the above equation and solving for the concentration.

For example, using the data for methoxyfenozide from Figure 3c:

$$\text{Methoxyfenozide} \quad = \quad \left(\frac{\text{Methoxyfenozide peak area} - 0}{2.82e7} \right)$$

($\mu\text{g/g}$)

$$\text{Methoxyfenozide} \quad = \quad \left(\frac{1.32e4 - 0}{2.82e7} \right)$$

($\mu\text{g/g}$)

$$\begin{array}{l} \text{Methoxyfenozide Conc.} \\ \text{(gross)} \end{array} = 0.000468 \mu\text{g/mL}$$

Convert the concentration of $\mu\text{g/mL}$ of methoxyfenozide found in the final sample prepared for analysis to $\mu\text{g/g}$ of methoxyfenozide in the original crop sample aliquot as follows:

$$\text{Methoxyfenozide } (\mu\text{g/g}) = 0.000468 \mu\text{g/mL} \times \left(\frac{\frac{100 \text{ mL}}{1 \text{ mL}} \times \frac{3 \text{ mL}}{1.5 \text{ mL}} \times 1 \text{ mL}}{5 \text{ g}} \right)$$

$$\text{Methoxyfenozide Conc.} = 0.0187 \mu\text{g/g}$$

- 10.2.2. Determine the net concentration of methoxyfenozide in each recovery sample by subtracting any peak area response found at the retention time of methoxyfenozide in the untreated control sample from that of the gross methoxyfenozide concentration in the recovery sample.

For example, using the data for methoxyfenozide from Figure 3:

$$\begin{array}{l} \text{Methoxyfenozide} \\ \text{(net } \mu\text{g/g)} \end{array} = \begin{array}{l} \text{Methoxyfenozide conc.} \\ \text{(gross } \mu\text{g/g)} \end{array} - \begin{array}{l} \text{Methoxyfenozide conc.} \\ \text{(control } \mu\text{g/g)} \end{array}$$

$$\begin{array}{l} \text{Methoxyfenozide} \\ \text{(net } \mu\text{g/g)} \end{array} = 0.0187 \mu\text{g/g} - 0.0000 \mu\text{g/g}$$

$$\begin{array}{l} \text{Methoxyfenozide Conc.} \\ \text{(net)} \end{array} = 0.0187 \mu\text{g/g}$$

- 10.2.3. Determine the percent recovery by dividing the net concentration of each recovery sample by the theoretical concentration added.

$$\text{Recovery} = \frac{\text{Concentration Found}}{\text{Concentration Added}} \times 100\%$$

$$\text{Recovery} = \frac{0.0187 \mu\text{g/g}}{0.0200 \mu\text{g/g}} \times 100\%$$

$$\text{Recovery} = 94\%$$

10.3. Determination of Methoxyfenozide in High Moisture Crops

10.3.1. Determine the gross concentration of methoxyfenozide in each treated sample by substituting the peak area obtained into the equation for the standard calibration curve and calculating the uncorrected residue result as described in Section 10.2.1.

10.3.2. For those samples that require correction for method recovery, use the average recovery of all the recovery samples from a given sample set to correct for method efficiency.

For example, using the average LOQ recovery data for methoxyfenozide from Table 2 for the samples analyzed on 23-Apr-2002:

$$\text{Methoxyfenozide (corrected } \mu\text{g/g)} = \text{Methoxyfenozide conc. (corrected } \mu\text{g/g)} \times \left(\frac{100}{\% \text{ Recovery}} \right)$$

$$\text{Methoxyfenozide (corrected } \mu\text{g/g)} = 0.0187 \mu\text{g/g} \times \frac{100}{91.6}$$

$$\text{Methoxyfenozide (corrected)} = 0.0204 \mu\text{g/g}$$

11. RESULTS AND DISCUSSION

11.1. Method Validation

11.1.1. Recovery Levels and Precision

A method validation study was conducted to determine the recovery levels and the precision of the method for the determination of methoxyfenozide in high moisture crops. The results are summarized below and in Table 3.

Matrix	Validation Range ($\mu\text{g/g}$)	Number of Samples (n)	Average Percent Recovery	Standard Deviation (s)
High Moisture Crops	0.02–1.00	48	88	8

11.1.2. Standard Curve Linearity

For the linear least squares regression equations describing the detector response as a function of the standard calibration curve concentrations, the correlation coefficients (r^2) were greater than or equal to 0.9998 for all of the calibration curve determinations during the method validation. The results indicate linearity of the detector response as a function of the standard calibration curve.

11.1.3. Calculated Limits of Quantitation and Detection

Following established guidelines (13.2.), the limits of quantitation (LOQ) and detection (LOD) for the determination of methoxyfenozide in high moisture crops were calculated using the standard deviation from the 0.02- $\mu\text{g/g}$ recovery results. The LOQ was calculated as ten times the standard deviation ($10s$), and the LOD was calculated as three times the standard deviation ($3s$) of the results of the analysis of 18 samples. The results are summarized below.

Matrix	Average Recovery ($\mu\text{g/g}$)	Standard Deviation (s)	Limit of Detection ($3s$)	Limit of Quantitation ($10s$)
High Moisture Crops	0.0178	0.0019	0.0056	0.019

The calculated LOQ supported the method LOQ of 0.02 $\mu\text{g/g}$. The calculated LOD was 0.006 $\mu\text{g/g}$. In actual residue samples, numerical results should be reported as less than the LOQ ($<0.02 \mu\text{g/g}$) for residues that are above the LOD but less than the validated LOQ. For results less than the LOD, numerical results should be reported as not detected.

11.2. Specificity of Method and Confirmation of Residue Identity

The method is specific for the determination of methoxyfenozide by virtue of the chromatographic separation and selective detection system used. Confirmation of the presence of methoxyfenozide is based on retention time comparison (high performance liquid chromatography). Further confirmation is not considered necessary due to the highly specific nature of the MS-MS transition monitored during quantitation.

11.3. Assay Time

A typical analytical run would consist of a minimum of eight standards encompassing the expected range of sample concentrations, a reagent blank, a control (a non-fortified sample), a minimum of two fortified controls (one of which must be at the LOQ), and 20 samples. This typical analytical run can be prepared in approximately six hours, followed by the chromatographic analysis.

There are several acceptable “stopping points” in the method, where sample preparation (Section 9.) may be suspended, upon completion of a step, without deleterious effects on the sample analysis. These are indicated below:

- a. Step 9.3.1. (weighing only, samples stored frozen)
- b. Step 9.3.5.
- c. Step 9.3.6.
- d. Step 9.3.11.

If samples are to be stored overnight, the vials should be capped with PTFE-lined caps and stored in a refrigerator (except after Step 9.3.1 as noted above).

11.4. Standardization of Strata X 33 Solid-Phase Column Elution Profile

Variation in the Strata X 33 SPE columns may influence the elution profile of methoxyfenozide. It is necessary to obtain an elution profile for each lot of SPE columns used to ensure optimum recovery and clean-up efficiency. The following procedure should be used:

- 11.4.1. To a 12-mL vial containing 1.0 mL of the 90% methanol/10% 0.1 N hydrochloric acid solution, add 10.0 μ L of the 10.0- μ g/mL spiking solution from Section 7.1.3.
- 11.4.2. Place a Phenomenex Strata X 33 SPE (30-mg) column on the vacuum manifold.
- 11.4.3. Condition the SPE column cell to be used with 1.0 mL methanol followed by 1.0 mL water. Pull the plate dry at full vacuum for 10 seconds between solvents.
- 11.4.4. Dilute the sample from Step 11.4.1 with 2.0 mL water and vortex mix.
- 11.4.5. Transfer 1.5 mL of the sample from Step 11.4.4 to the prepared SPE well, and slowly pull the sample through the column at a flow rate of approximately 1 mL/min with the aid of vacuum. Discard the eluate. Pull the plate dry at full vacuum for 10 seconds after elution.
- 11.4.6. Wash the prepared SPE well with 1 mL of the 60% water/40% methanol with 0.1% formic acid, collecting the eluate in a 2-mL deep-well collection plate. Dry the SPE cartridge for 5 minutes at full vacuum. Concentrate the eluted aliquot as described in Section 11.4.8., below; sample will not dry. Bring the final volume to 1.0 mL using of 70% water/30% acetonitrile with 0.1% formic acid, by comparison of the height of the liquid with the reconstituted acetonitrile fractions (Section 11.4.8.).
- 11.4.7. Elute the methoxyfenozide from the plate with three 0.75-mL aliquots of acetonitrile at approximately 1 mL/min collecting each eluate in a separate 2-mL deep-well collection rack.
- 11.4.8. Evaporate the acetonitrile to dryness at 50 °C using nitrogen (1 L/min). Reconstitute the samples in 1.0 mL of 70% water/30% acetonitrile with 0.1% formic acid and mix gently (vortex and sonicate).
- 11.4.9. Analyze the calibration standards (Section 7.2.) and samples by LC/MS/MS as described in Section 8.
- 11.4.10. Calculate the percent recovery for methoxyfenozide as described in Section 10.2. A typical elution profile is illustrated in Figure 4.

11.4.11. If the elution profile differs from that shown in Figure 4, modify the volumes of eluants to be discarded and collected in Section 9.3.8.

12. NOTES

12.1. Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are assumed to be readily available and are, therefore, not listed.

12.2. The data in this method was generated using a Sciex API 3000 in optimal condition. Operating conditions may be modified to obtain optimal separation or sensitivity; however, method performance will be compromised by increasing the injection volume to compensate for low instrument sensitivity.

13. REFERENCES

13.1. Freund, J. E.; Williams, F. J. *Dictionary/Outline of Basic Statistics*; Dover: New York, 1991; p 170.

13.2. Keith, L. H.; Crummett, W.; Deegan, J., Jr.; Libby, R. A.; Taylor, J. K.; Wentler, G. *Anal. Chem.* **1983**, *55*, 2210-2218.

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Table 1. Identity and Structure of Methoxyfenozide (RH-2485)

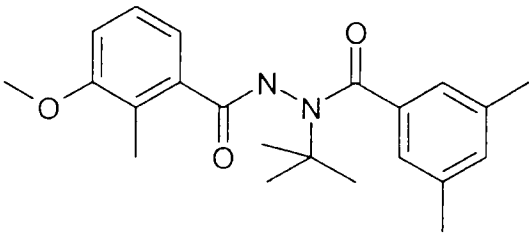
Common Name of Compound	Structure and CAS Name
<p>Methoxyfenozide (RH-2485)</p> <p>Molecular Formula: $C_{22}H_{28}N_2O_3$</p> <p>Nominal Mass: 368</p> <p>CAS Number: 161050-58-4</p>	

Table 2. Recovery of Methoxyfenozide (RH-2485) in High Moisture Crops

Sample Number	Sample Matrix	Date of Analysis ^a	Methoxyfenozide, µg/g		Percent Recovery
			Added	Found	
SN37507101	carrot	23-Apr-02	0	ND ^b	--
SN37509801	potato	23-Apr-02	0	ND	--
SN37512801	sugar beet roots	23-Apr-02	0	ND	--
SN37513601	sugar beet tops	23-Apr-02	0	ND	--
SN37527601	radish roots	25-Apr-02	0	ND	--
SN37528401	radish tops	25-Apr-02	0	ND	--
SN37526801	green onion	25-Apr-02	0	ND	--
SN37529201	bulb onion	25-Apr-02	0	ND	--
SN37507101	carrot	23-Apr-02	0.006	(0.00582) ^c	NA ^d
SN37509801	potato	23-Apr-02	0.006	(0.00548)	NA
SN37512801	sugar beet roots	23-Apr-02	0.006	(0.00578)	NA
SN37513601	sugar beet tops	23-Apr-02	0.006	(0.00538)	NA
SN37527601	radish roots	25-Apr-02	0.006	(0.00515)	NA
SN37528401	radish tops	25-Apr-02	0.006	(0.00533)	NA
SN37526801	green onion	25-Apr-02	0.006	(0.00557)	NA
SN37529201	bulb onion	25-Apr-02	0.006	(0.00485)	NA
SN37507101	carrot	23-Apr-02	0.02	0.0190	95
SN37507101	carrot	23-Apr-02	0.02	0.0188	94
SN37509801	potato	23-Apr-02	0.02	0.0185	93
SN37509801	potato	23-Apr-02	0.02	0.0189	95
SN37512801	sugar beet roots	23-Apr-02	0.02	0.0170	85
SN37512801	sugar beet roots	23-Apr-02	0.02	0.0184	92
SN37513601	sugar beet tops	23-Apr-02	0.02	0.0173	87
SN37513601	sugar beet tops	23-Apr-02	0.02	0.0187	94
SN37527601	radish roots	25-Apr-02	0.02	0.0157	79
SN37527601	radish roots	25-Apr-02	0.02	0.0157	79
SN37528401	radish tops	25-Apr-02	0.02	0.0173	87
SN37528401	radish tops	25-Apr-02	0.02	0.0162	81
SN37528401	radish tops	27-Jun-02	0.02	0.0220	110
SN37526801	green onion	25-Apr-02	0.02	0.0166	83
SN37526801	green onion	25-Apr-02	0.02	0.0176	88
SN37529201	bulb onion	25-Apr-02	0.02	0.0157	79
SN37529201	bulb onion	25-Apr-02	0.02	0.0154	77
SN37529201	bulb onion	27-Jun-02	0.02	0.0215	108

Table 2. (Cont.) Recovery of Methoxyfenozide (RH-2485) in High Moisture Crops

Sample Number	Sample Matrix	Date of Analysis ^a	Methoxyfenozide, $\mu\text{g/g}$		Percent Recovery
			Added	Found	
SN37507101	carrot	23-Apr-02	0.20	0.180	90
SN37509801	potato	23-Apr-02	0.20	0.187	94
SN37512801	sugar beet roots	23-Apr-02	0.20	0.180	90
SN37513601	sugar beet tops	23-Apr-02	0.20	0.184	92
SN37527601	radish roots	25-Apr-02	0.20	0.154	77
SN37527601	radish roots	27-Jun-02	0.20	0.172	86
SN37528401	radish tops	25-Apr-02	0.20	0.161	80
SN37528401	radish tops	27-Jun-02	0.20	0.192	96
SN37528401	radish tops	27-Jun-02	0.20	0.191	96
SN37528401	radish tops	27-Jun-02	0.20	0.189	95
SN37526801	green onion	25-Apr-02	0.20	0.167	84
SN37529201	bulb onion	25-Apr-02	0.20	0.159	79
SN37529201	bulb onion	27-Jun-02	0.20	0.199	100
SN37529201	bulb onion	27-Jun-02	0.20	0.201	101
SN37529201	bulb onion	27-Jun-02	0.20	0.199	100
SN37507101	carrot	23-Apr-02	1.00	0.888	89
SN37509801	potato	23-Apr-02	1.00	0.895	90
SN37512801	sugar beet roots	23-Apr-02	1.00	0.836	84
SN37513601	sugar beet tops	23-Apr-02	1.00	0.929	93
SN37527601	radish roots	25-Apr-02	1.00	0.798	80
SN37527601	radish roots	27-Jun-02	1.00	0.829	83
SN37528401	radish tops	25-Apr-02	1.00	0.807	81
SN37528401	radish tops	27-Jun-02	1.00	0.835	84
SN37528401	radish tops	27-Jun-02	1.00	0.849	85
SN37528401	radish tops	27-Jun-02	1.00	0.863	86
SN37526801	green onion	25-Apr-02	1.00	0.809	81
SN37529201	bulb onion	25-Apr-02	1.00	0.806	81
SN37529201	bulb onion	27-Jun-02	1.00	0.857	86
SN37529201	bulb onion	27-Jun-02	1.00	0.871	87
SN37529201	bulb onion	27-Jun-02	1.00	0.834	83

x = 88
s = 8
n = 48

^a The 'Date of Analysis' indicates the date that the samples were extracted.

^b ND = not detected. The residue was below the 0.006- $\mu\text{g/g}$ limit of detection.

^c () indicates that the concentration is less than the limit of quantitation of 0.02 $\mu\text{g/g}$. Values are reported with a lower degree of confidence than values above the limit of quantitation.

^d NA = not applicable. The residue was below the 0.02- $\mu\text{g/g}$ limit of quantitation.

Table 3. Summary of the Recovery of Methoxyfenozide (RH-2485) in High Moisture Crops

Matrix	Fortification Level (mg/kg)	Methoxyfenozide			
		Recovery Rate (%)		RSD (%)	n
		Mean	Range		
High Moisture Crops	0.02	89	77-110	10	18
	0.20	90	77-100	8	15
	1.00	85	80-93	4	15
	0.02-1.00	88	77-110	9	48

All calculations were done using Microsoft Excel 97 with full precision.

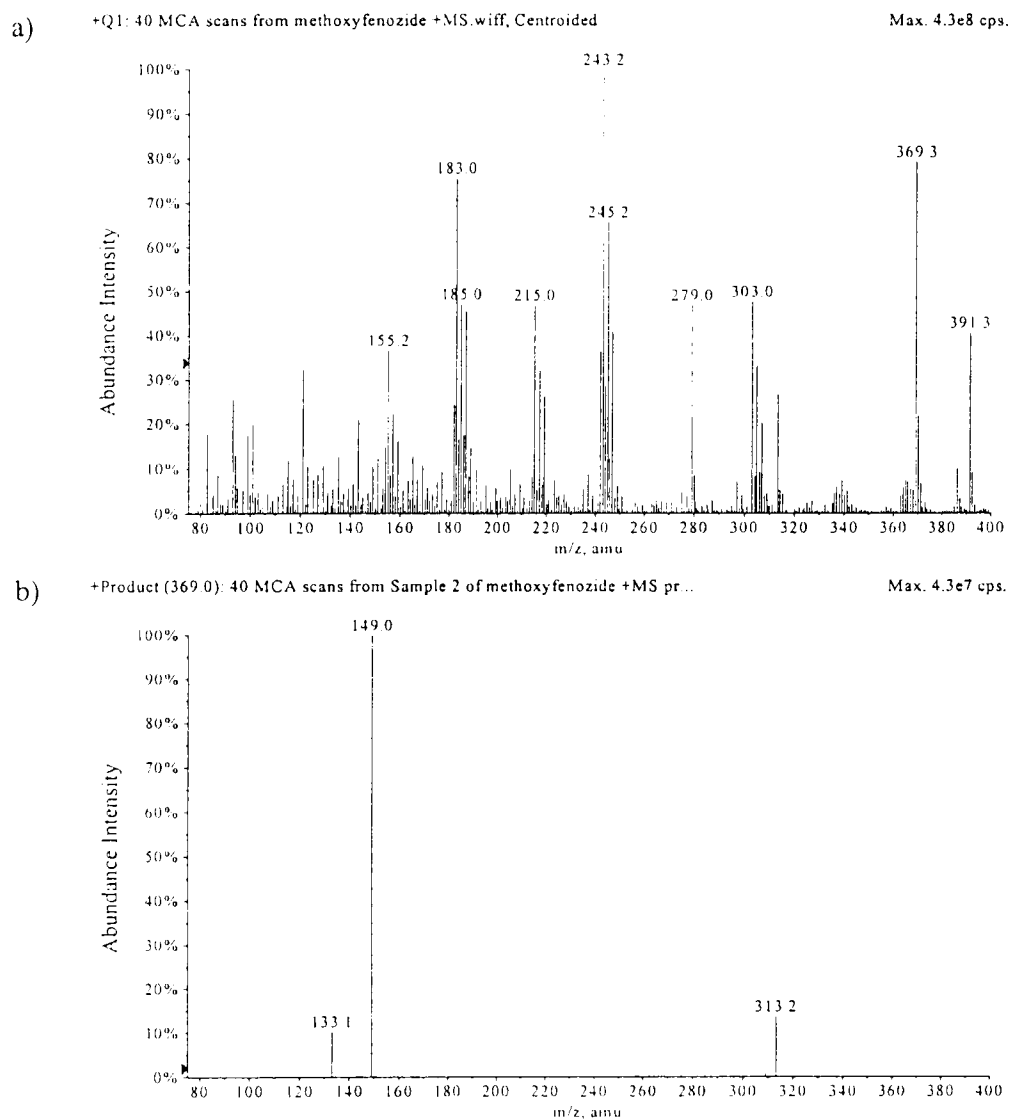
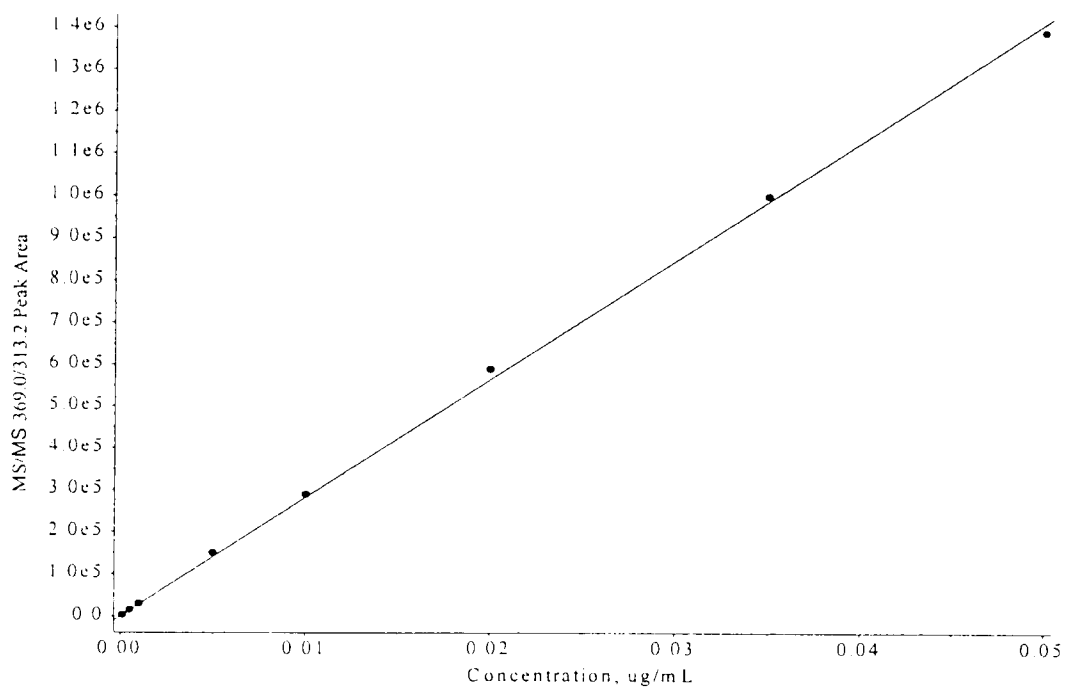


Figure 1. Mass Spectra for Methoxyfenozide: a) Mass Spectrum for Q1 Scan Using Positive Electrospray Ionization (M+H)⁺ at *m/z* 369, b) Product-ion Mass Spectrum of Methoxyfenozide Showing Fragment Ion at *m/z* 313

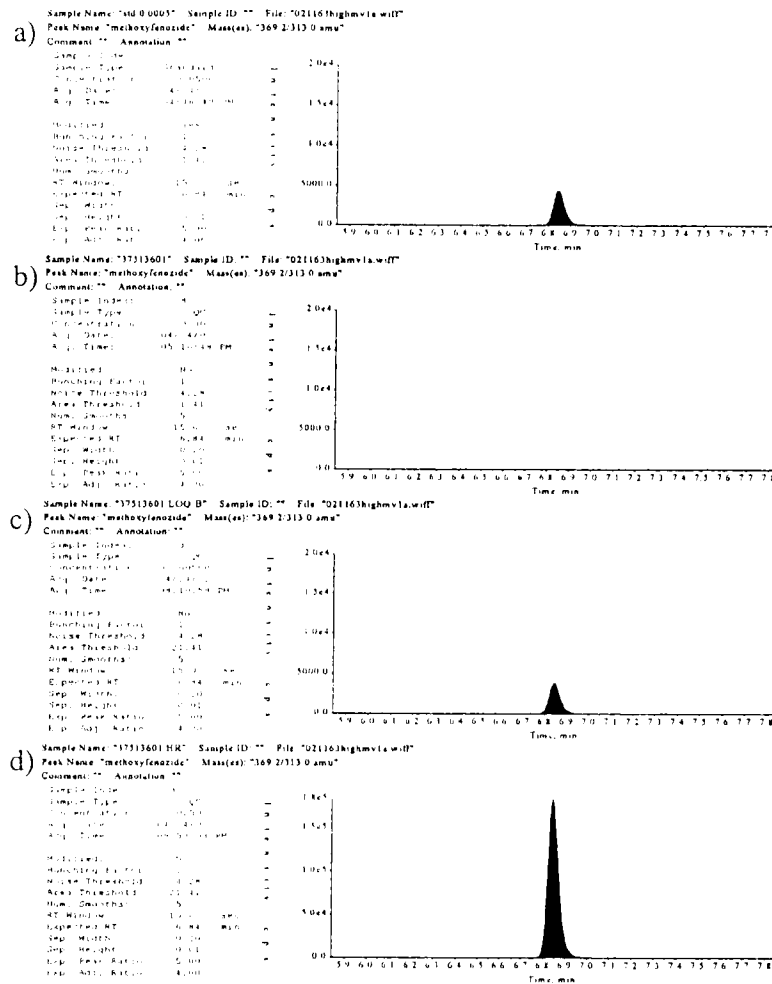
(methoxyfenozide): "Linear Through Zero" Regression ("No" weighting): $y = 2.82e+007 x$ ($r = 0.9998...$)



Methoxyfenozide Concentration µg/mL	Equivalent Sample Concentration µg/g	Methoxyfenozide Peak Area
0.0001	0.004	3.05E+03
0.0005	0.02	1.55E+04
0.001	0.04	3.06E+04
0.005	0.2	1.51E+05
0.01	0.4	2.89E+05
0.02	0.8	5.89E+05
0.035	1.4	9.99E+05
0.05	2.0	1.36E+06

Linear Equation: $Y = 2.82 \times 10^7 X$
 Correlation Coefficient (r^2): 0.9998

Figure 2. Typical Calibration Curve for the Determination of Methoxyfenozide in High Moisture Crops



a) 0.0005 µg/mL Standard	
Methoxyfenozide (369.0/313.2) Peak Area	1.51E+04
Equivalent Concentration (µg/g)	0.0200
Concentration Found (µg/g)	0.0214
Accuracy	107%

b) Control Sugar Beet Tops (37513601)	
Methoxyfenozide (369.0/313.2) Peak Area	0.00E+00
Concentration Added (µg/g)	0.0000
Concentration Found (µg/g)	0.0000
Percent Recovery	NA

c) 0.02-µg/g Sugar Beet Tops Recovery	
Methoxyfenozide (369.0/313.2) Peak Area	1.32E+04
Concentration Added (µg/g)	0.0200
Concentration Found (µg/g)	0.0187
Percent Recovery	94%

d) 1.0-µg/g Sugar Beet Tops Recovery	
Methoxyfenozide (369.0/313.2) Peak Area	6.55E+05
Concentration Added (µg/g)	1.0000
Concentration Found (µg/g)	0.929
Percent Recovery	93%

Figure 3. Typical Chromatograms of a) a 0.0005-µg/mL Standard Equivalent to 0.02 mg/kg of Methoxyfenozide in High Moisture Crops, and the Following Sugar Beet Tops Samples: b) Control Containing No Detectable Residue of Methoxyfenozide, c) 0.02-µg/g (LOQ) Recovery, and d) 1.0-µg/g Recovery

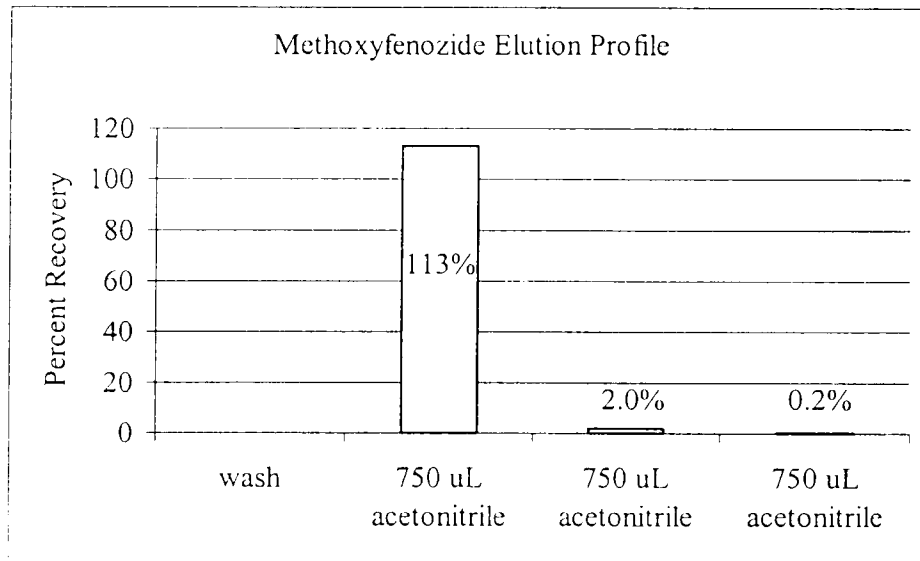


Figure 4. Typical Phenomenex Strata X 33 SPE Elution Profile for Methoxyfenozide