EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

OPINION ON

HC Orange n° 2

COLIPA n° B67

Scientific Committees
on consumer products
on emerging and newly identified health risks
on health and environmental risks

The SCCP adopted this opinion at its 14th plenary of 18 December 2007

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 Products (SCCP), 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 Evaluation Agency (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCP
Questions concerning the safety of consumer products (non-food products intended for the consumer).
In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

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http://ec.europa.eu/health/ph_risk/risk_en.htm

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1. BACKGROUND

Submission I for HC Orange n° 2 with the chemical name 1-(ß-aminoethylamino)-4-(ß-hydroxyethyloxy)-2-nitrobenzene was submitted in March 1990 by COLIPA [1, 2].

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) adopted at its plenary meeting on 20 May 1998 its opinion (XXIV/1292/97) with the final conclusion that:

"*1-(ß-aminoethylamino)-4-(ß-hydroxyethyloxy)-2-nitrobenzene has an acute oral toxicity of >5000 mg/kg bw in the rat. The substance can be classified as not irritating to skin and mucous membranes. The evidence of a sensitizing potential is equivocal, thus the substance may be regarded as a potential sensitizer, adequate labelling is recommended. Percutaneous absorption of a formulation was 0.055 % in the presence of hair and 0.084 % in the absence of hair. In the 90 day study 150 mg/kg/day is considered to be the NOAEL. In the teratogenicity study no signs of maternal or foetal toxicity were observed after administration of 100 mg/kg bw*."

The substance is currently regulated by the Cosmetics Directive (76/768/EC), Annex III, part 2 under entry 26 on the List of substances, provisionally allowed, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission II for this substance was submitted in July 2005 by COLIPA. According to this submission HC Orange n° 2 is used in semi-permanent hair colouring products 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 Orange n° 2 safe for use as a non-oxidative hair dye with a 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 Orange n° 2 in non-oxidative hair dye formulations?*

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

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Orange n° 2 (INCI)

3.1.1.2. Chemical names

1-(β-Aminoethyl)amino-4-(β-hydroxyethyl)oxy-2-nitrobenzene
1-(2-Aminoethyl)amino-4-(2-hydroxyethyl)oxy-2-nitrobenzene
2-[4-[(2-Aminoethyl)amino]-3-nitrophenoxy]ethanol

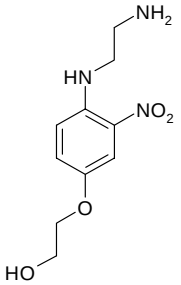
3.1.1.3. Trade names and abbreviations

Imexine FAB
COLIPA n° B67

3.1.1.4. CAS / EINECS number

CAS: 85765-48-6
ELINCS: 416-410-1 (Imexine FAB)

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{10}H_{15}N_3O_4$

3.1.2. Physical form

Red (purple) powder; almost odourless

3.1.3. Molecular weight

Molecular weight: 241

3.1.4. Purity, composition and substance codes

Deduced specifications for the Material used in the market

Purity by potentiometry: > 97.5 g/100g (Determination by spectrophotometry)
Purity (rel.) by HPLC: > 98.0%
Total impurities content: < 1.5 g/100g
Ash content: < 0.3 g/100g

Impurities (batch 0508076*)*
- 4-amino-3-nitrophenol: < 0.01 g/100g
- 4-chloro-3-nitrophenol: < 0.05 g/100g
- 2-(4-chloro-3-nitrophenoxy)-ethanol: < 0.20g/100g
- 2-(4-amino-3-nitrophenoxy)-ethanol: < 0.20g/100g
- 1,2-diaminoethane: < 0.01g/100g
Heavy Metals: < 20 mg/kg
- *As, Sb, Hg:* *< 5 mg/kg Each*
- *Cd:* *< 10 mg/kg*
- *Pb:* *< 20 mg/kg*

Batches used
The following table summarizes the results of characterization the 5 batches used in all the toxicological tests. In addition, Batch CFQ13911 of [ring-U-^{14}C]-HC Orange n° 2 (radiochemical purity ≥ 98.4%) was used in the skin absorption study [13].

	Batch				
	Op. 3	**Op. 7**	**Op. T13**	**Op. 11**	**0508076**
Appearance	Red (purple) powder				
Titre by potentiometry (g/100g)	99.7	99.1	99.5	99.4	97.9
Water content (g/100g)				0.52	0.11
Melting point (°C)				114 (1)	115 (2)
H.P.T.L.C. Profile	Conforms to the standard				
H.P.L.C. Profile [3] **(Relative UV purity %)**				In accordance with specs	98.2
Impurity content (g/100g)					
- A (H.P.L.C)				ND < 0.01	D < 0.01
- B (H.P.L.C)				ND < 0.05	D < 0.05
- C (H.P.L.C)				ND < 0.05	0.11
- D (H.P.L.C)				0.12	D < 0.05
- E (H.P.T.L.C.)					ND < 0.01
- X (H.P.L.C) [3]					0.59
- Z (H.P.L.C) [3]				D	0.66
Residual solvents (µg/g)					
- Isopropanol (GC				370	1000
- DMF (H.P.L.C)					ND < 100
Visible spectrum	The Vis. Spectra are comparable.				
Infra-red spectrum				In conformance with the proposed structure	
^{1}H and ^{13}C N.M.R. spectra				In accordance with the proposed structure	
Mass spectrometry				Compatible with the proposed structure	

ND: not detected - D: detected
- Impurity A: *4-amino-3-nitrophenol*
- Impurity B: *4-chloro-3-nitrophenol*
- Impurity C: *2-(4-chloro-3-nitrophenoxy)-ethanol*
- Impurity D: *2-(4-amino-3-nitrophenoxy)-ethanol*

- Impurity E: *1,2-diaminoethane*
- Impurity X: *2-{2-[4-(2-Aminoethylamino)-3-nitrophenoxy]-ethoxy}-ethanol*
- Impurity Z: 2-(4-{2-[4-(2-Hydroxyethoxy)-2-nitrophenylamino]-ethylamino}-3-nitrophenoxy) ethanol

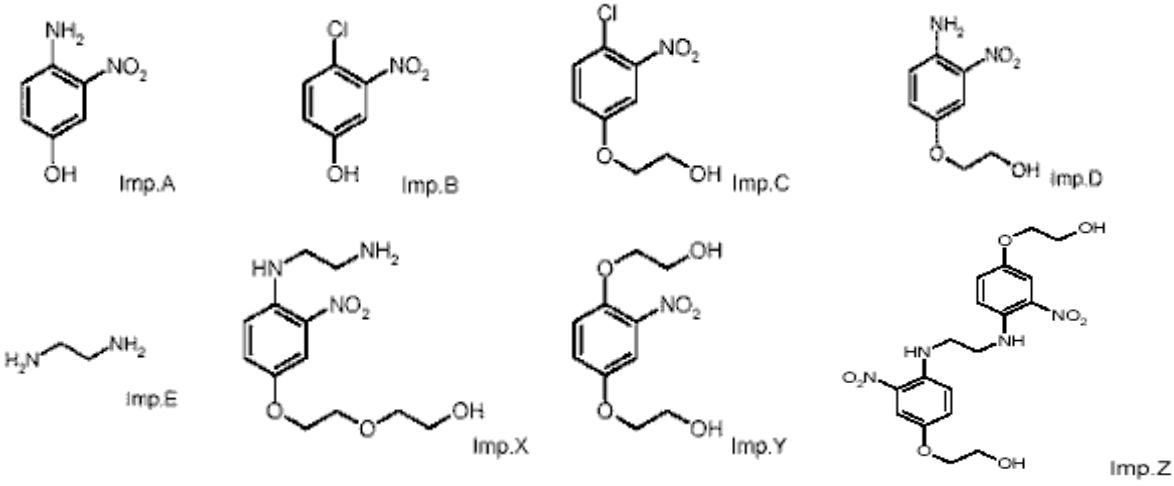
For information: HPLC purity of Y (UV Detection without response factor): 0.12%*
** 2-[4-(2-Hydroxyethoxy)-3-nitrophenoxy]-ethanol, detected in batch 0508076*

(1) Thermomicroscopic method
(2) DSC (differential scanning calorimetry)
(3) Determination of X and Z content against HC Orange N°2 considered as reference standard (100%)

3.1.5. Impurities / accompanying contaminants

Possible impurities which may originate from reagents and intermediate reaction products were checked in batches Op.11 and 0508076:

– Impurity A: 4-amino-3-nitrophenol
– Impurity B: 4-chloro-3-nitrophenol
– Impurity C: 2-(4-chloro-3-nitrophenoxy)-ethanol
– Impurity D: 2-(4-amino-3-nitrophenoxy)-ethanol
– Impurity E: 1,2-diaminoethane
– Impurity X: 2-{2-[4-(2-Aminoethylamino)-3-nitrophenoxy]-ethoxy}-ethanol
– Impurity Y: 2-[4-(2-Hydroxyethoxy)-3-nitrophenoxy]-ethanol.
– Impurity Z: 2-(4-{2-[4-(2-Hydroxyethoxy)-2-nitrophenylamino]-ethylamino}-3-nitrophenoxy) ethanol



Impurities contents
– Impurity A: Op.11: < 0.01g/100g (ND), 0508076: < 0.01g/100g (D)
– Impurity B: Op.11: < 0.05g/100g (ND), 0508076: < 0.05g/100g (D)
– Impurity C: Op.11: < 0.05 g/100g (ND), 0508076: 0.11g/100g
– Impurity D: Op.11: 0.12 g/100g (ND), 0508076: < 0.05 g/100g (D)
– Impurity E: Op.11: Not done on this batch, 0508076: < 0.01g/100g (ND),
– Impurity X: 0508076: 0.59 g/100g (D)
– Impurity Z: 0508076: 0.66 g/100g (ND)
– Impurity Y: HPLC purity (UV Detection without response factor): 0.12 %

3.1.6. Solubility

in water : 4.24 ± 0.16 g/L at 20°C ± 0.5°C according to EEC method A6

in ethanol : < 1 g/100 ml at 23°C after 24 hours
in DMSO : < 1 g/100 ml at 23°C after 24 hours

3.1.7. Partition coefficient (Log P_{ow})

Log $P_{O/W}$: 0.13 According to EEC method A8

3.1.8. Additional physical and chemical specifications

Melting point:	114-115 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
pH:	/
UV_Vis spectrum:	The ultra-violet light absorption, in the range 220 to 350 nm of a 0.005 g/l solution in ethanol (95 %), exhibits two maxima, at 238 nm (ε=0.529) and 286 nm (ε=0.099). The visible light absorption, in the range 350 to 600 nm of a 0.02 g/l solution in ethanol (95 %), exhibits a maximum at 466 nm (ε=0.510).

3.1.9. Homogeneity and Stability

The test item was stable in dosage forms at 0.1 and 250 mg/ml in DMSO and at 5 and 100 mg/ml in DMF over a 4-hour period at room temperature, protected from light and under inert gas atmosphere. It stayed homogeneous at 1 and 200 mg/ml in 0.5% CMC after day 0 and day 9.

The test item was stable in the dosage forms at 1 and 200 mg/ml in 0.5% CMC over a 6-hour period at room temperature and a 9-day period at +4 °C, protected from light and under inert gas atmosphere.

General Comments to physico-chemical characterisation

- The stability of the test substance in marketed products is not reported.
- The test substance is a secondary amine and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb. Data on nitrosamine content is not provided.

3.2. Function and uses

HC Orange n° 2 is used in non-oxidative (semi-permanent) hair colouring products at a maximum concentration of 1.0%.

3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 401
Species/strain:	Sprague-Dawley rats; fasted
Group size:	5 per sex
Test substance:	HC Orange n° 2
Batch:	SPES189058
Purity:	not provided
Dose:	5000 mg/kg bw
Route:	oral, in polyether glycol 400
Exposure:	single administration and a 14 days observation period
GLP:	in compliance

Animals (5/sex) were exposed to HC Orange n° 2 which was suspended in polyether glycol. A dose of 5000 mg/kg bw was administered once.

Results
After two weeks of observation (mortality/morbidity, clinical signs, body weight gain), animals were killed and subjected to gross necropsy examinations. No macroscopic abnormalities were observed.

Conclusion
The LD50 was greater than 5000 mg/kg bw.

Ref.: 1

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:	OECD 404 (1992)
Species:	Male New Zealand White rabbits
Group:	3
Substance:	IMEXINE FAB
Batch:	Op 11
Purity:	99.4%
Dose:	0.5 ml of test substance at 5% in the vehicle
Vehicle:	aqueous solution of carboxymethylcellulose at 0.5%
GLP:	in compliance

A single dose of 0.5 ml was applied to a 6 cm^2 clipped area of the skin of 3 male rabbits. The test substance was held in contact with the skin for 4 hours by means of a semi-occlusive dressing. Cutaneous reactions were observed approximately 1, 24, 48 and 72

hours after removal of the dressing and the daily until day 15. Any residual test substance was removed. The mean score of the values for oedema recorded for each animal after 24, 48 and 48 hours was calculated.

Results
No oedema was observed during the study.
An orange colouration of the treatment site by residual test substance had prevented the evaluation of erythema at grades 3 or 4 during at least 72 hours. Until the end of the observation period, evaluation of erythema was obscured by residual test substance. No necrosis or ulceration was observed.

Conclusion
The study authors concluded that the test substance, at a concentration of 5% in an aqueous solution of carboxymethylcellulose at 0.5%, could provoke moderate but not severe irritant effects.

Ref.: 2

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405 (1987)
Species:	Male New Zealand White rabbits
Group:	3
Substance:	IMEXINE FAB
Batch:	Op 11
Purity:	99.4%
Dose:	0.1 ml of test substance at 5% in the vehicle
Vehicle:	aqueous solution of carboxymethylcellulose at 0.5%
GLP:	in compliance

A single dose of 0.1 ml was instilled into the conjunctival sac of the left eye of 3 rabbits. The eyes were not rinsed after administration of the test substance. Ocular reactions were observed approximately 1, 24, 48 and 72 hours after administration. The mean score of the values recorded for each animal after 24, 48 and 48 hours was calculated.

Results
No ocular reactions were observed during the study.

Conclusion
The study authors concluded that the test substance, at a concentration of 5% in an aqueous solution of carboxymethylcellulose at 0.5%, was not irritant to the rabbit eye.

Ref.: 3

3.3.3. Skin sensitisation

Maximisation test in Guinea Pigs

Guideline:	OECD 406 (1981)	
Species:	albino Dunkin-Hartley guinea pig	
Group:	20 test and 10 control animals	
Substance:	IMEXINE FAB	
Batch:	Op 3	
Purity:	/	
Dose:	intradermal induction:	1% (w/v) in distilled water
		1% (w/v) in Freund's Complete Adjuvant plus distilled water in the ration of 1:1
	Topical induction:	50% (w/w) in distilled water
	Topical challenge:	50% (w/w) in distilled water

Vehicle: distilled water
GLP: in compliance

6 intradermal injections of 0.1 ml were made in the scapular region (3 left and 3 right).

These contained:
- Freund's complete adjuvant plus distilled water in a ratio of 1:1
- a 1% suspension (w/v) of HC Orange n° 2 in distilled water
- a 1% suspension (w/v) of HC Orange n° 2 in a 1:1 preparation of Freund's complete adjuvant in distilled water.

In the control animals, these injections did not contain HC Orange n° 2.

One week later, 0.2-0.3 ml of 50% HC Orange n° 2 in distilled water was administered topically on the same area and held in place for 48 hours under an occlusive dressing. Erythematous reactions were quantified 1 and 24 hours following removal of the patches.
Two weeks after the topical inductions, 0.1-0.2 ml test formulation (50% (w/w) in distilled water) was applied to the right flank of the test and control animals. The vehicle alone was applied to their left flank. The treatments were held in place for 24 hours under an occlusive dressing. Skin reactions were evaluated 24 and 48 hours after removal of the dressing.
Body weights were recorded at the beginning and the end of the study.

Results
No changes in body weight were noted during the study.

Induction phase
Orange-coloured staining of the skin caused by the test material was noted 1 and 24 hours after bandage removal. The degree of staining prevented accurate evaluation of erythema at all treated skin sites in test animals.

Challenge phase
Slight orange-coloured staining was noted at all test sites. This did not prevent accurate assessment of the skin responses.
Positive skin responses (redness grade 1 and 2) were noted at test material sites of eight test group animals at the 2h observation as well as incidents of loss of skin suppleness and/or well-defined oedema.
Scattered mild redness (grade 1) persisted at the test material site of one test group animal at the 48h observation. Common signs of desquamation and loss of skin flexibility were noted at 48h which precluded assessment of erythema at the test material skin sites of four test group animals.
No adverse reactions were noted at the test material and vehicle control sites of the control animals at 24 and 48h observation.

Conclusion
The study authors concluded that the test substance was a moderate skin sensitiser.

Ref.: 4

Local Lymph Node Assay (LLNA)

Guideline: OECD 429 (2002)
Species: CBA/J mouse, nulliparous and non-pregnant females
Group: 4 (preliminary test), 56 (main test)
Substance: HC Orange n° 2
Batch: 0508076
Purity: 97.9%
Concentration: 0, 0.5, 1, 2.5, 5, 10%

Vehicle: dimethylformamide (DMF)
Dose: 25 µL
Control: α-hexylcinnamaldehyde (HCA), 25% in DMF
GLP: in compliance

A preliminary irritation test was performed in order to define the concentrations of the test item to be used in the main test.
HC Orange n° 2 was tested in two independent experiments, both on 28 female mice allocated to seven groups of four animals each: 5 treatment groups, 1 negative control (vehicle) and one positive control (α-hexylcinnamaldehyde, 25% in DMF).
For the 1st experiment, the highest test concentration (10%) was selected. For the 2nd experiment, the tested concentrations were selected on the basis of the results of the first experiment.

In the 1st experiment, the test item was tested at concentrations of 0.5, 1, 2.5, 5, 10%. As equivocal results were obtained and in order to determine more precisely the EC3-value, the test item was tested in the second experiment at the same concentrations.
In each experiment, the test item, vehicle or control were applied over the ears (25 µL per ear) for three consecutive days. After 2 days of resting, the proliferation of the lymph node cells in the lymph node draining was measured by incorporation of tritiated methyl thymidine (day 6). The obtained values were used to calculate the stimulation indices (SI).
The irritation potential of the test item was assessed in parallel by measurement of the ear thickness on day 1, 2, 3 and 6.

Results
Since the test item was non-irritant in the preliminary test, the highest concentration retained for the main test was the maximal practical concentration.
No mortality or clinical signs were observed. No noteworthy increases in ear thickness were observed in the treated animals. A skin orange coloration of the ears, which could have masked slight to severe erythema, was observed in all treated animals.

In the first experiment, positive lymphoproliferative response was observed at the highest concentration but without clear evidence of a dose-response relationship. In the absence of local irritation, this response was attributed to delayed contact hypersensitivity. The calculated EC3-value was equal to 3.80%.

In the second experiment, a dose-related increase in the SI 'except at 5%) was noted and the threshold positive value of 3 was exceeded at concentrations ≥ 2.5%.

The results are presented in the table.

	Concentration (%)	Stimulation Index (SI)	
		1st experiment	2nd experiment
HC Orange n° 2	0.5	1.26	2.25
HC Orange n° 2	1	0.83	2.59
HC Orange n° 2	2.5	2.47	9.02
HC Orange n° 2	5	1.57	8.74
HC Orange n° 2	10	5.57	13.27
α-Hexylcinnamaldehyde	25	4.86	12.13

The EC3-value, calculated on the basis of the results obtained in the second experiment was 1.10%.

Conclusion
Under the experimental conditions, the test item induced contact hypersensitivity in the LLN assay. According to the EC3-value, HC Orange n° 2 was considered to be a strong skin sensitiser.

Ref.: 5

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428 (draft 2000)
Tissue:	abdominal human skin membranes, 3 female donors
Group size:	8 skin membranes
Diffusion cells:	flow-through automated diffusion cell
Skin integrity:	permeability coefficient for tritiated water (< $2.5.10^{-3}$ cm/h for all selected membranes)
Test substance:	HC Orange n° 2 [^{14}C]-HC Orange n° 2 (8.38 MBq/mg, 2.04 GBq/mmol)
Batch:	0508076 CFQ13911 Batch 1 (radio-labelled)
Purity:	97.9% 98.4% (HPLC) (radio-labelled)
Test item:	1.00% (w/w by LSC; 0.97% w/w by HPLC-UV) HC Orange n° 2 in a semi-permanent hair dye formulation
Doses:	20 mg/cm²
Receptor fluid:	phosphate buffered saline containing 0.01% sodium azide (w/v)
Solubility in water:	6 mg/ml (30 °C)
Stability:	1.3% variation in dose formulation after 24 hours
Method of Analysis:	Liquid scintillation counting
GLP:	in compliance

The percutaneous absorption of HC Orange n° 2 was evaluated on 8 skin preparations from 3 different donors. One cell was rejected due to a low recovery value.

The mean recovery of HC Orange n° 2 was 100.4%. Most of the HC Orange n° 2 was recovered in the skin wash after 30 minutes of exposure. The skin wash represented 98.5% of the applied dose and the dislodgeable dose 98.6% of the applied dose. After 24 hours, 1.38% of the applied dose was retained in the stratum corneum; those amounts are not considered to be percutaneously absorbed. Thus, at this time, they do not contribute to the systemic dose (SCCNFP, 2003). At the end of the experiment, 0.41% of the applied dose was found in the skin (epidermis + dermis).

The mean penetration of HC Orange n° 2 into the receptor fluid after 24 hours was 0.03 µgeq/cm², representing 0.01% of the applied dose. The mean maximal flux for the absorption of HC Orange n° 2 through human skin was 0.0046 µgeq/cm²/h. A small difference in penetration of HC Orange n° 2 was observed between the three donors. The skin preparations of donor 2 (highest penetration) were about 2.5 to 5-fold more permeable than the mean penetration through the 5 skin preparations of the 2 other donors.

The mean total absorption (dermal delivery), defined as the compound-relate radioactivity present in the receptor fluid, the receptor compartment wash and the skin (excluding tape strips), was 0.42% of the applied dose.

	Percentage of dose									
Replicate	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	***R7*** [5]	**R8**	**Mean**	**SD**
Skin wash	99.80	97.38	98.54	98.54	97.15	99.81	*79.06*	98.34	**98.51**	**1.04**
Cotton swabs	0.11	0.08	0.13	0.09	0.13	0.08	*0.04*	0.04	**0.10**	**0.03**
Donor compartment	0.02	0.04	0.03	0.04	0.02	0.02	*0.01*	0.01	**0.03**	**0.01**
Dislodgeable dose [1]	99.9	97.5	98.7	98.7	97.3	99.9	*79.1*	98.4	**98.6**	**1.0**
Tape strips	1.55	1.94	1.50	1.41 [4]	1.39	1.12	*0.71*	0.72	**1.38**	**0.38**
Unabsorbed dose [2]	101.5	99.4	100.2	100.1	98.7	101.0	*79.8*	99.1	**100.0**	**1.0**
Receptor fluid +										
Receptor wash	0.01	0.01	0.03	0.02	0.02	0.01	*0.01*	0.01	**0.01**	**0.01**
Skin	0.69	0.25	0.68	0.35	0.25	0.39	*0.42*	0.24	**0.41**	**0.20**
Total absorption [3]	0.70	0.26	0.71	0.37	0.27	0.40	*0.43*	0.25	**0.42**	**0.20**
Total recovery	102.2	99.7	100.9	100.5	99.0	101.4	*80.2*	99.4	**100.4**	**1.2**

	µgeq/cm²									
Replicate	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	***R7*** [5]	**R8**	**Mean**	**SD**
Skin wash	199.7	194.9	197.2	197.2	193.8	199.1	*157.7*	196.2	**196.9**	**2.13**
Cotton swabs	0.23	0.16	0.27	0.19	0.26	0.16	*0.07*	0.09	**0.19**	**0.06**
Donor compartment	0.03	0.08	0.05	0.08	0.05	0.03	*0.02*	0.02	**0.05**	**0.02**
Dislodgeable dose [1]	200.0	195.1	197.5	197.5	194.1	199.3	*157.8*	196.3	**197.1**	**2.1**
Tape strips	3.11	3.89	3.01	2.83 [4]	2.78	2.23	*1.41*	1.44	**2.76**	**0.76**
Unabsorbed dose [2]	203.1	199.0	200.5	200.3	196.9	201.6	*159.2*	197.7	**199.9**	**2.2**
Receptor fluid + Receptor wash	0.02	0.02	0.05	0.04	0.04	0.01	*0.01*	0.02	**0.03**	**0.02**
Skin	1.38	0.51	1.36	0.70	0.50	0.77	*0.84*	0.49	**0.81**	**0.40**
Total absorption [3]	1.40	0.53	**1.41**	0.74	0.54	0.79	*0.86*	0.50	**0.84**	**0.40**
Total recovery	204.5	199.6	202.0	201.0	197.4	202.3	*160.1*	198.2	**200.7**	**2.5**

[1] amount in skin wash, cotton swabs and donor compartment wash
[2] amount in dislodgeable dose and tape strips
[3] amount in receptor fluid, receptor compartment wash and skin (excluding tape strips)
[4] tape stripping stopped after 9 strips
[5] R7 was excluded from calculation due to low recovery value

Conclusion
Under the experimental conditions, the mean total absorption (dermal delivery) defined as the compound-related radioactivity present in the receptor fluid, the receptor compartment wash and the skin (excluding tape strips) was 0.84 µgeq/cm² or 0.42% of the applied dose.
Ref.: 13

Comment
As too few chambers were used, the A_{max} of 1.41 µg/cm² is used for the calculation of the Margin of safety.

3.3.5. Repeated dose toxicity

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

No data submitted

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

Guideline: OECD 408
Species/strain: Sprague-Dawley rats
Group size: 10 per sex per dose
Test substance: HC Orange n° 2
Batch: Op 11
Purity: 99.5%
Dose: 0, 50, 150, 500 mg/kg bw/day
Route: oral, in carboxymethylcellulose
Exposure: by oral gavage
GLP: in compliance

Three groups of 10 males ad 10 females received HC Orange n° 2 daily by oral gavage at doses 0, 50, 150, 500 mg/kg/day for 13 weeks. A negative control group consisted of 10 rats exposed to vehicle only. Animals were observed daily and body weight and food consumption were recorded weekly. An ophthalmologic evaluation in high exposure group was assessed before and after exposure. Haematology and clinical chemistry as well as urine analysis was carried out at week 13.
At the end of the exposure animals were killed and submitted to macroscopic examination. Macroscopic lesions and required tissues from control and high exposure groups were

examined microscopically. In low and medium exposure groups macroscopic lesions were studied only in lungs, liver, kidneys.

Results
No mortality occurred during the study. Clinical signs consisted of ptyalism and reddish/orange to red discolouration of extremities, fur and urine at all dose levels.
Lower body weight gain (10% and 8% in males and females respectively) was observed in both sexes at 500 mg/kg/day group. No changes in food consumption or efficacy of food utilization were observed. Lower blood glucose levels in both sexes at 500 mg/kg/day were recorded. The relative liver weight was higher in 150 mg/kg/day group in females and in both sexes at 500 mg/kg/day group. Significantly higher relative testicular weights were observed at all dose levels. No histological changes were correlated to these findings and they may be attributed to the lower final body weight of the animals.

Conclusion
Based on the lower body weight gain and lower blood glucose level at 500 mg/kg/day group the NOAEL was set at 150 mg/kg bw per day.

Ref.: 6

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD 471	
Strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	
Replicates:	3 replicates in 3 individual experiments both in the presence and absence of S9-mix.	
Test substance:	Imexine FAB	
Solvent:	DMSO	
Batch:	Op T 13	
Purity:	99.5%	
Concentrations:	Experiment 1:	50, 150, 500, 1500 and 5000 µg/plate without S9-mix 10, 30, 100, 300 and 1000 µg/plate with S9-mix
	Experiment 2:	50, 150, 500, 1500 and 5000 µg/plate without S9-mix 50, 150, 500, 1500 and 2500 µg/plate with S9-mix
	Experiment 3:	250, 500, 1000, 1500 and 2000 µg/plate with S9-mix (TA1535 only)
Treatment:	Experiment 1:	direct plate incorporation with 48 incubation without and with S9-mix
	Experiment 2:	direct plate incorporation with 48 - 72 h incubation
	without	S9-mix pre-incubation method with 60 minutes pre-incubation
	and	48 h incubation with S9-mix.
	Experiment 3:	pre-incubation method with 60 minutes pre-incubation
	and	48 h incubation with S9-mix (TA1535 only).
GLP:	In compliance	

Imexine FAB was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Test concentrations were based on the results of a preliminary toxicity study with concentrations up to the prescribed maximum concentration of 5000

µg/plate. Experiment 1 and experiment 2 without metabolic activation were performed with the direct plate incorporation method, experiment 2 with metabolic activation and experiment 3 with the pre-incubation method. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Negative and positive controls were in accordance with the OECD guideline.

Results
Toxicity was reported in experiment 1 without S9-mix at 1500 and 5000 µg/plate and with S9-mix at 1500 (TA102 only) and 5000 µg/plate for all strains; in experiment 2 with S9-mix at 2500 µg/plate for all strains. In experiment 2 with S9-mix for TA1535 increases in revertants were found at 500 and 1500 µg/plate. These positive findings were considered not biologically relevant as they were not dose dependent and not reproducible in the other experiments.
Further biologically relevant, dose dependent increases in the number of revertants were not observed in any strains at any of the doses tested both in the absence or presence of metabolic activation.

Conclusion
Under the experimental conditions used Imexine FAB was not genotoxic (mutagenic) in the gene mutation tests in bacteria both in the absence and the presence of S9 metabolic activation.

Ref.: 7

In vitro Mammalian Cell Gene Mutation Test (*tk* locus)

Guideline:	OECD 476
Cells:	L5178Y mouse lymphoma cells
Replicates:	duplicate cultures in 2 independent experiments
Test substance:	HC Orange n° 2
Solvent:	DMSO
Batch:	0508076
Purity:	97.9%
Concentrations:	Experiment 1: 0.63, 1.25, 1.88, 2.5, 3.75 and 4.5 mM without S9-mix 0.63, 1.25, 2.5, 3.75, 5 and 7.5 mM with S9-mix Experiment 2: 0.50, 1, 2, 3.86, 4 and 4.5 mM without S9-mix 0.88, 1.75, 3.5, 5.25, 6 and 7.0 mM with S9-mix
Treatment	3 h treatment without and with S9-mix; expression period 48 h and selection period of 11 - 12 days
GLP:	In compliance

HC Orange n° 2 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 with at least 6 concentrations up to the prescribed maximum concentration of 10 mM (2410 µg/ml) considering precipitation, adjusted relative total growth and relative suspension growth. In the main test, cells were treated for 3 h in the absence or presence of S9-mix followed by an expression period of 48 h 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 in the main experiments as adjusted relative total growth of the treated cultures relative to the adjusted relative total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
Exclusively in experiment 2 with S9-mix the highest dose tested showed the appropriate level of toxicity (10-20% adjusted relative total growth after the highest dose). In the other

experiments excessive toxicity was seen at the highest dose whereas the first analysable doses did not show enough toxicity.
A slight increase in the mutant frequency was found in experiment 2 at 1 mM. Since this increase was not observed at higher doses and not reproducible, it is considered not biologically relevant. A biological relevant, reproducible increase in the mutant frequency was not observed following treatment with HC Orange n° 2 at any dose level tested in the absence or presence of S9-mix in both experiment 1 and 2.

Conclusion
Under the experimental conditions used, HC Orange n° 2 was considered not mutagenic in the mouse lymphoma assay at the *tk* locus.

Ref.: 8

Comment
The appropriate level of toxicity was not reached in experiment 1 and experiment 2 without S9-mix pointing to insufficient exposure of the cells.

In vitro **Micronucleus Test**

Guideline:	OECD 487 (draft 2004)	
Cells:	human lymphocytes from 2 healthy, non-smoking male volunteers	
Replicates:	duplicates in 2 independent experiments	
Test substance:	HC Orange n° 2	
Solvent:	DMSO	
Batch:	0508076	
Purity:	97.9%	
Concentrations:	Experiment 1:	0, 323.5, 404.3 and 631.8 µg/ml (without S9-mix) 0, 1100, 1200 and 1300 µg/ml (with S9-mix)
	Experiment 2:	0, 650, 750 and 1000 µg/ml (without S9-mix) 0, 1200, 1250 and 1300 µg/ml (with S9-mix)
Treatment	Experiment 1:	24 h PHA followed by 20 + 28 h treatment (without S9-mix) 24 h PHA followed by 3 + 45 h treatment (with S9-mix)
	Experiment 2:	48 h PHA followed by 20 + 28 h treatment (without S9-mix) 48 h PHA followed by 3 + 45 h treatment (with S9-mix)
GLP:	In compliance	

HC Orange n° 2 has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in cultured human lymphocytes. Suitable ranges of test concentrations for the main experiment were based on the results of a cytotoxicity range-finding experiment measuring replication index (RI). To determine the test concentrations for micronucleus analysis in each separate experiment, the RI is measured in cultures treated with increasing concentrations of HC Orange n° 2. The top dose for micronucleus analysis was to be the one at which approximately 60% reduction in RI occurred or the highest dose tested. Two lower doses were selected so that a range of cytotoxicity from maximum to little or none is covered. Treatment periods were 20 h without and 3 h with S9-mix. Harvest times were 72 hours (experiment 1) or 96 hours (experiments 2) after the beginning of culture. The final 27-28 h of incubation was in the presence of cytochalasin B (at a final concentration of 6 µg/ml). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Justified negative and positive controls were used.

Results

Measurements on post-treatment media in the absence or presence of S9-mix indicated that HC Orange n° 2 had no effect on osmolarity or pH as compared to concurrent vehicle controls.
In the experiments with S9-mix the highest analysable concentrations did not reach the target 60% cytotoxicity. However, as the toxicity curve for this treatment was very steep and the highest analysable concentrations used probably are very close to the one that would induce 60% toxicity, the highest concentrations used in the present experiment are considered as suitable top concentrations for analysis.
Without metabolic activation, increases in micronucleated binuclear cells were observed in experiment 1 at the two lowest doses tested. As there was no evidence for a dose response relationship and the values fell within the historical vehicle control range, these increases were considered not to be biologically relevant. In experiment 2 an increase in micronucleated binuclear cells was observed at every dose tested. However, only for the two lower doses these increases were outside the historical vehicle control range. The reduced response at the highest dose tested may be due to cytotoxicity inducing cell cycle delay.
With metabolic activation, an increase in the number of micronucleated binuclear cells compared to concurrent control values was never observed at the different doses tested in both experiments.

Conclusion
Under the experimental conditions used HC Orange n° 2 induced an increase in micronucleated binuclear cells in the absence of metabolic activation following a 20 h treatment.
Consequently, HC Orange n° 2 is genotoxic (clastogenic and/or aneugenic) in cultured human peripheral lymphocytes *in vitro*.

Ref.: 9

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	Crl:CD (SD)BR rats
Group size:	5 rats/sex/group
Test substance:	HC Orange n° 2
Batch:	0508076
Purity:	97.9%
Dose level:	0, 200, 400 and 800 mg/kg bw
Route:	oral gavage, once
Vehicle:	0.5% aqueous carboxymethylcellulose
Sacrifice times:	24 h for all dose levels, vehicle control and positive control, 48 h for the highest dose and vehicle control.
GLP:	In compliance

HC Orange n° 2 has been investigated for the induction of micronuclei in bone marrow cells of rats. Test concentrations were based on a dose range finding assay in which clinical (toxic) signs and mortality was recorded. In the main experiment rats were exposed by oral gavage to single doses of 0, 200, 400 and 800 mg/kg bw. Bone marrow cells were collected 24 h or 48 h (highest dose and vehicle control only) after dosing. Satellite rats (3 rats/sex) allocated for determination of plasma level of HC Orange n° 2 were incorporated.
Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE ratio). All animals were observed for clinical signs and mortality immediately after dosing, 1 h after dosing and at least daily. Bone marrow preparations were stained and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
HC Orange n° 2 did not induce mortality. Clinical signs included red genital discharge, orange urine and orange stain of the tail. No statistically significant decreases in the PCE/NCE ratios were observed with HC Orange n° 2 at doses up to 800 mg/kg bw in either female or male rats. Plasma analysis demonstrated systemic exposure of the test animals after oral administration of HC Orange n° 2 at 800 mg/kg bw.
HC Orange n° 2 did not induce a biological relevant increase in micronucleated erythrocytes at any dose tested.

Conclusion
Under the experimental conditions used HC Orange n° 2 did not induce micronuclei in bone marrow cells of treated rats and, consequently, HC Orange n° 2 was not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of rats.

Ref.: 10

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Study 1

Guideline:	OECD 414
Species/strain:	Sprague-Dawley rats;
Group size:	100 female
Test substance:	HC Orange n° 2
Batch:	Op 11
Purity:	99.5%
Dose:	0, 100, 300, 1000 mg/kg/day
Route:	oral, in carboxymethylcellulose 0.5%
Exposure:	by oral gavage, 5 ml/kg/day
GLP:	in compliance

Three groups of 25 mated female rats of the Sprague-Dawley strain received the test item, by daily oral administration at 5, 20 or 500 mg/kg/day from day 6 to day 15 of gestation. A fourth group of 25 mated rats received the vehicle only. Dosages were based on the data of the dose range finding study in which doses of 300 and 1000 mg/kg/day were used. Animals were observed daily for mortality/morbidity and clinical signs recorded. Food consumption and body weight gain were recorded. Dams were sacrificed on day 20 post-coitum and subjected to necropsy. The number of alive and death foetuses, their distribution and site in the uterus, implantation and number of corpora lutea were determined. The weight and the sex of foetuses were recorded as well as soft tissue and skeletal examinations were carried out.

Results
No mortality of dams was observed. The main clinical signs of dams were ptyalism and loud breathing in the highest exposure group. A dose dependent reduction in mean food consumption and body weight gain was recorded at all dose groups. Total post implantation

loss was observed at 300 and 1000 mg/kg/day. The mean foetal body weight was lower in the 1000 mg/kg/day group. In the 300 and 1000 mg/kg/day group, a delayed ossification of foetuses was observed.

Conclusion
HC Orange n° 2 was ~~not~~ maternal toxic at all dose levels administered. The LOAEL for maternal toxicity was set at 100 mg/kg bw. The NOAEL was 100 mg/kg/day in terms of embryo-foetal developmental effects.

Ref.: 11

Study 2

Guideline:	OECD 414
Species/strain:	Sprague-Dawley rats;
Group size:	24 mated females per dose group
Test substance:	HC Orange n° 2
Batch:	0508076
Purity:	97.5%
Dose:	0, 5, 20, 500 mg/kg bw
Route:	oral, in carboxymethylcellulose 0.5%
Exposure:	by oral gavage, 5 ml/kg/day
GLP:	in compliance

Groups of 24 mated female rats of the Sprague-Dawley strain were exposed daily by oral administration at 5, 20 or 500 mg/kg/day from day 6 to day 19 *post-coitum*. A negative control group (24 females) received the vehicle (0.5% aqueous carboxymethylcellulose) only. Clinical signs and mortality/morbidity were checked daily. Body weight gain and food consumption were recorded. On day 20 *post-coitum*, the dams were sacrificed and subjected to a macroscopic examination. The following litter parameters were recorded: numbers of *corpora lutea*, implantation sites, early and late resorptions, dead and live foetuses. The foetuses were weighed, sexed and subjected to external and visceral, or skeletal examinations.

Results
There were no premature deaths during the study. No treatment-related clinical signs were observed, apart from orange/red-coloration of the urine in all females, a dose-related incidence of orange/red-coloured extremities, and red-coloration of the fur and ptyalism at the 500 mg/kg/day group. Maternal effects observed were reduced body weight gain and reduced food consumption at 500 mg/kg/day group. High incidence of orange-coloured contents of the stomach was observed at 500 mg/kg/day group. None of the litter parameters evaluated were affected. There were no treatment-related malformations or variations in any groups at external, soft tissue or skeletal examinations.

Conclusion
The NOAEL for maternal toxicity was 20 mg/kg/day, and the dose-level of 500 mg/kg/day was the NOEL for embryo-foetal toxicity.

Ref.: 12

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

(HC Orange n° 2)
(Non-oxidative / semi-permanent)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**1.41 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.987 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.016 mg/kg**
No observed adverse effect level (maternal toxicity, oral, rat)	**NOAEL**	**=**	**20 mg/kg bw**
Margin of Safety	**NOAEL / SED**	**=**	**1250**

3.3.14. Discussion

Physico-chemical properties
HC Orange n° 2 is used in non-oxidative (semi-permanent) hair colouring products at a maximum concentration of 1.0%.
The stability of the test substance in marketed products is not reported. The test substance is a secondary amine and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be <50 ppb. Data on nitrosamine content is not provided.

General toxicity
The LD50 for HC Orange n° 2 was greater than 5000 mg/kg bw.
The data of the 90-day study indicated a lower body weight gain and a lower blood glucose level in the 500 mg/kg bw/day dose group. Therefore, the NOAEL was set at 150 mg/kg bw per day.
Two studies were submitted on maternal and/or embryo-toxicity. In study 1, HC Orange n° 2 was maternal toxic at all dose levels administered. The NOAEL was 100 mg/kg bw/day in

terms of embryo-foetal developmental effects. In study 2, the NOAEL for maternal toxicity was 20 mg/kg/day; 500 mg/kg bw/day was considered to be the NOEL for embryo-foetal toxicity.
On the basis of both studies, the NOAEL was 100 mg/kg bw/day for embryo-foetal and 20 mg/kg bw/day for maternal toxicity.

Irritation / sensitisation
HC Orange n° 2, at a concentration of 5% in an aqueous solution of carboxymethylcellulose at 0.5%, showed moderately irritant effects. At a concentration of 5% in an aqueous solution of carboxymethylcellulose at 0.5%, it was not irritant to the rabbit eye.
HC Orange n° 2 was a moderate skin sensitiser in a Guinea pig maximisation test. It induced contact hypersensitivity in the LLNA assay. According to the EC3-value, HC Orange n° 2 was considered to be a strong skin sensitiser.

Dermal absorption
As too few chambers were used in the percutaneous absorption study on pig skin *in vitro*, the A_{max} of 1.41 µg/cm² is used for the calculation of the Margin of safety.

Mutagenicity
The three types of mutation: gene mutation, structural chromosome mutation and aneuploidy were appropriately covered for HC Orange n° 2. HC Orange n° 2 exposure did not result in gene mutations in bacteria or mammalian cells ($tk^{+/-}$ locus). HC Orange n° 2 was clastogenic and/or aneugenic *in vitro* inducing an increase in micronucleated cells *in vitro*. This positive result could, however, not be confirmed in an *in vivo* micronucleus test in bone marrow cells of rats.
Consequently, HC Orange n° 2 can be considered to have no relevant mutagenic potential *in vivo*. Additional tests are not necessary.

Carcinogenicity
No data submitted

4. CONCLUSION

This risk assessment relates to the use of HC Orange n° 2 in non-oxidative hair dye formulations only.

The SCCP is of the opinion that, apart from the risks associated with the use of a strong sensitiser, the use of HC Orange n° 2, at a maximum concentration of 1.0% on the head, does not pose any other risk to the health of the consumer.

HC Orange n° 2 is a secondary amine, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

5. MINORITY OPINION

Not applicable

6. REFERENCES

The references in italics (15-24) were not submitted by the applicant as full reports in the present dossier, since the studies reported therein were not considered to be adequate.

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