Analytical Method for the Determination of MKH 6561 and Seven Degradates in Soil

1.0 Summary

A method is described for the trace determination of MKH 6561 and its environmental degradates (MKH 6561 sulfonamide methyl ester, MKH 6561 sulfonamide acid, MKH 6561 carboxylic acid, *N*-methyl propoxy triazolinone, *N*-methyl propoxy triazolinone amide, saccharin and 4-hydroxy saccharin) in soil. After the addition of the corresponding isotopically-labeled internal standards, samples are extracted by accelerated solvent extraction (ASE), concentrated under nitrogen using a Turbovap® unit and analyzed by HPLC / Electrospray MS-MS (LC/ESI-MS-MS).

2.0 Materials

2.1 Equipment and Supplies

- Various general laboratory glassware and utensils
- Aluminum weigh dish (Fisher #08-732 or equivalent)
- Autosampler vials and caps (2-mL, Baxter #C4800-135 or equivalent)
- Borosilicate glass test tubes, 20 x 150 mm (Fisher #14-961-33 or equivalent)
- Plastic weighing pans (Fisher #02-204-18 or equivalent)
- VOA vials, 60 mL (I-Chem #236-0060 or equivalent)
- Acrodisc PTFE syringe filters, 13 mm X 0.45 µm (Gelman #4422 or equivalent)
- Accelerated solvent extractor (ASE) unit, Model 200 (Dionex Corp.) or equivalent
- ASE extraction cells, 33 mL size (Dionex #049562 or equivalent)
- Cellulose filters for ASE extraction cell caps (Dionex #049458 or equivalent)
- Analytical balance, 0.01-mg readout (Mettler A163 or equivalent)
- Top-loading balance, 0.01-g readout
- TurboVap LV evaporator (Zymark Corporation) or equivalent
- Phenomenex Columbus C18 HPLC column, 50 x 2 mm, 5 micron (Phenomenex #00B-4108-BO or equivalent)
- PE Sciex API III Plus or API 365 LC/Tandem mass spectrometer with Turbo Ion Spray interface and gradient HPLC (Shimadzu) system or equivalent

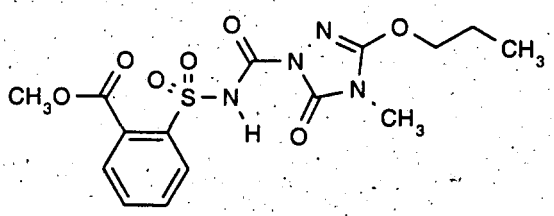
- Two-position electric actuator valve, Valco Instruments Co., Inc., #ECGW

2.2 Reagents and Solvents

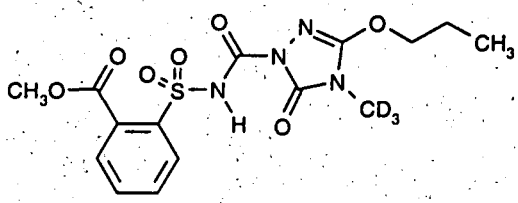
- Acetone (Fisher Optima grade, #A929-4 or equivalent)
- Acetonitrile (ACN; B&J HPLC-grade high purity solvent, Baxter #015-4 or equivalent)
- Water (B&J HPLC-grade high purity solvent, Baxter #365-4 or equivalent)
- Methanol (MeOH; B&J HPLC grade high purity solvent, Baxter #230-4 or equivalent)
- Calcium chloride (dihydrate, $CaCl_2 \cdot 2H_2O$, Mallinckrodt #4160 or equivalent)
- Hydromatrix (inert material for ASE, Varian #0019-8003 or equivalent)
- 2 M Ammonium hydroxide (NH_4OH, 30% NH_3; Baker #9721-3 or equivalent). Prepare by diluting 133 mL of 15M concentrate to 1 L with HPLC-grade water.
- Extraction solvent: ACN / water (1:1) containing 50 mM $CaCl_2$ and 10 mM NH_4OH. Prepare 1 L by dissolving 7.35 g of $CaCl_2 \cdot 2H_2O$ in 500 mL of HPLC-grade water. Add 5 mL of 2 M NH_4OH and dilute to 1 L volume with ACN.
- 0.1% Formic acid (v/v) in water: Prepare by adding 1 mL formic acid (J.T. Baker #0128-01) per L of water.
- 9:1 Water / MeOH with 0.1% acetic acid (v/v): Prepare by diluting 1 mL acetic acid (Mallinckrodt #3121) and 100 mL MeOH to 1 L with HPLC-grade water.
- MeOH with 0.1% phosphoric acid (v/v): Prepare by adding 1 mL phosphoric acid (Mallinckrodt #2796) to 1 L MeOH.

2.3 Analytical Standards

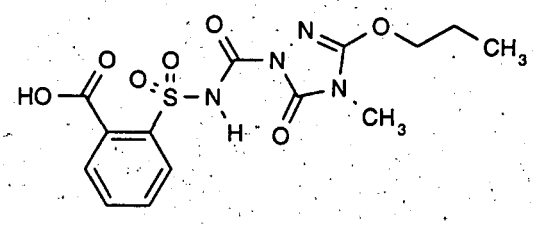
Name: MKH 6561 (sodium salt of MKH 5554, shown)
Ref. #: K-624 or equivalent
Formula: $C_{15}H_{18}O_7N_4S$
Mol. Wt.: 398.0
Purity: 99.2%
Nomen.: 4,5-Dihydro-3-propoxy-4-methyl-5-oxo-N-[[2-(carbomethoxy)phenyl] sulfonyl]-1H-1,2,4-triazole-1-carboxamide



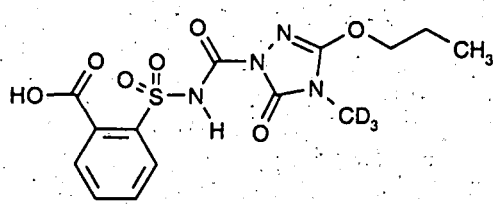
Name: MKH 6561-*N-methyl-d*$_3$ (sodium salt of MKH 5554, shown)
Ref. #: K-745 or equivalent
Formula: $C_{15}H_{15}O_7N_4SD_3$
Mol. Wt.: 401.0
Purity: 98.1% (chemical purity)
Nomen.: 4,5-Dihydro-3-propoxy-4-methyl-5-oxo-N-[[2-(carbomethoxy)phenyl] sulfonyl]-1H-1,2,4-triazole-1-carboxamide-*N*-methyl-d_3



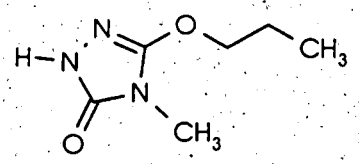
Name: MKH 6561 Carboxylic Acid
Ref. #: K-713 or equivalent
Formula: $C_{14}H_{16}O_7N_4S$
Mol. Wt.: 384.0
Purity: 96.7%
Nomen.: 2-[[[(4,5-dihydro-4-methyl-5-oxo-3-propoxy-1H-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]benzoic acid



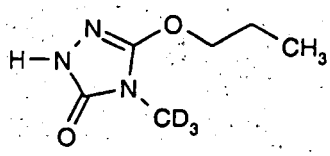
Name: MKH 6561 Carboxylic Acid-*N-methyl-d*$_3$
Ref. #: K-746 or equivalent
Formula: $C_{14}H_{16}O_7N_4SD_3$
Mol. Wt.: 387.0
Purity: 96.7% (chemical purity)
Nomen.: 2-[[[(4,5-dihydro-4-methyl-5-oxo-3-propoxy-1H-1,2,4-triazol-1-yl) carbonyl]amino]sulfonyl]benzoic acid-*N*-methyl-d_3



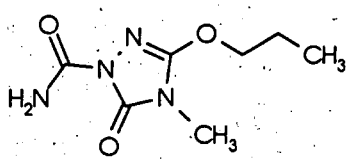
Name: *N*-Methyl Propoxy Triazolinone
Ref. #: K-748 or equivalent
Formula: $C_6H_{11}O_2N_3$
Mol. Wt.: 157.2
Purity: 99.5%
Nomen.: 4-methyl-3-propoxy-1,2,4-triazolin-5-one



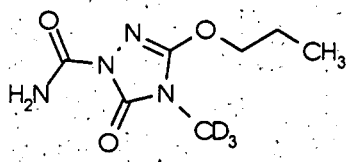
Name: *N*-Methyl Propoxy Triazolinone-*methyl-d*$_3$
Ref. #: K-744 or equivalent
Formula: $C_6H_8O_2N_3D_3$
Mol. Wt.: 160.0
Purity: 99.5% (chemical purity)
Nomen.: 4-methyl-3-propoxy-1,2,4-triazolin-5-one-methyl-d_3



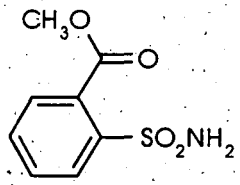
Name: *N*-Methyl Propoxy Triazolinone Amide (previously known as "Carboxamide Triazolinone")
Ref. #: K-798 or equivalent
Formula: $C_7H_{12}O_3N_4$
Mol. Wt.: 200.2
Purity: 97.3%
Nomen.: 4,5-dihydro-4-methyl-5-oxo-3-propoxy-1H-1,2,4-triazole-1-carboxamide



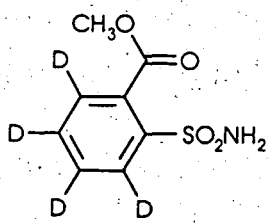
Name: *N*-Methyl Propoxy Triazolinone Amide-*methyl-d_3*
Ref. #: K-812 or equivalent
Formula: $C_7H_{12}O_3N_4D_3$
Mol. Wt.: 203.2
Purity: 98.1% (chemical purity)
Nomen.: 4,5-dihydro-4-methyl-5-oxo-3-propoxy-1H-1,2,4-triazole-1-carboxamide-N-methyl-d_3



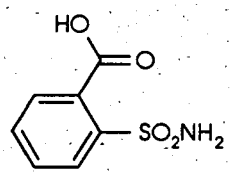
Name: MKH 6561 Sulfonamide Methyl Ester
Ref. #: K-715 or equivalent
Formula: $C_8H_9O_4NS$
Mol. Wt.: 215.0
Purity: 99.8%
Nomen.: 2-carbomethoxybenzene-sulfonamide



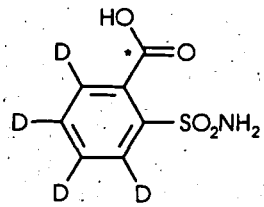
Name: MKH 6561 Sulfonamide Methyl Ester-*phenyl-3,4,5,6-d_4*
Ref. # : K-742 or equivalent
Formula: $C_8H_5O_4NSD_4$
Mol. Wt.: 219.0
Purity: 99.7% (chemical purity)
Nomen.: 2-carbomethoxybenzene-sulfonamide-*phenyl-3,4,5,6-d_4*



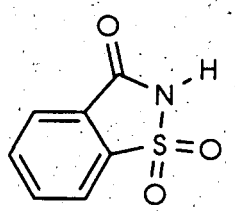
Name: MKH 6561 Sulfonamide Acid
Ref. #: K-712 or equivalent
Formula: $C_7H_7O_4NS$
Mol. Wt.: 201.0
Purity: 99.3%
Nomen.: 2-carboxybenzene-sulfonamide



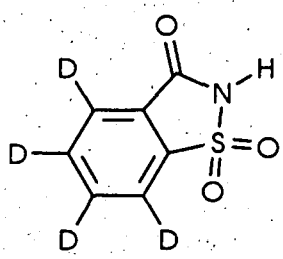
Name: MKH 6561 Sulfonamide Acid-*phenyl-3,4,5,6-d_4-carbonyl-^{13}C*
Ref. #: K-747 or equivalent
Formula: $C_6H_3O_4NS\ ^{13}C\ D_4$
Mol. Wt.: 206.0
Purity: 99% (chemical purity)
Nomen.: 2-carboxybenzene-sulfonamide-*phenyl-3,4,5,6-d_4-carbonyl-^{13}C*



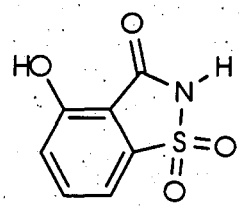
Name: Saccharin
Ref. #: K-727 or equivalent
Formula: $C_7H_5NO_4S$
Mol. Wt.: 183.2
Purity: 100.0%
Nomen.: *O*-benzoic sulfimide



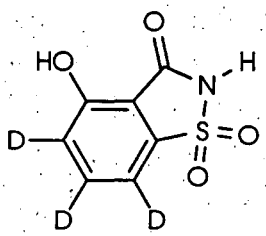
Name: Saccharin-*3,4,5,6-d_4*
Ref. #: K-743 or equivalent
Formula: $C_7HNO_4SD_4$
Mol. Wt.: 187.0
Purity: 99.3% (chemical purity)
Nomen.: *O*-benzoic sulfimide-*3,4,5,6-d4*



Name: 4-Hydroxy Saccharin
Ref. #: K-795 or equivalent
Formula: $C_7H_5NO_5S$
Mol. Wt.: 199.2
Purity: 99.8%
Nomen.: 4-hydroxy-1,2-benzothiazole-3(2H)-one-1,1-dioxide



Name: 4-Hydroxy Saccharin-*d_3*
Ref. #: K-817 or equivalent
Formula: $C_7H_5NO_5S$-*D_3*
Mol. Wt.: 202.2
Purity: 98.9% (chemical purity)
Nomen.: 4-hydroxy-1,2-benzothiazole-3(2H)-one-1,1-dioxide



2.4 Safety and Health

The toxicity or carcinogenicity of each chemical used in this method has not been precisely determined, and thus each compound must be treated as a potential health

hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available.

3.0 Procedures

3.1 Preparation of Standard Solutions and Reagents

3.1.1 Native Analyte Stock Solutions

Prepare separate 500-ppm stock solutions (nominally 0.5 mg/mL) of MKH 6561 (K-624 or equivalent), MKH 6561 carboxylic acid (K-713 or equivalent), *N*-methyl propoxy triazolinone (K-748 or equivalent), *N*-methyl propoxy triazolinone amide (K-798 or equivalent), MKH 6561 sulfonamide methyl ester (K-715 or equivalent), MKH 6561 sulfonamide acid (K-712 or equivalent), saccharin (K-727 or equivalent) and 4-hydroxy saccharin (K-795 or equivalent) standards by weighing 5-10 mg of each using an analytical balance (with 0.01-mg readout), adding the corresponding volume of ACN/water 1:1 (for MKH 6561 sulfonamide methyl ester, use MeOH containing 0.1% H_3PO_4) and mixing; for example, dissolve 7.10 mg of standard in 14.20 mL of solvent. Correct added volume for % purity if standard is <99%. Store solutions in a freezer when not in use.

3.1.2 Native Analyte Spiking Solutions

Prepare 2.5-ppm and 0.25-ppm mixed spiking solutions (nominally 2.5 and 0.25 ng/µL, respectively) containing MKH 6561 (K-624 or equivalent), MKH 6561 carboxylic acid (K-713 or equivalent), *N*-methyl propoxy triazolinone (K-748 or equivalent), *N*-methyl propoxy triazolinone amide (K-798 or equivalent), MKH 6561 sulfonamide acid (K-712 or equivalent), saccharin (K-727 or equivalent) and 4-hydroxy saccharin (K-795 or equivalent) standards first by removing the stock solutions from section 3.1.1 from the freezer and allowing them to equilibrate at room temperature. Do not include the MKH 6561 sulfonamide methyl ester (K-715 or equivalent), as it will be prepared as an individual solution. For the 2.5-ppm solution, add the appropriate volume of each stock solution (see compound list above) to obtain a 1:200 dilution in ACN/water 1:1 (for example, add 500 µL of each 500-ppm stock solution to a 100-mL volumetric flask, bring to volume and mix). To prepare the 0.25-ppm solution, for example, add 50 µL of each 500-ppm stock solution to a 100-mL volumetric flask, bring to volume and mix. The 0.25-ppm solution can also be prepared by making a 1:10 dilution of the 2.5-ppm solution. Store solutions in a freezer when not in use.

Prepare separate 2.5-ppm and 0.25-ppm spiking solutions (nominally 2.5 and 0.25 ng/µL, respectively) of the MKH 6561 sulfonamide methyl ester (K-715 or equivalent) by performing a dilution as above from the 500-ppm stock solution and diluting up to volume with MeOH containing 0.1% H_3PO_4.

3.1.3 Internal Standard Stock Solutions

Prepare 500-ppm stock solutions (nominally 0.5 mg/mL) MKH 6561-*N-methyl-d_3* (K-745 or equivalent), MKH 6561 carboxylic acid-*N-methyl-d_3* (K-746 or equivalent), *N*-methyl propoxy triazolinone-*methyl-d_3* (K-744 or equivalent), *N*-methyl propoxy triazolinone amide-*methyl-d_3* (K-812 or equivalent), MKH 6561 sulfonamide methyl ester-*phenyl-3,4,5,6-d_4* (K-742 or equivalent), MKH 6561 sulfonamide acid-*phenyl-3,4,5,6-d_4-carbonyl-^{13}C* (K-747 or equivalent), saccharin-*phenyl-3,4,5,6-d_4* (K-743 or equivalent) and 4-hydroxy saccharin-*d_3* (K-817 or equivalent) standards by weighing 5-10 mg of each using an analytical balance (with 0.01-mg readout), adding the corresponding volume of ACN/water 1:1 (for MKH 6561 sulfonamide methyl ester, use MeOH containing 0.1% H_3PO_4) and mixing; for example, dissolve 7.10 mg of standard in 14.20 mL of solvent. Correct added volume for % purity if standard is <99%. Store solutions in a freezer when not in use.

3.1.4 Internal Standard Spiking Solution

Prepare a 2.5-ppm mixed spiking solution (nominally 2.5 ng/µL) of MKH 6561-*N-methyl-d_3* (K-745 or equivalent), MKH 6561 carboxylic acid-*N-methyl-d_3* (K-746 or equivalent), *N*-methyl propoxy triazolinone-*methyl-d_3* (K-744 or equivalent), *N*-methyl propoxy triazolinone amide-*methyl-d_3* (K-812 or equivalent), MKH 6561 sulfonamide acid-*phenyl-3,4,5,6-d_4-carbonyl-^{13}C* (K-747 or equivalent), saccharin-*phenyl-3,4,5,6-d_4* (K-743 or equivalent) and 4-hydroxy saccharin-*d_3* (K-817 or equivalent) standards by removing the stock solutions from section 3.1.3 from the freezer, allowing them to equilibrate at room temperature, and then adding the appropriate volume of each 500-ppm stock solution (see compound list above) to obtain a 1:200 dilution in ACN/water 1:1 (for example, add 500 µL of each stock solution to a 100-mL volumetric flask, bring to volume and mix). Do not include the MKH 6561 sulfonamide methyl ester-*phenyl-3,4,5,6-d_4* (K-742 or equivalent) as it will be prepared as an individual solution.

Prepare a separate 2.5-ppm spiking solution (nominally 2.5 ng/µL) of MKH 6561 sulfonamide methyl ester-*phenyl-3,4,5,6-d_4* (K-742 or equivalent) by performing a dilution as above from the 500-ppm stock solution and diluting up to volume with MeOH containing 0.1% H_3PO_4.

3.2 Extraction of Soil Samples

Process soil samples as follows:

1. Weigh soil into a plastic weighing pan. See table below to determine sample weight.

2. Add hydromatrix and mix with the soil thoroughly using a spatula (see table below for sample weight). Transfer mixture to an ASE sample cell using a funnel.

	Soil Type	
	Light, Sandy Soils [1]	**Heavy, Clay soils [2]**
Sample wt	25 g	15 g
Hydromatrix wt	2 g	4 g
Final aliquot volume	1/4th of final extract volume	5/12th of final extract volume
Target sample aliquot in final extract	6.25 g	6.25 g
Amount of internal standard solution added	100 µL	60 µL
Amount of native analyte spiking solution added for QC recovery samples	50 µL	30 µL
Amount of sand used for reagent blank samples [3]	35 g	30 g

[1] Conditions used for Washington dissipation study (see Table 1 for soil characteristics).
[2] Conditions used for Kansas dissipation study (see Table 1 for soil characteristics).
[3] Reagent blanks are prepared by mixing sand with specified amount of hydromatrix and filling ASE cells to the same level as the samples.

3. Add the appropriate volume of the two 2.5-ng/µL internal standard spiking solutions from section 3.1.4 to the sample cell (amount depends on amount of soil extracted; see table above for specified volume), resulting in a 10 ppb fortification. Fortify recovery samples with the appropriate volume of the two 2.5-ng/µL native analyte spiking solutions, resulting in a 5-ppb fortification (amount depends on amount of soil extracted; see table above for specified volume). Cap the top of the cell firmly.

4. Place sample cell onto ASE unit and extract using the conditions listed in the table below. The purge time may need to be adjusted depending on the particular soil type being extracted (lengthen time for heavy clay soils). Adjust other parameters if

needed to obtain recovery of analytes (soil characteristics may affect recovery of analytes).

ASE Conditions		
Parameter	**Light, Sandy Soils [1]**	**Heavy, Clay soils [2]**
Solvent	ACN/water (1:1) with 50 mM $CaCl_2$ and 10 mM NH_4OH	ACN/water (1:1) with 50 mM $CaCl_2$ and 10 mM NH_4OH
Heat Cycle	5 min	5 min
Temp	80 °C	80 °C
Static Cycle	5 min	5 min
Flush	50%	75%
Purge	120 sec	120 sec

[1] Conditions used for Washington field dissipation study.
[2] Conditions used for Kansas field dissipation study.

Note: Proper care of the ASE and cells are required. After use and disassembly, the cells are cleaned in the following manner. The cell bodies are rinsed with de-ionized water using a brush followed by rinses of methanol and acetone. The cell caps are rinsed with de-ionized water, then sonicated in methanol followed by acetone. The cell caps are sonicated in each organic solvent for approximately 5 minutes.

5. Obtain the total extraction volume from the ASE report or screen.

6. Transfer an aliquot of the extract, equivalent to approximately 6.25 g of soil, into a 20 x 150 test tube and evaporate solvent under nitrogen to approximately 0.2 mL at 50 °C on a Turbovap unit.

7. Reconstitute residue in 1 mL of 9:1 water/MeOH containing 0.1% acetic acid.

8. Filter the extract through a 0.45-µm Acrodisc filter into an HPLC vial for analysis. If previous experience with a particular soil or soil depth dictates (i.e. filter clogs), split the sample into two aliquots for filtering (larger diameter filters could be used if the loss in the filter is acceptable).

3.3 HPLC/MS-MS Analysis

Instrumental conditions used are given below. Please note that the Washington study was conducted earlier and some of the HPLC parameters were changed or improved for the analysis of Kansas soil samples.

3.3.1 HPLC Conditions for Analysis of MKH 6561, *N*-Methyl Propoxy Triazolinone, *N*-Methyl Propoxy Triazolinone Amide and MKH 6561 Sulfonamide Methyl Ester

Column:	Phenomenex Columbus C18, 50 x 2 mm, 5 µ, or equivalent
Flow rate:	0.35 mL/min
Injection volume:	2 µL on Sciex API III Plus instrument and 7 µL on Sciex API 365 (can be adjusted up to 20 µL, depending on the condition of instrument)
Solvents/gradient:	Solvent A = water + 0.1% formic acid Solvent B = acetonitrile/water (9:1, v/v) + 0.1% formic acid + 5 mM ammonium acetate

Washington Samples		
Time (min)	% Solvent A	% Solvent B
0	100	0
3	100	0
4	80	20
8.5	30	70
9	100	0
14	100	0

Approximate retention times:

N-Methyl propoxy triazolinone	=	7.6 min.
N-Methyl propoxy triazolinone amide	=	7.7 min.
MKH 6561 Sulfonamide methyl ester	=	8.0 min.
MKH 6561	=	10.1 min.

Kansas Samples		
Time (min)	% Solvent A	% Solvent B
0	90	10
4	80	20
6	63	37
8	30	70
8.5	20	80
9	90	10
14	90	10

Approximate retention times:

N-Methyl propoxy triazolinone	=	4.3 min.
N-Methyl propoxy triazolinone amide	=	5.0 min.
MKH 6561 Sulfonamide methyl ester	=	5.5 min.
MKH 6561	=	10.1 min.

During the first 2.5 min., the column effluent is diverted to waste. The analytes *N*-methyl propoxy triazolinone, *N*-methyl propoxy triazolinone amide and MKH 6561 sulfonamide methyl ester are detected using positive-ion mode. After elution of these three analytes, instrument is switched to negative-ion mode for the detection of MKH 6561. Note: The column or conditions used may be modified if necessary, but should be clearly documented in the study raw data.

3.3.2 HPLC Conditions for Analysis of MKH 6561 Carboxylic Acid, MKH 6561 Sulfonamide Acid, Saccharin and 4-Hydroxy Saccharin

Column:	Phenomenex Columbus C18, 50 x 2 mm, 5 µ, or equivalent
Flow rate:	0.35 mL/min.
Injection volume:	10 µL on Sciex API III Plus instrument (can be adjusted up to 50 µL, depending on the condition of instrument) and 50 µL on Sciex API 365
Solvents/gradient:	Solvent A = Water + 0.1% formic acid Solvent B = acetonitrile/water (9:1, v/v) + 0.1% formic acid + 5 mM ammonium acetate

Washington Samples		
Time (min)	% Solvent A	% Solvent B
0	100	0
2	100	0
3	80	20
8.5	30	70
9	100	0
14	100	0

Approximate retention times:

MKH 6561 sulfonamide acid = 6.4 min.
Saccharin = 7.3 min.
4-Hydroxy saccharin = 8.1 min.
MKH 6561 carboxylic acid = 9.3 min.

Kansas Samples		
Time (min)	% Solvent A	% Solvent B
0	100	0
2	100	0
4	80	20
8.5	30	70
9	100	0
14	100	0

Approximate retention times:

MKH 6561 sulfonamide acid = 6.1 min.
Saccharin = 6.3 min.
4-Hydroxy saccharin = 7.1 min.
MKH 6561 carboxylic acid = 8.3 min.

During the first four minutes of the run, the column effluent was diverted to waste. All of the analytes are detected using negative-ion mode.

Note: The column or conditions used may be modified if necessary, but should be clearly documented in the study raw data.

3.3.3 MS-MS Conditions

The mass spectrometer (MS) must be optimized to monitor the daughter ions for each analyte at the mass unit resolution. The MS scan summaries of precursor → product transition pairs are presented in Table 2. Turbo-Ion Spray ionization technique is used for all analyses. Due to the large number of analytes in this method and difficulty in sufficiently separating many of them, two separate LC/MS analyses are made for each sample. This may be modified if instrument sensitivity is sufficient to allow simultaneous analysis of multiple analytes. Basic instrument parameters are listed below. Typical instrument conditions are listed in Tables 3 and 4 (Sciex API 365 instrument) and Tables 5 and 6 (Sciex API III Plus instrument):

Interface heater	60 °C
Nebulizer temp.:	500 °C
Nebulizer gas:	80 psi
Curtain gas:	1.2 L/min.
Auxiliary gas:	8 mL/min.

MS state file parameters used for the field dissipation analyses are listed in Table 3 and 4 (API 365 instrument) and Table 5 and 6 (API III instrument).

3.3.4 Calibration Curve

A minimum four point calibration curve should be prepared by analyzing at least four concentrations of standards (e.g. 6.25 ng/mL, 31.3 ng/mL, 62.5 ng/mL, 156.3 ng/mL and 312.5 ng/mL (optional)), which are 1-, 5-, 10-, 25- and 50- ppb standards of sample equivalents), each containing 62.5 ng/mL (10-ppb sample equivalent) of the internal standards by LC/MS-MS. These should be prepared from the standard solutions from sections 3.1.2 and 3.1.4 as detailed below (see Table 7 for solution parameters). The calibration curve standards will be injected at the beginning of each set of samples. Selected calibration standards will be re-injected at regular intervals during the run to monitor changes in instrumental sensitivity and reproducibility. All of these standard injections will be part of the calibration curve.

If samples contain >50 ppb of analyte (or greater than upper level of calibration curve), they should be re-injected using a calibration curve containing higher concentration standards to cover the concentration range.

The calibration curve should be linear ($r^2 \geq 0.98$), otherwise, dilution samples should be re-extracted for the analysis.

All standards should be stored in a refrigerator when not in use and should be routinely monitored for degradation.

1-ppb sample-equivalent standard (6.25 ng/mL): Prepare by adding 25 µL of the two 2.5-ppm native analyte spiking solutions from section 3.1.2 and 250 µL of the two 2.5-ppm internal standard spiking solutions from section 3.1.4 to a 10-mL volumetric flask, bringing to volume with 9:1 water/MeOH containing 0.1% acetic acid, and mixing.

5-ppb sample-equivalent standard (31.3 ng/mL): Prepare by adding 125 µL of the two 2.5-ppm native analyte spiking solutions from section 3.1.2 and 250 µL of the two 2.5-ppm internal standard spiking solutions from section 3.1.4 to a 10-mL volumetric flask, bringing to volume with 9:1 water/MeOH containing 0.1% acetic acid, and mixing.

10-ppb sample-equivalent standard (62.5 ng/mL): Prepare by adding 250 µL of the two 2.5-ppm native analyte spiking solutions from section 3.1.2 and 250 µL of the two 2.5-ppm internal standard spiking solutions from section 3.1.4 to a 10-mL volumetric flask, bringing to volume with 9:1 water/MeOH containing 0.1% acetic acid, and mixing.

25-ppb sample-equivalent standard (156.3 ng/mL): Prepare by adding 625 µL of the two 2.5-ppm native analyte spiking solutions from section 3.1.2 and 250 µL of the two 2.5-ppm internal standard spiking solutions from section 3.1.4 to a 10-mL volumetric flask, bringing to volume with 9:1 water/MeOH containing 0.1% acetic acid, and mixing.

50-ppb sample-equivalent standard (312.5 ng/mL, optional): Prepare by adding 1250 µL of the two 2.5-ppm native analyte spiking solutions from section 3.1.2 and 250 µL of the two 2.5-ppm internal standard spiking solutions from section 3.1.4 to a 10-mL volumetric flask, bringing to volume with 9:1 water/MeOH containing 0.1% acetic acid, and mixing.

3.3.5 Quantitation

The concentration (ppb) of each analyte is calculated by applying the area

ratio (native analyte area to internal standard area) to the calibration curve. This can be performed by the MS software, MacQuan1.5 version program (PE Sciex).

Linear Regression

Regression (calibration) curves for each analyte are calculated by plotting the peak area ratio of each native analyte and its deuterated internal standard (IS) versus the concentration of the analyte. A linear, 1/x weighted regression, is performed using the equation:

$$y = a + b\,x$$

where: y = area ratio of analyte to IS
x = concentration of analyte
a = intercept
b = slope

Calculation of Analyte Concentration:

After performing linear regression for the calibration curves (correlation coefficient must be ≥0.98) and determining the slope and intercept parameters, analyte concentrations are calculated using the equation:

$$\textit{Analyte Conc. (ppb)} = \frac{y\,(\textit{area ratio}) - a\,(\textit{intercept})}{b\,(\textit{slope})}$$

Concentrations of calibration standards should also be calculated along with the samples. Back calculated standards must have an accuracy of 80-120% of the theoretical concentrations.

Calculation of Percent Recovery in Spiked Validation Samples

Recovery of spiked validation samples is calculated as follows:

$$\% \text{ Recovery} = 100 \text{ X } \frac{\text{Conc}_{Nat}}{\text{Fortification Level}}$$

Where: $Conc_{NAT}$ = calculated amount (ppb in sample), uploaded from the mass spectrometer

Fortification level = the concentration (ppb) at which the matrix spike was prepared

3.3.6 Potential Trouble-Shooting

Potential problems faced in the analysis may include sensitivity problems, cross-talk, matrix interference, and analytical standard degradation.

Sensitivity Problems: Sensitivity for different analytes may vary significantly and it is difficult to optimize the MS instrument for all of them simultaneously. Particularly in the positive-ion mode (see section 3.3.1), the sensitivities of *N*-methyl propoxy-triazolinone and *N*-methyl propoxy-triazolinone amide are usually about 3 orders of magnitude higher than that of MKH 6561 sulfonamide methyl ester. Therefore, if there is not a balance of sensitivities for all compounds, there may be either not enough sensitivity for MKH 6561 sulfonamide methyl ester or the instrument could be too sensitive for the other two analytes to produce linear calibration curves. It is not recommended that the instrument parameters be switch between individual analyte acquisitions because the parameter switch time and stabilization time may cause instability in the instrument which could affect the sensitivity. Therefore, the instrumental conditions should be carefully optimized for the best compromise of results; i.e., suitable sensitivity and reproducibility.

Cross-Talk: Though LC/MS-MS techniques do not usually require complete separation of all analytes, "cross-talk" between analytes may still occur under certain conditions. For example, *N*-methyl propoxy triazolinone amide can potentially fragment before Q1 (first quadrupole) to produce its daughter ion (m/z 158), which is the parent ion of *N*-methyl propoxy triazolinone, thus resulting in interference. Therefore, the MS parameters should be adjusted to avoid such a fragmentation in Q1. The instrument should be operated at the mass unit resolution. Alternatively, the HPLC gradient can be modified to fully resolve these analytes.

Matrix Interference: Soil samples are extracted with solvent containing high concentrations of $CaCl_2$, so the extracts become gelatinous after evaporating the organic solvent. A switching valve is highly recommended after the column to divert the effluent to the waste during the first few minutes of the analytical run in order to remove inorganic salt(s). If a switching valve is not used, such inorganic material is deposited at the orifice when the sample is vaporized in the interface and may easily clog the inlet or further contaminate the instrument. The sensitivity would decrease quickly until it was unsatisfactory.

Depending on the soil, some organic compounds from the soil matrix may also interfere with quantification. Modifying the LC gradient can usually resolve such interference.

Analytical Standard Degradation: All standards and samples need to be stored in the freezer before analysis. Standard solutions of the MKH 6561 sulfonamide methyl ester and its deuterated internal standard should be prepared in acidic MeOH as described in section 3.1.2. There is evidence that MKH 6561 sulfonamide methyl ester can convert to saccharin under alkaline conditions, thus reducing the response for this analyte. If this problem is, it may be necessary to neutralize the extracts quickly after completion of the extraction.

3.4 Method Validation

3.4.1 Recovery Analyses

1. Weigh twelve samples into ASE cells as described in section 3.2 above.

2. Designate two samples as control matrix samples, five samples as 1-ppb spikes (LOQ), and five samples as 10-ppb spikes (10 X LOQ). A reagent blank (sand + hydromatrix) should also be included in each set (see section 3.2 for weights).

3. Fortify the 1-ppb samples with the appropriate volume of the 0.25-ppm native analyte spiking solutions (section 3.1.2) and the 10-ppb samples with the 2.5-ppm native analyte spiking solutions (section 3.1.2). The fortification parameters for the recovery analyses are given in Table 8. See also section 3.2 for correct sample weights and spiking volumes used for different types of soils.

4. Fortify each sample with the appropriate volume of the internal standard spiking solution (section 3.1.4; Table 8). See section 3.2 for correct sample weights and spiking volumes. Process and analyze the samples as described in sections 3.2 (starting at step #4) through 3.3.

3.4.2 Linearity Determination in Matrix

Prepare a five-point (minimum) HPLC/MS-MS matrix linearity curve using 0-, 2.5-, 10-, 25- and 50-ppb matrix standards prepared as follows. *Note: These concentrations are only examples, as other specific concentrations may be requested by the study director.*

1. Weigh five samples into ASE cells as described in section 3.2 above.

2. Process the samples as described in section 3.2, steps #4 through #6 (do not add internal standard solutions as described in step #3).

3. Fortify the extracts as follows (see Table 9 for sample fortification parameters):

   0-ppb sample-equivalent (0 ng/mL): Prepare by adding 100 µL of the two 2.5-ppm internal standard spiking solutions from section 3.1.4 to one of the control extracts. (Sample is not spiked with the native analyte spiking solutions).

   2.5-ppb sample-equivalent (15.6 ng/mL): Prepare by adding 62.5 µL of the two 0.25-ppm native analyte spiking solutions from section 3.1.2 and 100 µL of the two 2.5-ppm internal standard spiking solutions from section 3.1.4 to one of the control extracts.

   10-ppb sample-equivalent (62.5 ng/mL): Prepare by adding 250 µL of the two 0.25-ppm native analyte spiking solutions from section 3.1.2 and 100 µL of the two 2.5-ppm internal standard spiking solutions from section 3.1.4 to one of the control extracts.

   25-ppb sample-equivalent (156.3 ng/mL): Prepare by adding 62.5 µL of the two 2.5-ppm native analyte spiking solutions from section 3.1.2 and 100 µL of the two 2.5-ppm internal standard spiking solutions from section 3.1.4 to one of the control extracts.

50-ppb sample-equivalent (312.5 ng/mL): Prepare by adding 125 µL of the two 2.5-ppm native analyte spiking solutions from section 3.1.2 and 100 µL of the two 2.5-ppm internal standard spiking solutions from section 3.1.4 to one of the control extracts.

4. Complete the sample preparation as descibed in section 3.2, steps #7-8.

5. Analyze the matrix standards by LC/MS-MS in triplicate as described in section 3.3.

3.4.3 Determination of Limits of Detection (LOD) and Limits of Quantitation (LOQ)

The LOD is defined statistically as 3.143 (students t-value) times the sample standard deviation of replicate samples analyzed at a level approximating 1 to 5 times the estimated LOD. Although the LOQ is sometime defined as 3 to 5 times the LOD, it will defined here as the level at which acceptable recoveries (70 to 120%, RSD ≤20%) are demonstrated experimentally.

3.5 Moisture Determination

1. Thaw soil samples. Calibrate top-loading balance.

2. Weigh aluminum weighing dish (W_1) and record weight.

3. Tare balance, add ~10 g of soil, re-weigh and record weight of moist soil (W_2).

4. Place dish oven at approx. 120 °C for a minimum of 18 hours.

5. Re-weigh soil plus dish (W_3).

6. Calculations:

$$\% \, Moisture = \frac{W_2 - W_3}{W_2 - W_1}$$

Table 2. Mass spectrometer scan summary.

Scan Summary [1]						
Analyte	**Type**	**Ionization Mode**	**Parent Ion (*m/z*)**	**Daughter Ion (*m/z*)**	**Scan time (sec)**	**Collision Energy (mV)**
MKH 6561 Carboxylic Acid	Native	Neg.	383	182	0.15	16
MKH 6561 Carboxylic Acid-*N-methyl-d_3*	Int. Std.	Neg.	386	182	0.15	16
MKH 6561 Sulfonamide Acid	Native	Neg.	200	156	0.15	16
MKH 6561 Sulfonamide Acid-*phenyl-3,4,5,6-d_4-carbonyl-^{13}C*	Int. Std.	Neg.	205	160	0.15	16
Saccharin	Native	Neg.	182	106	0.15	16
Saccharin-*3,4,5,6-d_4*	Int. Std.	Neg.	186	106	0.15	16
4-Hydroxy Saccharin	Native	Neg.	198	135	0.15	16
4-Hydroxy Saccharin-*^{13}C,^{15}N*	Int. Std.	Neg.	201	138	0.15	16
N-Methyl Propoxy Triazolinone	Native	Pos.	158	116	0.20	-10
N-Methyl Propoxy-Triazolinone-*methyl-d_3*	Int. Std.	Pos.	161	119	0.20	-10
N-Methyl Propoxy Triazolinone Amide	Native	Pos.	201	158	0.20	-10
N-Methyl Propoxy Triazolinone Amide-*methyl-d_3*	Int. Std.	Pos.	204	161	0.20	-10
MKH 6561 Sulfonamide Methyl Ester	Native	Pos.	233 [2]	199	0.20	-10
MKH 6561 Sulfonamide Methyl Ester-*phenyl-3,4,5,6-d_4*	Int. Std.	Pos.	237 [2]	203	0.20	-10
MKH 6561	Native	Neg.	397	156	0.45	16
MKH 6561-*N-methyl-d_3*	Int. Std.	Neg.	400	159	0.45	16

[1] Based on API III plus instrument.

[2] Parent ion for MKH 6561 sulfonamide ester is not molecular ion, but is solvent adduct ion.

Table 3. PE Sciex API 365 instrument state files for analysis of *N*-methyl propoxy triazolinone, *N*-methyl propoxy triazolinone amide, MKH 6561 sulfonamide methyl ester and MKH 6561.

Positive-Ion Detection Parameters		Negative-Ion Detection Parameters	
Parameter	**Value**	**Parameter**	**Value**
IS	5000.000	IS	-4500.000
TEM	450.000	TEM	500.000
OR	0.000	OR	-20.000
RNG	80.000	RNG	-150.000
Q0	-9.500	Q0	5.000
IQ1	-11.000	IQ1	7.000
ST	-14.000	ST	10.000
RO1	-11.000	RO1	6.000
IQ2	-20.000	IQ2	15.000
RO2	-25.000	RO2	20.000
IQ3	-40.000	IQ3	35.000
RO3	-30.000	RO3	25.000
DF	-200.000	DF	200.000
CEM	2000.000	CEM	-2000.000
NEB	10	NEB	14
CUR	8	CUR	8
CAD	6	CAD	8
Aux. Gas	8 L/min	Aux. Gas	8 L/min.

Notes

During the first four minutes of the analytical run, the column effluent is diverted to waste. The analytes *N*-methyl propoxy triazolinone, *N*-methyl propoxy triazolinone amide and MKH 6561 sulfonamide methyl ester are detected using positive-ion mode. After elution of these three analytes, instrument is switched to negative-ion mode for the detection of MKH 6561.

These conditions may need to be modified slightly to obtain the best results on the instrument being used, but any changes should be clearly documented in the study raw data.

Table 4. PE Sciex API 365 instrument state files for analysis of MKH 6561 sulfonamide acid, saccharin, 4-hydroxy saccharin and MKH 6561 carboxylic acid.

Negative-Ion Detection Parameters	
Parameter	**Value**
IS	-4000.00
TEM	450.00
OR	-45.00
RNG	-210.00
Q0	10.00
IQ1	12.20
ST	16.50
RO1	11.00
IQ2	25.00
RO2	30.00
IQ3	45.00
RO3	35.00
DF	200.00
CEM	-2000.00
NEB	14
CUR	7
CAD	6
Aux. Gas	8 L/min

Notes

All of the analytes are detected using negative-ion mode.

These conditions may need to be modified slightly to obtain the best results on the instrument being used, but any changes should be clearly documented in the study raw data.

Table 5. PE Sciex API III instrument state files for analysis of *N*-methyl propoxy triazolinone, *N*-methyl propoxy triazolinone amide, MKH 6561 sulfonamide methyl ester and MKH 6561.

Positive-Ion Detection Parameters		Negative-Ion Detection Parameters	
Parameter	**Value**	**Parameter**	**Value**
DI	50.00	DI	50.00
ISV	4500.00	ISV	-3400.00
IN	650.00	IN	-650.00
OR	45.00	OR	-65.00
R0	30.00	R0	-30.00
M1	500.00	M1	500.00
RE1	114.00	RE1	111.00
DM1	0.06	DM1	0.05
R1	23.20	R1	-26.00
L7	25.00	L7	-19.00
R2	20.00	R2	-14.00
M3	500.00	M3	500.00
RE3	114.60	RE3	113.90
DM3	0.06	DM3	0.08
RX	5.00	RX	1.00
R3	15.00	R3	-9.00
L9	-250.00	L9	250.00
FP	-250.00	FP	250.00
MU	-4000.00	MU	4400.00
CGT (Ar)	242.70	CGT (Ar)	250.61
Interface Heater	60°C	Interface Heater	60°C
Nebulizer Gas (N_2)	80 psi	Nebulizer Gas (N_2)	80 psi
Curtain Gas (N_2)	1.2 L/min	Curtain Gas (N_2)	1.2 L/min
Turbo Gun Temp.	500°C	Turbo Gun Temp.	500°C
Turbo Gun Gas (N_2, Auxiliary Gas)	8 L/min	Turbo Gun Gas (N_2, Auxiliary Gas)	8 L/min

Notes

During the first four minutes of the analytical run, the column effluent is diverted to waste. The analytes *N*-methyl propoxy triazolinone, *N*-methyl propoxy triazolinone amide and MKH 6561 sulfonamide methyl ester are detected using positive-ion mode. After elution of these three analytes, instrument is switched to negative-ion mode for the detection of MKH 6561.

These conditions may need to be modified slightly to obtain the best results on the instrument being used, but any changes should be clearly documented in the study raw data.

Table 6. PE Sciex API III instrument state files for analysis of MKH 6561 sulfonamide acid, saccharin, 4-hydroxy saccharin and MKH 6561 carboxylic acid.

Negative-Ion Detection Parameters	
Parameter	**Value**
DI	50.00
ISV	-3400.00
IN	-650.00
OR	-65.00
R0	-30.00
M1	500.00
RE1	111.00
DM1	0.05
R1	-26.00
L7	-19.00
R2	-14.00
M3	500.00
RE3	113.90
DM3	0.08
RX	1.00
R3	-9.00
L9	250.00
FP	250.00
MU	4400.00
CGT (Ar)	250.61
Interface Heater	60°C
Nebulizer Gas (N_2)	80 psi
Curtain Gas (N_2)	1.2 L/min
Turbo Gun Temp.	500°C
Turbo Gun Gas (N_2, Auxiliary Gas)	8 L/min

Notes

All of the analytes are detected using negative-ion mode.

These conditions may need to be modified slightly to obtain the best results on the instrument being used, but any changes should be clearly documented in the study raw data.

Table 7. Sample fortification parameters for preparation of calibration curve.

Sample Fortification Level (ppb) [1]	Amount of Native Analytes Added to Prepare Solution [2]		Native Analyte Conc. in 10-mL Final Solutions [3]	Amount of Internal Standard Added to Prepare Solution [4]		Internal Standard Concentrations in Final Solutions [5]
	ng	µL Spike Solution	ng/mL	ng	µL Spike Solution	ng/mL
1.0	62.5	25	6.25	625	250	62.5
5.0	313	125	31.3	625	250	62.5
10	625	250	62.5	625	250	62.5
25 [5]	1563	625	156.3	625	250	62.5

[1] Concentration level in soil sample equivalents (6.25 g in final aliquot).

[2] Amount of the two 2.5-ppm native analyte spiking solutions from section 3.1.2.

[3] Volume of calibration solutions is 10 mL.

[4] Amount of the two 2.5-ppm internal standard spiking solutions from section 3.1.4.

[5] Equal to 10 ppb in soil sample equivalents.

[6] If sample concentrations of analytes are over calibration curve limit (>25 ppb), these samples can be re-analyzed by adding a higher level (50 ppb) and dropping the lowest level (1 ppb) for the calibration curve.

Table 8. Sample fortification parameters for recovery analyses.

Sample Fortification Level (ppb)	Amount of Native Analytes Added per Sample [1]		Native Analyte Concentrations in Final Solutions	Amount of Internal Standard Added per Sample [1]		Internal Standard Concentrations in Final Solutions [2]
	ng	µL Spike Solution	ng/mL	ng	µL Spike Solution [1,3]	ng/mL
0 [3]	0	0	0	250	100 [1]	62.5
1.0	25	100 [1,5]	6.25	250	100 [1]	62.5
10	250	100 [1,6]	62.5	250	100 [1]	62.5

[1] Amounts specified are for 25-g sample (6.25 g in final aliquot). If only 15 g of soil is used for a heavy soil, correct the volumes correspondingly (60 µL).

[2] Equal to 10 ppb in soil sample equivalents.

[3] Amount of the two 2.5-ppm internal standard spiking solutions from section 3.1.4.

[4] Control sample (not fortified with native analytes).

[5] Amount of the two 0.25-ppm native standard spiking solutions from section 3.1.2.

[6] Amount of the two 2.5-ppm native standard spiking solutions from section 3.1.2.

Table 9. Sample fortification parameters for matrix linearity.

Sample Fortification Level (ppb) [1]	Amount of Native Analytes Added per Sample		Native Analyte Concentrations in Final Solutions	Amount of Internal Standard Added per Sample		Internal Standard Concentrations in Final Solutions [2]
	ng	µL Spike Solution	ng/mL	ng	µL Spike Solution [3]	ng/mL
0	0	0	0	62.5	25	62.5
0.5	3.12	12.5 [4]	3.12	62.5	25	62.5
2.5	15.6	62.5 [4]	15.6	62.5	25	62.5
10	62.5	250 [4]	62.5	62.5	25	62.5
25	156.3	62.5 [5]	156.3	62.5	25	62.5

[1] Concentration level in soil sample equivalents (25-g sample; 6.25 g in final sample aliquot).

[2] Equal to 10 ppb in soil sample equivalents.

[3] Amount of the two 2.5-ppm internal standard spiking solutions from section 3.1.4.

[4] Amount of the two 0.25-ppm native analyte spiking solutions from section 3.1.2.

[5] Amount of the two 2.5-ppm native analyte spiking solutions from section 3.1.2.