Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Yellow No. 16
(Colipa No. B123)

Submission I

The SCCS adopted this opinion at its 12th plenary meeting

on 15 December 2015

Revision of 16 March 2016

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.
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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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This opinion has been subject to a commenting period of eight 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, HC Yellow No. 16 (B123), Regulation 1223/2009, CAS 1184721-10-5

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1. BACKGROUND

Submission I on hair dye HC Yellow No. 16 (B123) (T44P2) CAS No. 1184721-10-5 with the chemical name 2-chloro-4-[(1E)-(1-methyl-1H-pyrazol-5-yl)diazenyl]-phenol was transmitted by Cosmetics Europe in October 2014.

The new ingredient HC Yellow No. 16 (B123) is a non-reactive dye that will be used as a direct hair colouring agent up to 1.5% in non-oxidative as well as up to on-head concentration of 1% in oxidative hair dye formulations.

2. TERMS OF REFERENCE

(1) In light of the data provided, does the SCCS consider HC Yellow No. 16 (B123) safe when used as a direct hair colouring agent up to 1.5% in non-oxidative as well as up to on-head concentration of 1% in oxidative hair dye formulations?

(2) Does the SCCS have any further scientific concerns with regard to the use of HC Yellow No. 16 (B123) in cosmetic products?

3. OPINION

3.1 Chemical and Physical Specifications of HC Yellow No. 16

3.1.1 Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Yellow No. 16 (INCI)

3.1.1.2 Chemical names

2-chloro-4-[(1E)-(1-methyl-1H-pyrazol-5-yl)diazenyl]-phenol

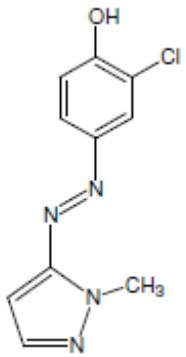
3.1.1.3 Trade names and abbreviations

T44P2

3.1.1.4 CAS / EC number

CAS: 1184721-10-5
EC: /

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

$C_{10}H_9ClN_4O$

3.1.2 Physical form

Yellow powder

3.1.3 Molecular weight

Molecular weight: 236.7 g/mol

3.1.4 Purity, composition and substance codes

Chemical characterisation of the sample Color 38-Yellow T44P2 Lot 11-001 was performed by ^{1}H-NMR, ^{13}C-NMR, IR and UV spectroscopy (in DMSO).

Purity of HC Yellow No. 16 and impurities in it were determined by HPLC-PDA. The detection was performed at 254 nm for all the batches and at 366 nm using HPLC-PDA for the batches FF#20080829 and 20.05.08.

Composition of HC Yellow No. 16 in the 4 batches was reported as described in the table below. The results at λ 254 nm are reported to be:

Batch	FF#20080829	T-9609-9610	20.05.08	11-001
% purity/retention time	99.15	99.26	98.42	99.59
Impurity 1(4.9)	0.05	0.18	1.07	0.27
Impurity 2 (12.26)	0.07	0.32	0	0
Impurity 3 (16.82)	0.1	0.1	0.47	0.14
Impurity 4 (21.9)	0.66	0.05	0	0
Water Content	<1%			
Ash content	<1%			

3.1.5 Impurities / accompanying contaminants

Heavy metal content:
- Arsenic < 5 ppm
- Antimony < 5 ppm
- Lead < 20 ppm
- Cadmium < 10 ppm
- Mercury < 5 ppm

SCCS comments on purity and impurities
- Study report on analytical methodology and information on the validation of the HPLC method used for purity/impurity testing was not submitted.
- IR, ^{13}C-NMR and ^{1}H-NMR data for the chemical identification of the main compound was submitted only for the batch 11-001. IR, ^{13}C-NMR and ^{1}H-NMR data for the batches FF#20080829, T-9609-9610 and 20.05.08 must be submitted.
- Reported %purity appears to be %HPLC peak areas at 254 nm for all the batches and at 366 nm for the batches FF#20080829 and 20.05.08. According to the spectra provided for the characterisation of HC Yellow No. 16, two major absorption bands at ~365 and 495 nm (representing yellow colour) were noted. The purity should be determined at the specific wavelength of the substance, for example at 365 nm for all the batches.
- In the absence of the analytical report, it is unclear how the peak purity of the major peak was calculated before determination; and whether a reference standard was used for the determination of the purity of HC Yellow No. 16 in various batches.

- The impurities of HC Yellow No. 16 were not chemically characterised. The synthetic route was provided in additional data. The applicant provided three possible impurities according to the synthetic route. In addition, the impurities should be characterised by LC-MS or GC-MS. Any impurity belonging to CMR (carcinogenic, mutagenic and reproduction toxic) classes, such as aniline derivative (4-amino-2-chlorophenol), benzene, tetrahydrofuran, dichloromethane, etc. must be quantified.
- The SCCS has been informed by the applicant that details on impurities will be submitted at a later date as the work is still on-going.
- Purity and impurity data presented in the final dossier for the batches 20.05.08 and FF20080829 are not consistent with the data presented in Ref 03 and Ref 06 for the same batches with respect to the number of impurities, retention time (tR) and % purity.
- On the basis of the above-mentioned shortcomings, reported purity and impurity data cannot be accepted.
- The methodology used for the determination of water content, ash content and heavy metal content was not described.

3.1.6 Solubility

Water: 0.5% (pH 9)
DMSO: > 10%
Ethanol: < 1%

SCCS comment
The method for water solubility determination is unclear because the study report of water solubility determination was not submitted. Water solubility should be determined by EC Method A.8. In addition it should be documented that the solubilised material in fact is the test substance and not the impurities in it.

3.1.7 Partition coefficient (Log Pow)

Log P_{ow}	+1.86 ± 0.83 (neutral)	Calculated
	- 1.29 ± 1.0 (mono-anionic form)	Calculated

SCCS comment
Log P_{ow} was not determined by EC Method A.8

3.1.8 Additional physical and chemical specifications

Melting point: /
Boiling point: /
Flash point: /
Vapour pressure: /
Density: /
Viscosity: /
pKa: /
Refractive index: /
UV_Vis spectrum (200-800 nm):
in DMSO: two maxima in absorbance at 365 and ~490 nm, depending on the concentration of the test substance
in HPLC eluent: λmax 201 nm and 365 nm
in alkaline hydrogen peroxide: λmax ca. 450 nm

SCCS comment
The nature of the solvent and the pH of the solution may affect the UV spectra of the test compound. This is the explanation for the differences in the UV spectra of HC Yellow No. 16 in various solvents (DMSO, HPLC mobile phase and alkaline hydrogen peroxide). No additional physicochemical specifications such as melting point and pka value of HC Yellow No. 16 are provided.

3.1.9 Homogeneity and Stability

A solution of 0.5% of HC Yellow No. 16 in MEA (monoethanolamine) buffer was prepared. The dye solution was mixed 1:1 with aqueous peroxide 6% at t=0 and t=45min, a 1 ml sample was taken, diluted to 10ml with water, filtered and analysed by HPLC The chromatograms at t=0 and t=45 minutes were compared. For reference, the dye solution 0.5% in MEA buffer mixed 1:1 with water, diluted to 1/10 in water and filtered was also analysed.
HPLC-PDA analysis of HC Yellow No. 16 mixed with alkaline peroxide was carried out at time t = 0 min and t = 45min. The analysis was carried out at 254nm and 368nm and the UV/vis spectra of the HC Yellow No. 16 peak at time = 0 min and time = 45 min was also compared with the HC Yellow No. 16 reference. The peak area was used to quantify stability.

HC Yellow No. 16 was shown to be stable in alkaline peroxide for at least 45 minutes.

SCCS comment
A stability study under alkaline peroxide conditions was performed using HPLC-PDA analysis at 254 and 368 nm. The batch used for this test is unknown to the SCCS. Also no information is given with respect to the purity and impurities of this batch.

General SCCS comments on physicochemical properties
- Chemical characterisation data should be provided regarding IR, ^{13}C-NMR and ^{1}H-NMR for all the batches used in the studies.
- The purity and impurity data cannot be accepted in the absence of a study report describing analytical methodology, validation of the method, reference standard used for the determination, etc. The impurities of HC Yellow No. 16 were not chemically characterised. HPLC-PDA impurity data presented in the final dossier are not in agreement with those presented in reference 03 and reference 06 for the batches 20.05.08 and FF20080829. In the additional data provided by the applicant, the main structure of the proposed possible impurities is the parent compound (HC yellow No 16). A potential impurity, although of low level, is free radical (as it is indicated from the suggested pattern). In addition, the impurities should be characterised by LC-MS and/or GC-MS and any impurity belonging to CMR (carcinogenic, mutagenic and reproduction toxic) classes, such as carcinogenic aniline derivative, benzene, tetrahydrofuran, dichloromethane, etc. must be quantified.
- Water solubility and Log Pow are not measured using EC Methods.
- Physicochemical specifications such as melting point and density of HC Yellow No. 16 are not provided.
- Stability of HC Yellow No. 16 in typical hair dye formulations is not reported.

3.2 Function and uses

HC Yellow No. 16 is intended to be used as a direct dye ingredient in oxidative and non-oxidative hair colouring products at on-head concentrations of up to respectively 1% and 1.5%.

3.3 Toxicological evaluation

3.3.1 Acute toxicity

3.3.1.1. Acute oral toxicity

No acute oral toxicity study was performed with HC Yellow No. 16.

In the 14-day dose range-finding study in rats, HC Yellow No. 16 was administered by oral gavage (in 0.5% methylcellulose) at dose levels of 0, 75, 150, 300 or 600 mg/kg bw/day. Deaths occurred at 600 mg/kg bw/day (1 male on day 3 and 4 females on days 5-7). Although this dose level was changed to 450 mg/kg/day from day 4 in males and day 7 in females, 3 males died (on days 5-6). Deaths also occurred at 300 mg/kg bw/day (1 male and 1 female on days 6 and 8, respectively). No deaths were reported at the lower dose levels (75 and 150 mg/kg bw/day). Several animals exhibited a decrease in locomotor activity, prone position, tremor, irregular respiration, hypothermia, anaemic change, and/or chromaturia (yellow urine) before death.

Ref.: 6

In the 90-day study in rats administered, HC Yellow No. 16 by oral gavage (in 0.5% methylcellulose) at dose levels of 0, 3, 15 or 75 mg/kg bw/day, no deaths occurred

Ref.: 7

Based on the mortalities in the 14-day range-finding rat study from 300 mg/kg bw/day, HC Yellow No. 16 is considered to possess a moderate acute toxic potential following oral administration.

3.3.1.2 Acute dermal toxicity

No data

3.3.1.3 Acute inhalation toxicity

No data

3.3.1.4 Acute intraperitoneal toxicity

No data

3.3.2 Irritation and corrosivity

3.3.2.1 Skin irritation

Guideline: OECD TG 404 (2002), EC 440/2008
Species/strain: New Zealand White Rabbits
Group size: 3 animals (1 male, 2 females)

Test substance:	T44P2
Batch:	FF#20080829
Purity:	99% (HPLC, 254 nm)
Dose:	0.5 g
Treatment period:	4 hours
Observation:	1, 24, 48, 72 hours and 7, 10, 14 days
GLP:	in compliance
Study period:	21 January- 10 February 2009

The test substance was applied by topical semi-occlusive application of 0.5 g (moistened with approximately 0.5 ml of purified water) to the intact left flank of each of three young adult New Zealand White rabbits (1 male and 2 females). The duration of treatment was four hours. The scoring of skin reactions was performed at 1, 24, 48 and 72 hours, as well as 7, 10 and 14 days after removal of the dressing.

Results
The test item did not elicit any skin reactions at the application site of any animal at any of the observation times (all scores for erythema/eschar and oedema were 0). The test item caused a light yellow staining of the treated skin 1 hour after exposure. Slight staining persisted up to day 14, the end of the observation period.

Conclusion
Based upon the referred classification criteria, HC Yellow No. 16 is considered to be "not irritating" to rabbit skin.

Ref.: 1

SCCS comment
Under the conditions of this study, the test substance is not irritant to rabbit skin.

3.3.2.2 Mucous membrane irritation / Eye irritation

Guideline:	OECD TG 405 (2002), EC 440/2008
Species/strain:	New Zealand White rabbits
Group size:	3 animals (1 male, 2 females)
Test substance:	T44P2
Batch:	FF#20080829
Purity:	99% (HPLC 254 nm)
Dose:	0.1g
Observation:	1, 24, 48, and 72 hours post administration
GLP:	in compliance
Study period:	10 - 20 February 2009

The test substance was applied by instillation of 0.1 g (undiluted) into the left eye of each of three young adult New Zealand White rabbits (1 male and 2 females). The right eye remained untreated and served as the reference control. The ocular reactions were assessed approximately 1, 24, 48 and 72 hours after instillation of the test substance.

Results
Swelling of the conjunctiva (chemosis) was observed in one female rabbit at the 1-hour reading. All animals were observed with slight reddening of the conjunctiva (score 1) at the 1-hour reading and which persisted up to 24 hours in the male rabbit. Slight reddening of the sclera was observed in all animals (score 1) at the 1-hour reading, which persisted up to 48 hours in one male and one female rabbit. Slight ocular discharge was noted in all animals at the 1-hour reading. No irritation reactions of cornea and iris were observed.

Yellow test item remnants were evident in the treated eye or conjunctival sac of all animals at the 1-hour reading. Slight yellow staining of the treated eye produced by the test item was observed in all animals at the 1-hour reading.

Conclusion
Based upon the referred classification criteria (Commission Directive 2001/59/EC of August 2001), HC Yellow No. 16 is considered to be "not irritating" to rabbit eye.

Ref.: 2

SCCS comment
Under the conditions of this study, the test substance is a slight irritant to the rabbit eye; classification according to the CLP criteria is not warranted.

3.3.3 Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD TG 429 (2002)
Species/strain:	female mice CBA/CaOlaHsd; 8-12 weeks old
Group size:	2 (pre-test) 4 per dose group (main test)
Test substance:	T44P2
Batch:	20.05.08
Purity:	98.0% (HPLC)
Vehicle:	dimethylsulfoxide (DMSO)
Concentration:	1%, 5%, 10%, 20%
Positive control:	independent experiment with α-Hexylcinnamaldehyde in acetone: olive oil (4+1)
GLP:	in compliance
Study period:	22 July - 13 August 2008

A non-GLP pre-test was performed with two animals per dose group (2.5, 5, 10 and 20%) to determine the highest tolerable concentration. At the tested concentrations, the mice did not show any signs of severe irritation or systemic toxicity. Based on the pre-test data the concentrations for the main study were chosen as follows: 1, 5, 10 and 20% in DMSO. The highest concentration (20%) was the highest technically achievable concentration in DMSO. On three consecutive days, 25 µl of test item or the vehicle control were applied topically to the dorsum of each ear. Five days after the first application, [^{3}H]-methylthymidine was intravenously injected into the tail vein. Mice were sacrificed and auricular lymph nodes were dissected, pooled for each experimental group and single cell suspensions were prepared. Lymphocyte proliferation was determined by the incorporation of [^{3}H]-methylthymidine. In the LLNA, a test item is regarded as a skin sensitiser if at least in one of the experimental groups a ≥3-fold increase of proliferation is induced compared to the vehicle control group, as indicated by the Stimulation Index (SI).

Results
The animals did not show any clinical signs during the course of the study and no cases of mortality were observed.
The test item induced SI values of 0.78, 0.93, 0.79, and 0.88 at concentrations of 1, 5, 10, and 20%, respectively. The EC3 value could not be calculated since all SI values were below 3.

Conclusion
HC Yellow No. 16, solved in DMSO, did not induce biologically relevant immune response in the LLNA after dermal application to mouse ear.

Ref.: 3

SCCS comment
HC Yellow No. 16 is not a skin sensitiser based on the LLNA results in which a maximum concentration of 20% was used.

3.3.4 Dermal / percutaneous absorption

***In vitro* percutaneous absorption under non-oxidative conditions**

Guideline:	OECD TG 428 (2004)
Test system:	frozen dermatomed pig ear skin (400 ± 80 µm)
Membrane integrity:	conductivity measurement
Replicates:	12 replicates (6 donors)
Test substance:	T44P2
Batch:	11-001
Purity:	98.55%
Test item:	cream with 1.5% of T44P2
Dose applied:	20 µL/cm^2 of the test preparation (approx. 300 µg T44P2/cm^2)
Exposed area:	1 cm^2
Exposure period:	30 minutes
Sampling period:	24 hours
Receptor fluid:	phosphate buffered saline (PBS)
Solubility in receptor fluid:	1500 ng/mL
Mass balance analysis:	provided
Tape stripping:	yes (4 pools of 5 strips each)
Method of Analysis:	LC-MS/MS
GLP:	in compliance
Study period:	6 May- 17 May 2013

Porcine ears were obtained from a slaughterhouse on the day of slaughter. The pigs were steam-cleaned beforehand. The outer ear region was cleaned with cold water. The dermatomed skin (400 ± 80 µm) was stored at -80 °C. Before use, the skin layers were thawed and the skin pieces were stamped out and the hair was removed. The surface of the skin in contact with the test preparation was 1.0 cm². Skin integrity, as measured by the conductivity across the skin samples, was proven before treatment (i.e. conductivity of <900 µS/cm). Only skin samples meeting these criteria were used. The skin was mounted in glass flow-through diffusion chambers with a diameter of 1.135 cm and the receptor solution was pumped through the receptor chamber at a rate of 0.8-1.1 ml/h. The receptor chamber temperature was kept at 32 ± 1 °C during the whole experiment. 12 replicates (6 donors) were investigated. The dermal delivery was monitored over 24 hours under non-occluded static conditions. At the beginning of the experiment, each donor chamber was filled with 1 ml receptor solution to determine the impedance of the skin. After measuring the impedance, the receptor solution was removed from the donor and the skin was dried. After 30 minutes incubation, the test item was removed from the skin by washing three times with shampoo solution. Receptor fluid was collected after 24 hours. The *stratum corneum* was separated by tape stripping from the remaining epidermis and dermis. The tape strips were collected in 4 pools and together with the remaining skin compartments extracted and analysed for their content of the test substance using LC-MS/MS.

Results
The solubility of the test substance in the receptor fluid was given to be at least 1500 ng/ml. The total recovery of 103 ± 6.37% of the applied dose confirmed the validity of the test. One chamber examined did not meet the acceptance criteria (> 85%) and was not used for the calculation. The results are summarised in the Table below:

	Cream with 1.5% T44P2					
Amount of T44P2	Expressed as µg/cm^2 of skin surface mean ± S.D. (n = 11)			Expressed as % of dose mean ± S.D. (n = 11)		
Amount applied	255	±	17.3	100	±	6.77
Unabsorbed dose	262	±	22.2	103	±	8.70
Adsorbed to *Stratum corneum* (isolated by stripping, after 24 hours)	0.966	±	1.628	0.379	±	0.639
Absorbable dose (Epidermis + Dermis (after 24 hours)	0.257	±	0.195	0.102	±	0.0774
Absorbed dose after 24 hours	0.154	±	0.180	0.0616	±	0.0724
Recovery	263	±	22.5	103	±	6.37
Dermal delivery (receptor fluid + epidermis + dermis, excluding tape strip)	0.412	±	0.289	0.164	±	0.117

Conclusion
Under the non-oxidative conditions of this study, it can be stated that the dermal delivery of HC Yellow No. 16 was 0.412 ± 0.289 µg/cm^2 (0.164 ± 0.117% of the applied dose).

Ref.: 4

SCCS comment
In accordance with the SCCS Notes of Guidance, the mean + 1 SD i.e. 0.701 µg/cm^2 will be used for the MoS calculation under non-oxidative conditions.

***In vitro* percutaneous absorption under oxidative conditions**

Guideline:	OECD TG 428 (2004)
Test system:	frozen dermatomed pig ear skin (400±80 µm)
Membrane integrity:	conductivity measurement
Replicates:	12 replicates (6 donors)
Test substance:	T44P2
Batch:	FF#20080829
Purity:	99%
Test item:	T44P2 in oxidative formulation (9.4 g 2% T44P2 gel mixed with 0.6 g 50% hydrogen peroxide lotion)
Dose applied:	20 µL/cm^2 of the test preparation (approx. 360 µg T44P2/cm^2)
Exposed area:	1 cm^2
Exposure period:	30 minutes
Sampling period:	24 hours
Receptor fluid:	phosphate buffered saline (PBS)
Solubility in receptor fluid:	1500 ng/mL
Mass balance analysis:	provided
Tape stripping:	yes (4 pools of 5 strips each)
Method of Analysis:	LC-MS/MS
GLP:	in compliance
Study period:	12 January - 14 February 2010

Porcine ears were obtained from the slaughterhouse on the day of slaughter. The pigs were steam-cleaned beforehand. The outer ear region was washed, carefully shaved and the skin was removed by dissection. Thickness of the dissected skin was approximately 400 ± 80 µm. The surface of the skin that was in contact with the test substance during the permeation assay was 1.0 cm^2. Skin integrity, as measured by the conductivity across the skin samples, was proven before treatment (i.e. conductivity of < 900 µS/cm). Only skin samples meeting these criteria were used. The skin was mounted in glass flow-through diffusion chambers with a diameter of 1.135 cm and the receptor solution (0.9% saline) was pumped through the receptor chamber at a rate of 0.8-1.1 ml/h. 12 replicates (6 donors) were investigated. The dermal delivery was monitored over 24 hours under non-occluded dynamic conditions. At the beginning of the experiment, each donor chamber was filled with 1 ml receptor solution to determine the impedance of the skin. After measuring the impedance, the receptor solution was removed from the donor and the skin was dried. After 30 minutes incubation, the test item was removed from the skin by washing three times with 10% shampoo solution. Receptor fluid was collected after 0.5, 2, 4, 8, 16, 20, 23 and 24 hours. The receptor chamber temperature was kept at 32 ± 1 °C during the whole experiment. The *stratum corneum* was separated by tape stripping from the remaining epidermis and dermis. The tape strips were collected in 4 pools and together with the remaining skin compartments extracted and analysed for their content of test substance using LC-MS/MS.

Results
The solubility of the test substance in the receptor and in the extraction solution is given to be at least 100 ng/ml. The total recovery of 99.8 ± 5.56% of the applied dose confirmed the validity of the oxidative test. Two chambers examined did not meet the acceptance criteria (> 85%) and were not used for the calculation. The results are summarised in the Table below:

	Gel 2% T44P2 to be mixed 1:1 with developer 6%					
Amount of T44P2	Expressed as µg/cm^2 of skin surface mean ± S.D. (n = 10)			Expressed as % of dose mean ± S.D. (n = 10)		
Amount applied	281	±	40.8	100	±	14.5
Absorbed dose after 24 hours	0.309	±	0.18	0.11	±	0.05
Adsorbed to *Stratum corneum* (isolated by stripping, after 24 hours)	0.43	±	0.25	0.15	±	0.09
Absorbable dose (Epidermis + Dermis (after 24 hours)	0.20	±	0.13	0.07	±	0.05
Dislodged dose, 30 min	280	±	48.3	99.4	±	5.66
Unabsorbed dose	280	±	48.2	99.7	±	17.15
Recovery	281	±	48.1	99.8	±	5.56
Dermal delivery (receptor fluid + epidermis + dermis, excluding tape strip)	0.51	±	0.21	0.18	±	0.07

The lowest detection limit under the conditions reported is 0.15 ng/ml and the lowest limit of quantification is 0.20 ng/ml in PBS and MeOH.

Conclusion
Under the oxidative conditions of this study, it can be stated that the dermal delivery of HC

Yellow No. 16 was 0.51 ± 0.21 µg/cm² (0.18 ± 0.07% of the applied dose).

Ref.: 5

SCCS comment
In accordance with the SCCS Notes of Guidance, the mean + 1 SD i.e. 0.72 µg/cm^2 will be used for the MoS calculation under oxidative conditions.

3.3.5. Repeated dose toxicity

3.3.5.1 Repeated Dose (14 days) oral toxicity

Guideline:	OECD TG 407 (2008)
Species/strain:	Rats, Crl:CD(SD)
Group size:	5/sex/group
Test substance:	T44P2
Batch:	T-9609-9610
Purity:	99.27%
Vehicle:	0.5% w/v aqueous methylcellulose solution
Dose levels:	0, 75, 150, 300(200), 600(450) mg/kg bw/day
Dose volume:	10 ml/kg bw/day
Route:	oral
Administration:	by gavage
GLP:	in compliance
Study period:	3 September – 16 December 2010

In a dose-range finding study, the test substance was administered by oral gavage to male and female rats (5/sex/group) at dose levels of 0, 75, 150, 300(200), and 600(450) mg/kg bw/day once daily for 14 days. Due to severe clinical signs (death and/or remarkable decreases in body weight and food consumption) during the first week of dosing, the dose levels were reduced from 600 mg/kg bw/day to 450 mg/kg bw/day from day 4 onwards in males and from day 7 onwards in females, and from 300 mg/kg bw/day to 200 mg/kg bw/day from day 8 onwards in males. Control animals were dosed with the vehicle alone. The dosing formulations were prepared just before each administration.

A micronucleus test was carried out using a portion of surplus femur obtained at the scheduled necropsy: see section 3.3.6.2.

Results
The stability of the test substance was confirmed with the certificate of analysis provided by the sponsor. The test substance is stable at room temperature for at least 2 years.

Deaths occurred at 600 mg/kg bw/day (1 male on day 3 and 4 females on days 5-7). Although this dose level was reduced to 450 mg/kg/day from day 4 in males and day 7 in females, 3 males died (on days 5-6). Deaths also occurred at 300 mg/kg bw/day (1 male and 1 female on days 6 and 8, respectively). Several animals exhibited a decrease in locomotor activity, prone position, tremor, irregular respiration, hypothermia, anaemic change, and/or chromaturia (yellow urine) before death. Remarkable decreases in body weight and food consumption were also noted.
No deaths were reported at the lower dose levels (75 and 150 mg/kg bw/day). A decrease in locomotor activity, emaciation, tremor, irregular respiration, and/or anaemic change were noted in one male at 300(200) mg/kg bw/day from day 7 and in one female at 600(450) mg/kg bw/day between days 5 and 10. Chromaturia was noted sporadically in one male and in one female each at 300(200) mg/kg bw/day and 600(450) mg/kg bw/day.

In the surviving animals, a statistically significantly lower body weight was observed in males at 300(200) mg/kg bw/day or more and in females at 150 mg/kg bw/day or more on day 3, However, individual body weight increased from day 6 in females at 150 mg/kg bw/day and from day 9 in males and females at 300(200) mg/kg bw/day or more.
Food consumption was decreased correspondingly, but was gradually alleviated and was not statistically significant on day 14.

Several changes related to the test substance were noted in urinalysis, haematology and clinical chemistry in males and/or females at 75 mg/kg bw/day and/or more.
Statistically significant increases in kidney and spleen weights were noted in males and females at 150 mg/kg bw/day or more. Liver weight increased in males at 300(200) mg/kg bw/day and in females at 300 mg/kg bw/day or more. Adrenal weight increased in males at 150 and 300(200) mg/kg bw/day. Thymus weight decreased in females at 300 mg/kg bw/day.
Histopathological changes were observed in the kidney, spleen, liver and bone marrow in males and/or females at 75 mg/kg bw/day and/or more.

Conclusion
On the basis of the results obtained in the 14-day dose range finding study, the no-observed adverse effect level (NOAEL) of T44P2 is lower than 75 mg/kg bw/day.

Ref.: 6

3.3.5.2 Sub-chronic (90 days) toxicity (oral)

Guideline:	OECD TG 408 (1998)
Species/strain:	Rats, Crl:CD(SD)
Group size:	15/sex/group (control and high-dose groups), 10/sex/group (other groups)
Test substance:	T44P2
Batch:	11-001
Purity:	99.2%
Vehicle:	0.5% w/v aqueous methylcellulose solution
Dose-levels:	0, 3, 15, 75 mg/kg bw/day
Dose volume:	10 ml/kg bw/day
Route:	oral
Administration:	by gavage
GLP:	in compliance
Study period:	22 September 2011 – 15 March 2012

The test substance was administered by oral gavage to male and female rats (10/sex/group) at dose levels of 0, 3, 15, and 75 mg/kg bw/day once daily for 90 days. Control animals were dosed with the vehicle alone. The dose levels were based on the findings in the 14-day dose-range finding study described above (Ref.: 6). An additional 5 males and 5 females were assigned to the control and the high dose groups to assess the reversibility of the effects after a 4-week recovery period.

Results
The stability of the substance was confirmed with the certificate of analysis provided by the sponsor. The test substance is stable at room temperature for at least 2 years.
The stability, homogeneity and concentration of the test substance in the dosing formulations were confirmed when it was stored for 8 days in a refrigerator followed by a 6-hour period at room temperature shielded from light at concentrations of 0.1 mg/ml and 15 mg/ml.

Ref.: 13

No deaths and no treatment-related clinical signs of toxicity were observed during the study.
No abnormalities were noted in any group in the function tests (sensory reactivity to stimuli and grip strength), or in the motor activity test, body weight, food consumption and ophthalmology.
In the final week of the dosing period, an increase in epithelial cells in urinary sediment was noted in two females at 75 mg/kg/day and an increase in urinary glucose in one female at 75 mg/kg/day. In males, no abnormalities were noted in any groups.
At the end of the recovery period, no treatment-related changes were noted.

At the end of the dosing period, statistically significant decreases in red blood cell count, haematocrit, haemoglobin concentration, and MCHC were noted in males and females at 75 mg/kg/day. Decreases in red blood cell count, haemoglobin concentration and MCHC were also noted in females at 15 mg/kg bw/day. Statistically significant increases in MCV, MCH, and reticulocyte ratio and count were noted in males and females at 75 mg/kg bw/day.
At the end of the recovery period, no treatment-related changes were noted.

At the end of the dosing period, a statistically significant increase in total bilirubin was noted in females at 75 mg/kg bw/day; no abnormalities were noted in males.
At the end of the recovery period, no treatment-related changes were noted.

At the end of the dosing period, statistically significant increases in the relative spleen weights were noted in males and females at 75 mg/kg bw/day, and in the relative kidney weights in males at 75 mg/kg bw/day.
At the end of the recovery period, no treatment-related changes were noted.

At the end of the dosing period, treatment-related histopathological changes were noted in the kidney, spleen, liver and bone marrow.
In the kidneys, increased incidence and severity (minimal to mild grade) of basophilic tubule, and cell infiltration (lymphocyte, interstitium; minimal grade) were noted in males; necrosis of proximal tubular epithelium (minimal grade) and regeneration of tubular epithelium (minimal grade) were noted in females; and interstitium mineralisation (minimal grade), hyaline cast (minimal grade), and brown pigment deposition in the tubular epithelium (minimal grade) were noted in males and in females, at 75 mg/kg bw/day.
In the spleen, increased incidences of extramedullary haematopoiesis of erythroid lineage (minimal grade) and of haemosiderin deposition in splenic macrophages (minimal grade) were noted in females at 15 mg/kg bw/day and in males and females at 75 mg/kg bw/day.
In the liver, increased incidences of focal hepatocyte necrosis (minimal grade) in males and of haemosiderin deposition in Kupffer cells (minimal grade) were noted in females at 75 mg/kg bw/day.
In the femoral bone marrow, an increase in erythrocytic cells (minimal grade) was noted in males and females at 75 mg/kg bw/day.
At the end of the recovery period, treatment-related changes were still noted in the kidney and spleen.

Conclusion
The NOAEL was judged to be 15 mg/kg bw/day for males and 3 mg/kg bw/day for females.
Ref.: 7

SCCS comment
The SCCS agrees with the NOAEL of 3 mg/kg bw/day; this NOAEL is used for the MoS calculation.

3.3.5.3 Chronic (> 12 months) toxicity

No data

3.3.6 Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD TG 471 (1997)	
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2 uvrA	
Replicates:	triplicates in two independent experiments	
Test substance:	T44P2	
Batch:	FF#20080829	
Purity:	99% (HPLC 254 nm)	
Solvent:	DMSO	
Concentrations:	experiment I:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
	experiment II:	39.06, 78.13, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate without and with S9-mix
Treatment:	direct plate incorporation with at least 48 h incubation, without and with S9-mix	
GLP:	in compliance	
Study period:	19 June – 7 July 2009	

T44P2 was investigated for the induction of gene mutations in strains of *Salmonella typhimurium* and *Escherichia coli* (Ames test). Liver S9 fraction isolated from 7-8 weeks old male Syrian golden 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. Since in this pre-experiment, evaluable plates were obtained for five concentrations or more in all strains used, the pre-experiment is reported as the main experiment. The experiments were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results

No precipitation occurred up to the highest concentration investigated.
Without metabolic activation, toxic effects, evident as a reduction in the number of revertants, occurred in experiment I at 2500 µg/plate and above in TA1537 and at 1000 µg/plate and above with TA98; in experiment II at 5000 µg/plate in TA1537 and in *E. coli*.
With metabolic activation, toxic effects were observed in experiment I at 1000 µg/plate and above in TA1537 and at 5000 µg/plate in TA1535 and TA98; in experiment II at 2500 µg/plate and above in TA1535 and at 5000 µg/plate in TA1537 and TA98.
A biologically relevant increase in revertant colony numbers of any of the tester strains was not observed following treatment with T44P2 at any concentration level, neither in the presence nor absence of metabolic activation.

Conclusion
Under the experimental conditions used, HC Yellow No. 16 was not genotoxic (mutagenic) in this gene mutation test in bacteria.

Ref.: 8

SCCS comment
T44P2 is an azo dye. In the SCCS Notes of Guidance, for azo dyes and diazo compounds in the gene mutation test in bacteria the use of a reductive metabolic activation system is recommended.

***In vitro* Mammalian Cell Gene Mutation Test (*hprt*-locus)**

Guideline:	OECD TG 476 (1997)	
Cells:	V79 cells	
Replicates:	duplicate cultures in two independent experiments	
Test substance:	T44P2	
Batch:	FF#20080829	
Purity:	99.09% (HPLC 254 nm); 98.83% (HPLC 366 nm)	
Solvent:	DMSO	
Concentrations:	experiment I:	9.4, 18.8, 37.5, 75 and 600 µg/ml without and with S9-mix
	experiment II	4.7, 9.4, 18.8, 37.5 and 75 µg/ml with S9-mix and 4 h treatment 9.4, 18.8, 37.5 75 and 600 µg/ml without S9-mix and 24 h treatment
Treatment:	experiment I:	4 h treatment both without and with S9-mix; expression period 7 days and a selection period of 8 days
	experiment II:	4 h treatment with S9-mix; expression period 7 days and a selection period of 8 days 24 h treatment without S9-mix; expression period 7 days and a selection period of 8 days
GLP:	in compliance	
Study period:	23 June – 24 August 2009	

T44P2 was assayed for gene mutations at the *hprt* locus of V79 cells in both the absence and presence of S9 metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-test on toxicity. Toxicity of T44P2 is indicated by a reduction of the cloning efficiency. Concentrations were tested without and with metabolic activation with concentrations between 4.7 and 600 µg/ml.
In the main tests, cells were treated for 4 h or 24 h (experiment II, without S9-mix only) followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Toxicity was measured in the main experiments as percentage cloning efficiency of the treated cultures relative to the cloning efficiency of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-test for toxicity, relevant cytotoxicity occurred at 9.4 to 37.5 µg/ml without metabolic activation. No relevant toxicity was noted at higher but precipitating concentrations. In the presence of metabolic activation, and following continuous treatment for 24 h, cytotoxic effects were observed at 37.5 µg/ml and above. Precipitation of T44P2 visible to the unaided eye occurred at 75 µg/ml and above under all experimental conditions.

The data on cloning efficiency did not indicate that the required 10-20% survival was reached at any concentration. In experiment I only the second cultures in the presence of metabolic activation had the required survival. In experiment II, the second cultures in the presence of metabolic activation, the higher concentrations were even too toxic.
In both experiments, no biologically relevant and concentration-dependent increase in the mutant frequency was observed, either in the presence or in the absence of metabolic activation. Moreover, a linear regression analysis did not indicate a biologically relevant trend of the mutant frequency by a probability value of <0.05 in any of the experimental groups. Some isolated increases were judged as irrelevant fluctuation since they were not reproduced in the parallel culture under identical conditions or at any other higher concentration.

Conclusion
Under the experimental conditions used, HC Yellow No. 16 did not induce gene mutations in this gene mutation test in mammalian cells and, consequently, HC Yellow No. 16 is not mutagenic in V79 cells.

Ref.: 9

Micronucleus Test in mammalian cells

Guideline:	draft OECD TG 487
Cells:	V79 cells
Replicates:	duplicate cultures in a single experiment
Test substance:	T44P2
Batch:	FF#20080829
Purity:	99.09% (HPLC 254 nm); 98.83% (HPLC 366 nm)
Solvent:	DMSO
Concentrations:	0, 138.8, 277.5 and 555 µg/ml without S9-mix 0, 17.3, 34.7, 69.4, 138.8 and 277.5 µg/ml with S9-mix
Treatment:	4 h treatment both without and with S9-mix; harvest time 24 h after the beginning of treatment
GLP:	in compliance
Study period:	9 - 23 June 2009

T44P2 has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in V79 cells. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. In a pre-test, the toxicity of T44P2 was tested up to the maximal required concentration of 10 mM, which is 1110 µg/ml. However, the highest concentration evaluated is the one that reduces cell growth to approximately 50% determined by the proliferation index. Two lower concentrations are evaluated as well. The treatment period in the main test was 4 h with and without S9-mix. The harvest time was 24 h after the beginning of culture. Negative and positive controls were in accordance with the draft guideline.

Results
Precipitation of T44P2 was observed at 277.5 µg/ml and above in the absence of S9-mix and at 138.8 µg/ml and above in the presence of S9-mix. Based on a reduction in proliferation index of approximately 50%, 555 µg/ml was chosen as maximal concentration without S9-mix and 277.5 µg/ml with S9-mix.
In the absence of metabolic activation, a single statistically significant increase in the number of cells with micronuclei was seen at the lowest concentration tested. This increase was clearly within the range of the historical control values and therefore is considered not biologically relevant. In the presence of metabolic activation, a statistically significant and concentration -dependent increase in the number of cells with micronuclei was found. All values were outside the range of the historical control values.

Conclusion
Under the experimental conditions used, HC Yellow No. 16 is genotoxic (clastogenic and/or aneugenic) in V79 cells.

Ref.: 10

Micronucleus Test in Human Lymphocytes

Guideline:	draft OECD TG 487	
Cells:	human lymphocytes	
Replicates:	duplicate cultures in 2 independent experiment	
Test substance:	T44P2	
Batch:	FF#20080829	
Purity:	99% (HPLC 254 nm)	
Solvent:	DMSO	
Concentrations:	experiment I:	0, 38.6, 67.6 and 118.4 µg/ml without and with S9-mix
	experiment II:	0, 21.3, 37.3 and 65.3 µg/ml without S9-mix 0, 25, 50, 75, 125 and 150 µg/ml with S9-mix
Treatment:	experiment I:	4 h treatment both with and without S9-mix; harvest time 40 h after the beginning of treatment
	experiment II:	20 h treatment without S9-mix; harvest time 40 h after the beginning of treatment 4 h treatment with S9-mix; harvest time 40 h after the beginning of treatment
GLP:	in compliance	
Study period:	12 May – 24 June 2010	

T44P2 has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in human lymphocytes. Blood samples were obtained from a healthy male (experiment I) and female (experiment II) donor not receiving medication. After collection and before use in the various experiments the human lymphocytes were subcultured in the presence of phytohemagglutinin for 48 h.
A preliminary cytotoxicity test on the reduction in the cytokinesis block proliferation index (CBPI) in comparison with controls with 4 h treatment and a harvest time of 40 h without and with S9-mix was performed using concentrations up to 1110 µg/ml in order to determine the toxicity of T44P2, the solubility during exposure and thus the test concentrations for the main micronucleus test. Since the cultures in this preliminary cytotoxicity test fulfilled the requirements for cytotoxic evaluation and the experimental conditions were identical to those required in the main test, this preliminary test was designed as experiment I.
The treatment period in the main test was either 4 h without and with S9-mix or 20 h without S9-mix. The harvest time was 40 h after the beginning of culture. The final 20 h before harvest was in the presence of cytochalasin B (at a final concentration of 4 µg/ml).
For assessment of cytotoxicity the relative CBPI was estimated as compared to the respective solvent control. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Negative and positive controls were in accordance with the draft guideline.

Results
In the preliminary cytotoxicity test precipitation of T44P2 was observed at the end of treatment at 67.6 µg/ml and above both in the absence and presence of S9-mix.
In the preliminary cytotoxicity test clear toxic effects were observed after 4 h treatment at 118.4 µg/ml and above in the absence and presence of metabolic activation. Considering the toxicity data of experiment I, 200 µg/ml and 400 µg/ml were chosen as top concentrations in experiment II.

After 4 h treatment, precipitation of the test item in culture medium was observed at 67.6 µg/ml and above in the absence and presence of S9-mix. After 20 h treatment without S9-mix precipitation was not seen at the concentrations tested.
In both experiments, a biologically relevant increase in the number of cells with micronuclei was not observed both without and with metabolic activation. Micronucleus inductions were close to the range of the solvent control and within the range of historical control data.

Conclusion
Under the experimental conditions used, HC Yellow No. 16 did not induce an increase in micronucleated cells and, consequently, is not clastogenic and/or aneugenic in human lymphocytes.

Ref.: 11

SCCS overall comment on micronucleus tests *in vitro*
Contradictory results were obtained in two *in vitro* micronucleus tests. While results on V79 cells were positive, a negative outcome was obtained with human lymphocytes. As precipitation in the test with human lymphocytes occurred already at 67.6 µg/ml and above, much lower concentrations were used for the main experiment compared with the positive micronucleus test on V79 cells.

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

Bone marrow micronucleus test in the rat

Guideline:	OECD TG 407 (2008)
Species/strain:	Crl:CD(SD) rats
Group size:	5 males and 5 females per test group
Test substance:	T44P2
Batch:	T-9609-9610
Purity:	99.27%
Vehicle:	0.5 w/v% methylcellulose solution
Dose level:	0, 75, 150, 300 (200) and 600 (450) mg/kg bw/day
Route:	oral gavage
Treatment duration:	once daily for 14 days
Sacrifice times:	/
GLP:	in compliance
Study period:	3 September – 16 December 2010

T44P2 was investigated for the induction of micronuclei in bone marrow cells of rats. The micronucleus study was integrated in a 14-day oral repeated dose toxicity study in rats.
Test doses were based on the results of a 14-day dose selection experiment in rats. The rats were treated by oral gavage once daily for 14 days.
The rats were treated with 0, 75, 150, 300 and 600 mg/kg bw/day. During the dosing period the rats were observed 3 times per day for clinical signs: before dosing, just after dosing and 3-4 h after dosing. Outside the dosing period, the animals were observed once a day in the morning. All common parameters from a repeated dose toxicity study were measured, e.g. body and organ weights, food consumption, haematology, blood chemistry etc.
A micronucleus test was carried out using a portion of surplus femur obtained at the scheduled necropsy. Toxicity and thus exposure of the target cells was determined by estimating the ratio between immature and total erythrocytes (IE/TE).
Bone marrow preparations were stained with acridine orange and examined microscopically for the IE/TE ratio and micronuclei.

Results

In the range-finding study for dose selection, rats died or lost weight at doses of 600 mg/kg/bw/day or higher.
In the main experiment, after starting treatment, 1 male died on day 3 and 3 females died on days 5 and 6 at 600 mg/kg bw/day and remarkable decreases in body weight and food consumption were noted at this dose. At 300 mg/kg bw/day, 1 male died on day 6 at 300 mg/kg bw/day and again remarkable decreases in body weight and food consumption were seen at 300 mg/kg bw/day. Consequently, in males the dose level 300 mg/kg bw/day was changed into 200 mg/kg bw/day on day 8 and later. The dose level 600 mg/kg bw/day was changed into 450 mg/kg bw/day on day 4 and later in males and on day 8 and later in females.
Sporadically clinical signs (decrease in locomotor activity, emaciation, tremor, irregular respiration and/or anemic change) were observed. A decrease in food consumption and bw was seen in males at 300 (200) mg/kg bw/day and at 150 mg/kg bw/day in females.
Since only one rat survived at the 600 mg/kg bw/day group, the micronucleus test was not conducted on this group.
In the present test, the analysis of the IE/TE ratio did not give any indications of an induced bone marrow cytotoxicity. However, the clinical signs observed in the present experiment indicate a bioavailability of T44P2.
Compared to the concurrent vehicle controls, no biologically relevant increase in the number of bone marrow cells with micronuclei was observed for any dose tested.

Conclusion
Under the experimental conditions used, HC Yellow No. 16 is not genotoxic (clastogenic and/or aneugenic) in this micronucleus test in bone marrow cells of rats.

Ref.: 6

3.3.7 Carcinogenicity

No data

3.3.8 Reproductive toxicity

3.3.8.1 Two generation reproduction toxicity

No data

3.3.8.2 Other data on fertility and reproduction toxicity

No data

3.3.8.3 Developmental Toxicity

Prenatal Developmental Toxicity Study in Rats: dose range finding study

Guideline:	OECD TG 414 (2001)
Species/strain:	Rats, Crl:CD(SD)
Group size:	6 females/group
Test substance:	T44P2
Batch:	T-9609-9610
Purity:	99.27%
Vehicle:	0.5 % w/v aqueous methylcellulose solution

Dose levels:	0, 10, 25, 50, 100, 150 mg/kg bw/day
Dose volume:	10 ml/kg bw/day
Route:	oral
Administration:	by gavage
GLP:	/
Study period:	10 September 2010 - 7 February 2011

In a dose-range finding study, the test substance was administered by oral gavage to mated female rats (6 females/group) at dose levels of 0, 10, 25, 50, 100 and 150 mg/kg bw/day from gestation day 6 to 19. Control animals were dosed with the vehicle alone. The dosing formulations were prepared just before each administration.

Results
The stability of the test substance was confirmed with the certificate of analysis provided by the sponsor. The test substance is stable at room temperature for at least 2 years.

No deaths, clinical signs of toxicity or gross pathological changes were observed in any group.
Decreased (not statistically significant) body weight, food consumption and gravid uterus weight were observed at 150 mg/kg bw/day.

There were no treatment-related effects in the number of corpora lutea, implantations, pre- and post-implantation loss index, early and late resorption index, dead foetus index, live foetuses, or sex ratio in any dose group.

Decreases in the body weight of the live male and female foetuses were recorded in 100 and 150 mg/kg bw/day dose groups but did not reach statistical significance.

No treatment-related placental or foetal anomalies (external, visceral or skeletal) were observed in any live foetus in any group. In the examination of the progress of ossification using sternebrae and sacrocaudal vertebrea, no effects were observed in any group.

Conclusion
Based on the experiment, dose levels of 15, 50 and 150 mg/kg body weight/day were proposed for the main study on embryo-foetal development.

Ref.: 12

Prenatal Developmental Toxicity Study in Rats

Guideline:	OECD TG 414 (2001)
Species/strain:	Rats, Crl:CD(SD)
Group size:	20/ group
Test substance:	T44P2
Batch:	11-001
Purity:	99.2%
Vehicle:	0.5% w/v aqueous methylcellulose solution
Dose levels:	0, 15, 50 and 150 mg/kg bw/day
Dose volume:	10 ml/kg bw/day
Route:	oral
Administration:	by gavage
GLP:	in compliance
Study period:	15 September 2011 – 21 March 2012

The test substance was administered by oral gavage to mated female rats (20 females/group) at dose levels of 0, 15, 50 and 150 mg/kg bw/day from gestation day 6 to

19. Control animals were dosed with the vehicle alone. All females were sacrificed on day 20 of gestation and the foetuses were removed by Caesarean section.

Results
The stability of the test substance was confirmed with the certificate of analysis provided by the sponsor. The test substance is stable at room temperature for at least 2 years.
The homogeneity and stability of the dosing formulations (0.1 and 15 mg/ml) was confirmed for 8 days in 3.8-7.5°C protected from light, followed by 6 hours at room temperature protected from light.
The concentration and homogeneity of the test substance in the dosing formulations were confirmed (1.5, 5 and 15 mg/ml).

One female each in the control and 15 mg/kg bw/day groups was not pregnant and the data from these animals were excluded from the evaluation.
No deaths, clinical signs of toxicity, significant differences in gravid uterus weight, or gross pathological changes were observed in any group.
Statistically significant decreases in body weight and food consumption were observed in the 50 and 150 mg/kg bw/day groups.

There were no treatment-related effects in the number of corpora lutea, implantations, pre- and post-implantation loss index, early and late resorption index, dead foetus index, live foetuses, or sex ratio of live foetuses in any dose group. No placental anomalies were observed in any group.

The body weights of live male and female foetuses in the 150 mg/kg bw/day group were statistically significantly lower than those in the control group.
No treatment-related foetal anomalies (external, visceral or skeletal) were observed in any live foetus in any group. In the examination of the progress of ossification using sternebrae and sacrocaudal vertebrea, no effects were observed in any group.

Conclusion
Based on the result described above, the maternal NOAEL of HC Yellow No. 16 was considered to be 15 mg/kg bw/day for general toxicity of the dams and 50 mg/kg bw/day for the embryo-foetal development. HC Yellow No. 16 did not reveal any teratogenic potential up to 150 mg/kg bw/day.

Ref.: 13

3.3.9 Toxicokinetics

3.3.9.1 Toxicokinetics in laboratory animals

Absorption, Distribution, Metabolism and Excretion (ADME) Following Single Percutaneous and Oral Adminstration in rat

Guideline:	OECD TG 417 (2010), OECD TG 427 (2004)
Species/strain:	Rats, Sprague Dawley (Crl:CD SD)
Group size:	4 male rats (6-8 weeks old)
Test substance:	T44P2
Batch:	11-001
Purity:	99.2%
Test item:	^{14}C-T44P2
Batch:	CFQ41176
Radiochemical purity:	99.70%; specific activity: 19.68 MBq/mg
Vehicle:	Oral administration - 0.5% w/v methylcellulose (MC) solution

	Dermal administration - 4% monoethanolamine/ethanol (50/50, v/v)
Dose levels:	Oral administration: 3, 15, 75 mg/kg bw (for determination of plasma radioactivity); 3 mg/kg bw for other measurements Dermal administration: 25 mg/kg bw
Route:	oral (gavage), dermal
Administration:	single administration
GLP:	in compliance
Study period:	2 December 2011 - 3 March 2012

Radiolabelled test substance (radiolabel: carbon in benzene ring, not further specified) was administered to male rats at a single percutaneous (25 mg/kg bw; normal and damaged skin for determination of plasma radioactivity, normal skin for other measurements) or oral dose (3, 15, 75 mg/kg bw for determination of plasma radioactivity; 3 mg/kg bw for other measurements) to investigate the plasma concentration, distribution in tissues, and excretion in urine, faeces and bile (in bile duct cannulated animals), as well as metabolite profiles in plasma, urine, faeces and bile.

Results

Radioactivity in each lot of the percutaneous dosing formulation and oral dosing formulation met the criteria: 90.0% to 110% (92.4% to 97.7% of the nominal concentration for percutaneous dosing; 97.1% to 107.2% of the nominal concentration for oral dosing).

Absorption:

Results for the determination of parameters in plasma are presented in the table:

	mg/kg (single	C_{max} (ng eq.)	t_{max} (h)	$t_{1/2}$ (h)	AUC_{0-t} (ng eq h/mL)
Dermal normal	25	80.79 ± 90.88	3.4 ± 5.8	1.5 ± 0.5*	2739 ± 384
Dermal damaged	25	1967 ± 1901	0.5 ± 0.0	4.2 ± 0.1 **	12300 ± 7970
Oral	3	940.2 ± 60.3	4.5 ± 1.9	83.8 ± 12.6	15460 ± 1460
	15	5386 ± 1397	5.0 ± 2.0	90.7 ± 25.3	67630 ± 10160
	75	21840 ± 2450	7.0 ± 3.5	93.9 ± 24.0	352800 ± 49500

* using concentrations from 0.5 h to 2 h

** using concentrations from 0.5 h to 12 h

In non-fasting male rats receiving ^{14}C HC Yellow No. 16 administered as a single oral dose of 3, 15 and 75 mg/kg bw, a dose dependency (lineary correlation) between the C_{max} and dose (R^2=0.9978) and also between the AUC_{0-t} and dose (R^2=0.9999) was observed. This indicates that absorption did not saturate with the doses administered following oral administration.

Distribution in tissue

The results indicated that no significant persistency of radioactivity was detected in any tissue.

Excretion in urine and faeces –normal rats:

Following percutaneous application to normal rats, up to 1.54 ± 0.71 and 0.44 ± 0.22% of the dose was excreted into urine and faeces, respectively, by 168 hours post-dose; the total recovery of radioactivity was 100.92 ± 1.96% of the dose.
Following oral administration of a single dose of 3 mg/kg bw to normal rats, 75.96 ± 1.95 and 18.83 ± 1.11% of the dose was excreted into urine and faeces, respectively, by 168 hours post-dose; the total recovery of radioactivity was 97.22 ± 1.35% of the dose.
These results indicated that the dose was always completely excreted by 168 hours post-dose, regardless of the dosing route.

Excretion in bile, urine and faeces – bile-duct cannulated rats:
Following oral administration of a single dose of 3 mg/kg bw to bile-duct cannulated rats, 37.03 ± 9.12, 47.26 ± 7.95 and 12.57 ± 1.09% of the dose was excreted into bile, urine and faeces, respectively, by 48 hours post-dose; the total recovery of radioactivity was 98.99 ± 0.50% of the dose.
Since animals with bile-duct cannulation showed lower faecal excretion of radioactivity than those without bile-duct cannulation, the radioactivity into faeces of rats without bile-duct cannulation was considered to have been excreted via bile after oral administration.
The amount excreted into faeces after oral administration in animals with cannulation was considered to be excreted directly into the faeces and therefore, not as being absorbed by the digestive tract. The absorbed rate via oral administration will be calculated as 86.34%.

Metabolic Profile

Plasma:
After a single percutaneous application of 25 mg/kg bw, six radioactive peaks were detected, with three radioactive peaks detected 0.5 hours post-dose and four radioactive peaks detected 4 hours post-dose. At 4 hours post-dose, the parent compound accounted for about 37%; three major metabolites accounted for about 23, 23 and 17%, respectively.
After a single oral administration of 3 mg/kg bw/day, twelve radioactive peaks were detected 4 hours post-dose. The parent compound accounted for about 21%.

Urine:
After a single percutaneous application of 25 mg/kg bw, ten radioactive peaks were detected at 24 hours post-dose; the parent compound accounted for about 0.02% of the dose. The two major metabolites accounted for 0.11 and 0.05% of the dose, respectively.
After a single oral administration of 3 mg/kg bw, seventeen radioactive peaks were detected at 24 hours post-dose; the parent compound accounted for about 0.9% of the dose. The three major metabolites accounted for about 29, 11 and 7.8% of the dose, respectively.

Faeces:
After a single percutaneous application of 25 mg/kg bw, five radioactive peaks were detected at 24 hours post-dose; the parent compound accounted for about 0.01% of the dose. The major metabolite accounted for about 0.1 % of the dose.
After a single oral administration of 3 mg/kg bw, seven radioactive peaks were detected at 24 hours post-dose; the parent compound accounted for about 0.3% of the dose. The major metabolite accounted for about 3 % of the dose.

Bile:
After a single oral administration of 3 mg/kg bw, five radioactive peaks were detected at 24 hours post-dose; the parent compound accounted for about 0.8% of the dose. The two major metabolites accounted for about 24 and 7 % of the dose, respectively.

Conclusion
No characteristic metabolite found in the urine, plasma or faeces after percutaneous administration, only. All other metabolites are qualitative similar and therefore, no significant differences in the metabolism of the test substance in the urine was observed, regardless the dosing route.

Based on the results of this study, the no-observed-adverse-effect level (NOAEL) of HC Yellow No. 16 of 3 mg/kg bw/day, as identified by a 90-day oral repeat dose study, was corrected and set at 2.6 mg/kg bw/day.

Ref.: 14

SCCS comment
Under the conditions of this toxicokinetic study, an oral absorption of 84% is considered based on the results from the bile-duct cannulated rats showing an excretion of about 37% and 47% of the dose in bile and urine, respectively. The oral absorption of 84% is used for the correction of the NOAEL for the MoS calculation.

3.3.9.2 Toxicokinetics in humans

No data

3.3.10 Photo-induced toxicity

3.3.10.1 Phototoxicity / photo-irritation and photosensitisation

No data

3.3.10.2 Photomutagenicity / photoclastogenicity

No data

3.3.11 Human data

No data

3.3.12 Special investigations

No data

3.3.13 Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY
(Oxidative conditions)
(In formulation, on head concentration 1%)

Absorption through the skin	**A**	**= 0.72 µg/cm^2**
Skin Area surface	**SAS**	**= 580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**= 0.418 mg**
Typical body weight of human		**= 60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**= 0.0070 mg/kg**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**= 3 mg/kg bw/d**

corrected value based on TK-study
Bioavailability 84%* **= 2.52 mg/kg bw/d**

Margin of Safety	**adjusted NOAEL/SED =**	**360**

* based on the toxicokinetic study (ref. 14).

CALCULATION OF THE MARGIN OF SAFETY
(Non-oxidative conditions)
(In formulation, on head concentration 1.5%)

Absorption through the skin	**A**	**= 0.701 µg/cm^2**
Skin Area surface	**SAS**	**= 580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**= 0.407 mg**
Typical body weight of human		**= 60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**= 0.0068 mg/kg**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**= 3 mg/kg bw/d**
corrected value based on TK-study		
Bioavailability 84%*		**= 2.52 mg/kg bw/d**

Margin of Safety	**adjusted NOAEL/SED =**	**370**

* based on the toxicokinetic study (ref. 14).

3.3.14 Discussion

Physicochemical properties
HC Yellow No. 16 is intended to be used as a direct dye ingredient in oxidative and non-oxidative hair colouring products at on-head concentrations of up to respectively 1% and 1.5%.
The reported purity and impurity data on HC Yellow No. 16 cannot be accepted in the absence of the study report describing analytical methodology, validation of the method, reference standard used for the determination, etc. In addition, further identification data regarding mass spectrometry, ^{13}C-NMR, ^{1}H-NMR for all the batches used in the studies should be submitted 2) The impurities of HC Yellow No. 16 were not chemically characterised. The synthetic route was provided in additional data. The applicant provided three possible impurities according to the synthetic route, one of which is a free radical and the other (main structure) is the test compound. HPLC-PDA impurity data presented in the final dossier are not consistent with data presented in Ref. 03 and Ref. 06 for the batches 20.05.08 and FF20080829 with respect to the number of impurities, retention time (tR) and % purity. In addition, the impurities should be characterised and any impurity belonging to CMR (carcinogenic, mutagenic and reproduction toxic) classes, such as aniline derivative (4-amino-2-chlorophenol), benzene, tetrahydrofuran, dichloromethane, etc. must be quantified.
Water solubility and Log P_{ow} are not measured using respected EC methods. Physicochemical specifications such as melting point and density of HC Yellow No. 16 are not provided. Stability of HC Yellow No. 16 in typical hair dye formulations is not reported.

General toxicity
No acute oral toxicity study with HC Yellow No. 16 was submitted. However, based on the mortalities in the 14-day dose range-finding rat study from 300 mg/kg bw/day, HC Yellow No. 16 is considered to possess a moderate acute toxic potential following oral administration.

Daily administration of 15 and 75 mg/kg bw/day HC Yellow No. 16 by oral gavage to female Sprague Dawley rats for 90 days resulted in changes in haematological parameters and histopathological changes in the spleen; similar changes were observed in male rats at 75 mg/kg bw/day. The NOAEL of 3 mg/kg bw/day is used for the MoS calculation.
In the developmental toxicity study in rats, daily administration of 50 and 150 mg/kg bw/day HC Yellow No. 16 by oral gavage to pregnant female Sprague Dawley rats during gestation days 6 to 19 resulted in maternal toxicity in form of decreased body weight. The body weights of live foetuses in the 150 mg/kg bw/day group were statistically significantly lower than those in the control group. The NOAEL for maternal toxicity is 15 mg/kg bw/day and the NOAEL for developmental toxicity is 50 mg/kg bw/day. Neither malformations nor variations occurred at the highest dose level of 150 mg/kg bw/day.

Irritation/sensitisation
Under the conditions of the experimental *in vivo* studies performed according to internationally accepted test guidelines, HC Yellow No. 16 is considered to be not irritant to rabbit skin and slightly irritating to the rabbit eye.
The skin sensitisation potential of HC Yellow No. 16 was evaluated in one LLNA study. This substance was tested at concentrations of 1, 5, 10 and 20% in DMSO. The highest dose tested was the maximum concentration that could be technically achieved in this vehicle. In none of the experimental groups was lymphocyte proliferation induced. Based on these results, SCCS concludes that HC Yellow No. 16 is not a skin sensitiser.

Dermal absorption
Two *in vitro* experiments, one under oxidative and one under non-oxidative conditions, were performed to measure the dermal absorption of HC Yellow No. 16. Under non-oxidative conditions, the dermal delivery of HC Yellow No. 16 was 0.412 ± 0.289 µg/cm² (0.164 ± 0.117% of the applied dose), whereas a dermal absorption of 0.51 ± 0.21 µg/cm² (0.18 ± 0.07% of the applied dose) was measured under oxidative conditions. For the calculation of the MoS, a dermal absorption of the mean + 1SD will be used: 0.701 µg/cm^2 for non-oxidative conditions and 0.72 µg/cm^2 for oxidative conditions.

Mutagenicity
Overall, the genotoxicity of HC Yellow No. 16 was investigated for the three endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy.
HC Yellow No. 16 did not induce gene mutations in bacteria nor at the *hprt* locus in a gene mutation test in mammalian cells. In two *in vitro* micronucleus tests, contradictory results were obtained. In one test, treatment with HC Yellow No. 16 resulted in an increase in the number of cells with micronuclei whereas another one was negative.
The clastogenicity found *in vitro* was not confirmed *in vivo*. HC Yellow No. 16 was studied in an *in vivo* micronucleus test which was integrated in a 14-day repeated dose toxicity study. Although 14-day treatment resulted in toxicity, an increase in the number of cells with micronuclei was not observed.
Consequently, HC Yellow No. 16 can be considered to have no genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No data were submitted.

Toxicokinetics
Under the conditions of the *in vivo* toxicokinetic study, an oral absorption of 84% is considered based on the results from the bile-duct cannulated rats showing an excretion of about 37% and 47% of the dose in bile and urine, respectively. An oral absorption of 84% is used for the correction of the NOAEL for the MoS calculation.

Human data
No data were submitted.

4. CONCLUSION

(1) In light of the data provided, does the SCCS consider HC Yellow No. 16 (B123) safe when used as a direct hair colouring agent up to 1.5% in non-oxidative as well as up to on-head concentration of 1% in oxidative hair dye formulations?

In the light of the data provided, SCCS considers that the use of HC Yellow No. 16 (B123) as an ingredient at 1% in oxidative hair dye formulations and at 1.5% in non-oxidative hair dye formulations is safe.

(2) Does the SCCS have any further scientific concerns with regard to the use of HC Yellow No. 16 (B123) in cosmetic products?

The purity of HC Yellow No. 16 and impurities in it are not adequately quantified.

Data on purity and impurities of HC Yellow No. 16 (B123) should be provided, together with purity specifications of the substance intended for use in cosmetic products.

5. MINORITY OPINION

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6. REFERENCES

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