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Study Title

ANALYTICAL METHOD FOR THE DETERMINATION OF TRIBENURON METHYL AND METABOLITES IN-L5296, IN-A4098, IN-D5119, AND IN-00581 IN WATER USING LC/MS/MS

Test Guidelines

European Commission, Directorate General Health and Consumer Protection. "Guidance Document on Residue Analytical Methods", SANCO/825/00 rev. 6, June 20, 2000.

ANALYTICAL METHOD FOR THE DETERMINATION OF TRIBENURON METHYL AND METABOLITES IN-L5296, IN-A4098, IN-D5119 AND IN-00581 IN WATER USING LC/MS/MS

1.0 SUMMARY

The purpose of this study was to develop an analytical method for the detection, quantitative analysis, and confirmation of tribenuron methyl (DPX-L5300) and metabolites IN-L5296, IN-A4098, IN-D5119, and IN-00581 in water. As a result of the poor stability of tribenuron methyl in water and the polarity of IN-D5119, two extraction and cleanup procedures were developed. The first procedure was developed for the quantitative analysis of tribenuron methyl, IN-L5396, and IN-A4098. A second procedure was developed for the analysis of IN-D5119 and IN-00581. Both procedures were validated using ground, surface, and drinking water.

Tribenuron methyl, IN-L5296, and IN-A4098 were extracted from the water samples by filtering 200 mL of water through an Oasis HLB solid phase extraction (SPE) cartridge. Following a wash step, the analytes were eluted in 15 mL of base-adjusted acetonitrile. One mL of water was added and the eluate was evaporated under a flow of nitrogen until the volume was less than 1 mL. The final volume was adjusted to 10 mL using water. Tribenuron methyl, IN-A4098, and IN-D5119 were separated from co-extracts by reversed phase liquid chromatography (LC) and were detected by positive ion electrospray mass spectrometry/mass spectrometry (MS/MS). The Limit of Quantitation (LOQ) was 0.050 µg/L (ppb). The Limit of Detection (LOD) was estimated to be 0.005 µg/L (ppb).

The analysis of IN-D5119 and IN-00581 required acidification of the sample prior to extraction. Following the addition of acetic acid, IN-D5119 and IN-00581 were extracted by filtering 50 mL of water through an Oasis HLB solid phase extraction (SPE) cartridge. The SPE was washed with 70:30 (v.v.) water:methanol, the analytes were eluted in 20 mL of 70:30 (v.v.) methanol: water. The extracts were evaporated under a flow of nitrogen until the volume was approximately 6 mL. The final volume was adjusted to 8 mL using water. IN-D5119 and IN-00581 were separated from co-extracts by reversed-phase liquid chromatography (LC) and were detected by negative ion electrospray mass spectrometry/mass spectrometry (MS/MS). The Limit of Quantitation (LOQ) was 0.10 µg/L (ppb). The Limit of Detection (LOD) was estimated to be 0.03 µg/L (ppb).

During method validation, acceptable recoveries were generated for water samples fortified at 1X and 10X the LOQ as indicated in the following table. Results from method validation are summarized in the following table.

2.0 INTRODUCTION

The structure, CAS name, CAS registry number, and various physical properties of tribenuron methyl (DPX-L5300) and metabolites IN-L5296, IN-A4098, IN-D5119, and IN-00581 can be found in Appendix 1. The method was validated on ground, surface, and drinking water.

In order to create an analytical procedure that was simple and efficient, the analyses was divided into compounds that ionize in the positive ion mode (tribenuron methyl, IN-L5296, and IN-A4098) and those that ionize in the negative ion mode (IN-D5119 and IN-00581). This division was necessary because the concentration of the polar carboxylic acid, IN-D5119, on an SPE cartridge required the addition of acid to the water sample prior to loading. Tribenuron methyl is unstable in aqueous acidic solutions (Reference 1). As a result, two analytical methods were developed.

Both methods followed the same general procedure. The analytes were concentrated onto a Waters Oasis solid-phase extraction cartridge (SPE). The SPE cartridge was washed prior to the elution of the analytes. The eluate volume was reduced under a flow of nitrogen and the final volume was adjusted using water. Detection and quantitative analysis was performed using electrospray LC/MS/MS analysis.

The LOQ for tribenuron methyl, IN-L5296 and IN-A4098 was 0.050 µg/L (ppb). The LOD was estimated to be 0.005 µg/L (ppb). The LOQ for IN-D5119 and IN-00581 was 0.10 µg/L (ppb). The LOD was estimated to be 0.03 µg/L (ppb). During method validation, acceptable recoveries for water samples fortified at 1X and 10X the LOQ were generated.

Due to the selective nature of the LC/MS/MS method, a separate confirmation method was not necessary. Confirmation using LC/MS/MS of possible residues were

based on the detection and relative ratios of two MS/MS ion fragments. Confirmation criteria and examples are discussed in this report.

3.0 MATERIALS

Equivalent equipment and materials may be substituted unless otherwise specified. Note any specification in the following descriptions before making substitutions. Substitutions should only be made *if equivalency/suitability has been verified with acceptable control and fortification recovery data.*

3.1 Equipment

Instrumentation

LC system, HP1100 (Hewlett-Packard, Wilmington, DE)

Mass Spectrometer System, Quattro II with ESI interface (Micromass Inc., Altrincham, UK)

VWR brand Vortex Geni 2 Mixer, Cat. No. 58815-178 (VWR Scientific Co., Bridgeport, NJ)

Biohit Proline Electronic Pipettors, Variable Volume with Tip Ejector, Vanguard, 5.0-100 µL Cat. No. 53495-200, 50-1000 µL Cat. No. 53495-205 and 0.10-5.0 mL Cat. No. 53495-290 (VWR Scientific Co., Bridgeport, NJ)

Evaporator - N-Evap® Model 111 laboratory sample evaporator/nitrogen manifold fitted with Teflon®-coated needles (Organomation Associates, South Berlin, MA). This unit is attached to a dry, clean nitrogen source.

Solid-Phase Extraction Equipment

Visiprep 12 port SPE vacuum manifold, PN 5-7030 (Supelco, Bellefonte, PA)

Solid-Phase Extraction Supplies:

Oasis® HLB cartridge, 1g/20cc, PN 186000117 (Waters, Milford, MA) - **Do not substitute**

Solid Phase Extraction Plastic Reservoir – 75-mL size, Catalog No. 1213-1012 (Varian, Harbor City, CA)

Reservoir Adapters – Catalog No. 1213-1003 (Varian, Harbor City, CA)

Chromatographic Supplies

HPLC Column: 4.6 mm i.d. × 15 cm, Phenomenex Aqua analytical column with 3-µm diameter packing Part # 00F-4311-E0 (Phenomenex, Torrance, CA)

HPLC Vials, Target DP Amber Kit, T/S/T Septa, 100 PK, Part # 5182-0556 (Hewlett-Packard, Wilmington, DE)

Labware

Glass Pipette, Kimax Brand Class A, 50-mL, Cat. No. 53046-349 (VWR Scientific Co., Bridgeport, NJ)

Pyrex Brand Single Metric Scale Graduated Cylinders, 10-mL and 100-mL capacity, Cat. No. 24709-715 and 24709-748, respectively (VWR Scientific Co., Bridgeport, NJ)

VWR brand Disposable Pasteur Pipettes, Borosilicate Glass, 9 in, Cat. No. 53283-914 equipped with 2 mL, 13 X 32 mm rubber bulbs, Cat. No. 56310-240 (VWR Scientific Co., Bridgeport, NJ)

Centrifuge tubes, Pyrex Brand 15-mL capacity, Cat. No. 21048-027 (VWR Scientific Co., Bridgeport, NJ)

Centrifuge tubes, PYREX Brand Conical Centrifuge Tubes with Standard Taper Stopper, 50-mL capacity, Cat. No. 21048-050 (VWR Scientific Co., Bridgeport, NJ)

Centrifuge tubes, Polystyrene 50-mL capacity, Cat. No. 21008-939 (VWR Scientific Co., Bridgeport, NJ)

Centrifuge tubes, Polystyrene 15-mL capacity, Cat. No. 21008-930 (VWR Scientific Co., Bridgeport, NJ)

Erlenmeyer Flask, polycarbonate, 250-mL and 125-mL capacity, Cat. No. 29152-168 and 29152-146 respectively (VWR Scientific Co., Bridgeport, NJ)

Miscellaneous

Syringe filter - Acrodisc PTFE 0.2 μm, 13-mm diameter Filter Unit, Cat. No. 28143-985 (VWR Scientific Co., Bridgeport, NJ)

6 Port Electrically Actuated Valve, Valco Instruments Co. Inc., PN 1384 (Alltech, Deerfield, IL)

3.2 Reagents and Standards

Equivalent reagents may be substituted for those listed below. To determine if impurities in substituted reagents interfere with analyses, appropriate amounts of the solvents should be taken through the entire method using the chromatographic conditions specified in this report.

Acetic Acid - Baker Analyzed® glacial acetic acid, #9524-00 (J. T. Baker, Inc. Danvers, MA)

Ammonium Hydroxide Solution - 28-30%, #AX-1303-13 (EM Science, Gibbstown, NJ)

Acetonitrile (ACN) - EM Omni Solv®, HPLC-grade acetonitrile, #AX0142-1 (EM Science, Gibbstown, NJ)

Formic Acid - Guaranteed Reagent 98% minimum, #FX0440-5 (EM Science, Gibbstown, NJ)

Hexanes - EM Omni Solv®, #HX0296-1 (EM Science, Gibbstown, NJ)

Methanol - EM Omni Solv®, HPLC-grade methanol, #MX0488-1 (EM Science, Gibbstown, NJ)

Water - EM Omni Solv®, HPLC-grade water, #WX0004-1 (EM Science, Gibbstown, NJ)

The analytical reference standards were prepared by DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company

3.3 Safety and Health

No unusually hazardous materials are used in this method. All appropriate material safety data sheets should be read and followed, and proper personal protective equipment used. An MSDS sheet for the analytes is available from DuPont Crop Protection, Global Technology Division, E. I. du Pont de Nemours and Company.

4.0 METHOD

4.1 Principles of the Analytical Method

In order to create an analytical procedure that was simple and efficient, the analyses was divided into compounds that ionize in the positive ion mode (tribenuron methyl, IN-L5296 and IN-A4098), and those that ionize in the negative ion mode (IN-D5119 and IN-00581). This division was necessary because the concentration of the carboxylic acid, IN-D5119, on an SPE cartridge required the addition of acid prior to loading. The instability of tribenuron methyl in aqueous acidic solutions would not allow acidification prior to loading (Reference 1).

Both methods follow the same general procedure. The analytes were concentrated from the water sample onto a Waters Oasis SPE. The SPE cartridge was washed prior to the elution of the analytes. The eluate volume was reduced under a flow of nitrogen and the final volume was adjusted using water. Detection and quantitative analysis was performed using LC/MS/MS analysis.

4.2 Analytical Procedure

4.2.1 Glassware and Equipment

Cleaning

Glassware should be scrubbed with a brush using a laboratory soap solution, rinsed two to five times with tap water, rinsed with distilled or deionized water and finally rinsed with acetone or another suitable solvent and allowed to air dry prior to each use.

4.2.2 Preparation of Solutions

The following solutions should be prepared weekly and stored at room temperature unless stated otherwise:

0.005 M aqueous Formic Acid Solution - Add 230-µL of formic acid to 1000-mL of water and mix the resulting solution to homogeneity.

1.0 M ammonium hydroxide. Add 6.9 mL of ammonium hydroxide solution (28-30% NH_3) to a volume of 93.1 mL of EM Science water. Mix the resulting solution to homogeneity.

Solution A -- Basic Acetonitrile - Add 20 mL of 1.0 M ammonia hydroxide to 980 mL of the acetonitrile volume and mix the resulting solution to homogeneity. This solution may be prepared monthly.

Solution B -- 0.1% aqueous acetic acid - Add 1.0-mL of acetic acid to 1000 mL of water and mix the resulting solution to homogeneity.

Solution C -- 70:30 water:methanol 0.1% acetic acid. Add 700 mL of water to 300 mL of methanol. Add 1.0-mL of acetic acid and mix the resulting solution to homogeneity. This solution may be prepared monthly.

Solution D -- 70:30 methanol: water. Add 700 mL of methanol to 300 mL of water and mix the resulting solution to homogeneity. This solution may be prepared monthly.

4.2.3 Preparation and Stability of Stock Standard

Use Class A volumetric flasks when preparing standard solutions.

Prepare standard stock solutions by accurately weighing 10 ± 0.01 mg of each analyte into individual 100-mL volumetric flask using an analytical balance. Record the accurate weight of the standard. Dissolve the standard in approximately 50 mL of HPLC-grade acetonitrile. After dissolving, bring the solution to a volume of 100 mL using HPLC-grade acetonitrile and invert the volumetric flask to mix the solution to homogeneity. This standard solution is stable for approximately 3 months when stored in a freezer at approximately -20°C immediately after each use. The concentration of each analyte in solution is 100 µg/mL.

4.2.4 Preparation and Stability of Intermediate and Fortification Standards

Use Class A volumetric flasks when preparing standard solutions.

Analysis of tribenuron methyl, IN-L5296 and IN-A4098

Prepare a 1.0-µg/mL tribenuron methyl, IN-L5296, and IN-A4098 fortification standard in acetonitrile by pipetting 1.00 mL of the 100.0-µg/mL stock standard into a 100-mL volumetric flask. Bring to volume using HPLC-grade acetonitrile and mix to homogeneity.

Analysis of IN-D5119 and IN-00581

Prepare a 1.0-µg/mL IN-D5119 and IN-00581 intermediate standard in methanol by pipetting 1.00 mL of the 100.0-µg/mL stock standard into a 100-mL volumetric flask. Bring to volume using HPLC-grade methanol and mix to homogeneity.

Prepare a 0.50-µg/mL IN-D5119 and IN-00581 fortification standard in methanol by pipetting 50.00 mL of the 1.0-µg/mL stock standard into a 100-mL volumetric flask. Bring to volume using HPLC-grade methanol and mix to homogeneity.

Alternate or additional solutions may be prepared as needed. All standard solutions prepared in acetonitrile or methanol are stable for approximately 3 months if stored in a freezer at approximately -20°C immediately after each use.

4.2.5 *Preparation and Stability of Calibration Standards*

Analysis of tribenuron methyl, IN-L5296 and IN-A4098

Prepare the calibration standards by pipetting volumes of the 1.00-µg/mL tribenuron methyl, IN-L5296, and IN-A4098 fortification standard solution shown in the following table into separate 10.0-mL volumetric flasks (alternative or additional standards may be prepared as needed):

Desired Standard Concentration (ng/mL)	Volume of 1.00-µg/mL Standard Required (mL)
20	0.20
10	0.10
2.0	0.020
1.0	0.010
0.50	0.0050

Add the appropriate amount of water to the volumetric flasks to dilute to 10.00 mL. These standard solutions should be freshly prepared with each sample set and stored approximately 4°C prior to use. Each of the calibration standards was vortexed for 30 seconds prior to injection.

Analysis of IN-D5119 and IN-00581

Prepare the calibration standards by pipetting volumes of the 0.50-µg/mL IN-D5119 and IN-00581 fortification standard solution shown in the following table into separate 10.0-mL volumetric flasks (alternative or additional standards may be prepared as needed):

Desired Standard Concentration (ng/mL)	Volume of 0.50-µg/mL Standard Required (mL)
3.0	0.060
2.0	0.040
1.0	0.020
0.50	0.010

Add the appropriate amount of water to the volumetric flasks to dilute to 10.00 mL. These standard solutions should be freshly prepared with each sample set and stored approximately 4°C prior to use. Each of the calibration standards was vortexed for 30 seconds prior to injection.

4.2.6 *Source of Samples*

Water control samples were obtained from local water sources. All water sources are provided in the table below. Bottle water was purchased from a local grocery Store.

Origin	Location
Lums Pond	Bear, Delaware
Brandywine River	Wilmington, Delaware
Kemblesville Well	Kemblesville, Pennsylvania
Bottle Spring	Great Bear, Greenwich, CT

All samples were refrigerated until use.

4.2.7 *Storage and Preparation of Samples*

Water samples should be stored at approximately 4°C. The water samples were shaken by hand prior to use to ensure homogeneity. No additional filtration or purification was performed prior to sample processing.

4.2.8 *Sample Fortification Procedure*

All fortifications were made directly to the water following the measurement of the sample.

Analysis of tribenuron methyl, IN-L5296 and IN-A4098

Fortified 200-mL samples were prepared using a 1.00-µg/mL fortification standard solution.

Fortification Level (µg/L)	Volume of Standard (mL)	Spiking standards Concentration (µg/mL)
0.050	0.010	1.00
0.50	0.100	1.00

The total amount of acetonitrile applied to the water should be less than 0.50 mL.

Analysis of IN-D5119 and IN-00581

Fortified 50-mL samples were prepared using a 0.50-µg/mL fortification standard solution.

Fortification Level (µg/L)	Volume of Standard (mL)	Spiking standards Concentration (µg/mL)
0.10	0.010	0.50
1.0	0.100	0.50

The total amount of methanol applied to the water should be less than 0.50 mL.

4.2.9 Analyte Extraction and Purification Procedures

Analysis of tribenuron methyl, IN-L5296, and IN-A4098

1. Accurately measure 200.0-mL (± 1%) of water into a 250-mL polycarbonate Erlenmeyer flask. Fortify sample if necessary. Cap and shake the samples vigorously.
2. Using an adapter, place a 75-mL reservoir above a 20-cc, 1-g Oasis HLB cartridge and attach it to an SPE manifold. Precondition the cartridge with 15 mL of methanol, discard the conditioning solution. **Do not let the cartridge go to dryness**. Then condition the cartridge with 15 mL of water. **Do not let the cartridge go to dryness**.
3. Gradually load the sample into the 75-mL reservoir. Using vacuum, pull the sample through the Oasis cartridges at a flow rate of 2-10 mL/min. Rinse the flask with 30 mL of water and load the water into the reservoir just before all of the sample would have passed through. Use vacuum to pull the cartridge dry for 3 minutes. Discard the eluate.
4. Wash the cartridge with 15 mL of hexane. Use vacuum to pull the cartridge dry for 3 minutes. Discard the eluate.
5. Elute the analytes with 15 mL of Solution A. Load Solution A onto the cartridge, vacuum or positive pressure may be required to start the flow but should be turned off once the flow has started. Once the dripping has stopped, use a small amount of nitrogen pressure[1] to empty the remaining liquid in the cartridge into a centrifuge tube. Collect the eluate in a 50-mL centrifuge tube.
6. Add 1.0 mL of water to each centrifuge tube and vortex. Evaporate the extract to approximately 0.5 mL using a flow of nitrogen in an N-Evap at 25-30°C. Using a pipette, adjust the final volume to 10 mL with water. Vortex the centrifuge tube and filter an aliquot of the extract using a disposable syringe through a 0.2-µm acrodisc filter into an HPLC vial. Analyze the solution by LC/MS/MS as described in the following section.

Extracts will be stable for approximately 24 hours if stored at 4°C.

[1] A nitrogen line fitted through a rubber stopper should be used to push out any remaining solvent. Care must be taken to use the minimal amount of nitrogen pressure possible.

<u>Analysis of IN-D5119 and IN-00581</u>

1. Accurately measure 50.0 mL (± 1%) of water into a 125-mL polycarbonate Erlenmeyer flask. Fortify sample if necessary. Add 50 µL of acetic acid to lower the water pH. Cap and shake the samples vigorously.
2. Using an adapter, place a 75-mL reservoir above a 20-cc, 1-g Oasis HLB cartridge and attach it to an SPE manifold. Precondition the cartridge with 15 mL of methanol, discard the conditioning solution. **Do not let the cartridge go to dryness**. Then condition the cartridge with 15 mL of solution B. **Do not let the cartridge go to dryness**.
3. Load the sample into the 75-mL reservoir. Using vacuum, pull the sample through the Oasis cartridges at a flow rate of 2-10 mL/min. Rinse the flask with 30 mL of solution B and load the rinse into the reservoir just before all of the sample would have passed through. Use vacuum to pull the cartridge dry for 3 minutes. Discard the eluate.
4. Wash the cartridge with 10 mL of solution C. Wash the cartridge with an additional 10-mL of water. Use vacuum to pull the cartridge dry for 3 minutes. Discard the eluate.
5. Elute the analytes with 20 mL of Solution D. Load Solution D onto the cartridge, vacuum or positive pressure may be required to start the flow but should be turned off once the flow has started. Once the dripping has stopped, use a small amount of nitrogen pressure[2] to empty the remaining liquid in the cartridge into a centrifuge tube. Collect the eluate in a 50-mL centrifuge tube.
6. Evaporate the extract to approximately 6.0 mL using a flow of nitrogen in an N-Evap at 25-30°C. Using a pipette, adjust the final volume to 8 mL with water. (For 10X fortifications adjust the volume to 20-mL.) Vortex the centrifuge tube and filter an aliquot of the extract using a disposable syringe through a 0.2-µm acrodisc filter into an HPLC vial. Analyze the solution by LC/MS/MS as described in the following section.

Extracts will be stable for approximately 24 hours if stored at 4°C.

4.3 Instrumentation for the Method

4.3.1 Chromatography

Reversed-phase chromatography was used to separate tribenuron methyl and metabolites from co-extracts. A Phenomenex aqua® column was selected. The column choice reflected experimental results indicating preferred separation from co-extractants. Alternative chromatographic conditions can be used, provided the analytical method is validated and provides acceptable recoveries as defined by regulatory method guidelines.

[2] A nitrogen line fitted through a rubber stopper should be used to push out any remaining solvent. Care must be taken to use the minimal amount of nitrogen pressure possible.

Analysis of Tribenuron Methyl, IN-L5296, and IN-A4098

System:	Hewlett-Packard HP1100 HPLC			
Column:	4.6 mm i.d. × 15 cm, Phenomenex Aqua			
Column Temperature:	40 °C			
Injection Volume:	0.075 mL			
Flow Rate:	1.00 mL/min			
Conditions:	A: 0.005 M aqueous Formic Acid B: Methanol			
	Time	%A	%B	Flow (mL/Min.)
	0.0	90	10	1.00
	1.0	90	10	1.00
	3.5	70	30	1.00
	12.0	20	80	1.00
	12.5	5	95	1.00
	14.5	5	95	1.00
	15.0	90	10	1.00
IN-A4098 Retention Time:	6.0 minutes			
IN-L5296 Retention Time:	7.6 minutes			
Tribenuron methyl Retention Time:	13.3 minutes			
Total Run Time:	22 min			

Analysis of IN-D5119 and IN-00581

System:	Hewlett-Packard HP1100 HPLC			
Column:	4.6 mm i.d. × 15 cm, Phenomenex aqua			
Column Temperature:	25 °C			
Injection Volume:	0.020 mL			
Flow Rate:	1.00 mL/min			
Conditions:	A: 0.005 M aqueous Formic Acid B: Methanol			
	Time	%A	%B	Flow (mL/Min.)
	0.0	90	10	1.00
	1.0	90	10	1.00
	3.5	70	30	1.00
	12.0	20	80	1.00
	12.5	10	90	1.00
	15.5	10	90	1.00
	16.5	90	10	1.00
IN-D5119 Retention Time:	7.1 minutes			
IN-00581 Retention Time:	8.8 minutes			
Total Run Time:	25 minutes			

A six-port electronically activated switching valve was used to direct the flow to waste prior to and following the elution of the compounds of interest. The use of this

valve reduces source contamination and enables additional samples to be analyzed prior to source cleaning. The valve switching times are given in the following table.

Time (Minutes)	Column Eluate Flow
0.00-5.0	Waste
5.00-14.2	MS source
14.2-End	Waste

Since electrospray LC/MS systems perform optimally at low flow rates, the eluate was split following the switching valve. Approximately 100 µL/min of eluate (10:1 split) flowed into the ion source with the remaining eluate flowing into a waste container.

4.3.2 *LC/MS/MS Analysis*

The quantitative analysis of tribenuron methyl and metabolites was performed using a Micromass Quattro II LC/MS/MS system. Quantitative analysis was based on the integration of a single ion transition. The system parameters were adjusted while a solution of each analyte was infused directly into the electrospray ion source. The solution composition was 50% methanol/50% water, so that it would approximate the composition of the mobile phase at the retention time of the analyte. The solution concentration was approximately 2 µg/mL. A summary of the experimental conditions is provided in the following table:

Micromass Quattro LC ESI-LC/MS/MS Mass Spectrometer Conditions

Analysis of Tribenuron Methyl, IN-L5296, and IN-A4098

Analytes	Ions Monitored	Cone Voltage	Collision Energy	Dwell (Seconds)
Tribenuron methyl	**395.8→ 154.9 ± 0.2 AMU**	**48V**	**15V**	**0.40**
	395.8→ 180.9 ± 0.2 AMU	48V	22V	0.40
IN-L5296	**155.0→ 71.0 ± 0.2 AMU**	**45V**	**18V**	**0.40**
	155.0→ 56.9 ± 0.2 AMU	45V	19V	0.40
IN-A4098	**141.0→ 57.0 ± 0.2 AMU**	**45V**	**17V**	**0.40**
	141.0→ 85.8 ± 0.2 AMU	45V	16V	0.40
Ion Mode:	Positive			
Electrospray Voltage:	4.0 kV			
Detector Voltage:	750 V			
Source Temperatures:	150 °C			
Collision Gas Pressure:	2.8 e-3 mBar			
Nebulizing Gas Flow:	15 L/h			
Drying Gas Flow:	300 L/h			

Analysis of IN-D5119 and IN-00581

Analytes	Ions Monitored	Cone Voltage	Collision Energy	Dwell (Seconds)
IN-D5119	**199.9→ 155.8 ± 0.2 AMU**	**40V**	**11V**	**0.40**
	199.9→ 91.8 ± 0.2 AMU	40V	20V	0.40
IN-00581	**181.9→ 41.8 ± 0.2 AMU**	**55V**	**25V**	**0.40**
	181.9→ 105.8 ± 0.2 AMU	55V	18V	0.40
Ion Mode:	Negative			
Electrospray Voltage:	3.0 kV			
Detector Voltage:	750 V			
Source Temperatures:	150 °C			
Collision Gas Pressure:	2.7 e-3 mBar			
Nebulizing Gas Flow:	15 L/h			
Drying Gas Flow:	300 L/h			

A complete list of the experimental parameters is given in Appendix 4. A typical LC/MS and LC/MS/MS full scan spectrum is shown in Figure 1 and Figure 2, respectively.

The instrument was operated in MS/MS-(MRM) positive and negative ion modes for quantitative analysis. Peak area was used for quantitation. **Quantitation was performed using the ion transition displayed in bold face print.** The relative ratio of the fragment ions was evaluated to confirm the presence of an analyte in an unknown sample.

4.3.3 *Calibration Procedure and Sample Analysis*

A 0.50-ng/mL chromatographic standard should be analyzed prior to the start of analyses to establish that the instrument is working properly. If a signal-to-noise ratio of approximately 5-10 to 1 is not attained, the instrument must be tuned or cleaned prior to sample analysis. Operating parameters must be tailored to the particular instrument used, especially if it is to be an alternate vendor's instrument, and should be checked daily. Note that some ion channels other than those used for development of this method may need to be added or eliminated when utilizing this method on other instrumentation. Each ion channel used for sample analysis/quantitation must be checked to insure it is free of interference. The control will be used to demonstrate that baseline interference is less than signal-to-noise 3:1. Begin each sample set by injecting a minimum of 2 calibration standards. The first injection should always be disregarded.

4.4 *Calculations*

4.4.1 *Methods*

Average Response Factor (RF_{Avg}) was calculated as follows:

$$RF_{Ave} = \frac{(\text{Conc. A} \div \text{Area A}) + (\text{Conc. B} \div \text{Area B}) + (\text{Conc. C} \div \text{Area C}) + (\text{Conc. D} \div \text{Area D}) + (\text{Conc. E} \div \text{Area E})}{\text{Total Number of Standards Injected}}$$

ppb found was calculated as follows:

$$\text{ppb Found} = \frac{(\text{Peak Area}) \times (RF_{Ave}) \times (\text{Final Volume}) \times (\text{Dilution Factor})}{(\text{Sample Volume})} \times \frac{1000\ \mu g/L}{1\ \mu g/mL}$$

In the event a peak was detected in the control, a corrected peak area was used to calculate ppb found for freshly fortified samples. The corrected peak area is the area of the fortified sample minus the area of the control sample.

The percent recovery found was calculated as follows:

$$\%\ \text{Recovery} = \frac{(\text{ppb Found})}{(\text{Fortification level})} \times 100$$

4.4.2 *Example*

For a well water sample fortified with IN-L5296 at 0.05 µg/L (0.05 ppb) [Date Extracted 19-April-01, 0.05 ppb Fortification (a)], the concentration found was calculated as follows:

Average Response Factor was calculated as follows:

$$RF_{Ave} = \frac{(0.0010\ \mu g/mL \div 3215\ AC) + (0.00050\ \mu g/mL \div 1600\ AC) + (0.0020\ \mu g/mL \div 6298\ AC) + (0.010\ \mu g/mL \div 29599\ AC) + (0.020\ \mu g/mL \div 58409\ AC)}{5}$$

(AC ≡ Area Counts)

$RF_{Avg} = 3.242730e^{-7}\ \mu g/mL/AC$

ppb found was calculated as follows:

$$\text{ppb Found} = \frac{(2892\ AC) \times (3.242730e-7\ \mu g/mL/AC) \times (10.0\ mL) \times (1)}{(200\ mL)} \times \frac{1000\ \mu g/L}{1\ \mu g/mL}$$

ppb Found = 0.0468898

(ppb values are reported to two significant figures in Table 1 of this report. Rounding was performed using the Microsoft Excel version 7.0 for Windows 95 rounding function)

The percent recovery found was calculated as follows:

$$\% \text{ Recovery} = \frac{(0.0468898\,\mu g/L)}{(0.050\,\mu g/L)} \times 100$$

% Recovery = 94%

(percent recoveries are rounded to the nearest whole number in Table 1, without rounding the concentration or ppb found)

6.2 Confirmation Criteria

In order for a sample set to be valid, the relative standard deviation of the ion ratios calculated from the calibration standards analyzed must be less than 20%. For the confirmation of possible tribenuron and metabolite residues in a water sample, the ion ratio must fall within ±30% of the average ratio for all calibration standards for a specific sample set.

If the ion ratio is outside the ±30% range, the signal was most likely generated from a compound that is unrelated to tribenuron methyl. The unknown compound also has the same ion by LC/MS and a similar fragmentation pattern. In addition to meeting the defined ion ratio criteria, the elution time of the compound of interest must fall within 2% of the elution time of the standards analyzed for that sample set.

6.3 Method Validation Results

The calibration standard ion ratios collected during method validation had a relative standard deviation less than 20%. For the analysis of calibration standards during method validation, all ion ratio data meet the 20% RSD criteria. For the analysis of calibration standards and fortified samples, the relative standard deviation was also less than 20%. All fortified samples analyzed meet the accuracy criteria of ±30% of the average ratio for all calibration standards for a selected sample set. Confirmation criteria for selected sets from method validation are shown in Appendix 3.

6.4 Example of Calculation

The values presented here for tribenuron methyl in Lums Pond Water are the method validation and are presented in Appendix 3. The calibration standards and samples are examples of ion ratio data collected during method validation.

Sample	Area (395.8→154.9)	Area (395.8→180.9)	Ratio (154.9/180.9)
0.001 µg/mL std	717	442	1.6
0.00050 µg/ml std	361	216	1.7
0.0020 µg/mL std	1305	755	1.7
0.010 µg/mL std	7135	4079	1.7
0.020 µg/mL std	14624	8433	1.7
Lums Pond + 0.05 ppb (a)	605	357	1.7
Lums Pond + 0.05 ppb (b)	621	355	1.7
Lums Pond + 0.50 ppb (a)	5845	3268	1.8
Lums Pond + 0.50 ppb (b)	7140	3962	1.8
Lums Pond + 0.50 ppb (c)	7034	3980	1.8

Average Ratio of Calibration Standards = 1.7

The ±30% range was calculated as:

Upper limit was 1.7 + (1.7*0.3) = 2.2

Lower limit was 1.7 - (1.7*0.3) = 1.2

The standard average ratio was represented to 2 significant figures. However, the value used to calculate the upper and lower limits was not rounded.

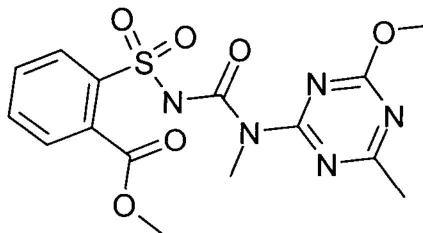
The ion ratios for the LOQ samples and the 10X LOQ samples fall within the upper and lower limits calculated. Based on the criteria outlined, the levels of tribenuron methyl found would be *confirmed* as a tribenuron methyl residue. Examples of typical samples analyzed during method validation are shown in Appendix 3.

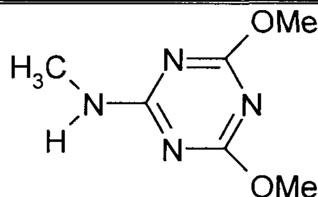
7.0 CONCLUSIONS

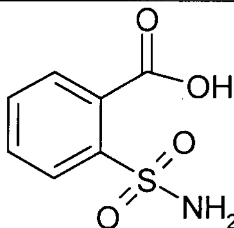
This method for determination of residues of tribenuron methyl and metabolites in water samples meets the guidelines of the European Commission, Directorate General Health and Consumer Protection. "Guidance Document on Residue Analytical Methods", SANCO/825/00 rev. 6, June 20, 2000.

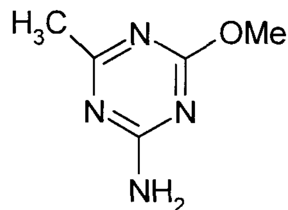
The LC/MS/MS method is free of interference above the LOD at the retention time of tribenuron methyl and metabolites in unfortified water samples. The method generated acceptable recoveries over concentration levels expected in the water tested.

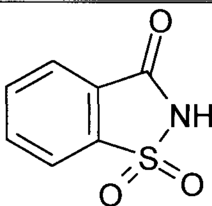
APPENDIX 1 STRUCTURE AND PROPERTIES OF TRIBENURON METHYL AND METABOLITES

Common Name	Tribenuron methyl
Structure	
DPX Number	DPX-L5300
Formula	$C_{15}H_{17}N_5O_6S$
Molecular Weight	395.39
Monoisotopic Weight	395.09
pKa	5.0

Common Name	None
Structure	
DPX Number	IN-L5296
Formula	$C_6H_{10}N_4O_2$
Molecular Weight	170.17
Monoisotopic Weight	170.08
pKa	none

Common Name	None
Structure	
DPX Number	IN-D5119
Formula	$C_7H_7NO_4S$
Molecular Weight	201.20
Monoisotopic Weight	201.01
pKa	Yes- Unknown

Common Name	None
Structure	
DPX Number	IN-A4098
Formula	$C_5H_8N_4O$
Molecular Weight	140.14
Monoisotopic Weight	140.07
pKa	None

Common Name	Saccharin
Structure	
DPX Number	IN-00581
Formula	$C_7H_5NO_3S$
Molecular Weight	183.19
Monoisotopic Weight	183.00
pKa	11 (CRC Handbook)