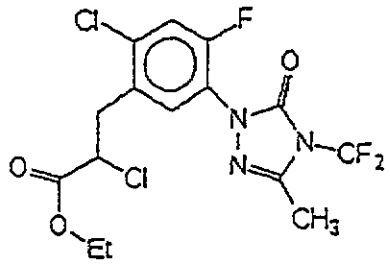
Independent Method Validation:
Determination of F8426 and Its Metabolites in/on Soil

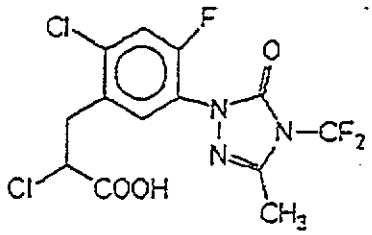
Reference Substances

The following standards were received at Maxim on March 26, 1996 from FMC Corporation, Agricultural Products Group, PO Box 8, Princeton, NJ 08543:

Chemical Name: ethyl α, 2-dichloro-5[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl]-4-fluorobenzene propanoate
Common Name: F8426
FMC Code Number: 116426
Reference Code: CR-30
Percent Purity: 97.6
Maxim Number: 20-817-005
Structure:



Chemical Name: α, 2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoic acid
Common Name: F8426-chloropropionic acid
FMC Code Number: 124161
Reference Code: CR-34
Percent Purity: 99.0
Maxim Number: 20-817-006
Structure:



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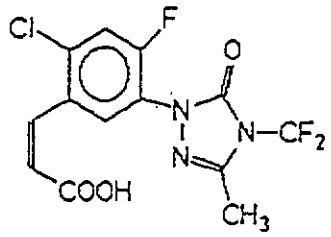
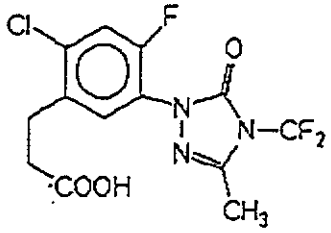
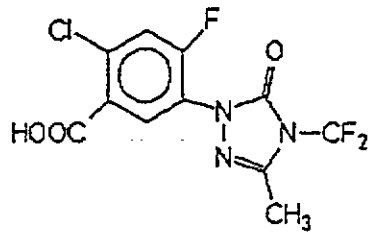
Chemical Name: 2-chloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoic acid
Common Name: F8426-cinnamic acid
FMC Code Number: 125151
Reference Code: CR-8
Percent Purity: 96.9
Maxim Number: 20-817-007
Structure:



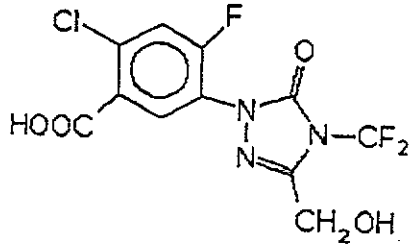
Chemical Name: 2-chloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoic acid
Common Name: F8426-propionic acid
FMC Code Number: 125165
Reference Code: CR-16
Percent Purity: 98.6
Maxim Number: 20-817-008
Structure:



Chemical Name: 2-chloro-5-[4-(difluoromethyl)]-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl]-4-fluorobenzoic acid
Common Name: F8426-benzoic acid
FMC Code Number: 97083
Reference Code: CR-10
Percent Purity: 97.0
Maxim Number: 20-817-004
Structure:



Chemical Name: 2-chloro-5-[4-(difluoromethyl)]-4,5-dihydro-3-hydroxymethyl-5-oxo-1H-1,2,4-triazol-1-yl]-4-fluorobenzoic acid
Common Name: 3-hydroxymethyl-F8426-benzoic acid
FMC Code Number: 125171
Reference Code: CR-27
Percent Purity: 91.0
Maxim Number: 20-817-009
Structure:



The analytical standards and standard solutions were stored in the refrigerator (>0°C to <10°C) when not in use.

4.0 Method of Analysis

4.1 A Draft FMC Method entitled "Analytical Methodology for The Determination of F8426 and Its Metabolites in/on Soil" was validated by fortifying duplicate control soil samples at 5.0 ppb and 100 ppb with F8426, F8426-ClPAc, F8426-CAc, F8426-PAc, F8426-BAc and 3-OH-F8426-BAc. The lower level was based on the limit of quantitation for soil. The higher level was fortified at 20x the limit of quantitation. Control soil (not fortified) was also analyzed in duplicate.

There was one modification to the method; the GC column used for quantitation was different than the one described in the method. See Section 5.0 Instrumentation for additional information.

4.2 A brief description of the method follows:
F8426 and its five metabolites were extracted from soil by refluxing with acetonitrile/water. After removing the acetonitrile by rotary evaporation, the aqueous was acidified, then subjected to solid phase extraction (C-18). The extract was then derivatized with diazomethane/ether followed by pyridine/acetic anhydride to methylate the acid metabolites. Quantitation

was determined by gas chromatography using a mass selective detector (MSD). Figure 1 shows a flow chart of the method. See Appendix III for a more detailed description of the method.

4.3 Preparation of Standards

Stock standard solutions were prepared by weighing an appropriate amount of each compound (about 0.1000 g) and diluting with acetonitrile to 100.0 mL in a volumetric flask. The concentration of the stock solutions were corrected for purity. A 100.0 μg/mL combination standard was prepared by aliquoting 10.0 mL of each stock solution into a 100 mL volumetric and bringing to volume with acetonitrile.

Fortification Solutions

For the 100 ppb fortification, a 16.0 μg/mL standard was prepared by aliquoting 8.0 mL of the 100 μg/mL combination solution into a 50 mL volumetric flask and bringing to volume with acetonitrile. The 0.80 μg/mL solution (for the 5.0 ppb fortifications) was prepared by aliquoting 5.0 mL of the 16.0 μg/mL combination solution and diluting to 100 mL with acetonitrile.

Quantitation Solutions

A 40.0 μg/mL combination standard solution was prepared by aliquoting 20.0 mL of the 100.0 μg/mL combination solution into a 50 mL volumetric flask and bringing to volume with acetonitrile. A 200 μL aliquot of this standard was added to 5.0 mL of ether for derivatization. The final volume of the derivatized combination standard was 10.0 mL (hexane) yielding a concentration of 0.80 μg/mL. Serial dilutions were made to give 0.40, 0.20, 0.10 and 0.05 μg/mL concentrations.

5.0 Instrumentation

Extracts were injected onto a Hewlett Packard 5890 Series II gas chromatograph equipped with a Hewlett Packard 5971 Mass Selective Detector using the following conditions:

5.1 Column: J&W DB-5 15m x 0.25 mm i.d.; 0.25 μ film thickness

5.2 Temperatures

Oven:

Initial Temperature: 150°C

Initial Time:	1.0 minute
Temperature Ramp:	25°C/minute to 250°C
Temperature Ramp A:	5°C./minute to 260°C
Hold Time A:	3.0 minutes
Temperature Ramp B:	30°C/minute to 295°C
Hold Time B:	4.0 minutes
Equilibration Time:	2.0 minutes
Detector:	300°C
Injection:	250°C

5.3 Purge Time: On at 0.50 minutes

5.4 Carrier Gas (Helium): Head Pressure set at 5.6 psi

5.5 Injection Volume: 2 μL (splitless)

5.6 Detector Mode: Single Ion Monitoring (SIM)

5.7 Ions Monitored and Retention Times

Compound	Retention Time	Ion Monitored
F8426	5.55	330
F8426-ClPAc	5.34	326
F8426-CAc	5.44	361
F8426-PAc	4.95	303
F8426-BAc	4.30	335
3-OH-F8426-BAc	5.55	351

6.0 Quantitation

Gas chromatographic data were processed using a Hewlett Packard ChemStation equipped with MS-DOS software, Version C.02.00. The data system constructed best-fit calibration curves for each compound by plotting the standard injected (in pg) versus the standard response (peak area). It then calculated the pg injected for each compound from every sample injected. QuattroPro spreadsheets were used to calculate ppb recovered and percent recoveries. Examples of the calculations used are shown below:

Construction of Curve

$y = mx + b$ where: y = response (peak area of the sample)
m = slope
x = concentration (pg injected)
b = y-intercept

Five (5) combination calibration standards were injected throughout the analytical set from which the curves were calibrated. The range of standards was 0.05 μg/mL to 0.80 μg/mL (100 pg to 1600 pg injected) and the injection volume was 2 μL. See Figures 2-25 for typical chromatograms and Figures 26-31 for linearity curves of each compound.

Determination of ppb Found and % Recovery from a Control Sample Fortified with F8426

$$\text{ppb Recovered} = \frac{pg\ injected}{mg\ injected}$$

Where: pg injected was the value obtained from standard curve
mg injected was the amount of soil matrix injected

The following sample will be used to illustrate the calculation:

Control soil fortified at 5.0 ppb with F8426

Notebook/Sequence No.: 6221204A (Figure 42)

Peak Area Responses of F8426 Analytical Standards

Standard ID	pg Injected	Area
S6221201	100	5117
S6221202	200	9656
S6221203	400	18385
S6221204	800	27752
S6221205	1600	74831

Response of Sample = 38115

Therefore: $y = mx + b$

$38115 = 45.409x + (-1005.42)$

$x = \frac{38115 - (-1005.42)}{45.409}$

$x = 861.5$

ppb F8426 Recovered $= \frac{861.5}{160}$

$= 5.38$

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

$= \frac{5.38}{5.0} \times 100$

$= 107.7$

It was not necessary to correct ppb Recovered since the controls did not contain detectable residues of F8426 or its metabolites.

8.0 Discussion

8.1 The Sponsor may want to expand Section V. ANALYTICAL PROCEDURE to clarify the following:

After transferring the filtrate to a 500 mL flask, it was not stated whether the vacuum flask was rinsed. It is standard laboratory procedure to rinse any sample transfer step. Maxim did not rinse the flask. It is recommended the rinse be included.

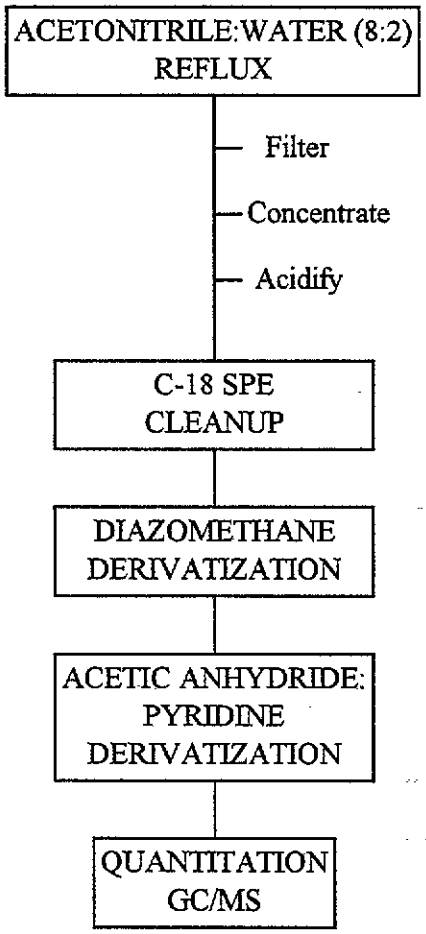
CONCENTRATION (ROTOVAP): Samples "bumped" without warning. A caution statement should be included that this may occur. Maxim decreased the water bath temperature and slowly rotated the flasks.

CLEANUP: It is recommended that the 5 mL column rinse should be used to rinse the flask that contained the sample extract. A statement should be included that the glass wool plug be removed prior to drying the cartridge with nitrogen and eluting with ether. Maxim did not remove the glass wool for either step.

8.2 An analytical set of six (6) samples as described in this report took approximately 16 person hours (two eight hour working days). This included processing of the data and preparation of spreadsheets.

8.3 As with any analytical method, each step is very important to achieve and maintain acceptable results. Maxim felt the critical step with this method was to establish the retention times for each standard by injecting individual and combination quantitation standards. A different GC column (DB-5 (J & W), ; 15 m; 0.25 mm i.d., 0.25 μm film thickness instead of an HP-5, 25 m, 0.32 mm i.d., 0.5 μm film thickness) column was used for quantitation because the foreline pressure and vacuum remained high resulting in inconsistent peak areas of the standards with the larger column. The option of using a different column should be included in the method.

Figure 1. Flow Diagram of the Analytical Method



SUMMARY

Draft FMC Method "Analytical Methodology for the Determination of F8426 and Its Metabolites in/on Soil" is described in more detail below. The method was validated independent of FMC to demonstrate the ruggedness of the method by fortifying control soil with F8426, F8426-chloropropionic acid (F8426-ClPAc), F8426-cinnamic acid (F-8426-CAc), F8426-propionic acid (F8426-PAc), F8426-benzoic acid (F8426-BAc) and 3-hydroxymethyl-F8426-benzoic acid (3-OH-F8426-BAc). Satisfactory results were obtained at Maxim Technologies, Inc..

1.0 Equipment

The following equipment was used to conduct the method validation:

Analytical Balance; Mettler AE 163
Top Loading Balance; Mettler PM4600
Diazomethane Generation; Mini Diazald® Apparatus Kit, Z10,889-8, Aldrich
N-Evap Evaporator; Organomation
Rotary Evaporator; Buchi
Filter Paper; GF/A (90 mm), Whatman glass microfibre filter
Hot Plate/ Stirrers
Solid Phase Extraction (SPE) Visiprep Vacuum Box
Syringes; Various volumes, Hamilton
Standard Laboratory Glassware

2.0 Reagents

The following reagents were used to conduct the method validation:

Acetone; OmniSolv, EM Science
Acetic Anhydride; Alltech
Acetonitrile; Omnisolv, EM Science
Diazald®, Aldrich
Dichloromethane; Omnisolv, EM Science
Ether; Omnisolv, EM Science
Hexane; Omnisolv, EM Science

Hydrochloric Acid; concentrated, VWR
Potassium Hydroxide; pellets; JT Baker
Pyridine, Anhydrous, Alltech
Solid Phase Extraction (SPE) Columns, C-18, 1 gram/6 mL, Varian
Sulfuric Acid, concentrated, Fisher
Water, Omnisolv, EM Science

3.0 Preparation of Stock and Fortification Standards

3.1 Stock Standard Solutions
These were prepared by weighing an appropriate amount of each compound (about 0.1000 g) and diluting with acetonitrile to 100.0 mL in a volumetric flask. The concentration of the stock solutions were corrected for purity. A 100.0 μg/mL combination standard was prepared by aliquoting 10.0 mL of each stock solution into a 100 mL volumetric and bringing to volume with acetonitrile.

3.2 Fortification Solutions
For the 100 ppb fortification, a 16.0 μg/mL standard was prepared by aliquoting 8.0 mL of the 100 μg/mL combination solution into a 50 mL volumetric flask and bringing to volume with acetonitrile. The 0.80 μg/mL solution (for the 5.0 ppb fortifications) was prepared by aliquoting 5.0 mL of the 16.0 μg/mL combination solution and diluting to 100 mL with acetonitrile.

4.0 Analytical Procedure

4.1 Glassware Preparation

Prior to use, all glassware was washed with soap and water followed by rinsing with 0.25 N HCl. The glassware was then rinsed with acetone.

4.2 Diazomethane/Ether Preparation

Diazomethane was prepared using the Mini-Diazald® Apparatus Kit (Aldrich). Details and methodology were supplied by Aldrich.

4.3 Sample Analysis

4.3.1 Soil (80 grams) was weighed into 500 mL flat-bottom flasks. For the fortification samples add a known amount of a combination analytical standard directly onto the surface of the soil (either with a

syringe or volumetric pipet). A stir bar was put in each flask.

4.3.2 To each flask, 200 mL of reflux solvent (acetonitrile:water; 8:2; v/v) was added and manually stirred. The flasks were placed on a hot plate with a magnetic stirrer and then connected to a cold water condenser. The samples were refluxed for one (1) hour and allowed to cool for fifteen minutes. The condensers were rinsed with about 10 mL of reflux solvent (this was collected in the flask).

4.3.3 The vacuum glassware was rinsed with reflux solvent and the samples were filtered using a Buchner funnel and GF/A filter paper (pre-wet). The 500 mL flat-bottom flask and filter cake were rinsed 2 x 25 mL with reflux solvent. The filtrates were transferred to clean 500 mL flat-bottom flasks. The samples were concentrated to about 30 mL on a rotary evaporator to remove the acetonitrile. The water bath temperature was approximately 35°C. Caution: Samples tend to "bump" without warning.

4.3.4 Solid Phase Extraction Cleanup
The C-18 columns were prepared by placing a small amount of glasswool into each, then attaching 75 mL reservoirs to the top of each column. The columns were put on the Visiprep and conditioned with 10 mL of methanol followed by 10 mL of 0.2% sulfuric acid. Do not allow the liquid to go below the top of the column packing.

The samples (from 3.3.3) were brought up to about 50 mL volume with distilled water and 1.0 mL of 10% sulfuric acid was added to each. They were transferred to the reservoirs and vacuum was applied to achieve a flow rate of about 3-4 mL per minute. After the sample passed through the column, it was rinsed with 5 mL of distilled water.

Strong vacuum was applied to the columns for five minutes followed by nitrogen flow (about 5 psi) for sixty minutes. The compounds were eluted from the columns with 5.0 mL of ethyl ether into a 15 mL graduated centrifuge tube.

4.3.5 The samples were derivatized by adding 1.0 mL of diazomethane/ether solution followed by gentle swirling. The samples were placed in the N-Evap (water temperature at 45°C) and concentrated to <0.1 mL, but not to dryness. To each sample was

added 0.5 mL of pyridine and 0.5 mL of acetic anhydride and swirl. The samples were heated for 30 minutes at 50°C ±2°C. Again the samples were concentrated to <0.1 mL using the N-Evap with water temperature at about 45°C. Do not concentrate to dryness. The samples were diluted with 10 mL of hexane then concentrated (using the N-Evap) to 1.0 mL for analysis by GC/MS.

5.0 Quantitation Standards

A 40.0 µg/mL combination standard solution was prepared by aliquoting 20.0 mL of the 100.0 µg/mL combination solution into a 50 mL volumetric flask and bringing to volume with acetonitrile. A 200 µL aliquot of this standard was added to 5.0 mL of ether for derivatization. The final volume of the derivatized combination standard was 10.0 mL (hexane) yielding a concentration of 0.80 µg/mL. Serial dilutions were made to give 0.40, 0.20, 0.10 and 0.05 µg/mL concentrations.

6.0 Instrumentation

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6.1	Column:	J&W DB-5 15m x 0.25 mm i.d.; 0.25 µ film thickness
6.2	Temperatures	
	Oven:	
	Initial Temperature:	150°C
	Initial Time:	1.0 minute
	Temperature Ramp:	25°C/minute to 250°C
	Temperature Ramp A:	5°C./minute to 260°C
	Hold Time A:	3.0 minutes
	Temperature Ramp B:	30°C/minute to 295°C
	Hold Time B:	4.0 minutes
	Equilibration Time:	2.0 minutes
	Detector:	300°C
	Injection:	250°C
6.3	Purge Time:	On at 0.50 minutes
6.4	Carrier Gas (Helium):	Head Pressure set at 5.6 psi
6.5	Injection Volume:	2 µL (splitless)

6.6 Detector Mode: Single Ion Monitoring (SIM)

6.7 Ions Monitored and Retention Times

Compound	Retention Time	Ion Monitored
F8426	5.55	330
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F8426-CAc	5.44	361
F8426-PAc	4.95	303
F8426-BAc	4.30	335
3-OH-F8426-BAc	5.55	351

7.0 Method of Calculation

Gas chromatographic data were processed using a Hewlett Packard ChemStation equipped with MS-DOS software, Version C.02.00. The data system constructed best-fit calibration curves for each compound by plotting the standard injected (in pg) versus the standard response (peak area). It then calculated the pg injected for each compound from every sample injected. QuattroPro spreadsheets were used to calculate ppb recovered and percent recoveries. Examples of the calculations used are shown below:

Construction of Curve

$y = mx + b$ where: y = response (peak area of the sample)
m = slope
x = concentration (pg injected)
b = y-intercept

Five (5) combination calibration standards were injected throughout the analytical set from which the curves were calibrated. The range of standards was 0.05 μg/mL to 0.80 μg/mL (100 pg to 1600 pg injected) and the injection volume was 2 μL.

Determination of ppb Found and % Recovery from a Fortified Control Sample

$$\text{ppb Recovered} = \frac{pg\ injected}{mg\ injected}$$

Where: pg injected was the value obtained from standard curve
mg injected was the amount of soil matrix injected

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