REPORT No. 2500268
Regulatory Document

DSM

Document Date:	09-May-2006
Authors:	Thiel A (NRD-CH), Köhl W (SCC GmbH), Kunz S (RCC-CCR) and Schulz M (RCC-CCR)
Title:	In Vitro Chromosome Aberration Test in Chinese Hamster V79 Cells with Ultrazine FG-R (Food Grade Lignosulphonate) (Study conducted at RCC-CCR; D-64380 Rossdorf, Germany. RCC-CCR study number 899102)
Project No.	6309
Compound No.	Ultrazine FG-R (Food Grade Lignosulphonate), Calcium Lignosulphonate, LS FG DP-955 FGR004

Summary

Ultrazine FG-R was assessed for its potential to induce structural chromosome aberrations in V79 cells *in vitro* in two independent experiments. Each experiment was performed in duplicate cultures thereby analysing a total of 200 metaphases per concentration for chromosomal aberrations.

In experiment I, cells were exposed to the test substance either in the presence or absence of metabolic activation (S9-mix) for 4 hours and analysed for chromosomal aberrations 18 hours post-exposure. In experiment II, cells were exposed for 18 or 28 hours without metabolic activation and analysed for chromosomal damage. In the presence of metabolic activation, cells were exposed for 4 hours followed by a further 24 hour period prior to analysis.

(continued next page)

This report consists of Pages I – III and 1-64

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Approved

Name	**Signature**	**Date**
Main Author A. Thiel		18.05.2006
Principal Scientist / Competence Mgr J. Bausch		
Research Center Head L. Pasamontes		
Project Manager B. Mussler		18.05.2006

The following concentrations were evaluated:

Experiment I, 4 h exposure + 14 h post-exposure, -S9:	625; 1,250; 2,500 µg/mL
Experiment I, 4 h exposure + 14 h post-exposure, +S9:	2,500; 3,750; 5,000 µg/mL
Experiment II,18 h exposure, -S9:	156.3; 312.5; 625 µg/mL
Experiment II, 28 hour exposure, -S9:	1,500 µg/mL
Experiment II, 4 hour exposure + 24 hour post-exposure, +S9:	2,500; 3,750; 5,000 µg/mL

Higher concentrations than the above indicated could not be analysed for chromosomal aberrations due to precipitation of the test article and/or low metaphase numbers.

Neither with nor without metabolic activation, clastogenicity was observed. Observed structural and numerical chromosomal aberrations were within the known range of the historical control data base. The sensitivity of the test system was shown by appropriate positive controls.

Conclusion:
Under the reported test conditions, Ultrazine FG-R did not induce structural nor numerical chromosomal aberrations in V79 cells in the presence and absence of metabolic activation. Thus, Ultrazine FG-R is not considered to be clastogenic.

Nomenclature and Structural Formula (if available)

Test Article Name:	Ultrazine FG-R (Food Grade Lignosulphonate)
Chemical Name:	Calcium Lignosulphonate
Batch No.:	FGR-004

FINAL REPORT

Study Title:

IN VITRO CHROMOSOME ABERRATION TEST IN CHINESE HAMSTER V79 CELLS WITH ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

Data Requirements / Test Guidelines:

based on.

Ninth Addendum to the OECD Guidelines for Testing of Chemicals, February 1998, adopted July 21, 1997, Guideline No. 473 "*In vitro* Mammalian Chromosome Aberration Test".

EPA Health Effects Test Guidelines, OPPTS 870.5375, EPA 712-C-98-223, August 1998
"*In Vitro* Mammalian Chromosome Aberration Test".

Commission Directive 2000/32/EC, L1362000, Annex 4A: "Mutagenicity – *In vitro* Mammalian Chromosome Aberration Test", dated May 19, 2000.

Study Director:

Dr. Markus Schulz (until February 28, 2006)

Dr. Susanne Kunz (from March 01, 2006)

Study Completion Date:

May 09, 2006

Test Facility:

RCC - Cytotest Cell Research GmbH (RCC-CCR)
In den Leppsteinswiesen 19,
D-64380 Rossdorf
Germany

Sponsor:

DSM Nutritional Products AG
Wurmisweg 576
CH-4303 Kaiseraugst
Switzerland

RCC-CCR Study No.: 899102

RCC

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1 STATEMENT OF COMPLIANCE

Study Number:	899102
Test Item:	Ultrazine FG-R (Food Grade Lignosulphonate)
Study Director:	Dr. Susanne Kunz
Title:	*In vitro* Chromosome Aberration Test in Chinese Hamster V79 Cells with Ultrazine FG-R (Food Grade Lignosulphonate)

This study performed in the test facility of RCC Cytotest Cell Research was conducted in compliance with Good Laboratory Practice Regulations:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), dated July 25, 1994 ("BGBl. I 1994", pp. 1703), last revision dated June 27, 2002.

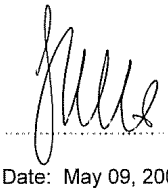
"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final].

Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

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

Study Director

R C C - C C R
Dr. Susanne Kunz



Date: May 09, 2006

2 STATEMENT OF QUALITY ASSURANCE UNIT

Study Number: 899102

Test Item: Ultrazine FG-R (Food Grade Lignosulphonate)

Study Director: Dr. Susanne Kunz

Title: *In vitro* Chromosome Aberration Test
in Chinese Hamster V79 Cells
with Ultrazine FG-R (Food Grade Lignosulphonate)

The general facilities and activities of RCC Cytotest Cell Research GmbH are inspected periodically and the results are reported to the responsible person and the Management.

Study procedures were inspected periodically. The study plan and this report were audited by the Quality Assurance Unit. The dates are given below.

Phases and Dates of QAU Inspections/ Audits		Dates of Reports to the Study Director and to Management
Study Plan (Draft):	September 27, 2005	September 27, 2005
Study Plan:	September 28, 2005	-
1st Amendment to Study Plan (Draft):	April 03, 2006	April 03, 2006
1st Amendment to Study Plan:	April 11, 2006	-
Process Inspection:	October 26, 2005 December 07, 2005 January 12, 2006	October 26, 2005 December 07, 2005 January 12, 2006
Draft Report:	April 03, 2006	April 03, 2006

This statement is to confirm that the present final report reflects the raw data.

Head of Quality Assurance Unit Frauke Hermann

F. Hermann

Date: May 09, 2006

3 PROJECT STAFF SIGNATURES

Study Director Dr. Susanne Kunz

Date: May 09, 2006

Management Dr. Wolfgang Völkner

Date: May 09, 2006

4 CONTENTS

5 PREFACE

5.1 General

Title:	*In vitro* Chromosome Aberration Test in Chinese Hamster V79 Cells with Ultrazine FG-R (Food Grade Lignosulphonate)
Sponsor:	DSM Nutritional Products AG Wurmisweg 576 CH-4303 Kaiseraugst Switzerland
Scientific Monitor:	SCC Scientific Consulting Company Chemisch-Wissenschaftliche Beratung GmbH Dr. Werner Köhl Mikroforum Ring 1 D-55234 Wendelsheim Germany
SCC Project No.:	612-002
Test Facility:	RCC Cytotest Cell Research GmbH (RCC-CCR) In den Leppsteinswiesen 19 D-64380 Rossdorf

5.2 Responsibilities

Study Director:	Dr. Markus Schulz (until February 28, 2006) Dr. Susanne Kunz (from March 01, 2006)
Management:	Dr. Wolfgang Völkner
Head of Quality Assurance Unit:	Frauke Hermann

5.3 Schedule

Date of the Study Plan:	September 28, 2005
Date of the 1st Amendment to Study Plan:	April 07, 2006
Experimental Starting Date:	October 12, 2005
Experimental Completion Date:	January 27, 2006
Date of the Draft Report:	March 13, 2006
Study Completion Date:	May 09, 2006

5.4 Good Laboratory Practice

The study was performed in compliance with:

”Chemikaliengesetz“ (Chemicals Act) of the Federal Republic of Germany, ”Anhang 1“ (Annex 1), dated July 25, 1994 (”BGBl. I 1994“, pp. 1703), last revision dated June 27, 2002.

”OECD Principles of Good Laboratory Practice“, as revised in 1997 [C(97)186/Final].

Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

5.5 Guidelines

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

Ninth Addendum to the OECD Guidelines for Testing of Chemicals, February 1998, adopted July 21, 1997, Guideline No. 473 "*In vitro* Mammalian Chromosome Aberration Test".

EPA Health Effects Test Guidelines, OPPTS 870.5375, EPA 712-C-98-223, August 1998 "*In Vitro* Mammalian Chromosome Aberration Test".

Commission Directive 2000/32/EC, L1362000, Annex 4A: "Mutagenicity – *In vitro* Mammalian Chromosome Aberration Test", dated May 19, 2000.

5.6 Archiving

RCC Cytotest Cell Research GmbH will archive the following data for 15 years:

Raw data, study plan, final report, and a sample of the test item.

Microscopic slides will be archived for at least 12 years.

No data will be discarded without the sponsor´s consent.

5.7 Deviations from the Study Plan

There were no deviations from the study plan.

6 SUMMARY OF RESULTS

The test item Ultrazine FG-R (Food Grade Lignosulphonate), dissolved in deionised water, was assessed for its potential to induce structural chromosome aberrations in V79 cells of the Chinese hamster *in vitro* in two independent experiments. The following study design was performed:

	Without S9 mix			With S9 mix	
	Exp. I	Exp. II		Exp. I	Exp. II
Exposure period	4 hrs	18 hrs	28 hrs	4 hrs	4 hrs
Recovery	14 hrs	-	-	14 hrs	24 hrs
Preparation interval	18 hrs	18 hrs	28 hrs	18 hrs	28 hrs

In each experimental group two parallel cultures were set up. A total of 100 metaphase plates per culture were scored for structural chromosome aberrations.

The highest concentration for the toxicity pre-test (5000 µg/mL) was chosen based on test item solubility in appropriate solvent and according to the limit dose set by respective guidelines.

Dose selection for the cytogenetic experiments was based on obtained toxicity data. The chosen treatment concentrations are described in Table 2 (page 19). The evaluated treatments and the results are summarised in Table 1 (page 12).

In Experiment I, without metabolic activation, precipitation was observed at 5000 µg/mL, following a 4 hrs exposure period and 18 hrs preparation time. At 3750 µg/mL, microscopically visible precipitation and/or low metaphase numbers were observed and cells were not evaluated. No cytotoxicity was observed up to the highest concentration evaluated (2500 µg/mL).

In Experiment I, with metabolic activation, neither toxic effects nor test item precipitation were noted at any test concentration, up to the limit dose of 5000 µg/mL.

In Experiment II, following 18 hrs exposure without S9 mix, no cytotoxicity was noted up to the highest evaluated concentration (625 µg/mL). At concentrations exceeding 625 µg/mL microscopically visible precipitation and/or low metaphase numbers were observed and cells were not evaluated. Toxic effects, indicated by reduced cell numbers below 50 % of control, were observed after 28 hrs continuous treatment in the absence of S9 mix at the highest applied concentration (1500 µg/mL). In the presence of S9 mix, no cytotoxicity was observed up to the required limit concentration.

Neither with nor without metabolic activation clastogenicity was observed at the concentrations evaluated. In Experiment I, in the absence of S9 mix, a dose-related increase in cells carrying chromosomal aberrations was observed (1.5 %, 2.0 % and 3.0 % aberrant cells, exclusive gaps, see Table 7), statistically significant from concurrent solvent control at the two highest concentrations (1250 µg/mL and 2500 µg/mL). However, these values were clearly within our historical control data range (0.0 - 4.0 % aberrant cells, exclusive gaps, see Table 14, page 37) and thus considered as being biologically irrelevant.

No relevant increase in the frequencies of polyploid metaphases was found after treatment with the test item as compared to the frequencies of the controls.

Appropriate mutagens were used as positive controls. They induced statistically significant increases ($p < 0.05$) in cells with structural chromosome aberrations.

6.1 Conclusion

Under the experimental conditions reported, the test item did not induce structural chromosome aberrations as determined by the chromosome aberration test in V79 cells (Chinese hamster cell line) *in vitro*.

Therefore, Ultrazine FG-R (Food Grade Lignosulphonate) is considered to be non-clastogenic in this chromosome aberration test in the absence and presence of metabolic activation.

Table 1: Summary of results of the chromosome aberration study with Ultrazine FG-R (Food Grade Lignosulphonate)

Exp.	Preparation interval	Test item concentration in µg/mL	Polyploid cells in %	Cell numbers in % of control	Mitotic indices in % of control	Aberrant cells in % incl. gaps*	excl. gaps*	with exchanges
Exposure period 4 hrs without S9 mix								
I	18 hrs	Solvent control[1]	2.4	100.0	100.0	0.5	0.0	0.0
		Positive control[2]	2.8	n.t.	98.4	13.5	**13.5[s]**	4.5
		625.0	2.1	70.9	96.3	1.5	1.5	0.0
		1250.0	2.4	106.4	97.2	2.0	**2.0[s]**	0.0
		2500.0	1.9	89.8	104.3	3.0	**3.0[s]**	0.0
Exposure period 18 hrs without S9 mix								
II	18 hrs	Solvent control[1]	3.6	100.0	100.0	3.0	2.5	0.0
		Positive control[3]	2.6	n.t.	79.4	28.5	**27.0[s]**	6.0
		156.3	3.1	96.8	101.5	4.5	3.0	0.0
		312.5	2.9	143.2	103.1	2.5	1.5	0.0
		625.0	2.5	100.8	108.5	2.0	1.5	0.5
Exposure period 28 hrs without S9 mix								
II	28 hrs	Solvent control[1]	1.4	100.0	100.0	2.5	2.0	0.0
		Positive control[3]	2.0	n.t.	91.2	18.5	**18.0[s]**	4.5
		1500.0	1.6	43.6	69.0	3.0	3.0	0.0
Exposure period 4 hrs with S9 mix								
I	18 hrs	Solvent control[1]	3.2	100.0	100.0	0.5	0.5	0.0
		Positive control[4]	1.0	n.t.	58.0	9.0	**9.0[s]**	2.0
		2500.0	1.7	76.8	63.5	2.5	1.5	0.0
		3750.0	1.7	67.3	75.9	2.0	1.0	0.0
		5000.0	1.8	126.3	72.3	0.5	0.0	0.0
II	28 hrs	Solvent control[1]	1.5	100.0	100.0	3.5	0.5	0.0
		Positive control[5]	1.2	n.t.	104.9	14.0	**12.5[s]**	2.5
		2500.0	1.5	53.0	110.8	1.0	1.0	0.0
		3750.0	0.7	74.6	109.7	3.5	2.5	0.5
		5000.0	1.1	70.2	110.4	2.0	2.0	0.0

* Inclusive cells carrying exchanges
n.t. Not tested
s Aberration frequency statistically significant higher than corresponding control values
[1] Deionised water 10 % (v/v)
[2] EMS 400.0 µg/mL
[3] EMS 300.0 µg/mL
[5] CPA 2.0 µg/mL
[4] CPA 1.4 µg/mL

7 INTRODUCTION

According to national and international law, chemicals have to be tested for a possible hazard to humans and the environment, before being introduced to the market. Genotoxicity studies provide important information for the assessment of the mutagenic potential of these substances (1, 4). The *in vitro* Chromosome Aberration Test performed in this study is an essential part of genotoxicity test batteries for substances.

This *in vitro* test is a test for the detection of structural chromosomal aberrations. Such aberrations are frequently lethal to the damaged cells (8, 10). However, cytogenetic damage in somatic cells is an indicator of a potential to induce more subtle chromosomal damage that may be compatible with cell division. Similar damage induced in germ cells may lead to heritable cytogenetic abnormalities. Heritable cytogenetic abnormalities are known to have deleterious effects in man, e.g. induction of neoplastic events or birth defects. Also, chromosome abnormalities in somatic cells may become one of the reasons why a transformed cell population may develop into cancer.

Chromosome aberrations are generally evaluated in first post treatment mitoses. The majority of chemical mutagens induced structural aberrations are of the chromatid type, but chromosome type aberrations also occur.

For treatment, cell populations in exponential growth are used to guarantee that there are cells in all stages of the cell cycle (i.e. an asynchronous population). Since the normal cell cycle time is 12 hrs (see page 16) and the guidelines require fixation times of about 1.5-fold of the normal cell cycle, a fixation time of around 18 hours is appropriate. As some chemicals induce extensive mitotic delay at clastogenic concentrations or show clastogenic potential only when cells have passed through more than one cell cycle after the beginning of treatment, an additional later sampling time (28 hrs) is included (3). Due to the limited capacity of the V79 cells for metabolic activation of potential mutagens, an exogenous metabolic activation system is included (7).

The frequency of polyploid and endoreduplicated cells is also scored.

To validate the test reference mutagens were tested concurrently to the test item.

7.1 Aims of the Study

This *in vitro* test was performed to assess the potential of Ultrazine FG-R (Food Grade Lignosulphonate) to induce structural chromosome aberrations. Evaluation of cytogenetic damage induced in V79 cells (cell line from the lung of the Chinese Hamster), in the absence and the presence of metabolic activation was performed in two independent experiments at one preparation interval (18 hrs) in Experiment I and at two preparation intervals (18 hrs and 28 hrs) in Experiment II.

8 MATERIALS AND METHODS

8.1 Test Item

Internal RCC-CCR Test Item Number: S 5644 22

The test item and the information concerning the test item were provided by the sponsor.

Identity:	Ultrazine FG-R (Food Grade Lignosulphonate)
Chemical Name:	Calcium Lignosulphonate
Batch No.:	FGR-004
Aggregate State at Room Temperature:	Solid
Colour:	Brown
Molecular Weight Average: (Sample within range)	52,000 Da (93.9 % in range 1,000 – 250,000 Da)
Purity:	95.5 % (dry solids)
Certificate of Analysis (date):	August 26, 2005
Stability in Solvent:	Not indicated by the sponsor
Storage:	Room temperature, moisture protected
Expiration Date:	August 26, 2007

On the day of the experiment (immediately before treatment), the test item was dissolved in deionised water. The final concentration of deionised water in the culture medium was 10 % (v/v). The solvent was chosen based on its solubility properties and its relative tolerance to the cell cultures.

8.2 Controls

8.2.1 Solvent Controls

Concurrent solvent controls (deionised water) were performed.

8.2.2 Positive Control Substances

Without metabolic activation

Name:	EMS; ethylmethane sulfonate
Supplier:	ACROS ORGANICS, B-2440 Geel
Purity:	≥ 98 %
Lot no.:	A019600501
Expiration Date:	June 2006
Dissolved in:	Nutrient medium
Final Concentration:	300 - 400 µg/mL (2.4 – 3.2 mM)

Solutions were prepared on the day of experiment. The stability of the positive control substance in solution was proven by the mutagenic response in the expected range.

With metabolic activation

Name:	CPA; cyclophosphamide
Supplier:	Aldrich Chemie, D-89555 Steinheim
Purity:	≥ 98 %
Lot no.:	113K1406 (Experiment I)
Expiration Date:	December 2007
Lot no.:	84K1328 (Experiment II)
Expiration Date:	September 2008
Dissolved in:	Saline (0.9 % [w/v])
Final Concentration:	1.4 - 2.0 µg/mL (5.0 – 7.0 µM)

The dilutions of the stock solutions were prepared on the day of experiment. The stability of CPA in solution at room temperature is good. At 25° C only 3.5 % of its potency is lost after 24 hours (6).

8.3 Test System

8.3.1 Reasons for the Choice of the Cell Line V79

The V79 cell line has been used successfully for many years in *in vitro* experiments. Especially the high proliferation rate (doubling time of clone V79/D3 in stock cultures: 12 hrs, determined on January 17, 2005) and a reasonable plating efficiency of untreated cells (as a rule more than 70 %), both necessary for the appropriate performance of the study, recommend the use of this cell line. The cells have a stable karyotype with a modal chromosome number of 22.

Lack of metabolic activity under *in vitro* conditions is a disadvantage of tests with cell cultures, as many chemicals only develop mutagenic potential when metabolised by the mammalian organism. However, metabolic activation of chemicals can be achieved by supplementing the cell cultures with liver microsome preparations (S9 mix).

8.3.2 Cell Cultures

Large stocks of the V79 cell line (supplied by Laboratory for Mutagenicity Testing, LMP, Technical University Darmstadt, D-64287 Darmstadt) were stored in liquid nitrogen in the cell bank of RCC Cytotest Cell Research GmbH, allowing the repeated use of the same cell culture batch in experiments. Before freezing each batch was screened for mycoplasm contamination and checked for karyotype stability. Consequently, the parameters of the experiments remain similar because of standardised characteristics of the cells.

Thawed stock cultures were propagated at 37° C in 80 cm² plastic flasks (GREINER, D-72632 Frickenhausen). About 5×10^5 cells per flask were seeded into 15 mL of MEM (Minimal Essential Medium; SEROMED; D-12247 Berlin) supplemented with 10 % fetal calf serum (FCS; PAA Laboratories GmbH, D-35091 Cölbe). The cells were subcultured twice weekly. The cell cultures were incubated at 37° C in a humidified atmosphere with 1.5 % carbon dioxide (98.5 % air).

8.4 Mammalian Microsomal Fraction S9 Mix

8.4.1 S9 (Preparation by RCC Cytotest Cell Research)

Phenobarbital/β-Naphthoflavone induced rat liver S9 microsomal fractions were used as the metabolic activation system. The S9 was prepared from 8 - 12 weeks old male Wistar HanIbm rats, weight approx. 220 - 320 g (supplied from RCC Ltd; Laboratory Animal Services, CH-4414 Füllinsdorf) induced by applications of 80 mg/kg b.w. Phenobarbital i.p. (Desitin; D-22335 Hamburg) and β-Naphthoflavone p.o. (Aldrich, D-89555 Steinheim) each on three consecutive days. The livers were prepared 24 hours after the last treatment. The S9 fractions were produced by dilution of the liver homogenate with a KCl solution (1:3 parts) followed by centrifugation at 9000 g. Aliquots of the supernatant were frozen and stored in ampoules at -80° C. Small numbers of the ampoules were kept at -20°C for up to one week.

The protein concentration was 36.4 mg/mL (Lot no. 240605) in the pre-test and in Experiment I and 32.6 mg/mL (Lot no. 021205) in Experiment II.

8.4.2 S9 Mix

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

During the experiment the S9 mix was stored in an ice bath. The S9 mix preparation was performed according to Ames et al. (1).

8.5 Range-finder

A pre-test on cell growth inhibition with 4 hrs and 24 hrs treatments was performed in order to determine the toxicity of the test item (2). Cytotoxicity was determined using concentrations separated by no more than a factor of 2 - √10. The general experimental conditions in this pre-test were the same as described below for the main cytogenetic experiment. The following method was used:

In a quantitative assessment, exponentially growing cell cultures (seeding about 40,000 cells/ slide, with regard to the culture time 48 hrs) were treated with the test item, simulating the conditions of the main experiment. A qualitative evaluation of cell number and cell morphology was made 4 hrs and 24 hrs after start of treatment. The cells were stained 24 hrs after start of treatment. Using a 400 fold microscopic magnification the cells were counted in 10 co-ordinate defined fields of the slides (2 slides per treatment group). The cell number of the treatment groups is given as % cells in relation to the control.

8.6 Dose Selection

The highest concentration used in the cytogenetic experiments was chosen with regard to the current OECD Guideline for in vitro mammalian cytogenetic tests, requesting for the top concentration clear toxicity with reduced cell numbers or mitotic indices below 50 % of control, whichever is the lowest concentration, and/or the occurrence of precipitation. In case of tolerance the maximum concentration should be 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest, if solution in an appropriate solvent is possible.
With respect to the current OECD Guideline 473, 5000 µg/mL of Ultrazine FG-R (Food Grade Lignosulphonate) was applied as top concentration for treatment of the cultures in the pre-test. Test item concentrations between 39.1 and 5000 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. No precipitation of the test item in culture medium was observed in the absence and presence of S9 mix.

Experiment I:

Using reduced cell numbers as an indicator for toxicity in the pre-test (see Table 4, page 27), clear toxic effects were observed after 4 hrs treatment with 5000 µg/mL in the absence of S9 mix and with 2500 µg/mL and above in the presence of S9 mix. Considering the toxicity data of the this pre-test, 5000 µg/mL (without S9 mix) and 3750 µg/mL (with S9 mix) were chosen as top concentrations in Experiment I. The experimental part with S9 mix was repeated with a top concentration of 5000 µg/mL due to the lack of cytotoxic effects (Experiment I* and I). With respect to the current OECD Guideline (see 8.6 above) the slides of the first experiment with S9 mix were not evaluated for chromosome aberrations.

Experiment II:

In the range finding experiment clearly reduced cell numbers were observed after 24 hrs treatment with 1250 µg/mL and above in the absence of S9 mix (see Table 4, page 27). Therefore, 1500 µg/mL was chosen as top treatment concentration for continuous exposure in the absence of S9 mix. Due to the lack of cytotoxicity (in Experiment II*), the experimental part with 18 hrs continuous exposure in the absence of S9 mix was repeated with a top test item concentration of 5000 µg/mL (Experiment II). With respect to the current OECD Guideline (see 8.6 above) the slides of the first experiment with 18 hrs continuous exposure were not evaluated for chromosome aberrations. In the presence of S9 mix 5000 µg/mL was chosen as top treatment concentration based on the results obtained in Experiment I.

The applied concentrations in the cytogenetic experiments are presented in Table 2, page 19. Evaluated concentrations are given in bold letters. The cytogenetic evaluation of higher concentrations in the respective intervals (without S9 mix) was impossible due to strong test item induced toxic effects (reduced cell numbers and/or low metaphase numbers, partially paralleled by poor metaphase quality) or test item precipitates on the slides.

Table 2: Doses applied in the Chromosome aberration test with Ultrazine FG-R (Food Grade Lignosulphonate)

Preparation interval	Exposure period	Exp.	Concentration in µg/mL					
			Without S9 mix					
18 hrs	4 hrs	I	312.5	**625.0**	**1250.0**	**2500.0**	3750.0#	5000.0P#
18 hrs	18 hrs	II*	46.9	93.8	187.5	375.0	750.0	1500.0
18 hrs	18 hrs	II	**156.3**	**312.5**	**625.0**	1250.0#	2500.0#	5000.0#
28 hrs	28 hrs	II			187.5	375.0	750.0	**1500.0**
			With S9 mix					
18 hrs	4 hrs	I*	156.3	312.5	625.0	1250.0	2500.0	3750.0
18 hrs	4 hrs	I	312.5	625.0	1250.0	**2500.0**	**3750.0**	**5000.0**
28 hrs	4 hrs	II	312.5	625.0	1250.0	**2500.0**	**3750.0**	**5000.0**

Evaluated experimental points are shown in bold characters

P Precipitation occurred (visible to the unaided eye)

* Was repeated due to the lack of cytotoxicity

Concentrations were not evaluable due to low metaphase numbers/quality and/or microscopically visible precipitation on the slides

8.7 Experimental Performance

8.7.1 Schedule

	Without S9 mix			With S9 mix	
	Exp. I	Exp. II		Exp. I	Exp. II
Exposure period	4 hrs	18 hrs	28 hrs	4 hrs	4 hrs
Recovery	14 hrs	-	-	14 hrs	24 hrs
Preparation interval	18 hrs	18 hrs	28 hrs	18 hrs	28 hrs

8.7.2 Seeding of the Cultures

Exponentially growing stock cultures more than 50 % confluent are treated with trypsin-EDTA-solution at 37° C for approx. 5 minutes. Then the enzymatic treatment is stopped by adding complete culture medium and a single cell suspension is prepared. The trypsin concentration for all subculturing steps is 0.5 % (w/v) in Ca-Mg-free salt solution (Invitrogen GIBCO, D-76131 Karlsruhe).

Prior to the trypsin treatment the cells are rinsed with Ca-Mg-free salt solution.
The Ca-Mg-free salt solution is composed as follows (per litre):

NaCl	8000 mg
KCl	200 mg
KH_2P0_4	200 mg
Na_2HP0_4	150 mg

The cells were seeded into Quadriperm dishes (Heraeus, D-63450 Hanau) which contained microscopic slides (at least 2 chambers per dish and test group). In each chamber 1×10^4 - 6×10^4 cells were seeded with regard to the preparation time. The medium was MEM with 10 % FCS (complete medium).

8.7.3 Treatment

Exposure period 4 hours

The culture medium of exponentially growing cell cultures was replaced with serum-free medium (for treatment with S9 mix) or complete medium (for treatment without S9 mix) with 10 % FCS (v/v), containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL medium were used. Concurrent solvent, and positive controls were performed. After 4 hrs the cultures were washed twice with "Saline G" and then the cells were cultured in complete medium for the remaining culture time.

The "Saline G" solution was composed as follows (per litre):

NaCl	8000 mg
KCl	400 mg
Glucose x H_20	1100 mg
Na_2HP0_4 x $7H_20$	290 mg
KH_2P0_4	150 mg

pH was adjusted to 7.2

Exposure period 18 and 28 hours

The culture medium of exponentially growing cell cultures was replaced with complete medium (with 10 % FCS) containing different concentrations of the test item without S9 mix. The medium was not changed until preparation of the cells.

All cultures were incubated at 37° C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

8.7.4 Preparation of the Cultures

Colcemid was added (0.2 µg/mL culture medium) to the cultures 15.5 hrs and 25.5 hrs, respectively after the start of the treatment. The cells on the slides were treated 2.5 hrs later, in the chambers with hypotonic solution (0.4 % KCl) for 20 min at 37° C. After incubation in the hypotonic solution the cells were fixed with a mixture of methanol and glacial acetic acid (3:1 parts, respectively). Per experiment two slides per group were prepared. After preparation the cells were stained with Giemsa (E. Merck, D-64293 Darmstadt).

8.7.5 Evaluation of Cell Numbers

For evaluation of cytotoxicity indicated by reduced cell numbers two additional cultures per test item concentration and solvent control group, not treated with colcemid, were set up in parallel. These cultures were stained after 18 hrs and 28 hrs, respectively, in order to determine microscopically the cell number within 10 defined fields per coded slide. The cell number of the treatment groups is given in percentage compared to the respective solvent control.

8.7.6 Analysis of Metaphase Cells

Evaluation of the cultures was performed (according to standard protocol of the "Arbeits-gruppe der Industrie, Cytogenetik" [5]) using NIKON microscopes with 100x oil immersion objectives. Breaks, fragments, deletions, exchanges, and chromosome disintegrations were recorded as structural chromosome aberrations. Gaps were recorded as well but not included in the calculation of the aberration rates. 100 well spread metaphase plates per culture were scored for cytogenetic damage on coded slides. In total 200 metaphases per test substance concentration were evaluated.

Only metaphases with characteristic chromosome numbers of 22 ± 1 were included in the analysis. To describe a cytotoxic effect the mitotic index (% cells in mitosis) was determined. In addition, the number of polyploid cells in 500 metaphase plates per culture was determined (% polyploid metaphases; in the case of this aneuploid cell line polyploid means a near tetraploid karyotype).

8.8 Data Recording

The data generated were recorded in the raw data file. The results are presented in tabular form, including experimental groups with the test item, solvent, and positive controls.

8.9 Acceptability of the Test

The chromosome aberration test performed in our laboratory is considered acceptable if it meets the following criteria:

a) The number of structural aberrations found in the solvent controls falls within the range of our historical laboratory control data: 0.0 - 4.0 % aberrant cells, exclusive gaps.

b) The positive control substances produce significant increases in the number of cells with structural chromosome aberrations, which are within the range of the laboratory´s historical control data:

Test group Final concentration	Aberrant cells in % (excl. gaps) Range	Test group Final concentration	Aberrant cells in % (excl. gaps) Range
Without S9 mix		**With S9 mix**	
EMS 200 – 400 µg/mL	7.0 - 63.0	CPA 0.7 – 1.4 µg/mL	7.0 – 49.0

8.10 Evaluation of Results

Following analyses were carried out:

- The percentage of cells with structural chromosome aberration(s) were evaluated.
- Different types of structural chromosome aberrations were listed with their numbers and frequencies for experimental and control cultures.
- Gaps were recorded separately and reported, but generally not included in the total aberration frequency.
- Concurrent measures of cytotoxicity for all treated and solvent control cultures in the main aberration experiment(s) were recorded.

A test item is classified as non-clastogenic if:

- the number of induced structural chromosome aberrations in all evaluated dose groups is in the range of our historical control data (0.0 - 4.0 % aberrant cells, exclusive gaps).

and/or

- no significant increase of the number of structural chromosome aberrations is observed.

A test item is classified as clastogenic if:

- the number of induced structural chromosome aberrations is not in the range of our historical control data (0.0 - 4.0 % aberrant cells, exclusive gaps).

and

- either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.

Statistical significance was confirmed by means of the Fisher´s exact test (9) ($p < 0.05$). However, both biological and statistical significance should be considered together. If the criteria mentioned above for the test item are not clearly met, the classification with regard to the historical data and the biological relevance is discussed and/or a confirmatory experiment is performed.

Although the inclusion of the structural chromosome aberrations is the purpose of this study, it is important to include the polyploids and endoreduplications. The following criteria is valid:

A test item can be classified as aneugenic if:

- the number of induced numerical aberrations is not in the range of our historical control data (0.0 - 8.5 % polyploid cells).

9 RESULTS AND DISCUSSION

The test item Ultrazine FG-R (Food Grade Lignosulphonate), dissolved in deionised water, was assessed for its potential to induce structural chromosome aberrations in V79 cells of the Chinese hamster *in vitro* in the absence and the presence of metabolic activation by S9 mix.

Two independent experiments (Exp. I and Exp. II) were performed. The chromosomes were prepared 18 hrs (Exp. I and II) and 28 hrs (Exp. II) after start of treatment with the test item. The exposure period was 4 hrs, with and without metabolic activation and 18 hrs (Exp. I and II) and 28 hrs (Exp. II), without metabolic activation.

In each experimental group two parallel cultures were set up. A total of 100 metaphase plates per culture, equal to a total of 200 per experimental group, were scored for structural chromosome aberrations.

In a range finding pre-test on toxicity cell numbers were scored 24 hrs after start of treatment as an indicator for cytotoxicity. Concentrations between 39.1 and 5000 µg/mL were applied. Clear toxic effects were observed after 4 hrs treatment with 5000 µg/mL in the absence of S9 mix and 2500 µg/mL and above in the presence of S9 mix. In addition, 24 hrs treatment with 1250 µg/mL and above in the absence of S9 mix induced strong toxic effects (see Table 4, page 27).

In the pre-experiment, no precipitation of the test item was observed. No influence of the test item on the pH value or osmolarity was observed (solvent control 284 mOsm, pH 7.4 versus 292 mOsm and pH 7.4 at 5000 µg/mL).

Dose selection for the cytogenetic experiment was based on obtained toxicity data. The chosen treatment concentrations are described in Table 2 (page 19). The evaluated treatments are shown in Table 3.

Table 3: Evaluated experimental points after treatment with Ultrazine FG-R (Food Grade Lignosulphonate)

Exp.	Preparation interval	Exposure period	Concentration in µg/mL			Preparation interval	Exposure period	Concentration in µg/mL		
			Without S9 mix					With S9 mix		
I	18 hrs	4 hrs	625.0	1250.0	2500.0	18 hrs	4 hrs	2500.0	3750.0	5000.0
II	18 hrs	18 hrs	156.3	312.5	625.0					
II	28 hrs	28 hrs			1500.0	28 hrs	4 hrs	2500.0	3750.0	5000.0

In Experiment I, without metabolic activation, precipitation was observed at 5000 µg/mL, following a 4 hrs exposure period and 18 hrs preparation time. The concentration of 3750 µg/mL was not evaluated due to test item precipitation observed microscopically. No cytotoxicity was observed up to the highest evaluated concentration (2500 µg/mL). With

metabolic activation, neither toxic effects nor test item precipitation were noted at any test concentration, up to the limit dose of 5000 µg/mL (see Table 5, page 28).

In Experiment II, following 18 hrs exposure without S9 mix, no cytotoxicity was noted up to the highest evaluated concentration (625 µg/mL). Higher concentrations (1250, 2500 and 5000 µg/mL) were not evaluable due to low metaphase numbers, partially paralleled by poor metaphase quality and/or microscopically visible precipitation on the slides.

In Experiment II toxic effects, indicated by reduced cell numbers below 50 % of control, were observed after 28 hrs continuous treatment in the absence of S9 mix at the concentration of 1500 µg/mL. In the presence of S9 mix, no cytotoxicity was observed up to the highest concentration tested, as required by respective guidelines (limit dose).

In both experiments, in the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying structural chromosome aberrations was observed (see Table 7, 8, 10 and 11, pages 30, 31, 33, and 34). The aberration rates of the cells after treatment with the test item (0.0 - 3.0 % aberrant cells, exclusive gaps) were within the range of our historical control data: 0.0 - 4.0 % aberrant cells, exclusive gaps (see Table 14, page 37).

In Experiment I, in the absence of S9 mix, a dose-related increase in cells carrying chromosomal aberrations was observed (1.5 %, 2.0 % and 3.0 % aberrant cells, exclusive gaps), statistically significant from concurrent controls at the two highest concentrations of 1250 µg/mL and 2500 µg/mL. However, as noted above, these values were clearly within our historical control data range (0.0 - 4.0 % aberrant cells, exclusive gaps) (see Table 14, page 37) and thus considered biologically irrelevant.

Table 6 and 9, page 29 and 32, show the occurrence of polyploid metaphases. In both experiments, no biologically relevant increase in the rate of polyploid metaphases was found after treatment with the test item (0.7 – 3.1 %), as compared to the rates of the solvent controls (1.4 – 3.6 %). The values were clearly within the range of our historical control data: 0.0 – 5.5 % polyploid cells (Table 15, page 38).

In both experiments, EMS (300 and 400 µg/mL, respectively) and CPA (1.4 and 2.0 µg/mL, respectively) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations, showing the required susceptibility of the test system.

In conclusion, it can be stated that under the experimental conditions reported, the test item Ultrazine FG-R (Food Grade Lignosulphonate) did not induce structural chromosome aberrations in V79 cells (Chinese hamster cell line), in the absence and presence of metabolic activation.

10 DISTRIBUTION OF THE REPORT

Scientific Monitor	4 x (1 x duplicate bound, 2 x copy unbound, 1x complete PDF-file)
Study Director	1 x (original)

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12 ANNEX I: TABLES OF RESULTS

12.1 Pre-Test on Toxicity

In the pre-test the toxicity of the test item was examined using the determination of the cell number. Cell numbers of two cultures (10 coordinate defined fields per culture) were determined for each experimental group.

Table 4: Cytotoxicity of Ultrazine FG-R (Food Grade Lignosulphonate) to cultures of Chinese hamster cell line V79.

Without S9 mix, 4 hrs exposure			**With S9 mix, 4 hrs exposure**		
Concentration in µg/mL	Number of cells	% of solvent control	Concentration in µg/mL	Number of cells	% of solvent control
Solvent control	425	100	Solvent control	904	100
39.1	512	120.4	39.1	774	85.6
78.1	452	106.4	78.1	818	90.4
156.3	439	103.3	156.3	808	89.3
312.5	456	107.3	312.5	690	76.3
625.0	424	99.8	625.0	509	56.3
1250.0	433	101.8	1250.0	527	58.2
2500.0	556	130.7	2500.0	375	41.5
5000.0	70	16.5	5000.0	86	9.5

Without S9 mix; 24 hrs exposure

Concentration in µg/mL	Number of cells	% of solvent control
Solvent control	662	100
39.1	603	91.1
78.1	602	90.9
156.3	690	104.2
312.5	554	83.7
625.0	524	79.1
1250.0	189	28.5
2500.0	39	5.9
5000.0	43	6.4

12.2 Experiments I and II: Determination of Toxicity

The toxicity of the test item was examined using the determination of the cell number. Cell numbers of two cultures (10 co-ordinate defined fields per culture) were determined for each experimental group, except the positive control.

Table 5: Number of cells in % of solvent control

Without metabolic activation (S9 mix)

Experiment I: 4 hrs exposure			Experiment II: continuous exposure		
Preparation interval	Concentration in µg/mL	Cells in % of solvent control	Preparation interval	Concentration in µg/mL	Cells in % of solvent control
18 hrs	312.5	93.6	18 hrs	**156.3**	96.8
"	**625.0**	70.9	"	**312.5**	143.2
"	**1250.0**	106.4	"	**625.0**	100.8
"	**2500.0**	89.8	"	1250.0[#]	82.4
"	3750.0[#]	73.8	"	2500.0[#]	53.1
"	5000.0[P#]	27.7	"	5000.0[#]	21.3
			28 hrs	187.5	101.4
			"	375.0	78.8
			"	750.0	86.1
			"	**1500.0**	43.6

With metabolic activation (S9 mix)

Experiment I: 4 hrs exposure			Experiment II: 4 hrs exposure		
Preparation interval	Concentration in µg/mL	Cells in % of solvent control	Preparation interval	Concentration in µg/mL	Cells in % of solvent control
18 hrs	312.5	85.6	28 hrs	312.5	71.3
"	625.0	93.7	"	625.0	75.3
"	1250.0	105.8	"	1250.0	63.0
"	**2500.0**	76.8	"	**2500.0**	53.0
"	**3750.0**	67.3	"	**3750.0**	74.6
"	**5000.0**	126.3	"	**5000.0**	70.2

Experimental groups evaluated for cytogenetic damage were printed in bold characters

[P] Precipitation occurred 4 hrs after start of treatment (visible to the unaided eye)

[#] Concentrations were not evaluable due to low metaphase numbers/quality and/or microscopically visible precipitation on the slides

12.3 Experiment I

Table 6: Number of polyploid cells and mitotic index;
preparation interval 18 hrs with and without S9 mix

Treatment group	Conc. per mL	S9 mix	Exposure period/ Recovery	Polyploid cells* Culture 1	Culture 2	Total	%	Mitotic indices** Absolute 1	Absolute 2	Mean	%***
Solv. control#	10.0 %	-	4 / 14 hrs	14	10	24	2.4	16.7	15.5	16.1	100.0
Pos. control##	400.0 µg	-	4 / 14 hrs	11	17	28	2.8	16.5	15.2	15.9	98.4
Test item	625.0 µg	-	4 / 14 hrs	9	12	21	2.1	16.5	14.5	15.5	96.3
"	1250.0 µg	-	4 / 14 hrs	10	14	24	2.4	15.9	15.4	15.7	97.2
"	2500.0 µg	-	4 / 14 hrs	7	12	19	1.9	16.9	16.7	16.8	104.3
Solv. control#	10.0 %	+	4 / 14 hrs	11	21	32	3.2	15.1	15.6	15.4	100.0
Pos. control###	1.4 µg	+	4 / 14 hrs	3	7	10	1.0	9.5	8.3	8.9	58.0
Test item	2500.0 µg	+	4 / 14 hrs	7	10	17	1.7	10.0	9.5	9.8	63.5
"	3750.0 µg	+	4 / 14 hrs	5	12	17	1.7	12.3	11.0	11.7	75.9
"	5000.0 µg	+	4 / 14 hrs	10	8	18	1.8	11.2	11.0	11.1	72.3

* The number of polyploid cells was determined of each test group in a sample of 500 cells per culture

** The mitotic index was determined in a sample of 1000 cells per culture of each test group in %

*** For the positive control groups and the test item groups, the relative values of the mitotic index are related to the solvent controls

Deionised water

EMS

CPA

Table 7: Structural chromosome aberrations Experiment I;
preparation interval 18 hrs without S9 mix: exposure period 4 hrs

Slide no.	Cells scored	% Aberrant cells incl. gaps*	% Aberrant cells excl. gaps*	% Aberrant cells with ex-changes	Aberrations: Gaps g	Gaps ig	Chromatid type b	Chromatid type f	Chromatid type d	Chromatid type ex	Chromosome type ib	Chromosome type if	Chromosome type id	Chromosome type cx	Other ma	Other cd
					Without S9 mix											
Solvent control: Deionised water 10 %																
1	100				0	0	0	0	0	0	0	0	0	0	0	0
2	100				0	1	0	0	0	0	0	0	0	0	0	0
1 + 2	200	0.5	0.0	0.0	0	1	0	0	0	0	0	0	0	0	0	0
Positive control: EMS 400.0 µg / mL																
1	100				0	0	8	0	0	2	0	0	0	0	0	0
2	100				0	0	9	1	0	7	1	1	0	0	0	0
1 + 2	200	13.5	13.5	4.5	0	0	17	1	0	9	1	1	0	0	0	0
Test item: 625.0 µg / mL																
1	100				0	0	0	0	0	0	1	0	0	0	0	0
2	100				0	0	2	0	0	0	0	0	0	0	0	0
1 + 2	200	1.5	1.5	0.0	0	0	2	0	0	0	1	0	0	0	0	0
Test item: 1250.0 µg / mL																
1	100				0	0	2	0	0	0	1	0	0	0	0	0
2	100				0	0	1	0	0	0	0	0	0	0	0	0
1 + 2	200	2.0	2.0	0.0	0	0	3	0	0	0	1	0	0	0	0	0
Test item: 2500.0 µg / mL																
1	100				0	0	3	0	0	0	0	0	0	0	0	0
2	100				0	0	1	0	0	0	1	1	0	0	0	0
1 + 2	200	3.0	3.0	0.0	0	0	4	0	0	0	1	1	0	0	0	0

* Inclusive cells carrying exchanges

Abbreviations

g = gap, ig = iso-gap (gaps are achromatic lesions of chromatid or chromosome type where no or only a minimal misalignment of chromosomal material is visible), b = break, ib = iso-break, f = fragment, if = iso-fragment, d = deletion, id = iso-deletion, ma = multiple aberration (= more than 4 events in one cell [excluding gaps]), ex = chromatid type exchange, cx = chromosome type exchange, cd = chromosomal disintegration (= pulverization)

Table 8: Structural chromosome aberrations Experiment I;
preparation interval 18 hrs with S9 mix: exposure period 4 hrs

Slide no.	Cells scored	% Aberrant cells incl. gaps*	% Aberrant cells excl. gaps*	% Aberrant cells with ex-changes	Aberrations: Gaps g	Gaps ig	Chromatid type b	Chromatid type f	Chromatid type d	Chromatid type ex	Chromosome type ib	Chromosome type if	Chromosome type id	Chromosome type cx	Other ma	Other cd
					With S9 mix											
Solvent control: Deionised water 10 %																
1	100				0	0	0	0	0	0	0	1	0	0	0	0
2	100				0	0	0	0	0	0	0	0	0	0	0	0
1 + 2	200	0.5	0.5	0.0	0	0	0	0	0	0	0	1	0	0	0	0
Positive control: CPA 1.4 µg / mL																
1	100				1	0	3	2	0	1	1	1	0	0	0	0
2	100				0	0	2	4	0	3	0	0	0	0	1	0
1 + 2	200	9.0	9.0	2.0	1	0	5	6	0	4	1	1	0	0	1	0
Test item: 2500.0 µg / mL																
1	100				2	1	1	0	0	0	0	0	0	0	0	0
2	100				0	0	2	0	0	0	0	0	0	0	0	0
1 + 2	200	2.5	1.5	0.0	2	1	3	0	0	0	0	0	0	0	0	0
Test item: 3750.0 µg / mL																
1	100				2	0	0	0	0	0	0	1	0	0	0	0
2	100				0	0	1	0	0	0	0	0	0	0	0	0
1 + 2	200	2.0	1.0	0.0	2	0	1	0	0	0	0	1	0	0	0	0
Test item: 5000.0 µg / mL																
1	100				1	0	0	0	0	0	0	0	0	0	0	0
2	100				0	0	0	0	0	0	0	0	0	0	0	0
1 + 2	200	0.5	0.0	0.0	1	0	0	0	0	0	0	0	0	0	0	0

* Inclusive cells carrying exchanges

Abbreviations

g = gap, ig = iso-gap (gaps are achromatic lesions of chromatid or chromosome type where no or only a minimal misalignment of chromosomal material is visible), b = break, ib = iso-break, f = fragment, if = iso-fragment, d = deletion, id = iso-deletion, ma = multiple aberration (= more than 4 events in one cell [excluding gaps]), ex = chromatid type exchange, cx = chromosome type exchange, cd = chromosomal disintegration (= pulverization)

12.4 Experiment II

Table 9: Number of polyploid cells and mitotic index;
preparation interval 18 and 28 hrs without S9 mix;
preparation interval 28 hrs with S9 mix

Treatment group	Conc. per mL		S9 mix	Exposure period/ Recovery	Polyploid cells* Culture 1	Culture 2	Total	%	Mitotic indices** Absolute 1	Absolute 2	Mean	%***
Solv. control#	10.0	%	-	18 / - hrs	21	15	36	3.6	20.6	18.2	19.4	100.0
Pos. control##	300.0	µg	-	18 / - hrs	20	6	26	2.6	14.0	16.8	15.4	79.4
Test item	156.3	µg	-	18 / - hrs	18	13	31	3.1	22.1	17.3	19.7	101.5
"	312.5	µg	-	18 / - hrs	12	17	29	2.9	21.0	19.0	20.0	103.1
"	625.0	µg	-	18 / - hrs	15	10	25	2.5	18.6	23.5	21.1	108.5
Solv. control#	10.0	%	-	28 / - hrs	6	8	14	1.4	18.7	15.5	17.1	100.0
Pos. control##	300.0	µg	-	28 / - hrs	11	9	20	2.0	15.4	15.8	15.6	91.2
Test item	1500.0	µg	-	28 / - hrs	6	10	16	1.6	11.6	12.0	11.8	69.0
Solv. control#	10.0	%	+	4 / 24 hrs	6	9	15	1.5	15.4	13.4	14.4	100.0
Pos. control###	2.0	µg	+	4 / 24 hrs	7	5	12	1.2	16.5	13.7	15.1	104.9
Test item	2500.0	µg	+	4 / 24 hrs	10	5	15	1.5	16.0	15.9	16.0	110.8
"	3750.0	µg	+	4 / 24 hrs	4	3	7	0.7	16.3	15.3	15.8	109.7
"	5000.0	µg	+	4 / 24 hrs	5	6	11	1.1	14.8	17.0	15.9	110.4

* The number of polyploid cells was determined of each test group in a sample of 500 cells per culture

** The mitotic index was determined in a sample of 1000 cells per culture of each test group in %

*** For the positive control groups and the test item groups, the relative values of the mitotic index are related to the solvent controls

Deionised water

EMS

CPA

Table 10: Structural chromosome aberrations Experiment II;
preparation interval 18 hrs without S9 mix: exposure period 18 hrs

Slide no.	Cells scored	% Aberrant cells incl. gaps*	% Aberrant cells excl. gaps*	% Aberrant cells with exchanges	Aberrations: Gaps g	Gaps ig	Chromatid type b	Chromatid type f	Chromatid type d	Chromatid type ex	Chromosome type ib	Chromosome type if	Chromosome type id	Chromosome type cx	Other ma	Other cd
					Without S9 mix											
Solvent control: Deionised water 10 %																
1	100				0	0	1	1	0	0	1	1	0	0	0	0
2	100				1	0	1	0	0	0	1	0	0	0	0	0
1 + 2	200	3.0	2.5	0.0	1	0	2	1	0	0	2	1	0	0	0	0
Positive control: EMS 300.0 µg / mL																
1	100				2	0	25	2	0	9	6	1	0	0	0	0
2	100				2	0	23	1	0	6	3	1	0	0	0	0
1 + 2	200	28.5	27.0	6.0	4	0	48	3	0	15	9	2	0	0	0	0
Test item: 156.3 µg / mL																
1	100				2	0	1	0	0	0	2	1	0	0	0	0
2	100				1	0	2	0	0	0	0	0	0	0	0	0
1 + 2	200	4.5	3.0	0.0	3	0	3	0	0	0	2	1	0	0	0	0
Test item: 312.5 µg / mL																
1	100				2	0	0	0	0	0	0	0	0	0	0	0
2	100				0	0	2	0	0	0	0	1	0	0	0	0
1 + 2	200	2.5	1.5	0.0	2	0	2	0	0	0	0	1	0	0	0	0
Test item: 625.0 µg / mL																
1	100				1	0	0	0	0	1	0	1	0	0	0	0
2	100				0	0	1	0	0	0	0	0	0	0	0	0
1 + 2	200	2.0	1.5	0.5	1	0	1	0	0	1	0	1	0	0	0	0

* Inclusive cells carrying exchanges

Abbreviations

g = gap, ig = iso-gap (gaps are achromatic lesions of chromatid or chromosome type where no or only a minimal misalignment of chromosomal material is visible), b = break, ib = iso-break, f = fragment, if = iso-fragment, d = deletion, id = iso-deletion, ma = multiple aberration (= more than 4 events in one cell [excluding gaps]), ex = chromatid type exchange, cx = chromosome type exchange, cd = chromosomal disintegration (= pulverization)

Table 11: Structural chromosome aberrations Experiment II;
preparation interval 28 hrs without S9 mix: exposure period 28 hrs;
preparation interval 28 hrs with S9 mix: exposure period 4 hrs

Slide	Cells	% Aberrant cells			Aberrations											
no.	scored	incl. gaps*	excl. gaps*	with ex-changes	Gaps g	Gaps ig	Chromatid type b	Chromatid type f	Chromatid type d	Chromatid type ex	Chromosome type ib	Chromosome type if	Chromosome type id	Chromosome type cx	Other ma	Other cd
					Without S9 mix											
Solvent control: Deionised water 10 %																
1	100				1	0	1	0	0	0	0	0	0	0	0	0
2	100				0	0	3	0	0	0	0	0	0	0	0	0
1 + 2	200	2.5	2.0	0.0	1	0	4	0	0	0	0	0	0	0	0	0
Positive control: EMS 300.0 µg / mL																
1	100				1	0	10	2	0	6	5	0	1	0	1	0
2	100				1	0	11	2	0	4	3	1	0	0	0	0
1 + 2	200	18.5	18.0	4.5	2	0	21	4	0	10	8	1	1	0	1	0
Test item: 1500.0 µg / mL																
1	100				0	0	2	1	0	0	0	1	0	0	0	0
2	100				0	0	1	0	0	0	0	0	1	0	0	0
1 + 2	200	3.0	3.0	0.0	0	0	3	1	0	0	0	1	1	0	0	0
					With S9 mix											
Solvent control: Deionised water 10 %																
1	100				2	0	1	0	0	0	0	0	0	0	0	0
2	100				4	0	0	0	0	0	0	0	0	0	0	0
1 + 2	200	3.5	0.5	0.0	6	0	1	0	0	0	0	0	0	0	0	0
Positive control: CPA 2.0 µg / mL																
1	100				0	0	2	3	0	1	1	4	0	0	3	0
2	100				3	0	1	2	0	1	2	6	0	3	1	0
1 + 2	200	14.0	12.5	2.5	3	0	3	5	0	2	3	10	0	3	4	0
Test item: 2500.0 µg / mL																
1	100				0	0	1	0	0	0	1	0	0	0	0	0
2	100				0	0	0	0	0	0	0	0	0	0	0	0
1 + 2	200	1.0	1.0	0.0	0	0	1	0	0	0	1	0	0	0	0	0
Test item: 3750.0 µg / mL																
1	100				0	0	2	0	0	1	0	0	0	0	0	0
2	100				1	1	2	0	0	0	0	0	0	0	0	0
1 + 2	200	3.5	2.5	0.5	1	1	4	0	0	1	0	0	0	0	0	0
Test item: 5000.0 µg / mL																
1	100				0	0	0	0	0	0	0	0	0	0	0	0
2	100				0	0	2	0	0	0	1	2	0	0	1	0
1 + 2	200	2.0	2.0	0.0	0	0	2	0	0	0	1	2	0	0	1	0

* Inclusive cells carrying exchanges

Abbreviations

g = gap, ig = iso-gap (gaps are achromatic lesions of chromatid or chromosome type where no or only a minimal misalignment of chromosomal material is visible), b = break, ib = iso-break, f = fragment, if = iso-fragment, d = deletion, id = iso-deletion, ma = multiple aberration (= more than 4 events in one cell [excluding gaps]), ex = chromatid type exchange, cx = chromosome type exchange, cd = chromosomal disintegration (= pulverization)

12.5 Biometry

Statistical significance at the five per cent level ($p < 0.05$) was evaluated by means of the Fisher´s exact test. Evaluation was performed only for cells carrying aberrations exclusive gaps.

Table 12: Biometry of Experiment I

Test group versus solvent control		Preparation interval	Exposure period	S9 mix	p-value
Test group	625.0 µg/mL	18 hrs	4 hrs	-	0.062
"	1250.0 µg/mL	18 hrs	4 hrs	-	0.031[s]
"	2500.0 µg/mL	18 hrs	4 hrs	-	0.008[s]
"	2500.0 µg/mL	18 hrs	4 hrs	+	0.187
"	3750.0 µg/mL	18 hrs	4 hrs	+	0.312
"	5000.0 µg/mL	18 hrs	4 hrs	+	n.c.
Positive control versus solvent control					
EMS	400.0 µg/mL	18 hrs	4 hrs	-	< 0.001[s]
CPA	1.4 µg/mL	18 hrs	4 hrs	+	< 0.001[s]

Table 13: Biometry of Experiment II

Test group versus solvent control		Preparation interval	Exposure period	S9 mix	p-value
Test group	156.3 µg/mL	18 hrs	18 hrs	-	0.386
"	312.5 µg/mL	18 hrs	18 hrs	-	n.c.
"	625.0 µg/mL	18 hrs	18 hrs	-	n.c.
"	1500.0 µg/mL	28 hrs	28 hrs	-	0.272
"	2500.0 µg/mL	28 hrs	4 hrs	+	0.312
"	3750.0 µg/mL	28 hrs	4 hrs	+	0.061
"	5000.0 µg/mL	28 hrs	4 hrs	+	0.108
Positive control versus solvent control					
EMS	300.0 µg/mL	18 hrs	18 hrs	-	< 0.001[s]
EMS	300.0 µg/mL	28 hrs	28 hrs	-	< 0.001[s]
CPA	2.0 µg/mL	28 hrs	4 hrs	+	< 0.001[s]

n.c. Not calculated as the aberration rate is equal or lower than the corresponding control rate
[s] Aberration rate is statistically significant higher than the control rate

13 ANNEX II

13.1 Chromosome Aberrations: Classification and Criteria

1. **Gaps**

   Gaps are small areas of the chromosome which are unstained. The chromatids remain aligned as normal and the gap does not extend along the chromatid for a distance greater than the width of a chromatid. If the gap occurs on one chromatid only it is a chromatid gap (g).

2. **Chromatid Breaks**

   Chromatid breaks (b) vary in appearance. The chromatid may remain aligned but show a gap which is too large to classify as a gap. Alternatively, the chromatid may be broken so that the broken fragment is displaced. In some cases, the fragment is not seen at all. A chromatid fragment (f) should be scored if the chromosome of origin cannot be identified. In addition, deletions can occur as a result of a break. The missing terminal end of a chromatid in the assessed metaphase is classified as deletion (d).

3. **Chromosome breaks**

   Chromosome breaks (ib) are breaks in both chromatids of the chromosome. A fragment with two chromatids is formed and this may be displaced by varying degrees. Breaks are distinguished from gaps by the size of the unstained region. A chromosome break is scored if the fragment is associated with a chromosome from which it was probably derived. However, fragments are often seen in isolation and are then scored as chromatid fragments (if). In addition, isodeletions can occur as a result of a isobreak. The missing terminal end of a chromosome in the assessed metaphase is classified as isodeletion (id).

4. **Exchanges**

   Exchanges are formed by faulty rejoining of broken chromosomes and may be of the chromosome or chromatid type. Chromatid exchanges (ex) have numerous different forms but are generally not further classified. Where multiple exchanges have occurred each exchange point is counted as one chromatid exchange. Chromosome exchanges (cx) generally appear as either a dicentric or a ring form, either of which can be associated with a fragment, which if possible should be scored as part of the exchange.

5. **Multiple Aberrations**

   If many aberrations are present in one metaphase, the exact details may not be scorable. This is particularly the case when chromosome pulverisation (cd) occurs. If the number of aberrations is greater than 4 then the cell is classified as multiple aberrant (ma).

6. **Chromosome Number**

   If the chromosome (centromere) number is 22 ± 1 then it is classified as a diploid cell and scored for aberrations. If less than 22 ± 1 chromosomes are counted then the cell is ignored under the assumption that some chromosomes may have been lost for technical reasons. If greater than 22 ± 1 chromosomes are scored then the count is recorded and the cell classified as an aneuploid cell. If multiple copies of the haploid chromosome number (other than diploid) are scored then the count is recorded and the cell classified as polyploid. If the chromosomes are arranged in closely apposed pairs, i.e. 4 chromatids instead of 2, the cell is scored as endoreduplicated (e).

14 ANNEX III

14.1 Historical laboratory control data

Table 14: Chinese hamster V79 cell cultures (2003 to 2004)

Without S9 mix										
		Aberrant cells (%)								
		Inclusive gaps			Exclusive gaps			With exchanges		
Test group Concentration	Cells scored	Range	Mean	Calculated range*	Range	Mean	Calculated range*	Range	Mean	Calculated range*
Negative control										
Culture medium MEM	48800	0.0-6.0	1.6	0.8-2.4	0.0-4.0	0.9	0.4-1.5	0.0-1.0	0.1	0.0-0.3
Aqueous solv. ** 10 % (v/v)	18800	0.0-4.5	1.6	0.8-2.3	0.0-3.5	1.0	0.4-1.5	0.0-1.0	0.1	0.0-0.4
Org. solvents*** 0.5 % (v/v)	24600	0.0-5.5	1.6	0.7-2.5	0.0-4.0	0.9	0.3-1.5	0.0-1.5	0.2	0.0-0.4
Total	**92200**	**0.0-6.0**	**1.6**	**0.8-2.4**	**0.0-4.0**	**0.9**	**0.4-1.5**	**0.0-1.5**	**0.1**	**0.0-0.3**
Positive control										
EMS 200–400 µg/mL	48800	8.5-66.0	18.7	11.8-25.6	7.0-63.0	16.9	10.1-23.7	0.5-30.0	6.8	3.4-10.2
With S9 mix										
		Aberrant cells (%)								
		Inclusive gaps			Exclusive gaps			With exchanges		
Test group Concentration	Cells scored	Range	Mean	Calculated range*	Range	Mean	Calculated range*	Range	Mean	Calculated range*
Negative control										
Culture medium MEM	35400	0.0-5.0	1.9	0.9-2.8	0.0-4.0	1.2	0.5-1.9	0.0-2.0	0.3	0.0-0.7
Aqueous solv. ** 10 % (v/v)	14000	0.0-5.0	2.0	0.9-3.1	0.0-4.3	1.4	0.6-2.2	0.0-2.0	0.3	0.0-0.7
Org. solvents*** 0.5 % (v/v)	17200	0.0-5.0	1.9	1.0-2.8	0.0-4.0	1.2	0.4-1.9	0.0-2.0	0.3	0.0-0.6
Total	**66600**	**0.0-5.0**	**1.9**	**0.9-2.8**	**0.0-4.3**	**1.2**	**0.5-2.0**	**0.0-2.0**	**0.3**	**0.0-0.7**
Positive control										
CPA 0.7–1.4 µg/mL	35200	8.0-50.0	14.5	10.6-18.5	7.0-49.0	12.9	9.3-16.5	0.0-19.0	4.6	2.4-6.8

* Mean ± standard deviation

** Aqueous solvents: deionised water and 0.9 % (w/v) saline

*** Organic solvents: acetone, DMSO, ethanol, and tetrahydrofurane

Table 15: V79 cell cultures from 2003 to 2004

Without S9 mix				
Polyploid cells (%)				
Test group Concentration	Cells scored	Range	Mean	Calculated range*
Negative control				
Culture medium MEM	48800	0.4-5.5	2.3	1.7-3.0
Aqueous solv. ** 10 % (v/v)	18800	0.4-4.6	2.3	1.6-3.0
Organic solv. *** 0.5 % (v/v)	24600	0.4-5.1	2.3	1.6-3.0
Total	**92200**	**0.4-5.5**	**2.3**	**1.7-3.0**
Positive control				
EMS 200–400 µg/mL	48800	0.1-5.0	2.1	1.4-2.7
With S9 mix				
Polyploid cells (%)				
Test group Concentration	Cells scored	Range	Mean	Calculated range*
Negative control				
Culture medium MEM	35400	0.3-4.9	2.2	1.5-2.8
Aqueous solv. ** 10 % (v/v)	14000	0.9-4.3	2.2	1.7-2.7
Organic solv. *** 0.5 % (v/v)	17200	0.3-5.6	2.3	1.6-2.9
Total	**66600**	**0.3-5.6**	**2.2**	**1.6-2.8**
Positive control				
CPA 0.7–1.4 µg/mL	35200	0.4-4.4	2.1	1.6-2.7

* Mean ± standard deviation

** Aqueous solvents: deionised water and 0.9 % (w/v) saline

*** Organic solvents: acetone, ethanol, DMSO, and tetrahydrofurane

15 ANNEX IV

15.1 Copy of GLP Certificate

Hessisches Ministerium für Umwelt,
ländlichen Raum und Verbraucherschutz

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 88/320/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 88/320/EEC at:

☒ Prüfeinrichtung/Test facillity ☐Prüfstandort/Test site

RCC Cytotest Cell Research GmbH
RCC Cytotest Cell Research GmbH
In den Leppsteinwiesen 19
64380 Roßdorf

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according chemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften	**2** Toxicity studies
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	**3** Mutagenicity studies
8 Analytische Prüfungen an biologischen Materialien	**8** Analytical studies on biological materials
9 Virussicherheitsprüfungen	**9** Virus validation studies

03.06.; 19.07.-22.07.2004
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Th. Zimmermann, Referent, Wiesbaden, den **Wiesbaden, den 06. Januar 200**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

Hess. Ministerium für Umwelt, ländlichen Raum und Verbraucherschutz,
Mainzer Straße 80 D65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority)

D-65189 Wiesbaden, Mainzer Straße 80
Telefon: 0611. 81 50
Telefax: 0611. 81 51 94 1
E-Mail: poststelle@hmulv.hessen.de

D-65187 Wiesbaden, Hölderlinstraße 1-3
Telefon: 0611. 81 70
Telefax: 0611. 81 72 18 1

16 ANNEX V

16.1 Copy of the Study Plan

(20 pages)

RCC-CCR STUDY NUMBER 899102

IN VITRO CHROMOSOME ABERRATION TEST IN CHINESE HAMSTER V79 CELLS WITH ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

STUDY PLAN

RCC

1 SIGNATURES

Study Director

Dr. Markus Schulz

M. Schulz

Date: September 28, 2005

Management

Dr. Wolfgang Völkner

Date: September 28, 2005

Head of Quality
Assurance Unit

Frauke Hermann

F. Hermann

Date: September 28, 2005

Scientific Monitor
(on behalf of the Sponsor)

Date: October 12, 2005

899102pe2 doc

2 CONTENTS

3 PREFACE

3.1 General

Title:	*In vitro* Chromosome Aberration Test in Chinese Hamster V79 Cells with Ultrazine FG-R (Food Grade Lignosulphonate)
Sponsor:	DSM Nutritional Products AG Wurmisweg 576 CH-4303 Kaiseraugst Switzerland
Scientific Monitor:	Dr. Werner Köhl SCC Scientific Consulting Company Chemisch-Wissenschaftliche Beratung GmbH Mikroforum Ring 1 D-55234 Wendelsheim Germany
SCC Project No.:	612-002
Test Facility:	RCC Cytotest Cell Research GmbH (RCC-CCR) In den Leppsteinswiesen 19 D-64380 Rossdorf Germany

3.2 Responsibilities

Study Director:	Dr. Markus Schulz
Deputy Study Director:	Dr. Susanne Kunz
Management:	Dr. Wolfgang Völkner
Head of Quality Assurance Unit:	Frauke Hermann

3.3 Schedule

Date of the Study Plan:	September 28, 2005
Proposed Experimental Starting Date:	October 2005
Proposed Experimental Completion Date:	End of January 2006
Proposed Date of Draft Report:	Beginning of February 2006

3.4 Good Laboratory Practice

The study will be performed in compliance with:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), dated July 25, 1994 ("BGBl. I 1994", pp. 1703), last revision dated June 27, 2002.

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final].

Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

3.5 Guidelines

This study will be conducted according to the procedures indicated by the following internationally accepted guidelines and recommendations:

Ninth Addendum to the OECD Guidelines for Testing of Chemicals, February 1998, adopted July 21, 1997, Guideline No. 473 "*In vitro* Mammalian Chromosome Aberration Test".

EPA Health Effects Test Guidelines, OPPTS 870.5375, EPA 712-C-98-223, August 1998 "*In Vitro* Mammalian Chromosome Aberration Test".

Commission Directive 2000/32/EC, L1362000, Annex 4A: "Mutagenicity – *In vitro* Mammalian Chromosome Aberration Test", dated May 19, 2000.

3.6 Amendment and Deviation Procedures

Amendments (planned changes) to the study plan will be issued and signed by the Study Director. The Sponsor will receive the original and a copy of the amendment. The original is to be countersigned upon agreement and returned to RCC Cytotest Cell Research GmbH. The amendment will be distributed (see Distribution) and added to all copies of the study plan.

Deviations (unplanned changes) to the study plan will be documented and maintained with the raw data. The report will reflect any deviations. The sponsor will be promptly informed of any deviations from the study plan.

3.7 Archiving

RCC Cytotest Cell Research GmbH will archive the following data for 15 years:

Raw data, study plan, final report, and a sample of the test item.

Microscopic slides will be archived for at least 12 years.

No data will be discarded without the sponsor´s consent.

4 INTRODUCTION

According to national and international acts chemicals have to be tested before introduction to the market for a possible hazard to humans and the environment. Genotoxicity studies provide important information for the assessment of the mutagenic potential of these substances (1, 4). The *in vitro* Chromosome Aberration Test performed in this study is an essential part of genotoxicity test batteries of substances.

This *in vitro* test is a test for the detection of structural chromosome aberrations (8, 10). These aberrations are frequently lethal to the damaged cells. However, cytogenetic damage in somatic cells is an indicator of a potential to induce more subtle chromosome damage that is compatible with cell division. Similar damage induced in germinal cells may lead to heritable cytogenetic abnormalities. Heritable cytogenetic abnormalities are known to have deleterious effects in man, e.g. induction of neoplastic events or birth defects. Also, chromosome abnormalities in somatic cells may become one of the reasons why a transformed cell population may develop cancerogenicity.

Chromosome aberrations are generally evaluated in first post treatment mitoses. While the majority of chemical mutagens induced aberrations are of the chromatid type, chromosome type aberrations also occur.

For treatment, cell populations should be in exponential growth to guarantee that there are cells in all stages of the cell cycle (i.e. an asynchronous population). Since the normal cell cycle time is 12 hrs (see page 10) and the guidelines require preparation times of about 1.5-fold of the normal cell cycle, a preparation time of about 18 hours is appropriate. Because there may be chemicals which induce very extensive mitotic delay at clastogenic concentrations or may be clastogenic only when cells have passed through more than one cell cycle since the beginning of treatment an additional later sampling time (28 hrs) should be included (3). Due to the limited capacity of the V79 cells for metabolic activation of potential mutagens an exogenous metabolic activation system is necessary (7).

The frequency of polyploid and endoreduplicated cells should also be scored so that the level in control cultures can be monitored and their induction by the test item can be recorded especially when a late sampling time is used.

To validate the test reference mutagens will be tested in parallel to the test item.

4.1 Aims of the Study

This *in vitro* test will be performed to assess the potential of Ultrazine FG-R (Food Grade Lignosulphonate) to induce structural chromosome aberrations. Evaluation of cytogenetic damage induced in V79 cells (cell line from the lung of the Chinese Hamster) in the absence and the presence of metabolic activation will be performed in one experiment at one preparation interval (18 hrs). In case of a negative or inconclusive result a second experiment will be performed after discussion with the sponsor. In case of a clear positive response, a second experiment is not required.

5 MATERIALS AND METHODS

5.1 Test Item

Internal RCC-CCR Test Item Number: S 5644 22

The test item and the information concerning the test item were provided by the sponsor.

Identity:	Ultrazine FG-R (Food Grade Lignosulphonate)
Chemical Name:	Calcium Lignosulphonate
Batch No.:	FGR-004
Aggregate State at Room Temperature:	Solid
Colour:	Brown
Molecular Weight Average: (Sample within range)	52,000 Da (93.9 % in range 1,000 – 250,000 Da)
Purity:	95.5 % (dry solids)
Certificate of Analysis (date):	August 26, 2005
Stability in Solvent:	Not indicated by the sponsor
Storage:	Room temperature, moisture protected
Expiration Date:	August 26, 2007

On the day of the experiment (immediately before treatment), the test item will be dissolved in a suitable solvent (e.g. acetone, ethanol, DMSO, or water). The final concentration of the solvent in the culture medium will not exceed 0.5 % (v/v) if an organic solvent is used. The solvent will be chosen to its solubility properties and its relative nontoxicity to the cell cultures.

5.2 Safety Precautions

Routine hygienic procedures will be sufficient to ensure personnel health and safety.

5.3 Controls

If possible, concurrent controls will be used for several RCC-CCR studies performed simultaneously.

5.3.1 Solvent Controls

Concurrent solvent controls will be performed.

5.3.2 Positive Control Substances

Without metabolic activation

Name:	EMS; ethylmethane sulfonate
Supplier:	ACROS ORGANICS, B-2440 Geel
Purity:	≥ 98 %
Lot no.:	Will be added in the final report
Expiry date:	Will be added in the final report
Dissolved in:	Nutrient medium
Final Concentration:	200 - 400 µg/mL (1.6 – 3.2 mM)

The solutions will be prepared on the day of experiment. The stability of the positive control substance in solution is proven by the mutagenic response in the expected range.

With metabolic activation

Name:	CPA; cyclophosphamide
Supplier:	Aldrich Chemie, D-89555 Steinheim
Purity:	≥ 98 %
Lot no.:	Will be added in the final report
Expiry date:	Will be added in the final report
Dissolved in:	Saline (0.9 % NaCl [w/v])
Final Concentration:	1.0 - 2.0 µg/mL (3.5 – 7.0 µM)

The dilutions of the stock solutions will be prepared on the day of experiment. The stability of CPA in solution at room temperature is good. At 25° C only 3.5 % of its potency is lost after 24 hours (6).

5.4 Test System

5.4.1 Reasons for the Choice of the Cell Line V79

The V79 cell line has been used successfully for many years in *in vitro* experiments. Especially the high proliferation rate (doubling time of clone V79/D3 in stock cultures: 12 hrs, determined on January 17, 2005) and a reasonable plating efficiency of untreated cells (as a rule more than 70 %) both necessary for the appropriate performance of the study, recommend the use of this cell line. The cells have a stable karyotype with a modal chromosome number of 22.

Lacking metabolic activities of cells under *in vitro* conditions are a disadvantage of tests with cell cultures as many chemicals only develop a mutagenic potential when they are metabolized by the mammalian organism. However, metabolic activation of chemicals can be achieved at least partially by supplementing the cell cultures with liver microsome preparations (S9 mix).

5.4.2 Cell Cultures

Large stocks of the V79 cell line (obtained from LMP; Technical University Darmstadt, D-64287 Darmstadt) are stored in liquid nitrogen in the cell bank of RCC Cytotest Cell Research GmbH. This allows the repeated use of the same cell culture batch in experiments. Before freezing each batch is screened for mycoplasm contamination and checked for karyotype stability. Consequently, the parameters of the experiments remain similar because of the reproducible characteristics of the cells.

Thawed stock cultures are propagated at 37° C in 80 cm^2 plastic flasks (GREINER, D-72632 Frickenhausen). About 5×10^5 cells per flask will be seeded in 15 mL of MEM (minimal essential medium; SEROMED; D-12247 Berlin) supplemented with 10 % fetal calf serum (FCS; PAA Laboratories GmbH, D-35091 Cölbe). The cells are subcultured twice weekly. The cell cultures are incubated at 37° C in a humidified atmosphere with 1.5 % carbon dioxide (98.5% air).

5.5 Mammalian Microsomal Fraction S9 Mix

5.5.1 S9 (Preparation by RCC Cytotest Cell Research)

Phenobarbital/β-naphthoflavone induced rat liver S9 is used as the metabolic activation system. The S9 is prepared from 8 – 12 weeks old male Wistar Hanlbm rats, weight approx. 220 – 320 g (supplied from RCC Ltd; Laboratory Animal Services, CH-4414 Füllinsdorf) induced by applications of 80 mg/kg b.w. phenobarbital i.p. (Desitin; D-22335 Hamburg) and β-naphthoflavone p.o. (Aldrich, D-89555 Steinheim) each on three consecutive days. The livers are prepared 24 hours after the last treatment. The S9 fractions are produced by dilution of the liver homogenate with a KCl solution (1:3 parts) followed by centrifugation at 9000 g. Aliquots of the supernatant are frozen and stored in ampoules at -80° C. Small numbers of the ampoules can be kept at –20° C for up to one week. The protein concentration in the S9 preparation is usually between 20 and 45 mg/mL.

5.5.2 S9 Mix

An appropriate quantity of S9 supernatant is thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. Cofactors will be added to the S9 mix to reach the following concentrations:

8 mM $MgCl_2$
33 mM KCl
5 mM Glucose-6-phosphate
4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

During the experiment the S9 mix is stored in an ice bath. The S9 mix preparation will be performed according to Ames et al. (1).

5.6 Dose Selection

Dose selection will be performed according to the current OECD Guideline for chromosomal aberration studies. If not toxic, the test item should be tested up to a maximum concentration of 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest. The highest treatment concentration chosen for the first cytogenetic experiment should inhibit the cell growth and/or the mitotic index by > 50 %. The solubility of the test item and changes in the pH value and the osmolarity does influence the dose selection.

5.7 Range-finder

A pre-test on cell growth inhibition will be performed in order to determine the toxicity of the test item (2), the solubility during exposure and changes in osmolarity and pH value at experimental conditions. The test item will be applied up to the maximum concentration mentioned above, unless solubility, pH value or osmolarity influence dose selection. Relatively insoluble substances will be tested up to the highest concentration possible, formulated in an appropriate solvent yielding a homogeneous suspension.

The range finder results will be provided to the scientific monitor to discuss the test item concentrations to be applied in the main experiments.

5.8 Cytogenetic Experiment

Dose selection will be performed considering to the current OECD Guideline for chromosomal aberration studies and the results of the pre-test. The solubility will be assessed at the start and the end of the exposure period, since solubility may change during exposure. The precipitate should not interfere with the scoring.

If not toxic, the test item should be tested up to a maximum concentration of 5 mg/mL, 5 µL/mL or 10 mM, as mentioned above.

For relatively insoluble substances that are not toxic at concentrations lower than the insoluble concentrations, the highest dose should be a concentration above the limit of solubility in the final culture medium at the end of the exposure period.

In some cases (e.g. when toxicity occurs only at higher than the lowest insoluble concentration), it is advisable to test at more than one concentration with visible precipitation.

The Chromosome Aberration Test will be conducted in two independent experiments, in the presence and the absence of S9 mix. The concentrations to be used in both studies will be confirmed by the scientific monitor.

According to the criteria mentioned above at least three adequately separated concentrations will be evaluated for cytogenetic damage, using at least two independent cultures per concentration.

If no toxicity at soluble concentrations is observed and precipitation occurs, at least four concentrations will be evaluated at preparation interval 18 hrs (the highest evaluable concentration, one concentration at the limit of solubility and two concentrations in the soluble range) and at least three concentrations will be evaluated at preparation interval 28 hrs (the highest evaluable concentration, one concentration at the limit of solubility, and one concentration in the soluble range).

5.9 Experimental Performance Pre-Test on Toxicity

A pre-test on cell growth inhibition with 4 hrs and 24 hrs treatment will be performed in order to determine the toxicity of the test item (2). Cytotoxicity will be determined using concentrations separated by no more than a factor of 2 - √10. The general experimental conditions in this pre-test will be the same as described below for the cytogenetic main experiment. The following method will be used:

In a quantitative assessment, exponentially growing cell cultures (seeding 10,000 – 60,000 cells/slide, with regard to the culture time 48 - 72 hrs) will be treated with the test item for simulating the conditions of the main experiment. A qualitative evaluation of cell number and cell morphology will be made after 4 hrs and 24 hrs after start of treatment. 24 hrs after start of treatment the cells will be stained and in 10 coordinate defined fields of the slides (2 slides per treatment group) the cells will be counted. Cytotoxicity is characterised by the number of cells (in percent) in comparison with the solvent control.

5.10 Experimental Performance Cytogenetic Experiment

5.10.1 Schedule

Table 1: Study Design

	Without S9-mix			With S9-Mix	
	Exp. I	Exp. II		Exp. I	Exp. II
Exposure period	4 hrs	18 hrs	28 hrs	4 hrs	4 hrs
Recovery	14 hrs	-	-	14 hrs	24 hrs
Preparation interval	18 hrs	18 hrs	28 hrs	18 hrs	28 hrs

5.10.2 Seeding of the Cultures

Exponentially growing stock cultures more than 50 % confluent are treated with trypsin-EDTA-solution at 37° C for approx. 5 minutes. Then the enzymatic treatment is stopped by adding complete culture medium and a single cell suspension is prepared. The trypsin concentration for all subculturing steps is 0.5 % (w/v) in Ca-Mg-free salt solution (Invitrogen GIBCO, D-76131 Karlsruhe).

Prior to the trypsin treatment the cells are rinsed with Ca-Mg-free salt solution.
The Ca-Mg-free salt solution is composed as follows (per litre):

NaCl	8000 mg
KCl	200 mg
KH_2P0_4	200 mg
Na_2HP0_4	150 mg

The cells are seeded into Quadriperm dishes (Heraeus, D-63450 Hanau) which contain microscopic slides (at least 2 chambers per dish and test group). Into each chamber 1×10^4 - 6×10^4 cells are seeded with regard to preparation time. The medium is MEM with 10 % FCS.

5.10.3 Treatment

Exposure period 4 hrs:

The culture medium of exponentially growing cell cultures is replaced with serum-free medium (for treatment with S9 mix) or complete medium (for treatment without S9 mix) with 10 % FCS (v/v), containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL medium will be used. Concurrent solvent and positive controls will be performed. After 4 hrs the cultures are washed twice with "Saline G" and then the cells will be cultured in complete medium for the remaining culture time.

The "Saline G" solution is composed as follows (per litre):

NaCl	8000 mg
KCl	400 mg
Glucose x H_20	1100 mg
Na_2HP0_4 x $7H_20$	290 mg
KH_2P0_4	150 mg

pH is adjusted to 7.2.

Exposure period 18 hrs and 28 hrs:

The culture medium of exponentially growing cell cultures is replaced with complete medium (with 10 % FCS) containing the test item without S9 mix. This medium will not be changed until preparation of the cells. Concurrent solvent and positive controls will be performed.

All cultures are incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

5.10.4 Preparation of the Cultures

15.5 hrs and 25.5 hrs after the start of the treatment colcemid is added to the cultures (0.2 µg/mL culture medium). 2.5 hrs later, the cells are treated on the slides in the chambers with hypotonic solution (0.4 % KCl) for 20 min at 37° C. After incubation in the hypotonic solution the cells are fixed with a mixture of methanol and glacial acetic acid (3:1 parts, respectively) for at least 60 min. After preparation the cells will be stained with Giemsa (Merck, D-64293 Darmstadt).

5.10.5 Evaluation of Cell Numbers

For evaluation of cytotoxicity indicated by reduced cell numbers two additional cultures per test item and solvent control group, not treated with colcemid, will be set up in parallel. These cultures will be stained after 18 hrs and 28 hrs, respectively, in order to determine microscopically the cell number within 10 defined fields per coded slide. The cell number of the treatment groups will be given in percentage compared to the respective solvent control.

5.10.6 Analysis of Metaphase Cells

Evaluation of the cultures will be performed (according to standard protocol of the "Arbeitsgruppe der Industrie, Cytogenetik" [5]) using NIKON microscopes with 100 x oil immersion objectives. Breaks, fragments, deletions, exchanges and chromosome desintegrations are recorded as structural chromosome aberrations. Gaps are recorded as well but not included in the calculation of the aberration rates. At least 100 well spread metaphase plates per culture are scored for cytogenetic damage on coded slides. In the case of strong clastogenicity the sample for evaluation can be reduced to 50 metaphase plates per culture in the respective test group. Only metaphases with the characteristic chromosome number of 22 ± 1 are included in the analysis. To describe a cytotoxic effect the mitotic index (% cells in mitosis) is determined. In addition, the number of polyploid cells in 500 metaphase cells per culture (% polyploid metaphases; in the case of this aneuploid cell line polyploid means a near tetraploid karyotype) is scored.

5.11 Data Recording

The data generated will be recorded in the raw data file. Individual culture data will be presented in tabular form, including experimental groups with the test item and the controls (positive and solvent controls).

5.12 Acceptability of the Test

The chromosome aberration test is considered acceptable if it meets the following criteria:

a) The number of aberrations found in the solvent controls falls within the range of historical laboratory control data: 0.0 - 4.0 % aberrant cells, exclusive gaps.

b) The positive control substances should produce significant increases in the number of cells with structural chromosome aberrations, which are within the range of the laboratory´s historical control data:

Table 2: RCC-CCR Laboratory´s historical positive control data

Test group Final concentration	Aberrant cells in % (excl. gaps) range	Test group Final concentration	Aberrant cells in % (excl. gaps) range
Without S9 mix		**With S9 mix**	
EMS 200 – 400 µg/mL	7.0 - 63.0 %	CPA 0.7 – 1.4 µg/mL	7.0 - 49.0 %

5.13 Evaluation of Results

Following analyses will be carried out:

– The percentage of cells with structural chromosome aberration(s) will be evaluated.

– Different types of structural chromosome aberrations will be listed with their numbers and frequencies for experimental and control cultures.

– Gaps are recorded separately and reported, but generally not included in the total aberration frequency.

– Concurrent measures of cytotoxicity for all treated and solvent control cultures in the main aberration experiment(s) will be recorded.

– Equivocal results will be clarified by further testing preferably using modification of experimental conditions (after prior consultation with the sponsor/scientific monitor).

A test item can be classified as non-clastogenic if:

– the number of induced structural chromosome aberrations in all evaluated dose groups is in the range of our historical control data (0.0 - 4.0 % aberrant cells, exclusive gaps).

and/or

– no significant increase of the number of structural chromosome aberrations is observed.

A test item can be classified as clastogenic if:

– the number of induced structural chromosome aberrations is not in the range of our historical control data (0.0 - 4.0 % aberrant cells, exclusive gaps).

and

– either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.

Statistical significance will be confirmed by means of the Fisher´s exact test (9) ($p < 0.05$). However, both biological and statistical significance should be considered together. If the criteria above mentioned for the test item are not clearly met, the classification with regard to the historical data and the biological relevance is discussed and/or a confirmatory experiment is performed.

Although the inclusion of the structural chromosome aberrations is the purpose of this study, it is important to include the polyploids and endoreduplications. The following criteria is valid:

A test item can be classified as aneugenic if:

- the number of induced numerical aberrations are not in the range of our historical control data (0.0 - 8.5 % polyploid cells).

6 REPORTING

A GLP-compliant draft report will be submitted to the scientific monitor for scientific review. Following receipt of the scientific monitor´s comments, a QA-audited final report will be issued.

The report will contain all relevant information about the test item (e.g. Certificate of Analysis, etc) as well as the study protocol.

The report will be in line with EPA-format requirements.

The report will cover all information required by the relevant OECD and EU guidelines.

The scientific monitor will receive 1 Original (bound) and two copies (unbound) and one complete pdf-file of the report.

7 DISTRIBUTION

Scientific monitor	4 × (1 x original, to be returned to RCC-CCR, 1 x duplicate; 1 x copy, and 1 x pdf-file)
Study Director	1 × (copy)
QAU	1 × (copy)

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9 ANNEX

9.1 Historical laboratory control data

Table 3: RCC-CCR Laboratory´s historical data
In vitro Chromosome Aberration Test in Chinese hamster V79 cells (2003 to 2004)

Without S9 mix										
		Aberrant cells (%)								
		Inclusive gaps			Exclusive gaps			With exchanges		
Test group Concentration	Cells scored	Range	Mean	Calculated range*	Range	Mean	Calculated range*	Range	Mean	Calculated range*
Negative control										
Culture medium MEM	48800	0.0-6.0	1.6	0.8-2.4	0.0-4.0	0.9	0.4-1.5	0.0-1.0	0.1	0.0-0.3
Aqueous solv. ** 10 % (v/v)	18800	0.0-4.5	1.6	0.8-2.3	0.0-3.5	1.0	0.4-1.5	0.0-1.0	0.1	0.0-0.4
Org. solvents*** 0.5 % (v/v)	24600	0.0-5.5	1.6	0.7-2.5	0.0-4.0	0.9	0.3-1.5	0.0-1.5	0.2	0.0-0.4
Total	**92200**	**0.0-6.0**	**1.6**	**0.8-2.4**	**0.0-4.0**	**0.9**	**0.4-1.5**	**0.0-1.5**	**0.1**	**0.0-0.3**
Positive control										
EMS 200–400 µg/mL	48800	8.5-66.0	18.7	11.8-25.6	7.0-63.0	16.9	10.1-23.7	0.5-30.0	6.8	3.4-10.2
With S9 mix										
		Aberrant cells (%)								
		Inclusive gaps			Exclusive gaps			With exchanges		
Test group Concentration	Cells scored	Range	Mean	Calculated range*	Range	Mean	Calculated range*	Range	Mean	Calculated range*
Negative control										
Culture medium MEM	35400	0.0-5.0	1.9	0.9-2.8	0.0-4.0	1.2	0.5-1.9	0.0-2.0	0.3	0.0-0.7
Aqueous solv. ** 10 % (v/v)	14000	0.0-5.0	2.0	0.9-3.1	0.0-4.3	1.4	0.6-2.2	0.0-2.0	0.3	0.0-0.7
Org. solvents*** 0.5 % (v/v)	17200	0.0-5.0	1.9	1.0-2.8	0.0-4.0	1.2	0.4-1.9	0.0-2.0	0.3	0.0-0.6
Total	**66600**	**0.0-5.0**	**1.9**	**0.9-2.8**	**0.0-4.3**	**1.2**	**0.5-2.0**	**0.0-2.0**	**0.3**	**0.0-0.7**
Positive control										
CPA 0.7–1.4 µg/mL	35200	8.0-50.0	14.5	10.6-18.5	7.0-49.0	12.9	9.3-16.5	0.0-19.0	4.6	2.4-6.8

* Mean ± standard deviation

** Aqueous solvents: deionised water and 0.9 % (w/v) saline

*** Organic solvents: acetone, DMSO, ethanol, and tetrahydrofurane

17 ANNEX VI

17.1 Copy of the 1st Amendment to Study Plan

(3 pages)

RCC-CCR STUDY NUMBER 899102

1ST AMENDMENT TO STUDY PLAN

(3 pages)

TITLE: ***IN VITRO*** **CHROMOSOME ABERRATION TEST IN CHINESE HAMSTER V79 CELLS WITH Ultrazine FG-R (Food Grade Lignosulphonate)**

SPONSOR: **DSM Nutritional Products AG**
Wurmisweg 576
CH-4303 Kaiseraugst
Switzerland

SCIENTIFIC MONITOR (ON BEHALF OF THE SPONSOR): **Dr. Werner Köhl**
SCC Scientific Consulting Company
Chemisch-Wissenschaftliche Beratung GmbH
Mikroforum Ring 1
D-55234 Wendelsheim
Germany

DATE OF AMENDMENT: April 07, 2006

CONCERNING: **3.2 Responsibilities**
- page 4 -

PRESENT:

Study Director:	Dr. Markus Schulz
Deputy Study Director:	Dr. Susanne Kunz
Management:	Dr. Wolfgang Völkner
Head of Quality Assurance Unit:	Frauke Hermann

N E W:

Study Director:	**Dr. Markus Schulz (until February 28, 2006)** **Dr. Susanne Kunz (from March 01, 2006)**
Management:	Dr. Wolfgang Völkner
Head of Quality Assurance Unit:	Frauke Hermann

REASON FOR THE ALTERATION:

Change of the study director.

DISTRIBUTION

Scientific monitor	4 x (1 x original to be returned to RCC-CCR, 1 x duplicate, 1 x copy, and 1 x pdf-file)
Study Director	1 x (copy)
QAU	1 x (copy)

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SIGNATURES

Study Director — Dr. Susanne Kunz

Date: April 07, 2006

Management — Dr. Wolfgang Völkner

Date: April 07, 2006

Head of
Quality Assurance Unit — Frauke Hermann

Date: April 11, 2006

Scientific Monitor
(on behalf of the Sponsor)

Date: 27 APR. 2006

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