[redacted]
Huntingdon
Life Sciences

[redacted]

ACUTE TOXICITY TO *DAPHNIA MAGNA*

Company Sanitized. Does not contain TSCA CBI

ACUTE TOXICITY TO *DAPHNIA MAGNA*

Sponsor

DuPont Speciality Chemicals,
Jackson Laboratory,
Chambers Works,
Deepwater,
NJ 08023,
USA.

Research Laboratory

Huntingdon Life Sciences Ltd.,
Eye,
Suffolk IP23 7PX,
ENGLAND.

Draft Report Issued 25 January 1999
Final Report Issued 4 March 1999

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

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 1997 (Statutory Instrument No. 654).

EC Council Directive, 87/18/EEC of 18 December 1986 (Official Journal No. L 15/29).

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

Eileen Daly

Eileen C. Daly, Nat. Diploma, NCEA Eire.
Study Director,
Huntingdon Life Sciences Ltd.

4th March 1999

Date

QUALITY ASSURANCE STATEMENT

The following have been inspected or audited in relation to this study

Study Phases Inspected	Date of Inspection	Date of Reporting
Protocol Audit	06 August 1998	06 August 1998
Process Based Inspections		
Formulation of test media	02 October 1998	02 October 1998
Sampling of test media	02 October 1998	02 October 1998
Experimental set-up	15 December 1998	15 December 1998
Daphnia observations	11 November 1998	11 November 1998
Report Audit	19 February 1999	19 February 1999

Protocol Audit: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

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

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results to reflect the raw data.

Helen Comb, B.Sc.(Hons.).
Principal Auditor,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

3 March 1999
Date

CONTRIBUTING SCIENTISTS

STUDY MANAGEMENT

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SUMMARY

The acute toxicity of [redacted] to *Daphnia magna* was assessed under static exposure conditions. Throughout this report, the exposure concentrations and the test results have been expressed in terms of the active ingredient [redacted]

The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 2 "Acute Toxicity for *Daphnia*" and the OECD Guideline for Testing of Chemicals No. 202, Part I "*Daphnia* Acute Immobilisation Test".

Groups of twenty *Daphnia*, less than 24 hours old, were exposed for 48 hours to [redacted] at nominal concentrations of 4.27, 9.39, 20.7, 45.5 and 100 mg/l (as active ingredient). The test media were prepared in Elendt M4 medium and to aid dissolution ultrasound treatment was employed.

The measured concentrations of [redacted] in samples unfiltered of media indicated that the intended exposure concentrations were adequately achieved (between 85 and 97% of their nominal values) and maintained (between 93 and 100% of their starting values). The overall mean measured levels were 4.01, 8.92, 18.6, 40.2 and 85.9 mg a.i./l.

Observations of the *Daphnia* in each control and test vessel were made after 24 and 48 hours. After 48 hours, 20% immobility had occurred at the highest concentration (85.9 mg a.i./l); the highest concentration at which no immobility had occurred was 40.2 mg a.i./l.

The 48-hour EC_{50} could not be calculated but must be >85.9 mg a.i./l and the "no-observed-effect concentration" (NOEC) was 40.2 mg a.i./l.

Under the EC General Classification and Labelling Requirements for Dangerous Substances and Preparations, [redacted] is not considered to require classification as the 48-hour EC_{50} is considered to be greater than the highest nominal concentration, 100 mg a.i./l (mean measured level of 85.9 mg a.i./l).

INTRODUCTION

The objective of the study was to determine the acute toxicity (48 hour median effect concentration - EC_{50}) of [redacted] to *Daphnia magna* at 20°C.

The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 2 "Acute Toxicity for *Daphnia*" and the OECD Guideline for Testing of Chemicals No. 202, Part I "*Daphnia* Acute Immobilisation Test".

The protocol was approved by Huntingdon Life Sciences Management on 7 July 1998, by the Sponsor on 17 July 1998 and by the Study Director on 3 August 1998.

The experimental phase of the study was conducted between 1 September and 20 November 1998 and the results of chemical analysis were issued by 8 December 1998.

Information provided by the Sponsor indicated that the solubility of [redacted] in water was 25% by weight at 35 - 40°C and that its [redacted] Throughout this report, the exposure concentrations and test results have been expressed in terms of the active ingredient. Also, the Sponsor indicated that at room temperature the test substance was a suspension in water and upon standing it would separate out into its component phases; accordingly, at the recommendation of the Sponsor, the suspension was warmed to 35 - 40°C (in a water bath) with gentle stirring to obtain an homogenous composition before use.

The sensitivity of juvenile *Daphnia* cultured in this laboratory is periodically assessed using the reference substance potassium dichromate. The results for the most recent test performed prior to this study indicated that its 48-hour EC_{50} to *Daphnia magna* was 0.57 mg/l; this was within the range typically obtained in this laboratory (0.3 to 0.8 mg/l).

TEST SUBSTANCE

Identity:	[redacted]
Chemical name:	[redacted]
Appearance:	Pale-yellow slurry
Storage conditions:	Room temperature in the dark
Lot number:	3
Expiry date:	2 years from date of receipt
Purity:	[redacted]
Sample received:	23 June 1998

EXPERIMENTAL PROCEDURE

TEST ORGANISM

Daphnia magna (Straus) used in this study were cultured in-house and were obtained from a strain originating from the Institute National de Recherche Chimique Appliqué (IRChA), France.

Stock cultures of *Daphnia magna* were maintained in glass vessels containing approximately 1.5 litres of Elendt M4 culture medium in a temperature-controlled laboratory at nominally 20 ± 2°C. A photoperiod of 16 hours light : 8 hours dark was maintained, with periods of subdued lighting at the beginning and end of each light phase. The culture medium was renewed at least twice each week.

Cultures were fed daily with a suspension of the unicellular green alga, *Chlorella vulgaris*, to provide 0.1 to 0.2 mg carbon per daphnid, per day. Culture conditions ensure that the stock animals reproduce by parthenogenesis.

The day before the start of the study, all juvenile *Daphnia* were removed from the laboratory cultures. The following morning, juveniles produced by the gravid (egg-bearing) adult *Daphnia* were removed from the culture vessels and held in a separate holding vessel; these animals, which were less than 24 hours old, were used in the test.

DILUTION MEDIUM

The test organisms were maintained and the tests conducted in Elendt M4 medium (Appendix 1). The medium was prepared in reverse osmosis water.

TEST SUBSTANCE PREPARATION

Method of preparation

Based on information provided by the Sponsor, the test substance was warmed (to *c.*38°C) in a water bath and gently swirled to provide a homogenous mixture before weighing.

At all concentrations, the test media were individually prepared by adding the appropriate weights of substance (17.1, 37.6, 82.8, 182 and 400 mg) to dilution medium in a volumetric flask (1 l); to aid dissolution, the contents of the flasks were treated with ultrasound for thirty minutes before being made up to volume with dilution medium.

Stability of test concentrations

The test concentrations of [redacted] were measured using an HPLC method of chemical analysis (see Appendix 2). The stability of the test substance in dilution medium under refrigerated storage conditions was determined before the start of the study (Table 2, Appendix 2).

At the start of the definitive test, four samples (100 ml) were taken from the freshly-prepared control and test media; after 48 hours, the contents of the test vessels from each group were pooled and further samples were taken for analysis. They were stored in a refrigerator until two samples from each set were transferred to Huntingdon Research Centre, Cambridgeshire; one sample per concentration was analysed and all of the other samples remained in storage at Eye Research Centre in case further analysis was required.

EXPOSURE CONDITIONS

Experimental design

A rangefinding test was conducted, followed by a definitive (limit) test with one test concentration (100 mg a.i./l) plus a control (dilution medium). Because 60% immobility occurred in the limit test it was repeated and 50% immobility was observed. Another test was then undertaken employing five test concentrations (4.27, 9.39, 20.7, 45.5 and 100 mg a.i./l) but this gave variable results. It was suspected that repeated warming (to 38°C) of the small volume of test substance in the sub-sample container used for the aquatic studies may have affected the chemical characteristics of the test substance. Therefore, a new sub-sample of the test substance was obtained and a definitive test was conducted with five test concentrations and the results are reported here.

Twenty *Daphnia*, four replicates of five animals per vessel, were exposed in each control and test group.

The first instar *Daphnia* were placed in groups of five, at random into glass dishes containing 100 ml of medium to give a loading of 20 ml medium per organism. The dishes were loosely covered.

Test concentrations

The preliminary tests employed test concentrations within the range 1 to 100 mg/l (as active ingredient). The definitive test concentrations, which were selected based on the results of the preliminary tests, were:

Nominal concentrations: 4.27, 9.39, 20.7, 45.5 and 100 mg active ingredient/l.

(Expressed in terms of the test substance as received: 17.1, 37.6, 82.8, 182 and 400 mg/l)

Medium renewal

Daphnia were exposed to the test or control conditions for a period of 48 hours without renewal of test media.

Environmental conditions

The temperature of the test area was maintained at 20 ± 2°C and constant to within ± 1°C during the study. Temperature was continuously monitored in an additional vessel containing the same volume of dilution medium. A photoperiod of 16 hours light : 8 hours dark was maintained, with periods of subdued lighting at the beginning and end of each light phase. No supplementary aeration was employed and no feed was given during the exposure period.

The temperature, pH and dissolved oxygen levels of control and test media were recorded at the start and at the end of the study.

CRITERION OF EFFECT

Daphnia were considered to be immobile if they were unable to swim within approximately 15 seconds following gentle agitation of the test vessel.

The numbers of mobile, immobile and floating *Daphnia* were counted 24 and 48 hours after the start of the study.

EVALUATION OF DATA

The "no-observed-effect concentration" (NOEC) was derived by direct inspection of the data on the immobility of the animals. An incidence rate of more than 10% is considered to be significant.

PROTOCOL DEVIATIONS

None.

MAINTENANCE OF RECORDS

All specimens, raw data and study related documents generated during the course of the study at Huntingdon Life Sciences, together with a copy of the final report will be lodged in the Huntingdon Life Sciences Archive.

Such specimens and records will be retained for a minimum period of five years from the date of issue of the final report. At the end of the five year retention period the Sponsor will be contacted and advice sought on the future requirements. Under no circumstances will any item be discarded without the Sponsor's knowledge.

RESULTS

Chemical analysis

The results of chemical analysis are given in Table 1 and an example chromatogram is illustrated in Figure 1. Concentrations are expressed in terms of the measured levels of the active ingredient.

Results for unfiltered samples of [redacted] indicated that the intended exposure concentrations were adequately achieved (between 85 and 97% of nominal values) and were maintained (between 93 and 100% of starting values). The overall mean measured levels were 4.01, 8.92, 18.6, 40.2 and 85.9 mg a.i./l.

Immobility

Observations of the *Daphnia* in each control and test vessel, made after 24 and 48 hours, are listed in Table 2.

After 48 hours, 20% immobility occurred at the highest exposure concentration (85.9 mg a.i./l) and the highest concentration at which no immobilisation had occurred was 40.2 mg/l.

The 48-hour EC_{50} could not be calculated but must be >85.9 mg a.i./l.

Under the EC General Classification and Labelling Requirements for Dangerous Substances and Preparations, [redacted] is not considered to require classification as the 48-hour EC_{50} is considered to be greater than the highest nominal concentration, 100 mg a.i./l (mean measured level of 85.9 mg a.i./l).

Environmental parameters

The measurements of water quality (temperature, pH, dissolved oxygen and total hardness) are summarised in Table 3; they remained within acceptable limits during the study.

The test media were clear and colourless.

CONCLUSIONS

The 48-hour EC_{50} of [redacted] for the immobilisation of *Daphnia magna* could not be calculated but must be >85.9 mg active ingredient/l.

The 'no-observed-effect concentration' of [redacted] with *Daphnia magna* was 40.2 mg active ingredient/l.

REFERENCE

Official Journal of the European Communities Commission Directive (1 March 1991). Annex VI General classification and labelling requirements for dangerous substances and preparations. Part II "*Classification on the Basis of Environmental Effect*" p.62 – 64.

FIGURE 1

Typical sample chromatogram - 37.6 mg/l (9.39 mg a.i./l) taken from Day 2 of the study

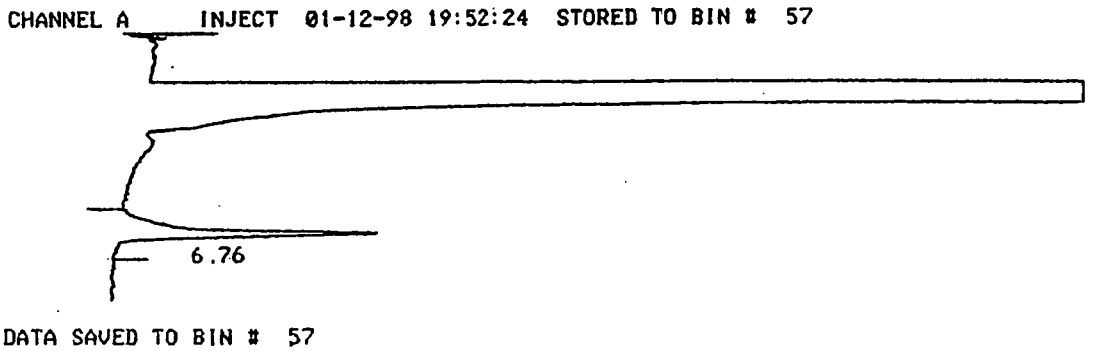


TABLE 1

Measured concentrations [redacted]

Nominal conc.,$ (mg/l)	Measured [redacted] concentrations (mg/l)			
	0 hours	48 hours	% ti	Overall mean
0	nd	nd	-	-
4.27	4.14 [97]	3.87 [91]	93	4.01 (94)
9.39	8.93 [95]	8.90 [95]	100	8.92 (95)
20.7	18.7 [90]	18.5 [89]	99	18.6 (90)
45.5	41.5 [91]	38.9 [85]	94	40.2 (88)
100	86.2 [86]	85.5 [86]	99	85.9 (86)

$: in terms of the active ingredient [redacted]
nd : none detected (< 0.5 mg active ingredient/l).
% ti : measured concentration after 48 hours expressed as a percentage of the starting concentration.
[ ] : measured concentration expressed as a percentage of the nominal concentration.
( ) : overall mean measured concentration expressed as a percentage of the nominal concentration.

TABLE 2

Cumulative immobilisation

Nominal conc.,$ mg/l (measured)	Cumulative numbers of immobile *Daphnia*											
	24 hours						48 hours					
	R_1	R_2	R_3	R_4	total	%	R_1	R_2	R_3	R_4	total	%
Control (nd)	0	0	0	0	0	0	0	0	0	0	0	0
4.27 (4.01)	0	0	0	0	0	0	0	0	0	0	0	0
9.39 (8.92)	0	0	0	0	0	0	0	0	0	0	0	0
20.7 (18.6)	0	0	0	0	0	0	0	0	0	0	0	0
45.5 (40.2)	0	0	0	0	0	0	0	0	0	0	0	0
100 (85.9)	0	0	0	0	0	0	2	2	0	0	4	20

$: as active ingredient [redacted]
nd : none detected (< 0.5 mg active ingredient/l)
R : replicate number

TABLE 3

Environmental parameters - temperature, pH and dissolved oxygen

Nominal conc.,$ mg/l (measured)	Temperature °C		pH		Dissolved oxygen %ASV	
	0 h	48 h	0 h	48 h	0 h	48 h
Control (nd)	19.7	19.8	7.8	7.3	95	92
4.27 (4.01)	20.0	19.9	7.8	7.8	93	91
9.39 (8.92)	20.0	20.0	7.7	7.6	92	92
20.7 (18.2)	19.9	19.9	7.6	7.8	92	92
45.5 (40.2)	20.2	19.9	7.5	7.8	91	91
100 (85.9)	19.8	20.0	7.2	7.8	89	89

$: as active ingredient [redacted]
nd : none detected (<0.5 mg active ingredient/l).
ASV : air saturation value.

The total hardness and alkalinity of the batch of Elendt M4 medium used were 240 mg/l and 65 mg/l as $CaCO_3$ respectively.

Continuous monitoring of an additional vessel containing diluent = 19.9 to 20.4°C.

APPENDIX 1

ELENDT M4 MEDIUM

1.	Trace elements	mg/l
	H_3BO_3	2.86
	$MnCl_2.4H_2O$	0.36
	LiCl	0.31
	RbCl	0.071
	$SrCl_2.6H_2O$	0.152
	NaBr	0.016
	$Na_2MoO_4.2H_2O$	0.063
	$CuCl.2H_2O$	0.0165
	$ZnCl_2$	0.013
	$CoCl_2.6H_2O$	0.010
	KI	0.0033
	$Na_2.SeO_3$	0.0022
	NH_4VO_3	0.00058
	Fe-EDTA solution	3.50
2.	Macro nutrients	mg/l
	$CaCl_2.2H_2O$	294
	$MgSO_4.7H_2O$	123
	KCl	5.80
	$NaHCO_3$	64.8
3.	Buffer nutrients	mg/l
	$Na_2.SiO_3.9H_2O$	10
	$NaNO_3$	0.274
	KH_2PO_4	0.143
	K_2HPO_4	0.184
4.	Vitamins	mg/l
	Thiamine hydrochloride	0.075
	Cyanocobalamine (B12)	0.0010
	Biotin	0.00075

The above analytical grade reagents are dissolved in reverse osmosis water.

APPENDIX 2

THE DETERMINATION OF [redacted] IN AQUEOUS MEDIA

SAMPLE ANALYSIS

The aqueous samples were diluted with sodium hydroxide and acetonitrile to bring the expected [redacted] concentrations within the calibration range. Determination of [redacted] was by high performance liquid chromatography (HPLC) using a conductivity detector[1].

CHROMATOGRAPHY INSTRUMENTATION AND CONDITIONS

A high performance liquid chromatography system comprising autosampler, pump, conductivity detector, anion suppressor and data collection system was used.

Column	
Type:	PLRP-S supplied by Polymer Laboratories
Dimensions (l × id):	250 × 4.6 mm
Temperature:	Ambient
Mobile phase	
Composition:	Acetonitrile : aqueous buffer solution (25 : 75% v/v)
Flow rate:	1.0 ml/min
Suppresser type:	
Regenerant composition:	50 mN sulphuric acid
Flow rate:	2.5 ml/min
Injection volume:	100 µl

Aqueous buffer solution: 2mM ammonium hydroxide/1mM sodium carbonate (made up in ultra high purity water) and filtered through 0.2 micron cellulose nitrate filter paper.

Under the above conditions [redacted] chromatographed as a single peak (see Figure 3).

[1] A method contained in a fax dated 23 July 1998 from Kavsy D Dastur, Dupont Specialty Chemicals was modified to comply with Huntingdon Life Sciences standard operating procedures and instrumentation.

CALIBRATION SOLUTIONS

Calibration solutions were prepared with the same batch of [redacted] used in the preparation of the toxicity test solutions.

Trout and Algae Studies

Working calibration solutions in the nominal range 40 to 500 mg/l (equal to 10 to 125 mg a.i./l) were prepared by volumetric dilution with acetonitrile: 100 mM sodium hydroxide (25: 75% v/v) of a primary standard prepared in ultra high purity water.

***Daphnia* Study**

Working calibration solutions in the nominal range 4.0 to 50.0 mg/l (equal to 1.0 to 12.5 mg a.i./l) were prepared by volumetric dilution with acetonitrile : 100 mM sodium hydroxide (25: 75% v/v) of a primary standard prepared in ultra high purity water.

CALCULATIONS

[redacted] concentrations were determined using mean bracketing standards.

The mean peak height responses were calculated for [redacted] in bracketing standard chromatograms. The [redacted] concentration in each sample was then calculated using the following equation:

$$\text{Concentration [redacted] (mg/l)} = \frac{Y \times A}{Z} \times F$$

Where
- Y = Detector response to [redacted]
- Z = Mean detector response to bracketing standard.
- A = Concentration of bracketing standard (mg/l).
- F = Factor to take into account sample processing.

The purity [redacted] of the test substance is given in the Test Substance Data Sheet [redacted]. Nominal and fortified concentrations are reported as [redacted] as supplied and in terms of the active ingredient.

VALIDATION OF THE ANALYTICAL PROCEDURE

The analytical procedure was validated by determining the linearity of response of the analytical system, specificity of chromatographic analysis, the limit of detection, and the method's accuracy and precision.
During the course of the study the performance of the method was monitored by the analysis of quality control samples.

TABLE 1

Validation recoveries of [redacted] from fortified samples of dilution media

Medium	Fortification level (mg/l)		Recovery as a % of fortification level
	As supplied	As active ingredient	
Dechlorinated tap water	Control	Control	ND
	387.9	96.98	95.8
	387.9	96.98	104
Algal	Control	Control	ND
	535.5	133.9	92.0
	530.0	132.5	101
	214.2	53.55	92.6
	212.0	53.00	95.5
Elendt M4	Control	Control	ND
	9.93	2.483	98.5
	9.93	2.483	95.9
	496.5	124.1	103
	496.5	124.1	103
Overall mean (RSD)			98.1 (4.2)

Active ingredient content of [redacted]
RSD: relative standard deviation.
ND: none detected; less than the limit of detection (trout and algal studies : 2.5 mg a.i./l; *Daphnia* study: 0.5 mg a.i./l).
The limit of detection is defined as the analyte concentration in a processed sample which would give a peak equal to 3 × local base-line noise.

TABLE 2

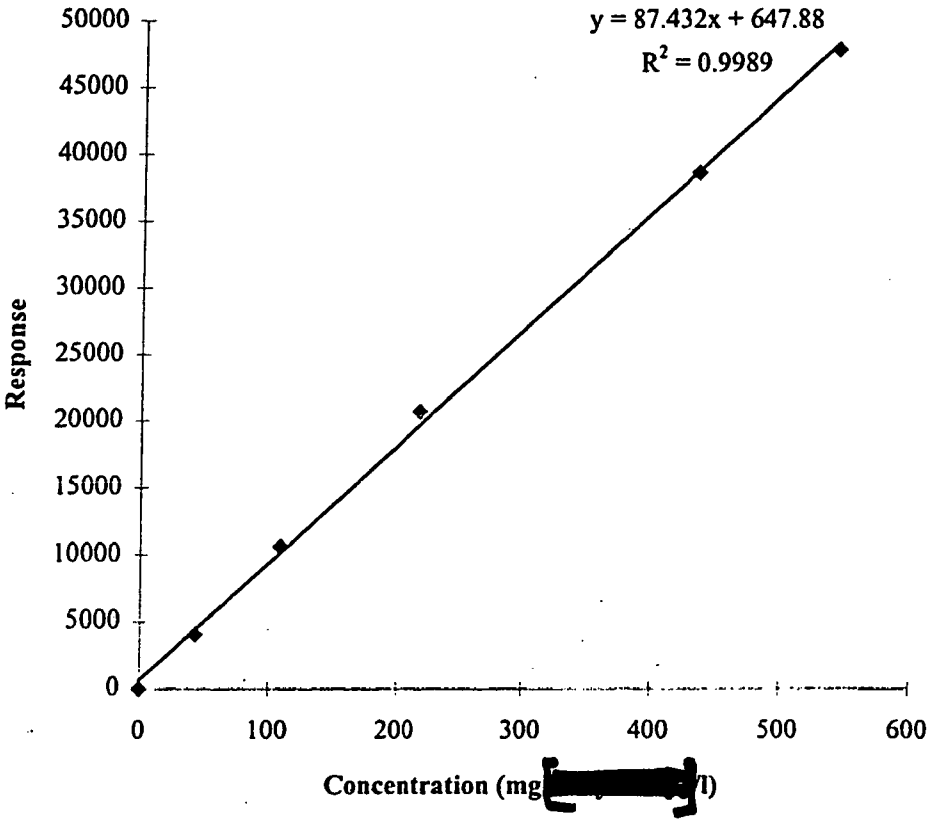
Stability of [redacted] in dilution medium

Storage conditions	Time-point	
	0 hours	20 hours
Procedural recovery [1]	110	-
Procedural recovery [1]	112	-
Light, sealed, room temperature [1]	-	103
Light, sealed, room temperature [1]	-	106
Dark, sealed, 4°C [1]	-	109
Dark, sealed, 4°C [1]	-	93.1
Procedural recovery [2]	-	109
Procedural recovery [2]	-	98.9

[1] Fortification level: 11.20 mg/l as [redacted]
[2] Fortification level: 11.65 mg/l as [redacted]
Results are given as percentage recoveries of [redacted] after storage for the indicated time.

FIGURE 1

Standard Calibration for [redacted]
(*ca* 40 - 500 mg/l)



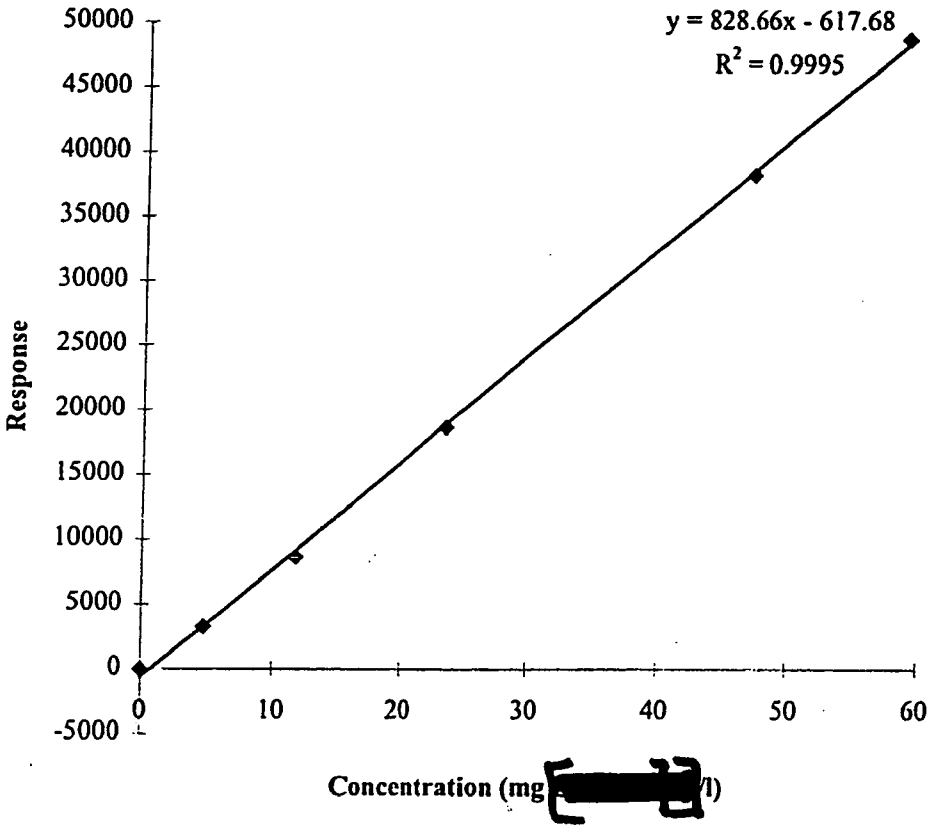
Run no.:DPT/439/013

Standard concentration (mg/l)	Peak height
0.0	NOP
43.57	4075
108.9	10668
217.9	20734
435.7	38644
544.7	47864

NOP: no observable peak

FIGURE 2

Standard Calibration for [redacted]
(*ca* 4 to 50 mg/l)



Run no.:DPT/440/002

Standard concentration (mg/l)	Peak height
0.0	NOP
4.720	3295
11.80	8657
23.60	18669
47.20	38224
59.00	48699

NOP: no observable peak

FIGURE 3

Typical calibration chromatography - 217.9 mg/l (= 54.47 mg/l as a.i.)

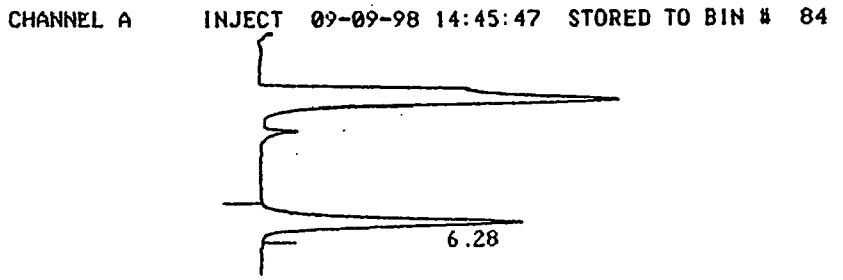


FIGURE 4

Typical chromatography - Unfortified algal medium

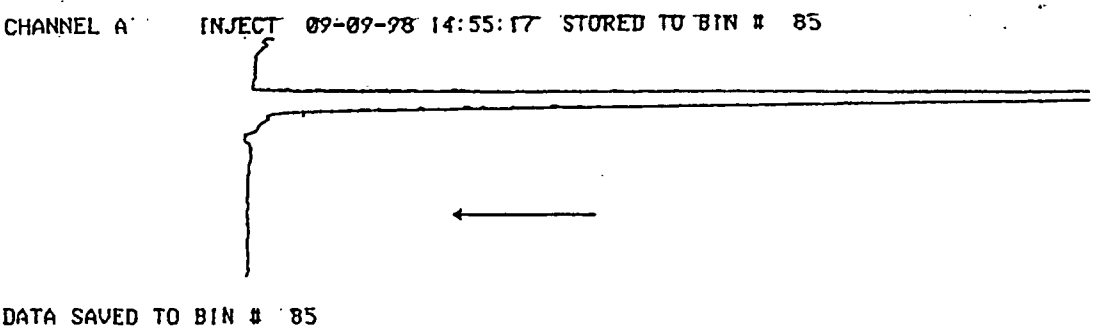


FIGURE 5

Typical chromatography
Sample of algal medium fortified at 535.5 mg/l (= 133.9 mg/l as a.i.)