SafePharm Laboratories

PFHA:

ALGAL INHIBITION TEST

SPL PROJECT NUMBER: 1742/020

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress.

This report has been audited by Safepharm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

	09 June 2003	Standard Test Method Compliance Audit
	06 July 2004	Test Material Preparation
	06 July 2004	Test System Preparation
	06 July 2004	Exposure
	09, 15 July 2004	Assessment of Response
	09, 22 July 2004	Chemical Analysis
§	03 September 2004	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: 16 DEC 2004

For Safepharm Quality Assurance Unit*

*Authorised QA Signatures:

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Deputy Head of Department:	JM Crowther MIScT MRQA
Senior Audit Staff:	JV Johnson BSc MRQA; G Wren ONC MRQA

GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

This report fully and accurately reflects the procedures used and data generated.

H Vryenhoef ..... Date: 16 DEC 2004

H Vryenhoef BSc
Study Director

The analytical data presented in this report were compiled by me or under my supervision and accurately reflect the data obtained.

..... Date: 16 DEC 2004

Dr J McKenzie CChem MRSC
Head of Analytical Services

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SUMMARY

Introduction. A study was performed to assess the effect of the test material on the growth of the green alga *Scenedesmus subspicatus.* The method followed that described in the OECD Guidelines for Testing of Chemicals (1984) No 201, "Alga, Growth Inhibition Test" referenced as Method C.3 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Methods. Following a preliminary range-finding test, *Scenedesmus subspicatus* was exposed to an aqueous solution of the test material at concentrations of 6.25, 12.5, 25, 50 and 100 mg/l (three replicate flasks per concentration) for 72 hours, under constant illumination and shaking at a temperature of 24 ± 1°C.

Samples of the algal populations were removed daily and cell concentrations determined for each control and treatment group, using a Coulter® Multisizer Particle Counter.

Results. Exposure of *Scenedesmus subspicatus* to the test material gave an E_bC_{50} (72 h) value of 90 mg/l* and an E_rC_{50} (0 - 72 h) value of 86 mg/l*. The No Observed Effect Concentration was 50 mg/l.

Chemical analysis of the test preparations at 0 and 72 hours showed measured test concentrations to range from 90% to 105% of nominal and so the results are based on nominal test concentrations only.

* It was not possible to calculate 95% confidence limits for the EC_{50} values as the data generated did not fit the models available for the calculation of confidence limits.

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ALGAL INHIBITION TEST

1. INTRODUCTION

This report contains a description of the methods used and results obtained during a study to investigate the effect of the test material on the growth of the green alga *Scenedesmus subspicatus*. The method followed the recommendations of the OECD Guidelines for Testing of Chemicals (1984) No 201, "Alga, Growth Inhibition Test" referenced as Method C.3 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Scenedesmus subspicatus is a freshwater unicellular alga, representative of primary producers found in natural waters and can therefore be considered as an important non-target organism in freshwater ecosystems.

The range-finding test was conducted between 27 April 2004 and 30 April 2004 and the definitive test between 27 July 2004 and 30 July 2004.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	PFHA
Description	:	colourless liquid
Lot number	:	C15009601
Date received	:	12 January 2004
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

2.2 Experimental Preparation

For the purpose of the definitive test, the test material was dissolved directly in culture medium.

An amount of test material (100 mg) was dissolved in culture medium and the volume adjusted to 500 ml to give a 200 mg/l stock solution from which serial dilutions were made to give further stock solutions of 100, 50, 25 and 12.5 mg/l. An aliquot (250 ml) of each stock solution was separately mixed with algal suspension (250 ml) to give the required test concentrations of 6.25, 12.5, 25, 50 and 100 mg/l.

The stock solutions and each of the prepared concentrations were inverted several times to ensure adequate mixing and homogeneity.

The concentration and stability of the test material in the test preparations were verified by chemical analysis at 0 and 72 hours (see Appendix 1).

3. METHODS

3.1 Test Species

The test was carried out using *Scenedesmus subspicatus* strain CCAP 276/20. Liquid cultures of *Scenedesmus subspicatus* were obtained from the Culture Collection of Algae and Protozoa (CCAP), Institute of Freshwater Ecology, The Ferry House, Far Sawrey, Ambleside, Cumbria. Cultures were maintained in the laboratory by the periodic replenishment of culture medium (Section 3.2). The culture was maintained in the laboratory at a temperature of 21 ± 1°C under continuous illumination (intensity approximately 7000 lux) and constant aeration.

3.2 Culture Medium

The culture medium used for both the range-finding and definitive tests was the same as that used to maintain the stock culture.

The culture medium is defined in Appendix 2.

3.3 Procedure

3.3.1 Range-finding test

The test concentrations to be used in the definitive test were determined by a preliminary range-finding test. The range-finding test was conducted by exposing *Scenedesmus subspicatus* cells to a series of nominal test concentrations of 1.0, 10 and 100 mg/l for a period of 72 hours.

The test was conducted in 250 ml glass conical flasks plugged with polyurethane foam bungs to reduce evaporation. Two replicate flasks were prepared for each control and test concentration. The test material was dissolved directly in culture medium.

An amount of test material (100 mg) was dissolved in culture medium and the volume adjusted to 500 ml to give a 200 mg/l stock solution from which serial dilutions were made to give further stock solutions of 20 and 2.0 mg/l. An aliquot (100 ml) of each stock solution was separately mixed with algal suspension (100 ml) to give the required test concentrations of 1.0, 10 and 100 mg/l.

The stock solutions and each of the prepared concentrations were inverted several times to ensure adequate mixing and homogeneity.

The control group was maintained under identical conditions but not exposed to the test material.

At the start of the range-finding test a sample of each test and control culture was removed and the cell density determined using a Coulter® Multisizer Particle Counter. The flasks were then plugged with polyurethane foam bungs and incubated (INFORS Multitron® Version 2 incubator) at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) and constantly shaken at approximately 150 rpm for 72 hours.

After 72 hours the cell density of each flask was determined using a Coulter® Multisizer Particle Counter.

3.3.2 Definitive test

Based on the results of the range-finding test the following test concentrations were assigned to the definitive test: 6.25, 12.5, 25, 50 and 100 mg/l.

3.3.2.1 *Preparation of the test material*

For the purpose of the definitive test the required amount of test material was added to each test vessel using the method described in Section 2.2.

3.3.2.2 *Exposure conditions*

As in the range-finding test 250 ml glass conical flasks were used. Three flasks each containing 100 ml of test preparation were used for the control and each treatment group.

The control group was maintained under identical conditions but not exposed to the test material.

Pre-culture conditions gave an algal suspension in log phase growth characterised by a cell density of 1.76×10^6 cells per ml. This suspension was diluted to a cell density of 2.01×10^4 cells per ml prior to use. At initiation of the test the culture contained a nominal cell density of 10^4 cells per ml.

The flasks were plugged with polyurethane foam bungs and incubated (INFORS Multitron® Version 2 incubator) at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) and constantly shaken at approximately 150 rpm for 72 hours.

Samples were taken at 0, 24, 48 and 72 hours and the cell densities determined using a Coulter® Multisizer Particle Counter.

3.3.2.3 *Physico-chemical measurements*

The pH of each control and test flask was determined at initiation of the test and after 72 hours exposure. The pH was measured using a WTW pH 320 pH meter. The temperature within the incubator was recorded daily.

3.3.2.4 *Verification of test concentrations*

Water samples were taken from the control and each test group (replicates R_1 - R_3 pooled) at 0 and 72 hours for quantitative analysis. Duplicate samples were taken at each occasion and stored at approximately -20°C for further analysis if necessary.

The method of analysis, stability, recovery and test preparation analyses are described in Appendix 1.

3.3.3 Evaluation of data

3.3.3.1 Comparison of areas under the growth curves

The area under the curve is taken to be an index of growth and was calculated using the following equation:

$$A = \frac{N_1 - N_0}{2} x\, t_1 + \frac{N_1 + N_2 - 2N_0}{2} x\,(t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} x\,(t_n - t_{n-1})$$

where

A	=	area
N_0	=	cell concentration at the start of the test
N_1	=	cell concentration at t_1
N_n	=	cell concentration at t_n
t_1	=	time of first measurement (hours from start)
t_n	=	time of nth measurement (hours from start)

Percentage inhibition of growth at each test concentration (I_A) was calculated by comparing the area under the test curve (A_t) with that under the control curve (A_c) using the following equation:

$$I_A = \frac{A_c - A_t}{A_c} x\,100$$

3.3.3.2 Comparison of growth rates

The average maximum growth rate (μ) for each culture was also calculated, from the straight section of the growth curve (Figure 1), using the following equation:

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

3.3.3.3 Statistical analysis

One way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955) was carried out on the area under the growth curve data at 72 hours for

the control and all test concentrations to determine any statistically significant differences between the test and control groups. All statistical analyses were performed using the SAS computer software package (SAS 1999 - 2001).

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Range-finding Test

The cell densities and percentage inhibition of growth values from the exposure of *Scenedesmus subspicatus* to the test material during the range-finding test are given in Table 1.

The results showed no significant effect on growth at the test concentrations of 1.0 and 10 mg/l. However, growth was observed to be reduced at 100 mg/l.

Based on this information test concentrations of 6.25, 12.5, 25, 50 and 100 mg/l were selected for the definitive test.

5.2 Definitive Test

5.2.1 Growth data

From the data given in Tables 2 and 3, it is clear that both the growth (r) and the biomass (b) of *Scenedesmus subspicatus* (CCAP 276/20) were affected by the presence of the test material over the 72-Hour exposure period.

The mean cell densities versus time for the definitive test are presented in Figure 1.

The percentage inhibition values (I_A), (Table 3) were plotted against test concentration (Figure 2), a line fitted using the Xlfit 3 software package (IDBS 2002) and the EC_{50} value with respect to the area under the growth curve, E_bC_{50} (72 h) determined from the equation for the fitted line.

The percentage inhibition values ($I\mu$), (Table 3) were plotted against test concentration (Figure 3), a line fitted using the Xlfit 3 software package (IDBS 2002) and the EC_{50} value with respect to the growth rate, E_rC_{50} determined from the equation for the fitted line.

Accordingly the following results were determined from the data:

E_bC_{50} (72 h) : 90 mg/l
E_rC_{50} (0 - 72 h) : 86 mg/l

where E_bC_{50} is the test concentration that reduced biomass by 50% and E_rC_{50} is the test concentration that reduced specific growth rate by 50%.

It was not possible to calculate 95% confidence limits for the EC_{50} values as the data generated did not fit the models available for the calculation of confidence limits eg Litchfield and Wilcoxon, Probit, Logistic and Weilbulls models (Litchfield and Wilcoxon 1949).

Statistical analysis of the area under the growth curve data was carried out for the control and and all test concentrations using one way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955). There were no statistically significant differences between the control and 6.25, 12.5, 25 and 50 mg/l test concentrations ($P \geq 0.05$), however the 100 mg/l test concentration was significantly different ($P < 0.05$) and therefore the "No Observed Effect Concentration" (NOEC) was 50 mg/l.

The following data show that the cell concentration of the control cultures increased by a factor of 35 during the test in line with the OECD Guideline that states the enhancement must be at least by a factor of 16 after 72 hours:

Mean cell density of control at 0 hours : 1.04×10^4 cells per ml
Mean cell density of control at 72 hours : 3.65×10^5 cells per ml

5.2.2 Observations on cultures

All test and control cultures were inspected microscopically at 72 hours. There were no abnormalities detected in any of the control or test cultures at 6.25, 12.5, 25 and 50 mg/l, however few intact cells were observed to be present in the test cultures at 100 mg/l.

5.2.3 Observations on test material solubility

At 0 hours all control and test cultures were clear colourless solutions. After the 72-Hour test period the control, 6.25, 12.5, 25 and 50 mg/l test cultures were green dispersions. The 100 mg/l test cultures remained as clear colourless solutions throughout the test period.

5.2.4 Physico-chemical measurements

The pH values of each test and control flask are given in Table 2. Temperature was maintained at 24 ± 1°C throughout the test.

The pH values of the control cultures (see Table 2) were observed to increase from pH 7.7 at 0 hours to pH 8.7 – 8.8 at 72 hours. The pH deviation in the control cultures was less than 1.5 pH units after 72 hours and therefore was within the limits given in the Test Guidelines.

The test material vessels showed a concentration dependent decline in pH values with lower pH values being observed in the higher test concentrations (see Table 2). Given the extremely low pH in the 100 mg/l test cultures it was considered likely that this contributed to the inhibitory effect that the test material exhibited.

5.2.5 Verification of test concentrations

Chemical analysis of the test solutions at 0 and 72 hours (see Appendix 1) showed measured test concentrations to range from 90% to 105% of nominal and so the results are calculated on nominal test concentrations only.

6. CONCLUSION

The effect of the test material on the growth of *Scenedesmus subspicatus* has been investigated over a 72-Hour period and gave an E_bC_{50} (72 h) value of 90 mg/l* and an E_rC_{50} (0 - 72 h) value of 86 mg/l*. The No Observed Effect Concentration at 72 hours was 50 mg/l.

7. REFERENCES

Dunnett, C W (1955) A Multiple Comparison Procedure for Comparing Several Treatments With a Control. *J Am Stat Assoc* **50**, 1096-1121.

SAS/STAT Proprietary Software Release 8.02 (1999 - 2001), SAS Institute Inc, Cary, NC, USA.

Sokal, R R and Rohlf, F J (1981) Biometry. New York: W H Freeman and Company.

Xlfit3 Version 3.0.5 Build 12 (2002), ID Business Solutions Ltd.

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* It was not possible to calculate 95% confidence limits for the EC_{50} values as the data generated did not fit the models available for the calculation of confidence limits.

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Table 1 Cell Densities and Percentage Inhibition of Growth from the Range-finding Test

Nominal Concentration (mg/l)		Cell Densities* (cells per ml)		
		0 Hours	72 Hours	% Inhibition (area under curve at 72 h)
Control	R_1	1.27E+04	4.82E+05	
	R_2	1.25E+04	4.83E+05	-
	Mean	1.26E+04	4.82E+05	
1.0	R_1	1.25E+04	4.67E+05	
	R_2	1.34E+04	4.70E+05	3
	Mean	1.29E+04	4.68E+05	
10	R_1	1.27E+04	4.09E+05	
	R_2	1.22E+04	4.04E+05	16
	Mean	1.25E+04	4.06E+05	
100	R_1	1.26E+04	2.71E+03	
	R_2	1.22E+04	3.41E+03	102
	Mean	1.24E+04	3.06E+03	

* Cell densities represent the mean number of cells per ml calculated from the mean of the cell counts from 3 counts for each of the replicate flasks.

R_1 and R_2 = Replicates 1 and 2

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Table 2 Cell Densities and pH Values in the Definitive Test

Nominal Concentration (mg/l)		pH	Cell Densities* (cells per ml)				pH
		0 h	0 h	24 h	48 h	72 h	72 h
Control	R_1	7.7	1.06E+04	4.13E+04	1.18E+05	3.24E+05	8.8
	R_2	7.7	1.05E+04	4.20E+04	1.10E+05	4.15E+05	8.8
	R_3	7.7	1.01E+04	5.50E+04	1.09E+05	3.56E+05	8.7
	Mean		1.04E+04	4.61E+04	1.12E+05	3.65E+05	
6.25	R_1	7.6	1.04E+04	4.81E+04	1.22E+05	3.17E+05	8.9
	R_2	7.6	9.95E+03	5.45E+04	1.22E+05	2.83E+05	8.9
	R_3	7.6	1.15E+04	4.04E+04	1.10E+05	2.63E+05	8.8
	Mean		1.06E+04	4.77E+04	1.18E+05	2.88E+05	
12.5	R_1	7.5	1.16E+04	4.92E+04	1.03E+05	3.10E+05	8.8
	R_2	7.5	1.01E+04	4.52E+04	1.07E+05	3.35E+05	8.8
	R_3	7.5	1.16E+04	5.40E+04	1.16E+05	3.77E+05	8.8
	Mean		1.11E+04	4.95E+04	1.08E+05	3.41E+05	
25	R_1	7.3	1.13E+04	4.67E+04	1.19E+05	1.95E+05	8.7
	R_2	7.3	9.57E+03	4.97E+04	1.20E+05	3.25E+05	8.7
	R_3	7.3	1.20E+04	5.26E+04	1.19E+05	2.54E+05	8.7
	Mean		1.09E+04	4.97E+04	1.19E+05	2.58E+05	
50	R_1	6.9	1.15E+04	5.17E+04	1.02E+05	4.75E+05	8.8
	R_2	6.9	1.11E+04	3.56E+04	1.05E+05	3.93E+05	8.8
	R_3	6.9	1.12E+04	3.98E+04	1.08E+05	3.77E+05	8.8
	Mean		1.12E+04	4.24E+04	1.05E+05	4.15E+05	
100	R_1	4.3	1.07E+04	1.22E+03	3.25E+03	3.22E+03	3.8
	R_2	4.3	1.05E+04	3.80E+02	1.74E+03	7.80E+02	3.9
	R_3	4.3	1.09E+04	6.36E+02	1.46E+03	6.72E+02	3.9
	Mean		1.07E+04	7.45E+02	2.15E+03	1.56E+03	

* Cell densities represent the mean number of cells per ml calculated from the mean of the cell counts from 3 counts for each of the replicate flasks.
R_1 - R_3 = Replicates 1 to 3

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Table 3 Inhibition of Growth Rate and Biomass

Nominal Concentration (mg/l)	Area Under Curve at 72 h	% Inhibition	Growth Rate (0 – 72 h)	% Inhibition
Control	7.55E+06	-	0.049	-
6.25	6.79E+06	10	0.046	6
12.5	7.21E+06	4	0.048	2
25	6.50E+06	14	0.044	10
50	7.84E+06	[4]	0.050	[2]
100	-5.54E+05	107	-0.027	155

[Increase in growth as compared to the controls]

E_bC_{50} (72 h) from Figure 2 = 90 mg/l
E_rC_{50} (0 - 72 h) from Figure 3 = 86 mg/l

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Figure 1 Mean Cell Densities v Time for the Definitive Test

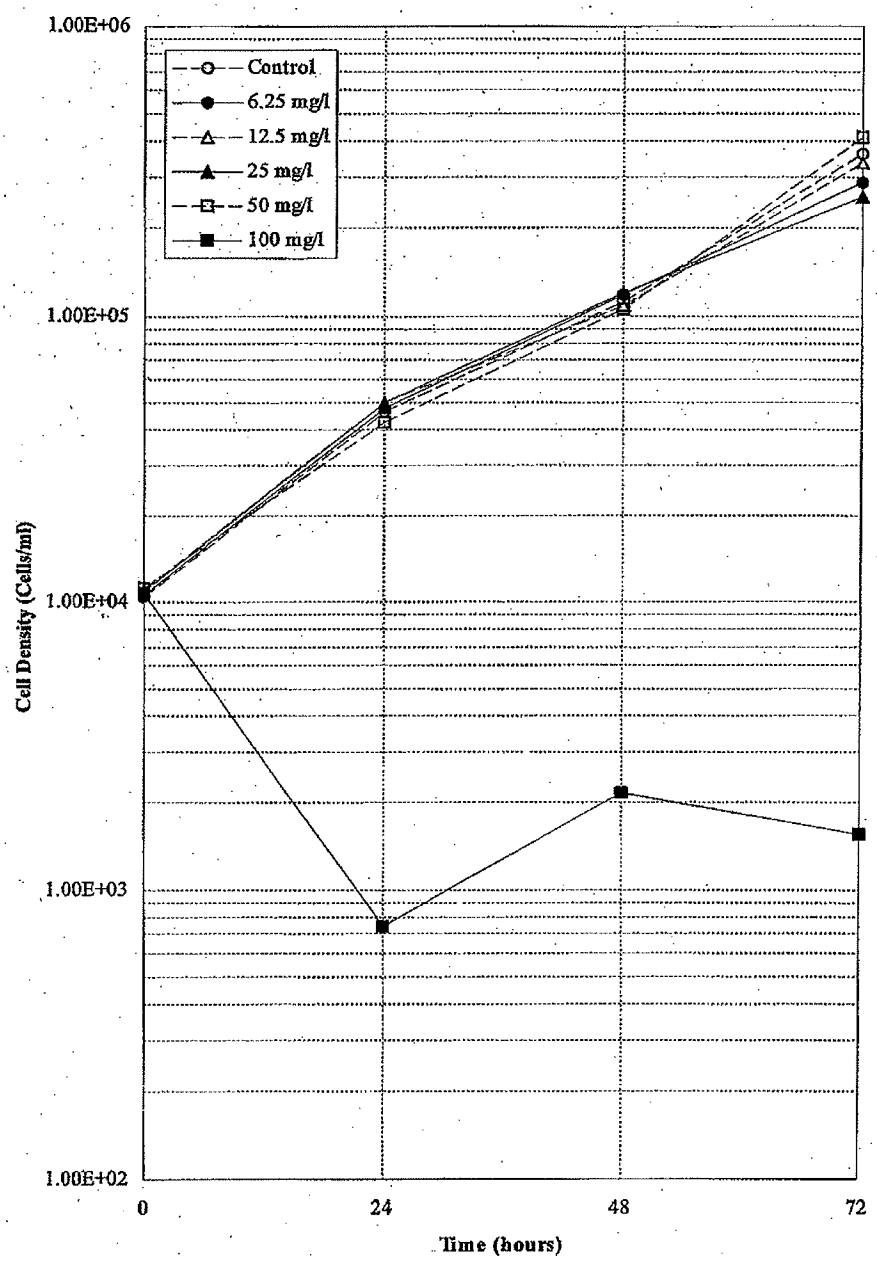

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Figure 2 Inhibition of Biomass Based on Nominal Test Concentrations

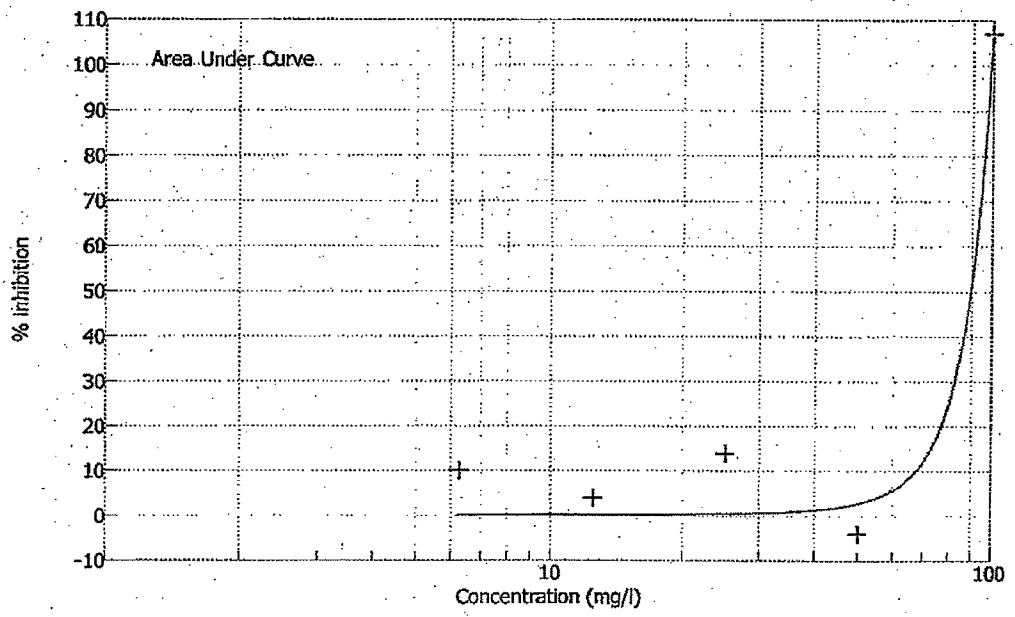

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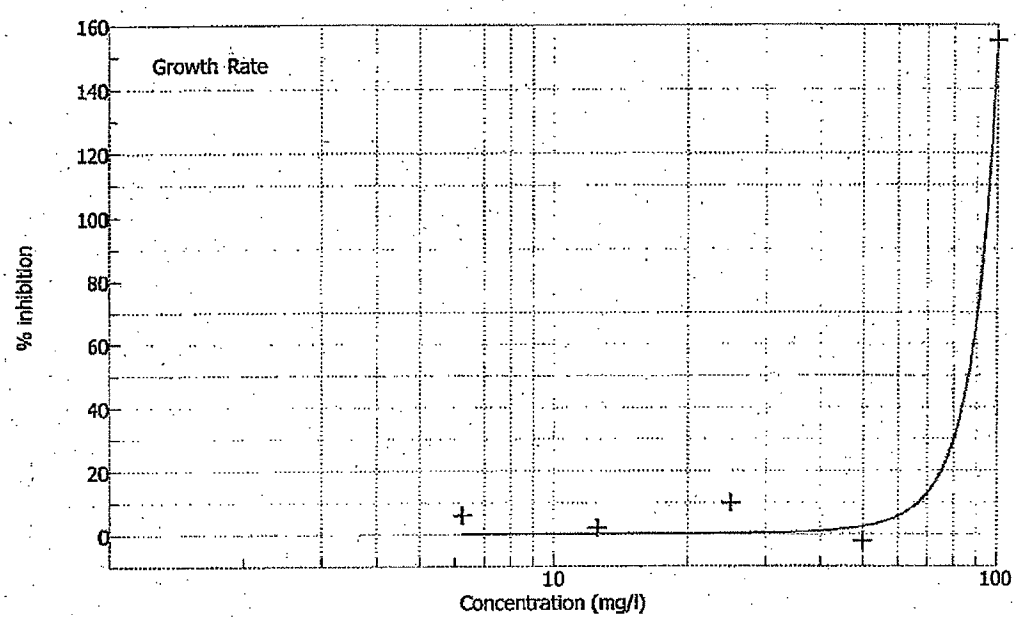
Figure 3 Inhibition of Growth Rate Based on Nominal Test Concentrations



Appendix 1 Verification of Test Concentrations

1. METHOD OF ANALYSIS

1.1 Introduction

The test material concentration in the test samples was determined by high performance liquid chromatography (HPLC) using an external standard. The test material gave a chromatographic profile consisting of a single peak.

The method was developed by the Department of Analytical Services, Safepharm Laboratories Limited.

1.2 Sample Preparation

A volume of test sample was diluted with methanol to give a final theoretical concentration of 1.0 mg/l.

1.3 Standards

Standard solutions of test material were prepared in methanol at a nominal concentration of 1.0 mg/l.

1.4 Procedure

The standards and samples were analysed by HPLC using the following conditions:

HPLC System	:	Agilent Technologies 1100 MSD, incorporating autosampler and workstation
Mass selective detector		
Source	:	electrospray
Fragmentation energy	:	50volts
Polarity	:	negative
Mode	:	single ion mode with 269 amu, 313 amu and 314 amu
Gas temperature	:	275°C
Drying gas	:	11 litre/minute

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Appendix 1 (continued) Verification of Test Concentrations

Nebuliser pressure	:	40 psi
Capillary voltage	:	2000 volts
Gain	:	1
Column	:	Luna C18, 5 µ, (250 x 4.6 mm id)
Column temperature	:	30°C
Mobile phase	:	methanol:0.1% ammonium carbonate (90:10 v/v)
Flow rate	:	0.5 ml/min
Injection volume	:	5 µl
Retention time	:	approximately 5 minutes

2. VALIDATION

2.1 Linearity

A range of standard solutions covering 0.10 to 2.0 mg/l (10% to 200% of the standard concentration) was analysed.

Linearity was confirmed (correlation factor, $R^2 = 0.9967$) ranging from 0 to 2.0 mg/l.

The results are presented graphically on page 23.

2.2 Recoveries

A range of preliminary test samples, accurately fortified at known concentrations of test material, was prepared and analysed.

The recovery samples were prepared by direct addition of the test material to a sample of test medium.

A further portion of a test sample was analysed following the addition of algal cells to assess the effects of algae on the recovery of test material from test medium.

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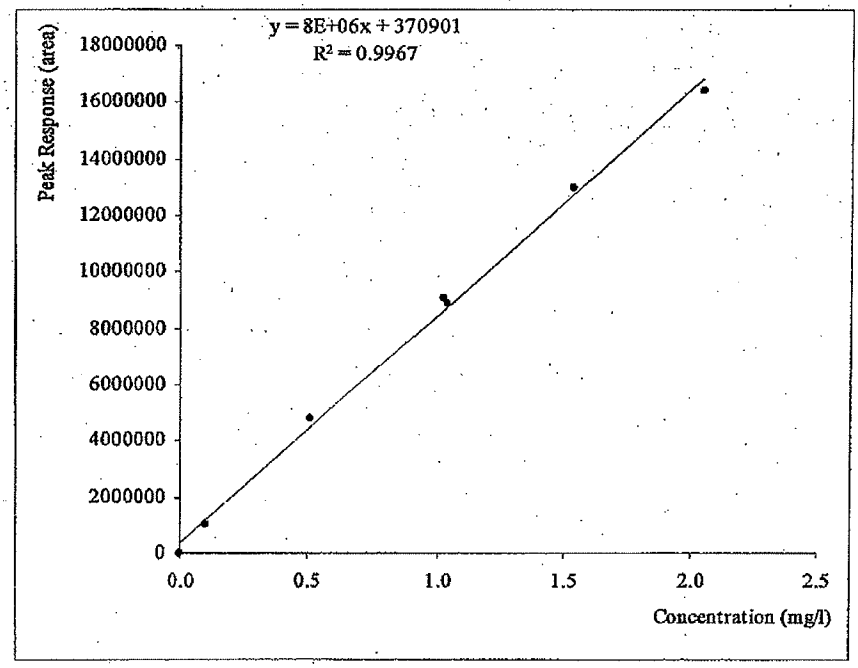
Appendix 1 (continued) Verification of Test Concentrations

Linearity of Detector Response



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Appendix 1 (continued) Verification of Test Concentrations

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
8.25	6.29	76	78
8.25	6.29	76	
33.0	25.9	79	
33.0	25.7	78	
132	104	79	
132	104	79	
8.25 plus algae	6.13	74	-
132 plus algae	103	78	

Although the results were just below the lowest accepted target of 80%, the low recovery was consistent over the test range and likely due to a systematic error of unknown origin.

The recoveries in test media from the Acute Toxicity to Rainbow Trout and *Daphnia magna* test (Safepharm Laboratories Project Numbers 1742/018 and 1742/019 respectively) indicated acceptable recovery. Therefore the method was considered acceptable, the test sample results have not been corrected for recovery.

The presence of algal cells was considered to have no significant effect on the recovery of the test material from the test medium.

2.3 Limit of Quantitation

The limit of quantitation has been assessed down to 0.035 mg/l.

3. STABILITY

A range of preliminary test samples was prepared, analysed initially and then after storage in sealed glass vessels at ambient temperature in light and dark conditions for approximately 72 hours (equivalent to the test exposure period). In addition a test sample was tested for stability without prior mixing (sonication) of the test sample bottle to assess for losses due to adsorption and/or insolubility.

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Appendix 1 (continued) Verification of Test Concentrations

Nominal concentration (mg/l)	6.25	25	100
Concentration found initially (mg/l)	6.29	25.8	104
Concentration found after storage in light conditions (mg/l)	6.34	25.9	106
Expressed as a percent of the initial concentration	101	100	102
Concentration found after storage in dark conditions (mg/l)	6.45	26.1	107
Expressed as a percent of the initial concentration	103	101	103
Concentration found after storage in dark conditions (mg/l) – unsonicated sample	6.40	NA	105
Expressed as a percent of the initial concentration	102	-	101

The test samples have been shown to be stable in the test medium.

The unsonicated stability vessel showed no evidence of insolubility or adherence to glass.

NA = Not applicable

Appendix 1 (continued) Verification of Test Concentrations

4. RESULTS

Sample	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration (%)
0 Hours	Control	<LOQ	-
	6.25	5.63	90
	12.5	12.4	99
	25	25.2	101
	50	49.3	99
	100	103	103
72 Hours	Control	<LOQ	-
	6.25	6.16	99
	12.5	13.1	105
	25	25.6	102
	50	50.3	101
	100	105	105

5. DISCUSSION

The detection system was found to have acceptable linearity. The analytical procedure had acceptable recoveries of test material in test medium. A method of analysis was validated and proven to be suitable for use.

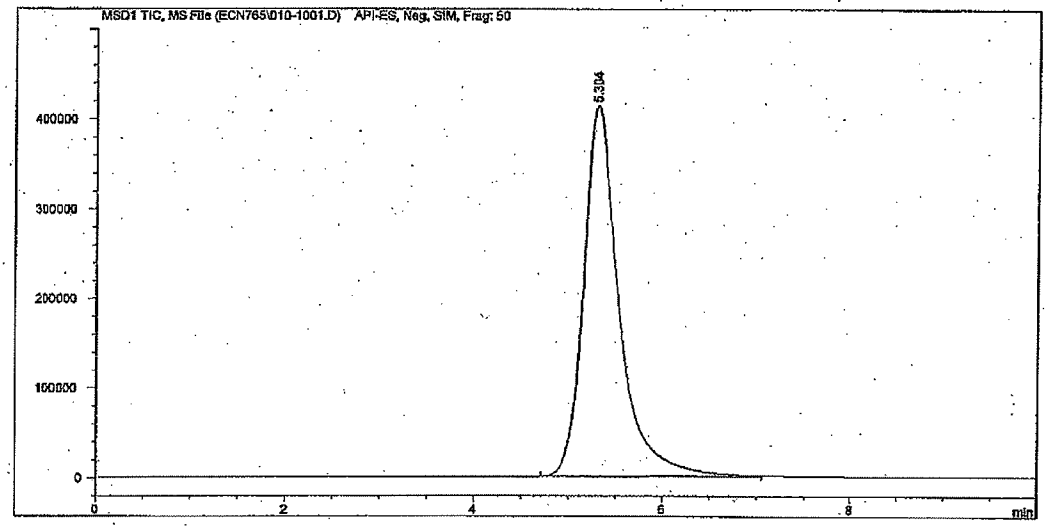
The test material was stable in the test medium for the duration of the test.

LOQ = Limit of quantitation

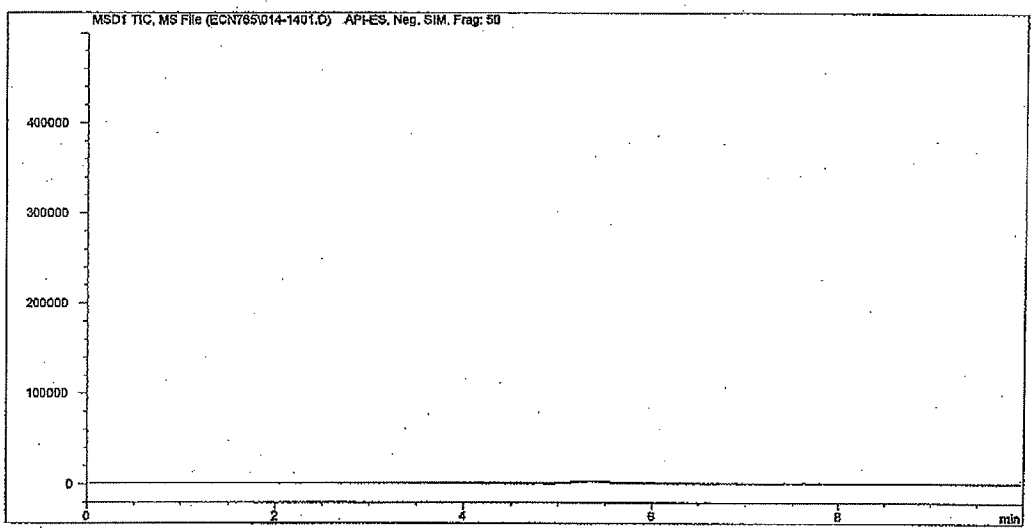
PFHA: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations

6. TYPICAL CHROMATOGRAPHY



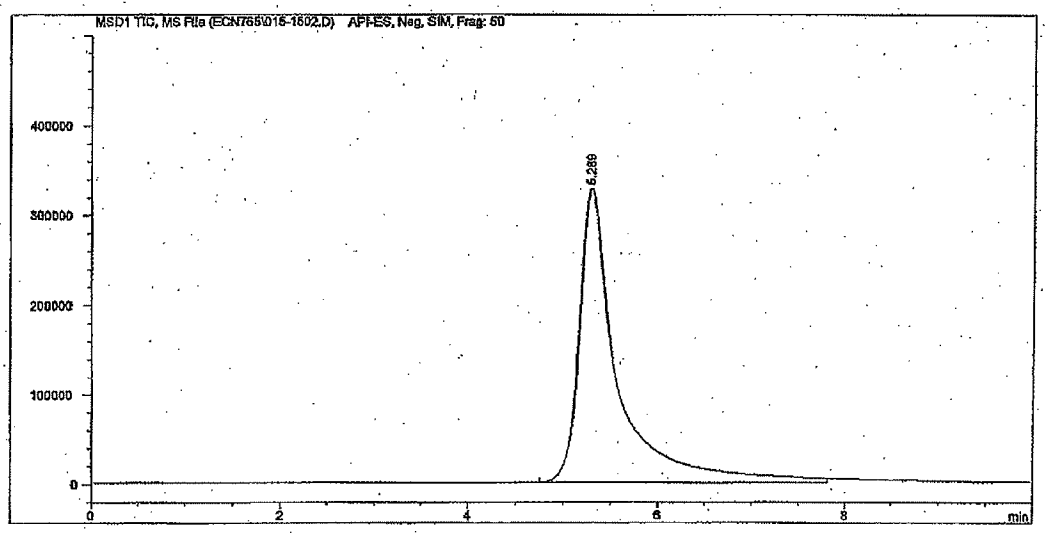
Standard 1.0 mg/l



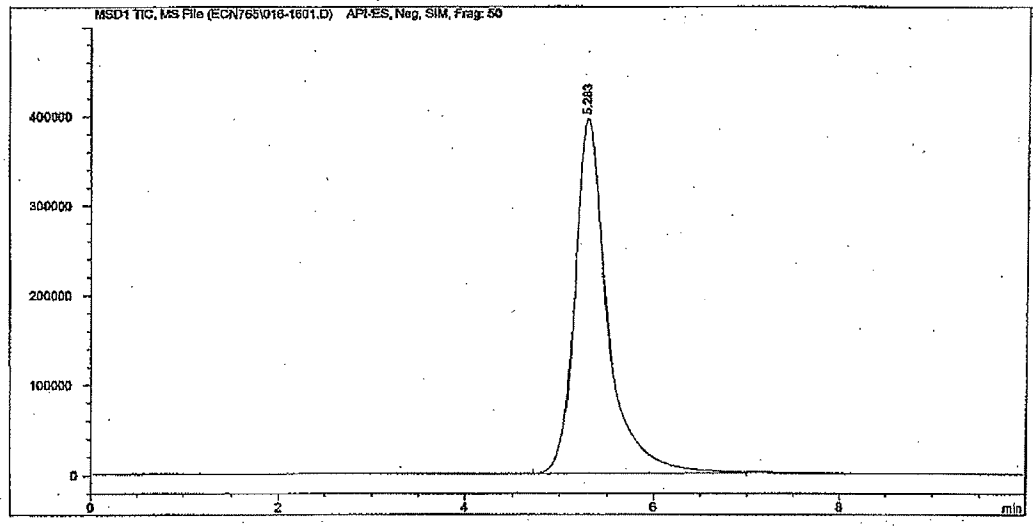
Control Sample

PFHA: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations



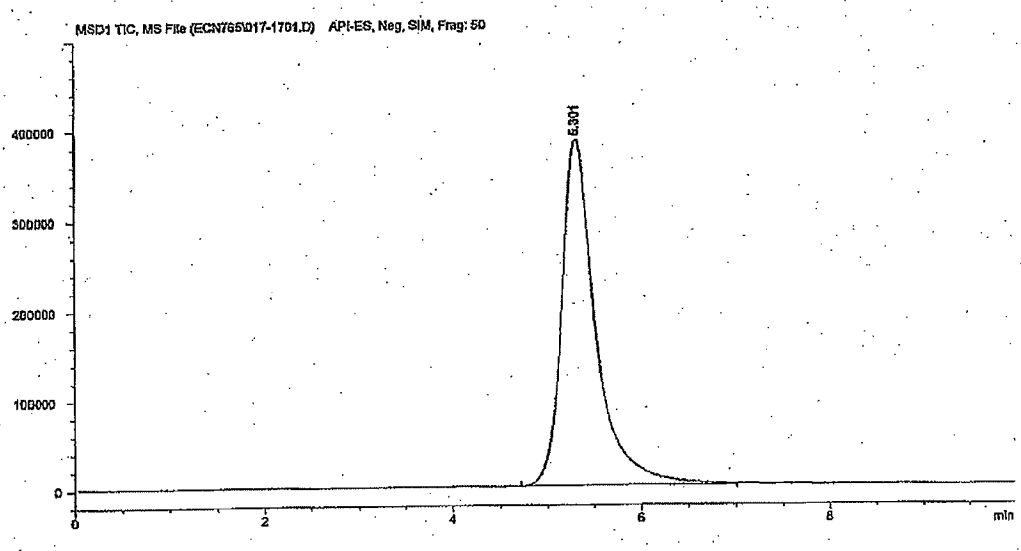
Test Sample 6.25 mg/l



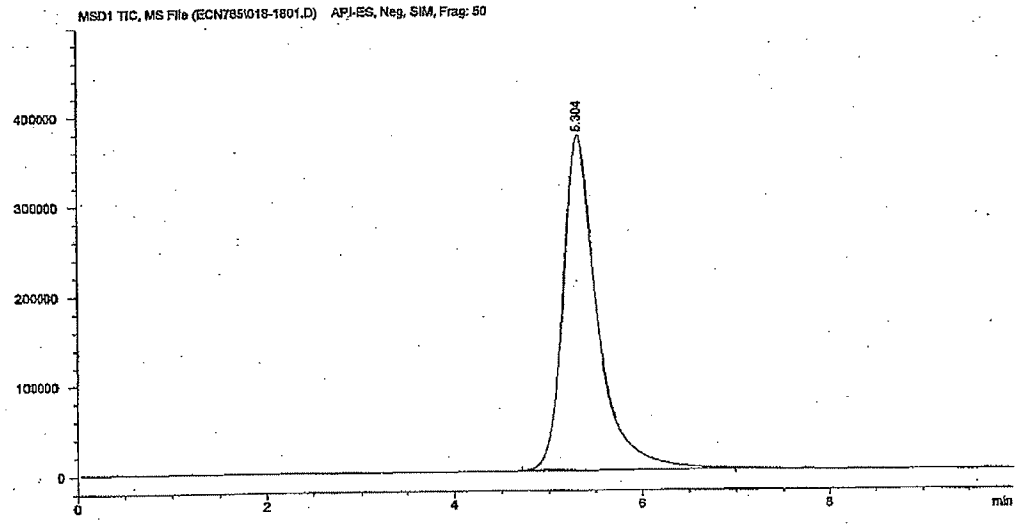
Test Sample 12.5 mg/l

PFHA: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations



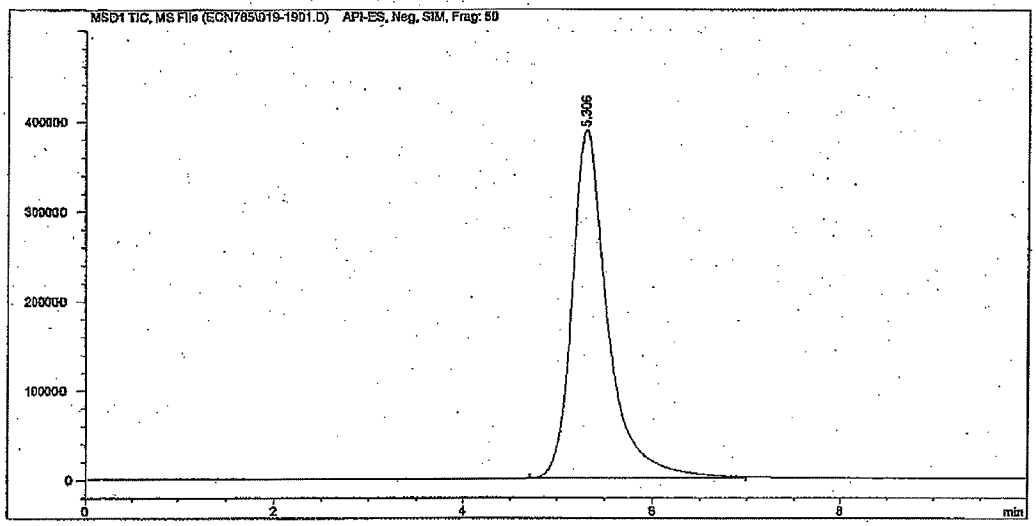
Test Sample 25 mg/l



Test Sample 50 mg/l

PFHA: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations



Test Sample 100 mg/l

PFHA: ALGAL INHIBITION TEST

Appendix 2 Culture Medium

$NaNO_3$	25.5	mg/l
$MgCl_2.6H_2O$	12.164	mg/l
$CaCl_2.2H_2O$	4.41	mg/l
$MgSO_4.7H_2O$	14.7	mg/l
K_2HPO_4	1.044	mg/l
$NaHCO_3$	15.0	mg/l
H_3BO_3	0.1855	mg/l
$MnCl_2.4H_2O$	0.415	mg/l
$ZnCl_2$	0.00327	mg/l
$FeCl_3.6H_2O$	0.159	mg/l
$CoCl_2.6H_2O$	0.00143	mg/l
$Na_2MoO_4.2H_2O$	0.00726	mg/l
$CuCl_2.2H_2O$	0.000012	mg/l
$Na_2EDTA.2H_2O$	0.30	mg/l
$Na_2SeO_3.5H_2O$	0.000010	mg/l

The culture medium was prepared using reverse osmosis purified deionised water (Elga Optima 15+ or Elga Purelab Option R-15 BP) and the pH adjusted to 7.5 ± 0.1 with 0.1N NaOH or HCl. The prepared media was sterilised by 0.2 µm membrane filtration.

Appendix 3 Statement of GLP Compliance in Accordance with Directive 88/320/EEC

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY
SafePharm Limited
Shardlow Business Park,
London Road,
Shardlow,
Derbyshire,
DE72 2GD

TEST TYPE
Analytical/Clinical
Chemistry
Environmental tox.
Environmental fate
Mutagenicity
Phys./Chem. tests
Toxicology

DATE OF INSPECTION

2nd December 2002

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

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

13/2/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority