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U.S. Environmental Protection Agency – East
TSCA Section 8(e)
Room 6428
1201 Constitution Avenue, NW
Washington, DC 20460-0001

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SUBJECT: Notice in Accordance with TSCA Section 8(e)

Algal Growth Inhibition Assay

has received the final report, February 11. 2011, regarding algal growth inhibition assay for the substance , which was the subject of PMN P94-1487. The full chemical name of this substance is considered CBI, generically identified as *thia alkanethiol.*

The conclusion of this test results indicated "no observed effect concentration" for growth rate was 0.00947 mg/L, for area under the growth curve was <0.00947 mg/L and the yield was 0.00947 mg/L.

Please note that the complete **chemical identity** of this substance is considered **Confidential Business Information**. Also enclosed is a sanitized copy of the report.

Sincerely,

William A. Olson, Ph.D.
Consultant

WAO:gbt
MTCA33-8E-2

Enclosure
CBI Report , 11 February 2011, 41 pages
Sanitized Report , 11 February 2011, 41 pages

Cc:

Company Sanitized

Sanitized

GST

ALGAL GROWTH INHIBITION ASSAY

HLS study number:	
Version ID:	Final report
Issue date:	11 February 2011

Sponsor and Test Facility Details

Sponsor

Test Facility

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Compliance with Good Laboratory Practice

GST: Algal Growth Inhibition Assay

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

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

EC Commission Directive 2004/10/EC of 11 February 2004 (Official Journal No L 50/44).

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.

11 February 2011
Date

Quality Assurance Statement

GST: Algal Growth Inhibition Assay

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	20 Aug 2008	20 Aug 2008
Protocol Amendment No.1	29 Sep 2009	29 Sep 2009
Protocol Amendment No.2	08 Oct 2009	08 Oct 2009
Protocol Amendment No.3	04 Feb 2010	04 Feb 2010
Protocol Amendment No.4	31 Mar 2010	31 Mar 2010
Report Audit	27 Aug 2010-02 Sep 2010	02 Sep 2010

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
Dose formulation	01 Feb 2010	01 Feb 2010
Experimental set-up	02 Feb 2010	02 Feb 2010
Sampling of test media for chemical analysis	05 Mar 2010	05 Mar 2010
Environmental measurements	13 Apr 2010	13 Apr 2010
Chromatography	29 Apr 2010	29 Apr 2010
Data Processing	29 Apr 2010	30 Apr 2010
Standard preparation	07 Jul 2010	07 Jul 2010
Fortification procedures	08 Jul 2010	08 Jul 2010

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

..... 11 February 2011

Date

Contributing Scientists

GST: Algal Growth Inhibition Assay

Study management

Aquatic Ecotoxicology and Biodegradation

Other Responsible Personnel

Aquatic Ecotoxicology and Biodegradation

Expert department

Environmental Analysis

Summary

The effect of GST on the growth of the unicellular green alga *Pseudokirchneriella subcapitata* was assessed under non-axenic conditions.

The study was conducted in accordance with EC Methods for Determination of Ecotoxicity, Annex to Directive 92/69/EEC Part C, Method 3 "Algal Inhibition Test" and Procedure 201 of the "Guidelines for Testing of Chemicals" of the Organisation for Economic Co-operation and Development: Freshwater Alga and Cyanobacteria, Growth Inhibition Test" (2006).

Due to the hydrolytic nature of the test substance, aspects of the study were designed following guidance outlined in OECD "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures" Number 23 (ENV/JM/MONO(2000)6), to ensure the test organism was exposed to both parent and degradate products.

Triplicate algal cultures, with an initial cell density of 1 x 10^4/mL, were exposed to GST dispersed in algal nutrient medium at nominal concentrations of 0.00970, 0.0231, 0.0470, 0.103, 0.227 and 0.5 mg/L. At each concentration, the test substance was dissolved in acetone before an aliquot was dispersed in OECD medium. The cultures were shaken in an orbital incubator under continuous illumination (mean values of 6570 to 6720 lux) at temperatures ranging from 22.1 to 23.1°C for 72 hours.

It was not possible to develop an analytical method with sufficient sensitivity to detect the required levels of GST in OECD medium. Therefore, an analytical method was developed to measure the levels of GST in acetone solvent stock solutions.

The measured levels of GST in samples of the solvent stock solutions ranged between 94 and 110% of their nominal values. Using the levels measured in the stock solutions and taking into account the dilution factor for preparation of the test media (1:10,000), the levels of GST in the aqueous media were calculated to be 0.00947, 0.0218, 0.0481, 0.105, 0.250 and 0.492 mg/L.

Cell numbers were counted daily to monitor growth. The test results are expressed in terms of growth rate, area under the growth curve and yield. The following values were derived from the data and are expressed in terms of the calculated GST concentrations in the test media.

Parameter	EC_{50} (mg/L)	EC_{10} (mg/L)	NOEC (mg/L)
Average specific growth rate	>0.492 (unavailable)	0.117 (0.0495 & 0.24)	0.00947
Area under the growth curve	0.164 (0.107 & 0.254)	0.0122 (0.00355 & 0.0339)	<0.00947*
Yield	0.264 (0.12 & 0.582)	0.0219 (0.000812 & 0.283)	0.00947

( ) : 95% confidence limits

*: statistical analysis of the data of the data for area under the growth curve indicated that at 0.00947 and 0.0218 mg/L the levels of inhibition (14.9 and 12.0% respectively) were significantly lower when compared to the solvent control, so a statistically derived NOEC was not identified for this parameter. However, an E_bC_{10} value calculated using the curve fitted to the data gave a value of 0.0122 mg/L. Since the E_bC_{10} was higher than the lowest level employed in the test, this value (0.0122 mg/L) is considered to represent the level of no effect to the test organism for area under the growth curve.

1. Introduction

This study was designed to assess the effect of GST on the growth of the unicellular green alga *Pseudokirchneriella subcapitata.*

The study was conducted in accordance with EC Methods for Determination of Ecotoxicity, Annex to Directive 92/69/EEC Part C, Method 3 "Algal Inhibition Test" and Procedure 201 of the "Guidelines for Testing of Chemicals" of the Organisation for Economic Co-operation and Development: Freshwater Alga and Cyanobacteria, Growth Inhibition Test" (2006).

The protocol was approved by and the Study Director on 18 August 2008 and by the Sponsor on 25 August 2008.

The experimental start and completion dates of the study were 29 August 2008 and 22 July 2010, respectively.

The study was conducted at

Information provided by the Sponsor indicated that at 25°C, under abiotic aqueous conditions at a pH of 7, the half-life of GST is less than 24 hours. Following information given in the OECD "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures" Number 23 (ENV/JM/MONO(2000)6), the study was designed to expose the test organisms to both parent and degradate products.

The results of the most recent laboratory reference test using potassium dichromate indicated that its 72-hour E_bC_{50} to *Pseudokirchneriella subcapitata* was 0.946 mg/L; this was within the range typically obtained in this laboratory (0.3 to 1 mg/L).

2. Test substance

Identity: GST

Chemical name:

Appearance:

Storage conditions:

Batch number:

Expiry date:

Purity/assay:

Water solubility:

Sample received (Huntingdon Site):

Certificate of analysis:

3. Experimental procedure

3.1 Test species

3.1.1 Name

Pseudokirchneriella subcapitata, Strain No. CCAP 278/4.

3.1.2 Source

Axenic, uni-cellular, liquid slope cultures of algae were obtained from the Culture Collection of Algae and Protozoa (CCAP), SAMS Research Services Ltd., Dunstaffnage Marine Laboratory, Dunbeg, Oban, Argyll, Scotland and arrived on 9 February 2010.

3.1.3 Pre-culture

The liquid slope cultures were stored in an illuminated refrigerator. Sterile algal nutrient medium (Appendix 2) was inoculated with cells aseptically removed from the slope culture; these primary liquid cultures (100 mL) were incubated for approximately three days in an orbital incubator under continuous illumination at nominal temperatures in the range 21 to 25°C. Subsequently, appropriate volumes of these primary cultures were aseptically transferred to fresh sterile algal nutrient medium to prepare secondary liquid cultures; these cultures were incubated, as stated above, for a further three days to provide an inoculum in the log phase of growth, characterised by a cell density of 1.05×10^6 cells/mL.

3.2 Culture medium

Sterile algal nutrient medium as recommended in Official Journal No. 761/2009 Part C.3 and OECD Procedure 201 (see Appendix 2).

3.3 Test substance preparation

The method of preparation used during the definitive test was based on the results of range finding tests and formulation trials that were conducted as part of another study being performed for the Sponsor on the same test substance (HLS Study Number MTU0428).

The test substance (87.2 µL) was dissolved in acetone (20 mL) to create a solvent stock solution at 5.0 mg/mL. This solution was serially diluted to provide intermediate solvent stock solutions at 2.27, 1.03, 0.470, 0.231 and 0.0970 mg/mL. An aliquot (equivalent to 100 µL/L) of the appropriate stock solution was added to dilution medium (500 mL) in a volumetric flask. The contents of each flask were shaken vigorously before use.

An aliquot (4.8 mL) of the secondary algal inoculum was added to a portion (500 mL) of the test medium at each concentration, to give an initial cell density of 1×10^4 cells/mL. An aliquot (100 mL) of the appropriate inoculated test medium was added to each of the test vessels.

As an intermediate vehicle was used to facilitate the preparation of the test medium, an additional control group containing acetone and dilution medium (100 µL/L) was included in the study. The solvent control vessel was filled with dilution medium containing the same concentration of auxiliary substance as present in the test concentration. The solvent selected for use in this study was based on the results of formulation trials conducted as part of HLS Study Number

The use of a solvent stock solution series followed recommendations in OECD Procedure 201 and Document Number 23 to promote dissolution of the test substance following trials which indicated a low level of aqueous solubility. Acetone was identified as the most suitable solvent and the study employed a control and solvent control group to ensure any effects on the test results could be identified.

3.4 Exposure conditions

3.4.1 Experimental design

The study comprised two range finding tests and a definitive test with five test concentrations, plus an algal nutrient medium control group and a solvent control (100 µL/L) group.

The rangefinding tests were conducted using media prepared from aqueous solutions. In the absence of a suitable analytical method, it was not possible to measure the levels of GST in aqueous samples and therefore exposure concentrations were not verified. Based on an agreement with the German regulatory authorities and following the results of HLS Study Number MTU0428, the definitive test was conducted using acetone stock solutions and samples of solvent stock solutions were analysed.

Six flasks were established for each control group and three flasks for each test group. All of the control and test flasks were incubated. The media remaining in the preparation flasks were used for water quality measurements at the start.

Before the start of the test, the required number of empty test vessels (250 mL conical flasks), were loosely stoppered with foam bungs, covered with aluminium foil that was secured by autoclave tape and sterilised by autoclaving (121°C for at least 15 minutes). After the addition of the inoculated test medium (100 mL), each flask was then loosely plugged with a foam bung.

The medium used for the control groups was prepared as described for the test media except that no test substance or solvent were added and a larger volume (700 mL) of medium was made.

3.4.2 Test concentrations

The first range finding test employed nominal test concentrations of 0.1, 1.0 and 10 mg/L. After 72 hours, algal growth was inhibited by 40, 96 and 99% respectively.

The second range finding test employed nominal test concentrations of 0.001, 0.01, 0.1 and 1.0 mg/L. After 72 hours, algal growth was inhibited by 70 and 95% at 0.1 and 1.0 mg/L respectively; no significant inhibition occurred at 0.001 or 0.01 mg/L.

The definitive test concentrations, selected based on the results of the range finding tests, were 0.00970, 0.0231, 0.0470, 0.103, 0.227 and 0.5 mg GST/L.

3.4.3 Stability of test concentrations

The test concentrations of GST in the acetone solvent stock solutions were measured using a High Performance Liquid Chromatographic method of analysis with UV detection (see Appendix 3). At the start of the definitive test, a single sample (10 mL) was taken from the freshly-prepared solvent stock solutions for analysis.

3.4.4 Environmental conditions

Conical flasks (250 mL) each containing control or test culture (100 mL) were placed in an illuminated orbital incubator according to a random number sequence. The cultures were incubated, without renewal of medium for 72 hours under continuous illumination (nominally 4440 to 8880 lux.) provided by 6 x 30 W "cool white" 1 metre fluorescent tubes. The temperature was maintained at 23 ± 2°C.

Temperature and pH of control and test flasks at the start and end of the test were recorded. Gaseous exchange and suspension of the algal cells were ensured by the action of the orbital shaker, oscillating at a nominal 150 cycles per minute. The minimum and maximum temperature and light intensity (four corner positions and in a central position of the random block design) within the test area were determined each day. To minimise the impact of differences in light intensity across the test area on algal growth, control and test flasks were re-positioned in the test area each day during the definitive test.

3.5 Measurement of growth

Samples were taken from control and test flasks at 24, 48 and 72 hours and the cell densities measured using a Coulter Z Series Particle Count and Size Analyser.

The estimate of cell numbers in each sample was based on the mean of three consecutive counts, corrected for background counts of uninoculated OECD medium. The presence of any abnormal cells was also noted during screening of each test level.

3.6 Evaluation of data

The data were compiled in an Excel spreadsheet and analysed using SAS 9.1 (SAS Institute 2002), using concentrations of GST calculated from the levels measured in samples of the solvent stocks and taking into account the dilution factor employed to prepare the test media (1:10,000).

The area under the curve was divided by initial count and total time to give AUCP (Area Under the Curve expressed as a Proportion of the initial cell count), where a value of 1 represents no growth and a value of 0 represents complete toxicity (all algae killed). In order to estimate the concentration at which 50% inhibition of growth occurred (EC_{50}), sigmoidal curves were fitted to AUCP and growth rate. For both variables, 0% inhibition was defined as the solvent control mean and 100% inhibition was defined as no growth. The minimum of the curve (for infinite concentration) was bounded between 0 and 1 for AUCP and between -1000 and 0 for growth rate. The formulae for these curves are given below:

$$\text{AUCP} = \begin{cases} Con & Control \\ Min + \dfrac{Con - Min}{1 + \dfrac{50(Con-1)ep}{50(Con+1) - 100Min}} & Otherwise \end{cases} \quad \text{where } ep = \exp\left(s \log\left(\frac{concentration}{EC50}\right)\right)$$

$$\text{Growth rate} = \begin{cases} Con & Control \\ Min + \dfrac{Con - Min}{1 + \dfrac{50Con\,ep}{50Con - 100Min}} & Otherwise \end{cases}$$

Con = an estimate of the solvent control mean
Min = an estimate of the minimum of the curve
s = slope estimate

Yield was calculated for each test culture as the final cell density (after 72 hours) minus the presumed initial cell density of 1×10^4 cells/mL. A mean yield value for each test concentration was calculated and the percentage inhibition was determined using the formula below:

$$\% \text{ Inhibition} = ((Y_C - Y_T) / Y_C) \times 100$$

Where Y_C = mean value for yield in the solvent control group
Y_T = value for yield in treatment replicate

All 95% confidence intervals for EC_{50} were calculated using the likelihood ratio method (Donaldson and Schnabel, 1985). The EC_{10} (with 95% confidence interval) was also estimated by reparameterising the above formulae.

For growth rate, AUCP and yield, Williams' test (1971, 1972) was also used to compare each treated group with the solvent control unless there was evidence of a non-monotonic dose-response relationship, in which case Dunnett's test (1955, 1964) was used.

For growth rate, AUCP and yield, the *t*-test was used to compare the control group with the solvent control.

3.7 Validity criteria

For the test to be valid:

- the cell concentration in control cultures should have increased by a factor of at least 16 within 72 hours;
- growth at one test concentration should be similar to the control group and one concentration should show a greater than 50% decrease in growth compared to the control (except where the test substance is not toxic to algae);
- the mean coefficient of variation for daily growth rates (days 0-1, 1-2 and 2-3) in the control cultures must not exceed 35%;
- the coefficient of variation of average specific growth rates during the test in replicate control cultures must not exceed 7%.

3.8 Protocol deviations

There were no deviations to the protocol.

4. Maintenance of records

All specimens (if appropriate), raw data and study related documents generated during the course of the study at [redacted] together with a copy of the final report will be lodged in the [redacted]

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

[redacted] will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

5. Results

5.1 Chemical analysis

The results of chemical analysis are given in Table 1 and example chromatograms are illustrated in Appendix 3.

The measured levels of GST in samples of the solvent stocks ranged between 94 and 110% of their nominal values. Using the measured levels in the stock solutions and taking into account the dilution factor employed for the preparation of the test media (1:10,000), the calculated test levels were 0.00947, 0.0218, 0.0481, 0.105, 0.250 and 0.492 mg/L and these values have been used in calculation of the test results.

5.2 Algal growth

Individual cell densities for each culture and the mean values are given in Table 2. The calculated area under the growth curve, average specific growth rate and yield values are given in Table 3 and are expressed in terms of percentage inhibition by comparing the test group value with that of the solvent control curve.

The test results have been expressed in terms of calculated test concentrations of GST. The following values were derived from the data:

Average specific growth rate

E_rC_{10} (0 - 72 h) : 0.117 mg/L (95% confidence limits, 0.0495 and 0.24 mg/L)

E_rC_{50} (0 - 72 h) : >0.492 mg/L (95% confidence limits unavailable)

No observed effect concentration (NOEC) : 0.00947 mg/L

Lowest observed effect concentration (LOEC) : 0.0218 mg/L

Area under the growth curve

E_bC_{10} (72 h) : 0.0122 mg/L (95% confidence limits, 0.00355 and 0.0339 mg/L)

E_bC_{50} (72 h) : 0.164 mg/L (95% confidence limits, 0.107 and 0.254 mg/L)

No observed effect concentration (NOEC) : <0.00947 mg/L*

Lowest observed effect concentration (LOEC) : 0.00947 mg/L

*: the results of statistical analysis indicated that at 0.00947 and 0.0218 mg/L the levels of inhibition (14.9 and 12.0% respectively) for area under the growth curve were significantly lower when compared to the solvent control, so a statistically derived NOEC was not identified for this parameter.

However, an E_bC_{10} value calculated using the curve fitted to the data gave a value of 0.0122 mg/L. Since the E_bC_{10} was higher than the lowest level employed in the test, this value (0.0122 mg/L) is considered to represent the level of no effect to the test organism for area under the growth curve.

Yield

E_yC_{10} (0 - 72 h) : 0.0219 mg/L (95% confidence limits: 0.000812 and 0.283 mg/L)

E_yC_{50} (0 - 72 h) : 0.264 mg/L (95% confidence limits, 0.12 and 0.582 mg/L)

No observed effect concentration (NOEC) : 0.00947 mg/L

Lowest observed effect concentration (LOEC) : 0.0218 mg/L

The mean coefficient of variation (CoV) for daily growth rates in solvent control cultures ranged between 3.78 and 7.06% during the definitive test and the CoV for the average specific growth rates of solvent control cultures was 1.41% during the 72 hour exposure period.

The mean coefficient of variation (CoV) for daily growth rates in control cultures ranged between 3.30 and 6.34% during the definitive test and the CoV for the average specific growth rates of control cultures was 0.647% during the 72 hour exposure period.

These results indicate that the validity criteria for this study guideline were met.

5.2.1 Observations

No microscopic abnormalities of the cells were detected.

5.2.2 Environmental parameters

The measurements of water quality (temperature and pH) in control and test flasks are summarised in Table 4; they remained within acceptable limits throughout the study.

The temperature of the incubator ranged between 22.1 and 23.1°C.

At the start of the test, the test media were colourless.

6. Conclusions

After 72 hours of exposure to GST, the E_rC_{50}, E_bC_{50} and E_yC_{50}, were >0.492, 0.164, and 0.264 mg/L respectively.

The E_rC_{10}, E_bC_{10} and E_yC_{10}, were 0.117, 0.0122 and 0.0219 mg/L, respectively.

The "no observed effect concentration" (NOEC) for growth rate was 0.00947 mg/L, for area under the growth curve was <0.00947 mg/L and for yield was 0.00947 mg/L.

7. References

DONALDSON, J.R. and SCHNABEL, R.B. (1985) Computational experience with confidence regions and confidence intervals for nonlinear least squares. *National Bureau of Standards*, Boulder, Colorado.

DUNNETT, C.W. (1955) A multiple comparison procedure for comparing several treatments with a control. *Journal of the American Statistical Association*, **50**, 1096-1121.

DUNNETT, C.W. (1964) New tables for multiple comparisons with a control. *Biometrics*, **20**, 482-491.

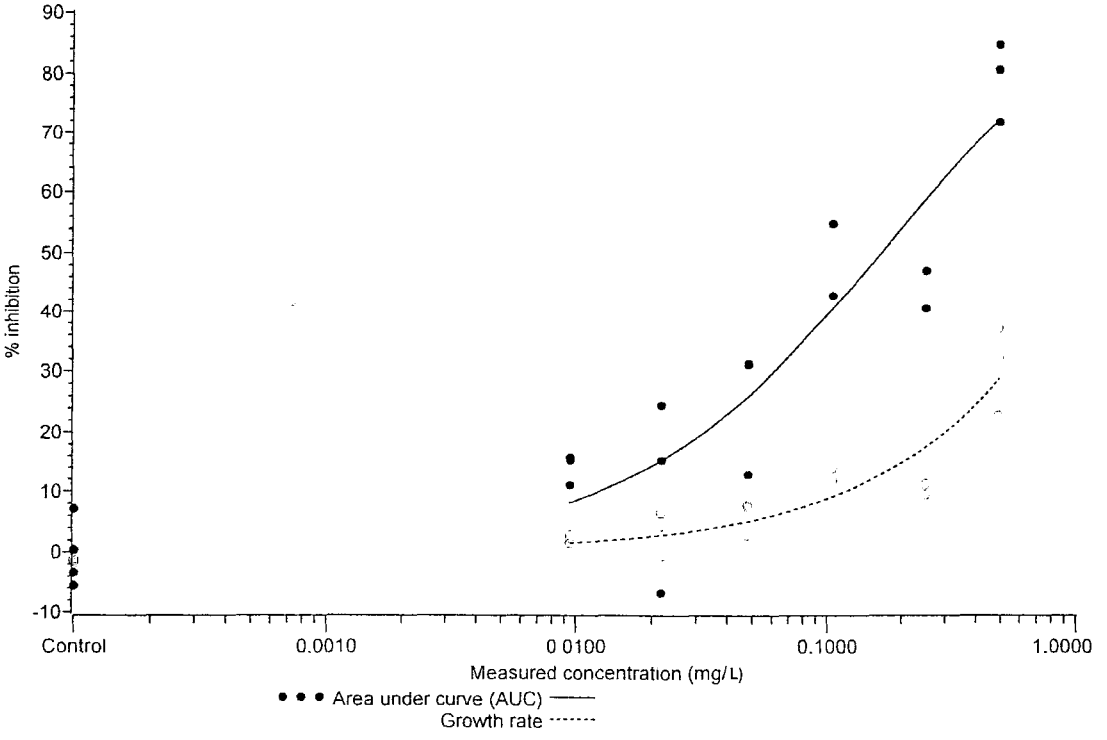
SAS INSTITUTE (2002) SAS OnlineDoc® *Version Nine*. SAS Institute Inc., Cary, NC, USA.

WILLIAMS, D.A. (1971) A test for differences between means when several dose levels are compared with a zero dose control. *Biometrics,* **27**, 103-117.

WILLIAMS, D.A. (1972) The comparison of several dose levels with a zero dose control. *Biometrics,* **28**, 519-531.

Figure 1 Inhibition of growth

a) Average specific growth rate and area under the growth curve



b) Yield

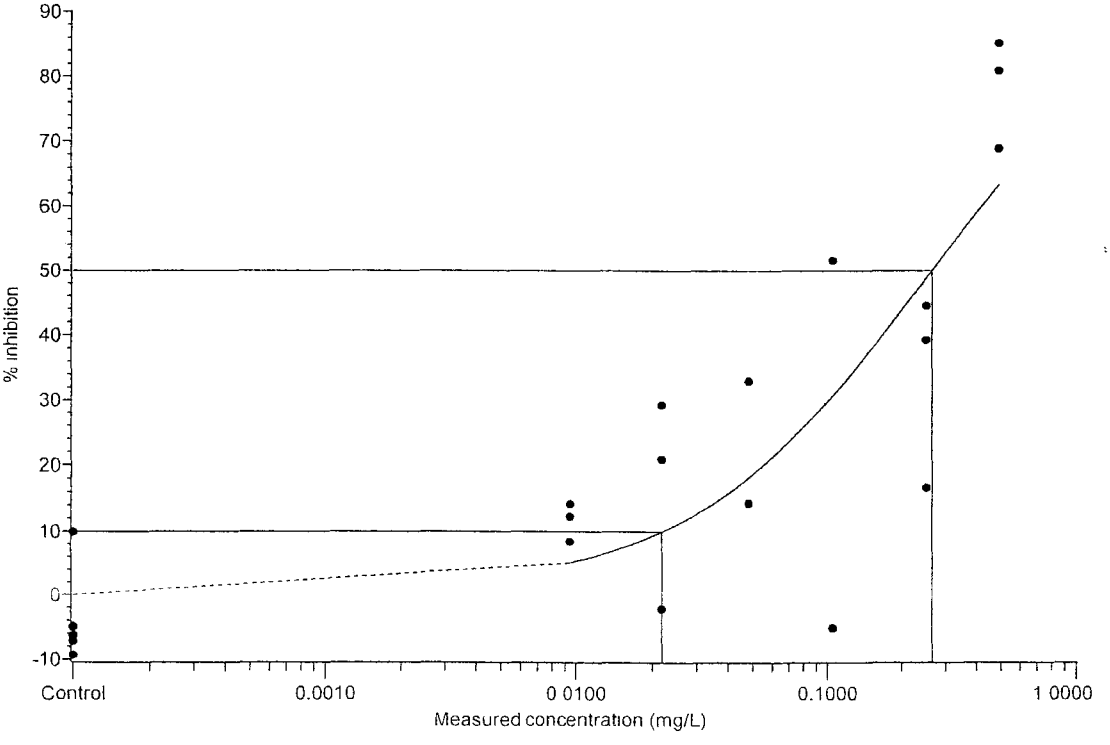


Table 1 Measured concentrations

Nominal GST conc., mg/mL	Measured GST concentrations (mg/mL) 0 hours	%N	Calculated measured concentration* mg/L
0.0970	0.0947	98	0.00947
0.231	0.218	94	0.0218
0.470	0.481	102	0.0481
1.03	1.05	102	0.105
2.27	2.50	110	0.250
5.0	4.92	98	0.492

%N measured concentration expressed as a percentage of the nominal concentration (calculated using unrounded values but expressed to 3 significant figures).

* solvent stock concentrations (mg/mL) were used to calculate the concentrations in aqueous media (mg/L) using the following equation:

concentration in solvent stock (mg/mL) x 0.1 (volume of solvent used/L)

Table 2 Cell densities

Exposure concentration (mg GST/L)		Replicate number	Cell densities (cells/mL)		
Nominal	Calculated		24 hours	48 hours	72 hours
Control	-	R_1	44283	336583	1648933
		R_2	45617	370317	1698467
		R_3	54850	392917	1566733
		R_4	52550	354583	1625333
		R_5	42850	336683	1591067
		R_6	50017	381617	1558867
		Mean	*48361*	*362117*	*1614900*
Solvent Control	nd	R_1	43050	462650	1869667
		R_2	50617	501283	1833833
		R_3	54817	480350	1869667
		R_4	51417	438350	1795933
		R_5	47050	433217	1817133
		R_6	49317	475017	1543000
		Mean	*49378*	*465144*	*1788206*
0.00970	0.00947	R_1	37617	396550	1501867
		R_2	37050	406683	1469333
		R_3	41250	416450	1568400
		Mean	*38639*	*406561*	*1513200*
0.0231	0.0218	R_1	54050	556217	1749200
		R_2	40750	409350	1213533
		R_3	35350	470983	1355833
		Mean	*43383*	*478850*	*1439522*
0.0470	0.0481	R_1	41750	442750	1466200
		R_2	39650	351750	1150500
		R_3	32850	365283	1133000
		Mean	*38083*	*386594*	*1249900*
0.103	0.105	R_1	69083[a]	676317[a]	1799100[a]
		R_2	28617	196050	833633
		R_3	30450	314150	928167
		Mean	*29533*	*255100*	*880900*
0.227	0.250	R_1	50850[a]	465850[a]	1427733[a]
		R_2	29717	197950	1043967
		R_3	30417	330417	954467
		Mean	*30067*	*264183*	*999217*
0.5	0.492	R_1	25483	121550	529033
		R_2	23017	104483	325133
		R_3	27350	80150	253200
		Mean	*25283*	*102061*	*369122*

- not applicable
nd not determined (solvent stock analysis only)
R_1-R_6 replicate number
a replicates removed from calculations due to consistently high cell counts throughout the test, which was attributed to potential bacterial growth within the test media and therefore considered to be anomalous

Note the initial cell density was estimated to be 1.30×10^4/mL.

Table 3 Inhibition of growth

Parameter	Nominal Concentration (mg/L)	Calculated Concentration (mg/L)	Sample size	Mean	% Inhibition	*p*
Growth rate to 72 hours	Control	-	6	0.071	1.9	0.254^{T}
	Solvent Control	nd	6	0.072	0.0	-
	0.00970	0.00947	3	0.070	3.2	0.130^{W}
	0.0231	0.0218	3	0.069	4.4	0.047^{*W}
	0.0470	0.0481	3	0.067	7.0	0.003^{**W}
	0.103	0.105	2	0.062	13.6	$<0.001^{***W}$
	0.227	0.250	2	0.064	11.2	$<0.001^{***W}$
	0.5	0.492	3	0.049	31.3	$<0.001^{***W}$
Growth rate 0 – 24 hours	Control	-	6	0.065	1.4	0.726^{T}
	Solvent Control	nd	6	0.066	0.0	-
	0.00970	0.00947	3	0.056	15.3	0.022^{*W}
	0.0231	0.0218	3	0.060	8.9	0.022^{*W}
	0.0470	0.0481	3	0.055	16.4	0.003^{**W}
	0.103	0.105	2	0.045	32.1	$<0.001^{***W}$
	0.227	0.250	2	0.046	30.9	$<0.001^{***W}$
	0.5	0.492	3	0.039	42.0	$<0.001^{***W}$
Growth rate 24 – 48 hours	Control	-	6	0.084	10.2	0.019^{*T}A
	Solvent Control	nd	6	0.094	0.0	-
	0.00970	0.00947	3	0.098	-4.9	$>0.999^{W}$
	0.0231	0.0218	3	0.100	-7.3	$>0.999^{W}$
	0.0470	0.0481	3	0.097	-3.3	$>0.999^{W}$
	0.103	0.105	2	0.089	5.1	0.541^{W}
	0.227	0.250	2	0.089	4.6	0.541^{W}
	0.5	0.492	3	0.058	38.4	$<0.001^{***W}$
Growth rate 48 - 72 hours	Control	-	6	0.062	-11.2	0.082^{T}
	Solvent Control	nd	6	0.056	0.0	-
	0.00970	0.00947	3	0.055	2.4	$>0.999^{D}$
	0.0231	0.0218	3	0.046	18.5	0.111^{D}
	0.0470	0.0481	3	0.049	13.0	0.403^{D}
	0.103	0.105	2	0.053	6.0	0.972^{D}
	0.227	0.250	2	0.057	-1.2	$>0.999^{D}$
	0.5	0.492	3	0.052	7.0	0.898^{D}

- not applicable
nd not determined (solvent stock analysis only)
p values are for the comparison with Solvent Control using Williams' test (W), Dunnett's test (D) and the *t*-test (T) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

A : the statistically significant level of inhibition between control and solvent control cultures for 24 – 48 hour growth rate (10.2%) is acknowledged but is not considered to impact on the results of the study as all statistical calculations are based on the growth in the solvent control cultures. The cell numbers achieved and the growth observed in the control cultures met all of the appropriate validity criteria for this study type (see section 5.2).

Table 3 Inhibition of growth (continued)

Parameter	Nominal Concentration (mg/L)	Calculated Concentration (mg/L)	Sample size	Mean	% Inhibition	*p*
Area under curve to 72 hours	Control	-	6	28.6	13.8	0.004**[T] A
	Solvent Control	nd	6	33.2	0.0	-
	0.00970	0.00947	3	28.2	14.9	0.018*[W]
	0.0231	0.0218	3	29.2	12.0	0.018*[W]
	0.0470	0.0481	3	24.6	25.9	<0.001***[W]
	0.103	0.105	2	16.8	49.4	<0.001***[W]
	0.227	0.250	2	18.5	44.4	<0.001***[W]
	0.5	0.492	3	6.9	79.3	<0.001***[W]
Yield	Control	-	6	1604900	9.7	0.180[T]
	Solvent Control	nd	6	1778206	0.0	-
	0.00970	0.00947	3	1503200	15.5	0.087[W]
	0.0231	0.0218	3	1429522	19.6	0.037*[W]
	0.0470	0.0481	2	1298350	27.0	0.015*[W]
	0.103	0.105	2	1306367	26.5	0.015*[W]
	0.227	0.250	3	1132056	36.3	<0.001***[W]
	0.5	0.492	3	369122.0	79.2	<0.001***[W]

- not applicable
nd not determined (solvent stock analysis only)
p values are for the comparison with Solvent Control using Williams' test (W), Dunnett's test (D) and the *t*-test (T) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

A : the statistically significant level of inhibition between control and solvent control for area under the growth curve is acknowledged but is not considered to impact on the results of the study as all statistical calculations are based on the growth in the solvent control cultures. The cell numbers achieved and the growth observed in the control cultures met all of the appropriate validity criteria for this study type (see section 5.2).

Table 4 Environmental parameters

a) Temperature and pH

Exposure concentration (mg GST/L)		Temperature °C		pH	
Nominal	Calculated	0 h	72 h	0 h	72 h
Control	-	22.6	23.2	7.81	8.21
Solvent Control	nd	22.6	23.1	7.80	7.68
0.00970	0.00947	22.5	23.3	7.78	7.49
0.0231	0.0218	22.5	22.8	7.78	7.35
0.0470	0.0481	22.6	23.0	7.78	7.26
0.103	0.105	22.5	23.1	7.78	7.11
0.227	0.250	22.6	22.6	7.78	7.05
0.5	0.492	22.7	23.0	7.78	6.98

- not applicable
nd not determined (solvent stock analysis only)

b) Light intensity

Incubator positions	Light intensity (lux)			
	Day 0	Day 1	Day 2	Day 3
Top, left	6420	6500	6480	6360
Bottom, left	6350	6310	6270	6440
Centre	7380	7320	7350	6860
Top, right	6640	6780	6700	6550
Bottom, right	6640	6690	6660	6640
Mean	*6686*	*6720*	*6692*	*6570*
Range (%)	*-5.0/+10.4*	*-6.1/+8.9*	*-6.3/+9.8*	*-3.2/+4.4*

Appendix 1 Certificate of Analysis

[redacted]

CERTIFICATE OF ANALYSIS

NAME OF SAMPLE : GST
LOT NO. OF SAMPLE : Lot number
DATE OF ANALYSIS : 2008/02/25

COMPOSITION

Identity	Conc.
#1	93.9%
#2	0.7%
#3	2.7%
#4	1.6%
#5	0.2%
#6	0.9%
TOTAL	100%

NAME :

SIGNATURE

DATE 2008 / 4 / 11

1

Appendix 2 Algal Nutrient Medium (OECD)

Four stock solutions were prepared according to the following table, using filtered, dechlorinated tap water which had been softened and treated by reverse osmosis, before microfiltration and purification (resistivity of 18 Megohm/cm). Stock solutions were sterilised by autoclaving (solutions 1-3) or by membrane filtration (solution 4) before being stored at 4°C in the dark.

Aliquots of stock solutions 1-3 were further diluted with the same diluent and autoclaved again to produce the working strength nutrient medium. Stock solution 4 was added to the medium on the day of use. The pH of the medium after equilibration with air is approximately 8.

Nutrient	Concentration in stock solution (g/L)	Volume of stock solution per litre of final medium (mL)	Final concentration in test solution (mg/L)
Stock solution 1: macro-nutrients			
NH_4Cl	1.5	10	15
$MgCl_2.6H_2O$	1.2		12
$CaCl_2.2H_2O$	1.8		18
$MgSO_4.7H_2O$	1.5		15
KH_2PO_4	0.16		1.6
Stock solution 2: Fe-EDTA			
$FeCl_3.6H_2O$	0.064	1	0.064
$Na_2EDTA.2H_2O$	0.1		0.1
Stock solution 3: trace elements			
H_3BO_3	0.185	1	0.185
$MnCl_2.4H_2O$	0.415		0.415
$ZnCl_2$	3×10^{-3}		3×10^{-3}
$CoCl_2.6H_2O$	1.5×10^{-3}		1.5×10^{-3}
$CuCl_2.2H_2O$	10^{-5}		10^{-5}
$Na_2MoO_4.2H_2O$	7×10^{-3}		7×10^{-3}
Stock solution 4: $NaHCO_3$			
$NaHCO_3$	50	1	50

Appendix 3 The Determination of GST in Acetone

General principle

Samples were diluted with acetonitrile:water (50:50 v:v). Quantitation was performed using liquid chromatography with UV detection (HPLC-UV).

Materials	Grade
Acetonitrile	HPLC
Potassium dihydrogen orthophosphate	AR
Water	HPLC

Preparation of reagents

Preparation of acetonitrile:water (50:50 v:v):
Acetonitrile (500 mL) and water (500 mL) were thoroughly mixed prior to use.

Preparation of 0.01M potassium dihydrogen orthophosphate:
Potassium dihydrogen orthophosphate (1.36 g) was added to water (1000 mL) and thoroughly mixed prior to use.

Preparation of 0.01M potassium dihydrogen orthophosphate:acetonitrile (50:50 v:v):
0.01M Potassium dihydrogen orthophosphate (500 mL) was added to acetonitrile (500 mL) and thoroughly mixed prior to use.

NOTE – to prepare different volumes of the above reagents, the ratio of the individual components was maintained.

Test substance solutions

GST was accurately weighed (corrected for purity) and dissolved in acetonitrile to give a stock standard solution of 10 mg/mL. The stock standard solution was diluted progressively with acetonitrile to give intermediate standard solutions.

An appropriate intermediate standard solution was diluted progressively with acetonitrile:water (50:50 v:v) to produce a series of calibration solutions in the range 0.5 to 10 µg/mL.

Sample analysis procedure

An aliquot (10 mL) of the sample was diluted by a factor of 5 with acetonitrile:water (50:50 v:v). Any further dilutions where required were performed using acetonitrile:water (50:50 v:v), prior to quantitation by HPLC-UV.

HPLC-UV conditions

Instrument:	HP 1100
Column:	Supelcosil LC-ABZ (25 cm x 4.6 mm x 5 μm)
Mobile phase:	0.01M potassium dihydrogen orthophosphate:acetonitrile (50:50 v:v) (isocratic)
Flow rate:	1 mL/min
Oven temperature:	40°C
Injection volume:	100 μL
Wavelength:	210 nm
Retention time:	approx. 12.5 minutes
LOQ:	0.005 mg/mL
LOD:	0.5 μg/mL (equivalent to 0.0025 mg/mL in acetone solution)

Calculation of results

Test samples were quantified using the following equation:

Residue found (mg/mL) = $x \times \frac{1}{M} \times D \times \frac{1}{1000}$

Where x (residue concentration in final solution) was calculated using the linear regression:

$y = m\,x + c$ where x (concentration in µg/mL) = $\frac{y - c}{m}$

c = intercept
m = slope
y = peak area of sample
M = matrix concentration (mL/mL)
D = dilution factor

Example calculation of GST detected in a sample of acetone treated at 0.47 mg/mL, (analytical identification 10/MTU/2340).

Linear regression $y = m\,x + c$

$y = 87.1380x - 20.1298$

where $y = 398.7$
m = 87.1380
c = -20.1298

Therefore, concentration of GST (x) $= \frac{398.7 + 20.1298}{87.1380}$

= 4.807 µg/mL

Matrix concentration = 0.2 mL/mL
Dilution factor = 20

GST detected (mg/mL) $= \frac{4.807\ \mu g/mL \times 20}{0.2\ mL/mL \times 10^{3}}$

= 0.481 mg/mL

RESULTS AND DISCUSSION

Calibration

The chromatographic response to GST was shown to be linear over the range of concentrations 0.5 to 10 µg/mL. Typical calibration data are presented in Table 1 and Figure 1. Typical chromatograms of calibration standards are presented in Figures 2 and 3.

Validation

The methodology for the determination of GST in acetone was validated at 0.005 and 5 mg/mL. The validation data is summarised in the following table:

Fortification level (mg/mL)	Mean recovery (%)	CV (%)
0.005	101	3.8
5	105	5.4
	(103)	(4 9)

( ) : overall values

Limit of quantitation (LOQ)

The limit of quantitation is defined as the lowest fortification level at which acceptable recovery data are obtained. The validation of the methodology for the determination of residues of GST in acetone, demonstrated that it can be accurately determined at a limit of quantitation (LOQ) of 0.005 mg/mL.

Limit of detection (LOD)

The limit of detection of the method is defined as the value of the lowest calibration standard chromatographed that gave a peak height to baseline noise ratio ≥3. For this study the limit of detection for GST was 0.5 µg/mL (equivalent to 0.0025 mg/mL in acetone).

Samples

Samples from the test were analysed in a suitably sized batch along with control samples fortified with GST which acted as procedural recovery samples. Procedural recoveries performed on control acetone were in the range 70 to 110% confirming the validity of the methodology on the day of analysis. Typical chromatograms of untreated, untreated fortified with GST and treated extracts are presented in Figures 4 to 6.

TABLE 1

Typical standard calibration for GST by HPLC-UV

Standard concentration (μg/mL)	Peak area
0	0
0.5	35.4
1	66.3
2	141.4
3	216.4
4	314.6
5	430.2
6	495.7
7	593.5
8	675.8
9	760.8
10	864.5

Calibration curve: $y = 87.1380\,x - 20.1298$ (batch 2)

$r = 0.9990$

Where x = concentration
y = peak area
r = regression coefficient

TABLE 2

Validation of GST in Acetone

Analytical identification	GST detected (mg/mL)	Recovery (%)	Mean (%)	Coefficient of variation (%)
Con A	ND			
Con B	ND			
F0.005 mg/mL A	0.00510	102		
F0.005 mg/mL B	0.00522	104		
F0.005 mg/mL C	0.00507	101	101	3.8
F0.005 mg/mL D	0.00510	102		
F0.005 mg/mL E	0.00482	94		
F5 mg/mL A	4.75	95		
F5 mg/mL B	5.32	106		
F5 mg/mL C	5.49	110	105	5.4
F5 mg/mL D	5.29	106		
F5 mg/mL E	5.31	106		

Overall mean = 103%
Overall coefficient of variation = 4.9%
ND – Not detected (<LOD of 0.0025 mg/mL)

FIGURE 1

Typical standard chemical calibration for GST by HPLC-UV

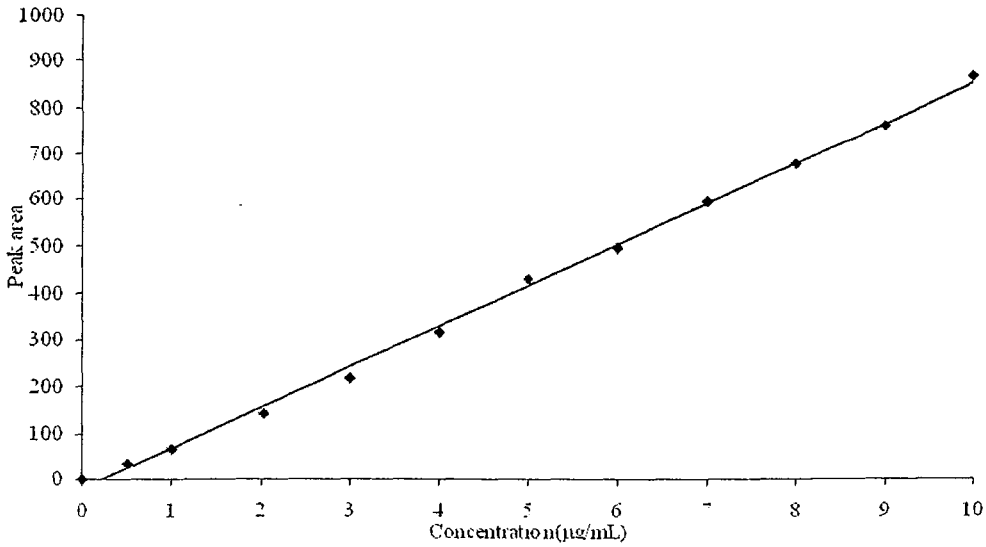


FIGURE 2

Typical chromatogram of a 0.5 µg/mL GST calibration standard

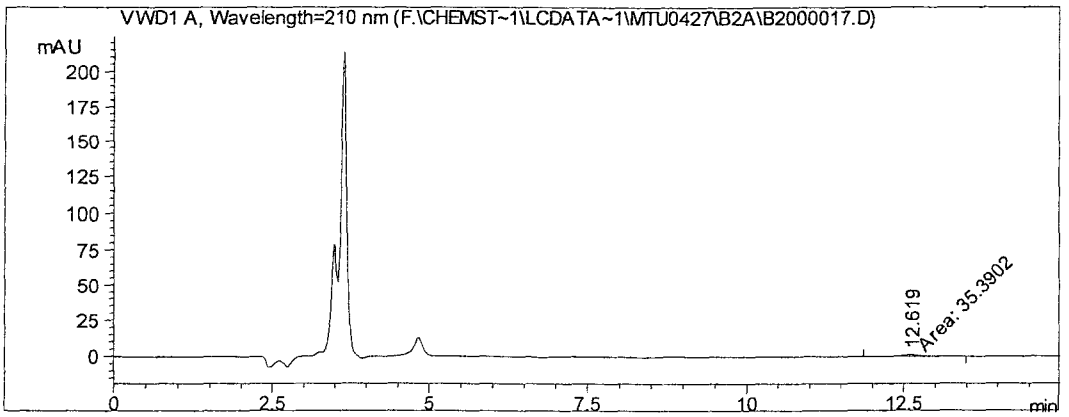


FIGURE 3

Typical chromatogram of a 10 µg/mL GST calibration standard

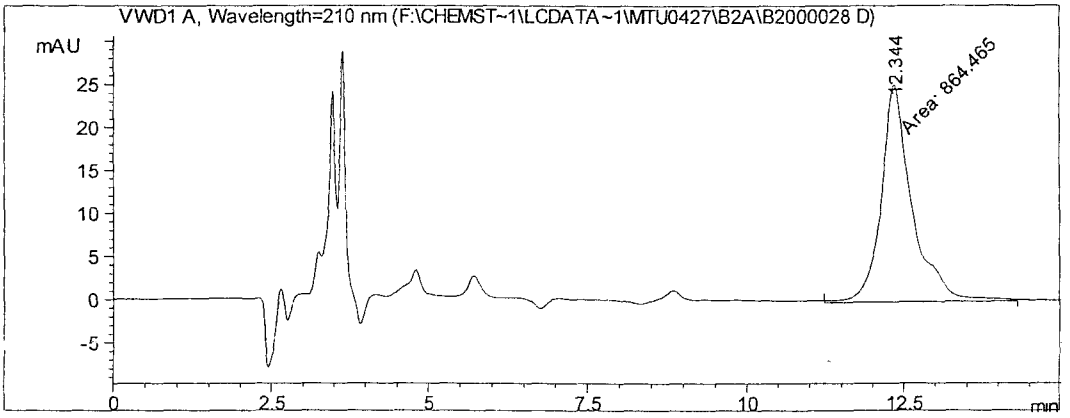


FIGURE 4

Chromatogram of an untreated acetone sample (analytical identification acetone Con A)

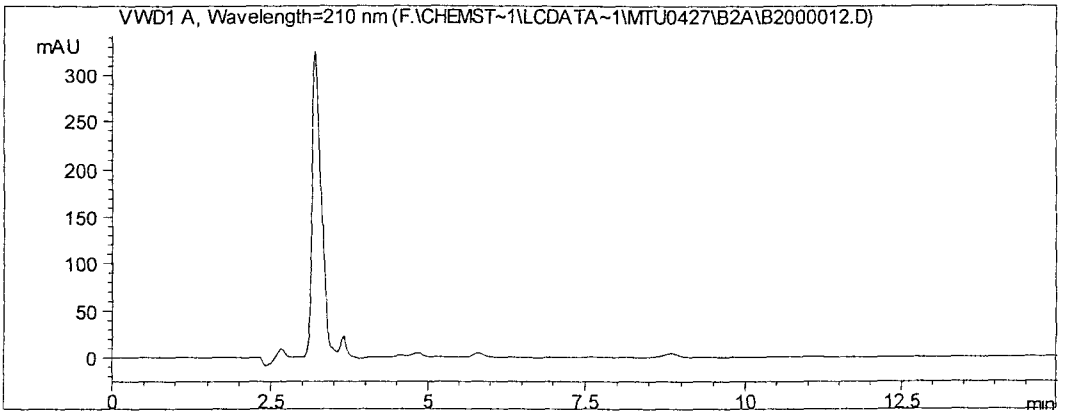


FIGURE 5

Chromatogram of an untreated acetone sample fortified with 5 mg/mL of GST (analytical identification acetone F5 A)

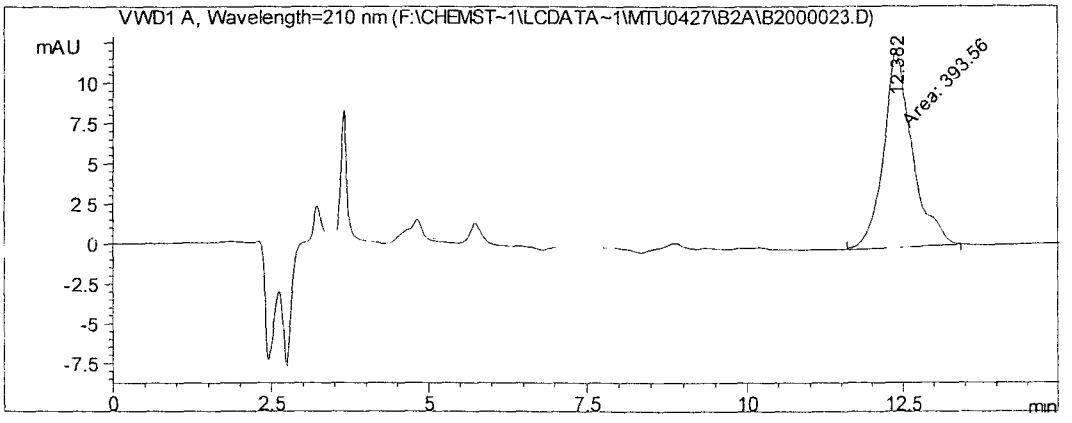
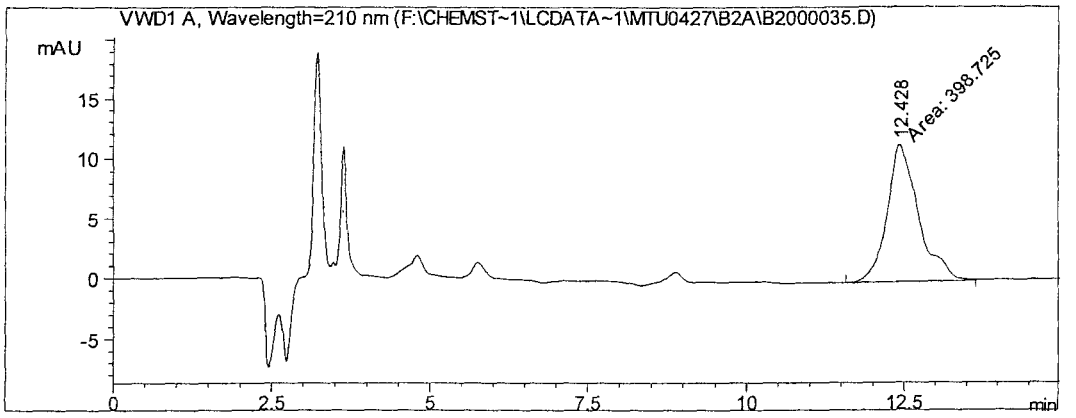


FIGURE 6

Chromatogram of a sample at 0.47 mg/mL
(analytical identification 10/GST/2340)



Appendix 4 Eye Research Centre GLP Compliance Statements

a) 2008

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT
OF THE

GOOD LABORATORY PRACTICE

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

TEST FACILITY	TEST TYPE
	Analytical Chemistry Ecosystems Environmental Fate Environmental Toxicity Mutagenicity Phys/Chem Testing Toxicology

DATE OF INSPECTION

28th January 2008

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the 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.

Appendix 4 Eye Research Centre GLP Compliance Statements

b) 2009

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE

GOOD LABORATORY PRACTICE

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

TEST FACILITY

TEST TYPE

Analytical/Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Phys/Chem Testing
Toxicology

DATE OF INSPECTION

17-19 February 2009

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the P 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.

Appendix 4 Eye Research Centre GLP Compliance Statements

c) 2010

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE

GOOD LABORATORY PRACTICE

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

TEST FACILITY

TEST TYPE

Analytical/Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Physico-chemical Testing
Residue Studies
Toxicology

DATE OF INSPECTION

26 January 2010

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the 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.

8EHQ-11-18262

333288

Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road * Woodbridge, VA 22192-5755
Telephone 703 590 7337 * Fax 703 580 8637
cfrsrv@aol.com

11 FEB 17 PM 2:38

February 18, 2011

U.S. Environmental Protection Agency – East
TSCA Section 8(e)
Room 6428
1201 Constitution Avenue, NW
Washington, DC 20460-0001

SUBJECT: Notice in Accordance with TSCA Section 8(e)

Prolonged Toxicity to *Daphnia Magna*

has received the final report, February 11, 2011, regarding prolonged toxicity to *Daphnia Magna* for the substance which was the subject of PMN P94-1487. The full chemical name of this substance is considered CBI, generically identified as *thia alkanethiol.*

The conclusion of this test resulted in finding that the NOEC for parental mortality to be 0.0321 mg/L.

Please note that the complete **chemical identity** of this substance is considered **Confidential Business Information**. Also enclosed is a sanitized copy of the report.

Sincerely,

William A. Olson, Ph.D.
Consultant

WAO:gbt
MTCA33-8E-3

Enclosure
CBI Report , 11 February 2011, 53 pages
Sanitized Report , 11 February 2011, 53 pages

Cc:

Company Sanitized

Sanitized

GST

PROLONGED TOXICITY TO *DAPHNIA MAGNA*

HLS study number:	( )
Version ID:	Final report
Issue date:	11 February 2011

Sponsor and Test Facility Details

Sponsor

Test Facility

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Compliance with Good Laboratory Practice

GST: Prolonged Toxicity to *Daphnia Magna*

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 Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994).
>
> OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98) 17.
>
> EC Commission Directive 2004/10/EC of 11 February 2004 (Official Journal No L 50/44).

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.

11 February 2011
Date

Quality Assurance Statement

GST: Prolonged Toxicity to *Daphnia Magna*

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	19 Aug 2008	19 Aug 2008
Protocol Amendment No.1	29 Sep 2009	29 Sep 2009
Protocol Amendment No.2	08 Oct 2009	08 Oct 2009
Protocol Amendment No.3	04 Feb 2010	04 Feb 2010
Protocol Amendment No.4	31 Mar 2010	31 Mar 2010
Report Audit	07 Dec 2010 - 09 Dec 2010	09 Dec 2010

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
Sampling of test media for chemical analysis	05 Jul 2010	05 Jul 2010
Counting of *Daphnia* juveniles/transfer of parents	06 Jul 2010 - 07 Jul 2010	07 Jul 2010
Standard preparation	07 Jul 2010	07 Jul 2010
Experimental set-up	27 Jul 2010	27 Jul 2010
Environmental measurements	12 Aug 2010	12 Aug 2010
Measurement of *Daphnia*	27 Aug 2010	27 Aug 2010
Chromatography	17 Sep 2010	17 Sep 2010
Results processing	23 Sep 2010	23 Sep 2010
Fortification procedures	21 Oct 2010	21 Oct 2010
Sample preparation (to include sample tracking)	01 Nov 2010	01 Nov 2010

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

.... 11 February 2011
Date

Contributing Scientists

GST: Prolonged Toxicity to *Daphnia Magna*

Study management

Aquatic Ecotoxicology and Biodegradation

Other responsible personnel

Aquatic Ecotoxicology and Biodegradation

Expert department

Environmental Analysis

Summary

The effect of GST on the reproduction of *Daphnia magna* was assessed under semi-static exposure conditions over a period of 21 days using the methods detailed in the OECD Guideline for Testing of Chemicals, No. 211 "*Daphnia magna*, reproduction test" (1998). Due to the hydrolytic nature of the test substance, aspects of the study were designed following guidance outlined in OECD "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures" Number 23 (ENV/JM/MONO(2000)6), to ensure the test organism was exposed to both parent and degradate products.

Groups of ten, individually-housed *Daphnia* were exposed for 21 days to the test substance prepared at nominal concentrations of 0.001, 0.0032, 0.01, 0.032 and 0.1 mg/L. The test media were prepared in Elendt M4 medium using a series of acetone stock solutions. The test media were renewed daily during the definitive test to ensure the *Daphnia* were exposed to maintained concentrations of the parent substance. As an auxiliary solvent was used to prepare the test media, a solvent control group (0.1 mL acetone/L) comprising twenty *Daphnia* and a diluent (Elendt M4) control group, comprising ten *Daphnia*, were employed in the study.

The test was initiated with first instar *Daphnia* that were less than 24-hours old. Daily records were maintained for mortality, floating and physical appearance, number of gravid animals, live and dead neonates, and the presence of aborted eggs and moulted carapaces. *Daphnia* were fed each day with a suspension of the unicellular green algae *Pseudokirchneriella subcapitata*.

It was not possible to develop an analytical method with sufficient sensitivity to detect the required levels of GST in Elendt M4 medium. Therefore, an analytical method was developed to measure the levels of GST in solvent stock solutions.

The measured levels of GST in samples of the solvent stock solutions ranged between 85 and 109% of their nominal values Using the levels measured in the stock solutions and taking into account the dilution factor for preparation of the test media (1:10,000), the levels of GST in the aqueous media were calculated to be 0.000893, 0.00317, 0.00930, 0.0321 and 0.101 mg/L

Parental mortality (Day 21)

Parental mortality (%) / calculated GST concentrations (mg/L)						
Control	Solvent Control	0.000893	0.00317	0.00930	0.0321	0.101
20	10	20	10	0	0	50

Based on these data, the EC_{50} value for the parental generation was calculated as *ca* 0.101 mg/L (0.110 mg/L); the EC_{20} and EC_{10} values were calculated as 0.0699 and 0.0590 mg/L respectively

The NOEC ("no observed effect concentration") for parental mortality was found to be 0.0321 mg/L

The LOEC ("lowest observed effect concentration") for parental mortality was found to be 0.101 mg/L.

No significant effects on either the physical appearance or mobility of the surviving adult *Daphnia* were observed.

Growth

Statistical analysis of the lengths of surviving adults after 21 days of exposure to GST indicated no adverse effects on growth.

The NOEC for length of surviving adults was found to be 0.101 mg/L.

The LOEC for length of surviving adults was found to be >0.101 mg/L.

Reproduction

Statistical analysis of the total number of live neonates produced by each surviving adult in the test groups compared to the solvent control group indicated that reproduction was significantly reduced at a GST concentration of 0.101 mg/L.

The NOEC for reproduction was found to be 0.0321 mg/L.

The LOEC for reproduction was found to be 0.101 mg/L.

Under the conditions of the test, the following EC_{10}, EC_{20} and EC_{50} values were derived:

	Reproduction (calculated GST concentrations; mg/L)
21-day EC_{10}	0.0696 [0.0277, 0.100]
21-day EC_{20}	0.0966 [0 0690, 0.196]
21-day EC_{50}	>0 101

[ ] : 95% confidence limits

Statistical analysis of the time taken to produce the first brood of neonates indicated no significant effect

The NOEC for first brood times was found to be 0.101 mg/L

The LOEC for first brood times was found to be >0.101 mg/L.

Statistical analysis of the numbers of dead neonates or aborted eggs produced by each surviving adult in the test groups compared to the solvent control group indicated that neonate survival was adversely affected at concentrations of 0.0321 and 0 101 mg/L.

The NOEC for production of dead neonates was found to be 0.00930 mg/L.

The LOEC for production of dead neonates was found to be 0.0321 mg/L

1. Introduction

The objective of this study was to examine the prolonged toxicity of GST to *Daphnia magna*. This was assessed by observations of the mortality of parental *Daphnia*, counts of the numbers of neonates produced during 21 days and the calculation of specific effect concentration rates (EC_{10}, EC_{20} and EC_{50}), the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) for reproduction.

The study was conducted using methods based on the OECD Guideline for Testing of Chemicals, No. 211 "*Daphnia magna*, reproduction test" (adopted 1998).

The protocol was approved by nd the Study Director on the 18 August 2008 and by the Sponsor on 25 August 2008.

The study was conducted at

The experimental phase of the study was conducted between 24 October 2008 and 2 September 2010, respectively.

Information provided by the Sponsor indicated that at 25°C, under abiotic aqueous conditions at a pH of 7, the half-life of GST is less than 24 hours. Following information given in the OECD "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures" Number 23 (ENV/JM/MONO(2000)6), the study was designed to expose the test organisms to both parent and degradate products.

The use of a solvent stock solution series followed recommendations in OECD Procedure 211 and Document Number 23 to promote dissolution of the test substance following formulation and analytical trials which indicated a low level of aqueous solubility of the parent substance under test conditions. Acetone was identified as the most suitable solvent and the study employed a control and solvent control group to ensure any effects on the test results could be identified.

2. Test substance

Identity: GST

Chemical name:

Batch number:

Appearance:

Storage conditions:

Expiry date:

Purity/Assay:

Water solubility:

Sample received (Huntingdon Site):

Certificate of analysis:

3. Experimental procedure

3.1 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 the *Daphnia* were maintained as parthenogenic cultures in covered one litre capacity glass vessels containing Elendt M4 culture medium (approximately 500 to 800 mL).

Cultures were held in a temperature-controlled laboratory at nominally 20 ± 2°C. A photoperiod of 16 hours light : 8 hours dark was maintained, with periods (one hour) of subdued lighting at the beginning and end of each light phase.

The culture medium was renewed at least three times each week. A maximum of fifteen adult *Daphnia* were maintained in each culture vessel.

Cultures were fed daily with a suspension of the unicellular green algae *Pseudokarchneriella subcapitata*. Algal cultures were grown in synthetic mineral salts medium. Concentrated algal cell suspensions were prepared by removing and centrifuging aliquots of algal culture and resuspending the algal pellets in small volumes of dilution medium. Appropriate volumes of these concentrated suspensions were added to each *Daphnia* culture to provide nominally 0.1 to 0.2 mg carbon per daphnid per day, except during the initial three days of culture when a slightly lower ration was given.

3.2 Procedure for obtaining neonate *Daphnia*

The day before the start of the test, all neonate daphnids were removed from the laboratory cultures. The following day, any neonates produced by the gravid (egg-bearing) adult *Daphnia* were removed from the culture vessel and held in a separate vessel. These animals, which were less than 24 hours old, were used in the test. The temperature of the holding water immediately prior to the addition of the neonates to the test vessels for the definitive test was 21.2°C.

3.3 Dilution medium

The test organisms were maintained and the test conducted in Elendt M4 medium (Appendix 2). The medium used in the study was prepared in de-ionised water

The total hardness and alkalinity of each batch of dilution medium were measured before use

3.4 Test substance preparation

The method of preparation used during the definitive test was based on the results of a range finding tests and a number of formulation trials.

The test media were prepared daily from solvent stock solutions that were prepared on Day 0, 7 and 14 of the definitive test. These stock solutions were stored refrigerated when not in use.

The test substance (87.2 µL, corrected for purity and density) was dissolved in acetone (10 mL) to provide a primary solvent stock solution at 10 mg/mL. This solution was serially diluted to provide intermediate solvent stock solutions at 1.0, 0.32, 0.1, 0.032 and 0.01 mg/mL. An aliquot (equivalent to 100 µL/L) of the appropriate stock solution was added to dilution medium (1000 mL) in a volumetric flask, to provide aqueous test media at 0.1, 0.032, 0.01, 0.0032 and 0.001 mg/L. The contents of each flask were shaken vigorously before use.

The control group was prepared using dilution medium but without the test substance. As an intermediate vehicle was used to facilitate the preparation of the test medium, an additional control group containing acetone and dilution medium (100 µL/L) was included in the study. The solvent selected for use in this study was based on the results of formulation trials conducted as part of this study.

3.5 Exposure conditions

3.5.1 Selection of exposure regime and test levels

The study comprised a rangefinding test, including a number of formulation trials, a 48 hour acute test to confirm immobility, and the definitive test.

Results of analysis indicated that the formulation method employed at the beginning of the rangefinding test did not achieve nominal GST concentrations. Therefore, a test based on a 48-hour EC_{50} acute toxicity to *Daphnia* study (OECD 201) was conducted prior to the definitive test, to confirm the expected EC_{50} value for *Daphnia magna* based on published data.

The range finding test was conducted at nominal concentrations of 0 0001, 0.001, 0.01, 0.1 and 1.0 mg/L.

After 12 days of exposure, 80% parental mortality was observed at a nominal concentration of 1.0 mg/L and no live neonates were produced at this concentration At a nominal concentration of 0.1 mg/L, neonate production was reduced (14%) after 12 days of exposure, when compared to the control.

The 48-hour EC_{50} test was conducted at nominal concentrations of 0.01, 0.1 and 1.0 mg/L After 48 hours exposure, no immobility was observed at 0.01 mg/L and immobility at 0.1 and 1.0 mg/L was 30 and 100% respectively.

3.5.2 Definitive test

Based on the results of the range finding and 48 hour acute tests, the definitive test employed nominal concentrations of 0.001, 0.0032, 0.01, 0.032 and 0.1 mg/L.

Control animals were exposed to dilution medium alone, and solvent control animals were exposed to dilution medium and acetone.

Ten, individually-housed, *Daphnia* were exposed for 21 days to each test group and dilution medium control, and twenty individuals to the solvent control group. *Daphnia* were added to the test vessels (glass jars, *ca.* 60 mL capacity) containing control or test medium, according to a random group order.

3.5.3 Stability of the test concentrations

The concentrations of GST in solvent stock solutions (used to prepare the test media) were measured using a HPLC-UV (High Performance Liquid Chromatographic with UV detection) method of analysis (see Appendix 3).

During the definitive test, samples (5 mL) of the solvent control and test solvent stock solutions were analysed on the first and last day each batch was used. The solvent stocks were used to prepare the aqueous test solutions for seven days consecutively

3.5.4 Medium renewal

Two sets of vessels were employed alternately during the study. One set was filled with freshly-prepared media at the start of the test and the *Daphnia* were added to each vessel.

The test media were renewed daily. On each occasion of renewal, any dead animals were discarded and the surviving parental *Daphnia* (mobile and immobile) were transferred to fresh control or test media in a second set of vessels. Any neonates, unhatched eggs or carapaces present in the expired media were counted and then discarded After completion of the analytical and environmental measurements, the vessels containing the expired media were emptied, rinsed thoroughly with purified water and left to drain until used for the next renewal.

3.5.5 Feeding

A concentrated suspension of *Pseudokirchneriella subcapitata* was added directly to the medium in each control and test vessel at the start and subsequently on each day during the definitive test. The volume of algal ration given during the definitive test was estimated to be in the range 0.1 to 0.2 mg carbon per daphnid per day, except during the initial two days of the test when a slightly lower ration was given (nominally 0.075 mg carbon per daphnid per day).

3.5.6 Environmental conditions

The temperature of the test area was continuously monitored in an additional vessel containing the same volume of dilution medium and was maintained at 19 6 to 22.6°C during the definitive test (see protocol deviations).

A photoperiod of 16 hours light : 8 hours dark was maintained, with periods (one hour) of subdued lighting at the beginning and end of each light phase. No supplementary aeration was employed and the pH of the test media was neither adjusted nor controlled during the study.

The temperature, pH and concentration of dissolved oxygen (DO) of the freshly-prepared control and test media were measured using the volumes remaining in the preparation flasks after the vessels had been filled. Measurements of pH and DO on expired media (24 hours old) were conducted on pooled contents of medium remaining in the vessels of each exposure group; measurements of temperature on expired media were conducted on the first remaining vessel of each exposure group.

The light intensity of the test area was determined to be 592 and 565 lux at the start and end of the test, respectively.

3.6 Criteria of Effect

Immobility was defined as the inability to free-swim in the test medium and death was defined as the cessation of all movement.

The numbers of mobile, floating (swimming at the media surface), immobile, dead and gravid (animals with eggs in the brood pouch) parental *Daphnia* were recorded daily, together with any general observations of their size and general appearance (if different from the controls).

At the end of the definitive test, after observations of the *Daphnia* were made, the body length (taken as the distance from the apex of the helmet to the base of the spine) of each surviving adult was measured using a microscope and stage mounted graticule.

From Day 6 (the first occasion when juveniles were observed), the numbers of live and dead neonates and the presence of any aborted (unhatched) eggs or dead juveniles in each vessel were recorded daily.

The vessels were also checked daily for carapaces moulted by the parental generation and, where present, these were removed from the vessels and counted.

3.7 Evaluation of data

The data were compiled in an Excel spreadsheet and the analysis was performed using SAS 9.1 (SAS Institute 2002), based on calculated GST concentrations.

On Day 12 of the definitive test, one replicate animal from the solvent control group (V24) was accidentally damaged during transfer. This animal subsequently died and therefore was removed from test calculations Consequently, the solvent control group is deemed to have been initiated with nineteen animals.

3.7.1 Mortality of the parental generation

The estimated effective concentration affecting the parental population were calculated using logistic regression.

3.7.2 Length of surviving adults

The body lengths of all surviving parental *Daphnia* in each group at the end of the test were compared to those in the solvent control group using a multiple comparisons Dunnett's test. The *t*-test was used to compare the control group with the solvent control.

3.7.3 Reproduction

The cumulative number of live neonates produced per adult in the solvent control group was compared with those in each test group using a multiple comparisons Williams' test (Williams; 1971, 1972). The EC_{10} and EC_{20} values were estimated by non-linear regression (logistic curve); the 95% confidence limits were derived by the likelihood ratio method The *t*-test was used to compare the control group with the solvent control.

The number of dead neonates or aborted eggs produced per adult in the solvent control group was compared with those in each test group and the control group using asymptotic linear by linear tests. An exact Wilcoxon rank sum test was used to compare the control group with the solvent control.

The time to the appearance of the first brood of neonates per adult *Daphnia* surviving until Day 21 of the test was analysed for each replicate. The solvent control group and all treated groups and the control group were compared using two-tailed Linear-by-linear Association test for a trend in time correlated with increasing dose (Agresti et al 1990).

3.8 Protocol deviations

On Day 17, the temperature of the expired media measured within the test vessels (22.3 to 22.6°C), exceeded the range stated in the protocol (maximum temperature of 22°C). The temperature of the test area was decreased by 1°C following this deviation.

During the definitive test, the maximum temperature in the additional vessel that was continuously monitored was 22.6°C, which exceeded the value stated in the protocol (22°C). During the conduct of the definitive test, on two occasions (Day 10 and Day 15) the minimum temperature recorded in the additional vessel (16.2 and 13.2°C respectively) also fell below the minimum value stated in the protocol (18°C) These deviations were considered a result of incorrect temperature monitoring equipment placement and a monitoring equipment failure respectively, as the ambient laboratory temperature remained within range. Following the second deviation on Day 15, the monitoring equipment was replaced.

None of these deviations was considered to have affected the results or integrity of the test as the validity criteria for this type of study were met.

3.9 Validity of the Test

For the study to be valid, the mortality of the parent *Daphnia* in the control groups must not exceed 20% at the end of the test and the mean number of live neonates produced per parent animal surviving at the end of the test in the control group must be ≥60. These criteria were achieved.

4. Maintenance of records

All specimens (if appropriate), raw data and study related documents generated during the course of the study at [redacted] together with a copy of the final report will be lodged in the [redacted]

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

[redacted] will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

5. Results

5.1 Chemical analysis

The results of chemical analysis are given in Table 1 and example chromatograms are given in Appendix 3.

The measured levels of GST in samples of the solvent stock solutions ranged between 85 and 109% of their nominal values. Using the mean measured levels in the stock solutions and taking into account the dilution factor employed for the preparation of the test media (1:10,000), aqueous test levels were calculated to be 0.000893, 0.00317, 0.00930, 0.0321 and 0.101 mg/L; these values have been used in calculation of the test results.

5.2 Survival of the parental *Daphnia*

The numbers of dead parental *Daphnia* observed during the definitive test are given in Table 2 and are summarised below.

Observation time (day)	% mortality / calculated GST concentration (mg/L)						
	Control	Solvent Control	0.000893	0.00317	0.00930	0.0321	0.101
2	0	5	0	0	0	0	0
14	20	10	10	0	0	0	50
21	20	10	20	10	0	0	50

Based on these data, the EC_{50} value for the parental generation was calculated as approximately 0.101 mg/L (0.110 mg/L), the EC_{20} and EC_{10} values were calculated as 0.0699 and 0.0590 mg/L respectively.

Based on parental mortality, the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) were 0.0321 mg/L and 0 101 mg/L, respectively.

5.3 Sub-lethal effects on the parental generation

Table 3 lists the body length of each surviving parental *Daphnia* at the end of the test. Statistical comparisons (Dunnett's test) showed that there was no significant effect at any test concentration when compared to the solvent control animals. Based on body length, the NOEC was 0.101 mg/L and the LOEC was >0.101 mg/L A statistically significant difference (p <0.05) was identified between the mean control (3.69 mm) and solvent control (3 82 mm) body lengths; this is not considered to be of biological significance as all surviving control animals fulfilled the appropriate validity criteria. This significance was attributed to the range of values observed in the control (3.5 to 3.8 mm) and the solvent control (3 5 to 4 1 mm) groups, but there was no reason identified for the results achieved.

5.4 Neonate production

The majority of *Daphnia* were observed to be gravid on Day 5 of the test, and neonate production was first observed on Day 6 by four *Daphnia*; the majority of first broods were produced on Day 8.

The numbers of live neonates produced during the test are given in Table 4 and a summary of the statistical analysis is given in Table 6a. This data is presented graphically as the dose response curve of mean number of neonates per surviving adult in Figure 1.

A summary of the cumulative total numbers of live neonates produced by surviving parental *Daphnia* on Day 21 is given below.

Calculated GST concentration (mg/L)	Cumulative number neonates/adult	
	mean	sd
Control	107	4 87
Solvent control	110	8 32
0.000893	107	3 91
0.00317	98.7	16.2
0.00930	108	11 5
0 0321	105	10.9
0 101	83 4	22 2

sd. standard deviation

Only the live neonates produced by parental *Daphnia* surviving at the end of the test (21-day effect concentrations) were included in the reproduction analysis. Under the conditions of the test, the following effective concentrations (EC_{10}, EC_{20} and EC_{50}) for reproduction were derived:

	Reproduction (calculated GST concentrations; mg/L)
21-day EC_{10}	0.0696 [0.0277, 0 100]
21-day EC_{20}	0.0966 [0.0690, 0.196]
21-day EC_{50}	>0 101

[ ] 95% confidence limits.

Statistical comparisons (Williams' test) using the total numbers of live neonates produced by each surviving adult on Day 21 showed that there was a significant reduction ($p<0.001$) in neonate production at a test concentration of 0.101 mg/L compared to the solvent control group. No effects on reproduction (live neonates) were observed at 0.000893 to 0.0321 mg/L, giving a NOEC of 0.0321 mg/L and a LOEC of 0 101 mg/L

The time taken to produce the first brood of neonates was not adversely affected at any test concentration (Table 6b); consequently the NOEC and LOEC for this parameter were 0.101 mg/L and >0.101 mg/L, respectively.

Statistical analysis (asymptotic linear by linear test) of the numbers of dead neonates or aborted eggs produced by each surviving adult in the test groups (Table 5) compared to the solvent control group indicated that neonate survival was adversely affected at concentrations of 0.0321 and 0.101 mg/L, giving a NOEC of 0.00930 mg/L and a LOEC of 0 0321 mg/L (see Table 6c).

Calculated GST concentration (mg/L)	Cumulative number dead neonates or aborted eggs/adult	
	mean	sd
Control	0	0
Solvent Control	0	0
0 000893	0	0
0.00317	0	0
0.00930	0	0
0 0321	0.300	0 675
0.101	1.20	1 10

sd. standard deviation.

5.5 Environmental conditions

Measurements of water quality of the test media taken during the test are given in Table 7 Measurements of pH and DO remained within acceptable limits during the study. The temperature of the test area, which ranged from 19.6 to 22.6°C during the 21-day exposure period, deviated from the range stated in the protocol. Measurements of temperature of the test media remained within acceptable limits during the study, with the exception of one occasion (Day 17), where the temperatures ranged between 22.3 and 22.6°C (see protocol deviations).

On the days of preparation, the test media were colourless.

6. Conclusions

Parental mortality: Under the conditions of the test, the EC_{50}, EC_{20} and EC_{10} values for the parental generation were identified as >0.101, 0.0699 and 0.0590 mg/L, respectively. The NOEC and LOEC for this parameter were 0.0321 mg/L and 0.101 mg/L, respectively.

Sub-lethal effects on parental generation: based on analysis of the lengths of surviving adults after 21 days of exposure, no adverse effects on growth were observed, giving a NOEC of 0.101 mg/L and a LOEC of >0.101 mg/L.

Reproduction: based on the total numbers of live neonates produced by each surviving parental *Daphnia* after 21 days of exposure, reproduction was significantly reduced at a GST concentration of 0.101 mg/L, giving a NOEC of 0.0321 mg/L and a LOEC of 0.101 mg/L.

Under the conditions of the test, the following EC_{10}, EC_{20} and EC_{50} values were derived:

	Reproduction (calculated GST concentrations; mg/L)
21-day EC_{10}	0.0696 [0.0277, 0.100]
21-day EC_{20}	0.0966 [0.0690, 0.196]
21-day EC_{50}	>0.101

[ ] . 95% confidence limits.

The time taken to produce the first brood of neonates was not adversely affected at any test concentration, consequently the NOEC and LOEC for this parameter were 0.101 mg/L and >0.101 mg/L, respectively.

Neonate survival (based on numbers of dead neonates or aborted eggs) was adversely affected at concentrations of 0.101 and 0.0321 mg/L, giving a NOEC of 0.00930 mg/L and a LOEC of 0.0321 mg/L.

7. References

AGRESTI, A., MEHTA, M.C. and PATEL, N.R. (1990) Exact inference for contingency tables with ordered categories. *Journal of the American Statistical Association*, **85**, 453-458.

BERKSON, J. (1944) Application of logistic function to bio-assay. *Journal of the American Statistical Association*. **39**, 357-365.

DUNNETT, C.W. (1955) A multiple comparison procedure for comparing several treatments with a control. *Journal of the American Statistical Association*, **50**, 1096-1121.

DUNNETT, C.W. (1964) New tables for multiple comparisons with a control. *Biometrics*, **20**, 482-491.

SAS INSTITUTE (2002) SAS OnlineDoc® Version Nine. SAS Institute Inc., Cary, NC, USA

WILLIAMS, D.A. (1971) A test for differences between means when several dose levels are compared with a zero dose control. *Biometrics*, **27**, 103-117.

WILLIAMS, D.A. (1972) The comparison of several dose levels with a zero dose control. *Biometrics*, **28**, 519-531.

Figure 1 Concentration response curve – mean number of live neonates per surviving adult after exposure for 21 days to GST

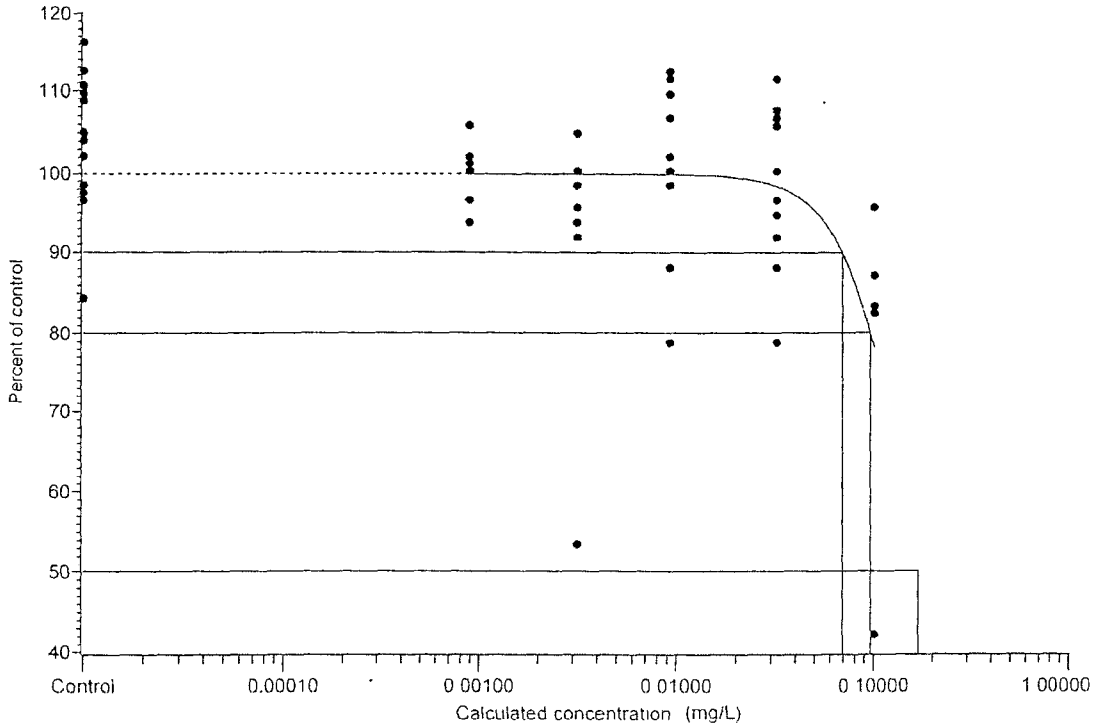


Table 1 Measured GST concentrations in solvent stock solutions

Nominal conc. (mg/mL)	Measured GST concentrations (mg/mL)										
	Day 0 F	Day 7 E	% initial	Day 7 F	Day 14 E	% initial	Day 14 F	Day 21 E	% initial	Calculated arithmetic mean (mg/L) *	% nominal
Solvent Control	ND	ND	-	ND	ND	-	ND	ND	-	ND	-
0 01	0 00850	0 00923	109	0.00895	0 00859	96	0.00989	0.00839	85	0.00893	89
0 032	0 0308	0.0333	108	0 0319	0.0323	101	0.0311	0.0306	98	0 0317	99
0.1	0 0965	0 0937	97	0 0965	0.0927	96	0.0917	0 0866	94	0.0930	93
0.32	0 350	0 326	93	0 323	0 331	102	0.314	0.282	90	0.321	100
1.0	0 944	1 04	110	1 02	1 07	105	1.06	0 916	86	1 01	101

F : freshly prepared solvent stock solution
E : expired solvent stock solution
ND : none detected (less than the limit of detection of 0.0025 mg/mL)
* : solvent stock concentrations (mg/mL) were used to calculate the concentrations in aqueous media (mg/L) using the following equation: concentration in solvent stock (mg/mL) x 0.1 (volume of solvent used/L)

Table 2 Cumulative parental mortality

Observation Time (days)	Control	Solvent Control	Calculated GST concentration, mg/L				
			0.000893	0.00317	0.00930	0.0321	0.101
1	0	0	0	0	0	0	0
2	0	1	0	0	0	0	0
3	0	1	0	0	0	0	0
4	0	1	0	0	0	0	0
5	0	1	0	0	0	0	3
6	0	1	0	0	0	0	5
7	1	1	0	0	0	0	5
8	1	1	0	0	0	0	5
9	1	1	0	0	0	0	5
10	1	1	1	0	0	0	5
11	1	1	1	0	0	0	5
12	1	1	1	0	0	0	5
13	1	2	1	0	0	0	5
14	2	2	1	0	0	0	5
15	2	2	1	0	0	0	5
16	2	2	1	0	0	0	5
17	2	2	1	0	0	0	5
18	2	2	2	1	0	0	5
19	2	2	2	1	0	0	5
20	2	2	2	1	0	0	5
21	2	2	2	1	0	0	5
Total	**2**	**2**	**2**	**1**	**0**	**0**	**5**

Table 3 Body lengths of surviving parental *Daphnia* on Day 21

Calculated GST conc. (mg/L)	Length of adult *Daphnia* (mm) Replicate number 1 (11)	2 (12)	3 (13)	4 (14)	5 (15)	6 (16)	7 (17)	8 (18)	9 (19)	10 (20)	Mean
Control	3 5	D	D	3.7	3 8	3.6	3.7	3.7	3.8	3.7	3 69
Solvent Control	3.9	3.6	3.9	3.7	3.8	3.7	3.9	3.9	4.1	3.8	3.82
	3 7	D	3.8	D*	3.5	3.9	D	4.0	3.8	4.0	
0.000893	3 9	4.0	4.0	3.9	3.7	3.9	D	3.8	3.9	D	3.89
0 00317	3.8	3 8	3 7	4.0	4 0	3 4	3.7	3.5	3.9	D	3 76
0.00930	3.8	3 9	4.0	3 8	4 0	3.9	3 9	3.7	4 0	3.8	3 88
0 0321	3.9	3.8	3 9	3.8	4 1	4 0	3.9	4.0	3 8	3.8	3 90
0.101	4.0	3 3	D	D	3 9	3.8	D	D	D	3.9	3.78

D . no data because the animal died before the end of the test
* . animal accidentally killed disregarded from test calculations
( ) . replicate identification for solvent control group

Table 4 Neonate production (live)

a) Control group

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	D	D	0	0	0	0	0	0	0
7	0	D	D	0	0	0	0	0	0	0
8	11	D	D	13	14	14	12	10	10	10
9	0	D	D	0	0	0	0	0	0	0
10	0	D	D	0	0	0	0	0	0	0
11	21	D	D	21	19	19	18	21	21	19
12	0	D	D	0	0	0	0	0	0	0
13	25	D	D	0	0	0	0	0	0	0
14	0	D	D	22	26	24	24	24	23	24
15	0	D	D	0	0	0	0	0	0	0
16	25	D	D	0	0	0	0	0	0	0
17	0	D	D	24	29	27	28	27	26	26
18	0	D	D	0	0	0	0	0	0	0
19	0	D	D	0	0	0	0	0	0	0
20	25	D	D	24	28	27	25	22	21	24
21	0	D	D	0	0	0	0	0	0	0
Total	**107**	**D**	**D**	**104**	**116**	**111**	**107**	**104**	**101**	**103**
Mean ± sd					**107 ± 4.87**					
% R					**2.86**					

D no data because the animal died before Day 21
sd standard deviation
% R % reduction compared to solvent control

Table 4 Neonate production (live) continued

b) Solvent Control group

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	0	0	0	0	0	0	0	0
7	0	0	8	0	0	10	0	0	0	0
8	13	15	0	12	14	2	13	11	11	10
9	0	0	0	0	0	0	0	0	0	0
10	0	0	14	0	0	20	1	0	0	0
11	23	17	0	19	21	0	19	18	19	18
12	0	0	17	0	0	0	0	0	0	0
13	0	0	0	0	0	25	0	0	0	0
14	26	22	0	22	27	0	25	25	27	24
15	0	0	19	0	0	0	0	0	0	0
16	0	0	0	0	0	30	0	0	0	0
17	28	26	0	27	30	0	26	28	30	28
18	0	0	29	0	0	0	0	0	0	0
19	0	0	0	0	0	32	0	0	0	0
20	27	24	0	25	28	0	32	21	29	24
21	0	0	32	0	0	0	0	0	0	0
Total	**117**	**104**	**111**	**105**	**120**	**109**	**116**	**103**	**116**	**104**

Observation time (days)	Replicate number 11	12	13	14*	15	16	17	18	19	20
6	0	D	0	-	0	7	D	0	0	0
7	0	D	0	-	0	0	D	0	9	0
8	13	D	13	-	14	0	D	14	0	13
9	0	D	0	-	0	0	D	0	0	0
10	14	D	0	-	0	0	D	0	17	0
11	2	D	21	-	21	0	D	20	0	20
12	0	D	0	-	0	20	D	0	22	0
13	22	D	0	-	0	0	D	0	0	0
14	0	D	26	-	22	0	D	27	0	27
15	0	D	0	-	0	26	D	0	23	0
16	28	D	0	-	0	0	D	0	0	0
17	0	D	27	-	29	0	D	27	0	8
18	0	D	0	-	0	28	D	0	32	0
19	50	D	0	-	0	0	D	0	0	0
20	0	D	31	-	19	0	D	24	0	22
21	0	D	0	-	0	29	D	0	30	0
Total	**109**	**D**	**118**	*	**105**	**103**	**D**	**112**	**124**	**90**
Mean ± sd					**110 ± 8.32**					
CoV					7.58					

sd	standard deviation
CoV	coefficient of variation for fecundity
D	no data because the animal died before Day 21
*	animal accidentally killed, disregarded from test calculations

Table 4 Neonate production (live) continued

c) 0.00893 mg/L

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	0	0	0	0	D	0	0	D
7	0	0	0	0	0	0	D	0	0	D
8	10	14	12	14	10	13	D	13	12	D
9	0	0	0	0	0	0	D	0	0	D
10	0	0	0	0	0	0	D	0	0	D
11	20	19	21	16	20	20	D	20	18	D
12	0	0	0	0	0	0	D	0	0	D
13	0	0	0	0	0	0	D	0	0	D
14	23	20	24	24	23	27	D	26	22	D
15	0	0	0	0	0	0	D	0	0	D
16	0	0	0	0	0	0	D	0	0	D
17	29	25	25	23	26	27	D	27	28	D
18	0	0	0	0	0	0	D	0	0	D
19	0	0	0	0	0	0	D	0	0	D
20	26	29	26	26	30	26	D	14	27	D
21	0	0	0	0	0	0	D	0	0	D
Total	**108**	**107**	**108**	**103**	**109**	**113**	**D**	**100**	**107**	**D**
Mean ± sd					**107 ± 3.91**					
% R					**2.63**					

d) 0.00317 mg/L

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	0	0	8	0	0	0	0	D
7	0	0	0	0	0	0	0	0	0	D
8	13	12	15	12	0	12	15	13	10	D
9	0	0	0	0	14	0	0	0	0	D
10	0	0	0	0	0	0	0	16	0	D
11	20	19	23	19	0	22	19	0	19	D
12	0	0	0	0	26	0	0	0	0	D
13	0	0	0	0	0	0	0	0	0	D
14	21	25	23	25	0	6	15	13	18	D
15	0	0	0	0	14	0	0	0	0	D
16	0	0	0	0	0	0	0	0	0	D
17	20	27	26	27	0	22	26	10	24	D
18	0	0	0	0	25	0	0	0	0	D
19	0	0	0	0	0	0	0	0	0	D
20	26	22	0	24	0	30	29	5	27	D
21	0	0	25	0	28	6	0	0	0	D
Total	**100**	**105**	**112**	**107**	**107**	**100**	**102**	**57**	**98**	**D**
Mean ± sd					**98.7 ± 16.2**					
% R					**10.1**					

D . no data because the animal died before Day 21
sd . standard deviation
% R . % reduction compared to solvent control

Table 4 Neonate production (live) continued

e) 0.00930 mg/L

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	0	0	0	0	0	0	0	8
7	0	0	0	0	0	0	0	0	0	0
8	11	11	12	13	12	12	11	13	11	0
9	0	0	0	0	0	0	0	0	0	13
10	0	0	0	0	0	0	0	0	0	0
11	19	24	21	21	19	22	21	20	20	0
12	0	0	0	0	0	0	0	0	0	21
13	0	0	0	0	0	0	0	1	0	0
14	12	13	14	24	26	25	24	21	25	0
15	0	0	0	0	0	0	0	0	0	20
16	0	0	0	0	0	0	0	0	0	0
17	23	29	26	32	29	30	29	26	33	0
18	0	0	0	0	0	0	0	0	0	25
19	0	0	0	0	0	0	0	0	0	0
20	29	32	32	27	28	30	29	0	31	0
21	0	0	0	0	0	0	0	3	0	28
Total	**94**	**109**	**105**	**117**	**114**	**119**	**114**	**84**	**120**	**107**
Mean ± sd					**108 ± 11.5**					
% R					**1.34**					

f) 0.0321 mg/L

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	6	0	0	0	0	0	0	0	0
7	0	1	0	0	0	0	0	0	0	0
8	12	0	10	9	9	13	12	13	10	13
9	0	0	0	0	0	0	0	0	0	0
10	0	14	0	0	0	0	0	20	0	18
11	17	0	18	18	23	20	18	0	21	0
12	0	17	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	20	0	22
14	2[illegible]	[illegible]	23	24	[illegible]	27	23	0	23	0
15	0	18	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	28	0	31
17	30	0	27	28	30	30	27	0	30	0
18	0	26	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	34	0	0
20	0	23	0	24	28	29	27	0	30	10
21	0	0	23	0	0	0	0	0	0	0
Total	**84**	**98**	**101**	**103**	**113**	**119**	**107**	**115**	**114**	**94**
Mean ± sd					**105 ± 10.9**					
% R					**4.52**					

sd . standard deviation
% R . % reduction compared to solvent control

Table 4 Neonate production (live) continued

g) 0.101 mg/L

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	D	D	0	0	D	D	D	0
7	0	0	D	D	0	0	D	D	D	0
8	4	8	D	D	0	11	D	D	D	0
9	0	0	D	D	12	0	D	D	D	8
10	0	0	D	D	0	0	D	D	D	0
11	17	14	D	D	19	17	D	D	D	1
12	0	0	D	D	0	0	D	D	D	11
13	0	0	D	D	0	0	D	D	D	0
14	19	17	D	D	17	14	D	D	D	15
15	0	0	D	D	0	0	D	D	D	0
16	0	0	D	D	0	0	D	D	D	0
17	23	6	D	D	24	25	D	D	D	25
18	0	0	D	D	0	0	D	D	D	0
19	0	0	D	D	0	0	D	D	D	0
20	30	0	D	D	30	22	D	D	D	3
21	0	0	D	D	0	0	D	D	D	25
Total	**93**	**45**	**D**	**D**	**102**	**89**	**D**	**D**	**D**	**88**
Mean ± sd					**83.4 ± 22.2**					
% R					**24.0**					

D . no data because the animal died before Day 21
sd . standard deviation
% R . % reduction compared to solvent control

Table 5 Neonate production (dead and aborted eggs)

a) Control group

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	D	D	0	0	0	0	0	0	0
7	0	D	D	0	0	0	0	0	0	0
8	0	D	D	0	0	0	0	0	0	0
9	0	D	D	0	0	0	0	0	0	0
10	0	D	D	0	0	0	0	0	0	0
11	0	D	D	0	0	0	0	0	0	0
12	0	D	D	0	0	0	0	0	0	0
13	0	D	D	0	0	0	0	0	0	0
14	0	D	D	0	0	0	0	0	0	0
15	0	D	D	0	0	0	0	0	0	0
16	0	D	D	0	0	0	0	0	0	0
17	0	D	D	0	0	0	0	0	0	0
18	0	D	D	0	0	0	0	0	0	0
19	0	D	D	0	0	0	0	0	0	0
20	0	D	D	0	0	0	0	0	0	0
21	0	D	D	0	0	0	0	0	0	0
Total	**0**	**D**	**D**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Mean					**0**					

D . no data because the animal died before Day 21

Table 5 Neonate production (dead and aborted eggs) continued

b) Solvent Control group

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

Observation time (days)	Replicate number 11	12	13	14*	15	16	17	18	19	20
6	0	D	0	-	0	0	D	0	0	0
7	0	D	0	-	0	0	D	0	0	0
8	0	D	0	-	0	0	D	0	0	0
9	0	D	0	-	0	0	D	0	0	0
10	0	D	0	-	0	0	D	0	0	0
11	0	D	0	-	0	0	D	0	0	0
12	0	D	0	-	0	0	D	0	0	0
13	0	D	0	-	0	0	D	0	0	0
14	0	D	0	-	0	0	D	0	0	0
15	0	D	0	-	0	0	D	0	0	0
16	0	D	0	-	0	0	D	0	0	0
17	0	D	0	-	0	0	D	0	0	0
18	0	D	0	-	0	0	D	0	0	0
19	0	D	0	-	0	0	D	0	0	0
20	0	D	0	-	0	0	D	0	0	0
21	0	D	0	-	0	0	D	0	0	0
Total	**0**	**D**	**0**	**-***	**0**	**0**	**D**	**0**	**0**	**0**
Mean					0					

D no data because the animal died before Day 21
* animal accidentally killed, disregarded from test calculations

Table 5 Neonate production (dead and aborted eggs) continued

c) 0.00893 mg/L

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	0	0	0	0	D	0	0	D
7	0	0	0	0	0	0	D	0	0	D
8	0	0	0	0	0	0	D	0	0	D
9	0	0	0	0	0	0	D	0	0	D
10	0	0	0	0	0	0	D	0	0	D
11	0	0	0	0	0	0	D	0	0	D
12	0	0	0	0	0	0	D	0	0	D
13	0	0	0	0	0	0	D	0	0	D
14	0	0	0	0	0	0	D	0	0	D
15	0	0	0	0	0	0	D	0	0	D
16	0	0	0	0	0	0	D	0	0	D
17	0	0	0	0	0	0	D	0	0	D
18	0	0	0	0	0	0	D	0	0	D
19	0	0	0	0	0	0	D	0	0	D
20	0	0	0	0	0	0	D	0	0	D
21	0	0	0	0	0	0	D	0	0	D
Total	**0**	**0**	**0**	**0**	**0**	**0**	**D**	**0**	**0**	**D**
Mean					**0**					

d) 0.00317 mg/L

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	0	0	0	0	0	0	0	D
7	0	0	0	0	0	0	0	0	0	D
8	0	0	0	0	0	0	0	0	0	D
9	0	0	0	0	0	0	0	0	0	D
10	0	0	0	0	0	0	0	0	0	D
11	0	0	0	0	0	0	0	0	0	D
12	0	0	0	0	0	0	0	0	0	D
13	0	0	0	0	0	0	0	0	0	D
14	0	0	0	0	0	0	0	0	0	D
15	0	0	0	0	0	0	0	0	0	D
16	0	0	0	0	0	0	0	0	0	D
17	0	0	0	0	0	0	0	0	0	D
18	0	0	0	0	0	0	0	0	0	D
19	0	0	0	0	0	0	0	0	0	D
20	0	0	0	0	0	0	0	0	0	D
21	0	0	0	0	0	0	0	0	0	D
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**D**
Mean					**0**					

D · no data because the animal died before Day 21

Table 5 Neonate production (dead and aborted eggs) continued

e) 0.00930 mg/L

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Mean					0					

f) 0.0321 mg/L

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	0	0	0	0	1	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0
21	0	0	2	0	0	0	0	0	0	0
Total	**0**	**0**	**2**	**0**	**0**	**0**	**1**	**0**	**0**	**0**
Mean ± sd					0.300 ± 0.675					

sd standard deviation

Table 5 Neonate production (dead and aborted eggs) continued

g) 0.101 mg/L

Observation time (days)	Replicate number 1	2	3	4	5	6	7	8	9	10
6	0	0	D	D	0	0	D	D	D	0
7	0	0	D	D	0	0	D	D	D	0
8	2	0	D	D	0	0	D	D	D	0
9	0	0	D	D	0	0	D	D	D	0
10	0	0	D	D	0	0	D	D	D	0
11	0	0	D	D	0	0	D	D	D	0
12	0	0	D	D	0	0	D	D	D	0
13	0	0	D	D	0	0	D	D	D	0
14	0	0	D	D	2	2	D	D	D	0
15	0	0	D	D	0	0	D	D	D	0
16	0	0	D	D	0	0	D	D	D	0
17	0	0	D	D	0	0	D	D	D	0
18	0	0	D	D	0	0	D	D	D	0
19	0	0	D	D	0	0	D	D	D	0
20	0	0	D	D	0	0	D	D	D	0
21	0	0	D	D	0	0	D	D	D	0
Total	**2**	**0**	**D**	**D**	**2**	**2**	**D**	**D**	**D**	**0**
Mean ± sd					**1.20 ± 1.10**					

D no data because the animal died before Day 21
sd standard deviation

Table 6 Summary of statistical analysis

a) Mean number of young per adult and lengths of surviving adults

Parameter	Calculated GST Concentration (mg/L)	Sample size	Mean	% Inhibition	*p*
Mean young per adult after 21 days	Control	8	106.6	2.9	0.521[T]
	Solvent Control	17	109.8	0 0	-
	0 000893	8	106.9	2.6	0.555[W]
	0.00317	9	98.7	10 1	0.281[W]
	0.00930	10	108.3	1.3	0.281[W]
	0 0321	10	104.8	4.5	0.281[W]
	0.101	5	83 4	24.0	<0 001***[W]
Length of surviving adults	Control	8	3 688	3 6	0.039*[T]
	Solvent Control	17	3 824	0 0	-
	0.000893	8	3 888	-1 7	0 822[D]
	0.00317	9	3.756	1 8	0 758[D]
	0.00930	10	3 880	-1 5	0 850[D]
	0 0321	10	3 900	-2.0	0.634[D]
	0.101	5	3.780	1.1	0 979[D]

p values are for the comparison with Control using Williams' test (W), Dunnett's test (D) and the *t*-test (T)
* $p < 0\ 05$
*** $p < 0.001$

b) Distribution of first broods by study day

Calculated GST Concentration (mg/L)	Day 6	7	8	9	10	*p*
Control	0	0	8	0	0	0 269
Solvent Control	1	3	13	0	0	
0.000893	0	0	8	0	0	
0 00317	1	0	8	0	0	
0 00930	1	0	9	0	0	
0 0321	1	0	9	0	0	
0.101	0	0	3	2	0	0 169

p values are by asymptotic linear by linear test for all groups up to and including the current row

Table 6 Summary of statistical analysis continued

c) Dead neonates - Summary of frequencies

Calculated GST Concentration (mg/L)	Number of dead neonates or aborted eggs			
	0	1	2	*p*
Control	10	0	0	1.000
Solvent Control	20	0	0	
0.000893	10	0	0	
0.00317	10	0	0	
0.00930	10	0	0	
0.0321	8	1	1	0.034*
0 101	7	0	3	0.002**

p values are by asymptotic linear by linear test for all groups up to and including the current row except for the control (compared to solvent control by exact Wilcoxon rank sum tests)
* $p < 0.05$
** $p < 0.01$

Table 7 Environmental parameters

Calculated GST concentration (mg/L)	Temperature °C		pH		Dissolved oxygen %ASV	
	Min	Max	Min	Max	Min	Max
Control	20 7	22.6	7.46	8.13	92	103
Solvent Control	20.6	22.4	7.33	8.06	77	103
0.000893	20.6	22.4	7.29	8.08	73	103
0.00317	20.6	22.5	7.24	8.06	73	102
0.00930	20.5	22.5	7.23	8.07	71	102
0.0321	20.5	22.4	7.27	8.06	68	103
0.101	20 5	22.3	7.30	8 06	80	103

ASV: Percent air saturation value as a measure of dissolved oxygen concentration

Continuous monitoring of temperature in an additional vessel containing dilution medium = 19 6 to 22.6°C

Total hardness of dilution medium = 250 to 270 mg/L as $CaCO_3$
Alkalinity of dilution medium = 43 to 63 mg/L as $CaCO_3$

Appendix 1 Certificate of Analysis

[redacted]

CERTIFICATE OF ANALYSIS

NAME OF SAMPLE : GST
LOT NO. OF SAMPLE : Lot numbe
DATE OF ANALYSIS . 2008/02/25

COMPOSITION

Identity	Conc
#1	93.9%
#2	0.7%
#3	2.7%
#4	1.6%
#5	0.2%
#6	0.9%
TOTAL	**100%**

NAME

SIGNATURE DATE 2008 / 4 / 11

1

Appendix 2 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
	$CuCl_2.2H_2O$	0.017
	$ZnCl_2$	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 deionised water produced by reverse osmosis and aerated prior to use The following parameters are monitored for each batch of medium prepared by the Department of Aquatic Ecotoxicology and Biodegradation: pH, dissolved oxygen, hardness and alkalinity.

Appendix 3 The Determination of GST in Acetone Stock Solutions

METHOD

General principle

Samples were diluted with acetonitrile:water (50:50 v:v). Quantitation was performed using liquid chromatography with UV detection (HPLC-UV).

Materials	Grade
Acetone	GD
Acetonitrile	HPLC
Potassium dihydrogen orthophosphate	AR
Water	HPLC

Preparation of reagents

Preparation of acetonitrile:water (50:50 v:v):
Acetonitrile (500 mL) and water (500 mL) were thoroughly mixed prior to use.

Preparation of 0.01M potassium dihydrogen orthophosphate:
Potassium dihydrogen orthophosphate (1.36 g) was added to water (1000 mL) and thoroughly mixed prior to use.

Preparation of 0.01M potassium dihydrogen orthophosphate:acetonitrile (50:50 v:v):
0.01M Potassium dihydrogen orthophosphate (500 mL) was added to acetonitrile (500 mL) and thoroughly mixed prior to use.

NOTE – to prepare different volumes of the above reagents, the ratio of the individual components was maintained.

Test substance solutions

GST was accurately weighed (corrected for purity) and dissolved in acetonitrile to give a stock standard solution of 10 mg/mL. The stock standard solution was diluted progressively with acetonitrile to give intermediate standard solutions.

An appropriate intermediate standard solution was diluted progressively with acetonitrile:water (50:50 v:v) to produce a series of calibration solutions in the range 0.5 to 10 μg/mL.

Sample analysis procedure

An aliquot (10 mL) of the sample was diluted by a factor of 5 with acetonitrile:water (50:50 v:v). Any further dilutions where required were performed using acetonitrile:water (50:50 v:v), prior to quantitation by HPLC-UV.

HPLC-UV conditions

Instrument:	HP 1100
Column:	Supelcosil LC-ABZ (25 cm x 4.6 mm x 5 μm)
Mobile phase:	0.01M potassium dihydrogen orthophosphate:acetonitrile (50:50 v:v)
Flow rate:	1 mL/min
Oven temperature:	40°C
Injection volume:	100 μL
Wavelength:	210 nm
Retention time:	approx. 12.5 minutes
LOQ:	0.005 mg/mL
LOD:	0.5 μg/mL (equivalent to 0.0025 mg/mL in acetone solution)

Calculation of results

Test samples were quantified using the following equation:

Residue found (mg/mL) = $x \times \frac{1}{M} \times D \times \frac{1}{1000}$

Where x (residue concentration in final solution) was calculated using the linear regression:

$y = m\,x + c$ where x (concentration in µg/mL) = $\frac{y - c}{m}$

c = intercept
m = slope
y = peak area of sample
M = matrix concentration (mL/mL)
D = dilution factor

Example calculation of GST detected in a sample of acetone treated at 0.1 mg/mL, (analytical identification 10/MTU/6043).

Linear regression $y = m\,x + c$

$$y = 91.0559\,x - 19.7309$$

where $y = 331.66$
m = 91.0559
c = -19.7309

Therefore, concentration of GST (x) $= \frac{331.66 + 19.7309}{91.0559}$

$= 3.859$ µg/mL

Matrix concentration = 0.2 mL/mL
Dilution factor = 5

GST detected (mg/mL) $= \frac{3.859 \text{ µg/mL} \times 5}{0.2 \text{ mL/mL} \times 1000}$

$= 0.0965$ mg/mL

RESULTS AND DISCUSSION

Calibration

The chromatographic response to GST was shown to be linear over the range of concentrations 0.5 to 10 µg/mL. Typical calibration data are presented in Table 1 and Figure 1. Typical chromatograms of calibration standards are presented in Figures 2 and 3.

Validation

The methodology for the determination of GST in acetone was validated at 0.005 and 5 mg/mL as part of ( )

Limit of quantitation (LOQ)

The limit of quantitation is defined as the lowest fortification level at which acceptable recovery data are obtained. The validation of the methodology for the determination of residues of GST in acetone, demonstrated that it can be accurately determined at a limit of quantitation (LOQ) of 0.005 mg/mL.

Limit of detection (LOD)

The limit of detection of the method is defined as the value of the lowest calibration standard chromatographed that gave a peak height to baseline noise ratio ≥3. For this study the limit of detection for GST was 0.5 µg/mL (equivalent to 0.0025 mg/mL in acetone).

Samples

Samples from the test were analysed in a suitably sized batch along with control samples fortified with GST which acted as procedural recovery samples. Procedural recoveries performed on control acetone were in the range 70 to 110% confirming the validity of the methodology on the day of analysis. Typical chromatograms of untreated, untreated fortified with GST and treated extracts are presented in Figures 4 to 6.

TABLE 1

Typical standard calibration for GST by HPLC-UV

Standard concentration (µg/mL)	Peak area
0	0
0.5	31.080
1	62.920
2	153.490
3	233.690
4	327.110
5	436.110
6	541.400
7	638 370
8	723.760
9	787.360
10	881.540

Calibration curve: $y = 91.0559\,x - 19\ 7309$ (batch 4)

$r = 0.9989$

Where x = concentration
y = peak area
r = regression coefficient

FIGURE 1

Typical standard chemical calibration for GST by HPLC-UV

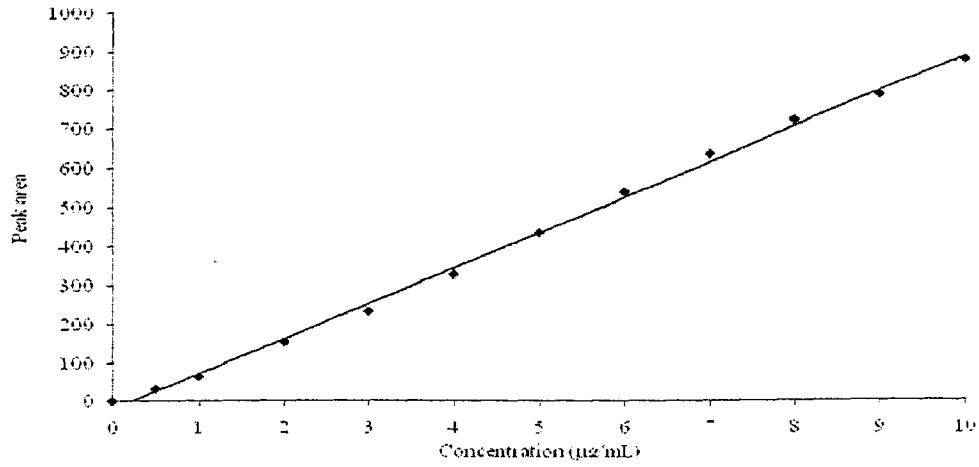


FIGURE 2

Typical chromatogram of a 0.5 µg/mL GST calibration standard

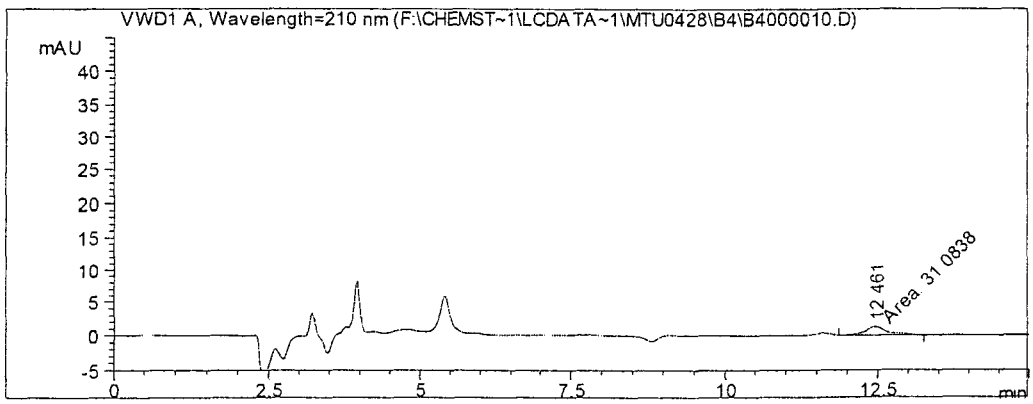


FIGURE 3

Typical chromatogram of a 10 µg/mL GST calibration standard

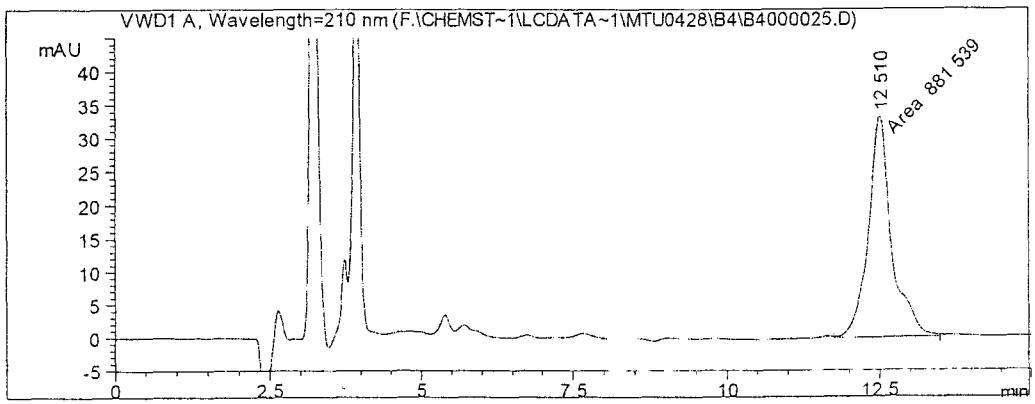


FIGURE 4

Chromatogram of an untreated acetone sample (analytical identification acetone Con A)

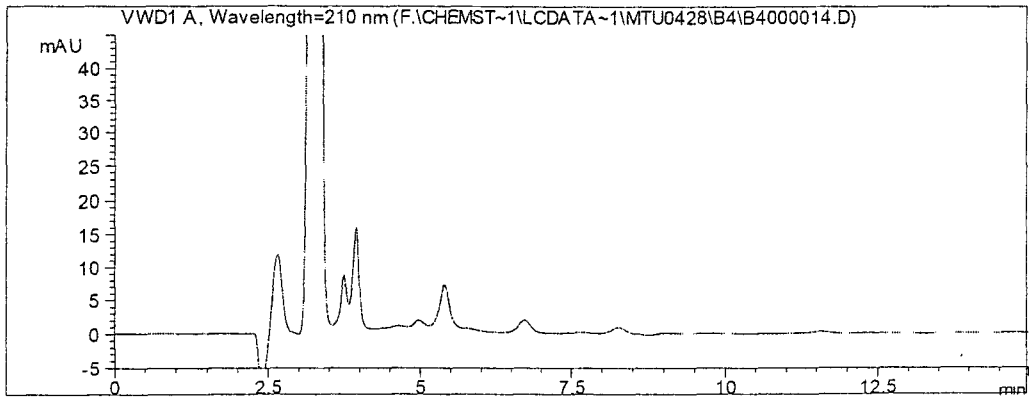


FIGURE 5

Chromatogram of an untreated acetone sample fortified with 0.1 mg/mL of GST (analytical identification acetone F0.1)

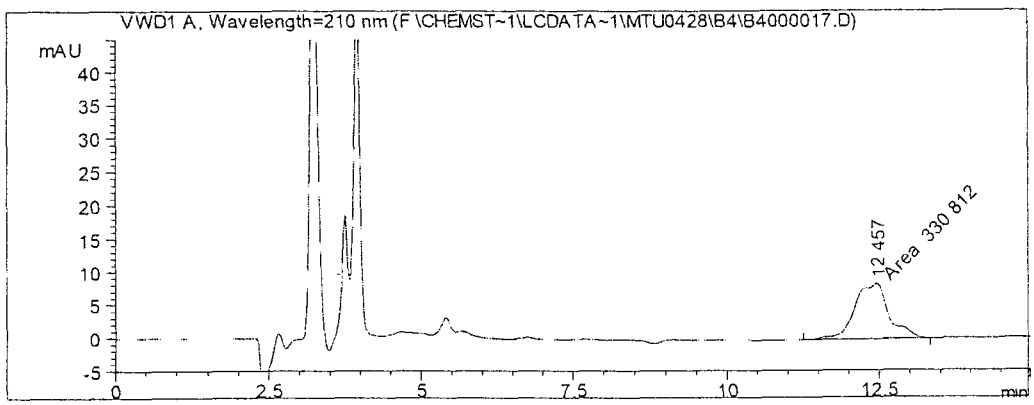


FIGURE 6

Chromatogram of a sample of an acetone stock solution of GST at 0.1 mg/mL taken on Day 0 (analytical identification 10/MTU/6043)

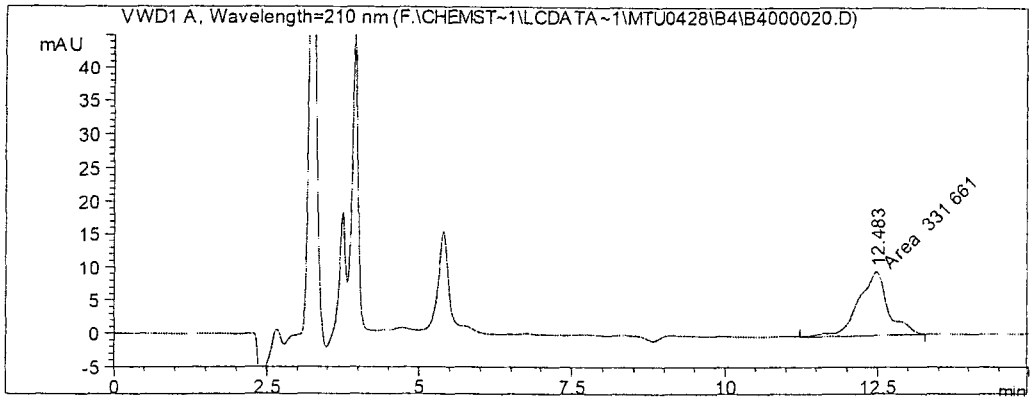
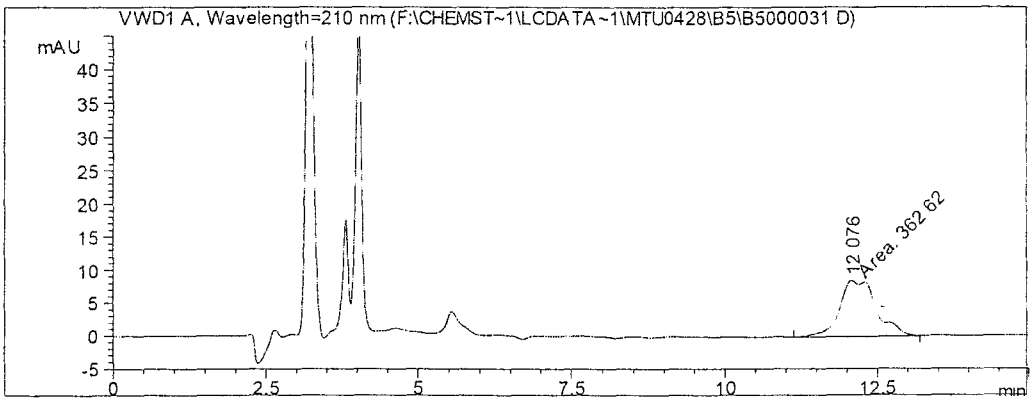


FIGURE 7

Chromatogram of a sample of an acetone stock solution of GST at 0.1 mg/mL taken on Day 7 (analytical identification 10/MTU/6359)



Appendix 4 Eye Research Centre GLP Compliance Statements

a) 2008

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE

GOOD LABORATORY PRACTICE

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

TEST FACILITY

TEST TYPE

Analytical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Phys/Chem Testing
Toxicology

DATE OF INSPECTION

28th January 2008

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the 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.

Appendix 4 Eye Research Centre GLP Compliance Statements continued

b) 2009

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE I

GOOD LABORATORY PRACTICE

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

TEST FACILITY

TEST TYPE

Analytical/Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Phys/Chem Testing
Toxicology

DATE OF INSPECTION

17-19 February 2009

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the 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.

c) 2010

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE U

GOOD LABORATORY PRACTICE

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

TEST FACILITY

TEST TYPE

Analytical/Clinical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Physico-chemical Testing
Residue Studies
Toxicology

DATE OF INSPECTION

26 January 2010

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the 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.