EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

HC Red n° 1

COLIPA N° B48

Adopted by the SCCP
during the 9th plenary meeting of 10 October 2006

TABLE OF CONTENTS

1. BACKGROUND

Submission I for HC Red N) 1 with the chemical name 2-Nitro-4-aminodiphenylamine was submitted in March 2003 by COLIPA[1,2].

Submission II for HC Red No. 1 was submitted by COLIPA in July 2005. According to this submission the substance is used in semi-permanent hair dye formulations at a maximum concentration of 1.0%.

Submission II presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider HC Red n° 1 safe for use as a non-oxidative hair dye with an on-head concentration of maximum 1.0 % taken into account the scientific data provided?*

2. *Does the SCCP recommend any further restrictions with regard to the use of HC Red n° 1 in any non-oxidative hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Red n° 1 (INCI name)

3.1.1.2. Chemical names

4-amino-2-nitrodiphenylamine
2-nitro-4-aminodiphenylamine
2-nitro-N'-phenyl-1,4-benzenediamine
2-nitro-N1-phenyl-benzene-1,4-diamine
2-nitro-N1-phenyl-p-phenylenediamine

3.1.1.3. Trade names and abbreviations

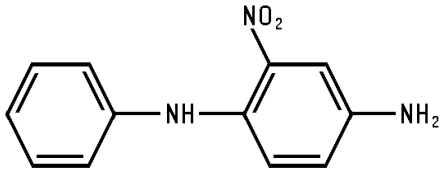
[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According to records of COLIPA

Trade name: /
COLIPA n°: B48
Other Code: GTS03974

3.1.1.4.	CAS / EINECS number

CAS : 2784-89-6
EINECS : 220-494-3

3.1.1.5.	Structural formula



3.1.1.6.	Empirical formula

Formula : $C_{12}H_{11}N_3O_2$

3.1.2.	Physical form

Green brown crystalline powder

3.1.3.	Molecular weight

Molecular weight : 229.24

3.1.4.	Purity, composition and substance codes

Batches used: A typical commercial lot of HC Red n° 1, Lot No 32, was used for toxicological evaluation during 2004 and 2005.

Chemical Identification by IR, ^{1H}NMR and ^{13C}NMR and Elemental Analysis

Purity by HPLC: > 98 % (vs external standard)
Purity by HPLC: 99.95% (vs external standard, 254nm) (Lot No 32, on May 12, 2004).
99.94% (reanalysis on January 20, 2005) after storage at room temperature demonstrating that this material was stable throughout the course of the toxicological investigations

3.1.5. Impurities / accompanying contaminants

Water content:	/
Loss on drying:	< 0.5% (0.264 % found in Lot No 32)
Residue on Ignition:	< 0.5% (< 0.1% found in Lot No 32)
Arsenic:	< 5 ppm
Antimony:	< 5 ppm
Lead:	< 20 ppm
Cadmium:	< 10 ppm
Mercury:	< 5 ppm
Residual solvents:	No data reported

Residual reagents, intermediates or by-products from the synthetic process

4-fluoro-3-nitroaniline:	< 100 ppm	
Aniline:	< 100 ppm	EU carcinogen category 3

Note
No data are given for another impurity identified as 4-acetamino-2-nitrophenylamine
Ref.: B48 Summary submission I, 2003

3.1.6. Solubility

Solubility measured after 15 minutes sonication.

Water:	0.003 – 0.005 mg/ml
Ethanol:	15.8 – 23.6 mg/ml
DMSO:	216 - 323 mg/ml

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 2.42 ± 0.10 (calculated ACD lab)

3.1.8. Additional physical and chemical specifications

- melting point: 99 – 105°C (103.2 – 103.5 ºC found in Lot No 32)
- flash point: /
- vapour pressure: /
- boiling point: /
- density at 20 °C: /
- viscosity: /
- pKa: /
- UV/visible absorption spectrum: λmax 272 nm, 497 nm
- Refractive index at 20 °C: /

3.1.9. Stability

Reanalysis of the bulk test article (Lot No 32), stored at room temperature for 8 months found the purity to be 99.94% demonstrating that this material was stable throughout the course of the toxicological investigations.

Stability in toxicological solvents:

PEG 400 solutions of HC Red n° 1 (0.4 and 200mg/ml) were shown to be stable (within ± 5% of the initial value) for 24 h when stored at room temperature and 15 days when stored at 5 ± °C.

DMSO solutions of HC Red n° 1 (0.05 and 100mg/ml) were shown to be stable (within ± 5% of the initial value) for 7 days when stored at ± 5 °C and for 16 days when stored at –20 °C.

General Comments on Physico-chemical characterisation

- Stability of the test material in marketed products is not reported.
- Log P_{ow}: calculated values can not be accepted as estimates of the true physical constants without justification, indicating that the reported values are realistic.

3.2. Function and uses

HC Red n° 1 is used in semi-permanent (non-oxidative) hair dye formulations to a maximum concentration of 1.0%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	/
Species/strain:	Sprague-Dawley rat
Group size:	5 males and 5 females per group
Observation:	14 days
Test substance:	HC Red n° 1
Batch:	/
Purity:	/
Dose level:	650 (females), 1250, 2500 (males) and 5000 mg/kg bw (administered by gavage suspended in 3% aqueous acacia)
GLP:	in compliance

Single oral doses of the test substance were administered to the animals by gavage. The animals were observed daily for 14 days and deaths and pharmacological and toxicological signs were recorded.

Results

dose (mg/kg bw)	male deaths/treated	female deaths/treated
625	-	0/5
1250	0/5	3/5
2500	0/5	-
5000	5/5	5/5

Conclusion
Under the conditions of the study the acute oral LD_{50} in the Sprague-Dawley strain of rats was between 2500 and 5000 mg/kg bw (males) and between 625 and 1250 mg/kg bw (females).
Ref.: 2

3.3.1.2. Acute dermal toxicity

/

3.3.1.3. Acute inhalation toxicity

/

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Primary skin irritation in rabbits

Guideline: /
Species/strain: New Zealand White Rabbit
Group: 6 animals (4 males, 2 females)
Substance: HC Red n° 1, according to Submission assumed commercial raw material
Purity: /
Concentration: unknown, slurry in water
Dose: 500 mg
GLP: /

The study was performed according to a Clariol Toxicology standard protocol for primary skin irritation test in rabbits, issued in 1986, with modified Draize scoring. A single dose was applied to 1 square inch of intact skin without occlusion for 24 h. Reading times were 24 and 72 h after application. No oedema or erythema was recorded. The total mean score was 0. The primary irritation index was mild, according to the test protocol.

Conclusion
Under the conditions of the study, a single open application of HC Red n° 1 did not cause irritation to the skin of rabbits.

Ref.: 4

3.3.2.2. Mucous membrane irritation

Guideline: /
Species: Rabbit (strain not specified)
Group: 4 (sex not stated)
Substance: HC Red n° 1
Batch: 414076
Purity: not stated
Dose: 100 mg undiluted material
GLP: not in compliance

The study was performed according to a Clariol Toxicology standard protocol for rabbit ocular irritation, issued in 1986, with modified Draize scoring. The test material was instilled into the conjunctival sac of one eye of each rabbit. The eyes of 2 animals were washed with 20 ml distilled water 20 seconds after instillation of the test material. Reading times were 1 hour and 1, 2 and 3 days after instillation.

Results
Deep conjunctival redness (grade 2 of 3) was observed in all animals at 1 hour. Conjuctival redness (grade 1 of 3) was observed in all animals 1 day, and in 1 animal 2 days after instillation. Eyelid swelling (grade 1 of 4) was observed 1 hour, 1 and 2 days after instillation.

Conclusion
Under conditions of the experiment, HC Red n° 1 caused primary eye irritation to the eye of rabbits.

Ref.: 3

3.3.3. Skin sensitisation

Animal data
Guinea pig studies

Guideline: /
Species/strain: Hartley Albino Guinea Pigs
Group: 10 animals (female)
Substance: HC Red n° 1, according to Submission assumed commercial raw material
Purity: /
Route: induction: intradermal injection and topical application under occlusive patch
challenge: topical application under occlusive patch
Dose: induction: intradermally, 2 rows of 3 injections each of 0.05 ml with 0.1% HC Red n° 1 in various combinations with Freund's Complete adjuvant; topically, occlusive patch of 25% HC Red n° 1, applied to a 2x4 cm area over the injection site;
challenge: 5% and 2% HC Red n° 1 applied to a 2x2 cm untreated skin area
Vehicle: propylene glycol
GLP: /

The study was performed with reference to the Guinea Pig Maximisation Test described by Magnusson and Kligman in 1969.
The intradermal and topical irritancy of HC Red n° 1 was established by a preliminary investigation. According to the reference, the final test level was the highest level that was tolerated locally. Before induction by topical application, however, the area was pretreated with 10% sodium lauryl sulphate in petrolatum. Reading times were 24, 48 and 72 h after removal of the test patches. No vehicle-treated control animals were included.

Results
The sensitisation rate was 100% (10/10 animals) as shown by challenge with 5% HC Red n° 1 and with 2% HC Red n° 1 one week later (re-challenge).

Conclusion
The study shows that the substance is an extremely potent skin sensitizer in the guinea pig.

Ref.: 5 and also ref 4 in submission I

Guideline:	/
Species/strain:	Hartley Albino Guinea Pigs
Group:	10 animals (female)
Substance:	HC Red n° 1, according to Submission assumed commercial raw material
Purity:	/
Route:	topical open application at induction and challenge
Dose:	induction: 0.5 ml of a 3% HC Red n° 1 suspension in the vehicle. A total of 18 applications; challenge: 0.5 ml of a 3% HC Red n° 1 suspension in the vehicle, one application
Vehicle:	10% isopropanol, 2% Tween 80, 2% hydroxyethylcellulose, sodium sulphide 0.05%, in deionized water; pH 7 (Schultz Hamburg Vehicle II)
GLP:	/

The study was performed by open epicutaneous testing with reference to a method by K. H. Schultz, Hamburg 24-5-76. Induction was performed on the left flank, after shaving approx. 6 cm^2, by applications 5 days/week for 3 weeks and 3 days on week 4. The interval between induction and challenge was 2 weeks. Challenge was performed on the opposite flank by one application to a 6 cm^2 area left for 24 h. Reading times were 24, 48 and 72 h after removal of the test material. Erythema and oedema were scored. No vehicle-treated control animals were included.

Results
The sensitisation rate was 70%, based on the finding of erythema in 7/10 animals.

Conclusion
The study indicates that the substance is a potent skin sensitizer.

Ref.: 6

Comment
The method is not a standard method.

Local lymph node assay (LLNA) studies

Guideline:	/
Species:	CBA/CaJ Mice
Group:	45 animals, 5 per group (female)
Substance:	HC Red n° 1 (supplier code TM#2044), according to Submission assumed commercial raw material, supplied by the sponsor (Bristol-Myers Squibb Worldwide Beauty Care)
Purity:	/
Concentration:	0.25, 0.5, 1.0 and 2.0% of HC Red n° 1
Dose:	25µl
Vehicle:	DMSO
Negative control:	vehicle
Positive control:	p-phenylenediamine (PPD) lot no. S0580797 at 0.25%, 0.5%, 1.0% and 2.0%
GLP:	/

The study was performed with reference to the Local Lymph Node Assay described by Kimber et al. 1995. The animals were treated on the dorsal surface of both ears once per day for 3 days. On day 5 (or day 6, according to Submission II) mice were injected intravenously with 20 µCi of ^{3}H-thymidine. After 5 hours, the draining auricular lymph nodes were removed. The lymph node cells were precipitated with 5% trichloro-acetic acid. Incorporation of ^{3}H-thymidine was measured in a ß-scintillation counter. The mean values obtained for each group were used to calculate stimulation indices (SI).

Results
The mean SI was 0.35 at 0.25% of HC Red n° 1, 1.06 at 0.50%, 1.04 at 1.0% and 1.52 at 2%. No individual test results or animal weight figures were reported. The mean SI for the positive control (PPD) was 7.10 at 2% PPD, and below 3 for the lower concentrations.

Conclusion
Under the conditions of this murine Local Lymph Node Assay, HC Red n° 1 did not induce skin sensitisation.

Ref.: 7

Comments
No rationale for choice of test concentrations was given. It is assumed, by the SCCP, that the highest concentration (2%) of the substance was too low and not in compliance with the requirements of the method. The overall low SI from tests with the positive control (PPD) indicates that the laboratory has difficulties with the test method, a conclusion supported by results in Ref. 8.

Guideline:	/
Species:	CBA/CaJ Mice
Group:	45 animals, 5 per group (female)

Substance:	HC Red n° 1 (supplier code TM#2044), according to Submission assumed commercial raw material, supplied by the sponsor (Bristol-Myers Squibb Worldwide Beauty Care)
Purity:	/
Concentration:	0.25, 0.5, 1.0 and 2.0% of HC Red n° 1
Dose:	25µl
Vehicle:	DMSO
Negative control:	vehicle
Positive control:	p-phenylenediamine (PPD) lot no. 9M16580 at 0.25, 0.5, 1.0 and 2.0%
GLP:	/

The study was performed with reference to the Local Lymph Node Assay, NIH Publication No. 99-4494. The animals were treated on the dorsal surface of both ears once per day on Days 1, 2 and 3. On Day 6, mice were injected intravenously with 20 µCi of ^{3}H-thymidine. After 5 hours, the draining auricular lymph nodes were removed. The lymph node cells were precipitated with 5% trichloro-acetic acid. Incorporation of ^{3}H-thymidine was measured in a ß-scintillation counter. The mean values obtained for each group were used to calculate stimulation indices (SI).

Results
None of the treatments caused any irritation. The mean SI was 3.49 at 0.25% of HC Red n° 1, 1.73 at 0.50%, 1.51 at 1.0% and 4.27 at 2%. The positive control (PPD) at 0.25%, 0.5%, 1.0% and 2.0% resulted in SI of 3.66, 9.13, 11.66 and 22.59, respectively.

Conclusions
It is concluded that the test substance HC Red n° 1 can induce skin sensitisation in the mice.

Ref.: 8

Comment
No rationale for test concentrations was given. It is concluded, by the SCCP, that the highest concentration (2%) of the substance was too low and not in accordance with the requirements of the method. The SI from tests with the positive control (PPD) was higher than in Ref. 7, and in better accordance with results from other studies.

Human data
Repeated insult patch test

Guideline:	/
Group:	105 volunteers (6 males and 99 females), 103 completed the study
Substance:	HC Red n° 1, according to Submission assumed commercial raw material
Purity:	/
Concentration:	3% slurry of HC Red n° 1 in a hair dye base, for induction and challenge
Route:	topical application under occlusive patch for induction and challenge
Dose:	0.2 ml applied to a 2 cm^2 infrascapular area for 24 h, for induction 3 times a week for 3 weeks; for challenge a single application
GLP:	not in compliance

A human repeated insult patch test was conducted with HC Red n° 1 mixed in a hair dye base. 8 other products were tested simultaneously in the same subjects. The interval between induction and challenge was 2 weeks. Reading times were 24 and 48 h after challenge patch removal.

Results
Strong positive reactions (+++ = definite erythema, definite oedema and vesiculation) were recorded in 1 subject at 48 h, and in 2 subjects at 72 h after application of challenge patch. One of them responded with +++ reaction during the induction phase, which was interpreted as evidence of presensitisation. Doubtful response (? = barely receptible erythema)) was recorded in 66 to 98 subjects on Days 1 to 9 of induction, and in 27 and 37 respectively at 48 h and 72 h after application of challenge patch.

Conclusions
The test substance (HC Red n° 1 in a hair dye base) is a skin sensitiser in humans.

Ref.: 9

Comment
The study is considered unethical.

3.3.4. Dermal / percutaneous absorption

***In Vitro* Percutaneous Absorption**

Guideline:	OECD 428 (2004)
Tissue:	Human skin (female), kept frozen at -20°C, dermatomed at 400 µm, body part not known
Tissue integrity:	Electrical resistance across skin membrane
Method:	Glass diffusion cells, exposed membrane area 2.54 cm^2
Test substance:	HC Red n° 1, 1%, in a non-oxidative hair dye base containing no other dye precursor, as gel. [^{14}C]-radiolabelled HC Red n° 1 incorporated into the dose to give $1x10^8$ to $1x10^9$ dpm/ml
Batch:	Commercial material, lot code #32, GTS 03974 (unlabelled substance)
Purity:	99.95%
Radiochemical purity:	98.2% (HPLC)
Dose applied:	20 mg/cm^2 corresponding to 200 µg HC Red n° 1 /cm^2
Receptor fluid:	4% polyoxyethylene 20 oleyl ether solution in phosphate buffered saline
Contact:	30 minutes, then washing of the skin surface, monitoring of the diffusion during 48 hours.
No. of replicates:	12 cells, of which 1 was excluded, were prepared with membrane from 5 subjects
Assay:	Liquid scintillation counting, dpm
GLP:	in compliance

The penetration and distribution of HC Red n° 1 from a nominal 1% w/w formulation was measured in vitro through human skin following the incorporation of [^{14}C]-HC Red n° 1. The mixed formulation was applied to skin membranes, mounted in glass diffusion cells, at 32±1° C. After a contact period of 30 minutes, the dose was washed from the surface with natural sponges soaked in 3% Teepol®. Samples of the receptor fluid were taken at recorded intervals (0.5, 1, 2, 4, 6, 24 and 48 hours after application), during which time the applications remained

unoccluded. At the end of the experiment, the surface of the skin was washed again and layers of stratum corneum removed using a tape stripping technique. The receptor fluid samples, sponges, tape strips, residual skin and donor chambers were analysed for radioactivity, which was representative of the HC Red n° 1 content. Penetration rates and distribution of HC Red n° 1 were calculated.

Results
The absorbed amount of HC Red n° 1 is shown in the table.

Table: Summary of HC Red n° 1 distribution in the test system

	Amount recovered (µg/cm2)			**Percent of dose recovered (%)**	
Test compartment	**mean**	**SD**	**range**	**mean**	**SD**
Flange	0.089	0.075		0.044	0.037
Donor chamber	0.164	0.120		0.082	0.060
Skin wash 0.5h	195	13.6		97.5	6.77
Skin wash 48h	1.12	0.794		0.560	0.396
Stratum corneum	0.199	0.111		0.099	0.056
Remaining epidermis/dermis	0.208	0.159	0.052 – 0.518	0.104	0.079
Receptor fluid	1.14	0.560	0.274 – 2.24	0.566	0.279
Systemically available *	**1.34**	**0.720**	**0.404 – 2.52**	**0.670**	**0.359**
Total	198	13.7		99.0	6.85

* total penetrated = sum of remaining epidermis and receptor fluid data

The total recovery was 99.0%. 1.34 ± 0.72 µg/cm^2 (0.67 ± 0.36 %) HC Red 1 was regarded as systemically available. The maximum absorption observed in the experiment was 2.52 µg/cm^2 and this will be used for calculating the MoS.

Ref.: 10

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral / dermal / inhalation toxicity

/

3.3.5.2. Sub-chronic (90 days) oral / dermal / inhalation toxicity

14-day oral dose range-finding study in rats

Guideline: OECD 408
Species/strain: Sprague-Dawley derived rats, strain Crl:CD (SD)\GS BR
Group size: 50 animals (5 males and 5 females / dose level)
Observation: 14 days
Test substance: HC Red n° 1
Batch: #32

Purity: 99.95%
Dose levels: 0, 50, 150, 300 or 600 mg/kg bw/day (administered in PEG 400 by gavage)
GLP: in compliance

The test article and vehicle were administered once daily, by oral gavage, for 14 consecutive days. General health/mortality and moribundity checks were performed twice daily. Detailed clinical observations were performed prior to dosing and on days 0, 3, 7, 10 and 13, and on the day of scheduled euthanasia. Cage-side observations were performed daily approximately 30 to 90 minutes after dose administration. Individual body weights were recorded one day prior to treatment and on days 0, 3, 7, 10 and 13. A final body weight was obtained prior to scheduled euthanasia. Food consumption was measured on days 0, 3, 7, 10 and 13. Blood and urine samples were collected from all animals on the day of scheduled euthanasia for evaluation of selected clinical pathology parameters. All animals were subjected to a gross necropsy examination upon death or scheduled euthanasia. Fresh organ weights were obtained for each surviving animal and selected tissues/organs were retained from all animals for possible future histopathological examination.

Results
Oral administration of HC Red n° 1 in the rat for 14 consecutive days at up to 600 mg/kg/day did not produce treatment-related mortality. The 150 and 600 mg/kg/day females that were found dead on day 4 were considered mechanical injuries secondary to the dosing procedure and not associated with test article toxicity. Test article-related changes were observed during this study. Several of these changes appeared consistent with oral administration of a concentrated dye. These included coloured staining of the body and urine. However, other findings were considered to be reflective of test article-induced toxicity and included dark material around the nose, decreased defecation, soft stools, dehydration, reduced weight gain and reduced food consumption. These changes were primarily observed in the 600 mg/kg/day males and females. Abnormalities in the clinical pathology parameters examined on this study were primarily observed in the 300 and 600 mg/kg/day males and 150, 300 and 600 mg/kg/day females and were suggestive of a slight regenerative anaemia. These included decreased erythrocytes, increased mean corpuscular volume, increased mean corpuscular haemoglobin, polychromatic red blood cells, increased alanine aminotransferase and increased total bilirubin. Other clinical chemistry changes included total protein, albumin, A/G ratio, and potassium. The most notable changes at necropsy included blackish-purple and/or enlarged spleen in the 300 and 600 mg/kg/day males and females. Spleen weights were increased in the 300 and 600 mg/kg/day males and the 150, 300 and 600 mg/kg/day females, and liver weights were increased in the 300 mg/kg/day males and the 150, 300 and 600 mg/kg/day females.

Conclusion
Based on the results of this study, oral administration of HC Red n° 1 in the rat for 14 consecutive days at up to 600 mg/kg/day did not produce treatment-related mortality. However, notable treatment-related changes were observed in the 150, 300 and 600 mg/kg/day dose groups. These generally consisted of clinical abnormalities, decreased weight gain/food consumption, abnormal clinical pathology, gross necropsy observations and increased liver and spleen weights. The no-observed-adverse-effect level (NOAEL) in this study is 50 mg/kg bw/d.
Ref.: 11

91-day oral subchronic toxicity study in rats

Guideline:	OECD 408
Species/strain:	Sprague-Dawley derived rats, strain Crl:CD (SD)\GS BR
Group size:	140 animals, 15 males and 15 females / dose level, 5 animals of both sexes of the control and high dose group were used as recovery group
Observation:	91 days
Test substance:	HC Red n° 1
Batch:	#32
Purity:	99.95%
Dose levels:	0, 2, 5, or 20 mg/kg bw/day (administered in PEG 400 by gavage)
GLP:	in compliance

The toxicity of HC Red n° 1 when administered to rats via oral gavage for at least 91 consecutive days was assessed. Sprague Dawley rats were treated with the test substance and the recovery group and the respective control group were observed for further 28 d. General health/mortality and moribundity checks were performed twice daily. Detailed clinical observations were performed weekly on all animals. Functional observation battery (FOB) observations were conducted on 10 rats/sex/group on days -1, 26, 40 and 89. Individual body weights and food consumption were recorded weekly. Ophthalmological examinations were performed on all animals once prior to in-life initiation and near in-life completion. Estrous cycle length and normality were evaluated by daily vaginal smears from all females on specified days. Blood and urine samples were collected from all animals for analysis of selected clinical pathology parameters on the day of scheduled euthanasia. All animals were subjected to a complete gross necropsy examination at scheduled euthanasia. Sperm was collected from each male rat following euthanasia for assessment of sperm enumeration, motility and morphology. Fresh organ weights were obtained at scheduled euthanasia from selected organs. A standardized list of tissues were obtained and preserved in 10% neutral buffered formalin for histopathological examination.

Results

Oral administration of the test substance was not associated with mortalities. In addition, treatment did not produce clinical abnormalities (exception: a dose-related increase in orange coloured urine; orange coloured urine on litter paper; orange staining on the anogenital/urogenital area and tail; and purple staining on the tail, nose-mouth area, forelimbs and dorsal region), neurological changes, variations in oestrous cyclicity, body weight effects or food consumption changes. There were a variety of statistically significant changes in the clinical chemistry parameters examined. For males, these included increased calcium (high and middle dose groups), total protein (high dose group) and globulin (high dose group). For females these included increased calcium (high dose group), total protein, albumin, globulin (high and middle dose groups), and sodium (high dose group). The toxicological significance of the increased albumin, globulin, total protein and sodium was unclear. These changes can be associated with dehydration or renal dysfunction. However, the findings were within the historical control range for this species, there was no evidence of dehydration and there were no notable changes in the renal histopathology or urinalysis parameters. Orange and/or red urine was observed in the substance-treated groups at the end of the treatment phase. In haematology at the end of the treatment or recovery phases statistically increased MCH was observed in the dose group 20 mg/kg bw/d (4 males). Increased leukocytes in females of the 5 (statistically significant) and 20 mg/kg/d group were also observed, and lymphocytes were also increased in these groups on study day 91/92. In addition, statistically decreased erythrocytes were observed in the high dose group (females) on day 119.

There were no toxicologically meaningful changes in the ophthalmology, gross necropsy, sperm evaluation or organ weight data. A statistically significant decrease in the percent motility was observed in the group 2 mg/kg bw/d at the end of the treatment period. This decrease is not considered treatment-related because a corresponding decrease was not observed in the higher dose groups. Statistically significant differences of organ weights at the end of the treatment period were limited to a decreased absolute and relative pituitary weight for females (5 mg/kg bw/d) and a decreased relative thymic weight for 4 males (20 mg/kg bw/d). There were no test article-related microscopic findings in this study: Gross necropsy observations at the end of the treatment period were consistent with oral administration of a chemical dye and included abnormal contents in the large intestine in females and stained tail and hair coat in both males and females treated with 20 mg/kg/day. There were no notable gross necropsy observations observed at the end of the recovery period for males or females.

Conclusion
According to the applicant the no-observed-adverse-effect level (NOAEL) in this study is 20 mg/kg bw/d.

Ref.: 12

Comment
Due to the decreased relative thymic weight at 20 mg/kg bw/d the SCCP sets the NOAEL at 5 mg/kg bw/d.

Dermal toxicity in rabbits following topical administration of a hair dye formulation containing 0.15 % HC Red n° 1

Guideline:	/
Species/strain:	New Zealand White Rabbits
Group size:	6 males and females per group
Test substance:	A semi-permanent hair dye formulation (P24) containing 0.15% HC Red n° 1 (see Table below)
Batch:	/
Purity:	not given
Dose:	1 ml per kg of a solution containing 0.15% HC Red n° 1
Treatment:	Topical administration twice weekly for 13 weeks
GLP:	not in compliance

The experiment involved altogether 12 different dye formulations and 3 negative control groups. The test substance was administered twice weekly for 13 weeks at a dose level of 1 ml test material / kg bw to alternating sides of the thoracic-lumbar area. 3 untreated groups served as negative controls. 1 hour following topical application, the test material was removed by shampooing and subsequent rinsing and drying. Body weight gain, survival rate, haematology, biochemistry and urinalysis were performed on week 0, 3, 7 and 13. The survivors were sacrificed and examined for gross abnormalities. Organ weights were determined and 25 tissues were inspected microscopically.

Results
No changes in body weight gains versus the control groups were observed. 5 control and 5 test animals died during the study due to complications resulting from cardiac puncture while collecting blood. It is not stated in which formulation group these deaths occurred. A statistically

significant increase in white blood cells in female animals and increases in % BUN in both males and females were found.

No gross abnormalities observed at autopsy. Across all the formulations tested there were a few instances where there were statistically significant differences in relative organ weights between a test group and the combined controls but these differences were not significant when the test group was compared with each control group separately. It is not clear whether such differences were observed in the formulation containing HC Red n° 1. No microscopic lesions were observed that were considered to be due to the administration of the hair dye formulations.

Ref.: 19

Comment
The study was published in 1976 and is of limited value.

Table: Content of hair dye formulation P24

Dye Ingredients, Chemical or Trade Name	**%**	**Base Ingredients, Chemical or Trade name**	**%**
HC Yellow No. 4	0.4	BHT	0.25
HC Yellow No. 5	0.1	Triethanolamine Dodecyl-benzene Sulphonate	0.5
HC Red No. 1	0.15	Oleic Acid	1.0
HC Blue No. 1	1.6	Lauric Diethanolamide	1.5
HC Blue No. 2	1.7	Polyoxyethylene Hydrogenated Tallow Amide (50 ETO)	1.9
HC Orange No. 1	0.15	Diethanolamine[2]	2.0
Acid Orange No. 3	0.2	Hydroxyethyl Cellulose	2.4
Disperse Blue No. 3	0.3	Carbitol	5.0
Disperse Violet No. 11	0.2	Water	qs100.0
Disperse Red No. 11	0.1		
Celliton Fast Navy Blue BRA[1]	0.2		
2-Nitro-4'-bis-(2-hydroxyethyl) aminodiphenylamine	0.4		
2-Nitro-4-methoxy-diphenylamine	0.1		

[1] A mixture of the following dyes: Disperse Yellow n° 1, Disperse Red n° 17, Disperse Blue n° 1, Disperse Violet n° 4

[2] According to Annex II no. 411 secondary alkanolamines are banned.

3.3.5.3. Chronic (> 12 months) toxicity

/

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial gene mutation assays

Guideline:	OECD 471
Species/strain:	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 *uvr*A (pKM101).
Replicates:	2 or 3 replicates in 2 individual experiments both in the presence and absence of S9.
Test substance:	GTS03974
Solvent:	DMSO
Batch:	32
Purity:	99.8%
Concentrations:	Experiment 1: 2.5 - 5000 µg/plate (with and without S9) Experiment 2: 7.5 - 5000 µg/plate (with and without S9) Experiment 3: 25 - 5000 µg/plate TA 1535 only (without S9)
Treatment:	In experiment 1 the pre-incubation method was used. In experiments 2 and 3 the direct plate incorporation method.
GLP:	in compliance

HC Red n° 1 was investigated for the induction of gene mutations in *S. typhimurium* (Ames test) and *E. coli* using the pre-incubation method with an incubation period of 20 minutes in experiment 1 and the direct plate incorporation method in experiments 2 and 3 with an exposure of 48 to 72 h. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity in experiment 1 in which a dose range was used up to the prescribed maximal dose of 5000 µg/plate. Toxicity was evaluated on the basis of a reduction in the number of revertant colonies and/or a thinning of the bacterial lawn. Negative and positive controls were in accordance with the OECD guideline.

Results
In experiment 1 precipitation of HC Red n° 1 was observed at 600 µg/plate (TA98) or 1800 µg/plate. Toxicity occurred at 200 µg/plate (-S9) or 600 µg/plate (+S9). HC Red n° 1 did not induce an increase in the number of revertant colonies as compared to concurrent vehicle controls in any of the *Salmonella* strains nor in *Escherichia coli*.
In experiment 2 precipitation and a moderately reduced background lawn was seen at 5000 µg/plate. A dose dependent increase in the number of revertants was again not found. In TA 1535 treatment without S9 metabolic activation with HC Red n° 1 resulted in a non-dose responsive increase in the number of revertants. This effect could not be confirmed in an additional repeat test.

Conclusion
Under the experimental conditions used HC Red n° 1 is not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 13

Guideline:	/
Species/strain:	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538.

Replicates:	3 replicates both in the presence and absence of S9.
Test substance:	HC Red n° 1
Solvent:	DMSO
Batch:	8-265H
Purity:	99.65%
Concentrations:	10 - 1000 µg/plate (with and without S9)
Treatment:	Direct plate incorporation method
GLP:	in compliance

Mutagenicity was investigated according to Maron and Ames (1983) Mutation Res., 113, 173-215.
HC Red n° 1 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) using direct plate incorporation method with an exposure of 46 to 72 h. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on historical data and on the level of toxicity in a range finding experiment. Toxicity was evaluated on the basis of a thinning of the bacterial lawn. Negative and positive controls were incorporated.

Results
Precipitation of HC Red n° 1 was not observed whereas toxicity occurred at 1000 µg/plate. HC Red n° 1 did not induce an increase in the number of revertant colonies as compared to concurrent vehicle controls in any of the five *Salmonella* strains.

Conclusion
Under the experimental conditions used HC Red n° 1 is not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 27

Comment
The negative result was, however, not confirmed in an independent experiment.

Guideline:	/
Species/strain:	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538.
Replicates:	2 replicates.
Test substance:	HC Red n° 1
Solvent:	DMSO
Batch:	2Q88
Purity:	/
Concentrations:	25 - 5000 µg/plate (with S9)
Treatment:	Direct plate incorporation method
GLP:	not in compliance

HC Red n° 1 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) probably using direct plate incorporation method. The study was not performed according any guideline nor according GLP. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was evaluated on the basis of "inhibition" of bacteria. Negative and positive controls were incorporated.

Results

Toxicity due to HC Red n° 1 exposure was found at 500 µg/plate (TA1537) or 1000 µg/plate and above. HC Red n° 1 did not induce an increase in the number of revertant colonies as compared to concurrent vehicle controls in any of the five Salmonella strains.

Conclusion
Under the experimental conditions used HC Red n° 1 is not genotoxic (mutagenic) in this gene mutation tests in bacteria. The negative result was not confirmed in an independent experiment.
Ref.: 26

Comment
Due to the poor reporting, this study is considered inadequate

In vitro* Mouse Lymphoma assay (*tk*$^{+/-}$ locus*)

Guideline:	OECD 476
Cells:	Mouse lymphoma cell line L5178Y
Replicates:	3 replicates per concentration
Test substance:	GTS03974
Solvent:	DMSO
Batch:	32
Purity:	99.8%
Concentrations:	Experiment 1: 1.5 - 140 µg/ml (without S9) 5 - 30 µg/ml (with S9) Experiment 2: 0.5 - 100 µg/ml (without S9)
Treatment	Experiment 1 exposure for 4h; in experiment 2 for 24 h; expression period 7 days
GLP:	in compliance

The test substance was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a preliminary toxicity test in which L5178Y cells were exposed to HC Red n° 1 for 4 h or 24 h both in the absence and presence of S9. Cells were treated for 4 h (experiment 1) or extendedly for 24 h (exp 2) followed by an expression period of 10-14 days to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured as suspension growth of the treated cultures relative to the growth of the solvent control cultures after 48 h. Mutant colonies were counted for each culture with ≥ 20% total relative growth including at least one culture with ≥ 10% but ≤ 20% relative total growth. Colony sizing was performed over a range from 0.2 to 1.1 mm. Negative and positive controls were in accordance with the OECD guideline.

Results
Exposure to HC Red n° 1 had no effect on osmolarity or pH as compared to concurrent vehicle controls. Both in the absence of S9 (4 h and 24 h treatment) and in the presence of S9 (4 h treatment) the appropriate level of toxicity (10 - 20% survival in the highest concentration tested) was reached. Visual precipitation was never seen at any concentration in treatment medium.
A concentration related increase in mutant frequency compared to concurrent vehicle controls was not found after 4 h treatment in the absence or presence of S9 metabolic activation nor when the treatment was extended up to 24 h without S9 metabolic activation.

Conclusion
Under the experimental conditions used, the test substance is not genotoxic (mutagenic/clastogenic) at the *tk* locus of mouse lymphoma cells.

Ref.: 15

***In vitro* chromosome aberration test**

Guideline:	OECD 473	
Cells:	Chinese Hamster Ovary (CHO) cells	
Replicates:	Duplicate cultures in 2 independent experiments	
Test substance:	GTS03974	
Solvent:	DMSO	
Batch:	32	
Purity:	99.8%	
Concentrations:	Experiment 1:	25 – 100 μg/ml (without S9)
	Experiment 2:	20 – 80 μg/ml (without S9)
		50 – 120 μg/ml (with S9)
Treatment:	Experiment 1:	4 h treatment without S9; harvest time 20 h after start of treatment.
	Experiment 2: harvest	20 h treatment without S9 and 4 h treatment with S9; time 20 h after start of treatment.
GLP:	In compliance	

HC Red n° 1 has been investigated in 2 independent experiments in the absence and presence of metabolic activation for the induction of chromosomal aberrations in CHO cells. Test concentrations were based on the results of a preliminary toxicity test assessing the effect of HC Red n° 1 on cell growth relative to the concurrent solvent control. Cells were treated for 4 h in the absence or presence of S9 metabolic activation or for 20 h in the absence of S9 metabolic activation. Harvest time was 20 hours after the beginning of treatment. Two hours before harvest, each culture was treated with colcemid solution (final concentration of 0.1 μg/ml) to block cells at metaphase of mitosis. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.
Cytotoxicity was determined by measuring the reduction in mitotic index (MI) and in the % cell growth inhibition. Chromosome (metaphase) preparations were stained with 5% Giemsa and examined microscopically for MI and chromosomal aberrations. Negative and positive controls were in accordance with the OECD guideline.

Results
Treatment with HC Red n° 1 did not affect osmolarity and pH compared to those of the concurrent vehicle controls. Precipitation was observed in treatment medium at concentrations ≥ 100 μg/ml. Although the MI did not show a 50% reduction in the highest doses tested, HC Red n° 1 induced sufficient toxicity, as shown by around up to 60% decrease in relative growth inhibition at the highest concentration tested compared to the concurrent solvent controls, indicating to sufficient exposure of the cells.
Treatment with HC Red n° 1 in the absence of S9 metabolic activation for either 4 or 20 h resulted in a concentration dependent statistical significant increase in cells with structural chromosomal aberrations. Also in the presence of S9 metabolic activation, a statistical

significant concentration dependent increase in the frequency of cells with chromosomal aberrations was found
HC Red n° 1 treatment did, however, not lead to an increase in the frequency of polyploid and/or endoreduplicated cells, both examples of numerical chromosomal aberrations.

Conclusion
Under the experimental conditions used, the test substance induced an increase in structural chromosomal aberrations and, consequently, is genotoxic (clastogenic) in CHO cells *in vitro*.

Ref.: 14

Guideline:	/
Cells:	Chinese Hamster Ovary (CHO) cells
Replicates:	Duplicate cultures
Test substance:	HC Red n° 1
Solvent:	DMSO
Batch:	/
Purity:	/
Concentrations:	7.5 – 25 µg/ml (without S9) 50 – 100 µg/ml (with S9)
Treatment:	18 h treatment without S9 and 4 h treatment with S9; harvest time 20 h after start of treatment.
GLP:	in compliance

HC Red n° 1 has been investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in CHO cells. Test concentrations were based on the results of a preliminary toxicity test assessing toxicity as indicated by the loss of growth potential of the cells. Cells were treated for 4 h in the absence or presence of S9 metabolic activation or for 24 h in the absence of S9 metabolic activation. Harvest time was 20 hours after the beginning of treatment. Two hours before harvest, each culture was treated with colcemid solution to block cells at metaphase of mitosis. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Chromosome (metaphase) preparations were stained with Giemsa and examined microscopically for chromosomal aberrations. Negative and positive controls were in accordance with the OECD guideline.

Results
HC Red n° 1 treatment did not have an effect on osmolarity. The pH of the medium could not be evaluated due to the dark purple colour of HC Red n° 1.
Although the MI was decreased in the cultures treated in the absence of S9 metabolic activation, a 50 % reduction in the highest doses tested was not reached. As toxicity was seen at a concentration of 50 µg/ml, an additional concentration between 25 and 50 µg/ml appeared essential but is missing. An effect on MI of HC Red n° 1 in cultures treated in the presence of S9 metabolic activation was exclusively seen at the highest concentration tested.
In the presence of S9 a dose dependent increase in cell with chromosomal aberrations was observed. At the highest dose (100 µg/ml) the increase was statistical significant. HC Red n° 1 treatment in the absence of S9 did not result in an increase in cells with chromosomal aberrations.
HC Red n° 1 treatment did not lead to an increase in the frequency of polyploid cells.

Conclusion

Under the experimental conditions used HC Red n° 1 induced in the presence of S9 metabolic activation an increase in structural chromosomal aberrations and, consequently, is genotoxic (clastogenic) in CHO cells *in vitro*.

Ref.: 28

Comment
The test is not performed according an OECD guideline and the purity is unknown.
The experiment with 4 h treatment and 20 h harvest in the presence of S9 metabolic activation is reported in the next test.

***In vitro* chromosome aberration test**

Guideline:	/
Cells:	Chinese Hamster Ovary (CHO) cells
Replicates:	Duplicate cultures
Test substance:	HC Red n° 1
Solvent:	DMSO
Batch:	/
Purity:	/
Concentrations:	75 – 175 µg/ml (with S9)
Treatment:	4 h treatment with S9; harvest time 20 h after start of treatment.
GLP:	in compliance

HC Red n° 1 has been investigated in the presence of metabolic activation for the induction of chromosomal aberrations in CHO cells. In fact, this experiment is to determine whether or not the positive result in the presence of metabolic activation described in the study above (Tice, study 92046, 1993) could be confirmed. Test concentrations were based on the results of a preliminary toxicity test described in the earlier mentioned report. Cells were treated for 4 h in the presence of S9 metabolic activation. Harvest time was 20 hours after the beginning of treatment. Two hours before harvest, each culture was treated with colcemid solution to block cells at metaphase of mitosis. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Cytotoxicity was determined by measuring the reduction in mitotic index (MI) and in the % cell growth inhibition. Chromosome (metaphase) preparations were stained with Giemsa and examined microscopically for chromosomal aberrations. Negative and positive controls were in accordance with the OECD draft guideline.

Results
Information on the effect of HC Red n° 1 treatment on osmolarity and pH effects is lacking. The MI dose-dependently decreased showing a 50 % reduction in the highest doses tested. Treatment with HC Red n° 1 in the presence of S9 resulted in a statistical significant increase in cell with chromosomal aberrations. Strikingly, over the concentration range tested the response appeared to have plateaued. HC Red n° 1 treatment did not lead to an increase in the frequency of polyploid cells but a significant decrease at the highest concentration tested.

Conclusion
Under the experimental conditions used HC Red n° 1 induced in the presence of S9 metabolic activation an increase in structural chromosomal aberrations and, consequently, is genotoxic (clastogenic) in CHO cells *in vitro*.

Ref.: 29

Comment
The test is not performed according an OECD guideline and the purity is unknown.

Rat liver *in vitro* UDS assay

Guidelines:	OECD 482
Cells:	Primary hepatocytes from Fischer 344 rats
Replicates:	Triplicates
Test Substance:	HC Red n° 1
Solvent:	DMSO
Batch:	/
Purity:	/
Concentrations:	1 – 25 µg/ml
Treatment:	18 – 20 h followed by fixation and autoradiography
GLP:	in compliance

HC Red n° 1 has been investigated for the induction of unscheduled DNA synthesis in primary hepatocytes of F344 rats.
Hepatocytes were isolated by *in situ* perfusion with the proteolytic enzyme collagenase. The isolated hepatocytes were allowed to attach to plastic cover slips for 2 h and then treated with HC Red n° 1 in the presence of ^{3}H-thymidine (final concentration 10 µC/ml) for 18 – 20 h. Evaluation of autoradiography was done after 7 days exposure.
Test concentrations were based on visual microscopical inspection of cover slips from each dose for toxicity. 25 µg/ml was chosen as the highest dose since it reduced the number of hepatocytes and increased the number of nuclei without cytoplasm. Unscheduled synthesis was determined in 50 hepatocytes per dose, calculated by substracting the cytoplasmic grain count from the nuclear grain count. Negative and positive controls were in accordance with the OECD guideline.

Results
Data on toxicity were lacking. No dose level of HC Red n° 1 revealed UDS induction in the treated primary hepatocytes as compared to the concurrent vehicle control. In addition the % of hepatocytes in repair ranged from 2 to 11% in the highest dose tested which is in the range of the concurrent vehicle control.

Conclusion
Under the experimental conditions used HC Red n° 1 did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in primary rat hepatocytes *in vitro*.

Ref.: 30

Comment
Purity of HC RED n° 1 is unknown. Also data on cytotoxicity are lacking. The value of this UDS test is limited.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	OECD 474
Species/strain:	ICR mice
Group size:	5 male mice
Test substance:	GTS03974
Batch:	32
Purity:	99.8%
Concentrations:	500, 1000 and 2000 mg/kg bw as single doses
Route:	Oral gavage
Vehicle:	polyethylene glycol 400
Sacrifice times:	24 h for all concentrations, 48 h for the vehicle control and the highest dose.
GLP:	in compliance

HC Red n° 1 has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on the level of mortality and toxicity in a dose range finding experiment. Since no differences in toxicity occurred between male and female mice, only male mice were used. Mice were exposed to single doses of 500, 1000 and 2000 mg/kg bw by oral gavage. 24 h or 48 h (highest dose and concurrent vehicle control only) after dosing bone marrow cells were collected. Bone marrow toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and 1000 erythrocytes.
Although not analysed plasma samples were collected from satellite groups of 3 male mice to determine toxicokinetic parameters after treatment with the highest HC Red n° 1 dose. These mice were bled 1, 2, 4, 6 and 8 h after dosing.
Bone marrow preparations were stained with May-Gruenwald-Giemsa and examined microscopically for micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the dose range finding study, systemic toxic effects were seen after HC Red n° 1 exposure: piloerection, lethargy and red urine. In the micronucleus study there was no treatment related mortality. Lethargy, signs of red skin tone and red urine were seen in mice exposed to the highest doses.
A reduction in the ratio between polychromatic and 1000 erythrocytes PCE/EC ratio up to about 15% was observed in the HC Red n° 1-treated groups relative to the vehicle controls, indicating to exposure of the target cells investigated.
A statistical significant increase in the number of cells with micronuclei was not found in any HC Red n° 1-treated groups relative to the concurrent vehicle control.
Conclusion
Under the experimental conditions used HC Red n° 1 did not induce micronuclei in bone marrow cells of treated mice and, consequently, HC Red n° 1 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 16

3.3.7. Carcinogenicity

Topical administration, mice

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	50 animals per sex

Test substance:	One semi-permanent hair dye formulation (P24) containing 0.15% HC Red n° 1
Batch:	/
Purity:	not given
Dose:	0.05 ml of a solution containing 0.15% HC Red n° 1
Route:	Topical, 1 application weekly
Exposure:	23 months
GLP:	not in compliance

The experiment involved altogether 12 different dye formulations and 3 negative control groups.

Dye applied topically to a 1 cm^2 area on a clipped (24 hours prior to application) site in the interscapular region. Mice received a dose of 0.05 ml topically without occlusion once weekly from 8 – 10 weeks of age for 23 months. The animals were observed daily for mortality and signs of toxicity, and were weighed monthly. A continuous weekly record was maintained for any skin lesions noted. After 9 months of treatment, 10 males and 10 females per group were necropsied and the study was terminated after 23 months. Skin and internal organs were evaluated histologically.

There were no overt sign of systemic toxicity in any of the dye-treated groups. Five male and 9 female survived 23 months compared to 3 males and 8 females in the control group. There were no significant differences in absolute or relative liver or kidney weights in groups of 10 male and 10 female mice necropsied after 9 months. Average body weights were comparable in all groups throughout the study. There were no statistically significant differences in the distribution of tumours among treated and control groups.

It is concluded that no evidence of carcinogenic activity was seen.

Ref.: 20

Topical administration, rats

Guideline:	/
Species/strain:	Male and female weanling Sprague Dawley rats, 60 per sex per group
Group size:	60 animals per sex and dose
Test substance:	One semi-permanent hair dye formulation (P24) containing 0.15 % HC Red n° 1
Batch:	/
Purity:	not given
Dose:	0.5 ml of a solution containing 0.15% HC Red n° 1
Route:	Topical. 1 application twice weekly
Exposure:	114 weeks
GLP:	not in compliance

The experiment involved altogether 10 different dye formulations and 3 negative control groups.

Groups of 60 male and 60 female were obtained from the first mating (F_{1a}) of a multi-generation reproduction study in rats treated with one semi-permanent hair dye formulation containing 0.15% HC Red n° 1. The F_0 parents had received topical application of the hair dye formulation from the time of their weaning to the weaning of their offspring. The dye formulations were

administered topically to the shaved (24 hours prior to application) neck and back area twice weekly. An initial dosage level of 0.2 ml/rat was increased incrementally by 0.1 ml per week until 0.5 ml was achieved. There were three independent control groups each containing 60 males and 60 females, which received no treatment.

The rats were observed daily for overt signs of toxicity and for mortality. Detailed observations were recorded weekly. Individual body weights were recorded weekly for the first 14 weeks and monthly thereafter. Group food consumption was recorded weekly. Haematological, biochemical and urinalysis studies were done on 5 males and 5 females per group at 3, 12, 18, and 24 months of study. After 12 months of treatment, 5 males and 5 females from each group were sacrificed and necropsied. Histopathological evaluations were performed on 18 tissues (plus tumour masses) including treated skin.

Changes in body weight and food consumption have been similar for control and treated rats. Survival just prior to terminal sacrifice (at week 117 119) the survival was 11 males and 15 females for the exposed group. Survival was 15 males and 14 – 18 females for the control groups. After 114 weeks, the mean body weight in the treated group was 740 g in males and 496 g in females. Control group values ranged from 682 to 759 g in males and 477 to 513 g in females.

Isolated variations in haematological values included decrease in total erythrocytes, haemoglobin and haematocrit in one treated female at 12 months and in one treated male at 24 months. Gross observations considered to possibly be test material related were skin lesions including ulceration, scabbing, abscesses and thickening, colouring of the fur and skin at the application site and increased incidences of enlarged and/or firm livers. The incidence of parathyroid hyperplasia was higher in treated male and female rats than in control groups, which was considered possibly compound related. The incidence of hyperkeratosis and dermatitis was considered higher in treated animals than in controls and was considered possibly compound related.

The most common tumour observed was pituitary adenoma. The statistically significant variations in incidence of pituitary adenoma between rats of different control groups as well as their statistically significant decrease in an experimental group when compared with two control groups, tends to discount any biological significance of these findings. In addition, the incidences of this tumour in rats of both sexes were comparable to that routinely observed in aging rats of this strain at the testing facility. The incidence of mammary lobular hyperplasia was statistically significantly greater in control group 3 females than in control group 1 females. When treated females were compared to control group 1 females the incidence of mammary adenocarcinoma/carcinoma was statistically significantly increased. However when treated females were compared with control group 2 and 3 females or with all control females combined, there was no significant increase in this tumour type. Therefore this finding is not considered to be of biological significance.

It is concluded that no evidence of carcinogenic activity was seen.

Ref.: 21

Comments on carcinogenicity

One study with HC Red n° 1 in a semi-permanent hair dye formulation involving topical application of mice and one involving topical application on rats have been identified. The concentration of HC Red n° 1 was in both studies 0.15% (the maximum concentration on the

human scalp is 1.0%). A number of different hair dye formulations were tested in the same studies. Although some of the formulations contained 2,4-diaminoanisol (classified as carcinogen category 2 in EU), none of the formulations induced tumours in the mice or rats. Thus, no conclusion with regard to carcinogenicity can be made from the studies.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

Multigeneration reproduction study in rats following topical administration of a hair dye formulation containing HC Red n° 1

Guideline:	/
Species/strain:	Charles River CD rats
Group size:	80 per group (40 males and 40 females)
Test substance:	A semi-permanent hair dye formulation (P24) containing 0.15% HC Red n° 1 (detailed composition is given in a Table in section 3.3.5.2.)
Batch:	/
Purity:	not given
Dose:	2 ml per kg of a solution containing 0.15% HC Red n° 1
Treatment:	Topical administration on days 1, 4, 7, 10, 13, 16 and 19 of gestation
GLP:	not in compliance

The experiment involved altogether 4 different dye formulations and 3 negative control groups. A dose of initially 0.2 ml and increasing by increments of 0.1 ml per application weekly until reaching 0.5 ml per rat of the test material was applied to the dorsoscapular area of the animals twice a week. Test material was applied to the parental generation (F_0) until they reached 100 days of age after which they were mated. The F_0 parents were reduced to 20 males and 20 females and rebred to produce F_{1b} litters. Twenty males and twenty females from the F_{1b} litter were then administered test material until they reached 100 days of age and were mated twice to produce the F_{2a} and F_{2b} litters. Twenty males and twenty females from the F_{2b} litter were then administered test material until they reached 100 days of age and mated to produce the F_{3a}, F_{3b} and F_{3c} litters. 3 clipped but untreated groups served as negative controls. Body weight, food consumption, survival and reproductive performance were recorded.

Results
No significant difference between test and control groups was observed regarding body weight, food consumption and survival. In all groups of F_2 rats, sialadenitis was observed for some rats followed by an increased incidence of respiratory congestion.

Reproductive performance of F_0, F_1 and F_2 parental rats showed no differences between test and control groups in fertility, gestation and live birth indices. However, the F_2 parents had markedly reduced fertility indices for three separate matings to produce the F_{3a-c} litters. But there were no significant differences between the control groups and the test groups with regard to fertility. Therefore it was concluded that this test formulation did not cause reduction in fertility. No significant differences between body weights, litter size and survival rats between the test and control groups of the offspring was observed.

Within 45% of the female rats that failed to produce offspring, had microscopically evident reproductive tract changes.

Table: Fertility Outcome

Source	**Generation**	**Fertility controls**	**Fertility P25**
Table 25	3_a-litters	10/20 (50), 6/20 (30), 2/20 (10)	7/20 (35)
Table 28	3_b-litters	7/20 (35), 7/20 (35), 3/20 (15)	9/20 (45)
Table 31	3_c-litters	6/19 (32), 3/20 (15), 3/20 (15)	5/19 (26)
Table A1	3_d-litters	control treated females + untreated males 3/20 (15)	
		control treated males + untreated females 16/20 (80)	
		P25- treated females + untreated males 3/20 (15)	
		P25-treated males + untreated females 12/20 (60)	

Ref.: 22

Comment
The study is of limited value due to illness (sialadenitis) of the animals.

3.3.8.2. Teratogenicity

Oral gavage developmental toxicity study in rats, range finding study

Guideline: /
Species/strain: Crl:CD (SD)IGS BR/VAF/Plus rat
Group size: 8 mated females per group
Observation: 14 days
Test substance: HC Red n° 1 in PEG 400
Batch: GTS03974
Purity: 99.8%
Dose level: 0, 25, 75, 125 and 250 mg/kg bw/d by (oral) gavage
Treatment period: once daily from day 6 to 20 of gestation
GLP: in compliance

This study was conducted to provide information for the selection of dosages to be used in the developmental toxicity study of HC Red n° 1. Forty presumed pregnant rats were randomly assigned to five dosage groups, eight rats per group. The test substance was administered orally once daily to rats on days 6 through 20 of gestation at dosages of 0, 25, 75, 125 and 250 mg/kg/day. Viabilities, clinical observations, body weights and feed consumption were recorded. All rats were sacrificed on day 21. The gravid uterus was weighed and subsequently examined for the number and distribution of corpora lutea, implantation sites and uterine contents. Foetuses were weighed and examined for gross external alterations and sex.

Results
All rats survived to scheduled sacrifice. Clinical observations considered test substance related included sparse hair coat, discoloured skin or urine (25, 75, 125 and 250 mg/kg/day dosage groups); purple substance on the fur (75 mg/kg/day dosage group); discoloured fur (75 and 125

mg/kg/day dosage groups); excess salivation, soft or liquid faeces (75, 125 and 250 mg/kg/day dosage groups); and localized alopecia (250 mg/kg/day dosage group). Necropsy observations related to the test substance included a black spleen in the 75, 125 and 250 mg/kg/day dosage groups and discoloured adipose tissue and dark red fluid in the urinary bladder in the 250 mg/kg/day dosage group. Although not dosage dependent, body weight gains were reduced in all test substance groups. The average maternal body weight was reduced in the 250 mg/kg/day dosage group beginning on day 9 and persisted until scheduled sacrifice. Gravid uterine weights were reduced in the 250 mg/kg/day dosage group, as well as the corrected maternal body weights on day 21. Corrected maternal body weight gains remained reduced for the dosage period in the 25, 75 and 125 mg/kg/day dosage groups, but a loss in body weight was observed in the 250 mg/kg/day dosage group. Absolute and relative feed consumption were reduced in the 125 and 250 mg/kg/day dosage groups during the entire dosage and gestation periods; absolute feed consumption values were 89.1%, 98.9%, 88.7% and 75.1% of the vehicle control group value during the dosage period. Pregnancy occurred in 7 to 8 rats in each dosage group. Postimplantation loss (i.e., early resorptions and percentage of resorbed conceptuses per litter) was increased in the 250 mg/kg/day dosage group. Reflecting this increase in postimplantation loss, the averages for litter size and live foetuses were reduced in the 250 mg/kg/day dosage group. Foetal body weights were also reduced in the 250 mg/kg/day dosage group. There were no foetal gross alterations. Based on these data, dosages of 25, 75 and 125 mg/kg/day of HC Red n° 1 were selected for the main study.

Ref.: 17

Oral gavage developmental toxicity study in rats

Guideline:	OECD 414
Species/strain:	Crl:CD (SD)IGS BR/VAF/Plus rat
Group size:	25 mated females per group
Observation:	14 days
Test substance:	HC Red n° 1 in PEG 400
Batch:	GTS03974
Purity:	99.8%
Dose level:	0, 25, 75 and 125 mg/kg bw/d by (oral) gavage
Treatment period:	once daily from day 6 to 20 of gestation
GLP:	in compliance

One hundred presumed pregnant Crl:CD®(SD)IGS BR VAF/Plus® rats, 25 per group, were randomly assigned to four dosage groups. Solutions of the test substance, HC Red n° 1, and/or the vehicle, 100% polyethylene glycol 400 (PEG 400), were administered orally once daily to rats on days 6 through 20 of gestation at dosages of 0, 25, 75 and 125 mg/kg/day. Viabilities, clinical observations, body weights and feed consumption values were recorded. All rats were sacrificed on day 21 of gestation. The gravid uterus was excised, weighed and subsequently examined for the number and distribution of corpora lutea, implantation sites and uterine contents. A gross necropsy was performed. Foetuses were weighed and examined for gross external alterations, sex and either soft tissue or skeletal alterations.

Results

No deaths related to the test substance occurred. One female rat in the 125 mg/kg/day dosage group began delivering and was sacrificed on day 20. Occurrences of orange, red or purple urine were significantly increased in all dosage groups, as well as purple fur and urine-stained

abdominal fur in the 75 and 125 mg/kg/day dosage groups. The number of rats with black spleens was significantly increased in the 125 mg/kg/day dosage group, and two rats in the 75 mg/kg/day dosage group also had black spleens. These discolorations were considered related to the colour of the test substance. One rat in the 125 mg/kg/day dosage group also had a rough spleen. On day 21 of gestation, mean body weight in the 125 mg/kg/day dosage group and corrected maternal body weight in the 75 and 125 mg/kg/day dosage groups were significantly reduced. Body weight losses were observed in all of the treated groups on 6 to 9 and body weight gains were significantly reduced in the 125 mg/kg/day dosage group on days 18 to 21, as well as for the entire dosage and gestation periods. An overall body weight loss was observed in the 125 mg/kg/day dosage group for the entire dosage period, and net body weight gain for the gestation period was also significantly reduced in the 75 and 125 mg/kg/day dosage groups. Absolute and relative feed consumption values were significantly reduced in the 75 and 125 mg/kg/day dosage groups on days 6 to 9 and 12 to 15, as well as for the entire dosage and gestation periods. Absolute feed consumption values were significantly reduced in the 25 mg/kg/day dosage group on days 6 to 9, and absolute and relative feed consumption values were also significantly reduced in the 125 mg/kg/day dosage group on days 18 to 21. Caesarean-sectioning and litter parameters were unaffected by substance treatment as high as 125 mg/kg/day, and no foetal alterations were considered treatment related.

The maternal no-observable-adverse-effect-level (NOAEL) for HC Red n° 1 was 25 mg/kg/day; at doses above this level, there were significant effects on maternal weight gain during the dosage period. There were no adverse effects observed on developmental parameter. Based on these observations, the developmental NOAEL is 125 mg/kg bw/d.

Ref.: 18

Teratology following topical administration of a hair dye formulation containing HC Red n° 1

Guideline:	/
Species/strain:	Charles River CD rats
Group size:	20 mated females per group
Test substance:	A semi-permanent hair dye formulation (P24) containing 0.15% HC Red n° 1 (detailed composition is given in a Table in section 3.3.5.2.)
Batch:	/
Purity:	not given
Dose:	2 ml per kg of a solution containing 0.15% HC Red n° 1
Treatment:	Topical administration on days 1, 4, 7, 10, 13, 16 and 19 of gestation
GLP:	not in compliance

The experiment involved altogether 6 different dye formulations, 1 positive control and 3 negative control groups.
A dose of 2 ml/kg of the test material was applied to the dorsoscapular area of female rats on days 1, 4, 7, 10, 13, 16 and 19 of gestation. As positive control one group was treated with 250 mg/kg bw/d acetylsalicylic acid on days 6-16 of gestation. Three untreated groups served as negative control. As maternal parameters body weight change and food consumption were determined. At necroscopy the number of corpora lutea, implantations, resorptions and abortions was counted and the sex ratio was determined. The number of live foetuses, dead and resorbed foetuses and foetal abnormalities were evaluated.

Results:
No significant differences in maternal and foetal parameters were observed.

Ref.: 19

Comment
The study is of limited value.

3.3.9. Toxicokinetics

/

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

/

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

/

3.3.11. Human data

/

3.3.12. Special investigations

/

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Maximum absorption through the skin	A (µg/cm^2)	=	2.52 µg/cm^2
Typical body weight of human		=	60 kg
Skin area surface	SAS (cm^2)	=	700 cm^2
Dermal absorption per treatment	SAS x A x 0.001	=	1.764 mg
Systemic exposure dose (SED)	SAS x A x 0.001/60	=	0.0294 mg/kg
No observed adverse effect level (rat, subchronic, oral)	NOAEL	=	5 mg/kg
Margin of Safety	**NOAEL/SED**	**=**	**170**

3.3.14. Discussion

Physico-chemical specifications
Stability of the test material in marketed products is not reported. Calculated values of Log P_{ow} can not be accepted as estimates of the true physical constants without justification, indicating that the reported values are realistic.

General toxicity
The acute oral LD_{50} in the Sprague-Dawley strain of rats was between 2500 and 5000 mg/kg bw (males) and between 625 and 1250 mg/kg bw (females).
In a subchronic toxicity study due to the decreased relative thymic weight at 20 mg/kg bw/d the SCCP sets the NOAEL at 5 mg/kg bw/d.
In a teratogenicity study, the maternal no-observable-adverse-effect-level (NOAEL) for HC Red n° 1 was 25 mg/kg/day; at doses above this level, there were significant effects on maternal weight gain during the dosage period. There were no adverse effects observed on developmental parameter. Based on these observations, the developmental NOAEL is 125 mg/kg bw/d.
Several toxicological studies including teratogenicity, subchronic toxicity and reproductive toxicity were performed using topical administration of a hair dye formulation containing *inter alia* 0.15% HC Red n° 1. These studies are of limited value.

Irritation/sensitisation
HC Red n° 1 was shown to be a skin sensitiser in guinea pigs (by Guinea pig maximation test and an open epicutaneous test), in mice (by LLNA) and in humans (by Repeated insult patch test). According to the Guinea pig maximisation test (ref. 5), HC Red n° 1 was an extremely potent skin sensitiser. The quality of the LLNA studies is questioned by the SCCP, assuming that the test concentrations were too low. The Human repeated insult patch test is considered by the SCCP to be unethical.

In Submission II, the applicant states that HC Red n° 1 is a skin sensitiser, and that "This is considered a manageable risk in the context of hair dye products as these products are

appropriately labelled to inform of the potential for allergy along with instructions to conduct a preliminary skin allergy test prior to every product use." The SCCP considers that in the case of extremely potent sensitisers such information on the label does not protect consumers from the risk of sensitisation or elicitation of allergic contact dermatitis.

Dermal absorption
Percutaneous Absorption was investigated i*n vitro* using human skin. The penetration and distribution of [^{14}C]-HC Red n° 1 from a 1% w/w formulation was measured. 1.34 ± 0.72 µg/cm^2 (0.67 ± 0.36%) HC Red 1 was regarded as systemically available. The maximum absorption observed in the experiment was 2.52 µg/cm^2 and this figure was used for calculating the MoS.

Mutagenicity
HC Red n° 1 did not induce gene mutations in three gene mutation tests in bacteria and in a gene mutation test in mammalian cells (*tk* locus). Moreover, HC Red n° 1 was also negative in an *in vitro* unscheduled DNA synthesis test which is often considered a surrogate for a gene mutation test. HC Red n° 1 did, however, induce chromosomal aberrations under *in vitro* conditions as found in three *in vitro* chromosomal aberration tests. Strikingly, in the mouse lymphoma assay where next to gene mutations also the genotoxic endpoint chromosome aberrations is covered, provided that colony sizing is performed, indications for chromosome aberrations were not found. The clastogenic effects of HC Red n° 1 could, however, not be confirmed in an *in vivo* bone marrow micronucleus test in mice. Apparently the genotoxicity of HC Red n° 1 observed *in vitro* does not lead to genotoxic effects in experimental animals under appropriate test conditions.
Since HC Red n° 1 did not produce gene mutations in mammalian cells and since the *in vitro* clastogenicity is overruled by the negative data *in vivo* in mice, the results of the tests performed indicate that HC Red n° 1 has no mutagenic potential *in vivo*.

In addition and according to the dossier a dominant lethal test has been conducted. No evidence of germ cell genotoxicity was noted in this study. Also an *in vivo* rat bone marrow micronucleus has been reported. This *in vivo* micronucleus test is negative as well. The quality of the study is under debate since target cell exposure is not satisfactory demonstrated.
Moreover, if these studies might come available they will not change the conclusion based on the available reports.

Carcinogenicity
No conclusions with regard to carcinogenicity can be drawn from a mouse and a rat study, testing HC Red n° 1 in a semi-permanent hair dye formulation by topical application.

4. CONCLUSION

The SCCP is of the opinion that the use of HC Red n° 1 in semi-permanent hair dye formulation at a maximum final concentration of 1.0% does not pose a risk to the health of the consumer, apart from its sensitising property.

Studies on the genotoxicity/mutagenicity of finished hair dye formulations should be undertaken following the relevant SCCNFP/SCCP opinions and in accordance with its Notes of Guidance.

5. MINORITY OPINION

Not applicable

6. REFERENCES

1a Test Article Bulk Characterisation for Procter & Gamble Study, 2746-53892, June 2005
1b Method Validation and Formulation Sample Analysis for Charles River Laboratories Discovery and Development Services Worcester Division Study VKAA-AB00, 2746-53892, June 2005
2. S. Rodriguez. Acute Toxicity of HC Red No. 1 in Male and Female Rats. Clairol Report C6674-003, February 12, 1987
3. R. Loehr, Rabbit Eye Irritation with HC Red No. 1, Clairol Report C6676-019, January 9, 1987
4. S. Rodriguez. Primary Skin Irritation in Rabbits with HC Red No. 1. Clairol Report C6675-07, January 8, 1987
5. R. Loehr. Screening Test for Delayed Contact Sensitisation in the Albino Guinea Pig with HC Red No. 1. Clairol Notebook #4206, pp. 37-40, February 16, 1979
6. R. Loehr. Evaluation of Sensitisation Potential of Hair Dye Ingredients. Clairol Report, July 25, 1979
7. Chapdelaine. Local Lymph Node Assay #0787XB05.008. Study 99015. Report to Clairol, Chrysalis Preclinical Services, June 30, 2000
8. J. Chapdelaine. Local Lymph Node Assay #0787XB05.010. Study 00006. Report to Clairol, Chrysalis Preclinical Services, July 14, 2000
9. R.C. Reardon, M.L. Reed, and J. Mruczek. Repeated Insult Patch Test: Study 871004. Report to Clairol, TKL Research, Inc., April 21, 1987
10. HC Red 1: *In Vitro* Penetration From A Formulation Through Human Skin, P&G Study # 2746-53899, June 2005
11. A 14-Day Range-Finding Oral Toxicity Study In Rats With GTS03974, P&G Study # 2746-53895, June 2005
12. A 91-Day Oral Gavage Toxicity Study With GTS03974 In Rats With A 28-Day Recovery Phase, P&G study # 2746-53896, July 2005
13. Bacterial Mutation Test (Ames), P&G Study # 2746-53901, June 2005
14. *In Vitro* Chromosome Aberration Assay, P&G Study # 2746-53897, June 2005
15. *In Vitro* Mouse Lymphoma (L5167Y/TK+/-) Mutation Assay, P&G Study # 2746-53898, July 2005
16. *In Vivo* Micronucleus Assay, P&G Study # 2746-53894, June 2005
17. Oral (Gavage) Dosage-Range Developmental Toxicity Study Of GTS03974 In Rats, P&G Study # 2746-53982, June 2005
18. Oral (Gavage) Developmental Toxicity Study Of GTS03974 In Rats, P&G Study # 2746-53893, July 2005
19. C. Burnett, E.I. Goldenthal, S.B. Harris, F.X. Wazeter, J. Strausberg, R. Kapp, and R. Voelker. Teratology and Percutaneous Toxicity Studies on Hair Dyes. J. Toxicol. Env. Health, 1: 1027-1040, 1976
20. C. Burnett, M.M. Jacobs, A. Seppala, and P. Shubik. Evaluation of the Toxicity and Carcinogenicity of Hair Dyes. J. Toxicol. Env. Health, 6: 247-257, 1980
21. E. Goldenthal. Lifetime Toxicity/Carcinogenesis Study in Rats. Report to Clairol, Inc., International Research and Development Corporation, April 10, 1979

22. F.X. Wazeter and E.I. Goldenthal. Multigeneration Reproduction Study in Rats. Report to Clairol, International Research Development Corporation, November 4, 1977

7. ACKNOWLEDGEMENTS

Members of the working group are acknowledged for their valuable contribution to this opinion. The members of the working group are:
Dr. C. Chambers
Prof. R. Dubakiene
Prof. V. Kapoulas
Prof. C. Lidén
Prof. J.-P. Marty
Prof. T. Platzek (Chairman, Rapporteur)
Dr. S.C. Rastogi
Prof. T. Sanner
Dr. J. van Engelen
Dr. I.R. White

External experts

Dr. M.-L. Binderup	Danish Institute for Food and Veterinary Research, Denmark
Dr. H. Norppa	Institute of Occupational Health, Finland
Dr. K. Peltonen	EVIRA, Finland
Dr. J. van Benthem	RIVM, the Netherlands