

1.0 INTRODUCTION

1.1 Scope of the Method

Diflufenzopyr (BAS 654 H, Reg. No. 362255) and its metabolites, M1 (Reg. No. 363181), M2 (Reg. No. 395202), M6 (Reg. No. 395198), and M9 (Reg. No. 395207) were determined by LC-MS/MS in water.

A residue analytical method for the detection and quantification of the active ingredient BAS 654 H (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 was needed for monitoring purposes with a limit of quantification (LOQ) of 0.5 µg/kg (ppb).

BAS 654 H is an auxin transport inhibitor herbicide developed by BASF. The previous method of analysis D9906, was originally developed (Reference 1) at BASF Corporation for the analysis of BAS 654 H and its Phthalazinone (M1) metabolite in water by GC/MS. The current method was developed at ADPEN Laboratories, Inc. for the analysis of BAS 654 H, M1, M2, M6, M9 in water by LC-MS/MS.

The LOQ and LOD of the method is 0.5 ppb and 0.1 ppb respectively, for the active ingredient, BAS 654 H and the metabolites M1, M2, M6 and M9. The method has been successfully tested and validated in water.

The method was validated at two fortification levels (0.5 and 5.0 µg/kg) for well and surface water types. For each fortification level and matrix, five replicates were analyzed. Additionally, a method blank and two replicates of control samples were examined.

1.2 Principle of the Method

Water samples containing Diflufenzopyr (BAS 654 H) and metabolites (M1, M6, and M9) are filtered and analysed without additional preparation for direct injection into an LC-MS/MS.

Water samples fortified with M2 were extracted with dichloromethane in the presence of salt. The extract was concentrated and reconstituted with acetonitrile and 50 mM ammonium carbonate to an appropriate final volume. The residues were determined using LC-MS/MS.

Primary and confirmatory mass transitions (m/z) for all analytes were analyzed using LC-MS/MS.

1.3 Specificity

The residues of Diflufenzopyr (BAS 654 H, Reg. No. 362255) and its metabolites, M1 (Reg. No. 363181), M2 (Reg. No. 395202), M6 (Reg. No. 395198), and M9 (Reg. No. 395207) were identified and quantified as individual compounds.

2.0 MATERIALS AND METHODS

2.1 Test systems

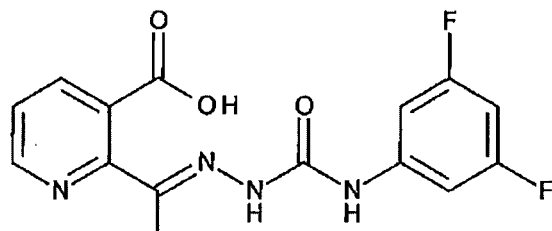
The following test systems were considered in this study of validation:

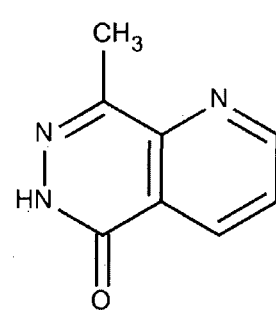
Well Water (BD Well)

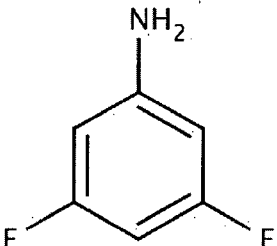
Surface Water (Goose River)

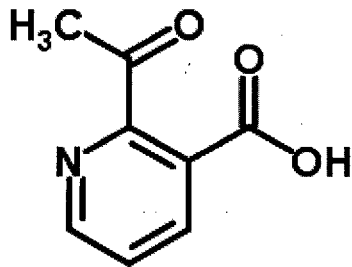
Certificates of water characterization, both well and surface, can be found in Appendix 9.5.

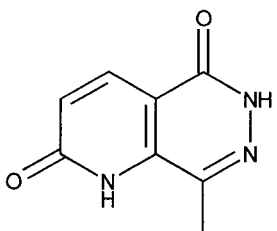
2.2 Test and Reference Items

BASF Reg. No.:	362255
Common Name:	Diflufenzopyr
BAS Code Name:	BAS 654 H
IUPAC Name:	2-[1-[4-(3-5-difluorophenyl)semicarbazono]ethyl]nicotinic acid
	Purpose: Active Ingredient
	CAS No.: 109293-97-2
	Batch No.: L67-200
	Purity: 97.6%
	Molecular Formula: C ₁₅ H ₁₂ F ₂ N ₄ O ₃
Molecular Weight: 334.3 g/mol Expiration Date: March 1, 2014 March 1, 2018	

BASF Reg. No.:	363181
Common Name:	None
BAS Code Name:	M1
IUPAC Name:	8-methylpyrido[2,3-d]pyridazin-5(6H)-one
	Purpose: Metabolite and end analyte
	CAS No.: 90004-07-2
	Batch No.: L74-44
	Purity: 100.0%
	Molecular Formula: C ₈ H ₇ N ₃ O
Molecular Weight: 161.2 g/mol Expiration Date: August 1, 2015	

BASF Reg. No.:	395202	
Common Name:	None	
BAS Code Name:	M2	
IUPAC Name:	3,5-difluoroaniline	
	Purpose: Metabolite and end analyte	
	CAS No.: 372-39-4	
	Batch No.: RS-35DFA-093093	
	Purity: 98.8%	
	Molecular Formula: C ₆ H ₅ F ₂ N	
Molecular Weight: 129.1 g/mol		Expiration Date: June 1, 2023

BASF Reg. No.:	395198	
Common Name:	None	
BAS Code Name:	M6	
IUPAC Name:	2-acetyl nicotinic acid	
	Purpose: Metabolite and end analyte	
	CAS No.: 89942-59-6	
	Batch No.: 1213-65	
	Purity: 99.5%	
	Molecular Formula: C ₈ H ₇ NO ₃	
Molecular Weight: 165.1 g/mol		Expiration Date: April 1, 2020

BASF Reg. No.:	395207	
Common Name:	None	
BAS Code Name:	M9	
IUPAC Name:	8-methylpyrido[2,3-d]pyridazine-2,5(1H,6H)-dione	
	Purpose: Metabolite and end analyte	
	CAS No.: None	
	Batch No.: 01748-022	
	Purity: 99.3%	
	Molecular Formula: C ₈ H ₇ N ₃ O ₂	
Molecular Weight: 177.2 g/mol		Expiration Date: February 1, 2024

BASF has retained a reserve sample of these chemicals and has documents at the BASF Crop Protection, Research Triangle Park, North Carolina specifying the location of the synthesis and characterization information for these compounds.

2.3 Equipment, Materials and Methods

2.3.1 Equipment

Suggested sizes, suppliers, and manufacturers of the equipment for this method are below.

Equipment	Size, Description	Manufacturer
Balance, analytical	Model XP26	Metler Toledo
Balance, top-loading	Model CP3202 S	Sartorius
Bottle, amber glass	Qorpak, 10 mL, 25, 50, and 100 mL with Teflon®-lined screw cap	Qorpak
Columns, analytical	Kinetex PFP, 100 x 2.1 mm, 1.7 µm SN:681573-3	Phenomenex
Separatory funnels	250 mL	Various
Cylinder, graduated	Various sizes	Various
Pipet, Volumetric	0.2, 1, and 5 mL (adjustable volumes)	Various
Nitrogen-evaporator	Model 112	Organomation
Rotavap	R121	Buchi
Spatula	Various	Various
Tube, centrifuge	50-mL disposable polypropylene graduated	Various
Tube, centrifuge	15-mL disposable polypropylene graduated	Various
Vials, HPLC	2.0 mL	Agilent
Vortex mixer	Genie 2	Fisher Scientific Co
HPLC-MS/MS	AB SCIEX 5500 Triple Quad MS/MS	AB SCIEX

2.3.2 Chemicals

Chemical	Grade	Manufacturer/Supplier
Acetonitrile (ACN)	HPLC	EMD
Dichloromethane	HPLC	EMD
Formic acid	98%	EMD
MeOH	HPLC	EMD
Ammonium formate	Reagent grade	Fluka
Ammonium carbonate	ACS Reagent	Fisher Scientific
Sodium chloride	Reagent grade	EMD
Sodium sulfate	Anhydrous	Fisher Scientific
HPLC Water	HPLC	EMD
DI Water	Lab DI water	ADPEN

Solutions and Solvent Mixtures

Description	Composition
50 mM Ammonium carbonate	Molecular weight of ammonium carbonate is 96 g/mol. In 1 L of 50 mM solution there are 4.8 g of the substance. E.g., 6.5 g of ammonium carbonate are: $6.5 \text{ g} / 96 \text{ (g/mol)} = 0.0684 \text{ mol}$. $0.0684 \text{ mol} / 0.0500 \text{ (mol/L)} = 1.37 \text{ L}$. Therefore, the 6.5 g of ammonium carbonate are dissolved and brought to a final volume of 1.37 L, by the help of a graduated cylinder. Solvent is stored at ambient temperature and is prepared fresh every 14 days. Calibration and fortification solutions prepared in 50 mM ammonium carbonate are stored in a refrigerator and stable for 14 days.
HPLC mobile phase A for BAS 654 H, M1, M6 and M9	1% Formic acid, 10 mM ammonium formate in water. Add 10 mL formic acid and 0.63 g ammonium formate into 1 L water into 1-L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase B for BAS 654 H, M1, M6 and M9	1% Formic acid, 10 mM ammonium formate in MeOH. Add 10 mL formic acid and 0.63 g ammonium formate into 1 L MeOH into 1-L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase A for M2	1% Formic acid in water
HPLC mobile phase B for M2	1% Formic acid in ACN

2.3.3 Standard Solutions

Stock Standard Solutions

Analytes are dissolved with appropriate solvent in volumetric flasks as specified in the table below.

Analyte	Solvent	Comment
BAS 654 H	acetone	Sensitive to acids. Analyte is most stable in acetone.
M1	ACN	Store at RT.
M2	ACN	Store at Refrigerator.
M6	ACN	Analyte dissolves in pure ACN readily. DMSO is not required.
M9	DMSO	Saturation observed at approximately 1000 ng/ μ L. Prepare stock solution at approximately 500-700 ng/ μ L. Store at RT.

Stock solutions of BAS 654 H, M1, M2 and M6 are prepared individually at approximately 1000 ng/ μ L. Appropriate volumes of each stock standard is used to prepare the intermediate standard solutions.

Intermediate Standard Solutions

Analytes are diluted and mixed in volumetric flasks as specified in the table below.

Stock Standard Solution	Intermediate Standard Solution	Concentration, ng/ μ L
BAS 654 H in acetone	Mixed solution of BAS 654 H, M1, M6 and M9 in 50 mM $(\text{NH}_4)_2\text{CO}_3$ in water. M2 prepared separately in 50 mM $(\text{NH}_4)_2\text{CO}_3$ in water.	20 for BAS 654 H, M1, M6, and M9
M1 in ACN		
M2 in ACN		
M6 in ACN		
M9 in DMSO		50 for M2

Fortification Standard Solutions

Analytes are diluted and mixed in volumetric flasks as specified in the table below.

Intermediate Standard Solution	Fortification Standard Solution	Concentration, pg/ μ L
Mixed solution of BAS 654 H, M1, M6 and M9 in 50 mM $(\text{NH}_4)_2\text{CO}_3$ in water. M2 prepared separately in 50 mM $(\text{NH}_4)_2\text{CO}_3$ in water.	Mixed solution of BAS 654 H, M1, M6 and M9 in 50 mM $(\text{NH}_4)_2\text{CO}_3$ in water. M2 prepared separately in 50 mM $(\text{NH}_4)_2\text{CO}_3$ in water.	1000 and 100 for M2 250 and 25 for BAS 654 H, M1, M6 and M9

Calibration Standard Solutions

Prepare mixed calibration solutions for LC-MS/MS analysis by diluting the solutions at level 20 ng/ μ L in volumetric flasks. Dilute to the mark with 50 mM $(\text{NH}_4)_2\text{CO}_3$, as specified in the table below.

Analysis of BAS 654 H, M1, M6 and M9

Concentration, pg/ μ L	Aliquot, mL	FV, mL	Final Concentration, pg/ μ L
250	0.4	10	10
25	2	10	5
25	0.8	10	2
10	1	10	1
5	1	10	0.5
2	1	10	0.2
1	1	10	0.1

Analysis of M2

Concentration, pg/ μ L	Aliquot, mL	FV, mL	Final Concentration, pg/ μ L
1000	2	20	100
1000	0.5	10	50
1000	0.3	10	30
100	2	10	20
100	1.5	10	15
100	1	10	10
50	1	10	5

2.3.4 Standard Solution Storage Stability

Standard solutions are kept refrigerated. BASF recommends that the stock solutions (1 mg/mL) be made fresh every three months. Diluted BAS 654 H in acetone should be stored refrigerated no longer than one month.

3.0 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Water samples received from the field are stored refrigerated (less than 10 °C) before analysis.

3.2 Weighing and Fortification

Add appropriate amount of water sample into a vessel. Add appropriate fortification standard solutions, as specified in the table below.

Analyte	Fortification Level, ppb	Fortification Solution Volume, mL	Fortification Solution Concentration, ppb
M2	0.5	0.5	100
	5.0	0.5	1000
BAS 654 H, M1, M6, M9	0.5	0.2	25
	5.0	0.2	250

3.3 Procedure for BAS 654 H, M1, M6, and M9

For the analysis of BAS 654 H, M1, M6 and M9, 10-mL sample is fortified, filtered through a 0.45 µm PTFE syringe filter and vialled for analysis by LC-MS/MS.

3.4 Procedure for M2

For the analysis of M2, 20 g of sodium chloride is dissolved in 100 mL of sample water and solution is extracted with three portions of methylene chloride, 20 mL each. An additional extraction can be made to slightly improve recoveries if necessary. The extract was dried with sodium sulfate and concentrated under vacuum, where ACN was added and concentrated again to remove the methylene chloride. The concentrated ACN extract was transferred to 15-mL test tubes and evaporated to about 1 mL on a nitrogen evaporator at 50-60 °C. The sample was reconstituted with 50 mM $(\text{NH}_4)_2\text{CO}_3$ to a final volume of 2 mL for the blank, control and LOQ samples and to 20 mL for the 10xLOQ samples, filtered through a 0.45 µm syringe filter and vialled for analysis by LC-MS/MS.

3.5 Influence of Matrix Effects on Analysis

During method development, matrix suppression was experienced. Of all the analytes M2 was most effected with recoveries in the mid 70-s for well water and mid 60-s for surface water. A smaller effect is observed for BAS 654 H and M6, where the recoveries from well water are about 10-15% higher than in surface water. M1 and M9 are practically not affected. Samples can be analyzed using calibration standard solutions prepared in solvent.

3.6 Stability of Extracts

Extracts of BAS 654 H, M1, M6 and M9 are not stable and should be injected immediately after samples are prepared for injection. Extracts of M2 are stable. Procedural recoveries can be used to prove the stability over a longer time interval, if necessary.

4.0 INSTRUMENTAL ANALYSIS

4.1 Instrumentation and Conditions

HPLC Instrument Parameters For BAS 654 H, M1, M6, and M9 (Quantitation)

HPLC System	Agilent 1290 HPLC System with large injection volume			
Column:	Phenomenex Kinetex PFP, 100 x 2.1 mm, 1.7 μ m			
Injection:	40.0 μ L			
Column Temperature:	30.0 $^{\circ}$ C			
Mobile Phase:	A = 10 mM ammonium formate + 1% formic acid in HPLC grade water B = 10 mM ammonium formate + 1% formic acid in HPLC grade methanol			
Gradient	Time (min)	Flow rate (μ L/min)	Composition	
			% A	% B
	0.00	300	100	0
	2.50	300	100	0
	5.50	300	65	35
	6.50	300	65	35
	7.50	300	30	70
	8.80	300	30	70
	9.00	300	10	90
	11.00	300	10	90
	11.10	300	100	0
12.20	300	100	0	

HPLC Instrument Parameters For M2 (Quantitation)

HPLC System	Agilent 1290 HPLC System with large injection volume			
Column:	Phenomenex Kinetex PFP, 100 x 2.1 mm, 1.7 μ m			
Injection:	40.0 μ L			
Column Temperature:	30.0 $^{\circ}$ C			
Mobile Phase:	A = 1% formic acid in HPLC grade water B = 1% formic acid in HPLC grade ACN			
Gradient	Time (min)	Flow rate (μ L/min)	Composition	
			% A	%B
	0.00	300	95	5
	0.50	300	95	5
	3.00	300	50	50
	6.50	300	50	50
	7.00	300	0	100
	7.40	300	0	100
	7.50	300	95	5
8.60	300	95	5	

MS/MS Instrument Parameters For BAS 654 H, M1, M6 and M9.

Mass Spectrometer:	AB Sciex 5500 Triple Quad	
Turbo ion spray	500°C	
Software Version:	Analyst 1.5.2	
Expected Retention Times	M9	M6
	~4.8 min	~5.8 min
Transitions: (m/z)*	178.1→ 150.1 (quantitation)	165.9→ 148.1 (quantitation)
	178.1→ 119.1 (confirmation)	165.9→91.9 (confirmation)
Ionization Mode*	Positive	Positive
Expected Retention Times	M1	BAS 654 H
	~6.8 min	~9.7 min
Transitions: (m/z)*	162.2→ 102.9 (quantitation)	335.0→ 206.1 (quantitation)
	162.2→ 88.7 (confirmation)	335.0→ 162.2 (confirmation)
Ionization Mode*	Positive	Positive

* Proposed as quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time.

Note: Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of the fortification experiments are in the acceptable range.

In general a divert valve is used to reduce the matrix load on the detection system.

Instrument conditions, e.g. injection volumes, columns, gradient steps or mass transitions may be modified, but any changes must be recorded in the raw data. Changes are acceptable, when the recoveries of the fortification experiments are in the acceptable range.

Other parameters like gas flows and voltages are depended of the equipment used and therefore not listed. Those parameters may need to be adapted for the used instrument.

MS/MS Instrument Parameters (M2 only)

Mass	AB Sciex 5500 Triple Quad
Turbo ion spray	500 °C
Software Version:	Analyst 1.5.2
Expected Retention	M2
	~4.4 min
Transitions: (m/z)*	130.1→ 83.0 (quantitation)
	130.1→ 110.1 (confirmation)
Ionization Mode*	Positive

* Proposed as quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time.

4.2 Calibration Procedures

The residue found (pg) is calculated using the peak areas found and the calibration curve. At least five calibration levels need to be injected. The calibration curve is obtained by direct injection of standards of BAS 654 H, M1, M6 and M9 in the range of 0.1 to 10 ng/mL, except for

M2 which has a range of 5 to 50 ng/mL in separate injection. In a given injection run, the same injection volume is used for all samples and standards.

4.3 Calculation of Residues and Recoveries

Calculation of results is based on area measurements. The recoveries and residues of BAS 654 H, M9, M6, M1 and M2 in ppb are calculated with the following formulas:

a) Calibration curve: $y = mx + b$ Solving for x: $x = \frac{y-b}{m}$

Where,
 $m =$ slope
 $b =$ intercept
 $x =$ amount found (pg)
 $y =$ peak area of analyte in the sample

The following equations were used within LIMS for residue and recovery calculations:

b) Amount of sample injected (μL) = $\frac{\text{sample volume (mL)} \times \text{injection size } (\mu\text{L})}{\text{final sample vol. (mL)}}$

Where,
 Final sample volume (mL)
 = [((Initial Extract Volume (mL) / Aliquot Taken (mL)) \times Final Extract Volume (mL))
 \times Dilution Factor (DF)]

c) Amount found (ppb) = $\frac{\text{pg found}}{\mu\text{L of sample injected}}$

d) Percent recovery (%) = $\frac{\text{Amount found (ppb)} - \text{Amount found in control (ppb)}}{\text{Amount fortified (ppb)}} \times 100$

Example: Recovery calculations of Diflufenzopyr (m/z 335.0 \rightarrow 206.1) in well water fortified at LOQ level, 0.5 ppb. See WO-14040109.

a) Calibration curve:
 $y = 2821.02552 \cdot x + 1021.32689$

Solving for x:

$$53851 = 2821.02552 \cdot x + 1021.32689$$

$$x = (53851 - 1021.32689) / 2821.02552 = 18.73$$

b) amount of sample injected () $\frac{0}{0} \text{ m}$

c) amount found (ppb) $\frac{.3 \text{ pg}}{0} \text{ 0.6 ppb}$

d Recovery () $\frac{0.6 \text{ ppb}}{0.00 \text{ ppb}}$ 00 g

5.2 Summary of Method

Type of method: LC-MS/MS

Test systems: Well Water (BD Well)
Surface Water (Goose River)

Analytes and selected mass transitions (*m/z*):

BAS 654 H	335.0 → 206.1
	335.0 → 162.2
M1	162.2 → 102.9
	162.2 → 88.7
M2	130.1 → 83.0
	130.1 → 110.1
M6	165.9 → 148.1
	165.9 → 91.9
M9	178.1 → 150.1
	178.1 → 19.1

Analytical procedure: As described in Section 3.0 of Analytical Method No. R0039

Confirmatory technique: The method allows for the determination of BAS 654 H, M9, M6, M1 and M2 using LC-MS/MS, which is a highly selective and self-confirmatory detection technique. However, a secondary ion and/or different chromatographic conditions can be used for confirmation.

Limit of detection: 0.1 µg/kg

Limit of quantification (LOQ): 0.5 µg/kg (lowest fortification level)

Levels of fortification: 0.5 µg/kg and 5 µg/kg of each analyte

Time required: A set of 13 samples requires about 12 hours of work to complete the two procedures.

7.0 EXTRACTABILITY

BAS 654 H and its metabolytes show remarkable preference for water. They could not be extracted with organic solvent, with the exception of M2. These observations can be explained with the presence of strongly polar groups that result in ionic or zwitterionic species. The attempts to use SPE columns of different nature did not provide sufficient recoveries for the same reason. The sensitivity of the modern instruments allows for the analysis of BAS 654 H, M1, M6 and M9 without concentration and simple filtration.

M2 is only a weak base and does not form zwitterions, contrary to the other analytes. Correspondingly, M2 is extracted well from a large volume of sample water and concentrated into a small final volume. The large concentration factor compensates for the otherwise poor instrument sensitivity toward M2. The current concentration factor is 50 and can be increased if required.

Figure 9.2.1 Method Flowchart – Analysis of BAS 654 H, M1, M6, and M9

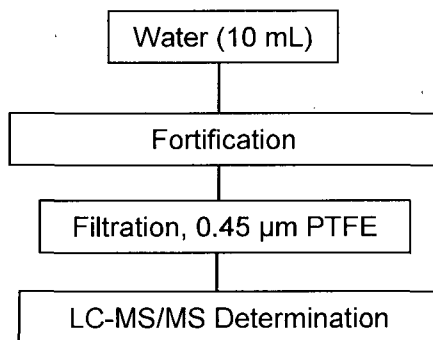


Figure 9.2.2 Method Flowchart – Analysis of M2

