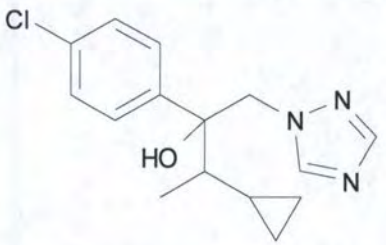
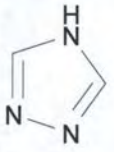
1.0 INTRODUCTION

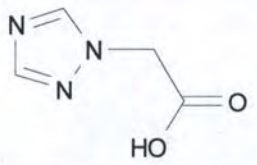
1.1 Scope and Chemical Structures

This method is utilized for the residue determination of cyproconazole, 1,2,4-triazole, and triazole acetic acid in soil samples at a lower limit of method validation (LLMV) of 1.0 ppb (ng/g). Thus, in this study the LLMV corresponds to the limit of quantification (LOQ). The limit of detection (LOD) is 25 pg for all the analytes and is defined as the lowest amount of standard injected (on-column injected amount) and used to construct the respective calibration plots.

The chemical structures of the analytes are as follows:

Name/Synonym:	Cyproconazole (SAN619)
CAS Name:	1H-1,2,4-triazole-1-ethanol, alpha-(4-chlorophenyl)-alpha-(1-cyclopropylethyl)-
CAS Number:	94361-06-5
Structure:	

Name/Synonym:	1,2,4-triazole
CAS Name:	1H-1,2,4-triazole
CAS Number:	288-88-0
Structure:	

Name/Synonym:	Triazole acetic acid
CAS Name:	1H-1,2,4-Triazole-1-acetic acid
CAS Number:	28711-29-7
Structure:	

1.2 Method Summary

Soil samples (10 g) are extracted two times by shaking with solvent at room temperature for 20 minutes. The extracts are combined upon centrifugation and filtration to separate the suspended solids. The volatile organics of the combined extracts are removed under vacuum with a rotary evaporator at a bath temperature of 35°C. The resulting concentrated aqueous extracts are quantitatively transferred to a plastic centrifuge tube and are diluted with deionized water to a final volume of 10 mL. Aliquots of this final extract are taken for solid phase extraction (SPE) workup. Cleanup for cyproconazole and 1,2,4-triazole are accomplished by a mixed mode cation exchange SPE, while triazole acetic acid is processed by a mixed mode anion exchange SPE. Upon SPE cleanup and subsequent concentration, an aliquot of the final sample extract is transferred to an LC sample vial and subjected to LC/MS/MS analysis. Two LC/MS/MS analyses are performed: ESI positive mode for cyproconazole and triazole; and ESI negative mode for triazole acetic acid. Residue quantification is carried out using external standard calibrations.

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted except in cases where it is noted that no substitution is allowed.

2.2 Reagents

All solvents and other reagents must be of high purity, *e.g.* glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents and analytical standards used in this method along with details of preparation of solutions is included in Appendix 2.

2.3 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials:

1. Ensure good ventilation.
2. Wear gloves and laboratory coat.
3. Prevent inhalation and contact with mouth or skin.
4. Wash any contaminated area immediately.

Individual primary stock solutions at the 100 µg/mL concentration level are prepared by dissolving 10.0 mg of each compound into individual 100-mL volumetric flasks followed by dilution to the mark with methanol. The amounts weighed for each compound should be corrected for its respective % purity.

Alternatively, the appropriate volume of methanol is added to a known amount of standard material using the equation below. The concentration of the analytical standard is corrected for its chemical purity.

$$V(mL)=\frac{wt.(mg)\times P}{C(\mu g/mL)}\times 10^{3}$$

Where "*V*" is the volume of methanol needed; "*wt.*" is the weight, in mg, of the solid analytical standard; "*P*" is the purity, in decimal form, of the analytical standard; "*C*" is the desired concentration of the final solution, in µg/mL; and 10^3 is a conversion factor. In this second case, the standard material is weighed directly into the amber glass storage bottle.

2.3.1 Fortification Standards

A mixed standard solution at the 1.0 µg/mL concentration is prepared by combining 1.0 mL of each individual primary stock standard into a 100-mL volumetric flask and filling to the mark with 5/95 methanol/water (v/v; HPLC grade). Serial dilutions of this mixed standard solution are prepared in 5/95 methanol/water (v/v; HPLC grade) to create mixed fortification standards. Prepare the standard levels such that no more than 1.0 mL of standard is used to fortify soil samples. For example, a 10-g soil sample fortified at 1.0 ppb could be fortified using 1.0 mL of a 0.010 µg/mL fortification solution.

2.3.2 Injection Standards

Since two injections are made per sample, two sets of injection standards should be prepared. One set of injection standards should be prepared by combining 1.0 mL of the cyproconazole and the 1,2,4-triazole primary stock solutions into a 100-mL volumetric flask and filling to the mark with 5/95 methanol/water (v/v; HPLC grade). This will yield a 1.0 µg/mL solution. Serial dilutions of this mixed standard solution are prepared in 5/95 methanol/water (v/v; HPLC grade) to create mixed injection standards. Prepare the standard levels in the appropriate concentration range starting with a standard at half the concentration equivalent

to the LLMV (0.50 ppb) and ending at an upper concentration equivalent to 60 ppb. These mixed calibration standards are used for analytical purposes for injections made in the positive mode from the Varian Certify SPE cartridge cleanup procedures (Section 3.3.2).

A separate set of injection standards are prepared by placing 1.0 mL of the triazole acetic acid (TAA) primary stock solution into a 100-mL volumetric flask and filling to the mark with 5/95 methanol/water (v/v; HPLC grade). This will yield a 1.0 µg/mL solution. Serial dilutions of this standard solution are prepared in 5/95 methanol/water (v/v; HPLC grade) to create TAA injection standards. Prepare the standard levels in the appropriate concentration range starting with a standard at half the concentration equivalent to the LLMV (0.50 ppb) and ending at an upper concentration equivalent to 60 ppb. These TAA calibration standards are used for analytical purposes for injections made in the negative mode from the Waters MAX SPE cartridge cleanup procedures (Section 3.3.1).

All standard solutions are stored in amber glass bottles in a refrigerator at approximately 4°C to prevent concentration changes due to photodecomposition of the analytes or solvent evaporation. Freshly mixed working standard solutions are typically prepared every three months and fresh stock standard solutions are prepared every six months. In general, the expiration dates of the stock and working standard solutions are not extended beyond the expiration date of the solid standard unless stability considerations or other pertinent information dictate otherwise.

2.4 Safety Precautions and Hazards

Whereas most of the chemicals in this method have not been completely characterized, general laboratory safety precautions are advised (e.g., safety glasses, gloves, etc.). The user(s) should consult the relevant MSDS for commonly used reagents and materials.

Special Note: Formic acid is dangerously caustic to skin and should be handled with extreme care.

3.0 ANALYTICAL PROCEDURE

Note: Due to the low detection limit of the method it is important that precautions be taken to avoid cross contamination in the laboratory.

Specifically:

- Wherever possible disposable glassware/plastic-ware has been specified, new glassware/plastic-ware should be used for each batch of samples.
- Each solvent used in the method should be checked to verify that it is free from contamination (if contamination is suspected).
- Existing glassware should be solvent (methanol or acetone) rinsed, after washing and before use in the method.

3.1 Sample Preparation

It is important that a homogeneous soil sample be available for analysis. All samples should be prepared using an approved method of preparation for residue analysis prior to use.

3.2 Extraction

a) Weigh representative amounts of soil (10 ± 0.1 g) into separate 50-mL disposable plastic centrifuge tubes. At least one untreated control and two control samples fortified with known amounts of analytes of interest should be analyzed with each sample set, using the same procedure, to verify method performance. No more than 1.0 mL of fortification solution should be added. Allow fortified control samples to equilibrate for at least 5 minutes before proceeding to the extraction.

b) Add 25 mL of 80/20 methanol/water, cap and shake on a mechanical shaker at a speed that visibly agitates the samples for a minimum of 20 minutes. Tubes should be placed in a flat or horizontal orientation.

c) Centrifuge samples at 5000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for about five minutes. Decant and filter the supernatant liquid through a Whatman 2V filter paper into a 250-mL flat (or round) bottom flask.

Note: With some soils, particularly those with a high clay content, the solution may still be visibly cloudy even after centrifugation. This is normal and will not affect results.

d) Repeat extraction using another 25 mL of 80/20 methanol/water. Add extraction solvent to the solid soil remaining in the centrifuge tube from the first extraction at 3.2 (b). Cap and shake by hand or vortex to mix. If shaking cannot break up the compacted soil, use a suitable implement (e.g., a spatula) to facilitate this process. Shake on a mechanical shaker at a speed that visibly agitates the samples for a minimum of 20 minutes. Once again, tubes should be placed in a flat or horizontal orientation.

e) Centrifuge samples at 5000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 5 minutes. Decant and filter the supernatant liquid into the flask containing the first extract from 3.2 (c).

f) After most of the extract has filtered, rinse the filter paper with 10 mL of methanol and collect the rinsate into the same flask containing extracts from 3.2 (c) and 3.2 (e).

g) Concentrate the resulting solution to aqueous (~ 3 mL) under vacuum with a rotary evaporator at a bath temperature of 35°C.

h) Quantitative transfer the resulting concentrates with de-ionized (or HPLC grade) water to a clean 15-mL plastic centrifuge tube. Dilute to a final volume of 10 mL

with de-ionized (or HPLC grade) water. Mix well with shaking or vortex and proceed to solid phase extraction cleanup as described in section 3.3. Method stopping point suggested at this point.

Note: Method stopping point suggested at this point if needed. It has been demonstrated, during the method development, that the soil extracts or the excess portions of the final soil extracts (prior to the SPE procedures) can be stored under refrigeration up to two weeks for further processing without re-extraction of the soil.

3.3 Solid Phase Extraction (SPE) Cleanup

Sample cleanup is accomplished by two separate procedures. The first is the use of Waters Oasis MAX solid phase extraction (SPE) cartridge for triazole acetic acid. The second is the use of Varian Bond Elut Certify solid phase extraction (SPE) cartridge for cyproconazole and 1,2,4-triazole. Both procedures are described below. Do not allow cartridges to dry during the process. Allow one solvent to flow through the SPE (no liquid layer on top of bed) before adding the next solvent. The flow rate should be kept at a rate of less than 20 drops per minute. Flow efficiency can be improved by controlled vacuum on the SPE extraction box or controlled positive pressure on the SPE cartridge; however, sample loading is performed using gravity flow.

3.3.1 Solid Phase Extraction Cleanup by Waters Oasis MAX Cartridges

a) Condition the SPE cartridges as follows:
   1. Methanol; one cartridge full
   2. 2% Formic acid in methanol; 5-mL, one time
   3. De-ionized (or HPLC grade) water; 5-mL, two times
   4. 1% Ammonium hydroxide in methanol (**freshly prepared**); 5-mL, two times
   5. 1% Ammonium hydroxide in water (**freshly prepared**); 3-mL, two times
   6. Close the valve and add an additional 400 µL of 1% ammonium hydroxide in water

b) Load 2.0-mL aliquot of the soil extract from 3.2(h) by gravity

c) Wash the cartridge as follows:
   1. De-ionized (or HPLC grade) water; 2-mL, two times
   2. De-ionized (or HPLC grade) water; 5-mL additional
   3. 50/50 Methanol/water; 5-mL, one time
   4. 1% Ammonium hydroxide in methanol (**freshly prepared**); 2-mL, three times
   5. Methanol; 2-mL, one time

d) Elute with 2% formic acid in methanol; 2-mL, four times and **collect** into a clean 15-mL plastic centrifuge tube

e) Evaporate the eluent to dryness under a gentle stream of nitrogen at a bath temperature of 40°C

f) Reconstitute to a final volume of 2-mL (or an appropriate final volume for higher concentration residue samples) with 5/95 methanol/water

g) Vortex and transfer into an HPLC vial for LC/MS/MS analysis

3.3.2 Solid Phase Extraction Cleanup by Varian Bond Elut Certify Cartridges

a) Condition the SPE cartridges as follows:
   1. Methanol; two cartridges full
   2. 0.5% Ammonium hydroxide in 90/10 methanol/water (**freshly prepared**); 2-mL, two times
   3. De-ionized (or HPLC grade) water; 2-mL, two times
   4. 5% Formic acid in methanol; 2-mL, two times
   5. 2% Formic acid in water; 2-mL, one time

b) Load 2.0-mL aliquot of the soil extract from 3.2(h) by gravity

c) Wash the cartridge as follows:
   1. De-ionized (or HPLC grade) water; 2-mL, two times
   2. Methanol; 2-mL, two times

d) Elute with 0.5% ammonium hydroxide in 90/10 methanol/water (**freshly prepared**); 2-mL, three times and **collect** into a clean 15-mL plastic centrifuge tube

e) Evaporate the eluent to aqueous (< 0.6 mL) under a gentle stream of nitrogen at a bath temperature of 40°C

f) Reconstitute to a final volume of 2-mL (or an appropriate final volume for high concentration residue samples) with 5/95 methanol/water

g) Vortex and transfer into an HPLC vial for LC/MS/MS analysis

3.4 Time Required for Analysis

The methodology was performed with a batch of 18 samples during validation. One person can complete the sample preparation of 18 samples in one day (8 hour work period). The analytical sequence is typically performed overnight on a LC/MS/MS system.

3.5 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks unless otherwise specified in the analytical procedure. However, the SPE cleanup procedures should not be stopped for overnight and weekend breaks until the final sample extracts are obtained (steps 3.3.1(d) or 3.3.2(d)) and volatiles partially evaporated (steps 3.3.1(e) or 3.3.2(e)). Should it be necessary to store the extracts overnight, the fresh recovery sample results will indicate whether acceptable results are obtained. Samples should be stored in sealed containers at refrigerated temperatures when the analyses cannot be completed in a single working day. Acceptable method recoveries will validate any work flow interruptions.

3.6 Preparation of Calibration Standards for LC/MS/MS

Standards for external calibration should be prepared in 5:95 methanol:ultra pure water. In general, it is recommended that a minimum of five levels of calibration standards are to be used for calibration plots establishment. In this study, the following concentration levels

were prepared for calibration plots: 60 pg/µL, 40 pg/µL, 20 pg/µL, 10 pg/µL, 5.0 pg/µL, 2.0 pg/µL, 1.0 pg/µL and 0.5 pg/µL.

LC calibration standards should be stored in amber glass bottles under refrigeration conditions. An expiration date of three months is recommended unless additional study data are generated that show a longer expiration date is appropriate.

4.0 FINAL DETERMINATION

The following instrument conditions have been found to be suitable for the targeted analytes analysis in this laboratory. Other instruments may also be used, however, optimization may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum instrument operation.

4.1 LC System Description and Operating Conditions

LC Instrumentation:

The Thermo Electron Surveyor Plus LC system consists of an analytical pump unit (a quaternary solvent system) and an autosampler. The solvent degasser, column oven and sample tray temperature control are integral parts to the LC system. The system is controlled and data processed by Thermo Electron Xcalibur™ Software.

LC Operating Conditions:

Injection Volume: 50 µL
Sample Compartment Temp.: refrigerated at 15°C (if possible)
Column Temperature: 25°C
Column: Zorbax SB-Aq, 4.6 x 75 mm, 3.5 µm (Agilent Catalog No. 866953-914)
Column filter: ColumnSaver (MAC-MOD Catalog No. MMCS210)
Mobile Phase A: 0.1% formic acid in HPLC grade water
Mobile Phase B: 0.1% formic acid in HPLC grade methanol

Mobile Phase Program for **Triazole Acetic Acid (TAA)** analysis in conjunction with Negative Mode MRM MS/MS detection:

Step	Time (min)	%A	%B	Flow Rate (mL/min)	Gradient
0	0.0	98	2	0.5	---
1	5.0	98	2	0.5	---

The typical retention time for TAA using this instrument and these conditions is approximately 2.8 minutes. The retention time may vary depending upon chromatographic conditions and systems.

Mobile Phase Program for **1,2,4-Triazole (T) and Cyproconazole (CCZ)** analysis in conjunction with Positive Mode MRM MS/MS detection:

Step	Time (min)	%A	%B	Flow Rate (mL/min)	Gradient
0	0.0	98	2	0.5	---
1	2.0	98	2	0.5	---
2	3.0	10	90	0.5	linear
3	8.0	10	90	0.5	---
4	8.1	98	2	1.0	linear
5	10.0	98	2	1.0	---
6	10.1	98	2	0.5	---
7	11.0	98	2	0.5	---

The typical retention times for 1,2,4-Triazole and Cyproconazole using this instrument and these conditions are approximately 2.6 and 6.0 minutes, respectively. The retention time may vary depending upon chromatographic conditions and systems.

Note: To help minimize instrument contamination, a timed event controlled switching valve may be used to divert the LC stream to waste during periods of no data collection.

4.2 Mass Spectrometer Conditions

A Thermo Electron TSQ Quantum Ultra mass spectrometer was used to establish and validate the method. The system is controlled and data processed by Thermo Electron Xcalibur™ Software. Triazole acetic acid is analyzed using electrospray source with negative ionization/detection mode while 1,2,4-triazole and cyproconazole are analyzed using electrospray source with the positive ionization/detection mode. Separate analytical sequences were used to achieve the instrumental maximum sensitivity.

Following are the instrumental parameters applied for this method. The analyst should make necessary adjustments and tuning to these parameters to obtain optimum operational conditions based on the actual instrument used for the specific study.

Ion Source Parameters:

	Negative Mode	Positive Mode
Spray Voltage (V)	3000	3800
Vaporization Temperature (°C)	350	350
Sheath Gas Pressure (psi)	50	60
Ion Sweep Gas Pressure (psi)	5.0	5.0
Aux Gas Pressure (psi)	20	50
Capillary Temperature (°C)	350	350
Tube Lens Offset	-83	104
Source CID (V)	10	0
Collision Pressure (mTorr)	0.9	0.9
Quad MS/MS Bias (V)	0.0	0.0

MRM (SRM) operating conditions and MS/MS transitions:

Analyte	MW (exact)	MS/MS Transition	Scan Width	Dwell (sec.)	CE (Volts)	Q1 PW	Q3 PW
Triazole Acetic Acid	127.04	126.1 → 82.2	0.01	0.10	12	0.7	0.7
1,2,4-Triazole	69.03	70.1 → 43.2	0.01	0.10	20	0.7	0.7
Cyproconazole	291.11	292.1 → 125.0	0.01	0.10	30	0.7	0.7

Data collection window for negative mode is 2 – 4 minutes.
Data collection window for positive mode is 1 – 8 minutes.

5.0 CALCULATION OF RESULTS

Determination of Residues in Samples:

Inject the samples from 3.3.1(g) and 3.3.2(g) into the analysis system along with a selected range of calibration standards. Quality control (QC) standards are injected intermittently throughout the set to evaluate system stability. Calibrate the instrument with injections of at least five (or more) concentration levels of the standard solutions and generate a calibration curve for each analyte using proper regression parameters (*e.g.* linear regression with 1/X weighing). The data system (*e.g.* Xcalibur™) uses the calibration plot and the respective peak responses (*e.g.*, area or height) to calculate the amount of analyte in a sample. If the analyte response in the sample exceeds 10% of the response for the highest concentration standard injected, the sample should be diluted with 5:95 (v/v) MeOH:purified water and re-analyzed.

Determination of Residues in Fortified Samples:

Validate the method performance for each set of samples analyzed by including a control sample and two or more control samples fortified with known amounts of the desired analytes prior to the extraction procedure. The fortification levels for external recoveries should approximate the expected residue levels in the study samples.

Recovery data are generally considered acceptable when the mean values, for each fortification level, are between 70% and 120% with a relative standard deviation of ≤20%.

Calculations:

Calculations may be performed by computer program (preferred) or manually as shown below.

Calculate the analyte concentration (in ppb) for field-incurred residues using the equation:

$$RES\ (ppb) = \frac{Analyte\ \ found\ \ (ng)}{SWI\ \ (mg)} \times 1000$$

where *RES* is the residue value in ppb, *Analyte Found (ng)* is calculated from the standard calibration curve, and *SWI* is the sample weight injected in milligrams (mg).

NOTE: If the analyte found is calculated in units of picograms (pg, instead of ng), the 1000 multiplication factor is not needed.

The amount (mg), of sample weight injected (*SWI*) can be calculated using the equation:

$$SWI(mg) = \frac{FW\,(g)\,x\,IV\,(\mu L)}{FV(mL)}$$

where *FW* = final sample weight (g), *IV* = LC injection volume (µL) and *FV* = final volume in which sample is dissolved (mL).

The final sample weight (*FW*) is calculated by the equation:

$$FW(g) = \left[\frac{SWE(g)\,x\,A1(mL)}{EV\,(mL)+\{SWE(g)\,x\,M\,(\%)/100\}}\right] x \left[\frac{A2(mL)}{INV\,(mL)}\right]$$

where *FW* = final weight (g), *SWE* = sample weight extracted (g), *A1* = aliquot 1 volume (mL), *EV* = total extraction solvent volume (mL), *M* = sample moisture in percent, *A2* = aliquot 2 volume (mL), if needed, *INV* = interim volume (mL) is the total volume from which the second aliquot is taken.

NOTES: For method performance (recovery) samples, the *M*% (moisture) value is set to zero since the fortifications are based upon their wet weights. If no sample dilutions are performed, the second term in the equation (i.e., *A2/INV*) is equal to one.

Corrections may be made to the residue value (*RES*) calculated above. At the discretion of the study director, this value may be corrected to account for the average recovery and/or sample moisture.

The recovery factor, expressed as a percentage (*R%*), is calculated using the following equation.

$$R\% = \frac{RES\ fortified\ (ppb) - RES\ control\ (ppb)}{ppb\ \ analyte\ \ added} \times 100$$

To correct a residue value to its dry weight value, the following equation may be used:

$$SDW\,(ppb) = \left[\frac{CR\,(ppb)}{\frac{(100 - M(\%))}{100}} \right]$$

where *SDW* = soil dry weight residue (ppb), *CR* = corrected soil residue, and *M* = soil moisture (%). For study samples, soil moistures should be determined following the appropriate SOP.

The recovery corrected soil residue can be determined by the equation:

$$CR\,(ppb) = \left[\frac{RES\ (ppb)}{AR\,(\%)} \right] x\,100$$

where *CR* = recovery corrected residue (ppb), *RES* = residue found (ppb) and *AR* = average recovery (%).

When the average percentage recovery is greater than 100%, the sample residue values should not be corrected.

6.0 INTERFERENCES AND CONFIRMATION

Due to the high selectivity of the detection technique, interference arising from the sample matrix has not been observed. Final determination by LC/MS/MS is considered to be highly specific; therefore, no confirmatory conditions are included.

It is recommended that each batch of solvent or reagents be checked for potential contamination prior to use. Further, it is highly recommended that the performance of solid phase extraction cartridges be checked prior to use with selected level of calibration standards. This method uses disposable labware, where possible. All reusable glassware should be detergent washed then rinsed with HPLC grade methanol or acetone prior to use.

7.0 MODIFICATIONS AND POTENTIAL PROBLEMS

It is possible that contaminants from chemicals, solvents, glassware, etc. may interfere with the analysis and give a false positive result. It is recommended that reagent blank samples be included in a sample set if contamination is suspected. During method development, minor carryover of cyproconazole residues immediately after high level standards or samples injections have been observed with some types of LC injectors. If carryover issues are suspected, the injection of blank samples consisting of 80:20 (v/v) methanol:water within the analysis can be used to flush the system.

Although the SPE cartridges used for this method have been demonstrated to be suitable for up to 100 ppb residue levels, SPE cartridge sample overload or sample break through may still exist. If cartridge overload or sample break through is suspected, it is highly recommended that SPE performances be checked with a known level of calibration standard and adjust loading concentration or replacing the SPE lot accordingly.

Any modifications to this method must be documented in the study raw data.

APPENDIX 5 METHOD FLOWCHART

Extraction

Weigh a 10 ± 0.1 g of representative soil sample in 50-mL Centrifuge Tube

↓

Extract twice with 80/20 MeOH/H_2O (v/v) on a shaker for 20 minutes

↓

Combine the extracts upon centrifugation and filtration

↓

Concentrate to aqueous and adjust volume to 10 mL with H_2O

SPE: Oasis MAX	**SPE: Bond Elut Certify**
Conditioning (see Section 3.3.1 (a))	Conditioning (see Section 3.3.2 (a))
↓	↓
Load 2.0 mL of soil extracts	Load 2.0 mL of soil extracts
↓	↓
Wash SPE cartridge (see Section 3.3.1 (c))	Wash SPE cartridge (see Section 3.3.2 (c))
↓	↓
Elute with 2% formic acid in MeOH and collect	Elute with 0.5% NH_4OH in 90/10 MeOH/H_2O and collect
↓	↓
Evaporate the eluate to dryness under N_2	Evaporate the eluate to aqueous under N_2
↓	↓
Reconstitute to final volume	Reconstitute to final volume
↓	↓
Transfer to LC vial	Transfer to LC vial
↓	↓
Analyzed on LC/MS/MS	Analyzed on LC/MS/MS