1.0 INTRODUCTION

1.1 Scope of the Study

The purpose of this study was to validate ADPEN analytical method M1408 for the detection and quantification of residues of prosulfuron and its metabolites in soil and water by LC-MS/MS. The study was conducted in accordance with EPA Guideline OCSPP 850.6100 and SANCO/825/00 rev.8.1

The method was validated at two fortification levels for water, LOQ (0.01 ppb) and 10x LOQ (0.1 ppb) and two fortification levels for soil, LOQ (0.1 ppb) and 100x LOQ (10 ppb). Five replicates were analyzed at each fortification level. Additionally, a method blank and two replicates of unfortified samples were analyzed. For each analyte, two mass transitions were evaluated, with the exception of CGA150829, which only has one mass transition.

Matrix-matched and solvent-matched standard solutions were also analyzed within the study to verify possible matrix effects.

1.2 Principle of the Method

For residues analysis of prosulfuron and its metabolites water samples are shaked well and filtered with a 0.2 µm syringe filter into an autosampler vial prior to LC-MS/MS analysis. For soil sample preparation 10.0 g of soil is extracted using 25 mL of buffer solution (85:15, MeOH:water + 10mM sodium phosphate). The samples are shaken, centrifuged, concentrated, reconstituted and filtered prior to LC-MS/MS anlaysis.

1.3 Specificity

Specificity was demonstrated by comparison of the sample chromatograms with the chromatograms of standard solution samples using a highly specific MS/MS method. The retention time of the analytes, prosulfuron, CGA300406, CGA-159902 and CGA-150829, in sample extracts matched those of the standard solutions.

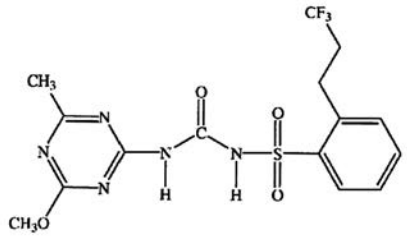
2.0 MATERIALS AND METHODS

2.1 Test systems

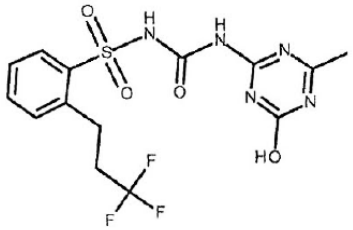
Soil and water were the test systems considered in this validation study.

2.2 Test and Reference Items

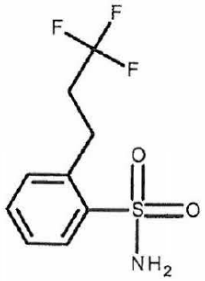
2.2.1 Prosulfuron

Common Name:	Prosulfuron
Product Code:	CGA152005
CA Index Name:	Benzenesulfonamide, N-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]-2-(3,3,3-trifluoropropyl)-
IUPAC Name:	1-(4-methoxy-6-methyl-triazin-2-yl)-3-[2-(3, 3, 3-trifluoropropyl}-phenylsulfonyl]-urea
CAS No.:	94125-34-5
Batch/Lot Identification:	731405
Molecular Formula:	$C_{15}H_{16}F_3N_5O_4S$
Molecular Weight:	419.4 g/mol
Purity:	99.0%
Expiration Date:	End of Febraury 2019
Storage:	< 30 °C
Source:	Syngenta Crop Protection, LLC., Greensboro, NC
Structural Formula:	

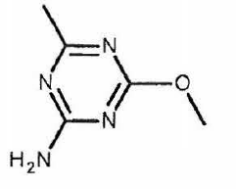
2.2.2 CGA300406

Compound Code:	CGA300406
Batch/Lot Identification:	NEH-XV-88
Purity:	96.9%
Expiration Date:	June 30, 2016
Storage:	Refrigerator
Source:	Syngenta Crop Protection, LLC., Greensboro, NC
Structural Formula:	

2.2.3 CGA159902

Compound Code:	CGA159902
Batch/Lot Identification:	BPM-XII-17
Purity:	98.3%
Expiration Date:	June 30, 2016
Storage:	Refrigerator
Source:	Syngenta Crop Protection, LLC., Greensboro, NC
Structural Formula:	

2.2.4 CGA150829

Compound Code:	CGA150829
Batch/Lot Identification:	CDC-III-91
Purity:	99.3%
Expiration Date:	June 30, 2016
Storage:	Refrigerator
Source:	Syngenta Crop Protection, LLC., Greensboro, NC
Structural Formula:	

All specifications given on the certificate of analysis, provided by the sponsor/supplier, are essential for correct identification of the reference items for use under GLP. They have not been verified by the test facility and no claim of GLP compliance will be made for these data, except where this is explicitly claimed on the certificate of analysis. Reference items were supplied by Syngenta Crop Protection, LLC., Greensboro, NC.

2.3 Materials and Methods

2.3.1 Equipment

Equipment	Size, Description	Manufacturer
Balance, Analytical	Model XP26	Mettler Toledo
Beaker	Various Sizes	Pyrex, Kimax or equivalent
Bottle, Amber Glass	10 to 50 mL with Teflon®-lined screw cap	Qorpak
Centrifuge	Capable of Achieving 3700 G	Acuspin 3R, Fisher Scientific
Centrifuge Tube, PP	15 mL	BD, Falcon, VWR or equivalent
	50 mL	
Cylinder, Graduated	10 – 1000 mL	Various
Vials, Analytical	2 mL	Agilent or equivalent
UPLC	1290	Agilent
Mass Spectrometer	6500 Triple Quad	AB Sciex
Pipette, Volumetric (Adjustable)	10 – 100 µL	Eppendorf
	20 – 200 µL	
	100 – 1000 µL	
	500 – 2500 µL	
	500 – 5000 µL	
Ultrasonic Bath	Model 97043-980	VWR
Omni Bead Reptor 24	Bead Mill Homogenizer	Omni International
Mixer, Vortex	MaxiMix Plus	Barnstead Thermolyne
UPLC Column	Acquity-HSS T3 C18, 150×2.1 mm, 1.8µm	Waters

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	EMD	AX0145-1
Formic Acid	98%	EMD	X04406
Water	HPLC	EMD	WX0008-1
Methanol	HPLC	EMD	MX0475-1
Sodium phosphate	BioUltra	Sigma	71636
Ammonium acetate	HPLC	EMD	AX1222-5

Note: Equivalent reagents and chemicals from other suppliers may be substituted. Sodium phosphate buffer solution is non-volatile and detrimental to the instrument, ammonium acetate is strongly recommended as a substitute.

2.3.2.2 Solutions and Solvent Mixtures

Description	Code	Composition
HPLC mobile phase A	LC1	**0.1% Formic Acid in Water** Add 1 mL of formic acid into a 1 L volumetric flask and bring to the mark with HPLC water. Mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	**0.1% Formic Acid in Acetonitrile** Add 1 mL of formic acid into a 1 L volumetric flask and bring to the mark with ACN. Mix well to ensure complete homogeneous solution.
Extraction buffer	N/A	MeOH:water (85:15, v/v) + 10 mM ammonium acetate (or 10 mM sodium phosphate)

Note: If necessary, the solutions may also be prepared in different volumes as long as the proportions are not modified. Sodium phosphate buffer is non-volatile and detrimental to the instrument, ammonium acetate is strongly recommended as a substitute.

2.3.2.3 Standard Solutions

Stock Solutions

Approximately 100 µg/mL stock solutions for each analayte were prepared by dissolving 1–2 mg of test substance into 10 mL of acetonitrile or methanol. All standard preparations were corrected for chemical purity. All stock and intermediate solutions were stored under freezer conditions (nominally ≤ −5 °C). All working solutions were stored under refrigerator conditions (nominally 0–10 °C).

Intermediate Solutions

Mixed-analyte intermediate solutions were prepared in acetonitrile from individual stock solutions by volumetric dilution. All intermediate solutions were stored under freezer conditions (nominally ≤ -5 °C).

Fortification Solutions

Mixed-analyte fortification solutions were prepared by serial dilutions of mixed-analyte intermediate solutions and used for the fortification of recovery samples in water and soil. A preparation scheme to include all the analytes is presented in the table below.

Parent solution concentration (ng/µL)	Aliquot volume (mL)	Dilution with acetonitrile to a final volume of (mL)	Final Fortification Concentration (ng/µL)
10	1	10	1.0
1	0.10	10	0.01
0.01	1	10	0.001

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

Calibration Standard Solutions

Water Analysis

Standard calibration solutions for LC-MS/MS analysis were prepared by using the mixed intermediate solutions prepared from the section “Fortification Solutions”. Final standard

calibration solutions were prepared in filtered water for solvent-matched calibations as exemplified in the table below. A homogeneous solution was ensured by vortexing.

Preparation of standard solutions for calibration

Analytes	Parent Solution (ng/mL)	Aliquot Volume (mL)	Final Volume (mL)	Final concentration (ng/mL)
Prosulfuron CGA300406 CGA159902 CGA150829	10000	0.2	10	200
	200	0.5	10	10.0
	10	0.3	10	0.30
	10	0.1	10	0.10
	0.30	1.0	10	0.03
	0.10	1.0	10	0.01
	0.10	0.5	10	0.005
	0.01	2.0	10	0.002

***Note:** If matrix-matched standards are needed for successful analysis, they should contain at least 90% of matrix load and are prepared in matrix solutions. If necessary, the volume of solution prepared may be changed as long as the proportions are not modified.

Soil Analysis

Standard calibration solutions for LC-MS/MS analysis were prepared by using the mixed intermediate solutions prepared from the section “Fortification Solutions Preparation”. Final standard calibration solutions were prepared in control extract for matrix-matched calibrations; as exemplified in the table below. A homogeneous solution was ensured by vortexing.

Preparation of standard solutions for calibration

Analytes	Parent Solution (ng/mL)	Aliquot Volume (mL)	Diluent Volume (mL)	Final Volume (mL)	Final concentration (ng/mL)
Prosulfuron CGA300406 CGA159902 CGA150829	100	0.24	3.76	4.0	6.00
	6	0.50	1.00	1.5	2.00
	6	0.15	1.35	1.5	0.60
	2	0.30	1.20	1.5	0.40
	2	0.15	1.35	1.5	0.20
	0.6	0.25	1.25	1.5	0.10
	0.4	0.15	1.35	1.5	0.04
	0.2	0.15	1.05	1.2	0.025
	0.04	0.30	1.20	1.5	0.008

***Note:** If matrix-matched standards are needed for successful analysis, they should contain at least 90% of matrix load and are prepared in matrix solutions. If necessary, the volume of solution prepared may be changed as long as the proportions are not modified.

2.3.2.4 Standard Solution Storage Stability

The storage stability of stock and intermediate standard solutions prepared in acetonitrile and kept at frozen temperatures was established during the development of Syngenta method number AG-600 (Reference 1), GRM035.02A (Reference 2), and GRM035.03A (Reference 3). The storage stability of working standard solutions prepared in HPLC water and kept at refrigerated temperatures was also established.

2.3.3 Analytical Procedure

2.3.3.1 Weighing and Fortification

Water Samples

For control samples, 10 mL of water sample was placed into a 15 mL polypropylene test tube. For fortified samples, add the appropriate amount of fortification standard solution.

Sample Type	Sample Size (mL)	Concentration of Fortification Solution (ng/mL)	Volume of Fortification Solution added (mL)	Level of Fortification (ppb)
Control	10	-	-	0
Fortification (LOQ)*	10	1	0.1	0.01
Fortification (10× LOQ)	10	10	0.1	0.1

*Limit of Quantitation

Soil Samples

For control samples, 10 g of soil sample was placed into a 50 mL polypropylene test tube. For fortified samples, add the appropriate amount of fortification standard solution.

Sample Type	Sample Size (g)	Concentration of Fortification Solution (ng/mL)	Volume of Fortification Solution (mL)	Level of Fortification (ppb)
Control	10	-	-	0
Fortification (LOQ)*	10	10	0.1	0.1
Fortification (100× LOQ)	10	1000	0.1	10

*Limit of Quantitation

2.3.3.2 Extraction of Sample Material

Water Samples

An aliquot of water sample was filtered using a 0.2 µm syringe filter in order to eliminate particulate matter. The initial 0.1 – 0.2 mL of filtrate was disposed and the remaining sample aliquot filtered into an autosampler vial for analysis by LC-MS/MS.

Soil Samples

10 g of soil sample was placed in a 50 mL polypropylene test tube. 25 mL of extraction buffer solution (MeOH:water +10 mM sodium phosphate) was added to the samples. Samples were shaken on the Omni Bead Ruptor for 1 min at speed setting of 4 m/s. Samples were placed on the centrifuge for 30 min at 4150 rpm. The extracts were concentrated under nitrogen using evaporation at 50 °C. Samples were reconstituted to 5 mL using HPLC grade water, centrifuged again, and filtered. Samples were vialed for analysis by LC-MS/MS.

2.3.3.3 Influence of Matrix Effects on Analysis

During method development, matrix suppression was experienced in both water and soil. Prosulfuron was most effected by the influence of matrix with recoveries at ~160% in soil. A smaller effect is observed for other analytes; therefore, samples were analyzed with calibration standard solutions prepared in control matrix (matrix-matched standards).

3.0 INSTRUMENTAL ANALYSIS

3.1 Instrumentation and Conditions

Mass Spectrometer:	API 6500 (Instrument #27)			
Inlet [HPLC System]:	Agilent 1290 Infinity HPLC System			
Software Version:	Analyst 1.6.2			
Column:	Acquity-HSS T3 C18; 1.8 µm, 2.1 X 150 mm			
Injection:	40 µL	Column Temperature: 50 °C		
Mobile Phase:	A = 0.1% formic acid in water B = 0.1% formic acid in acetonitrile			
Gradient	Time (min.)	Composition		
		% A	% B	
	0.00	99.0	1.0	
	0.50	99.0	1.0	
	2.40	40.0	60.0	
	4.25	35.0	65.0	
	4.26	0.0	100.0	
	5.25	0.0	100.0	
	5.26	99.0	1.0	
	5.75	99.0	1.0	
Flow Rate:	600 µL/minute			
Expected Retention Times	Prosulfuron	CGA300406	CGA159902	CGA150829
	~4.45 min	~3.62 min	~3.80 min	~2.46 min

<u>Mass Detector Conditions for Prosulfuron, CGA300406, CGA159902:</u>

Detector:	AB Sciex Triple Quad 6500
Ionization mode:	Turbo Spray
Source polarity:	Negative
Curtain gas (CUR):	10 (arbitrary units)
Gas 1 (GS1):	60 (arbitrary units)
Gas 2 (GS2):	60 (arbitrary units)
Temperature (TEM):	550 °C
Collision gas setting (CAD):	9
Ionspray voltage (IS):	-4500
Entrance potential (EP):	-10

Mass Detector Conditions for CGA150829:

Detector:	AB Sciex Triple Quad 6500
Ionization mode:	Turbo Spray
Source polarity:	Positive
Curtain gas (CUR):	10 (arbitrary units)
Gas 1 (GS1):	60 (arbitrary units)
Gas 2 (GS2):	60 (arbitrary units)
Temperature (TEM):	550 °C
Collision gas setting (CAD):	9
Ionspray voltage (IS):	5500
Entrance potential (EP):	10

Mass Source and Detection Parameters

Compound	Parent (*m/z*)	Collision energy (V)	Declustering potential (DP)	Collision cell exit potential (CXP)	Fragment ions (*m/z*)	Dwell time (msec)	Ion type
Prosulfuron	418.0	-25.54	-33.7	-7.84	138.9	150	Primary
	418.0	-70.63	-33.7	-5.22	106.8	150	Confirmatory
CGA300406	404.1	-19.17	-41.9	-5.72	125.1	150	Primary
	404.1	-56.43	-41.9	-9.31	82.0	150	Confirmatory
CGA159902	252.1	-21.27	-105.0	-6.18	212.1	150	Primary
	252.1	-36.0	-105.0	-5.12	102.1	150	Confirmatory
CGA150829	141.0	45.0	101.0	8.0	57.1	150	Primary

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.

3.2 Calibration Procedures

Calculation of results was based on peak area measurements using a calibration curve. A calibration curve for matrix-matched standards was obtained by direct injection by LC-MS/MS of at least five calibration standards of prosulfuron and its metabolites in the range of 0.008 to 0.60 ng/mL for soil. A calibration curve for matrix-matched standards was obtained by direct injection by LC-MS/MS of six calibration standards of prosulfuron and its metabolites in the range of 0.002 to 0.30 ng/mL for water. The same injection volume was used for all samples and standards in all sequences.

3.3 Calculation of Residues and Recoveries

a) Calibration curve: $y = mx + b$ Solving for x: $x = \frac{y-b}{m}$

Where, m = slope
b = y-intercept
x = Amount found (ng)
y = Peak area

b) Amount of sample injected (g) $= \frac{\text{injection size (µL)}}{\text{final sample volume (mL)}} \times \text{sample weight (g)} \times \frac{1\ mL}{1000\ \mu L}$

c) Residue found (ppb) $= \frac{\text{ng found}}{\text{Amount of sample injected (g)}}$

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

Example: Prosulfuron (m/z 418.00 → 138.90) in Soil fortified at 0.1 ppb (LOQ) (Sample ID: 15033001-Recovery1-1) in WO-15033001:

a) Calibration curve: $y = (5.67e + 07)x + (1.23e + 04)$

b) Solving for x (ng): $x = \frac{87070 - (1.23e+04)}{(5.67e+07)} = 0.00132$ ng

c) Amount of sample injected (g) $= \frac{40\ \mu L}{25.0\ mL} \times 10.02\ g \times \frac{1\ mL}{1000\ \mu L} = 0.01603$ g

d) Residue found (ppb) $= \frac{0.00132\ ng}{0.01603\ g} = 0.08234$ ppb

e) Recovery (%) $= \frac{0.08234 - 0.00000}{0.100} \times 100 = 82\%$

5.0 SUMMARY OF METHOD

Type of method:
Method Validation for prosulfuron and its metabolite analysis in water and soil by LC-MS/MS

Test systems: Soil and water.

Analyte mass transitions:

Compound	Transitions (*m/z*)	Source polarity
Prosulfuron	418.0→138.9* 418.0→106.8	Negative
CGA-300406	404.1→125.1* 401.1→82.0	Negative
CGA-159902	252.1→212.1* 252.1→102.1	Negative
CGA-150829	141.0→57.1*	Positive

*The primary ion transition; however, any of these transitions could be used for quantitation in case interference is observed at the same retention time.

Analytical procedure :
Water is extracted by filtering through syringe filter and analysis by LC-MS/MS. Soil is extracted by disruption, centrifugation, and evaporation of solvent extract and analysis by LC-MS/MS. The technical procedure is presented in Appendix 6.

Confirmatory technique:
For the analysis of prosulfuron and its metabolites with the primary and confirmatory mass transitions, the detector responses were determined to be linear within the range from 0.002 ng/mL to 0.3 ng/mL in water with $r \geq 0.99$ and from 0.008 to 0.6 ng/mL in soil with $r \geq 0.99$. Due to the high selectivity and specificity of LC-MS/MS, an additional confirmatory technique is not necessary for CGA150829. Quantification is based on the monitoring of two mass transitions for all other analytes. Recovery data was reported for each mass transition and matrix considered, as shown in the detailed analytical data in Appendix 5.

Limit of detection (LOD):
20% of the LOQ in each matrix equivalent to 0.002 ppb in water for all analytes and 0.02 ppb in soil for prosulfuron, CGA300406, CGA159902, and 60% of the LOQ for CGA150829, 0.06 ppb in soil.

Limit of quantification (LOQ):
0.01 and 0.1 ppb (lowest fortification level) for all analytes in water and soil, respectively.

Levels of fortification:
0.01 ppb (LOQ) and 0.1 ppb (10x LOQ) for all analytes in water, and 0.1 ppb (LOQ) and 10 ppb (100x LOQ) for all analytes in soil.

Time required:
A set of 13 water samples requires approximately 6 hours of work and a set of 13 soil samples requires approximately 8 hours of work.

6.0 DISCUSSION

Recovery findings: Method M1408 was sucessfully validated to determine residues of prosulfuron and its metabolites in soil at the LOQ and 100× LOQ and water at the LOQ and 10× LOQ. Overall mean recovery values of the validation experiments were determined to be between 70% and 120%.

Repeatability: The overall relative standard deviations (%RSD) for all fortification levels were below 20**%** at the primary and confirmatory mass transition.

Controls: In untreated control samples prosulfuron and its metabolite were not detectable (below 20% of LOQ for all analytes in water and 60% of LOQ for CGA150829 in soil).

Specificity: The method M1408 is highly specific for prosulfuron and its metabolites (mass transitions from parent ions to two typical fragments in MS/MS mode) in soil and water No interfering peaks were found at the retention times for all analytes.

Linearity: For analysis of prosulfuron and its metabolites at the primary and confirmatory mass transitions, the detector responses were determined to be linear within the range from 0.002 ng/mL to 0.3 ng/mL in water with $r \geq 0.99$ and from 0.008 to 0.6 ng/mL in soil with $r \geq 0.99$.

Limit of Quantification: The LOQ was found to be 0.01 and 0.1 ppb (lowest fortification level) for all analytes in water and soil, respectively.

The analytical method for the determination of prosulfuron and its metabolites, CGA300406, CGA159902, and CGA150829 in water and soil has been successfully performed and demonstrated the method M1408 fulfills the requirements guidelines, OCSPP 850.6100 and SANCO/825/00 rev 8.1 and is therefore fully applicable to Independent lab validation and field dissipation monitoring.

7.0 REFERENCES

[1] Williams, W., et. al. Syngenta Method No. AG-600. CIBA-GEIGY Corporation. May 21, 1992.

[2] Oppilliart, S. Syngenta Method No. GRM035.02A. Syngenta Ltd. February 23, 2010.

[3] Oppilliart, S. Syngenta Method No. GRM035.03A. Syngenta Crop Protection, LLC. February 23, 2010

1.0 INTRODUCTION

Prosulfuron (CGA152005) is a sulfuonylurea used as a herbicide in rice production in the United States of America. This method (ADPEN-M1408) was developed at ADPEN Laboratories, Inc. for the analysis of residues of prosulfuron and its metabolites, CGA300406, CGA159902 and CGA150829, in paddy water and soil/sediment, and based in part on Syngenta Methods AG-600 and GRM035.03A (References 1 and 2). The method has a limit of quantitation of 0.01 ppb (10 ppt) in water and 0.1 ppb (100 ppt) in soil for all analytes.

2.0 MATERIALS

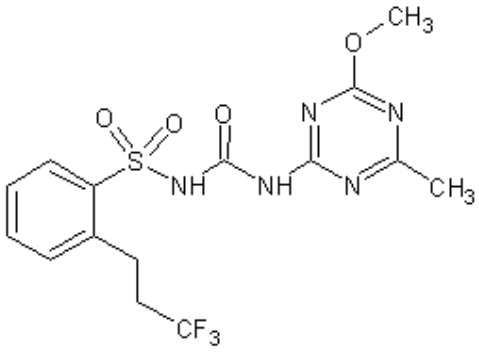
2.1 Safety

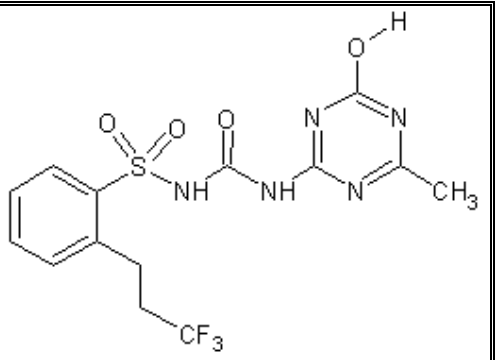
The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Store work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

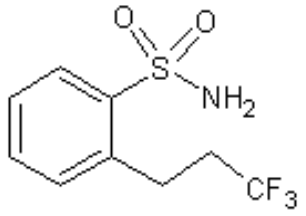
Disposal of samples and chemicals must be done in compliance with on-site safety policies and procedures.

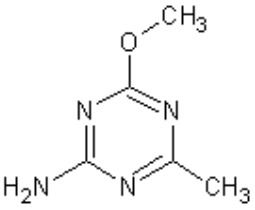
2.2 Test and Reference Items

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

Common Name:	Prosulfuron	
IUPAC Name:	1-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-3-[2-(3,3,3-trifluoropropyl) phenylsulfonyl]urea	
Code Name:	CGA152005	
CAS Number:	94125-34-5	
Molecular Formula:	$C_{15}H_{16}F_3N_5O_4S$	
Molecular Weight:	419.38	
Lot Number:	731405	
Purity (%):	99.0	
Expiration Date:	02-28-2019	

IUPAC Name:	1-(4-hydroxy-6-methyl-1,3,5-triazin-2-yl)-3-[2-(3,3,3-trifluoropropyl) phenylsulfonyl]urea	
Code Name:	CGA300406	
Molecular Formula:	C15H14F3N5O4S	
Molecular Weight:	405.35	
Lot Number:	NEH-XV-88	
Purity (%):	96.9	
Expiration Date:	06-30-2016	

IUPAC Name:	2-(3,3,3-trifluoropropyl) phenylsulfonylurea	
Code Name:	CGA159902	
CAS Number:	94125-42-5	
Molecular Formula:	C9H10F3NO2S	
Molecular Weight:	253.24	
Lot Number:	BPM-XII-17	
Purity (%):	98.3	
Expiration Date:	06-30-2016	

IUPAC Name:	2-amino-4-hydroxy-6-methyl-1,3,5-triazine	
Code Name:	CGA150829	
CAS Number:	1668-54-8	
Molecular Formula:	C5H8N4O	
Molecular Weight:	140.14	
Lot Number:	CDC-III-91	
Purity (%):	99.3	
Expiration Date:	06-30-2016	

2.3 Equipment

Equipment	Size, Description	Manufacturer
Balance, Analytical	Model XP26	Mettler Toledo
Beaker	Various Sizes	Pyrex, Kimax or equivalent
Bottle, Amber glass	10 to 50 mL with Teflon®-lined screw cap	Qorpak
Centrifuge	≥3700 G	Fisher Scientific, Accuspin 3R
Centrifuge Tube, Polypropylene	15 mL	BD, Falcon, VWR or equivalent
	50 mL	
Cylinder, Graduated	10 - 1000 mL	Various
UPLC	1290	Agilent
Mass Spectrometer	6500 Triple Quad	AB Sciex
Mixer, vortex	MaxiMix Plus	Barnstead Thermolyne
Omni Bead Ruptor 24	Bead Mill homogenizer	Omni International

Equipment (continued)

Equipment	Size, Description	Manufacturer
Pipette, Volumetric (adjustable)	10-100 µL	Eppendorf
	20-200 µL	
	100-1000 µL	
	500-2500 µL	
	500-5000 µL	
Ultrasonic Bath	Model 97043-980	VWR
UPLC Column	Acquity-HSS T3 C18, 150 x 2.1 mm, 1.8 µm	Waters
Vials, analytical	2 mL	Agilent or equivalent
Vials, filtration	2-mL 0.2um PTFE (35530-PC-100)	Thomson Instrument Company

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	EMD	AX0145-1
Formic Acid	98%	EMD	X04406
Water	HPLC	EMD	WX0008-1
Methanol	HPLC	EMD	MX0475-1
Sodium phosphate	BioUltra	Sigma	71636
Ammonium acetate	HPLC	EMD	AX1222-5

Note: Equivalent reagents and chemicals from other suppliers may be substituted. Sodium phosphate is detrimental to LC-MS/MS instrumentation; therefore, ammonium acetate is a suitable substitute.

2.4.2 Solutions and Solvent Mixtures

Description	Code	Composition
HPLC mobile phase A	LC1	**0.1% Formic Acid in Water** Add 1 mL of formic acid into a 1 L volumetric flask and bring to the mark with HPLC water. Mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	**0.1% Formic Acid in Acetonitrile** Add 1 mL of formic acid into a 1 L volumetric flask and bring to the mark with ACN. Mix well to ensure complete homogeneous solution.
Extraction buffer	N/A	85% MeOH, 15% aqueous solution of 10 mM Na_2HPO_4
Extraction buffer	N/A	85% MeOH, 15% aqueous solution of 10 mM NH_4OAc

Note: If necessary, solutions may be prepared in different volumes as long as the proportions are not modified. The method validation was conducted using the sodium phosphate buffer; however, ammonium acetate is preferred, as sodium phosphate can be detrimental to the longevity of the mass spectrometer.

2.4.3 Standard Solutions

Stock Solutions

Prepare 100 µg/mL stock solutions by weighing 1 mg of analyte and transferring quantitatively to a 10 mL volumetric flask with the help of solvent. Dilute to the mark with the appropriate solvent (see table below). Ensure a complete homogeneous solution (e.g. by sonication or vortexing). A correction for purity is made to the standard concentration if chemical purity is ≤ 99.6%. If the purity is > 99.6 %, the correction is optional. **Special note should be made of the stability of the concentrated stock solution of CGA300406**.

Analyte	Solvent	Expiration
Prosulfuron	ACN	1 year
CGA300406	ACN	1 day
CGA159902	ACN	1 year
CGA150829	MeOH	4 months

Intermediate Stock Solutions

Intermediate stock solutions of three analytes, prosulfuron, CGA159902 and CGA150829, can be prepared as a mixture in ACN and given an expiration of 4 months, in accord with the shortest expiration period of CGA150829 (prosulfuron and CGA159902 have an expiration of 6 months, if prepared separately). For practical reasons, stock solutions of CGA300406 should be prepared separately as it degrades quickly and has an expiration period of no more than 1 week.

Analyte	Solvent	Expiration
Prosulfuron	ACN	4 months
CGA300406	ACN	1 week
CGA159902	ACN	4 months
CGA150829	ACN	4 months

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.

Fortification Solutions

Mixed-analyte fortification solutions can be prepared by combining stock or intermediate solutions of each analyte in a volumetric flask using the following example scheme. Dilute to the mark with HPLC water as specified in the table below and vortex to ensure a complete homogeneous solution. Preparation in acetonitrile may be necessary at higher concentrations. The following is an example preparation scheme to include all the analytes:

Analytes	Parent Solution	Aliquot Volume (mL)	Final Volume (mL)	Final Concentration	Matrix and Fortification Level
Prosulfuron CGA300406 CGA159902 CGA150829	10 ng/mL	1.0	10	1.0 ng/mL	water, LOQ
	1000 ng/mL	1.0	10	100 ng/mL	water, 100xLOQ,
	100 ng/mL	1.0	10	10 ng/mL	soil, LOQ
	10 µg/mL	1.0	10	1000 ng/mL	soil, 100xLOQ

Note: The standard concentrations and fortification levels are presented, assuming the water sample size is 10 mL and the soil sample size is 10 g.

Note: Different concentration scheme may be used, if other fortification levels are needed.

Calibration Standard Solutions

Water Analysis:
Prepare mixed calibration solutions for LC-MS/MS analysis of water samples by using the solutions prepared above as described in the table below. Dilute to the mark with appropriate solvent (water or filtered control matrix) and vortex to ensure a complete homogeneous solution.

The following is a suggested preparation scheme to include all the analytes:

Analytes	Parent Solution	Aliquot Volume (mL)	Final Volume (mL)	Final Concentration
Prosulfuron CGA300406 CGA159902 CGA150829	100 ng/mL	1.0	10	10 ng/mL
	10 ng/mL	1.0	10*	1 ng/mL (1000 pg/mL)
	10 ng/mL	0.3	10*	300 pg/mL
	1000 pg/mL	1.0	10*	100 pg/mL
	300 pg/mL	1.0	10*	30 pg/mL
	100 pg/mL	1.0	10*	10 pg/mL
	100 pg/mL	0.5	10*	5 pg/mL
	10 pg/mL	2	10*	2 pg/mL

Note: If matrix-matched standards are used (see Section 3.5), they should contain at least 90% of matrix load. Therefore, while following the dilution scheme in the above

table, all solutions at a final concentration of 1000 pg/mL or less are diluted with filtered environmental water from a clean control sample. The highest calibration point, 300 pg/mL, contains 97% matrix load, and the lower concentrations contain 99% matrix load or more.

Soil Analysis:
Prepare mixed-analyte calibration solutions for LC-MS/MS analysis of soil samples by using solutions prepared above, and the example scheme in the table below. Dilute to the mark with appropriate solvents (water or control matrix) and vortex to ensure a complete homogeneous solution.

The following is a suggested scheme to prepare mixed matrix-matched standards:

Analytes	Parent Solution	Aliquot Volume (mL)	Diluent volume (mL)	Final volume	Final Concentration
Prosulfuron CGA300406 CGA159902 CGA150829	10 ng/mL	0.3	2.7*	3 mL	1 ng/mL
	1 ng/mL	0.9	0.6*	1.5 mL	600 pg/mL
	1 ng/mL	0.6	0.9*	1.5 mL	400 pg/mL
	1 ng/mL	0.3	1.2*	1.5 mL	200 g/mL
	1 ng/mL	0.15	1.35*	1.5 mL	100 pg/mL
	400 pg/mL	0.15	1.35*	1.5 mL	40 pg/mL
	200 g/mL	0.15	1.05*	1.2 mL	25 pg/mL
	40 pg/mL	0.3	1.2*	1.5 mL	8 pg/mL
	Total diluent volume:		10.35		

Note: The diluent for the preparation of the matrix matched standard is the final extract of a control soil sample and is limited in volume by the procedure. For this reason the calibration standards are prepared directly in the 2-mL autosampler vials. The highest calibration point is 600 pg/mL. Additional amount of control extract diluent is needed for the dilution of the 100xLOQ fortified samples.

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

2.4.4 Stability of Standard Solutions

Prosulfuron and CGA300406 are sensitive to acids, aqueous solutions of high ionic strength and light, and will degrade quickly. However, the presence of formic acid in the standard solution accelerates the hydrolysis processes and should not be added to any of the standard solutions.

Analyte	Stock solutions		Intermediate solutions		Working solutions	
	Solvent	Expiration	Solvent	Expiration	Solvent	Expiration
Prosulfuron	ACN	1 year	ACN	4 months [a]	Water	1 week
CGA300406	ACN	1 day	ACN	1 week		
CGA159902	ACN	1 year	ACN	4 months [a]		
CGA150829	MeOH	4 months	ACN	4 months [a]		

Stock and intermediate solutions should be prepared and stored in a freezer individually. The concentrated solution of CGA300406 (prepared from neat material), expires in one day. The intermediate solution, prepared from the concentrated solution, is stable for one week. The other three analytes are more stable and their intermediate solutions are used repeatedly.

Working solutions, for fortification and calibration, which contain all four analytes are stored in a refrigerator and expire within one week as per Reference 1.

Matrix matched (working) standards are prepared by dilution of the standard solutions in HPLC water with control sample extract. The content of the filtered control sample should be no less than 90%. The stability of the matrix matched standards is unknown at this time. They should be prepared just prior to instrument analysis and used immediately. The stability of matrix matched standards may be conducted to determine their expiration time.

3.0 ANALYTICAL PROCEDURE

3.1 Sample Storage

Until analysis, water samples are stored in clean glass or plastic bottles in a refrigerator at ca. +4 °C. Soil samples are stored in a freezer at less than -18 °C.

3.2 Sample Preparation

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

3.3 Weighing and Fortification

3.3.1 **Water**

Measure 10.0 mL of water sample in a 15-mL polypropylene test tube. For the fortification samples, add an appropriate amount of standard solution to the respective control. For example, to prepare a 0.010 ppb fortification sample, pipet 100 µL of the 1 ng/mL standard fortification solution to 10 mL of control sample.

The following fortification scheme may be used for water matrices:

Sample Type	Sample Size	Concentration of Spiking Solution (ng/mL)	Volume of Spiking Solution (µL)	Level of Fortification (ppb)
Control	10 mL	-	-	0
Fortification (LOQ)*	10 mL	1	100	0.0100
Fortification (100×LOQ)	10 mL	100	100	1.00

*Limit of Quantification

3.3.2 Soil

Weigh 10.0 g of soil sample in a 50-mL polypropylene centrifuge tube. For the fortification samples, add an appropriate amount of standard solution to the respective control. For example, to prepare a 0.10 ppb fortification sample, pipet 100 µL of the 10 ng/mL standard fortification solution to 10 g of control sample.

The following scheme may be used for soil matrices:

Sample Type	Sample Size	Concentration of Spiking Solution (ng/mL)	Volume of Spiking Solution (µL)	Level of Fortification (ppb)
Control	10 g	-	-	0
Fortification (LOQ)*	10 g	10	100	0.100
Fortification (100×LOQ)	10 g	1000	100	10.0

*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

3.4.1 Water

Transfer a small aliquot of the water sample (~0.45 mL) into a 0.2 µm filtration vial to eliminate particulate matter. Mechanically or manually depress the filter plunger into the vial. Filtration vial can go directly into an autosampler tray for analysis by LC-MS/MS.

3.4.2 Soil

Add 25 mL of extraction solvent, 85% methanol and 15% extraction buffer in water, to the soil sample. Close and tighten the cap of the centrifuge tube, and insert the tube into the Omni Bead Ruptor rack. Initiate extraction program set for one interval for 1 min at a speed setting of 4 m/s.

Centrifuge the sample for 30 min at 4150 rpm (3700 g-force). Higher speeds and/or longer time may be necessary. The centrifuged samples should separate into clear supernatant and solid cake precipitate.

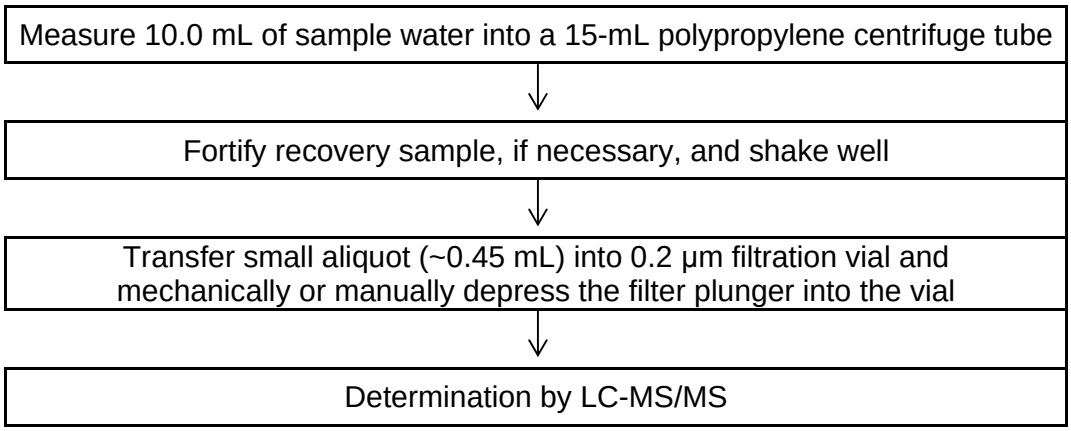
Transfer 5 mL of supernatant into a 15-mL centrifuge tube.
Note: Transfer 15 mL of the control supernatant into a clean 50-mL centrifuge tube to be used for preparation of matrix-matched standards and for dilutions, if required.

Concentrate the extract under nitrogen (Turbo Vap or N-Evap) at 40-50 °C to aqueous residue (approximately 0.75 mL for 5-mL sample and approximately 2 mL for the 15-mL control sample). Reconstitute to 5 mL using HPLC grade water (15 mL for the control diluent).

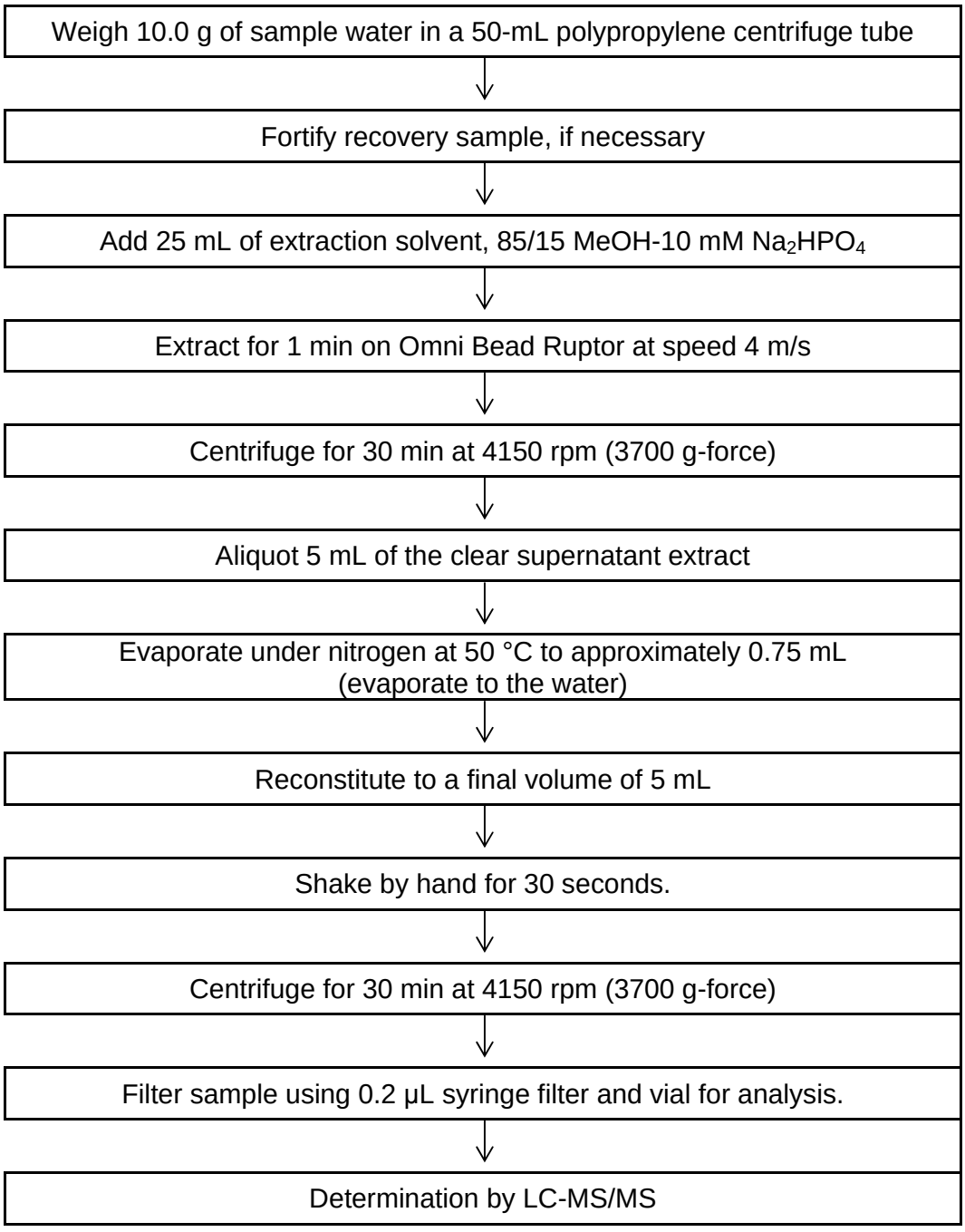
Shake sample by hand for 20 seconds and centrifuge for 30 min at 4150 rpm (3700 g-force). Pass the sample through a 0.2 µm syringe filter and vial for analysis.
Note: The extra control sample solution is passed through a 0.2 µm syringe filter and placed in a clean container; this solution is used as diluent for matrix matched standards.

Note: Samples fortified at a concentration higher than the LOQ should be diluted down to the LOQ.

3.5 Flowchart for the Analysis of Water Samples



3.1 Flowchart for the Analysis of Soil Samples



3.2 Influence of Matrix Effects on Analysis

Depending on the soil and water type, matrix effects have been found to cause significant suppression or enhancement of analytes when analyzed by LC-MS/MS. If significant suppression occurs, (i.e., matrix effects > 20%) matrix-matched standards should be utilized.

Analyses of water and soil samples may require the use of matrix matched standards. 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 needs to be tested prior to analyses. Calibration standard solutions (prepared in solvent) are compared against their respective calibration standards prepared in control matrix extracts.

A comparison of the percent change (%Δ) between the solvent-based and matrix-matched calibration can be made using the following formula:

$$\%\Delta = \frac{\left| Area_{SS} - Area_{MMS} \right|}{Area_{SS}} * 100$$

For the type of water and soil tested during validation, matrix effects showed significant variation for the different analytes and fluctuate between analyses. Significant matrix effects were observed for CGA300406. Prosulfuron can be also affected.

3.3 Stability of Extracts

The extracts of the analytes in the final volume solution may not be stable; therefore, sample extracts should be analyzed immediately unless extract stability can be determined.

4.0 QUANTITATION AND CALCULATION

4.1 Set-up of the Analytical Run

A sequence for measurement generally consists of:

- Calibration standards
- Control samples
- Procedural recovery samples

Reagent or solvent 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. At least 5 calibration levels are needed for quantitation.

4.2 Instrumental Analysis

4.2.1 Instrumentation and Conditions for the Analysis of Water

Instrument:	AB Sciex Instruments API 6500			
Inlet [UPLC System]:	Agilent 1290 UPLC System			
Software Version:	Analyst 1.6.2			
Column:	Acquity HSS T3; 1.8 µm, 2.1 X 150 mm			
Injection Size:	40 µL			
Column Temperature:	50 °C			
Mobile Phase:	A = 0.1% formic acid in water B = 0.1% formic acid in acetonitrile			
Gradient	Time, min	Flow rate, µL/min	Composition	
			% A	%B
	0	600	99	1
	0.5	600	99	1
	2.4	600	40	60
	4.25	600	35	65
	4.26	600	0	100
	5.25	600	0	100
	5.26	600	99	1
	5.75	600	99	1

Analytes:	Prosulfuron	CGA300406	CGA159902	CGA150829
Retention Times:	4.16 min	3.38 min	3.55 min	2.19 min
Primary Transitions (m/z):	418.0→138.9	404.1 → 125.1	252.1 → 212.1	141.0 → 57.1
Secondary Transitions (m/z):	418.0→106.8	404.1 → 82.0	252.1 → 102.1	N/A
Ionization Mode	Negative	Negative	Negative	Positive
	Negative Mode switches to Negative at 3.0 min.			
	Turbo ion spray (550°C)			

Notes:
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 the Analysis of Soil

Instrument:	AB Sciex Instruments API 6500			
Inlet [UPLC System]:	Agilent 1290 UPLC System			
Software Version:	Analyst 1.6.2			
Column:	Acquity HSS T3; 1.8 µm, 2.1 X 150 mm			
Injection Size:	40 µL			
Column Temperature:	50 °C			
Mobile Phase:	A = 0.1% formic acid in water B = 0.1% formic acid in acetonitrile			
Gradient	Time, min	Flow rate, µL/min	Composition	
			% A	%B
	0	600	99	1
	0.5	600	99	1
	6	600	40	60
	8	600	35	65
	8.01	600	0	100
	8.75	600	0	100
	8.76	600	99	1
	10	600	99	1

Analytes:	Prosulfuron	CGA300406	CGA159902	CGA150829
Retention Times:	7.10 min	5.72 min	5.95 min	2.86 min
Primary Transitions (m/z):	418.0→138.9	404.1 → 125.1	252.1 → 212.1	141.0 → 57.1
Secondary Transitions (m/z):	418.0→106.8	404.1 → 82.0	252.1 → 102.1	N/A
Ionization Mode	Negative	Negative	Negative	Positive
	Negative Mode switches to Negative at 4.5 min.			
	Turbo ion spray (550°C)			

Notes:
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.

4.2.3 Calibration Procedures

Calculation of results is based on peak area measurements using a calibration curve. At least 5 calibration levels need to be injected. The calibration curve is obtained by direct injection of prosulfuron and accompanying metabolites standards for LC-MS/MS in the range of 2 to 300 pg/mL for water samples and 8 to 600 pg/mL for soil samples. In a given injection run, the same injection volume is used for all samples and standards.

Linear least square fit calibration curves with 1/x weighing factor are preferred for evaluation. Other functions (e.g. quadratic) may be used if required.

4.2.4 Calculation of Residues and Recoveries

Calculation of results is based on area measurements. For procedural recoveries, the sample size will be considered 10 mL in the final calculation of residues [ng/L for water]. The method requires that the sample weight to be 10.0 mL of filtered water for fortification samples. The recovery is the ratio of the concentration found and the theoretical concentration of analyte, times 100.
The residues of analyte in ng/mL are calculated as shown in equations **I** and **II**:

I. Using linear regression analysis the concentration of analytes, C_A, is calculated as follows:

$$C_A = \frac{Response - Intercept}{Slope}$$

The calculation of C_A is accomplished automatically by the instrument data system.

II. Residue is the amount of analyte in ng/mL per sample:

$$Residue = \frac{FV \times C_A}{V_S \times AF \times CF}$$

FV = Final volume of the extract, mL
C_A = Concentration of analyte as read from the calibration curve [ng/mL]
V_S = Volume of the sample extracted, mL
AF = Aliquot factor
CF = Unit conversion factor, if necessary

For the analysis of water, where FV=V_S and AF = 1, Residue = C_A, times unit conversion factor if necessary.

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

III. Recovery, R%, is calculated by the use of the following formula:

$$R,\% = \frac{\text{Residue}_{\text{fortified sample}} - \text{Residue}_{\text{control}}}{\text{Amount}_{\text{theoretical}}} \times 100$$

For the analysis of water, the Residue equals the C_A and the $\text{Amount}_{\text{theoretical}}$ is the theoretical concentration. Alternative formula may be used:

$$R,\% = \frac{C_{\text{found in fortified sample}} - C_{\text{control}}}{C_{\text{theoretical}}} \times 100$$

Where C are the concentrations of analytes as found by the Analyst program.

5.0 METHOD MANAGEMENT AND TIME REQUIREMENTS

The analysis of one set of water samples (= 30 unknown samples, 2 fortified samples for recovery experiments, 1 control sample) requires 0.7 working days (6 hours) per laboratory assistant. Larger number of samples can be analyzed using automated equipment.

The analysis of one set of soil samples (= 20 - 30 unknown samples, 2 fortified samples for recovery experiments, 1 control sample) requires 1 working day (8 hours) per laboratory assistant.

This time does not include the instrument time, the calculation of the results, and the preparation of the equipment, as well as the reporting of all raw data under GLP.

6.0 CONCLUSION AND METHOD CAPABILITIES

Representative calibration curves and chromatograms are provided in Appendix A for water and Appendix B for soil.

Limit of Quantitation (LOQ) and Limit of Detection (LOD)
The limit of quantitation is defined as the lowest fortification level successfully tested. The limit of quantitation is ppt for all analytes in soil. The limit of detection was estimated at 20% of the limit of quantitation, equivalent to 0.002 ng/mL for water, for all analytes, and 0.008 ng/mL for soil samples with one exception. For CGA150829 the LOD is 0.025 ng/mL, this is due to lower sensitivity. The lowest standard for each analyte in the calibration curve has good detectability (signal to noise ratio better than or equal to 3:1).

Selectivity
The tested matrices showed no significant interferences (< 20 %) at the retention time of the analytes.

Confirmatory Techniques
The LC-MS/MS final determination for prosulfuron and its metabolites is a highly selective detection technique. For prosulfuron, CGA300406 and CGA159902, quantitation is possible at two different transitions and no additional confirmatory technique is required. For CGA150829 only one transition was available due to lower sensitivity.

Potential Problems
Strong matrix enhancement and suppression effects were observed during method development, especially for CGA300406 and in lesser degree for prosulfuron with or without cleanup. The utilization of matrix matched standards resolves the problem and eliminates the need of sample cleanup.

7.0 REFERENCES

[1] Method Number AG-600. Analytical Method for the Determination of CGA-152005 and Its Metabolites CGA-159902 and CGA-300406 (Desmethyl CGA-152005) In Water and Soil by High Performance Liquid Chromatography with Mass Spectrometric Detection Including Validation Data, CIBA-GEIGY Corporation, May 21, 1992.

[2] Method Number GRM035.03A. Triasulfuron -Analytical Method for the Determination of Residues of Triasulfuron and its Metabolite CGA150829 in Water. Final Determination by LC-MS/MS.