1.0 INTRODUCTION

1.1 Scope and chemical structures

Analytical method GRM033.04A is suitable for the determination of cyproconazole residues (Figure 1) in water specimens. The limit of quantification has been established at 0.05 µg/L (or 0.05 ppb).

This method satisfies EU guidelines OECD Guidance Document ENV/JM/MONO(2007)17, US EPA guidelines EPA OPPTS 850.7100 and EC Guidance Documents SANCO/3029/99

1.2 Method summary

Enviromental water specimens are analysed directly by high performance liquid chromatography using triple quadrupole mass spectrometry (LC-MS-MS).

The limit of quantification of the method is 0.05 µg/L(or 0.05ppb) for cyproconazole in drinking water, surface water and ground water.

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 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

Weigh out accurately; using a five-figure balance, sufficient cyproconazole analytical standard to allow dilution in acetonitrile to give 200 µg/mL stock solutions in volumetric flasks.

Alternatively, the appropriate volume of solvent 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 a volumetric flask.

2.3.2 Fortification Solutions

Sample fortification solutions containing cyproconazole should then be prepared by serial dilution in acetonitrile. It is recommended that the following solutions are prepared: 1.0 µg/mL, 0.1 µg/mL and 0.01 µg/mL.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

No significant suppression or enhancement of the instrument response for cyproconazole has been observed in the water types tested using the procedures described in Section 3 during method validation and non-matrix matched standards should normally be used for calibration.

Standards must be prepared in ultra pure water. Standards are prepared as follows: the required standard in acetonitrile is added into a polypropylene centrifuge tube (15 ml size) and diluted to 10 mL with ultra pure water.

A calibration curve should be generated to quantify cyproconazole residues. Standards over an appropriate concentration range should be prepared as described above, using the requisite volumes of cyproconazole in acetonitrile.

Any matrix effects observed may be compensated for by use of matrix matched standards at the discretion of the study director, or by dilution of the final sample with ultra pure water should instrument sensitivity permit.

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored in a refrigerator or freezer 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 expiry 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	AceticAcid
Harmful Vapour	✓	✓
Highly Flammable	✓	✗
Harmful by Skin Absorption	✓	✓
Irritant to respiratory system and eyes	✓	✓
Causes burns	✗	✓
Syngenta Hazard Category	SHC-C, S	SHC-C, S
OES Short Term (mg/m^3)	105	37
OES Long Term (mg/m^3)	70	25

NA = Not known

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

3.0 ANALYTICAL PROCEDURE

A summary of the method is included in flow-chart form in Appendix 4.

3.1 Sample Fortification

In order to verify method performance and allow recovery corrections to be made (if appropriate), fortified control samples should be included with each sample set. To each pre-weighed control water sample, add the appropriate amount of standard solution containing cyproconazole in acetonitrile (not more than 0.1 ml). At least one untreated control and two fortified control samples should be analysed with each sample set.

3.2 Extraction

a) If samples are received frozen, they should be allowed to defrost thoroughly before analysis. Once completely thawed, the bulk water samples should be shaken thoroughly prior to analysis. Samples may be analysed directly without any prior sample concentration or clean-up procedures. Transfer aliquots directly into a suitable autosampler vial, ready for final determination by LC-MS/MS. The sample concentration is 1.0 g/ml.

b) Transfer 10 ml of the water sample to be analysed into a polypropylene centrifuge tube (15 ml size). Fortify the recovery samples with the appropriate amount of cyproconazole in acetonitrile. Cap the tube securely and shake gently to mix thoroughly.

c) Transfer an aliquot of the samples using a plastic disposable pipette to suitable autosampler vial ready for final determination by LC-MS/MS.

3.3 Preparation of Calibration Standards

Non-matrix matched calibration standards must be prepared in ultra pure water. Standards are prepared as follow: the required standard in acetonitrile is added into a polypropylene centrifuge tube (15 ml size) and diluted to 10 mL with ultra pure water.

3.4 Time required for analysis

The methodology is normally performed with a batch of up to 20 samples. One person can complete the analysis of up to 20 samples in 1 day (8 hour working period).

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. 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 following instrumentation and conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though optimisation 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. The method has been developed for use on the Applied Biosystems API 4000 LC-MS/MS.

Final determination by LC-MS/MS with 2 transitions is considered to be highly specific and no further confirmatory conditions are included.

4.1 Instrument description

Pump	:	Agilent 1100 series quaternary pump model number G1311A
Degasser	:	Agilent 1100 series model number G1322A
Column Oven	:	Agilent 1100 series model number G1316A fitted with column switching valve
Detector	:	Applied Biosystems API 4000 triple quadrupole mass spectrometer with Analyst™ software version 1.4.2
Autosampler	:	Agilent 1100 series model number G1313A
Gas Supply	:	Peak Scientific NM20ZA gas station

4.2 Chromatography conditions

Column	:	Hichrom KR100 5 C18 5 µm particle size, 50 x 3.2 mm i.d
Column Oven Temperature	:	40 °C
Injection volume	:	10-50 µL
Stop Time	:	5.0 minutes
Injection protocol	:	Analyse calibration standard after no more than 4 sample injections.
Mobile phase	:	Solvent A = Acetonitrile Solvent B =0.2 % acetic acid in Ultra pure water

4.3 Mobile Phase Gradient

Under these conditions the retention time of cyproconazole is approximately 2.2 minutes.

Time (min)	% Solvent A	% Solvent B	Flow (mL/min)
0:01	30	70	1.0
3.00	80	20	1.0
3.50	80	20	1.0
3.60	30	70	1.0
5.00	30	70	1.0

4.4 Mass spectrometer conditions

Interface	:	TIS Turbo Ionisation Spray
Polarity	:	Positive
CAD		12
CUR		20
GS1		25
GS2		20
Ion Spray Voltage (IS):		5500
TEM (°C)		450
Interface Heater		On
Scan type	:	Multiple reaction monitoring (MRM)

Analyte	Parent ion (m/z)	Daughter ion (m/z)	DP (V)	EP (V)	CXP (V)	CE (V)
Cyproconazole	292.19	70.2 (quantification)	60	10	23	40
		125.0 (confirmatory)	60	10	10	40

MRM Conditions		**Cyproconazole Primary Transition**	**Cyproconazole Confirmatory Transition**
Q1 *m/z*	:	292.19	70.2
Q3 *m/z*	:	292.19	125.0
Dwell time	:	200 ms	200 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit

5.0 CALCULATION OF RESULTS

5.1 Single point calibration procedure

Residues may be calculated in µg/L 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 cyproconazole 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 the analytes.

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

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

d) Calculate the residues in the sample, expressed as mg/kg, 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 (ng/mL)
Sample Conc. = Sample concentration (g/mL)

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

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \ (\mu g/L)$$

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 3).

5.2 Multi point calibration procedure

Residues may be calculated in µg/L for each sample as follows.

a) Prepare mixed standard solutions of cyproconazole over a concentration range appropriate to the expected residues in the samples (for example, 50% LOQ to 20 x LOQ). An appropriate number of different concentrations within this range should be prepared (at least four).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to the analyte. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions.

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 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) Alternatively (depending on the regression analysis software available) a quadratic equation may be used to fit the data. In this case the following general equation should be re-arranged and used to calculate residues:

$$y = a + bx + cx^2$$

Where y is the instrument response value, x is the standard concentration and a, b, c are constants.

f) Calculate the residues of cyproconazole in the sample, expressed as mg/kg, as follows

$$\text{Residue ((ug/L)} = \frac{\text{Analyte found } (n\text{g/mL})}{\text{Sample conc. (g/mL)}}$$

Where analyte found (µg/L) 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. storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \ (\mu g/L)$$

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analysed 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 analysed with each batch of samples.

At least two recovery samples (control samples accurately fortified with known amounts of cyproconazole) should also be analysed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found. The fortification levels should be appropriate to the residue levels expected.

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

7.0 SPECIFICITY

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

7.1 Matrix interference

LC-MS/MS is a highly specific detection technique. Interference arising from the matrices tested has not been observed.

7.2 Reagent and solvent interference

Using high purity solvents and reagents no interference has been found.

7.3 Labware interference

This method uses disposable labware. All reusable glassware should be detergent washed and then rinsed with HPLC-grade methanol, acetone or acetonitrile prior to use.

APPENDIX 3 LC-MS/MS TUNING PROCEDURE

Calibration of Instrument

The instrument must be mass-calibrated on a regular basis using polypropylene glycol (PPG) solutions according to the manufacturer's instructions. Calibrate both mass-resolving quadrupoles (Q1 and Q3).

Tuning instrument for API4000

Infuse a standard solution of Cyproconazole (0.1 to 1.0 µg/mL in mobile phase, see Section 4.2) directly into the mass spectrometer interface at a rate at of about 10 µL/min. Roughly adjust interface parameters (sprayer position, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high parent ion signal at m/z = 292 under positive ionisation conditions.

Using the Analyst 1.4 software quantitative optimisation routine, tune the instrument for Cyproconazole, ensuring that the correct ions are selected (initial Q1 292 and product ions m/z = 70 & 125). Alternatively, the instrument ion optics and collision energy may be tuned manually for Cyproconazole, to ensure maximum sensitivity.

Note: If problems are encountered in tuning the instrument for these ions, the ions should be entered in the method as detailed in Section 4.3 and tuning performed manually.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injections of Cyproconazole standards in mobile phase and at the flow rate to be used. Tune the interface parameters (sprayer position, spray and heater gas flows, spray, orifice, and focusing ring voltages) and the collision gas flow for maximum sensitivity.

In positive ionisation mode, cations of Cyproconazole generated in the ion source are selected and subjected to further fragmentation by collisional activation. The most sensitive daughter ions (m/z = 70 and m/z = 125) are then selected and used for quantitative analysis.

APPENDIX 4 METHOD FLOW CHART