

## ABSTRACT

The purpose of the study was to demonstrate that BASF Analytical Method R0039, "The Determination of Residues of Diflufenzopyr (Reg. No. 362255) and its Metabolites, M1 (Reg. No. 363181), M2 (Reg. No. 395202), M6 (Reg. No. 395198), and M9 (Reg. No. 395207) in Water using LC-MS/MS", could be performed successfully at an outside facility with no prior experience with the method (Reference 1).

The analytical method was successfully implemented at Alliance Pharma for ground water matrices. Diflufenzopyr (Reg. No. 362255) and its metabolites were recovered within acceptance limits in the aforementioned matrix.

For diflufenzopyr, and its metabolites M1, M6, and M9, ground water samples were fortified with 0.050 mL of 100 ng/mL or with 0.050 mL of 1000 ng/mL fortification solutions to obtain the LOQ (0.5 µg/L) and 10 x LOQ (5 µg/L) concentrations, respectively. Then the samples were vortexed and analyzed via LC-MS/MS.

For metabolite M2, ground water samples were fortified with 0.500 mL of 100 ng/mL or with 0.500 mL of 1000 ng/mL fortification solution to obtain the LOQ (0.5 µg/L) and 10 x LOQ (5 µg/L) concentrations, respectively. The samples were extracted with dichloromethane several times, dried with Na<sub>2</sub>SO<sub>4</sub>, and were concentrated. The extract was then diluted with ACN, concentrated again, and was reconstituted with 50 mM ammonium carbonate. Then the samples were vortexed and analyzed via LC-MS/MS.

The limit of quantitation (LOQ) of the method is 0.5 µg/L in ground water. The limit of detection (LOD) of the method is 0.1 µg/L in ground water.

For validation, the ground water specimens were fortified with diflufenzopyr and its metabolites, and then were analyzed according to the established method validation guidelines. Each analytical set consisted of one method blank, two control samples, five fortifications at LOQ, and five fortifications at 10 x LOQ. Apparent residues of diflufenzopyr and all metabolites were below the LOD in all control samples.

The method validation was successfully completed for the analysis of diflufenzopyr and its metabolites M1, M2, M6, and M9 in the ground water matrix tested. The LC-MS/MS conditions described in the analytical method were modified to allow enough time for equilibration of the 100x2.1 mm column from 90% mobile phase B concentration back to 0%. Also, the calibration standards for diflufenzopyr, M1, M6, and M9 were made in deionized water instead of 50mM sodium carbonate because the different pH of ground water samples and standards led to very different retention time for the M6 and M9 analytes between standards and samples. The confirmatory transition of M1 was changed to 93.1 from 162.2-88.7. The results were acceptable for both primary and secondary ion transitions.

Recovery results of the method validation for BASF Analytical Method R0039 are shown below:

## 1.0 INTRODUCTION

### 1.1 Purpose of the Study

The purpose of the study was to demonstrate that BASF Analytical Method R0039, "The Determination of Residues of Diflufenzopyr (Reg. No. 362255) and its Metabolites, M1 (Reg. No. 363181), M2 (Reg. No. 395202), M6 (Reg. No. 395198), and M9 (Reg. No. 395207) in Water using LC-MS/MS", could be performed successfully at an outside facility with no prior experience with the method.

### 1.2 Summary of the Results

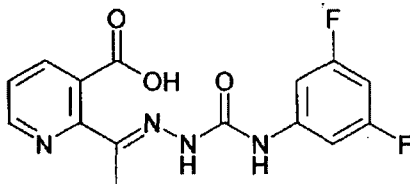
The independent laboratory validation of this BASF method was successfully completed. Detailed results for diflufenzopyr and its metabolites are presented in Tables 1 – 10 for ground water. Certificates of analysis are presented in Appendix 1, and the detailed reports representing the analytical data are presented in Appendix 3.

## 2.0 REFERENCE SUBSTANCE AND SAMPLING HISTORY

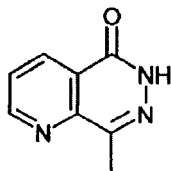
### 2.1 Reference Materials

Reference substance diflufenzopyr and its metabolites M1, M2, M6, and M9 were used for individual fortifications and LC-MS/MS calibration. Concentrated (stock), fortification, and calibration standards were prepared according to the analytical method. Example standard solution preparations are presented in Table 3. Standard solutions prepared for this study were stored at 4°C. A brief description of reference standards used in this study is presented below.

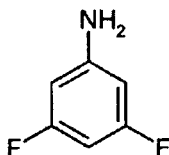
<b>BASF Code Name:</b>	BAS 654 H
<b>Common Name:</b>	Diflufenzopyr
<b>Batch No.:</b>	L67-200
<b>BASF Registry Number:</b>	362255
<b>CAS Number:</b>	1918-00-9
<b>IUPAC Name:</b>	2-(methyl-«(3,5-difluorophenylamine)-carbonyl)hydrazone)-methyl)-3-pyridine carboxylic acid
<b>Molecular Formula:</b>	C <sub>15</sub> H <sub>12</sub> F <sub>2</sub> N <sub>4</sub> O <sub>3</sub>
<b>Molecular Weight:</b>	334.28 g/mol
<b>Purity:</b>	97.6%
<b>Expiration Date:</b>	Mar 01, 2018
<b>Chemical Structure:</b>	



**BASF Code Name:** M1  
**Common Name:** Phthalazinone  
**Batch No.:** L74-44  
**BASF Registry Number:** 363181  
**CAS Number:** 90004-07-2  
**IUPAC Name:** 8-Methyl-5(6H)-pyrido(2,3-d)pyridazinone  
**Molecular Formula:** C<sub>8</sub>H<sub>7</sub>N<sub>3</sub>O  
**Molecular Weight:** 161.16 g/mol  
**Purity:** 100%  
**Expiration Date:** Aug 01, 2015  
**Chemical Structure:**

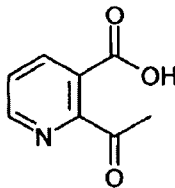


**BASF Code Name:** M2  
**Common Name:** 3,5-Difluoroaniline  
**Batch No.:** RS-35DFA-093093  
**BASF Registry Number:** 395202  
**IUPAC Name:** 3,5-Difluoroaniline  
**Molecular Formula:** C<sub>6</sub>H<sub>5</sub>F<sub>2</sub>N  
**Molecular Weight:** 161.16 g/mol  
**Purity:** 98.8%  
**Expiration Date:** Jun 01, 2023  
**Chemical Structure:**

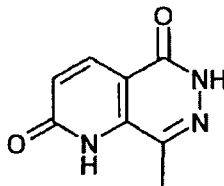


**BASF Code Name:** M6  
**Common Name:** 2-Acetyl nicotinic acid  
**Batch No.:** 1213-65  
**BASF Registry Number:** 395198  
**CAS Number:** 89942-59-6  
**IUPAC Name:** 2-Acetyl nicotinic acid  
**Molecular Formula:** C<sub>8</sub>H<sub>7</sub>NO<sub>3</sub>  
**Molecular Weight:** 165.15 g/mol  
**Purity:** 99.5%  
**Expiration Date:** Apr 01, 2020

**Chemical Structure:**



<b>BASF Code Name:</b>	M9 (2-keto M1)
<b>Common Name:</b>	Phthalazindione
<b>Batch No.:</b>	01748-022
<b>BASF Registry Number:</b>	395207
<b>CAS Number:</b>	NA
<b>IUPAC Name:</b>	8-Methylpyrido(2,3-d)pyridazine-2,5(1 H,6H)-dione
<b>Molecular Formula:</b>	C <sub>8</sub> H <sub>7</sub> N <sub>3</sub> O <sub>2</sub>
<b>Molecular Weight:</b>	177.16 g/mol
<b>Purity:</b>	99.3%
<b>Expiration Date:</b>	Feb 01, 2024
<b>Chemical Structure:</b>	



## 2.2 Test System

Ground water matrices were sent from ADPEN Laboratories, Inc. on March 25, 2014 and received by Alliance Pharma on March 26, 2014. The water characterization report is presented in Appendix 2.

## 3.0 TECHNICAL PROCEDURE

BASF Analytical Method R0039: "The Determination of Residues of Diflufenzopyr (Reg. No. 362255) and its Metabolites, M1 (Reg. No. 363181), M2 (Reg. No. 395202), M6 (Reg. No. 395198), and M9 (Reg. No. 395207) in Water using LC-MS/MS".

### 3.1 Summary of Analytical Procedure for BAS 654 H, M1, M6, and M9

10 mL of ground water was fortified with 0.050 mL of 100 ng/mL or with 0.050 mL of 1000 ng/mL fortification solution to obtain the LOQ (0.5 µg/L) and 10 x LOQ (5 µg/L), respectively. The samples were vortexed and analyzed via LC-MS/MS.

The primary (quantitative) and secondary (confirmatory) transition ions monitored are presented below:

Analyte	Transition ( <i>m/z</i> )		Ionization Mode	Retention Time (min.)
	Primary	Secondary		
Diflufenzopyr	335.0 → 206.1	335.0 → 162.2	Positive	~9.7
M1	162.2 → 102.9	162.2 → 93.1	Positive	~6.8
M6	165.9 → 148.1	165.9 → 91.9	Positive	~6.0
M9	178.1 → 150.1	178.1 → 119.1	Positive	~5.2

### 3.2 Summary of Analytical Procedure for M2

100 mL of ground water was placed in a 250-mL separatory funnel and was fortified with 0.500 mL of 100 ng/mL or with 0.500 mL of 1000 ng/mL fortification solution to obtain the LOQ (0.5 µg/L) and 10 x LOQ (5 µg/L), respectively. The samples were shaken for 10 minutes and 20 mL of dichloromethane (DCM) was added to each sample. Then 15 mL of NaCl dry powder was added, the sample was shaken to dissolve, and then was allowed to separate. The lower DCM layer was drained into a 125-mL round bottom flask. The DCM extract was then passed through a layer of Na<sub>2</sub>SO<sub>4</sub> to absorb any moisture. The liquid remaining in the separatory funnel was washed two more times with 20 mL of DCM and the extracts were added to the same round bottom flask. The DCM extract was concentrated to 2-4 mL under vacuum, then 20 mL of ACN was added and the sample was concentrated to 2-4 mL under vacuum to remove all DCM. The concentrated extract was transferred to a polypropylene test tube with 3 portions of ~3 mL ACN. The extract was concentrated to ~1 mL under nitrogen at 50-55°C. The extract was then reconstituted to 2 mL with 50 mM ammonium carbonate.

The samples were vortexed, filtered through a 0.45 µm syringe filter, and analyzed via LC-MS/MS.

The primary (quantitative) and secondary (confirmatory) transition ions monitored are presented below:

Analyte	Transition ( <i>m/z</i> )		Ionization Mode	Retention Time (min.)
	Primary	Secondary		
M2	130.1 → 83.0	130.1 → 110.1	Positive	~5.6

### 4.0 LIMITS OF QUANTITATION AND DETECTION

The LOQ and LOD for residues diflufenzopyr in ground water are 0.05 and 0.01 µg/L, respectively.

### 5.0 CALIBRATION, CALCULATIONS, AND STATISTICS

Quantitation of residues in all samples was achieved using an external calibration curve calculated by linear regression of instrument responses for the reference substances at multiple concentrations.

Individual standard curves were prepared for diflufenzopyr and its metabolites by injecting standard solutions at appropriate concentrations. Calibration standard concentrations for diflufenzopyr, M2, M6, and M9 ranged from 0.1 – 15 ng/mL (40 µL injected). Calibration standard concentrations for M2 ranged from 5 – 100 ng/mL (10 µL injected). Calibration standards were interspersed with sample injections. Analyst® 1.6 software created the standard curve based on linear regression using  $1/x^2$  weighting. The regression functions were used to calculate the best-fit line by plotting the analyte found (ng/mL) on the x-axis versus the detector's peak response (peak area) on the y-axis. Typical calibration curves are presented in Figures 1-5. Representative chromatograms of calibration standards are presented in Figure 6-10.

Peak integration and quantitation were performed within Analyst® 1.6 software using the calibration curve equation to determine the amount of analyte found (ng/mL) during sample analysis. Recovery results and additional sample concentrations were calculated for each set of samples using Microsoft® Excel spreadsheet data reports, which are presented in Appendix 3.

The following equations were used for residue and recovery calculations for diflufenzopyr in ground water matrices:

$$\text{Concentration [ng/mL]} = \frac{\text{Concentration in the final volume [ng/mL]} \times \text{Response} - \text{Intercept}}{\text{Slope}} = C_A$$

$$\text{Aliquot Factor (A}_F\text{)} = \frac{\text{Residue in the sample } [\mu\text{g/L}]}{\text{Aliquot taken}} \times \text{Final Volume}$$

$$\text{Residue } [\mu\text{g/L}] = \frac{V_{\text{end}} \times C_A}{V_{\text{sample}} \times A_F}$$

$$\text{Recovery \%} = \frac{\text{Recovery in the sample } [\%]}{\text{Residue in fortified sample} - \text{Residue in control}} \times \text{Amount of analyte fortified} \times 100\%$$

**Example: Diflufenzopyr, 335.0 → 206.1; ground water sample LOQ1 fortified at 0.5 µg/L:**

**The following values were used in this calculation:**

Response of fortified sample	75858
Response of control sample	0
Slope:	134000
Intercept:	240
Sample Volume ( $V_{\text{sample}}$ ):	10.0 mL
Final Volume ( $V_{\text{end}}$ ):	10.0 mL
Aliquot factor $A_F$ :	1 (= 100%)
Conversion factor ng $\rightarrow$ $\mu\text{g}$ :	1000

$$\text{Concentration (ng/mL)} = \frac{75858 - 240}{134000} = 0.56 \text{ ng/mL}$$

$$\text{Aliquot Factor (A}_F\text{)} = \frac{10.0 \text{ mL}}{10.0 \text{ mL}} = 1$$

**Residue ( $\mu\text{g/L}$ )**

$$= \frac{10.0 \text{ mL} \times 0.564 \text{ ng/mL}}{10.0 \text{ mL} \times 1} = 0.564 \text{ ng/mL} = 0.564 \mu\text{g/L}$$

$$\text{Recovery \%} = \frac{(0.564 \mu\text{g/L} - 0.000 \mu\text{g/L}) \times 100 \%}{0.500 \mu\text{g/L}} = 113\%$$

**Example: M2, 130.1  $\rightarrow$  83.0; ground water sample LOQ1 fortified at 0.5  $\mu\text{g/L}$ :**

**The following values were used in this calculation:**

Response of fortified sample	535454
Response of control sample	0
Slope:	22300
Intercept:	-10500
Sample Volume ( $V_{\text{sample}}$ ):	100.0 mL
Final Volume ( $V_{\text{end}}$ ):	2.0 mL
Aliquot factor $A_F$ :	1 (= 100%)
Conversion factor ng $\rightarrow$ $\mu\text{g}$ :	1000

$$\text{Concentration (ng/mL)} = \frac{535656 + 10500}{22300} = 24.49 \text{ ng/mL}$$

$$\text{Aliquot Factor (A}_F\text{)} = \frac{2.0 \text{ mL}}{2.0 \text{ mL}} = 1$$

**Residue ( $\mu\text{g/L}$ )**

$$= \frac{2.0 \text{ mL} \times 24.49 \text{ ng/mL}}{100.0 \text{ mL} \times 1} = 0.490 \text{ ng/mL} = 0.490 \mu\text{g/L}$$

$$\text{Recovery \%} = \frac{(0.490 \mu\text{g/L} - 0.000 \mu\text{g/L}) \times 100 \%}{0.500 \mu\text{g/L}} = 98 \%$$

Statistical treatment of the data included calculation of means, standard deviations (SD), and percent relative standard deviations (%RSD). These calculations were performed using Microsoft® Excel. Results were rounded only for reporting purposes. No calculations were made with rounded numbers.



Fortification Scheme Used for Analysis of Diflufenzopyr, M1, M6, & M9

Sample Data		Fortification data					Final Volume (mL)
Sample Type	Vol. (mL)	µg/L Fortified	Analyte	Vol. of Standard Used	Standard Conc.	Standard Number	
Control	10.00	Control	None	NA	NA	NA	10.0
0.5 µg/L fortification	10.00	0.5	all <sup>4</sup>	0.05 mL	100 ng/mL	Fort-0.5µg/L -A	10.0
5 µg/L fortification	10.00	5	all <sup>4</sup>	0.05 mL	1000 ng/mL	Fort-5µg/L-A	10.0

Fortification Scheme Used for Analysis of M2

Sample Data		Fortification data					Final Volume (mL)
Sample Type	Vol. (mL)	µg/L Fortified	Analyte	Vol. of Standard Used	Standard Conc.	Standard Number	
Control	100.00	Control	None	NA	NA	NA	100
0.5 µg/L fortification	100.00	0.5	all <sup>4</sup>	0.5 mL	100 ng/mL	Fort-0.5µg/L -A	100
5 µg/L fortification	100.00	5	all <sup>4</sup>	0.5 mL	1000 ng/mL	Fort-5µg/L-A	100

1. Stock solutions are used for up to 90 days, and dilutions of the stock solution are used for up to 30 days.
2. ACN = Acetonitrile, DMSO = Dimethylsulfoxide.
3. Not used for injection.
4. all = diflufenzopyr, M1, M2, M6, & M9

**Table 12 Instrument Conditions and Parameters**

**Quantitation for Ground water for M2**

Instrument:	Sciex API 5500 Mass Spectrometer		
Inlet [HPLC System]:	Shimadzu LC-30AD Series		
Software Version:	Analyst 1.6		
Column:	Kinetex PFP 1.7 $\mu$ m 2.1 x x100 mm		
Injection:	40 $\mu$ L		
Mobile Phase:	A = 0.1% Formic Acid in Water B = 0.1% Formic Acid in ACN		
Gradient	<b>Time (minute)</b>	<b>Composition (%)</b>	
		<b>A</b>	<b>B</b>
	0.01	95	5
	0.5	95	5
	3	50	50
	6.5	50	50
	7	0	100
	7.4	0	100
	7.5	95	5
10.0	Stop		
Flow Rate:	300 $\mu$ L/minute		
Analytes	Expected Retention Times (minutes)	Transitions (m/z) :	
		Quantitation ion	Secondary ion
M2	~5.6	130.1 $\rightarrow$ 83.0	130.1 $\rightarrow$ 110.1
Ionization Mode:	Positive ion; Electrospray (500°C)		

**Quantitation for Ground water for Diflufenzopyr, M1, M6, and M9**

Instrument:	Sciex API 5500 Mass Spectrometer		
Inlet [HPLC System]:	Shimadzu LC-30AD Series		
Software Version:	Analyst 1.6		
Column:	Kinetex PFP 1.7 $\mu$ m 2.1 x x100 mm		
Injection:	40 $\mu$ L		
Mobile Phase:	A = 10 mM Ammonium Formate in 1% Formic Acid in H <sub>2</sub> O B = 10 mM Ammonium Formate in 1% Formic Acid in Methanol		
Gradient	<b>Time (minute)</b>	<b>Composition (%)</b>	
		<b>A</b>	<b>B</b>
	0.01	100	0
	2.5	100	0
	5.5	65	35
	6.5	65	35
	7.5	30	70
	8.8	30	70
	9.0	10	90
	11.0	10	90
	11.1	100	0
14.5	Stop		
Flow Rate:	300 $\mu$ L/minute		
Analytes	Expected Retention Times (minutes)	Transitions (m/z) :	
		Quantitation ion	Secondary ion
Diflufenzopyr	~9.7	335.0 $\rightarrow$ 206.1	335.0 $\rightarrow$ 162.2
M1	~6.8	162.2 $\rightarrow$ 102.9	162.2 $\rightarrow$ 93.1
M6	~6.0	165.9 $\rightarrow$ 148.1	165.9 $\rightarrow$ 91.9
M9	~5.2	178.1 $\rightarrow$ 150.1	178.1 $\rightarrow$ 119.1
Ionization Mode:	Positive ion; Electrospray (500°C)		