## ABSTRACT

The objective of this validation study was to demonstrate the applicability and repeatability of BASF Analytical Method No. D1308/02 for the determination of residues of afidopyropen (BAS 440 I) and its metabolites M440I001, M440I002, M440I003, M440I005, M440I016, M440I024, and M440I057 in soil by LC-MS/MS.

**Principle of the method.** Residues of afidopyropen in soil samples (2.5 g each) are extracted (twice) by mechanically shaking with 25 mL of acetonitrile:water (70:30, v/v) and centrifuged. Residues in an aliquot of the combined extracts are cleaned-up and partitioned by shaking, in the presence of a mixture of "QuEChERS" [Quick, Easy, Cheap, Effective, Rugged, and Safe] salts (sodium chloride, magnesium sulfate, and citrate buffering agents), into the organic layer, and centrifuged. The residues in the retained acetontrile layer are further purified with the addition of a second "QuEChERS" salt mixture (containing $MgSO_4$ to remove residual water and primary secondary amine [PSA] sorbent) and centrifuged. An aliquot of the organic phase is evaporated to dryness and reconstituted in acidified acetonitrile:water (30:70, v/v).

The residues are determined by high performance liquid chromatography (HPLC) with detection by positive ion electrospray ionization tandem mass spectrometry (MS/MS-ESI), monitoring for quantitation purposes ion transitions m/z 594→148 for parent afidopyropen; m/z 458→148 for M440I001; m/z 526→148 for M440I002; m/z 526→148 for M440I003; m/z 524→148 for M440I005; m/z 542→218 or m/z 524→218 for M440I016; m/z 610→218 for M440I024; and m/z 524→148 for M440I057. Typically for confirmatory purposes, the following alternate ion transitions are monitored, again in the positive ionization mode: m/z 594→202 for parent afidopyropen; m/z 458→106 for M440I001; m/z 526→202 for M440I002; m/z 526→202 for M440I003; m/z 524→80 for M440I005; m/z 542→164 for M440I016; m/z 610→122 for M440I024; and m/z 524→202 for M440I057. The results are calculated by direct comparison of the sample peak responses to those of external standards.

There are three chromatography options available to optimize analysis, the two main options being “Method A” and “Method C,” both of which were validated in this study. The options are identical except Method A consists of a shorter run time (same bonded and mobile phase constituents, but different gradients). Method A does not include M440I057 as a target analyte, as this option was developed prior to the identification of M440I057 as a residue of interest and M440I057 co-elutes (same retention time) with its structural isomer, M440I005, in the gradient set forth under Method A. Under Method C, the isomers are separated by their retention times on the HPLC column enabling separate quantitation of each of these analytes.

**Test conditions.** For validation, untreated soil samples were fortified with each analyte and analyzed according to the established method validation guidelines. The analytical sets consisted of a reagent blank, two controls, five replicates fortified with analyte at the method limit of quantitation, 0.001 mg/kg (ppm), and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 0.01 mg/kg. For each analyte, the two 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.

## **ABSTRACT** (continued)

**Limit of Quantification (LOQ) and Limit of Detection (LOD).** The limit of quantitation (LOQ) was defined by the lowest fortification level successfully tested. The validated LOQ for residues of afidopyropen in soil is 0.001 mg/kg, for each analyte. The limit of detection (LOD) is set at 0.000155 ppm (rounded to 0.0002 mg/kg or 20% of LOQ) during method validation. The LOD is defined as the absolute amount (0.001 ng) of analyte injected into the LC-MS/MS using lowest standard of the calibration.

**Selectivity.** The method determines residues of afidopyropen in soil by LC-MS/MS. No interfering peaks were found at the retention times for these analytes. The multiple reaction monitoring (MRM) transitions used to identify each analyte were determined by product ion spectra. The experiment to evaluate any potential matrix effects showed that the matrix load in the samples from each soil type had no significant influence on analysis (matrix effects generally <20%); therefore, the validation samples were analyzed only using solvent-based calibration standard solutions.

**Linearity.** Acceptable linearity was observed for the standard range and the two mass transitions tested for each analyte: The method-detector response was linear over the 0.05 to 1.0 ng/mL range ($r = \geq 0.9889$).

**Standard stability.** The available storage stability data indicate that stock solutions of afidopyropen and metabolites prepared in acetone (or methanol:acetone, 50:50 v/v, for M440I001 and M440I005) are stable held under refrigeration for at least 3 months (1 month for M440I057, the longest interval tested to-date). In addition, the mixed intermediate (fortification) solutions prepared by combining aliquots of the stock solutions for each analyte and diluting with acetonitrile and the calibration standards prepared by serial dilution of the intermediate standards using acidified acetonitrile:water (30:70, v/v) have been shown to be stable when stored under refrigeration for at least 1 month. During the course of this study, the test/reference substance solutions were stored in a refrigerator and all solutions were used within the demonstrated time period of stability.

**Extract stability.** The method validation fortification sample extracts were analyzed within 1 day of extraction. The generally acceptable method recoveries obtained during analysis demonstrate the storage stability of residues of afidopyropen in the extracts in the brief period prior to analysis. In addition, the recoveries from stored solutions generated during extract stability experiments performed in conjunction with this study, which included tests on the initial extracts and HPLC final volume stored under refrigeration, indicated that each analyte, for the representative matrix tested (loamy sand soil) is stable in extracts for at least the time period tested, 7 to 9 days, sufficient to support the storage intervals and conditions incurred by the extracts in the subject study and to provide stability information for future work with the method.

# 1. INTRODUCTION

## 1.1 Background and Purpose of Study

The objective of this validation study was to demonstrate the applicability and repeatability of BASF Analytical Method No. D1308/02, used for the determination of residues of afidopyropen (BAS 440 I) in soil by LC-MS/MS.

# 2. MATERIALS AND METHODS

## 2.1 Test Systems

The soil samples used in this study were clay loam sample number R1300860005 (18-24 inch soil depth) and loamy sand sample number R1300890001 (0-3 inch soil depth). These samples had been collected under BASF Study 394796 (Reference 1) and were characterized by Agvise Laboratories. The relevant portions of the GLP soil characterization reports are provided in Appendix L. All samples were received frozen from the field and were stored frozen at BASF Crop Protection prior to analysis. Each analysis set was uniquely identified with a Master Sheet Number, which consisted of the study number plus a unique number (e.g., 394795-04). The test system samples were assigned unique numbers and these were recorded in each analytical set or “Master Sheet” (e.g., soil fortification sample 394795-04-4, from Master Sheet No. 394795-04). The actual sample numbers used for the analysis were identified in the raw data and in this final report.

## 2.2 Test and Reference Substances

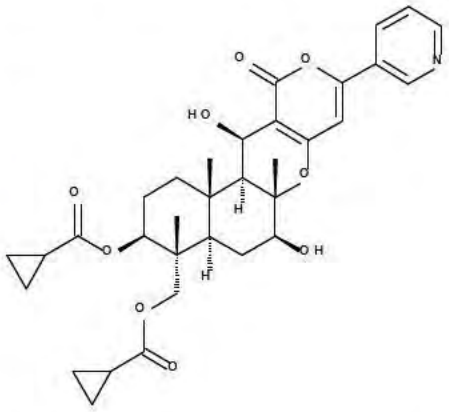
The test/reference standards shown below were synthesized by BASF Aktiengesellschaft (Limburgerhof, Germany) and used during the analytical portion of this study. The test/reference substances were maintained frozen until use in this study. BASF Aktiengesellschaft determined characterization and purity prior to the substance being used in this study. Details of these determinations are available to BASF and are located at Landwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany.

The test/reference substances in solution were used in the study to generate data for both instrument and method performance. Quantitation of residues in all samples was achieved using calibration curves calculated by linear regression of instrument responses for the reference substances. The performance of the instrument was evaluated during each injection set.

### 2.2.1 Afidopyropen

| | |
|---|---|
| Common Name | Afidopyropen |
| BAS-Code | BAS 440 I |
| BASF Reg. No. | 5599022 |
| CAS-No. | 915972-17-7 |
| Molecular Formula | $C_{33}H_{39}NO_9$ |
| Molecular Weight | 593.7 g/mol |
| 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 |
| Lot No. | L82-65 |
| Purity (%) | 98.1 |
| Expiration Date | June 1, 2016 |

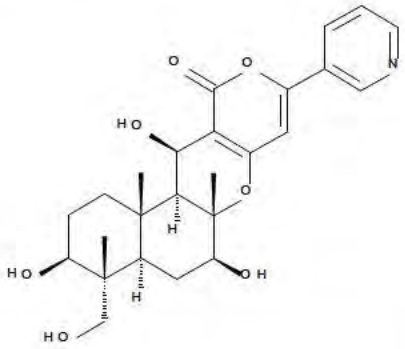
Chemical structure:



### 2.2.2 Metabolite M440I001

| | |
|---|---|
| Common Name | Not assigned |
| BAS-Code | M440I001 |
| BASF Reg. No. | 5741530 |
| CAS-No. | None assigned |
| Molecular Formula | $C_{25}H_{31}NO_7$ |
| Molecular Weight | 457.5 g/mol |
| 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 |
| Lot No. | L82-66 |
| Purity (%) | 93.9 |
| Expiration Date | February 01, 2016 |

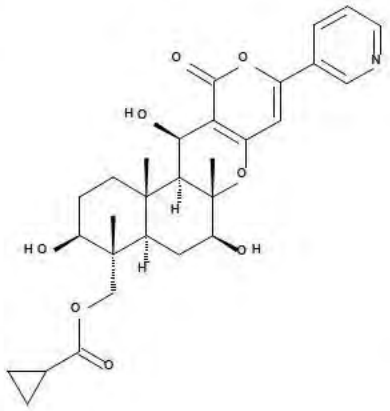
Chemical structure:



### 2.2.3 Metabolite M440I002

| | |
|---|---|
| Common Name | Not assigned |
| BAS-Code | M440I002 |
| BASF Reg. No. | 5741532 |
| CAS-No. | None assigned |
| Molecular Formula | $C_{29}H_{35}NO_8$ |
| Molecular Weight | 525.6 g/mol |
| 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-4yl]methyl cyclopropanecarboxylate |
| Lot No. | L82-67 |
| Purity (%) | 92.5 |
| Expiration Date | February 1, 2016 |

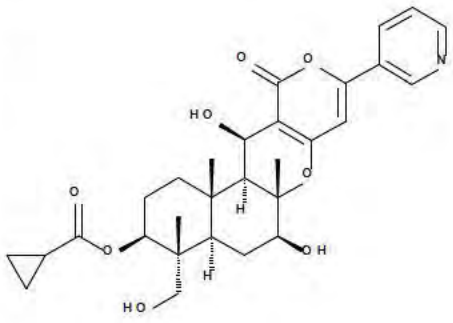
Chemical structure:



### 2.2.4 Metabolite M440I003

| | |
|---|---|
| Common Name | Not assigned |
| BAS-Code | M440I003 |
| BASF Reg. No. | 5741533 |
| CAS-No. | None assigned |
| Molecular Formula | $C_{29}H_{35}NO_8$ |
| Molecular Weight | 525.6 g/mol |
| 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 |
| Lot No. | L82-72 |
| Purity (%) | 98.6 |
| Expiration Date | September 1, 2016 |

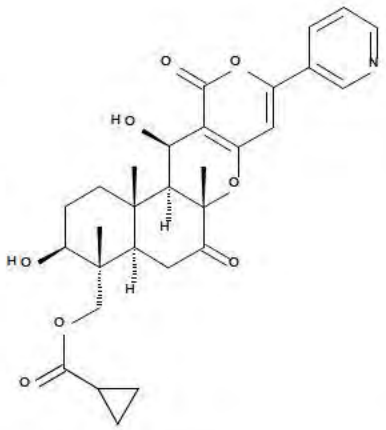
Chemical structure:



### 2.2.5 Metabolite M440I005

| | |
|---|---|
| Common Name | Not assigned |
| BAS-Code | M440I005 |
| BASF Reg. No. | 5824382 |
| CAS-No. | None assigned |
| Molecular Formula | $C_{29}H_{33}NO_8$ |
| Molecular Weight | 523.6 g/mol |
| 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 |
| Lot No. | L82-73 |
| Purity (%) | 90.9 |
| Expiration Date | March 1, 2016 |

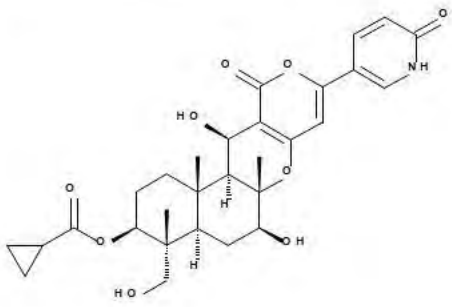
Chemical structure:



### 2.2.6 Metabolite M440I016

| | |
|---|---|
| Common Name | Not assigned |
| BAS-Code | M440I016 |
| BASF Reg. No. | 5845597 |
| CAS-No. | None assigned |
| Molecular Formula | $C_{29}H_{35}NO_9$ |
| Molecular Weight | 541.6 g/mol |
| IUPAC Name | (3S, 4R, 4aR, 6S, 6aS, 12R, 12aS, 12bS)-6, 12-dihyroxy-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 |
| Lot No. | L82-148 |
| Purity (%) | 88.9 |
| Expiration Date | May 1, 2016 |

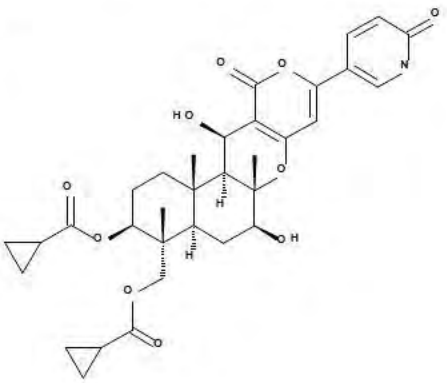
Chemical structure:



### 2.2.7 Metabolite M440I024

| | |
|---|---|
| Common Name | Not assigned |
| BAS-Code | M440I024 |
| BASF Reg. No. | 5886215 |
| CAS-No. | None assigned |
| Molecular Formula | $C_{33}H_{39}NO_{10}$ |
| Molecular Weight | 609.7 g/mol |
| 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-dihyropyridin-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 |
| Lot No. | L82-149 |
| Purity (%) | 91.3 |
| Expiration Date | May 1, 2016 |

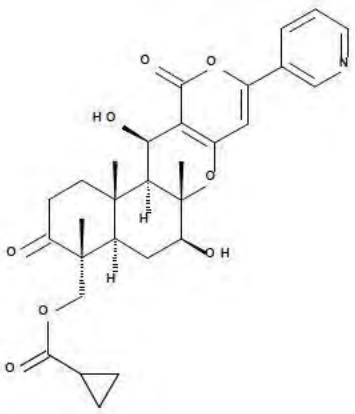
Chemical structure:



### 2.2.8 Metabolite M440I057

| | |
|---|---|
| Common Name | Not assigned |
| BAS-Code | M440I057 |
| BASF Reg. No. | 6010129 |
| CAS-No. | None assigned |
| Molecular Formula | $C_{29}H_{33}NO_8$ |
| Molecular Weight | 523.6 g/mol |
| IUPAC 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 |
| Lot No. | L82-164 |
| Purity (%) | 97.4% |
| Expiration Date | January 1, 2017 |

Chemical structure:



Stock solutions of afidopyropen and the metabolites were prepared in acetone, except for M440I001 and M440I005, which were prepared in methanol:acetone (50:50, v/v). The mixed intermediate/fortification solutions containing each analyte were prepared by combining aliquots of the stock solutions for each analyte and diluting with acetonitrile. The calibration standards were prepared by serial dilution of the intermediate standards using acidified (1% formic acid content) acetonitrile:water (30:70, v/v). The stability of the analytes in standard solutions has been determined in a related afidopyropen validation study performed on water (Reference 2) and in conjunction with this study. In this study, to determine the stability of select metabolites in solutions, aged stock, intermediate, and/or calibration standards were analyzed against freshly prepared standard solutions.

During the course of this study, the test/reference substance solutions were stored under refrigeration. Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data. Example standard dilution and use information, as performed in the subject study, are provided in Appendix K.

### 2.3 Route of Administration

In this method validation study, the test substances were applied to the test system as analytical standard solutions (in acetonitrile) by micropipette to ensure precise delivery of a small amount of the test substances.

### 2.4 Analytical Method

#### 2.4.1 Principle of the Method

Using BASF Analytical Method No. D1308/02, residues of afidopyropen in soil matrices are extracted with appropriate solvents, cleaned-up by centrifugation and liquid-liquid partitioning, and then quantified using LC/MS/MS. The method procedures validated in this study is provided in Appendix B. A description of the methodology follows.

Briefly, residues of afidopyropen in soil samples (2.5 g each) are extracted (twice) by mechanically shaking with 25 mL of acetonitrile:water (70:30, v/v) and centrifuged. Residues in an aliquot of the combined extracts are cleaned-up and partitioned by shaking, in the presence of a mixture of "QuEChERS" salts (sodium chloride, magnesium sulfate, and citrate buffering agents), into the organic layer, and centrifuged. The residues in the retained acetontrile layer are further purified with the addition of a second "QuEChERS" salt mixture (containing $MgSO_4$, to remove residual water, and PSA sorbent) and centrifuged. An aliquot of the organic phase is evaporated to dryness and reconstituted in acidified acetonitrile:water (30:70, v/v) and then analyzed by HPLC/MS/MS.

#### 2.4.2 Specificity/Selectivity

The residues of afidopyropen are determined by HPLC/MS/MS, monitoring in the positive mode for quantitation purposes ion transitions m/z 594→148 for parent afidopyropen; m/z 458→148 for M440I001; m/z 526→148 for M440I002; m/z 526→148 for M440I003; m/z 524→148 for M440I005; m/z 542→218 or m/z 524→218 for M440I016; m/z 610→218 for M440I024; and m/z 524→148 for M440I057. Typically for confirmatory purposes, the following alternate ion transitions are monitored, again in the positive ionization mode: m/z 594→202 for parent afidopyropen; m/z 458→106 for M440I001; m/z 526→202 for M440I002; m/z 526→202 for M440I003; m/z 524→80 for M440I005; m/z 542→164 for M440I016; m/z 610→122 for M440I024; and m/z 524→202 for M440I057. The results are calculated by direct comparison of the sample peak responses to those of external standards.

There are three chromatography options available to optimize analysis, the two main options being “Method A” and “Method C,” both of which were validated in this study. The options are identical except Method A consists of a shorter run time (mobile phase gradient water:acetonitrile, each acidified with 1% formic acid, 95:5 to 10:90, v/v, over ~5 minutes, flow rate 700 uL/minute) and Method C consists of a longer run time (same components, 85:15 to 5:95, v/v, over ~10 minutes, flow rate 600 uL/minute). Method A does not include M440I057 as a target analyte, the method was developed prior to the identification of M440I057 as a residue of interest; nevertheless, M440I057 co-elutes with M440I005, its structural isomer, in the gradient set forth under Method A. Under Method C, the isomers are separated by their retention times on the HPLC column, enabling quantitation of the contribution of each of these analytes.

As HPLC/MS/MS is regarded as a highly-specific detection method when two ion transitions have been validated, an additional confirmatory method or technique is not necessary. The multiple reaction monitoring (MRM) transitions used to identify afidopyropen were determined by product ion scan (see Appendix J).

## 2.5 Validation of Method

For validation, untreated soil samples were fortified with each analyte and analyzed according to the established method validation guidelines. To test the repeatability of the method, the analytical sets consisted of a reagent blank, and for each matrix, two controls, five replicates fortified with each analyte at the method limit of quantitation, 0.001 mg/kg (ppm), and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 0.01 mg/kg. For each analyte, the two mass transitions described above were evaluated.

## 2.6 Influence of Matrix Effects on Analysis

In conjunction with the subject study, matrix-matched standards and solvent-based standards were analyzed in a separate experiment to evaluate any potential matrix effects on LC/MS/MS analysis. This involved comparing calibration standards prepared in control matrix against calibration standard solutions prepared with acidified acetonitrile:water (30:70, v/v). The matrix-matched standards were made by evaporating control sample extracts (worked-up through the method) to dryness and reconstituting with each of three solvent-based standards (concentrations, 0.1, 0.25, and 0.5 ng/mL). Each set of matrix-matched standards (for each soil matrix) was bracketed by a block of calibration standards with additional injections of tested standard levels occurring as appropriate during the run. All standard injections within that matrix set, were used in calculations involving matrix effects.

The data generated were evaluated by comparing the average area response of the standards for typically three injections of each type (with and without matrix) for each of the three standard concentration levels. Acceptability (i.e., matrices had no significant influence on the analysis) requires a difference in area of <20%, calculated as the "Mean Area Change (%)". For each matrix/ion transition, an overall average "Mean Area Change (%)" across the three tested concentrations was calculated to make a general assessment of acceptability with respect to matrix effects.

**ABSTRACT**

The active ingredient (a.i.) afidopyropen (BAS 440 I) is an insecticide currently being developed by BASF Crop Protection, Inc., for use in field crops , leafy and fruiting vegetable crops, and vineyard/orchards. This method will detect BAS 440 I and its metabolites M440I001, M440I002, M440I003, M440I005, M440I016, M440I024, and M440I057 down to 0.001 mg/kg in soil.

A brief description of the method is provided below:

A 2.5 gram soil sample is extracted twice with acetonitrile-water (70:30, v/v). An aliquot 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.

An aliquot of the acetonitrile supernatant is treated with magnesium sulfate and PSA to remove trace water and remove interference. An aliquot of the resulting extract is then evaporated to dryness at room temperature using a Nitrogen evaporator. The sample is reconstituted in 0.1% formic acid in acetonitrile-water (30:70, v/v).

The residues are determined using LC-MS/MS.

The method has a limit of quantitation of 0.001 mg/kg in soil matrices. The limit of detection is set to 0.0002 mg/kg.

# 1 INTRODUCTION

# 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.

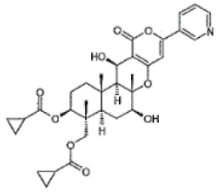
### 2.2.1 BAS 440 I

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

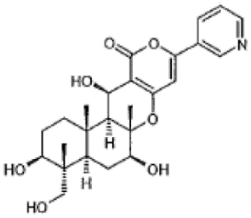
### 2.2.2 M440I001

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

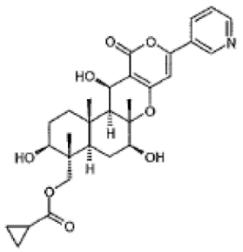
### 2.2.3 M440I002

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

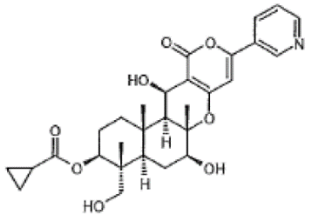
### 2.2.4 M440I003

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

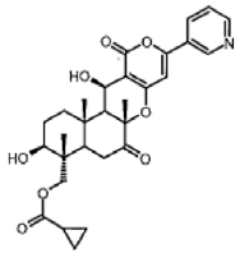
Structural formula:



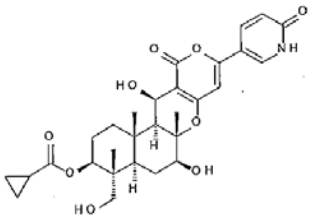
Empirical formula: $C_{29}H_{35}NO_8$

Molecular weight: 525.6

### 2.2.5 M440I005

| | |
|---|---|
| Reg-No.: | 5824382 |
| Common name: | M440I005 |
| Chemical name: | [(3S,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 |
| 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 |

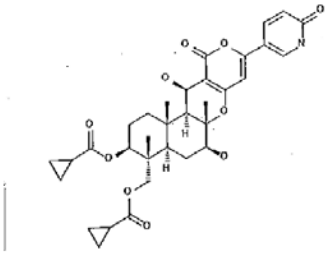
### 2.2.7 M440I024

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

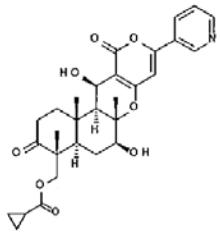
### 2.2.8 M440I057

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®<br>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 |
|---|---|---|
| Stock Solvent | S1 | acetone-methanol (50:50, v/v)<br>In a 1-liter container, mix 500 mL methanol and 500 mL acetone. Other amounts may be made in the same proportion. Store at room temperature. |
| Extraction solvent | E1 | acetonitrile-water (70:30, v/v)<br>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)<br>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<br>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<br>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.

## 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. Centrifuge at approximately 3500 rpm for 5 minutes.

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.<br>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 been determined.

## 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 | 50⁰ C | | |
| Injection Volume | 10 µL (or greater) | | |
| Mobile Phase | A: Water 0.1% Formic Acid<br>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*<br>594 → 202 | positive | approx. 3.0 min |
| M440I001 | 458 → 148*<br>458 → 202<br>458 → 106 | positive | approx. 3.0 min |
| M440I002 | 526 → 148*<br>526 → 202 | positive | approx. 3.3 min |
| M440I003 | 526 → 148*<br>526 → 202 | positive | approx. 3.5 min |
| M440I005 | 524 → 148*<br>524 → 80 | positive | approx. 3.5 min. |
| M440I016 | 524 → 218*<br>542 → 218 | positive | approx. 3.5 min. |
| M440I024 | 610 → 218*<br>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 cannot 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 | 50⁰ C | | |
| Injection Volume | 10 µL (or greater) | | |
| Mobile Phase | A: Water 0.1% Formic Acid<br>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*<br>594 → 202 | positive | approx. 2.7 min |
| M440I001 | 458 → 148*<br>458 → 202<br>458 → 106 | positive | approx. 1.7 min |
| M440I002 | 526 → 148*<br>526 → 202 | positive | approx. 2.1 min |
| M440I003 | 526 → 148*<br>526 → 202 | positive | approx. 2.4 min |
| M440I005 | 524 → 148*<br>524 → 80 | positive | approx. 2.3 min. |
| M440I016 | 542 → 218*<br>542 → 164 | positive | approx. 2.3 min. |
| M440I024 | 610 → 218*<br>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 | 45⁰ C | | |
| Injection Volume | 10 µL (or greater) | | |
| Mobile Phase | A: Water 0.1% Formic Acid<br>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*<br>594 → 202 | positive | approx. 9.8 min |
| M440I001 | 458 → 148*<br>458 → 106 | positive | approx. 1.6 min |
| M440I002 | 526 → 148*<br>526 → 202 | positive | approx. 4.3 min |
| M440I003 | 526 → 148*<br>526 → 202 | positive | approx. 7.7 min |
| M440I005 | 524 → 148*<br>524 → 80 | positive | approx. 6.4 min |
| M440I016 | 542 → 218*<br>542 → 164 | positive | approx. 7.4 min |
| M440I024 | 610 → 218*<br>610 → 122 | positive | approx. 9.7 min |
| M440I057 | 524 → 148*<br>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]** $= \frac{\text{Response} - \textit{Intercept}}{\textit{Slope}} = C_A$

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

| | | |
|---|---|---|
| $V_{end}$ | = | 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.

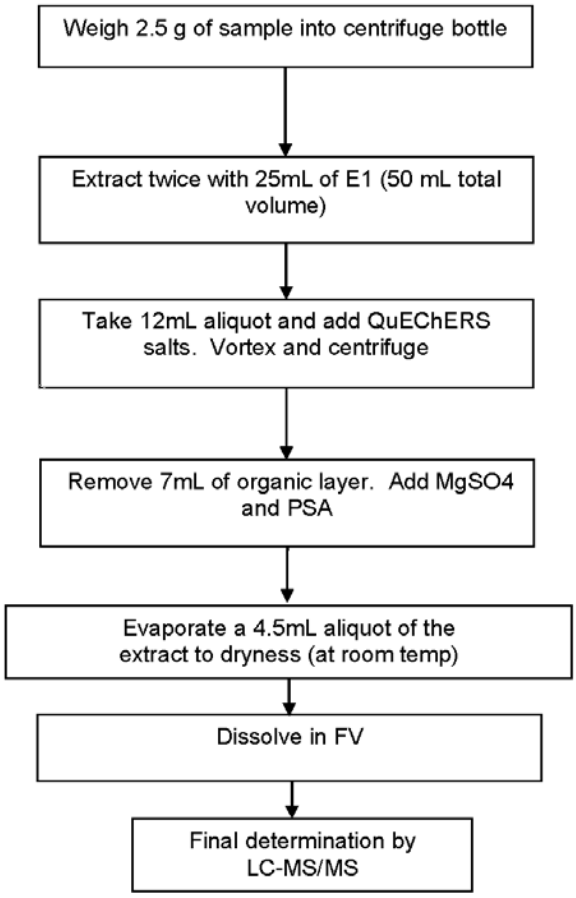
The recoveries of spiked compounds are calculated according to equation III:

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

### 4.3 Method Validation Recoveries

See final report for method 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.

## DEFINITIONS AND ACRONYMS

| | |
|---|---|
| **Sample Set:** | A group of samples that are extracted and cleaned up at the same time using the same method represented. |
| **Untreated Sample:** | A sample that has not been treated with the test substance. |
| **Control Sample:** | Usually an untreated sample used for fortification experiments (can be acquired from same study or from a different source). |
| **Unknown Sample:** | The samples with unknown residues. |
| **Treated Sample:** | A sample that has been treated with the test substance. |
| **Blank:** | Solvent, solution or mobile phase injected together with a sample set. |
| **Reagent Blank:** | A complete analysis conducted using solvents and reagents only in absence of any sample. Also known as blank of reagents or procedural blank.<br>This sample is analyzed within the sample set in order to evaluate possible contamination on chemicals/reagents. |
| **Procedural Recovery:** | A control sample to which a known amount of analyte has been added before sample work up. This sample is then carried through the method and analyzed with the unknown samples in order to determine the reliability of the method. |
| **Instrument Recovery:** | A control sample which is carried through the method and to which a known amount of analyte has been added before injection. This sample is analyzed within the sample set in order to evaluate the matrix effect in the instrument. |
| **Analytical Run:** | A group of samples that undergo a determinative measurement on an analytical instrument (such as GC, HPLC, CE, GC/MS, or LC/MS/MS) in a defined and continuous sequence under identical instrumental conditions. |
| **Limit of Quantitation (LOQ):** | Lowest tested concentration of the analyte in a sample that can be determined with acceptable accuracy and precision according to the method. |
| **Limit of Detection (LOD):** | Concentration of analyte equivalent to a defined percentage of the limit of quantitation of the method (e.g., 20% of LOQ).<br>At this concentration, the analyte must be qualitatively detectable in sample matrix (analyte peak height at least 3–5 x baseline noise). |

**Typical Recovery Calculation for LC/MS/MS Quantitation**

Sample No. 394795-04-5. Control soil fortified at the LOQ with afidopyropen (and other analytes), Master Sheet No. 394795-4.

$$\text{Concentration of analyte (ng/mL)} = \frac{\text{peak area - intercept}}{\text{slope}}$$

| | Afidopyropen |
|---|---|
| Peak Area = | 20,976.0 |
| Intercept = | -387.0832 |
| Slope = | 75890.7338 |
| Conc. (ng/mL) = | 0.2815 |

The concentration of analyte in mg/kg is calculated as shown in equation:

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

Where:

$V_{end}$ = Final volume [mL]
$C_A$ = Concentration of analyte as read from the calibration curve [ng/mL]
G = Weight of the sample extracted
$A_F$ = Aliquotation factor
1000 = Factor remaining after all unit conversions

| | Afidopyropen |
|---|---|
| $V_{end}$ = | 1.0 mL |
| $A_F$ = | 12.9% |
| G = | 2.50 |
| Conc. (ng/mL) = | 0.2815 |
| Residue (mg/kg) = | 0.00087 |

$A_F$ = Aliquot taken from acetonitrile extraction vol. (4.5 mL) / [ACN extraction vol. (35 mL) x Final volume (1 mL) ]

Net residue (mg/kg of analyte) = Residue (mg/kg of analyte) - Residue in Control (mg/kg)

$$\text{Recovery of analyte (\%)} = \frac{\text{Residue (mg/kg of analyte) - Residue in Control (mg/kg)}}{\text{Amount Fortified (mg/kg)}} \times 100$$

| | Afidopyropen |
|---|---|
| Amount fortified (mg/kg) = | 0.001 |
| Residue (mg/kg) = | 0.00087 |
| Residue in control = | 0.0000 |
| %Recovery | 87% |

Use full calculator precision in any intermediate calculations. Round only the final value.