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Chlorothalonil
**Chlorothalonil (R44686) - Analytical Method for the Determination
of Residues of Chlorothalonil and R182281 in Crops**
Analytical Method

DATA REQUIREMENT(S):

EPA OPPTS 860.1340
SANC0/3029/99 rev.4
SANC0/825/00 rev.6

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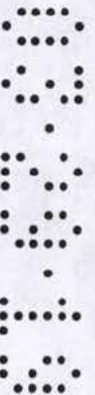
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1. Introduction and Summary

1.1 Scope

The analytical procedure described is suitable for the determination of residues of chlorothalonil (Figure 1) and its metabolite R182281 (Figure 2) in crop matrices using an external standardisation procedure. The limit of quantification has been set at 0.01 mg kg⁻¹.

Figure 1

Code no.	: R44686
Common Name	: Chlorothalonil
IUPAC Name	: Tetrachloroisophthalonitrile
CAS-Registry No	: 1897-45-6
Molecular Mass	: 265.9

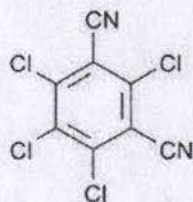
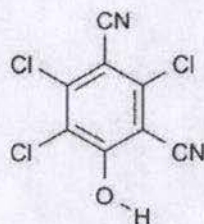


Figure 2

Code no.	: R182281
Other no.	: SDS 3701
IUPAC Name	: 2,4,5-Trichloro-6-hydroxyisophthalonitrile
CAS-Registry No	: 28343-61-5
Molecular Mass	: 247.5



1.2 Method Summary

Crop samples are extracted by homogenisation with acetone:5 M sulphuric acid solution (95:5 v/v) and then centrifuged. For chlorothalonil determination, aliquots are diluted with water followed by a solid phase extraction (SPE) clean-up. Chlorothalonil is analysed by gas liquid chromatography with mass selective detection (GC-MSD). For the determination of R182281, aliquots are diluted with acetonitrile:water. Final determination of R182281 is by high performance liquid chromatography with triple-quadrupole mass spectrometric detection (LC-MS/MS).

2. Materials

The recommended equipment and reagents are described in Appendices 1 and 2. Equipment with equivalent performance specifications and reagents of comparable purity can be substituted provided that they can be shown to be suitable.

2.1 Apparatus

See Appendix 1 for a list of apparatus used during this method.

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. See Appendix 2 for a list of reagents used in this method.

2.3 Preparation of Analytical Standards

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.

Analytical standards of chlorothalonil and R182281 should be prepared and stored in acid-washed amber volumetric flasks. The amber glassware prevents potential photolytic degradation of chlorothalonil and R182281. The acid washing removes

any detergent residues present, which may cause instability problems (Reference 1). Glassware should be soaked for a minimum of one hour in 1M hydrochloric acid solution, and then rinsed in ultra-pure water followed by acetone for preparing R182281 standards and a further rinse with toluene if preparing chlorothalonil. Finally the glassware should be left to dry before use.

Weigh out accurately, using a five figure balance, sufficient chlorothalonil analytical standard to allow dilution in toluene to give a $1000 \mu\text{g mL}^{-1}$ stock solution in a volumetric flask. The chlorothalonil stock solution is then serially diluted in toluene to $0.01 \mu\text{g mL}^{-1}$. These standards may be used for fortification of recovery samples prior to extraction and for GC-MSD final determination (see section 3.2 and 3.5). It is recommended that a $1000 \mu\text{g mL}^{-1}$ stock solution is prepared for chlorothalonil. This allows the use of small standard volumes for high fortification levels to minimise any immiscibility issues.

Weigh out accurately, using a five-figure balance, sufficient R182281 analytical standard to allow dilution in acidified acetone to give a $200 \mu\text{g mL}^{-1}$ stock solution in a volumetric flask. This standard should then be diluted by serial dilution to $0.01 \mu\text{g mL}^{-1}$ in acidified acetone. The acidified acetone is prepared by adding 1 mL of concentrated hydrochloric acid (98%) to 2.5 L of acetone. These standards should be used for the fortification of the samples prior to extraction and for preparation of calibration standards for LC-MS/MS analysis as described in sections 3.2 and 3.5.

When not in use, always store the standard solutions in a refrigerator at $\leq 7^\circ\text{C}$ to prevent decomposition and/or concentration of the standard. Chlorothalonil analytical standards should be replaced with freshly prepared standards after six months (Reference 2). R182281 analytical standards should be replaced with freshly prepared standards after four months.

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 safety manual (e.g. Syngenta Laboratory Safety Manual), which contains recommendations and procedures for handling chemicals or a monograph such as 'Hazards in the Chemical Laboratory', Edited by S G Luxon, The Chemical Society, London (Reference 3).

Solvent Hazards

	Acetone	Methanol	Acetonitrile	Toluene
Harmful Vapour	✓	✓	✓	✓
Highly Flammable	✓	✓	✓	✓
Harmful by Skin Absorption	x	✓	✓	✓
Syngenta Hazard Category	SHC-B, S	SHC-C, S	SHC-C, S	SHC-D, S
OES Short Term (mg m ⁻³)	3560	310	105	560
OES Long Term (mg m ⁻³)	1780	260	70	188

N/A = not available

Reagent Hazards

	Sulphuric Acid	Hydrochloric Acid	Acetic Acid
Harmful Vapour	✓	✓	✓
Highly Flammable	x	x	x
Harmful by Skin Absorption	✓ Causes severe burns	✓ Causes severe burns	✓ Causes severe burns
Syngenta Hazard Category	SHC-C, S	SHC-C, S	SHC-C, S
OES Short Term (mg m ⁻³)	N/A	N/A	37
OES Long Term (mg m ⁻³)	1	7	25

N/A = not available

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

Chlorothalonil has been assigned to Syngenta Hazard Classification D.

At present there is insufficient data available to assign a Syngenta Hazard Category for R182281. It should be treated as a class SHC-C compound until further information indicates otherwise.

The toxicity classification scale rates highly toxic chemicals as class SHC-E and non-toxic chemicals as class SHC-A. An additional S classification indicates a severe eye and skin irritant.

2.5 Time Required for Analysis

The methodology is normally performed with a batch of up to 15 samples. One person can complete the analysis of up to 20 samples in 1 day (8 working hour period).

2.6 Work Stoppages

The analytical procedure can be stopped at various points for overnight and weekend breaks except where specified in the analytical procedure. Acceptable external standard recoveries will validate the work stoppages. Samples should be stored in sealed vessels at a temperature of $\leq 7^{\circ}\text{C}$.

2.7 Modifications and Potential Problems

For preparation of aqueous HPLC mobile phases the use of bottled HPLC grade water is preferred. This gives a reduced MS/MS background signal when compared with water from a laboratory water purification system.

When monitoring non matrix-matched standards against matrix-matched standards or samples a retention time difference may be observed.

3. Analytical Procedure

3.1 Sample Preparation

Samples should be prepared using an approved method of sample preparation for residue analysis, such as Syngenta standard operating procedure ESJH/910/-- for crops (Reference 4).

To avoid losses of chlorothalonil from the samples during preparation certain precautions are required (Reference 5 and 6). Wet crops (e.g. tomatoes) are prepared by homogenisation in the presence of 1 M H_2SO_4 solution (10 mL acid to 100 g crop) and dry ice. Dry crops (e.g. cereal straw) are prepared by chopping in a knife mill (without acid) and dry seed crops (e.g. pulses, cereal grain, peanuts) are not prepared in any way.

3.2 Extraction

- a) Weigh representative amounts of crop matrix equivalent to 10 g (i.e. 11 g for wet crops prepared with acid) into screw-capped plastic centrifuge bottles (250 mL size) and for pulses and nuts with a larger grain size into 500 mL screw-capped glass blending jars (e.g. Eberbach 500 mL screw-topped glass jar). At least one untreated control and two control samples fortified with known amounts of chlorothalonil in toluene and R182281 in acidified acetone (not more than 0.5 mL for each analyte) should be analysed alongside each batch of samples to demonstrate acceptable method performance.

Add acetone:5 M sulphuric acid solution (95:5 v/v, 100 mL minus the water content of the samples, and minus a further 1 mL for crops prepared with acid).

Homogenise at high speed for 3 – 5 minutes using a top-drive homogeniser for the majority of crops. For pulses and nuts with a larger grain size the use of a Waring blender or similar (1 L size) with a 500 mL screw-capped glass container containing standard blending blades with Teflon seals is necessary (see Appendix 1 for supplier details). The blending container should be cleaned with water after use then rinsed with acetone and left to dry before re-use. The use of detergent for cleaning should be avoided as any residues remaining on glassware may cause instability problems with chlorothalonil.

Note: Estimate the percentage water content in each matrix type and hence the total volume of water in the 10 g sub-sample. E.g. for a 10 g sub-sample with 90% natural water content add 100 mL – (10 x 90/100) mL = 91 mL extraction solution. It is sufficient to round the natural water content to the nearest ten percent value. Any volume contraction from mixing organic solvents with water and evaporation loss during extraction is considered to be negligible.

The water content of matrices can be obtained from published sources (e.g. Reference 5). The relevant information may also be obtained from the following USDA web site: http://www.nal.usda.gov/fnic/cgi-bin/nut_search.pl

Alternatively, where information is not available from the above sources, it may be necessary to determine the moisture content following a suitable moisture content determination procedure e.g. SOP ESJH/309/-- (Reference 6).

- b) Centrifuge samples at 3500 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 5 minutes.

The sample concentration is now 0.1 g mL⁻¹.

3.3 Sample Clean-up For Chlorothalonil

- a) Transfer aliquots of the crop extracts equivalent to 0.2 g into a disposable glass test tube (10 mL size). Add water (6 mL) to the aliquots and mix.
- b) Take one C8 (EC) solid phase extraction cartridge (size 100 mg, 3 mL) for each sample to be analysed and place on a suitable vacuum manifold (e.g. IST Vacmaster). Add methanol (3 mL) and draw through under vacuum to the level of the top frit at a rate of approximately 2 mL min⁻¹, discarding the column eluate. Do not allow the cartridges to become dry. Add water (3 mL) to the top of each cartridge and draw through under vacuum to the level of the top frit at the same rate, again discarding the column eluates. Do not allow the cartridges to become dry.

- c) Transfer the sample aliquots from section 3.3.1 (a) onto the prepared columns and allow to percolate onto the cartridges under gravity or low vacuum (approximately 200 mbar). Discard the column eluates.
- d) Add water:acetonitrile (70:30, 1.5 mL) to the top of each cartridge and draw through under vacuum to the level of the top frit at a rate of approximately 2 mL min⁻¹. Discard the wash. Remove any remaining droplets of water adhering to the inside of the cartridge with absorbent tissue and dry the cartridges under high vacuum (≤ 500 mbar) for approximately 15-20 minutes.

Note : Where achievable vacuums are less than specified or apparatus does not allow sufficient air flow through the cartridges, longer drying times may be required.

- e) When the cartridges are dry, place pre-calibrated graduated collection tubes (15 mL) under each port, as required, in the manifold rack. Add toluene (2 mL) to the top of each cartridge to elute chlorothalonil, and draw through under vacuum at a rate of approximately 2 mL min⁻¹, removing the excess solvent from the column.

Note: It is recommended that the graduations on each batch of test tubes are checked prior to use. Pipette toluene (4 mL) into the tube to confirm that the solvent meniscus corresponds to the 4 mL graduation mark on the tube.

- f) Adjust the final volume to 4 mL with toluene. Sample concentration is now 0.05 g mL⁻¹. Transfer 1 mL aliquots from each sample into GC autosampler vials. Cap and crimp the vials and analyse for chlorothalonil by GC-MSD using negative-ion chemical ionisation. Use of calibration standards in the presence of matrix is recommended. The preparation of matrix matched calibration standards is described in Section 3.5.

Note: The above SPE procedure has been developed using columns from the stated manufacturer; however, it is possible to carry out the procedure using similar columns from other manufacturers. In all cases, it is strongly recommended that the elution profile of the chosen batch of columns is checked prior to commencing analysis. This will eliminate any variation between manufacturers' products and between batches.

3.4 Sample Clean-up For R182281

- a) Transfer aliquots of the extracts (0.1 mL, equivalent to 0.01 g) from section 3.2 (b) into suitable LC-MS/MS autosampler vials. Add acetonitrile:water (50:50 v/v, 0.9 mL) to give a final volume of 1.0 mL. Cap and crimp the vials and mix well. The sample concentration is now 0.01 g mL⁻¹.

- b) If samples are visibly cloudy or contain particulate material (e.g. samples with high lipid content) it is recommended that they are filtered through Waters GHP 0.45 μm Acrodisc syringe filters prior to analysis by LC-MS/MS.

3.5 Calibration Standards

Suppression or enhancement of instrument response to chlorothalonil and R182281 in the presence of matrix was generally greater than 10% in this laboratory on the crop matrices tested. Therefore, samples should be quantified using matrix matched calibration standards.

Alternatively, samples may be further diluted with the appropriate solvent where instrument sensitivity allows, avoiding the use of matrix-matched standards.

Matrix-matched calibration standards may be prepared from analytical standards prepared in Section 2.3, as follows:

For example, to prepare a $0.001 \mu\text{g mL}^{-1}$ chlorothalonil matrix-matched standard, take 0.99 mL of the control sample in toluene at Section 3.3.1 (f) and place in a suitable autosampler vial. Add 10 μL of a $0.1 \mu\text{g mL}^{-1}$ standard in toluene. Ultrasonicate thoroughly and transfer to a suitable autosampler vial. The concentration of the matrix-matched calibration standard is now $0.001 \mu\text{g mL}^{-1}$.

To prepare a $0.001 \mu\text{g mL}^{-1}$ R182281 matrix-matched standard for example, take a further 0.01 g control aliquot at Section 3.4 (a). Take the sample through the analytical procedure to Section 3.4 (b). Add 10 μL of a $0.1 \mu\text{g mL}^{-1}$ standard in acidified acetone to a suitable autosampler vial. Add 0.99 mL of the sample in acetonitrile:water (50:50 v/v) from Section 3.4. Ultrasonicate thoroughly and transfer to a suitable autosampler vial. The concentration of the matrix-matched calibration standard is now $0.001 \mu\text{g mL}^{-1}$.

4. Final Determination

The following instruments and conditions have been found to be suitable for this analysis in this laboratory. Other instruments can be equally used, however 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.

4.1 Chlorothalonil Final Determination by GC-MSD

Instrument Description

The following instrumentation has been found to be satisfactory for the analysis of chlorothalonil, using an Agilent 6890 gas liquid chromatograph fitted with an Agilent 5973 series mass selective detector working in negative chemical ionisation mode.

Chromatography Conditions

Chromatography conditions

Column	:	BPX-50: 15.0 m x 0.25 mm x 0.25 µm film thickness
Injection Port	:	Split/splitless injector operating in splitless mode with Carbofrit™ 4 mm gooseneck inlet liner
Carrier gas and head pressure	:	Helium at a constant flow of 1 ml min ⁻¹ with a pressure pulse of 30 psi for 1 minute
Splitless time	:	1.0 minute
Injection volume	:	1 µl
Injector temperature	:	250°C
Transfer line temperature	:	280°C
Temperature programme	:	120°C hold for 1 minute then programme at 20°C per minute to 300°C.

MSD Conditions

Ionisation mode	:	Negative ion CI using methane gas
Electron energy	:	70 eV

Compound Name	Low Mass Resolution	SIM MODE	
		Chlorothalonil	Yes
		Qualifier 1	264 m/z
		Qualifier 2	268 m/z
		Retention Time	6.07 min

The ions selected for the quantification of chlorothalonil are those that are the most abundant and are free of interference from co-extractives. Typical chromatograms are shown in Appendix 4. Also included are the full-scan spectra, showing the fragmentation of chlorothalonil. Ratios of the target ion compared to the qualifier ions 1 and 2 are approximately 100:80:45 respectively. GC-MSD with 3 fragment ions of $m/z < 100$ is considered to be highly specific and no confirmatory conditions are included.

4.2 R182281 Final Determination by LC-MS/MS

Instrument Description

The following instrumentation has been found to be satisfactory for the analysis of R182281:

Pump	: Agilent 1100 series quaternary pump model number G1311A
Degasser	: Agilent 1000 series model number G1322A
Column Oven	: Agilent 1100 series model number G1316A
Detector	: Applied Biosystems API 3000 triple quadrupole mass spectrometer with Analyst™ software version 1.4.1
Auto sampler	: CTCPAL
Gas Supply	: Peak Scientific NM20ZA gas station

Chromatography Conditions

Column	: Phenomenex Ultracarb ODS (30) 5 μ m 150 mm x 4.6 mm i.d.
Column Oven Temperature	: 40°C
Flow rate	: 1.5 mL min ⁻¹
Injection volume	: 40 μ L
Injection protocol	: Analyse calibration standard after 2 to 4 sample injections
Stop Time	: 5.0 minutes
Mobile phase	: 1 : Acetonitrile 2 : 0.2% (v/v) acetic acid in HPLC water

Mobile Phase Gradient

Time (min)	% Mobile Phase 1	% Mobile Phase 2
0.0	50.0	50.0
2.0	95.0	5.0
2.5	95.0	5.0
2.6	50.0	50.0
5.0	50.0	50.0

Column Divert programme

Time (min)	Valve position
0.0	To waste
0.75	To mass spectrometer

Note : The column eluate is diverted to waste for 0.75 minutes to prevent ionic material from the sample contaminating the mass spectrometer front plate.

A secondary pump providing flow of mobile phase to the mass spectrometer when the column eluate is switched to waste is unnecessary.

The flow of eluate into the mass spectrometer should be optimised using an in-line flow splitter. A flow rate of approximately 200 – 400 $\mu\text{L min}^{-1}$ is recommended. This should produce a small wet spot visible on the MS front plate at the start of the injection.

Under these conditions the retention time of R182281 is 2.3 minutes.

API 3000 Mass Spectrometer Conditions

Interface	:	TurboIonSpray	
Polarity	:	Negative	
Nebuliser gas (NEB)	:	Air set at 13 (arbitrary units)	
Curtain gas (CUR)	:	Nitrogen set at 11 (arbitrary units)	
Temperature (TEM)	:	450°C	
Ionspray voltage	:	-3500 V	
Collision gas setting (CAD)	:	Nitrogen set at 6 (arbitrary units)	
Scan type	:	Multiple Reaction Monitoring (MRM)	
Analyte		R182281 (primary transition)	R182281 (confirmatory transition)
Q1 <i>m/z</i>	:	244.9	244.9
Q3 <i>m/z</i>	:	181.9	174.9
Dwell time	:	200	200
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	-36 V	-40
Focusing potential (FP)	:	-220 V	-220
Entrance potential (EP)	:	-10 V	-10
Collision energy (CE)	:	-36 V	-36V
Collision cell exit potential (CXP)	:	-11 V	-11 V
Electron multiplier setting (CEM)	:	-1800 V	-1800 V

Protonated molecular ions generated in the ion source (m/z 244.9) are selected and subjected to further fragmentation by collisional activation. The ions (m/z 244.9 \rightarrow 181.9, corresponding to the loss of nitrile and hydroxy fragments) in the resulting daughter spectra are then monitored and used for quantitative analysis. LC-MS/MS using the above transition is considered to be highly specific. A second transition (m/z 244.9 \rightarrow 174.9, corresponding to the loss of two chlorine atoms) may be monitored if further confirmation of the presence of R182281 is required.

Note: The product ion scans were not generated as part of the validation study.

5. Calculation of Results

Residues may be calculated using an external standardisation procedure.

Residues of chlorothalonil should be quantified using the target ion (266 m/z). If this is not possible because of chromatographic interference, or if further confirmation of the presence of chlorothalonil is required, residue calculations may be carried out using the qualifier ions (264 m/z and 268 m/z).

Residues of R182281 should be quantified using the primary transition (m/z 244.9 \rightarrow 181.9). If this is not possible because of chromatographic interference, or if further confirmation of the presence of R182281 is required, residue calculations may be carried out using the confirmatory transition (m/z 244.9 \rightarrow 174.9).

Chlorothalonil and R182281 residues may be calculated in mg kg^{-1} 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 chlorothalonil or R182281 at an appropriate concentration into the GC-MSD or LC-MS/MS operated under conditions as described in Section 4. When a consistent response is obtained, measure the peak area obtained for chlorothalonil or R182281.
- b) Make an injection of each sample solution and measure the peak heights or areas of the peaks corresponding to chlorothalonil or R182281.
- c) Re-inject the standard solution after a maximum of four injections of sample solutions.

- d) Calculate the chlorothalonil or R182281 residue in the sample, expressed as mg kg^{-1} , using a mean standard response from each of the injections bracketing the sample as follows:

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

PK area (SA) = Peak response for sample

PK area (STD) = Average peak response for bracketing standards

Standard Conc. = Concentration of calibration standard ($\mu\text{g mL}^{-1}$)

Sample Conc. = Sample concentration (g mL^{-1})

If residues need to be corrected for average percentage recovery, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average Percentage Recovery}} \quad (\text{mg kg}^{-1})$$

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

6. Control and Recovery Samples

The levels of external recoveries should be determined by the residue levels expected. A minimum of one control and two external recovery experiments should be run alongside each set of samples analysed (that is untreated samples accurately fortified with a known amount of chlorothalonil and R182281 prior to extraction).

Control and external recovery should be analysed with each set of samples as detailed in Section 3. Acceptable recovery values demonstrate reliable method performance during the analysis and may be used to correct any chlorothalonil or R182281 residues found. Recovery data is generally considered acceptable when the mean values are between 70% and 110% and with a coefficient of variation of $\leq 20\%$.

7. Specificity

If unexpected interference is observed at final determination, it is recommended that a reagent blank be taken through the analytical procedure to trace the source of the problem. Reagent blanks may be taken through the analytical procedure on a routine basis.

7.1 Matrix Interference

The detection techniques used are highly selective and no interference arising from co-eluting compounds from the crop matrices tested has been observed.

7.2 Reagent and Solvent Interference

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

7.3 Labware Interference

The method mainly uses disposable labware. No interference from labware has been found.

8. Method Validation

8.1 Recovery Data and Repeatability

The procedures described in Section 3 have been validated on a range of crops. Validation is reported in Syngenta Report RJ3286B (Reference 7) for chlorothalonil and Syngenta report T013840-05-REG (Reference 8) for R182281.

8.1.1 Stability of Extracts

Chlorothalonil (Reference 9) and R182281 (Reference 8) stored in final extracts, at a temperature of <7°C in glass vials, are stable for a period of at least 7 days.

Chlorothalonil and R182281 in primary extracts stored at a temperature of <7°C, are stable for a period of at least 30 days (Reference 9).

8.2 Limit of Quantification and Limit of Detection

8.2.1 Limit of Quantification (LOQ)

The limit of quantification of the method is defined as the lowest analyte concentration in a sample at which the methodology has been validated and a mean recovery of 70 - 110% with a c.v. of ≤ 20% has been obtained.

Generally, for accurate quantification, the response for an analyte peak should be no lower than four times the mean amplitude of the background noise in an untreated sample at the corresponding retention time.

The limit of quantification has been set at 0.01 mg kg^{-1} for both analytes.

8.2.2 **Limit of Detection (LOD)**

The limit of detection of the method is defined as the lowest analyte concentration detectable above the mean amplitude of the background noise in an untreated sample at the corresponding retention time. An estimate of the LOD can be taken as four times background noise. Note that the LOD may vary between runs and from instrument to instrument.

The limit of detection of this method was estimated at 0.002 mg kg^{-1} for chlorothalonil and $0.0009 \text{ mg kg}^{-1}$ for R182281.



Appendix 6 : API 3000 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 manufactures instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

Tuning of API 3000 MS/MS Instrument for R182281

Infuse a standard solution of R182281 (0.1 to $10 \mu\text{g mL}^{-1}$) in acetonitrile:ultra pure water (50:50 v/v) directly into the mass spectrometer interface at a rate at of $5 - 20 \mu\text{L min}^{-1}$. Roughly adjust the interface parameters (sprayer position, spray, heater and auxiliary gas flows, in addition to spray, orifice, and focusing ring voltages) for a sufficiently high parent ion signal at m/z 244.9.

Using the Analyst software quantitative optimisation programme, tune the instrument for R182281, ensuring that the correct ions are selected (initial Q1 $m/z = 244.9$ and product ion $m/z = 181.9$). Confirmatory ion ($m/z = 174.9$) may also be tuned if required. If desired, manual tuning of the ion optics and collision energy can be carried out to ensure maximum sensitivity.

Connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injection of R182281 standards using a mobile phase of 50:50 (v/v) ultra pure water:acetonitrile + 0.2 % acetic acid at the required flow rate and at the intended split ratio. 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.

Note: The product ion scans were not generated as part of the validation study data.