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Report to:
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Daphnia magna reproduction test with p-xylene

Study director: **Estelle Bjørnestad, M.Sc.**
Project No.: **52852**
GLP Study No.: **91337/449**
Date: **2005.01.04/TKH**

Sponsor:
CEFIC
Aromatic Producers Association
Avenue E. Van Nieuwenhuyse 4, bte 2
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Monitor of the study:
TNO Nutrition and Food Research
Study Monitor: Drs Casper Hamwijk
Utrechtseweg 48
3704 HE Zeist
The Netherlands

Title: *Daphnia magna* reproduction test with p-xylene

GLP Study No.: 91337/449

Project No.: 52852

Test Period: 2004.08.25 - 2004.09.15

Test facilities:

***Daphnia magna* Reproduction test:**
DHI Water & Environment
Dept. of Environmental Risk Assessment
Agern Allé 5
DK-2970 Hørsholm
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Chemical analysis
TNO Nutrition and Food Research
Analytical Sciences Department
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NL-3704 HE Zeist
The Netherlands

Authentication:

We the undersigned hereby declare that the ecotoxicological investigation described in this report "*Daphnia magna* reproduction test with p-xylene" was carried out under our supervision, and in accordance with the OECD Principles of Good Laboratory Practice (as revised in 1997).

The study has been carried out in accordance with the procedures described in the report, which represents a true and accurate record of the results obtained.

DHI:

Study Director:	Estelle Bjørnestad, M.Sc.	2005.01.04
Approved by:	Torben Madsen, Ph.D.	2005.01.05
Technician:	Connie Seierø	2005.01.04

TNO:

Principal Investigator analytical chemistry:	Ton Schouten	2005.01.07

Quality Assurance Statement
DHI Water & Environment
Agern Allé 5
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Inspection of GLP studies is performed according to the following principles dependent on the type of study.

In short term studies (less than 1 week) conducted frequently, critical phases are inspected 2-3 times per year or on request from the sponsor.

In long term studies and short-term studies conducted non-frequently, inspections are made at critical phases of the individual study.

The performance of this study has been secured by DHI Water & Environment's Quality Assurance Unit.

The dates of inspection and audit are given below:

Date	**Activity**
2004.06.21	Protocol received
2004.09.15	Study audit
2005.01.04	Final report audited

This test report accurately describes the methods and procedures used in the study and accurately reflect the raw data of the study.

Louise Schlüter (Quality Assurance) 2005.01.04

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1. Summary

The freshwater crustacean *Daphnia magna* was tested with p-xylene in a reproduction test at the following nominal test concentrations: 0.168; 0.336; 0.672; 1.34; 2.69; 5.38 and 10.75 mg/l. The total test duration was 21 day. The test was carried out twice. The first study was performed from 17 June to 8 July 2004. It was discarded due to the very low number of offspring in the controls and further the high percentage of dead parent animals (40%) in the controls. Therefore, the study was repeated from 25 August to 15 September 2004. The test concentrations were the same as those used in the first study.

In order to verify the actual test concentrations, samples were taken from each test concentration at regular intervals during the whole study. The samples were sent to the principal investigator at TNO Nutrition and Food Research for chemical analysis. The chemical analysis showed that the actual measured test concentrations were as follows: 0.109; 0.209; 0.427; 0.771; 1.57; 3.16 and 5.30 mg/l.

Given as the actual measured test concentrations, the observed test results were as follows:

- The first offsprings were observed after 8 days in the control and in test concentrations up to 1.57 mg/l. At 3.16 mg/l, the first offspring was seen after 12 days and in the highest test concentration of 5.30 mg/l, no offspring was seen as all parent animals were dead after 5 days of exposure.
- The data processing showed that NOEC was 1.57 mg/l, LOEC was 3.16 mg/l, EC10-21d was 1.91 mg/l and EC50-21d was 2.90 mg/l.

2. Study objective

The objective of this study was to investigate the effects of the test substance on the reproductive output of the freshwater crustacean *Daphnia magna* during 21 days of exposure.

The study was performed in accordance with the OECD Guideline for testing of chemicals, *Daphnia magna* Reproduction Test /1/. The test was carried out in compliance with the OECD Principles of Good Laboratory Practice (GLP) as revised in 1997 /2/.

3. Test substance

Product name:	p-xylene
Product number:	95682
Product brand:	FLUKA
CAS number:	106-42-3
Molecular formula:	C_8H_{10}
Molecular weight:	106.17
Expiry date:	27 May 2005
Batch No.:	LOT 429739/1
Assay (GC area %):	99.4% REL

Remarks on GC:	0.01% toluene, 0.10% ethylbenzene, 0.48% m-xylene
Appearance:	Colourless clear liquid
Density D20/4:	0.861
Refractive Index N20/D:	1.496
Residue (evaporation)	0.0001%
Infrared spectrum:	Corresponds
Date of QC-release:	02/OCT/01

The test substance was provided by TNO Nutrition and Food Research. It was received at DHI on 3 June 2004 and stored at 20°C until use.

4. Preparation of stock and test solutions

A stock solution of 112 mg/l (65 µl/500 ml) of p-xylene was prepared at the beginning of the test and at each renewal of test media.

The range of test concentrations was determined on the basis of the results from a preliminary acute range-finding test, which showed a 100% mortality at 10.8 mg/l (nominal) and 5% mortality at 5.4 mg/l (nominal). The following test concentrations were chosen for the reproduction test: 0.168; 0.336; 0.672; 1.34; 2.69; 5.38 and 10.75 mg/l (nominal).

5. Test method

The objective of this study was to assess the effects of p-xylene on the reproductive output of the freshwater crustacean *Daphnia magna* during 21 days of exposure.

A strain of the freshwater crustacean *Daphnia magna* (Straus), *Cladocera*, *Crustacea* collected in the Langedam, Birkerød, has been cultured at DHI Water & Environment (the former Water Quality Institute) since 1979 according to the DHI Standard Operation Procedure and ISO Standard /3/.

Young females <24 hours taken from this culture were exposed to the test substance added to test medium at the selected range of test concentrations. At the end of the test, the total number of live offsprings produced per parent animal alive at the end of the test was assessed.

The test was performed in glass flasks with a final volume of 120 ml closed with a teflon lid. At the initiation of the test, 120 ml of test solution and one female <24 hours old was added to each flask. The alga *Pseudokirchneriella subcapitata* was added directly to the test flasks as feed, approx. 2×10^7 cells ≈ 0.2 mgC/animal/day until day 8, after which 0.4 mgC/animal/day was applied.

The test was performed as a static renewal test, i.e. the total volume in each flask was renewed three times a week. pH and oxygen saturation were recorded before and after each renewal of test medium. The measurements are included in Annex 5.

The reproductive output of the animals exposed to the test substance was compared to that of the controls in order to determine the no observed effect concentration (NOEC) and the EC10 and EC50 were also calculated.

The survival of parent animals and time to production of first brood were also reported.

The test was carried out at 20 ± 1°C in a climate room with normal laboratory light having a daily light/dark period of 16:8 hours.

6. Chemical analysis

In order to verify the actual exposure concentrations, samples were taken from each test concentration at regular intervals during the test. Sub-samples of 10 ml will be collected in 25 ml screwcap vials and 10 ml of acetonitrile will be added to each vial before closing the lids. Although duplicate samples were taken of each of the test solutions, only one of these duplicate samples was analysed. The other one is a spare sample and will be disposed of two months after the approval of the test report.

At the end of the test, all samples were sent to TNO Nutrition and Food Research for chemical analysis under the responsibility of the Principal Investigator. Annex 3 gives a list of the collected samples.

The concentration of the test substance in the samples was determined using HPLC method. Quantification of the test substance was obtained by comparing the peak area of the test substance peak in the samples with those in the calibration solutions containing known amounts of the test substance in blank medium. The analytical method was validated by analysing an appropriate standard solution three times. The method meets the following criteria:

- Linearity
  The correlation coefficient of the calibration curve (≥ 5 points) was greater than 0.996.
- Repeatability of the retention time
  The relative standard deviation in the retention time of the test substance when an appropriate standard solution was injected three times was less than 2%.
- Repeatability of the concentration
  The relative standard deviation was less than 10% when a test solution (an appropriate standard solution) was analysed three times.
- Selectivity
  No peak was found in the blank sample with a retention time of 95-105% of that of the test substance. However, it was not possible to detect a concentration of 10% of the lowest dose sample. The theoretical p-xylene concentration in the analysis sample from the lowest dose group was 0.084 mg/l (as all samples were 1:1 diluted with acetonitrile, see Annex 6: 3.2). The calculated LOD (3x noise) was 0.011 mg p-xylene/l, which was about 13% of this concentration (nominal concentration of the undiluted test medium 0.168 mg p-xylene/l).

For the preparation of calibration solutions, the Principle Investigator used test substance from the batch, of which a portion was sent to DHI for the *Daphnia* reproduction test.

The Principal Investigator at TNO Nutrition and Food Research was responsible for the chemical analysis. The results are given in Annex 6.

7. Data processing

The no observed effect concentration (NOEC) was calculated by use of Dunnett's procedure /4/. The EC10 and EC50 values (10% and 50% effect) for reproduction and their confidence intervals were calculated by use of TOXEDO /5/. The results of the data processing are presented in Annex 1.

8. Results of the reproduction test

The results from the test are presented in Annex 2 in tables listing the number of offspring, dead and live parent animals for each test vessel and for each observation time. The number of live offspring produced by live parent animal was counted and the mean number of live offspring produced by each live parent per exposure concentration was calculated.

The results from the chemical analysis performed by TNO are given in Annex 6.

In Annex 4, both the nominal test concentrations and the corresponding actual measured test concentrations are shown, together with the calculated recovery for each sample. The actual measured test concentrations were thereafter used in the data processing.

Figure 8.1 shows the calculated recovery of the nominal test concentrations in percentage of the start concentration for the individual samples.

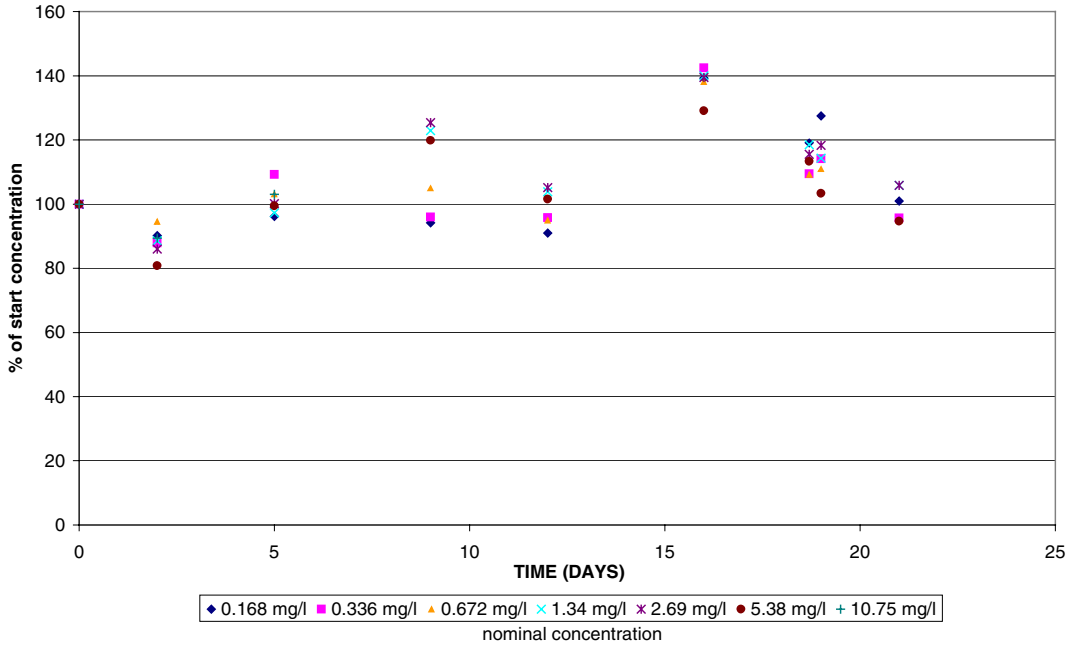

Figure 8.1 The calculated recovery of the nominal test concentrations in percentage of the start concentration for the individual samples

The first offsprings were observed after 8 days in the control and in the test concentrations up to 2.69 mg/l (nominal) corresponding 1.57 mg/l actual measured test concentration. At 5.38 mg/l (nominal), the first offspring was seen after 12 days and in the highest test concentration of 10.75 mg/l (nominal) no offspring was seen and all parent animals were dead after 5 days of exposure. The corresponding actual measured test concentrations were 3.16 mg/l and 5.30 mg/l, respectively.

The data processing showed that NOEC was 1.57 mg/l, LOEC was 3.16 mg/l, EC10-21d was 1.91 mg/l and EC50-21d was 2.90 mg/l (actual measured test concentrations).

9. Validation

The validation criteria of the test guideline for the control are fulfilled:

- The mortality of the parent animals in the control does not exceed 20% at the end of the test
- The mean number of live offsprings produced per control parent animal surviving at the end of the test is ≥ 60.

10. Amendments to the protocol

Amendment No. 1 of 2004.07.06 regarding HSGC analysis.

The actual concentration of test substance in the collected samples will be determined by use of a validated HPLC method instead of HSGC, as it is believed to be more appropriate.

The method meets the following criteria:

- Linearity: The correlation coefficient of the calibration graph should be greater than or equal to 0.996
- Repeatability of the analysis: The relative standard deviation should be smaller than 10% when the test calibration solution is analyzed three times
- Selectivity: No peak should be found in a blank test medium with a retention time of 95%-105% of that of the test substance. If the blank sample shows a peak with a retention time in the above range and the area of this peak is > 10% of the lowest concentration level, the result for the sample containing test substance will be corrected for the level found in the blank sample

Amendment No. 2 of 2004.08.24 regarding the repetition of the study.
This reproduction study was finalised on 8 July as planned, but it was discarded due to the very low number of offspring in the controls and further the high percentage of dead adults (40%) in the controls. Therefore, the study will be repeated and the results of the original study will not be reported.

Chapter 10:
The sampling procedure has been modified, as HPLC analysis will be used to verify the concentration of p-xylene in the test solutions.

Sub-samples of 10 ml will be collected in 25 ml screwcap vials and 10 ml of acetonitrile will be added to each vial before closing the lids.

Chapter 11:
A stock solution of 112 mg/l (65 µl/500 ml) of the substance will be prepared at the beginning of the test and at each renewal of test media.
The range of test concentrations is determined on the basis of the results from the preliminary acute range-finding test. The test concentrations will be the following: 0.168; 0.336; 0.672; 1.34; 2.69; 5.38 and 10.75 mg/l.

Chapter 14:
The study will be repeated from the 25 August to 15 September 2004.

11. Archives

All data generated and all other records and information relevant to the quality and integrity of the study will be retained. They will be filed in the archives of DHI Water & Environment after termination of the study and retained for a period of 10 years after issue of the final report.

A reference sample of the test substance will be retained for a period of two years after completion of the study provided that it is sufficiently stable. DHI reserves the right to

return the test substance to the sponsor if, in the opinion of DHI, they cannot safely be disposed of by conventional procedures.

At the end of the 10-year period, the sponsor will be consulted regarding the disposal or continued storage of raw data.

Raw data with respect to the chemical analysis will be filed in Raw data and all other information relevant to the quality and integrity of the chemical analyses will be retained in the archives of TNO Nutrition and Food Research, for a period of at least 10 years after reporting of the study. After this period, the sponsor will be contacted to decide on the fate of the data.

12. References

/1/ OECD Guideline for Testing of Chemicals 211, 21st September 1998. *Daphnia magna* Reproduction Test.

/2/ OECD Principles of Good Laboratory Practice (as revised in 1997). ENV/MC/CHEM (98)17.

/3/ ISO 6341: Water quality – Determination of the inhibition of the mobility of *Daphnia magna* Starus (*Cladocera, Crustacea)* Acute toxicity test. Third edition, 1996-04-01.

/4/ US-EPA (1994): Dunnett Program Version 1.5. US Environmental Protection Agency, Cincinnati.

/5/ VKI (1999). TOXEDO Ver. 1.5. Program for statistical estimation of EC values, based on experimental data from ecotoxicological assays.

Annex 1: Data processing

Inhibition of the reproduction of *Daphnia magna* with p-xylene

Statistical parameters calculated from continuous responses based on continuous mean
Test type: Reproduction test

Control values

Concentration in mg/l	Reproduction number of offspring	Inhibition in per cent
Control 1	84	-
Control 2	81	-
Control 3	101	-
Control 4	99	-
Control 5	113	-
Control 6	80	-
Control 7	113	-
Control 8	92	-
Control 9	70	-
Control 10	100	-
Control mean	93	0

Experimental data

Concentration in mg/l	Reproduction number of offspring	Inhibition in percent
0.109	100	0
0.109	90	4
0.109	111	0
0.109	0	100
0.109	104	0
0.109	98	0
0.109	102	0
0.109	62	34
0.109	125	0
0.209	114	0
0.209	91	2
0.209	123	0
0.209	78	16
0.209	87	7
0.209	115	0
0.209	103	0
0.209	102	0
0.209	104	0
0.209	96	0
0.427	111	0
0.427	109	0
0.427	81	13
0.427	87	7
0.427	132	0
0.427	107	0
0.427	64	31
0.427	98	0
0.427	112	0
0.427	79	15
0.771	110	0
0.771	118	0
0.771	131	0
0.771	119	0
0.771	116	0

0.771	137	0
0.771	66	29
0.771	95	0
0.771	109	0
1.570	125	0
1.570	104	0
1.570	116	0
1.570	49	47
1.570	80	14
1.570	107	0
1.570	99	0
1.570	122	0
1.570	92	1
1.570	111	0
3.160	45	52
3.160	29	69
3.160	45	52
3.160	17	82
3.160	42	55
3.160	43	54
3.160	33	65
3.160	42	55
3.160	37	60
3.160	41	56
5.300	0	100
5.300	0	100
5.300	0	100
5.300	0	100
5.300	0	100
5.300	0	100
5.300	0	100
5.300	0	100
5.300	0	100
5.300	0	100

Dunnett's procedure:

NOEC: 1.57 mg/l

EC10: 1.91 mg/l
EC50: 2.90 mg/l

Annex 2: The primary data of the reproduction test

CONTROL

Day	0	2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total	sd.
Date	25/8	27/8	30/8	31/8	1/9	2/9	3/9	4/9	5/9	6/9	7/9	8/9	9/9	10/9	11/9	12/9	13/9	14/9	15/9		
A	0	0	0		0	+	13			12		26		0			26		7	**84**	
B	0	0	0		0	0	11			12		18		0			19		21	**81**	
C	0	0	0		0	+	12			11		25		0			27		26	**101**	
D	0	0	0		0	+	16			14		18		0			24		27	**99**	
E	0	0	0		0	0	13			16		0		34			27		23	**113**	
F	0	0	0		0	+	10			16		26		0			17		11	**80**	
G	0	0	0		0	+	18			18		21		0			22		34	**113**	
H	0	0	0		0	+	11			16		19		0			18		28	**92**	
I	0	0	0		0	0	0	+		9		17		21			22		1	**70**	
J	0	0	0		0	+	15			16		18		0			19		32	**100**	
Total	0	0	0		0	0	119			140		188		55			221		210	**933**	
Average per female	**0**	**0**	**0**		**0**	**0**	**11.9**			**14**		**18.8**		**5.5**			**22.1**		**21.0**	**93.3**	**14.4**

0.168 mg/l (nominal)

Day	0	2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total	sd.
Date	25/8	27/8	30/8	31/8	1/9	2/9	3/9	4/9	5/9	6/9	7/9	8/9	9/9	10/9	11/9	12/9	13/9	14/9	15/9		
A	0	0	0		0	0	0	0	0	0	D										
B	0	0	0		0	+	12			15		28		0			21		24	**100**	
C	0	0	0		0	+	10			16		26		0			23		15	**90**	
D	0	0	0		0	+	13			14		26		0			25		33	**111**	
E	0	0	0		0	0	0	0	0	0	0	0	0	0			0		0	**0**	
F	0	0	0		0	+	14			16		25		0			20		29	**104**	
G	0	0	0		0	0	11			11		24		0			20		32	**98**	
H	0	0	0		0	+	11			17		18		0			22		34	**102**	
I	0	0	0		0	0	0	+		12		19		20			11		0	**62**	
J	0	0	0		0	+	15			17		28		0			31		34	**125**	
Total	0	0	0		0		86			118		194		20			173		201	**792**	
Average per female	**0**	**0**	**0**		**0**		**8.6**			**11.8**		**21.6**		**2.22**			**19.2**		**22.3**	**88.0**	**37.1**

0.336 mg/l (nominal)

Day	0	2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total	sd.
Date	25/8	27/8	30/8	31/8	1/9	2/9	3/9	4/9	5/9	6/9	7/9	8/9	9/9	10/9	11/9	12/9	13/9	14/9	15/9		
A	0	0	0		0	+	11			15		28		0			22		38	**114**	
B	0	0	0		0	+	9			11		26		0			19		26	**91**	
C	0	0	0		0	0	10			13		28		0			32		40	**123**	
D	0	0	0		0	+	12			15		18		0			6		27	**78**	
E	0	0	0		0	+	12			11		27		0			29		8	**87**	
F	0	0	0		0	+	15			17		22		0			26		35	**115**	
G	0	0	0		0	+	15			17		11		0			27		33	**103**	
H	0	0	0		0	+	13			15		21		0			25		28	**102**	
I	0	0	0		0	+	9			16		20		26			33		0	**104**	
J	0	0	0		0	+	14			17		11		0			22		32	**96**	
Total	0	0	0		0		120			147		212		26			241		267	**1013**	
Average per female	**0**	**0**	**0**		**0**		**12.0**			**14.7**		**21.2**		**2.6**			**24.1**		**26.7**	**101**	**13.8**

0.672 mg/l (nominal)

Day	0	2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total	sd.
Date	25/8	27/8	30/8	31/8	1/9	2/9	3/9	4/9	5/9	6/9	7/9	8/9	9/9	10/9	11/9	12/9	13/9	14/9	15/9		
A	0	0	0		0	+	16			16		25		1			23		30	**111**	
B	0	0	0		0	+	15			17		9		20			28		20	**109**	
C	0	0	0		0	+	8			14		12		0			22		25	**81**	
D	0	0	0		0	+	8			13		17		0			21		28	**87**	
E	0	0	0		0	0	10			21		34		0			40		27	**132**	
F	0	0	0		0	+	14			19		20		0			23		31	**107**	
G	0	0	0		0	+	6			11		20		0			23		4	**64**	
H	0	0	0		0	+	15			21		19		0			15		28	**98**	
I	0	0	0		0	0	8			10		24		0			28		42	**112**	
J	0	0	0		0	+	7			12		24		0			20		16	**79**	
Total	0	0	0		0		107			154		204		21			243		251	**980**	
Average per female	**0**	**0**	**0**		**0**		**10.7**			**15.4**		**20.4**		**2.1**			**24.3**		**25.1**	**98.0**	**20.1**

1.34 mg/l (nominal)

Day	0	2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total	sd.
Date	25/8	27/8	30/8	31/8	1/9	2/9	3/9	4/9	5/9	6/9	7/9	8/9	9/9	10/9	11/9	12/9	13/9	14/9	15/9		
A	0	0	0		0	+	15			14		25		0			25		31	**110**	
B	0	0	0		0	+	13			16		23		0			32		34	**118**	
C	D																				
D	0	0	0		0	+	16			17		26		0			32		40	**131**	
E	0	0	0		0	0	10			15		26		0			33		35	**119**	
F	0	0	0		0	+	14			14		25		0			28		35	**116**	
G	0	0	0		0	+	9			23		31		37			37		0	**137**	
H	0	0	0		0	0	0	0	+	8		17		0			18		23	**66**	
I	0	0	0		0	+	11			9		21		0			24		30	**95**	
J	0	0	0		0	0	8			13		26		0			28		34	**109**	
Total	0	0	0		0		96			129		220		37			257		262	**1001**	
Average per female	**0**	**0**	**0**		**0**		**10.7**			**14.3**		**24.4**		**4.11**			**28.6**		**29.1**	**111**	**20.9**

2.69 mg/l (nominal)

Day	0	2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total	sd.
Date	25/8	27/8	30/8	31/8	1/9	2/9	3/9	4/9	5/9	6/9	7/9	8/9	9/9	10/9	11/9	12/9	13/9	14/9	15/9		
A	0	0	0		0	+	6			19		31		31			1		37	**125**	
B	0	0	0		0	0	8			14		23		0			30		29	**104**	
C	0	0	0		0	+	13			15		30		0			29		29	**116**	
D	0	0	0		0	0	2			3		0		2			14		28	**49**	
E	0	0	0		0	+	14			15		22		0			26		3	**80**	
F	0	0	0		0	+	6			14		28		0			29		30	**107**	
G	0	0	0		0	0	0	+		16		27		28			28		0	**99**	
H	0	0	0		0	+	11			19		27		0			28		37	**122**	
I	0	0	0		0	0	0	+		12		23		28			29		0	**92**	
J	0	0	0		0	+	12			16		29		28			1		25	**111**	
Total	0	0	0		0		72			143		240		117			215		218	**1005**	
Average per female	**0**	**0**	**0**		**0**		**7.2**			**14.3**		**24.0**		**11.7**			**21.5**		**21.8**	**101**	**22.6**

5.38 mg/l (nominal)

Day	0	2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total	sd.
Date	25/8	27/8	30/8	31/8	1/9	2/9	3/9	4/9	5/9	6/9	7/9	8/9	9/9	10/9	11/9	12/9	13/9	14/9	15/9		
A	0	0	0		0	0	0	0	0	0	+	11		6			14		14	**45**	
B	0	0	0		0	0	0	0	0	5		6		0			11		7	**29**	
C	0	0	0		0	0	0	0	0	6		0		14			14		11	**45**	
D	0	0	0		0	0	0	0	0	5		5		0			6		1	**17**	
E	0	0	0		0	0	0	0	0	4		12		11			15		0	**42**	
F	0	0	0		0	0	0	0	0	5		9		14			15		0	**43**	
G	0	0	0		0	0	0	0	0	4		8		12			9		0	**33**	
H	0	0	0		0	0	0	0	0	3		12		0			13		14	**42**	
I	0	0	0		0	0	0	0	0	5		12		0			13		7	**37**	
J	0	0	0		0	0	0	0	0	4		11		13			13		0	**41**	
Total	0	0	0		0	0	0	0	0	41		86		70			123		54	**374**	
Average per female	**0**	**0**	**0**		**0**	**0**	**0**	**0**	**0**	**4.1**		**8.6**		**7**			**12.3**		**5.4**	**37.4**	**8.9**

10.75 mg/l (nominal)

Day	0	2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total	sd.
Date	25/8	27/8	30/8	31/8	1/9	2/9	3/9	4/9	5/9	6/9	7/9	8/9	9/9	10/9	11/9	12/9	13/9	14/9	15/9		
A	0	0	D																		
B	0	0	D																		
C	0	0	D																		
D	0	0	D																		
E	0	0	D																		
F	0	0	D																		
G	0	0	D																		
H	0	0	D																		
I	0	0	D																		
J	0	0	D																		
Total	0	0																			
Average per female	**0**	**0**																			

\+ Offspring present
D Dead

Annex 3: Sampling

List of collected samples from the Daphnia reproduction study

Each sample is marked with two labels. One with the identification of the study, test substance code (DHI 04-449), nominal concentration, replicate (A or B), test time, file number, Study Director initials, start date and the technician. The other label identifies the sample by the number stated in the table below.

Date		25 Aug.	27 Aug.	30 Aug.	3 Sep.	6 Sep.	10 Sep.	13 Sep.		15 Sep.
T=		0	2	5	9	12	16	19		21
Nominal concentration [mg/l]	Replicate	new	new	old	new	old	new	old	new	old
Control	A	1	2	3	4	5	6	7	8	9
	B	67	68	69	70	71	72	73	74	75
0.168	A	10	11	12	13	14	15	16	17	18
	B	76	77	78	79	80	81	82	83	84
0.336	A	19	20	21	22	23	24	25	26	27
	B	85	86	87	88	89	90	91	92	93
0.672	A	28	29	30	31	32	33	34	35	36
	B	94	95	96	97	98	99	100	101	102
1.34	A	37	38	39	40	41	42	43	44	45
	B	103	104	105	106	107	108	109	110	111
2.69	A	46	47	48	49	50	51	52	53	54
	B	112	113	114	115	116	117	118	119	120
5.38	A	55	56	57	58	59	60	61	62	63
	B	121	122	123	124	125	126	127	128	129
10.75	A	64	65	66	-	-	-	-	-	-
	B	130	131	132	-	-	-	-	-	-

- : no samples taken

Annex 4: Nominal and corresponding measured concentrations

TIME (days)	0	2	5	9	12	16	18.7	19	21	Mean	sd.
	new	new	old	new	old	new	old	new	old		
Algae [ml]	0	0	0.16	0	1.05	0	1.4	0	3.3		
Dilution factor	0.500	0.500	0.499	0.500	0.496	0.500	0.494	0.500	0.486		
0 mg/l	0	0	0	0	0	0	0	0	0		
0.168 mg/l - Nominal concentration											
Measured conc. [mg/l]	0.051	0.046	0.049	0.048	0.046	0.071	0.060	0.065	0.050		
Actual measured conc. [mg/l]	**0.102**	**0.092**	**0.098**	**0.096**	**0.093**	**0.142**	**0.121**	**0.130**	**0.103**	**0.109**	**0.019**
Recovery	60.7	54.8	58.4	57.1	55.3	84.5	72.3	77.4	61.3		
% of start	100	90	96	94	91	139	119	127	101		
0.336 mg/l - Nominal concentration											
Measured conc. [mg/l]	0.099	0.087	0.108	0.095	0.094	0.141	0.107	0.113	0.092		
Actual measured conc. [mg/l]	**0.198**	**0.174**	**0.216**	**0.190**	**0.190**	**0.282**	**0.217**	**0.226**	**0.189**	**0.209**	**0.034**
Recovery	58.9	51.8	64.4	56.5	56.5	83.9	64.5	67.3	56.4		
% of start	100	88	109	96	96	142	109	114	96		
0.672 mg/l - Nominal concentration											
Measured conc. [mg/l]	0.202	0.191	0.208	0.212	0.19	0.279	0.218	0.224	0.186		
Actual measured conc. [mg/l]	**0.404**	**0.382**	**0.417**	**0.424**	**0.383**	**0.558**	**0.441**	**0.448**	**0.383**	**0.427**	**0.058**
Recovery	60.1	56.8	62.0	63.1	57.1	83.0	65.7	66.7	57.0		
% of start	100	95	103	105	95	138	109	111	95		
1.34 mg/l - Nominal concentration											
Measured conc. [mg/l]	0.35	0.31	0.34	0.43	0.36	0.49	0.41	0.4	0.36		
Actual measured conc. [mg/l]	**0.700**	**0.620**	**0.681**	**0.860**	**0.727**	**0.980**	**0.830**	**0.800**	**0.741**	**0.771**	**0.113**
Recovery	52.2	46.3	50.8	64.2	54.2	73.1	61.9	59.7	55.3		
% of start	100	89	97	123	104	140	119	114	106		
2.69 mg/l - Nominal concentration											
Measured conc. [mg/l]	0.71	0.61	0.71	0.89	0.74	0.99	0.81	0.84	0.73		
Actual measured conc. [mg/l]	**1.420**	**1.220**	**1.422**	**1.780**	**1.493**	**1.980**	**1.640**	**1.680**	**1.502**	**1.571**	**0.233**
Recovery	52.8	45.4	52.9	66.2	55.5	73.6	61.0	62.5	55.9		
% of start	100	86	100	125	105	139	115	118	106		
5.38 mg/l - Nominal concentration											
Measured conc. [mg/l]	1.51	1.22	1.5	1.81	1.52	1.95	1.69	1.56	1.39		
Actual measured conc. [mg/l]	**3.020**	**2.440**	**3.004**	**3.620**	**3.068**	**3.900**	**3.421**	**3.120**	**2.861**	**3.161**	**0.458**
Recovery	56.1	45.4	55.8	67.3	57.0	72.5	63.6	58.0	53.2		
% of start	100	81	99	120	102	129	113	103	95		
10.75 mg/l - Nominal concentration											
Measured conc. [mg/l]	2.72	2.43	2.8								
Actual measured conc. [mg/l]	**5.440**	**4.860**	**5.608**							**5.303**	**0.529**
Recovery	50.6	45.2	52.2								
% of start	100	89	103								

Annex 5: Measurements of pH and oxygen

Date/time		25/8		27/8		30/8		1/9		3/9		6/9		8/9		10/9		13/9		15/9	
T=		0		2		5		7		9		12		14		16		19		21	
Nominal concentration [mg/l]	Test solution	pH	O_2 [%]	pH	O_2 [%]	pH	O_2 [%]	pH	O_2 [%]	pH	O_2 [%]	pH	O_2 [%]	pH	O_2 [%]	pH	O_2 [%]	pH	O_2 [%]	pH	O_2 [%]
Control	New	8.2	98	8.1	98	8.3	98	8.0	98	8.0	98	8.0	>100	8.0	>100	8.0	97	7.8	99		
	Old			8.0	98	8.3	>100	8.0	>100	7.9	>100	8.1	>100	7.7	82	8.0	98	7.6	73	8.0	>100
0.168	New	8.2	97	8.1	97	8.2	98	8.1	98	7.9	98	8.0	>100	8.1	>100	8.0	96	7.8	97		
	Old			8.2	100	8.5	>100	8.1	>100	7.8	89	8.3	>100	7.7	83	8.0	89	7.6	51	7.7	84
0.336	New	8.3	96	8.1	98	8.2	98	8.1	98	7.9	99	8.1	>100	8.1	98	8.0	93	7.8	99		
	Old			8.2	100	8.5	>100	8.1	>100	7.8	82	8.1	>100	7.7	81	8.1	88	7.6	59	7.6	73
0.672	New	8.3	96	8.2	98	8.2	98	8.1	98	7.9	99	8.1	>100	8.1	>100	8.0	94	7.8	99		
	Old			8.3	100	8.6	>100	8.2	99	7.8	87	8.2	>100	7.7	83	8.2	92	7.5	44	7.5	70
1.34	New	8.3	96	8.2	98	8.2	98	8.1	98	7.9	98	8.1	>100	8.1	>100	8.0	94	7.9	100		
	Old			8.4	100	8.6	>100	8.2	>100	7.9	86	8.3	>100	7.7	82	8.1	90	7.5	38	7.5	75
2.69	New	8.3	96	8.2	99	8.2	98	8.2	98	7.9	98	8.1	>100	8.1	98	8.0	94	7.8	100		
	Old			8.4	100	8.7	>100	8.3	>100	7.9	90	8.5	>100	7.7	87	8.1	94	7.5	55	7.8	97
5.38	New	8.3	96	8.2	98	8.2	98	8.2	98	7.9	98	8.1	>100	8.1	98	8.0	98	7.8	100		
	Old			8.4	100	8.8	>100	8.3	>100	7.9	98	8.5	>100	7.9	85	8.1	92	7.8	95	8.3	>100
10.75	New	8.3	96	8.2	99																
	Old			8.4	100	8.8	>100														

Annex 6: Chemical analysis

TNO Chemistry

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toegepast-natuurwetenschappelijk
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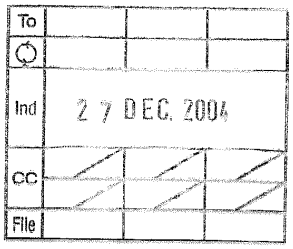

Date
December 20, 2004

Subject
Final report

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Enclosure(s)
report V5819/03, annex 6 DHI study 91337/449

Dear Estelle,

I herewith send you the hard copy of final report V5819/03, annex 6 to DHI study 91337/449 "Daphnia magna reproduction test with p-xylene, analytical confirmation of exposure concentrations. (ANNEX 6)"
I already sent you a pdf (20/12/04).
I hope we will collaborate again very soon!.

Kind regards,

Ton Schouten

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TNO Report

V 5819/03, Annex 6 to DHI study 91337/449 |

Daphnia magna reproduction test with p-xylene. Analytical confirmation of exposure concentrations. (ANNEX 6)

Date	10th December, 2004
Authors	A. Schouten
Sponsor	CEFIC Aromatic Producers Association Study outsourced to DHI Water and Environment Chemical analysis by TNO
TNO project number	30512/01.05
TNO study code	5819/035819/03
Sponsor's study code	91337/449
Status	Final
Previous versions	
Number of pages	17

Statement of GLP compliance

I, the undersigned, hereby declare that this report constitutes a complete, true and accurate representation of the study and its results. All study activities performed by TNO Nutrition and Food Research were carried out in compliance with the current OECD Principles of Good Laboratory Practice (Organisation for Economic Co-operation and Development, Paris, ENV/MC/CHEM (98) 17).

A. Schouten.
Principal investigator
Analytical Sciences Department

Date: 10 December 2004

Approved by:

M.W.H. den Reijer (MSc)
Management of Analytical Sciences Department

Date: 10 December 2004

Quality Assurance Statement

Report title : *Daphnia magna reproduction test with p-xylene. Analytical confirmation of exposure concentrations (ANNEX 6)*

Report number : V 5819/03, Annex 6 to DHI study 91337/449

Report date : 10 December 2004

The study plan was audited as follows:

Date of audit	Date of report
13 July 2004 (including amendment 1)	13 July 2004
14 September 2004 (amendment 2)	14 September 2004

The experimental phase of this part of study was audited as follows:

Date of audit	Phase	Date of report
22 September 2004	preparation of test substance solutions instrumental (test)analysis	22 September 2004
22 September 2004	dilution of test substance solutions sample pre-treatment	22 September 2004

This analytical report was audited as follows:

Start date of audit	Date of report
8 October 2004 (draft)	8 October 2004
15 December 2004 (final)	15 December 2004

I, the undersigned, hereby declare that this analytical report provides an accurate record of the procedures employed and the results obtained in this part of the study; all audits were reported to the principal investigator and test site management on the dates indicated. Summaries of the audit results were reported to the study director, test facility management and lead QA.

G.S. Oostenbrug, PhD
Quality Assurance Auditor
TNO Nutrition and Food Research

Date: 15 December 2004

List of abbreviations

GLP	Good Laboratory Practice
HPLC-UV	High Performance Liquid Chromatography / Ultra violet detection
OECD	Organisation for Economic Co-operation and Development
RSD	Relative Standard Deviation

Contents

1 Introduction

This analytical annex describes the chemical analysis of the samples from the *Daphnia magna* reproduction test with p-xylene (CAS # 106-42-3).
The objective of this part of the study was to validate the analytical method for the determination of p-xylene in the test mixture (water/ acetonitrile 1:1 *v/v)*, followed by determination of the concentration of p-xylene in the samples taken during the study (TNO study 5819/03, DHI study 91337/449).

2 Responsible personnel and facilities

2.1 Sponsor

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Principal investigator analytical chemistry:
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3 Experimental

3.1 Reference substance

On 19 May 2004 TNO Food and Nutrition Research received a sample (1 l in a brown glass bottle) of p-xylene, a colourless liquid, (TNO formulation container 1 (25220, TNO test substance number 040085-001), CAS # 106-42-3. The reference material was obtained from Sigma Aldrich, lot 429739/1, purity 99.4% (according certificate of analysis). The reference material was stored at ambient temperature and protected from light. Upon receipt the expiry date was set at May 2005.

3.2 Study samples

According to the study director, sub-samples of 10 ml were collected in a 25–ml screw cap vial and 10 ml of acetonitrile was added to each vial before closing the lids. After termination of the toxicity study the refrigerated samples were transported to the TNO Analytical Science Department. After receipt on 21 September 2004 the samples were stored at 2- 10°C until analysis on 22 and 23 September 2004.

3.3 Analysis

3.3.1 Principle

The concentration of p-xylene in test medium was determined using High Performance Liquid Chromatography (HPLC) with UV detection. Quantification of p-xylene was achieved by comparing the peak areas in the chromatograms of the study samples with those in the chromatograms of calibration solutions.

3.3.2 Validation criteria

The method was validated, by analyzing an appropriate standard solution three times. The method should meet the following criteria:

- Linearity: the correlation coefficient of the calibration curve (≥ 5 points) should be greater than or equal to 0.996.
- Repeatability of the retention time: the relative standard deviation (RSD) in the retention time of the test substance when an appropriate standard solution is injected three times should be less than 2 %.
- Repeatability of the concentration: the relative standard deviation should be smaller than 10 % when a test calibration solution is analyzed three times.
- Selectivity: no peak should be found in a blank test medium with a retention time of 95% - 105% of that of the test substance. In case the blank sample shows a peak with a retention time in the above range and the area of this peak is >10% of the corresponding peak in the chromatogram of low-dose sample, the results for the formulation samples containing test substance will be corrected for the level found in the blank.

3.3.3 Sample preparation

Samples were acclimatized (ambient temperature) and manually shaken (10 sec.). An aliquot of the sample was transferred into an HPLC autosampler vial.

3.3.4 Preparation of the validation samples

Three validation samples containing 0.8080 mg p-xylene per litre HPLC mobile phase (3.3.5) were prepared by the Analytical Sciences Department on 22 and 23 September 2004. A 0.50 ml aliquot of a stock solution of 202.0 mg p-xylene / 100 ml HPLC mobile phase that was prepared on 22 September 2004 was diluted, with 4.50 ml HPLC mobile phase solution. This solution was diluted 250 times (20 µl p-xylene solution + 5 ml HPLC mobile phase). Validation samples, including a blank sample (sample 5819/03-A1), were analyzed as described in section and 3.3.5.

3.3.5 Chromatography

The validation samples, study samples and calibration solutions, prepared as described in section 3.3.6, were analysed using HPLC.
The following chromatographic conditions were used:

Column	: Phenomenex Luna 5µ C18 (2), 150/4.6 mm
Mobile phase	: acetonitrile/water = 62/38 (*v/v*), degassed by sonication during 15 minutes
Injection volume	: 20 µl
Flow	: 1 ml/min
Column temperature	: 30 °C
Detection	: 220 nm
Integration	: PC 1000

3.3.6 Calibration

Calibration solutions were prepared by alternately diluting 25, 50, 100, 250, 500, 1000 and 2000 times two freshly prepared stock solutions (approximately 200 mg p-xylene per litre of mobile phase) to obtain concentrations between 0.10 and 8 mg p-xylene / l HPLC mobile phase.[1]
The calibration solutions were analyzed as described in section 3.3.5. A calibration graph was constructed by plotting the peak area of the chromatograms of the calibration solutions against the concentration. The concentration of p-xylene in the samples was calculated using the calibration graph.

3.4 Deviations of the protocol

Protocol P5819/03 (DHI study code 91337/449) section 12 and Amendment 1:
It was not possible to detect a concentration of 10 % of the lowest dose sample. The theoretical p-xylene concentration in the analysis sample was 0.084 mg /l (as all samples were 1:1 diluted with acetonitrile, see 3.2). The calculated LOD (3x noise) was 0.011 mg p-xylene/ l, which was about 13 % of that concentration.
This deviation did not affect the outcome of the analysis.

[1]On 23 September 2004 two additional calibration solutions with a concentration of 0.03974 and 0.02020 mg/l were prepared and analysed.

4 Results

4.1 Validation of the analytical method

4.1.1 Linearity

The calibration coefficient was >0.996 and therefore the calibration graph was considered to be rectilinear. A typical calibration graph is presented in Figure 1 (section 8.2 of this annex).

4.1.2 Selectivity

No peak was found in a blank test medium extract with a retention time of 95 % - 105 % of that of the test substance. However, it was not possible to detect a concentration of 10 % of the lowest dose sample. The theoretical p-xylene concentration in the analysis sample from the lowest dose group was 0.084 mg /l (as all samples were 1:1 diluted with acetonitrile, see 3.2). The calculated LOD (3x noise) was 0.011 mg p-xylene/ l, which was about 13 % of this concentration (nominal concentration of the undiluted test medium 0.168 mg p-xylene/ l).

4.1.3 Repeatability

The peak areas and the retention times and their respective RSD (n=3) in the repeatability experiment with the validation samples prepared on 22 and 23 September 2004 respectively and analysed on that days are shown in Table 1 and 2. The RSD in the peak area (as a measure for the concentration) of p-xylene in the three validation samples was 0.4 and 0.2 %, respectively. The RSD in the retention time was 0.01% on both days. These values met the validation criteria.

Table 1 Repeatability of the analysis of p-xylene as determined from the validation samples prepared and analysed on 22 September 2004.

Concentration prepared ($mg.l^{-1}$)	Peak area (counts)	Retention time p-xylene peak (min)
0.8080	48204	8.010
	48264	8.011
	47925	8.011
Mean (n=3)	48131	8.01
RSD (n=3)	0.4 %	0.01%

Table 2 Repeatability of the analysis of p-xylene as determined from the validation samples prepared and analysed on 23 September 2004

Concentration prepared ($mg.l^{-1}$)	Peak area (counts)	Retention time p-xylene peak (min)
0.8080	47759	8.012
	47901	8.013
	47683	8.013
Mean (n=3)	47781	8.01
RSD (n=3)	0.2 %	0.01%

4.2 Results

The results of the analysis of the study samples are presented in Table 3. First analysis occurred on 22 September 2004. However, unexpectedly the peak areas of the samples of the lowest dose group were smaller than the peak area of the lowest calibration point. Therefore the results for sample A10 to A18 were obtained by extrapolation. The results of the reanalysis on 23 September 2004 (linearity of the calibration checked with two additional calibration points) show that extrapolation was acceptable.

Table 3 Concentration of p-xylene in the study samples

Sample code 5819/03	Intended dose level ($mg.l^{-1}$)	Date	Days	Old/new	Measured concentration ($mg.l^{-1}$)[2, 3]	Reanalysis ($mg.l^{-1}$)[2, 3]
A 1	0	25/08/04	0	New	< LOD	
A 2	0	27/08/04	2	New	< LOD	
A 3	0	30/08/04	5	Old	< LOD	
A 4	0	03/09/04	9	New	< LOD	
A 5	0	06/09/04	12	Old	< LOD	
A 6	0	10/09/04	16	New	< LOD	
A 7	0	13/09/04	19	Old	< LOD	< LOD
A 8	0	13/09/04	19	New	< LOD	
A 9	0	15/09/04	21	Old	< LOD	
A 10	0.168	25/08/04	0	New	0.051	0.043
A 11	0.168	27/08/04	2	New	0.046	0.044
A 12	0.168	30/08/04	5	Old	0.049	0.052
A 13	0.168	03/09/04	9	New	0.048	0.043
A 14	0.168	06/09/04	12	Old	0.046	0.048
A 15	0.168	10/09/04	16	New	0.071	0.066
A 16	0.168	13/09/04	19	Old	0.060	0.052
A 17	0.168	13/09/04	19	New	0.065	0.053
A 18	0.168	15/09/04	21	Old	0.050	0.042
A 19	0.336	25/08/04	0	New	0.099	
A 20	0.336	27/08/04	2	New	0.087	
A 21	0.336	30/08/04	5	Old	0.108	
A 22	0.336	03/09/04	9	New	0.095	
A 23	0.336	06/09/04	12	Old	0.094	
A 24	0.336	10/09/04	16	New	0.141	
A 25	0.336	13/09/04	19	Old	0.107	
A 26	0.336	13/09/04	19	New	0.113	
A 27	0.336	15/09/04	21	Old	0.092	
A 28	0.672	25/08/04	0	New	0.202	
A 29	0.672	27/08/04	2	New	0.191	
A 30	0.672	30/08/04	5	Old	0.208	
A 31	0.672	03/09/04	9	New	0.212	

[2] Measured concentrations were *not* corrected for the 1:1 dilution with acetonitril.
[3] First analysis occurred on 22 September 2004, reanalysis on 23 September 2004

Sample code 5819/03	Intended dose level ($mg.l^{-1}$)	Date	Days	Old/new	Measured concentration ($mg.l^{-1}$)[2, 3]	Reanalysis ($mg.l^{-1}$)[2, 3]
A 32	0.672	06/09/04	12	Old	0.190	
A 33	0.672	10/09/04	16	New	0.279	
A 34	0.672	13/09/04	19	Old	0.218	
A 35	0.672	13/09/04	19	New	0.224	
A 36	0.672	15/09/04	21	Old	0.186	
A 37	1.34	25/08/04	0	New	0.35	
A 38	1.34	27/08/04	2	New	0.31	
A 39	1.34	30/08/04	5	Old	0.34	
A 40	1.34	03/09/04	9	New	0.43	
A 41	1.34	06/09/04	12	Old	0.36	
A 42	1.34	10/09/04	16	New	0.49	
A 43	1.34	13/09/04	19	Old	0.41	
A 44	1.34	13/09/04	19	New	0.40	
A 45	1.34	15/09/04	21	Old	0.36	
A 46	2.69	25/08/04	0	New	0.71	
A 47	2.69	27/08/04	2	New	0.61	
A 48	2.69	30/08/04	5	Old	0.71	
A 49	2.69	03/09/04	9	New	0.89	
A 50	2.69	06/09/04	12	Old	0.74	
A 51	2.69	10/09/04	16	New	0.99	
A 52	2.69	13/09/04	19	Old	0.81	
A 53	2.69	13/09/04	19	New	0.84	
A 54	2.69	15/09/04	21	Old	0.73	
A 55	5.38	25/08/04	0	New	1.51	
A 56	5.38	27/08/04	2	New	1.22	
A 57	5.38	30/08/04	5	Old	1.50	
A 58	5.38	03/09/04	9	New	1.81	
A 59	5.38	06/09/04	12	Old	1.52	
A 60	5.38	10/09/04	16	New	1.95	
A 61	5.38	13/09/04	19	Old	1.69	
A 62	5.38	13/09/04	19	New	1.56	
A 63	5.38	15/09/04	21	Old	1.39	
A 64	10.75	25/08/04	0	New	2.72	
A 65	10.75	27/08/04	2	New	2.43	
A 66	10.75	30/08/04	5	Old	2.80	

5 Conclusion

The results as presented in this Analytical report may unconditionally be used in study 5819/03 (DHI study 91337/449).

6 Documentation and retention of records

6.1 Documentation

The documentation of this study consists of the study protocol, correspondence, report and raw data or true copies of these.

6.2 Retention of records

The following documents relating to the analytical part of the study will be retained for 10 years after completion of the final report in the archives of TNO Nutrition and Food Research:

1. Copies of approved study protocol, and its amendments and the final sub report.
2. Raw data or true copies of these.
3. Correspondence.
4. All other information related to this part of the study.

On request, after this period of 10 years the documents will be transferred to the sponsor.

7 References

1. Organisation for Economic Co-operation and Development, Paris. OECD Principles of Good Laboratory Practice (as revised in 1997). ENV/MC/CHEM (98) 17

8 Addenda

8.1 Annexes

Copy of the endorsement of GLP compliance

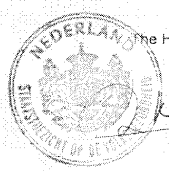
ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC the conformity with the OECD Principles of GLP was assessed on 7-11 June 2004 at

TNO Nutrition and Food Research
Utrechtseweg 48, P.O. Box 360
3700 AJ ZEIST

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Toxicity, mutagenicity, biodegradation, residues, analytical and clinical chemistry, kinetics and metabolism, and occupational toxicity.



The Hague, 19 August 2004

Dr Th. Helder
GLP Compliance Monitoring Department

Inspectorate for Health Protection and Veterinary Public Health
Food and Consumer Product Safety Authority

8.2 Typical calibration graph

Figure 1. Typical calibration graph of the test substance (concentrations between 0.10 and 8 mg p-xylene / l HPLC mobile phase)

y = 58138 x + 436.42 , correlation r = 1.0000