1.2 Method Summary

Twenty (20) g sub-samples of soil are extracted two times by mechanical shaking (20 minutes) with acetonitrile:10mM ammonium acetate adjusted to pH 5 (50:50; v/v).[1] The extracts are combined upon centrifugation and the extraction volume adjusted to 50-mL. Aliquots are syringe filtered (0.2µm PTFE). Alternatively, sample clean-up is performed by solid-phase extraction (SPE) using Oasis MAX (6cc, 150mg) cartridges. Final determination is carried out by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS) using electrospray ionization techniques. The enantiomeric ratio determination is calculated by direct integration of individual enantiomers and normalized to 100 for the enatimeric pair. The SPE concentration and clean-up is typically required when significant disproportional distribution of individual enantiomers and/or insufficient instrumental sensitivity is encountered.

The limit of quantification of the method is 0.001 mg/kg for individual stereoisomer (0.001 ppm, 1.0 ppb).

[1] **Note:** The extraction practices applied in this method is the same as described in Method GRM044.03A, except a *p*H 5.0 buffer is used instead of *p*H 5.5 buffer to further improve chemical stability for fluazifop butyl during extraction.

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted.

2.2 Reagents

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in Appendix 2.

2.3 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves and laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area immediately.

2.3.1 Stock Solutions

Prepare individual 100 µg/mL stock solutions for fluazifop-butyl (PP9, R117009, CGA128175; racemic mixture), fluazifop (PP6, R115625; racemic mixture), fluazifop-P-butyl (PP5, R154875; *R*-isomer enriched), fluazifop-P (R156172; *R*-isomer enriched), R159618 (fluazifop-butyl; *S*- isomer enriched) and R159697 (fluazifop; *S*-isomer enriched) by one of the following methods.

Weigh out accurately, using a five figure balance, sufficient individual analytical standards into a "Class A" volumetric flask (100 mL; amber preferred). Dilute to the mark with acetonitrile to give a concentration of 100 µg/mL stock solutions of PP9 (R117009, CGA128175; racemic mixture), PP6 (R115625; racemic mixture), PP5 (R154875; *R*-isomer enriched), R156172 (*R*-isomer enriched), R159618 (*S*- isomer enriched) and R159697 (*S*-isomer enriched). Note that the amount weighed for each compound should be corrected for its respective % purity and % enantiomeric purity (if enantiomerically enriched).

Alternatively, the appropriate volume of acetonitrile to add to a known amount of standard material may be determined using the equation below. The standard concentration is corrected for its chemical purity.

$$V = \frac{W \times P}{C} \times 1000$$

P = Standard purity in decimal form (P(%)/100)
V = Volume of acetonitrile required
W = Weight, in mg, of the solid analytical standard
C = Desired concentration of the final solution, (µg/mL)
1000 = Unit conversion factor

In this case, the standard material is weighed (or accurately measured if in solution form) directly into an appropriate storage vessel.

2.3.2 Fortification Solutions

Sample fortification solutions containing individual analyte (PP9 or PP6) should be prepared by serial dilution in acetonitrile from stock solutions. It is recommended that the following solutions are prepared: 10.0 µg/mL, 1.0 µg/mL and 0.2 µg/mL. Mixed standards of fluazifop and fluazifop-butyl **should be avoided** due to possible chemical instability of fluazifop-butyl during extraction practices.

By using this fortification standard preparation, *e.g.*, a 20 g soil sample fortified at 1.0 ppb (individual enatiomer) can be accomplished by fortifying 200 µL of 0.2 µg/mL (*i.e.* ng/µL) PP9 (or PP6) fortification solution into the soil sample.

For verification purposes, if required, specific configuration (*R/S*) ratio of fortification solutions can be prepared by proper mixing of individual stock solutions. For example, mixing of equal volume of PP5 (R/S = 98/2) and PP9 (R/S = 50/50; racemic) at 10.0 µg/mL will produce a fortification solution consisting of R/S ratio of 74/26 at 10.0 µg/mL.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

Due to possible degradation of the fluazifop-butyl (enantiomeric enriched or racemic), this analyte is prepared as a separate calibration standard set from fluazifop (enantiomeric enriched or racemic). The working calibration standards are prepared individually from the 100 µg/mL stock standards by transferring 10.0 mL of the appropriate stock solutions into a 100-mL volumetric flask and diluting to the mark with acetonitrile resulting in intermediate working standards at 10 µg/mL concentration levels in acetonitrile. Similarly, further dilution of these working standards, individually, with acetonitrile:10 mM ammonium acetate pH 5 (30:70; v/v) results in intermediate working standards at 1.0 µg/mL concentration levels. The two sets of LC-MS/MS calibration standards (PP9 and PP6) are prepared by serial dilutions of the intermediate working standards at 1.0 µg/mL concentration levels in acetonitrile:10mM ammonium acetate pH 5 (30:70; v/v). A minimum of five concentration levels, at a recommended range from 0.10 to 20 pg/µL (equivalent to 0.05 to 10 pg/µL of individual enantiomers) should be prepared for LC-MS/MS quantification via external standard calibrations.

A calibration curve should be generated to quantify fluazifop-butyl (R and S) and fluazifop (R and S) residues. Standards covering an appropriate concentration range should be prepared as described above, using the requisite volumes of PP9 (R117009) and PP6 (R115625) in acetonitrile:10 mM ammonium acetate pH 5 (30:70; v/v). The following concentration levels of standards were prepared for calibration using the instrumentation found in Section 4: 0.10 pg/µL, 0.20 pg/µL, 0.50 pg/µL, 1.0 pg/µL, 2.0 pg/µL, 5.0 pg/µL, 10 pg/µL and 20 pg/µL.

Typical chromatograms from LC-MS/MS analysis of the standard solutions are shown in Figure 2; indicating ms/ms transitions of *m/z* (326.1 → 254.1) and *m/z* (326.1 → 226.1) for fluazifop and *m/z* (384.1 → 282.1) and *m/z* (384.1 → 328.1) for fluazifop-butyl.

No significant matrix effects, suppression or enhancement of the instrument response for PP9 and PP6 have been observed in the soil samples tested using the procedures described in Section 3 during method development and non-matrix calibration standards should normally be used for quantitation.

Any matrix effects observed may be compensated for by use of matrix-matched standards at the discretion of the study director, or by further dilution of the final sample with acetonitrile:10 mM ammonium acetate pH 5 (30:70; v/v) should instrument sensitivity permit.

2.3.4 Standard Solution Storage and Expiration

All stock solutions, calibration standards and fortification standards should be stored in amber glass bottles under refrigeration conditions (approximately 4°C) when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration of six months for stock solutions (enantiomeric enriched or racemic) in acetonitrile is recommended unless additional data are generated to support a longer expiration period.

For the buffer stabilized fluazifop-butyl (enantiomeric enriched or racemic) solutions, an expiration of one month for calibration and for fortification standards is recommended. For the buffer stabilized fluazifop (enantiomeric enriched or racemic) solutions, an expiration of three months for calibration standards and fortification standards is recommended. The expiration of calibration and fortification standards should not be extended unless additional study data are generated that show a longer expiration date is appropriate.

2.4 Safety Precautions and Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate MSDS or a monograph such as 'Hazards in the Chemical Laboratory', edited by S. G. Luxon, The Chemical Society, London (Reference 1).

Solvent and Reagent Hazards

	Acetonitrile	Methanol	Formic acid	Conc. NH_3
Harmful Vapour	✓	✓	✓	✓
Highly Flammable	✓	✓	✗	✓
Harmful by Skin Absorption	✓	✓	✓	✓
Irritant to respiratory system and eyes	✓	✓	✓	✓
Causes severe burns	✗	✗	✓	✓
OES Short Term (mg/m^3)	105	310	N/A	24*
OES Long Term (mg/m^3)	70	260	9	17*

* Based on NH_3
N/A: not known

Suitable personal protective equipment should be worn when handling chemicals and reagents. The appropriate MSDS should be consulted for each reagent and a local risk assessment should be carried out. In all cases avoid breathing vapour. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

A summary of the method is included in flow-chart form in Appendix 4.

3.1 Sample Preparation

All samples should be prepared using an approved method of preparation to obtain a homogeneous sample prior to analysis.

3.2 Sample Fortification

In order to verify method performance and allow recovery corrections to be made (if appropriate), fortified control samples should be included with each sample set. To each pre-weighed control soil sample, add the appropriate amount of standard solution containing analytes of interest (fluazifop-butyl and fluazifop) in acetonitrile. Let each sample stand for at least five minutes after fortification to allow the spiking solution to soak into the matrix before proceeding with the extraction procedure. At least one untreated control and two fortified control samples should be analysed with each sample set to verify method performance.

3.3 Extraction

A summary of the method is included in flow-chart form in Appendix 4.

a) Weigh representative amounts of soil (20 g) into 50-mL polypropylene centrifuge tubes. No more than 1.0 mL of fortification solution should be added. Allow fortified control samples to equilibrate for at least 5 minutes before proceeding to the extraction.

Note: Due to the chemical instability of PP9 (or PP5) in soil at room temperature, the PP9 (or PP5) fortified recovery samples should be extracted within 20 minutes after fortification to obtain meaningful procedural recoveries.

b) Add 30 mL of acetonitrile:10 mM ammonium acetate pH 5 (50:50; v/v) into the centrifuge tube, cap and shake on a mechanical shaker at a speed that visibly agitates the samples for 20 minutes. ***Tubes should be placed in a horizontal orientation.***

c) Centrifuge samples at approximately 5000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for about 10 minutes.

d) Transfer supernatant to a clean 50-mL centrifuge tube.

Note: With some soils, particularly those with high clay content, the solution may remain visibly cloudy after centrifugation. This is normal and will not affect results.

e) Repeat extraction using another 20 mL of acetonitrile:10 mM ammonium acetate pH 5 (50:50; v/v) for the remaining solid (soil) in the centrifuge tube from the first extraction. Cap and shake by hand or vortex to dislodge and disrupt the remaining pellet. If shaking is insufficinet use a suitable implement (*e.g.*, a spatula) to facilitate this process. Shake on a mechanical shaker at a speed that visibly agitates the samples for 20 minutes. ***Tubes should be placed in a horizontal orientation.***

f) Centrifuge samples at 5000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for about 10 minutes.

g) Transfer and combine the supernatant into the corresponding 50-mL centrifuge tube containing the first extract.

h) Adjust the final volume of the combined supernatants to 50-mL with 10mM ammonium acetate pH 5 (Buffer A) and mix well with vigorous shaking or vortexing.

i) Filter approximately 1-mL of resulting extract through a 0.2 µm PTFE syringe filter (13mm; see Appendix 1) into a 1-mL glass vial.

j) Transfer a 500-µL aliquot of the filtered extract into an HPLC vial containing 500-µL of 10 mM ammonium acetate pH 5 (Buffer A). This results in a two times (2x) dilution of the final extract.

Note: The two times (2x) dilution rate is required to maintain proper chromatographic retention of analytes (Specifically fluazifop) and minimizing possible interferences from the matrices and labware observed for fluazifop-butyl.

Higher dilution ratio and re-analysis may be required when (1) samples contain residues greater than 100 ppb and/or (2) earlier analytical runs indicated possible interferences which may affect residue determinations. In these cases, acetonitrile:10 mM ammonium acetate adjusted to pH 5 (50:50; v/v) is recommended for further dilution.

k) Vortex to mix, sample is ready for final determination by LC-MS/MS.

3.4 Solid Phase Extraction Procedure.

A solid phase extraction procedure may be necessary where there is insufficient sensitivity on the instrument to accurately determine enatiomeric ratio or quantify residues at the LOQ level by dilution & injection approach, or where matrix effects are significant.

The sample cleanup and concentration are accomplished by the use of Waters Oasis® MAX solid phase extraction (150 mg, 6-mL) cartridge. In general, avoid cartridges drying during the process unless specified otherwise. Allow one solvent to flow through the SPE (no liquid layer on top of bed) before adding the next solvent. The flow rate should be kept at a rate of less than 20 drops per minute (approximately 1 mL/min). Flow efficiency can be improved by controlled vacuum on the SPE extraction box or controlled positive pressure on the SPE cartridge, however, **gravity flow** is highly recommended for the sample loading and final elution. The SPE procedures are described as below:

a) Condition the SPE cartridges as follows:
   1. Methanol; 3-mL, two times.
   2. Acetonitrile; 3-mL, two times.
   3. Methanol; 3-mL, two times.
   4. 0.01% NH_4OH in water (v/v); 3-mL, two times.
   5. Water; 3-mL, two times.

b) Transfer 5.0 mL of the final soil extract from Section 3.3(h) into a 15-ml polypropylene centrifuge tube followed by addition of 5.0 mL 10 mM ammonium actetae pH 5 (Buffer A) and mix well.

c) Load the resulting diluted soil extract onto the SPE cartridge; portion-wise and quantitatively. Slight positive pressure or vacuum may be applied if needed; however, the flow rate should be less than 20 drops per minutes (approximately 1 mL/min). Discard the eluents.

d) Wash the cartridge as follows and discard the washes.
   1. Rinse the polypropylene tube with 2.0 mL of 0.1% formic acid in methanol:ultra-pure water (20:80; v/v) and transfer rinsate to the SPE cartridge.
   2. Rinse the polypropylene tube with 3 mL ultra-pure water and transfer rinsate to the SPE cartridge, repeat with additional 3 mL ultra-pure water.

e) Elute the cartridge with acidified (1% formic acid) acetonitrile:acetone (25:75; v/v) using 2-mL repeated three times (total of 6-mL), and collect into a clean 15-mL polypropylene centrifuge tube.

f) Evaporate the eluent to dryness under a gentle stream of nitrogen or air at a bath temperature of approximately 40°C.

g) Add 0.3-mL acetonitrile to the centrifuge tube and vortex to rinse the sides of the tube.

h) Dilute to a final volume of 1-mL with 10 mM ammonium acetate pH 5 (Buffer A) or to an appropriate final volume with acetonitrile:10 mM ammonium acetate adjusted to pH 5 (30:70; v/v). for high concentration residue samples. Dilution solvent should be chosen to maintain an approximate acetonitrile:10 mM ammonium acetate adjusted to pH 5 (30:70; v/v). ratio when added to the acetonitrile from 3.4 (g).

i) Vortex and transfer into suitable autosampler vial for LC-MS/MS analysis.

Note: If analyte concentration is expected to be high, further dilution with acetonitrile:10 mM ammonium acetate adjusted to pH 5 (30:70; v/v) may be necessary prior to LC-MS/MS analysis for residue determination.

3.5 Experimental Precautions

a) The SPE procedure has been developed using cartridges from the stated manufacturer. Similar cartridges from other manufacturers may be used. In all cases however, it is strongly recommended that the elution profile of the chosen batch of cartridges is checked prior to commencing analysis to assess any variation in manufacturers' products and between batches.

b) Bottled HPLC grade ultra pure water is used to prepare the LC mobile phase, which produces a lower background noise in the MS/MS chromatograms than water taken from a laboratory water purification system.

c) To prevent contamination of the instrument and to minimize possible carry-over issues, it is recommended that high level recoveries (> 0.1 mg/kg) and samples with expected residues greater than 0.1 mg/kg should be diluted so that the final individual analyte concentration does not exceed 0.010 µg/mL. It may also be useful to include blank injections of acetonitrile:ultra pure water (30:70; v/v) after high level samples to clear any observed carry-over greater than 10% of the LOQ.

3.6 Time Required for Analysis

One skilled analyst can typically complete the sample preparation of a set of 23 samples within approximately 8 working hours with dilute-and-shoot procedures. The analytical sequence was typically performed overnight on a LC-MS/MS system.

3.7 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks unless otherwise specified in the analytical procedure. Acceptable method recoveries will

validate any work flow interruptions. Samples should be stored refrigerated in sealed containers where the analysis cannot be completed in a single day

4.0 FINAL DETERMINATION

An integrated Thermo Electron TSQ Quantum Ultra mass spectrometer was used to establish the method. The system is controlled and data is processed by Thermo Electron Xcalibur™ Software. Other instruments may also be used, however optimization may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum instrument operation.

Following are the typical instrumental parameters applied for this method during method validation using a Thermo Electron TSQ Quantum Ultra mass spectrometer. The analyst should make necessary adjustments and tuning to these parameters to obtain optimum operational conditions based on the actual instrument used for the specific study.

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

4.1 Instrument Description

HPLC System	:	Surveyor Plus LC System – A quaternary solvent system equip with MS Pump Plus
Autosampler	:	Surveyor MS Plus
Detector	:	Thermo Electron TSQ Quantum Ultra mass spectrometer with Xcalibur™ Software
Collision Gas	:	Purified Argon in compressed cylinder
Gas Supply	:	House Nitrogen supply

4.2 Chromatography Conditions

Column	:	Chiralpak AS-RH, 4.6 x 150 mm, 5.0 µm (Chiral Technologies Part No. 20724)
Column Oven Temperature	:	25°C
Injection volume	:	50 µL
Stop Time	:	13 minutes
Injection protocol	:	Analyze calibration standard after 4 to 5 sample injections
Sample Tray Temperature	:	15°C
Mobile phase	:	Solvent A: 0.1% formic acid in Optima grade water Solvent B: HPLC grade 2-propanol Solvent C: 0.1% formic acid in HPLC grade Acetonitrile

Mobile Phase Composition

Time (min)	%A	%B	%C	Flow Rate (µL/min)
0.0	50	5	45	600
3.0	50	5	45	600
4.0	35	5	60	600
12.0	35	5	60	600
12.1	50	5	45	600
13.0	50	5	45	600

The typical retention times for the analytes are listed in Section 4.3 when using this instrumentation and conditions. The retention time may vary depending upon chromatographic conditions and systems.

Note: To help minimizing instrument contamination, a timed event controlled switching valve may be used to divert the LC stream to waste during periods of no data collection.

4.3 Mass Spectrometer Conditions

Ion Source Parameters (HESI-II Probe):

	Negative Mode	Positive Mode
Spray Voltage (V)	2500	3200
Vaporization Temperature (°C)	300	300
Sheath Gas Pressure (psi)	30	30
Ion Sweep Gas Pressure (psi)	3.0	3.0
Aux Gas Pressure (psi)	10	10
Capillary Temperature (°C)	300	300
Tube Lens Offset	tuned value(s)	tuned value(s)
Skimmer Offset (V)	0	0
Collision Pressure (mTorr)	1.0	1.0

Note: The mass spectrometer tuning parameters shown here are for reference only. The analyst should always consult with instrument operation manual to obtain optimum conditions for all the analytes prior to residue analysis.

MRM (SRM) Operating Parameters:

MS/MS Transitions

Analyte	MS/MS Transition*	Scan Width	Dwell (sec.)	CE (Volts)	Q1 PW	Q3 PW	RT (min.)
Fluazifop (*S-isomer*; R159697)	Negative mode						
Primary	326.10 → 254.10	0.01	0.05	16	0.7	0.7	5.8
Confirmatory	326.10 → 226.10	0.01	0.05	25	0.7	0.7	5.8
Fluazifop (*R-isomer*; R156172)	Negative mode						
Primary	326.10 → 254.10	0.01	0.05	16	0.7	0.7	6.5
Confirmatory	326.10 → 226.10	0.01	0.05	25	0.7	0.7	6.5
Fluazifop-Butyl (*S-isomer*; R159618)	Positive mode						
Primary	384.10 → 282.10	0.01	0.05	22	0.7	0.7	10.7
Confirmatory	384.10 → 328.10	0.01	0.05	18	0.7	0.7	10.7
Fluazifop-Butyl (*R-isomer*; PP5)	Positive mode						
Primary	384.10 → 282.10	0.01	0.05	22	0.7	0.7	10.7
Confirmatory	384.10 → 328.10	0.01	0.05	18	0.7	0.7	10.7

Data collection windows:
4.5 – 8.5 minutes is in negative mode and 8.5 – 13.0 minutes is in positive mode.

* The specified mass difference of 0.001 amu for precursor ions in quantification and confirmation detections is required for channel separation of signals on the Thermo Electron TSQ Quantum Ultra mass spectrometer with Xcalibur™ software. The MS/MS transitions listed were the most sensitive and stable transitions for the corresponding analytes based on the optimal tuning parameters obtained prior to method validation with Thermo Electron TSQ Quantum Ultra instrument. Alternative MS/MS transitions may be used if different comparable instrument is applied or encounter interferences. Analysts should consult with instrument operation manuals for the specifics and adjustments when using instruments from different manufacturers to obtain optimum results.

4.4 Confirmatory Procedures

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

5.0 CALCULATION OF RESULTS

5.1 Multi point Calibration Procedure

Residues of analytes may be calculated in µg/kg (*i.e* ppb) for each sample as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (for example, 30% LOQ to 20x LOQ). An appropriate number of different concentrations within this range should be prepared (at least five).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to the analytes of interest. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of five injections of sample solutions

c) Generate calibration curve parameters using an appropriate regression package.

d) The following equation can be rearranged and used to calculate residues as follows:

$$y = mx + c$$

Where y is the instrument response value, x is the standard concentration, m is the gradient of the line of best fit (or "X-variable 1" in MS Excel) and c is the intercept value. An example of this equation generated using the experimental values of *m* and *c* should be included in the raw data, as should the "R-Squared" (R^2) value for the regression.

Re-arrangement for *x* gives

$$x = \frac{y - c}{m}$$

e) Calculate the residue for the analyte of interst in the sample, expressed as µg/kg, as follows

$$\text{Residue}\ (\mu\text{g/kg}) = \frac{\text{Analyte found}\ (\mu\text{g/mL})}{\text{Sample conc.}\ (\text{g/mL})} \times \frac{1{,}000\ g}{1\ kg}$$

Where analyte found (µg/mL) is calculated from the standard calibration curve and sample conc. is the final sample concentration in *g/mL*.

If residues need to be corrected for average percentage recovery; *e.g.* for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}}\ (\mu\text{g/kg})$$

Single point calibration is generally not recommended. It is recommended that a calibration curve is generated with each analytical run to demonstrate the linearity of instrument response (Reference 2).

5.2 Enantiomeric Ratio Determination by Residue Results

The enatiomeric ratio can be reasonably determined from quantification results of **individual enantiomers** at a minimum concentration of 0.1 pg/µL (*i.e.* 5.0 pg on-column). However, it is generally advised that the enantiomer ratio should be determined at higher concentration levels to ensure accuracy, particularly when high disproportion enantiomer ratio is expected. For example, a sample concentration at 2.0 pg/µL would allow to determine enatiomeric ratio of 95/5 (R/S or S/R) with reasonable accuracy.

To determine the enatiomeric ratio for fluazifop-butyl (*e.g.* using amount found in picograms):

$$\textit{Total}_{\text{fluazifop-butyl}} = \textit{S}_{\text{fluazifop-butyl}} + \textit{R}_{\text{fluazifop-butyl}}$$

$$\textit{S : R} = (\textit{S}_{\text{fluazifop-butyl}} / \textit{Total}_{\text{fluazifop-butyl}}) \times 100 : (\textit{R}_{\text{fluazifop-butyl}} / \textit{Total}_{\text{fluazifop-butyl}}) \times 100$$

To determine the enatiomeric ratio for fluazifop (*e.g.* using amount found in picograms):

$$\textit{Total}_{\text{fluazifop}} = \textit{S}_{\text{fluazifop}} + \textit{R}_{\text{fluazifop}}$$

$$\textit{S : R} = (\textit{S}_{\text{fluazifop}} / \textit{Total}_{\text{fluazifop}}) \times 100 : (\textit{R}_{\text{fluazifop}} / \textit{Total}_{\text{fluazifop}}) \times 100$$

5.3 Enantiomeric Ratio Determination by Peak Area

Alternatively, the enantiomeric ratio can be determined with reasonable accuracy by peak area ratio of a corresponding pair of enantiomers at a mimimum **individual enantiomeric concentration** within the range from 0.2 pg/µL to 10 pg/µL when relative response factors of enantiomer pairs have been determined. The relative response factors of the enantiomeric pairs can be estimated by averaging area ratio of S/R isomers from 5 consecutive analyses at analyte concentrations of 2.0 pg/µL using PP9 and PP6 (S:R ratio of 1:1) as reference standards within the same analytical set. The samples are to be bracketed with the reference standard within the analytical set.

Relative response factor determination (fluazifop-butyl or fluazifop) from racemic reference standards at recommended concentrations can be estimated by following equation based on *R*-isomer (or *S*-isomer) as reference (i.e. $RF_{\text{R-isomer}} = 1.000$ or *vice versa*):

$$\textit{RF}_{\text{S-isomer}} = \textit{S-isomer}_{\text{average area count}} / \textit{R-isomer}_{\text{average area count}}$$

Note: ***RF*** values are recommended to rounded to third decimal places for accuracy considerations

Therefore,

Enantiomeric ratio for fluazifop-butyl:

$$\textit{S/R} = ((\textit{Area}_{\textit{S}\text{-fluazifop-butyl}} / \textit{Total Area}_{\text{fluazifop-butyl}}) / \textit{RF}_{\textit{S}\text{-fluazifop-butyl}}) / ((\textit{Area}_{\textit{R}\text{-fluazifop-butyl}} / \textit{Total Area}_{\text{fluazifop-butyl}}) / \textit{RF}_{\textit{R}\text{-fluazifop-butyl}})$$

Where, $\textit{Total Area}_{\text{fluazifop-butyl}} = (\textit{Area}_{\textit{S}\text{-fluazifop-butyl}}) + (\textit{Area}_{\textit{R}\text{-fluazifop-butyl}})$

$$\textit{S : R} = (\textit{S} / (\textit{S}+\textit{R})) \times 100 : (\textit{R} / (\textit{S}+\textit{R})) \times 100$$

Enantiomeric ratio for fluazifop:

$$S/R = ((Area_{S\text{-fluazifop}} / Total\ Area_{fluazifop}) / RF_{S\text{-fluazifop}}) / ((Area_{R\text{-fluazifop}} / Total\ Area_{fluazifop}) / RF_{R\text{-fluazifop}})$$

Where, $Total\ Area_{fluazifop} = (Area_{S\text{-fluazifop}}) + (Area_{R\text{-fluazifop}})$

$$S:R = (S / (S+R)) \times 100 : (R / (S+R)) \times 100$$

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analyzed with each set of samples to verify that the sample used to prepare recovery samples is free from contamination. A minimum of one control should be analysed with each batch of samples

At least two recovery samples (control samples accurately fortified with known amounts fluazifop-butyl and fluaziop in acetonitrile) should also be analysed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found. The fortification levels should be appropriate to the residue levels expected.

Recovery efficiency is generally considered acceptable when the mean values are between 70% and 120% and with a relative standard deviation of $\leq 20\%$.

Where the method is used for monitoring purposes, control and recovery samples are not required where suitable control samples are not available.

7.0 SPECIFICITY

It is recommended that reagent blank samples be included in a sample set if contamination is suspected.

7.1 Matrix

LC-MS/MS is a highly specific detection technique. No significant matrix effects were observed in the soil types tested during method development/validation and non-matrix standards should generally be used for quantification.

Any matrix effects observed may be compensated for by use of matrix matched standards at the discretion of the study director, or by dilution of the final sample with acetonitrile:10 mM ammonium acetate pH 5 (30:70; v/v) should instrument sensitivity permit.

7.2 Reagent and Solvent Interference

Using high purity solvents and reagents no interference has been found.

7.3 Labware Interference

This method uses mainly disposable labware. All reusable glassware should be detergent washed and then rinsed with HPLC grade methanol, acetone or acetonitrile prior to use.

APPENDIX 3 LC-MS/MS Tuning Procedure

Calibration of Instrument

The instrument must be mass calibrated on a regular basis using polytyrosine-1,3,6 solutions according to the manufacturer's instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

Tuning Instrument for Fluazifop-butyl (PP9) and Fluazifop (PP6)

Infuse a standard solutions of fluazifop-butyl (0.1 to 1.0 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 5-20 µL/min. Roughly adjust interface parameters (sprayer position and temperature, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high parent ion signal at *m/z* 384.1 for fluazifop-butyl in positive ionization mode.

Similarily, infuse a standard solutions of fluazifop (0.1 to 1.0 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 5-20 µL/min. Roughly adjust interface parameters (sprayer position and temperature, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high parent ion signal at *m/z* 326.1 for fluazifop in negative ionization mode.

Using the Xcalibur™ Software optimization routine to tune the instrument for fluazifop-butyl and fluazifop, individual, and ensuring that the correct ion is selected. If desired, manual tuning of the ion optics and collision energy can be carried out to ensure maximum sensitivity.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injection of standards using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position and temperature, spray and heater gas flows, spray, orifice, and focusing ring voltages and the collision gas pressure) for maximum sensitivity.

For fluazifop-butyl, in positive ionization mode, the protonated molecular ion generated in the ion source (*m/z* 384.1) is selected and subjected to further fragmentation by collision induced fragmentation. The two most sensitive product ions (*m/z* 282.1 and *m/z* 328.1) are selected and used for quantitative and confirmative analysis.

For fluazifop, in negative ionization mode, the de-protonated molecular ion generated in the ion source (*m/z* 326.1) is selected and subjected to further fragmentation by collision induced fragmentation. The two most sensitive product ions (*m/z* 254.1 and *m/z* 226.1) are selected and used for quantitative and confirmative analysis.

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

The enatiomeric enriched analytes can be tuned in the same manner.

APPENDIX 4 Method Flow Chart