SafePharm Laboratories

C6-2AL:

ACUTE TOXICITY TO RAINBOW TROUT
(*Oncorhynchus mykiss*)

SPL PROJECT NUMBER: 1742/021

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QUALITY ASSURANCE REPORT

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

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

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

	02 December 2003	Standard Test Method Compliance Audit
	21 February 2005	Test Material Preparation
	14, 21 February 2005	Test System Preparation
	14 February 2005	Exposure
	14, 17 February 2005	Assessment of Response
	08, 14, 16, 28 February 2005	Chemical Analysis
§	18 April 2005	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: - 9 JUN 2005

For Safepharm Quality Assurance Unit*

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***Authorised QA Signatures:**

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GLP COMPLIANCE STATEMENT

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

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

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

..... Date: - 8 JUN 2005

P M Wetton BSc
Study Director

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

..... Date: - 8 JUN 2005

Dr J McKenzie CChem MRSC
Head of Analytical Services

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SUMMARY

Introduction. A study was performed to assess the acute toxicity of the test material to rainbow trout (*Oncorhynchus mykiss*). The method followed that described in the OECD Guidelines for Testing of Chemicals (1992) No 203, "Fish, Acute Toxicity Test" referenced as Method C.1 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Methods. Following a preliminary range-finding test, fish were exposed, in groups of seven, to an aqueous solution of the test material over a range of nominal concentrations of 1.3, 2.3, 4.2, 7.3 and 13 mg/l for a period of 96 hours at a temperature of approximately 14°C under semi-static test conditions. The test material solutions were prepared by stirring an excess (50 mg/l) of test material in dechlorinated tap water at approximately 1500 rpm at a temperature of approximately 14°C for 24 hours prior to removing any undissolved test material by filtration (0.2 µm) through a pre-conditioned filter to produce a saturated solution with a nominal test concentration of 13 mg/l. The nominal concentration of the saturated solution was based on the results of chemical analysis of a saturated solution prepared during a preliminary dosing trial. A series of dilutions were made from this saturated solution to prepare the remainder of the test series. The number of mortalities and any sub-lethal effects of exposure in each test and control vessel were determined 3 and 6 hours after the start of exposure and then daily throughout the test until termination after 96 hours.

Results. The 96-Hour LC_{50} based on nominal test concentrations was 9.0 mg/l with 95% confidence limits of 7.7 – 10 mg/l. The No Observed Effect Concentration was 2.3 mg/l.

Analysis of the freshly prepared test media throughout the test showed measured values to range from 0.109 mg/l to 4.80 mg/l and analysis of the old or expired test media showed measured values to range from 0.0872 mg/l to 4.22 mg/l. A large amount of variability was shown in the measured concentrations with values in the range of 8% to 56% of nominal for the fresh test media and 7% to 43% of nominal for the old or expired test media. However, on each sampling occasion the measured values were shown to increase with increasing nominal test concentration and the measured concentrations shown for the media sampled at the end of each renewal period showed a slight decline compared to the measured test concentrations of the samples taken at the beginning of each renewal period.

The variability shown in the measured test concentrations was considered to be due to the variability in the concentration of the saturated solutions produced at each preparation period. Measured values of 2.33, 4.80, 1.23 and 8.05 mg/l were shown for the saturated solution at 0, 24, 48 and 72 hours respectively. This variability was considered to be due to differences in the water quality from day to day such as pH and dissolved salts affecting the solubility of the test material, plus minor differences in stirring the saturated solution although every effort was made to ensure consistency in stirring.

Although the measured test concentrations were shown to be variable, it was considered inappropriate to repeat the test as the range-finding and definitive tests showed the same biological response and a No Observed Effect Concentration and LC_{50} values were obtained during the definitive test.

Given that the measured test concentrations were low and variable and a slight decline was shown in the old or expired test media compared to the fresh test media it was considered justifiable to base the results on the time-weighted mean measured test concentrations of the test media to give a "worst case" analysis of the data. The time-weighted mean measured test concentrations were calculated to be 0.276, 0.486, 1.02, 1.83 and 3.27 mg/l. The 96-Hour LC_{50} based on the time-weighted mean measured test concentrations was 2.3 mg/l with 95% confidence limits of 1.9 – 2.6 mg/l. The No Observed Effect Concentration was 0.49 mg/l.

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ACUTE TOXICITY TO RAINBOW TROUT (*Oncorhynchus mykiss*)

1. INTRODUCTION

This report contains a description of the methods used and results obtained during a study to investigate the acute toxicity of the test material to rainbow trout. The method followed the recommendations of the OECD Guidelines for Testing of Chemicals (1992) No 203 "Fish, Acute Toxicity Test" referenced as Method C.1 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Rainbow trout is a freshwater fish representative of a wide variety of natural habitats, and can therefore be considered as an important non-target organism in freshwater ecosystems.

The range-finding test was conducted between 22 September 2004 and 27 September 2004 and the definitive test between 13 February 2005 and 18 February 2005.

In view of the difficulties associated with the evaluation of aquatic toxicity of poorly water soluble test materials, a modification of the standard method for the preparation of aqueous media was performed. An approach endorsed by several important regulatory authorities in the EU and elsewhere (ECETOC 1996 and OECD 2000), is to expose organisms to a saturated solution of the test material in cases where the test material is of high purity and is poorly soluble in water and in the permitted auxiliary solvents and surfactants. Using this approach, a saturated solution was prepared by stirring an excess (50 mg/l) of test material with dechlorinated tap water for 24 hours and then removing the undissolved test material by filtration through a pre-conditioned filter (0.2 µm) to give a saturated solution with a nominal concentration of 13 mg/l.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	C6-2AL
Description	:	clear colourless liquid
Chemical name	:	1H, 1H, 2H, 2H-tridecafluoro-1-octanol
Lot number	:	001002
Date received	:	sample used for range-finding test : 12 January 2004 sample used for definitive test : 27 January 2005
Storage conditions	:	room temperature in the dark

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

2.2 Experimental Preparation

Due to the low aqueous solubility and high purity of the test material the test concentrations used in the definitive test were prepared by diluting a saturated solution prepared from initial test material dispersions at a concentration of 50 mg/l.

An amount of test material (1150 mg) was dispersed in 22.5 litres of dechlorinated tap water with the aid of propeller stirring at approximately 1500 rpm at 14°C for a period of 24 hours. After 24 hours the stirring was stopped and the undissolved test material removed by filtration (0.2 µm Gelman SuporCap filter, first approximate 1 litre discarded in order to pre-condition the filter) to give a saturated solution with a nominal concentration of 13 mg/l*. This nominal 13 mg/l* test concentration was prepared in duplicate or triplicate to ensure a sufficient volume was available for testing and dilution. Aliquots (2.5, 4.42, 8.08 and 14 litres) of this nominal 13 mg/l* test concentration were each separately dispersed into a final volume of 25 litres of dechlorinated tap water and stirred using a flat bladed mixer for approximately 1 minute to give the remainder of the test series of nominal test concentrations of 1.3, 2.3, 4.2 and 7.3 mg/l.

The concentration and stability of the test material in the test preparations were verified by chemical analysis at 0 (fresh media), 24, 48, 72 (old and fresh media) and 96 hours (old media) (see Appendix 1).

3. METHODS

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3.1 Test Species

The test was carried out using juvenile rainbow trout (*Oncorhynchus mykiss*). Fish were obtained from Brow Well Fisheries Limited, Hebden, near Skipton, Yorkshire, UK and maintained in-house since 06 January 2005. Fish were maintained in a glass fibre tank with a "single pass" water renewal system. Fish were acclimatised to test conditions from 2 February 2005 to 14 February 2005. The lighting cycle was controlled to give a 16 hours light and 8 hours darkness cycle with 20 minute dawn and dusk transition periods.

The water temperature was controlled at approximately 14°C with a dissolved oxygen content of greater than or equal to 9.3 mg O_2/l. These parameters were recorded daily. The stock fish were fed commercial trout pellets which was discontinued approximately 24 hours prior to the start of the definitive test. There was zero mortality in the 7 days prior to the start of the test and the fish had a mean standard length of 4.7 cm (sd = 0.3) and a mean weight of 1.28 g (sd = 0.22) at the

* Concentration based on the results of preliminary analysis of a saturated solution of the test material

end of the definitive test. Based on the mean weight value this gave a loading rate of 0.36 g bodyweight/litre.

The diet and diluent water are considered not to contain any contaminant that would affect the integrity and outcome of the study.

3.2 Test Water

The test water used for both the range-finding and definitive tests was the same as that used to maintain the stock fish.

Laboratory tap water was dechlorinated by passage through an activated carbon filter (Purite Series 500) and partly softened (Elga Nimbus 1248D Duplex Water Softener) giving water with a total hardness of approximately 100 mg/l as $CaCO_3$. After dechlorination and softening the water was passed through a series of computer controlled plate heat exchangers to achieve the required temperature. Typical water quality characteristics for the tap water as supplied, prior to dechlorination and softening, are given in Appendix 2.

3.3 Procedure

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3.3.1 Media preparation trials

Initial media preparation trials were conducted using a solvent spike method of introduction of the test material into the test diluent at a nominal concentration of 10 mg/l. However, the results from analysis of these initial preparations were inconclusive showing great variation in the measured concentrations and where the test media were untreated prior to analysis the nominal test concentration of 10 mg/l was not achieved. Therefore, this method of preparation was considered to be unsuitable for testing.

Further media preparation trials were therefore conducted by preparing saturated solutions of the test material. In the first saturated solution preparation trial an amount of test material (550 mg) was dispersed in 11 litres of dechlorinated tap water to give an initial dispersion of 50 mg/l. This was then stirred by propeller stirrer at approximately 1500 rpm at a temperature of approximately 14°C for 24 hours. On completion of stirring samples were taken from the saturated solution for chemical analysis. Undissolved test material was removed by filtration (0.2 µm filters with the initial 1 litre and 2 litres discarded to pre-condition the filters) and by centrifugation for 30 minutes (10000 g and 40000 g) prior to analysis of the samples.

Prior to beginning the definitive test a second preparation trial was conducted, preparing the lowest, middle and highest test concentrations to be used in the definitive test from a saturated

solution of the test material. An amount of test material (1125 mg) was dispersed in 22.5 litres of dechlorinated tap water to give an initial dispersion of 50 mg/l. This was then stirred by propeller stirrer at approximately 1500 rpm at a temperature of approximately 14°C for 24 hours. The initial dispersion was prepared in duplicate to allow sufficient saturated solution for dilutions to be made to give the test series. On completion of stirring the undissolved test material was removed by filtration (0.2 µm filter with the initial 1 litre discarded to pre-condition the filter) to give a saturated solution of the test material with a nominal concentration of 13 mg/l*. Volumes (2.5, 8.08 and 25 litres) of the saturated solution were dispensed to glass test vessels and the final volume adjusted to 25 litres to give the nominal test range of 1.3, 4.2 and 13 mg/l. Samples were taken from each of the prepared test concentrations for analysis after being left on laboratory benching under test conditions for 24 hours (old or expired test media). During the 24 hour period no auxiliary aeration was supplied to the test vessels, the vessel lids were sealed and a 25 litre test volume was used giving a minimal headspace, due to the suspected volatile nature of the test material.

3.3.2 Range-finding test

The test concentrations to be used in the definitive test were determined by a preliminary range-finding test.

In the range-finding test fish were exposed to a series of nominal test concentrations of 0.13, 1.3 and 13 mg/l*. Due to the low aqueous solubility and high purity of the test material the test concentrations used in the range-finding test were prepared by diluting a saturated solution prepared from initial test material dispersions at a concentration of 50 mg/l.

Amounts of test material (550 and 1125 mg) were each separately dispersed in 11 and 22.5 litres of dechlorinated tap water respectively with the aid of propeller stirring at approximately 1500 rpm at a temperature of approximately 14°C for a period of 24 hours. After 24 hours the stirring was stopped and the undissolved test material removed by filtration (0.2 µm Gelman SuporCap filter, first approximate 1 litre discarded in order to pre-condition the filter) to give a saturated solution with a nominal concentration of 13 mg/l*. Aliquots (250 ml and 2.5 litres) of this nominal 13 mg/l test concentration were each separately dispersed into a final volume of 25 litres of dechlorinated tap water and stirred using a flat bladed mixer for approximately 1 minute to give the remainder of the test series of nominal test concentrations of 0.13 and 1.3 mg/l.

Each of the prepared test concentrations was mixed thoroughly to ensure homogeneity.

* Concentration based on the results of preliminary analysis of a saturated solution of the test material.

In the range-finding test 3 fish were added to each nominal 20 litre test and control vessel containing 25 litres of media and maintained at approximately 14°C in a temperature controlled room with a photoperiod of 16 hours light and 8 hours darkness with 20 minute dawn and dusk transition periods for a period of 96 hours under static test conditions. The test vessels were completely filled to reduce headspace and sealed with no auxiliary aeration to prevent losses of test material due to the suspected volatile nature of the test material.

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

Each vessel was covered to reduce evaporation. After 3, 6, 24, 48, 72 and 96 hours any mortalities or sub-lethal effects of exposure were determined by visual inspection of the test fish.

3.3.3 Definitive test

Based on the results of the range-finding test the test material solutions for the definitive test were prepared by stirring an excess (50 mg/l) of test material in dechlorinated tap water at approximately 1500 rpm at a temperature of 14°C for a period of 24 hours. After 24 hours the stirring was stopped and any undissolved test material was removed by filtration (0.2 µm) through a pre-conditioned filter to give a saturated solution of the test material at a nominal concentration of 13 mg/l* from which dilutions were made to produce the remaining test groups of 1.3, 2.3, 4.2 and 7.3 mg/l.

3.3.3.1 Preparation of the test material

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

3.3.3.2 Exposure conditions

As in the range-finding test glass exposure vessels containing 25 litres of test media were used for each test concentration. At the start of the test seven fish were placed in each test vessel at random, in the test preparations. The test vessels were maintained at approximately 14°C in a temperature controlled room with a photoperiod of 16 hours light and 8 hours darkness with 20 minute dawn and dusk transition periods for a period of 96 hours. The test vessels were completely filled and sealed, and received no auxiliary aeration due to the suspected volatile nature of the test material. The fish were not individually identified and received no food during exposure.

* Concentration based on the results of preliminary analysis of a saturated solution of the test material.

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

A semi-static test regime was employed in the test involving a daily renewal of the test preparations to ensure that the concentrations of the test material remained near nominal and to prevent the build up of nitrogenous waste products.

Any mortalities and sub-lethal effects of exposure were recorded at 3, 6, 24, 48, 72 and 96 hours after the start of exposure. The criteria of death were taken to be the absence of both respiratory movement and response to physical stimulation.

3.3.3.3 Physico-chemical measurements

The water temperature, pH and dissolved oxygen concentrations were recorded daily throughout the test. The measurements at 0 hours, and after each test media renewal at 24, 48 and 72 hours, represent those of the freshly prepared test preparations while the measurements taken prior to each test media renewal, and on termination of the test after 96 hours, represent those of the used or 24-Hour old test preparations. The pH was measured using a WTW pH 320 pH meter, the dissolved oxygen concentration was measured using a YSI 550 dissolved oxygen meter and the temperature was measured using a Hanna Instruments HI 93510 digital thermometer.

3.3.3.4 Verification of test concentrations

Water samples were taken from the control and all surviving test groups at 0 (fresh media), 24, 48, 72 (old and fresh media) and 96 hours (old media) for quantitative analysis.

Duplicate samples were taken and stored at approximately -20°C for further analysis if necessary.

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

3.3.3.5 Evaluation of data

An estimate of the LC_{50} values at 3, 6 and 24 hours were given by inspection of the mortality data. The LC_{50} value and associated confidence limits at 96 hours were calculated by the trimmed Spearman-Karber method (Hamilton *et al* 1977) using the ToxCalc computer software package (ToxCalc 1999) and at 48 and 72 hours the LC_{50} values were calculated using the geometric mean method as follows:

LC_{50} value = $\sqrt{C_1 \times C_2}$

where:

C_1 = concentration showing 0% mortality
C_2 = concentration showing 100% mortality

When only one partial response is shown the trimmed Spearman-Karber method is appropriate.

If there are no mortalities between 0% and 100% mortality, then the geometric mean of the highest test concentration showing no lethality and the lowest test concentration showing 100% lethality is calculated. The concentrations resulting in 0% and 100% mortality will be the 95% confidence limits.

The time-weighted mean measured test concentrations were calculated as follows:

$$TWM = \frac{\text{Total area}}{\text{Total number of days of the test}}$$

$$\text{where Total area} = \frac{C_0 - C_1}{\ln(C_0) - \ln(C_1)} \text{ x days}$$

TWM = time-weighted mean measured test concentration (mg/l)
C_0 = measured concentration at the start of each renewal period (mg/l)
C_1 = measured concentration at the end of each renewal period (mg/l)
Days = number of days in the renewal period

4. ARCHIVES

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

5. RESULTS

5.1 Media preparation trials

The results of the initial media preparation trials conducted using a solvent spike method of preparation showed great variation in the measured concentrations and where the test media were untreated prior to analysis, the nominal test concentration of 10 mg/l was not achieved. Therefore, this method of preparation was considered to be unsuitable for testing.

Further media preparation trials were conducted by preparing the test media as a saturated solution. Chemical analysis of this saturated solution showed measured concentrations of 13.3, 12.4, 13.1 and 13.0 mg/l for samples that were filtered with a 1 litre pre-conditioning volume, filtered with a 2 litre pre-conditioning volume, centrifuged at 10000 *g* and centrifuged at 40000 *g* respectively. These results indicated that a saturated solution contained approximately 13 mg/l of test material and that the use of filtration with a 1 litre pre-conditioning volume was appropriate for use in testing. To confirm this, the lowest, middle and highest test concentrations were prepared from a saturated solution using this method of preparation. Chemical analysis showed measured concentrations of 143%, 100% and 110% of the nominal values for the 1.3, 4.2 and 13 mg/l nominal test concentrations respectively confirming the suitability of this method of preparation for testing.

5.2 Range-finding Test

Cumulative mortality data from the exposure of rainbow trout to the test material during the range-finding test are given in Table 1 and sub-lethal effects of exposure are given in Table 2.

The results showed no mortalities at the test concentrations of 0.13 and 1.3 mg/l. However, mortalities were observed at 13 mg/l*. After 24 hours exposure three out of three fish were observed to be moribund at 13 mg/l*. These fish were killed due to the approach of the substantial severity limit (Animals (Scientific Procedures) Act 1986) and classed as mortalities for the 48-Hour time point.

Based on this information the test material solutions for the definitive test were prepared by stirring an excess (50 mg/l) of test material in dechlorinated tap water for a period of time and then removing any undissolved test material by filtration to give a saturated solution with a nominal concentration of 13 mg/l*. This saturated solution was then further diluted, as necessary, to produce the remaining test concentrations of 1.3, 2.3, 4.2 and 7.3 mg/l.

* Concentration based on the results of preliminary analysis of a saturated solution of the test material.

5.3 Definitive Test

5.3.1 Mortality data

Cumulative mortality data from the exposure of rainbow trout to the test material during the definitive test are given in Table 3 and the relationship between percentage mortality and concentration at 96 hours is given in Figure 1.

Inspection of the mortality data at 3, 6 and 24 hours and analysis of the mortality data by the trimmed Spearman-Karber method (Hamilton *et al* 1977) at 96 hours and the geometric mean method at 48 and 72 hours based on the nominal test concentrations gave the following results:

Time (h)	LC_{50} (mg/l)	95% Confidence limits (mg/l)
3	> 13	–
6	> 13	–
24	> 13	–
48	9.7	7.3 – 13**
72	9.7	7.3 – 13**
96	9.0	7.7 – 10

The results of the definitive test showed the highest test concentration resulting in 0% mortality to be 4.2 mg/l, the lowest test concentration resulting in 100% mortality to be 13 mg/l and the No Observed Effect Concentration (NOEC) to be 2.3 mg/l. The No Observed Effect Concentration is based upon zero mortalities and the absence of any sub-lethal effects of exposure at this concentration (Section 5.3.2).

The relationship between the median lethal concentration (LC_{50}) and time is presented graphically in Figure 2.

5.3.2 Sub-lethal effects

Sub-lethal effects of exposure were observed at test concentrations of 4.2 mg/l and above. These responses were swimming at the surface, swimming at the bottom, increased pigmentation, swimming at the surface with increased pigmentation, swimming at the bottom with increased pigmentation, and the presence of moribund fish (see Table 4).

** Concentrations resulting in 0% and 100% mortalities respectively

After 29 hours exposure seven out of seven fish were observed moribund at 13 mg/l. These fish were killed due to the approach of the substantial severity limit (Animals (Scientific Procedures) Act 1986) and classed as mortalities for the 48-Hour time point.

5.3.3 Observations on test material solubility

The test material preparations were observed to be clear colourless solutions throughout the duration of the test.

5.3.4 Physico-chemical measurements

The results of the physico-chemical measurements are given in Appendix 3. Temperature was maintained at approximately 14°C throughout the test. While there were no treatment related differences for oxygen concentration, concentration dependent differences in pH were observed throughout the test.

5.3.5 Verification of test concentrations

Analysis of the test preparations was conducted at 0 (fresh test media), 24, 48, 72 (old and fresh test media) and 96 hours (old test media). Analysis of the fresh test media (see Appendix 1) showed measured values to range from 0.109 mg/l to 4.80 mg/l and analysis of the old or expired test media (see Appendix 1) showed measured values to range from 0.0872 mg/l to 4.22 mg/l. A large amount of variability was shown in the measured concentrations with values in the range of 8% to 56% of nominal for the fresh test media and 7% to 43% of nominal for the old or expired test media. However, on each sampling occasion the measured values were shown to increase with increasing nominal test concentration and the measured concentrations shown for the media sampled at the end of each renewal period showed a slight decline compared to the measured test concentrations of the samples taken at the beginning of each renewal period.

The variability shown in the measured test concentrations was considered to be due to the variability in the concentration of the saturated solutions produced for each preparation period. Measured values of 2.33, 4.80, 1.23 and 8.05 mg/l were shown for the saturated solutions at 0, 24, 48 and 72 hours respectively. This variability was considered to be due to differences in the water quality from day to day such as pH and dissolved salts affecting the solubility of the test material, plus minor differences in stirring the saturated solution although every effort was made to ensure consistency in stirring.

Although the measured test concentrations were shown to be variable, it was considered inappropriate to repeat the test as the range-finding and definitive tests showed the same

biological response to the test material and a No Observed Effect Concentration and LC_{50} values were obtained during the definitive test.

Given that the measured test concentrations were low and variable and a slight decline was shown in the old or expired test media compared to the fresh test media it was considered justifiable to base the results on the time-weighted mean measured test concentrations of the test media to give a "worst case" analysis of the data. The time-weighted mean measured test concentrations were calculated to be 0.276, 0.486, 1.02, 1.83 and 3.27 mg/l.

Inspection of the mortality data at 3, 6 and 24 hours and analysis of the mortality data by the trimmed Spearman-Karber method (Hamilton *et al* 1977) at 96 hours and the geometric mean method at 48 and 72 hours based on the time-weighted mean measured test concentrations gave the following results:

Time (h)	LC_{50} (mg/l)	95% Confidence limits (mg/l)
3	> 3.3	–
6	> 3.3	–
24	> 3.3	–
48	2.4	1.8 – 3.3**
72	2.4	1.8 – 3.3**
96	2.3	1.9 – 2.6

The results of the definitive test based on the time-weighted mean measured test concentrations showed the highest test concentration resulting in 0% mortality to be 1.0 mg/l, the lowest test concentration resulting in 100% mortality to be 3.3 mg/l and the No Observed Effect Concentration (NOEC) to be 0.49 mg/l. The No Observed Effect Concentration is based upon zero mortalities and the absence of any sub-lethal effects of exposure at this concentration.

The use of time-weighted mean measured test concentrations was considered to have significantly affected the results of the definitive test.

6. CONCLUSION

The acute toxicity of the test material to the freshwater fish rainbow trout (*Oncorhynchus mykiss*) has been investigated and based on nominal test concentrations gave a 96-Hour LC_{50} value of 9.0 mg/l with 95% confidence limits of 7.7 – 10 mg/l. The No Observed Effect Concentration was 2.3 mg/l.

** Concentrations resulting in 0% and 100% mortalities respectively

Based on the time-weighted mean measured test concentrations the acute toxicity of the test material to rainbow trout gave a 96-Hour LC_{50} value of 2.3 mg/l with 95% confidence limits of 1.9 – 2.6 mg/l. The No Observed Effect Concentration was 0.49 mg/l.

7. REFERENCES

Hamilton, M A, Russo, R C and Thurston, R V (1977) Trimmed Spearman-Karber Method for Estimating Median Lethal Concentration in Toxicity Bioassays. *Environ Sci Technol* **11**, 714-719.

ToxCalc Version 5.0.23C (1999), Tidepool Scientific Software, McKinleyville, CA 95519, USA.

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Table 1 Cumulative Mortality Data in the Range-finding Test

Nominal Concentration (mg/l)*	Cumulative Mortality (Initial Population = 3)					
	3 Hours	6 Hours	24 Hours	48 Hours	72 Hours	96 Hours
Control	0	0	0	0	0	0
0.13	0	0	0	0	0	0
1.3	0	0	0	0	0	0
13	0	0	0**	3	3	3

* Concentrations based on the results of preliminary analysis of a saturated solution of the test material.
** After 24 hours exposure three out of three fish were observed to be moribund at 13 mg/l. These fish were killed due to the approach of the substantial severity limit (Animals (Scientific Procedures) Act 1986) and classed as mortalities for the 48-Hour time point.

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Table 2 Sub-lethal Effects of Exposure in the Range-finding Test

Nominal Concentration (mg/l)*	Sub-lethal Effects	Time (Hours)					
		3	6	24	48	72	96
Control	No abnormalities detected						
0.13	No abnormalities detected						
1.3	No abnormalities detected						
13	Loss of equilibrium Moribund	3/3	3/3	3/3**	A/D		

A/D = All fish dead
* Concentrations based on the results of preliminary analysis of a saturated solution of the test material.
** After 24 hours exposure three out of three fish were observed to be moribund at 13 mg/l. These fish were killed due to the approach of the substantial severity limit (Animals (Scientific Procedures). Act 1986) and classed as mortalities for the 48-Hour time point.

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Table 3 Cumulative Mortality Data in the Definitive Test

Nominal Concentration (mg/l)	Time-weighted Mean Measured Concentration (mg/l)	Cumulative Mortality (Initial Population = 7)						% Mortality
		3 Hours	6 Hours	24 Hours	48 Hours	72 Hours	96 Hours	96 Hours
Control	Control	0	0	0	0	0	0	0
1.3	0.28	0	0	0	0	0	0	0
2.3	0.49	0	0	0	0	0	0	0
4.2	1.0	0	0	0	0	0	0	0
7.3	1.8	0	0	0	0	0	1	14
13	3.3	0	0	0*	7	7	7	100

* After 29 hours exposure seven out of seven fish were observed moribund at 13 mg/l. These fish were killed due to the approach of the substantial severity limit (Animals (Scientific Procedures) Act 1986) and classed as mortalities for the 48-Hour time point.

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Table 4 Sub-lethal Effects of Exposure in the Definitive Test

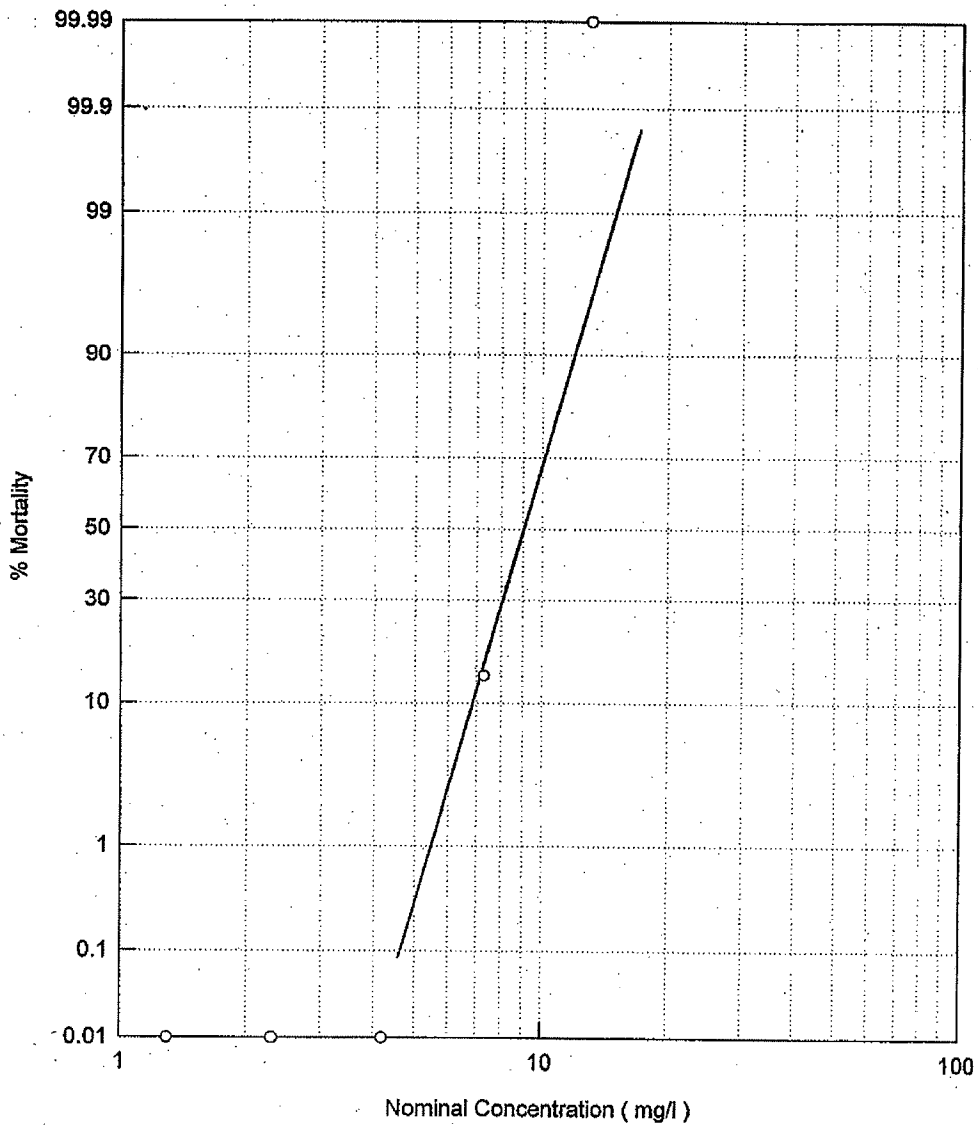
Nominal Concentration (mg/l)	Time-weighted Mean Measured Concentration (mg/l)	Sub-lethal Effects	Time (Hours)					
			3	6	24	48	72	96
Control	Control	No abnormalities detected						
1.3	0.28	No abnormalities detected						
2.3	0.49	No abnormalities detected						
4.2	1.0	Increased pigmentation						2/7
7.3	1.8	Swimming at the surface with increased pigmentation Swimming at the bottom with increased pigmentation				3/7 1/7	3/7 1/7	4/6 2/6
13	3.3	Swimming at the surface Swimming at the bottom		4/7	4/7* 2/7	A/D		

A/D = All fish dead

* After 29 hours exposure seven out of seven fish were observed moribund at 13 mg/l. These fish were killed due to the approach of the substantial severity limit (Animals (Scientific Procedures) Act 1986) and classed as mortalities for the 48-Hour time point.

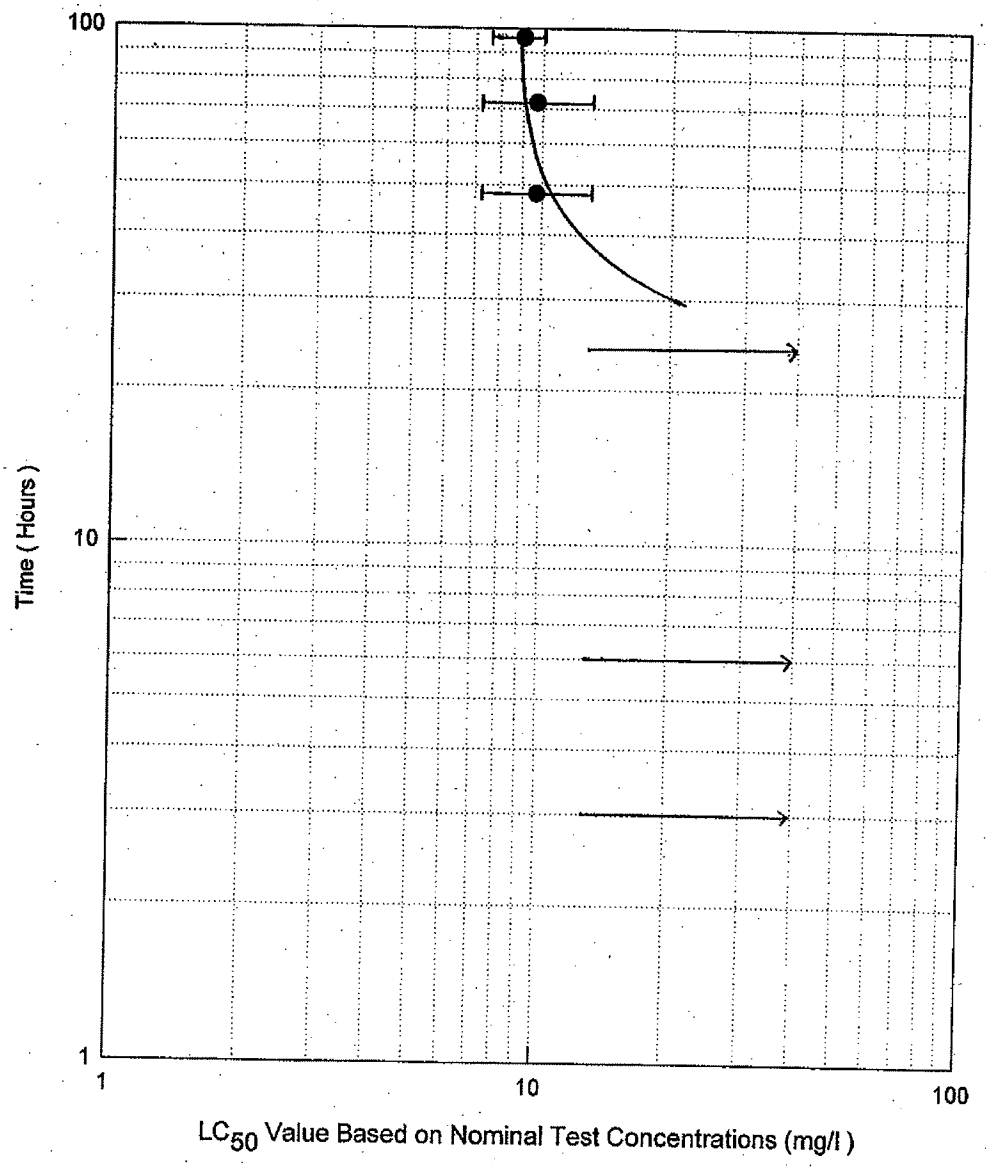
C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Figure 1 **Concentration-Mortality Curve**



C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Figure 2 **Toxicity Curve**



C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 Verification of Test Concentrations

1. METHOD OF ANALYSIS

1.1 Introduction

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

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

1.2 Sample Preparation

A Strata X (33 µ, 60 mg) solid phase extraction (SPE) cartridge was sequentially pre-conditioned with methanol and water*. A volume of test sample was eluted through the cartridge. The test material was then eluted from the cartridge with methanol and made to volume to give a final theoretical concentration of between approximately 9 and 13 mg/l.

1.3 Standards

Standard solutions of test material were prepared in methanol at a nominal concentration of 10 mg/l.

1.4 Procedure

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

GC System	:	Agilent Technologies 5890 or 6890, incorporating autosampler and workstation
Column	:	ZB-5 (30 m x 0.53 mm id, 5 µm film)
Oven temperature program	:	initial 60°C for 2 minutes 10°C/minute to 150°C

* Prepared by ELGA Purelab Option R-15 water purification

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations

Injector temperature	:	200°C
Detector temperature	:	250°C
Carrier gas and pressure	:	nitrogen at approximately 5 psi
Injection volume	:	1 µl
Injection mode	:	splitless purge on at 1 minute
Detector	:	flame ionisation detector (FID)
Retention time	:	approximately 7 minutes

2. VALIDATION

2.1 Linearity

A range of standard solutions covering 1 to 98 mg/l (10% to 980% of the standard concentration) was analysed.

Linearity was confirmed (R^2 =0.9998) in the range from 0 to 98 mg/l.

The results are presented graphically on page 27.

2.2 Recoveries

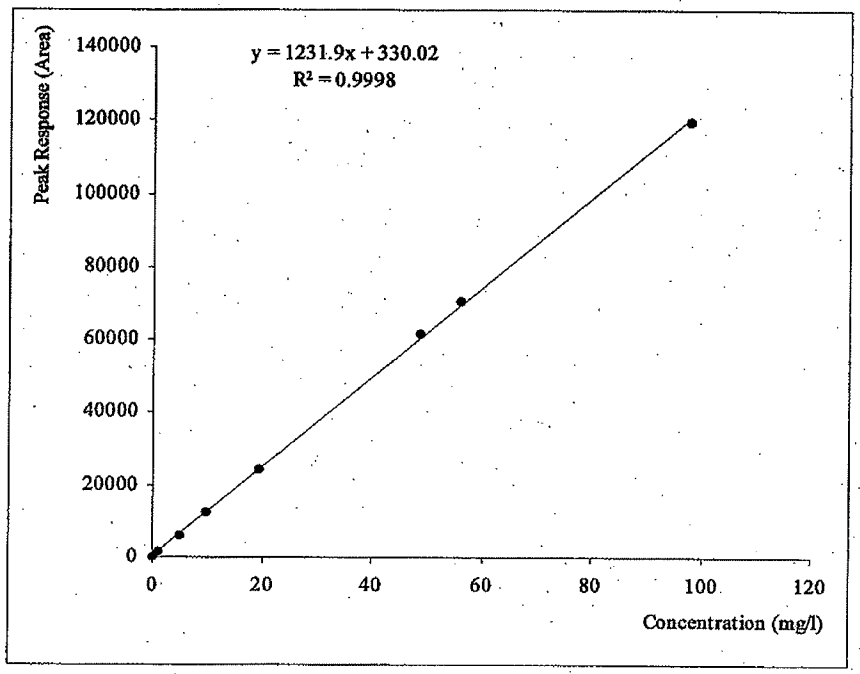
A range of preliminary test samples, accurately fortified at known concentrations of test material, was prepared and analysed.

The recovery samples were prepared by addition of a standard solution of test material to a sample of test medium. A standard solution was accurately prepared by dissolving the test material in methanol. An accurate volume of the standard solution was added to a known volume of test medium to achieve the required concentrations of test material in water.

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations

Linearity of Detector Response



C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
1.48	1.07	72	70
1.48	1.00	68	
4.17	1.10	26	25
4.17	1.01	24	
4.75	0.689	15	13
4.75	0.583	12	
14.8	11.2	75	70
14.8	9.55	64	

The method had low recoveries of test material from test medium. Procedural recoveries were prepared and run alongside the test samples.

2.3 Limit of Quantitation

The limit of quantitation has been assessed down to 0.033 mg/l.

3. STABILITY

A range of preliminary test samples was prepared, analysed initially and then after storage in sealed glass vessels at ambient temperature in light and dark conditions for approximately 24 hours (equivalent to the period of media renewal). In addition a test sample was tested for stability without prior mixing (sonication) the test sample bottle to assess for losses due to adsorption and/or insolubility.

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations

Nominal concentration (mg/l)	1.3	4.2	4.2	13
Concentration found initially (mg/l)	1.04	1.05	0.636	10.4
Concentration found after storage in light conditions (mg/l)	0.607	0.407	0.203	4.63
Expressed as a percent of the initial concentration	59	39	32	45
Concentration found after storage in dark conditions (mg/l)	0.649	0.424	0.217	2.58
Expressed as a percent of the initial concentration	63	40	34	25
Concentration found after storage in dark conditions (mg/l) - unsonicated sample	0.581	NA	NA	2.22
Expressed as a percent of the initial concentration	56	-	-	21

The test samples have been shown to be unstable in the test medium in light and dark conditions.

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

4. PRELIMINARY SAMPLE PREPARATION

An initial preliminary saturated solution was prepared. This was treated prior to analysis by filtration (0.2 µm filter) with one and two litres discarded to pre-condition the filters and after centrifugation at 10000 *g* and 40000 *g* for 30 minutes. These were analysed and gave the following results.

Sample	Concentration Found (mg/l)
Filtered, 1 litre discarded	13.3
Filtered, 2 litres discarded	12.4
Centrifuged, 10000 *g*	13.1
Centrifuged, 40000 *g*	13.0

To confirm that the saturated solution was approximately 13 mg/l a further preliminary saturated solution was prepared and the lowest, middle and highest test concentrations were prepared by dilution in test media. The results are given below.

NA = Not applicable

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations

Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration (%)
1.3	1.86	143
4.2	4.21	100
13	14.4	110

The above results were corrected for procedural recoveries.

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations

5. RESULTS

5.1 Test Samples

Sample	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration (%)
0 Hours	Control	<LOQ	-
	1.3	0.179	14
	2.3	0.333	14
	4.2	0.673	16
	7.3	1.11	15
	13	2.33	18
24 Hours Old Media	Control	<LOQ	-
	1.3	0.137	11
	2.3	0.225	10
	4.2	0.454	11
	7.3	0.790	11
	13	1.78	14
24 Hours Fresh Media	Control	<LOQ	-
	1.3	0.414	32
	2.3	0.698	30
	4.2	1.34	32
	7.3	2.37	32
	13	4.80	37
48 Hours Old Media	Control	<LOQ	-
	1.3	0.290	22
	2.3	0.499	22
	4.2	1.02	24
	7.3	2.04	28
	13	4.22	32

LOQ = Limit of quantitation

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations

Sample	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration (%)
48 Hours	Control	<LOQ	-
Fresh Media	1.3	0.109	8
	2.3	0.181	8
	4.2	0.362	9
	7.3	0.594	8
	13	1.23	9
72 Hours	Control	<LOQ	-
Old Media	1.3	0.0872	7
	2.3	0.154	7
	4.2	0.302	7
	7.3	0.541	7
72 Hours	Control	<LOQ	-
Fresh Media	1.3	0.589	45
	2.3	1.01	44
	4.2	2.24	53
	7.3	4.09	56
	13	8.05	62
96 Hours	Control	<LOQ	-
Old Media	1.3	0.425	33
	2.3	0.811	35
	4.2	1.78	42
	7.3	3.17	43

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations

5.2 Procedural Recoveries

Sample	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Fortification (%)
0 Hours	Control	<LOQ	-
	1.3	0.276	21
	2.3	0.554	24
	4.2	1.14	27
	7.3	2.37	32
	13	9.30	71
24 Hours	Control	<LOQ	-
	1.3	0.587	39
	2.3	1.22	46
	4.2	2.07	43
	7.3	3.78	45
	13	11.5	77
48 Hours	Control	<LOQ	-
	1.3	0.297	17
	2.3	0.583	19
	4.2	1.02	18
	7.3	2.15	22
	13	9.30	54
72 Hours	Control	<LOQ	-
	1.3	0.522	39
	2.3	0.799	33
	4.2	1.46	34
	7.3	3.54	47
	13	10.6	78
96 Hours	Control	<LOQ	-
	1.3	0.384	24
	2.3	0.637	22
	4.2	1.29	25
	7.3	2.66	30
	13	10.7	67

LOQ = Limit of quantitation

Appendix 1 (continued) Verification of Test Concentrations

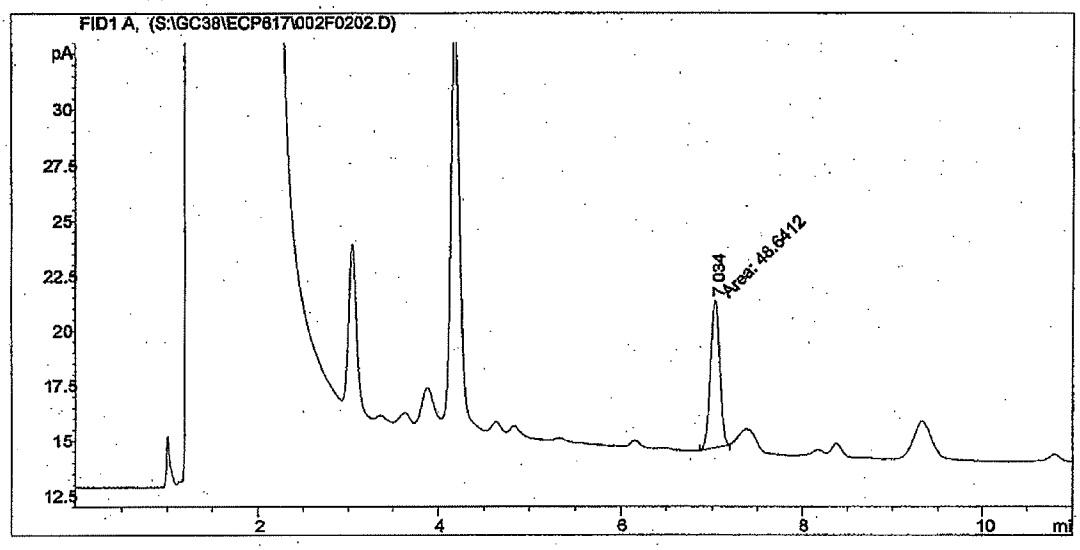
6. DISCUSSION

The detection system was found to have acceptable linearity. The analytical procedure had low recoveries of test material in test medium. Procedural recoveries were carried out alongside test samples. The top level the results showed recoveries in the region of 70% of fortification. At the lower levels, which were produced by dilution of the top level, recoveries of approximately half to a third of this top level were obtained. This was believed to be due to a procedural artefact from the solvent spiking procedure, and the results could not be related to the test samples. Therefore results have not been corrected for procedural recoveries.

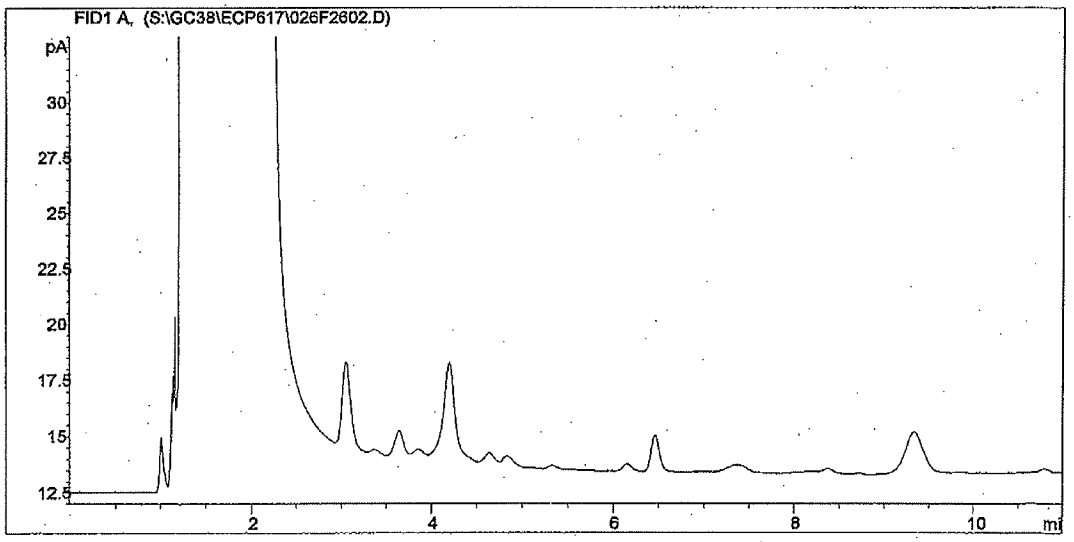
C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations

7. TYPICAL CHROMATOGRAPHY



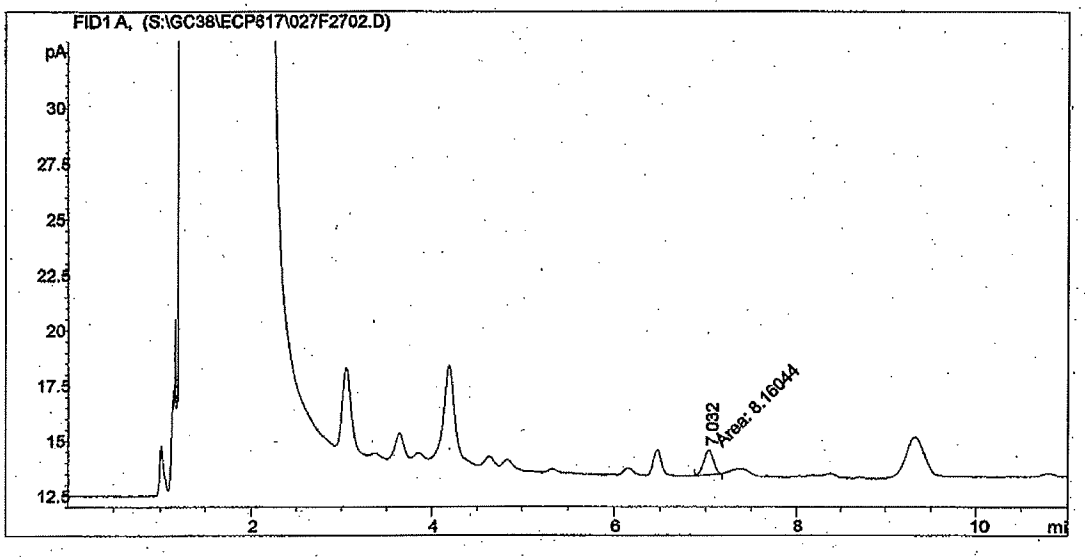
Standard 10 mg/l



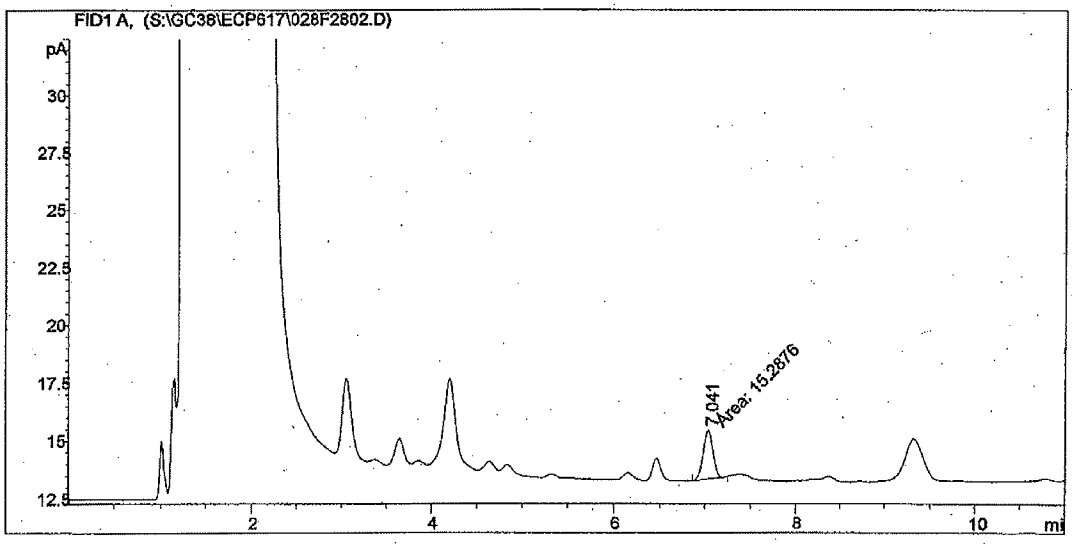
0 Hours Control Sample

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



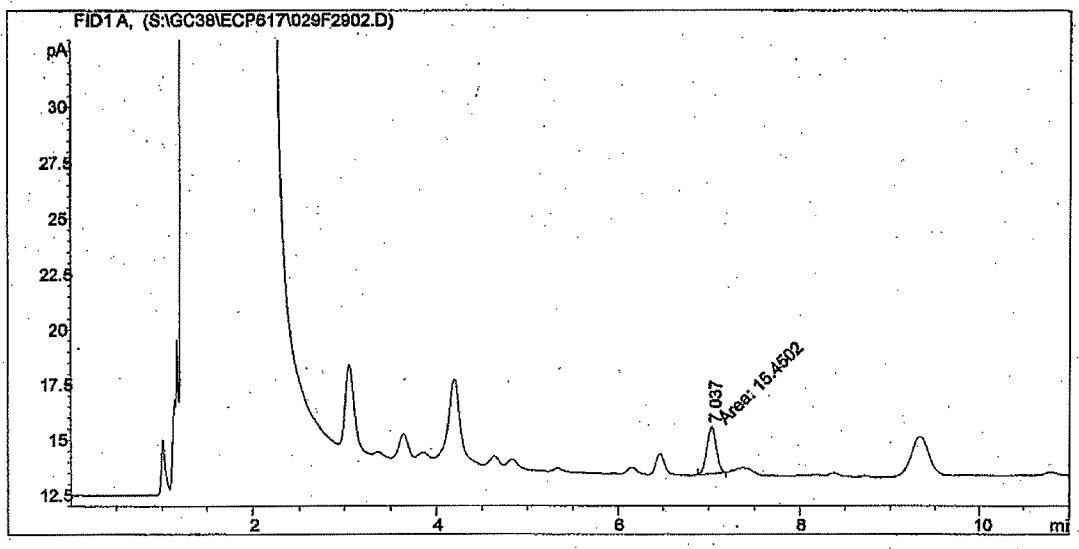
0 Hours Test Sample 1.3 mg/l



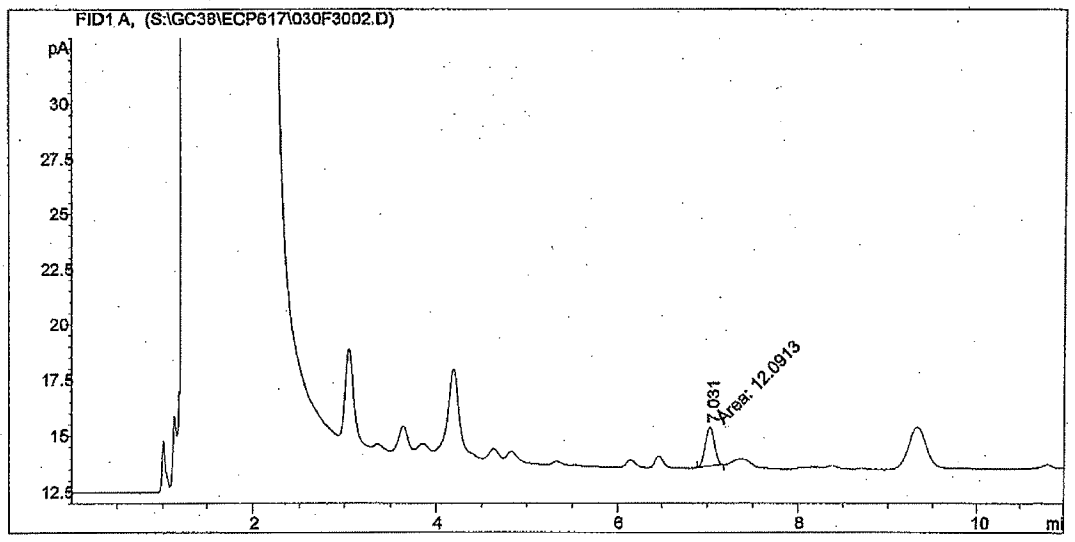
0 Hours Test Sample 2.3 mg/l

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



0 Hours Test Sample 4.2 mg/l

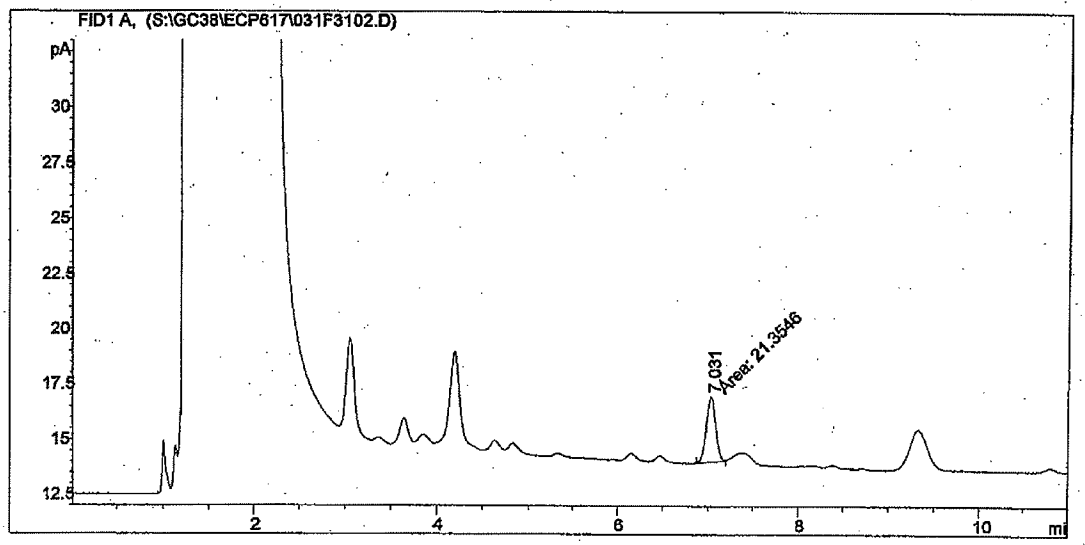


0 Hours Test Sample 7.3 mg/l

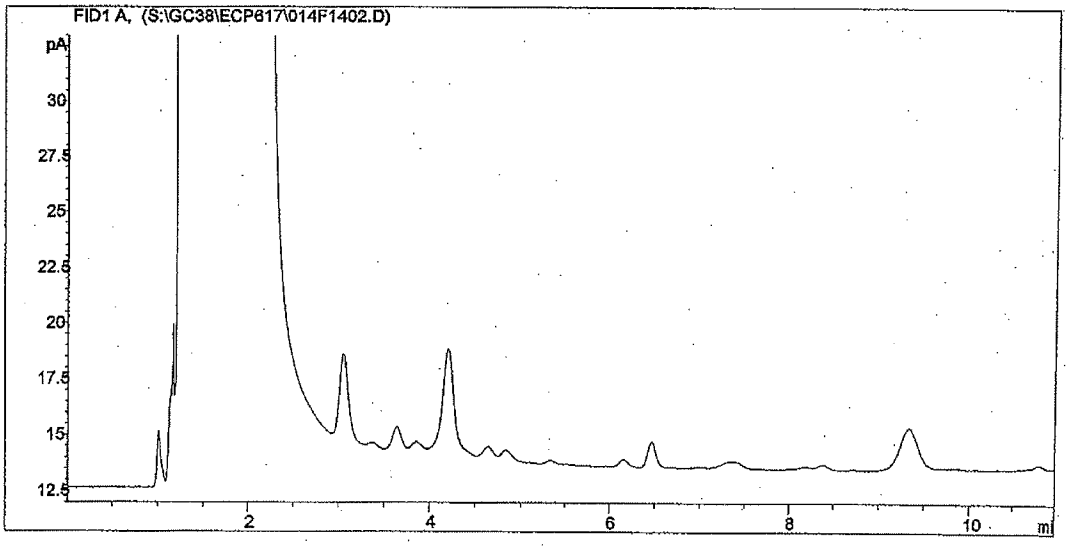
CONTAINS CONFIDENTIAL BUSINESS INFORMATION

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



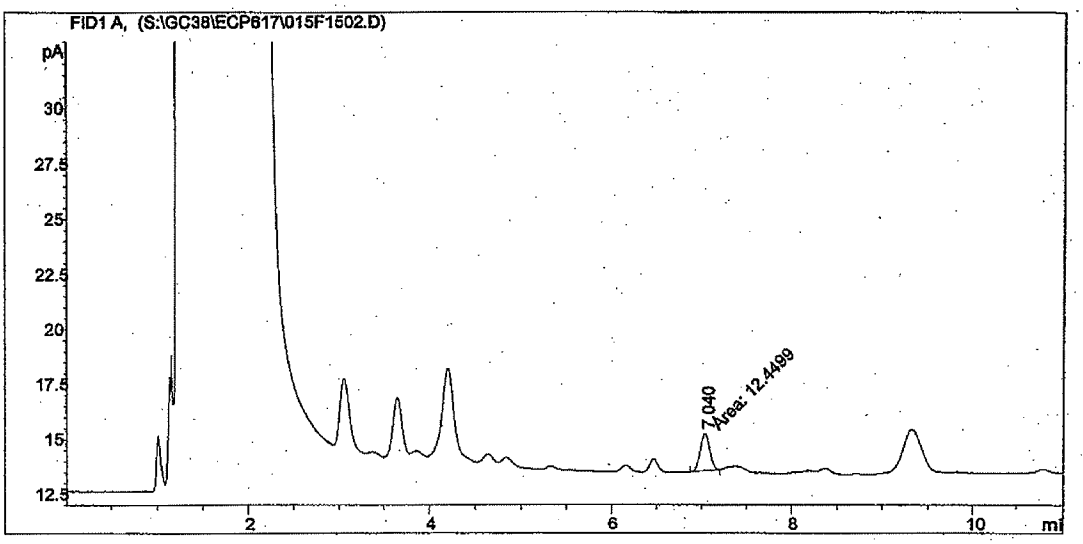
0 Hours Test Sample 13 mg/l



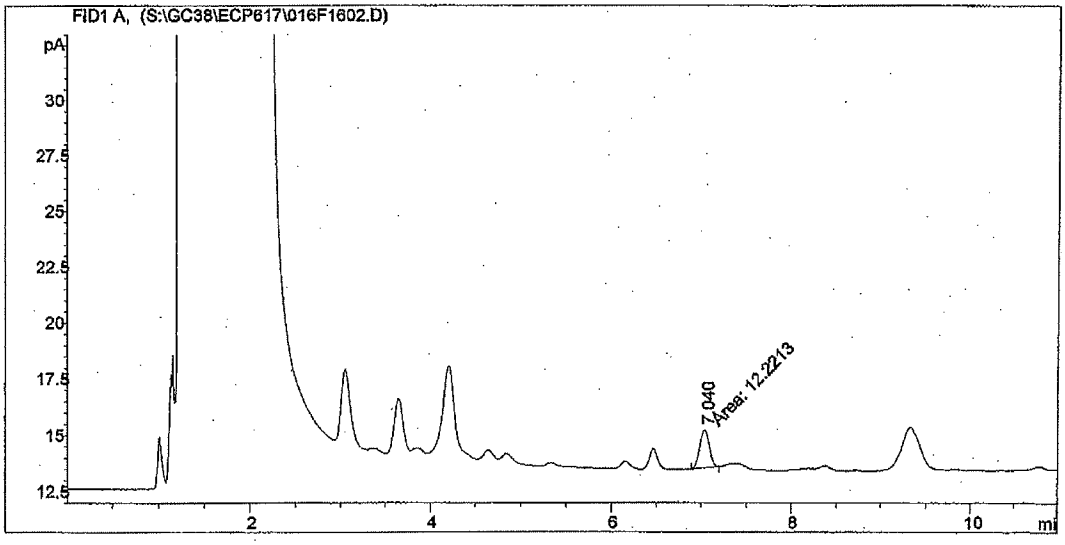
0 Hours Procedural Recovery Solvent Control

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



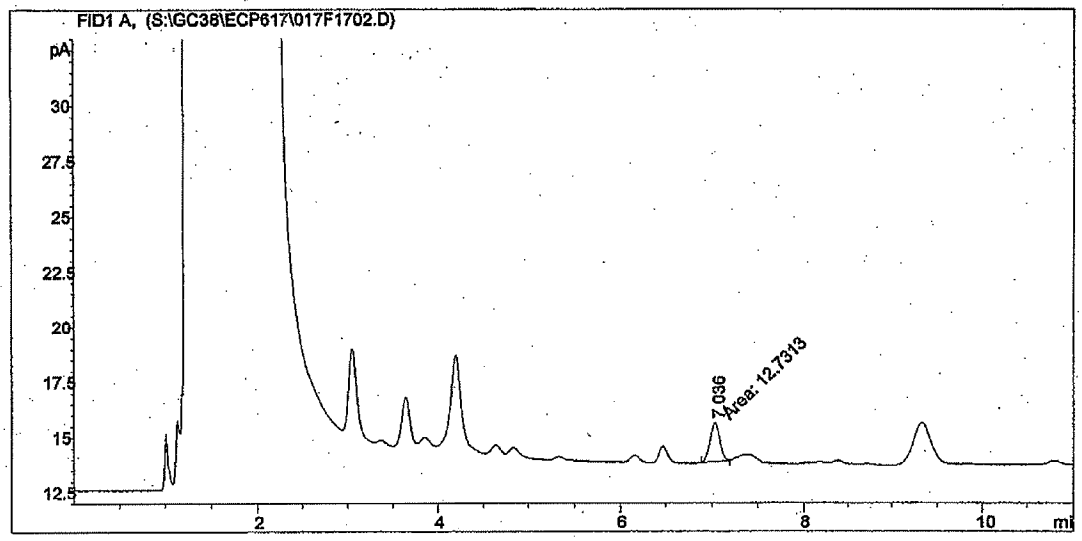
0 Hours Procedural Recovery 1.3 mg/l



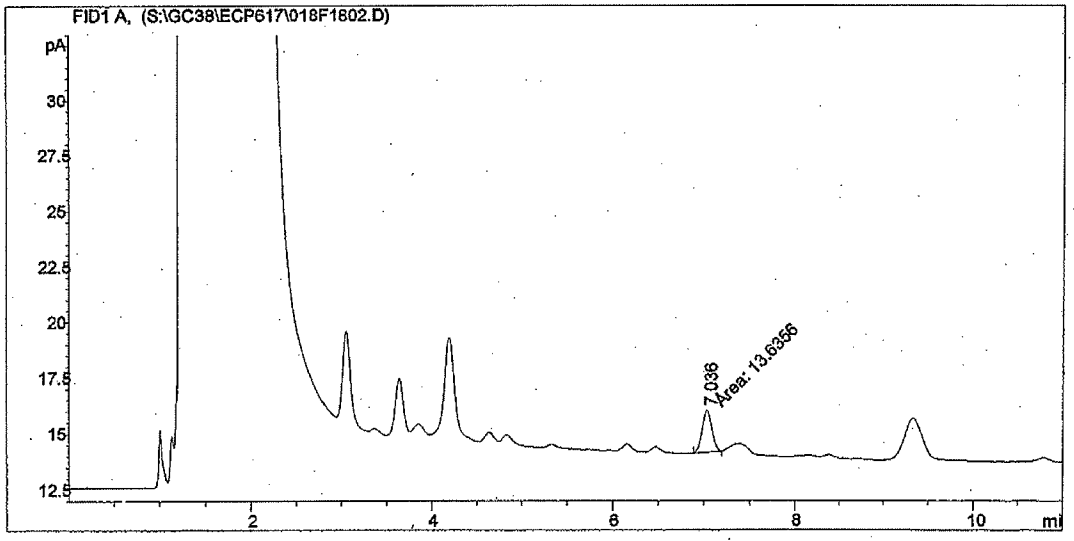
0 Hours Procedural Recovery 2.3 mg/l

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



0 Hours Procedural Recovery 4.2 mg/l

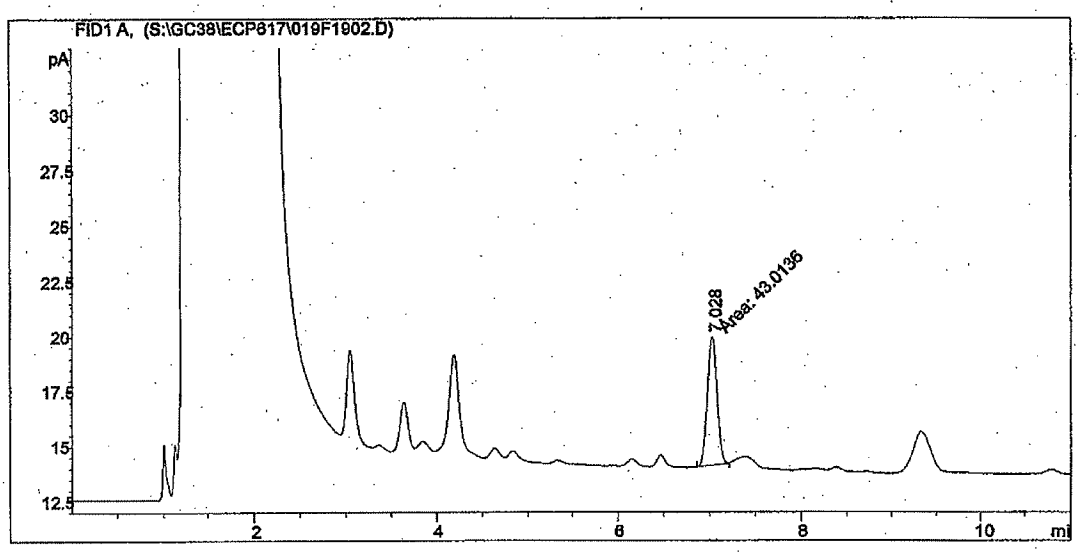


0 Hours Procedural Recovery 7.3 mg/l

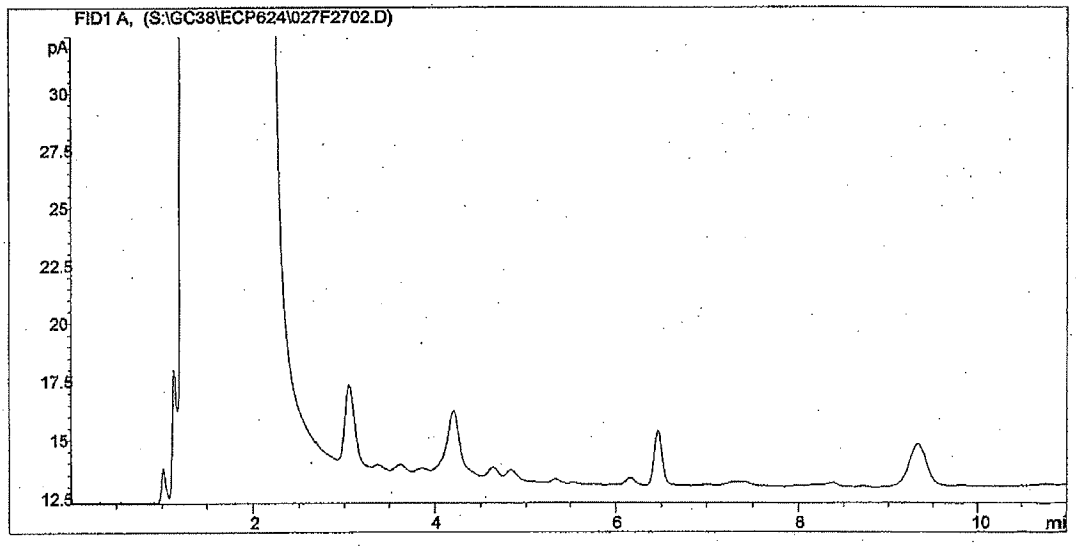
CONTAINS CONFIDENTIAL BUSINESS INFORMATION

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



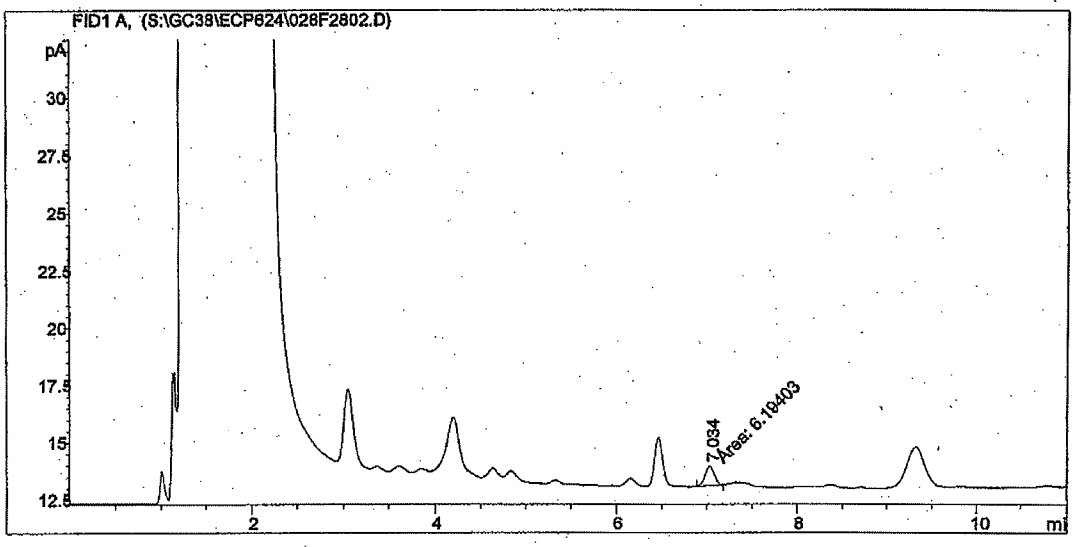
0 Hours Procedural Recovery 13 mg/l



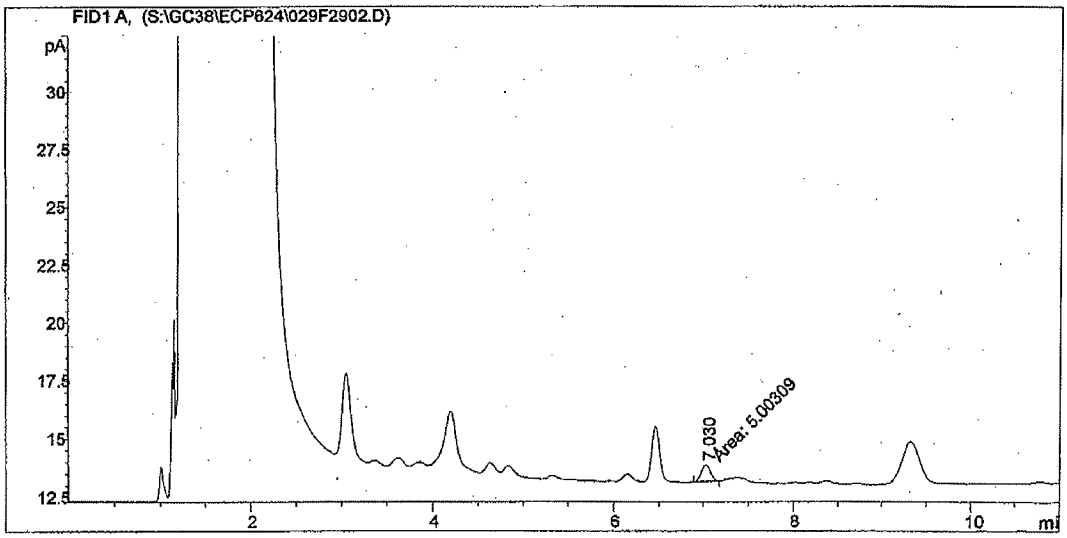
24 Hours Old Media Control Sample

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



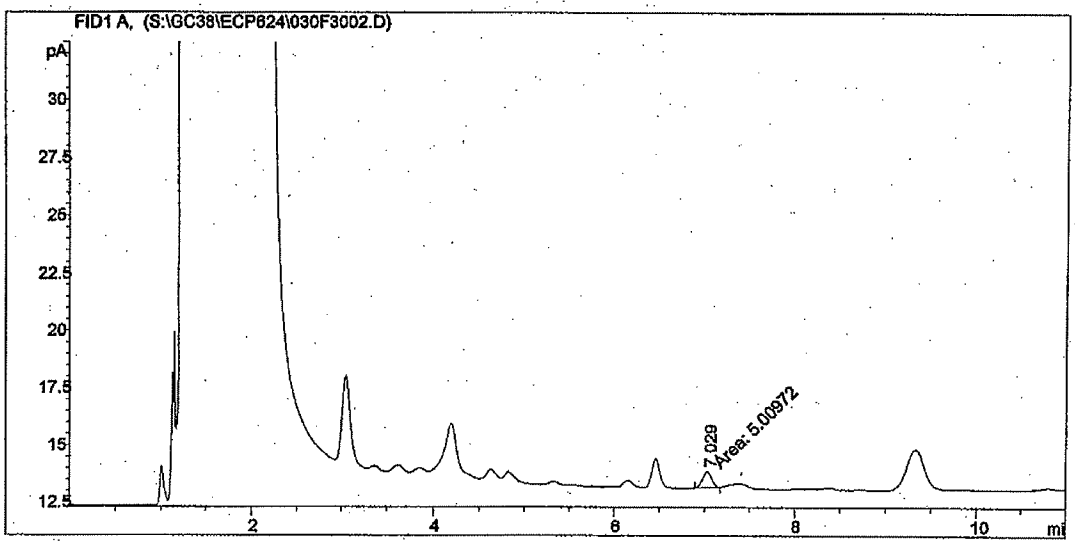
24 Hours Old Media Test Sample 1.3 mg/l



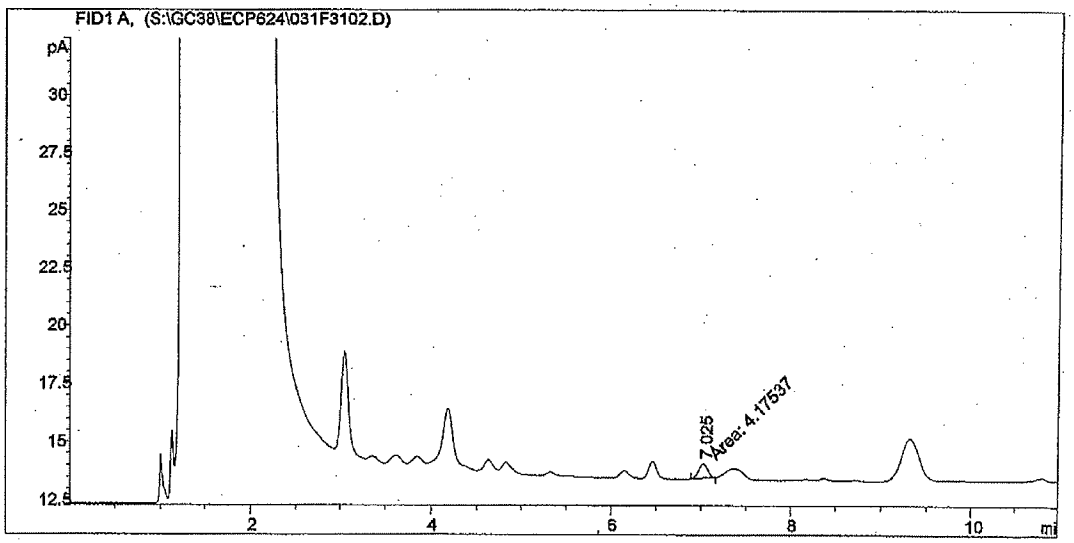
24 Hours Old Media Test Sample 2.3 mg/l

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



24 Hours Old Media Test Sample 4.2 mg/l

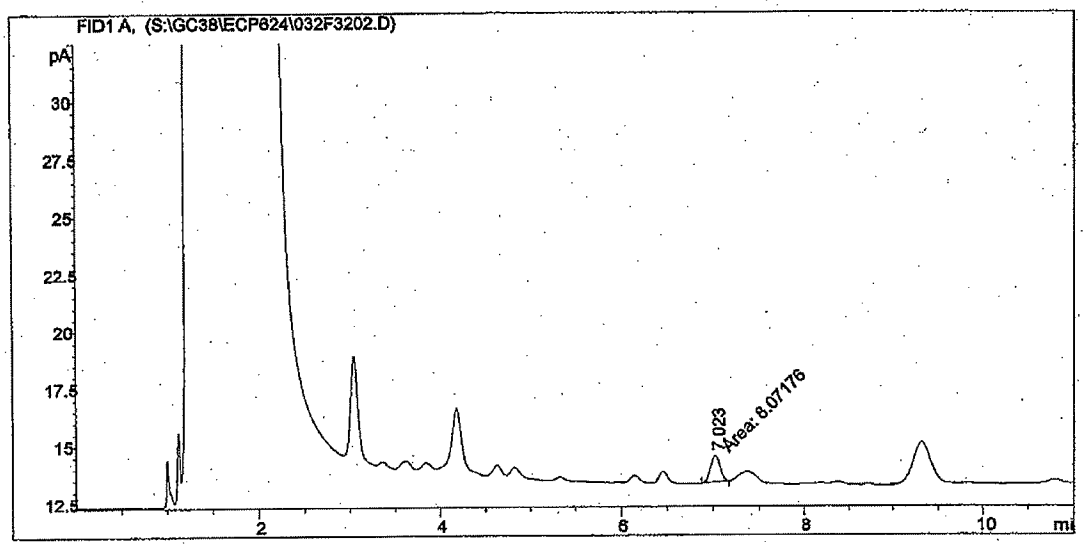


24 Hours Old Media Test Sample 7.3 mg/l

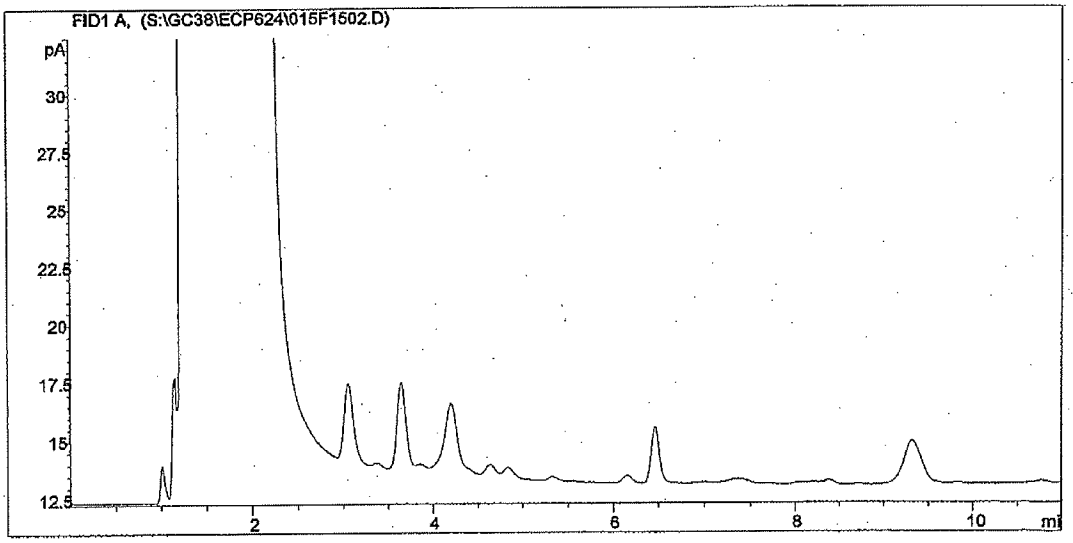
CONTAINS CONFIDENTIAL BUSINESS INFORMATION

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



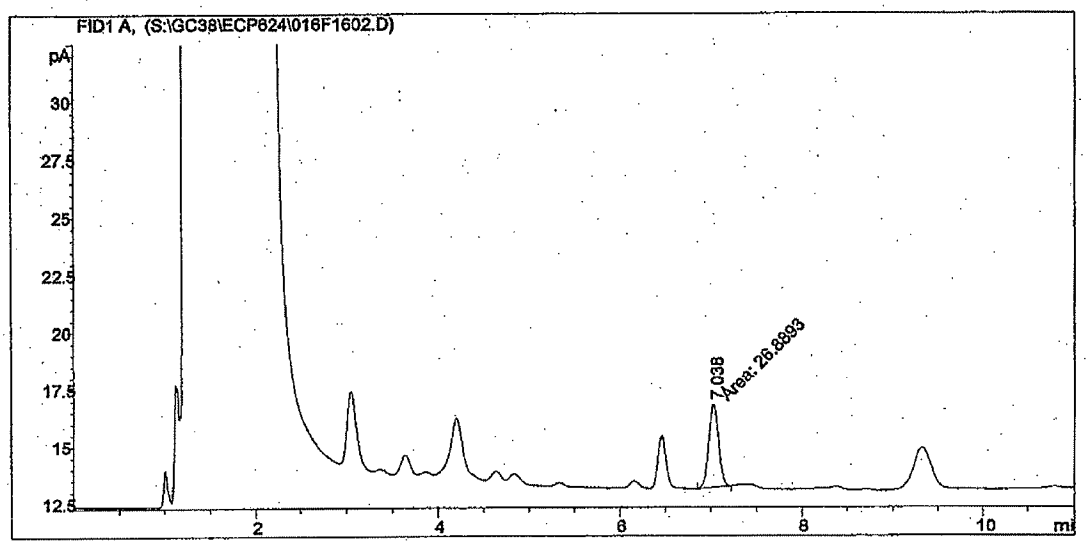
24 Hours Old Media Test Sample 13 mg/l



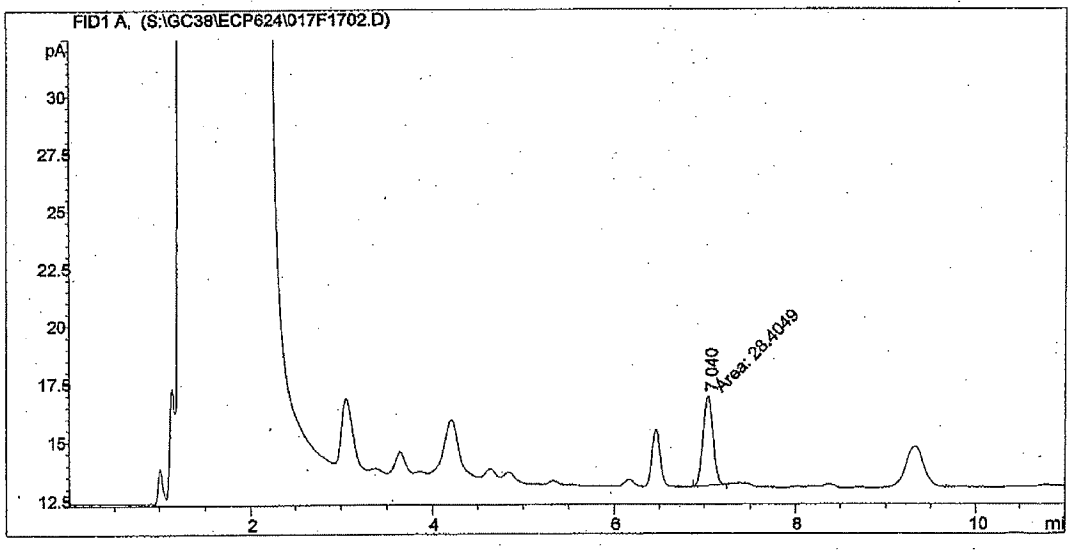
24 Hours Procedural Recovery Solvent Control

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



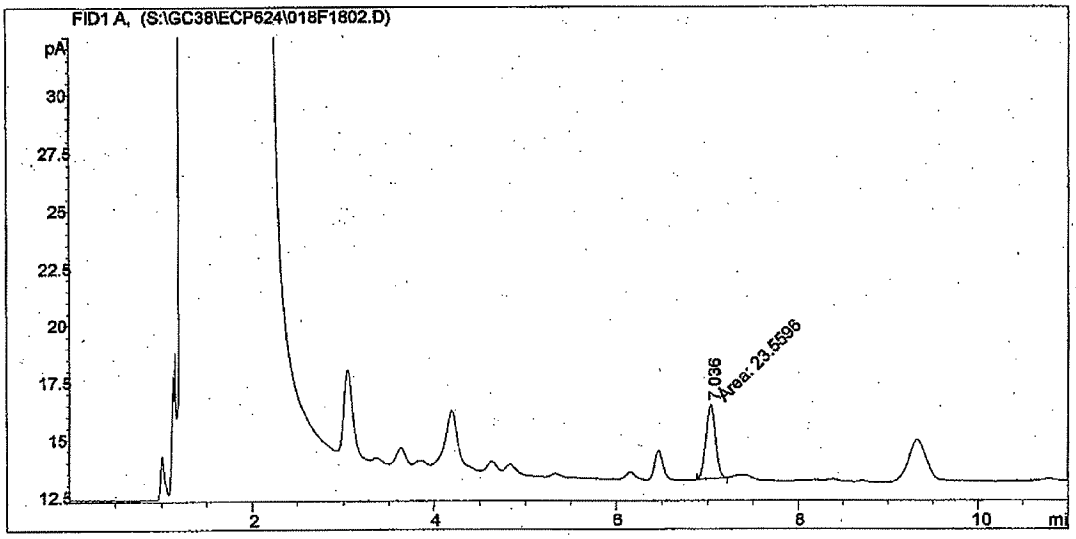
24 Hours Procedural Recovery 1.3 mg/l



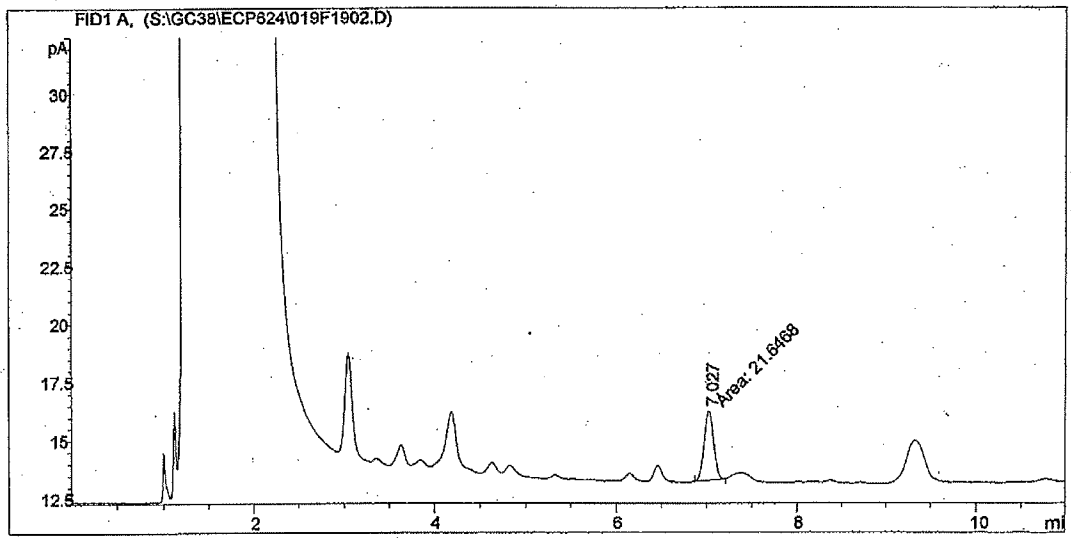
24 Hours Procedural Recovery 2.3 mg/l

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



24 Hours Procedural Recovery 4.2 mg/l

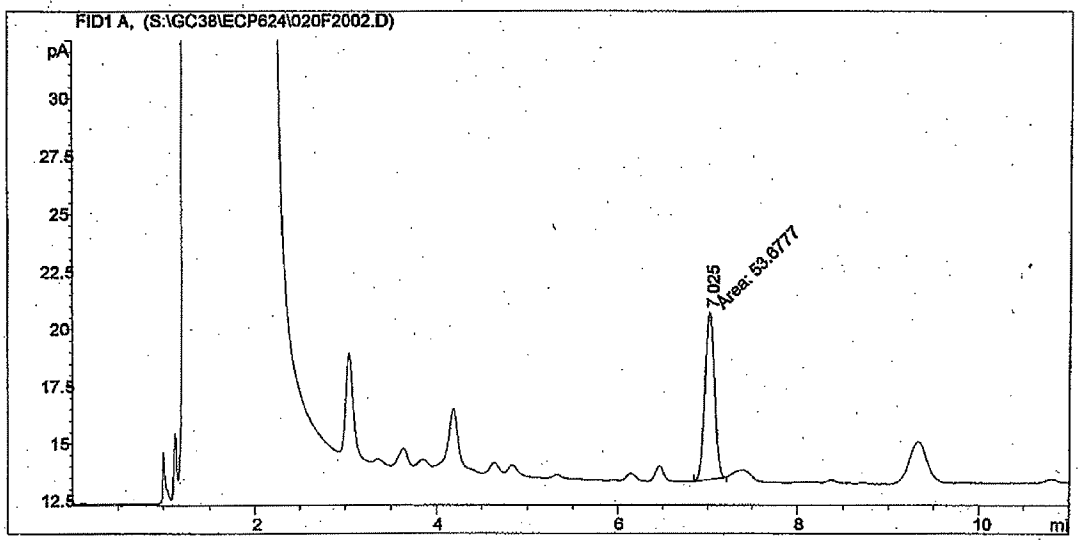


24 Hours Procedural Recovery 7.3 mg/l

CONTAINS CONFIDENTIAL BUSINESS INFORMATION

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 1 (continued) Verification of Test Concentrations



24 Hours Procedural Recovery 13 mg/l

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 2 Typical Water Quality Characteristics

WATER SUPPLY ZONE	SINFIN CHELLASTON SHARDLOW								
REPORTING PERIOD:	01/01/2003 TO 31/12/2003								
PARAMETER	NO. OF SAMPLES PLANNED PER ANNUM	NO. OF SAMPLES TAKEN IN YEAR	PCV RELAXED	NO. OF SAMPLES CONTRAVENING PCV	% OF SAMPLES CONTRAVENING PCV	CONCENTRATION OR VALUE (ALL SAMPLES)			
						MIN	MEAN	MAX	UNITS
QUALITATIVE TASTE	60	60		0	0.000	0.000	0.000	0.000	
QUALITATIVE ODOUR	60	60		0	0.000	0.000	0.000	0.000	
CONDUCTIVITY	60	61	>1500	0	0.000	359.000	439.164	610.000	µS/cm
TURBIDITY	36	36	>4.0	0	0.000	<0.200	<0.200	1.000	NTU
TEMPERATURE	60	61	>25.0	0	0.000	5.400	12.887	22.400	deg C
pH	6	6	9.5	0	0.000	7.100	7.617	8.00	pH unit
NITRATE	6	6	>50.00	0	0.000	9.800	12.283	14.600	mg NO_3/l
NITRITE	6	6	>0.10	0	0.000	<0.010	<0.010	<0.010	mg NO_2/l
NITRATE NITRITE CALCULAT	6	6		0	0.000	<1.000	<1.000	<1.000	
AMMONIUM	6	6	>0.50	0	0.000	<0.050	<0.050	<0.050	mg NH_4/l
IRON	36	36	>200	2	5.556	<20.000	55.000	250.000	µg Fe/l
ALUMINIUM	6	6	>200	0	0.000	<20.000	<20.000	62.000	µg Al/l
QUANTITATIVE TASTE	6	6	>3	0	0.000	0.000	0.000	0.000	
QUANTITATIVE ODOUR	6	6	>3	0	0.000	0.000	0.000	0.000	
MANGANESE	6	6	>50	0	0.000	<5.000	<7.000	28.000	µg Mn/l
COLOUR	6	6	>20	0	0.000	<2.000	<2.000	<2.000	mg/l Pt/C
CHLORODIBROMOMETHANE	4	4		0	0.000	5.800	9.700	12.100	µg/l
BROMODICHLOROMETHANE	4	4		0	0.000	8.200	13.125	18.800	µg/l
CHLOROFORM	4	4		0	0.000	12.900	18.850	30.800	µg/l
TRIHALOMETHANES	4	4	>100.0	0	0.000	31.100	43.475	63.600	µg/l
TETRACHLOROMETHANE	4	4	>3.0	0	0.000	<0.100	<0.100	<0.100	µg/l
TRICHLOROETHENE	4	4	>30.0	0	0.000	<1.000	<1.000	<1.000	µg/l
TETRACHLOROETHENE	4	4	>10.0	0	0.000	<1.000	<1.000	<1.000	µg/l
COPPER	1	1	>3000.0	0	0.000	<100.000	<100.000	<100.000	µg Cu/l
LEAD	1	1	>50	0	0.000	<1.000	<1.000	<1.000	µg Pb/l
ZINC	1	1	>5000.0	0	0.000	37.000	37.000	37.000	µg Zn/l
SIMAZINE	1	1	>0.100	0	0.000	<0.010	<0.010	<0.010	µg/l
ATRAZINE	1	1	>0.100	0	0.000	<0.010	<0.010	<0.010	µg/l
PESTICIDES	1	1	>0.50	0	0.000	<0.010	<0.010	<0.010	µg/l
POLYCYCLIC AROMATIC HYDR	1	1	>0.200	0	0.000	0.750	0.075	0.075	µg/l
INDENO (1,2,3-CD) PYRENE	1	1		0	0.000	<3.000	<3.000	<3.000	ng/l
BENZO 3,4 PYRENE	1	1	>10.0	0	0.000	<1.000	<1.000	<1.000	ng/l
BENZO 3,4 FLUORANTHENE	1	1		0	0.000	<2.000	<2.000	<2.000	ng/l
BENZO 11,12 FLUORANTHENE	1	1		0	0.000	<2.000	<2.000	<2.000	ng/l
BENZO 1,12 PERYLENE	1	1		0	0.000	<2.000	<2.000	<2.000	ng/l
FLUORANTHENE	1	1		0	0.000	75.000	75.000	75.000	ng/l
BROMOFORM	4	4		0	0.000	1.400	1.800	2.000	µg/l
CHLORIDE	1	1	>400	0	0.000	27.000	27.000	27.000	mg Cl/l
SULPHATE	1	1	>250	0	0.000	73.000	73.000	73.000	mg SO_4/l
CALCIUM	1	1	>250	0	0.000	51.000	51.000	51.000	mg Ca/l
MAGNESIUM	1	1	>50.0	0	0.000	8.000	8.000	8.000	mg Mg/l
SODIUM	1	1	>150	0	0.000	28.000	28.000	28.000	mg Na/l
POTASSIUM	1	1	>12.0	0	0.000	3.000	3.000	3.000	mg K/l
OXIDISABILITY	1	1	>5.0	0	0.000	1.100	1.100	1.100	mg O_2/l
TOTAL ORGANIC CARBON	1	1		0	0.000	1.700	1.700	1.700	mg C/l
BORON	1	1	>1000	0	0.000	<50.000	<50.000	<50.000	µg B/l
SURFACTANTS	1	1	>200	0	0.000	<20.000	<20.000	<20.000	µg/l
PHOSPHORUS	1	1	>2200	0	0.000	1950.000	1950.000	1950.000	µg P/l
FLUORIDE	1	1	>1500	0	0.000	304.000	304.000	304.000	µg F/l
BARIUM	1	1	>1000	0	0.000	89.000	89.000	89.000	µg Ba/l
SILVER	1	1	>10.0	0	0.000	<0.300	<0.300	<0.300	µg Ag/l
ARSENIC	1	1	>50	0	0.000	<1.000	<1.000	<1.000	µg As/l
CADMIUM	1	1	>5.0	0	0.000	<0.500	<0.500	<0.050	µg Cd/l
CYANIDE	1	1	>50	0	0.000	<5.000	<5.000	<5.000	µg Cn/l
CHROMIUM	1	1	>50	0	0.000	<1.000	<1.000	<1.000	µg Cr/l
MERCURY	1	1	>1.0	0	0.000	<0.100	<0.100	<0.100	µg Hg/l
NICKEL	1	1	>50	0	0.000	2.000	2.000	2.000	µg Ni/l
ANTIMONY	1	1	>10.0	0	0.000	<1.000	<1.000	<1.000	µg Sb/l
SELENIUM	1	1	>10.0	0	0.000	<1.000	<1.000	<1.000	µg Se/l
ALKALINITY	1	1		0	0.000	113.000	113.000	113.000	mg HCO_3/l
HARDNESS TOTAL	1	1		0	0.000	65.000	65.000	65.000	mg Ca/l
ENTEROCOCCI CONFIRMED	4	4		0	0.000	0.000	0.000	0.000	No 100 ml
COLIFORMS CONFIRMED	60	60	>0	0	0.000	0.000	0.000	0.000	No 100 ml
E.COLI CONFIRMED	60	60	>0	0	0.000	0.000	0.000	0.000	No 100 ml
COLONY COUNT AT 37°C	52	52		0	0.000	0.000	29.038	1430.00	No 1 ml
COLONY COUNT AT 21°C	52	52		0	0.000	0.000	61.885	2340.00	No 1 ml
CHLORINE FREE	60	60		0	0.000	0.010	0.129	0.370	mg/l
CHLORINE TOTAL	60	60		0	0.000	0.030	0.221	0.540	mg/l

PCV = Prescribed Concentration or Value

C6-2AL: ACUTE TOXICITY TO RAINBOW TROUT

Appendix 3 Physico-Chemical Measurements

Nominal Concentration (mg/l)	Time (Hours)											
	0 Hours (Fresh Media)				24 Hours (Old Media)				24 Hours (Fresh Media)			
	pH	mg O_2/l	%ASV*	T°C	pH	mg O_2/l	%ASV*	T°C	pH	mg O_2/l	%ASV*	T°C
Control	7.6	10.2	97	13.3	7.4	8.6	83	13.5	7.5	10.1	96	13.3
1.3	7.6	10.2	99	13.7	7.4	7.9	77	13.6	7.5	10.0	97	13.6
2.3	7.7	10.2	99	13.7	7.5	8.5	83	13.6	7.5	10.0	97	13.6
4.2	7.8	10.1	98	13.6	7.5	8.2	80	13.5	7.5	10.1	98	13.5
7.3	8.0	10.2	99	13.7	7.6	8.3	81	13.5	7.6	10.0	97	13.6
13	8.4	10.2	99	14.0	7.9	8.1	79	13.5	7.8	10.0	97	13.7

Nominal Concentration (mg/l)	Time (Hours)											
	48 Hours (Old Media)				48 Hours (Fresh Media)				72 Hours (Old Media)			
	pH	mg O_2/l	%ASV*	T°C	pH	mg O_2/l	%ASV*	T°C	pH	mg O_2/l	%ASV*	T°C
Control	7.4	8.8	84	13.2	7.5	10.1	96	13.4	7.4	8.9	86	13.5
1.3	7.3	8.2	78	13.2	7.5	10.2	97	13.4	7.3	8.3	81	13.5
2.3	7.4	8.7	83	13.2	7.5	10.1	96	13.4	7.3	8.7	84	13.5
4.2	7.4	8.6	82	13.2	7.5	10.1	96	13.4	7.4	8.5	83	13.5
7.3	7.5	8.7	83	13.2	7.6	10.2	99	13.5	7.4	8.6	83	13.5
13	7.7	9.2	88	13.3		A/D						

Nominal Concentration (mg/l)	Time (Hours)							
	72 Hours (Fresh Media)				96 Hours (Old Media)			
	pH	mg O_2/l	%ASV*	T°C	pH	mg O_2/l	%ASV*	T°C
Control	7.5	10.2	97	13.3	7.4	9.0	86	13.4
1.3	7.5	10.1	96	13.4	7.4	8.7	84	13.5
2.3	7.5	10.0	97	13.6	7.4	9.2	89	13.5
4.2	7.5	10.2	99	13.6	7.4	8.9	86	13.5
7.3	7.5	10.2	99	13.6	7.3	8.3	81	13.6
13		A/D						

*ASV = Dissolved oxygen concentration expressed as a percentage of Air Saturation Value
A/D = All fish dead

Appendix 4 Statement of GLP Compliance in Accordance with Directive 88/320/EEC

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

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

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

DATE OF INSPECTION

2nd December 2002

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

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

13/2/03

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