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Title: Determination of Tebuthiuron and its Metabolites 104, 106, 107, and 108 in Well Water By Liquid Chromatography- Atmospheric Pressure Chemical Ionization Mass Spectrometry

1. Scope:

This section method (SM) is applicable to the analysis of Tebuthiuron and the following metabolites: 104 (N-(5-(1,1-Dimethylethyl)-1,3,4-thiadiazol-2-yl)-N-methylurea), 106 (N-(5-(1,1-Dimethylethyl)-1,3,4-thiadiazol-2-yl)-urea), 107 (2-Dimethylethyl-5-methylamino-1,3,4-thiadiazol) and 108 (2-Dimethylethyl-5-amino-1,3,4-thiadiazole) in well water using HPLC/MS/MS. The reporting limit for all analytes is 0.05 ppb.

2. Principle:

A conditioned Water's Oasis® MCX Cartridge is used to retain the analytes from a well water sample. Then the cartridge is placed under vacuum to eliminate the remaining water. The analytes are eluted with 5% ammonium hydroxide in methanol. The eluant is then filtered, concentrated, dissolved in methanol and analyzed by APCI/LC/MS/MS.

3. Safety:

All general laboratory safety rules for sample preparation and analysis shall be followed.

4. Interferences:

The metabolite 104 and the metabolite 107 co-elute. And some of the molecular ions (215^+) of the metabolite 104 decompose to ion (172^+) on the ionization source. The ion (172^+) is also the molecular ion of the metabolite 107. Therefore, a positive finding of 107 may be due to the presence of the 104. To correct the identification and quantification problem of the metabolite 107, we use the mobile phase of 45% acetonitrile and 55% water with 0.1% formic acid and the same column to separate the metabolite 104 and the metabolite 107.

5. Apparatus and Equipment:

- 5.1 Nitrogen Evaporator (Meyer N-EVAP Organomation Model #112 or equivalent)
- 5.2 Balance (Mettler PC 4400 or equivalent)
- 5.3 Vortex-vibrating mixer
- 5.4 Solid phase extraction manifold, Supelco Visiprep TM24 or equivalent

- 5.5 Solid phase extraction manifold accessories: vacuum source, vacuum chamber, vacuum controller, cartridge fittings (tube adapters) and connectors, sample delivery tubing with stainless steel weight, sample collection tubes and rack.
- 5.7 High Performance Liquid Chromatograph equipped with an ion trap (HPLC-MS)

6. Reagents and Supplies:

- 6.1 Tebuthiuron CAS#34014-18-1
- 6.2 Metabolite104 CAS#
- 6.3 Metabolite 106 CAS#
- 6.4 Metabolite 107 CAS#
- 6.5 Metabolite 108 CAS#
- 6.6 Methanol, MS grade, Burdick & Jackson or equivalent
- 6.7 Water, MS grade, Burdick & Jackson or equivalent
- 6.8 Formic acid, HPLC grade
- 6.9 Acetonitrile, MS grade, Burdick & Jackson or equivalent
- 6.10 Ammonium formate 1.0 M
- 6.11 Ammonium Hydroxide, reagent grade or equivalent.
- 6.12 Elution reagent: 5% ammonium hydroxide in methanol.
- 6.13 Hydrochloric acid 6 N
- 6.14 Mobil phase A: For 500 mL, mix 470 ± 2mL water, 25 ± 0.5 mL methanol, 4.50 ± 0.25 mL 1 M ammonium formate and 0.5 ± 0.05 mL formic acid.
- 6.15 Mobil phase B: For 500mL, mix 450 ± 2mL methanol and 45 ± 0.5 mL water with 4.50 ± 0.25 mL 1 M ammonium formate and 0.5 ± 0.05 mL formic acid.
- 6.16 Solid phase extraction cartridges: Waters Oasis® MCX 6 cc (150 mg), 60-micron particle size cartridge.
- 6.17 Nylon Acrodisc®, 0.2 micron, Gelman Sciences
- 6.18 Syringe and plunger for filtration, 10mL
- 6.19 Graduated test tube, 15 mL (calibrated at 0.5mL with methanol)
- 6.20 LCMS Columns:
 - Analytical column: Waters SymmetryShieldRP₁₈ 5 µm, 3.9 x 150 mm column (part # 186000108) or equivalent
 - Guard column: Waters SymmetryShieldRP₁₈ 5 µm, 3.9 x 20 mm cartridge (Part # 186000107) or equivalent
 - Guard column holder: Waters Sentry guard holder universal. (Part # wat064610)

7. Standards Preparation:

- 7.1 All 1.0 mg/mL individual standards were obtained from the Standards Repository.

The individual standards of 10 µg/mL were prepared from the 1.0mg/mL standards with methanol. The combination working standard of 1.0 ng/µL was prepared from the individual 10 ng/µL standards in methanol. It was diluted to the following concentrations: 0.05, 0.1, 0.25 and 0.5 µg/mL. They were used for instrument calibration.

7.2 Keep all standards in the designated refrigerator for storage.

7.3 The expiration date of each standard is six months from the preparation date or the expiration date of the stock standards whichever is first.

8. **Sample Preservation and Storage:**

Store all samples waiting for extraction in a separate refrigerator (32-40 °F).

9. **Test Sample Preparation:**

9.1 Background Preparation

The Department of Pesticide Regulation (DPR) provided the well water for background to be used in method validation and QC.

9.2 Preparation of blank and spike

Matrix blank: Weigh out 500 g of background water and follow the test sample extraction procedure.

Matrix spike: Weigh out 500 g of background water. Spike a client requested amount of herbicides into the background water and let it stand for 1 minute. Follow the test sample extraction procedure.

9.3 Test Sample Extraction

9.3.1 Remove sample from refrigerator and allow them to come to ambient temperature.

9.3.2 Weigh 500 ± 0.5 g of water sample into a 600 mL beaker.

9.3.3 Adjust pH to 2.5 – 3.0 with 3 N HCL.

- 9.3.4 Connect MCX cartridge to the vacuum manifold.
- 9.3.7 Condition the cartridge with ~15 mL of methanol at a flow rate ~ 8 mL/minutes followed by ~ 15 mL of D.I. water by applying vacuum.
- 9.3.8 Turn off the vacuum when the D.I. water has just passed through the cartridge. Refill MCX cartridge with D.I. water. Attach the sample delivery tube to the cartridge and place weighted tube end into water sample.
- 9.3.9 Allow the sample to pass through the conditioned cartridge by applying vacuum. Adjust the flow rate to ~ 8 mL/minute
- 9.3.10 After the entire water sample has passed through the cartridge, increase the vacuum to ~ 20 psi for about 2 minutes. Detached the sample delivery tube from MCX cartridge. Shake out any excess water in the cartridge reservoir.
- 9.3.11 Place the graduated test tubes into the vacuum manifold.
- 9.3.12 Elute and collect all analytes into the test tube with 15 ± 0.5 mL of 5% ammonium hydroxide in methanol at a flow rate of ~8 mL/minutes.
- 9.3.15 Concentrate the eluant to ~0.2 mL in a water bath at 38 ± 2 °C under a gentle stream of nitrogen. Bring to a final volume of 0.5 mL with methanol. Vortex for 30 seconds. Transfer the extract into two autosampler vials with inserts. Analyzed by APCI/LC/MS/MS.

10. **Instrument Calibration:**

- 10.1 The calibration standard curve consists of a minimum of three levels. The lowest level must be at or below the corresponding reporting limits.
- 10.2 The LCMS calibration curves were obtained using linear regression.

11. **Analysis:**

- 11.1 HPLC-MS

11.1.1 HPLC Instrument: Waters model 2695 HPLC and auto-sampler with column heater and remote control through Thermo Finnigan Xcalibur system.

Column: Waters SymmetryShield RP₁₈ 5 µm, 3.9 x 150 mm column
 Column Temperature: 40 °C

Mobile Phase: Gradient

<u>Time(min)</u>	<u>Flow rate</u>	<u>Mobile Phase A</u>	<u>Mobile Phase B</u>
0	0.75	85.0	15.0
3.0	0.75	85.0	15.0
4.0	0.75	50.0	50.0
10.0	0.75	50.0	50.0
12.0	0.75	45.0	55.0
14	0.75	5.0	95.0
18.5	0.75	5.0	15.0
21.5	0.75	85.0	15.0
24	0.75	85.0	15.0

Injection Volume: 15 µL

11.1.2 Liquid Chromatograph Mass spectrometer (LC-MS) and Operating Parameters

Model: Finnigan Model DECA ion trap MS
 Ion Source Type: Atmospheric pressure Ionization (APCI)
 Source Polarity: Positive
 APCI Vaporizer Temp: 450 °C
 Capillary Temperature: 220 °C
 Sheath Gas flow rate: 80 (arb)
 Auxiliary Gas flow: 10 (arb)
 Mode of operation: MS/MS

Compound Name	Retention Time (min.)	Molecular Mass	Mass Range (collected)	Product Ions
Metabolite 108	7.42	157.23	60-240	158
Metabolite 106	8.33	200.26	55.0-220	158, 184
Metabolite 107	8.90	172.26	50-200	89, 172
Metabolite 104	8.91	214.28	55-250	158,172,215
Tebuthiuron	10.98	228.31	100-250	172,229

Note: The column conditions, temperature, mobile phase, etc. may slightly shift retention time.

Compound Name	Segment / Scan #	Segment Time	Parent Mass (m/z)	Isolation Width (m/z)	Normalized Collision Energy(%)	Activation Q
Metabolite 108	1/1	7.9	MS			
	1/2		158	4	30	0.250
Metabolite 106	2/1	0.80	MS			
	2/2		201	3	32	0.250
Metabolite 107	2/3 (3/2)		172	3	35	0.250
Metabolite 104	3/1	1.34	MS			
	3/3		215	3	30	0.250
Tebuthiuron	4/1	3.96	MS			
	4/2		229	3	35	0.250

12. Quality Control:

12.1 Method Detection Limits (MDL)

Method Detection Limit (MDL) refers to the lowest concentration of the analyte that a method can detect reliably. To determine the MDL, 7 well water samples are spiked at 0.100 µg and processed through the entire method along with a blank. The standard deviation derived from the spiked sample recoveries was used to calculate the MDL for each analyte using the following equation:

$$\text{MDL} = tS$$

Where t is the Student t test value for the 99% confidence level with n-1 degrees of freedom and S denotes the standard deviation obtained from n replicate analyses. For the n=7 replicates used to determine the MDL, t=3.143.

The results for the standard deviations and MDL are in Appendix 1.

12.2 Reporting Limit (RL)

Reporting limit (RL) refers to a level at which reliable quantitative results may be obtained. The MDL is used as a guide to determine the RL. The RL is chosen in a range 1-5 times the MDL, as per client agreement. The reporting limit for this method is 0.05 ppb.

12.3 Method Validation

The method validation consisted of five sample sets. Each set included four levels of fortification and a method blank. All spikes and method blanks were processed through the entire analytical method. Spike levels and recoveries for the triazines are shown in Appendix 2.

12.4 Control Charts and Limits

Control charts were generated using the data from the method validation for each analyte. The upper and lower warning and control limits are set at ± 2 and 3 standard deviations of the % recovery, respectively, shown in Appendix 2.

12.5 Acceptance Criteria

12.5.1 Each set of samples will have a matrix blank and a spiked matrix sample.

12.5.2 The retention time should be within ± 2 per cent of that of the standards.

12.5.3 The recoveries of the matrix spikes shall be within the control limits.

12.5.4 The sample shall be diluted if results fall outside of the calibration curve.

13. Calculations:

Quantitation is based on an external standard (ESTD) calculation using either the peak area or height. The LCMS software used a linear curve fit, with all levels weighted equally. Alternatively, at the chemist's discretion, concentrations may be calculated using the response factor for the standard whose value is < 30% to the level in the sample.

$$\text{ppb} = \frac{(\text{sample peak area or ht}) \times (\text{std conc}) \times (\text{std vol. Injected}) \times (\text{final vol of sample})(1000 \mu\text{L/mL})}{(\text{std. peak area or ht}) \times (\text{sample vol injected}) \times (\text{sample wt (g)})}$$

14. Reporting Procedure:

Sample results are reported out according to the client's analytical laboratory specification sheets.

15. Discussion and References:

15.1 The segment durations in the mass spectrometer settings determine the retention time windows for each analyte. As the HPLC column performance may change over time because of irreversible contamination, phase stripping, etc., it may be necessary to adjust these windows before beginning a sequence for the observed retention times of the analytes. Installation of a new guard column or analytical column may also necessitate adjustments of window times. These retention time windows should be verified before each sequence, and adjusted as necessary.

15.4 References:

15.4.1 SOP # EM 501.4

15.4.2 SOP #EM 501.5

15.4.3 SOP # EM 62.9

Appendix 1

The Determination of Method Detection Limit (MDL) and Reporting Limit (RL) in Well Water

Results:	Well Water				
Spike\Analytes	Tebuthiuron	Metabolite 104	Metabolite 106	Metabolite 107	Metabolite 108
0.1 ppb spike1	0.093	0.101	0.098	0.085	0.081
0.1 ppb spike2	0.090	0.110	0.101	0.093	0.085
0.1 ppb spike3	0.095	0.116	0.107	0.093	0.101
0.1 ppb spike4	0.100	0.138	0.102	0.102	0.101
0.1 ppb spike5	0.088	0.103	0.096	0.094	0.082
0.1 ppb spike6	0.089	0.099	0.090	0.092	0.081
0.1 ppb spike7	0.098	0.115	0.100	0.112	0.099
Average	0.093	0.112	0.099	0.096	0.090
STDEV	0.0046	0.0134	0.0053	0.0087	0.0098
MDL	0.014	0.042	0.017	0.027	0.031
RL	0.05	0.05	0.05	0.05	0.05

Appendix 2

Method Validation Data in Well Water

Results:								Well Water	
Analyte	Spike ppb	Recovery Set 1	(%) set 2	set 3	set 4	set 5		%	
Tebuthiuron	0.2	96.5	99.5	87.0	87.0	82.5	Mean:	90.8	
	0.5	102	87.2	88.2	95.6	80.8	SD:	8.1	
	0.75	103	100	93.1	87.9	74.0	UCL:	114.9	
	1.0	103	87.4	89.0	90.4	81.9	UWL:	106.9	
							LWL:	74.7	
						LCL:	66.6		
Metabolite104	0.2	115	124	127	102	103	Mean:	112.8	
	0.5	128	122	94.4	115	103	SD:	12.0	
	0.75	123	136	118	102	101	UCL:	148.8	
	1.0	119	92.5	116	106	109	UWL:	136.8	
							LWL:	88.7	
						LCL:	76.7		
Metabolite106	0.2	99.5	101	82.0	92.5	79.5	Mean:	91.3	
	0.5	99.6	92.8	87.8	100	78.0	SD:	9.6	
	0.75	104	105	87.5	91.7	71.2	UCL:	120.2	
	1.0	100	98.1	89.8	87.0	80.0	UWL:	110.6	
							LWL:	72.1	
						LCL:	62.5		
Metabolite 107	0.2	94.5	89.5	82.0	88.0	71.5	Mean:	83.2	
	0.5	93.8	71.4	79.4	89.4	71.2	SD:	8.0	
	0.75	87.1	84.3	88.1	84.8	66.3	UCL:	107.3	
	1.0	91.5	82.3	83.4	87.4	77.8	UWL:	99.2	
							LWL:	67.1	
						LCL:	59.1		

Results: **Well Water**

Analyte	Spike ppb	Recovery Set 1	(%) set 2	set 3	set 4	set 5		%
Metabolite 108	0.2	82.5	75.5	80.5	89.5	72.0	Mean:	79.9
	0.5	86.8	77.2	73.4	98.2	73.2	SD:	7.8
	0.75	86.8	74.4	75.2	81.6	63.6	UCL:	103.3
	1.0	84.0	79.8	77.2	89.7	76.9	UWL:	95.5
							LWL:	64.3
							LCL:	56.5

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