ABSTRACT

The purpose of this study was to demonstrate that BASF Analytical Method D1308/02 “Method for the Determination of Residues of Afidopyropen (BAS 440 I, Reg No. 5599022) and its metabolites M440I001 (Reg No. 5741530), M440I002 (Reg No. 5741532), M440I003 (Reg No. 5741533), M440I005 (Reg No. 5824382), M440I016 (Reg No. 5845597), M440I024 (Reg No. 5886215), and M440I057 (Reg No. 6010129) in soil by LC-MS/MS”, could be performed successfully at an outside facility with no prior experience with the method (Reference 1).

Principle of the method. The residues of BAS 440 I and its metabolites M440I001, M440I002, M440I003, M440I005, M440I016, M440I024, and M440I057 are extracted from 2.5 grams of soil by adding 25 mL of 70:30 acetonitrile:water (v/v) twice. An aliquot (12 mL) is taken and the acetonitrile layer is partitioned in the presence of various salts (MgSO4, NaCl, citric acid, disodium salt sesquihydrate, citric acid, trisodium salt dihydrate) for sample clean-up. The approximately 7 mL acetonitrile supernatant is treated with magnesium sulfate and PSA to remove trace water. A 4.5 mL aliquot of the resulting extract is then evaporated to dryness at room temperature and reconstituted to a 1 mL final volume for LC-MS/MS determination.

Test conditions. For validation, untreated soil samples were fortified with BAS 440 I and its metabolites and analyzed according to the established method validation guidelines. The analytical sets for each matrix typically consisted of a reagent blank, two controls, five replicates fortified with each analyte at the method limit of quantitation (LOQ) and five replicates fortified at a higher level, corresponding to 10× the LOQ. The mass transitions described above were evaluated. In conjunction with the subject study, matrix- and solvent-matched standards were analyzed in a separate experiment to evaluate any potential matrix effects.

Limit of Quantification (LOQ) and Limit of Detection (LOD). The limit of quantitation (LOQ) is defined as the lowest fortification level tested. For soil, the LOQ is 0.001 mg/kg. The limit of detection (LOD) is set at 0.0002 mg/kg, which is at 20% of the LOQ during this ILV. The LOD is defined as the absolute amount (0.0000128 ng – Method A and 0.0000064 ng – Method C) of analyte injected into the LC-MS/MS using lowest standard of the calibration.

Selectivity. Instrument Method A determines residues of BAS 440 I, M440I001, M440I002, M440I003, M440I005, M440I016, and M440I024, and Instrument Method C determines residues of BAS 440 I, M440I001, M440I002, M440I003, M440I005, M440I016, M440I024, and M440I057 in soil matrices by LC-MS/MS. No interfering peaks were found at the retention times for any analyte. The experiment to evaluate any potential matrix effects showed that the matrix load in the samples from each commodity had no significant influence on analysis (matrix effects < 20%) and is presented in the results and discussion section; therefore, the validation samples were analyzed only using solvent-based calibration standard solutions (Reference 1).

Linearity. Acceptable linearity was observed for the standard range and the two mass transitions tested: The method-detector response was linear over the 0.05–2.5 ng/mL range (r = ≥0.990), for soil analyses.

1. INTRODUCTION

1.1 Scope of the Method

BASF Analytical Method No. D1308/02 was developed to determine the residues of BAS 440 I and its metabolites M440I001, M440I002, M440I003, M440I005, M440I016, M440I024, and M440I057 in soil matrices using LC-MS/MS at BASF Crop Protection (BASF) in Research Triangle Park, North Carolina. This method was independently validated at ADPEN Laboratories, Inc (ADPEN).

The independent lab validation was conducted using two fortification levels limit of quantitation (0.001 ppm) and ten times of limit of quantitation (0.01 ppm) for one soil type. For each fortification level and matrix, five replicates were analyzed. Additionally, one reagent blank and two replicates of unfortified samples were examined.

1.2 Principle of the Method

The soil samples (2.5 g) were fortified with acetonitrile and thoroughly mixed. An aliquot of resulting solution was analyzed to determine the residues of BAS 440 I and its metabolites using LC-MS/MS. The transitions for BAS 440 I and it metabolites were monitored in positive mode for primary and confirmation quantification.

1.3 Specificity

To demonstrate the specificity of the analytical method, an additional confirmatory mass transition was monitored simultaneous to the primary quantitation transition for analysis of all analytes. Primary and confirmatory transitions for each analyte are listed below:

Analyte	Transition (*m/z*)	
	Primary	Confirmatory
BAS 440 I	594.20 → 202.30	594.20 → 148.20
M440I001	458.20 → 148.20	458.20 → 106.10
M440I002	526.20 → 148.10	526.20 → 202.10
M440I003	526.21 → 148.00	526.21 → 202.20
M440I005	524.20 → 148.30	524.20 → 80.00
M440I016 (Method A)	524.20 → 218.10	542.20 → 218.10
M440I016 (Method C)	542.20 → 218.10	542.20 → 164.00
M440I024	610.50 → 218.10	610.50 → 122.00
M440I057	524.10 → 148.20	524.10 → 202.20

The method was able to accurately determine residues of BAS 440 I and its metabolites and no interference was observed at the retention time of the analyte peaks. No matrix suppression or enhancement was found to affect the analytes in soil.

2. REFERENCE SUBSTANCE AND SAMPLING HISTORY

2.1 Test Systems

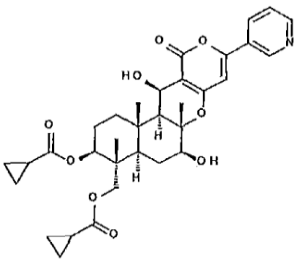
The test system considered in this study was soil.

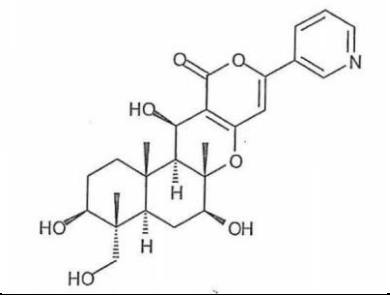
The control soil sample was provided by BASF, and was received on October 15, 2015. Upon arrival at the laboratory, the sample was opened, inspected, and checked against enclosed shipping forms. The test system was received frozen and stored under frozen conditions at all times, unless necessary for laboratory analysis. The test system was characterized at AGVISE Laboratories (604 Highway 15 West, Northwood, ND 58267). A copy of the characterization data for the sample is provided in Appendix E.

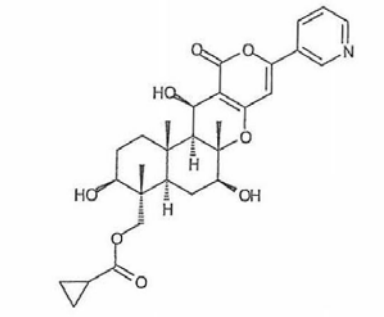
2.2 Test and Reference Substances

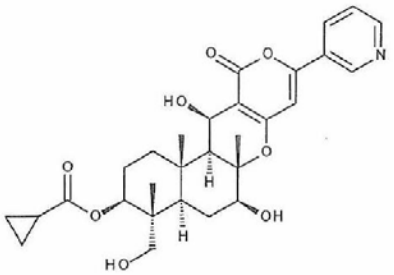
The standard substance was stored in a freezer (≤ -5°C) until use. BASF has retained a reserve sample of this chemical, and has documentation specifying the location of the synthesis and characterization information available at BASF Crop Protection, Research Triangle Park, North Carolina.

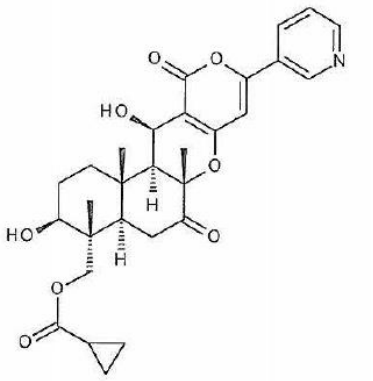
The reference substances of BAS 440 I, M440I001, M440I002, M440I003, M440I005, M440I016, M440I024, and M440I057 were provided by the sponsor and received on August 12, 2015 and September 23, 2015. The certificates of analysis for all substances are presented in Appendix B. A summary of the reference substances are presented below.

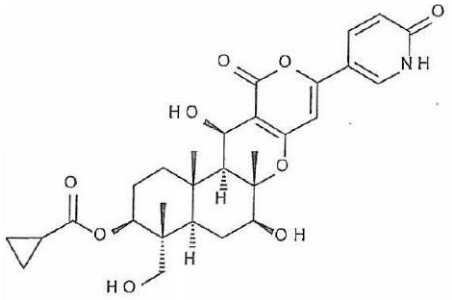
BASF Code Name:	BAS 440 I
Common Name:	Afidopyropen
Batch Number:	COD-002022
BASF Registry Number:	5599022
CAS Number:	915972-17-7
IUPAC Name:	[(3S,4R,4aR,6S,6aS,12R,12aS,12bS)-3 (cyclopropanecarbonyloxy)-6,12-dihydroxy-4,6a,12b-trimethyl-11-oxo-9-(pyridin-3-yl)-1,2,3,4,4a,5,6,6a, 12a,12b-decahydro 11H,12H-benzo[f]pyrano[4,3-b]chromen-4-yl]methyl cyclopropanecarboxylate
Molecular Formula:	$C_{33}H_{39}NO_9$
Molecular Weight:	593.7 g/mol
Purity:	94.4%
Expiration Date:	November 30, 2016
Chemical Structure:	

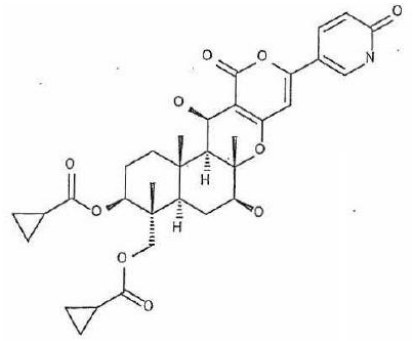
BASF Code Name:	M440I001
Batch Number:	L82-66
BASF Registry Number:	5741530
IUPAC Name:	(3S,4R,4aR,6S,6aS,12R,12aS,12bS)-3,6,12-trihydroxy-4-(hydroxymethyl)-4,6a,12b-trimethyl-9-(pyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H,11H-benzo[f]pyrano[4,3-b]chromen-11-one
Molecular Formula:	$C_{25}H_{31}NO_7$
Molecular Weight:	457.5 g/mol
Purity:	93.9%
Expiration Date:	February 1, 2016
Chemical Structure:	

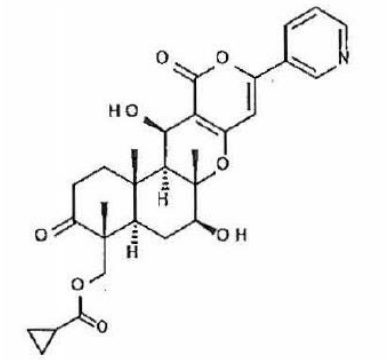
BASF Code Name:	M440I002
Batch Number:	L82-67
BASF Registry Number:	5741532
IUPAC Name:	[(3S,4R,4aR,6S,6aS,12R,12aS,12bS)-3,6,12-trihydroxy-4,6a,12b-trimethyl-11-oxo-9-(pyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H,11H-benzo[f]pyrano[4,3-b]chromen-4-yl]methyl cyclopropanecarboxylate
Molecular Formula:	$C_{29}H_{35}NO_8$
Molecular Weight:	525.6 g/mol
Purity:	92.5%
Expiration Date:	February 1, 2016
Chemical Structure:	

BASF Code Name:	M440I003
Batch Number:	L82-72
BASF Registry Number:	5741533
IUPAC Name:	(3S,4R,4aR,6S,6aS,12R,12aS,12bS)-6,12-dihydroxy-4-(hydroxymethyl)-4,6a,12b-trimethyl-11-oxo-9-(pyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H,11H-benzo[f]pyrano[4,3-b]chromen-3-yl cyclopropanecarboxylate
Molecular Formula:	$C_{29}H_{35}NO_8$
Molecular Weight:	525.6 g/mol
Purity:	98.6%
Expiration Date:	September 1, 2016
Chemical Structure:	

BASF Code Name:	M440I005
Batch Number:	L82-73
BASF Registry Number:	5824382
IUPAC Name:	[(3S,4R,4aR,6aS,12R,12aS,12bS)-3,12-dihydroxy-4,6a,12b-trimethyl-6,11-dioxo-9-(pyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a, 12b-decahydro-2H,11H-benzo[f]pyrano[4,3-b]chromen-4-yl]methyl cyclopropanecarboxylate
Molecular Formula:	$C_{29}H_{33}NO_8$
Molecular Weight:	523.6 g/mol
Purity:	90.9%
Expiration Date:	March 1, 2016
Chemical Structure:	

BASF Code Name:	M440I016
Batch Number:	L82-148
BASF Registry Number:	5845597
IUPAC Name:	(3S,4R,4aR,6S,6aS,12R,12aS,12bS)-6,12-dihydroxy-4-(hydroxymethyl)-4,6a,12b-trimethyl-11-oxo-9-(6-oxo-1,6-dihydropyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H,11H-benzo[f]pyrano[4,3-b]chromen-3-yl cyclopropanecarboxylate
Molecular Formula:	$C_{29}H_{35}NO_9$
Molecular Weight:	541.6 g/mol
Purity:	88.9%
Expiration Date:	May 1, 2016
Chemical Structure:	

BASF Code Name:	M440I024
Batch Number:	L82-149
BASF Registry Number:	5886215
IUPAC Name:	[(3S,4a,4aR,6S,6aS,12R,12aS,12bS)-3 [(cyclopropylcarbonyl)oxy]-6,12-dihydroxy-4,6a,12b-trimethyl-11-oxo-9-(6-oxo-1,6-dihydropyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H,11H-benzo[f]pyrano[4,3-b]chromen-4-yl]methyl cyclopropanecarboxylate
Molecular Formula:	$C_{33}H_{39}NO_{10}$
Molecular Weight:	609.7 g/mol
Purity:	91.3%
Expiration Date:	May 1, 2016
Chemical Structure:	

BASF Code Name:	M440I057
Batch Number:	L82-164
BASF Registry Number:	6010129
IUPAC Name:	[(4R,4aR,6S,6aS,12R,12aS,12bS)-6,12-dlhydroxy-4,6a,12b-trimethyl-3,11-dioxo-9-(pyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a, 12b-decahydro-2H,11H-benzo[f]pyrano[4,3-b] chromen-4-yl]methyl cyclopropanecarboxylate
Molecular Formula:	$C_{29}H_{33}NO_8$
Molecular Weight:	523.6 g/mol
Purity:	97.4%
Expiration Date:	January 1, 2017
Chemical Structure:	

2.3 Test System

Soil was homogenized and provided by BASF. The soil sample was sent from BASF on October 14, 2015 and received at ADPEN on October 15, 2015.

The Laboratory Information Management System (LIMS) provided a unique laboratory analysis code (e.g., 151019002-001) for the soil sample, which is cross-referenced on the detailed analytical data reports to the assigned unique sample number.

3. ANALYTICAL METHOD

BASF Analytical Method D1308/02, “Method for the Determination of Residues of Afidopyropen (BAS 440 I, Reg No. 5599022) and its metabolites M440I001 (Reg No. 5741530), M440I002 (Reg No. 5741532), M440I003 (Reg No. 5741533), M440I005 (Reg No. 5824382), M440I016 (Reg No. 5845597), M440I024 (Reg No. 5886215), and M440I057 (Reg No. 6010129) in soil by LC-MS/MS” was used for the analysis of the samples.

The residues of BAS 440 I and its metabolites M440I001, M440I002, M440I003, M440I005, M440I016, M440I024, and M440I057 are extracted from 2.5 grams of soil by adding 25 mL of 70:30 acetonitrile:water (v/v) twice. An aliquot (12 mL) is taken and the acetonitrile layer is partitioned in the presence of various salts (MgSO4,, NaCl, citric acid, disodium salt sesquihydrate, citric acid, trisodium salt dihydrate) for sample clean-up. The approximately 7 mL acetonitrile supernatant is treated with magnesium sulfate and PSA to remove trace water. A 4.5 mL aliquot of the resulting extract is then evaporated to dryness at room temperature and

reconstituted to a 1 mL final volume for LC-MS/MS determination. Instrument parameters for Method A and C are described in Tables 32 and 33.

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

Analyte	Instrument Method	Transition (*m/z*)		Ionization Mode	Retention Time (min)
		Primary	Confirmatory		
BAS 440 I	A	594.20 → 202.30	594.20 → 148.20	Positive	4.3
	C				11.1
M440I001	A	458.20 → 148.20	458.20 → 106.10		3.5
	C				1.6
M440I002	A	526.20 → 148.10	526.20 → 202.10		3.8
	C				4.7
M440I003	A	526.21 → 148.00	526.21 → 202.20		4.0
	C				8.5
M440I005	A	524.20 → 148.30	524.20 → 80.00		4.0
	C				7.2
M440I016	A	524.20 → 218.10	542.20 → 218.10		4.0
	C	542.20 → 218.10	542.20 → 164.00		8.3
M440I024	A	610.50 → 218.10	610.50 → 122.00		4.2
	C				10.8
M440I057	C	524.10 → 148.20	524.10 → 202.20		7.0

Table 32 Instrument Conditions and Parameters (Method A)

HPLC Conditions			
Chromatographic System:	Agilent 1290 UPLC		
Column:	Acquity UPLC BEH Phenyl; 1.7 µm, 2.1 x 100 mm		
Temperature:	45 °C		
Flow rate (µL/min)	700		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	95.0	5.0
	2.00	95.0	5.0
	4.00	25.0	75.0
	4.50	10.0	90.0
	5.00	10.0	90.0
	5.10	95.0	5.0
	6.00	95.0	5.0
Mobile Phase A:	0.1% formic acid in water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	20 µL		

MS/MS Conditions						
Detection System:	AB SCIEX 6500					
Ionization:	Turbo Spray					
Polarity:	Positive					
Curtain gas (CUR):	10.00, 20.00					
Temperature (TEM):	500 °C					
Collision gas setting (CAD):	8.00					
GS1:	30.00, 14.00					
GS2:	30.00, 0.00					
Entrance potential (EP):	7.00					
Scan type:	MRM					
MRM Conditions	Transition (m/z)	Dwell (msec)	DP	CE	CXP	Retention Time (min)
BAS 440 I Reg. No. 5599022	594.20 → 202.30	100.00	116.80	46.20	6.20	4.27
	594.20 → 148.20			85.90	13.20	
M440I001 Reg. No. 5741530	458.20 → 148.20	100.00	106.60	45.00	7.10	3.46
	458.20 → 106.10			70.00	17.50	
M440I002 Reg. No. 5741532	526.20 → 148.10	100.00	17.90	50.30	8.80	3.80
	526.20 → 202.10			41.00	13.70	
M440I003 Reg. No. 5741533	526.21 → 148.00	100.00	106.60	48.90	8.90	3.96
	526.21 → 202.20			43.80	10.40	
M440I005 Reg. No. 5824382	524.20 → 148.30	100.00	106.60	55.10	6.90	3.92
	524.20 → 80.00			120.20	14.00	
M440I016 Reg. No. 5845597	524.20 → 218.10	200.00	86.90	44.60	11.90	3.95
	542.20 → 218.10					
M440I024 Reg. No. 5886215	610.50 → 218.10	100.00	80.00	43.80	13.10	4.18
	610.50 → 122.00			94.70	19.30	

Note: Method A does not analyze for M440I057 due to coelution with M440I005.

Table 33 Instrument Conditions and Parameters (Method C)

HPLC Conditions			
Chromatographic System:	Agilent 1290 UPLC		
Column:	Acquity UPLC BEH Phenyl; 1.7 µm, 2.1 x 100 mm		
Temperature:	45 °C		
Flow rate (µL/min)	600		
Gradient:	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	85.0	15.0
	0.05	85.0	15.0
	8.50	75.0	25.0
	10.25	55.0	45.0
	11.00	5.0	95.0
	11.95	5.0	95.0
	12.00	85.0	15.0
	13.00	85.0	15.0
Mobile Phase A:	0.1% formic acid in water		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Injection Volume:	10 µL		

MS/MS Conditions						
Detection System:	AB SCIEX 6500					
Ionization:	Turbo Spray					
Polarity:	Positive					
Curtain gas (CUR):	10.00					
Temperature (TEM):	500 °C					
Collision gas setting (CAD):	8.00					
GS1:	30.00					
GS2:	30.00					
Entrance potential (EP):	7.00					
Scan type:	MRM					
MRM Conditions	Transition (m/z)	Dwell (msec)	DP	CE	CXP	Retention Time (min)
BAS 440 I Reg. No. 5599022	594.20 → 202.30	100.00	116.80	46.20	6.20	11.10
M440I001 Reg. No. 5741530	458.20 → 148.20	200.00	106.60	45.00	7.10	1.58
	458.20 → 106.10			70.00	17.50	
M440I002 Reg. No. 5741532	526.20 → 148.10	200.00	17.90	50.30	8.80	4.75
	526.20 → 202.10			41.00	13.70	
M440I003 Reg. No. 5741533	526.21 → 148.00	50.00	81.10	48.90	8.90	8.54
	526.21 → 202.20			43.80	10.40	
M440I005 Reg. No. 5824382	524.20 → 148.30	50.00	71.50	55.10	6.90	7.20
	524.20 → 80.00			120.20	14.00	
M440I016 Reg. No. 5845597	542.20 → 218.10	200.00	86.90	44.60	11.90	8.24
	542.20 → 164.00			54.90	10.30	
M440I024 Reg. No. 5886215	610.50 → 218.10	200.00	80.00	43.80	13.10	10.80
	610.50 → 122.00			94.70	19.30	
M440I057 Reg. No. 6010129	524.10 → 148.20	50.00	112.20	49.30	9.10	6.97
	524.10 → 202.20			42.10	11.80	

1 INTRODUCTION

BAS 440 I is an insecticide used against several insects in various crops.

Version	TP Date	Change
1	Aug 2013	Creation of the method.
1	May 15, 2014	Addition of clean-up and concentration steps to improve recoveries.
1	July 14, 2014	Addition of metabolites M440I016 and M440I024. Correct LC Method B from Methanol to Acetonitrile, gradient, and retention times.
1	June 23, 2015	Add Reg No. of analytes to the title
2	August 20, 2015	Adding metabolite M440I057 and new gradient to separate all analytes. Updated example calculations.
2	September 23, 2015	Correct typos and add clarifications
2	October 19, 2015	Correct typo in title and abstract

2 MATERIALS

2.1 Safety

The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Store work clothing separately. Keep away from food, drink, and animal feed stuffs. No eating, drinking, smoking, or tobacco use at the place of work. Hands and/or face should be washed before breaks and at the end of the shift. Details are given in the Materials Safety Data Sheets (MSDS) of the individual substances. All procedures involving organic solvents should be performed in a well-ventilated hood. Disposal of samples and chemicals must be done in compliance with on-site safety policies and procedures.

2.2 Test and Reference Items

Test and reference items should be stored according to the information provided in the certificate of analysis.

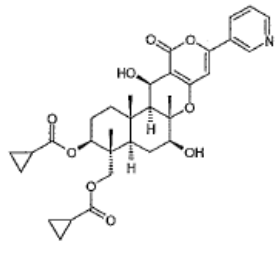
2.2.1 BAS 440 I

Reg. No.: 5599022

Common name: BAS 440 I

Chemical name: [(3S, 4R, 4aR, 6S, 6aS, 12R, 12aS, 12bS)-3-(cyclopropanecarbonyloxy)-6,12-dihydroxy-4, 6a 12b-trimethyl-11-oxo-9-(pyridin-3-y))-1,2,3,4,4a,5,6,6a, 12a, 12b-decahydro-11H,12H-benzo[f]pyrano[4,3-b]chromen-4-yl] methylcyclopropanecarboxylate

Structural formula:



Empirical formula: $C_{33}H_{39}NO_9$

Molecular weight: 593.7

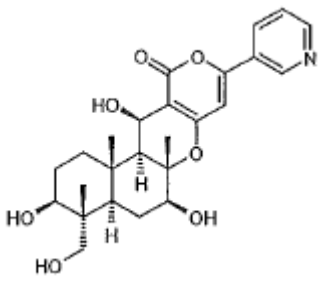
2.2.2 M440I001

Reg. No.: 5741530

Common name: M440I001

Chemical name: (3S,4R,4aR,6S,6aS,12R,12aS,12bS)-3,6,12-trihydroxy-4-(hydroxymethyl)-4,6a,12b-trimethyl-9-(pyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H, 11H-benzo[f]pyrano[4,3-b]chromen-11-one

Structural formula:



Empirical formula: $C_{25}H_{31}NO_7$

Molecular weight: 457.5

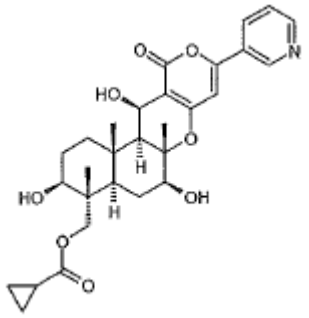
2.2.3 M440I002

Reg-No.: 5741532

Common name: M440I002

Chemical name: [(3S,4R,4aR,6S,6aS,12R,12aS,12bS)-3,6,12-trihydroxy-4-6a, 12b-trimethyl-11-oxo-9-(pyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H, 11H- benzo[f]pyrano[4,3-b]chromen-4yl]methyl cyclopropanecarboxylate

Structural formula:



Empirical formula: $C_{29}H_{35}NO_8$

Molecular weight: 525.6

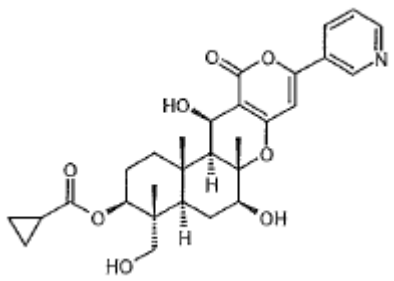
2.2.4 M440I003

Reg-No.: 5741533

Common name: M440I003

Chemical name: (3S,4R,4aR,6S,6aS,12R,12aS,12bS)-6,12-dihydroxy-4-(hydroxymethyl)-4,6a,12b-trimethyl-11-oxo-9-(pyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H, 11H-benzo[f] pyrano [4,3-b]chromen-3-yl cyclopropanecarboxylate

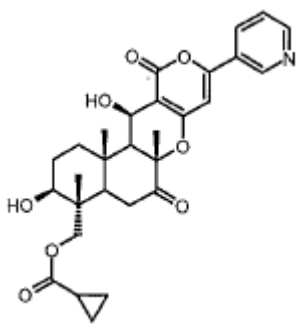
Structural formula:



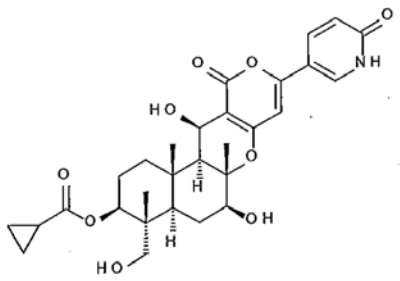
Empirical formula: $C_{29}H_{35}NO_8$

Molecular weight: 525.6

2.2.5 M440I005

Reg-No.:	5824382
Common name:	M440I005
Chemical name:	[(3S,6aS,12R,12bS)-3,12-dihydroxy-4,6a,12b-trimethyl-6,11-dioxo-9-(pyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H,11H-benzo[f]pyrano[4,3-b]chromen-4-yl]methyl cyclopropanecarboxylate
Structural formula:	
Empirical formula:	$C_{29}H_{33}NO_8$
Molecular weight:	523.6

2.2.6 M440I016

Reg-No.:	5845597
Common name:	M440I016
Chemical name:	(3S,4R, 4aR,6S,6aS,12R,12aS,12bS)-6,12-dihydroxy-4-(hydroxymethyl)-4,6a,12b-trimethyl-11-oxo-9-(6-oxo-1,6-dihydropyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H, 11H-benzo[f] pyrano [4,3-b]chromen-3-yl cyclopropanecarboxylate
Structural formula:	
Empirical formula:	$C_{29}H_{35}NO_9$
Molecular weight:	541.6

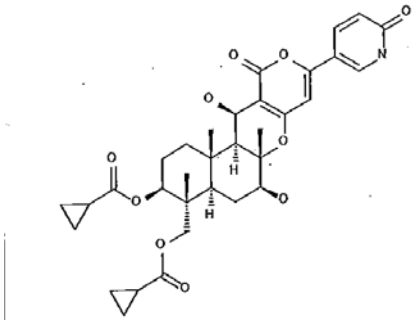
2.2.7 M440I024

Reg-No.: 5886215

Common name: M440I024

Chemical name: (3S,4a, 4aR,6S,6aS,12R,12aS,12bS)-3-[(cyclopropylcarbonyl)oxy] -6,12-dlhydroxy-4, 6a, 12b-trimethyl-11-oxo-9-(6-oxo-1,6-dihydropyndin-3-yl)- 1,3,4,4a, 5,6,6a, 12,12a,12a,12b-decahydro-2H,11 H-benzo[f]pyrano[4,3-b]chromen-4-yi]methyl cyclopropanecarboxylate

Structural formula:



Empirical formula: $C_{33}H_{39}NO_{10}$

Molecular weight: 609.7

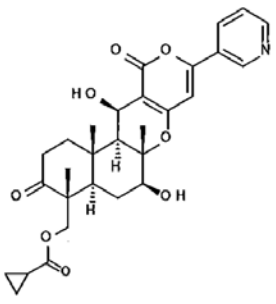
2.2.8 M440I057

Reg-No.: 6010129

Common name: M440I057

Chemical name: [(4R,4aR,6S,6aS,12R,12aS,12bS)-6,12-dihydroxy-4,6a,12b-trimethyl-3,11-dioxo-9-(pyridin-3-yl)-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2H,11H-benzo[f]pyrano[4,3-b]chromen-4-yl]methyl cyclopropanecarboxylate

Structural formula:



Empirical formula: $C_{29}H_{33}NO_8$

Molecular weight: 523.6

2.3 Equipment

Equipment	Size, Description	Manufacturer/ Supplier
Amber bottle with Teflon-lined screw caps	2 OZ	VWR
Analytical Balance	PM 4800 Delta Range	Mettler Toledo
Centrifuge	Refrigerated Centrifuge Model CS-6KR	Beckmann
Centrifuge tubes, glass	50 mL	Pyrex
Centrifuge tubes, Teflon	50 mL	VWR
Conical Centrifuge Tube	50 mL	VWR
Culture Tubes	16 X 100 mm	Fisher
Culture Tubes,caps	16 mm	VWR
Flask, Erlen Meyer, 24/40	1000 mL	Various
Flask, Flat Bottom	250 mL	Various
Graduated Tubes	10 mL	Kimax
Injection Vials	2 mL Amber	National Scientific
Mechanical shaker	KS501 digital	IKA Labortechnik
MicroMan pipettes	10 µL – 1000 µL	Gilson
Stephan Floor Chopper	Homoloid Machine, Model J.	Fitzpatrick, Co.
Syringe filter	PTFE Acrodisc® 0.22 µm pore size	Pall Gelman
Square Jar	Clear French Square Jars with PTFE caps	Fisher
Ultrasonic Bath	Branson 1210	Branson
UPLC-MS/MS Instrument	API 4000	PE Sciex
UPLC/MS column	UPLC BEH Phenyl 1.7mm 2.1 X 100mm	Waters
UPLC/MS column	Acquity UPLC BEH 1.7mm 2.1 X 50mm	Waters
Volumetric Flasks	5, 10, 25, 50, 100 mL	VWR
Wide neck glass bottle	250 mL	Kimble

Note: The equipment and instrumentation listed above may be substituted by that of similar specifications. The applicability is confirmed if the recoveries of the fortification experiments are in the expected concentration range.

2.4 Reagents

2.4.1 Chemicals

Chemical	Grade	Manufacturer/ Supplier	Catalog Number
Acetonitrile	High Purity	B & J	015-4
Acetone	HPLC Grade	EMD	AX0115P-1
Methanol	High Purity	B & J	230-4
Water	High Purity	B & J	365-4
Formic Acid	98%	E.M. Science	FX0440-7
Magnesium Sulfate	Anhydrous	Fisher Scientific	M65-500
Sodium Chloride	Reagent Grade	Sigma Aldrich	310166-1KG
PSA	40um	Agilent	12213024
Citric acid, disodium salt sesquihydrate	99%	Acros Organics	250240010
Citric acid, trisodium salt dihydrate	99%	Acros Organics	227130010
QuEChERS Preweighed Extraction Salts		UCT (United Chem)	ECQUUS12CT
QuEChERS dSPE		UCT (United Chem) or Restek	ECMPS15CT or 26236

Note: Equivalent reagents and chemicals from other suppliers may be substituted. The preweighed Quechers tubes listed can be used for analysis

2.4.2 Solutions and Solvent Mixtures

Description	Code	Composition
Extraction solvent	E1	acetonitrile-water (70:30, v/v) In a 4-liter container, mix 1200 mL water and 2800 mL acetonitrile. Other amounts may be made in the same proportion. Store at room temperature.
Final volume	FV	0.1% formic acid in acetonitrile-water (30:70, v/v) Add 1 mL of concentrated formic acid to 700 mL of water in a 1-liter volumetric flask. Dilute to 1 liter with acetonitrile. Other amounts may be made in the same proportion. Store at room temperature.
HPLC mobile phase A	LC1	0.1% formic acid in water Add 500 mL of water and 1 mL of concentrated formic acid into a 1L Erlenmeyer flask, bring up to volume and mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	0.1% formic acid in acetonitrile Add 500 mL of acetonitrile and 1 mL of concentrated formic acid into a 1L Erlenmeyer flask, bring up to volume and mix well to ensure complete homogeneous solution.

Note: If necessary, the solutions may also be prepared in different volumes as long as the proportions are not modified.

2.4.3 Standard Solutions

Stock Solutions

Prepare a 1.0 mg/mL stock solution by weighing an appropriate amount of the analytes into a flask and add the required volume of acetone (except for M440I001 and M440I005 which requires methanol-acetone (50:50, v/v). For example, to prepare 10 mL of 1.0 mg/mL stock solution of the analyte in acetone, weigh 10 mg the analyte into a 10 mL volumetric flask. Dissolve and dilute to mark with acetone. Ensure a complete homogeneous solution (e.g., by sonication or vortexing).

Independence of standard calibration and fortification solutions should initially be confirmed to show correct preparation of the solutions. This can be achieved for example using one of the following approaches:

- Two stock solutions are independently prepared. One is used for preparation of fortification solutions, the other for calibration standard solutions.
- Fortification and calibration standard solutions should be prepared from one stock solution in separate dilution series.

For subsequent preparations of solutions, freshly prepared solutions can be compared directly to previous standard solutions.

A correction for purity is done if the purity is ≤ 95%. If the purity is > 95%, correction is optional.

Note: BAS 440 I, M440I001, and M440I003 require care to go into solution at a 1 mg/mL level. Sonication is necessary. Lower concentrations (0.5 mg/mL) can be used.

Fortification Solutions

Prepare standard solutions for fortification from the stock solution (see above). Dilute volumetrically with appropriate solvents as shown in the table below and ensure a complete homogeneous solution (e.g., by sonication or vortexing).

Preparation of mixed Fortification solutions (all analytes)

Take solution (µg/mL)	Volume (mL)	Dilute with ACN to a final volume of (mL)	Concentration (µg/mL)
1000 (Stock)	1	10	100
100	2.5	25	10
10	0.25	25	0.1

Note: A different concentration scheme may be used, if other fortification levels are needed for the analysis. If necessary, the volume of solution prepared may be changed as long as the proportions are not modified.

Calibration Standard Solutions

Prepare standard calibration solutions for LC-MS/MS analysis by using the solutions that were prepared in Section “Stock Solutions” or “Fortification Solutions” in volumetric flasks. Dilute volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution (e.g., by sonication or vortexing).

Preparation of standard solutions for calibration

Take solution (ng/mL)	Volume (µL)	Dilute with (FV) to a final volume of (mL)	Concentration (ng/mL)
100	1000	10	10
100	250	10	2.5
10	1000	10	1
10	500	10	0.5
2.5	1000	10	0.25
1	1000	10	0.1
0.5	1000	10	0.05

Note: A different concentration scheme may be used and additional standards may be prepared as needed.
If necessary, the volume of solution prepared may be changed as long as the proportions are not modified.

Matrix-Matched Standard Solutions

If matrix matched standards are shown to be necessary, prepare matrix-matched calibration solutions for LC-MS/MS analysis by using the solutions that were prepared above in Section “Calibration Standard Solutions” using the following procedure:

Evaporate the 5 mL aliquot (Section 3.5a) of a control (untreated) sample extract to dryness. Reconstitute in the appropriate volume (1 mL or LOQ volume) of an injection standard. This procedure should be done for all needed standard concentrations.

2.4.4 Stability of Standard Solutions

Stability for stock solutions are considered to be 3 months for the stock solutions and 1 month for fortification and injection solutions. Stability will be proven in the validation.

3 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Samples have to be sufficiently homogenized beforehand, in order to assure that the aliquot taken for residue analysis is representative for the whole sample.

3.2 Sample Storage

Sample storage stability is established in a separate study.

3.3 Weighing and Fortification

For treated samples and control samples, weigh 2.5 ± 0.1 g of soil sample into a 250 mL square jar.

For fortified samples, use the following fortification scheme.

Sample Type	Sample Weight	Concentration of Spiking Solution [ng/mL]	Volume of Spiking Solution [µL]	Level of Fortification in ppb [µg/kg]
Control	2.5 g	-	-	0
Fortification (LOQ)	2.5 g	100	25	1*
Fortification (100× LOQ)	2.5 g	10,000	25	100
Treated	2.5 g	-	-	-

* Limit of quantification

Note: Volume of spiking solution added to generate the fortified sample should not exceed 10% of sample weight or volume.

3.4 Extraction of Sample Material

a) Add **25 mL** of acetonitrile-water (70:30, v/v) (E1) to the weighed sample in an concical centrifuge tube.

b) Firmly cap the vessel. Shake on a mechanical shaker for approximately 15 minutes at 300 rpm or vortex for approximately half the time.

c) Centrifuge at approximately 3500 rpm for 5 minutes

d) Detach the cap and decant the entire extract in a glass square bottle.

e) Add another **25 mL** of acetonitrile-water (70:30, v/v) (E1) to the weighed sample in the extraction vessel. Firmly cap the vessel. Shake on a mechanical shaker for approximately 15 minutes at 300 rpm or vortex for approximately half the time.

f) Detach the cap and decant the entire extract into the square bottle from step (d).

g) Briefly shake the combined extract and hold for step 3.5.

3.5 Sample Clean-up

3.5.1. ACN Layer Partition

a) Transfer a 12 mL aliquot to a glass culture tube

b) Add the approximate weights (or preweighed) of the following salts – 0.8 g of $MgSO_4$, 0.2 g of NaCl, 0.1g of Citric Acid Disodium Salt Sesquihydrate, 0.2 g of Citric Acid Trisodium Salt Dihydrate.

c) Vortex sample for approximately 1 minute.

d) Centrifuge sample for approximately 2 minutes at 3500 rpm

e) Aliquot approximately 7 mL of the upper layer (ACN) to another glass culture tube.

Note: Plastic containers may give low recoveries.

Glass tubes may break at high centrifuge speeds. Care should be given and lower speeds maybe needed

3.5.2. PSA Clean-up

a) Add the approximate weights (or preweighed) of material to the 7mL aliquot: 0.9 g of MgSO4 and 0.15 g of PSA

b) Vortex sample for approximately 1 minute.

c) Centrifuge sample for approximately 2 minutes at 3500 rpm

d) Aliquot 4.5 mL into a glass culture tube.

Note:	In some cases a 4.5 mL aliquot is hard to remove from this step. If a smaller aliquot is taken, then the final volume should be changed proportionally. (ie. A 3.375 mL aliquot would be reconstituted in step 3.6 to 0.75 mL.)

3.5.3. Evaporation

a) Using a Nitrogen evaporator at room temperature, evaporate samples to dryness

Note:	Evaporating ACN at room temperature using a Nitrogen evaporator can take approximately 2 hours.

3.6 Preparation for Measurement

a) Reconstitute the dried samples in 1mL of FV (0.1% formic acid in acetonitrile-water (30:70, v/v). Sonicate and vortex (less than 1 minute)

b) For samples with analyte concentrations outside the standard curve, dilute with FV as appropriate.

Note:	Different final volume maybe used as long as the LOQ concentration is within the curve and the lowest standard in the curve is at most 20% of the LOQ concentration. Proper sonication is necessary for successful reconstitution

3.7 Influence of matrix effects on analysis

During method development, it was demonstrated that the matrix load tested had no significant influence on the analysis (i.e., matrix effects < 20%).

3.8 Stability of Extracts and Final Volumes

Extract stability has not been tested.

4 QUANTIFICATION AND CALCULATION

4.1 Set-up of the analytical run

A sequence for measurement generally consists of:
- Calibration standards
- Control samples
- Procedural recovery samples
- Unknown samples
- Instrument recovery sample

Reagent Blanks or blanks can also be injected if necessary. Each injection set should begin and end with an injection of a calibration standard. Standards should be interspersed with samples. Each calibration standard should be at least injected twice. At least five calibration levels need to be injected.

4.2 Instrumental analysis

4.2.1 Instrumentation and Conditions

LC-MS/MS Method A
Analysis of all analytes except M440I057

	Parameter		
Chromatographic System	Waters Acquity UPLC		
Analytical-column	Acquity UPLC BEH Phenyl 1.7mm 2.1 X 100mm		
Column Temperature	50^0 C		
Injection Volume	10 µL (or greater)		
Mobile Phase	A: Water 0.1% Formic Acid B: Acetonitrile 0.1% Formic Acid		
Flow Rate	700 µL/min		
Steps (including wash and equilibration)	Time (min)	Phase (A)	Phase (B)
	0.0	95	5
	2.0	95	5
	4.0	20	80
	4.5	10	90
	5.0	10	90
	5.1	95	5
	6.0	95	5
Detection System	AB SCIEX 5500		
Ionisation	Turbo Ion Spray (ESI)		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
BAS 440 I	594 → 148* 594 → 202	positive	approx. 3.0 min
M440I001	458 → 148* 458 → 202 458 → 106	positive	approx. 3.0 min
M440I002	526 → 148* 526 → 202	positive	approx. 3.3 min
M440I003	526 → 148* 526 → 202	positive	approx. 3.5 min
M440I005	524 → 148* 524 → 80	positive	approx. 3.5 min.
M440I016	524 → 218* 524 → 164	positive	approx. 3.5 min.
M440I024	610 → 218* 610 → 122	positive	approx. 3.7 min.

Note: Items in parathesis used in method development, not in validation

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

Note: M440I005 and M440I057 have the same molecular weight. **LC-MS/MS Methods A and B do not separate these two analytes and therefore these analytes can not be analyzed together using these methods.**

LC-MS/MS Method B
Analysis of all analytes except M440I057 (not validated)

	Parameter		
Chromatographic System	Waters Acquity UPLC		
Analytical-column	Acquity UPLC BEH C-18 1.7mm 2.1 X 50mm		
Column Temperature	50^0 C		
Injection Volume	10 µL (or greater)		
Mobile Phase	A: Water 0.1% Formic Acid B: Acetonitrile 0.1% Formic Acid		
Flow Rate	700 µL/min		
Steps (including wash and equilibration)	Time (min)	Phase (A)	Phase (B)
	0.0	95	5
	0.3	95	5
	3.0	20	80
	4.0	5	95
	5.0	5	95
	5.1	95	5
	6.0	95	5
Detection System	AB SCIEX 4000 Q-Trap		
Ionisation	Turbo Ion Spray (ESI)		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
BAS 440 I	594 → 148* 594 → 202	positive	approx. 2.7 min
M440I001	458 → 148* 458 → 202 458 → 106	positive	approx. 1.7 min
M440I002	526 → 148* 526 → 202	positive	approx. 2.1 min
M440I003	526 → 148* 526 → 202	positive	approx. 2.4 min
M440I005	524 → 148* 524 → 80	positive	approx. 2.3 min.
M440I016	524 → 218* 524 → 164	positive	approx. 2.3 min.
M440I024	610 → 218* 610 → 122	positive	approx. 2.6 min.

Note: Items in parathesis used in method development, not in validation

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

LC-MS/MS Method C
Analysis of all analytes

	Parameter		
Chromatographic System	Waters Acquity UPLC		
Analytical-column	Acquity UPLC BEH Phenyl 1.7mm 2.1 X 100mm		
Column Temperature	45^0 C		
Injection Volume	10 µL (or greater)		
Mobile Phase	A: Water 0.1% Formic Acid B: Acetonitrile 0.1% Formic Acid		
Flow Rate	600 µL/min		
Steps (including wash and equilibration)	Time (min)	Phase (A)	Phase (B)
	0.00	85	15
	0.05	85	15
	8.50	75	25
	9.50	5	95
	10.45	5	95
	10.50	85	15
	11.00	85	15
Detection System	AB SCIEX 5500		
Ionisation	Turbo Ion Spray (ESI)		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
BAS 440 I	594 → 148* 594 → 202	positive	approx. 9.8 min
M440I001	458 → 148* 458 → 106	positive	approx. 1.6 min
M440I002	526 → 148* 526 → 202	positive	approx. 4.3 min
M440I003	526 → 148* 526 → 202	positive	approx. 7.7 min
M440I005	524 → 148* 524 → 80	positive	approx. 6.4 min
M440I016	524 → 218* 524 → 164	positive	approx. 7.4 min
M440I024	610 → 218* 610 → 122	positive	approx. 9.7 min
M440I057	524 → 148* 524 → 202	positive	approx. 6.1 min

Note: Items in parathesis used in method development, not in validation

* 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.

Note: Due to M440I005 and M440I003 having molecular weights only 2 amu apart, there is some isotopic overlap if not separated by retention time. However this was shown to be an insignificant contribution.

Note: M440I005 and M440I057 have the same molecular weight. **LC-MS/MS Methods A and B do not separate these two analytes and therefore these analytes can not be analyzed together using these methods.**

4.2.2 Calibration procedures

Calculation of results is based on peak area measurements using a calibration curve. At least five calibration levels need to be injected (e.g., required for enforcement). The calibration curve is obtained by direct injection of the analytes for LC-MS/MS, usually in the range of 0.05 ng/mL to 2.5 ng/mL. In a given injection run, the same injection volume is used for all samples and standards.

Linear calibration functions are preferred for evaluation. If other functions are used (e.g., quadratic), this should be fully justified.

4.2.3 Calculation of Residues and Recoveries

Calculation of results is based on area measurements. For the procedural recoveries, the sample weight will be considered 2.5 g in the final calculation of residues [µg/kg]. The method requires that the sample weight to be 2.5 ± 0.1 g for fortification samples. The recovery is the percentage of the fortified amount (µg or ng), which is recovered through the method and the weights cancel out, as shown in the equation below, during the final calculation step.

Calculation of results is based on area measurements. The recoveries and residues are calculated using the following formulas.

I. Concentration [ng/mL] $= \dfrac{\text{Response} - \mathit{Intercept}}{\mathit{Slope}} = C_A$

II. Residue [mg/kg] $= \dfrac{V_{end} \times C_A}{G \times A_F \times 1000}$

V_{end}	=	Final volume of the extract after all dilution steps [mL]
C_A	=	Concentration of analyte as read from the calibration curve [ng/mL]
G	=	Weight of the sample extracted [g]
A_F	=	Aliquot factor (see note)
1000	=	Factor remaining after all unit conversions

The aliquot factor (**A_F**) is defined as:

$$A_F = \frac{\text{"Aliquot from Extract"}}{\text{Total Volume of Extract}} \times \frac{\text{Volume of ACN for evaporation}}{\text{ACN volume in "Aliquot from Extract"}}$$

During the method, the water in the extraction solvent is partitioned from the acetonitrile by the addition of salts. The analyte remains in the acetonitrile layer.

$$A_F = \frac{12\ \text{mL}}{50\ \text{mL}} X \frac{4.5\ \text{mL}}{8.4\ \text{mL}}$$

$$A_F = \frac{54\ \text{mL}}{420\ \text{mL}} = 0.1286$$

Thus, the aliquot factor is 0.129 (or 12.9%) for the method.

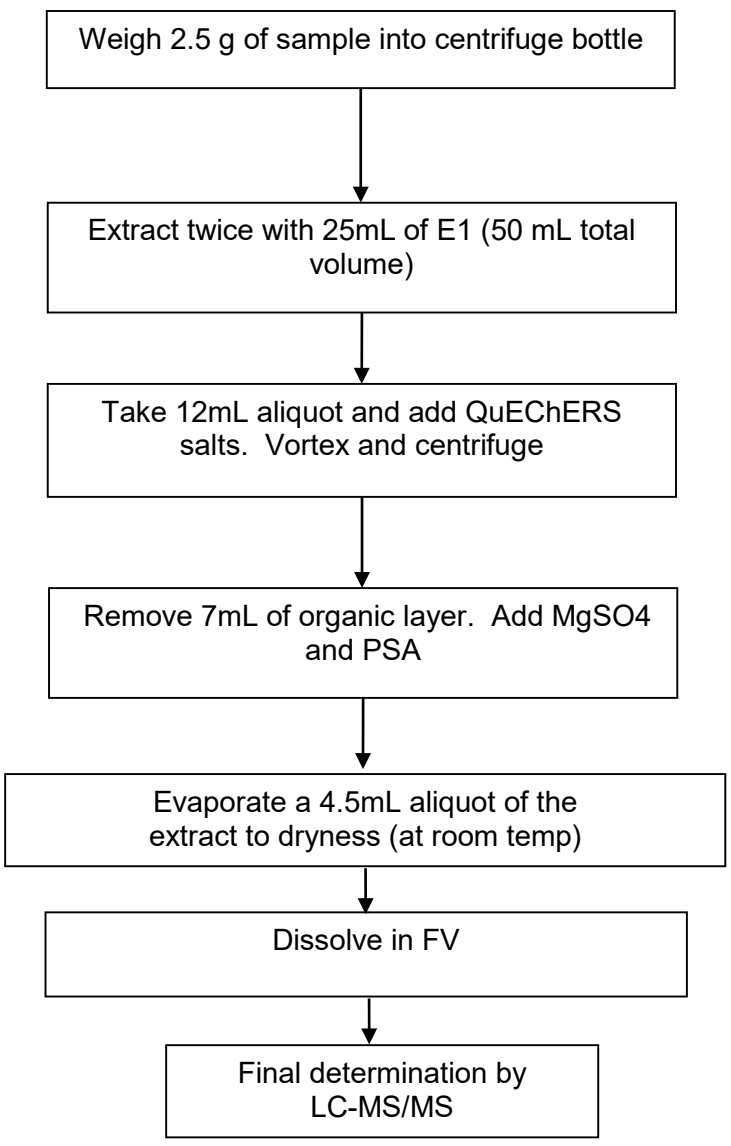
The recoveries of spiked compounds are calculated according to equation **III**:

III. Recovery % $= \dfrac{(\text{Residue in fortified sample} - \text{Residue in control}) \times 100}{\text{Amount of analyte fortified}}$

4.3 Method Validation Recoveries

To be attached after validation

5 FLOWCHART


Weigh 2.5 g of sample into centrifuge bottle
Extract twice with 25mL of E1 (50 mL total volume)
Take 12mL aliquot and add QuEChERS salts. Vortex and centrifuge
Remove 7mL of organic layer. Add MgSO4 and PSA
Evaporate a 4.5mL aliquot of the extract to dryness (at room temp)
Dissolve in FV
Final determination by LC-MS/MS

6 METHOD MANAGEMENT AND TIME REQUIREMENTS

The analysis of one series of samples (one reagent blank, two controls, and 10 fortified samples for recovery experiments) requires 1 working day (8 hours) per laboratory assistant. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

7 CONCLUSION AND METHOD CAPABILITIES

Limit of Quantification (LOQ) and Limit of Detection (LOD)
The limit of quantification is defined as the lowest fortification level successfully tested. The limit of quantification and limit of detection are 1 and 0.2 µg/kg for BAS 440 I and its metabolites. The lowest standard for each analyte in the calibration curve has good detectability (signal to noise ratio greater than 3:1).

Selectivity
No interfering peaks are found at the retention times for BAS 440 I and its metabolites. Justification of selection of transitions using product ion spectra will be attached later.

Confirmatory Techniques
The HPLC-MS/MS final determination for BAS 440 I and its metabolites. is a highly selective detection technique. For every compound the quantitation is possible at two different transitions. Therefore, no additional confirmatory technique is required.

Potential Problems
If matrix suppression or enhancement is observed, matrix-matched standards should be used.

8 REFERENCES

Added later

9 APPENDIX

9.1 Example of Calculation

Example: Soybean sample fortified at 0.01 mg/kg for BAS 440 I:

Concentration in the final volume [ng/mL]

$$\textbf{Concentration [ng/mL]} = \frac{\text{Response} - \textit{Intercept}}{\textit{Slope}} = C_A$$

Residue in the sample [ppm = mg/kg = µg/g = ng/mg]

$$\textbf{Residue [mg/kg]} = \frac{V_{end} \times C_A}{G \times A_F \times 1000}$$

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

The following values were used in this calculation:

Response of fortified sample	31950
Response of control sample	0
Slope:	110674
Intercept:	2245
Sample Weight (g):	2.5
Final Volume (V_{end}):	1
Aliquot factor A_F:	0.129 (12.9%)

$$\textbf{Concentration (ng/mL)} = \frac{31950 - 2245}{110674} = 0.268 ng/ml$$

Residue (mg/kg) =

$$\frac{1 ml \times 0.268 ng/ml \times 1 g}{2.5 g \times 0.129 \times 1000 mg} = 0.00083 ng/mg \text{ (or mg/kg)}$$

$$\textbf{Recovery \%} = \frac{(0.00083 mg/kg - 0 mg/kg) \times 100}{0.001 mg/kg} = 83\%$$

Note: Aliquot factor is 0.129. See Section 4.2.3 for explanation.