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Contractor	RCC LTD		
Document Title	INITIAL SUBMISSION: INFLUENCE OF TK 11014 (IRGANOX L 135) ON SURVIVAL AND REPRODUCTION OF DAPHNIA MAGNA IN A SEMI-STATIC TEST OVER THREE WEEKS, WITH COVER LETTER DATED 5/24/2000		
Chemical Category	3,5-DI-T-BUTYL-4-HYDROXYHYDROCINNAMIC ACID, C7-9 BRANCHED A*		

A 03

Ciba Specialty Chemicals
North America
Additives

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May 24, 2000

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Attention: Section 8(e)
Office of Pollution Prevention and Toxics
Environmental Protection Agency
401 M Street, SW
Washington, DC 20460-0001

Via Federal Express

MR 36097

Subject: TSCA 8(e) Notice - TK 11014 (Irganox L-135)

Dear Document Control Officer:

This letter and enclosed report do not contain Confidential Business Information.

In accordance with EPA's March 16, 1978 Policy Statement on Section 8(e) reporting under the Toxic Substances Control Act (TSCA), the EPA's June, 1991 TSCA Section 8(e) Reporting Guide, Ciba Specialty Chemicals Corporation wishes to bring to the attention of the Environmental Protection Agency the results observed in a *Daphnia* survival/reproduction study conducted with Irganox L-135, which is 3,5-Di-*t*-butyl-4-hydroxyhydrocinnamic acid, C₇₋₉ branched alkyl esters CASRN 125643-61-0

We are enclosing a copy of the study entitled, "*Influence of TK 11014 (Irganox L 135) on Survival and Reproduction of Daphnia Magna in a Semistatic Test Over Three Weeks*". The highest treatment concentration tested was 100 mg/L nominal with a measured water-soluble concentration of less than 0.03 mg/L. This was the only concentration which reduced survival of *adult Daphnia* (~30%). The second highest concentration, a 1:3.2 dilutions of the 100 mg/L nominal level, was the lowest concentration that reduced production of young daphnids. Three other dilutions of the 100 mg/L stock solution (1:10, 1:32, and 1:100) had no adverse effect on survival or reproduction.

Based upon current EPA guidelines, it is felt these results warrant reporting under TSCA 8(e). Please call the undersigned if you have any questions concerning this submittal.

Respectfully,

Ciba Specialty Chemicals Corporation

Thomas Barber
Manager, Regulatory Compliance

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540 White Plains Road
Tarrytown, New York 10591

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Study Title

**INFLUENCE OF
TK 11014 (IRGANOX L 135)
ON SURVIVAL AND REPRODUCTION OF
DAPHNIA MAGNA
IN A SEMISTATIC TEST
OVER THREE WEEKS**

Data Requirement / Test Guideline:

OECD 211

Study Director:

Dr. Armin Peither

Study Completion Date:

February 29, 2000

Performing Laboratory:

RCC Ltd
Environmental Chemistry &
Pharmanalytics Division
CH-4452 Itingen / Switzerland

Sponsor:

CIBA SPECIALTY CHEMICALS INC.
Additives Division
P.O. Box
CH-4002 Basel / Switzerland

Study Project No.:

744840

GLP CERTIFICATE

EIDGENÖSSISCHES DEPARTEMENT DES INNERN
DÉPARTEMENT FÉDÉRAL DE L'INTÉRIEUR
DIPARTIMENTO FEDERALE DELL'INTERNO
SWISS FEDERAL DEPARTMENT OF THE INTERIOR

Statement of GLP Compliance

It is hereby confirmed that

during the period of

October 22, 26 -30, 1998
November 5, 1998

the following test facilities of

RCC Ltd
4452 Itingen
Switzerland

were inspected by the Federal Office of Public Health, the Federal Office of Environment, Forests and Landscape and the Intercantonal Office for the Control of Medicines with respect to the compliance with the Swiss GLP Guidelines.

Test Facilities

Areas of Expertise

- Toxicology Division

TOX

- Environmental Chemistry &
Pharmanalytics Division

ACC, ECT, ENF, PCT, RES,
OTH (Animal metabolism)

- Biotechnology & Animal
Breeding Division

OTH (Microbiology)

The inspection was performed in agreement with the OECD Guidelines for National GLP Inspections and Audits. It was found that the aforementioned test facilities were operating in compliance with the Swiss Principles of Good Laboratory Practice (Good Laboratory Practice (GLP) in Switzerland, Procedures and Principles, March 1986) at the time they were inspected.

Federal Department of the Interior

Ruth Dreifuss

The President of the Swiss Confederation

Bern, March 1999

GOOD LABORATORY PRACTICE**STATEMENT OF COMPLIANCE**

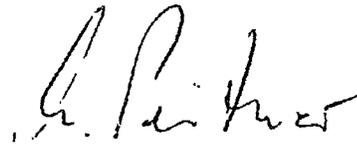
RCC Project Number: 744840
Test Item: TK 11014 (Irganox L 135)
Study Director: Dr. Armin Peither
Study Title: Influence of TK 11014 (Irganox L 135) on survival and reproduction of *Daphnia magna* in a semistatic test over three weeks

This study (with the exception of the pre-experiments as mentioned in the report) was conducted in compliance with Good Laboratory Practice Regulations and meets the requirements as listed below.

- OECD Principles of Good Laboratory Practice, as revised in 1997 [C(97)186/Final].
- Good Laboratory Practice (GLP) in Switzerland, Procedures and Principles, March 1986.

There were no circumstances that may have affected the quality or integrity of the data.

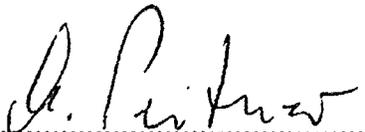
Study Director: Dr. Armin Peither


.....
Date: February 29, 2000

SIGNATURE PAGE

Study Director:

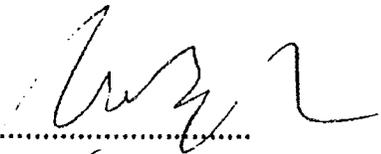
Dr. Armin Peither


.....
Date: February 29, 2000

Managing Director:



Mr. Markus Arenz


.....
Date: Feb 29, 2000

QUALITY ASSURANCE UNIT

RCC Ltd, Environmental Chemistry & Pharamalytics Division, CH-4452 Itingen / Switzerland

STATEMENT

RCC Project Number: 744840

Test Item: TK 11014 (Irganox L 135)

Study Director: Dr. Armin Peither

Study Title: Influence of TK 11014 (Irganox L 135) on survival and reproduction of *Daphnia magna* in a semistatic test over three weeks

Study procedures (with the exception of the pre-experiments as mentioned in the report) were periodically inspected. The protocol and this report were audited by the Quality Assurance Unit. The dates are given below.

Dates of QAU Inspections / Audits		Dates of Reports to the Study Director and to Management
November 22, 1999	Protocol	November 22, 1999
November 24, 1999	Test medium preparation	November 25, 1999
February 17/21, 2000	Final report	February 21, 2000

Quality Assurance:

for Mrs. Ursula Burri

Ursula Burri
Date: Feb 23, 2000

CERTIFICATION OF GLP AND VERIFICATION OF THE REPORT

The Statement of Compliance with Good Laboratory Practice found in this report, and signed by the Study Director is truthful and accurate, and this report as provided by the testing facility is complete and unaltered.

For the Sponsor:

.....
Date: 28.3.2000

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GENERAL INFORMATION

GENERAL

Study Title: Influence of TK 11014 (Irganox L 135) on survival and reproduction of *Daphnia magna* in a semistatic test over three weeks

Test Item: TK 11014 (Irganox L 135)

Test System: *Daphnia magna*

Sponsor: Ciba Specialty Chemicals Inc.
Additives Division
P.O. Box
CH-4002 Basel / Switzerland

Monitoring Scientist: Dr. Severin Müller

Testing Facility: RCC Ltd
Environmental Chemistry & Pharamanalytics Division
Zelgliweg 1
CH-4452 Itingen / Switzerland

RCC Project No.: 744840

Analytical Identification No.: 753254

RESPONSIBILITIES

Study Director: Dr. Armin Peither

Deputy Study Director: Dr. Ulrich Memmert

Technical Coordinator: Mrs. Anne Enderlin

Principal Investigator Analytics: Dr. Jörn Schreitmüller

Head of RCC Quality Assurance: Mrs. Iris Wüthrich

SCHEDULE

Start of Experiments: December 1, 1999

Completion of Experiments: January 7, 2000

Study Completion Date: February 29, 2000

ARCHIVING

RCC Ltd, CH-4452 Itingen/Switzerland will archive the raw data, protocol, report and test item reference sample of the present study for at least ten years. Thereafter, no raw data, protocol, report and test item reference sample will be withdrawn without the sponsor's prior consent.

GUIDELINES

This study followed the procedures indicated by the following internationally accepted Guideline and Recommendation:

OECD Guidelines for Testing of Chemicals, No. 211, *Daphnia magna* Reproduction Test, September 21, 1998.

SUMMARY OF PROTOCOL AMENDMENTS

First Amendment to Protocol

Concerning:	Alteration:	Reason:
2.2 Test Item	Purity of the test item	Update

SUMMARY

The influence of the test item TK 11014 (Irganox L 135) on the reproduction and survival rate of *Daphnia magna* was investigated in a semistatic test over 21 days following the OECD Guidelines for Testing of Chemicals, No. 211, (1998): "*Daphnia magna* Reproduction Test".

Due to the low water solubility of the test item, a supersaturated emulsion of the test item with a loading rate of 100 mg/l was continuously stirred at room temperature in the dark over 6.5 days. This emulsion was filtered and the undiluted filtrate with the maximum concentration of dissolved test item was used as the highest test medium concentration and as stock solution for four dilutions in a geometric series differing by a constant factor of 3.2. Additionally, a control was tested in parallel.

At all preparation dates, the test item concentrations in the analyzed test medium (the undiluted filtrate of the supersaturated emulsion) were below the limit of analytical quantification of 0.03 mg/l. Therefore, all reported biological results are related to the 'undiluted filtrate' and its 'dilutions', respectively.

Taking into account the survival rates and the reproduction rates of the test animals, the dilution 1:3.2 was the highest treatment of TK 11014 (Irganox L 135) tested without toxic effects after the exposure period of 21 days (21-day NOEC). Thus, the 21-day NOEC was approximately threefold below the water solubility of TK 11014 (Irganox L 135).

The undiluted filtrate was the lowest treatment tested with toxic effects (21-day LOEC) due to the significant mortality rate and due to the statistically significantly reduced mean reproduction rate of *Daphnia magna* at the water solubility limit of the test item.

The 21-day EC50 for the reproduction rate of *Daphnia magna* could not be calculated since the mean reproduction rate in the undiluted filtrate was reduced by only 20%.

1 INTRODUCTION / RATIONALE

The objective of this study was to evaluate toxic effects of the test item TK 11014 (Irganox L 135) on the survival and reproduction rate of *Daphnia magna* during an exposure period of three weeks. For this purpose, the daphnids were continuously exposed in a semistatic test to aqueous test media containing the test item at various concentrations. The mortality, the number of young born and signs of intoxication were compared with corresponding parameters in the control.

The test species *Daphnia magna* and the method of application are recommended by the testing guideline.

2 MATERIALS AND METHODS

2.1 DEFINITIONS

NOEC (No Observed
Effect Concentration):

The highest test concentration at which no significant effect on the reproduction rate and on the survival rate of the parent animals or any other toxic effect is observed.

LOEC (Lowest Observed
Effect Concentration):

The lowest test concentration at which the mean number of alive offspring produced per parent is significantly lower than in the control, and/or the mortality rate of the parent animals is significantly higher than in the control, or any other significant toxic effect on the test animals is observed.

ECx (Reproduction rate):

The calculated concentration of test item which results in a x% inhibition of the reproduction rate compared with the control.

2.2 TEST ITEM

The test item and the following information concerning the test item were provided by the sponsor:

Identity:	TK 11014
Product name:	Irganox L 135
Batch no.:	0004749S
Expiration date:	January 25, 2002
Purity:	≥ 98%
Stability in water:	For days
Solubility in water:	< 0.03 mg/l (at 20°C)
Density:	0.96 g/l at 20°C
Vapor pressure:	1.5×10^{-3} Pa (at 20°C)
Aggregate state under storage conditions:	Liquid
Color:	Yellow to brown
Storage conditions:	At room temperature at about 20 °C, in the dark

2.3 ANALYTICAL STANDARD

The test item was used as analytical standard.

2.4 TEST SYSTEM

The study was performed with females of a clone of the species *Daphnia magna* Straus. The clone was originally supplied by the University of Sheffield/UK in 1992, defined from the supplier as clone 5. Since this date the clone is bred in the laboratories of RCC Ltd under identical temperature and light conditions as in the test, and in the same kind of test water as used in the test. At the start of the test the young daphnids used were less than 24 hours old and were not first brood progeny.

2.5 STUDY DESIGN

2.5.1 Experimental Conditions

The study was started with 10 daphnids per test concentration and control. Each test animal was kept individually in a 100 ml glass beaker containing 80 ml test medium. The beakers were covered with glass plates. The test vessels were labeled with the RCC project number and all necessary additional information to assure unmistakable identification. The test was performed in a temperature-controlled room.

Water temperature: 20–21 °C during the test period

Light conditions: Photoperiod of 16 hours light and 8 hours darkness, light intensity during light period within the range of 300–800 Lux.

Test duration: 21 days

Test water: The test was conducted in reconstituted water^(*) with an initial pH of 7.9 ± 0.3. Before use, the test water was aerated until oxygen saturation. During the test, the test media have not been aerated.

^(*) In purified water the following analytical grade salts and additives have been added to following nominal concentrations (reconstituted water, "M7"):

<u>Main Compounds</u>		<u>Trace Elements</u> ^(a)		<u>Vitamins</u>	
CaCl ₂ ·2H ₂ O	293.80 mg/l	B	(H ₃ BO ₃) 125.0 µg/l	Thiamine HCl	75.0 µg/l
MgSO ₄ ·7H ₂ O	123.30 mg/l	Fe	(FeSO ₄ ·7H ₂ O) ^(b) 50.0 µg/l	Cyanocobalamine (B ₁₂)	1.0 µg/l
KCl	5.80 mg/l	Mn	(MnCl ₂ ·4H ₂ O) 25.0 µg/l	Biotin (B ₇)	0.75 µg/l
NaHCO ₃	64.80 mg/l	Li	(LiCl) 12.5 µg/l		
Na ₂ SiO ₃ ·9H ₂ O	10.00 mg/l	Rb	(RbCl) 12.5 µg/l		
NaNO ₃	0.27 mg/l	Sr	(SrCl ₂ ·6H ₂ O) 12.5 µg/l		
KH ₂ PO ₄	0.14 mg/l	Br	(NaBr) 3.1 µg/l		
K ₂ HPO ₄	0.18 mg/l	Mo	(Na ₂ MoO ₄ ·2H ₂ O) 6.3 µg/l		
		Cu	(CuCl ₂ ·2H ₂ O) 1.6 µg/l		
		Zn	(ZnCl ₂) 6.3 µg/l		
		Co	(CoCl ₂ ·6H ₂ O) 2.5 µg/l		
		J	(KJ) 2.5 µg/l		
		Se	(Na ₂ SeO ₃) 1.0 µg/l		
		V	(NH ₄ VO ₃) 0.3 µg/l		
		EDTA	(Na ₂ EDTA·2H ₂ O) ^(b) 625 µg/l		

Water Hardness: 2.5 mmol/l (= 250 mg/l) as CaCO₃

Alkalinity: 0.9 mmol/l

^(a) concentrations of the target element, the source compounds are given in brackets

^(b) stock solutions of FeSO₄·7H₂O and Na₂EDTA·2H₂O are poured together and autoclaved immediately

2.5.2 Dosage and Concentrations

According to information given by the sponsor and according to a pre-experiment (without GLP) at RCC the solubility limit of the test item in water respectively test water is < 0.03 mg/l.

Therefore, a filtrate of a supersaturated emulsion of the test item and dilutions of the filtrate were prepared. The undiluted filtrate and the dilutions 1:3.2, 1:10, 1:32 and 1:100 were tested. Additionally, a control was tested in parallel (test water without test item).

Prior to each test medium renewal, the test media were freshly prepared as follows: A supersaturated emulsion with a loading rate of 100 mg/l was prepared by weighing 250 mg (range: 250 - 251 mg) of the test item into 1000 ml test water. This mixture was treated by ultrasonification for 15 minutes and was made up to 2500 ml test water. No auxiliary solvent or emulsifier was used. Then, the mixture was stirred for 6.5 days at room temperature in the dark. The long stirring period was chosen to dissolve a maximum of the test item in the test water. After stirring, the undissolved fraction of the test item was allowed to separate from the water phase for another half a day.

After separation a part of the test item was swimming at the water-surface (oily film), while a part was homogeneously distributed in the water phase. This supersaturated emulsion was separated from the oily film by sucking it of with a Teflon-tube and was filtered through a membrane filter (Schleicher & Schuell, Type NC45, pore size $0.45 \mu\text{m}$).

The undiluted filtrate of the supersaturated emulsion with the maximum concentration of dissolved test item was used as the highest concentrated test medium. Additionally, adequate volumes of the filtrate were diluted with test water for the preparation of test media with lower test item concentrations. No additional dilution step was inserted. In this way the following treatments were prepared: the undiluted filtrate of the emulsion with a loading rate of 100 mg/l and the dilutions 1:3.2, 1:10, 1:32 and 1:100 of the filtrate (1 part of the filtrate added to 2.2, 9, 31, and 99 parts of test water, respectively).

The actual concentrations of the test item in the test media were analytically examined (see Section 2.6.3). The test media were prepared just before introduction of daphnids (= start of the test).

The test concentrations were based on the results of a range-finding test, the results of an earlier acute toxicity test (RCC Project 708557) and on results of pre-experiments to the solubility of the test item. A loading rate in excess of 100 mg/l test item was not tested.

The range-finding test and the pre-experiments to the selection of suitable methods for the preparation of the emulsion and the test media were not performed in compliance with the GLP-Regulations and therefore are excluded from the Statement of Compliance. However, the raw data, respectively copies of raw data of these tests will be archived under the RCC project number of the present study.

2.5.3 Test Medium Renewal and Feeding of Daphnids

In this semistatic test, the test media of all test concentrations and of the control were renewed on Days 2, 5, 7, 9, 12, 14, 16 and 19 of the exposure period (every Monday, Wednesday and Friday). By that, a total of 9 test medium renewals were performed. At these dates the surviving test animals were carefully transferred by glass tubes from the old test vessels into the freshly prepared test media of the corresponding concentrations.

The test animals were fed on each working day (Monday through Friday) with a food mixture containing one part of green algae of the species *Scenedesmus subspicatus* (freshly grown in the laboratories of RCC) and one part of fish food suspension*. The carbon content of the algal and fish food suspensions was determined using a Shimadzu TOC 500 Analyser. The food amounts were based on the measured concentrations of total organic carbon (TOC) in the food suspensions.

The amounts of TOC fed per test animal and day:

Day	0/1:	0.10 mg	TOC / daphnia / day
Day	2/5-8/12-13:	0.15 mg	TOC / daphnia / day
Day	14-15:	0.20 mg	TOC / daphnia / day
Day	9/20:	0.25 mg	TOC / daphnia / day
Day	16/19	0.30 mg	TOC / daphnia / day

* Fish food suspension:

10 g of a commercial fish diet (TETRA MIN Hauptfutter, obtained from TETRA-Werke, D-49324 Melle, Germany) were powdered and suspended in 500 ml test water. The suspension was allowed to stand for 4 hours, then 400 ml of the supernatant were taken and boiled. This suspension was stored deep frozen in small quantities until use.

2.6 EVALUATIONS

2.6.1 Mortality of Adults and Number of Young

The mortality of adults and the number of young were recorded three times per week before renewal of test media. Dead animals and offspring were removed at the renewal of the test media. The reproduction rate was calculated as the total number of living offspring produced per parent female alive at the end of the test.

The NOEC and the LOEC of the reproduction rate were statistically evaluated by testing the mean reproduction rate at the test concentrations on statistically significant differences to the control value by the multiple Williams-test (Ref. 1, 2) after a one-way analysis of variance (ANOVA).

The EC50 of the reproduction rate after 21 days could not be calculated due to the low toxic effect of the test item on the reproduction rate up to the highest test concentration, and was therefore determined directly from the raw data.

2.6.2 Water Quality Criteria

The pH-values and dissolved oxygen concentrations in the test media were measured in all test concentrations and in the control at the beginning and end of each test medium renewal period. Only the pH-values and oxygen concentrations of the lowest and highest test concentration and the control are reported since in all test media pH-values and oxygen concentrations were in the same range.

The water temperature was measured in one control beaker at the beginning and at the end of each test medium renewal period. Additionally, the air-temperature in the testing room was continuously recorded by a temperature recorder.

The appearance of the test media was visually recorded at each of the above mentioned intervals.

2.6.3 Analysis of the Test Item Concentrations

For the analytical measurements, duplicate samples were taken from the freshly prepared undiluted emulsion filtrate at each test medium preparation date. Since the water solubility limit of the test item was below the limit of quantification, no samples were taken from the dilutions of the filtrate.

The duplicate samples from the start of the test were analyzed immediately after preparation. The duplicate samples from the other sampling dates were deep-frozen (at about -20 °C) immediately after sampling and were analyzed after the end of the test.

3 RESULTS AND DISCUSSION

Analytical results

At the start of the test medium renewal periods, the test item concentration in the analyzed test medium (the undiluted filtrate of the supersaturated emulsion) was below the limit of quantification of 0.03 mg/l (see Table 2 in the attached analytical report). Therefore, all reported biological results are related to the 'undiluted filtrate' and its 'dilutions', respectively.

Survival of adults

In the control and at the dilutions from 1:100 to 1:3.2 the survival rate of the test animals at the end of the test was 100% (Table 1). In the undiluted filtrate the mortality rate was 30%. Thus, the survival rate of *Daphnia magna* after 21 days was significantly (>10%) reduced first in the undiluted filtrate.

Reproduction rate

Table 2 shows the total number of alive offspring reproduced by all adults within 21 days of continuous exposure to TK 11014 (Irganox L 135) (cumulative values). The first young offspring released from their parent animals were recorded in the control and in all treatments (the undiluted filtrate and its dilutions) at the observation on Day 9.

The reproduction rates calculated for each individual test animal, which survived until the end of the test, are given in Table 3. The mean reproduction rate at the different treatments and the control is given in Figure 1. The mean reproduction rate of the daphnids in the control was 77.8 ± 13.5 alive offspring per adult (mean \pm SD). According to the results of a Williams-Test (one-sided smaller, $\alpha = 0.05$) no significant toxic effect of the test item on the mean reproduction rate was determined at the dilutions 1:100 to 1:3.2. First in the undiluted filtrate, the mean reproduction rate of surviving daphnids was statistically significantly reduced to in the mean 79.7% alive offspring compared to the control.

The 21-day EC50 for the reproduction rate of *Daphnia magna* could not be calculated since the mean reproduction rate in the undiluted filtrate was reduced by only 20%.

Signs of intoxication

With exception of the reported mortality and the reduced reproduction rates, no particular signs of intoxication were observed at the test animals during the test.

NOEC and LOEC

Taking into account the survival rates and the reproduction rates of the test animals, the dilution 1:3.2 was the highest treatment of TK 11014 (Irganox L 135) tested without toxic effects after the exposure period of 21 days (21-day NOEC). Thus, the 21-day NOEC was approximately threefold below the water solubility of TK 11014 (Irganox L 135).

The undiluted filtrate was the lowest treatment tested with toxic effects (21-day LOEC) due to the significant mortality rate and due to the statistically significantly reduced mean reproduction rate of *Daphnia magna* at the water solubility limit of the test item.

Dissolved oxygen, pH, and water temperature

During the test period, the pH-values in the measured test media ranged from 7.6 to 8.0 (Table 4). The dissolved oxygen concentrations (Table 5) were at least 8.0 mg/l (measured at the dilution 1:10, not shown in Table 5). The water temperature ranged from 20 to 21°C during the test period.

Appearance of the test media

No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the test period.

4 TABLES

Table 1: Number of surviving test animals, exposed to the test item.

Exposure Day	Treatments					
	Control	Dilution 1:100	Dilution 1:32	Dilution 1:10	Dilution 1:3.2	Undiluted Filtrate
0	10	10	10	10	10	10
1	10	10	10	10	10	10
2	10	10	10	10	10	10
5	10	10	10	10	10	10
7	10	10	10	10	10	10
9	10	10	10	10	10	9
12	10	10	10	10	10	7
14	10	10	10	10	10	7
16	10	10	10	10	10	7
19	10	10	10	10	10	7
21	10	10	10	10	10	7
% surviving on Day 21	100	100	100	100	100	70

Table 2: The total number of alive, young daphnids reproduced by all adults (cumulative values).

Exposure Day	Treatments					
	Control	Dilution 1:100	Dilution 1:32	Dilution 1:10	Dilution 1:3.2	Undiluted Filtrate
0	0	0	0	0	0	0
1	0	0	0	0	0	0
2	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
9	41	29	28	35	39	4
12	190	188	178	176	153	66
14	209	188	181	178	164	96
16	344	342	326	319	269	142
19	530	547	531	527	471	252
21	778	796	783	790	746	439
% of control ¹	100	102.3	100.6	101.5	95.9	56.4

¹: based on the value of the last exposure day

Table 3: The number of alive offspring reproduced per surviving adult within 21 days of exposure.

Replicate No.	Treatments					
	Control	Dilution 1:100	Dilution 1:32	Dilution 1:10	Dilution 1:3.2	Undiluted Filtrate
1	89	71	76	78	76	66
2	70	88	83	76	86	*
3	74	84	84	83	81	55
4	77	85	91	84	70	46
5	68	71	80	67	63	61
6	90	87	69	81	77	*
7	82	71	79	82	76	68
8	89	81	79	87	73	68
9	48	83	66	80	75	*
10	91	75	76	72	69	70
mean	77.8	79.6	78.3	79.0	74.6	62.0
± SD	13.5	6.9	7.2	6.0	6.4	8.7
n	10	10	10	10	10	7
CV %	17.4	8.7	9.2	7.6	8.6	14.1
Mean in %	100	102.3	100.6	101.5	95.9	79.7
STAT	-	n.s.	n.s.	n.s.	n.s.	s.

CV %: coefficient of variation in %: $(SD_x / \text{mean}_x) \cdot 100\%$
 STAT: results of a Williams-test with the mean values of alive offspring (one-sided smaller, $\alpha = 0.05$)
 n.s.: mean value not significantly lower than in the control
 s.: mean value significantly lower than in the control
 *: test animal died during the test period

Table 4: pH-values in the test media at the start and the end of the test media renewal periods.

Exposure Period (Days)	pH-values					
	Start			End		
	Treatments					
	control	Dilution 1:100	Undiluted Filtrate	control	Dilution 1:100	Undiluted Filtrate
0-2	7.9	7.9	8.0	7.9	7.9	7.9
2-5	8.0	8.0	8.0	7.8	7.8	7.9
5-7	7.9	7.9	7.9	7.8	7.8	7.8
7-9	7.9	7.9	7.9	7.8	7.9	7.8
9-12	7.9	7.9	7.9	7.8	7.9	7.8
12-14	7.9	7.9	7.9	7.8	7.8	7.8
14-16	8.0	8.0	8.0	7.8	7.8	7.8
16-19	7.8	7.8	7.8	7.7	7.7	7.7
19-21	7.8	7.8	7.8	7.6	7.6	7.7

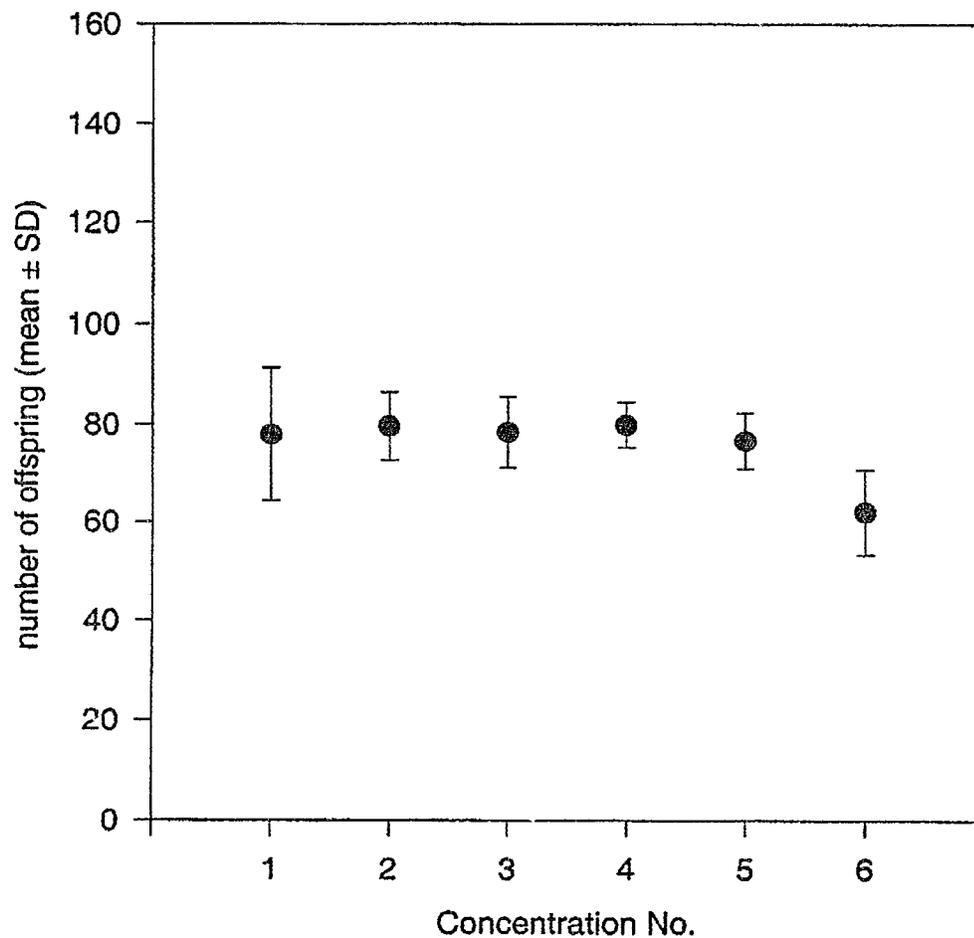
Table 5: Dissolved oxygen concentrations in the test media at the start and the end of the test media renewal periods.

Exposure Period (Days)	Oxygen concentrations (mg/l)					
	Start			End		
	Treatments					
	control	Dilution 1:100	Undiluted Filtrate	control	Dilution 1:100	Undiluted Filtrate
0-2	8.9	8.9	8.9	8.3	8.3	8.3
2-5	8.8	8.8	8.6	8.4	8.3	8.4
5-7	8.7	8.7	8.6	8.5	8.4	8.4
7-9	8.9	8.8	8.6	8.6	8.5	8.6
9-12	8.8	8.8	8.8	8.5	8.5	8.4
12-14	8.9	8.9	8.9	8.6	8.5	8.5
14-16	8.9	8.9	8.8	8.5	8.6	8.5
16-19	8.8	8.9	8.8	8.6	8.6	8.6
19-21	8.8	8.8	8.8	8.2	8.1	8.2

5 FIGURE

Figure 1: Number of alive offspring (mean \pm SD) per parent animal surviving at the end of the test.

Conc.1: Control; Conc.2: Dilution 1:100; Conc.3: Dilution 1:32;
Conc.4: Dilution 1:10; Conc.5: Dilution 1:3.2; Conc.6: Undiluted Filtrate;



6 REFERENCES

- 1) WILLIAMS, D. A. (1971):
A test for differences between treatment means when several dose levels are compared with a zero dose control.
Biometrics 27, 103-117
- 2) WILLIAMS, D. A. (1972):
The comparison of several dose levels with a zero dose control.
Biometrics 28, 519-531

ATTACHMENT

ANALYTICAL REPORT

**DETERMINATION OF THE CONCENTRATIONS OF
TK 11014 (IRGANOX L 135)
IN TEST MEDIUM OF A SEMI-STATIC
REPRODUCTION TOXICITY TEST
WITH *DAPHNIA MAGNA***

RCC PROJECT 744840

ANALYTICAL REPORT

PERFORMING LABORATORY:

RCC Ltd
Environmental Chemistry &
Pharmanalytics Division
CH-4452 Itingen/Switzerland

ANALYTICAL ID. NO. 753254

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GOOD LABORATORY PRACTICE**STATEMENT OF COMPLIANCE**

RCC Project No.: 744840
Analytical Id. No.: 753254
Test Item: TK 11014 (Irganox L 135)
Principal Investigator Analytics: Dr. J. Schreitmüller
Title of the Study: Determination of the concentrations of TK 11014 (Irganox L 135) in test medium of a semi-static reproduction toxicity test with *Daphnia magna*

This study (with the exception of the pre-experiments as mentioned in the report) was conducted in compliance with Good Laboratory Practice Regulations.

Principal Investigator Analytics: Dr. J. Schreitmüller

.....
Date: February 25, 2000

PREFACE

GENERAL INFORMATION

Title of the Study: Determination of the concentrations of TK 11014 (Irganox L 135) in test medium of a semi-static reproduction toxicity test with *Daphnia magna*

Test Item: TK 11014 (Irganox L 135)

Sponsor: Ciba Specialty Chemicals Inc.
Additives Division
P.O. Box
CH-4002 Basel/Switzerland

Monitoring Scientist: Dr. Severin Müller

Testing Facility: RCC Ltd
Environmental Chemistry & Pharamanalytics Division
Zelgliweg 1
CH-4452 Itingen/Switzerland

RCC Project No.: 744840

Analytical Id. No.: 753254

PROJECT STAFF

Principal Investigator
Analytics: Dr. J. Schreitmüller

.....
Date: February 23, 2000

Technician: R. Kerkez, R. Weber

Reporting: M. Viererbe

SCHEDULE

Start of Experiments: November 30, 1999
End of Experiments: January 07, 2000

1 OBJECTIVE

In this report the results obtained for the concentrations of TK 11014 (IRGANOX L 135) in test medium are described.

The quantification of the test item was performed by HPLC analysis and UV/VIS-detection.

2 MATERIALS AND METHODS

The implementation of the analytical method was performed on basis of a method provided by the sponsor ¹.

2.1 TEST ITEM

The test item as described in the biological part of this study was also used for analytical purposes.

2.2 ANALYTICAL PROCEDURE

2.2.1 Storage

Series I

The samples were analysed after the sampling procedure (treatment samples and biological control samples of sampling day 0).

Series II

The samples were stored deep frozen and protected from light until analysis was performed (treatment samples and biological control samples of sampling days 2 to 19).

2.2.2 Reagents and Solvents

Acetonitrile	Baker, no. 9017
Purified water for HPLC	in-house prepared by a water purification system (Millipore)
Test water	as described in the biological part of this study

¹ These experiments were not performed according to the regulations of GLP. However, the raw data or copies of the raw data will be archived under the project number of the main study.

2.2.3 Standard Solutions used for Sample Quantification

Series I

11.62 mg of the test item was dissolved in a mixture of acetonitrile/purified water (50:50; v:v) and made up to the mark in a 200 ml volumetric flask to prepare a stock solution of 58.1 mg/l. Defined volumes of this stock solution were diluted with the aforementioned mixture to obtain standard solutions in the range of 0.02905 to 5.81 mg/l of the test item.

Series II

13.2 mg of the test item was dissolved in a mixture of acetonitrile/purified water (50:50; v:v) and made up to the mark in a 100 ml volumetric flask to prepare a stock solution of 132 mg/l. Defined volumes of this stock solution were diluted with the aforementioned mixture to obtain standard solutions in the range of 0.0099 to 0.066 mg/l of the test item.

These solutions were used to calibrate the HPLC-system.

2.2.4 Preparation of Spiked Test Water Samples

To demonstrate the validity of the method untreated test water was spiked with the test item.

Series I

10.47 mg of the test item was dissolved in acetonitrile and made up to the mark in a 100 ml volumetric flask to prepare a stock solution of 104.7 mg/l. Defined volumes of this stock solution were diluted with test water to obtain standard solutions of the test item with a concentration of 0.03141 mg/l.

Series II

26.3 mg of the test item was dissolved in acetonitrile and made up to the mark in a 100 ml volumetric flask to prepare a stock solution of 263 mg/l. Defined volumes of this stock solution were diluted with test water to obtain standard solutions of the test item with a concentration of 0.0263 mg/l.

In addition, test water without test item was analysed (analytical blank).

These solutions were subjected to the same treatment as a sample.

2.2.5 Analysis of Samples

Series I

The samples from the biological test were shaken mechanically to obtain homogeneous sample solutions.

Series II

The samples from the biological test were thawed at 25° C (waterbath) and shaken mechanically to obtain homogeneous sample solutions.

Aliquots of the samples were analysed by HPLC and UV/VIS-detection.

For results obtained see Table 2.

2.3 HPLC CONDITIONS

Auto sampler:	Varian 9300	
Pump:	Varian 9010 (Series I)	[Merck L-6200 (Series II)]
Detector:	Varian 9050 (Series I)	[Merck L-4000 (Series II)]
Workstation:	Varian LC 9020 STAR	
Column:	LiChrospher 100 RP 18; 125 x 4.6 mm; 5 μ m	
Eluent:	acetonitrile/purified water (88:12; v:v)	
Flow rate:	1.5 ml/min	
Temperature:	room temperature	
Injection volume:	500 μ l	
Detection wave length:	210 nm	
Retention time:	approx. 4.5 min	

2.4 EVALUATION OF RESULTS

Injected samples were quantified by peak areas with reference to the respective calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentration in mg/l. The correlation was performed using a linear function given below (equation 1). For results obtained see Table 1. From this curve the concentration x of the test item in an injected sample was calculated by the following equation:

$$y = b \cdot x + a \quad (1)$$

where

- y = Peak area of test item in injected sample [counts]
- x = Concentration of test item in injected sample [mg/l]
- a = y-axis intercept
- b = Slope

The recovery of the test item in a sample was calculated by equation 2:

$$R = \frac{x}{c_{nom}} \cdot 100 \% \quad (2)$$

where

- R = Recovery
- x = Concentration of test item in sample found by equation 1 [mg/l]
- c_{nom} = Nominal concentration of test item in sample [mg/l]

3 RESULTS AND DISCUSSION

The results obtained for the concentrations of TK 11014 (Irganox L 135) in test medium are presented in Table 2. For the preparation of the treatment samples the test item was suspended in test water. The suspension was filtrated and the undiluted filtrate was taken as the test medium (see biological part of this study).

An example of the calibration data for test item-standards is given in Table 1. The R^2 fits were at least 0.9990 (optimum 1.0000). This reflects the linearity of the HPLC-system within the given calibration ranges.

Typical HPLC chromatograms are shown in the attached Figures 1 to 5.

Concurrent with the sample analysis recoveries of spiked test water samples in the relevant concentrations (0.0263 and 0.03141 mg/l of the test item) were performed in duplicate. The mean concentrations were found to be 82 % and 92 % of the spiked values, with an average of 87 % ($n = 4$). Therefore, no correction for possible losses during the analytical procedure is necessary.

The limit of quantification (LOQ) for the test item in test medium was derived from the content of the spiked samples at which an acceptable recovery was obtained: the value was at about 0.03 mg/l.

The biological control samples and an analysed analytical blank (test water) did not affect the HPLC-chromatogram at the retention time of the test item.

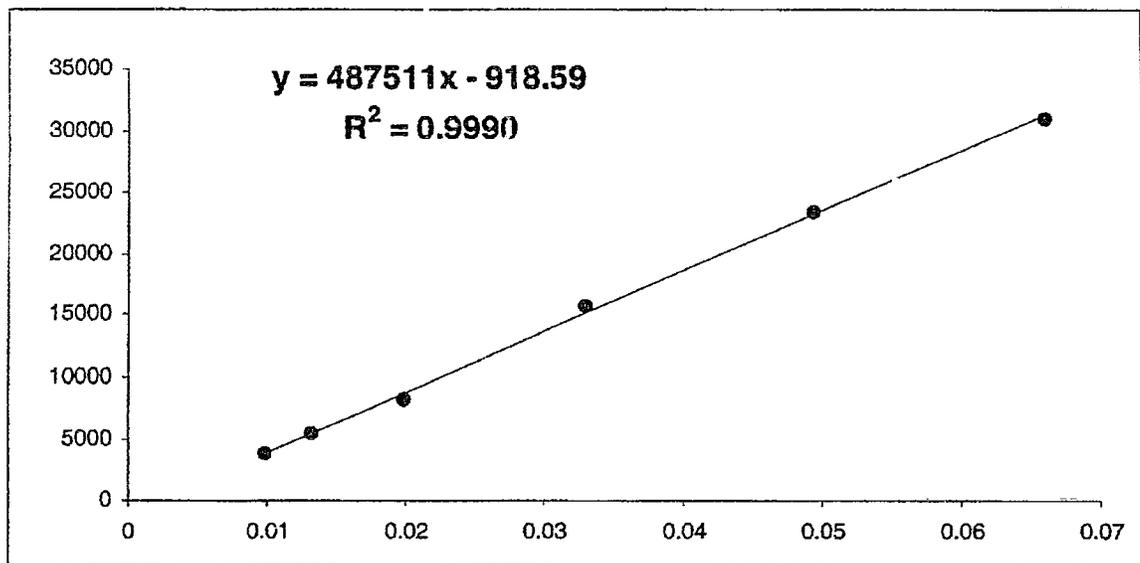
The concentrations of TK 11014 (Irganox L 135) found in the treatment samples were always below the LOQ of 0.03 mg/l.

The tabulated values of the samples represent rounded results obtained by calculation using the exact raw data.

4 TABLES

Table 1: Example of calibration data of test item-standards

Standard [mg/l]	Peak area measured [counts]	Deviation from calculated value [%]
0.0099	3916	0.2
0.0132	5549	0.6
0.0198	8248	-5.9
0.0330	15710	3.4
0.0495	23361	0.6
0.0660	31014	-0.8



where

y = Peak area of test item in injected solution [counts]

x = Concentration of test item in injected solution [mg/l]

Table 2: Results obtained for the concentrations of the test item in test medium

Nominal concentration of TK 11014 (Irganox L 135) [mg/l]	Sampling date [day]	Age of sample [hours]	RCC sample code	TK 11014 (Irganox L 135) measured			
				[mg/l]	[% of nominal]	mean [mg/l]	[% of nominal]
Treatment samples							
undiluted filtrate	0	0	D-3	< 0.03	n.a.		
	0	0	D-4	< 0.03	n.a.	< 0.03	n.a.
	2	0	D-7	< 0.03	n.a.		
	2	0	D-8	< 0.03	n.a.	< 0.03	n.a.
	5	0	D-11	< 0.03	n.a.		
	5	0	D-12	< 0.03	n.a.	< 0.03	n.a.
	7	0	D-15	< 0.03	n.a.		
	7	0	D-16	< 0.03	n.a.	< 0.03	n.a.
	9	0	D-19	< 0.03	n.a.		
	9	0	D-20	< 0.03	n.a.	< 0.03	n.a.
	12	0	D-23	< 0.03	n.a.		
	12	0	D-24	< 0.03	n.a.	< 0.03	n.a.
	14	0	D-27	< 0.03	n.a.		
	14	0	D-28	< 0.03	n.a.	< 0.03	n.a.
	16	0	D-31	< 0.03	n.a.		
	16	0	D-32	< 0.03	n.a.	< 0.03	n.a.
19	0	D-35	< 0.03	n.a.			
19	0	D-36	< 0.03	n.a.	< 0.03	n.a.	
Biological control samples							
0	0	0	D-1	< 0.03	n.a.	n.a.	n.a.
	0	0	D-2	< 0.03	n.a.	n.a.	n.a.
	2	0	D-6	< 0.03	n.a.	n.a.	n.a.
	5	0	D-10	< 0.03	n.a.	n.a.	n.a.
	7	0	D-14	< 0.03	n.a.	n.a.	n.a.
	9	0	D-18	< 0.03	n.a.	n.a.	n.a.
	12	0	D-22	< 0.03	n.a.	n.a.	n.a.
	14	0	D-26	< 0.03	n.a.	n.a.	n.a.
	16	0	D-30	< 0.03	n.a.	n.a.	n.a.
19	0	D-34	< 0.03	n.a.	n.a.	n.a.	
Spiked test water samples							
0.02630		0	DZ4	0.021	80		
		0	DZ5	0.022	85	0.022	82
0.03141		0	DZ1	0.030	96		
		0	DZ2	0.028	89	0.029	92
Analytical blank							
0		0	DZ3	< 0.03	n.a.		

n.d. = no test item detected

n.a. = not applicable

5 FIGURES

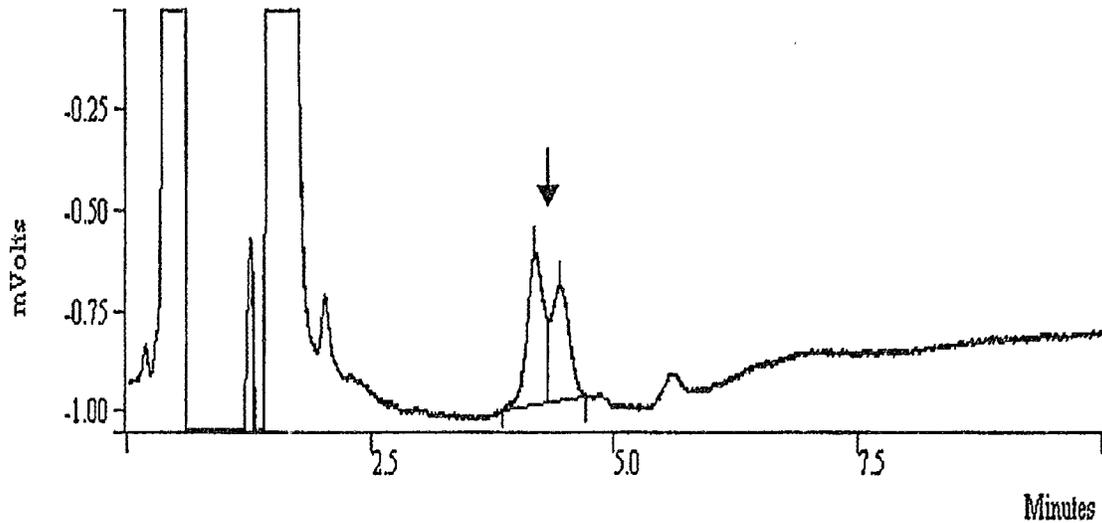


Figure 1: HPLC-chromatogram of standard solution (mid-level)
Concentration: 0.0198 mg/l of the test item

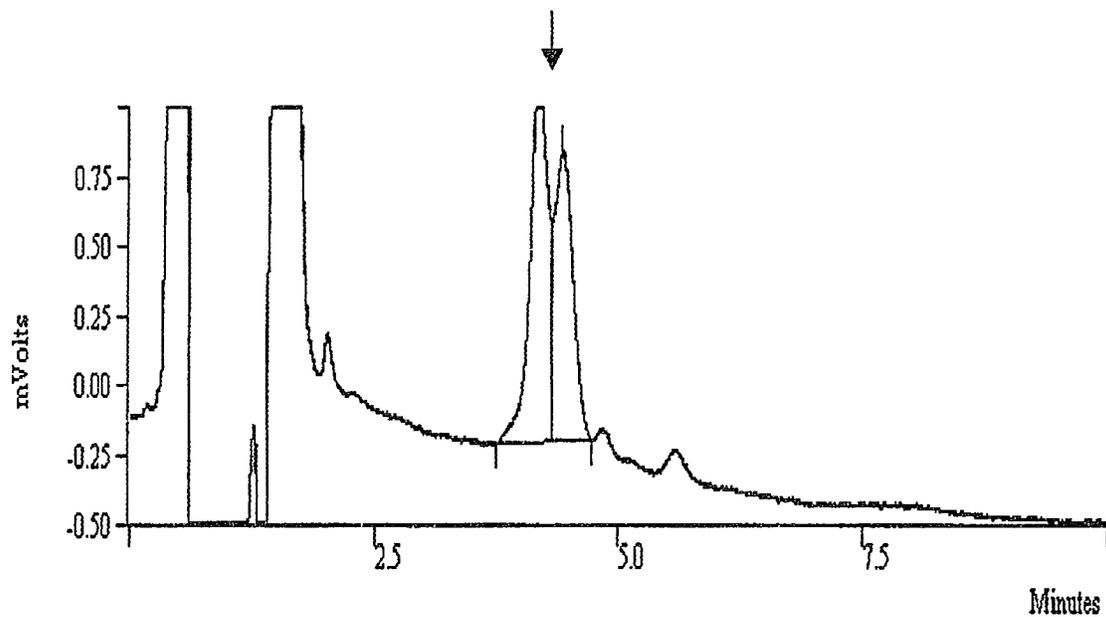


Figure 2: HPLC-chromatogram of standard solution (high-level)
Concentration: 0.066 mg/l of the test item

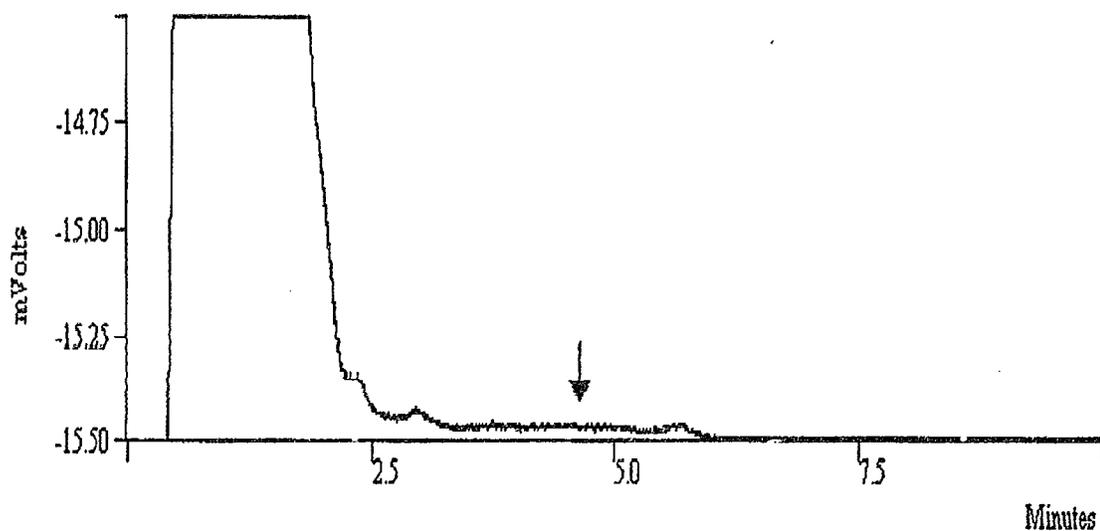


Figure 3: HPLC-chromatogram of biological control sample
Sample Id. No.: D-6
Sampling day 2

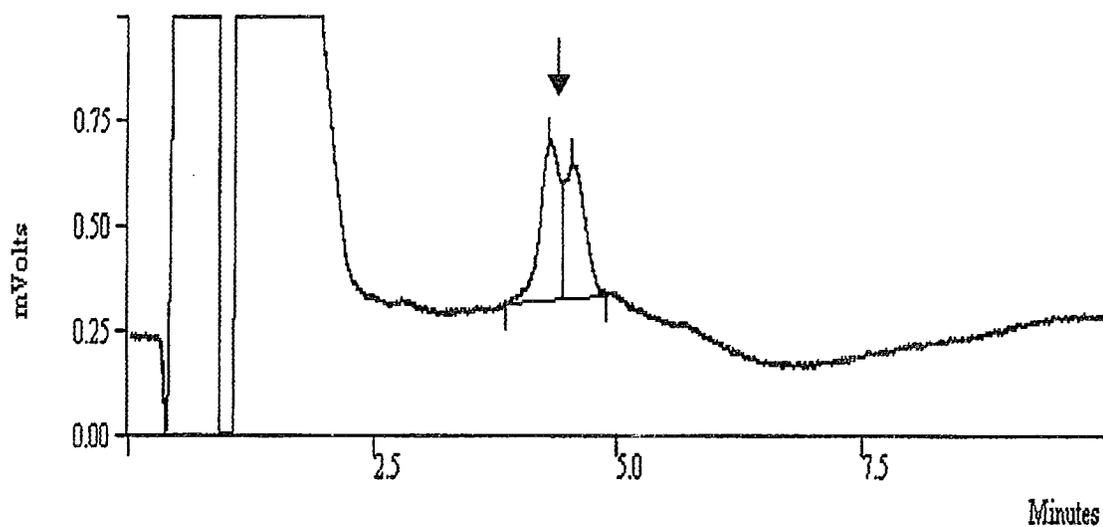


Figure 4: HPLC-chromatogram of spiked test water sample
Sample Id. No.: DZ5
(spiked with 0.0263 mg/l of the test item)
Recovery: 85 % of the nominal concentration

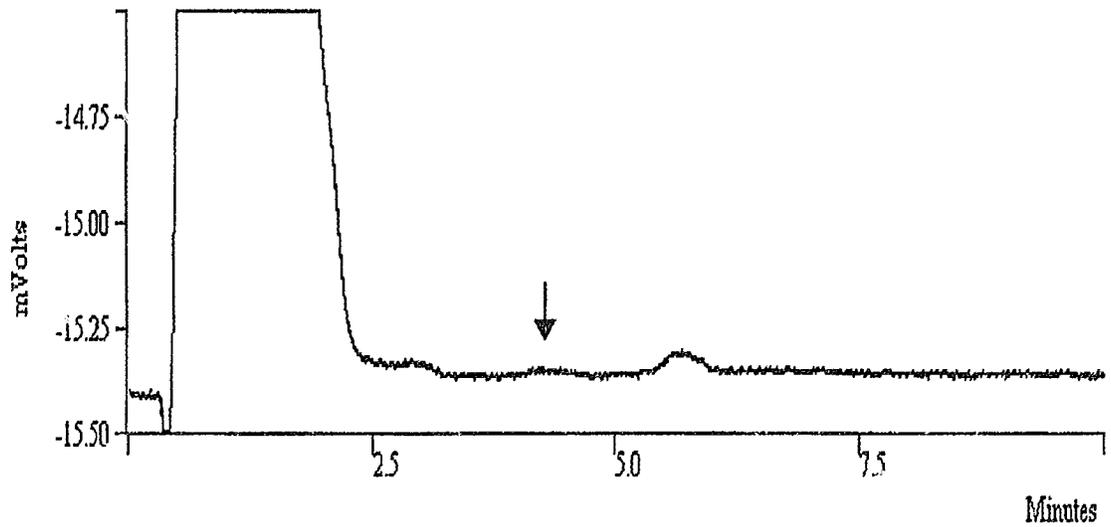


Figure 5: HPLC-chromatogram of non-aged treatment sample
Sample Id. No.: D-15
Sampling day 7
(undiluted filtrate; concentration below the limit of quantification of 0.03 mg/l)