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Givaudan

March 11, 2011

Via email and USPS

Matthew G. Leopard, Director
Information Management Division
USEPA Headquarters
Ariel Rios Building
1200 Pennsylvania Avenue, N. W.
Mail Code: 7407M
Washington, DC 20460

8EHQ-06-16409

Re: Withdrawal of Confidentiality Claims

Dear Mr. Leopard,

We are writing in response to three letters from you dated February 8, 2011 regarding claims of confidentiality for the identity of five chemical substances that were the subject of reports under Section 8(e) of the Toxic Substances Control Act ("TSCA") dated March 27, 2006, July 1, 2008, and April 21 and May 19, 2009. Givaudan hereby withdraws all claims of confidentiality made with respect to the Section 8(e) reports in question. This withdrawal relates only to the five reports referenced in your letters, not to any other submission that Givaudan may have made under TSCA.

Please note this withdrawal of confidentiality is intended only to allow EPA to reveal to the public the information that was claimed confidential at the time of the reports in question. Givaudan is not relinquishing any property rights in the studies in question.

If you have any questions regarding this letter, please contact our counsel, Thomas B. Johnston, McKenna Long & Aldridge LLP, at (202) 496-7656.

Sincerely,

Greg Adamson, Ph.D.
Vice President Regulatory Affairs & Product Safety
Givaudan Fragrances Corporation

cc: Mr. Scott M. Sherlock (OPPTS)
Mr. Ryan Wallace (OPPTS)

8911000017 6

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Givaudan

Delivery via Federal Express

March 27, 2006

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TSCA Confidential Business Information Center (7407M)
EPA East – Room 6428 Attn: Section 8(e)
U.S. Environmental Protection Agency
1201 Constitution Avenue, NW
Washington, DC 20004-3302

~~TSCA CONFIDENTIAL BUSINESS INFORMATION~~ RPW
~~— DOES NOT CONTAIN NATIONAL — SECURITY INFORMATION (E.O. 12065)~~

Dear Sir/Madam:

The enclosed report entitled "*in vitro* Chromosome Aberration Test in Chinese Hamster V79 Cells with Safraleine" provides the results of a mutagenicity study performed with 2,3,3-TRIMETHYLINDAN-1-ONE, CAS# 54440-17-4. Please note this report was received by us on March 1, 2006.

Dose selection for the cytogenetic experiments was performed considering the toxicity data. The chosen treatment concentrations are described in the report itself.

In the absence and the presence of S9 mix, concentrations showing clear cytotoxicity were not scorable for cytogenetic damage.

In the presence of S9 mix, a statistically significant and biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item. A dose-dependent increase in chromosomal aberrations was found in the absence and the presence of S9 mix. Additionally, the number of cells carrying exchanges was increased in the experimental part with S9 mix in the highest evaluated concentration.

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.

In conclusion, it can be stated that under the experimental conditions reported, the test item induced structural chromosome aberrations as determined by the chromosome aberration test in V79 cells (Chinese hamster cell line) *in vitro*.

Therefore, Safraleine is considered to be clastogenic in this chromosome aberration test in the presence of metabolic activation.

It should be noted that Safraleine showed no effects in a bacterial mutation assay (according to OECD 471 Guidelines) both in the presence and absence of metabolic activation.

Givaudan Fragrances Corporation
1775 Windsor Road Teaneck NJ 07666 USA
T 201 833 7500 F 201 833 8165 www.givaudan.com

8EHQ-06-15409

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This report is being submitted in accordance with the guidelines contained in the TSCA Section 8(e) Reporting Guide and subsequent guidance documents.

Please note that certain information in the enclosed study are claimed TSCA Confidential Business Information (CBI). Therefore, we are also submitting an excised copy of the study for the public record.

Should EPA have any questions or comments concerning this submission please contact me.

Sincerely,
Givaudan Fragrances Corporation

Barbara Lewis

Barbara Lewis, Ph.D.
Manager Regulatory Affairs
201-833-7561

Enclosures

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RCC-CCR STUDY NUMBER 905407

IN VITRO

CHROMOSOME ABERRATION TEST IN CHINESE HAMSTER V79 CELLS WITH SAFRALEINE

FINAL REPORT

Study Completion Date:

February 14, 2006

293992

1 COPY OF GLP CERTIFICATE

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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 facility ☐ Prüfstandort/Test site

RCC Cytotest Cell Research GmbH
RCC Cytotest Cell Research GmbH
In den Leppstainwiesen 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
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)
8 Analytische Prüfungen an biologischen Materialien
9 Virussicherheitsprüfungen

2 Toxicity studies
3 Mutagenicity studies
8 Analytical studies on biological materials
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 2005
(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)

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3 PREFACE

3.1 General

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Title:	*In vitro* Chromosome Aberration Test in Chinese Hamster V79 Cells with SAFRALEINE
Sponsor:	Givaudan Suisse SA 5, chemin de la Parfumerie CH-1214 Vernier Switzerland
Study Monitor:	Ms. Sophie Dubois
Test Facility:	RCC Cytotest Cell Research GmbH (RCC-CCR) In den Leppsteinswiesen 19 D-64380 Rossdorf Germany
Contracting Institute:	RCC Ltd CH-4452 Itingen Switzerland
Reference Number:	A25863

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:	August 30, 2005
Experimental Starting Date:	September 28, 2005
Experimental Completion Date:	November 11, 2005
Date of the Draft Report:	December 13, 2005
Study Completion Date:	February 14, 2006

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3.4 Project Staff Signatures

Study Director	Dr. Markus Schulz M. Schulz Date: February 14, 2006
Management	Dr. Wolfgang Völkner G. Völkner Date. February 14, 2006

3.5 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].

3.6 Guidelines

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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".

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

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.

3.8 Deviations from the Study Plan

There were no deviations from the study plan.

4 STATEMENT OF COMPLIANCE

~~CONFIDENTIAL~~ RPW

Study Number.	905407
Test Item:	SAFRALEINE
Study Director.	Dr. Markus Schulz
Title:	*In vitro* Chromosome Aberration Test in Chinese Hamster V79 Cells with SAFRALEINE

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].

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

Study Director

RCC Cytotest Cell Research GmbH
Dr. Markus Schulz

M. Schulz

Date: February 14, 2006

5 STATEMENT OF QUALITY ASSURANCE UNIT

Study Number: 905407

Test Item: SAFRALEINE

Study Director: Dr. Markus Schulz

~~CONFIDENTIAL~~ RPW

Title: *In vitro* Chromosome Aberration Test in Chinese Hamster V79 Cells with SAFRALEINE

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):	August 04, 2005	August 04, 2005
Study Plan:	August 31, 2005	-
Process Inspection:	October 26, 2005	October 26, 2005
Draft Report:	January 03, 2006	January 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: February 14, 2006

6 SUMMARY OF RESULTS

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The test item SAFRALEINE, dissolved in ethanol, was assessed for its potential to induce structural chromosome aberrations in V79 cells of the Chinese hamster *in vitro* in one experiment. The following study design was performed:

	With and without S9 mix
Exposure period	4 hrs
Recovery	14 hrs
Preparation interval	18 hrs

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

The highest applied concentration in the pre-test on toxicity (1750 µg/mL; approx. 10 mM) was chosen with regard to the molecular weight of the test item with respect to the current OECD Guideline 473.

Dose selection for the cytogenetic experiments was performed considering the toxicity data. The chosen treatment concentrations are described in Table 2 (page 16). The evaluated experimental points and the results are summarised in Table 1 (page 10).

In the absence and the presence of S9 mix, concentrations showing clear cytotoxicity were not scorable for cytogenetic damage.

In the presence of S9 mix, a statistically significant and biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item. A dose-dependent increase in chromosomal aberrations was found in the absence and the presence of S9 mix. Additionally, the number of cells carrying exchanges was increased in the experimental part with S9 mix in the highest evaluated concentration.

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

In conclusion, it can be stated that under the experimental conditions reported, the test item induced structural chromosome aberrations as determined by the chromosome aberration test in V79 cells (Chinese hamster cell line) *in vitro*.

Therefore, SAFRALEINE is considered to be clastogenic in this chromosome aberration test in the presence of metabolic activation.

Table 1: Summary of results of the chromosome aberration study with SAFRALEINE

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				
18 hrs	Solvent control[1]	1.8	100.0	100.0	1.5	1.5	0.0
	Positive control[2]	3.1	n.t.	77.7	20.5	**19.5**[S]	7.0
	7.8	n.d.	133.7	100.0	n.d.	n.d.	n.d.
	15.6	n.d.	164.2	103.3	n.d.	n.d.	n.d.
	31.3	2.7	112.3	99.6	0.5	0.5	0.0
	62.5	1.7	103.5	90.1	3.0	2.5	0.5
	125.0	3.3	115.2	92.6	3.5	3.5	0.0
	250.0	n.d.	3.7	0.0	n.e.	n.e.	n.e.
			Exposure period 4 hrs with S9 mix				
18 hrs	Solvent control[1]	2.0	100.0	100.0	1.0	1.0	0.5
	Positive control[3]	2.7	n.t.	100.4	14.5	**14.0**[S]	3.5
	3.9	n.d.	114.5	109.9	n.d.	n.d.	n.d.
	7.8	2.3	103.8	105.6	3.5	2.5	0.5
	15.6	2.8	108.4	107.3	4.0	3.5	0.5
	31.3	2.4	77.3	79.0	25.0	**25.0**[S]	9.5
	62.5	n.d.	37.5	0.0	n.e.	n.e.	n.e.
	125.0	n.d.	21.1	0.0	n.e.	n.e.	n.e.

* Inclusive cells carrying exchanges
n.d. Not determined
n.e. Not evaluable
n.t. Not tested
S Aberration frequency statistically significant higher than corresponding control values
1 Ethanol 0.5 % (v/v)
2 EMS 400.0 µg/mL
3 CPA 1.4 µg/mL

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7 INTRODUCTION

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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 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 develops, possibly leading to neoplastic processes.

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

For treatment, cell cultures 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 13) 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. Because there may be chemicals which induce a very extensive mitotic delay at clastogenic concentrations or may be clastogenic only when cells have passed through more than one cell cycle after 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 included (7).

The frequency of polyploid/endoreduplicated cells should also be noted 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 were tested in parallel to the test item.

7.1 Aims of the Study

This *in vitro* test was performed to assess the potential of SAFRALEINE 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 one experiment at 18 hrs preparation interval.

8 MATERIALS AND METHODS

8.1 Test Item

RPW

Internal RCC-CCR Test Item Number:	S 5726 11

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

Identity:	SAFRALEINE
Batch No.:	9000611694
Aggregate State at Room Temperature:	Liquid
Colour:	Colourless to pale yellow
Molecular Weight:	174.2 g/mol
Purity:	99.5 %
Solubility in Water:	446 mg/L
Stability in Solvent:	Not indicated by the sponsor
Storage:	2 - 8 °C, protected from light
Expiration Date:	June 25, 2007

On the day of the experiment (immediately before treatment), the test item was dissolved in ethanol (E. MERCK, D-64293 Darmstadt; purity 99.8 %). The final concentration of ethanol in the culture medium was 0.5 % (v/v). The solvent was chosen to its solubility properties and its relative nontoxicity to the cell cultures.

8.2 Controls

8.2.1 Solvent Controls

Concurrent solvent controls (ethanol) were performed.

8.2.2 Positive Control Substances

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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:	400 µg/mL (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
Expiration Date:	December 2007
Dissolved in:	Saline (0.9 % [w/v])
Final Concentration:	1.4 µg/mL (5.0 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.

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).

8.3.2 Cell Cultures

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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 standardized 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 was used as the metabolic activation system. The S9 was 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 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).

8.4.2 S9 Mix

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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 treatment 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 cytogenetic main 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 for 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 coordinate 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 nontoxicity the maximum concentration should be 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest, if formulability in an appropriate solvent is possible.
With respect to the molecular weight of the test item, 1750 µg/mL of SAFRALEINE (approx. 10 mM) were applied as top concentration for treatment of the cultures in the pre-test. Test item concentrations between 13.7 and 1750 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. In the pre-test on toxicity, precipitation of the test item was observed after 4 hrs treatment at 437.5 µg/mL and above with and without S9 mix.

Using reduced cell numbers as an indicator for toxicity in the pre-test, clear toxic effects were observed after 4 hrs treatment with 218.8 µg/mL and above in the absence of S9 mix and with 54.7 µg/mL and above in the presence of S9 mix. Considering the toxicity data of the pre-test, 250 µg/mL (without S9 mix) and 125 µg/mL (with S9 mix) were chosen as top concentrations in the main experiment. Since the test item was considered to be clastogenic after 4 hrs treatment the second experiment performed was not evaluated. The applied concentrations in the cytogenetic experiment are presented in Table 2 below.

Table 2: Doses applied in the Chromosome aberration test with SAFRALEINE

Preparation interval	Exposure period	Concentration in µg/mL					
		Without S9 mix					
18 hrs	4 hrs	7.8	15.6	**31.3**	**62.5**	**125.0**	250.0
		With S9 mix					
18 hrs	4 hrs	3.9	**7.8**	**15.6**	**31.3**	62.5	125.0

Evaluated experimental points are shown in bold characters

The cytogenetic evaluation of the highest concentrations in the respective experimental parts (with and 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).

8.7 Experimental Performance

8.7.1 Schedule

	With and without S9 mix
Exposure period	4 hrs
Recovery	14 hrs
Preparation interval	18 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

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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
$Na_2HP0_4 \times 7H_20$	290 mg
KH_2P0_4	150 mg

pH was adjusted to 7.2

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 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

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For evaluation of cytotoxicity indicated by reduced cell numbers two additional cultures per test item and solvent control group, not treated with colcemid, were set up in parallel. These cultures were stained after 18 hrs, 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 "Arbeitsgruppe 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.
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

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

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 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:

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

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

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The test item SAFRALEINE, dissolved in Ethanol, 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.

According to the OECD Guideline only one experiment was performed and evaluated, since the test item was considered to be mutagenic after 4 hrs treatment. The chromosomes were prepared 18 hrs after start of treatment with the test item. The exposure period was 4 hrs with and without metabolic activation.

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

In a range finding pre-test on toxicity cell numbers 24 hrs after start of treatment were scored as an indicator for cytotoxicity. Concentrations between 13.7 and 1750 µg/mL were applied. Clear toxic effects were observed after treatment with 218.8 µg/mL and above in the absence of S9 mix and with 54.7 µg/mL and above in the presence of S9 mix (see Table 4, page 23).

In the pre-experiment, precipitation of the test item in culture medium was observed after treatment with 437.5 µg/mL and above in the absence and presence of S9 mix. No influence of the test item on the pH value or osmolarity was observed (solvent control 442 mOsm, pH 7.4 versus 390 mOsm and pH 7.4 at 1750 µg/mL).

Table 3: Evaluated experimental points after treatment with SAFRALEINE

Preparation interval	Exposure period	Concentration in µg/mL			Preparation interval	Exposure period	Concentration in µg/mL		
		Without S9 mix					With S9 mix		
18 hrs	4 hrs	31.3	62.5	125.0	18 hrs	4 hrs	7.8	15.6	31.3

In the main experiment, in the absence and presence of S9 mix, no clear toxic effects indicated by reduced cell numbers (see Table 5, page 24) and/or mitotic indices (see Table 6, page 25) of below 50 % of control were observed up to the highest scorable test item concentration. However, concentrations showing clear cytotoxic effects were not scorable for cytogenetic damage.

In this study, in the absence of S9 mix, a dose-dependent increase of aberrant metaphase cells (0.5 %, 2.5 % and 3.5 %) was observed in the concentration range scored: 31.3 to 125 µg/mL (see Table 7, page 26). However, these values were clearly within our historical control data range (0.0 - 4.0 % aberrant cells, exclusive gaps), and, therefore, this observation has to be regarded as biologically irrelevant.
In contrast, in the presence of S9 mix, a dose-dependent increase in cells carrying structural chromosome aberrations (2.5 %, 3.5 %, and 25 %) was observed in the concentration range scored: 7.8 to 31.3 µg/mL. The response (25 % aberrant cells, exclusive gaps) at the highest evaluated concentration (31.3 µg/mL) was statistically significant (see Table 8, page 27) and clearly exceeded our laboratory's historical control data range (0.0 – 4.0 % aberrant cells,

exclusive gaps). Additionally, the number of cells carrying exchanges (9.5 %) was distinctly increased at this test item concentration compared with the respective solvent control (0.5 %). Therefore, the dose-dependency and the statistical significance have to be reagarded as being biologically relevant.

Table 6, page 25, shows the occurrence of polyploid metaphases. In this experiment, no biologically relevant increase in the rate of polyploid metaphases was found after treatment with the test item (1.7 – 3.3 %) as compared to the rates of the solvent controls (1.8 – 2.0 %).

EMS (400 µg/mL) and CPA (1.4 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations.

In conclusion, it can be stated that under the experimental conditions reported, the test item SAFRALEINE induced structural chromosome aberrations in V79 cells (Chinese hamster cell line) in the presence of metabolic activation.

10 DISTRIBUTION OF THE REPORT

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Sponsor	2x (1x copy, 1x electronic copy as PDF-file)
Contracting Institute	1x (electronic copy as PDF-file)
Study Director	1x (original)

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12 ANNEX I: TABLES OF RESULTS

12.1 Pre-Test on Toxicity

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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 SAFRALEINE 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	802	100.0	Solvent control	431	100.0
13.7	975	121.6	13.7	684	158.7
27.3	932	116.3	27.3	260	60.2
54.7	784	97.8	54.7	172	39.9
109.4	651	81.2	109.4	94	21.7
218.8	0	0.0	218.8	3	0.6
437.5[P]	0	0.0	437.5[P]	27	6.3
875.0[P]	0	0.0	875.0[P]	0	0.0
1750.0[P]	0	0.0	1750.0[P]	0	0.0

Without S9 mix; 24 hrs exposure

Concentration in µg/mL	Number of cells	% of solvent control
Solvent control	691	100.0
13.7	591	85.5
27.3	714	103.3
54.7	561	81.2
109.4	359	52.0
218.8	0	0.0
437.5[P]	0	0.0
875.0[P]	0	0.0
1750.0[P]	0	0.0

[P] Precipitation occurred at the end of treatment

12.2 Main Experiment: Determination of Toxicity

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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, except the positive control.

Table 5: Number of cells in % of solvent control

Without metabolic activation (S9 mix)			With metabolic activation (S9 mix)		
Preparation interval	Concentration in µg/mL	Cells in % of solvent control*	Preparation interval	Concentration in µg/mL	Cells in % of solvent control
18 hrs	7.8	133.7	18 hrs	3.9	114.5
"	15.6	164.2	"	**7.8**	103.8
"	**31.3**	112.3	"	**15.6**	108.4
"	**62.5**	103.5	"	**31.3**	77.3
"	**125.0**	115.2	"	62.5	37.5
"	250.0	3.7	"	125.0	21.1

Experimental groups evaluated for cytogenetic damage were printed in bold characters

12.3 Main Experiment

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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#	0.5 %	-	4 / 14 hrs	9	9	18	1.8	11.8	12.4	12.1	100.0
Pos. control##	400.0 µg	-	4 / 14 hrs	16	15	31	3.1	9.1	9.7	9.4	77.7
Test item	31.3 µg	-	4 / 14 hrs	14	13	27	2.7	12.0	12.1	12.1	99.6
"	62.5 µg	-	4 / 14 hrs	7	10	17	1.7	8.9	12.9	10.9	90.1
"	125.0 µg	-	4 / 14 hrs	17	16	33	3.3	10.9	11.5	11.2	92.6
Solv. control#	0.5 %	+	4 / 14 hrs	8	12	20	2.0	11.8	11.5	11.7	100.0
Pos. control###	1.4 µg	+	4 / 14 hrs	16	11	27	2.7	11.1	12.3	11.7	100.4
Test item	7.8 µg	+	4 / 14 hrs	10	13	23	2.3	12.1	12.5	12.3	105.6
"	15.6 µg	+	4 / 14 hrs	20	8	28	2.8	12.1	12.9	12.5	107.3
"	31.3 µg	+	4 / 14 hrs	10	14	24	2.4	8.9	9.5	9.2	79.0

* 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

\# Ethanol

EMS

CPA

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

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Slide no.	Cells scored	% Aberrant cells incl. gaps*	excl. gaps*	with exchanges	Aberrations: Gaps g	ig	Chromatid type b	f	d	ex	Chromosome type ib	if	id	cx	Other ma	cd
					Without S9 mix											
Solvent control: Ethanol 0.5 %																
1	100				0	0	1	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	1.5	1.5	0.0	0	0	2	0	0	0	0	1	0	0	0	0
Positive control: EMS 400.0 µg / mL																
1	100				2	0	23	2	0	8	4	1	0	0	1	0
2	100				1	0	9	0	0	6	0	0	0	0	0	0
1 + 2	200	20.5	19.5	7.0	3	0	32	2	0	14	4	1	0	0	1	0
Test item: 31.3 µg / mL																
1	100				0	0	1	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	0.5	0.0	0	0	1	0	0	0	0	0	0	0	0	0
Test item: 62.5 µg / mL																
1	100				1	0	0	1	0	0	1	0	0	0	0	0
2	100				1	0	2	0	0	1	0	0	0	0	0	0
1 + 2	200	3.0	2.5	0.5	2	0	2	1	0	1	1	0	0	0	0	0
Test item: 125.0 µg / mL																
1	100				0	0	3	0	0	0	0	0	0	0	0	0
2	100				0	0	3	1	0	0	0	0	0	0	0	0
1 + 2	200	3.5	3.5	0.0	0	0	6	1	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)

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

Slide no.	Cells scored	% Aberrant cells incl. gaps*	excl. gaps*	with exchanges	Aberrations: Gaps g	Gaps ig	Chromatid type b	f	d	ex	Chromosome type ib	if	id	cx	Other ma	cd
					With S9 mix											
Solvent control: Ethanol 0.5 %																
1	100				0	0	0	0	0	1	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	0.5	0	0	0	0	0	1	1	0	0	0	0	0
Positive control: CPA 1.4 µg / mL																
1	100				1	0	4	1	0	5	2	0	0	0	0	0
2	100				0	0	8	7	0	4	3	2	0	0	0	0
1 + 2	200	14.5	14.0	3.5	1	0	12	8	0	9	5	2	0	0	0	0
Test item: 7.8 µg / mL																
1	100				1	0	2	0	0	0	0	0	0	0	0	0
2	100				1	0	2	0	0	1	0	0	0	0	0	0
1 + 2	200	3.5	2.5	0.5	2	0	4	0	0	1	0	0	0	0	0	0
Test item: 15.6 µg / mL																
1	100				1	0	3	0	0	2	0	1	0	0	0	0
2	100				0	0	2	0	0	0	0	0	0	0	0	0
1 + 2	200	4.0	3.5	0.5	1	0	5	0	0	2	0	1	0	0	0	0
Test item: 31.3 µg / mL																
1	100				0	0	14	6	0	11	8	3	0	0	0	0
2	100				2	0	11	4	0	9	5	2	0	0	0	0
1 + 2	200	25.0	25.0	9.5	2	0	25	10	0	20	13	5	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 Biometry

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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 9: Biometry of the Main Experiment

Test group versus solvent control		Preparation interval	Exposure period	S9 mix	p-value
Test group	31.3 µg/mL	18 hrs	4 hrs	-	n.c.
"	62.5 µg/mL	18 hrs	4 hrs	-	0.252
"	125.0 µg/mL	18 hrs	4 hrs	-	0.111
"	7.8 µg/mL	18 hrs	4 hrs	+	0.143
"	15.6 µg/mL	18 hrs	4 hrs	+	0.053
"	31.3 µg/mL	18 hrs	4 hrs	+	< 0.001[s]
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]

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

14 ANNEX III

14.1 Historical laboratory control data

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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