ROTENONE

PHYSICO-CHEMICAL PROPERTIES

Sponsor

VESO, Veterinärmedicinsk Oppdragscenter AS
Pb. 8109 Dep.
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NORWAY

Research Laboratory

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Final: 30 May 2007

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Rotenone

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The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994).

EC Commission Directive 2004/10/EC of 11 February 2004.

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

The experimental work undertaken at London Metropolitan University (measurement of NMR spectrum) was performed at the direction of the Huntingdon Life Sciences Study Director. The laboratories are not part of the UK GLP compliance programme, however, they have been inspected by the Huntingdon Life Sciences Quality Assurance Department. No claim of compliance is made for the work conducted at London Metropolitan University.

These principles of Good Laboratory Practice are accepted by the regulatory authorities of the United States of America and Japan on the basis of intergovernmental agreements.

The stability (expiry) of the sample was the responsibility of the Sponsor.

.....A. L. Comb.....

A. L. Comb, B.Sc., Ph.D.
Study Director
Huntingdon Life Sciences Ltd.

.....30 May 2007.....

Date

QUALITY ASSURANCE STATEMENT

Rotenone

Physico-Chemical Properties

The following inspections and audits have been carried out in relation to this study:

Study Phase	**Date(s) of Inspection**	**Date of Reporting to Study Director and Management**
Protocol Audit	27 June 2006	27 June 2006
Report Audit	3 - 17 April 2007	17 April 2007

Process based inspections: At or about the time this study was in progress inspections of procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated below:

Process Based Inspections	**Date(s) of Inspection**	**Date of Reporting to Management**
Spectrophotometry	30 November - 4 December 2006	4 December 2006
Physical characteristics	5 December 2006	5 December 2006
Solubility	13 - 15 December 2006	15 December 2006
Vapour pressure	3 - 4 January 2007	4 January 2007
Chromatography	9 January 2007	9 January 2007
Phase transition	1 February 2007	2 February 2007

In addition, an inspection of the facility where this study was conducted was carried out on an annual basis. These inspections were promptly reported to Company Management.

.....

T. Whatling, M.I.A.T., M.R.Q.A.
Principal Auditor
Department of Quality Assurance
Huntingdon Life Sciences Ltd.

30.5.2007
.....

Date

RESPONSIBLE PERSONNEL

Rotenone

Physico-Chemical Properties

The following staff were responsible for the conduct of the work and reporting of the results.

A. L. Comb, B.Sc., Ph.D.
(Study Director, Product Chemistry)

H. Harper, M.Sc.
(Scientist, Pesticide Residues)

C. Pointer
(Scientist, Product Chemistry)

C. Steil, B.Sc.
(Scientist, Product Chemistry)

SUMMARY

A study was performed to determine a series of physico-chemical properties of Rotenone.

The package of tests undertaken is designed to comply with the requirements of the European Biocides Directive.

Summary of properties

The ultraviolet/visible absorption spectra, infrared absorption spectrum, nuclear magnetic resonance spectrum and mass spectrum were consistent with the assigned structure.

EEC method no.	OECD method no.	Test	Result
A1	102	Melting temperature	157 to 175.5°C, with decomposition
A2	103	Boiling temperature	Decomposed without boiling above 190°C. Decomposition was considered to have started during melting.
A3	109	Relative density (D_4^{20})	1.34
A4	104	Vapour pressure	6×10^{-6} Pascals at 25°C
A5	115	Surface tension	Not applicable, water solubility was less than 1 mg/l
A6	105	Water solubility	0.289 mg/l
		Organic solvent solubility	
		methanol	2.76 g/l
		acetone	70.6 g/l
		xylene	29.6 g/l
		1,2-dichloroethane	> 250 g/l
		ethyl acetate	53.2 g/l
		n-heptane	0.0771 g/l
		n-octanol	1.12 g/l

INTRODUCTION

A study was performed to determine a series of physico-chemical properties of Rotenone.

The package of tests undertaken is designed to comply with the requirements of the European Biocides Directive.

The protocol was approved by the Study Director and Huntingdon Life Sciences Management on 22 June 2006 and by the Sponsor on 28 June 2006.

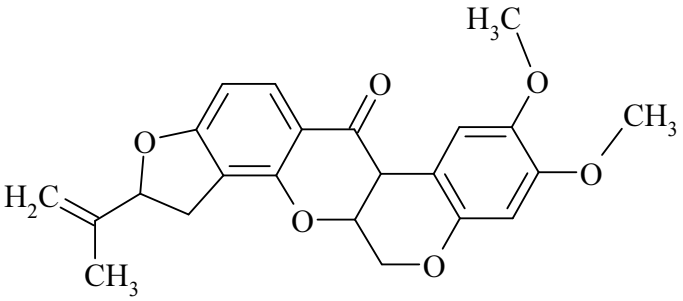
The experimental start and completion dates were 29 September 2006 and 14 February 2007 respectively.

Location of study	:	Eye Research Centre Eye Suffolk IP23 7PX
The nuclear magnetic resonance spectrum will be subcontracted to	:	London Metropolitan University Institute for Health Research & Policy 166–220 Holloway Road London N7 8DE

The NMR spectrometry was performed under the supervision of Dr A. Bligh (Responsible Analyst).

Primary data from the tests performed at Huntingdon Life Sciences and London Metropolitan University, and a copy of the final report, are stored in the archives of Huntingdon Life Sciences.

TEST SUBSTANCE

Identity:	Rotenone
Chemical name:	(2R,6aS,12aS)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno-[3,4-b]furo[2,3-h]chromen-6-one
Structure:	
Action/Intended use:	Botanical insecticide
Appearance:	Off-white fine powder
Storage conditions:	Ambient
Batch number:	046K1189
Purity:	98%
Date received:	8 August 2006

ULTRAVIOLET/VISIBLE ABSORPTION SPECTRUM (OECD Method 101)

APPARATUS

Unicam 8755 UV-Visible Spectrophotometer

Calibration: Wavelength scale calibrated using holmium glass filter.

Absorbance scale calibrated against acidified potassium dichromate solution.

PROCEDURE

The ultraviolet/visible (UV/vis) absorption spectra for Rotenone in purified water/methanol, 0.1M aqueous hydrochloric acid/methanol and 0.1M aqueous sodium hydroxide/methanol mixtures were measured under the following conditions.

Concentration:	11.9 mg/l
Cell type:	Quartz
Cell path length:	1 cm
Scan range:	200 - 800 nm
Scan speed:	50 nm/min
Slit width:	1 nm

The corresponding neutral, acidic and basic solvent systems were used as references as appropriate.

RESULTS

The spectra from 200 to 800 nm are shown in Figures 1 to 3.

The following absorption wavelength maxima (λ_{max}) and molar absorption coefficients (ε) for Rotenone were obtained:

Solvent	λ_{max} **(nm)**	**Absorbance**	ε **(dm^3/mol/cm)**
Methanol:purified water	206	1.218	40500
(3:7 v/v)	217	0.816	27100
	236	0.408	13600
	297	0.514	17100
	313	0.398	13200
Methanol:0.14M HCl	205	1.249	41600
(3:7 v/v)	217	0.832	27700
	236	0.416	13800
	297	0.523	17400
	314	0.396	13200
Methanol:0.14M NaOH	222	0.889	29600
(3:7 v/v)	232	0.674	22400
	297	0.436	14500
	352	0.121	4030

The pH values of the test solutions were 5.8, 1.3 and 12.8 respectively.

The peaks below 220 nm for the basic spectrum were discounted since these were due to the solvent cut-off point of the sodium hydroxide solution.

CONCLUSION

The ultraviolet/visible spectra were consistent with the assigned structure of Rotenone.

FIGURE 1

Ultraviolet/visible absorption spectrum of Rotenone in methanol:water (3:7 v/v)

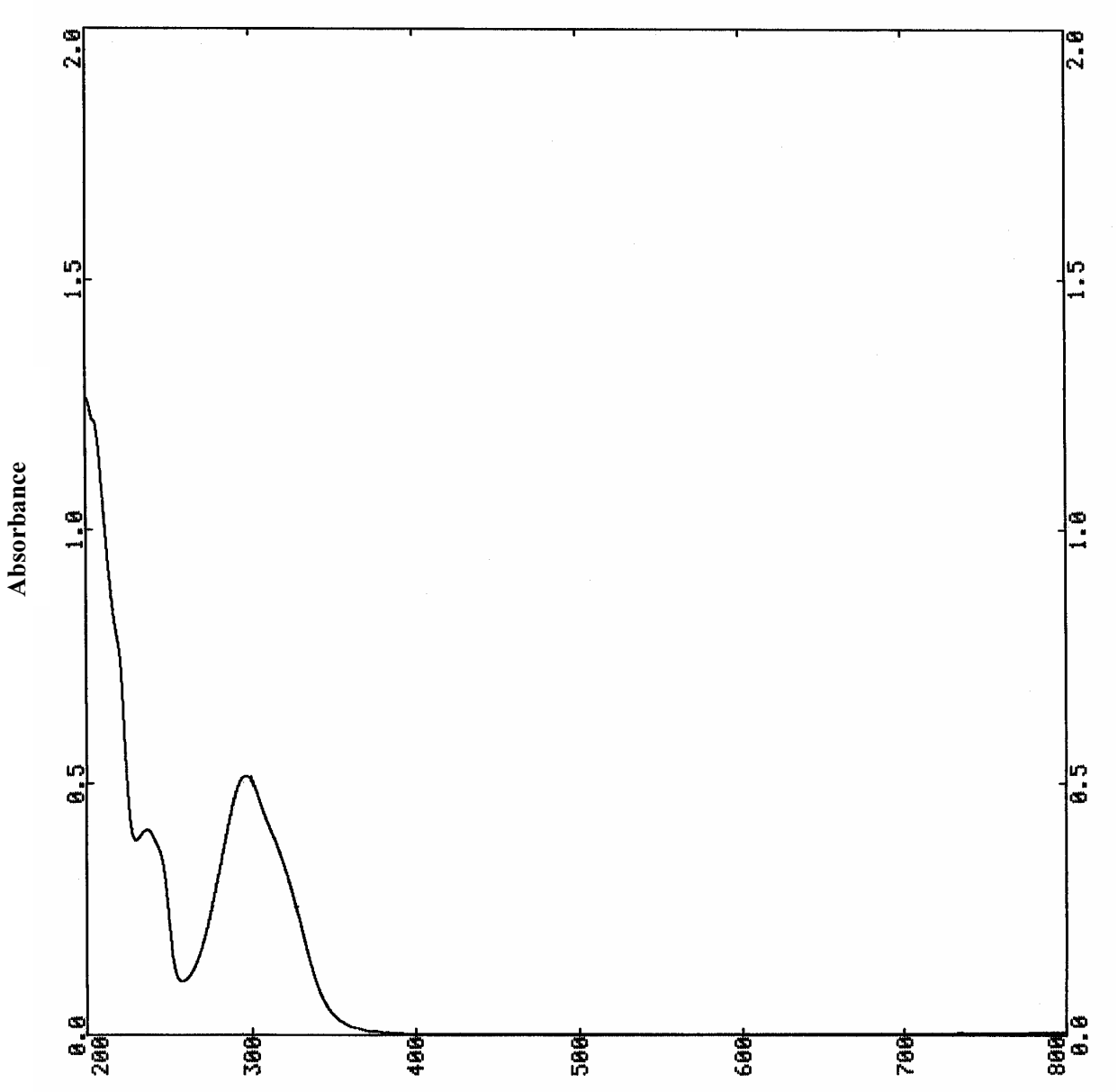


FIGURE 2

Ultraviolet/visible absorption spectrum of Rotenone in methanol:0.14M hydrochloric acid (3:7 v/v)

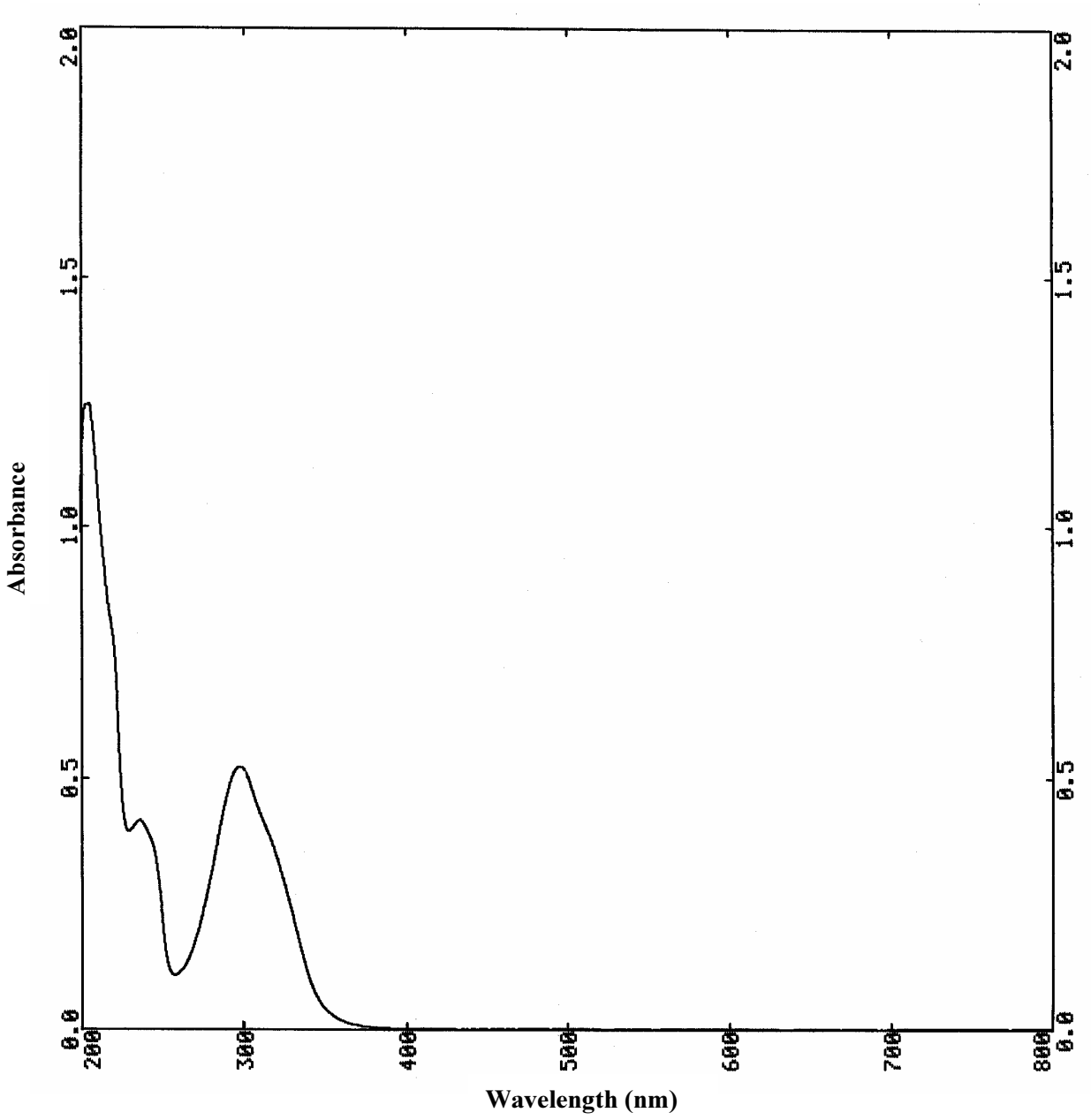
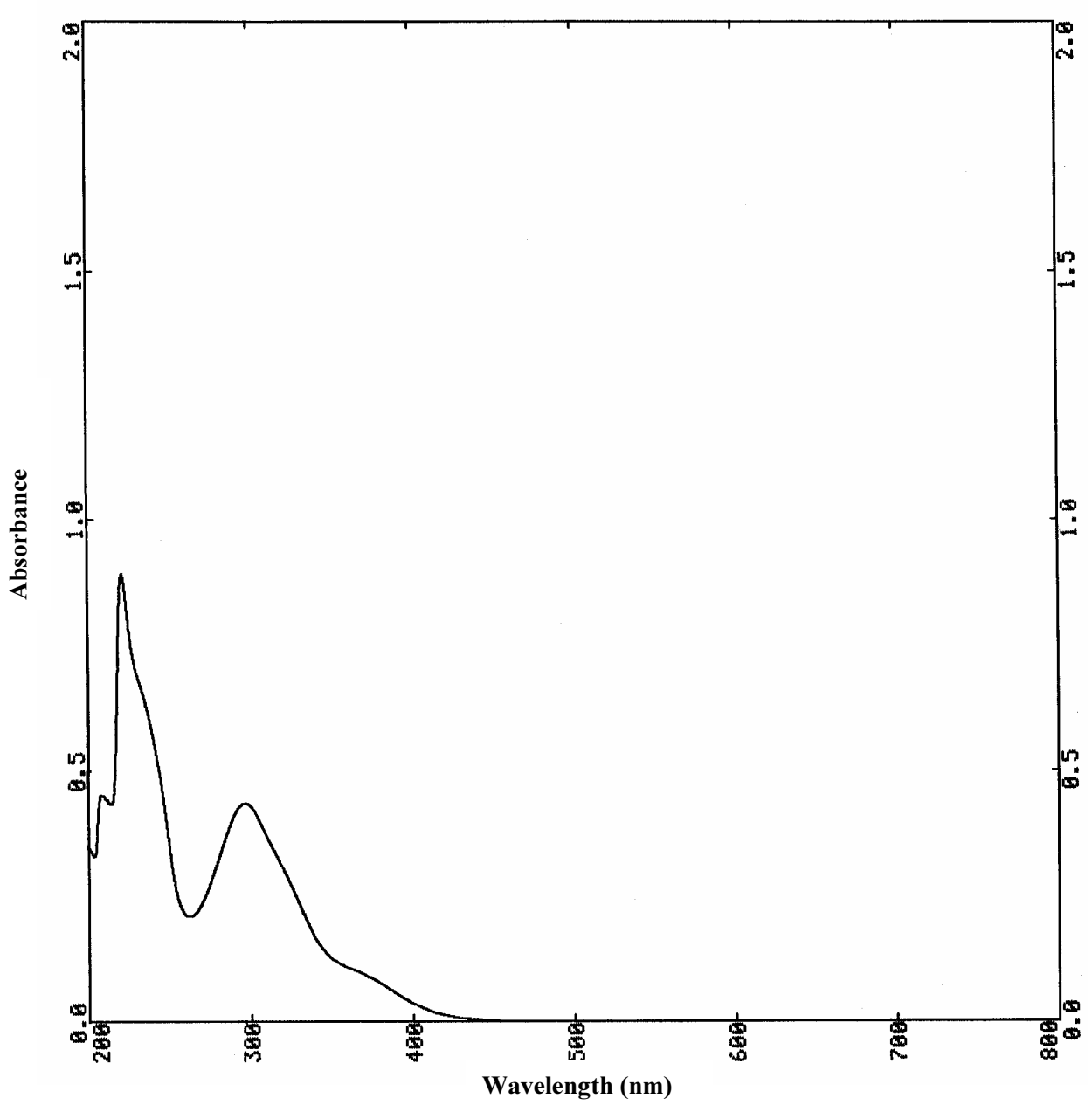


FIGURE 3

Ultraviolet/visible absorption spectrum of Rotenone in methanol:0.14M sodium hydroxide (3:7 v/v)



INFRARED SPECTRUM

APPARATUS

Mattson Galaxy 3020 Fourier Transform - Infrared (FT-IR) Spectrometer.

Calibration: Wavelength scale calibrated against a polystyrene film.

PROCEDURE

The infrared (IR) absorption spectrum of Rotenone as a potassium bromide disc over the scan range 500 to 4000 cm^{-1} was recorded (Figure 4). The instrumental parameters were as follows:

Resolution:	4.0 cm^{-1}
Number of scans:	64
Gain:	1

RESULTS

The spectrum showed the presence of characteristic absorption bands as follows:

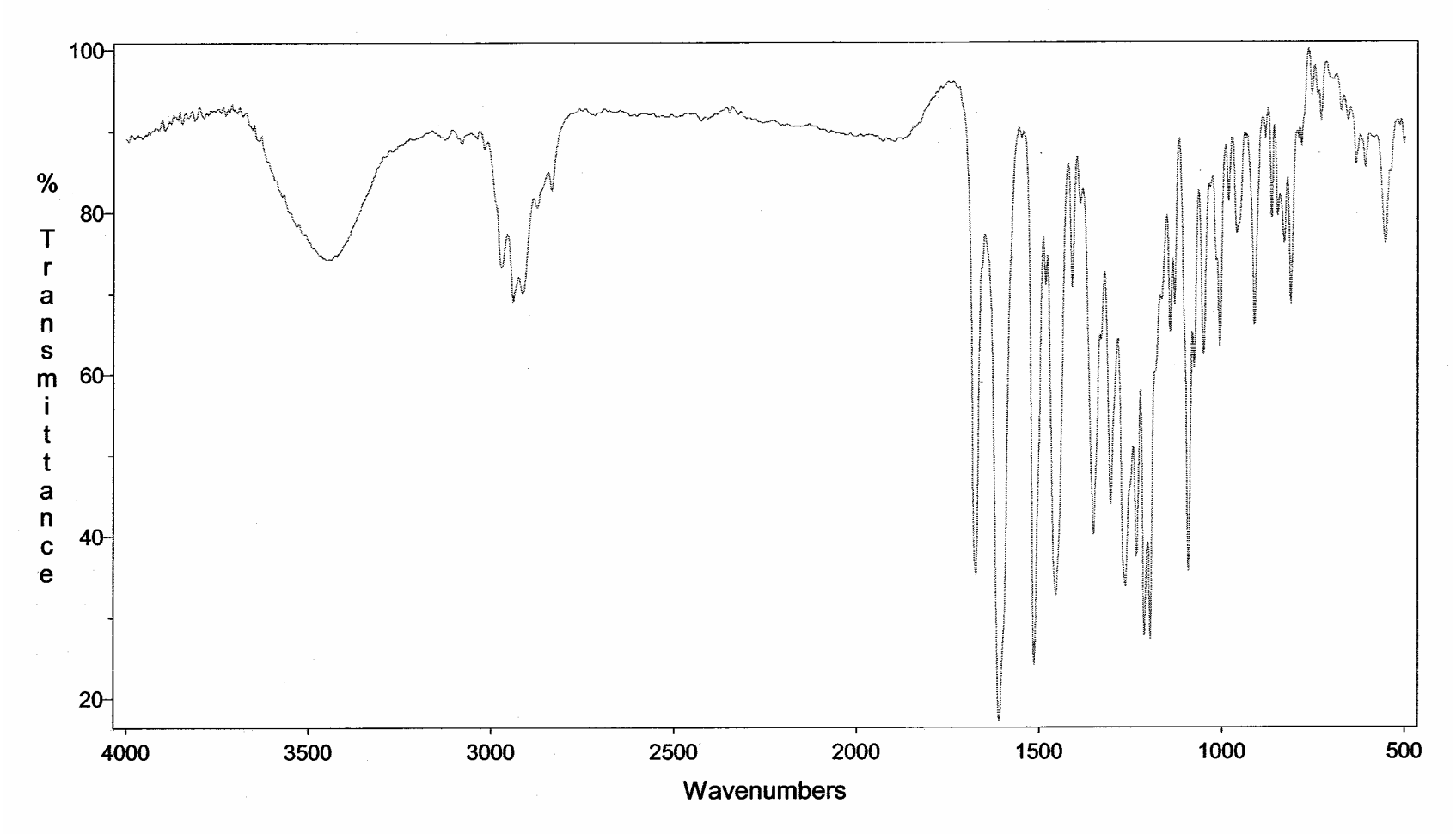
Frequency (cm^{-1})	Observation	Assignment
3300 - 3600	Broad, medium band	Water
3000 - 3100	Very weak bands	C-H (aromatic) stretch $=CH_2$ stretch
2840 - 3000	Medium bands	C-H (alkyl) stretches
1673	Strong, sharp band	C=O stretch
1610	Strong, sharp bands	C=C stretch
1000 - 1600	Strong bands	Region includes: C-C (aromatic) stretch CH_2, CH_3 (alkyl) deformations $=CH_2$ in plane deformations C-O-C stretches C-O (aromatic) stretch C-H (aromatic) in plane deformation
<1000	Medium/weak bands	Region includes: C-H (aromatic) out of plane deformations $=CH_2$ out of plane deformations Skeletal vibrations

CONCLUSION

The infrared spectrum was consistent with the assigned structure of Rotenone.

FIGURE 4

Infrared spectrum of Rotenone



NUCLEAR MAGNETIC RESONANCE SPECTRUM

APPARATUS

Bruker Avance 500 MHz Nuclear Magnetic Resonance (NMR) Spectrometer.

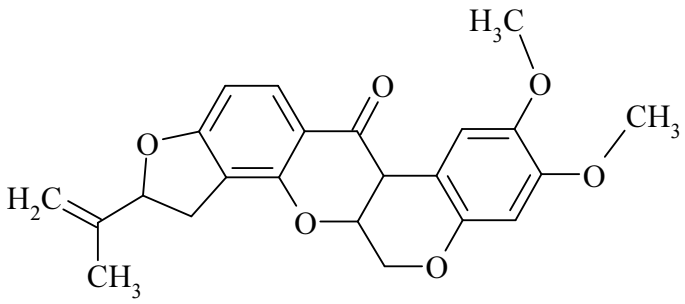
Calibration: The spectrometer internally calibrates relative to tetramethylsilane (TMS).

PROCEDURE

A solution of Rotenone in deuterated chloroform was prepared. A proton NMR spectrum (Figures 5 to 7) was obtained using the conditions listed on the spectrum.

RESULTS

Structure:



Details of the proton spectral data are given in the following table:

Chemical shift (ppm) relative to TMS	Assignment
1.8	CH_2=C-C**H**$_3$
3.8	O-C**H**$_3$
4.93	C**H**$_2$=C-CH_3
6.45	aromatic (CHC(OCH_3)C(OCH_3)C**H**C(O))
6.5	aromatic (CC**H**CHC(C=O))
6.78	aromatic (C**H**C(OCH_3)C(OCH_3)CHC(O))
7.26	solvent
7.85	aromatic (CCHC**H**C(C=O))

The remaining signals between 2.8 and 5.4 ppm are due to the alkyl protons, but have not been individually assigned due to the complexity of the spectrum.

CONCLUSION

The NMR spectrum was consistent with the assigned structure of Rotenone.

FIGURE 5

Nuclear magnetic resonance spectrum of Rotenone

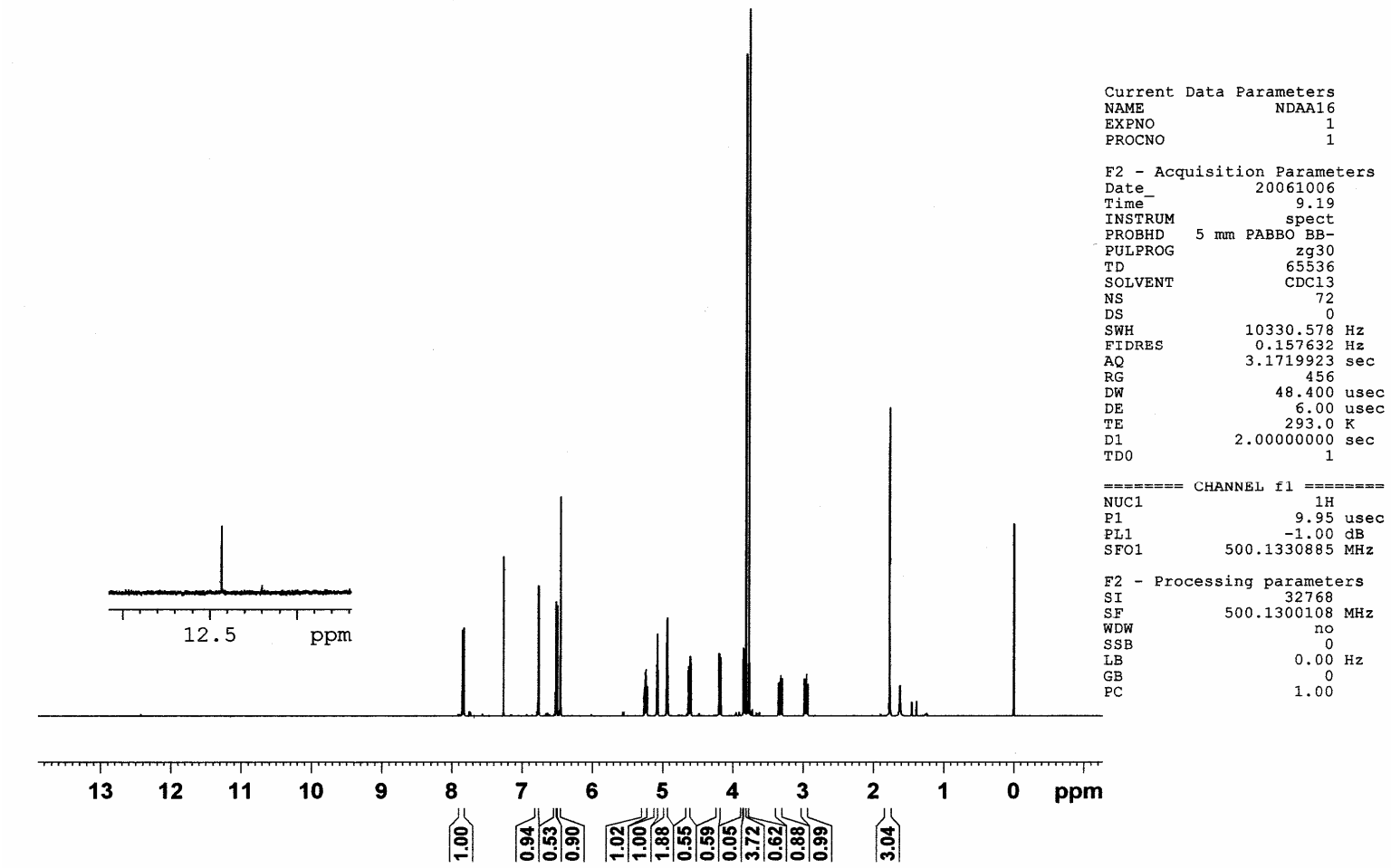


FIGURE 6

Nuclear magnetic resonance spectrum of Rotenone (expanded in region 2.8 to 5.4 ppm)

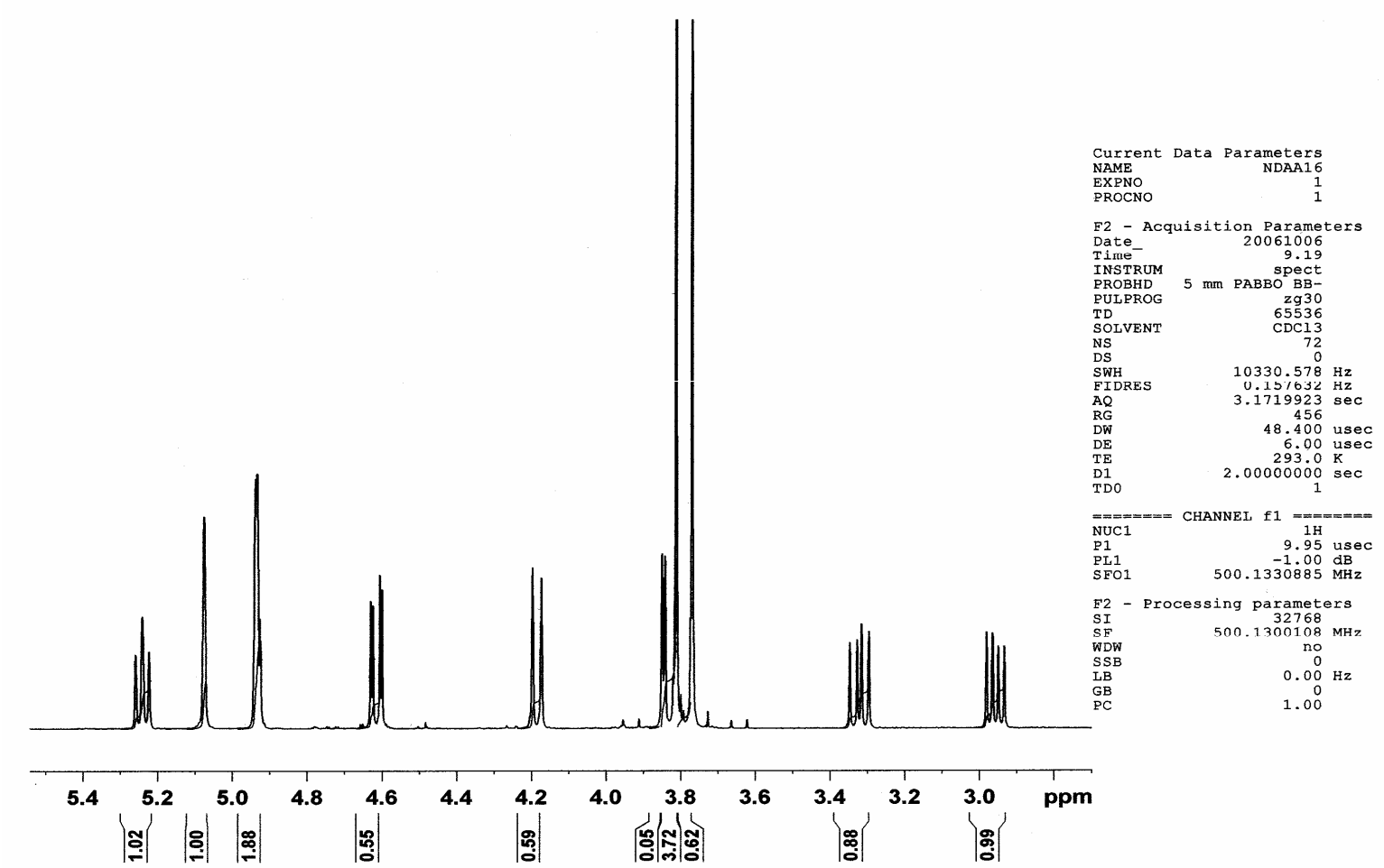
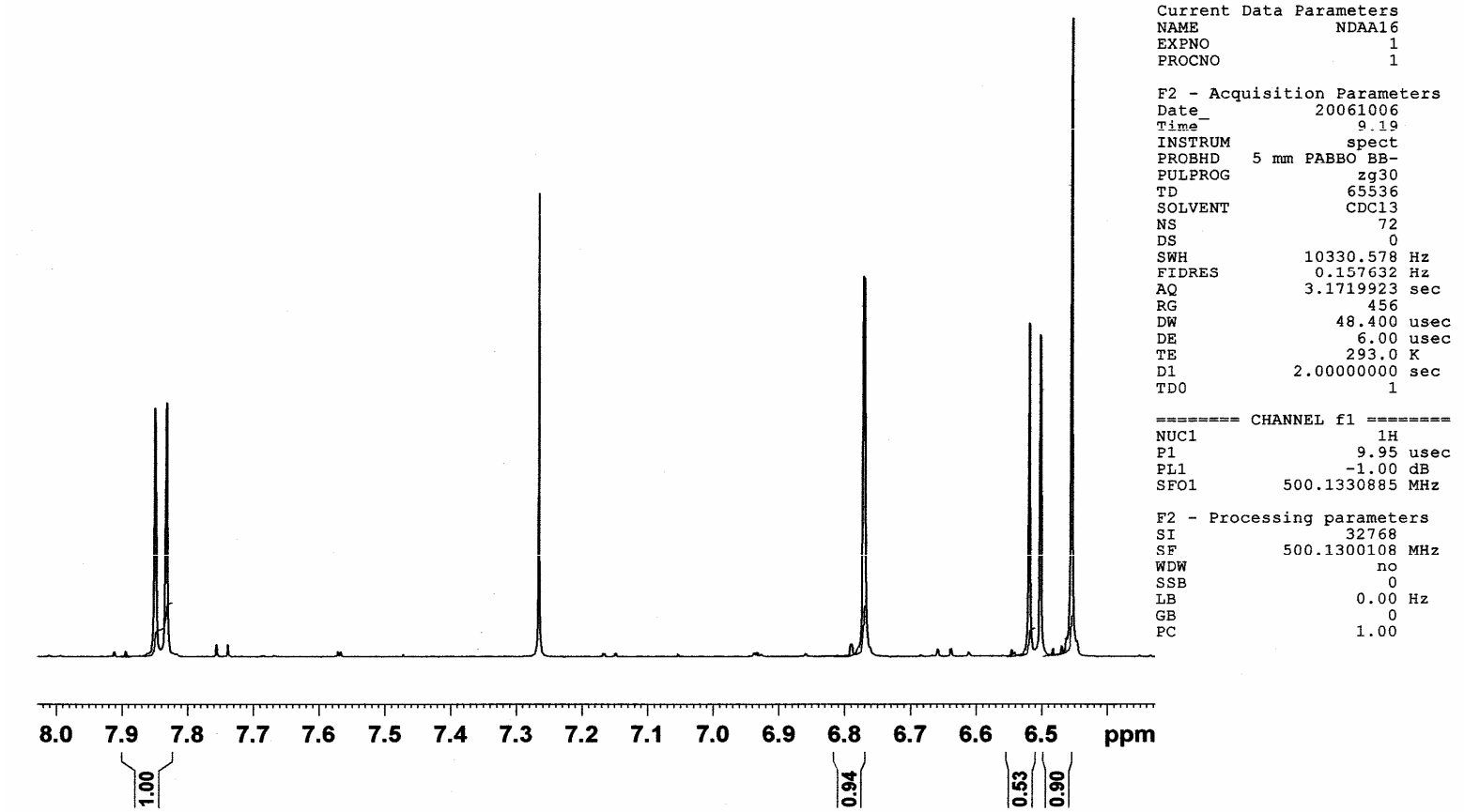


FIGURE 7

Nuclear magnetic resonance spectrum of Rotenone (expanded in region 6.4 to 8 ppm)



MASS SPECTRUM

APPARATUS

Finnigan-Masslab TRIO 1000 Mass Spectrometer.

PROCEDURE

A solution of Rotenone was prepared in acetone and was analysed by gas chromatography-mass spectrometry (GC-MS) in order to obtain the mass spectrum. Data was acquired with the instrument in electron impact (EI) ionisation mode.

The following GC conditions were used:

Instrument:	Hewlett Packard 5890 Gas Chromatograph
Column:	ZB-1 (30 m x 0.25 mm internal diameter x 0.25 μm film thickness)
Carrier gas:	Helium at 1 ml/minute
Oven temperature	
Initial:	200°C for 2 minutes
Ramp:	30°C/min to 350°C
Final:	350°C for 5 minutes
Injection technique:	Splitless
Injection temperature:	300°C
Injection volume:	1 μl

The mass spectrometer conditions were as follows:

Ionisation energy:	70 eV
Source temperature:	200°C
Scan range:	80 - 500 amu
Scan time:	0.1 sec/scan

RESULTS

The electron impact mass spectrum of Rotenone is shown in Figure 8. Due to the thermal instability of the test substance only a weak spectrum of the compound was obtained.

The following peaks were identified in the spectrum, together with tentative assignments:

Peak - m/z	Assignment
394	molecular ion
379	loss of CH_3 from molecular ion
363	loss of O-CH_3 from molecular ion
351	loss of CH_2=CH-CH_3 and proton from molecular ion

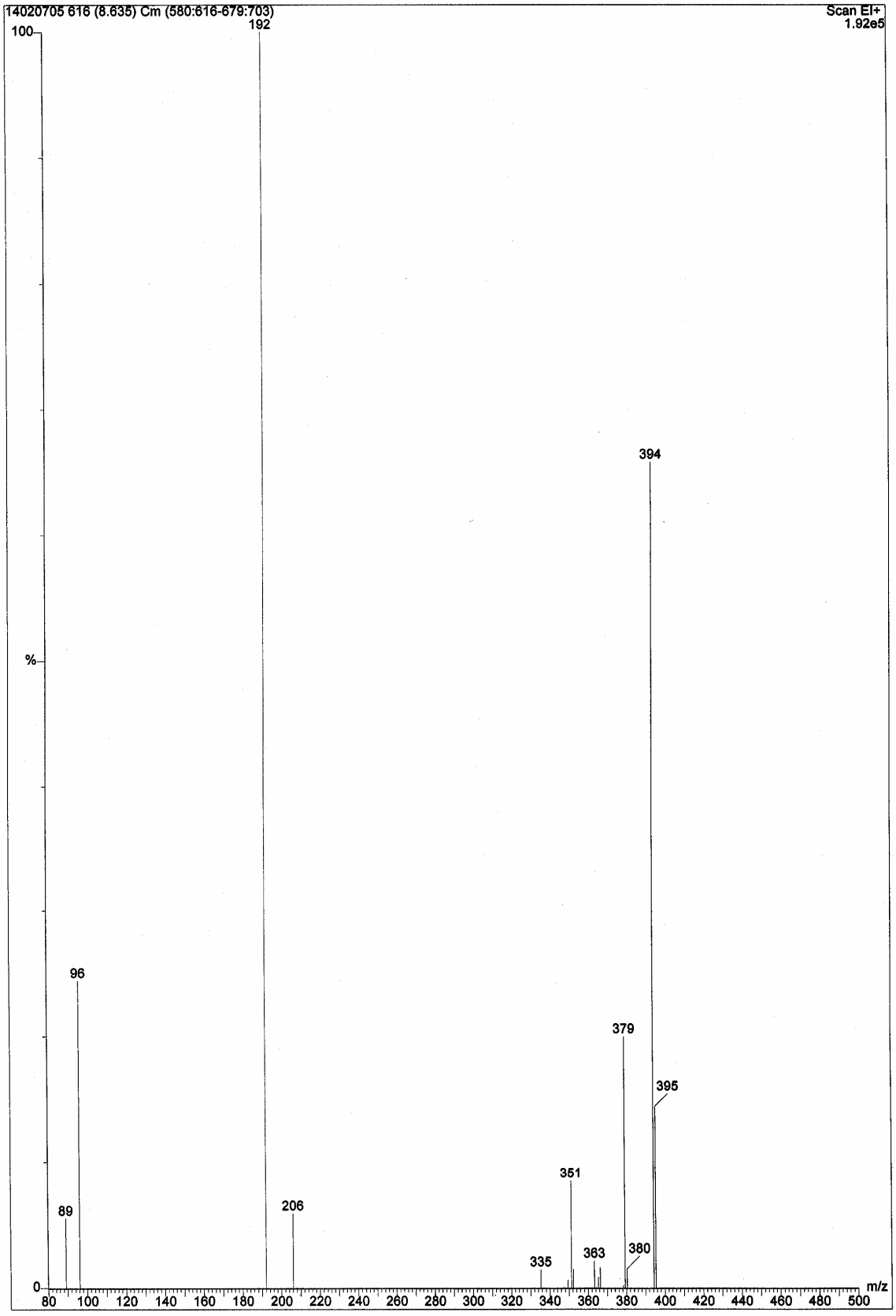
It was not possible to make assignments to the other peaks due to the potential for complex rearrangements.

CONCLUSION

The mass spectrum was consistent with the assigned structure of Rotenone.

FIGURE 8

Mass spectrum of Rotenone



MELTING TEMPERATURE
(EEC Method A1, OECD Method 102)

METHOD

The melting temperature was determined using the metal block method.

DEFINITION AND UNITS

The melting temperature of a substance is defined as the temperature (°C) at which the phase transition from solid to liquid state at normal atmospheric pressure takes place.

APPARATUS

Melting point apparatus Model B-545, Buchi.

Calibration: The apparatus is regularly calibrated by determination of a series of melting points of reference materials.

PROCEDURE

Dried test substance was pulverised to a fine powder and a small amount tightly packed into a capillary tube to a height of 3 mm. Following an initial estimation of melting point, the melting point apparatus was set at approximately 10°C below the expected melting temperature. The capillary tube was placed in the apparatus and heated at 1°C/minute until melting was complete.

The procedure was performed in duplicate using a fresh sample on each occasion.

RESULTS

The results of duplicate melting point determinations on Rotenone were as follows:

Melting stage*	Temperature (°C) Sample I	Sample II
A	157.0	157.0
B	158.0	158.0
C	170.5	171.0
D	174.5	174.5
E	175.5	175.5

*as referenced in OECD Method 102.

It was noted that following melting the samples had changed from an off-white solid to a yellow/orange liquid.

CONCLUSION

The melting range of Rotenone was found to be 157 to 175.5°C.

The colour change observed during melting indicated that the test substance may have decomposed on melting.

BOILING TEMPERATURE
(EEC Method A2, OECD Method 103)

METHOD

The boiling temperature was determined by a modified Siwoloboff method.

DEFINITION AND UNITS

The normal boiling temperature is defined as the temperature (°C) at which the vapour pressure of a liquid is the same as the Standard Pressure.

APPARATUS

Melting/boiling point apparatus: Model B-545, Buchi.

Calibration: The apparatus is regularly calibrated by determination of boiling points of reference materials.

PROCEDURE

A boiling tube (3.2 mm diameter) was filled with the sample, heated until liquid and a boiling capillary was immersed, open end first.

Observations were then recorded as the temperature of the sample was raised.

RESULTS

Barometric pressure: 1011 mbar

A significant colour change from yellow to dark yellow was observed at 190°C, indicating decomposition. No sign of boiling was noted.

CONCLUSION

The boiling temperature of Rotenone was not determinable, as the test substance decomposed at temperatures above 190°C. From observations recorded during the melting temperature test, it was likely that the test substance decomposed on melting.

RELATIVE DENSITY
(EEC Method A3, OECD Method 109)

METHOD

The relative density of Rotenone was determined relative to purified water using a pyknometer at 20°C. 0.1 v/v aqueous Tween 80 was employed as the displacing liquid.

DEFINITION AND UNITS

The relative density (D_4^T) of solids and liquids is defined as the ratio of the mass of a volume of substance to be examined, determined at T°C, and the mass of the same volume of water at 4°C.

APPARATUS

Analytical balance:	Model RC 210P, Sartorius Instruments
Pyknometer:	Glass, nominal 10 cm^3 capacity at 20°C, fitted with capillary stopper (BS 4699)

REAGENTS

Water:	Purified by reverse osmosis and deionising; Elga Prima/Maxima
Displacement liquid:	0.1% v/v aqueous Tween 80

SUITABILITY OF DISPLACEMENT LIQUID

The suitability of 0.1% v/v Tween 80 as a displacement liquid was confirmed by the observation that 10 mg of Rotenone did not dissolve in 10 ml of this vehicle (solubility <0.1% w/v). The relative density of 0.1% v/v Tween 80 has been determined to be 0.998 at 20°C, i.e. the same as pure water.

PROCEDURE

Test temperature 20°C

A clean, dry pyknometer was accurately weighed (w_1) and then test substance (approximately 1 g) was added. The pyknometer was re-weighed (w_2) and then the test substance covered with displacement liquid. After vigorous shaking of the stoppered pyknometer, to suspend the test substance, air bubbles were removed by ultrasonification. The pyknometer was filled with displacement liquid, stoppered and weighed as before (w_3).

The pyknometer was then cleaned by rinsing with 0.1% v/v Tween 80, prior to filling to the limits of its capacity with displacement liquid. It was then carefully stoppered without trapping air and re-weighed (w_4).

Two tests were performed concurrently using separate pyknometers.

Parameters:

mass of pyknometer empty (g) = w_1

mass of pyknometer + test substance (g) = w_2

mass of pyknometer + test substance + 0.1% v/v Tween 80 (g) = w_3

mass of pyknometer + 0.1% v/v Tween 80 (g) = w_4

Calculations:

mass of 0.1% v/v Tween 80 to fill pyknometer (g) = $w_4 - w_1 = W_1$

mass of test substance (g) = $w_2 - w_1 = W_2$

mass of 0.1% v/v Tween 80 to fill pyknometer containing W_2 g test substance (g) = $w_3 - w_2 = W_3$

mass of 0.1% v/v Tween 80 equivalent to W_2 g test substance (g) = $W_1 - W_3 = W_4$

volume of W_2 g test substance (ml) = $W_4 / \rho_w^T = V_s$

relative density of test substance = $W_2/(V_s \times \rho_w^4) = D_4^T$

where ρ_w^T is the density of water and 0.1% v/v Tween 80 at the temperature of determination (0.998 g/ml)

ρ_w^4 is the density of water at 4°C (1.000 g/ml)

D_4^T is the relative density of the test material at the test temperature compared to water at 4°C

RESULTS

Parameter	Determination 1	Determination 2
w_1	14.15643	14.71328
w_2	15.16648	15.73373
w_3	24.84189	25.97706
w_4	24.57592	25.72129
W_1	10.41949	11.00801
W_2	1.01005	1.02045
W_3	9.67541	10.24333
W_4	0.74408	0.76468
V_s	0.74557	0.76621
D_4^T	1.35	1.33
$\bar{x}\ D_4^T$	1.34	

CONCLUSION

The relative density (D_4^{20}) of Rotenone was found to be 1.34.

VAPOUR PRESSURE
(EEC Method A4, OECD Method 104)

METHOD

The vapour pressure was determined using a vapour pressure balance.

DEFINITION AND UNITS

The vapour pressure of a substance is defined as the saturation pressure above a solid or liquid substance. At the thermodynamic equilibrium, the vapour pressure of a pure substance is a function of temperature only. The SI unit (International System of units) of pressure is the pascal (Pa).

APPARATUS

The vapour pressure balance was constructed by the Department of Facilities Management at Huntingdon Life Sciences. A furnace, containing test substance, is separated from one pan of the microbalance (1 g head, C.I. Electronics) by means of a moveable shutter. This entire assembly is housed in a bell-jar which can be evacuated to a vacuum of $<10^{-5}$ Torr by means of a diffusion pump and a rotary pump connected in series. The pressure within the system is measured by Pirani and ion gauges and the temperature of the furnace by a Type K thermocouple. The signals from the microbalance and thermocouple are sent to a chart recorder.

PROCEDURE

The microbalance was calibrated with a NAMAS calibrated 1 mg weight. It was found that 1 µg produced a deflection of 2.764×10^{-3} V.

A quantity of test substance (approximately 0.16 g) was added to the furnace. The apparatus was then assembled and evacuated to a pressure of less than 1×10^{-5} Torr (1.3×10^{-3} Pa).

After stabilisation at a given temperature, the shutter was opened to allow a stream of vapour to impact upon one balance pan. The temperature and pan deflection were recorded on a chart recorder. The trace obtained enabled the calculation of mass difference. The furnace temperature was then raised in increments and further measurements taken.

Four runs were performed between temperatures of 35 and 131ºC. The same sample was used for each test, with the pressure being kept at approximately 1×10^{-5} Torr (1.3×10^{-3} Pa) or below throughout the test.

CALCULATIONS

Assuming no condensation, the vapour pressure is related to the observed mass difference by the relationship:

$$\text{Vapour pressure} = \frac{\Delta m.g}{A} \qquad \text{Equation 1}$$

In this study, condensation occurred and consequently since the mass difference can, by reduced momentum transfer, be decreased by a factor up to two from that appropriate to equation 1. The vapour pressure can also be obtained from the kinematic theory relationship for effusion or complete condensation:

$$\text{Vapour pressure} = \sqrt{\frac{2\pi.RT}{M}} \cdot \frac{C}{A} \qquad \text{Equation 2}$$

The efficiency of condensation may be determined from the relative magnitudes of the measured mass difference and condensation rate. For a degree of condensation rate, the measured condensation rate must be divided by the degree of condensation (α). Likewise the experimental mass difference must be modified by a factor of $2/(2-\alpha)$, which gives an enhancement factor of two in the vapour pressure calculation when the molecules do not bounce back.

By equating equations 1 and 2 at a given vapour pressure, it is possible to allow for the evaluation of of α at each data point:

$$\frac{\Delta m}{C} = \frac{2-\alpha}{2\alpha} \cdot \sqrt{\frac{2\pi.RT}{M}} \cdot \frac{1}{g} \qquad \text{Equation 3}$$

Rearrangement of equation 3 gives equation 4 from which α can be calculated directly

$$\alpha = \left[\left(\frac{\Delta m.g}{C} \cdot \sqrt{\frac{M}{2\pi.RT}} \right) + 0.5 \right]^{-1} \qquad \text{Equation 4}$$

The effect of α on the two methods for estimating vapour pressure yields identical results. Confidence in this system is demonstrated by the observation that the values of α may approach but never exceed unity (when α=1 there is 100 % condensation).

The vapour pressure-temperature relationship is as follows:

$$\log_{10} V_p = \frac{\text{slope}}{T} + \text{intercept} \qquad \text{Equation 5}$$

Consequently, a plot of $\log_{10} V_p$ versus 1/T(K) should be linear, and by extrapolation, the vapour pressure at 298.15K can be calculated.

Glossary of terms used in equations 1 to 5

A	=	Surface area of the aperture (5.952×10^{-6} m^2)
C	=	Condensation rate (kg/s)
g	=	Acceleration due to gravity (9.813 m/s^2)
M	=	Relative molecular mass (kg/mol)
Δm	=	Mass difference (kg)
R	=	Universal gas constant (8.314 J/mol/K)
T	=	Temperature (K)
V_p	=	Vapour pressure (Pa)

RESULTS

A total of four runs were conducted, however, the data from runs 1 and 2 were not reported since the data were comparatively high and variable, which was likely to be due to degassing of the sample.

The subsequent results are detailed in Tables 1 and 2, with plots of $\log_{10}$Vp versus 1/T in Figure 9.

A summary is shown below:

	Run 3	**Run 4**
Correlation:	-0.9706	-0.9601
Slope:	-3012	-2932
Intercept:	4.913	4.647
Log Vp at 25°C:	-5.19	5.19
V_p (Pa) at 25°C:	6.47×10^{-6}	6.50×10^{-6}

The mean vapour pressure at 25°C was 6×10^{-6} Pa.

CONCLUSION

The vapour pressure of Rotenone was found to be 6×10^{-6} Pa at 25°C.

TABLE 1

Determination of the vapour pressure of Rotenone (Run 3)

Temperature (°C)	Mass difference (µg)	Condensation rate (µg/s)	Vapour pressure (Pa)		α	Corrected vapour pressure (Pa)		1/Temperature (1/K)	Log vapour pressure
			From mass difference	From condensation		From mass difference	From condensation		
80.5	0.20	-	0.00033	-	-	0.00033	-	0.00283	-3.48
85.0	0.20	-	0.00033	-	-	0.00033	-	0.00279	-3.48
90.0	0.26	-	0.00043	-	-	0.00043	-	0.00275	-3.37
95.0	0.24	0.005	0.00040	0.00017	0.360	0.00049	0.00049	0.00272	-3.31
100.0	0.29	0.010	0.00048	0.00037	0.551	0.00066	0.00066	0.00268	-3.18
105.5	0.35	0.001	0.00058	0.00006	0.091	0.00061	0.00061	0.00264	-3.22
110.0	0.47	0.013	0.00078	0.00050	0.485	0.00103	0.00103	0.00261	-2.99
115.0	0.56	0.016	0.00093	0.00059	0.483	0.00122	0.00122	0.00258	-2.91
120.5	0.77	0.030	0.00128	0.00114	0.619	0.00185	0.00185	0.00254	-2.73
125.0	0.98	0.044	0.00162	0.00170	0.687	0.00247	0.00247	0.00251	-2.61
130.5	1.54	0.069	0.00254	0.00268	0.691	0.00387	0.00387	0.00248	-2.41

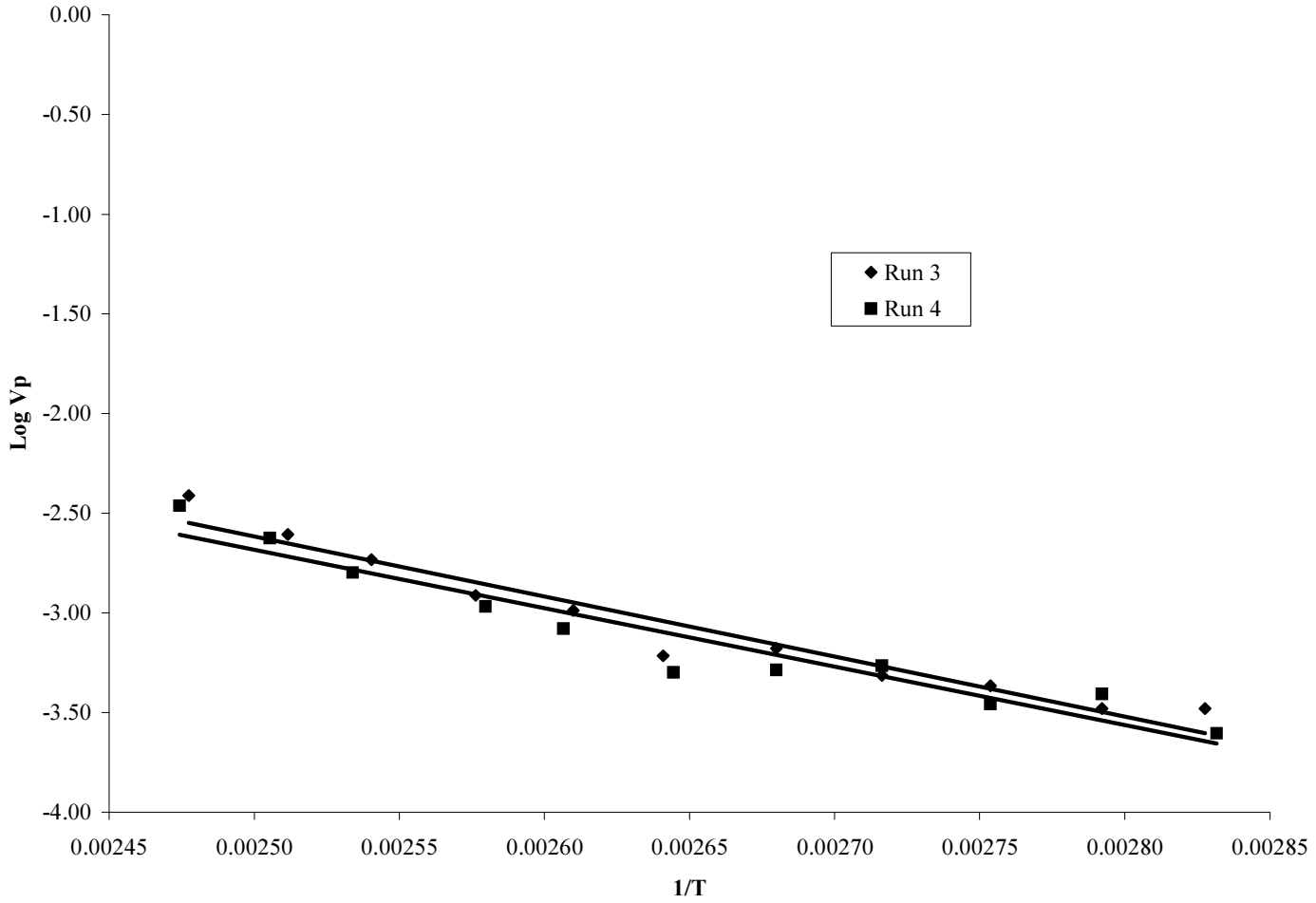
TABLE 2

Determination of the vapour pressure of Rotenone (Run 4)

Temperature (°C)	Mass difference (µg)	Condensation rate (µg/s)	Vapour pressure (Pa)		α	Corrected vapour pressure (Pa)		1/Temperature (1/K)	Log vapour pressure
			From mass difference	From condensation		From mass difference	From condensation		
80.0	0.15	-	0.00025	-	-	0.00025	-	0.00283	-3.60
85.0	0.19	0.004	0.00031	0.00015	0.391	0.00039	0.00039	0.00279	-3.41
90.0	0.21	-	0.00035	-	-	0.00035	-	0.00275	-3.46
95.0	0.27	0.005	0.00045	0.00019	0.352	0.00054	0.00054	0.00272	-3.27
100.0	0.27	0.004	0.00045	0.00014	0.265	0.00052	0.00052	0.00268	-3.29
105.0	0.26	0.004	0.00043	0.00014	0.286	0.00050	0.00050	0.00264	-3.30
110.5	0.36	0.012	0.00060	0.00047	0.567	0.00083	0.00083	0.00261	-3.08
114.5	0.48	0.015	0.00080	0.00056	0.523	0.00108	0.00108	0.00258	-2.97
121.5	0.65	0.027	0.00108	0.00102	0.644	0.00159	0.00159	0.00253	-2.80
126.0	0.90	0.045	0.00149	0.00176	0.741	0.00237	0.00237	0.00251	-2.63
131.0	1.35	0.063	0.00222	0.00245	0.712	0.00345	0.00345	0.00247	-2.46

FIGURE 9

Graphical representation of the vapour pressure for Rotenone



SURFACE TENSION
(EEC Method A5, OECD Method 115)

The surface tension test was not applicable to the test substance, Rotenone, since the water solubility of the test substance was less than 1 mg/l.

WATER SOLUBILITY
(EEC Method A6, OECD Method 105)

DEFINITION AND UNITS

The solubility in water is specified by the saturation mass concentration of the substance in water, and is a function of temperature. Solubility is specified in units of mass per volume of solution. The SI unit is kg/m^3; g/l may also be used.

PRELIMINARY TEST

A preliminary test was conducted by shaking a known mass of the test substance with purified water and visually assessing dissolution. The solubility of Rotenone was estimated to be less than 10 mg/l and therefore definitive tests were performed by a flow through column elution method.

PREPARATION OF TEST SYSTEMS

A stainless steel column (25 cm x 4.6 mm internal diameter, fitted with 2 μm frits) was packed with a sample (1 ml) of glass beads coated with the test substance:

Support material:	Glass beads, 40 mesh (BDH Chemicals Ltd.)
Loading of Rotenone onto support:	Glass beads (5 g) were mixed with a solution of the test substance in 1,2-dichloroethane (5 g/L, 50 ml). The solvent was removed under vacuum to give 50 mg Rotenone/1 g support

The column was packed so that there was a small glass wool plug either side of the glass beads.

A control column containing glass beads only was also prepared.

PROCEDURE

The charged column was connected to a HPLC pump equipped with stainless steel capillaries and purified water was added to the solvent reservoir. The system was equilibrated at the test temperature of 20°C.

The flow through the column was started at 0.4 ml/minute and the first five bed volumes of water from the column were discarded. Samples (5 ml) of the eluent were then collected for analysis, so that they were separated from each other by time intervals corresponding to the passage of at least ten bed volumes. Upon collection of each sample, the solutions were examined for any Tyndall effect and were found to be clear. Aliquots (1 ml) of each sample collected were diluted to 2 ml with methanol prior to analysis by a high performance liquid chromatography (HPLC) method.

Samples were taken until equilibration was demonstrated by at least five successive samples.

The procedure was then repeated on the packed column using half the flow rate of the first (0.2 ml/minute).

The pH value of each sample was measured.

There was concern that the test substance degraded while in solution and therefore all of the sample preparations were performed in amber glassware in the dark using a Kodak 6B filter safelight.

HPLC CONDITIONS

Instrument:	Hewlett Packard 1050 Liquid Chromatograph
Column:	YMC Pack ODS-AM (15 x 4.6 mm internal diameter)
Column temperature:	Ambient
Mobile phase composition:	Acetonitrile:water (50:50 v/v)
Flow rate:	1.5 ml/min
Injection volume:	50 µl
Detector:	UV set at 280 nm
Retention time:	Approximately 7 minutes

VERIFICATION SAMPLES

Blank and fortified control samples were processed and analysed as for the test samples.

PREPARATION OF CALIBRATION

A stock calibration solution of concentration 400 mg/l was prepared by weighing test substance (20 mg) into a 50 ml volumetric flask and dissolving in and diluting to volume with methanol.

Calibration solutions in the range 0.04 to 4 mg/l were prepared by dilutions of the stock solution with methanol:water (50:50 v/v). The concentrations were corrected to account for the purity of the test substance.

BRACKETING STANDARD SOLUTION

An intermediate sample from the chemical calibration was analysed concurrently with the test samples as a bracketing standard solution.

CALCULATIONS

The concentration of Rotenone in the analysed solution (C_A) was calculated from standards introduced before and after samples (bracketing standards) by the following equation:

$$C_A \text{ (mg/l)} = \frac{\text{sample peak area x standard concentration (mg/l)}}{\text{mean peak area of bracketing standards}}$$

The concentration of Rotenone in the test solutions (C_B) was calculated from the following equation:

$$C_B \text{ (mg/l)} = C_A \text{ (mg/l)} \times \text{dilution factor}$$

where the dilution factor was 2.

RESULTS

The detector calibration was found to be linear over the range 0 to 4 mg/l of standard solutions in methanol:water (50:50 v/v) with a regression coefficient of 1.0000 (Table 3, Figure 10).

The recovery of Rotenone from fortified control samples was deemed to be acceptable, and thus no correction was necessary to the determined sample concentrations. No significant interfering peaks were evident in blank control solutions.

Table 4 presents a summary of the results of the test and shows that the water solubility of Rotenone was 0.289 ± 0.016 mg/l. Table 5 presents the primary data for this test.

CONCLUSION

The water solubility of Rotenone was found to be 0.289 mg/l.

TABLE 3

Standard calibration for Rotenone

Standard concentration (mg/l)	Peak area
0.03981	1.5357
0.07962	3.4793
0.3981	14.810
0.7962	29.908
1.592	60.712
2.388	91.496
3.185	122.71
3.981	153.81

Linear regression (including x = 0, y = 0): y = 38.6x - 0.247, r = 1.0000

x = concentration
y = peak area

FIGURE 10

Standard calibration for Rotenone

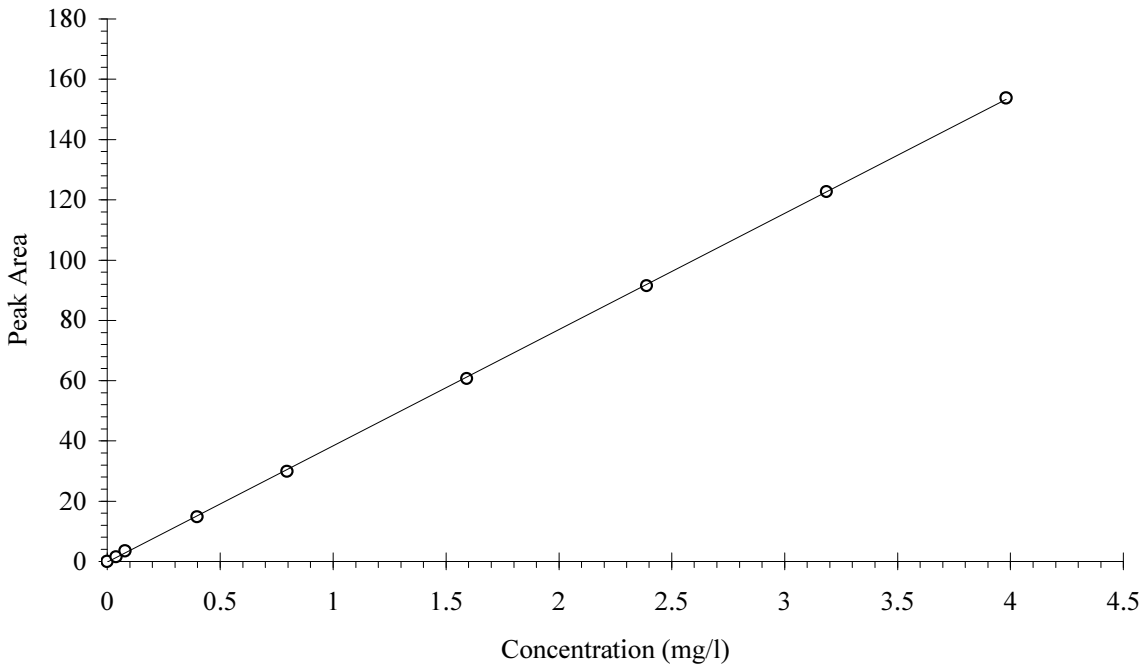


TABLE 4

Measurements of water solubility in purified water at 20°C

1. Column flow rate 0.4 ml/minute

Sample number	Concentration (mg/l)	pH
1A	0.2663	6.6
1B	0.2655	6.6
1C	0.2979	6.8
1D	0.2829	6.8
1E	0.3230	6.6
1F	0.3062	6.6

Water solubility = 0.290 ± 0.023 mg/l (C. of V. = 7.9%)

2. Column flow rate 0.2 ml/minute

Sample number	Concentration (mg/l)	pH
2A	0.2931	6.9
2B	0.2937	6.8
2C	0.2867	6.8
2D	0.2947	6.8
2E	0.2853	6.7
2F	0.2752	6.6

Water solubility = 0.288 ± 0.007 mg/l (C. of V. = 2.6%)

Overall mean water solubility = 0.289 ± 0.016 mg/l (C. of V. = 5.6%)

TABLE 5

HPLC analysis of samples from test in purified water

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (mg/l)
0.3981 mg/l std	15.258	-	-	-
Sample 1A	5.1096	0.1332	2	0.2663
Sample 1B	5.0941	0.1328	2	0.2655
Sample 1C	5.7162	0.1490	2	0.2979
Sample 1D	5.4272	0.1414	2	0.2829
0.3981 mg/l std	15.294	-	-	-
Sample 1E	6.1478	0.1615	2	0.3230
Sample 1F	5.8265	0.1531	2	0.3062
0.3981 mg/l std	15.011	-	-	-
0.3981 mg/l std	15.294	-	-	-
Sample 2A	5.5779	0.1465	2	0.2931
Sample 2B	5.5896	0.1469	2	0.2937
0.3981 mg/l std	15.011	-	-	-
0.3981 mg/l std	14.702	-	-	-
Sample 2C	5.2077	0.1434	2	0.2867
Sample 2D	5.3527	0.1473	2	0.2947
Sample 2E	5.1822	0.1427	2	0.2853
Sample 2F	4.9983	0.1376	2	0.2752
0.3981 mg/l std	14.222	-	-	-

ORGANIC SOLVENT SOLUBILITY

METHOD

The solubility of Rotenone was determined in a range of organic solvents by a flask shake method based on EEC Method A6.

DEFINITION AND UNITS

The solubility of a test substance is specified by the saturation mass concentration of the substance in a given solvent, and is a function of temperature. Solubility is specified in units of mass per volume of solvent or solution and will be reported in grams/litre (g/l).

PROCEDURE

The solubility of Rotenone was determined in each of the following solvents: methanol, acetone, xylene, 1,2-dichloroethane, ethyl acetate, n-heptane and n-octanol.

Preliminary test

Known weights of test substance were shaken with increasing amounts of each solvent until the compound completely dissolved. The following table lists the results from the preliminary investigations.

Solvent	Solubility (g/l)
methanol	2.5 - 3.3
acetone	71 - 83
xylene	33 - 50
1,2-dichloroethane	> 250
ethyl acetate	56 - 63
n-heptane	< 0.1
n-octanol	< 0.1

Since the solubility in 1,2-dichloroethane was greater than 250 g/l then no further testing was required. The solubility of Rotenone in the remaining solvents was subsequently determined by the definitive flask shake method.

Definitive test

For each solvent system under investigation, the following procedure was adopted.

Replicate samples of Rotenone were accurately weighed into glass vials and the appropriate solvent was added. Each vial was sealed, shaken at 20°C and then duplicate vials were removed for analysis after 4 and 24 hours. During the test with n-octanol, duplicate samples were also prepared and mixed for 48 hours prior to analysis since it appeared that equilibrium was not achieved after 24 hours.

The following table lists the quantities of test substance and solvents used during the definitive tests.

Solvent	Amount of Rotenone (mg)	Amount of solvent (ml)
methanol	90	5
acetone	1040	5
xylene	625	5
ethyl acetate	800	5
n-heptane	20	10
n-octanol	20	10

On analysis of the methanol, acetone, xylene, ethyl acetate and n-octanol samples, portions of the contents were filtered (nylon, 0.2 µm) and aliquots of the filtrates were diluted to volume with methanol:water (50:50 v/v). The final solutions were analysed by a high performance liquid chromatography (HPLC) method.

The following table lists the volumes of the filtrates diluted and the final volume of the diluted samples.

Solvent	Volume of filtrate aliquot (ml)	Diluted volume (ml)
methanol	0.25	25
acetone	0.05	100
xylene	0.05	100
ethyl acetate	0.05	100
n-octanol	1	50*

* consisted of a 1 to 5 ml dilution followed by a 1 to 10 ml dilution.

On analysis of the n-heptane samples, portions of the contents were filtered (nylon, 0.2 µm) and aliquots (2 ml) of the filtrates were evaporated to dryness under nitrogen at 40°C. The residues were redissolved and diluted to volume (2 ml) with methanol:water (50:50 v/v) prior to analysis by the HPLC method.

There was concern that the test substance degraded while in solution and therefore all of the sample preparations were performed in amber glassware in the dark using a Kodak 6B filter safelight.

HPLC CONDITIONS

Instrument:	Hewlett Packard 1050 Liquid Chromatograph
Column:	YMC Pack ODS-AM (15 x 4.6 mm internal diameter)
Column temperature:	Ambient
Mobile phase composition:	Acetonitrile:water (50:50 v/v)
Flow rate:	1.5 ml/min
Injection volume:	20 µl
Detector:	UV set at 280 nm
Retention time:	Approximately 7 minutes

VERIFICATION SAMPLES

Blank and fortified control samples were processed and analysed as for the test samples.

PREPARATION OF CALIBRATION

A stock calibration solution of concentration 820 mg/l was prepared by weighing test substance (42 mg) into a 50 ml volumetric flask and dissolving in and diluting to volume with methanol.

Calibration solutions in the range 1.6 to 82 mg/l were prepared by dilutions of the stock solution with methanol:water (50:50 v/v). The concentrations were corrected to account for the purity of the test substance.

BRACKETING STANDARD SOLUTION

An intermediate sample from the chemical calibration was analysed concurrently with the test samples as a bracketing standard solution.

CALCULATIONS

The concentration of Rotenone in the analysed solution (C_A) was calculated from standards introduced before and after samples (bracketing standards) by the following equation:

$$C_A \text{ (mg/l)} = \frac{\text{sample peak area x standard concentration (mg/l)}}{\text{mean peak area of bracketing standards}}$$

The concentration of Rotenone in the test solutions (C_B) was calculated from the following equation:

$$C_B \text{ (g/l)} = C_A \text{ (mg/l)} \times \text{dilution factor}/1000$$

where 1000 is the factor to convert the units from mg/l to g/l

RESULTS

The detector calibration was found to be linear over the range 0 to 82 mg/l of standard solutions in methanol:water (50:50 v/v) with a regression coefficient of 1.0000 (Table 6, Figure 11).

The recovery of Rotenone from fortified control samples was deemed to be acceptable, and thus no correction was necessary to the determined sample concentrations. No significant interfering peaks were evident in blank control solutions.

Tables 7 to 12 present summaries of the results of the definitive tests and show the following solubilities of Rotenone:

Solvent	Solubility (g/l)
methanol	2.76
acetone	70.6
xylene	29.6
ethyl acetate	53.2
n-heptane	0.0771
n-octanol	1.12

Tables 13 to 18 present the primary data for the tests.

CONCLUSION

The solubility of Rotenone was found to be: 2.76 g/l in methanol, 70.6 g/l in acetone, 29.6 g/l in xylene, greater than 250 g/l in 1,2-dichloroethane, 53.2 g/l in ethyl acetate, 0.0771 g/l in n-heptane and 1.12 g/l in n-octanol.

TABLE 6

Standard calibration for Rotenone

Standard concentration (mg/l)	Peak area
1.648	30.537
4.121	76.199
8.242	156.02
16.48	315.81
32.97	633.87
49.45	947.83
65.93	1276.1
82.42	1592.9

Linear regression (including x=0, y=0) $y = 19.3x - 2.69$, $r = 1.0000$

x = concentration
y = peak area

FIGURE 11

Standard calibration for Rotenone

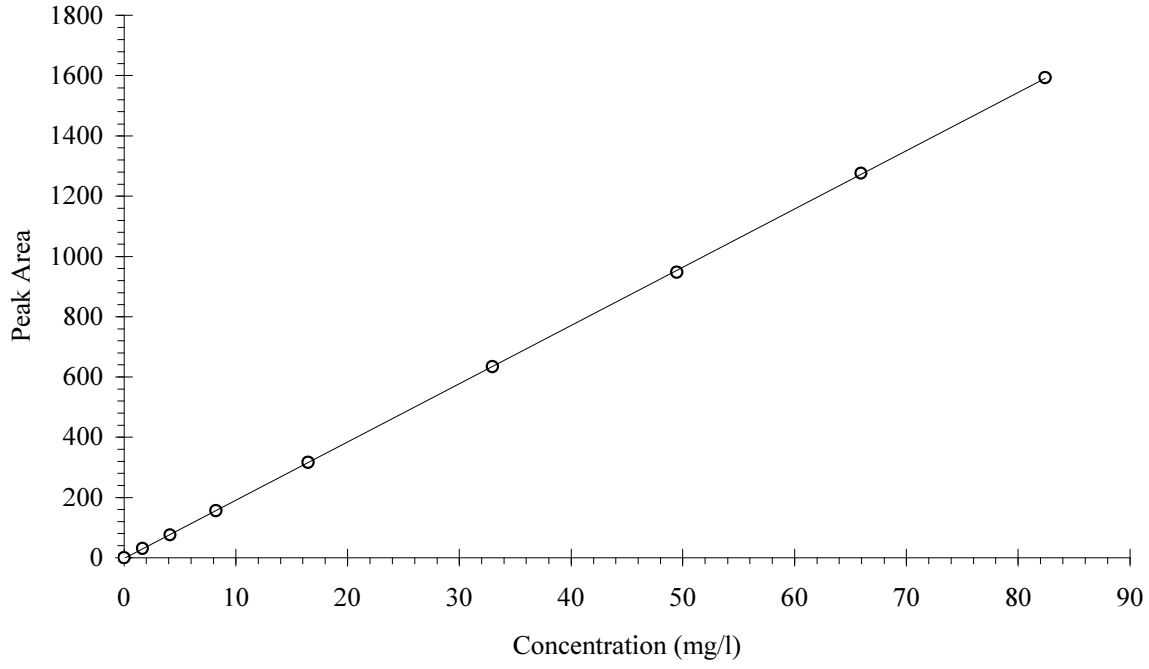


TABLE 7

Measurements of solubility in methanol

Time (hours)	Concentration (g/l)	Mean concentration (g/l)
4	2.793, 2.836	2.815
24	2.797, 2.617	2.707

Mean solubility* = 2.76 ± 0.10 g/l
(C. of V. = 3.5%)

* Determined as mean of all samples

TABLE 8

Measurements of solubility in acetone

Time (hours)	Concentration (g/l)	Mean concentration (g/l)
4	73.80, 72.95	73.37
24	70.80, 64.73	67.77

Mean solubility* = 70.6 ± 4.1 g/l
(C. of V. = 5.8%)

* Determined as mean of all samples

TABLE 9

Measurements of solubility in xylene

Time (hours)	Concentration (g/l)	Mean concentration (g/l)
4	30.35, 28.71	29.53
24	29.70, 29.48	29.59

Mean solubility* = 29.6 ± 0.7 g/l
(C. of V. = 2.3%)

*Determined as mean of all samples

TABLE 10

Measurements of solubility in ethyl acetate

Time (hours)	Concentration (g/l)	Mean concentration (g/l)
4	51.78, 55.61	53.69
24	51.71, 53.85	52.78

Mean solubility* = 53.2 ± 1.9 g/l
(C. of V. = 3.5%)

* Determined as mean of all samples

TABLE 11

Measurements of solubility in n-heptane

Time (hours)	Concentration (g/l)	Mean concentration (g/l)
4	0.07836, 0.06829	0.07332
24	0.08052, 0.08128	0.08090

Mean solubility* = 0.0771 ± 0.0060 g/l
(C. of V. = 7.8%)

* Determined as mean of all samples

TABLE 12

Measurements of solubility in n-octanol

Time (hours)	Concentration (g/l)	Mean concentration (g/l)
4	0.8897, 0.9697	0.9297
24	1.054, 1.104	1.079
48	1.222, 1.085	1.154

Mean solubility* = 1.12 ± 0.07 g/l
(C. of V. = 6.6%)

*Determined as mean of 24 and 48 hour samples

TABLE 13

HPLC analysis of samples from test in methanol

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (g/l)
32.97 mg/l std	631.55	-	-	-
4 hour sample A	534.85	27.93	100	2.793
4 hour sample B	543.11	28.36	100	2.836
24 hour sample A	535.56	27.97	100	2.797
24 hour sample B	501.11	26.17	100	2.617
32.97 mg/l std	631.20	-	-	-

TABLE 14

HPLC analysis of samples from test in acetone

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (g/l)
32.97 mg/l std	631.40	-	-	-
4 hour sample A	706.82	36.90	2000	73.80
4 hour sample B	698.67	36.47	2000	72.95
24 hour sample A	678.08	35.40	2000	70.80
24 hour sample B	619.99	32.37	2000	64.73
32.97 mg/l std	631.70	-	-	-

TABLE 15

HPLC analysis of samples from test in xylene

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (g/l)
32.97 mg/l std	633.32	-	-	-
4 hour sample A	290.51	15.17	2000	30.35
4 hour sample B	274.85	14.36	2000	28.71
24 hour sample A	284.25	14.85	2000	29.70
24 hour sample B	282.19	14.74	2000	29.48
32.97 mg/l std	629.07	-	-	-

TABLE 16

HPLC analysis of samples from test in ethyl acetate

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (g/l)
32.97 mg/l std	631.70	-	-	-
4 hour sample A	495.99	25.89	2000	51.78
4 hour sample B	532.64	27.80	2000	55.61
24 hour sample A	495.31	25.85	2000	51.71
24 hour sample B	515.80	26.92	2000	53.85
32.97 mg/l std	631.55	-	-	-

TABLE 17

HPLC analysis of samples from test in n-heptane

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (g/l)
32.97 mg/l std	631.20	-	-	-
4 hour sample A	1499.7	78.36	1	0.07836
4 hour sample B	1306.9	68.29	1	0.06829
24 hour sample A	1541.1	80.52	1	0.08052
24 hour sample B	1555.5	81.28	1	0.08128
32.97 mg/l std	630.80	-	-	-

TABLE 18

HPLC analysis of samples from test in n-octanol

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (g/l)
15.34 mg/l std	234.67	-	-	-
4 hour sample A	268.14	17.79	50	0.8897
4 hour sample B	292.27	19.39	50	0.9697
24 hour sample A	317.76	21.09	50	1.054
24 hour sample B	332.68	22.08	50	1.104
15.34 mg/l std	227.67	-	-	-
48 hour sample A	351.42	24.44	50	1.222
48 hour sample B	311.96	21.69	50	1.085
15.34 mg/l std	213.53	-	-	-

APPENDIX 1

CERTIFICATE OF ANALYSIS

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Rotenone, 95-98%
Product Number	R8875
Product Brand	Sigma
CAS Number	83-79-4
Molecular Formula	$C_{23}H_{22}O_6$
Molecular Weight	394.42

TEST	SPECIFICATION	LOT 046K1189 RESULTS
APPEARANCE	WHITE TO YELLOW WITH A TAN CAST POWDER	LIGHT YELLOW POWDER
SOLUBILITY	CLEAR TO SLIGHTLY HAZY YELLOW SOLUTION AT 50MG/ML IN CHLOROFORM	SLIGHTLY HAZY YELLOW
ELEMENTAL ANALYSIS	66.5 TO 71.5%CARBON	69.4%
SOLVENT CONTENT	REPORT RESULT	NONE DETECTED BY NMR
SPECIFIC ROTATION	-114 TO -122DEG (C=1.39 IN CHLOROFORM AT 25DEGC)	-115 DEG
PURITY BY THIN LAYER CHROMATOGRAPHY	NLT 95%	98%
QC ACCEPTANCE DATE		MAY 2006

Rodney Burbach, Supervisor
Analytical Services
St. Louis, Missouri USA

APPENDIX 2

EYE RESEARCH CENTRE GLP COMPLIANCE STATEMENT 2005

Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Occold** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Toxicology** **Phys/Chem Testing**

DATE OF INSPECTION

12th April 2005

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Bryan J. Wright 9/6/05

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority