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STANDARD OPERATING PROCEDURE

RAM 243/06

Residue Analytical Method for the Analysis of Azoxystrobin and R230310 in Crops.

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Reviewer	: **R C Silcock**		

Authorised by : H Swaine **H Swaine** **Section Manager** 12 April 2000 **Date**

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This SOP RAM supersedes RAM 243/05

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1 **SCOPE**

The analytical procedures described are suitable for the determination of residues of the fungicide azoxystrobin (Figure 1), and its geometrical isomer, R230310 (Figure 2) in cereals, wine, soft fruits, leafy crops, root crops, rice, dried beans, peas, chilli, processed cereal crops and processed soft fruit crops.

To date, in these laboratories, the method has been applied to cereal, wine, soft fruit, leafy crop, root crop, dried bean, pea, rice, chilli, processed cereal crop and processed soft fruit crop samples and the limits of determination of the methods are 0.02 mg kg^{-1} in forage and straw, 10 µg $litre^{-1}$ in wine and 0.01 mg kg^{-1} for all other crops.

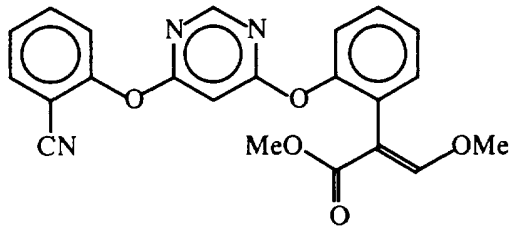


Figure 1 : Methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).

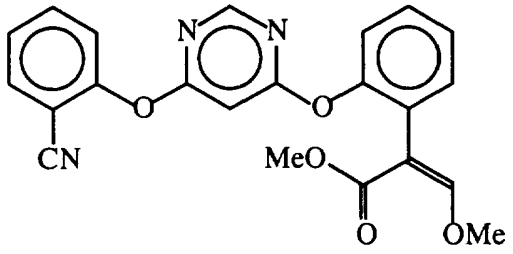


Figure 2 : Methyl (Z)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).

2 SUMMARY

Azoxystrobin and R230310 residues in banana, rice, leafy crop, dried bean, cereal and processed cereal crop samples are extracted in 90:10/ acetonitrile:water. An aliquot of the extract is cleaned up by adsorption chromatography on a Florisil column. The eluate is evaporated to dryness and taken up in a known volume of acetone for analysis by gas chromatography with nitrogen-phosphorus detection (GC-NPD).

Azoxystrobin and R230310 residues in wine, citrus fruit, root crop, chilli, soft fruit and processed soft fruit samples are extracted in 90:10/ acetonitrile:water except wine and citrus juice which is partitioned into dichloromethane. An aliquot of the extract is cleaned up by adsorption chromatography on a silica sorbent. The eluate is evaporated to dryness and taken up in a known volume of acetone for analysis by GC-NPD.

An alternative method for the final determination of azoxystrobin and R230310 in vines is high performance liquid chromatography using ultra-violet detection. After clean up by adsorption chromatography on a silica sorbent, the eluate is evaporated to dryness and taken up in a known volume of mobile phase for analysis by HPLC-UV.

3 PROCEDURE

The sections to be followed for each crop type are :-

Cereals (grain), rice (grain), processed cereals, dried beans and peas - a, b, c, d, gi, hi.

Cereals (straw and forage), rice (straw) - a, b, c, e, gi, hi.

Soft fruits (except bananas), processed soft fruits, chilli and root crops - a, c, d, gii, hii.

Leafy crops and bananas - a, c, e, gi, hi.

Wine - a, c, gii, hii.

Citrus juice - a, c, f, gii, hii.

3.1 Extraction

a) Thoroughly mix the sample and weigh or measure a representative aliquot (cereal grain, rice grain, processed cereals, soft fruits, processed soft fruits, dried beans, peas and root crops 20 g; cereal straw and rice straw 5 g; cereal forage and leafy crops 10 g; wine and citrus juice 20 cm^3) into a centrifuge bottle, round bottom flask (wine) or beaker (citrus juice).

b) Dry crops : If the samples are very dry, pre-wet with acetonitrile (10-20 cm^3) and soak for 15 minutes prior to fortification.

c) Fortify all samples (excluding controls) with a suitable known amount of internal standard (R216206, Figure 3 in Appendix 4). Fortify two control samples with an accurately known amount of azoxystrobin, R230310 and R216206 as recovery checks. For HPLC-UV analysis (grape and wine) omit the internal standard and fortify recovery checks accurately with known amount of azoxystrobin and R230310 only.

d) Homogenise the sample for two minutes in 90:10/acetonitrile:water (in a final volume of 60 cm^3). Filter the extract under vacuum through a Whatman No 1 filter paper into a round bottom flask. Rinse the residuum with further extraction solvent. Adjust to a suitable known volume (e.g.100-120 cm^3) with acetonitrile.

e) Homogenise the sample for two minutes in 90:10/acetonitrile:water (in a final volume of 80 cm^3). Place a Whatman No. 5 filter paper into a Büchner funnel followed by a Whatman No. 1 (except bananas, use No. 1 only). Decant off the solvent, under vacuum, into a round bottom flask. Add a further 50 cm^3 extraction solvent to the centrifuge bottle and homogenise for a further two minutes. Filter and rinse the residuum with further extraction solvent. Adjust to a suitable known volume (eg.130-150 cm^3) with acetonitrile.

f) Neutralise to pH 7, using a pH meter, with 5M sodium hydroxide (~2-4 cm^3). Transfer the sample to a 100 cm^3 separatory funnel. Measure out 90:10 acetonitrile:water (60 cm^3), rinse the sample beaker and add to the separatory funnel. Shake and filter, under vacuum, through a Whatman No.1 filter paper into a round bottom flask. Adjust to a suitable known volume (e.g. 80-100cm^3) with acetonitrile.

g) i) Take a funnel, plug with glass wool and add anhydrous sodium sulphate (~10 g). Pre wet with ethyl acetate (~10 cm^3), pass an aliquot (0.5 g straw, forage and leafy crops; 2 g grain, bananas, dried beans and peas) through and rinse with ethyl acetate (20 cm^3), collecting in a round bottom flask.

ii) Take a 1 g aliquot (crops) or 5 cm^3 aliquot (wine and citrus juice) and add to a 100 cm^3 separatory funnel containing an equivalent volume of dichloromethane plus half equivalent-volume of 5% sodium chloride solution. Partition and collect the dichloromethane layer through a funnel containing anhydrous sodium sulphate, pre wet with dichloromethane (~5 cm^3), into a round bottom flask. Rinse the sodium sulphate plug with further dichloromethane (~5 cm^3), collecting in the round bottom flask. If emulsions occur a second partition with dichloromethane may be required.

h) All samples: Evaporate the aliquots to dryness on a rotary evaporator at $\leq 40^{o}C$ and:

i) Redissolve in 20% ethyl acetate:hexane (2 cm^3) for Florisil column clean-up and ultrasonicate.

ii) Redissolve in 50:50/hexane:dichloromethane (2 cm^3) for silica column clean-up and ultrasonicate.

3.2 **Solid Phase Extraction Clean-Up (Silica, Si)**

a) Soft fruit, processed soft fruit, chilli, root crop, wine and citrus juice samples are cleaned up on a silica column.

Place a disposable silica (Si, 0.5 g) column in the Supelco™ assembly. Add 50:50/hexane:dichloromethane (3 cm^3) and allow to drip under gravity.

b) Transfer the sample extract from Section 3.1(hii) above onto the column, and allow to drip under gravity.

c) Wash the round bottom flask that contained the aliquot with 95:5/dichloromethane:ethyl acetate (2 cm^3) and load onto the cartridge. Allow to drip under gravity. Elute the cartridge with 70:30/dichloromethane:ethyl acetate (4 cm^3), allowing to drip under gravity, then dry the column by pushing the eluate through under positive pressure into the collection tubes.

d) Evaporate the eluates to dryness at ≤40°C under a stream of clean dry air. Redissolve the residuum in a known volume of acetone for analysis by GC-NPD or in mobile phase for analysis by HPLC - UV (grapes and wine only).

e) Standards used for HPLC - UV analysis should be prepared by pipetting the required amount e.g. 2 cm^3 of a 0.1 μg cm^3 standard, into an HPLC vial, blowing to dryness and taking up in 2 cm^3 mobile phase.

3.3 **Adsorption Column Chromatographic Clean-Up (Florisil)**

a) Cereal, processed cereal, banana, leafy crop, rice, dried bean and pea samples are cleaned up on a Florisil column.

b) Preparation of Florisil Columns

Place a small glass wool plug in the bottom of a 10 mm diameter chromatography column (pre-rinsed with acetone and hexane) and add n-hexane (15 cm^3). Slowly, with gentle tapping, add 5% water deactivated Florisil (2 g) followed by granular anhydrous sodium sulphate (1 g). Allow the hexane to percolate onto the column.

Note - Prior to use, each batch of column packing material must be calibrated as follows : Take a mixed azoxystrobin, R230310 and R216206 standard solution in acetone e.g. 0.1 μg cm^{-3}. Evaporate to dryness and redissolve in 20% ethyl acetate:hexane (2 cm^3), ultrasonicate. Transfer the aliquot to the top of the column and allow it to percolate onto the column. Wash with 20% ethyl acetate:hexane (15 cm^3). Then elute with 70% ethyl acetate:hexane and collect two fractions (10 cm^3) and one fraction (5 cm^3) of the eluate. Evaporate the fractions to dryness and take up in a known volume of acetone. Analyse the fractions by GC-NPD to determine the elution pattern.

c) Transfer the sample extract from Section 3.1(hii) above and allow to percolate onto the column. Elute the column using the procedure determined

from the column calibration. Collect the ethyl acetate:hexane eluate in a round bottom flask.

d) Evaporate the eluates to dryness on a rotary evaporator at ≤40°C. Redissolve the residuum in acetone to give a final concentration of 2 g cm^{-3} : grain, processed cereals, bananas and dried beans; 0.5 g cm^{-3} : straw and leafy crops; and 0 25 g cm^{-3} : forage and transfer to GC vials for analysis by GC-NPD

4 GAS CHROMATOGRAPHY WITH NITROGEN PHOSPHORUS DETECTION (GC-NPD)

The conditions for the analysis by GC-NPD will depend upon the equipment available. The operating manuals for the instruments should always be consulted to ensure safe optimum use. The following conditions have been found to be satisfactory using a Varian 3400 series gas chromatograph fitted with a Varian 8100 series autosampler:

4.1 GC-NPD Conditions

(i) Columns .	Rtx 200 (trifluoropropylmethyl polysiloxane) fused silica wall coated open tubular capillary 15 m x 0.32 mm (0.5 μm film thickness).
(ii) Oven temperatures :	70°C (hold 1 minute); program at 30°C $minute^{-1}$ to 220°C; program at 10°C $minute^{-1}$ to 280°C (hold 5 minutes); program at 30°C $minute^{-1}$ to 300°C (hold 10 minutes). These conditions may need to be adjusted according to crop type.
(iii) Injector :	Septum programmable injector (SPI): 40°C (hold 0.1 minute); program at 200°C $minute^{-1}$ to 250°C (hold 26.5 minutes). Injection volume = 2 μl.
(iv) Gas Flow Rates :	Helium (carrier) : 3 cm^3 min^{-1} Helium (makeup): 27 cm^3 min^{-1} Air: 175 cm^3 min^{-1} Hydrogen: 4.5 cm^3 min^{-1}
(v) Detector :	Temperature at 300°C. Bead setting: 3.0 - 3.3 amps (depending on condition of bead). Attenuation: 64. Range: 12

Under these conditions, for Rtx 200 capillary column, the retention times of azoxystrobin, R230310 and R216206 were approximately 13.2, 13.5 and 14.2 minutes respectively.

Note : These conditions should be adhered to as closely as possible. Conversion of the isomers (R230310 -> azoxystrobin) has been seen to occur under lower carrier gas flow rates at high temperatures.

4.2 **Calculation of Azoxystrobin and R230310 Residue Results**

Note - The internal standardisation procedure determines the concentration of azoxystrobin and R230310 residues in the final extract relative to that of a known concentration of internal standard which is added by accurate fortification of the sample prior to extraction. Correction for percentage recovery throughout the procedure is thereby inherent for each individual sample; in addition, any small volume errors, particularly those associated with the final GC injected solution are similarly corrected.

The calculation used for the determination of azoxystrobin and R230310 residues by internal standardisation using R216206 may be performed using a 'single point ratio calibration'. It should be noted that such calibrations are only feasible when the internal standard chosen meets certain criteria (Reference 1).

a) Make repeated injections of 2 µl of a standard solution containing a mixture of azoxystrobin, R230310 and R216206 each at 0.1 µg cm^{-3} into the GC operated under conditions described in Section 4.1.

When a consistent response is obtained measure the peak heights or areas obtained for azoxystrobin and R216206 and calculate the azoxystrobin/internal standard peak ratio. Similarly, measure the peak heights or areas obtained for R230310 and R216206 and calculate the R230310/internal standard peak ratio.

b) Make an injection of each sample solution and measure the peak heights or areas of the peaks corresponding to azoxystrobin, R230310 and R216206 and similarly calculate the peak ratios.

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

d) Calculate the residue in the sample, expressed as µg g^{-1} (crops) or µg $litre^{-1}$ (wine and citrus juice), by proportionation of the azoxystrobin and R230310/internal standard peak height or peak area ratio measured for the sample against that for the analytical standard solution.

$$\text{Residue} = \frac{\text{response ratio in sample}}{\text{response ratio in standard}} \times \frac{\text{conc. of analyte in standard}}{\text{conc. of R216206 in standard}} \times \text{R216206 fortification level}$$

where analyte = azoxystrobin or R230310

5 **HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING UV DETECTION (HPLC- UV)**

The conditions for the analysis by HPLC-UV will depend upon the equipment available. The operating manuals for the instruments should always be consulted to ensure safe optimum use. The following conditions have been found to be satisfactory using the instruments detailed below.

5 1 **HPLC-UV Conditions**

a)	Instrument	:	HPLC fitted with a UV detector e.g. Severn 6500 series, pump e.g Waters 501 series, autosampler and integrator or data handling system.
b)	Column	:	Spherisorb 5 ODS2 column 25 cm length x 4.6 mm internal diameter.
c)	Mobile Phase	:	Acetonitrile/water (55:45 - 60:40), flow rate 1 cm^3 $minute^{-1}$, depending on interfering peaks.
d)	Injector	:	Waters WISP 712 series (100 µl injection volume).
e)	Detector	:	255 nm

Under these conditions, using acetonitrile/water (57:43), the retention time of azoxystrobin and R230310 are 8.3 and 6.8 minutes respectively.

5.2 **Calculation of Azoxystrobin and R230310 Residue Results**

a) Make repeated injections of 100 µl of a standard solution containing azoxystrobin and R230310 at 0.1 µg cm^{-3} into the HPLC operated under the conditions described in 5.1 above. When a consistent response is obtained measure the peak heights or areas obtained for the standard.

b) Make an injection of each sample solution and measure the peak height or area of the peaks corresponding to azoxystrobin and R230310.

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

d) Calculate the residue in the sample, expressed as µg g^{-1} (grapes) or µg $litre^{-1}$ (wine) by proportionation of the azoxystrobin or R230310 peak height or peak area measured for the sample against that for the analytical standard solution.

$$\text{Residue} = \frac{\text{peak height / area in sample}}{\text{peak height / area in standard}} \times \frac{\text{concentration of standard}}{\text{concentration of sample solution}} \times \frac{\text{volume injected (std)}}{\text{volume injected (sample)}}$$

$$= \frac{\text{response}}{\text{response}} \times \frac{\mu g\ cm^{-3}}{g\ cm^{-3}} \times \frac{\mu L}{\mu L} = \mu g\ g^{-1} = mg\ kg^{-1}$$

6 CONTROL AND RECOVERY EXPERIMENTS

At least one untreated sample must be analysed alongside any set of samples, using exactly the same method. This ensures that no unobserved contamination of the samples occurred prior to, or during, the analysis. At least two control samples, accurately fortified with a suitable known amount of azoxystrobin, R230310 and R216206, should be analysed alongside every batch of treated samples. Fortification amounts should be based on anticipated residue levels. When no residues are expected, the recoveries should be fortified at low levels, typically 0.02-0.05 $\mu g\ g^{-1}$ or 10-50 μg $litre^{-1}$.

7 LIMIT OF DETERMINATION (QUANTITATION)

The limit of determination of the method can be assessed by carrying out recovery experiments at low levels of fortification (0.01 - 0.05 $\mu g\ g^{-1}$ or 10 - 50 $\mu g\ litre^{-1}$). In these laboratories the limits of determination have been set at 0.02 mg kg^{-1} in forage and straw, 10 $\mu g\ litre^{-1}$ in wine and citrus juice and 0.01 mg kg^{-1} for all other crops. Care must be taken when working at the limit of determination to minimise the risk of contamination.

8 LIMIT OF DETECTION

The limit of detection of azoxystrobin, R230310 and R216206 was investigated by fortifying control straw extracts that had been taken through the analytical method. The limit of detection was defined as the lowest concentration that gave a response 4 times background noise and was equivalent to 0.04 ng injected.

9 METHOD VALIDATION STUDIES

9.1 Recovery Data

In these laboratories to date the method has been applied to the analysis of cereal, wine, soft fruit, leafy crop, root crop, dried beans, pea, rice, chilli, processed cereal crop and processed soft fruit crop samples. Recoveries from a validation study for cereal and vine samples fortified over a range of azoxystrobin and R230310 concentrations are shown in Tables 1 and 3 (Reference 2). Typical recoveries taken from regulatory studies carried out from 1993 - 1996 for crop samples are shown in Tables 2, 5-15 and for vine samples, analysed by HPLC-UV, are shown in Table 4.

Table 1 : Cereal Recovery Data from Validation Study

Substrate	Fortification Level ($mg\ kg^{-1}$)	Azoxystrobin % Recovery	R230310 % Recovery
Straw	0.02	103, 110, 96, 85, 89, 85	77, 90, 82, 70, 92, 81
	0.2	98, 105, 102	98, 100, 96
	2.0	104, 101, 102	98, 96, 102
Grain	0.01	106, 100, 102, 84, 94, 90	127, 109, 106, 110, 110, 110
	0.1	98, 98, 97	98, 96, 98

NOTE: % Recovery values corrected for internal standard (R216206) recovery.

			Azoxystrobin	R230310
Straw :	Mean recovery	=	99%	90%
	Standard deviation	=	8	9
	Relative standard deviation	=	8%	10%
Grain :	Mean recovery	=	97%	107%
	Standard deviation	=	6	8
	Relative standard deviation	=	6	7%

Table 2 : Cereal Recovery Data from Regulatory Studies

Substrate	Fortification Level (mg kg^{-1})	Azoxystrobin % Recovery	R230310 % Recovery
Straw	0.2	116	95
	1.0	106, 100, 97, 103	96, 98, 96, 92
	2.0	96, 100, 101, 101	94, 95, 100, 96
Forage	0.2	94	93
	0.5	105, 97, 98, 98, 99 100, 102, 105, 97	104, 105, 98, 97, 100, 91, 108, 104, 105
	1.0	100, 98, 104, 98, 101	106, 107, 98, 99, 106
	2.0	102, 93, 98.97, 100	110, 102, 102, 88
		100, 102	101, 103, 106
Grain	0.05	108, 108, 100, 100, 100, 96, 100, 100, 106, 104, 96, 100	92, 86, 98, 92, 108, 102, 102, 90, 106, 88, 96
	0.1	99	101

NOTE : % Recovery values corrected for internal standard (R216206) recovery.

			Azoxystrobin	R230310
Straw :	Mean recovery	=	102%	96%
	Standard deviation	=	6	2
	Relative standard deviation	=	6%	2%
Forage :	Mean recovery	=	99%	102%
	Standard deviation	=	3	6
	Relative standard deviation	=	3%	6%
Grain :	Mean recovery	=	101%	96%
	Standard deviation	=	4	7
	Relative standard deviation	=	4%	7%

Table 3 : Vine Recovery Data from Validation Study

Substrate	Fortification Level	Azoxystrobin % Recovery	R230310 % Recovery
Grape	0.01 mg kg^{-1}	100, 100, 100, 100	100, 100, 100, 100
	0.02 mg kg^{-1}	110, 105, 100, 100	105, 105, 105, 100
	0 05 mg kg^{-1}	104, 104, 102, 102	106, 104, 100, 100
	0.10 mg kg^{-1}	105, 101, 101, 106	103, 98, 98, 102
	0.50 mg kg^{-1}	98, 99, 98, 99	95, 98, 97, 95
Wine	10 µg l^{-1}	106, 103, 97, 99	101, 106, 99, 102
	20 µg l^{-1}	100, 102, 98, 96	103, 100, 94, 97
	50 µg l^{-1}	96, 96, 101, 102	97, 99, 101, 106
	100 µg l^{-1}	100, 99, 99, 97	101, 101, 103, 97
	500 µg l^{-1}	101, 100, 100, 101	97, 97, 98, 99

NOTE : % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Grape :	Mean recovery	=	100%	98%
	Standard deviation	=	5	6
	Relative standard deviation	=	5%	6%
Wine :	Mean recovery	=	100%	100%
	Standard deviation	=	3	3
	Relative standard deviation	=	3%	3%

Table 4 : Vine Recovery Data from Regulatory Studies

Substrate	Fortification Level	Azoxystrobin % Recovery	R230310 % Recovery
Grape	0.1 mg kg^{-1}	71, 64, 90, 92	82, 75, 84, 90
	0 2 mg kg^{-1}	100, 105, 82, 85, 86	110, 105, 81, 85, 81
	0.50 mg kg^{-1}	68, 80	71, 72
	1.0 mg kg^{-1}	97, 94, 78, 100, 108, 92, 92, 89, 95	94, 94, 80, 100, 93, 85, 85, 89, 93
Wine	100 µg l^{-1}	70, 85, 76	68, 82, 79

			Azoxystrobin	R230310
Grape :	Mean recovery	=	86%	87%
	Standard deviation	=	12	11
	Relative standard deviation	=	14%	13%
Wine :	Mean recovery	=	77%	76%
	Standard deviation	=	6	6
	Relative standard deviation	=	8%	8%

Table 5 : Banana Recovery Data from Regulatory Studies

Substrate	Fortification Level (mg kg^{-1})	Azoxystrobin % Recovery	R230310 % Recovery
Whole Fruit	0.02	110, 110, 110	115, 105, 110
	0.05	98, 96, 102, 104, 104, 108, 114, 110, 100, 102, 102, 106, 98, 100, 98, 100	100, 100, 112, 112, 114, 126, 106, 112, 108, 110, 110, 112, 96, 104, 94, 96
	0.10	101, 101, 98, 98, 103, 103, 100, 95, 101, 95	103, 99, 100, 99, 106, 108, 107, 109, 113, 103
Pulp	0.02	100, 95	105, 100
	0 05	106, 102, 96, 98, 96, 100, 94, 102, 98, 94, 104, 102, 104, 96, 102	108, 114, 102, 116, 100, 114, 116, 114, 110, 106, 116, 108, 116, 108, 108
	0.10	100, 99, 101, 103, 100, 100	99, 100, 102, 102, 102, 107
Skin	0.02	100, 105, 120	115, 115, 110
	0.05	94, 108, 102, 108, 114, 110, 102, 98, 104, 110, 110, 104, 112, 100, 112, 104, 98	100, 104, 112, 118, 108, 106, 116, 114, 100, 118, 104, 110, 100, 102, 106, 98, 92
	0.10	107, 107, 85, 90, 111, 107, 99, 97, 99, 98	101, 99, 93, 95, 102, 97,

NOTE : % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Whole Fruit :	Mean recovery	=	102%	107%
	Standard deviation	=	5	7
	Relative standard deviation	=	5%	7%
Pulp :	Mean recovery	=	100%	108%
	Standard deviation	=	3	6
	Relative standard deviation	=	3%	6%
Skin :	Mean recovery	=	104%	105%
	Standard deviation	=	7	8
	Relative standard deviation	=	7%	7%

Table 6 : Cucumber and Tomato Recovery Data from Regulatory Studies

Substrate	Fortification Level (mg kg^{-1})	Azoxystrobin % Recovery	R230310 % Recovery
Cucumber	0.1	98, 99, 101, 100, 101, 103, 102	108, 101, 107, 112, 108, 104, 110
	0.50	97	111
Tomato	0.50	100, 100, 104, 102	118, 118, 114, 116
	0.2	107, 99	107, 103

NOTE : % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Cucumber :	Mean recovery	=	100%	108%
	Standard deviation	=	2	4
	Relative standard deviation	=	2%	3%
Tomato :	Mean recovery	=	102%	113%
	Standard deviation	=	3	6
	Relative standard deviation	=	3%	6%

Table 7 : Melon Recovery Data from Regulatory Studies

Substrate	Fortification Level (mg kg^{-1})	Azoxystrobin % Recovery	R230310 % Recovery
Skin	0.2	103, 104, 100, 109	103, 103, 99, 109
	0.50	98, 105	104, 110
Pulp	0.02	115, 100	125, 100
	0.05	100, 98	112, 124
	0.1	95, 100	101, 101

NOTE : % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Skin :	Mean recovery	=	103%	105%
	Standard deviation	=	4	4
	Relative standard deviation	=	4%	4%
Pulp :	Mean recovery	=	101%	111%
	Standard deviation	=	7	12
	Relative standard deviation	=	7%	11%

Table 8 : Apple Recovery Data from Regulatory Studies

Substrate	Fortification Level (mg kg^{-1})	Azoxystrobin % Recovery	R230310 % Recovery
Apple	0.1	96, 101	96, 108
	0.50	105, 98, 99, 103, 106, 107, 105, 103, 104, 102, 97, 107, 100, 101, 101	106, 105, 104, 97, 101, 104, 94, 100, 104, 103, 102, 102, 104, 105, 105
	1.0	94, 98, 105, 103, 103, 104	98, 105, 107, 112, 103, 109
	2.0	101, 107	101, 112

NOTE. % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Apple :	Mean recovery	=	102%	104%
	Standard deviation	=	4	4
	Relative standard deviation	=	4%	4%

Table 9 : Leafy and Root Crop Recovery Data from Regulatory Studies

Substrate	Fortification Level (mg kg^{-1})	Azoxystrobin % Recovery	R230310 % Recovery
Leafy crop	0.05	104, 114, 94, 96	94, 92, 98, 104
	0.10	110, 106	108, 109
	0.20	99	108
Root crop	0.05	98, 102	94, 90
	0.2	102	97

NOTE: % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Leafy crop :	Mean recovery	=	103%	102%
	Standard deviation	=	7	7
	Relative standard deviation	=	7%	7%
Root crop :	Mean recovery	=	101%	102%
	Standard deviation	=	2	7
	Relative standard deviation	=	2%	7%

Table 10 : Dried Beans Recovery Data from Regulatory Studies

Substrate	Fortification Level ($mg\ kg^{-1}$)	Azoxystrobin % Recovery	R230310 % Recovery
Grain	0.01	99, 99, 99, 103	101, 93, 102, 101
	0.05	98, 98, 106, 110	96, 94, 96, 102
	0.10	106, 105, 100, 99	104, 103, 103, 101
	0.20	106, 103	101, 103

NOTE: % Recovery values corrected for internal standard (R216206) recovery

			Azoxystrobin	R230310
Grain :	Mean recovery	=	102%	100%
	Standard deviation	=	4	4
	Relative standard deviation	=	4%	4%

Table 11 : Peach Recovery Data from Regulatory Studies

Substrate	Fortification Level ($mg\ kg^{-1}$)	Azoxystrobin % Recovery	R230310 % Recovery
Peach	0.10	101, 102, 103, 101, 109, 103, 97, 101, 100, 98, 102, 98, 104, 101, 106, 104	114, 109, 104, 103, 107, 107, 107, 109, 111, 110, 113, 111, 109, 109, 106, 114
	0.20	97, 104, 99, 104, 96, 101, 101, 100, 98, 101, 96, 95, 90, 96	109, 113, 111, 113, 106, 108, 110, 107, 104, 104, 102, 103, 107, 110

NOTE: % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Peach :	Mean recovery	=	100%	108%
	Standard deviation	=	4	3
	Relative standard deviation	=	4%	3%

Table 12 : Processed Cereal Recovery Data from Regulatory Studies

Substrate	Fortification Level ($mg\ kg^{-1}$)	Azoxystrobin % Recovery	R230310 % Recovery
Grain	0.10	108, 103	93, 98
Bran	0.10	115, 115	98, 99
Middlings	0.10	101	99
Shorts	0.10	103	101
Germ	0.10	107	99
Flour	0.10	101	102

NOTE: % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Processed: cereal	Mean recovery	=	107%	99%
	Standard deviation	=	6	3
	Relative standard deviation	=	5%	3%

Table 13 : Processed Soft Fruit Recovery Data from Regulatory Studies

Substrate	Fortification Level ($mg\ kg^{-1}$)	Azoxystrobin % Recovery	R230310 % Recovery
Tomato Fruit	0.05	104, 102, 104	108, 108, 106
Pomace	0.50	99, 100	99, 102
Paste	0.10	101	111

NOTE: % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Processed : soft fruit	Mean recovery	=	102%	106%
	Standard deviation	=	2	4
	Relative standard deviation	=	2%	4%

Table 14 : Chilli Recovery Data from Regulatory Studies

Substrate	Fortification Level (mg kg^{-1})	Azoxystrobin % Recovery	R230310 % Recovery
Chilli	0.01	98, 94, 81, 87	114, 108, 112, 101
	0.05	91, 101	108, 113
	0.1	100, 101, 114, 107	112, 106, 106, 98
	0.20	103, 100, 98, 100	106, 107, 105, 104

NOTE. % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Chilli .	Mean recovery	=	98%	107%
	Standard deviation	=	8	5
	Relative standard deviation	=	8%	4%

Table 15 : Rice Recovery Data from Regulatory Studies

Substrate	Fortification Level (mg kg^{-1})	Azoxystrobin % Recovery	R230310 % Recovery
Grain	0.1	105, 103, 103	101, 105, 105
	0.2	111, 101, 101, 100, 100, 101, 100, 102	107, 105, 101, 101, 110, 103, 102, 105
	0.25	100, 98	102, 102
	1.0	100, 101, 100, 104, 102, 103	107, 108, 102, 104, 99, 95
Straw	0.50	95	104
	1.0	97, 99, 102, 100, 103, 100, 101, 99, 103, 105	100, 98, 99, 93, 101, 106, 108, 108, 97, 114
	2.0	99	101
	5.0	97	101

NOTE : % Recovery values corrected for internal standard (R216206) recovery .

			Azoxystrobin	R230310
Grain :	Mean recovery	=	102%	103%
	Standard deviation	=	3	4
	Relative standard deviation	=	3%	3%
Straw :	Mean recovery	=	100%	102%
	Standard deviation	=	3	6
	Relative standard deviation	=	3%	5%

Table 16 : Orange Juice Recovery Data from Regulatory Studies

Substrate	Fortification Level ($mg\ kg^{-1}$)	Azoxystrobin % Recovery	R230310 % Recovery
Juice	0.01	113, 109, 105, 106	104, 107, 100, 97
	0.05	98, 103, 101, 100	105, 104, 105, 102

NOTE : % Recovery values corrected for internal standard (R216206) recovery.

			Azoxystrobin	R230310
Orange juice :	Mean recovery	=	104%	105%
	Standard deviation	=	5	1
	Relative standard deviation	=	5%	1%

9.2 **Linearity Statement**

For accurate quantitation of residue concentrations, all analyses should be carried out within the linear range of the detector response. In these laboratories the detector response, using GC-NPD, for azoxystrobin, R230310 and R216206 was shown to be linear from 0.02 $\mu g\ cm^{-3}$ to 1.0 $\mu g\ cm^{-3}$ standard concentration. The equation of the line, y=mx+c, for each compound is shown below.

Azoxystrobin : y = 39257x + 284

R230310 : y = 33615x + 304

R216206 : y = 34691x + 203

The detector response for azoxystrobin, using HPLC-UV, was shown to be linear from 0.01 $\mu g\ cm^{-3}$ to 0.5 $\mu g\ cm^{-3}$ standard concentration. The equation of the line, y=mx+c, is shown below. An example is shown in Appendix 1.

Azoxystrobin: y = 29613x + 48

10 **EXAMPLES OF TYPICAL CHROMATOGRAMS** - see Appendices 2 and 3

11 REFERENCES

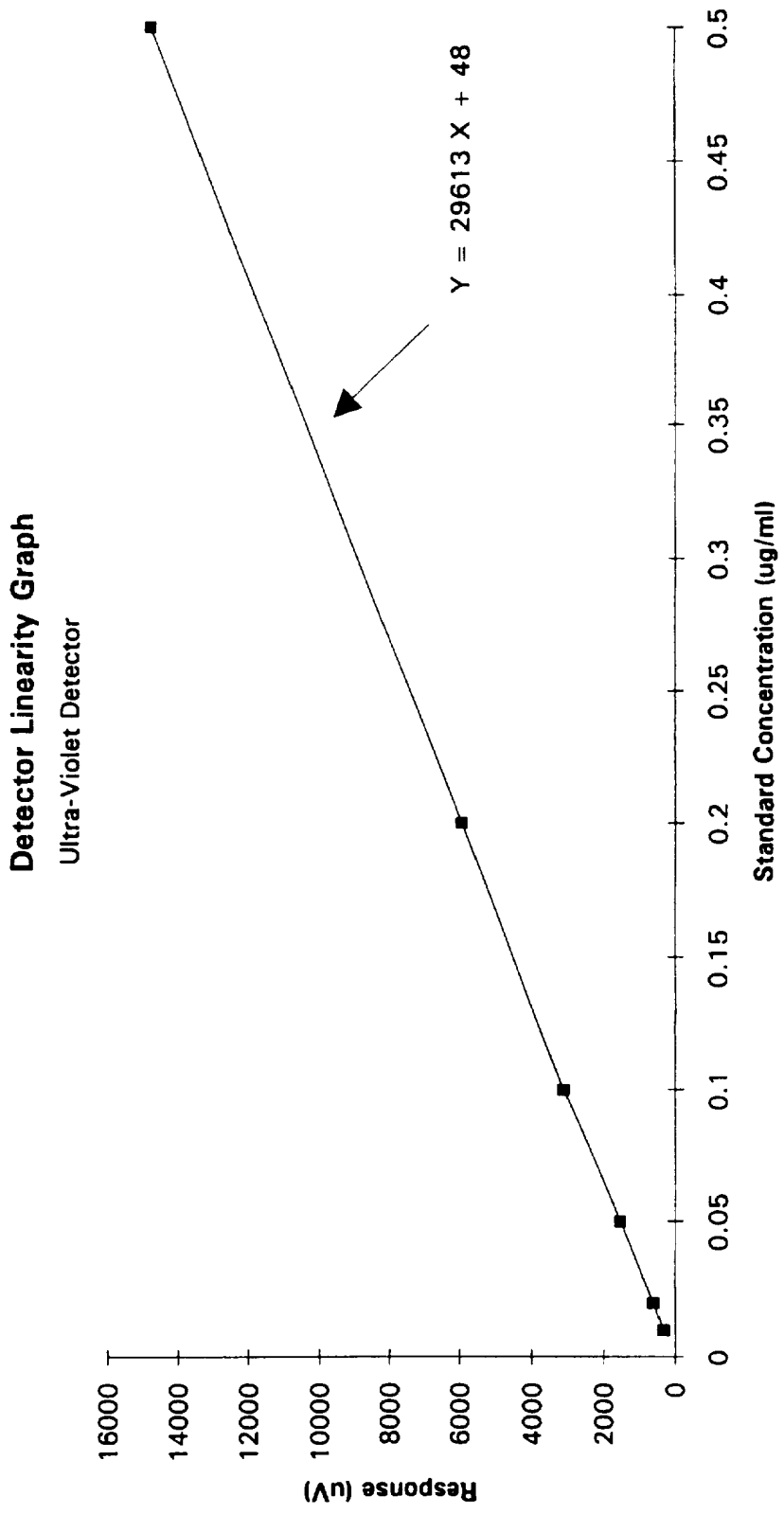
1. Cardone M J, Palermo P J and Sybrandt L B : Potential Error in single point ratio calculations based on linear calibration curves with a significant intercept, Anal Chem., 52, pp 1187-1191, 1980.

2. Burke S R, Sapiets A (1995) ICIA5504 and R230310 : Validation of a method for the determination of Residues in Cereals and Vines. Final Report. Zeneca Agrochemicals Report Number RJ1729B.

Filename	:	RAM24306.doc
Location	:	GROUP/WP/SOPRAM
Reference	:	SRB/SM/CG
Date	:	20 December 1999

Appendix 1

Detector Linearity Graph for HPLC-UV


Detector Linearity Graph
Ultra-Violet Detector
Y = 29613 X + 48
Response (uV)
16000
14000
12000
10000
8000
6000
4000
2000
0
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
Standard Concentration (ug/ml)

Appendix 2

Typical Gas Chromatograms for Azoxystrobin and R230310 Residue Determination in Cereals

Figure 1 : 0.5 $\mu g\ cm^{-3}$ azoxystrobin, R230310 and R216206 standard.

Figure 2 : Untreated straw sample at 0.5 g cm^{-3}.

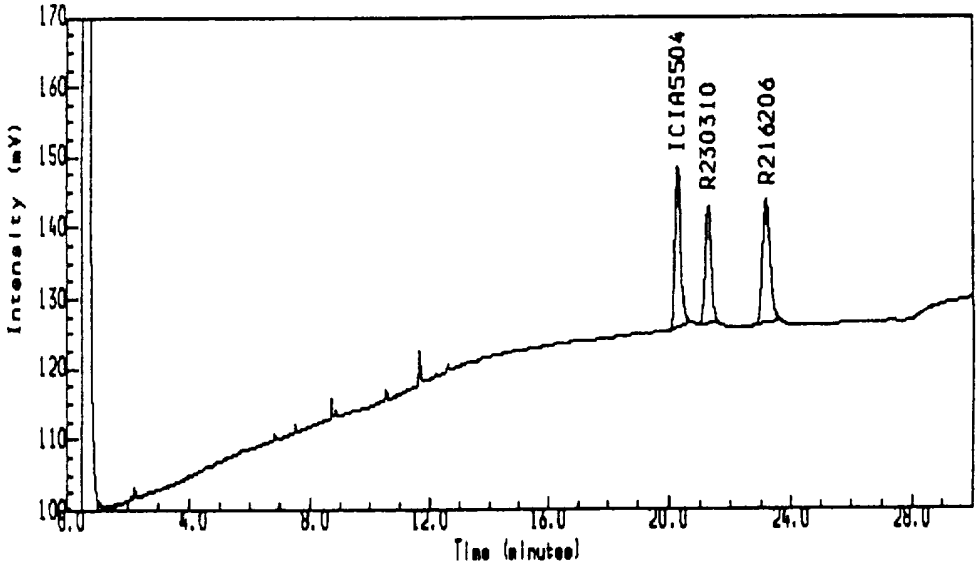
Figure 3 : Untreated straw sample at 0.5 g cm^{-3} fortified at 2.0 $\mu g\ g^{-1}$.
Recovery = 100% azoxystrobin, 92% R230310
(corrected for internal standard recovery)

Figure 1 : 0.5 μg cm^{-3} azoxystrobin, R230310 and R216206 standard.

[RESIDUE] 16 AS5512A,1,1
Reported on 3-AUG-1994 at 11:37

Injection Report

Acquired on 26-JUL-1994 at 17:28



Sample Name : D9579/10B
Sample Id : 5512/94/1
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

Peak	RT mins	Hght uV	Area uVs	MG/KG	Peak name	Width
1	20.267	22555	283747	0.5006	ICIA5504	14.1
2	21.269	16507	221336	0.4989	R230310	14.1
3	23.168	17332	284522		R216206	16.6

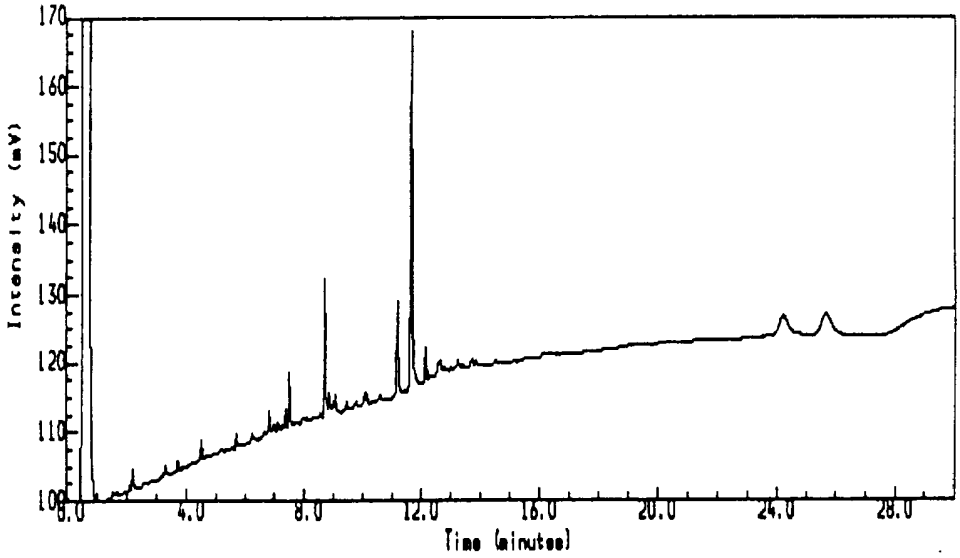
Totals			
Unknowns	0	0	N/A
Quantified	56393	789605	0.9995
Grand Total	56393	789605	0.9995

Figure 2 : Untreated straw sample at 0.5 g cm^{-3}.

[RESIDUE] 16 AS5512A,4,1
Reported on 4-AUG-1994 at 08:47

Injection Report

Acquired on 26-JUL-1994 at 19:44



Sample Name : 623/4/1 94
Sample Id : 5512/94/4
Sample Type : Control Amount=1.00000
Bottle No : 1

PEAK INFORMATION

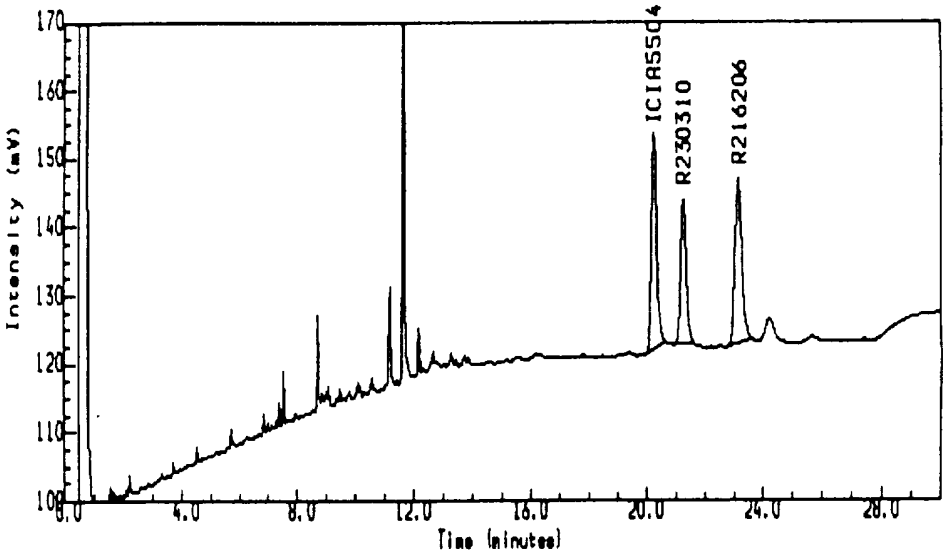
No peaks detected

Figure 3 : **Untreated straw sample at 0.5 g cm^{-3} fortified at 2.0 $\mu g\ g^{-1}$. Recovery = 100% azoxystrobin, 92% R230310 (corrected for internal standard recovery).**

[RESIDUE] 16 AS5512A,5,1
Reported on 3-AUG-1994 at 11:38

Injection Report

Acquired on 26-JUL-1994 at 20:29



Sample Name : R1 623/5/1 94
Sample Id : 5512/94/5
Sample Type : Recovery Amount=1.00000
Bottle No : 1

PEAK INFORMATION

Peak	RT mins	Hght uV	Area uVs	MG/KG	Peak name	Width
1	20.267	31360	395022	2.0035	ICIA5504	12.8
2	21.269	21076	281132	1.8335	R230310	14.1
3	23.168	24086	398468		R216206	16.6

Totals			
Unknowns	0	0	N/A
Quantified	76523	1074622	3.8370
Grand Total	76523	1074622	3.8370

Appendix 3

Typical HPLC Chromatograms for Azoxystrobin and R230310 Residue Determination in Vines

Figure 1 : **0.5 μg cm^{-3} azoxystrobin and R230310 standard.**

Figure 2 : **Untreated grape sample at 0.5 g cm^{-3}.**

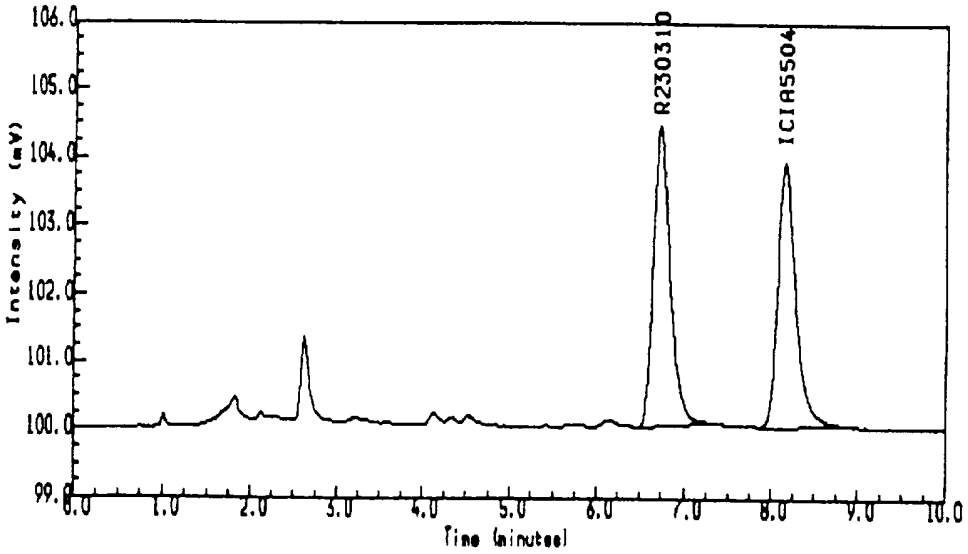
Figure 3 : **Untreated grape sample at 0.5 g cm^{-3} fortified at 0.5 μg g^{-1}. Recovery = 92% azoxystrobin, 91% R230310.**

Figure 1 : 0.5 μg cm^{-3} azoxystrobin and R230310 standard.

[RESIDUE] 19 AS5384A,1,1
Reported on 1-JUL-1994 at 09:05

Injection Report

Acquired on 28-JUN-1994 at 16:08



Sample Name : D9579/5P
Sample Id : 5384/94/1
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

Peak	RT mins	Hght uV	Area uVs	MG/KG	Peak name	Width
1	6.731	4397	56522	0.500	R230310	12.2
2	8.160	3907	56527	0.501	ICIA5504	14.1

Totals

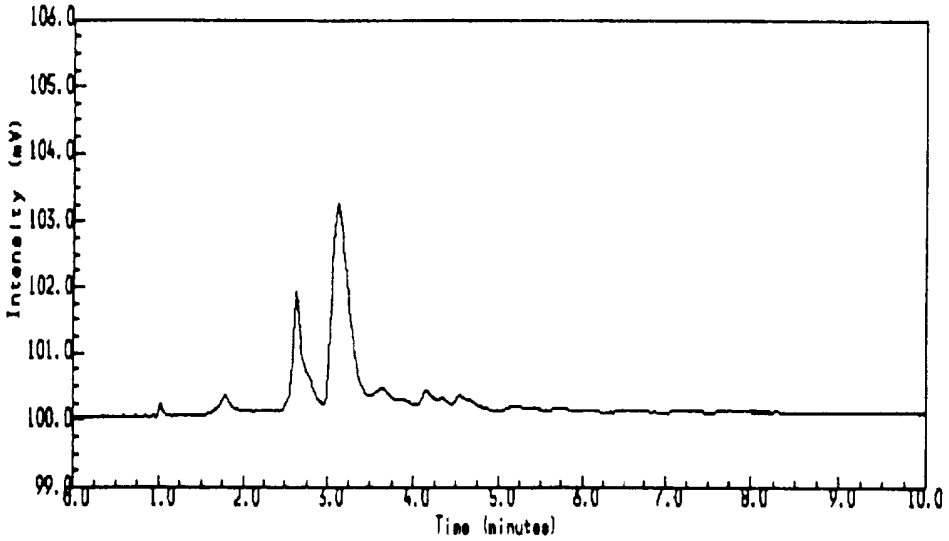
Unknowns	0	0	N/A
Quantified	8304	113049	1.001
Grand Total	8304	113049	1.001

Figure 2 : **Untreated grape sample at 0.5 g cm^{-3}.**

[RESIDUE] 19 AS5384A,2,1
Reported on 1-JUL-1994 at 09:05

Injection Report

Acquired on 28-JUN-1994 at 16:50



Sample Name : 544/1/1/94
Sample Id : 5384/94/2
Sample Type : Control Amount=1.00000
Bottle No : 1

PEAK INFORMATION

Peak RT mins	Hght uV	Area uVs	MG/KG	Peak name	Width

Totals			
Unknowns	30	354	N/A
Quantified	0	0	0.000
Grand Total	30	354	0.000

ANALYSIS SUMMARY

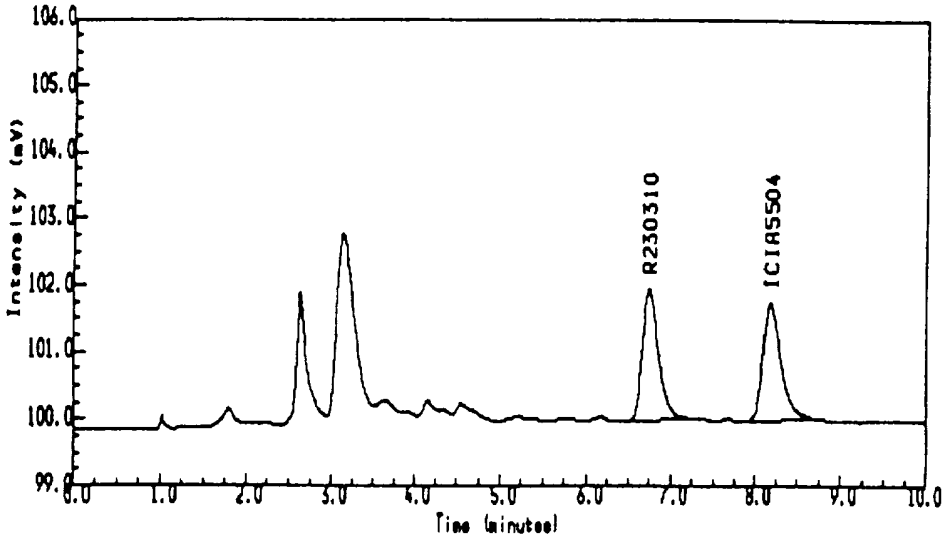
Method..... WG5504
Run sequence..... WG5384
Calibration..... WG5504
External standard calibration using area

Figure 3 : Untreated grape sample at 0.5 g cm^{-3} fortified at 0.5 $\mu g\ g^{-1}$.
Recovery = 92% azoxystrobin, 91% R230310.

[RESIDUE] 19 AS5384A,3,1
Reported on 1-JUL-1994 at 09:05

Injection Report

Acquired on 28-JUN-1994 at 17:31



Sample Name : R1 544/2/1/94
Sample Id : 5384/94/3
Sample Type : Recovery Amount=1.00000
Bottle No : 1

PEAK INFORMATION

Peak	RT mins	Hght uV	Area uVs	MG/KG	Peak name	Width
1	6.752	1962	2563	0.454	R230310	12.8
2	8.192	1748	2949	0.458	ICIA5504	14.1

Totals

Unknowns	0	0	N/A
Quantified	3710	51512	0.912
Grand Total	3710	51512	0.912

Appendix 4

Materials/Safety

1 **Apparatus**

a) Glass centrifuge bottles (250 cm^3 capacity) for sample extraction.

b) 100 cm^3 separatory funnels.

c) Teflon funnels.

d) High speed homogeniser, e g. Silverson Homogeniser, available from Silverson Machines Limited, Chesham, Buckinghamshire, UK.

e) Filtration apparatus: Büchner funnel, adapter, filter paper (Whatman No.1 & No. 5, 9 cm)

f) Round bottom flasks (250, 100 cm^3 capacity).

g) Rotary evaporator e.g Büchi

h) Supelco^{lm} vacuum manifold system

i) Glass reactivials (7 cm^3 capacity).

j) Vials for GC and HPLC analysis.

k) A gas chromatograph fitted with a nitrogen phosphorus detector e.g. Varian 3400 series, autosampler and integrator or data handling system.

l) HPLC system with a UV detector, autosampler and integrator or data handling system.

m) pH meter e.g Whatman PHA 400 pH/mV meter.

2 **Reagents**

a) Solvents: acetone, acetonitrile, ethyl acetate, hexane and dichloromethane (distilled in glass).

b) Solid phase extraction sorbents (Si) available from Analytichem International.

c) Florisil (100-200 US mesh) for chromatographic use available from Fisons Scientific Equipment, Loughborough, UK. The Florisil is prepared by drying in an oven at 110°C for 24 hours, cooling and weighing into 100 g batches. 1 cm^3 of ultra pure water is added, a glass rod is placed in with the Florisil and this is tumbled for one hour. This is repeated until 5 cm^3 has been added to give 5% water deactivated Florisil.

d) Granular anhydrous sodium sulphate (Analar grade). BDH Chemicals Ltd., Poole, England, UK. The sodium sulphate is heated to 110°C for 12 hours and allowed to cool before use

e) Sodium hydroxide (Analar reagent). FSA Laboratory Supplies, Bishop Meadow Road, Loughborough, LE11 ORG, England, UK.

f) GC capillary column, Rt_x 200 (trifluoropropylmethyl polysiloxane) capillary 15 m x 0 32 mm internal diameter (0.5 µm film thickness), available from Thames Chromatography, Maidenhead, Berkshire, England, UK.

g) HPLC column, Spherisorb 5 ODS2 25 cm x 4.6 mm internal diameter Hichrom Ltd., 1 The Markham Centre, Station Road, Theale, Reading, Berkshire, RG7 4PE, England, UK.

h) Glass wool - contaminants are removed by soaking the glass wool in hexane (redistilled) overnight. Leave uncovered in a fumehood until all the hexane has evaporated and dry in an oven at 110° C overnight.

i) pH4 and pH7 buffer available from ABB Kent-Taylor Limited, Oldends Lane, Stonehouse, Gloucestershire, England GL10 3TA.

j) A sample of azoxystrobin, R230310 and R216206 (Figure 3) of known purity

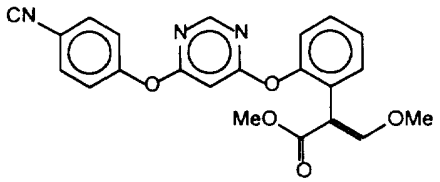


Figure 3 : Methyl (E)-2-{2-[6-(4-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).

3 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. ZENECA Laboratory Safety Manual) which contains recommendations and procedures for handling chemicals or a monograph such as 'Hazards in the Chemical Laboratory', Edited by G D Muir, The Chemical Society, London.

a) Solvent Hazards

	Acetone	Ethyl Acetate	Acetonitrile	Hexane	Dichloromethane
Harmful vapour	Yes	Yes	Yes	Yes	Yes
Highly flammable	Yes	Yes	Yes	Yes	No
Harmful by skin absorption	No	Yes	Yes	No	No
TLV mg/m^3	2400	1400	70	180	350

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

Sodium hydroxide is highly corrosive. Avoid contact with skin and eyes.

b) Azoxystrobin has a divisional toxicity class of 4. Azoxystrobin has a mammalian toxicity (acute oral LD_{50}) in rat greater than 5000 mg kg^{-1}.

4 **Preparation of Analytical Standards**

Weigh out accurately using a five figure balance, sufficient of azoxystrobin, R230310 and R216206 solid to allow dilution in acetone to give 1000 μg cm^{-3} stock solutions in volumetric flasks Make serial dilutions from the stock to give 100 μg cm^{-3} standard solution. Prepare 10 μg cm^{-3}, 1 0 μg cm^{-3} and 0 1 μg cm^{-3} mix standard solutions of azoxystrobin, R230310 and R216206 in acetone to be used for fortification of recovery samples. Prepare 10 μg cm^{-3}, 1.0 μg cm^{-3} and 0.1 μg cm^{-3} standard solutions of R216206 in acetone to be used for fortification of treated samples.

When not in use, always store the standard solutions, securely stoppered, in a refrigerator at ≤7°C to prevent decomposition and/or concentration of the solvent strength. Analytical standards should be freshly prepared from the solid material after six months of use.