1 INTRODUCTION

1.1 Scope of the Method

Dimethenamid-P (BAS 656 H) is an herbicide used against several weeds in various crops.

The BASF-analytical method L0109/02 is based on methods developed earlier [1 -2]. A new version became necessary to include the metabolite M31 (Reg No. 360712). The new method offers the possibility to determine residues of BAS 656 H and its metabolites M23 (Reg No. 360715), M27 (Reg No. 360714) and M31 (Reg No. 360712) in soil and sediment.

The described method allows the specific determination of BAS 656 H and its metabolites with a limit of quantitation (LOQ) of 0.005 mg/kg for the parent compound and the metabolites in soil and sediment.

This method was developed at BASF SE, Agricultural Center Limburgerhof, Germany.

The purpose of this study was to demonstrate the validity of the method by performing recovery experiments with spiked soil samples. Spiked soil samples were analysed for BAS 656 H and its metabolites M23, M27 and M31. The recovery trials were carried out in both soil and sediment.

The method was validated at two fortification levels (0.005 and 0.05 mg/kg) for two soils (LUFA 2.2 and LUFA 5M soils) and one sediment (Berghäuser Altrhein sediment). For each fortification level and matrix, five replicates were analysed. Additionally, at least two replicates of unfortified control samples were examined per analytical sample set. For each analyte, two mass transitions were also evaluated.

As matrix effects were observed during method development, matrix-matched standards were used for the calibration. Determination is achieved by HPLC-MS/MS.

All the analyses were performed by two persons, with the same equipment, in the same laboratory and within a short interval of time.

1.2 Principle of the Method

Soil samples (5 g) are placed into a plastic centrifuge tube and extracted with 20 mL of solution S1 [methanol/ water (60/40, v/v)] by mechanical shaking for 30 min at 225 rpm. Subsequently, the sample is centrifuged and the liquid phase is decanted and the extraction is repeated with another 20 mL of S1. The extracts were combined and dilute to 50 mL with water. An additional dilution with 50 mL of water was performed (Final Volume = 100 mL) before LC-MS/MS measurement.

1.3 Specificity

Dimethenamid-P (BAS 656 H) and its metabolites (M23, M27 and M31) were identified and quantified as individual compounds.

2 MATERIALS AND METHODS

2.1 Test systems

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

Test System 1: **LUFA 2.2 Soil**

Test System 2: **LUFA 5M Soil**

Test System 3: **Sediment, Berghäuser Altrhein Sediment**

Test System 4: **Solvent, Methanol**

Test System 5: **Solvent, methanol/ water (20/80, v/v)**

The description and characterization of the used soils is given in the respective attached certificates (Figure A.74 to Figure A.76)

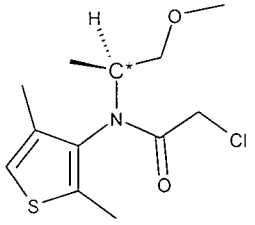
2.2 Test and Reference Items

Test and reference items should be stored according to the information provided in the certificate of analysis (Figure A.77 to Figure A.80).

2.2.1 BAS 656 H

Common Name	**Dimethenamid-P**
BASF Reg. No.	363851
CAS-No.	163515-14-8
Molecular Formula	$C_{12}H_{18}ClNO_2S$
Molecular Weight	275.8
IUPAC Name	(S)-2-chloro-N-(2,4-dimethyl-3-thienyl)-N-(2-methoxy-1-methylethyl)acetamide
Batch No.	BEAU201204
Purity (%)	96.4
Test Substance Type	PAI
Storage Advice	keep at room temperature (typically +25°C) or cooler
GLP	yes
Expiration Date	01.Jul.2016

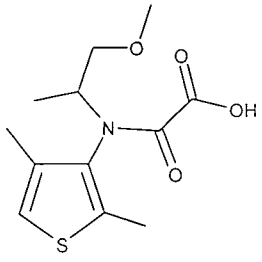
Chemical structure:



2.2.2 Metabolite M23

BASF Reg. No.	360715
CAS-No.	
Molecular Formula	$C_{12}H_{17}NO_4S$
Molecular Weight	271.3
IUPAC Name	N-(2,4-dimethyl-thiophen-3-yl)-N-(2-methoxy-1-methyl-ethyl)-oxalamic acid
Batch No.	L81-76
Purity (%)	98.8
Test Substance Type	ME
Storage Advice	keep at room temperature (typically +25°C) or cooler
GLP	yes
Expiration Date	01.Oct.2018

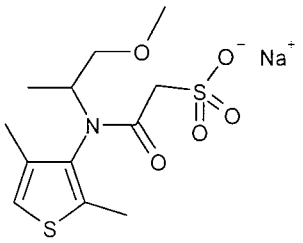
Chemical structure:



2.2.3 Metabolite M27

BASF Reg. No.	360714
CAS-No.	
Molecular Formula	$C_{12}H_{18}NNaO_5S_2$
Molecular Weight	343.4
IUPAC Name	sodium [(2,4-dimethyl-thiophen-3-yl)-(2-methoxy-1-methyl-ethyl)-carbamoyl]-methanesulfonate
Batch No.	1213-32
Purity (%)	97.4
Test Substance Type	ME
Storage Advice	keep at room temperature (typically +25°C) or cooler, Hygroscopic; keep away from humidity
GLP	yes
Expiration Date	01.Feb.2014

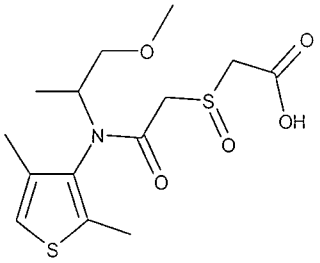
Chemical structure:



2.2.4 Metabolite M31

BASF Reg. No.	360712
CAS-No.	
Molecular Formula	$C_{14}H_{21}NO_5S_2$
Molecular Weight	347.5
IUPAC Name	[[(2,4-Dimethyl-thiophen-3-yl)-(2-methoxy-1-methyl-ethyl)-carbamoyl]-methanesulfinyl]-acetic acid
Batch No.	L81-132
Purity (%)	100
Test Substance Type	ME
Storage Advice	keep at room temperature (typically +25°C) or cooler
GLP	yes
Expiration Date	01.Jul.2019

Chemical structure:



2.2.5 Stability of Test and Reference Items

2.2.5.1 Stability of Fortification and Calibration Standard Solutions

In this study, it was investigated the stability of each analyte in the fortification (1 µg/mL) and standard solutions (1 ng/mL), which were prepared in methanol and methanol/water (20/80, v/v), respectively.

For this purpose, these solutions were stored at 4 °C for four weeks. After two and four weeks, the concentration of BAS 656 H and the respective metabolites (M23, M27 and M31) was measured against freshly prepared standards within one analytical queue.

Quantification of the analytes was done for both mass transitions. Recovery data for each matrix and analyte/mass transition are presented in the results (section 3.1).

2.2.5.2 Stability of Extracts

Since sample extracts at day 0 (starting values) had to be stored prior to final measurement for a short-time period, the stability of the analytes in the extracts was also tested at fortification level of 0.05 mg/kg.

Data were obtained from the stored extracts after 7 days at 4 °C.

At each sampling time point, the concentration of BAS 656 H and the respective metabolites (M23, M27 and M31) was measured against freshly prepared standards within one analytical queue.

Quantification of the analytes was done for both mass transitions. Procedural recoveries were used to prove the stability of the analytes in the soil extracts. Recovery data for each analyte/mass transition are presented in the results (section 3.2).

2.3 Materials and Methods

2.3.1 Equipment

Equipment	Size, Description	Manufacturer
Analytical Balance	Analytical, AT261	Mettler. Giessen (Germany)
Mechanical shaker	Edmund Bühler SM25	Joh. Otto, 72379 Hechingen
Centrifuge	5810R	Eppendorf. 22331 Hamburg
Pipette Gilson microman®	Various volumes	Gilson Medical Electronics S.A., F 95400 Villier-le-Bel, France
Brand HandyStep electronic		
Centrifuge tubes plastic	50 mL	
screw caps with PTFE seals	GL 18	
Autosampler vials with snap caps	1.8 mL, N 11-1	Macherey-Nagel GmbH, D 52313 Düren
Liquid Dispensers	5 mL, 100 mL	Walter Graf u. Co, GmbH &Co, 97861 Wertheim/Main FRG
Volumetric flasks	Various volumes	
Volumetric cylinder	1000 mL	

2.3.2 Reagents

2.3.2.1 Chemicals

Chemical	Grade	Manufacturer/Supplier	Catalog No.
Methanol	Gradient Grade	Merck, Darmstadt, FRG	No. 106011
Water, e.g. Baker® or Millipore®	Gradient Grade	J.T.Baker / Millipore/Waters	

2.3.2.2 Solutions and Solvent Mixtures

Description	Code	Composition
Extraction solvent	S1	Methanol/water, 60/40, v/v Add 600 mL of methanol and 400 mL of water into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Solvent system 2	S2	Methanol/water, 20/80, v/v Add 200 mL of methanol and 800 mL of water into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution
HPLC mobile phase A	LC1	0.1% Formic acid in water Add 1000 mL of water and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	0.1% Formic acid in methanol Add 1000 mL of methanol and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

2.3.2.3 Standard Solutions

Stock Solutions

The stock solution (1 mg/mL) was prepared by weighing an appropriate amount of each analyte into a flask and adding the required volume of methanol. Since metabolite M27 was used as sodium salt form, calculations were previously performed to weight the equivalent amount of metabolite as free acid form, in which the reported results refers to.

Further dilution steps of stock solution

Take solution (µg/mL)	Volume (mL)	Dilute with methanol to a final volume of (mL)	Concentration (µg/mL)
Combine 1 mL of each stock solution		10	100
100	1	10	10
10	1	10	1
1.0	1	10	0.1

Fortification Solutions

Standard solutions for fortification were prepared by mixing standard solution from the stock solution (see above). Afterwards, dilution series were made up by using the appropriate solvent, as exemplified in the table below. The use of sonication or vortexing was also considered for ensuring a complete homogeneous solution.

Preparation of mixed Fortification solutions

Take solution (µg/mL)	Volume (mL)	Dilute with methanol to a final volume of (mL)	Concentration (µg/mL)
10	1	10	1.0
1.0	1	10	0.1

Calibration Standard Solutions

Standard calibration solutions for LC-MS/MS analysis were prepared using the solutions, which were prepared in the previous section "stock solutions", and by diluting them with methanol/ water 20/80, v/v (solution S2) as needed. These solutions were made up as follows:

Preparation of standard solutions for calibration

Take solution (ng/mL)	Volume (mL)	Dilute with S2* to a final volume of (mL)	Concentration (ng/mL)
100	1.00	10	10
10#	1.00	10	1.00
10#	0.75	10	0.75
1.00#	5.00	10	0.50
0.50#	5.00	10	0.25
0.25#	4.00	10	0.10
0.50#	1.00	10	0.05

#from the dilution series

*Where the use of matrix-matched standards was needed (i.e., instrument recovery samples), the calibration standard solutions were prepared using a matrix solution (i.e., from the extracts of a control sample during the analytical procedure). The matrix-matched standards were made in a way in which the matrix load is at least 90% of the matrix load in the unknown samples. In addition, the matrix load was the same for all calibration standard solutions.

2.3.3 Set-up of the Analytical Run

Reagent blanks or blanks were injected as necessary. Each analytical run started and ended with an injection of a calibration standard. Standards were interspersed with samples. Six calibration levels were injected.

2.4 Instrumental Analysis

2.4.1 Analytical Procedure

2.4.1.1 Weighing and Fortification

For treated and control samples, 5 g of soil were transferred into a plastic centrifuge tube.

For fortified samples, 5 g of the matrix were also transferred into a plastic centrifuge tube. Then, fortification solutions were added on the matrix, as shown in the following table:

Preparation of Fortification

Sample Type	Sample Weight	Concentration of Spiking Solution [µg/mL]	Volume of Spiking Solution [mL]	Level of Fortification [mg/kg]
Control	5 g	-	-	0.000
Fortification (LOQ)	5 g	0.1	0.25	0.005*
Fortification (10xLOQ)	5 g	1.0	0.25	0.05
Treated	5 g	-	-	-

* Limit of quantification (LOQ)

2.4.1.2 Extraction of Sample Material

Soil samples (5 g) are placed into a plastic centrifuge tube and extracted with 20 mL of solution S1 [methanol/ water (60/40, v/v)] by mechanical shaking for 30 min at 225 rpm. Subsequently, the sample is centrifuged and the liquid phase is decanted and the extraction is repeated with another 20 mL of S1. The extracts were combined and dilute to 50 mL with water. An additional dilution with 50 mL of water was performed (Final Volume = 100 mL) before LC-MS/MS measurement.

2.4.1.3 Preparation for Measurement

An aliquot of the combined extract (V_{End} = 100 mL, V_{End} = Final Volume) was transferred into a HPLC vial.

In the case of fortifications, control and treated samples in the range of LOQ, no further dilutions were necessary. However, in samples with higher residues, dilutions with appropriate amounts of S2 were performed.

2.4.2 Instrumentation and Conditions

	Parameter		
Chromatographic System	CTC Pal Autosampler, Sciex Triple Quadrupole LC/MS/MS API 4000		
Analytical-column	Zorbax Eclipse XDB C18, 150 x 4.6 mm, 5 µm particle size		
Pre-column:	No		
Column Temperature	40°C		
Injection Volume	50 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Methanol / formic acid, 1000/1, v/v		
Flow Rate	1000 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.0	80	20
	5.0	20	80
	10.0	20	80
	10.1	80	20
	15.0	80	20
Detection System	PE Sciex API 4000 Mass Spectrometer		
Ionisation	Turbo spray (ESI)		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
BAS 656 H	276 → 244* 276 → 168	positive	approx. 7.3 min
360715 (M23)	270 → 198* 270 → 166	negative	approx. 5.6 min.
360714 (M27)	320 → 121* 320 → 80	negative	approx. 6.1 min
360712 (M31)	346 → 240* 346 → 198	negative	approx. 5.8 min.

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

2.4.3 Calibration Procedures

Calculation of results was based on peak area measurements using a calibration curve. Six calibration levels were injected. The calibration curve was obtained by direct injection of BAS 656 H, M23, M27 and M31 mix standards for LC-MS/MS in the range of 1 ng/mL to 0.05 ng/mL. In all injection runs, the same injection volume was used for all samples and standards.

2.4.4 Rounding of Decimal Places

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than were used in the actual calculation to increase readability and to indicate the approximate precision of the reported results. Minor differences in the results obtained with such "rounded" values in comparison to those obtained with higher precision values are well within the limits of the experimental accuracy and therefore of no practical concern.

2.4.5 Calculation of Residues and Recoveries

For the procedural recoveries, the sample weight was considered 5 g in the final calculation of residues [mg/kg]. The method requires also that the sample weight was 5 ± 0.1 g for fortification samples.

I. Concentration Final Volume [ng/mL] $= \frac{\text{Response} - \text{Intercept}}{\text{Slope}} = C_A$

II. Residues [mg/kg] $= \frac{V_F \times C_A}{G \times A_F \times 1000}$

V_F	=	Final volume of the extract after all dilution steps [mL]
C_A	=	Concentration of analyte obtained from the calibration curve [ng/mL]
G	=	Weight of the sample extracted [g]
A_F	=	Aliquotation factor = 1.0 (=100%)
1000	=	Factor remaining after all unit conversions

Recovery is the percentage of the fortified amount (µg or ng), which is recovered through the method. The recoveries of spiked compounds are calculated according to equation **III**:

III. Recovery corrected (%) $= \frac{(\text{Residue in fortified sample} - \text{Residue in control}) \times 100}{\text{Nominal concentration in the sample matrix}}$

Appendix 6.2: Additional Information on the Method

Figure A.13: Method Flowchart