1997.019

Attachment 1

445951-04

ZENECA Ag Products
ZENECA Inc.
Wilmington, DE 19850-5458

VOLUME 4

Study Title

RESIDUE ANALYTICAL METHOD FOR THE ANALYSIS OF AZOXYSTROBIN IN ANIMAL TISSUE , EGG AND MILK

Data Requirement

Guideline Ref.: 860.1330

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Study Completion Date

May 15, 1998

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Laboratory Project ID

RAM 255

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I, the undersigned, declare that the objectives laid down in the protocol were achieved and that the data generated are valid. The report fully and accurately reflects the procedures used and the raw data generated in the above study.

The study was conducted in compliance with the United Kingdom Good Laboratory Practice Regulations 1997. These regulations are in accordance with the Organisation for Economic Co-operation and Development Principles of Good Laboratory Practice OCDE/GD(92)32.

These international standards are acceptable to the United States Environmental Protection Agency and this study, therefore, satisfies the requirements of 40 CFR Part 160.

S. R. Burke
Study Director

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Zeneca Ag Products

RAM 255 : EPA ENFORCEMENT METHOD

RESIDUE ANALYTICAL METHOD FOR THE ANALYSIS OF AZOXYSTROBIN IN ANIMAL TISSUE, EGG AND MILK.

Authors : S R Burke and A Sapiets Issuing Section : Dietary Exposure

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1 SCOPE

The analytical procedures described are suitable for the determination of residues of the fungicide azoxystrobin (Figure 1) in animal tissue, egg and milk.

To date, in these laboratories, the method has been applied to animal tissue and egg samples with a limit of determination of 0.01 $\mu g\ g^{-1}$. Milk has a limit of determination of 0.001 $\mu g\ g^{-1}$.

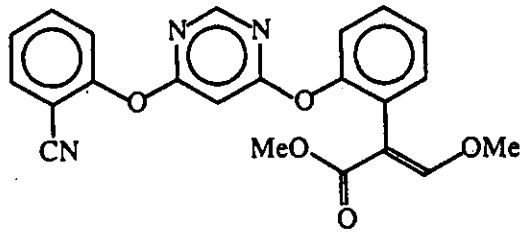


Figure 1 : Azoxystrobin : Methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (IUPAC).

Using this method, a batch of ten samples can be analysed within a 48 hour period.

The analytical procedure can be stopped at various points for overnight and weekend breaks unless specifically noted in the analytical procedure. Samples should be stored in sealed vessels at <7°C.

2 SUMMARY

Azoxystrobin residues in tissue and egg samples are extracted in acetonitrile. An aliquot of the extract is cleaned up by gel permeation chromatography (GPC) eluting through Alumina-n and Florisil solid phase extraction cartridges. The eluate is evaporated to dryness and taken up in a known volume of toluene for analysis by gas chromatography with nitrogen-phosphorus detection (GC-NPD).

Azoxystrobin residues in milk samples are extracted in acetonitrile and partitioned into dichloromethane. The extract is again cleaned up by GPC eluting through Alumina-n and Florisil solid phase extraction cartridges. The eluate is evaporated to dryness and taken up in a known volume of toluene for analysis by GC-NPD.

3 PROCEDURE

3.1 Extraction

a) Thoroughly mix the sample and weigh a representative aliquot (10 g) into a centrifuge bottle (tissue and egg) or beaker (milk).

b) Animal tissue and egg : Homogenise for approximately five minutes in acetonitrile (40 - 60 cm^3). Centrifuge the sample at 3000 rpm for 3 minutes before decanting the extraction solvent into a round bottom flask. Adjust to a suitable known volume (e.g. 50 -100 cm^3) with acetonitrile.

Milk : Transfer the milk sample to a separatory funnel and add 30 cm^3 acetonitrile for each 10 g of milk. Shake for approximately 2 minutes and decant the extraction solvent into a second separatory funnel.

c) Tissue and egg : Take an aliquot equivalent to 2 g and evaporate to dryness in a round bottom flask, having added an equivalent amount of ethyl acetate to prevent bumping. Redissolve in 75:25 ethyl acetate:toluene (4 cm^3), ultrasonicate, and transfer to a glass vial for GPC clean-up.

Milk : Partition with an equivalent volume of dichloromethane plus half equivalent volume of 5% sodium chloride solution, shaking for approximately 2 minutes. Place a funnel filled with anhydrous sodium sulphate onto a round bottom flask. Collect the organic layer into the round bottom flask. Wash the anhydrous sodium sulphate with further dichloromethane (5 cm^3) and collect in the same round bottom flask. Evaporate to dryness and redissolve in 75:25 (4 cm^3) ethyl acetate:toluene, ultrasonicate, and transfer to a glass vial for GPC clean-up.

3.2 Gel Permeation Chromatography (GPC)

The conditions for sample clean-up by GPC will depend upon the equipment available. The operating manuals for the instruments should always be consulted to ensure safe and optimum use. The following conditions have been found to be satisfactory using a Gilson Model 233XL available from Anachem Ltd.

Prior to use, each GPC column should be calibrated, as described in the instrument manual, in order to determine the dump and collect times for the compounds to be analysed. Also the solvent should be pumped through the GPC column for at least two hours prior to use.

(i) Column	:	Bio-Beads SX-3 (50 g) packed in a 60 cm x 25 mm glass column.
(ii) Solvent System	:	75:25 Ethyl Acetate:Toluene
(iii) Flow Rate	:	5 cm^3 min^{-1}.
(iv) Sample Loop Volume	:	2 cm^3

Azoxystrobin is eluted between 25 and 30 minutes (approximately 25 cm^3 collection volume).

3.3 **Solid Phase Extraction Clean-up (Alumina-n, Florisil)**

a) Clamp a glass reservoir (glass syringe barrels are a suitable alternative) and attach an Alumina-n (1 g) solid phase extraction cartridge beneath, followed by a Florisil (0.5 g) solid phase extraction cartridge.

b) Wash the cartridges with 75:25 ethyl acetate:toluene (20 cm^3).

c) Transfer the collected fraction from the scintillation vials for each sample into the reservoir and through the solid phase extraction cartridges. Collect into a round bottom flask (100 cm^3).

d) Rinse the cartridge with further 75:25 ethyl acetate:toluene (10 cm^3) pushing through any solvent remaining in the reservoir, into the round bottom flask.

e) Evaporate the eluate to dryness on a rotary evaporator at ≤40°C and redissolve in an appropriate volume of toluene to give final sample concentrations of 1 g cm^{-3} for animal tissue and egg and 5 g cm^{-3} for milk before 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 Varian 3400 series gas chromatograph fitted with a Varian 8100 series autosampler. Conditions may need to be adjusted to compensate for the age of the column.

4.1 Gas Chromatography Conditions

(i) Column : Rt_x-200 (trifluoropropylmethyl polysiloxane), fused silica wall coated open tubular 15 metres x 0.32 mm internal diameter (0.5 µm film thickness)

(ii) Oven temperature : 70°C (hold 1.5 minutes); program at 30°C $minute^{-1}$ to 220°C; program at 10°C $minute^{-1}$ to 300°C (hold 10 minutes). These conditions may need to be adjusted according to tissue type.

(iii) Injector : Septum programmable injector (SPI): 40°C (hold 0.1 minute); program at 180°C $minute^{-1}$ to 250°C (hold 25 minutes);. Injection volume = 2 µl.

(iv) Gas Flow Rates :

Helium (carrier)	:	2.5 cm^3 min^{-1}
Helium (makeup)	:	27.5 cm^3 min^{-1}
Air	:	175 cm^3 min^{-1}
Hydrogen	:	3.5 cm^3 min^{-1}

(v) Detector : Temperature at 320°C. Bead setting: 3.0 - 3.3 amps (depending on condition of bead). Attenuation: 64. Range: 12

Under these conditions the retention time of azoxystrobin is approximately 12.6 minutes.

A SPI injector should be used as better sensitivity is obtained than when using a split/splitless injector. If necessary an on column injector can be substituted.

4.2 **Calculation of Azoxystrobin Residue Results**

a) Make repeated injections of 2 µl of a standard solution containing azoxystrobin at 0.05 - 0.1 µg cm^{-3} into the GC operated under conditions described in 4.1 above. When a consistent response is obtained measure the peak heights or areas obtained for the standard.

b) Make a 2 µl injection of each sample solution and measure the peak heights or areas of the peaks corresponding to azoxystrobin.

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

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

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

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

5 **LIMIT OF DETERMINATION (QUANTITATION)**

In these laboratories the limits of determination have been set at 0.01 µg g^{-1} animal tissue and egg, and 0.001 µg g^{-1} milk. Care must be taken when working at the limit of determination to minimise the risk of contamination.

6 **LIMIT OF DETECTION**

The limit of detection is set at 4 x baseline noise.

7 **RECOVERY DATA**

In these laboratories to date the method has been applied to the analysis of animal tissue, egg and milk samples. Typical recoveries of the analytical method for azoxystrobin, fortified over a range of concentrations, are shown below.

Table 1 : Results of Milk Analyses

Fortification Level	Azoxystrobin % Recovery
0.001 µg g^{-1}	115, 91, 119, 93, 95, 94, 95, 89, 74, 76, 85, 100
0.005 µg g^{-1}	111, 95,
0.01 µg g^{-1}	97, 96
0.02 µg g^{-1}	98, 98

		Azoxystrobin
Mean recovery	=	96%
Standard deviation	=	11
Relative standard deviation	=	11%

Table 2 : Results of Egg Analyses

Fortification Level	Azoxystrobin % Recovery
0.01 µg g^{-1}	87, 93, 85, 95
0.02 µg g^{-1}	95, 100, 80, 80
0.05 µg g^{-1}	78, 80, 84, 84
0.10 µg g^{-1}	86, 82, 85, 80

		Azoxystrobin
Mean recovery	=	86%
Standard deviation	=	7
Relative standard deviation	=	8%

Table 3 : Results of Animal Tissue Analysis

Substrate	Fortification Level	Azoxystrobin % Recovery
Liver	0.01 µg g^{-1}	88, 108, 108, 89, 93, 106, 115, 122, 79, 96, 102, 96
	0.02 µg g^{-1}	78, 104
	0.05 µg g^{-1}	102, 91
	0.10 µg g^{-1}	100, 89
Muscle	0.01 µg g^{-1}	101, 106, 86, 98
	0.1 µg g^{-1}	98, 95
Fat	0.01 µg^{-1}	124, 96, 85, 97
	0.1 µg g^{-1}	97, 97

		Azoxystrobin
Liver: Mean recovery	=	98%
Standard deviation	=	12
Relative standard deviation	=	12%
Muscle: Mean recovery	=	97%
Standard deviation	=	7
Relative standard deviation	=	7%
Fat: Mean recovery	=	99%
Standard deviation	=	13
Relative standard deviation	=	13%

8 **EXAMPLES OF TYPICAL CHROMATOGRAMS** - see Appendix 1

Filename : EPA255.doc
Location : E:GROUP\WP\EPAMETHD
Reference : SRB/AP
Date : 26 February 1998

Appendix 1

Typical Gas Chromatograms for Azoxystrobin Residue Determination in Milk and Muscle

Figure 2 : 0.05 μg cm^{-3} azoxystrobin standard.

Figure 3 : Untreated milk sample at 10 g cm^{-3}.

Figure 4 : Untreated milk sample at 5 g cm^{-3} fortified at 0.01 μg g^{-1}.
Recovery = 97%

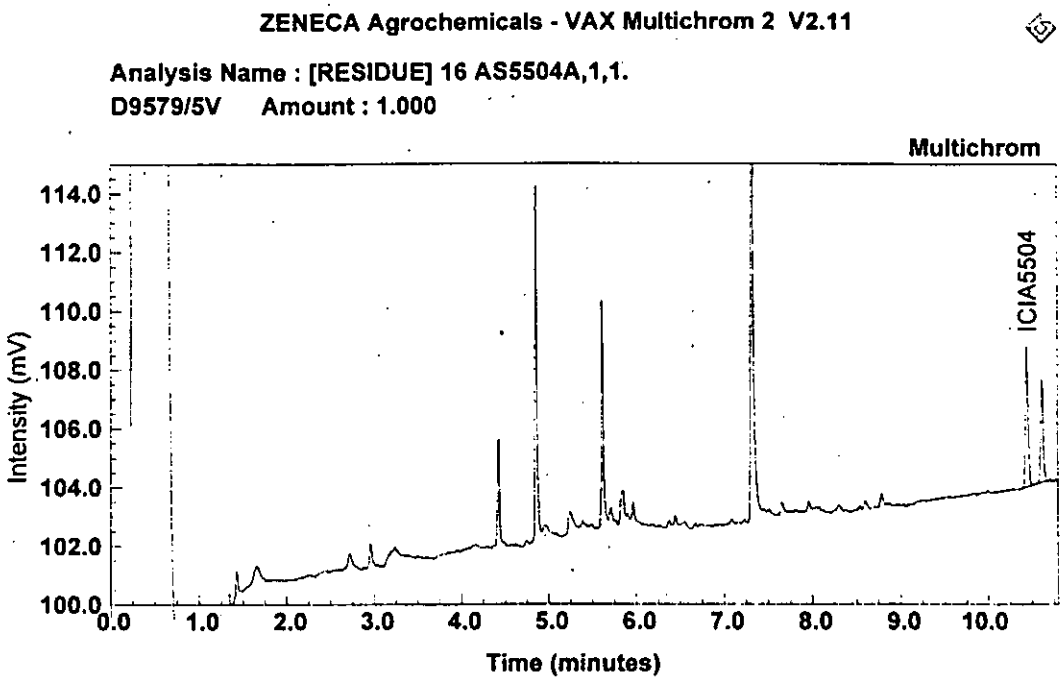
Figure 5 : Untreated milk sample at 10 g cm^{-3} fortified at 0.001 μg g^{-1}.
Recovery = 92%

Figure 6 : 0.1 μg cm^{-3} azoxystrobin.

Figure 7 : Untreated muscle sample at 1.0 g cm^{-3}.

Figure 8 : Untreated muscle sample at 1.0 g cm^{-3} fortified at 0.1 mg kg^{-1}.
Recovery = 104%

Figure 2 : **0.05 μg cm^{-3} azoxystrobin standard.**



Injection Report

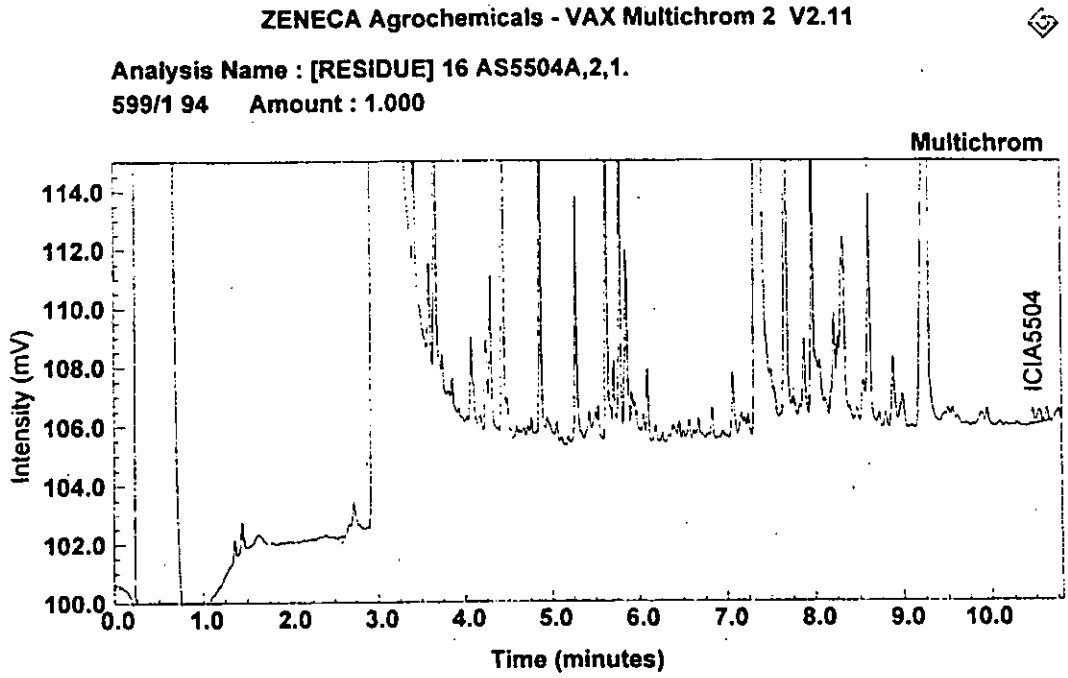
Acquired on 18-AUG-1994 at 16:50

Sample Name : D9579/5V
Sample Id : 5504/94/1
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

Peak	RT mins	Height μv	Area μvs	mg/kg	Peak name	Width	RF slope	RF intercept
1	10.453	4802	9567	0.05035	ICIA5504	2.6	1.0148	0.0000

Figure 3 : **Untreated milk sample at 10 g cm^{-3}.**



Injection Report

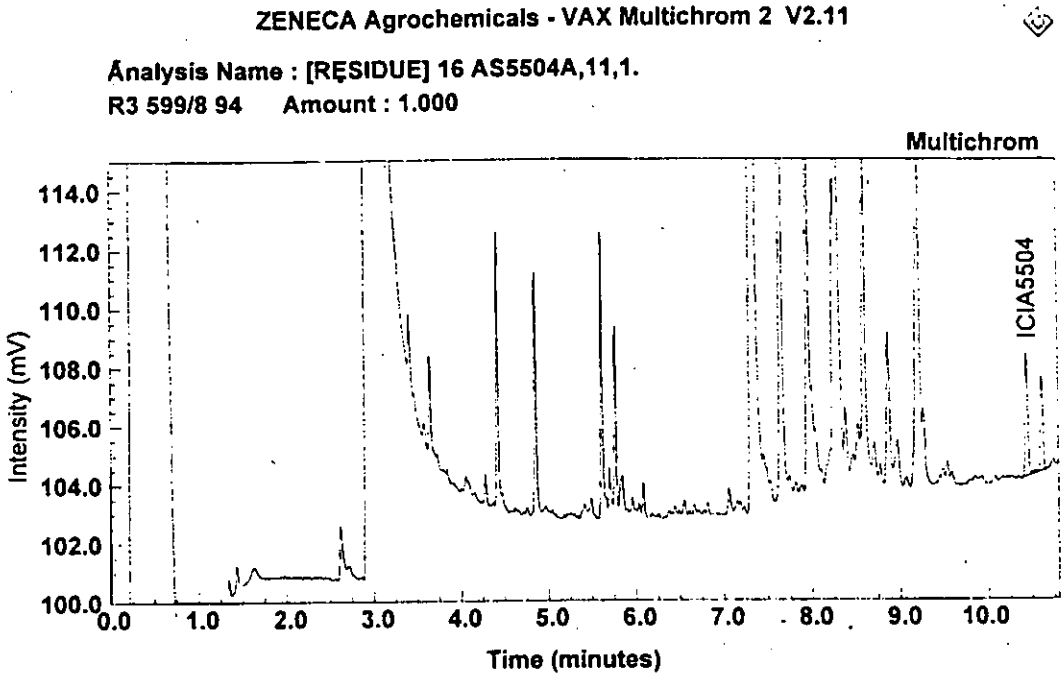
Acquired on 18-AUG-1994 at 17:12

Sample Name : 599/1 94
Sample Id : 5504/94/2
Sample Type : Control Amount=1.00000
Bottle No : 1

PEAK INFORMATION

Peak	RT mins	Height µv	Area µvs	mg/kg	Peak name	Width	RF slope	RF intercept
1	10.475	561	3703	0.00000	ICIA5504	2.6	1.0148	0.0000

Figure 4 : **Untreated milk sample at 5 g cm^{-3} fortified at 0.01 µg g^{-1}. Recovery = 97%**



Injection Report

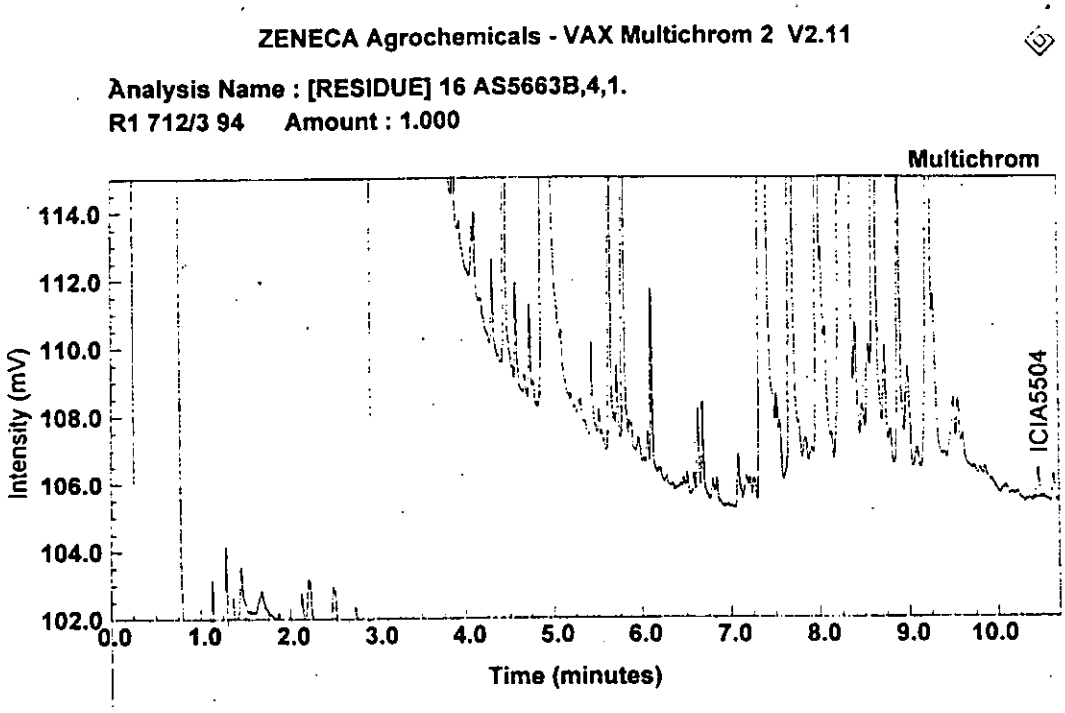
Acquired on 18-AUG-1994 at 20:32

Sample Name : R3 599/8 94
Sample Id : 5504/94/11
Sample Type : Recovery Amount=1.00000
Bottle No : 1

PEAK INFORMATION

Peak	RT mins	µv Height	µv Area	mg/kg	Peak name	Width	RF slope	RF intercept
1	10.443	4183	8351	0.00970	ICIA5504	2.6	1.0148	0.0000

Figure 5 : **Untreated milk sample at 10 g cm^{-3} fortified at 0.001 µg g^{-1}. Recovery = 92%**



Injection Report

Acquired on 26-AUG-1994 at 22:40

Sample Name : R1 712/3 94
Sample Id : 5663/94/4
Sample Type : Recovery Amount=1.00000
Bottle No : 1

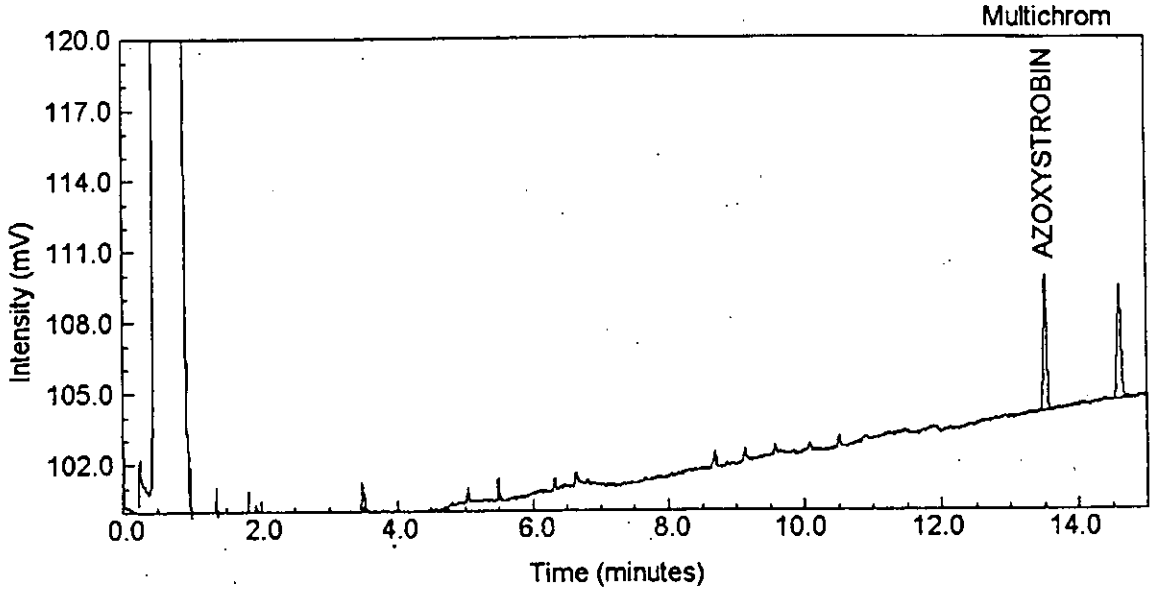
PEAK INFORMATION

Peak	RT mins	µv Height	µv Area	mg/kg	Peak name	Width	RF slope	RF intercept
1	10.464	891	1856	0.00092	ICIA5504	2.2	1.0075	0.0000

Figure 6 : **0.1 µg cm^{-3} azoxystrobin.**

ZENECA Agrochemicals - VAX Multichrom 2 V2.11

Analysis Name : [RESIDUE] 11 AS9238A,1,1.
D9579/57E Amount : 1.000



Injection Report

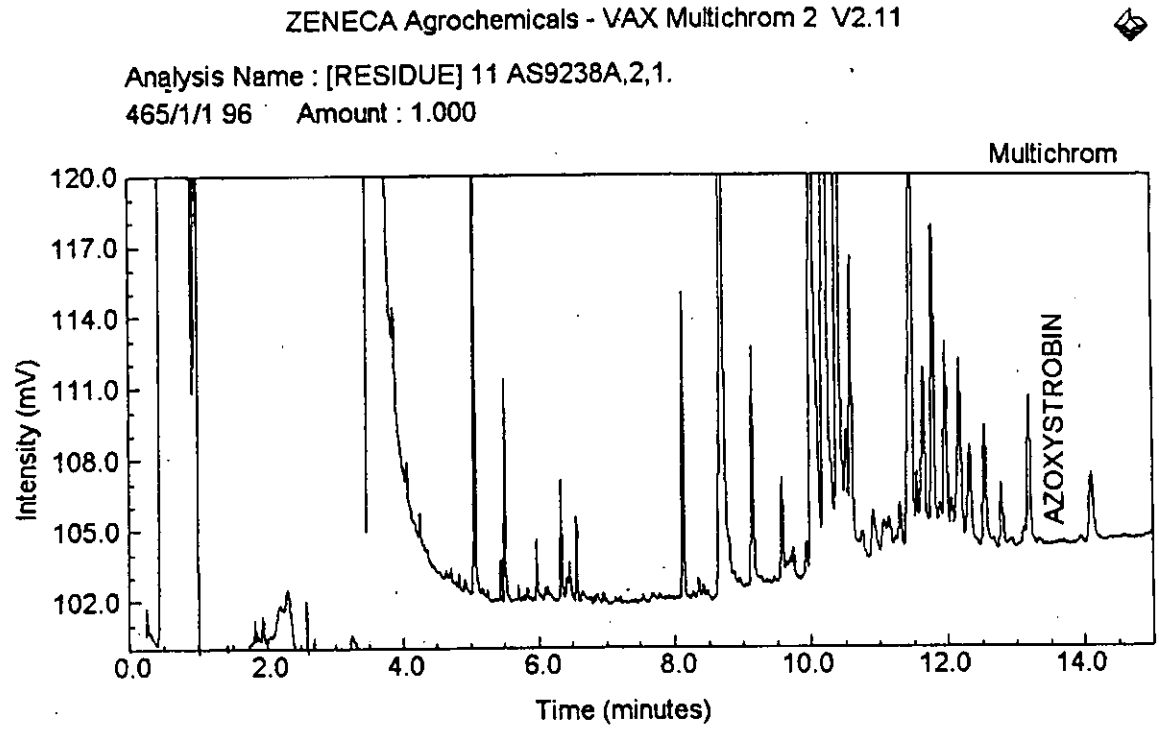
Acquired on 2-MAY-1996 at 23:42

Sample Name : D9579/57E
Sample Id : 9238/96/1
Sample Type : Standard Amount=1.00000
Bottle No : 1

PEAK INFORMATION

Peak	RT mins	Height	mg/kg	Peak name	Width	RF slope	RF intercept
1	13.515	5745	0.10273	AZOXYSTROBIN	3.2	1.1659	0.0000

Figure 7 : **Untreated muscle sample at 1.0 g cm^{-3}.**



Injection Report

Acquired on 3-MAY-1996 at 00:18

Sample Name : 465/1/1 96
Sample Id : 9238/96/2
Sample Type : Control Amount=1.00000
Bottle No : 1

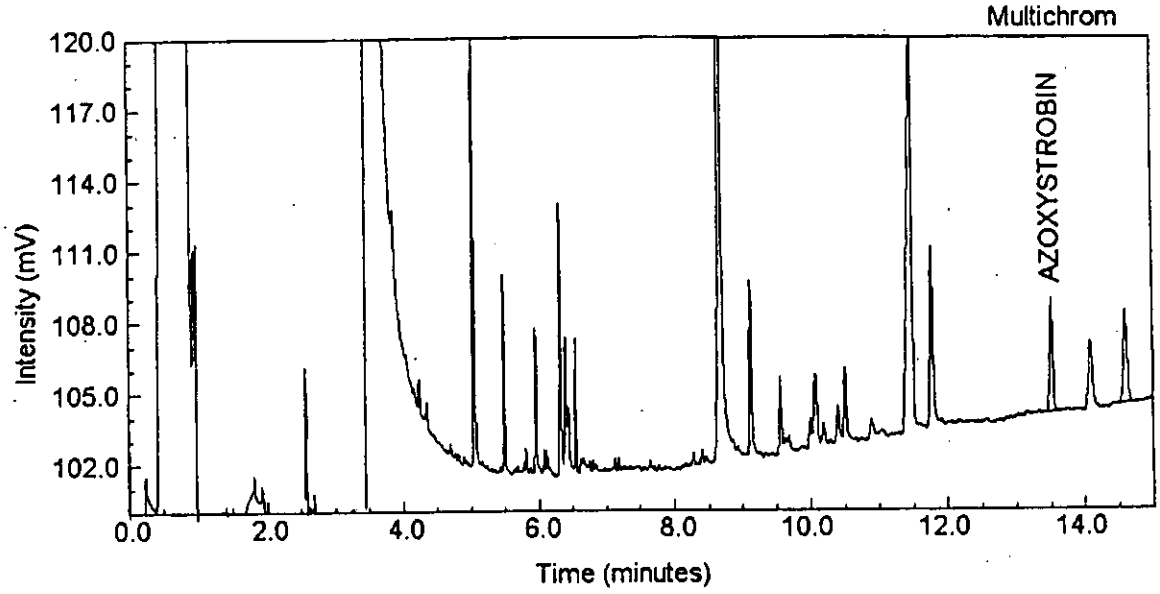
PEAK INFORMATION

Peak	RT mins	Height	mg/kg	Peak name	Width	RF slope	RF intercept
1	13.573	61	0.00000	AZOXYSTROBIN	3.8	1.1659	0.0000

Figure 8 : **Untreated muscle sample at 1.0 g cm^{-3} fortified at 0.1 mg kg^{-1}. Recovery = 104%**

ZENECA Agrochemicals - VAX Multichrom 2 V2.11

Analysis Name : [RESIDUE] 11 AS9238A,3,1.
R1 465/2/1 96 Amount : 1.000



Injection Report

Acquired on 3-MAY-1996 at 00:54

Sample Name : R1 465/2/1 96
Sample Id : 9238/96/3
Sample Type : Recovery Amount=1.00000
Bottle No : 1

PEAK INFORMATION

Peak	RT mins	Height	mg/kg	Peak name	Width	RF slope	RF intercept
1	13.520	4746	0.10362	AZOXYSTROBIN	3.2	1.1659	0.0000

Appendix 2

Materials/Safety

1 **Apparatus**

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

b) High speed homogeniser, e.g. Janke and Kunkel ultraturrax T25, available from Fisher Scientific, International Headquarters, 50 Fraden Road, Springfield, New Jersey 07081-3193, USA.

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

d) Separating funnels (250, 100 cm^3 capacity).

e) Small glass funnels for filtration through sodium sulphate.

f) Rotary evaporator e.g. Buchi

g) A gel permeation chromatography instrument e.g. Gilson 233XL, available from Gilson, 3000 West Bettline Highway, Box 620027, Middleton, Wisconsin 63562-0027, USA.

h) HP vials for GC analysis.

i) A gas chromatograph fitted with a nitrogen phosphorus detector and a septum programmable injector or on column injector system e.g. Varian 3400 series, autosampler and integrator or data handling system, available from Varian Chromatography Systems, 2700 Mitchell Drive, Walnut Creek, California 94598, USA.

j) A 60 cm x 2.5 cm glass column for use with the gel permeation chromatography instrument, available from Gilson, 3000 West Bettline Highway, Box 630027, Middleton, Wisconsin 63562-0027, USA.

k) GC capillary column, Rt_x-200 (trifluoropropylmethyl polysiloxane) fused silica wall coated open tubular 15 metres x 0.32 mm internal diameter (0.5 mm film thickness), available from Restek Corporation, 110 Benner Circle, Bellefonte, Pennsylvania 16823, USA. Restek #15036

2 **Reagents**

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

b) Solid phase extraction sorbents (Alumina-n and Florisil) available from Jones Chromatography USA Inc., PO Box 280329, Lakewood, Colorado 8022-0329, USA.

c) Bio-Beads SX-3, 200 - 400 mesh, available from Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, California 94547, USA.

d) Granular anhydrous sodium sulphate (Analar grade) available from J T Baker Inc., 222 Red School Lane, Phillipsburg, NJ 08865, USA.

e) Sodium chloride (Analar grade). Fisher Scientific, International Headquarters, 50 Fraden road, Springfield, New Jersey 07081-3193, USA.

f) 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.

g) A sample of azoxystrobin of known purity, available from ZENECA Agrochemicals, UK.

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 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

	Ethyl acetate	Acetonitrile	Hexane	Toluene	Dichloromethane
Harmful vapour	Yes	Yes	Yes	Yes	Yes
Highly flammable	Yes	Yes	Yes	Yes	No
Harmful by skin absorption	Yes	Yes	No	Yes	No
TLV mg/m^3	1400	70	1800	1780	350

In all cases avoid breathing vapour. 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 solid to allow dilution in toluene to give a 1000 μg cm^{-3} stock solution in a volumetric flask. 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} standard solutions of azoxystrobin in toluene to be used for GC analysis.

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.