Scientific Committee on Consumer Safety

SCCS

OPINION ON

Acid Orange 7

COLIPA n° C15

The SCCS adopted this opinion at its 6th plenary meeting

of 18 June 2014

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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This opinion has been subject to a commenting period of four weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged. Revised opinions carry the date of revision.

Keywords: SCCS, scientific opinion, hair dye, Acid Orange 7, C15, CAS 633-96-5, EC 211-199-0, Regulation 1223/2009.

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TABLE OF CONTENTS

ACKNOWLEDGMENTS 3

1. BACKGROUND 5

2. TERMS OF REFERENCE 5

3. OPINION 6

3.1. Chemical and Physical Specifications 6

3.1.1. Chemical identity 6
3.1.2 Physical form 6
3.1.3 Molecular weight 6
3.1.4 Purity, composition and substance codes 7
3.1.5 Impurities / accompanying contaminants 8
3.1.6 Solubility 8
3.1.7 Partition coefficient (Log Pow) 8
3.1.8 Additional physicochemical specifications 8
3.1.9. Stability 8

3.2. Function and uses 8

3.3. Toxicological Evaluation 9

3.3.1. Acute toxicity 9
3.3.2. Irritation and corrosivity 9
3.3.3. Skin sensitisation 11
3.3.4. Dermal / percutaneous absorption 13
3.3.5. Repeated dose toxicity 19
3.3.6. Mutagenicity / Genotoxicity 22
3.3.7. Carcinogenicity 25
3.3.8. Reproductive toxicity 25
3.3.9. Toxicokinetics 27
3.3.10. Photo-induced toxicity 27
3.3.11. Human data 27
3.3.12. Special investigations 27
3.3.13. Safety evaluation (including calculation of the MoS) 28
3.3.14. Discussion 28

4. CONCLUSION 29
5. MINORITY OPINION 29

1. BACKGROUND

Submission I and II for the hair dye Acid Orange 7 (INCI) (COLIPA No. C015) with the chemical name Sodium 4-[(2-hydroxy-1-naphthyl)azo]benzene sulfonate (CAS No 633-96-5 and EC No 211-199-0) were transmitted in September 2003 and July 2005 respectively by Cosmetics Europe.

Acid Orange 7 is identical with CI 15510 also used as a colouring agent "allowed in all cosmetic products except those intended to be applied in the vicinity of the eyes, in particular eye make-up and eye make-up remover".

According to Submission II Acid Orange 7 is a non-reactive dye, used as direct hair colouring agent up to an on-head concentration of 0.5% in non-oxidative as well as in oxidative hair dye formulations. Following Submission II, in March 2011 the Scientific Committee for Consumer Safety (SCCS) concluded that:

"*Based on the low margin of safety, the SCCS is of the opinion that the use of Acid Orange 7 as a hair dye ingredient up to a final on-head concentration of 0.5% under oxidative and non-oxidative conditions poses a risk to the health of the consumer.*

No dermal absorption study was performed under oxidative conditions. No data on the stability in an oxidative environment has been provided.

The safety of the use of Acid Orange 7 (CI15510) as a cosmetic colorant should be assessed." (SCCS/1382/10)[1]

Based on these conclusions, in February and July 2013 Cosmetics Europe1 has transmitted new data (Submission III) to clarify some analytical aspects and a new study on dermal absorption to provide clarification on the evaluation of Systemic Exposure Dose to Acid Orange 7 at 0.5% and 0.8% under non-oxidative and oxidative conditions respectively. In addition, Procter & Gamble[2] has transmitted an *in vitro* study to assess the dermal absorption of Acid Orange 7 under similar use conditions.

2. TERMS OF REFERENCE

1) In light of the new data provided, does the SCCS consider Acid Orange 7 (C015) safe at on-head concentrations up to 0.5% under non-oxidative conditions?

2) Does the SCCS consider Acid Orange 7 (C015) safe at on-head concentrations up to 0.8% under oxidative conditions?
If not, does the SCCS suggest maximum concentrations under oxidative and non-oxidative conditions for which Acid Orange 7 (C015) could be considered safe for consumers?

3) Does the SCCS have any further scientific concerns with regard to the use of Acid Orange 7 (C015) in cosmetic products particularly as it is used as colorant agent with the name of CI 15510?

[1] http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_057.pdf

[2] Procter & Gamble Reference No. 220109-85753

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Acid Orange 7 (INCI)

3.1.1.2. Chemical names

Sodium 4-[(2-hydroxy-1-naphthyl)azo]benzene sulfonate
4-[(2-hydroxy-1-naphthyl)azo]benzenesulfonic acid, monosodium salt

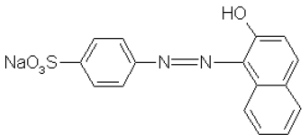
3.1.1.3 Trade names and abbreviations

Orange 205
Acid Orange 7 monosodium salt
Orange II
D&C Orange 4
CI 15510
COLIPA C015

3.1.1.4 CAS / EC number

CAS: 633-96-5
EC: 211-199-0

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

Formula: $C_{16}H_{11}N_2NaSO_4$

3.1.2 Physical form

Orange powder, odourless

3.1.3 Molecular weight

Molecular weight: 350.3 g/mol

3.1.4 Purity, composition and substance codes

Chemical Identification of Acid Orange 7 (Batch No. 2097AF, Lot No. AJ3559, Purity approx. 90%) was performed by UV-Vis, IR, NMR and MS. The sample used for HPLC analysis is identified as D&C Orange 4.

Purity: 90% (total colour content)
Relative chromatographic purity
(HPLC - UV/VIS peak area method): 99-100% at 210 nm, 254 nm, 480 nm)
Batch Comparison

Lot n°	**R0073770**	**AJ3559**	**AK5453**	**AL1478**
Batch	**0201212137**	**2097 AF**		**DC04/7**
		FDA certified	FDA certified	FDA certified
Total colour	99% (HPLC peak area)	90%	91%	96%
Volatile matter		6.7%		2.7%
NaCl		2.7%		1.2%
Na2SO4		0.05		0.05
Water insoluble Matter		0.10%		0.03%
2-Naphthol	0.055%	0.06%		0.02%
Sulfanilic acid	0.012%	0.07%		0.03%
Mercury	<10 ppm	/		/
Lead		/		/
Arsenic		/		/

SCCS comments

- The colour content of various batches, reported in the table above, may not represent actual Acid Orange 7 content. Purity was determined by an US-FDA recommended method, which determines colour content at a specific wavelength. Thus, impurities having the same chromophore groups as the active substance will also be represented as purity. The HPLC content of Acid Orange 7 in the batch 0201212137 was not determined using a standard reference material.
- Colour content in the batch AK5453 is only 91%, but impurities in this batch are not reported.

In the recent submission (submission III), chemical composition of Acid Orange 7 (without reference of batch no.) has been described as in the following table:

Dye Purity (HPLC by area)	greater than 99%
Dye Content (NMR)	greater than 87%
Solvent Content (loss on drying)	less than 13%
Sulphated Ash Content	less than 1%
2-Naphthol	less than 0.15%
Sulfanilic Acid	less than 0.1%
4,4′-(Diazoamino)dibenzenesulfonic acid	less than 0.1%
Total Heavy Metal Content	Arsenic less than 5 ppm Antimony less than 5 ppm Lead less than 20 ppm Cadmium less than 10 ppm Mercury less than 5 ppm

SCCS comment

No documentation was provided for the 87% dye content determined by NMR, or for the data on impurities.

3.1.5 Impurities / accompanying contaminants

See 3.1.4

3.1.6 Solubility

Water:	11% at 30°C
Saline:	2%
DMSO:	10%
Formulation:	2%

SCCS comment
Method for the determination of water solubility was not reported

3.1.7 Partition coefficient (Log Pow)

Log $P_{o/w}$: 1.4 (EC Method A.8)

3.1.8 Additional physicochemical specifications

Melting point:	164 °C (decomposition)
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV/Visible spectrum:	/λ max at 229 nm and 485 nm

3.1.9. Stability

Aqueous solutions were stable ≥72 hours.
Solutions/suspensions of Acid Orange 7 (0.025 mg/ml – 32 mg/ml) in 1% aqueous carboxymethylcellulose, used in 13-week chronic toxicity and developmental toxicity studies, were stable for an 11-day storage period.

SCCS comment
Stability of Acid Orange 7 under oxidative conditions of use has not been reported

General SCCS comments on physico-chemical characterisation

- The content of Acid Orange 7 in the test materials has not been measured using a standard reference material. The reported dye content of various batches may not represent actual Acid Orange 7 content.
- Colour content in the batch AK5453 is only 91%, but impurities in this batch are not reported.
- Stability of Acid Orange 7 under oxidative conditions of use has not been reported
- Stability of Acid Orange 7 in typical hair dye formulations is not reported.

3.2. Function and uses

Acid Orange 7, a non-reactive dye, is used as a direct hair colouring agent up to an on-head concentration of 0.5% in non-oxidative as well as in oxidative hair dye formulations.

Acid Orange 7 is listed as CI 15510 in Annex IV, part 1 – list of colouring agents allowed for use in cosmetic products – to Directive 76/768/EEC on cosmetic products; field of application 2: colouring agents allowed in all cosmetic products except those intended to be applied in the vicinity of the eyes, in particular eye make-up and eye make-up remover.

3.3. Toxicological Evaluation

The evaluation is taken from SCCS/1382/10, except the new dermal absorption study.

Part of the basic toxicological data was presented in the form of original articles, mostly published in peer-reviewed journals. As the substance has already been in use for decades, part of the toxicological data was generated between 1950 and 1980; another part between 1981 and 2000, and some data on mutagenicity in 2003.

Moreover, the applicant of the dossier carried out a literature search using the ChemI Plussystem, a search system including data bases as MEDLINE, TOXNET NLM Gateway etc. A statement is given that all hits of relevance for risk assessment of Acid Orange 7 were included in the presented files.

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

LD50 (rats)	> 10,000 mg/kg bw	
LD50 (♀ mice)	> 10,000 mg/kg bw	Ref.: 1
LD50 (♀ +♂ rats)	> 10,000 mg/kg bw.	Ref.: 2, 3, 4
LD50 (♀ +♂ rats)	11,300 mg/kg bw.	Ref.: 5
Dogs	1,000 mg/kg bw.	Ref.: 5

Conclusion
The test substance is of very low acute oral toxicity.

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline: /
Species/strain: albino rabbits (1.5 – 2.0 kg)
Size: 4 males
Test item: Orange II
Batch: /
Purity: /
Dose: 100 mg
GLP: /

No experimental data are presented. The protocol followed the modified method of Draize (Ref. 6). Results were published in the literature in 1981 (Ref. 7).

Result
Orange II produced very slight redness of abraded skin in some of the rabbits when observed at only 24 hours after application. The authors concluded that this was a false positive reaction.
Under the conditions in this experiment, the material is classified as non-irritant to the skin.
Ref.: 6, 7

SCCS comment
Assessment of skin irritation is not complying with the actual standards. The substance is considered as a mild skin irritant.

3.3.2.2. Mucous membrane irritation

Primary eye irritation, undiluted test compound

Guideline: /
Species/strain: rabbit
Group size: 3 males
Test substance: Orange II
Batch: /
Purity: /
Dose level: 100 mg
GLP: /

No experimental data are presented. Protocol and results were published in the literature in 1981 (Ref. 7).

Result
Based on the result of the experiment the authors conclude that Orange II can be classified as non-irritant to the eyes.
Ref.: 7

Primary eye irritation, diluted test compound

Guideline: /
Species/strain: rabbit
Group size: 6
Test substance: Orange II.
Batch: /
Purity: /
Dose level: 10% solution, twice daily, 5 days/week, 4 weeks

GLP: /

No experimental data are presented. Protocol and results were published by Burnett and Opdyke in 1971, and were briefly cited in Ref. 7.

Result
Orange II was not irritant to the eyes in this test.

Ref.: 7

SCCS comment
Assessment of eye irritation is not complying with the actual standards. Based on the data submitted, eye irritation potential cannot be evaluated.

3.3.3. Skin sensitisation

Magnusson & Kligman Maximisation test

Guideline:	OECD 406 (1981)
Species/strain:	Dunkin-Hartley guinea pig
Group size:	20 females in test group, 10 females in control group
Observ. Period:	25 days
Test substance:	Orange 205
Batch:	/
Purity:	> 85%
GLP:	in compliance

A pre-test was performed in order to assure an optimum technical application procedure.
The main study was performed as follows:
Induction: intradermal induction of sensitisation (day 1) in the test group was performed with Freund´s Complete Adjuvant (FCA) and physiological saline (1:1), test item at 25% in liquid paraffin, 25% dilution of the test item in FCA plus liquid paraffin; ratio 1:1. One week later, the epidermal induction of sensitisation was conducted under occlusion with the test item at 50% in paraffin for 48 hours.

Challenge: two weeks after topical induction, the challenge was performed by application of the test item at 10% and 5% in liquid paraffin under occlusive patch for 24 hours at a different part of the skin. Cutaneous reactions were evaluated at 24 and 48 hours after removal of the dressings.

Result
After the challenge, no skin reactions were observed.

Conclusion
Based on the result of this adjuvant test in guinea pigs, the test article was considered as a non-sensitiser.

Ref.: 10

Local Lymph Node Assay

Guideline:	OECD 429 (2002)
Species/strain:	Mice CBA/J
Group size:	5 females per group

Test substance: D&C Orange 4
Batch: 0201212137ICM Barrier, sample no R0073770
Purity: > 99.2%
Concentrations: a) 0.3, 1, 3, 9% (w/v) in DMSO
b) 0.3, 1, 3 and 5.4% (w/v) in water/acetone (1:1) mixed with olive oil (3:1)
Positive control: p-phenylenediamine (PPD) 1% (w/v) in DMSO
GLP: in compliance

On three consecutive days, 25 µl of test item, vehicle and positive control were applied topically to the dorsal surface of each ear lobe. 5 days after first application [3H]methylthymidine was intravenously injected into a tail vein. 5 hours later, mice were sacrificed by carbon dioxide inhalation and the draining auricular lymph nodes taken and weighed. Single cell suspension was prepared for each animal. Cells were washed with PBS and precipitated with 5% trichloro-acetic acid (TCA). 18 hours later the pellets were suspended in TCA and transferred into the scintillation cocktail. The proliferation capacity of lymph node cells was determined by the incorporation of [3H]-methylthymidine. A test item is regarded as a sensitiser in the LLNA if the exposure to at least one concentration of the test item resulted in an incorporation of 3HTdR at least 3-fold or greater than that recorded in control mice, as indicated by the stimulation index (S.I.).

Results

Treatment	Concentration	Stimulation index
Test item in DMSO	0.3% 1% 3% 9%	1.0 1.5 2.1 1.2
Test item in acetone/water mixed with olive oil	0.3% 1% 3% 9%	1.3 1.4 1.4 1.0
PPD in DMSO	1%	7.9

Conclusion
Based on the result in this LLNA in mice, D&C Orange 4 is not a skin sensitiser under defined experimental conditions in the two vehicles tested (DMSO or acetone/water mixed with olive oil).

Ref.: 11

Human studies

Two human studies were performed.

Firstly, a patch test was performed among 10 volunteers (5 were tested with the individual dye and other 5 with the hair dye formulations). The hair dye formulation consisted of the hair dye in a base formulation containing water, propylene carbonate, alcohol denat, lactic acid, PVMA/MA decadiene crosspolymer, dimethicone copolyol, fragrance, sodium hydroxide. The formulation was tested at 1, 3, and 10% in petrolatum. The individual hair dye was tested at 0.2, 0.6 and 2% in petrolatum.

In the second study, 40 hairdressers with a known allergy to PPD (p-phenylenediamine) and/or DTS (toluene-2,5-diamine) and/or ONPPD (ortho-nitro-p-phenylenediamine) were selected for the cross-reaction study. They were tested with the highest non-irritant concentration of the single hair dye and with the highest non-irritant concentration of the formulation.

Exclusion criteria were applied in both studies based on the use of antihistamines or systemic corticosteroids, moderate to severe skin exposure to the sun or to artificial UV light 1 week before investigation, severe systemic allergic reactions in the past or dermatitis on the skin of the back or severe dermatitis on other parts of the body.

Results
In the first study no irritant reaction to any of the concentrations were observed, either to the individual hair dyes or to the hair dye formulations. Based on these results, the highest concentration was used to perform the study for phase 2.

No positive reactions to the hair dye and to the hair dye formulation were observed in the second study with the hairdressers.

Ref.: 33

SCCS comment
These studies show that cross-sensitivity does not occur in individuals with known contact allergy to the main indicators for hair dye contact allergy.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428 (1999)
Test system:	pig ear skin (400-500 µm), 12 samples, skin integrity checked by conductivity measurement
Contact time:	30 minutes then washing with a shampoo, diffusion monitored for 24 hours
Test substance:	D&C orange 4 used at 0.5% in a hair dye gel formulation, batch T1E20000642 (composition not stated)
Control:	"neutral gel" (composition not stated)
Batch:	2097AF (raw material)
Purity:	90% (raw material)
Application:	200 µl (200 mg) / cm²
Diffusion cell:	flow through system (1 cm²)
Receptor fluid:	saline pH 3.0
Assay:	HPLC
GLP:	in compliance

Porcine ear obtained from the slaughter house immediately after slaughter and before steam cleaning were used for this experiment. The outer ear region was washed, carefully shaved and the skin was removed by dissection. Thickness of the dissected skin was approximately 400-500 µm. The skin was mounted in a glass flow-through diffusion chamber with an area open to diffusion of 1 cm². Each donor chamber was filled with 200 µl (200 mg) of the test item and covered (occluded) with Parafilm™. Saline, pH 3.0, was pumped through the receptor chambers, with a flow rate of 1-2 ml/hour, and was collected in plastic vials which were replaced according to the sampling times and stored at – 20 °C. The whole test system was set up in an incubator adjusted to 32 °C. After 30 min of contact the test item was removed from skin with shampoo solution.
Following the washing procedure the donor chambers were filled with 1ml of saline pH 3.0 for monitoring skin integrity during the 24 hours of diffusion). The collecting vials were changed after 0.5, 1, 2, 4, 6, 8 and 24 hours and D&C orange 4 was analyzed.

At the end of the experiment, the epidermal membrane was separated from the full thickness skin by heat. This technique as described by the applicant removes the horny layer and part of the upper stratum germinativum from the rest of the skin (lower stratum germinativum and upper dermis). After skin extraction, the item bound in the tissues was quantified. Since the epidermis was separated from the dermis by heat separation method,

the amount of dye found in the upper skin is considered by the applicant not to have passed the skin. The amount of penetrated test item found in the receptor solution plus that found in the lower skin extracts are considered as penetrated and absorbed.

Result
No measurable permeation through the skin occurred at any point of time within the time frame of both experiments. The lowest detection limit under the conditions reported is 0.08 µg/ml in the first and 0.078 µg/ml in the second experiment. The maximal possible calculated amount of the test item diffusing across the skin barrier is 1.2 µg/cm² in the first and 1.1 µg/cm2 in the second experiment. Together with the lower skin extract, the worst case consideration of penetrated test item is 2.4 µg/cm² (0.26% of the applied dose) in the first and 4.2 µg/cm² (0.37% of the applied dose) in the second experiment. It has to be emphasised that the values of the absorption without the lower skin are calculated and do not reflect real penetration. The mean recovery of the test item was 107.9% in the first and 92.9% in the second experiment.

Ref.: 23

SCCS comments

* The applied dose of 200 mg/cm² is too high
* The use of a receptor phase at pH 3 is "non-physiologic" and unjustified
* During the complete diffusion period (24 hours) the stratum corneum is in contact with 1 ml of pH 3 saline that is according to the applicant "compatible" with the test product. No information is provided on the effect of this permanent liquid in contact with the horny layer on the skin extraction or diffusion of the test compound.
* The heat separation is considered removing part of the stratum germinativum, in this case most of the epidermis is not taken into account for the estimation of the percutaneous absorption. No histology was performed. It is not acceptable to consider the stratum germinativum (the basal layer) as part of the "horny layer", i.e. a structure from which the tested compound will be exfoliated.
* The amount absorbed should be for both experiments (worst situation):
  - 1.2 µg (for the receptor fluid) + 2.4 µg (for the dermis) + 9.0 µg (for the epidermis) = 12.6 µg absorbed in 24 hours after a contact of 30 minutes
  - 1.1 µg (for the receptor fluid) + 3.1 µg (for the dermis) + 18.0 µg (for the epidermis) = 22.2 µg absorbed in 24 hours after a contact of 30 minutes
  - for the two studies associated, the mean total absorbed through the skin would be 17.4 µg in 24 hours after a contact of 30 minutes
* The study is not in accordance with the SCCS requirements.

Cutaneous absorption of D&C Orange 4, influence of carriers

Guideline:	/
Test system:	full back and flank pig skin (1000 µm), skin integrity checked by triated water
Contact time:	30 minutes then washing with a shampoo, diffusion monitored for 24 hours
Test substance:	D&C orange 4 in several formulations (at 0.5% in a gel and at 0.13% in a foam)
Batch:	/
Purity:	/
Application:	100 mg/cm²
Diffusion cell:	flow through system
Receptor fluid:	saline
Assay:	HPLC
GLP:	/

Split thickness pig skin samples from back and flank skin of a male animal (Schweizer Edelschwein) were used for this experiment, 1000 µm in thickness (stratum corneum, stratum germinativum and part of the dermis containing blood vessels). The surface of the skin which was in contact with the test substance during permeation assay was 4 or 9 cm². The dermal absorption of Orange 4 was investigated from various formulations (gel and foam). 100 mg/cm² of the gel containing 0.5% Orange 4 was applied to the skin (0.5 mg Orange 4 /cm²) for 30 minutes and subsequently washed off with water and shampoo. 100 mg of the foam formulation containing 0.13 mg of Orange 4 (= 0.13%) was applied to 9.1 cm² under similar conditions. In the flow-through system, the physiological receptor fluid in the acceptor chamber was constantly renewed (2.5-5 ml/h). The test item was sufficiently soluble in the receptor fluid (> 1mg/ml), thus not acting as a barrier to absorption. The receptor fluid was sampled after 16, 24 hours or in some experiments up to 88 hours. The content extracted from the skin (epidermis and upper dermis separated) after 24, 72 or 88 hours was determined in the same way.

Result
According to the applicant, the content of Orange 4 found in every single fraction of the receptor fluid was low, despite the late depot often measured on the skin surface or in upper layers of the stratum corneum. Taken together, these findings indicate that the part of Orange 4 which remains on or in the horny layer after the washing steps remains mainly on or in this layer, and that there is poor delivery into the receptor fluid over the observation period ranging from 24 to 88 hours in different experiments. Therefore the amounts found in the epidermis (skin surface) will not be considered for the calculation of the biologically available amount. In the case of the foam formulation, the worst case assumption considers a maximal amount of 12.9 ng/cm² as biologically available, while the same considerations for the other experiments lead to amounts of 557 ng/cm² and 1813 ng/cm² respectively as biologically available. For the risk assessment approach, a worst case assumption of 1813 ng/cm^2 is justified.

Ref.: 27

SCCS comment
This report is a compilation of two studies. The original reports with the complete experimental data are not in the documentation. Because of the lack of information, this report is considered inadequate for the evaluation of the dermal absorption of D&C Orange 4.

Dermal absorption study, submission II 2005

Guideline:	OECD Guideline 428
Tissue:	fresh dermatomed pig ear skin samples 300 µm thickness
Group size:	17 (1 was excluded) 3 experiment x 6 cells
Skin integrity:	conductivity < 900 µS
Diffusion cell:	glass flow-through diffusion cells
Test substance:	D&C Orange 4
Batch:	T1 E 2000064 4
Purity:	90%
Test item:	SC Clear + 0.5% D&C Orange 4 (Lot AJ 3559)
Dose:	20 µl per cm²
Dose of test substance:	100 µg/cm²
Receptor fluid:	Saline (0.9% NaCl)
Solubility receptor fluid:	/
Stability receptor fluid:	≥ 72 hours in water
Method of Analysis:	HPLC analysis and UV/VIS
GLP:	in compliance
Study date:	July 2005

The relevant component of SC Clear + 0.5% D&C Orange 4 was assessed for its potential for dermal absorption on porcine skin.
Three independent experiments were performed with 6 diffusion cells per experiment, of which, due to skin integrity problems, one cell could not be included in the calculations.
Thus, a total of 17 cells were used for the quantification of dermal absorption.
The study was performed on fresh dermatomed pig skin samples mounted on diffusion cells between donor and receptor chambers. The conductivity across the skin samples of each cell was determined before treatment and after the last sampling as a measure of skin integrity. 20 µL of the formulation was applied to each skin sample for 30 minutes.

Results

	Experiment I					
Chamber	1	2	3	4	5	6
Dermal absorption µg/cm^2	1.63	1.23	4.61	1.57	3.05	0.943
Dermal absorption %	1.84	1.33	4.87	1.74	3.25	1.07
	Experiment II					
Chamber	1	2	3	4	5	6
Dermal absorption µg/cm^2	0.359	0.213	0.662	0.285	0.754	0.872
Dermal absorption %	0.43	0.25	0.80	0.33	0.89	1.01
	Experiment III					
Chamber	1	2	3*	4	5	6
Dermal absorption µg/cm^2	0.927	0.367	2.77	0.081	1.12	0.533
Dermal absorption %	1.02	0.42	3.21	0.09	1.27	0.6
Mean dermal absorption Experiments I-III ± SD	**1.13 ± 1.15 µg/cm^2 1.25 ± 1.25 %**					

* value not used for the calculation of the mean dermal absorption, since the integrity of the skin sample was not given

Under the reported conditions, the dermal absorption of D&C Orange 4 is 1.13 ± 1.15 µg/cm² or 1.25 ± 1.20% (mean value of 17 diffusion cells (17 donors)).

Ref.: 31

SCCS comments

As a new dermal absorption study (described below) has been submitted, the results of the study described above are ignored.

New dermal absorption study, submission III 2013

Guideline:	OECD Guideline 428
Tissue:	Human skin samples (frozen) from 5 donors (3 abdomen and 2 breast, age 18-54 years), 200-400 µm thickness
Diffusion cell:	Glass flow-through diffusion cells
Group size:	4 experiments each with 12 diffusion cells mounted with 0.64 cm^2 split thickness human skin
Skin temperature:	32±1°C
Skin integrity:	Tritiated water barrier integrity test in which 250 µL tritiated water (*ca* 100,000 dpm) was applied on the skin; skin samples exhibiting absorption greater than 0.6% of the applied dose were excluded
Test substance:	Acid Orange 7
Batch (non-radiolabelled):	No. R0073770 was used in oxidative hair dye formulations and No. 25 was used non-oxidative hair dye formulations.
Purity:	99.36% (Batch No. R0073770) and 94.4% (Batch No.25)

Radiolabelled test substance: [^{14}C]-Acid Orange 7, Batch No. CFQ41617; radiochemical purity 58 mCi/mmol (molecular weight 352.2) and 99.3%.

Test item:	Oxidative hair dye formulations Magma (Batch No. DTF0938063AF02) with 0.5% on head C015 and Magma (Batch No. DTF0938062AF02) with 0.8% on head C015 when mixed (1:1.5) with developer Welloxon 12%. 0.2% and 0.5% C015 containing non-oxidative hair dye formulation were prepared by incorporating hair dye in Elumen Nulmasse, Batch No. T1 E 2010072 2.
Dose:	ca. 20 mg/cm²
Receptor fluid:	Phosphate buffered saline with 0.01% (w/v) sodium azide
Solubility receptor fluid:	(solubility in water 11%)
Stability receptor fluid:	(≥ 72 hours in water)
Method of Analysis:	Liquid scintillation counting
GLP:	in compliance
Study period:	November 2012-May 2013

Preparation of test items with radiolabelled Acid Orange 7:

Test Preparation 1: Magma with 0.8% on Head (2.55274 g) was added to 500 µCi [^{14}C]-Acid Orange 7 in a glass vial. The contents were vortex-mixed for 1 min, mixed by magnetic stirrer bar for 1 min, then vortex-mixed for a further 1 min. Immediately before dosing, Welloxon 12% developer at 1:1.5 (w/w) (3.84362 g) was added and mixed to a homogeneous formulation.

Test Preparation 2: Magma with 0.5% on Head (2.04169 g) was added to 200 µCi [^{14}C]-Acid Orange 7 in a glass vial. The contents were vortex-mixed for 1 min, mixed by magnetic stirrer bar for 1 min, then vortex-mixed for a further 1 min. Immediately before dosing, Welloxon 12% developer at 1:1.5 (w/w) (3.06104 g) was added, and mixed into the solution by microspatula for 5 min to a homogeneous formulation.

Test Preparation 3: Non-radiolabelled Acid Orange 7 (28.85 mg) was added to 500 µCi [^{14}C]-Acid Orange 7 in a glass vial. Elumen Nullmasse (6.37662 g) was added to the vial. The contents were mixed to a homogeneous formulation.

Test Preparation 4: Non-radiolabelled Acid Orange 7 (also known as D&C Orange No. 4), (9.69 mg) was added to 500 µCi [14C]-Acid Orange 7 in a glass vial. Elumen Nullmasse (6.38549 g) was added to the vial. The contents were mixed to a homogeneous formulation.

Twelve samples of human skin were mounted in flow-through diffusion chambers and the receptor solution was pumped through the receptor chamber at a rate of 1.5 ml/h ± 0.15 ml/h. Twelve replicates from 5 donors were investigated for each test preparation. At the beginning of the experiment each donor chamber was filled with tritiated water and the donor chamber was occluded. Penetration of tritiated water into the receptor fluid over 1 h was assessed to determine the barrier integrity of the skin. Any human skin sample exhibiting greater than 0.6% absorption of tritiated water was excluded from subsequent absorption measurements. At the end of the 1 h period, residual tritiated water was removed from the skin surface by rinsing with water. The skin was then dried with tissue paper.
Percutaneous absorption of each of the Test Preparation1-4 was tested using human skin membranes from 5 donors. *Ca.* 20 mg/cm^2 of the Test Preparations were evenly applied over the surface of the stratum corneum of twelve samples of skin. The donor chambers were not occluded. After 30 min of exposure, the Test Preparation was removed from the skin by eleven washings with 320 µl sodium dodecyl sulphate solution (2% w/v) and the skin was further rinsed with ten 320 µl washes with water. Receptor fluid was collected in

30 min fractions from 0 to 1 h post application, hourly from 1 to 6 h post application and then at 2-hour intervals from 6 to 72 h post application.
At 72 h post dose, the washing procedure was repeated as above. The skin was then removed from the flow-through cells and dried. The stratum corneum was removed by tape stripping and the unexposed skin (skin under the cell flange) was cut off from the exposed skin. The exposed epidermis was then heat separated from the dermis.

Results
A summary of the mean results is provided in the table below:

Test Preparation	1 (oxidative)	2 (oxidative)	3 (non-oxidative)	4 (non-oxidative)
Target Concentration (%, w/w)	0.8	0.5	0.5	0.2
Concentration by Radioactivity (%, w/w)	0.85	0.53	0.51	0.20
Total no. of donors	5	5	5	5
Total no. of replicates dosed	12	12	12	12
Total no. of replicates contributing to Mean & SD	12	12	12	12
Dislodgeable Dose 30 min (% Applied Dose)	102.07	99.62	92.49	80.55
Total Dislodgeable Dose (% Applied Dose)	102.15	99.70	95.54	84.70
Stratum Corneum (% Applied Dose)	0.07	0.11	4.17	11.19
Total Unabsorbed Dose (% Applied Dose)	102.21	99.81	99.74	95.90
Epidermis (% Applied Dose)	0.01	0.02	0.79	2.55
Dermis (% Applied Dose)	<0.01	<0.01	0.06	0.08
Total Absorbed Dose (% Applied Dose)	0.01	0.04	0.06	0.02
Mass Balance (% Applied Dose)	102.24	99.87	100.65	98.55
Dislodgeable Dose 30 min (µg equiv./cm^2)	178.92	101.14	94.42	33.15
Total Dislodgeable Dose (µg equiv./cm^2)	179.06	101.23	97.53	34.86
Stratum Corneum (µg equiv./cm^2)	0.12	0.11	4.25	4.61
Total Unabsorbed Dose (µg equiv./cm^2)	179.18	101.34	101.82	39.47
Epidermis (µg equiv./cm^2)	0.02	0.02	0.80	1.05
Dermis (µg equiv./cm^2)	<0.01	<0.01	0.07	0.03
Total Absorbed Dose (µg equiv./cm^2)	0.02	0.04	0.06	0.01
Mass Balance (µg equiv./cm^2)	179.23	101.40	102.75	40.56

Dislodgeable dose 30 min = skin wash 30 min + tissue swab 30 min + pipette tips 30 min
Total dislodgeable dose = dislodgeable dose 30 min + donor chamber wash + skin wash 72 h + tissue swab 72 h + pipette tips 72 h
Total unabsorbed dose = total dislodgeable dose + stratum corneum + unexposed skin
Total absorbed dose = cumulative receptor fluid + receptor rinse + receptor chamber wash
Mass balance = total unabsorbed dose + total absorbed + epidermis + dermis

Ref. 1, submission III

SCCS comment

According to the SCCS Notes of Guidance, the dermis was added to the total absorbed dose.

Dermal absorption of Acid Orange 7 in
Test Preparation 1 (0.8% on head oxidative hair dye formulation): 0.03 ±0.01 µg/cm^2
Test Preparation 2 (0.5% on head oxidative hair dye formulation): 0.05 ±0.05 µg/cm^2
Test Preparation 3 (0.5% non- oxidative hair dye formulation): 0.13 ±0.12 µg/cm^2
Test Preparation 4 (0.2% non-oxidative hair dye formulation): 0.04 ±0.00 µg/cm^2

For the calculation of MoS, the mean+ 1 SD dermal absorption will be used as described below:
- 0.04 µg/cm^2 (0.03 +0.01 µg/cm^2) Acid Orange 7 for 0.8% on head oxidative hair dye formulation
- 0.25 µg/cm^2 (0.13 +0.12 µg/cm^2) Acid Orange 7 for 0.5% non-oxidative hair dye formulation

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (14 days) oral / dermal / inhalation toxicity

14-days oral toxicity (range-finding study 1)

Guideline: /
Species/strain: SPF rats, strain HanIbm: WIST
Group size: 5/5 (each concentration)
Test substance: Acid Orange 7 in aqueous sol. + 1% CMC
Batch: 2097AF, Lot No. AJ 3559
Purity: 90%
Dosages: 0, 10, 60, 100, 300, 1000 mg/kg bw/day by oral gavage
GLP: /

40 rats (20 males and 20 females, strain HanIbm:WIST, SPF) were used for this assay. At the beginning of the study, the body weights of the animals ranged between 88-114 g for males and between 77-104 for females.

The test material (purity 90%, Batch 2097AF; Lot no. AJ3559) was homogenized in bi-distilled water containing 1% CMC.
The animals (5 males, 5 females, each concentration) were treated by oral gavage, once daily, 7 days per week for 14 days at doses of 0, 10, 60, 100, 300, 1000 mg/kg bw.

Clinical signs, food consumption and body weights were recovered periodically during the pretesting and treatment periods. All animals were killed, necropsied and examined post mortem.

Results
All animals survived until scheduled necropsy. Discoloration of faeces resulting from the oral administration of Acid Orange 7 was observed. No changes were observed in the body and body weight gains of the rats, nor in the food consumption. A significant increase in the absolute and relative spleen weight was observed in the rats treated at the doses of 60, 100, 300 and 1000 mg/kg bw/day. At 10 mg/kg bw/day this increase was also observed but was not statistically significant. Enlarged spleens were macroscopically observed in all rats treated at the dose of 100, 300 and 1000 mg/kg bw/day. Extramedullary hemopoiesis was observed in all rats treated at the dose of 10 and 60 mg/kg bw/day (no histological examination was performed on other groups). This was considered to be related to the tested compound.

Conclusion
On the basis of the results obtained in the 14-day dose range finding study, a proposal of dose levels for 90 day sub-chronic toxicity study is not possible. Therefore, an additional 14-day range finding study with the dose levels of 0, 2.5, 5, 10 mg/kg bw/day was envisaged.

Ref.: 12

14-day oral toxicity (range finding study 2)

Guideline: /
Species/strain: SPF rats, strain HanIbm: WIST
Group size: 5/5 (each concentration)
Test substance: Acid Orange 7 in aqueous sol. + 1% CMC
Batch: No. 2097AF, Lot No. AJ 3559
Purity: 90%
Dosages: 0, 2.5, 5, 10 mg/kg bw/day; by oral gavage
GLP: /

40 rats (20 males and 20 females, strain HanIbm:WIST, SPF) were used for this assay. At the beginning of the study, the body weights of the animals ranged between 94-118 g for males and between 85-106 for females.

The test material (purity 90%, Batch 2097AF; Lot no AJ3559) was homogenized in bi-distilled water containing 1% CMC.
The animals (5 males, 5 females, each concentration) were treated by oral gavage, once daily, 7 days per week for 14 days at doses of 0, 2.5, 5, 10 mg/kg bw.
Clinical signs, food consumption and body weights were recovered periodically during the pre-testing and treatment periods. At the end of the dosing, blood samples were withdrawn for haematology and plasma chemistry analyses. All animals were killed, necropsied and examined post mortem.
Histological examinations were performed on organs and tissues from all animals.

Results
All animals survived until scheduled necropsy and no clinical signs were observed during the study. No changes were observed in the body weight gains of the rats,or food consumption. Methemoglobin levels were significantly increased in the female treated at the dose of 10 mg/kg bw/day after 14 days treatment. This increase was also observed in males at the same dose but was not statistically significant. Heinz bodies were not reported in these animals. Bilirubin levels were also increased in the male rats treated at 10 mg/kg bw/day but this increase was not considered to be related to the tested compound. No test compound related findings in organ weight were observed. No microscopic findings related to Acid Orange 7 were reported.

Conclusion
On the basis of the results obtained in the 14-day dose range finding study, the following dose levels for the 90-day subchronic study were proposed: 0, 2.5, 5, 10 mg/kg bw/day.

Ref.: 13

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

13-week oral toxicity

Guideline:	OECD 408 (1998)
Species/strain:	SPF rats, strain HanIbm: WIST
Group size:	10/10 (each concentration)
Test substance:	Acid Orange 7 in aqueous sol. + 1% CMC
Batch:	No. 2097AF, Lot No. AJ 3559
Purity:	90%
Dosages:	0, 2.5, 5, 10 mg/kg bw/day; by oral gavage
GLP:	in compliance

80 rats (40 males and 40 females, strain WISTAR, HanIbm:WIST (SPF)) were used for this assay. The body weight of the animals in this test ranged between 123-163 g (mean 141 g) for males and between 98-143 g (mean 123 g) for females.

The test material was homogenized in bi-distilled water containing 1% CMC. According to the result of the 14-day dose range finding study (RCC 746460), the following concentrations were used for the 90-day oral gavage study: 0, 2.5, 5, 10 mg/kg bw/day. The animals (10 males, 10 females, each concentration) were treated by oral gavage, once daily, 7 days per week for at least 13 weeks.

Results
All rats survived the duration of the study. No clinical signs were observed. No test-related differences in grip strength were noted in all treated animals, when compared with the

controls. A dose-related decrease in locomotor activity was observed in males treated at the dose of 5 and 10 mg/kg bw/day and in females at the dose of 10 mg/kg bw/day.

Changes in haematology were evident in males treated with 5 and 10 mg/kg bw/day. Changes in haematological parameters consisted in increased methemoglobin levels in males up to 2.5 mg/kg bw/day and females up to 5 mg/kg bw/day, decreased haemoglobin levels in males (5 and 10 mg/kg bw/day) increased reticulocyte counts (relative and absolute) in all test article treated males and a general shift towards high fluorescent reticulocytes in high-dosed males. The changes noted in methemoglobin levels and reticulocte counts in males treated with 2.5 mg/kg bw/day were within the upper levels of the historical control data.

When compared with similarly high values at 5 mg/kg bw/day, there was no correlation to pathomorphological (extramedullary hemopoiesis in the spleen) or other haematological parameters at 2.5 mg/kg bw/day.
In males treated with 10 mg/kg bw/day, increased organ/body weight ratio in the spleen was noted, which correlated to microscopic findings (extramedullary hemopoiesis) in the spleen in all animals treated with 5-10 mg/kg bw/day.
No test article-related macroscopic findings were observed during necropsy.
With regard to the results obtained in this study, especially to changes in the haematological parameters of males, the No Observed Adverse Effect Level (NOAEL) of Acid Orange 7 was considered to be in the general range of around 2.5 mg/kg bw/day. This dose led to borderline effects on met-hemoglobin and reticulocytes counts without concurrent effects on the spleen.

Conclusion
The NOAEL for females was set to 2.5 mg/kg bw/ day by the study authors.

Ref.: 14

SCCS comment
It should be noted that at this dose, some slight early haematological effects (increased met-haemoglobin levels in males and increased reticulocytes count) have been observed. Even if they are in the upper limits of the historical control values, this finding could be test article related. Therefore, the dose of 2.5 mg/kg bw /day is considered as the LOAEL.

90–day dermal toxicity

3 animals per group (rabbits) were used for this assay.

The test material (FDA certified material) was applied to the depilated skin of rabbits. Daily applications were made to intact and abraded skin for 21 days and to intact skin for 90 days. The colour was applied at two concentrations of 0.1 and 1.0%, dissolved in water as well as USP White Ointment. Control groups were treated with respective media.

Results
There was no mortality or any evidence of systemic toxicity. Haematological values, growth responses and urinary components were normal. Gross autopsies disclosed no dose-related findings.

Ref.: 15

3.3.5.3. Chronic (> 12 months) toxicity

18-month dermal toxicity (Literature data)

200 mice (100 male and 100 female) served as controls and 100 mice were assigned to the test article treatment (Swiss Webster mice).
Initial weights of the animals ranged from 17-25 g.
FDA certified colour was used.
The colour was dissolved in distilled water. Mice were painted once weekly with 0.1 ml of the solution or suspension containing 1% of dye on actual pigment basis to a depilated area of 6 cm^2.
Survival, body weight and palpable growths were followed for 18 month. Each mouse was observed daily for behaviour, survival and visible growth.
All mice were necropsied after they died or were sacrificed. Organs were fixed in 10% formalin solution after recording any gross pathological findings. Histological examinations were carried out on all tumours and all visibly abnormal organs.

Results
The authors conclude that no adverse reactions or pathological changes were observed following weekly dermal applications of D&C Orange 4.

Ref.: 16, 17

SCCS comment
The study cannot be regarded as sufficient for evaluation. For additional studies see also 3.3.7. Carcinogenicity.

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial gene mutation assay

Guideline:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA
Replicates:	triplicates in 2 individual experiments
Test substance:	D&C Orange 4
Solvent:	DMSO
Batch nr:	2097AF
Purity:	90% (certified total colour content)
Concentration:	0, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
Treatment:	pre-incubation method with 30 minutes pre-incubation and at least 48 h incubation
GLP:	in compliance
Study date:	27 May 1999 – 7 September 1999

D&C Orange 4 was investigated for the induction of gene mutations in strains of *Salmonella typhimurium* and *Escherichia coli* (Ames test). Liver S9-fraction from untreated 7-8 weeks old male Syrian hamsters was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-experiment for toxicity and mutation induction with all strains both without and with S9-mix. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or clearing of the bacterial background lawn. Because relevant toxic effects were only observed in one of the strains at the maximal concentration, 5000 µg/plate was used as the top concentration. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in all strains used, the pre-experiment is reported as experiment I. Both experiments were performed with the pre-incubation method. Negative and positive controls were in accordance with the OECD guideline.

Results
No precipitation occurred up to the highest concentration tested. Toxic effects evident as clearing of the bacterial background lawn were observed in experiment I in TA98 at the highest dose (5000 µg/plate) and in experiment II in TA98 at 2500 and 5000 µg/plate and in TA1535 at 1000 and 2500 µg/plate for both strains exclusively in the presence of S9-mix. A biologically relevant increase in revertant colonies was not observed in any of the strains tested at any dose level in the absence or presence of S9-mix in both experiments. An isolated increase in strain TA98 exceeding the threshold of twice the number of revertants of the corresponding solvent control was observed in the first experiment at 333 µg/plate in the presence of S9-mix. Since this increase was not reproduced in the second experiment under identical experimental conditions controls, it was considered not biologically relevant.

Conclusion
Under the experimental conditions used D&C Orange 4 was not mutagenic in this gene mutation tests in bacteria, both in the absence and the presence of S9 metabolic activation.
Ref.: 20

In Vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476 (1997)	
Species/Strain:	L5178Y Mouse lymphoma cells	
Replicates:	duplicates in 2 independent experiments	
Test substance:	D&C Orange 4	
Solvent:	deionised water	
Batch no.:	2097 AF	
Purity:	90% (certified total colour content)	
Concentrations:	Experiment I:	0, 13.8, 27.5, 55, 110 and 220 µg/ml without and with S9-mix
	Experiment II:	0, 28.1, 56.3, 112.5, 225, 450 and 900 µg/plate without S9-mix
Treatment	Experiment I:	4 h treatment without and with S9-mix; expression period 72 h and selection period of 10-15 days
	Experiment II:	24 h treatment without S9-mix; expression period 72 h and selection period of 10-15 days
GLP:	in compliance.	
Study date:	25 May 1999 – 13 December 1999	

D&C Orange 4 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Liver S9-fraction from untreated 7-8 weeks old male Syrian hamsters was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-test on toxicity measuring relative suspension growth and relative total growth. In the main tests, cells were treated for 4 h (experiment I) or 24 h (experiment II) followed by an expression period of 72 h to fix the DNA damage into a stable *tk* mutation. Toxicity was measured in the main experiments as percentage relative total growth of the treated cultures relative to the total growth of the solvent control cultures. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. Negative and positive controls were in accordance with the OECD guideline.

Results
The recommended toxic range of approximately 10-20% survival compared to the concurrent negative controls was never covered in any of the experiments.
A dose dependent and biologically relevant increase in the mutant frequency was not observed up to the maximal concentrations tested. The ratio of small *versus* large colonies was not shifted as compared to the solvent control.

Conclusion
Under the experimental conditions used, D&C Orange 4 was not mutagenic in this mouse lymphoma assay at the *tk* locus.

Ref.: 21

SCCS comment
The appropriate level of toxicity (reduction of the relative total growth to 10-20%) was not reached pointing to insufficient exposure of the cells. However, higher concentrations, separated with a factor 2, in an additional experiment resulted in no surviving cells.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

In vivo Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474 (1997)
Species/strain:	NMRI mice
Group size:	5 mice/sex/group
Test substance:	D&C Orange 4
Batch no:	DC04/7
Purity:	96% (certified total colour content)
Dose level:	0, 500, 1000 and 2000 mg/kg bw
Route:	oral gavage
Vehicle:	deionised water
Sacrifice times:	24 h after treatment for all concentrations, 48 h for the high dose only.
GLP:	in compliance
Study date:	23 July 2002 – 19 August 2003

D&C Orange 4 has been investigated for the induction of micronuclei in bone marrow cells of mice. Test doses were based on the results of a pre-experiment with 2000 mg/kg bw on acute toxicity at various intervals of 1, 2-4, 6, 24, 30 and 48 h after start of treatment. In the main experiment, mice were exposed after starvation for 18 h orally to 0, 500, 1000 and 2000 mg/kg bw. Bone marrow cells were collected 24 h or 48 h (high dose only) after dosing. The mice of the high dose group were examined for acute toxic symptoms at intervals of around 1, 2-4, 6 and 24 h after treatment. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE). Bone marrow preparations were stained with May-Grünwald/Giemsa and examined microscopically for the PCE/NCE ratio and micronuclei. Additional animals (2 mice/dose/sex) were dosed, after starvation for 18 h, with the highest dose and the vehicle for blood sampling. Blood was sampled 1 and 4 h after treatment to analytically demonstrate the bioavailability of D&C Orange 4. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-experiment on acute toxicity with an exposure of 2000 mg/kg bw D&C Orange 4 exclusively, ruffled fur was found up to 4 h after administration. In the main experiment reduction of spontaneous activity, eyelid closure and ruffled fur were found at 2000 mg/kg bw up to 6 or 24 h (ruffled fur only).
Treatment with D&C Orange 4 did not result in decreased PCE/NCE ratios compared to the untreated controls indicating that D&C Orange 4 had no cytotoxic properties in the bone marrow. However, the quantitative analysis of the test item in the plasma of the treated animals showed significant amounts of D&C Orange 4 after 1 h. This level dropped after 4 h. Biologically relevant or statistically significant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 nor 48 h after treatment and neither for males nor for females.

Conclusion
Under the experimental conditions used, D&C Orange 4 did not induce a biologically relevant increase in the number of PCEs with micronuclei in bone marrow cells of treated mice and, consequently, D&C Orange 4 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 22

3.3.7. Carcinogenicity

Skin painting, Mice

Skin painting studies in Swiss Webster mice were carried out with a series of 11 coal-tar-derived colours including Acid Orange 7. The treatment groups contained 50 males and 50 females and the control groups contained 100 males and 100 females. Mice were painted once weekly in an area that precluded oral exposure with 0.1 ml containing 1.0% Acid Orange 7 to a depilated 6 cm^2 area. Survival, body weight, and palpable growth were followed for an 18-month period.
Microscopic examination which initially involved 50% of the treated animals was extended to include all tumours and grossly abnormal tissues and organs. There were no significant differences between treatment and control groups.

Ref.: 16

Other studies

Orange 4 is poorly absorbed from the intestine in mammals, but it is metabolized to sulphanilic acid and 1-amino-2-naphthol by micro-organisms in the gastro-intestinal tract which break down the azo linkage. Two papers, describing the same study, report that bladder implantation of pellets of 1-amino-2-naphthol hydrochloride in paraffin wax for 40 weeks caused bladder cancer in mice.
1-Amino-2-naphthol hydrochloride showed no evidence of mutagenicity in *Salmonella typhimurium* bacteria with or without a rat liver activation system.

Ref.: 29, 30

Human studies

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Dose Range Finding Study for effects on Embryo-Foetal Development

Guideline:	/
Species/strain:	SPF rats, strain HanIbm: WIST
Group size:	20 mated rats
Test substance:	Acid Orange 7 in aqueous sol. + 1% CMC
Batch:	No. 2097AF, Lot No. AJ 3559
Purity:	90%
Dosages:	0, 100, 300, 1000 mg/kg bw/day; by oral gavage (day 6 to 17 p.c.)

GLP: /

20 mated females, 5 per group (strain rat HanIbm:WIST, SPF) were used for this assay. The body weights of the animals ranged between 199-233 g at the beginning of the study.
Test material (purity 90%; Batch 2097AF, Lot AJ3559) was dissolved in bi-distilled water containing 1% carboxymethylcellulose sodium salt.
20 mated females rats were treated orally by gavage with a single dose of the test article once daily from day 6 through to day 17 post coitum at dose levels of 0, 100, 300, 1000 mg/kg bw/day.
A standard dose volume of 10 ml/kg bw with a daily adjustment to the actual body weight was used. Control animals were dosed with the vehicle alone.
Females were sacrificed on day 21 post coitum and the foetuses were removed by Caesarean section.

Results
Maternal toxicity: Two females from the 1000 mg/kg bw/day group died after four or five administrations. Prior to death, the females felt cold and showed general poor conditions. Reduced food consumption and body weight gain, orange-brown discoloured urine and faeces, ruffled fur and prostrations were also observed in this tested group. At 300 mg/kg bw/day, a slight decrease in food consumption and body weight gain was also observed in the first half of the treatment period. Discoloration of urine and faeces were also observed at the dose of 300 and 100 mg/kg bw/day. In the females from all tested groups, the spleen appeared enlarged and significant dose-related increased spleen weights were observed in all treated rats.

Foetal toxicity: No abnormalities were observed in any foetuses. In the foetuses of the group treated at 1000 mg/kg bw/day, a slight decrease in the mean body weight was observed.

Conclusion
With the exception of a slight reduced mean foetal body weight at 1000 mg/kg bw/day, no test article related differences were noted amongst the control group and any dose group. During external examination of foetuses, no abnormal findings were noted in any group.
Because of the distinct effects on the spleen weight of dams already at a dose level of 100 mg/kg bw/day, suitable dose levels for the main study for effects on embryo-foetal development in the rat could not be established.

Ref.: 18

Study for the Effects on Embryo-Foetal Development

Guideline:	OECD 414 (1981)
Species/strain:	SPF rats, strain HanIbm: WIST
Group size:	22 mated rats (each concentration)
Test substance:	Acid Orange 7 in aqueous sol. + 1% CMC
Batch:	No. 2097AF, Lot No. AJ 3559
Purity:	90%
Dosages:	0, 5, 40, 320 mg/kg bw/day; by oral gavage (day 6 to 17 p.c.)
GLP:	in compliance

88 mated female rats at the age at pairing of minimum 11 weeks, 22 per group, (strain WISTAR, HanIbm:WIST [SPF]) were used for this assay. The body weight of the animals in this test was between 169-244 g.

The test material was homogenized in bi-distilled water containing 1% CMC. The mixture of the test article and vehicle were prepared daily before administration. According to the

result of dose range finding studies (Ref. 13, 18) the following concentrations were used for the study: 0, 5, 40, 320 mg/kg bw/day.
The animals (22 mated female rats, each concentration) were treated by oral gavage, once daily from day 6 through to day 17 post coitum (last treatment).

A standard dose volume of 10 ml/kg bw was used. Females were sacrificed on day 21 post coitum and the foetuses were removed after Caesarean section. The examination of the dams and foetuses was performed in accordance with international recommendations.

Results
Maternal toxicity: All females survived and no clinical signs were noted in the treated rats. Reduced food consumption and body weight gain, orange-brown discoloured urine and faeces and ruffled fur were observed in the high dose group. At 40 mg/kg bw/day, a slight decrease in food consumption was also observed. Total resorptions were observed in 2 females in the high dose group. In the females treated at the dose of 40 and 320 mg/kg bw/day, a significant dose-related increased spleen weights was observed.

Foetal toxicity: No abnormalities related to the treatment were observed in any foetuses. In the foetuses of the group treated at 1000 mg/kg bw/day (dose not used), a slight decrease in the mean body weight was observed.

Conclusion
Based on the result, the No-Observable-Adverse-Effect-Level (NOAEL) of Acid Orange 7 was considered to be 5 mg/kg bw/day for the maternal organism and 320 mg/kg bw/day for the foetal organism.

Ref.: 19

3.3.9. Toxicokinetics

No data submitted

3.3.10.Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11.Human data

No data submitted

3.3.12.Special investigations

No data submitted

3.3.13.Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Acid Orange 7
(non-oxidative conditions)

Absorption through the skin	**A (mean + 1 SD)**	**= 0.25 µg/cm²**
Skin Area surface	**SAS**	**= 580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**= 0.145 mg**
Typical body weight of human		**= 60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**= 0.0024 mg/kg bw**
Lowest Observed Adverse Effect Level (13-week, oral, rat)	**LOAEL**	**= 2.5 mg/kg bw/d**
Adjusted by factor 3		**= 0.83 mg/kg bw/d**
Bioavailability 50%		**= 0.415**
MoS	**NOAEL / SED**	**= 172**

3.3.14.Discussion

Physico-chemical properties
Acid Orange 7 is intended for use in oxidative and non-oxidative hair dye formulations as a direct dye at a maximum concentration of 0.5%. Acid Orange 7 is also permitted to be used as a cosmetic colorant, listed as CI 15510 in Annex IV of the EU Cosmetic Directive.
The reported Acid Orange 7 content in various batches of test material should be considered semi-quantitative determination, because the content is determined only in terms of colour content. Thus, impurities with the same colour (chromophore groups) will also be measured together with the active substance. Impurities in one of the batches of Acid Orange 7 (colour content 91%) were not reported.
Stability of Acid Orange 7 under oxidative conditions of use has not been reported. In addition, the stability in of Acid Orange 7 in typical hair dye formulations is not reported.

Toxicity
Acid Orange 7 is of low acute oral toxicity.
The NOAEL of a 13-week oral toxicity study in rats was set to 2.5 mg/kg bw/d by the study authors. It should be noted that at this dose, some slight early haematological effects (increased met-haemoglobin levels in males and increased reticulocytes count) have already been observed. Even if these are in the upper limits of the historical control values, this finding could be test article-related. Therefore the dose of 2.5 mg/kg bw/d should be considered as a LOAEL.
Studies of effects on embryo-foetal development lead to a NOAEL of 5 mg/kg bw/d for maternal toxicity and 320 mg/kg bw/d for foetal toxicity.

Irritation and sensitisation
The assessment of skin and eye irritation does not comply with the current standards. According to the studies available, Acid Orange 7 is considered a mild skin irritant. Based on the data submitted, eye irritation potential cannot be evaluated.
Based on the result in the available studies, Acid Orange 7 is not a skin sensitiser.

Percutaneous absorption
For the calculation of MoS, the mean+ 1 SD dermal absorption will be used as described below:

- 0.04 µg/cm^2 (0.03 +0.01 µg/cm^2) Acid Orange 7 for 0.8% on head oxidative hair dye formulation
- 0.25 µg/cm^2 (0.13 +0.12 µg/cm^2) Acid Orange 7 for 0.5% non- oxidative hair dye formulation

Mutagenicity/genotoxicity
Overall, the genotoxicity of Acid Orange 7 is investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. Gene mutations were investigated in an *in vitro* test whereas chromosome aberrations and aneuploidy were tested in an *in vivo* test only. Acid Orange 7 was neither mutagenic in a gene mutation test in bacteria nor in an *in vitro* gene mutation test in mammalian cells. Acid Orange 7 did not induce an increase in bone marrow cells with micronuclei in an *in vivo* micronucleus test in mice. Consequently, Acid Orange 7 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
The sensitivity of the skin painting carcinogenicity test is low and it is unlikely that it would have identified a carcinogenic potential. An experiment where a metabolite of Orange 4 was implanted in the urinary bladder of mice is probably of little relevance to the use of the substance as a hair dye.

4. CONCLUSION

The SCCS is of the opinion that the use of Acid Orange 7 as a hair dye ingredient up to a final on-head concentration of 0.5% under non-oxidative conditions does not pose a risk to the health of the consumer.

As the stability of Acid Orange 7 under oxidative conditions is not known, no final decision can be taken on its safe use in oxidative hair dye formulations.

The SCCS has no information on the use concentrations of Acid Orange 7 as a colorant in other cosmetic products. The aggregate exposure is not known and, therefore, the safety of Acid Orange 7 as a cosmetic colorant cannot be assessed.

5. MINORITY OPINION

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