DIFENOCONAZOLE: METHOD

TITLE

Residue Method for the Determination of Residues of Difenoconazole (CGA 169374) in Various Crops and Processed Crop Fractions. Final Determination by LC-MS/MS

DATA REQUIREMENT

EPA Guideline OPPTS 860.1340

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PERFORMING LABORATORY

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VOLUME 1 OF 1 OF STUDY

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The method contained in this volume was not conducted in accordance with EPA Good Laboratory Practices, 40 CFR Part 160.

Study Director: There is no GLP study director for this volume.

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REM 147.08

RESIDUE METHOD FOR THE DETERMINATION OF RESIDUES OF DIFENOCONAZOLE (CGA169374) IN VARIOUS CROPS AND PROCESSED CROP FRACTIONS. FINAL DETERMINATION BY LC-MS/MS.

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Issuing Section : **Dietary Safety**

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Group Leader

: 11/11/04
Date

Page 1 of 50 Pages

Summary of revisions to previous version

Version	Summary of Revisions
08	Method updated from AG-575A. Filtration, partition, multiple SPE steps and packed GC-NPD final determination replaced with single Oasis HLB SPE step with LC-MS/MS final determination.

Contents

1. Introduction and Summary

1.1 Scope

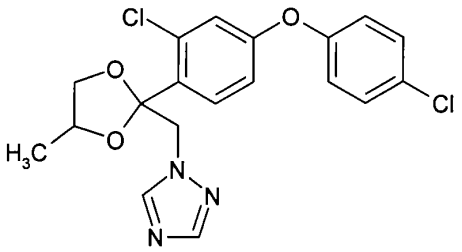
The analytical procedure described is suitable for the determination of residues of difenoconazole (Figure 1) in crop matrices using an external standardisation procedure. The limit of quantification has been set at 0.01 mg kg^{-1} for all matrices.

Figure 1 : Difenoconazole (CGA169374)

IUPAC Name : cis,trans-3-chloro-4-[4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether

Molecular Mass : 406

CAS Registry No. : 119446-68-3



1.2 Method Summary

Samples are extracted under reflux with methanol: concentrated ammonium hydroxide (80:20 v/v). Extracts are allowed to cool and settle and aliquots (1 mL = 0.1 g) are taken and diluted with ultra-pure water. Sample clean up is carried out by solid-phase extraction (SPE) using Oasis™ HLB cartridges. Final determination 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 or 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.

Weigh out accurately, using a five-figure balance, sufficient difenoconazole analytical standard to allow dilution in methanol to give a 200 $\mu g\ mL^{-1}$ stock solution in a volumetric flask. This standard should then be diluted by serial dilution to 0.01 $\mu g\ mL^{-1}$ in methanol. These standards should be used for sample fortification and for preparation of calibration standards for final LC-MS/MS determination (Section 3.5). Note: for fortification of vegetable oils (e.g. olive oil), serial dilutions in acetone should be made to the required concentration level if spiking volumes are greater than 100µL.

When not in use, always store the standard solutions in a refrigerator at ≤7°C to prevent decomposition and/or concentration of the standard. Unless further stability information is obtained, 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 1).

Solvent Hazards

	Methanol	Dichloromethane	Hexane	Ethyl Acetate	Acetonitrile
Harmful Vapour	✓	✓	✓	✓	✓
Highly Flammable	✓	×	✓	✓	✓
Harmful by Skin Absorption	✓	✓	✓	✓	✓
Syngenta Divisional Toxicity Class	SHC-C	SHC-D	SHC-B	SHC-B	SHC-C
OES Short Term ($mg\ m^{-3}$)	310	870 (MEL)	3600	N/A	105
OES Long Term ($mg\ m^{-3}$)	260	350 (MEL)	70	1400	70

N/A = Not available

Reagent Hazards

	Formic Acid	Ammonium Hydroxide
Harmful Vapour	✓	✓
Highly Flammable	×	×
Harmful by Skin Absorption	✓	✓
Syngenta Divisional Toxicity Class	SHC-C	SHC-C
OES Short Term ($mg\ m^{-3}$)	N/A	24
OES Long Term ($mg\ m^{-3}$)	5	17

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

Difenoconazole has been assigned a Syngenta toxicity classification of SHC-B.

The toxicity classification scale rates highly toxic chemicals as SHC-E and non-toxic chemicals as class SHC-A.

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 15 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 ≤7°C.

2.7 Modifications and Potential Problems

a) For preparation of aqueous HPLC mobile phases it has been found beneficial to use bottled HPLC grade water. This gives a reduced MS/MS background signal when compared to water from a laboratory water purification system.

b) If samples contain excessive particulate material and do not settle sufficiently to allow the aliquot to be taken easily at Section 3.3 (a), transfer a small portion (e.g. 10 mL ± 0.5 mL) to a 15 mL screw capped, graduated, plastic centrifuge tube. Centrifuge at 3500 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 5 minutes. Continue with the method as at 3.3 (a).

c) As the method does not incorporate a wash of the reflux condensers in the actual methodology, it is recommended that condensers are thoroughly rinsed with water and methanol after each analysis to prevent sample contamination.

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 2).

3.2 Extraction.

a) Note: the extraction system is retained from method AG-575A (Reference 3).

Weigh representative amounts of crop (10 g) into round bottomed flasks (250 mL size) (500 mL size for straw samples). At least one untreated control and two control samples fortified with known amounts of difenoconazole in methanol (not

more than 0.5 mL) should be analysed alongside each batch of samples to demonstrate acceptable method performance.

Add methanol:concentrated ammonium hydroxide (80:20 v/v;100 mL minus the water content of the samples). Add a few anti-bumping granules, stopper the flask and weigh each sample prior to heating under reflux for 2 hours.

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 due to mixing organic solvents with water during extraction is considered to be negligible.

The water content of matrices can be obtained from published sources (e.g. Reference 4). 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 such sources, it may be necessary to determine the moisture content experimentally, following a suitable moisture content determination procedure e.g. SOP ESJH/309/-- (Reference 5).

b) After 2 hours, remove the samples from the heat source and allow them to cool to room temperature. Keep the condensers attached to the round bottomed flasks during this cooling period.

c) Stopper each flask and weigh each sample. If any loss of weight has occurred, add extraction solvent (methanol:concentrated ammonium hydroxide, 80:20 v/v) until the original weight is reached.

The sample concentration is now 0.1 g mL^{-1}.

3.3 Sample Dilution

a) Using an autopipette, carefully transfer aliquots of the crop extracts from the supernatant layer, equivalent to 0.1 g (1.0 mL), into appropriate vessels (e.g. 15 mL screw capped, graduated, plastic centrifuge tubes). Add ultra pure water (10 mL ± 0.5 mL) to give a final volume of 11 mL ± 0.5 mL.

b) Cap the tubes securely and shake to ensure thorough mixing.

3.4 Solid Phase Extraction

a) Take one Oasis™ HLB cartridge (size 60 mg, 3 mL) for each sample to be analysed and place on a suitable vacuum manifold (e.g. IST Vac master). Add methanol (2 mL) on to the cartridges 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 ultra pure water (2 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 cartridges to become dry.

b) Transfer the samples from section 3.3 (b) on to the cartridges and draw through under vacuum at a rate of approximately 2 mL min^{-1} discarding the column eluates. Residues of difenoconazole are retained on the cartridge.

c) Add ultra pure water (1 mL) to the tubes that contained the samples. Rinse the tubes and transfer to the SPE cartridges. Draw through under vacuum to the level of the top frit at a rate of approximately 2 mL min^{-1}, discarding the column eluate.

d) Remove any remaining droplets of water adhering to the inside of the cartridges with absorbent tissue and dry under high vacuum (≤500 mbar) for a minimum of 20 minutes.

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

e) When dry, add hexane (2 mL) to the top of the SPE cartridges. Draw through under vacuum at a rate of approximately 2 mL min^{-1} discarding the column eluates.

f) Place suitable collection tubes (e.g. 10 mL test tubes) under each port, as required, in the manifold rack.

g) Add dichloromethane:ethyl acetate (80:20 v/v) (2 mL) to the SPE cartridges and draw through under vacuum at a rate of approximately 2 mL min^{-1}, collecting the column eluates. Apply a high vacuum (≤500 mbar) for approximately 5 seconds to draw off any remaining droplets of eluate. Residues of difenoconazole are eluted in this fraction.

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 be checked

prior to commencing analysis to rule out any variation between manufacturers' products and between batches.

h) Evaporate the eluate fractions to dryness under a stream of clean, dry air. An elevated temperature of up to 45°C may be used to aid this process.

i) Dissolve the samples in acetonitrile (2 mL) followed by ultra pure water (2 mL). Ultrasonicate thoroughly after the addition of each solvent. Transfer aliquots of the samples to suitable autosampler vials for analysis by LC-MS/MS. The sample concentration is now 0.025 g mL^{-1}. If the expected residues are outside the linear range of the instrument, samples may be diluted further by the addition of the appropriate amount of acetonitrile:ultra pure water (50:50 v/v).

3.5 LC-MS/MS Calibration Standards

Calibration standards for LC-MS/MS analysis are prepared by dilution of standards prepared in Section 2.3. with acetonitrile: ultra pure water (50:50 v/v). For example, to prepare a 0.001 μg mL^{-1} calibration standard, transfer 100 μL of a 0.01 μg mL^{-1} standard in methanol to a suitable autosampler vial. Add acetonitrile:ultra pure water (50:50 v/v; 0.9 mL) to give a 0.001 μg mL^{-1} calibration standard.

Suppression or enhancement of the LC-MS/MS response to difenoconazole in the presence of matrix was less than 10% in this laboratory on the crop matrices tested. This is considered to be negligible and samples should be quantified using non-matrix matched standards.

If a suppression of greater than 10% is observed then a matrix-matched standard may be used to compensate for these effects at the discretion of the study director. For example, to prepare a 0.001 μg mL^{-1} difenoconazole matrix matched standard, take a further 0.1 g control aliquot at section 3.3 (a). Take the sample through the analytical procedure to Section 3.4 (i). Add 40 μL of a 0.1 μg mL^{-1} standard in methanol to a suitable autosampler vial. Add 1.96mL of methanol followed by ultra pure water (2ml) from Section 3.4 (i). The concentration of the matrix-matched calibration standard is now 0.001 μg mL^{-1}.

Alternatively, further sample dilution at section 3.4 (i) may be used to eliminate any observed matrix effects where instrument sensitivity allows.

4. Final Determination by LC-MS/MS

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 Instrument Description

Pump	:	Agilent 1100 series quarternary 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 1.3
Auto sampler	:	CTCPAL
Gas Supply	:	Peak Scientific NM20ZA gas station

4.2 Chromatography Conditions

Column	:	KR100 5C18 5µm 50 mm x 3.2 mm id
Column Oven Temperature	:	40°C
Flow rate	:	1.0 mL min^{-1}
Injection volume	:	10 µL
Injection protocol	:	Analyse calibration standard after 3 to 4 sample injections
Stop Time	:	3.5 minutes
Mobile phase	:	**1**: Acetonitrile **2**: 0.2% (v/v) formic acid in UP water

Mobile Phase Gradient

Time (min)	% Mobile Phase 1	% Mobile Phase 2
0.0	50.0	50.0
2.0	90.0	10.0
2.4	90.0	10.0
2.5	50.0	50.0
3.5	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 a 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 $\mu L\ min^{-1}$ 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 difenoconazole is approximately 2.0 minutes.

4.3 API 3000 Mass Spectrometer Conditions

Interface	:	TurboIonSpray
Polarity	:	Positive
Nebuliser gas (NEB)	:	Air set at 13 (arbitrary units)
Curtain gas (CUR)	:	Nitrogen set at 12 (arbitrary units)
Temperature (TEM)	:	450°C
Ionspray voltage	:	5500 V
Collision gas setting (CAD)	:	Nitrogen set at 4 (arbitrary units)
Scan type	:	MRM
		Difenoconazole
Q1 *m/z*	:	406.0
Q3 *m/z*	:	251.1
Dwell time	:	300 ms
Resolution Q1	:	Unit
Resolution Q3	:	Unit
Declustering potential (DP)	:	61 V
Focusing potential (FP)	:	360 V
Entrance potential (EP)	:	10 V
Collision energy (CE)	:	35 V
Collision cell exit potential (CXP)	:	22 V
Electron multiplier setting (CEM)	:	1000 V

Protonated molecular ions generated in the ion source (difenoconazole *m/z* 406.0) are selected and subjected to further fragmentation by collisional activation. The most abundant ions (*m/z* 251.1, corresponding to the chlorinated diphenyl ether fragment) in the resulting daughter spectra are then monitored and used for quantitative analysis. No confirmatory conditions are included as final determination by LC-MS/MS is considered to be highly specific. If further confirmation is required a second daughter ion (*m/z* 187.9 or 111.3) may be monitored. Typical chromatograms are shown in Appendix 4. Initial and final product scans showing the fragmentation and daughter ions for difenoconazole are presented in Appendix 6.

5. Calculation of Results

Residues may be calculated using an external standardisation procedure.

Difenoconazole 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 an appropriate concentration of difenoconazole into the LC-MS/MS operating under conditions as described in Section 4. When a consistent response is obtained, measure the peak area obtained for difenoconazole.

b) Make an injection of each sample solution and measure the peak heights or areas of the peaks corresponding to difenoconazole .

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

d) Calculate the difenoconazole 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 ($\mu g\ mL^{-1}$)

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

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

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

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

6. Control and Recovery Experiments

Fortification levels for procedural recoveries should be appropriate to 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 difenoconazole prior to extraction).

Control and external recovery experiments should be completed as section 3 for each set of samples analysed. Acceptable recovery values demonstrate reliable method performance during the analysis and may be used to correct any difenoconazole residues found.

Recovery data is generally considered acceptable when the mean values are between 70% and 110% and with a relative standard deviation (RSD) 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.

7.1 Matrix

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

7.4 Protocol for High Level Standard and Sample Residue Injection

It is recommended when analysing standards and sample residues at high concentration (e.g. $\geq$0.01 µg mL^{-1}) that carry-over effects into subsequent injections are checked. Blank samples containing mobile phase may be injected after high concentration samples and standards to prevent carry over.

8. Method Validation

8.1 Recovery Data and Repeatability

A method validation study demonstrating acceptable recovery data and repeatability has been carried out on the procedures described in Section 3. This is reported in RJ3560B (Reference 6). A summary of the method validation data is presented in Appendix 3.

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 % RSD of $\leq$ 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 LC-MS/MS determination.

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}.

8.3 Detector Linearity

For accurate quantification of residue concentrations, analyses should be carried out within the linear range of detector responses. Detector linearity graphs are given in Appendix 5.

In these laboratories the linearity of the API 3000 LC-MS/MS detector response for difenoconazole standards prepared in acetonitrile:ultra pure water (50:50 v/v) was tested in the range from 0.000075 to 0.003 $\mu g\ mL^{-1}$ concentration (equivalent to 0.75 - 30 pg injected on column when using a 10 µL injection volume) and in the range from 0.000175 – 0.03 $\mu g\ mL^{-1}$ concentration (equivalent to 1.75 – 300 pg injected on column when using 10µL injection volume) and was found to be linear.

Standards were injected in triplicate and the mean response plotted against amount injected, using Microsoft Excel 2000. The intercept was set to zero and a linear trendline fit applied. The data were also plotted with no intercept set. The two plots were compared statistically by application of a t-test, performed using the Simple Linear Regression Programme Version 2.0. A t-value of 1.475 and 0.4646 for difenoconazole was obtained with n-2 degrees of freedom. The tabular t value at the 10% level of significance, with n-2 degrees of freedom, is 2.353. Since the computed t value is smaller than the tabular t value, at the 10% level of significance, the intercept α is not significantly different from zero and the two response curves are statistically similar. It is therefore acceptable to use single point calibrations for residue calculations (Reference 7).

If residues beyond the tested concentration range are expected, dilute the extract appropriately to bring it within the tested linear range prior to quantification.

8.4 Limitations

The method has been tested on oilseed rape seed, olives and olive oil, cereal grain, apples, cherries, grapes, sugar beet roots and leaves, broccoli, leeks, tomatoes and tomato purée. It can be reasonably assumed that the method can be applied to other crop matrices not tested provided successful recovery tests at the relevant levels validate the suitability of the method.

9. Conclusions

The method described is suitable for the analysis of difenoconazole and residues in crop matrices. Only commercially available laboratory equipment and reagents are required. One person can complete the analysis of a batch of up to 15 samples in 1.5 days (12 working hour period). Untreated and fortified samples should be extracted and analysed with each set of samples to demonstrate absence of any interference and adequate recovery, if possible. The limit of quantification has been set at 0.01 mg kg^{-1} with final analysis by LC-MS/MS.

10. References

1. Luxon S G (1992): Hazards in the Chemical Laboratory 5th Edition. The Royal Society of Chemistry. Thomas Graham House, The Science Park, Cambridge CB4 4WF, UK. ISBN 0-85186-229-2.

2. Syngenta Standard Operating Procedure SOP ESJH/910/--: Preparation of Crop Samples For Residue Analysis.

3. Grunenwald M. (1991): Syngenta Method no. AG-575A: Analytical Method for the Determination of CGA-169374 in Wheat Raw Agricultural Commodities by Gas Chromatography With Nitrogen/Phosphorus Detection.

4. Watt B K and Merril A L (1975): Composition of Foods, raw, processed and prepared, Agricultural Handbook No.8, Agricultural Research Service, United States Department of Agriculture, US Government Printing Office, Washington, D C, 20402.

5. Syngenta Standard Operating Procedure SOP ESJH/309/-- : Crop Moisture Determination.

6. Ely S V and Ryan J (2004): Difenoconazole : Validation of a Residue Analytical Method for the Determination of Residues in Various Crops and Processed Crop Fractions. Syngenta report number RJ3560B.

7. Cardone M J, Palermo P J and Sybrandt L B : Potential error in single point ratio calculations based on linear calibration curves with a significant intercept. Anal Chem., 52 pp 1187-1191, 1980.

Appendices

Appendix 1 : Apparatus

UK Suppliers

Tecator homogeniser for initial preparation of samples available from Philip Harris Scientific, 618 Western Avenue, Park Royal, London W3 0TE, UK. Part number M48-525.

General laboratory glassware, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK.

Heating mantles for sample reflux available from VWR International Ltd., Merck House, Poole, BH15 1TD, UK.

Laboratory centrifuge e.g. MSE Mistral 1000 series, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK. Part number CEK-151-010W.

Nalgene™ polypropylene centrifuge tubes, 15 mL capacity with 0.1 mL graduations. Available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK. Part number CFT-430M.

Disposable test tubes (15 mL capacity) available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK. Part number CEK-151-010W.

Isolute Vacmaster-20™ sample processing station, available from Argonaut Ltd., Tir-y-Berth Industrial Estate, New Road, Hengoed, Mid Glamorgan CF8 8AU, UK.

Oasis™ HLB solid phase extraction columns, 3 mL 60 mg size, available from Waters Ltd., 730 – 740 Centennial Court, Centennial Park, Elstree, Hertfordshire, WD6 3SZ, UK.

Techne Dri-block 3D sample concentrator, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK. Part number BLD-750-010Y.

Ultrasonic bath e.g. Ultrawave U300/D, available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK. Part number BMA-100-020P.

Crimp cap auto sampler vials and caps available from Agilent Technologies UK Limited, Chemical Analysis Group, Lakeside Heath, Cheadle Royal Business Park, Stockport, Cheshire. SK8 3GR, UK.

API 3000 LC-MS/MS system equipped with a TurboIonSpray source, available from Applied Biosystems, Kelvin Close, Birchwood Scientific Park North, Warrington, Cheshire WA3 7PB, UK.

HPLC column, KR100 5C18 50 mm x 3.2 mm i.d., 5 µm particle size, available from Phenomenex, Queens Avenue, Hurdsfield Ind Estate, Macclesfield, Cheshire, SK10 2BN, UK.

Agilent 1100 HPLC system equipped with a quaternary pump, vacuum degasser and column compartment with column switching valve, available from Agilent Technologies UK Limited, Chemical Analysis Group, Lakeside Heath, Cheadle Royal Business Park, Stockport, Cheshire SK8 3GR, UK.

CTC HTS PAL auto sampler, available from Presearch Ltd, System House, 59-61 Knowlpiece, Hitchin, Herts SG4 0TY, UK.

Peak Scientific NM20ZA gas station, available from available from Peak Scientific Instruments Ltd, Fountain Crescent, Inchinnan Business Park, Inchinnan, Renfrew, PA4 9RE, UK.

US Suppliers

Equipment for the initial preparation of samples e.g. Tecator homogeniser available from Perstorp Analytical inc., 12101 Tech Road, Silver Spring, Maryland 20904.

General laboratory glassware, available from Fisher Scientific, Liberty Lane, Hampton, NH 03842, USA.

Heating mantles for sample reflux available from VWR International, 1310 Goshen Parkway, West Chester, PA 19380

Laboratory centrifuge e.g. Heraeus Instruments model 17RS, available from Heraeus Instruments, 111-A Corporate Blvd, South Plainfield, NJ 07080, USA.

Nalgene™ polypropylene centrifuge tubes, 15 mL capacity with 0.1 mL graduations. Available from Nalge Company, 75 Panorama Creek Drive, PO Box 20365, Rochester, NY 14602-0365.

Disposable test tubes (10 mL capacity) available from Fisher Scientific, Liberty Lane, Hampton, NH 03842, USA.

Isolute Vacmaster-20™ sample processing station, available from Argonaut USA Ltd., PO Box 280 329, Lakewood, Colorado, 8022-0329.

Oasis™ HLB solid phase extraction columns, 3 mL 60 mg size, available from Waters Corporation, 34 Maple Street, Milford, Massachusetts, 01757 - 3696, USA.

Techne Dri-block 3D sample concentrator, available from Fisher Scientific, Liberty Lane, Hampton, NH 03842, USA.

Ultrasonic bath available from Fisher Scientific, Liberty Lane, Hampton, NH 03842, USA.

Crimp cap auto sampler vials and caps available from Agilent Technologies, 395 Page Mill Road, Palo Alto, CA 94304 USA.

API 3000 LC-MS/MS system equipped with a TurboIonSpray source, available from Applied Biosystems, 850 Lincoln Center, Foster City, CA 94404-1128, USA.

HPLC column, KR100 5C18 50 mm x 3.2 mm i.d., 5 µm particle size, available from Phenomenex, 2320 W. 205th St. Torrance, CA 90501-1456

Agilent 1100 HPLC system equipped with a quaternary pump, vacuum degasser and column compartment with column switching valve, available from Agilent Technologies, 395 Page Mill Road, Palo Alto, CA 94304 USA.

CTC HTS PAL autosampler, available from LEAP Technologies Inc., P.O. Box 969, Carrboro, NC 27510

Peak Scientific NM20ZA gas station, available from Peak Scientific Instruments, 1300 West Belmont Ave, Chicago, Il 60657

Appendix 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.

UK Suppliers

Acetonitrile, dichloromethane, ethyl acetate, methanol, hexane super purity grade and bottled HPLC grade water, available from Rathburn Chemicals Ltd., Walkerburn, Scotland EH43 6AU, UK.

Formic acid and concentrated ammonium hydroxide (35% solution) available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK.

Anti bumping granules available from Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK.

Ultra pure water from a laboratory water purification system e.g. Elga Maxima available from Elga Ltd., High Street, Lane End, High Wycombe, Buckinghamshire HP14 3JH, UK.

Difenoconazole analytical standard available from Syngenta, GLP Testing Facility EZA, Syngenta, CH-4333 Munchwilen, Switzerland.

US Suppliers

Acetonitrile, dichloromethane, ethyl acetate, methanol, hexane super purity grade and bottled HPLC grade water available from B & J Brand Solvents, from Scientific Products Division of Baxter Healthcare Corporation, USA (Tel: 312-689-8410).

Formic acid and concentrated ammonium hydroxide (35% solution) available from Fisher Scientific, Liberty Lane, Hampton, NH 03842, USA.

Anti bumping granules available from Fisher Scientific, Liberty Lane, Hampton, NH 03842, USA.

Ultra-pure water from a laboratory water purification system available from Waters Corporation, Milford, MA, USA.

Difenoconazole analytical standard available from Syngenta Crop Protection Inc. P.O. Box 18300, Greensboro, NC 27419-8300, USA.

Appendix 3 : Method Validation Data

Table 1. : Difenoconazole Recovery Data Obtained During Method Validation

Matrix	Fortification Level (mg kg^{-1})	Recovery (%)	Mean (%)	RSD (%)	Range (%)
Oilseed Rape Seed	0.01*	84, 81, 83, 94, 93	87	7	81 - 94
	0.10	91, 91, 90, 94, 93	92	2	90 - 94
	Overall		89	5	81 - 94
Olive Fruit	0.01*	115, 108, 103, 110, 93	106	8	93 - 115
	1.0	109, 104, 98, 97, 100	102	5	97 - 109
	Overall		104	7	93 - 115
Olive Oil	0.01*	94, 97, 103, 98, 96	98	3	94 - 103
	1.0	81, 94, 92, 84, 87	88	6	81 - 94
	Overall		93	7	81 - 103
Sugar Beet Leaves	0.01*	92, 75, 78, 84, 93	84	10	75 - 93
	1.0	96, 102, 95, 90, 95	96	4	90 - 102
	Overall		90	9	75 - 102
Sugar Beet Roots	0.01*	88, 90, 93, 83, 84	88	5	83 - 93
	0.20	84, 86, 91, 92, 93	89	4	84 –93
	Overall		88	4	83 - 93
Wheat Grain	0.01*	92, 107, 94, 89, 95	95	7	89 – 107
	0.10	96, 96, 95, 77, 92	91	9	77 – 96
	Overall		93	8	77 - 107

*Limit of quantification, defined by the lowest validated fortification level

Table 2. : Difenoconazole Recovery Data Obtained During Method Validation

Matrix	Fortification Level (mg kg^{-1})	Recovery (%)	Mean (%)	RSD (%)	Range (%)
Cherry	0.01*	81, 84, 85, 90, 90	86	5	81 - 90
	0.20	95, 84, 82, 91, 94	89	7	82 -95
	Overall		88	6	81 - 95
Apples	0.01*	78, 95, 84, 96, 101	91	10	78 - 101
	0.30	90, 84, 84, 83, 89	86	4	83 - 90
	Overall		88	8	78 - 101
Tomato Fruit	0.01*	85, 85, 81, 87, 89	85	3	81- 89
	0.50	76, 79, 87, 89, 85	83	7	76 -89
	Overall		84	5	76 - 89
Tomato Puree	0.01*	82, 91, 86, 95, 97	90	7	82 - 97
	1.0	101, 80, 97, 93, 96	93	9	80 - 101
	Overall		92	8	80 - 101
Grapes	0.01*	92, 99, 100, 101, 115	101	8	92 -115
	0.10	120, 119, 100, 99, 96	107	11	96 - 120
	Overall		104	10	92 - 120
Broccoli (Whole Plant)	0.01*	119, 90, 80, 88, 104	96	16	80 - 119
	0.10	98, 97, 105, 94, 103	99	5	94 - 105
	Overall		98	11	80 - 119
Leeks (Whole Plant)	0.01*	93, 86, 84, 83, 86	86	5	83 - 93
	0.20	89, 78, 89, 89, 89	87	6	78 - 89
	Overall		87	5	78 - 93

*Limit of quantification, defined by the lowest validated fortification level

Appendix 4 : Representative Chromatograms

Figure 2 : 0.00125 μg mL^{-1} Difenoconazole Standard.

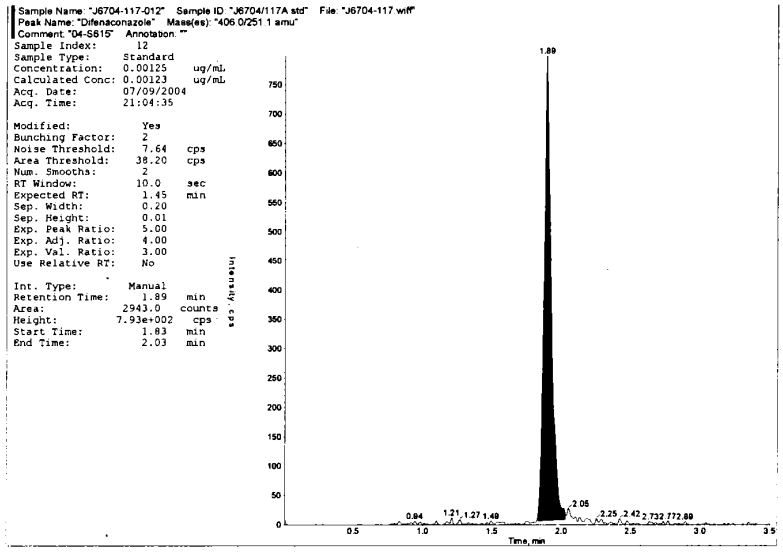


Figure 3 : Untreated Oilseed Rape Seed. Sample Concentration = 0.025 g mL^{-1}.

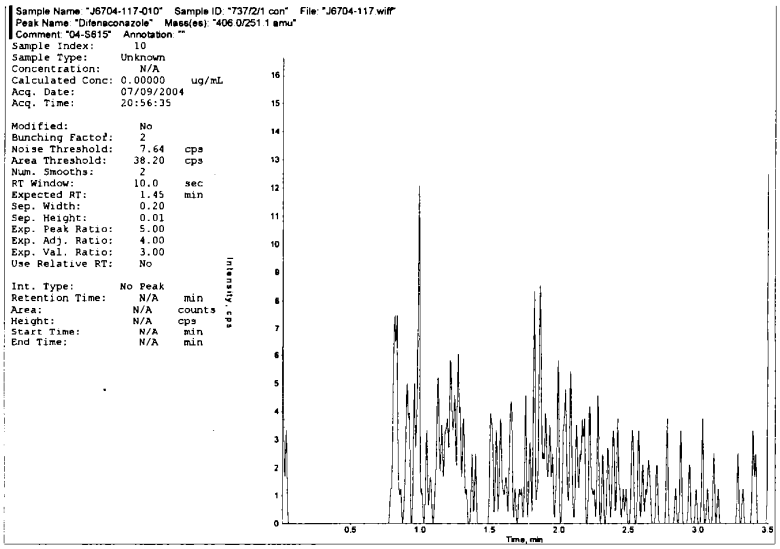


Figure 4 : Untreated Oilseed Rape Seed Fortified at 0.01 mg kg $^{-1}$. Sample Concentration = 0.025 g mL $^{-1}$. Recovery = 93%.

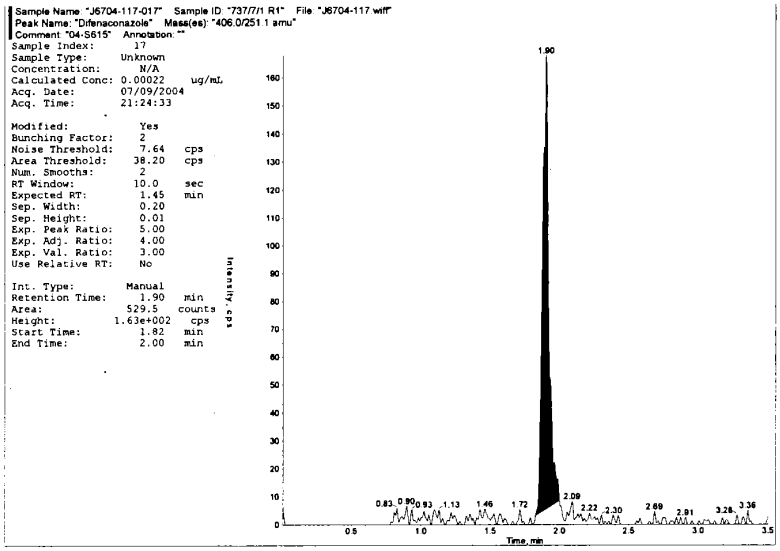


Figure 5 : 0.0025 μg mL^{-1} Difenoconazole Standard.

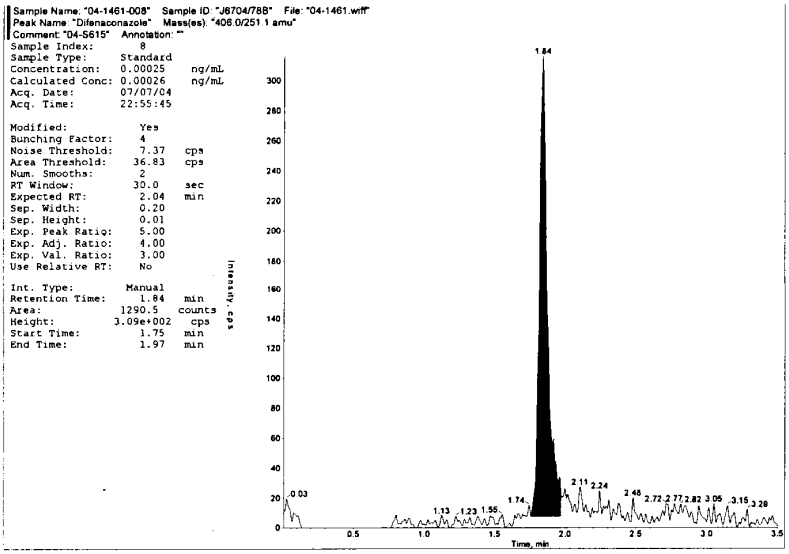


Figure 6: Untreated Olive Fruit. Sample Concentration = 0.025 g mL^{-1}.

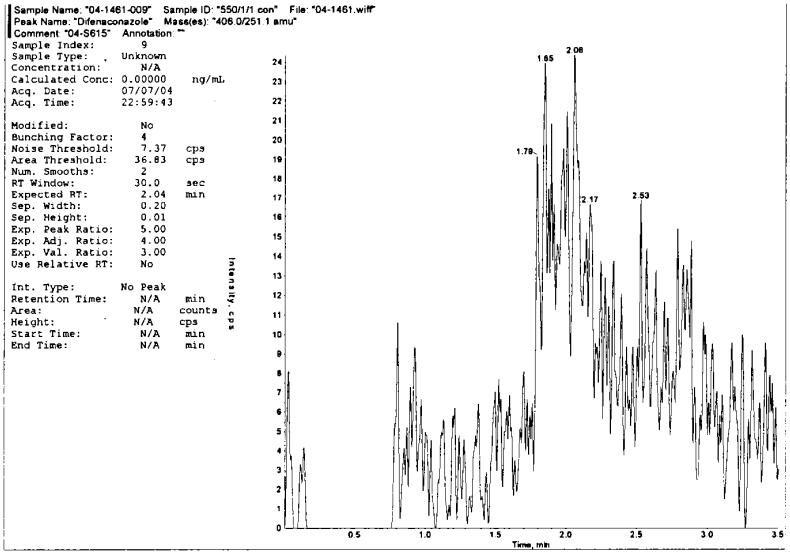


Figure 7 : Untreated Olive Fruit Fortified at 0.01 mg kg^{-1}. Sample Concentration = 0.025 g mL^{-1}. Recovery = 93%.

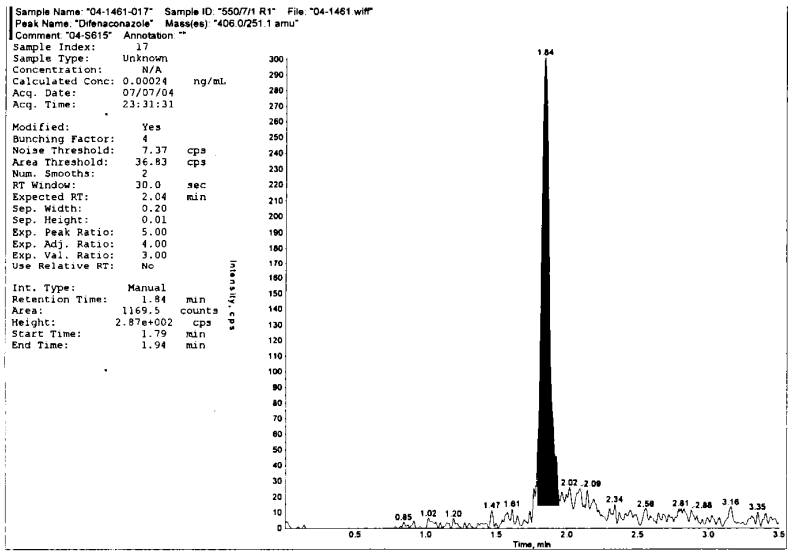


Figure 8 : 0.00025 μg mL^{-1} Difenoconazole Standard.

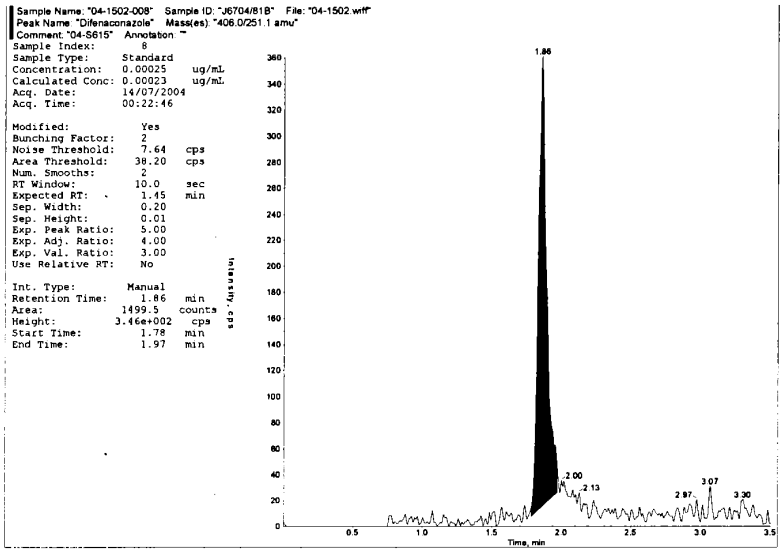


Figure 9 : Untreated Olive Oil. Sample Concentration = 0.025 g mL^{-1}.

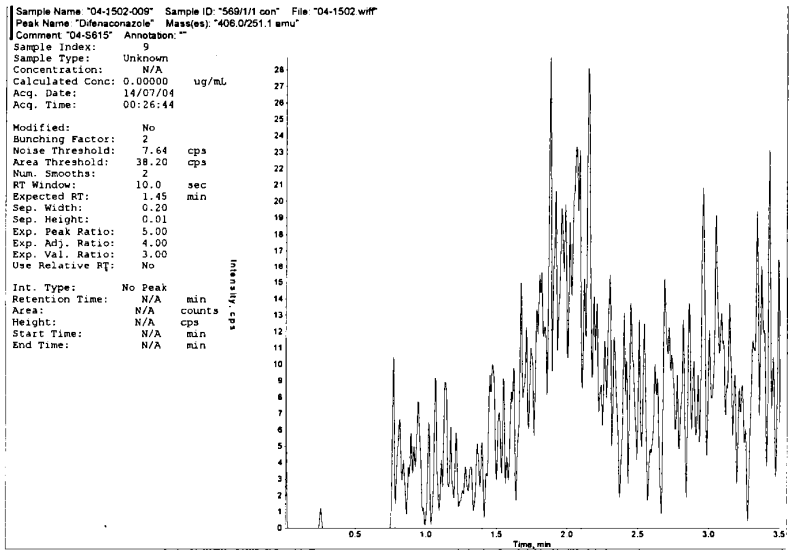


Figure 10 : Untreated Olive Oil Fortified at 0.01 mg kg $^{-1}$. Sample Concentration = 0.025 g mL $^{-1}$. Recovery = 94%.

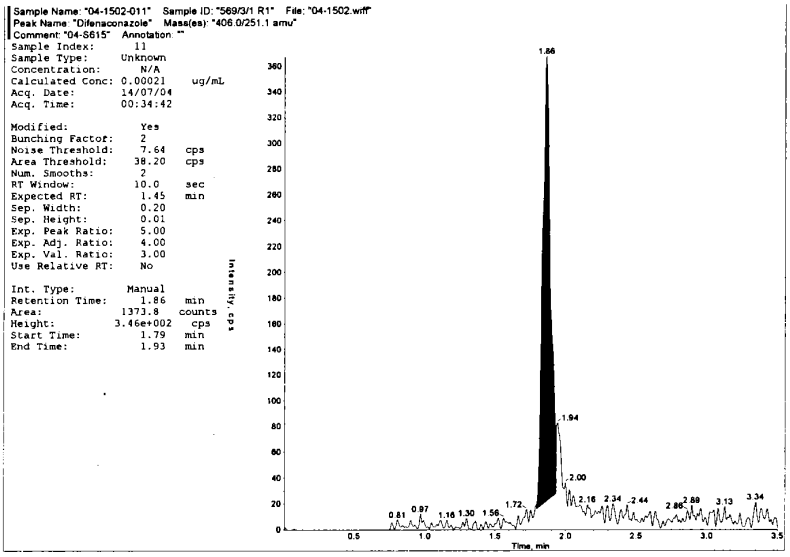


Figure 11 : 0.0125 μg mL^{-1} Difenoconazole Standard.

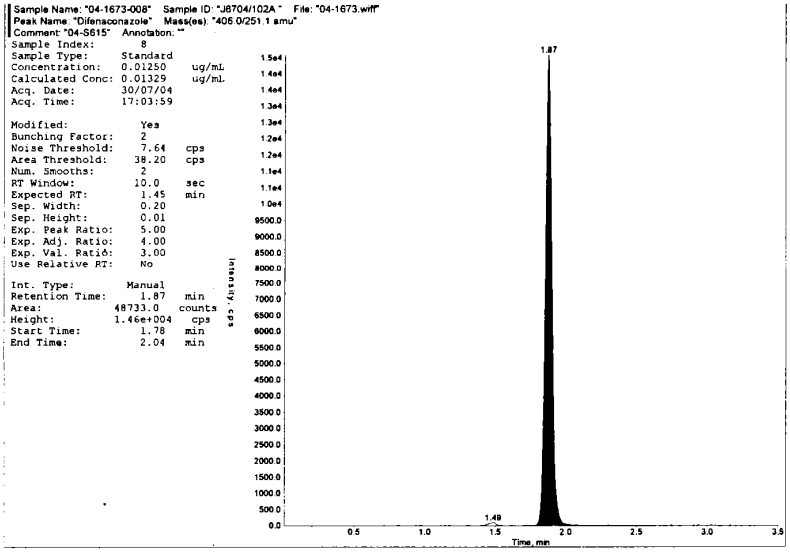


Figure 12: Untreated Sugar Beet Leaves. Sample Concentration = 0.025 g mL^{-1}.

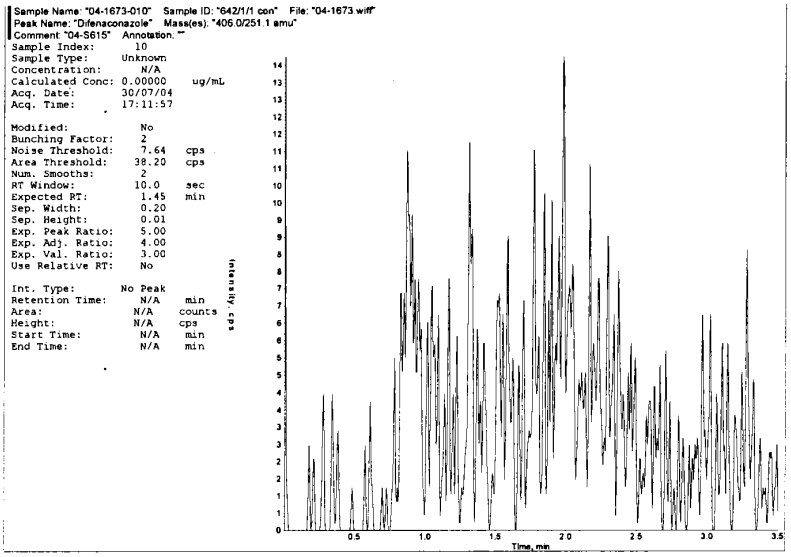


Figure 13 : Untreated Sugar Beet Leaves Fortified at 0.01 mg kg $^{-1}$. Sample Concentration = 0.025 g mL $^{-1}$. Recovery = 92%.

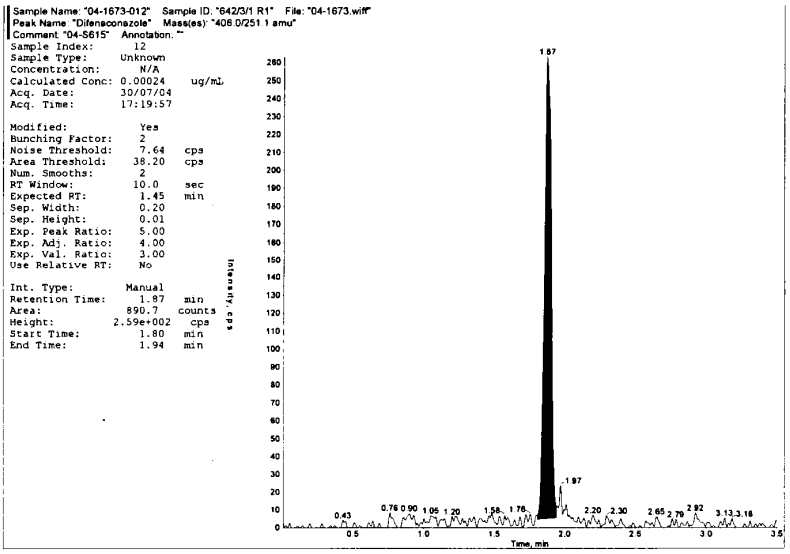


Figure 14 : 0.0025 μg mL^{-1} Difenoconazole Standard.

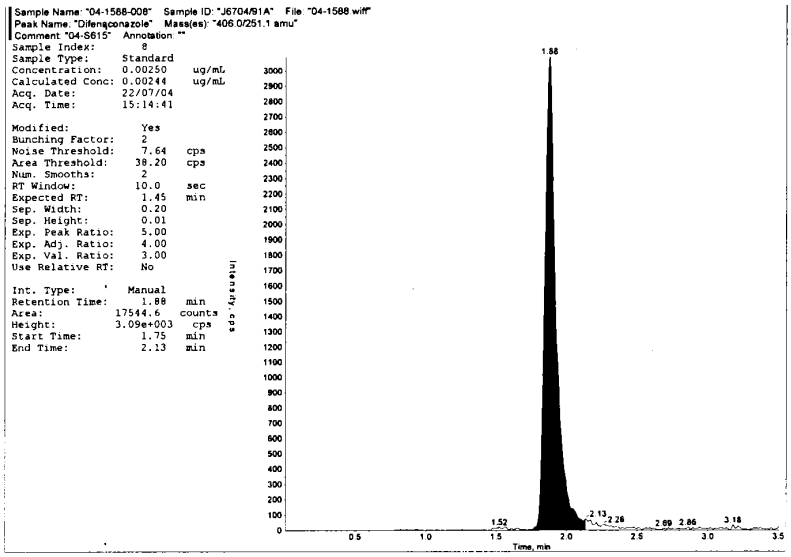


Figure 15 : Untreated Sugar Beet Roots. Sample Concentration = 0.025 g mL^{-1}.

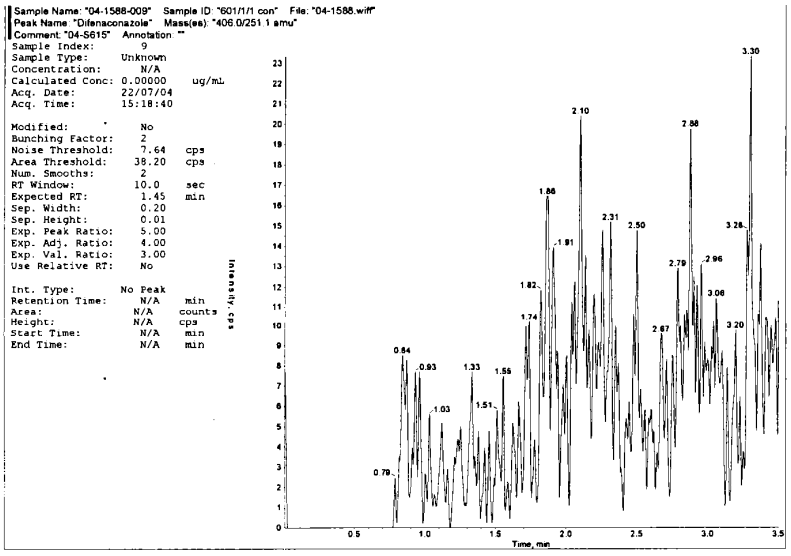


Figure 16: Untreated Sugar Beet Roots Fortified at 0.01 mg kg $^{-1}$. Sample Concentration = 0.025 g mL^{-1}. Recovery = 88%.

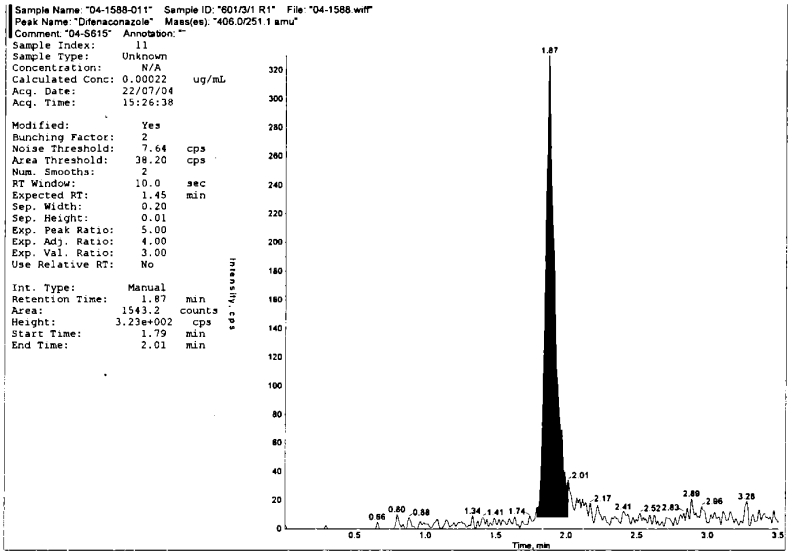


Figure 17 : 0.00125 μg mL^{-1} Difenoconazole Standard.

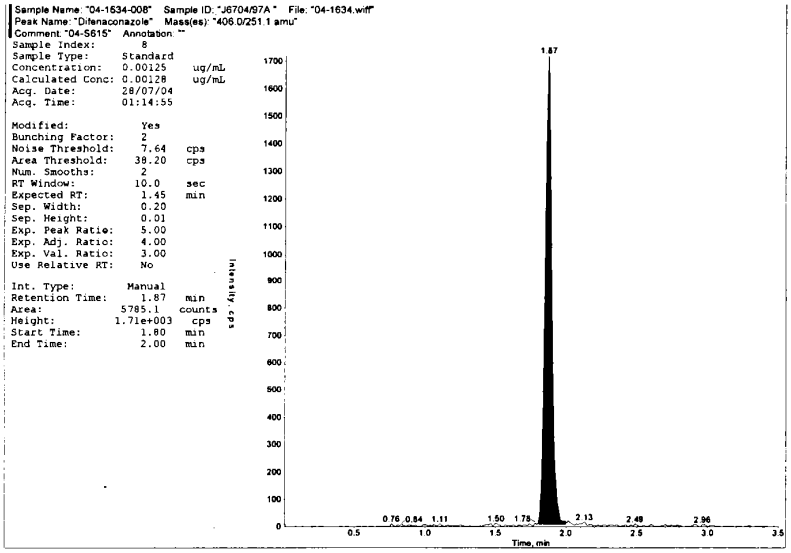


Figure 18: Untreated Wheat Grain. Sample Concentration = 0.025 g mL^{-1}.

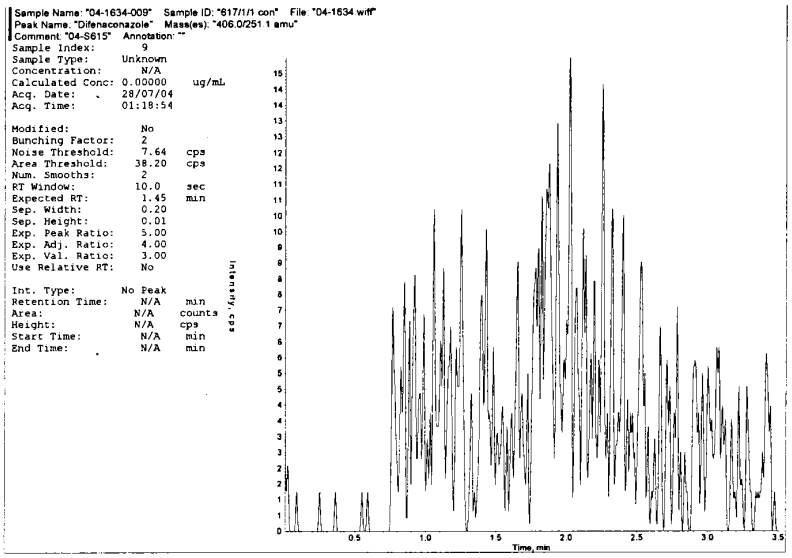


Figure 19 : Untreated Wheat Grain Fortified at 0.01 mg kg^{-1}. Sample Concentration = 0.025 g mL^{-1}. Recovery =92%.

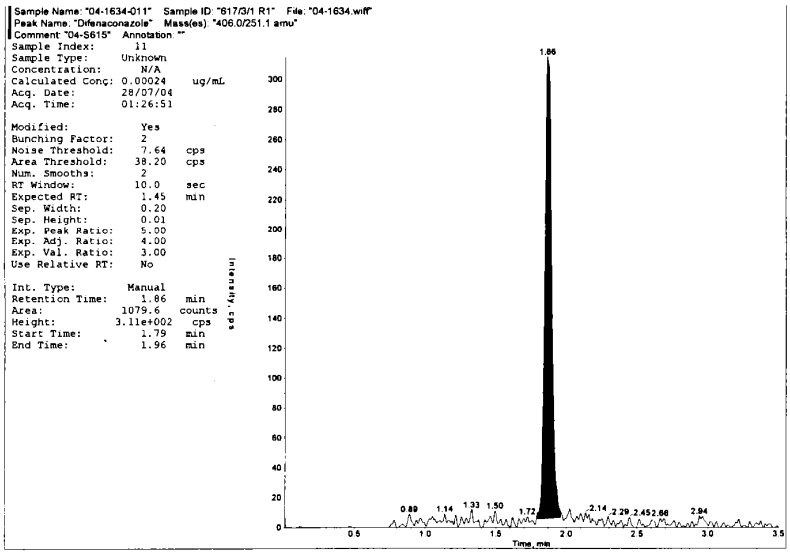


Figure 20: 0.0025 μg mL^{-1} Difenoconazole standard.

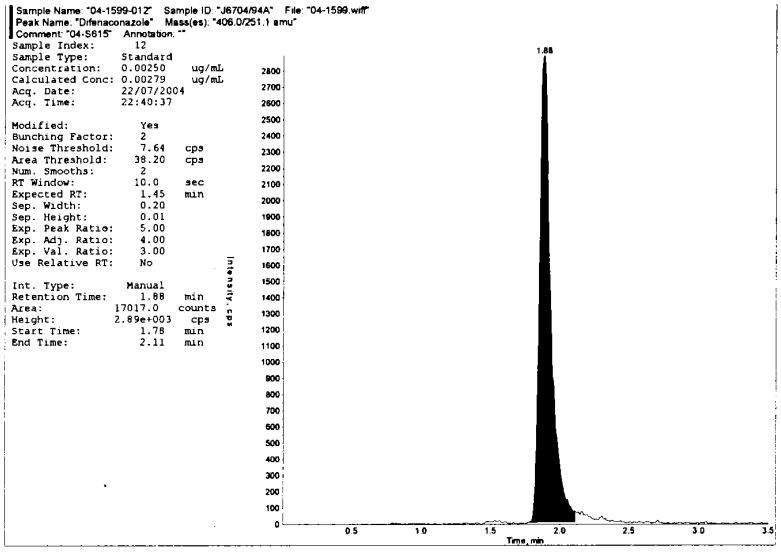


Figure 21 : Untreated Cherry Fruit. Sample concentration = 0.025 g mL^{-1}.

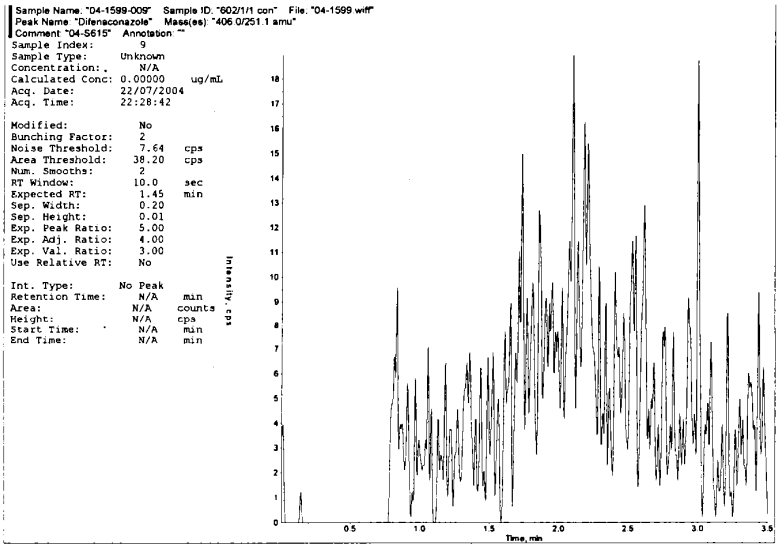


Figure 22 : Untreated Cherry Fruit Fortified at 0.01 mg kg $^{-1}$. Sample Concentration = 0.025 g mL $^{-1}$. Recovery = 84%.

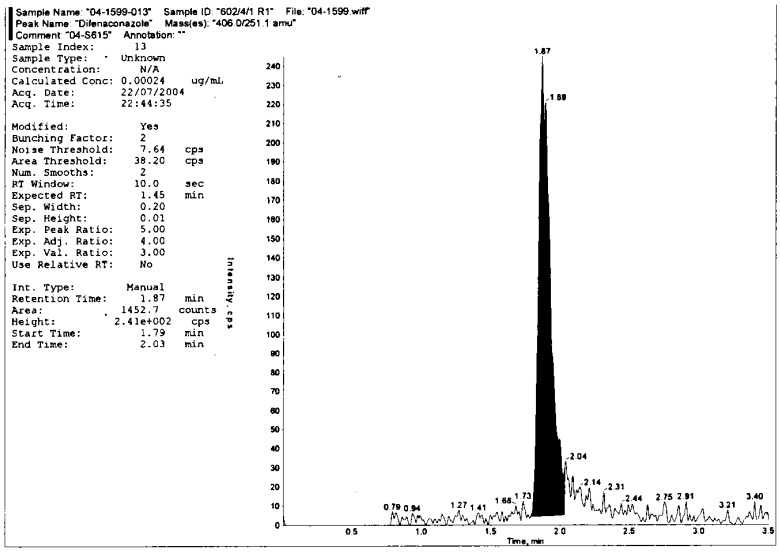


Figure 23 : 0.00375 μg mL^{-1} Difenoconazole standard .

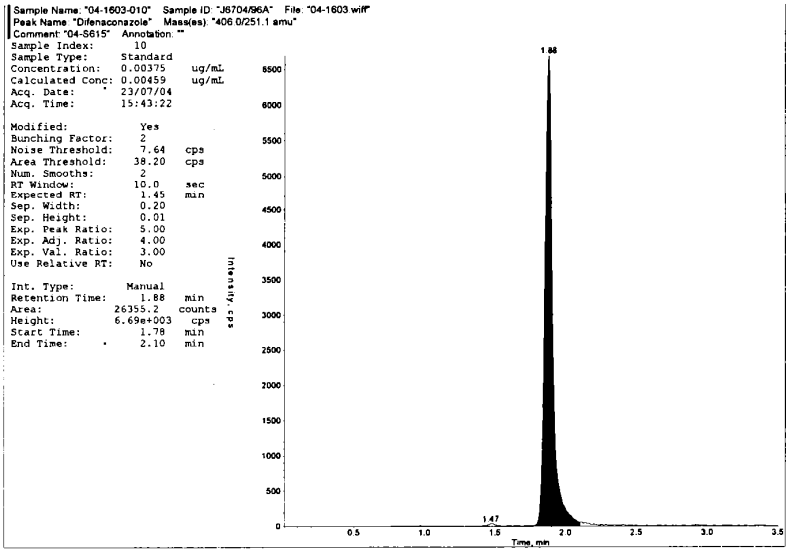


Figure 24 : Untreated Apples Fruit. Sample Concentration = 0.025 g mL^{-1}

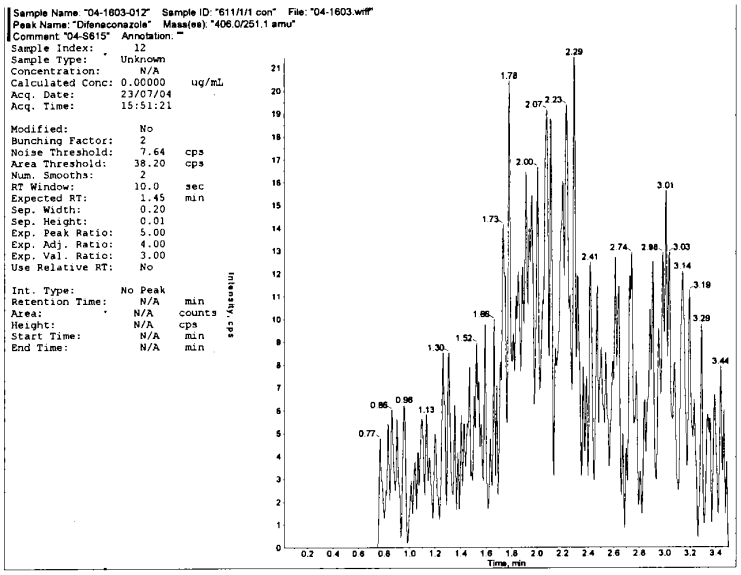


Figure 25 : Untreated Apple Fruit Fortified at 0.01 mg kg^{-1}. Sample concentration = 0.025 g mL^{-1}. Recovery = 95%.

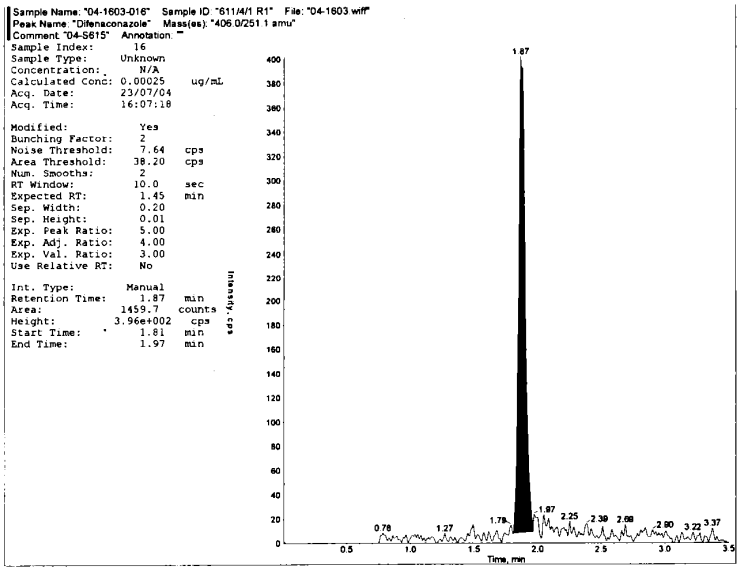


Figure 26 : 0.00625 μg mL^{-1} Difenoconazole Standard.

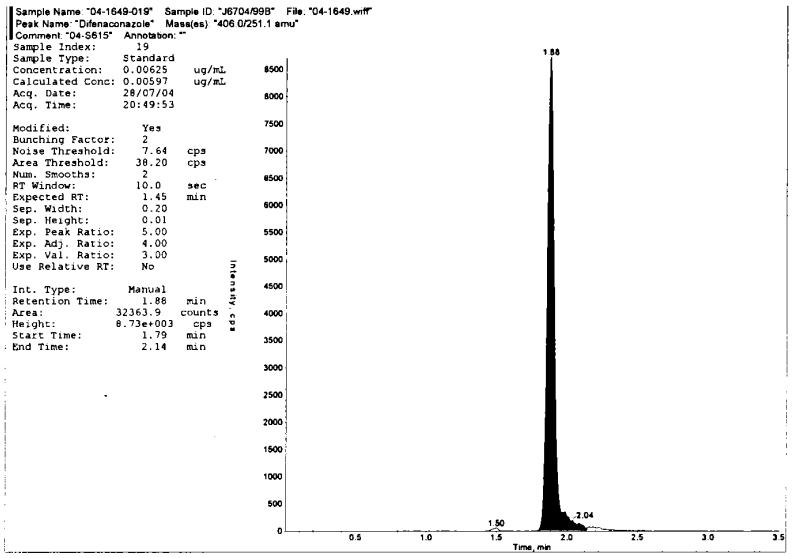


Figure 27 : Untreated Tomato. Sample Concentration = 0.025 g mL^{-1}

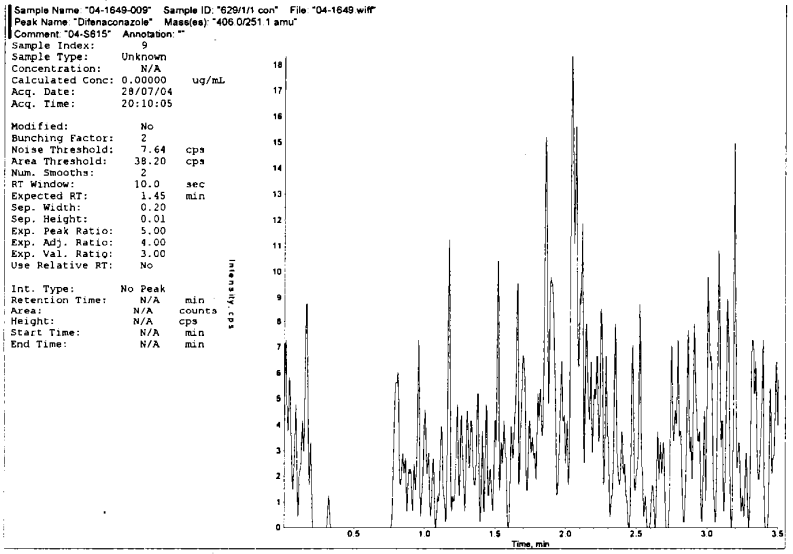


Figure 28 : Untreated Tomato Fortified at 0.01 mg kg $^{-1}$. Sample Concentration = 0.025 g mL $^{-1}$. Recovery = 85%.

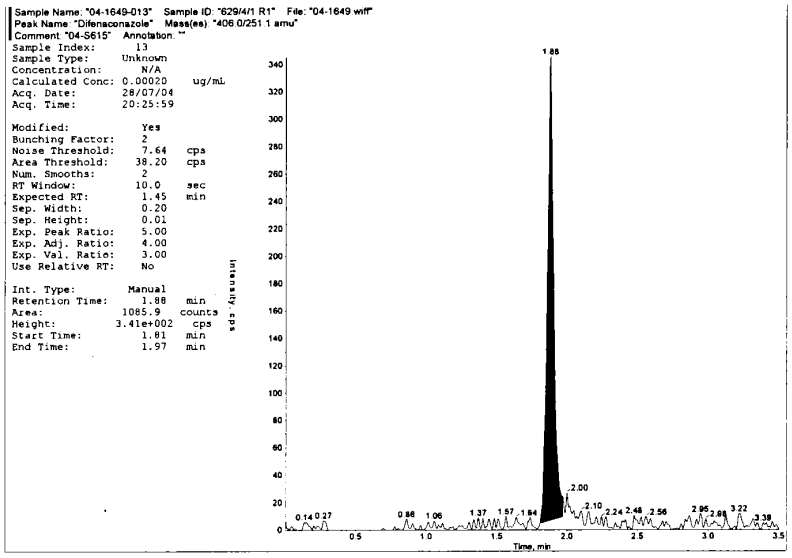


Figure 29 : 0.00025 μg mL^{-1} Difenoconazole Standard

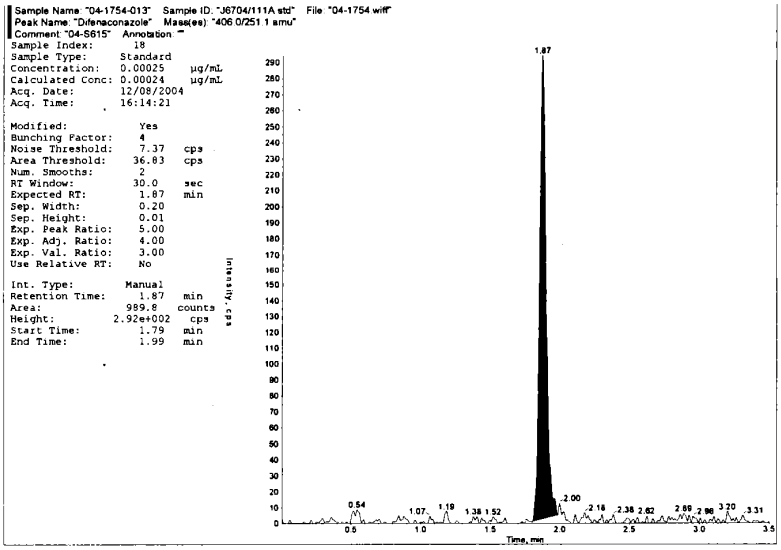


Figure 30 : Untreated Tomato Puree. Sample concentration = 0.025 g mL^{-1}.

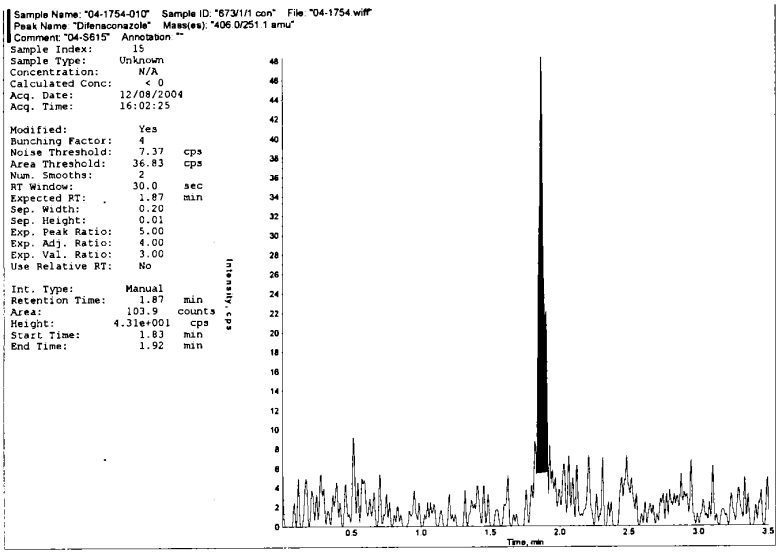


Figure 31 : Untreated Tomato Puree Fortified at 0.01 mg kg^{-1}. Sample Concentration = 0.025 g mL^{-1}. Recovery = 91%.

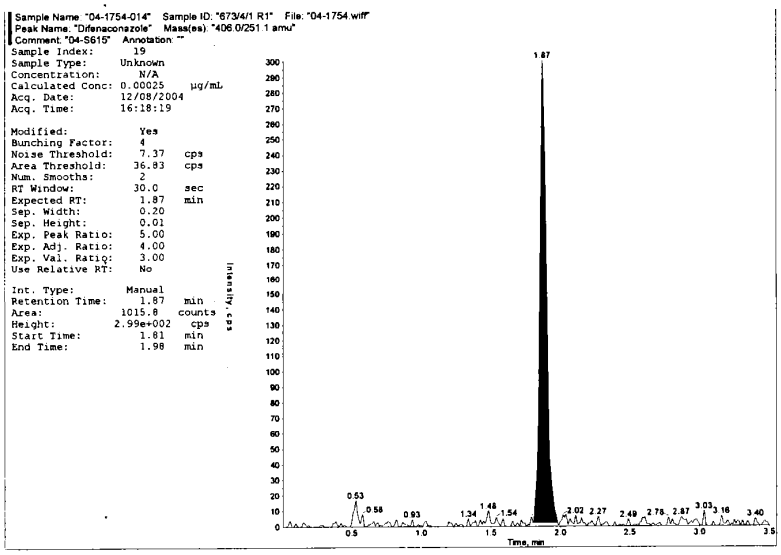


Figure 32 : 0.00125 μg mL^{-1} Difenoconazole Standard.

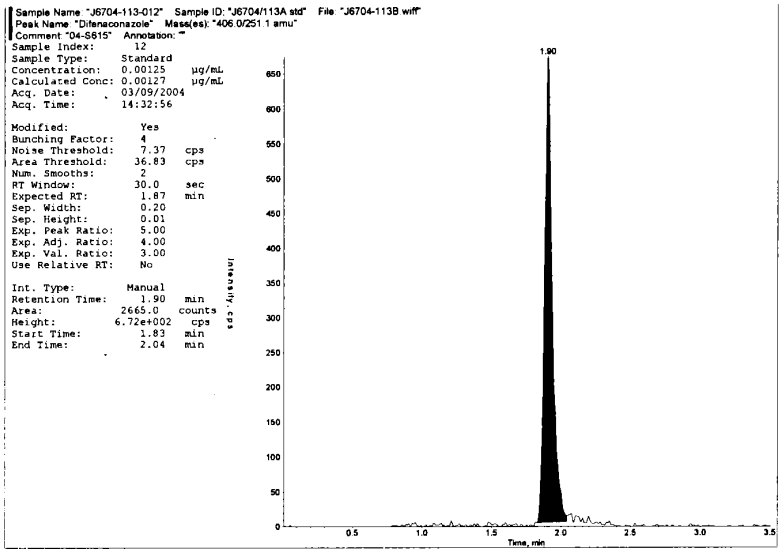


Figure 33 : Untreated Grapes. Sample Concentration = 0.025 g mL^{-1}.

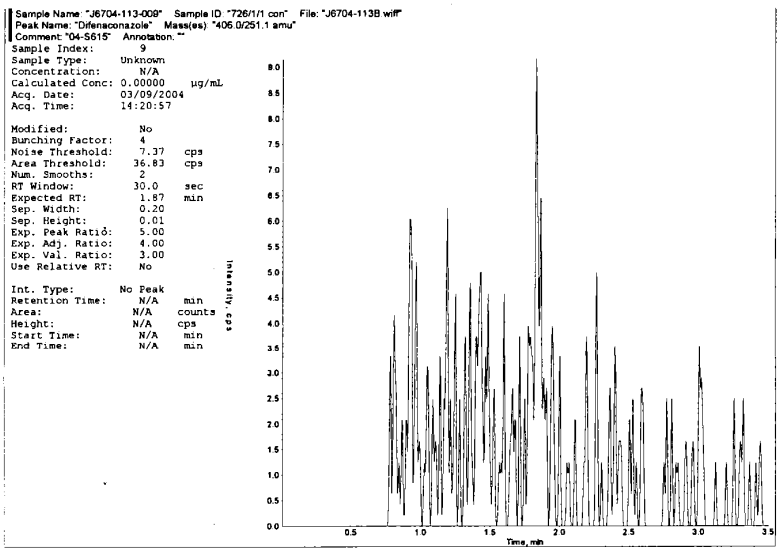


Figure 34: Untreated Grapes Fortified at 0.01 mg kg $^{-1}$. Sample Concentration = 0.025 g mL $^{-1}$. Recovery = 99%.

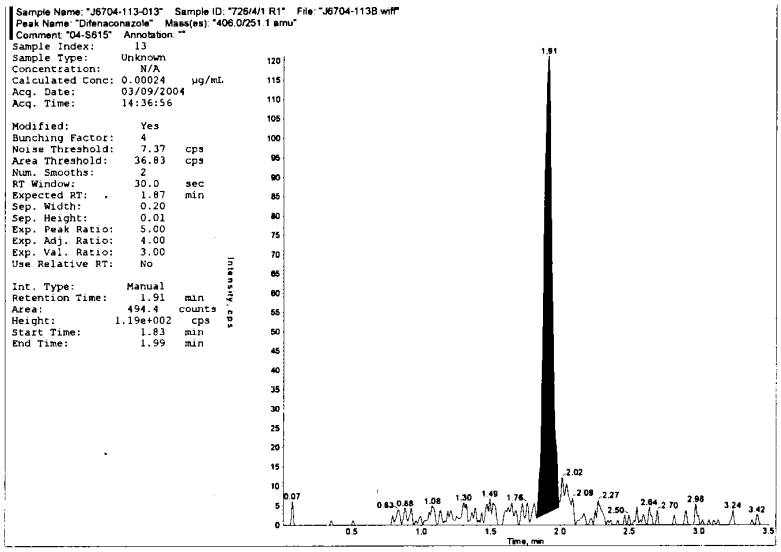


Figure 35 : 0.00125 µg mL^{-1} Difenoconazole standard.

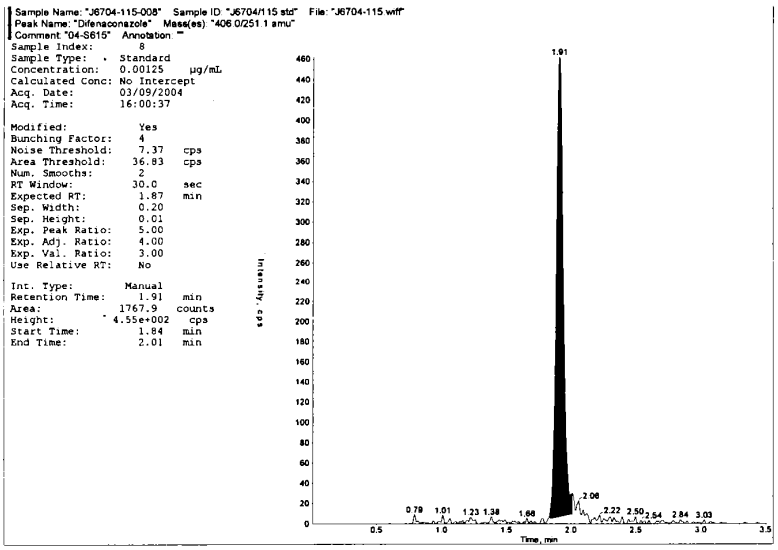


Figure 36 : Untreated Broccoli. Sample Concentration = 0.025 g mL^{-1}.

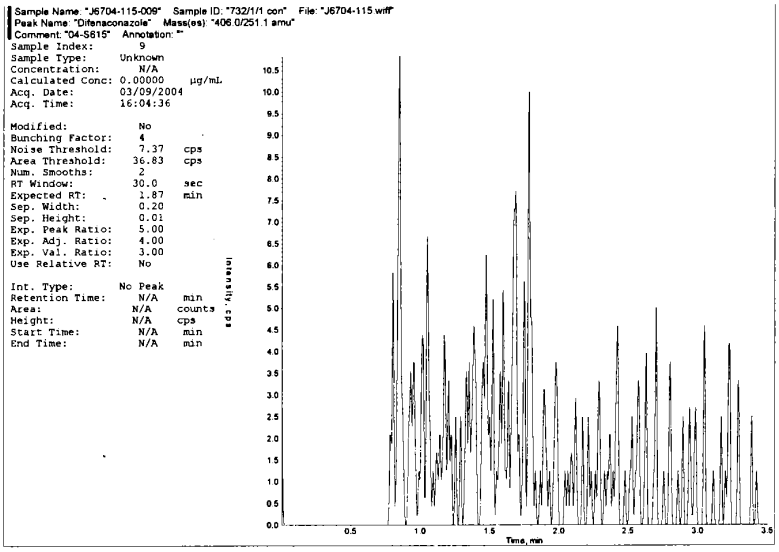


Figure 37 : Untreated Broccoli Fortified at 0.01 mg kg $^{-1}$. Sample Concentration = 0.025 g mL $^{-1}$. Recovery = 90%.

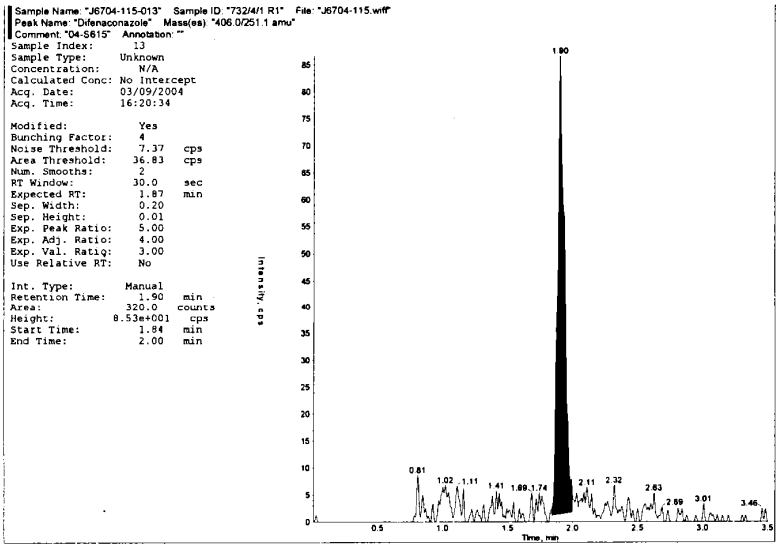


Figure 38 : 0.0025 μg mL^{-1} Difenoconazole Standard.

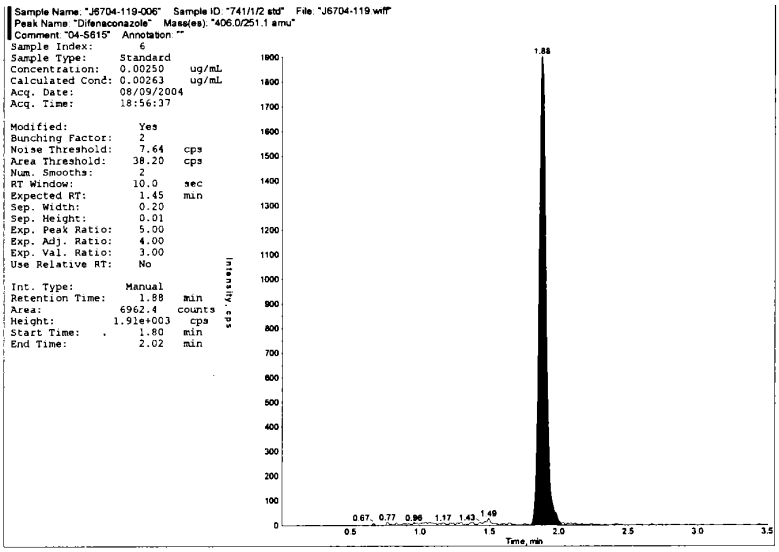


Figure 39 : Untreated Leeks. Sample Concentration = 0.025 g mL^{-1}

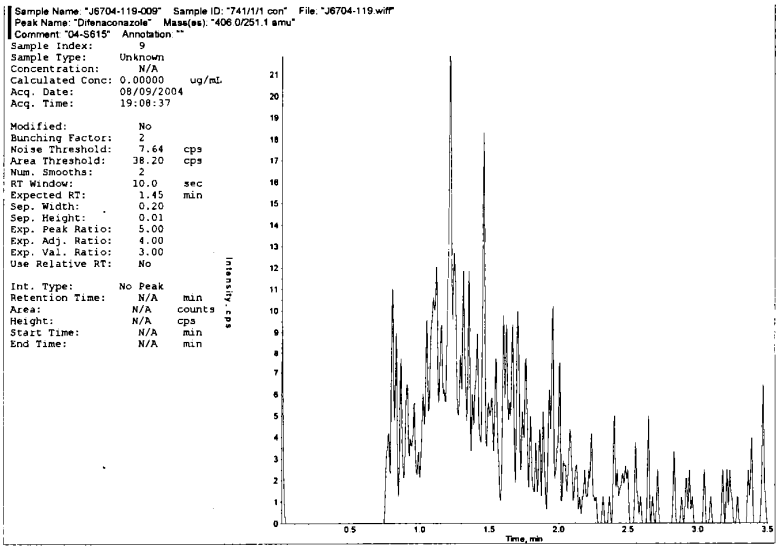
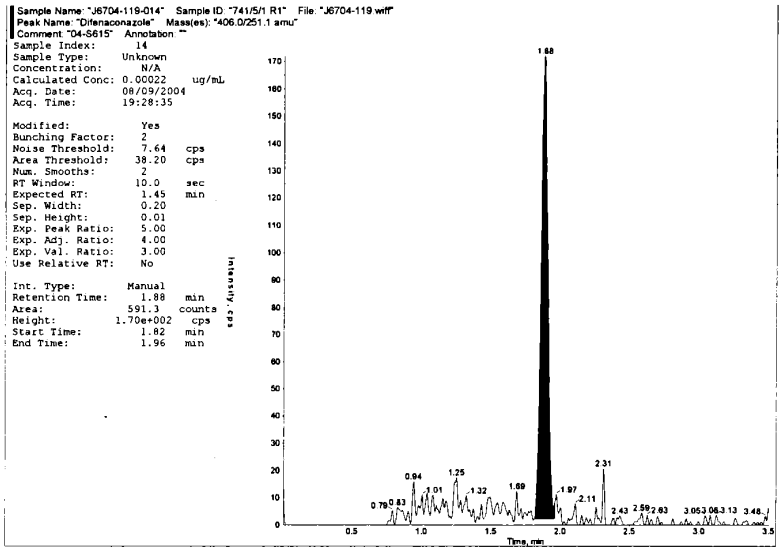


Figure 40 : Untreated Leeks Fortified at 0.01 mg kg $^{-1}$. Sample Concentration = 0.025 g mL $^{-1}$. Recovery = 84%.



Appendix 5: Detector Linearity Graphs

Figure 41 : **LC-MS/MS Detector Calibration Graph for Difenoconazole, Intercept set to Zero**

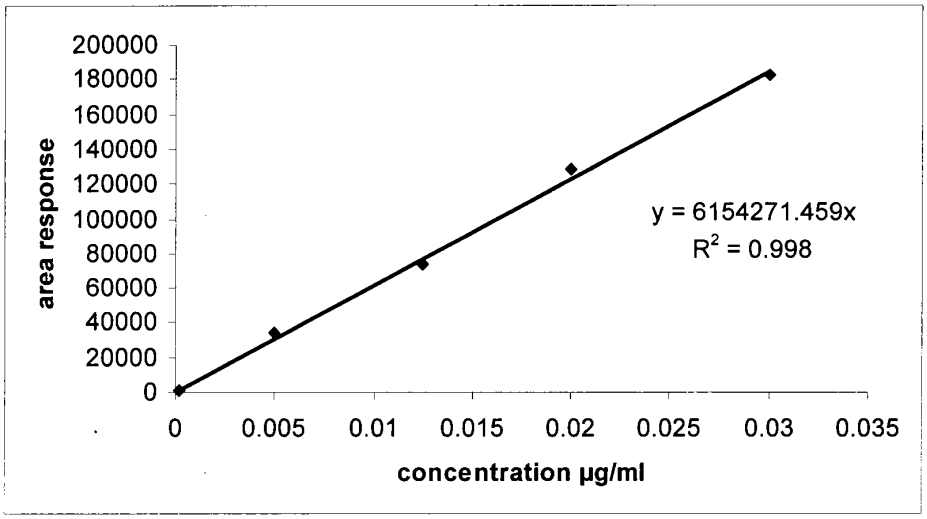
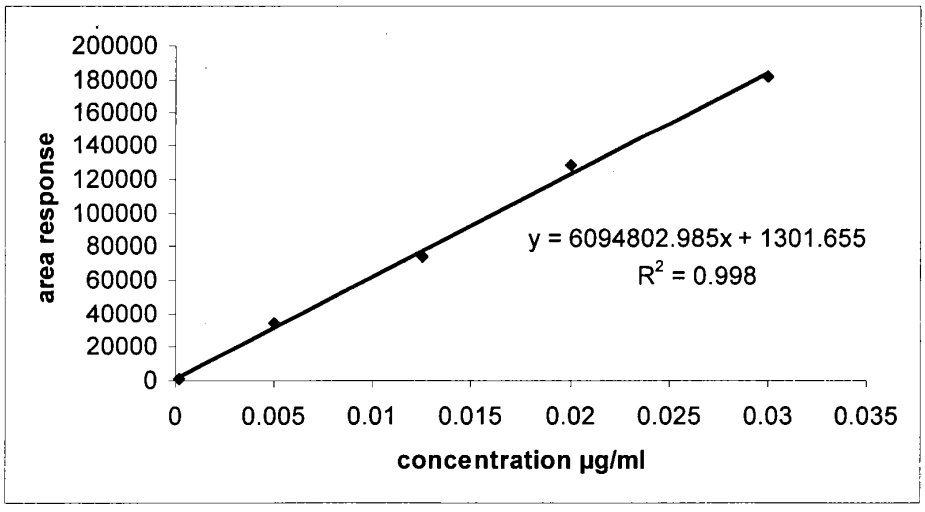


Figure 42 : **LC-MS/MS Detector Calibration Graph for Difenoconazole, No Intercept set**



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 Difenoconazole

Infuse a standard solution of difenoconazole (0.1 to 1.0 μg mL^{-1}) in acetonitrile:water 50:50 v/v directly into the mass spectrometer interface at a rate at of 5 – 20 μL 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* 406.0.

Using the Analyst software quantitative optimisation programme, tune the instrument for difenoconazole, ensuring that the correct ions are selected (difenoconazole initial Q1 *m/z* = 406.0 and product ion m/z = 251). 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 difenoconazole standards using a mobile phase of acetonitrile:water 50:50 v/v + 0.2 % formic 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.

Figure 43 : Difenoconazole Initial Product Scan (positive ionisation)

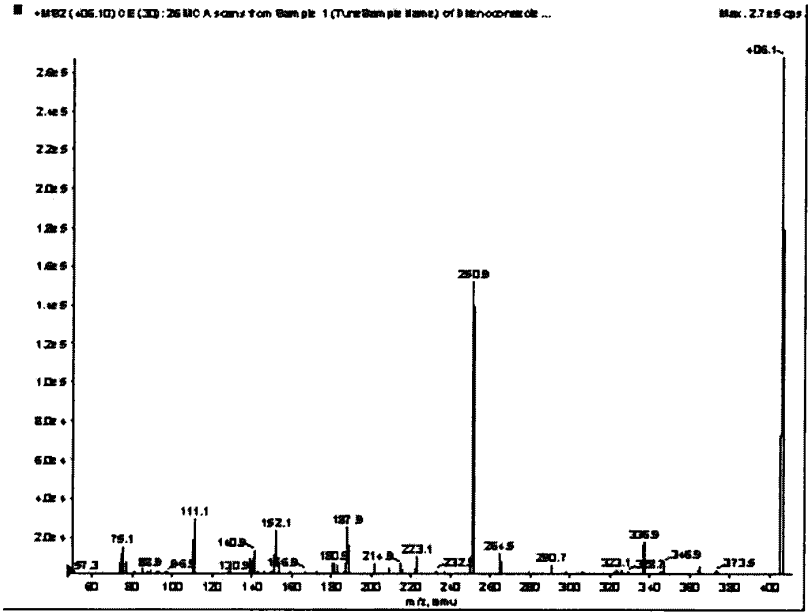


Figure 44 : Difenoconazole Final Product Scan Daughters of m/z = 406.0 (positive ionisation)