

Wildlife International, Ltd.

ATTACHMENT I

**Method Outline for the Analysis of MTI 446 and its
Metabolites UF and DN in/on Raw Agricultural Commodities Following
Homogenization and Subsampling**

1. Sample Extraction: MTI-446, UF and DN

Prepare samples and procedural recoveries by weighing 25.0 g of matrix into labeled 500-mL glass screw cap bottles. Fortify procedural recoveries using an appropriate stock solution(s). Unfortified matrix will serve as the matrix blank.

Add 150 mL of 80% CH₃CN: 20% H₂O followed by 0.5 mL of concentrated HCL to each sample. Blend the samples using a high-speed Ultra Turrax (T-25) sample homogenizer for approximately 1 minute.

Place samples on a shaker table and agitate for 30 minutes at a setting of 150 rpm.

Filter the suspension by suction through a pad of Celite (approximately 10 g) into a 1L-round bottom flask. Rinse the filter cake with an additional 100 mL of 80% CH₃CN: 20% H₂O and combine in its respective round bottom flask.

Transfer the filtrate into a 500-mL separatory funnel. Rinse the round bottom flask with 100 mL of hexane and combine it in its respective separatory funnel. Shake the separatory funnels for approximately 1 minute. Drain the lower CH₃CN/H₂O fraction into a 1L-round bottom flask and discard the hexane. Repeat the hexane- CH₃CN/H₂O partition a second time with 50 mL of hexane and combine the lower CH₃CN/H₂O fraction in its respective round bottom flask.

Rotary evaporate each sample to its aqueous remainder at a bath temperature of approximately 50-60°C.

Adjust the pH of the aqueous remainder to approximately pH 8 by drop-wise addition of buffer solution A (0.5M sodium carbonate - sodium hydrogen carbonate).

Transfer the aqueous solution to a 100-mL graduated cylinder and adjust the final volume to 80.0 mL using NANOpure® H₂O.

2. Sample Clean Up: MTI-446 and the UF Metabolite (Extrelut NT3)

Transfer 40 mL of the aqueous solution from Section 1 above into a 50-mL volumetric flask containing 15 g of NaCl and bring to volume with NANOpure® H₂O. Transfer the mixture to a French square bottle, place on shaker table and agitate for approximately 60 minutes at approximately 150 rpm.

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Using a Class A volumetric pipette, remove a 3.0-mL aliquot of the aqueous solution and transfer onto an Extrelut NT3 extraction column. Allow the extract to adsorb into the column for approximately 30-60 minutes.

Elute the analytes into 250-mL round bottom flasks using 120 mL of ethyl acetate.

Rotary evaporate the ethyl acetate extracts to dryness at approximately 40-50C°.

Reconstitute the residues using 3.0 mL of NANOpure® H₂O. Mix well by shaking.

OPTIONAL: If particulates are observed in the reconstituted solution, filter an aliquot of extract through a 0.45-µm Acrodisc filter.

3. Sample Clean-up: DN Metabolite only (Bond Elut CBA)

Measure the remaining 40 mL of aqueous solution from Section 1 and transfer it to a plastic 50-mL centrifuge tube or equivalent. Add 5.0 mL of buffer solution A and 5.0 mL of NANOpure® H₂O. Mix well by shaking.

OPTIONAL: If particulates are observed in the reconstituted solution, filter an aliquot of extract through a 0.45-µm Acrodisc filter.

Prepare an appropriate number of Bond Elut CBA SPE columns by rinsing and conditioning with 3 mL of methanol followed by 4 mL of buffer solution B (0.05 M sodium carbonate - sodium hydrogen carbonate). Do not allow the columns to go to dryness.

Using a Class A volumetric pipette, remove a 4.0-mL aliquot of the final aqueous solution from above, transfer it to the prepared CBA cartridge and allow it to pass through at approximately 1-2 mL/minute. Rinse the cartridge with 10 mL of NANOpure® H₂O, followed by 6 mL of methanol. Discard all eluates. Dry the cartridge under high vacuum.

Elute the analytes with 4.0 mL of 0.1N HCL measured using a Class A volumetric pipette into a 15-mL graduated tubes. Adjust to a 4.0 mL final volume if necessary using 0.1N HCL. Mix well by shaking.

OPTIONAL: If particulates are observed in the reconstituted solution, filter an aliquot of extract through a 0.45-µm Acrodisc filter

4. Combining of MTI-446/UF and DN Extracts for HPLC/MS/MS Analysis

Volumetrically combine an aliquot of the MTI-446/UF metabolite final aqueous extract from Section 2 with an equal-volume aliquot of the DN metabolite final aqueous extract from Section 3 (1:1, v/v). Mix well by shaking. Transfer an aliquot of the final combined extract to an autosampler vial and submit for LC/MS/MS analysis.

Table I. Analytical Instrumentation and Equipment for the Determination of MTI-446 and Metabolites DN and UF

Instrumentation:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a PE Sciex API 3000 Triple Quadrupole Mass Spectrometric Detector (LC/MS/MS) and Heated Nebulizer Ion Source																												
Analytical Column:	Phenomenex Luna C18; 150 x 3 mm, 5µm																												
Guard Column	Phenomenex Security Guard C18 (4 mm x 3 mm, 5µm)																												
Mobile Phases:	A1: 5% Methanol: 95% Water: 0.1% IPCC-MS-3 B1: Methanol: 0.1% IPCC-MS-3 Gradient Elution Program: <table border="1"> <thead> <tr> <th>Time</th> <th>%A1</th> <th>%B1</th> <th>Flow Rate (µL/min)</th> </tr> </thead> <tbody> <tr> <td>0.00</td> <td>100</td> <td>0</td> <td>500</td> </tr> <tr> <td>2.00</td> <td>100</td> <td>0</td> <td>500</td> </tr> <tr> <td>7.00</td> <td>20</td> <td>80</td> <td>500</td> </tr> <tr> <td>10.0</td> <td>20</td> <td>80</td> <td>500</td> </tr> <tr> <td>10.1</td> <td>100</td> <td>0</td> <td>800</td> </tr> <tr> <td>15.0</td> <td>100</td> <td>0</td> <td>500</td> </tr> </tbody> </table>	Time	%A1	%B1	Flow Rate (µL/min)	0.00	100	0	500	2.00	100	0	500	7.00	20	80	500	10.0	20	80	500	10.1	100	0	800	15.0	100	0	500
Time	%A1	%B1	Flow Rate (µL/min)																										
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10.1	100	0	800																										
15.0	100	0	500																										
Injection Volume:	50 µL																												
Total Run Time:	16.1 minutes																												
Mass Spectrometer Conditions:	Ionization Mode: Positive Scan Type: MRM Duration: 16.1 minutes Scan Rate: 1.520 scans/second Dwell Time: 500 mseconds/mass range Pause Time: 5 mseconds Mass Range: <table border="1"> <thead> <tr> <th></th> <th><u>MTI-446</u></th> <th><u>DN</u></th> <th><u>UF</u></th> </tr> </thead> <tbody> <tr> <td>Q1 Mass (amu):</td> <td>203.2</td> <td>158.2</td> <td>159.2</td> </tr> <tr> <td>Q3 Mass (amu):</td> <td>128.7</td> <td>101.9</td> <td>101.9</td> </tr> <tr> <td>R02:</td> <td>-28</td> <td>-26</td> <td>-26</td> </tr> <tr> <td>R03:</td> <td>-30</td> <td>-28</td> <td>-28</td> </tr> <tr> <td>ST3:</td> <td>-34</td> <td>-32</td> <td>-32</td> </tr> </tbody> </table> MS Voltage, Temperature and Gas Settings: NC: +5.0 Temp.: 450°C OR: +21 RNG: +70 QO: -10 IQ1: -11 ST: -15 R01 -11 IQ2: -20 DF: -350 CEM: +2000 NEB Gas: 13; CUR Gas: 9; CAD Gas: 4		<u>MTI-446</u>	<u>DN</u>	<u>UF</u>	Q1 Mass (amu):	203.2	158.2	159.2	Q3 Mass (amu):	128.7	101.9	101.9	R02:	-28	-26	-26	R03:	-30	-28	-28	ST3:	-34	-32	-32				
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MTI-446 Retention Time:	Approximately 7.4 mins																												
DN Retention Time:	Approximately 8.1 mins																												
UF Retention Time:	Approximately 6.5 mins																												