ABSTRACT

The objective of this validation study was to demonstrate the applicability and repeatability of BASF Analytical Method No. D1603/01 for the determination of residues of broflanilide (BAS 450 I) and its metabolites DM-8007, DC-DM-8007, DC-8007 and S(PFP-OH) -8007 in soil by LC-MS/MS.

Principle of the method. Residues of broflanilide in soil samples are extracted by shaking with methanol followed by methanol:water (70:30, v/v). Residues in an aliquot of the combined extracts are diluted with methanol:water (50:50, v/v), filtered and analyzed. The residues are determined by high performance liquid chromatography (HPLC) with detection by positive ion electrospray ionization tandem mass spectrometry (MS/MS-ESI), monitoring ion transitions m/z 663→643 for parent broflanilide; m/z 545→525 for DC-DM-8007; m/z 559→539 for DC-8007; m/z 649→242 for DM-8007; and m/z 661→641 for S(PFP-OH)-8007, and alternate ion transitions m/z 665→645 for parent broflanilide; m/z 547→527 for DC-DM-8007; m/z 561→541 for DC-8007; m/z 651→242 for DM-8007; and m/z 661→621 for S(PFP-OH)-8007. The results are calculated by direct comparison of the sample peak responses to those of external standards.

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, 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.

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 broflanilide in soil is 0.001 mg/kg, for each analyte, which is lower than the lowest relevant endpoint in soil ecotoxicology (LC50 > 1000 mg/kg of active ingredient in dry soil). The limit of detection (LOD) was 0.0002 mg/kg or 20% of the LOQ during method validation. The LOD for each analyte was shown to be detectable as the absolute amount of analyte injected (0.0002 ng on-column) with acceptable signal to noise ratio (S/N > 3:1).

Selectivity. The method determines residues of broflanilide 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 <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 0.01 to 0.2 ng/mL range (r = ≥0.9942, for method validation sets).

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. D1603/01, used for the determination of residues of broflanilide (BASF code “BAS 450 I”, or synonym “MCI-8007”) in soil by LC-MS/MS.

2. MATERIALS AND METHODS

2.1 Test Systems

The soil samples used in this study were sample number R1501030015 (Clay, 18-24 inch soil depth) and sample number R1501000019 (Sandy, 0-2 inch soil depth). These samples had been collected under BASF Study 710464 (Reference 1) and were characterized by Agvise Laboratories. The relevant portions of the GLP soil characterization reports are provided in Appendix K. 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., 815843-04). The test system samples were assigned unique numbers and these were recorded in each analytical set or “Master Sheet” (e.g., soil fortification sample 815843-04-4, from Master Sheet No. 815843-04). 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

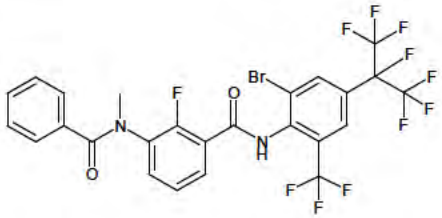
The test/reference standards, shown below, were synthesized by Mitsui Chemical Agro, Inc. (MCAG, Tokyo, Japan) and were maintained at room temperture until use in this study. Japan Analytical Chemistry Consultants Co., Ltd., on behalf of MCAG, determined characterization and purity prior to the substances being used in this study. Details of these determinations are available to BASF and are located at Japan Analytical Chemistry Consultants Co., Ltd. (Funado Itabashi, Tokyo, Japan). BASF has retained a reserve sample of these chemicals and has documentation at BASF Corporation, BASF Crop Protection (Research Triangle Park, North Carolina, USA).

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 Broflanilide

Common Name	Broflanilide
BAS Code Name	BAS 450 I
Synonym	MCI-8007
IUPAC Name	*N*-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluoro-3-(N-methylbenzamido) benzamide
BASF Reg. No.	5672774
Molecular Formula	$C_{25}H_{14}BrF_{11}N_2O_2$
Molecular Weight	663.29
Lot No.	089-100112-1
Purity:	99.67%
Expiration Date	April 9, 2017

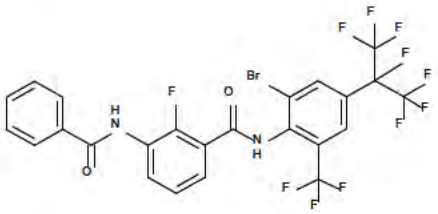
Chemical structure:



2.2.2 Metabolite DM-8007

Common Name	Not assigned
BAS Code Name	DM-8007
IUPAC Name	3-benzamido-*N*-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluorobenzamide
BASF Reg. No.	5856361
Molecular Formula	$C_{24}H_{12}$ $BrF_{11}N_2O_2$
Molecular Weight	649.3
Lot No.	296-007-81-1
Purity:	98.84%
Expiration Date	June 28, 2017

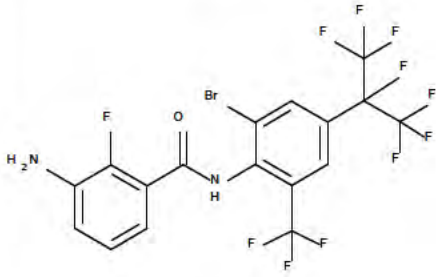
Chemical structure:



2.2.3 Metabolite DC-DM-8007

Common Name	Not assigned
BAS Code Name	DC-DM-8007
IUPAC Name	3-amino-*N*-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluorobenzamide
BASF Reg. No.	5936906
Molecular Formula	$C_{17}H_8BrF_{11}N_2O$
Molecular Weight	545.1
Lot No.	296-009-094-2
Purity:	98.58%
Expiration Date	June 28, 2017

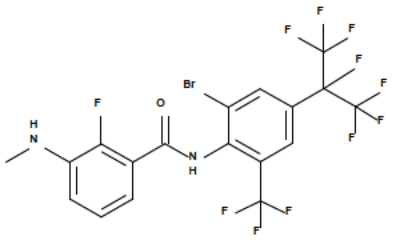
Chemical structure:



2.2.4 Metabolite DC-8007

Common Name	Not assigned
BAS Code Name	DC-8007
IUPAC Name	*N*-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluoro-3-(methylamino)benzamide
BASF Reg. No.	5936907
Molecular Formula	$C_{18}H_{10}BrF_{11}N_2O$
Molecular Weight	559.2
Lot No.	296-012-009-1
Purity:	99.07%
Expiration Date	June 28, 2017

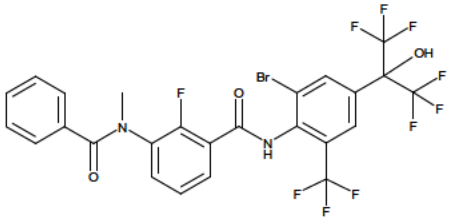
Chemical structure:



2.2.5 Metabolite S(PFP-OH)-8007

Common Name	Not assigned
BAS Code Name	S(PFP-OH)-8007
IUPAC Name	*N*-[2-bromo-4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluoro-3-(*N*-methlybenzamido) benzamide
BASF Reg. No.	5959598
Molecular Formula	$C_{25}H_{15}BrF_{10}N_2O_3$
Molecular Weight	661.3
Lot No.	267-012-094-3
Purity:	99.06%
Expiration Date	June 28, 2017

Chemical structure:



Stock solutions of broflanilide and the metabolites were prepared in acetonitrile. 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 methanol:water (50:50, v/v). The stability of the analytes in standard solutions has been determined in related studies on broflanilide (References 2 and 3).

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 analytical facility data and are archived periodically. Example standard dilution and use information, as performed in the subject study, are provided in Appendix J.

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. D1603/01, residues of broflanilide in soil are extracted with appropriate solvents, cleaned-up by centrifugation/filtration, and then quantified using LC/MS/MS. The method procedures validated in this study are provided in Appendix B. Briefly, residues of broflanilide in soil samples (5 g each) are extracted by shaking with methanol followed by methanol:water (70:30, v/v). Residues in an aliquot of the combined extracts are diluted with methanol:water (50:50, v/v), filtered and analyzed by LC-MS/MS.

2.4.2 Specificity/Selectivity

The residues of broflanilide are determined by LC/MS/MS monitoring in the positive mode for quantitation purposes ion transitions m/z 663→643 for parent broflanilide; m/z 545→525 for DC-DM-8007; m/z 559→539 for DC-8007; m/z 649→242 for DM-8007; and m/z 661→641 for S(PFP-OH)-8007. Typically for confirmatory purposes, the following alternate ion transitions are monitored, again in the positive ionization mode: m/z 665→645 for parent broflanilide; m/z 547→527 for DC-DM-8007; m/z 561→541 for DC-8007; m/z 651→242 for DM-8007; and m/z 661→621 for S(PFP-OH)-8007. The results are calculated by direct comparison of the sample peak responses to those of external standards.

As LC/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 broflanilide were determined by product ion scan (see Appendix I).

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 methanol:water (50:50, v/v). The matrix-matched standards were made by diluting a 1 ng/mL solvent standard with control sample extract (worked-up through the method) and methanol water (70:30, v:v), varying volumes to generate standards (concentrations, 0.025, 0.05, and 0.1 ng/mL). Matching solvent standards were prepared in a similar fashion without the use of control extract. Each set of matrix-matched standards (for each soil matrix) and one set of matching solvent standards were bracketed by a block of calibration standards with additional injections of tested standard levels occurring as appropriate during the run. All experimental 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.

2.7 Stability of Extracts

The stability of each analyte in stored extract solutions was determined in conjunction with the subject method validation study. To establish stability, reserved initial extracts from several control and method validation recovery soil samples that had been stored under refrigeration were cleaned-up and analyzed according to the method. Additionally, stability in the final volume, the solution prepared for LC/MS/MS injection was established for each matrix by reanalyzing several control and method validation recovery samples, as described above, which had been stored under refrigeration at the final volume stage. Quantification of the analytes in the stored samples for this experiment was performed for the primary mass transitions.

Extract and Final Volume Stability
The method validation fortification sample extracts were analyzed within 1 day of extraction. The acceptable method recoveries obtained during analyses demonstrate the storage stability of residues of broflanilide 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 stored under refrigeration and HPLC final volume held under refrigeration, indicated that each analyte is stable in soil extracts for at least the time period tested, 7 days, sufficient to support the storage intervals and conditions incurred by the extracts in the subject study, as shown in Table 3.

4. CALCULATIONS AND RAW DATA

An example calculation is included in Appendix C (page 55). Detailed analytical data such as supporting raw data necessary for re-calculations, standards and calibration curve data are provided in Appendix D (page 57). Example standard curves are provided in Appendix G (page 95). Example chromatographs are provided in Appendix H (page 104).

5. STATISTICS AND DATA INTEGRITY

Statistical treatment of the data included simple descriptive statistics, such as determinations of averages, standard deviation and/or RSD for the procedural recoveries and area counts and calculation of the calibration curve and correlation coefficient (r) by linear regression of the instrument responses for the reference standards. The statistical calculations throughout this report were performed using an automated computer spreadsheet (Microsoft Excel®) and were rounded for presentation purposes. Slight differences may be noted in hand calculations using the recoveries presented in the tables. These are due to rounding and have no effect on the scientific conclusions presented in this report. The detailed analytical data may be consulted for confirmation of the calculated results.

Several measures were taken to ensure the quality of the study results. The quality assurance unit at BASF inspected the analytical procedures for compliance with Good Laboratory Practices that included adherence to the protocol. The dates inspected are detailed in the quality assurance unit statement. Study samples and test and reference items were maintained in secured (i.e. pad-locked) storage with limited access. Freezer and refrigerator temperatures were continuously monitored by electronic means.

6. SUMMARY OF METHOD

Summaries of the method parameters and characteristics are provided in Table 4 and Table 5.

7. INDEPENDENT LABORATORY VALIDATION

The method was the subject of a successful independent laboratory validation (Reference 4).

The laboratory provided the following observations and recommendations to improve use of the method.

In the section „Instrumentation and Conditions for BAS 450 I and its Metabolites“, an alternative confirmatory mass transition for S(PFP-OH)-8007 should be included. The signal for transition m/z 661 → 621 was found to be too low for reliable quatitation. A product ion scan identified an alternative transition of 663 → 643; this transition was successfully used for confirmation in the ILV.

11. REFERENCES

1. Jacob, M., et. Al., (2017). Terrestrial Field Dissipation of the Insecticide BAS 450 I Following Broadcast Applications of BAS 450 00 I (SC). WEI Study Number: 227.32. BASF Study Number 710464. BASF Reg. Doc. No. 2017/7008695

2. Jose, W. (2017). Validation of BASF Method Number D1417/01 for the Determination of Residues of BAS 450 I and its Metabolites S(PFP-OH)-8007 and DM-8007 in Wheat (grain), Dry Beans (Seed), Tomato (Whole Fruit), Citrus (Whole Fruit), Soybean (Seed), and Coffee (Grain) using LC-MS/MS. – GENCS. BASF Study Number 772495. BASF Reg. Doc. No. 2016/3004081.

3. Delinsky, D. (2017). "Validation of Method D1608/01: Method for the Determination of BAS 450 I (Reg. No. 5672774) and Its Metabolites DM-8007 (Reg. No. 5856361), DC-DM-8007 (Reg. No. 5936906), DC-8007 (Reg. No. 5936907) and S(PFP-OH)-8007 (Reg. No. 5959598) in Surface and Drinking Water by LC-MS/MS". BASF Study Number 725931. BASF Reg. Doc. No. 2017/7000331.

4. Neeley, M. (2017). Independent Laboratory Validation of the Following Method Entitled: BASF Analytical Method D1603/01: "Method for the Determination of Residues of BAS 450 I (Reg. No. 5672774) and Its Metabolites DM-8007 (Reg. No. 5856361), DC-DM-8007 (Reg. No. 5936906), DC-8007 (Reg. No. 5936907) and S(PFP-OH)-8007 (Reg. No. 5959598) in Soil by LC-MS/MS (at LOQ of 1 ppb)". BASF Study Number 776691. BASF Reg. Doc. No. 2017/7008393.

5. Chanh Ta and Amy Strobush (2017), Aerobic Soil Metabolism of 14C-Broflanilide (MCI-8007 or BAS 450 I), BASF study number 818049; BASF Registration Document Number, 2017/7008279

12. TABLES

The summary tables are found on the pages which follow.

Table 4. Summary Parameters for the Analytical Method Used for the Quantitation of Residues of Broflanilide in Soil

Method ID	BASF Analytical Method No. D1603/01
Analyte(s)	Residues of broflanilide, including the metabolites DC-DM-8007, DC-8007, DM-8007, and S(PFP-OH)-8007, in soil
Extraction solvent/technique	Residues of broflanilide in soil samples (5 g each) are extracted by shaking with methanol followed by methanol:water (70:30, v/v). Residues in an aliquot of the combined extracts are diluted with methanol:water (50:50, v/v), filtered and analyzed by LC-MS/MS.
Cleanup strategies	Centrifugation and filtration (0.45 µm PTFE)
Instrument/Detector	All analyses were performed on a Waters Acquity LC/MS/MS system (Sciex 5500 with electrospray ionization) equipped with an Xbridge BEH phenyl column (100 x 2.1 mm, 2.5 µm), using a mobile phase gradient of water:methanol, each acidified with 0.1% formic acid, 70:30 to 5:95, v/v, over ~6 minutes, flow rate 600 uL/minute), and monitoring (in the positive mode) ion transitions m/z 663→643 for parent broflanilide; m/z 545→525 for DC-DM-8007; m/z 559→539 for DC-8007; m/z 649→242 for DM-8007; and m/z 661→641 for S(PFP-OH)-8007, and alternate ion transitions m/z 665→645 for parent broflanilide; m/z 547→527 for DC-DM-8007; m/z 561→541 for DC-8007; m/z 651→242 for DM-8007; and m/z 661→621 for S(PFP-OH)-8007.
Standardization method	Linear regression (1/x weighting). Direct comparison of the sample peak responses to those of external standards.
Stability of std solutions	Stock solutions of broflanilide and the metabolites prepared in acetonitrile have been demonstrated stable, when held under refrigeration, for at least 3 months. In addition, each analyte has been shown to be stable when stored under refrigeration for at least 1 month in 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 methanol:water (50:50, v/v).
Retention times (minutes)	Parent broflanilide, 5.64; DC-DM-8007, 5.15; DC-8007, 5.46; DM-8007, 5.65; and S(PFP-OH)-8007, 5.30.

Table 5. Characteristics for the Analytical Method Used for the Quantitation of Residues of Broflanilide in Soil Matrices

Analyte	Residues of broflanilide, including the metabolites DC-DM-8007, DC-8007, DM-8007, and S(PFP-OH)-8007), in soil
Equipment ID	Acquity ultra-HPLC chromatographic system with an Xbridge BEH Phenyl column (100 x 2.1 mm, 2.5 µm) is used with a mobile phase gradient of acidified water and acidified methanol (70:30 to 5:95, v/v, over ~6 minutes, flow rate 600 uL/minute).
Limit of quantitation (LOQ)	The validated LOQ for residues of broflanilide in soil is 0.001 mg/kg, for each analyte, which corresponds to a concentration in the final volume of 0.05 ng/mL.
Limit of detection (LOD)	0.0002 mg/kg (20% of the LOQ)

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Working Procedure:

Method for the determination of Residues of BAS 450 I (Reg. No. 5672774) and Its Metabolites DM-8007 (Reg. No. 5856361), DC-DM-8007 (Reg. No. 5936906), DC-8007 (Reg. No. 5936907) and S(PFP-OH)-8007 (Reg. No. 5959598) in Soil by LC-MS/MS (at LOQ of 1ppb)

BASF Method Number D1603/01

Final

Authors
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Date
August 15, 2017

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USA

Number of Pages
21

ABSTRACT

BASF Method D1603/01 is developed to determine the residues of BAS 450 I (Reg. No. 5672774) and its metabolites DM-8007 (Reg. No. 5856361), DC-DM-8007 (Reg. No. 5936906), DC-8007 (Reg. No. 5936907) and S(PFP-OH)-8007 (Reg. No. 5959598) in soil using LC-MS/MS at BASF Crop Protection, Research Triangle Park, N.C.

A 5 g soil sample aliquot is extracted by shaking with methanol followed by methanol-water (70:30, v/v). An aliquot of the combined extract is diluted with methanol-water (50:50, v/v), filtered and the residues are analyzed by LC-MS/MS.

The limit of quantitation of BAS 450 I and all metabolites is 0.001 mg/kg in all matrices. The limit of detection for each analyte is set to 20% of LOQ, equivalent to 0.0002 mg/kg.

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. 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). At this concentration, the analyte must be qualitatively detectable in sample matrix (analyte peak height at least 3-5 x baseline noise).

1 INTRODUCTION

BAS 450 I is a new insecticide that will be used for various crops. The analytical method D1603/01 offers the possibility to determine residues of BAS 450 I (Reg. No. 5672774) and its metabolites DM-8007 (Reg. No. 5856361), DC-DM-8007 (Reg. No. 5936906), DC-8007 (Reg. No. 5936907) and S(PFP-OH)-8007 (Reg. No. 5959598) in soil.

Method D1603/01 was successfully validated in different soil types (Reference 1). The method has a limit of quantitation of 0.001 mg/kg for BAS 450 I (Reg. No. 5672774) and its metabolites DM-8007 (Reg. No. 5856361), DC-DM-8007 (Reg. No. 5936906), DC-8007 (Reg. No. 5936907) and S(PFP-OH)-8007 (Reg. No. 5959598) in soil matrices.

This method was developed at BASF Crop Protection, Research Triangle Park, NC.

2 MATERIALS

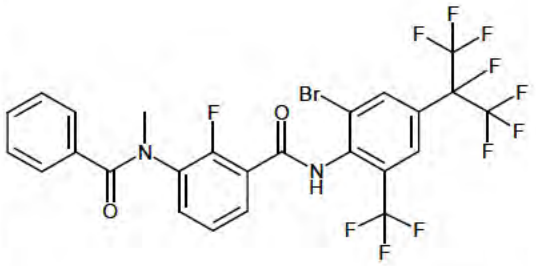
2.1 Safety

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. Ensure work clothing is stored 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.

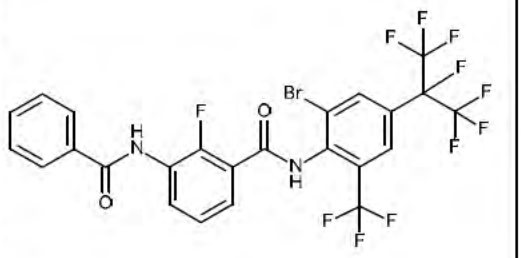
2.2 Test and Reference Items

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

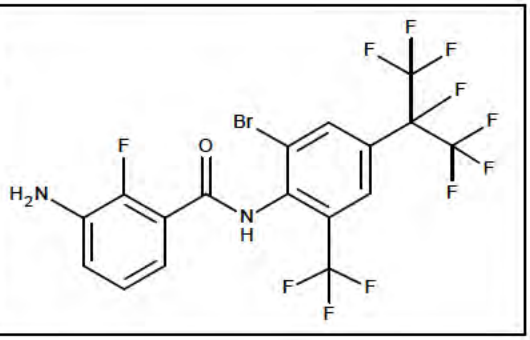
BAS-Code	BAS 450 I
Common Name	Broflanilide
Chemical Name	N-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluoro-3-(N-methylbenzamido)benzamide
BASF Reg. No.	5672774
CAS-No.	None
Molecular Formula	$C_{25}H_{14}BrF_{11}N_2O_2$
Molecular Weight	663.29



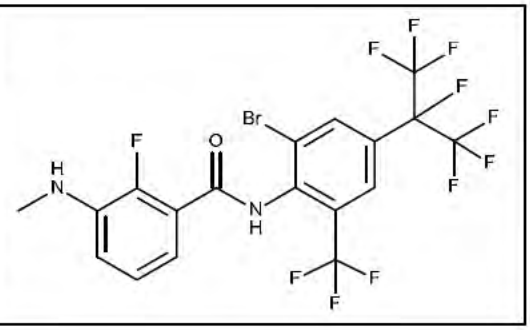
BAS-Code	None
Common Name	DM-8007
Chemical Name	3-benzamido-*N*-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluorobenzamide
BASF Reg. No.	5856361
CAS-No.	None
Molecular Formula	$C_{24}H_{12}BrF_{11}N_2O_2$
Molecular Weight	649.25

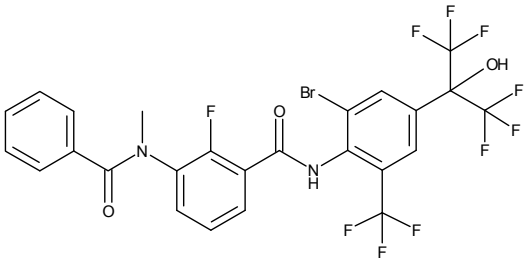


BAS-Code	None
Common Name	DC-DM-8007
Chemical Name	3-amino-*N*-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluorobenzamide
BASF Reg. No.	5936906
CAS-No.	None
Molecular Formula	$C_{17}H_8BrF_{11}N_2O$
Molecular Weight	545.15



BAS-Code	None
Common Name	DC-8007
Chemical Name	N-[2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluoro-3-(methylamino)benzamide
BASF Reg. No.	5936907
CAS-No.	None
Molecular Formula	$C_{18}H_{10}BrF_{11}N_2O$
Molecular Weight	559.17



BAS-Code	None	
Common Name	S(PFP-OH)-8007	
Chemical Name	*N*-[2-bromo-4-(1,1,1,3,3,3-hexafluorofluoro-2-hydroxypropan-2-yl)-6-(trifluoromethyl)phenyl]-2-fluoro-3-(*N*-methlybenzamido)benzamide	
BASF Reg. No.	5959598	
CAS-No.	None	
Molecular Formula	$C_{25}H_{15}BrF_{10}N_2O_3$	
Molecular Weight	661.3	

2.3 Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Amber Bottles	60 mL, Boston Round bottle with PTFE-faced PE lined cap attached	VWR	89042-908
Balance, Top-Load	150 g, CP153	Sartorius	
Beakers	Various Sizes	PYREX Brand, VWR Scientific Products	13922-029
Centrifuge	Allegra 6R	Beckman	
Centrifuge Tubes (disposable)	50 mL	VWR	89039-660
Culture Tubes (glass)	16x100mm, Lime glass disposable	VWR	60825-425
Culture Tube caps	Safe-T-Flex Caps	VWR	60828-768
Filters, Syringe Tip	13mm Syringe Filter, 0.45 µm PTFE membrane	PALL Life Sciences	4555
Graduated Cylinder	10 mL, PYREX	VWR	89090-636
LC-MS/MS injection vials	1.5 mL, Target DP	VWR, Thermo Scientific	00162506
LC Column	XBridge BEH Phenyl, 2.5 µm, 2.1x100 mm	Waters	186006067
LC System	Acquity	Waters	
Mass Spectrometer	API 5500	Sciex	
Microman Pipettes	1000 µL 250 µL 50 µL	Gilson	M1000 M250 M50
Microman Pipette tips	1000 µL tips 250 µL tips 50 µL tips	Gilson	CP1000 CP250 CP50
Pasteur Pipettes, disposable	2 mL, 14.6 cm Borosilicate Glass	VWR	14673-010
Scintillation Vials	20 mL	VWR	66022-060
Shaker	KS501 digital	IKA Labortechnik	0002526401
Spatula		Various	
Syringes, Disposable	1 mL	BD	BD301025
Volumetric Flasks	10 mL, 50 mL, 100 mL	Various	
Volumetric Pipettes	Various, class-A	Various	
Vortex Mixer	Genie 2	Fisher Scientific Co	12-812
Vortexer	Multi-tube vortexer, VX-2500	VWR	444-7063

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 No.
Acetonitrile	HPLC Grade	EMD	AX0145P-1
Methanol	HPLC Grade	EMD	MX0475P-1
Water	HPLC Grade	BDH ARISTAR PLUS	87003-652
Formic Acid	≥95%	Sigma-Aldrich	F0507

Note: Equivalent reagents and chemicals from other suppliers may be substituted.

2.4.2 Solutions and Solvent Mixtures

Description	Code	Composition
Extraction Solvent	S1	**Methanol-water, 70:30, v/v** Add 700 mL of methanol and 300 mL of water into a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Final Volume Solvent	FV1	**Methanol-water, 50:50, v/v** Add 500 mL of methanol and 500 mL of water into a 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase A	LC1	**0.1% Formic Acid in Water** Add 1000 mL of water and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	**0.1% Formic Acid in Methanol** Add 1000 mL of Methanol and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

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 individually by weighing an appropriate amount of the analyte into a flask and add the required volume.
For example, to prepare 10 mL of 1.0 mg/mL stock solution of BAS 450 I in acetonitrile, weigh 10 mg of BAS 450 I into a 10 mL volumetric flask. Dissolve and dilute to mark with acetonitrile. Ensure a complete homogeneous solution (e.g. by sonication or vortexing). The stock solutions for all other analytes are made in a similar fashion.

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.

Fortification Solutions

Prepare standard solutions for fortification by dilution of the above stock solution. 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 mixed Fortification solutions

Take solution (µg/mL)	Volume (mL)	Dilute with acetonitrile to a final volume of (mL)	Concentration (µg/mL)
1000	0.5	50	10
10	5	50	1.0
1.0	5	50	0.10

Note: A different concentration scheme may be used and other concentration levels may be prepared as needed.

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". 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 (mL)	Dilute with FV1* to a final volume of (mL)	Concentration (ng/mL)
100	0.5	50	1.0
1.0	20.0	100	0.20
0.2	25	50	0.10
0.2	12.5	50	0.05
0.2	5.0	50	0.02
0.2	2.5	50	0.01

* In case matrix-matched standards (= instrument recovery samples) are needed for successful analysis, calibration standard solution are prepared in matrix solution, i.e., final volume of a control sample carried through the analytical procedure. Matrix-matched standards should be prepared in a way that the matrix load is at least 90% of the matrix load in the unknown samples. In addition the matrix load should be the same in all calibration standard solutions.

Note: A different concentration scheme may be used and additional standards may be prepared as needed.

Additional Information:

- Use amber bottles with PTFE-faced PE lined screw caps as storage containers for all standard solutions.

3 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Samples must 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

Soil Samples are to be kept frozen until analysis.

3.3 Weighing and Fortification

For treated samples and control samples, weigh 5 g of soil sample into a 50 mL plastic centrifuge tube. For fortification samples, volumetrically add an appropriate volume of standard solution to the respective control. For example, pipette 50 µL of the 0.1 µg/mL solution for a 1 ppb fortification.

The following scheme may be used:

Sample Type	Sample Weight	Concentration of Spiking Solution	Volume of Spiking Solution	Level of Fortification
Control	5 g	-	-	0.00 µg/kg
Fortification (LOQ*)	5 g	100 ng/mL	0.05 mL	1 µg/kg
Fortification (10xLOQ)	5 g	1.0 µg/mL	0.05 mL	10 µg/kg
Treated	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

Weigh 5 g of soil into a 50 mL plastic centrifuge tube, add exactly 10 mL of methanol, vortex to mix, shake for 30 minutes at 300 rpm on a mechanical shaker and then centrifuge at 3000 for 5 minutes. Decant supernatant into a 50 mL plastic centrifuge tube. Add exactly 10 mL of **S1** to soil marc, vortex to mix, shake for 30 minutes at 300 rpm on a mechanical shaker and then centrifuge at 3000 for 5 minutes. Combine both supernatants into the same 50 mL plastic centrifuge tube. Mix well.

3.5 Sample Clean-up

No sample clean-up necessary proceed to section 3.6.

3.6 Preparation for Measurement

All samples – Remove an aliquot from the sample and perform a 1:5 dilution using **FV1** (methanol-water, 50:50, v/v). For example, transfer 1 mL aliquot from extract (Section 3.4) to a culture tube and add 4 mL of **FV1**, cap and vortex to mix. Samples are ready for injection.

High fortification and high residue samples - further dilute with **FV1** (methanol-water, 50:50, v/v) as necessary, to fit in the calibration curve.

Syringe filter all samples using 0.45µm PTFE syringe filters directly into HPLC injection vials, passing the first approximately 0.2 – 0.3 mL to waste.

3.7 Influence of matrix effects on analysis

It has been demonstrated that the matrix load in the samples from the soil matrices had no significant influence on the analysis (i.e., matrix effects < 20%). Therefore, samples can be analyzed using calibration standard solutions prepared in solvent **FV1** (see 2.4.3). If matrix effects are shown to be significant, matrix matched standards can be used for analysis.

3.8 Stability of Extracts and Final Volumes

All analytes have been shown to be stable in extracts and final volumes for at least 7 days (Reference 1).

3.9 Moisture Determination

The procedural recoveries will not be corrected for moisture content of the sample. Results of soil analysis are reported on a “dry weight” basis for residue determination. Therefore field treated soil sample weights must be corrected for moisture content by any method the laboratory customarily uses. The moisture determination will be conducted for the treated samples with residue value above LOD. An example of a moisture determination procedure is provided below:

The percent moisture is determined using an automated moisture determination equipment (Mettler Toledo HR83) using the formula below:

Moisture content [%] = ((Weight moist soil - Weight dry soil)/Weight moist soil) x 100

4 QUANTIFICATION AND CALCULATION

4.1 Set-up of the analytical run

A sequence for measurement generally consists of:
- o Calibration standards
- o Control samples
- o Procedural recovery samples
- o Unknown samples
- o 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 5 calibration levels need to be injected.

4.2 Instrumental analysis

4.2.1 Instrumentation and Conditions for BAS 450 I and Its Metabolites

	Parameter		
Chromatographic System	Waters Acquity		
Analytical-column	XBridge BEH Phenyl 2.5um, 2.1x100mm		
Column Temperature	50°C		
Injection Volume	20 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Methanol / formic acid, 1000/1, v/v		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00	70	30
	0.10	70	30
	4.00	40	60
	6.00	5	95
	7.20	5	95
	7.25	70	30
	8.00	70	30
Detection System	Sciex 5500		
Ionisation	Electrospray (ESI)		
Ionisation Temperature	700 °C		

Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
BAS 450 I (Reg. No.5672774)	663 → 643* 665 → 645	positive	approx. 5.6 min
DC-DM-8007 (Reg. No. 5936906)	545 → 525* 547 → 527	positive	approx. 5.2 min
DC-8007 (Reg. No. 5936907)	559 → 539* 561 → 541	positive	approx. 5.5 min
DM-8007 (Reg. No. 5856361)	649 → 242* 651 → 242	positive	approx. 5.7 min
S(PFP-OH)-8007 (Reg. No. 5959598)	661 --> 641* 661 --> 621	positive	approx. 5.3 min

* 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.

4.2.2 Instrumentation and Conditions for BAS 450 I and Its Metabolites used for Independent Laboratory Validation

	Parameter		
Chromatographic System	Waters Acquity		
Analytical-column	XBridge BEH Phenyl 2.5um, 2.1x100mm		
Column Temperature	50°C		
Injection Volume	20 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Methanol / formic acid, 1000/1, v/v		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00 0.10 4.00 6.00 7.20 7.25 8.00	70 70 40 5 5 70 70	30 30 60 95 95 30 30
Detection System	Sciex 5500		
Ionisation	Electrospray (ESI)		
Ionisation Temperature	700 °C		

Analyte	**Transitions (m/z)**	**Polarity**	**Expected Retention Time**
BAS 450 I (Reg. No.5672774)	663 → 643* 665 → 645	positive	approx. 5.6 min
DC-DM-8007 (Reg. No. 5936906)	545 → 525* 547 → 527	positive	approx. 5.2 min
DC-8007 (Reg. No. 5936907)	559 → 539* 561 → 541	positive	approx. 5.5 min
DM-8007 (Reg. No. 5856361)	649 → 242* 651 → 242	positive	approx. 5.7 min
S(PFP-OH)-8007 (Reg. No. 5959598)	661 --> 641* 663 --> 643	positive	approx. 5.3 min

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

4.2.3 Calibration procedures

Calculation of results is based on peak area measurements using a calibration curve. At least 5 calibration levels need to be injected (e.g., required for enforcement). The calibration curve is obtained by direct injection of BAS 450 I/metabolites standards for LC-MS/MS in the range of 0.2 ng/mL to 0.01 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.

4.2.4 Calculation of Residues and Recoveries

Calculation of results is based on area measurements.
For the procedural recoveries, the sample weight will be considered 5 g in the final calculation of residues [mg/kg]. The method requires that the sample weight to be 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 cancels out, as shown in the equation below, during the final calculation step.

The residues of BAS 450 I in mg/kg are calculated as shown in equations **I** and **II**:

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	=	Aliquotation factor
1000	=	Factor remaining after all unit conversions

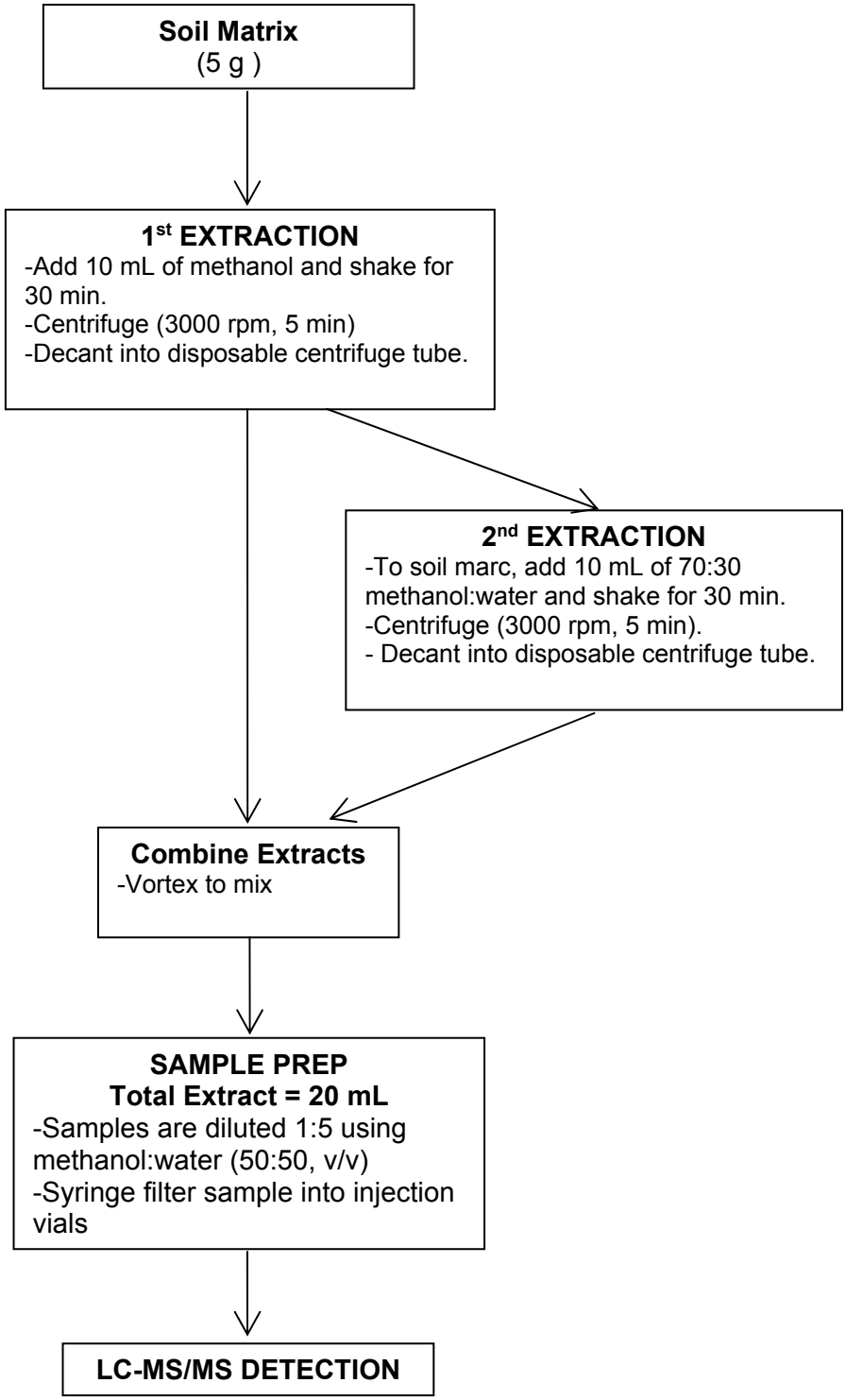
The recoveries of spiked compounds are calculated according to equation **III**:

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

IV. Soil residues based on soil dry weight

Residue [mg/kg] (Dry residue) = $= \frac{\text{Wet Sample Residue [mg/kg]} \times 100}{(100 - \text{"moisture content [\%]"})}$

5 FLOWCHART


Soil Matrix
(5 g)
1st EXTRACTION
-Add 10 mL of methanol and shake for 30 min.
-Centrifuge (3000 rpm, 5 min)
-Decant into disposable centrifuge tube.
2nd EXTRACTION
-To soil marc, add 10 mL of 70:30 methanol:water and shake for 30 min.
-Centrifuge (3000 rpm, 5 min).
- Decant into disposable centrifuge tube.
Combine Extracts
-Vortex to mix
SAMPLE PREP
Total Extract = 20 mL
-Samples are diluted 1:5 using methanol:water (50:50, v/v)
-Syringe filter sample into injection vials
LC-MS/MS DETECTION

6 METHOD MANAGEMENT AND TIME REQUIREMENTS

The analysis of one series of samples (= 13 unknown samples, 2 fortified samples for recovery experiments, 1 blank sample) 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 is 0.001 mg/kg for all analytes. The limit of detection was estimated at 20% of the limit of quantification, equivalent to 0.0002 mg/kg for BAS 450 I/metabolites. The lowest standard for each analyte in the calibration curve has good detectability (signal to noise ratio greater than 3:1).

Selectivity
The tested untreated soil samples showed no significant interferences (< 20 or 30 %) at the retention time of the analytes.

Confirmatory Techniques
The LC-MS/MS final determination for BAS 450 I 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
A PVDF filter is not suitable for use with this method, however, GHP and nylon filters may be found to be acceptable.

The glassware used for the method should be thoroughly rinsed with methanol followed by acetone to prevent contamination.

8 REFERENCES

1. Delinsky, D. “Method for the Determination of Residues of BAS 450 I (Reg. No. 5672774) and Its Metabolites DM-8007 (Reg. No. 5856361), DC-DM-8007 (Reg. No. 5936906), DC-8007 (Reg. No. 5936907) and S(PFP-OH)-8007 (Reg. No. 5959598) in Soil by LC-MS/MS (at LOQ of 1 ppb)”; BASF Study Number 815843; BASF Registration Document Number: 2017/7001823

2. Veiga, A. and Jose, W. (2017) “Validation of BASF Method Number D1417/01 for the determination of residues of BAS 450 I and its metabolites S(PFP-OH)-8007 and DM-8007 in wheat (grain), dry beans (seed), tomato (whole fruit), citrus (whole fruit), soybean (seed) and coffee (grain) using LC-MS/MS”; BASF Study Number, 772495; BASF Registration Document Number: 2016/3004081

3. D. Delinsky, 2017: Validation of BASF Method D1608/01: " Method for the Determination of BAS 450 I (Reg. No. 5672774) and Its Metabolites DM-8007 (Reg. No. 5856361), DC-DM-8007 (Reg. No. 5936906), DC-8007 (Reg. No. 5936907) and S(PFP-OH)-8007 (Reg. No. 5959598) in Surface and Drinking Water by LC-MS/MS". BASF Study Number 725931; BASF Reg. Doc. No. 2017/7000331.

9 APPENDIX

9.1 Example of Calculation

Example: BAS 450 I; soil sample fortified at 0.001 mg/kg:

Concentration in the final volume [ng/mL]

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

Residue in the sample [mg/kg]

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

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	1366
Response of control sample	56
Slope:	2.81e4
Intercept:	54.4
Sample Weight (G):	5 g
Final Volume (V_{end}):	5 mL
Aliquotation factor A_F:	0.05 (= 5%)
Conversion factor ng → µg:	1000

Concentration (ng/mL) $= \frac{1366 - 54.4}{2.81e4} = 0.0467\ ng/ml$

Residue (mg/kg)

$= \frac{5\ ml \times 0.0467\ ng/ml}{5\ g \times 0.05 \times 1000} = 0.000934\ \mu g/g = 0.000934\ mg/kg$

Recovery % $= \frac{(0.000934\ mg/kg - 0.00000\ mg/kg) \times 100}{0.0010\ mg/kg} = 93.4\%$

Typical Recovery Calculation for LC/MS/MS Quantitation

Sample No. 815843-04-4. Control soil fortified at the LOQ with broflanilide (and other analytes), Master Sheet No. 815843-4.

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

	Broflanilide
Peak Area =	35742
Intercept =	604
Slope =	6.44E+05
Conc. (ng/mL) =	0.0546

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

	Broflanilide
V_{end} =	5.0 mL
A_F =	5.0%
G =	5.00
Conc. (ng/mL) =	0.0546
Residue (mg/kg) =	0.001092

A_F = Aliquot taken from combined extract (1.0 mL) / combined extract volume (20 mL)

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

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

	Broflanilide
Amount fortified (mg/kg) =	0.001
Residue (mg/kg) =	0.001092
Residue in control =	<0.0002
%Recovery	109%

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

Protocol Amendments and Deviations

1) Correction of typographical error in Guideline section of the original study protocol

Original: EPA Residue Chemistry Test Guidelines
OPPTS 850.6100 Environmental Chemistry Methods and Associated Independent Laboratory Validation

New: Ecological Effects Test Guidelines OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation

2) Replaced technical procedure with final working procedure.
   a. To specifically change the notes about preparation of standards to remove statements allowing a change in prepared volume as long as proportions are maintained
   b. to add additional information such as stability information
   c. correct various typographical errors.

This change did not have an adverse effect on the outcome of this study.