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.

2.2 Reagents

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC/OPTIMA 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 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.
4. Wash any contaminated area immediately.

2.3.1 Stock Solutions

Prepare a 100 µg/mL stock solution for each analyte by one of the following methods:

Weigh out accurately, using a five figure balance, sufficient prometryn analytical standard into an amber "Class A" volumetric flask (100-mL). Dilute to the mark with acetonitrile (methanol for simazine) and mix well to give a 100 µg/mL stock solution of prometryn. Standards should be prepared in amber bottles and stored under refrigeration.

Alternatively, the appropriate volume of acetone to add to a known amount of standard material may be determined using the equation below. The standard concentration is corrected for its chemical purity.

$$V = \frac{W \times P}{C} \times 1000$$

P = Standard purity in decimal form (P(%)/100)
V = Volume of acetonitrile required
W = Weight, in mg, of the solid analytical standard
C = Desired concentration of the final solution, (µg/mL)
1000 = Unit conversion factor

In this case, the standard material is weighed directly into an appropriate storage vessel.

2.3.2 Fortification Solutions

Combined sample fortification solutions containing each analyte should be prepared by serial dilution in acetonitrile from each individual stock solution. It is recommended that the following combined fortification solutions are prepared: 1.0 µg/mL, 0.10 µg/mL and 0.01 µg/mL.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

Combined calibration standards are prepared in acetonitrile:water + 0.1% acetic acid (40/60 v/v). An aliquot from combined fortification solutions can be serially diluted in preparation of calibration standards. Using the instrumentation found in Section 4.0, the following concentration range of standards were prepared and used for calibration: 0.10 pg/µL - 10 pg/µL.

A calibration curve should be generated to quantify analyte residues. Standards over an appropriate concentration range should be prepared with a minimum of five levels.

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored in amber bottles and refrigerated when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration date of six months is recommended unless additional data are generated to support a longer expiration date.

2.4 Safety Precautions and Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate MSDS or a monograph such as 'Hazards in the Chemical Laboratory', edited by S. G. Luxon, The Chemical Society, London (Reference 1).

Solvent and Reagent hazards

	Acetonitrile	Acetic acid	Formic Acid
Harmful Vapor	✓	✓	✓
Highly Flammable	✓	×	×
Harmful by Skin Absorption	✓	✓	✓
Irritant to respiratory system and eyes	✓	✓	✓
Causes severe burns	×	✓	✓
Syngenta Hazard Category (SHC)	SHC-C, S	SHC-C, S	C, S
OES Short Term (mg/m^3)	105	37	N./A
OES Long Term (mg/m^3)	70	25	9

N/A not known

Syngenta Hazard Classification for triazines are SHC-C,S. The Syngenta Hazard Category scale rates highly toxic chemicals as category SHC-E and non-toxic chemicals as category SHC-A. An additional hazard category of S indicates the compound is a severe skin and eye irritant.

In all cases avoid breathing vapor. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

A summary of the method is included in flow-chart form as shown in Appendix 4. In order to verify method performance and allow recovery corrections to be made (if appropriate), fortified control samples should be included in each sample set. At least one untreated control and two control samples fortified with known amounts of each analyte should be analyzed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired.

3.1 Sample Preparation

a) If soil samples are received frozen (high moisture content) they should be allowed to defrost completely at room temperature. All samples should be prepared using an approved method of preparation to obtain a homogeneous sample prior to analysis.

b) Transfer 10 g of soil sample to be analyzed into a 125 mL round bottom flask. Sample fortification is carried out at this time using combined fortification standards in acetonitrile, if required. Allow fortification solvent to evaporate before proceeding.

c) To each sample add 60 mL of acetonitrile:water (90/10 v/v) and sufficient boiling chips. Place samples on reflux/condenser apparatus and reflux for 1 hour (boil time).

d) Transfer sample to a 125 mL nalgene bottle and centrifuge at 3500 rpm for 5 minutes.

e) Filter @1mL of supernatant through 50mg CEC18 sorbent (UCT), using either a 1 mL tube or 96-well plate format. Collect 0.6 mL in LC vial or deep well titer plate.

f) Add an additional 0.4 mL of 0.1% aqueous acetic acid to bring final volume to 1.0 mL. Final solvent concentration should be equivalent to acetonitrile:water + 0.1% acetic acid (40/60 v/v) or if volume is less or more than 0.5 mL, add an equal amount of 0.1% aqueous acetic acid, mix thoroughly and submit for LC-MS/MS analysis.

3.2 Problems and Modifications

The SPE procedure has been developed using cartridges from the stated manufacturer. Similar cartridges from other manufacturers may be used. In all cases however, it is strongly recommended that the elution profile of the chosen batch of cartridges is checked prior to commencing analysis to assess any variation in manufacturers' products and between batches.

3.3 Time Required for Analysis

The methodology is limited to the amount of reflux apparatus that is available. Using an apparatus of 12 reflux/condensers, one skilled analyst can complete the analysis of 60 samples in 1 day (8 hour working period).

3.4 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekends unless otherwise specified in the analytical procedure. Acceptable method recoveries will validate any work flow interruptions. Samples should be stored refrigerated in sealed containers where the analysis cannot be completed in a single day.

4.0 FINAL DETERMINATION

The method has been developed for use on an Applied Biosystems API5500. The following instrumentation and conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though 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 use.

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

4.1 Instrument Description

LC System	:	Waters Acquity UPLC System (I-Class)
Detector	:	Applied Biosystems Sciex 5500 QTRAP triple quadrupole mass spectrometer with Analyst software version 1.5.1.

4.2 Chromatography Conditions for Triazines

Column 1	:	ACE 5 C18, 3.0 x 50mm, 5.0 µm
Column Oven Temperature	:	30°C
Injection volume	:	10 µL
Flow Rate	:	1.0 mL/minute
Run Time	:	3.5 minutes
Injection protocol	:	Analyze calibration standard after 3 to 4 sample injections
Mobile phase	:	A (Solvent 1): 0.1% acetic acid in ultra-pure water B (Solvent 2): 0.1% acetic acid in ACN

Mobile Phase Composition

Time (mins)	% A	% B
0.00	95	5
1.50	5	95
2.20	5	95
2.50	95	5
3.5	95	5

4.3 API 5500QTRAP Mass Spectrometer Conditions

Interface	:	TurboIonSpray
Polarity	:	Positive
Curtain gas (CUR)	:	Nitrogen set at 20 (arbitrary units)
Temperature (TEM)	:	500°C
Ionspray voltage	:	1000
Collision gas setting (CAD)	:	Medium
Gas 1 (GS1)	:	Air set at 50 (arbitrary units)
Gas 2 (GS2)	:	Air set at 50 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		Atrazine Primary Transition	Atrazine Confirmatory Transition
Q1 *m/z*	:	216.1	216.1
Q3 *m/z*	:	174.2	132.0
Dwell time	:	50 ms	50 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	60 V	60 V
Entrance potential (EP)	:	10 V	10 V
Collision energy (CE)	:	35 V	35 V
Collision cell exit potential (CXP)	:	10 V	10 V

MRM Conditions		Simazine Primary Transition	Simazine Confirmatory Transition
Q1 *m/z*	:	202.1	202.1
Q3 *m/z*	:	132.0	103.7
Dwell time	:	50 ms	50 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	60 V	60 V
Entrance potential (EP)	:	10 V	10 V
Collision energy (CE)	:	35 V	35 V
Collision cell exit potential (CXP)	:	10 V	10 V

MRM Conditions		Propazine Primary Transition	Propazine Confirmatory Transition
Q1 *m/z*	:	230.1	230.1
Q3 *m/z*	:	146.0	188.0
Dwell time	:	50 ms	50 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	60 V	60 V
Entrance potential (EP)	:	10 V	10 V
Collision energy (CE)	:	35 V	35 V
Collision cell exit potential (CXP)	:	10 V	10 V

MRM Conditions		Prometryn Primary Transition	Prometryn Confirmatory Transition
Q1 *m/z*	:	242.2	242.2
Q3 *m/z*	:	158.1	200.1
Dwell time	:	50 ms	50 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	60 V	60 V
Entrance potential (EP)	:	10 V	10 V
Collision energy (CE)	:	35 V	35 V
Collision cell exit potential (CXP)	:	10 V	10 V

Typical chromatograms are shown in the Figures Section. Chromatograms for other soil types are similar.

4.4 Confirmatory Procedures for Triazines

Final determination by LC-MS/MS with primary and confirmatory transitions is considered to be highly specific; hence no further confirmatory conditions are required.

5.0 CALCULATION OF RESULTS

5.1 Multi Point Calibration Procedure

Analyte residues may be calculated in µg/kg for each sample as follows:

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (for example, 50% LOQ to 10 x LOQ). An appropriate number of different concentrations within this range should be prepared (at least five levels).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to respective target ions. Quality Control standard solutions should be interspersed throughout the analysis to monitor any matrix effects.

c) Generate calibration curve parameters using an appropriate regression package.

d) The following equation can be rearranged and used to calculate residues as follows:

$$y = mx + c$$

Where y is the instrument response value, x is the standard concentration, m is the gradient (slope) of the line of best fit ("X-variable 1" in MS Excel) and c is the intercept value. An example of this equation generated using the experimental values of m and c should be included in the raw data, as should the "R-Squared" value for the regression.

Re-arrangement for x gives

$$x = \frac{y - c}{m}$$

e) Calculate analyte residues in the sample, expressed as mg/kg, as follows
f)

$$\text{Residue (mg/kg)} = \frac{\text{Analyte found } (\mu\text{g/mL})}{\text{Sample conc. (g/mL)}}$$

Where analyte found (µg/mL) is calculated from the standard calibration curve and sample conc. is the final sample concentration in g/mL.

If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \text{ (mg/kg)}$$

5.2 Single Point Calibration Procedure

Analyte residues may be calculated in mg/kg (ppm) for each sample using a mean standard response from each of the injections bracketing the sample as follows.

a) Make repeated injections of a standard containing each analyte at an appropriate concentration into the LC-MS/MS operated under conditions as described in Section 4. When a consistent response is obtained, measure the peak areas obtained for prometryn.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to each analyte.

c) Re-inject the standard solution after a maximum of four injections of sample solutions.

d) Calculate the analyte residues in the sample, expressed as mg/kg (ppm) using a mean standard response from each of the injections bracketing the sample as follows:

$$\text{Residue (mg/kg)} = \frac{\text{PK area (SA)}}{\text{PK area (STD)}} \times \frac{\text{Standard Conc.}}{\text{Sample Conc.}}$$

PK area (SA) = Peak response for sample
PK area (STD) = Average peak response for bracketing standards
Standard Conc. = Concentration of standard (µg/mL)
Sample Conc. = Sample concentration (g/mL)

If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \text{ (mg/kg)}$$

Although single point calibration may be used to quantify residues it is recommended that a calibration curve is generated with each analytical run to demonstrate the linearity of instrument response (Reference 2).

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analyzed with each set of samples to verify that the sample used to prepare recovery samples is free from contamination. A minimum of one control should be analyzed with each batch of samples. Control samples from the same matrix are recommended to monitor any instrumental matrix effects present.

At least two recovery samples (control samples accurately fortified with known amounts of analyte), including one at the method LOQ and one at the expected residue level, should also be analyzed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found in the sample. The fortification levels should be appropriate to the residue levels expected in the sample.

Recovery efficiency is generally considered acceptable when the mean values are between 70% and 110% and with a relative standard deviation of $\leq 20\%$.

When the method is used for monitoring purposes, control and recovery samples are not required where suitable control samples are not available.

7.0 SPECIFICITY

It is recommended that reagent blank samples be included in a sample set if contamination is suspected.

FIGURE 1 Atrazine Chemical Structure

Common Name : Atrazine

Code Name : G30027

CAS Number : 1912-24-9

CA Index Name : 6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine

IUPAC Name : 6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine

Molecular Formula : $C_8H_{14}ClN_5$

Molecular Weight : 215.7

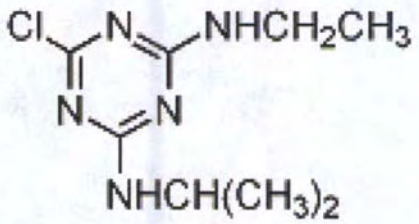


FIGURE 2 Simazine Chemical Structure

Common Name : Simazine

Code Name : G27692

CAS Number : 122-34-9

CA Index Name : 6-chloro-*N,N'*-diethyl-1,3,5-triazine-2,4-diamine

IUPAC Name : 6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine

Molecular Formula : $C_7H_{12}ClN_5$

Molecular Weight : 201.7

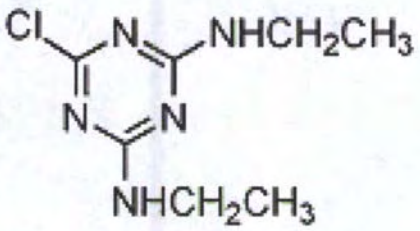


FIGURE 3 Propazine Chemical Structure

Common Name : Propazine

Code Name : G30028

CAS Number : 139-40-2

CA Index Name : 6-chloro-*N,N'*-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine

IUPAC Name : 6-chloro-N^2,N^4-diisopropyl-1,3,5-triazine-2,4-diamine

Molecular Formula : $C_9H_{16}ClN_5$

Molecular Weight : 229.7

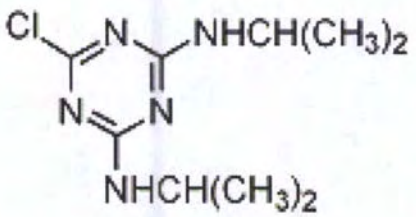


FIGURE 4 Prometryn Chemical Structure

Common Name : Prometryn

Code Name : G-34-161

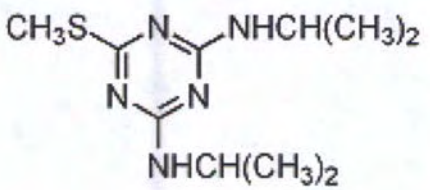
CAS Number : 7287-19-6

CA Index Name : *N,N'*-bis(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine

IUPAC Name : N^2,N^4-diisopropyl-6-methylthio-1,3,5-triazine-2,4-diamine

Molecular Formula : $C_{10}H_{19}N_5S$

Molecular Weight : 241.4



APPENDICES SECTION

APPENDIX 1 Apparatus

Recommended Suppliers

Equipment	Description	Supplier
General lab glassware	General lab glassware	www.thermoscientific.com
General lab plastic-ware	General lab plastic-ware	www.thermoscientific.com
SPE Extraction Column	1mL/50mg p/n CEC181L1	www.unitedchem.com
SPE Extraction 96-Well	WSHCEC18105	www.unitedchem.com
Autosampler vials	Snap cap, 2 mL size	www.thermoscientific.com
HPLC column	ACE 5 C18, 3.0 x 50mm	www.ace-hplc.com

APPENDIX 2 Reagents/Chemicals

Recommended Suppliers

Reagent	Description	Supplier
Ultra-pure water	Optima/HPLC grade	www.thermoscientific.com
Acetonitrile	Optima/HPLC grade	www.thermoscientific.com
General Lab Chemicals	A.C.S. grade	www.thermoscientific.com
Analytical standard	GLP certified	Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

a) acetonitrile:water (90/10 v/v); prepared by diluting 900 mL acetonitrile to 1L using ultra-pure water.

b) acetonitrile:water + 0.1% acetic acid (40/60 v/v); prepared by diluting 600 mL of ultra-pure water to 1L using Optima/HPLC Grade acetonitrile and adding 1 mL reagent grade acetic acid.

c) "0.1%" acetic acid in ultra-pure water, prepared by mixing 1.0 mL of reagent grade acetic acid with 1,000 mL of ultra-pure water.

d) "0.1%" acetic acid in acetonitrile, prepared by mixing 1.0 mL of reagent grade acetic acid with 1,000 mL of Optima/HPLC Grade acetonitrile..

APPENDIX 3 LC-MS/MS Tuning Procedure

Calibration of Instrument

The instrument must be mass calibrated on a regular basis using a recommended tuning solution according to the manufacturer's instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

Tuning Instrument

Infuse a standard solution of each analyte (0.1 to 1.0 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 10-20 µL/min. Roughly adjust interface parameters (sprayer position and temperature, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high parent ion signal for each analyte in positive ionization mode.

Using the systems software optimization routine, tune the instrument for each analyte ensuring that the correct ion is selected. If desired, manual tuning of the ion optics and collision energy can be carried out to ensure maximum sensitivity.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injections of individual analytes or a combined standard using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position, temperature, gas flows, and voltages) and the collision gas pressure for maximum sensitivity.

Final determination by LC-MS/MS is considered to be highly specific.

APPENDIX 4 Method Flow Chart for LC-MS/MS

Transfer sample (10 g) to a 125 mL round bottom

↓

Fortify recovery sample, if needed. Add boiling chips

↓

Add 60 mL acetonitrile:water (90/10 v/v) and reflux 1 hour

↓

Centrifuge 3500 rpm for 5 minutes

↓

Pass 1.0 mL supernatant through 50mg of CEC18 sorbent

↓

Dilute 0.6 mL of filtrate with 0.4 mL of 0.1% aqueous acetic acid, mix thoroughly

↓

Submit for LC-MS/MS analysis