Abstract

The objective of this validation study was to demonstrate the applicability and repeatability of method D1303/02 for the determination of quizalofop-p-ethyl (BAS 9152 H, Reg. No. 4059806) and its two metabolites quizalofop-p and 3-OH-quizalofop-acid in soil using LC-MS/MS. This method was developed in BASF Corporation, RTP, NC and was validated at Primera Analytical Solutions Corp. (PASC) in Princeton, New Jersey.

Principle of the Method. For the analysis of quizalofop-p-ethyl (BAS 9152 H), quizalofop-p, and 3-OH-quizalofop-acid, a 5 g (or 0.1 g) soil sample aliquot was extracted by shaking twice with acetonitrile - 6% phosphoric acid in water (80:20, v/v). An aliquot (8%) from the extract was diluted with acetonitrile-water (90:10, v/v) for the residue determination of quizalofop-p-ethyl and quizalofop-p using LC-MS/MS. A separate aliquot (40%) from the original extract was diluted with water for the residue determination of 3-OH-quizalofop-acid using LC-MS/MS.

Test Conditions. The method was validated at two fortification levels for each analyte: 0.005 mg/kg and 0.05 mg/kg for quizalofop-p-ethyl and quizalofop-p, and 0.001 mg/kg and 0.01 mg/kg for 3-OH-quizalofop-acid. For each fortification level and matrix, five replicates were analyzed. Additionally, at least two replicates of unfortified samples were analyzed with each sample set. For quizalofop-p-ethyl and quizalofop-p, two mass transitions (*m/z* 373→299 and *m/z* 375→301; *m/z* 345→299 and *m/z* 345→100) were evaluated using one chromatographic condition. For the metabolite 3-OH-quizalofop-acid, one mass transition (*m/z* 359→166) was used for both primary and confirmatory quantitation. A secondary chromatographic method was used for confirmation.

Matrix-matched standards and solvent-based standards were also analyzed and compared within this study to evaluate the matrix effects.

Limit of Quantitation (LOQ) and Limit of Detection (LOD). The limit of quantitation (LOQ) was defined by the lowest fortification level successfully tested. The LOQ is 0.005 mg/kg for quizalofop-p-ethyl and quizalofop-p, and is 0.001 mg/kg for 3-OH-quizalofop-acid. The limit of detection (LOD) is set at 20% of the LOQ, which is 0.001 mg/kg for quizalofop-p-ethyl and quizalofop-p and 0.0002 mg/kg for 3-OH-quizalofop-acid.

Selectivity. The method was able to determine residues of quizalofop-p-ethyl (BAS 9152 H, Reg. No. 4059806) and its metabolites (quizalofop-p and 3-OH-quizalofop-acid) individually in soil. No interfering peaks were found at the retention time for quizalofop-p-ethyl or for its metabolites individually (quizalofop-p and 3-OH-quizalofop-acid). No matrix suppression or enhancement was found for quizalofop-p-ethyl or for its metabolites.

1. Introduction

1.1. Scope of the Method

Quizalofop-p-ethyl (BAS 9152 H) is an herbicide that was developed for use in a broad spectrum of crops in the US. Quizalofop-p and 3-OH-quizalofop-acid are two metabolites of interest for the compound.

A residue analytical method for the detection and quantitation of the active ingredient quizalofop-p-ethyl (BAS 9152 H, Reg. No. 4059806) in soil was needed for monitoring purposes with a limit of quantitation (LOQ) of 0.005 mg/kg. The detection and quantitation of the two metabolites, quizalofop-p and 3-OH-quizalofop-acid, were also developed together with quizalofop-p-ethyl with a limit of quantitation (LOQ) of 0.005 mg/kg and 0.001 mg/kg, respectively.

As described below, the BASF Method No. D1303/02 allows for the determination of the three analytes with the required limit of quantitation in soil. This method was developed at BASF Crop Protection in Research Triangle Park, North Carolina and was validated at Primera Analytical Solutions Corp. in Princeton, NJ. To demonstrate the validity of the method, recovery trials with fortified soil samples were performed.

The method was validated at two fortification levels, LOQ and 10×LOQ (0.005 mg/kg and 0.05 mg/kg for quizalofop-p-ethyl and quizalofop-p, and 0.001 mg/kg and 0.01 mg/kg for 3-OH-quizalofop-acid) in soil samples. For each fortification level and matrix, five replicates were analyzed. Additionally, two replicates of unfortified samples were analyzed with each sample set. For quizalofop-p-ethyl and quizalofop-p, two mass transitions (*m/z* 373→299 and *m/z* 375→301; *m/z* 345→299 and *m/z* 345→100) were evaluated using one chromatographic condition, as outlined in Section 4.1.1. For 3-OH-quizalofop-acid, one mass transition (*m/z* 359→166) was evaluated using two chromatographic conditions, as outlined in Section 4.1.2. Two separate sample sizes (5 and 0.1 g) were validated in this study using different soil types

Matrix-matched and solvent-based standards were also analyzed within this study to check for possible matrix effects.

1.2. Principle of the Method

For the analysis of quizalofop-p-ethyl (BAS 9152 H), quizalofop-p, and 3-OH-quizalofop-acid, a 5 g (or 0.1 g) soil sample aliquot was extracted by shaking twice with acetonitrile - 6% phosphoric acid in water (80:20, v/v). An aliquot (8%) from the extract was diluted with acetonitrile-water (90:10, v/v) for the residue determination of quizalofop-p-ethyl and quizalofop-p using LC-MS/MS. A separate aliquot (40%) from the original extract was diluted with water for the residue determination of 3-OH-quizalofop-acid using LC-MS/MS.

1.3. Specificity

The method was able to accurately determine residues of quizalofop-p-ethyl, quizalofop-p, and 3-OH-quizalofop-acid. No interference was observed at the retention times for any of the three peaks. No matrix-suppression or enhancement was found in to affect any of the analytes. The use of matrix-matched standards was not necessary as shown in this method validation study.

2. Materials and Methods

2.1. Test systems

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

Test System 1: Loamy Sand Soil (BASF Study 394796, RCN R130088)

Test System 2: Silt Loam Soil (BASF Study 437860, RCN R130034)

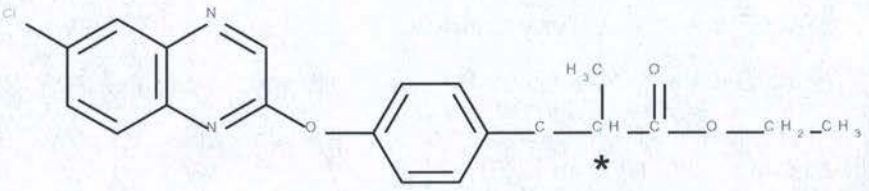
The description and characterization for the soil used is provided in the respective attached certificates (Appendix 5).

2.2. Test and Reference Items

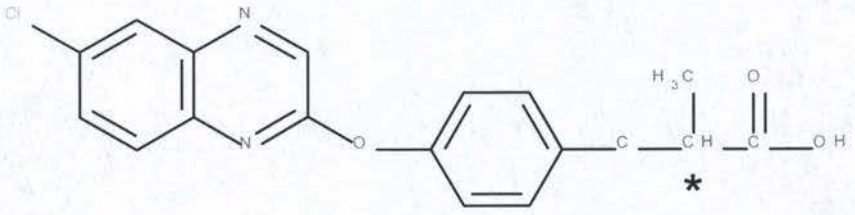
Standard substances were stored in a freezer (≤ -5°C) until use.

BASF has retained reserve samples of these chemicals, and has documentation specifying the location of the synthesis and characterization information for each of these compounds available at BASF, Research Triangle Park, North Carolina. The certificate of analysis for the reference standards are shown in Appendix 5.

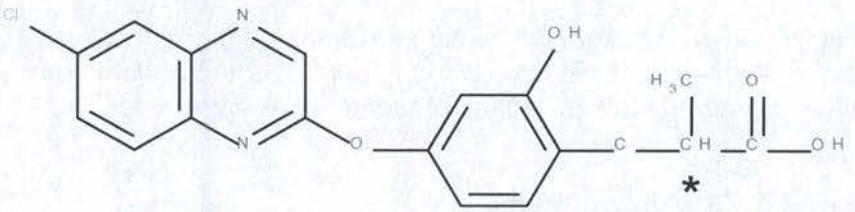
2.2.1. Quizalofop-p-ethyl (BAS 9152 H)

Common Name	Quizalofop-p-ethyl (QPE)
BASF Reg. No.	N/A
CAS No.	100646-51-3
Molecular Formula	$C_{19}H_{17}ClN_2O_4$
Molecular Weight	372.8
IUPAC Name	ethyl (R)-2-[4-(6-chloroquinoxanlin-2-yloxy)-phenoxyl]propionate
Batch No.	302D-S110926
Purity (%)	99.9%
Test Substance Type	PAI
Storage Advice	Dark and Cool (below 10°C is recommended)
GLP	Yes
Expiration Date	September 26, 2016
Chemical Structure	

2.2.2. Quizalofop-p

Common Name	Quizalofop-p (QP)
BASF Reg. No.	N/A
CAS No.	94051-08-8
Molecular Formula	$C_{17}H_{13}ClN_2O_4$
Molecular Weight	344.7
IUPAC Name	(R)-2-[4-(6-chloroquinoxanlin-2-yloxy)-phenoxyl]propionic acid
Batch No.	302D-ACID-S050325
Purity (%)	99.8%
Test Substance Type	Metabolite
Storage Advice	Dark and Cool (below 10°C is recommended)
GLP	Yes
Expiration Date	Dec. 5, 2017
Chemical Structure	

2.2.3. 3-OH-Quizalofop-acid

Common Name	R(+)-3-OH-quizalofop-acid (3-OH-QA)
BASF Reg. No.	N/A
CAS No.	N/A
Molecular Formula	C17H13ClN2O5
Molecular Weight	360.8
IUPAC Name	(R)-2-[4-(6-chloro-3-hydroxyquinoxanlin-2-yloxy)-phenoxyl]propionic acid
Batch No.	3-OH-302D-ACID-M941088
Purity (%)	95.9%
Test Substance Type	Metabolite
Storage Advice	Dark and Cool (below 10°C is recommended)
GLP	Yes
Expiration Date	March 3, 2017
Chemical Structure	

2.2.4. Stability of Test and Reference Items

2.2.4.1. Stability of Stock Standard and Calibration Standard Solutions

The stability of stock solutions of quizalofop-p-ethyl and quizalofop-p (prepared in acetonitrile), and 3-OH-quizalofop-acid (prepared in methanol) were investigated within this study. For this purpose, the stock standard solutions were stored refrigerated (4°C) for up to three months. At one month (29 days) and three months (95 days), the stored stock standards were diluted to 0.2 ng/mL, and were measured against freshly prepared standards (zero-day).

The stability of calibration solutions of quizalofop-p-ethyl and quizalofop-p (prepared in water-acetonitrile 10:90 v/v), and 3-OH-quizalofop-acid (prepared in water-acetonitrile 45:55 v/v) were investigated within this study. For this purpose, the standard solutions were stored refrigerated (4°C) for up to one month (29 days). The concentrations of the three analytes were measured against freshly prepared standards (zero-day).

Quantitation was done for both mass transitions of quizalofop-p-ethyl and quizalofop-p. For 3-OH-quizalofop-acid, both chromatographic methods were evaluated at one mass transition. Standard stability data is presented in Appendix 1.

2.2.4.2. Stability of Extracts

In order to store the sample solutions before final measurement, the stability of the analytes in extracts after initial extraction and in final solutions before measurement was tested. The sample extracts used for storage stability were fortification samples at the LOQ level.

Data were obtained from the refrigerated (4°C) stored extract solutions and final solutions after 7 days. The concentrations of quizalofop-p-ethyl, quizalofop-p, and 3-OH-quizalofop-acid were measured against freshly prepared standards within one analytical queue.

Quantitation of the analytes was performed using both mass transitions for quizalofop-p-ethyl and quizalofop-p. Similarly, both chromatographic conditions were used for 3-OH-quizalofop-acid. The recoveries of procedural fortifications were used to prove the stability of the analytes in the soil extracts and the final solutions. The extract stability data and final solution stability data is presented in Appendix 1, Tables 1.4 and 1.5.

2.3. Materials and Methods

2.3.1. Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Balance, Top Loader	PE3600, DeltaRange®	Mettler	----
Balance, Analytical	MT5	Mettler	----
Centrifuge	Sorval® T6000D	Sorvall	----
Mechanical shaker	KS501 Digital	IKA Labortechnik	----
Teflon® Centrifuge Tubes	50 mL	VWR Scientific Products	21009-477
Vortex	231	Fisher Scientific Products	14216-184
Volumetric, pipettes	2.5 mL, 5 mL, 20 mL, 25 mL	Fisher Scientific – Class A	13-650-2A
Plastic Micro Tubes	1.4 mL Alphanum tubes	Thermo Scientific	4253
HPLC vials	2.0 mL	Aegla	
Cap Mats	Cap mat for 96 well plates	Waters	186000856
Auto-vortexer	VX-2500 Multi-tube vortexer	VWR	58816-115
UPLC-MS/MS	Acquity UPLC System	Waters	----
	API 5000	AB Sciex	----
HPLC Column	Acquity UPLC® BEH C18, 1.7 µm, 2.1 x 50 mm	Waters	186002885
HPLC Column	Acquity UPLC® BEH Phenyl, 1.7 µm, 2.1x100 mm	Waters	186006067
HPLC Column	Acquity UPLC® HSS T3, 1.8 µm, 2.1x100 mm	Waters	176001132

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.

2.3.2. Reagents

2.3.2.1. Chemicals

Chemical	Grade	Manufacturer/Supplier	Catalog No.
Acetonitrile	HPLC Grade	Fisher Scientific PHARMCO*	No. 30721 30000HPLC
Water	HPLC Grade	BDH ARISTAR PLUS Millipore Milli-Q Gradient A10*	87003-652 ---
Methanol	HPLC Grade	EMD J.T. Baker*	MX0475-P1 9093-03
Phosphoric Acid	85%	Sigma Aldrich BDH*	015-011-00-6 BDH-3118
Formic acid	98% GR ACS	EMD Sigma*	FX0440-7 14265
Celite® 545	N/A	J.T. Baker EMD*	3371-05 CX0574-3
Ammonium Formate	Optima LC-MS Grade BioUltra	Fisher Scientific Fluka*	A115-50 09735

*Chemicals and reagents used in the method validation performed at PASC

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

2.3.2.2. Solutions and Solvent Mixtures

Description	Code	Composition
Extraction solvent	S1	Acetonitrile-6% Phosphoric Acid in water (80:20,v/v) 1) Preparation of 6% Phosphoric Acid in water (by volume): Add 71 mL of 85% phosphoric acid and 929 mL of water into a 1L Erlenmeyer flask and mix well to ensure a complete homogeneous solution 2) Add 800 mL of acetonitrile and 200 mL of 6% Phosphoric Acid in water from above into a, 1L Erlenmeyer flask and mix well to ensure a complete homogeneous solution.
Final volume solvent for Quizalofop-p-ethyl and Quizalofop-p	S2	Water-Acetonitrile (10:90,v/v) Add 100 mL of water and 900 mL of acetonitrile into a 1L Erlenmeyer flask and mix well to ensure a complete homogeneous solution.
Final volume solvent for 3-OH-Quizalofop -acid)	S3	Water-Acetonitrile (45:55,v/v) Add 450 mL of water and 550 mL of acetonitrile into a 1L Erlenmeyer flask and mix well to ensure a complete homogeneous solution.
LC mobile phase A	LC1	0.1% Formic Acid in Water (by volume) Add 999 mL of water and 1 mL of concentrated formic acid into a 1-L Erlenmeyer flask and mix well to ensure a complete homogeneous solution.
LC mobile phase B	LC2	0.1% Formic Acid in Acetonitrile (by volume) Add 999 mL of acetonitrile and 1 mL of concentrated formic acid into a 1-L Erlenmeyer flask and mix well to ensure a complete homogeneous solution.
HPLC mobile phase C	LC3	4 mM Ammonium Formate with 0.1% Formic Acid in Water Add 0.25 g of ammonium formate to 500 mL of water in a 1L graduated cylinder. Once fully dissolved add 1 mL of formic acid and bring the volume to 1 L with water.
HPLC mobile phase D	LC4	4 mM Ammonium Formate with 0.1% Formic Acid in Methanol Add 0.25 g of ammonium formate to 500 mL of methanol in a 1L graduated cylinder. Once fully dissolved add 1 mL of formic acid and bring the volume to 1 L with methanol.

Note: If necessary, the solutions may also be prepared in different volumes as long as the proportions are not modified.
Only LC3 and LC4 were used in this validation.

2.4. Standard Solutions

Amber bottles with Teflon-lined screw caps were used as storage containers for all standard solutions.

2.4.1. Stock Solutions

Stock standard solutions containing 1 mg/mL of quizalofop-p-ethyl (BAS 9152 H) and its metabolites individually (quizalofop-p and 3-OH-quizalofop-acid) were prepared separately by weighing an appropriate amount of reference item or standard into a volumetric flask and adding the required volume.

For example, 5 mg of quizalofop-p-ethyl was weighed into a 5 mL volumetric flask. The reference substance was dissolved and diluted to mark with acetonitrile. A completely homogeneous solution was ensured with a combination of sonication and vortexing.

The stock solutions for quizalofop-p-ethyl and quizalofop-p were made in acetonitrile and the stock solution for 3-OH-quizalfop-acid was made in methanol.

A correction for purity was not performed in this study. A correction for purity is done if the purity is ≤ 95%. If the purity is >95%, correction is optional.

Note: Standard calibration and fortification solutions should initially be confirmed to show correct preparation of the solutions. This can be achieved by using one of the following approaches:

1. Two stock solutions are independently prepared. One is used for preparation of fortification solutions, the other for calibration standard solutions.
2. 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.

2.4.2. Fortification Solutions

Samples were analyzed for quizalofop-p-ethyl and quizalofop-p in a different injection than for 3-OH-quizalofop-acid. Therefore, two different sets of fortification solutions were required for analysis.

Fortification Solutions for Quizalofop-p-ethyl and Quizalofop-p

Fortification solutions containing both quizalofop-p-ethyl and quizalofop-p were prepared at concentrations of 10, 1, and 0.1 µg/mL using the example dilution scheme in the table below. Solutions were diluted volumetrically with acetonitrile and were vortexed to ensure a complete homogeneous solution.

Preparation of Mixed Fortification Standard Solutions (quizalofop-p-ethyl and quizalofop-p)

Take solution (µg/mL)	Volume (mL)	Dilute with acetonitrile to a final volume of (mL)	Concentration (µg/mL)
1000 (of both analytes)	0.25	25	10.0
10.0	2.5	25	1.0
1.0	2.5	25	0.10

Fortification Solutions for 3-OH-quizalofop-acid

Fortification solutions containing 3-OH-quizalofop-acid were prepared at concentrations of 10, 2.0, 1.0, 0.2, 0.1, and 0.02 µg/mL using the example dilution scheme in the table below. Solutions were diluted volumetrically with methanol and were vortexed to ensure a complete homogeneous solution.

Preparation of Fortification Standard Solutions (3-OH-quizalofop-acid)

Take solution (µg/mL)	Volume (mL)	Dilute with methanol to a final volume of (mL)	Concentration (µg/mL)
1000	0.25	25	10.0
10.0	5	25	2.0 (for microanalysis)
10.0	2.5	25	1.0
1	5	25	0.2 (for microanalysis)
1	2.5	25	0.10
0.1	5	25	0.02 (for microanalysis)

Note: A different concentration scheme may be used if other fortification levels are needed for the analysis.
If necessary, the volume of solution prepared me be changed as long as the proportions are not modified.
If necessary, the fortification and calibration solution may be prepared separately as long as the same solutions and the proportions are not modified.
Do not prepare quizalofop-p-ethyl in methanol.

2.4.3. Calibration Standard Solutions

Calibration standards were prepared for LC-MS/MS analysis, in flasks, by using the solutions that were prepared in Section 2.4.2 (Fortification Solutions). Calibration solutions were diluted volumetrically with appropriate solvents. The solutions were vortexed to ensure a complete homogeneous solution.

The calibration solutions for quizalofop-p-ethyl and quizalofop-p were made and diluted with final volume solvent S2, while the calibration solutions for 3-OH-quizalofop-acid were made and diluted with final volume solvent S3. Solutions were prepared following the example dilution schemes in the tables below.

Preparation of Mixed Standard Solutions for Calibration (quizalofop-p-ethyl and quizalofop-p)

Take Solution Concentration of Each Analyte (ng/mL)	Volume (mL)	Dilute with S2 to a Final Volume (mL)	Final Concentration of Each Analyte (ng/mL)
100	0.25	25.0	1.0
1.0	10.0	50.0	0.20
0.20	25.0	50.0	0.10
0.10	25.0	50.0	0.050
0.050	20.0	50.0	0.020
0.020	25.0	50.0	0.010

Preparation of Standard Solutions for Calibration (3-OH-quizalofop-acid)

Take Solution Concentration (ng/mL)	Volume (mL)	Dilute with S3 to a Final Volume (mL)	Final Concentration (ng/mL)
100	0.25	25.0	1.0
1.0	10.0	50.0	0.20
0.20	25.0	50.0	0.10
0.10	25.0	50.0	0.050
0.050	20.0	50.0	0.020
0.020	25.0	50.0	0.010

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

2.4.4. Matrix-Matched Calibration Standard Solutions

Matrix-matched standard solutions were prepared with both soil matrix R130034 and R13008 in order to study the matrix effect on analyte response. Matrix-matched standards were prepared for standards at 0.5×LOQ, LOQ, and 2.5×LOQ in the following manner:

1. Fortification and calibration solutions, prepared in Section 2.4.2 and 2.4.3 above, were used to prepare the precursor matrix-matched solutions by following the example dilution schemes in the tables below.

Preparation of Mixed Matrix-Matched Standard Precursor Solutions (quizalofop-p-ethyl and quizalofop-p)

Take Solution Concentration of Each Analyte (ng/mL)	Volume (mL)	Dilute with S2 to a Final Volume (mL)	Final Concentration of Each Analyte (ng/mL)
100	0.625	50.0	1.25
1.0	25.0	50.0	0.50
0.50	25.0	50.0	0.25

Preparation of Matrix-Matched Standard Precursor Solutions (3-OH-quizalofop-acid)

Take Solution Concentration (ng/mL)	Volume (mL)	Dilute with S3 to a Final Volume (mL)	Final Concentration (ng/mL)
100	0.625	50.0	1.25
1.0	25.0	50.0	0.50
0.50	25.0	50.0	0.25

2. Three control solutions were prepared for each soil matrix and were brought up to final solutions, as outlined in Section 3.6.
3. All three extracts for each matrix were combined and vortexed to homogenize.
4. The matrix-matched standards were prepared according to the example dilution schemes in the tables below, using the precursor standards from Step 1 and the combined control extracts from Step 3 to make the solutions.
5. For comparison, the neat standard was prepared in the same way using S2 or S3 for the respective analyte.

Preparation of Mixed Matrix-Matched Standard Solutions (quizalofop-p-ethyl and quizalofop-p)

Final Concentration of Each Analyte in Matrix (ng/mL)	Volume of Combined Control Extract Taken (mL)	Precursor Standard Concentration of Each Analyte in S2 (ng/mL)	Volume of Precursor Standard (mL)
0.125 (2.5×LOQ)	0.9	1.25	0.1
0.05 (LOQ)	0.9	0.5	0.1
0.025 (0.5×LOQ)	0.9	0.25	0.1

Preparation of Matrix-Matched Standard Solutions (3-OH-quizalofop-acid)

Final Concentration in Matrix (ng/mL)	Volume of Combined Control Extract Taken (mL)	Precursor Standard Concentration in S3 (ng/mL)	Volume of Precursor Standard (mL)
0.125 (2.5×LOQ)	0.9	1.25	0.1
0.05 (LOQ)	0.9	0.5	0.1
0.025 (0.5×LOQ)	0.9	0.25	0.1

3. Analytical Procedure

3.1. Sample Preparation

Samples were sufficiently homogenized beforehand, in order to assure that the aliquot taken for residue analysis was representative of the whole sample.

3.2. Sample Storage

Soil samples were kept frozen until analysis. Freezer storage stability of quizalofop-p-ethyl and its metabolites in soil will be determined in BASF study 437861.

3.3. Weighing and Fortification

3.3.1. Weighing and Fortification, 0.1 g Sample Size

For control and treated samples, 0.1 ± 0.01 g of soil was weighed into an Alpha tube in a 96-well plate.

For fortified samples, 0.1 ± 0.01 g of control soil was weighed into an Alpha Tube. Fortification solutions were added according to the table below. When analyzing all three analytes, two fortification solutions were added to each fortification sample. The samples were fortified with quizalopfop-p-ethyl and quizalofop-p first, followed by a separate fortification of 3-OH-quizalofop-acid.

Sample Type	Sample Weight (g)	Analytes	Concentration of Spiking Solution (µg/mL)	Volume of Spiking Solution (mL)	Level of Fortification (µg/g)
Control	0.1	None	-	-	0.00
Fortification (LOQ)*	0.1	Quizalofop-p-ethyl and Quizalofop-p	0.1	0.005	0.005
		3-OH-Quizalofop-acid	0.02	0.005	0.001
Fortification (10xLOQ)	0.1	Quizalofop-p-ethyl and Quizalofop-p	1.0	0.005	0.05
		3-OH-Quizalofop-acid	0.2	0.005	0.01

*Limit of quantitation (LOQ)

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

3.3.2. Weighing and Fortification, 5 g Sample Size

For control and treated samples, 5 ± 0.1 g of soil sample was weighed into a 40 mL Teflon centrifuge tube.

For fortified samples, 5 ± 0.1 g of control soil was weighed into a 40 mL Teflon centrifuge tube. Fortification solutions were added according to the table below. When analyzing all three analytes, two fortification solutions were added to each fortification sample. The samples were fortified with quizalopfop-p-ethyl and quizalofop-p first, followed by a separate fortification of 3-OH-quizalofop-acid.

Sample Type	Sample Weight (g)	Analytes	Concentration of Spiking Solution (µg/mL)	Volume of Spiking Solution (mL)	Level of Fortification (µg/g)
Control	5	None	-	-	0.00
Fortification (LOQ)*	5	Quizalofop-p-ethyl and Quizalofop-p	0.1	0.25	0.005
		3-OH-quizalofop-acid	0.1	0.05	0.001
Fortification (10xLOQ)	5	Quizalofop-p-ethyl and Quizalofop-p	1.0	0.25	0.05
		3-OH-quizalofop-acid	1.0	0.05	0.01

* Limit of quantitation (LOQ)

3.4. Extraction of Sample Material

3.4.1. Extraction of Sample Material [Quizalofop-p-ethyl, Quizalofop-p, and 3-OH-quizalofop-acid; 0.1 g Sample Size]

Approximately 0.03 g of Celite® was added to the pre-weighed sample, followed by exactly 0.3 mL of extraction solvent S1. The tube was capped and the sample was vortexed for approximately 10 minutes using a multi-tube vortexer. The 96-well plate was inverted and vortexed for another 10 minutes, followed by centrifuging for 5 minutes at approximately 4000 rpm.

Exactly 0.6 mL of acetonitrile was added to a separate Alpha Tube, which was then capped and set aside.

Exactly 0.15 mL of the supernatant was transferred into a glass micro tube, which was then capped. Exactly 0.3 mL of extraction solvent S1 was added into the weighed sample tube. The vial was capped and was vortexed on a multi-tube vortexer for approximately 10 minutes. The 96-well plate was inverted and vortexed for another 10 minutes, followed by centrifuging for 5 minutes at approximately 4000 rpm.

Exactly 0.13 mL of the first extract was transferred from the glass micro tube back to the original weighed sample Alpha Tube. The glass micro tube was quantitatively washed by adding 0.1 mL of acetonitrile, from the Alpha tube which had been set aside, then

transferring exactly 0.085 mL of the rinsate back to the weighed sample tube. The rinsing process was repeated with another 0.1 mL of acetonitrile. This brought the volume of liquid in the weighed sample tube to 0.75 mL. The samples were vortexed for 15 seconds, the 96-well plate was inverted and vortexed for another 15 seconds, then the samples were centrifuged for 5 minutes at 4000 rpm.

Exactly 0.6 mL of the supernatant was transferred into the Alpha Tube, which had been set aside and contained a remaining 0.4 mL of acetonitrile. The aliquots were vortexed for 15 seconds, and the procedure continued as outlined in Section 3.6.

3.4.2. Extraction of Sample Material [Quizalofop-p-ethyl, Quizalofop-p, and 3-OH-quizalofop-acid; 5 g Sample Size]

Approximately 1.5 g of Celite® was added to the pre-weighed sample, followed by exactly 15 mL of extraction solvent S1. The tube was capped and the sample was shaken horizontally for approximately 30 minutes using a mechanical shaker set at 300 rpm. The sample was then centrifuged for about 5 minutes at approximately 4000 rpm.

Exactly 29 mL of acetonitrile was added to a separate 50 mL glass centrifuge tube, which was then capped and set aside.

Exactly 7.5 mL of the supernatant was transferred into a culture tube, which was then capped. Exactly 15 mL of extraction solvent S1 was added into the weighed sample tube. The sample tube was capped and was horizontally shaken again on a mechanical shaker set at 300 rpm for approximately 30 minutes. The sample was then centrifuged for 5 minutes at approximately 4000 rpm.

Exactly 7 mL of the first extract was transferred from the culture tube back to the original weighed sample tube. The culture tube was quantitatively washed by adding 4.5 mL of acetonitrile, from the 50 mL glass centrifuge tube which had been set aside, then transferring exactly 4 mL of the rinsate back to the weighed sample tube. The rinsing process was repeated with another 4.5 mL of acetonitrile. This brought the volume of liquid in the weighed sample tube to 37.5 mL. The samples were manually shaken for 10 seconds, then centrifuged for 5 minutes at 4000 rpm.

Exactly 30 mL of the supernatant was transferred into the 50 mL glass centrifuge tube, which had been set aside and contained a remaining 20 mL of acetonitrile. The aliquots were vortexed for 10 seconds, and the procedure continued as outlined in Section 3.6.

3.5. Sample Clean-up

No Sample clean-up was necessary.

3.6. Preparation for Measurement

3.6.1. Preparation for Measurement [Quizalofop-p-ethyl, Quizalofop-p; 0.1 and 5 g Sample Size]

Exactly 0.1 mL of the extract was taken and was diluted with 0.7 mL of S2 for analysis on the LC-MS/MS.

3.6.2. Preparation for Measurement [3-OH-Quizalofop-acid; 0.1 and 5 g Sample Size]

Exactly 0.5 mL of the extract was taken and was diluted with 0.3 mL of water for analysis on the LC-MS/MS.

3.7. Influence of Matrix effects on Analysis

In order to test the influence of the matrix effects on the analysis, the response of each analyte in the matrix compared to pure standards was studied. Therefore, the average area response of the standards with and without matrix at three concentration level (1/2 LOQ, LOQ, and 2.5LOQ) was compared.

The matrix-matched standards and their counterparts were made using the procedure described in Section 2.4.4.

Three injections were made for each concentration. The difference in the average area was calculated with the following formula:

$$\%\ Area = \frac{\left| Area\ (solvent\ standards) - Area\ (matrix\ standards) \right|}{Area\ (solvent\ standards)} \times 100$$

4. Instrument Analysis

4.1. Instrumentation and Conditions

4.1.1. Instrumentation and Conditions for Quizalofop-p-ethyl and Quizalofop-p

	Parameter		
Chromatographic System	Waters UPLC Acquity system		
Analytical-column	Acquity UPLC BEH C18 1.7 µm 2.1 x 50 mm		
Column Temperature	50 °C		
Injection Volume	20 µL		
Mobile Phase A Mobile Phase B	4 mM Ammonium Formate with 0.1% Formic Acid with Water 4 mM Ammonium Formate with 0.1% Formic Acid with Methanol		
Flow Rate	500 µL/min		
Gradient (including wash and equilibration)	Time (min) 0.0 0.5 1.0 3.0 3.5 3.6 4.0	Phase A 95 95 50 5 5 95 95	Phase B 5 5 50 95 95 5 5
Detection System	AB SCIEX 5000 Mass Spectrometer		
Ionization	Electrospray (ESI)		
Ionization Temperature	500 °C		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
Quizalofop-p-ethyl	373→299* 375→301	positive	Approx. 2.6 min
Quizalofop-p	345→299* 345→100	positive	Approx. 2.3 min.

* Proposed as quantitation transition.

4.1.2. Instrumentation and Conditions for 3-OH-quizalofop-acid

Method A: Used as the primary chromatographic method

	Parameter		
Chromatographic System	Waters UPLC Acquity system		
Analytical-column	Acquity UPLC BEH Phenyl 1.7 µm 2.1x100 mm		
Column Temperature	50 °C		
Injection Volume	30 µL		
Mobile Phase A Mobile Phase B	4 mM Ammonium Formate with 0.1% Formic Acid with Water 4 mM Ammonium Formate with 0.1% Formic Acid with Methanol		
Flow Rate	500 µL/min		
Steps (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.0	95	5
	0.5	95	5
	3.0	5	95
	3.5	5	95
	3.6	95	5
	4.0	95	5
Detection System	AB SCIEX 5000 Mass Spectrometer		
Ionization	Electrospray (ESI)		
Ionization Temperature	550 °C		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
3-OH-Quizalofop-acid	359→166	negative	Approx. 3.2 min

Method B: Used as the secondary chromatographic method

	Parameter		
Chromatographic System	Waters UPLC Acquity sustem		
Analytical-column	Acquity UPLC HSS T3 1.8 µm 2.1x100mm		
Column Temperature	50 °C		
Injection Volume	30 µL		
Mobile Phase A Mobile Phase B	4 mM Ammonium Formate with 0.1% Formic Acid with Water 4 mM Ammonium Formate with 0.1% Formic Acid with Methanol		
Flow Rate	600 µL/min		
Steps (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.0	95	5
	0.5	95	5
	3.0	5	95
	3.5	5	95
	3.6	95	5
	4.0	95	5
Detection System	AB SCIEX 5000 Mass Spectrometer		
Ionization	Electrospray (ESI)		
Ionization Temperature	550 °C		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
3-OH-Quizalofop-acid	359→166	negative	Approx. 3.1 min

Note: Instruments with similar specifications may be substituted for 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 in 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 dependent of the equipment used and are therefore not listed. Those parameters may need to be adapted for the instrument used.

4.2. Calibration Procedures

Calculation of results was based on peak area measurements using a calibration curve. The calibration curve was obtained by direct injection of quizalofop-p-ethyl and its metabolites, quizalofop-p and 3-OH-quizalofop-acid, at six known concentration levels across the range of 0.01 ng/mL to 1.0 ng/mL. In all injection runs, the same injection volume was used for all samples and standards.

The residues of quizalofop-p-ethyl, quizalofop-p, and 3-OH-quizalofop-acid were evaluated by linear regression with 1/x weighting.

4.3. Rounding Numbers

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

4.4. Statistical Analysis of Data

Mean recoveries were calculated on the data generated where appropriate. Full computer/calculator precision was used in any intermediate calculations, and only the final value was rounded. Slight differences may be noted in hand calculations versus calculations in the individual data tables presented in this report due to rounding and significant figures presented in calibration curve data provided by the mass spectroscopy laboratory. Simple descriptive statistics were performed on the data (average and/or standard deviation), as considered appropriate. Statistical treatment of the data included simple descriptive statistics, such as determinations of averages for the procedural recoveries and area counts and calculation of the calibration curve and coefficient of variation (r) by linear regression of the instrument responses for the reference standards.

4.5. Calculation of Residues and Recoveries

The calculation of results was based on area measurements.

For the procedural recoveries, the sample weight was considered to be 5 g or 0.1 g in the final calculation of residues [mg/mk]. The methods required that the sample weight be 5 ± 0.1 g (or 0.1 ± 0.01 g) for fortification samples. The recovery was the percentage of the fortified amount (µg or ng), which was recovered through the method and the weight cancelled out, as shown in the equation below, during the final step of calculation.

The residues of quizalofop-p-ethyl and its metabolites in mg/kg are calculated using equations a and b:

a) $$\text{Concentration (ng/mL)} = \frac{\text{Response}-\text{Intercept}}{\text{Slope}} = C_A$$

Note: The concentration in ng/mL is automatically calculated by the analyst software using the formula above.

b) $$\text{Residue (mg/kg)} = \frac{V_{end} \times C_A}{G \times A_F \times 1000}$$

Where, 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
1000 = Factor remaining after unit conversions

c) $$\text{Recovery (\%)} = \frac{(\text{Residue in Fortified Sample})-(\text{Residue in Control Sample})}{\text{Amount fortified}} \times 100$$

Example: Quizalofop-p-ethyl (BAS 9152 H) in loamy sand soil fortified at LOQ (0.005 mg/kg), and quantitated at the primary mass transition (*m/z* 373→299) (Sample ID LOQ-1_RCN_R130088 in results file Validation_091514.rdb)

a) Calibration curve: $y = 394000x + 1210$

$$\text{Concentration (ng/mL)} = \frac{19438-1210}{394000} = 0.0463 \text{ ng/mL}$$

b) $$\text{Residue (mg/kg)} = \frac{(62.5 \text{ mL}) \times (0.0463 \text{ ng/mL})}{(5.0 \text{ g}) \times (0.125) \times 1000} = 0.00463 \text{ mg/kg}$$

c) $$\text{Recovery (\%)} = \frac{0.00463 \text{ mg/kg} - 0.000000 \text{ mg/kg}}{0.005 \text{ mg/kg}} \times 100 = 92.6\%$$

Note:	Slight rounding differences may be noted when using a hand calculator. Full computer/calculator precision was used in any intermediate calculations. Only the final value was rounded.

5.4. Summary of Method

Type of Method: LC-MS/MS

Test Systems:
1. Loamy Sand Soil (BASF Study 394796, RCN R130088)
2. Silt Loam Soil (BASF Study 437860, RCN R130034)

Analytes and Selected Mass Transitions (*m/z*):

Quizalofop-p-ethyl	373→299* 375→301
Quizalofop-p	345→299* 345→100
3-OH-quizalofop-acid	359→166**

*Used as the primary transition for quantitation
**The same mass transition was used with a primary and secondary chromatographic method

Analytical Procedure: The analytes were extracted from the sample using acetonitrile -6% Phosphoric Acid in water (80:20,v/v).

Confirmatory Technique: Due to the high selectivity and specificity of LC-MS/MS, an additional confirmatory technique is not necessary.

Method of Quantitation: The quantitation is based on the monitoring of two mass transitions for quizalofop-p-ethyl and quizalofop-p, and one mass transition and two chromatographic methods for 3-OH-quizalofop-acid. Recovery data was reported for each mass transition and chromatographic method considered, as shown in Appendix 6.

Limit of Detection (LOD): 0.001 mg/kg quizalofop-p-ethyl and quizalofop-p, and 0.0002 mg/kg for 3-OH-quizalofop-acid.

Limit of Quantitation (LOQ): 0.005 mg/kg for quizalofop-p-ethyl and quizalofop-p, 0.001 mg/kg of 3-OH-quizalofop-acid corresponding to a concentration of 0.05 ng/mL in the final extract.

Levels of Fortification: 0.005 mg/kg (LOQ) and 0.05 mg/kg (10×LOQ) for quizalofop-p-ethyl and quizalofop-p
0.001 mg/kg (LOQ) and 0.01 mg/kg (10×LOQ) for 3-OH-quizalofop-acid

Time Required: A set of 13 samples requires about 16 hours of work (LC-MS/MS determination and calculation of the results included)

9. Potential Problems

1. The glassware used for the method should be thoroughly rinsed with acetonitrile to prevent contamination.
2. Certain LC vials with silicon polymer may introduce interference peaks during LC-MS/MS analysis of Quizalofop-p-ethyl and its metabolites.
3. If matrix suppression or enhancement is observed, matrix-matched standards should be used.

10. Recommendation from Independent Laboratory Method Validation (ILV)

The independent laboratory validation of the BASF method (D1303/02) was successfully completed in the first trial for soil. Some minor method modifications/findings and clarifications were required for successful completion of the method validation and are described below.

1 The HPLC columns identified in the Section 2.3 of the reference method did not agree with the HPLC columns identified in Section 4.2. The sponsor provided clarification that the columns identified in Section 4.2 were the correct HPLC columns to be used for instrumental analysis.

2 The HPLC gradients identified in Section 4.2 included 0.4 minutes of equilibration time at the end of each gradient. During the ILV it was found that additional equilibration time at the end of the method improved calibration linearity for all monitored transitions. It is suggested from the ILV that language be added to the method to allow for additional equilibration time to compensate for available analytical equipment. The 3-OH-quizalofop-acid was measured with 2.0 additional minutes of equilibration time added to the end of each run.

3 The HPLC column identified for the determination of quizalofop-p-ethyl and quizalofop-p was an Acquity UPLC BEH C18 (1.7 µm, 2.1 × 50mm). During the ILV, it was found that the chromatography for quizalofop-p was poor with the 50 mm HPLC column. The quantitation transition chromatography showed a tailing interference peak, and the confirmation transition chromatography showed poor peak shape. The HPLC column used during the ILV was an Acquity UPLC BEH C18 (1.7 µm, 2.1 × 100 mm), and the additional theoretical plates from the longer column resolved the chromatographic issues observed for both transitions.

4 The chromatographic system and detection system identified in the reference method were different from the chromatograph system and detection system used during the ILV. The reference method was completed using a Waters UPLC Acquity System as the chromatographic system, but at the ILV facility an Agilent 1290 chromatographic system was used. Further, the reference method was completed using an AB Sciex 5500 Mass Spectrometer detection system, but at the ILV facility an AB Sciex 6500 Q-trap Mass Spectrometer detection system was used. The summary of the instrument parameters used in ILV are summarized in page 47 :

Instrumentation and Conditions

	Parameter		
Chromatographic System	Agilent 1290		
Analytical-column	Acquity BEH C18; 1.7 µm, 2.1 X 100 mm		
Column Temperature	50°C		
Injection Volume	Typically, 20 µL		
Mobile Phase A	4 mM Ammonium Formate with 0.1% Formic Acid in Water		
Mobile Phase B	4 mM Ammonium Formate with 0.1% Formic Acid in Methanol		
Flow Rate	600 µL/min		
Gradient	Time (min)	% Phase A	% Phase B
	0.0	95	5
	0.5	95	5
	1.0	50	50
	3.0	5	95
	3.5	5	95
	3.6	95	5
	4.0	95	5
Detection System	AB Sciex Instruments 6500 Q-Trap		
Software Version:	Analyst 1.6		
Analyte	**Transition (*m/z*)**	**Polarity**	**Expected Retention Time**
Quizalofop-p-ethyl	373.0→299.0* 375.0→300.9	Positive	Approx. 3.3 min
Quizalofop-p	345.0→299.0* 345.0→100.0	Positive	Approx. 2.9 min

11. Protocol Changes

No changes or adjustments were made to the protocol.

12. Data Retention and Archiving

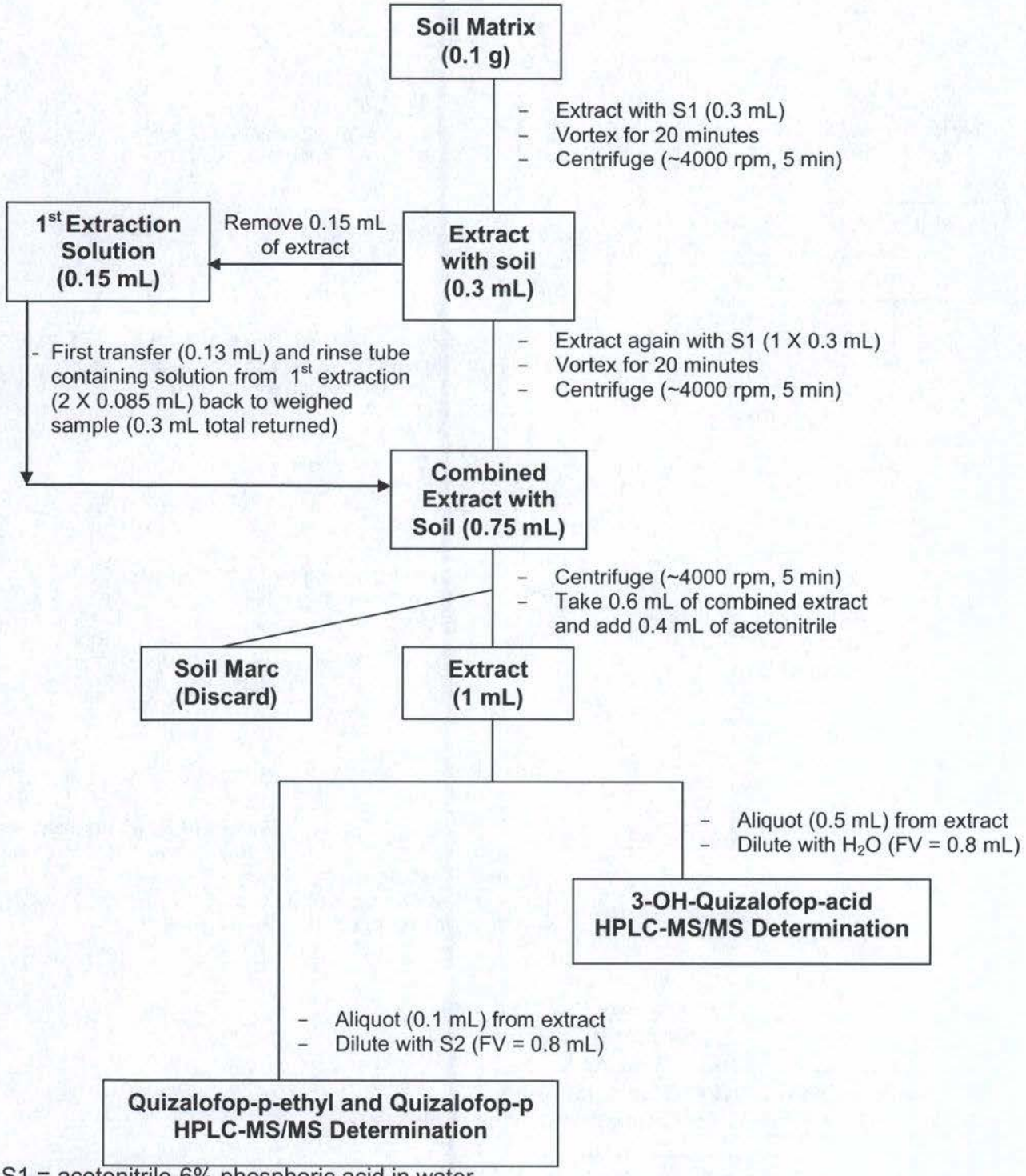
The raw data, analytical phase report, and all study related records pertaining to the analytical phase of the application verification samples will be archived at:

BASF Crop Protection
26 Davis Drive
Research Triangle Park, NC 27709

References

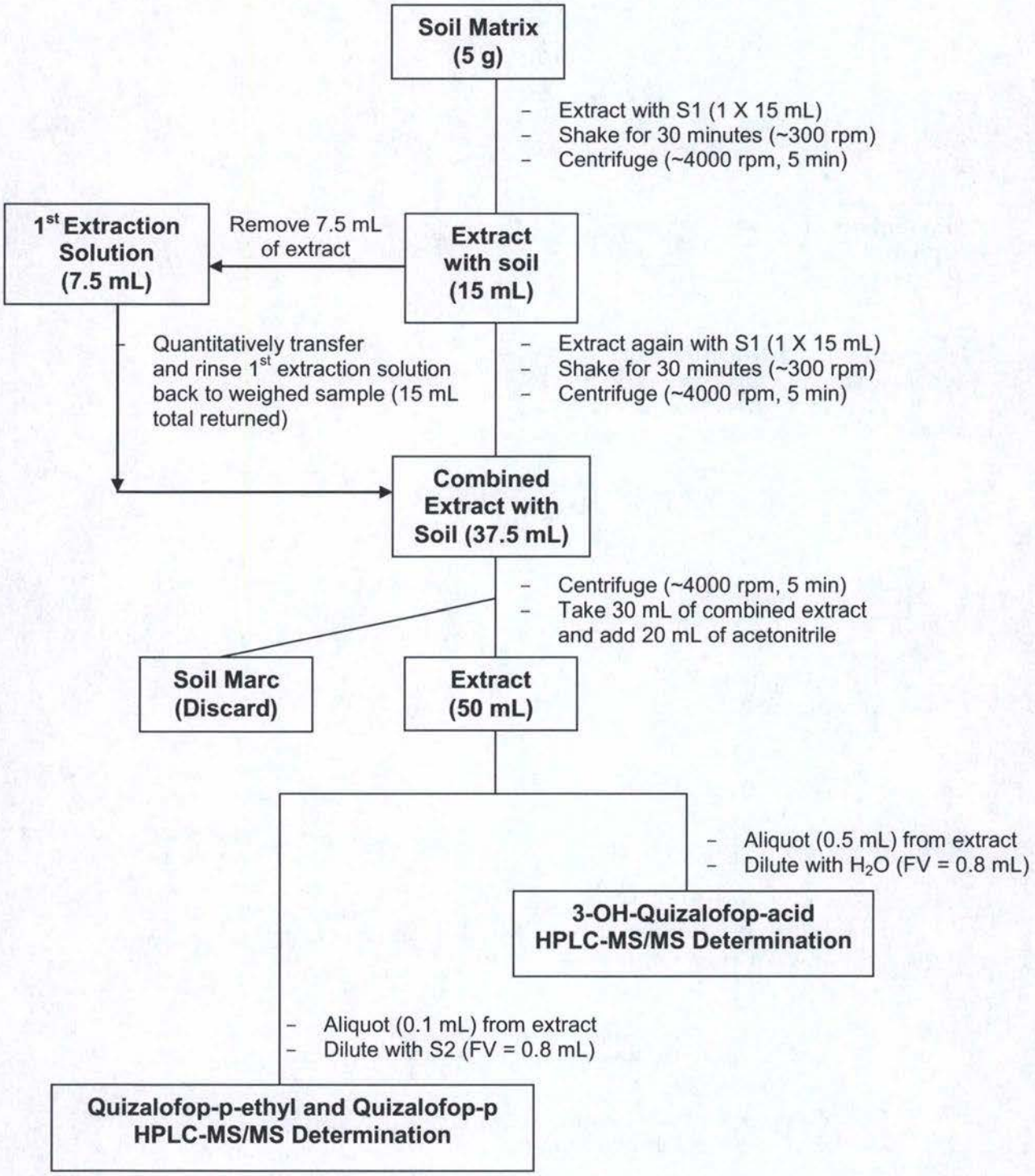
[1] Nejad, H. and Warren, R. (2015). Quizalofop-p-ethyl: Soil Extractability and Accountability Comparison Data (Soxhlet Versus Shaking) BASF Corporation, unpublished, BASF Reg. DOC ID. 2013/7002718

Figure 1 Analysis of Quizalofop-p-ethyl, Quizalofop-p, and 3-OH-quizalofop-acid in Soil, 0.1 g Samples



S1 = acetonitrile-6% phosphoric acid in water
S2 = Water-acetonitrile (10:90, v/v)

Figure 2 Analysis of Quizalofop-p-ethyl, Quizalofop-p and 3-OH-quizalofop-acid in Soil, 5 g Samples



S1 = acetonitrile-6% phosphoric acid in water
S2 = Water-acetonitrile (10:90, v/v)