Scientific Committee on Consumer Safety

SCCS

OPINION ON

5-Amino-6-chloro-o-cresol (INCI)

(3-Amino-2-chloro-6-methylphenol)

COLIPA n° A94

The SCCS adopted this opinion at its 17th plenary meeting

of 11 December 2012

About the Scientific Committees

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

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

SCCS

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

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Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on 5-amino-6-chloro-o-cresol, 11 December 2012

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

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1. BACKGROUND

Submission I for 5-amino-6-chloro-o-cresol was submitted in February 1996 by COLIPA[1].

Submission II for 5-amino-6-chloro-o-cresol was submitted by COLIPA in December 2005.

The Scientific Committee on Consumers Safety expressed its opinion (SCCS/1225/09) with the following conclusions:
Because of the low margin of safety for the use in both oxidative and non-oxidative hair dye formulations, the SCCS is of the opinion that the use of 5-amino-6-chloro-o-cresol as a hair dye ingredient up to a final on-head concentration of 2.0% under oxidative and non-oxidative conditions poses a risk to the health of the consumer.

In April 2012 Cosmetics Europe sent a new dossier with results of a dermal penetration study on the ingredient used in colouring products.
The information, including data on dermal penetration and stability under use conditions, are subject of the attached submission III.

2. TERMS OF REFERENCE

1. *Does SCCS consider 5-amino-6-chloro-o-cresol (A094) safe for use as oxidative and non-oxidative hair dye with a concentration on-head of maximum 1.0% taking into account the scientific data provided?*

2. *And/or does the SCCS recommend any further restrictions with regard to the use of 5-amino-6-chloro-o-cresol (A094) in oxidative and non -oxidative hair dye formulations?*

[1] COLIPA/Cosmetic Europe - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

5-Amino-6-chloro-o-cresol (INCI)

Remark
The INCI name is ambiguous. The CAS name is added to the title.

3.1.1.2 Chemical names

Phenol, 3-amino-2-chloro-6-methyl- (9CI) (CAS name)
Phenol, 3-amino-2-chloro-6-methyl-, hydrochloride (9CI) (CAS name)
2-Chloro-6-methyl-3-aminophenol
2-Methyl-5-amino-6-chlorophenol
3-Amino-2-chloro-6-methylphenol
6-Methyl-3-amino-2-chlorophenol

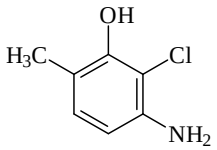
3.1.1.3 Trade names and abbreviations

Ro 543 (hydrochloride)
Ro 1200 (free base)
COLIPA A 094

3.1.1.4 CAS / EC number

CAS:	84540-50-1	(free base)
	80419-48-3	(hydrochloride)
EC:	283-144-9	(free base)
	/	(HCl)

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

Formula: $C_7H_8Cl\ NO$
$C_7H_8Cl\ NO\ .\ HCl$

3.1.2 Physical form

Beige fine powder

3.1.3 Molecular weight

Molecular weight: 157.6 (free base)
194.1 (hydrochloride)

3.1.4 Purity, composition and substance codes

Purity by NMR assay: > 98% (w/w) (free base)
Purity by HPLC assay: > 98% (area)
Solvent content (water): < 0.5% (w/w)

	Batch 5A6COC-DO131 = SAT 030633 = SAT 040281	**Batch 2665/196**
Identity	3-Amino-2-chloro-6-methylphenol [1]	3-Amino-2-chloro-6-methylphenol HCl [2]
Purity	99.6% (area), HPLC 98.7% (w/w), NMR	87% (area), HPLC
Solvent content (water)	< detection limit (0.1% w/w)	3.28 – 3.49% (w/w)
Impurities 3-Amino-6-methylphenol [3] 3-Amino-4-chloro-6-methylphenol 3-Amino-4-chloro-6-methylphenol HCl [4]	 900 ppm - < detection limit (100 ppm)	 6% (w/w) 2.5% (w/w)

[1] verified by ^{1}H-, ^{13}C-NMR-spectroscopy, DEPT-spectrum in D_2O / NaOD, IR-spectrometry and UV-spectrometry
[2] verified by elemental analysis
[3] 4-Amino-2-hydroxytoluene (COLIPA A27, opinion SCCP/1001/06: does not pose a risk to the health of the consumer at 1.5%, apart from its sensitising potential)
[4] 3-Amino-4-chloro-6-methylphenol HCl (5-Amino-4-chloro-o-cresol hydrochloride (COLIPA A117, opinion SCCP1120/07: does not pose a risk to the health of the consumer at a maximum on-head concentration of 1.5%)

Declaration by the applicant concerning A094, Batch used in acute oral toxicity study

The batch of COLIPA A094 used in the acute oral toxicity test is not fully analytically described. However, information is available from the laboratories that have synthesized this batch concerning the identity and purity of the material produced at that time. From this information it can be concluded that the former not fully described batch is representative and its specification is quite similar to the fully characterized batch 5A6COC-DO131.

Comment
1. The impurities of batch 5A6COC-DO131(5A6) are between 1.3 and 0.4%. Only about 0.1% has been categorized as 3-Amino-6-methylphenol. It is identified by HPLC and retention time only (ref 2).
2. About 1% of the impurities of batch 5A6 are not identified (ref 2).
3. The identification of the impurities in batch 2665/196 (about 10%) has been done only by HPLC (ref 3).
4. Batch 2665/196 has not been characterised by NMR, MS or IR. Thus, the absolute purity is not known.

3.1.5 Impurities / accompanying contaminants

See point 3.1.4.

3.1.6 Solubility

Water: < 10 g/l at room temperature

Ethanol: < 100 g/l at room temperature
DMSO: > 100 g/l at room temperature

Comment
The water solubility was not determined by the EC method.

3.1.7 Partition coefficient (Log Pow)

Log P_{ow}: 1.644 (determined by EU test method A.8)

Ref.: 16

1.44 (calculated)

3.1.8 Additional physical and chemical specifications

Melting point:	82-86 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
pH:	/
UV_Vis spectrum:	/

3.1.9 Homogeneity and Stability

A94 is a solid material at room temperature. Every study ascertained that the test material was stable at room temperature in the dark. Where solutions of A94 were used, stability was tested.

Experiments have been made on the stability of the test substance in propylene glycol. These solutions are stable for 7 days in the refrigerator. Homogeneity and accuracy of these formulations were checked 3 times in the chronic toxicity experiment (ref 12).
Solutions of the test substance in water which are used for the teratogenicity study were prepared daily by gravimetry and volumetry. These solutions were analysed once during the examination interval using an analytical method which has not been described. This analysis was done directly after preparation of the solutions and 2h later. This way it was proven that the solutions are stable for 2h (ref 14).

General Comments on physico-chemical characterisation

- The impurities of batch 5A6COC-DO131(5A6) are between 1.3 and 0.4%. Only about 0.1% has been categorized as 3-Amino-6-methylphenol. It is identified by HPLC and retention time only (ref 2).
- About 1% of the impurity of batch 5A6 is not identified (ref 2)
- The identification of the impurities in batch 2665/196 (about 10%) has been done only by HPLC (ref 3).
- Batch 2665/196 has not been characterised by NMR, MS or IR. Thus, the absolute purity is not known.
- The stability in a typical formulation was not reported
- The information on impurities of batch 2665/196 is insufficient.

3.2 Function and uses

5-Amino-6-chloro-o-cresol and it salts is used as a precursor for hair dyeing products. It reacts with primary intermediates to form the final dye-stuff. The reaction can be accelerated by addition of an oxidizing agent (e.g. hydrogen peroxide), but can also be achieved by air oxidation.

The final concentration of 5-amino-6-chloro-o-cresol on head can be up to 1.0% (calculated for the free base).

3.3 Toxicological Evaluation

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

Guideline:	/
Species/strain:	rat, TNO-Wistar
Group size:	60 male rats (10, respectively 20 rats per dose)
Test substance:	2-Chloro-6-methyl-3-aminophenol hydrochloride (Ro 543)
Batch:	/
Purity:	/
Dose:	501, 1000, 1250, 1580 and 1900 mg/kg bw
Vehicle:	*aqua dest*.
Dosing:	20 ml/kg bw
Route:	oral, gavage
GLP:	/
Study period:	1992

Male adult rats of the TNO-Wistar strain with a mean body weight of 200g were starved 16 h before and 3 h after treatment. Ro 543 (2-chloro-6-methyl-3-amino-aminophenol-hydro-chloride) was dissolved in *aqua dest*. to yield concentrations in the range 2.5 to 9.95 %. The dosage volume of 20 ml/kg bw was administered by gavage. Five doses in the range 0.501 to 1.99 g/kg bw were administered to groups of 10 and 20 animals respectively (20 animals for the 1250 mg/kg bw dose, all other doses 10 animals). All together 60 animals were used. During the observation period of 14 days record was kept of mortalities and signs of toxicity twice a day. At the day of administration repeated observations have been made.

Results
Already at the lowest dose of 0.5 g/kg bw, all animals had signs of reaction to treatment like apathy, staggering, accelerated breathing and prone position. Later on the animals suffered from hampered breathing. The excreted urine was coloured yellow-orange. No death occurred at a dose of 0.5 g/kg bw and one death at a dose of 1.00 g/kg bw. In the dose range of 1.25 to 1.90 g/kg bw, 8 and 9 deaths respectively were observed. Death occurred from within one to twenty four hours after treatment.

Conclusion
From these data, the acute median lethal dose (LD50) and its 95% confidence limits to rats of 2-chloro-6-methyl-3-aminophenol were calculated to1.36 (1.21-1.54) g/kg bw.

Ref.: 4

Comments

The test substance (Ro543) used in the acute oral toxicity test is not characterised (see section 3.1.4.). The study was not conducted according to GLP and the respective OECD guideline. Only one sex was tested.

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 (2002)
Species:	Albino rabbit, New Zealand White (SPF-Quality)
Group:	3 males
Substance:	A094 / SAT 030633
Batch:	5A6COCD0131
Purity:	99.6%
Dose:	0.5 g test substance moistened with 0.7 ml Milli-U water
Vehicle:	water (Milli-U)
Application:	4 hours, 2x3 cm semi-occlusive dressing
Observation:	1, 24, 48 and 72 after exposure
GLP:	in compliance
Study period:	5 – 21 November 2003

Approximately 24 hours prior to the treatment, the dorsal fur was shaved, to expose an area of about 150 cm^2. An aliquot of 0.5 g of the moistened test substance was applied to the intact shaved back skin of each animal. The patch was removed four hours after semi-occlusive contact.
Animals were examined for signs of erythema, eschar and oedema formation. The skin reactions were assessed approx. 1 hour, 24, 48 and 72 hours after termination of the exposure and the effects were scored according to the relevant OECD guideline.

Results
No reaction was seen at any time point.

Conclusion
Under the conditions of the study, the undiluted test substance was neither irritating nor corrosive when applied to the intact rabbit skin under semi-occlusive patch conditions.

Ref.: 5

3.3.2.2 Mucous membrane irritation

Guideline:	OECD 405 (2002)
Species:	Albino rabbit, New Zealand White (SPF-Quality)
Group:	3 males
Substance:	A094 / SAT 030633
Batch:	5A6COCD0131
Purity:	99.6%
Dose:	38.3 mg test substance (approximately 0.1 ml)
Vehicle:	/
Application:	instillation in the conjunctival sac

Observation: 1, 24, 48, 72 hours and 7 and/or 14 days after instillation
GLP: in compliance
Study period: 24 November – 8 December 2003

38.3 mg (equivalent of 0.1 ml) of A094 was placed into the conjunctival sac of one eye of the test animals. The substance remained in permanent contact with the eyes until rinsing with warm tap water, 24 hours after instillation. The other eyes served as controls.
The eye irritation reactions were scored approx. 1 hour, 24, 48 and 72 hours and 7 days after instillation of the test solution.

Results
The instillation of the undiluted A094 into the eyes resulted in effects on the cornea, iris and conjunctivae. The corneal injury consisted of opacity (maximum grade 1) and epithelial damage (maximum 55% of the corneal area). The corneal injury had resolved within 24 hours in one animal, and within 7 days in the other animals. Iridial irritation grade 1 was observed in one animal only 24 hours after instillation. The irritation of the conjunctivae consisted of redness and chemosis and had completely resolved within 7 days in two animals and within 14 days in the other animal.

Conclusion
Under the conditions of the study, the undiluted test material was irritating to the rabbit eye.

Ref.: 6

3.3.3 Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline: OECD 429 (2002)
Species: mouse, CBA strain, inbred, SPF-Quality (nulliparous and non-pregnant)
Group: 30 females (six groups of 5 animals each)
Substance: A094 / SAT 030633
Batch: 5A6COCD0131
Purity: 99.6%
Concentrations: 5, 25 and 50% (w/w)
Dose: 25 µl
Vehicle: ethanol:water (7:3 v/v)
Control: α-hexylcinnamaldehyde (August 2003)
GLP: in compliance
Study period: 5 – 26 January 2004

Initially four groups of five animals each were treated with the vehicle and three test substance concentrations, respectively. Based on the results, two additional groups were treated with the vehicle and the highest concentration. The test item was topically applied to the dorsal surface of the ears to analyse the sensitization activity by measuring the proliferative response of lymph node cells.
A homogenous dilution of the test item in a mixture of ethanol:water (7:3 v/v) was made shortly before each dosing. The highest technically achievable, non-irritating test item concentration was found in a pre-test with four mice. Based on these test results 5%, 25% and 50% solutions were chosen for the main study. The vehicle was chosen due to the chemical reactivity/instability of the test substance with other organic solvents like acetone or dimethylformamide.

The application volume, 25 µl, was spread over the entire dorsal surface of each ear lobe once daily for three consecutive days. The control group was treated with the vehicle exclusively. Five days after the first topical application, all mice were administered with radio-labelled thymidine (^{3}HTdR) by intravenous injection via the tail vein.

Approximately five hours after ^{3}HTdR application all mice were euthanized. The draining lymph nodes were excised and pooled for each experimental group. After preparation of the lymph nodes, disaggregation, and overnight precipitation of macromolecules, these precipitations were re-suspended and transferred to scintillation vials.

The level of ^{3}HTdR incorporation was then measured by scintillation counting. The proliferative response of lymph node cells is expressed as the ratio of ^{3}HTdR incorporation into lymph node cells of treated animals relative to that recorded in control mice (stimulation index).

An appropriate reference (α-hexylcinnamaldehyde) was used as positive control to demonstrate the sensitivity of the test system.

The proliferative capacity of pooled lymph node cells was determined by quantifying the incorporation of ^{3}H-methyl thymidine. A test item is regarded as a sensitizer if the exposure to at least one concentration resulted in an at least 3-fold increase in incorporation of ^{3}HTdR compared with concurrent controls, as indicated by the stimulation index (S.I.).

Results
No skin irritation was noted on the ear dorsum of the treated mice at any concentration.

Concentration	Stimulation Index
Test item	
5%	1.0
25%	0.9
50% (initial group)	4.0
50% (additional group)	1.0
α-Hexylcinnamaldehyde	
5%	2.5
10%	7.5
25%	24.4

The SI of 4.0 calculated for the initially treated group at 50% was not confirmed in the additional group treated at 50% that showed a SI of 1.0. Pooling the individual stimulation indices, an overall SI of 2.5 was calculated for the 50% concentration.

Conclusion
There was no clear evidence that A094 could elicit a SI ≥ 3. Based on the criteria of the test system, A094 was considered to be a non-sensitizer when tested up to the highest achievable concentration of 50% (w/v) in ethanol:water (7:3 v/v) in mice.

Ref.: 7

Comment
The discrepancy between the two experiments using a 50% concentration of A094 is unexplained. A094 is considered to be a non-sensitiser.

3.3.4 Dermal / percutaneous absorption

New study, submission III, 2012

Guideline:	OECD 428 (2004)
Tissue:	dermatomed (750-800 µm) pig skin, 4 pigs, 2 of each gender
Skin integrity:	transcutaneous electrical resistance (TER of at least 4kΩ)
Group size:	12 samples
Diffusion cells:	static diffusion cells, 3.14 cm² application area
Test substance:	5-amino-6-chloro-o-cresol [^{14}C]-5-amino-6-chloro-o-cresol, 62 mCi/mmol (388.47 µCi/mg)

Batch:	5A6COCD0131 CFQ41200 (radio-labelled)
Purity:	99.6 area% (HPLC) 97.8% (HPLC, radio-chemical purity)
Test item:	cream with 1% A094, Henkel code n° SAT 110012, batch FFJ10SP0032/03 cream with 2% A094, Henkel code n° SAT 110013, batch FFJ10SP0032/04 cream with 3% A094, Henkel code n° SAT 110014, batch FFJ10SP0032/05
Receptor fluid:	phosphate buffered saline
Solubility receptor fluid:	*circa* 2.5 g/L
Dose:	20 mg/cm²
Method of Analysis:	liquid scintillation counting
GLP:	in compliance
Study period:	1 March – 11 October 2011

The dermal bioavailability of A094 following topical application of three formulations, with radio-diluted [^{14}C]-A094 concentrations of 1, 2 and 3% (w/w), to excised dermatomed pig skin was reported.
Immediately prior to application, the formulations were mixed 1:1 (w/w) with developer without hydrogen peroxide and with developer with hydrogen peroxide (6%, w/w).The final applied concentration of A094 was 0.5%, 1% and 1.5% (w/w) respectively. The skin surface was washed at 30 min, for each test preparation, to reflect the in-use conditions. The skin was again washed at 24 h post dose as a final wash off before test run termination.
The hair dye formulation was applied at an application volume of *ca.* 20 mg/cm² to dermatomed pig skin mounted into static diffusion cells in vitro.

Results
Test item concentrations of 1, 2 and 3% were investigated. Below, only the 2% concentration (1% on head) and 1% concentration (0.5% on head for non-oxidative conditions) are reported in detail.
Distribution of [^{14}C]-A094 (µg equiv./cm²) at 24 h Post Dose Following Topical Application of [^{14}C]-A094 (1%, w/w) in Test Preparation 3 (Hair Dye Formulation without Hydrogen Peroxide) to Dermatomed Pig Skin.

	Cell Number and Animal Number													
	Cell 25 P026	Cell 26 P026	Cell 27 P026	Cell 28 P029	Cell 29 P029	Cell 30 P029	Cell 31 P028	Cell 32 P028	Cell 33 P028	Cell 34 P027	Cell 35 P027	Cell 36 P027	Mean	SD
Skin Wash 30min	158.49	154.76	151.96	158.93	168.53	166.68	153.66	134.32	152.65	157.49	168.45	161.78	157.31	9.35
Tissue Swab 30min	6.99	5.78	11.15	10.57	3.26	4.90	11.40	5.57	4.03	7.12	2.31	2.56	6.30	3.25
Pipette Tip 30min	0.85	0.39	1.03	0.98	0.47	1.32	1.34	0.27	2.38	0.75	0.23	2.93	1.08	0.83
Dislodgeable Dose 30min	166.32	160.93	164.14	170.48	172.26	172.90	166.41	140.16	159.06	165.36	170.99	167.28	164.69	8.84
Skin Wash 24h	2.61	4.43	2.30	4.14	3.01	2.89	3.63	2.33	2.59	2.19	1.98	1.12	2.77	0.94
Tissue Swab 24 h	0.18	1.14	0.19	0.54	0.23	0.20	0.32	0.28	0.38	0.39	0.40	0.35	0.38	0.26
Pipette Tip 24h	0.00	0.01	0.01	0.03	0.00	0.01	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.01
Donor Chamber Wash	0.25	0.44	*0.00	*0.05	*0.09	*0.09	*0.10	0.34	*0.01	*0.10	*0.07	*0.05	°0.13	°0.14
Total Dislodgeable Dose	169.37	166.95	166.64	175.25	175.59	176.09	170.47	143.11	162.05	168.03	173.45	168.80	167.98	8.90
Unexposed Skin	0.27	0.36	0.28	0.26	0.28	0.14	0.35	0.54	0.34	0.23	0.07	0.14	0.27	0.12
Total Unabsorbed	169.64	167.31	166.92	175.51	175.87	176.23	170.82	143.65	162.39	168.26	173.52	168.94	168.25	8.81
Stratum Corneum 1-5	1.38	2.96	0.79	0.37	0.44	0.46	0.56	0.44	0.79	0.61	1.32	1.68	0.98	0.76
Stratum Corneum 6-10	0.38	0.51	0.38	0.08	0.22	0.11	0.16	0.71	0.33	0.33	0.44	0.42	0.34	0.18
Stratum Corneum 11-15	0.27	0.35	0.64	0.06	0.07	0.06	0.16	0.92	0.22	0.29	0.15	0.11	0.28	0.26
Stratum Corneum 16-20	0.16	0.40	0.17	0.04	0.08	0.05	0.15	0.82	0.17	0.33	0.09	0.04	0.21	0.22
Dermal Adsorption (Total Stratum Corneum)	2.19	4.22	1.97	0.54	0.81	0.68	1.05	2.89	1.53	1.56	1.99	2.25	1.81	1.04
Dermal Absorption (Exposed Skin)	4.22	5.16	4.11	8.62	7.30	7.84	8.06	8.52	8.97	6.74	5.58	5.89	6.75	1.72
Receptor Fluid	*14.92	*16.25	*13.60	*11.54	*8.26	*9.04	*15.58	*28.64	*26.73	*12.09	*8.80	*24.99	°15.87	°7.12
Receptor Chamber Wash	0.34	0.46	*0.11	0.23	0.23	3.19	0.40	3.51	0.16	0.95	0.17	1.01	°0.90	°1.18
Percutaneous Penetration	15.26	16.70	13.71	11.77	8.49	12.23	15.99	32.15	26.89	13.04	8.97	26.00	16.77	7.54
Dermal Bioavailability	19.49	21.87	17.83	20.39	15.80	20.07	24.04	40.67	35.86	19.78	14.55	31.89	23.52	8.22
Mass Balance	191.32	193.39	186.72	196.44	192.47	196.97	195.91	187.21	199.77	189.60	190.07	203.08	193.58	5.01

*=Results directly calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background

Distribution of [^{14}C]-A094 (µg equiv./cm²) at 24 h Post Dose Following Topical Application of [^{14}C]-A094 (1%, w/w) in Test Preparation 4 (Hair Dye Formulation with 6% Hydrogen Peroxide) to Dermatomed Pig Skin

	Cell Number and Animal Number													
	Cell 37 P026	Cell 38 P026	Cell 39 P026	Cell 40 P029	Cell 41 P029	Cell 42 P029	*Cell 43 P028*	Cell 44 P028	Cell 45 P028	Cell 46 P027	Cell 47 P027	Cell 48 P027	Mean	SD
Skin Wash 30min	172.84	165.82	174.41	187.02	178.56	164.35	*138.54*	164.85	151.63	148.21	166.92	156.11	166.43	11.58
Tissue Swab 30min	2.43	1.25	7.10	5.37	1.51	7.26	*6.51*	4.05	3.66	12.87	2.75	8.22	5.14	3.50
Pipette Tip 30min	0.86	0.05	0.15	0.68	1.07	0.15	*1.21*	0.87	1.87	0.97	0.10	0.17	0.63	0.57
Dislodgeable Dose 30min	176.14	167.12	181.66	193.08	181.15	171.76	*146.27*	169.77	157.16	162.04	169.77	164.50	172.20	10.24
Skin Wash 24h	1.67	1.43	2.91	4.55	2.55	3.47	*4.23*	4.48	3.51	4.99	3.18	4.50	3.39	1.19
Tissue Swab 24 h	0.20	0.17	0.23	0.51	0.33	0.56	*0.82*	0.62	0.77	0.69	0.33	0.89	0.48	0.25
Pipette Tip 24h	*0.00	0.00	0.02	0.00	0.01	0.01	*0.07*	0.02	0.01	0.01	0.02	0.04	*0.01	*0.01
Donor Chamber Wash	*0.04	*0.02	*0.10	0.28	0.20	*0.14	*0.11*	0.16	*0.04	0.30	*0.14	0.41	*0.17	*0.12
Total Dislodgeable Dose	178.04	168.74	184.92	198.43	184.23	175.93	*151.49*	175.05	161.47	168.03	173.45	170.34	176.24	10.10
Unexposed Skin	0.23	0.17	0.65	0.19	0.13	0.16	*0.17*	0.12	0.28	0.12	0.08	0.13	0.21	0.16
Total Unabsorbed	178.27	168.92	185.57	198.62	184.36	176.09	*151.66*	175.17	161.76	168.16	173.53	170.47	176.45	10.14
Stratum Corneum 1-5	2.12	1.47	0.39	2.95	1.79	2.44	*6.87*	8.75	5.17	2.58	3.66	3.49	3.16	2.24
Stratum Corneum 6-10	0.78	1.59	0.97	0.55	0.40	0.38	*1.90*	2.10	2.32	1.38	1.09	1.36	1.17	0.65
Stratum Corneum 11-15	0.33	0.25	0.88	0.31	0.26	0.22	*1.14*	1.25	1.66	0.76	0.62	0.59	0.65	0.47
Stratum Corneum 16-20	0.20	0.27	0.36	0.15	0.18	0.12	*0.71*	0.92	1.14	0.45	0.36	0.37	0.41	0.33
Dermal Adsorption (Total Stratum Corneum)	3.43	3.58	2.59	3.96	2.62	3.16	*10.63*	13.02	10.28	5.18	5.73	5.81	5.40	3.35
Dermal Absorption (Exposed Skin)	3.49	4.38	4.26	7.56	3.07	4.03	*6.37*	7.79	4.97	3.84	3.17	3.57	4.56	1.63
Receptor Fluid	*9.17	*14.17	*12.51	*5.07	*4.24	*4.83	*10.85*	*12.85	*10.47	*11.24	*8.59	*9.34	*9.32	*3.40
Receptor Chamber Wash	0.23	0.17	0.14	*0.08	*0.03	*0.10	*0.19*	0.24	0.30	0.18	0.19	0.23	*0.17	*0.08
Percutaneous Penetration	9.40	14.34	12.65	5.15	4.27	4.93	*11.04*	13.10	10.77	11.42	8.78	9.57	9.49	3.45
Dermal Bioavailability	12.90	18.72	16.91	12.71	7.34	8.96	*17.41*	20.88	15.74	15.26	11.95	13.15	14.05	4.00
Mass Balance	194.60	191.22	205.07	215.29	194.32	188.21	*179.70*	209.07	187.78	188.60	191.21	189.43	195.89	9.50

Cell 43 rejected - mass balance not in the acceptance range of 100±10%

*=Results directly calculated from data less than 30 d.p.m. above background

*=Mean includes results calculated from data less than 30 d.p.m above background

Distribution of [14C]-A094 (µg equiv./cm²) at 24 h Post Dose Following Topical Application of [^{14}C]-A094 (0.5%, w/w) in Test Preparation 1 (Hair Dye Formulation without Hydrogen Peroxide) to Dermatomed Pig Skin

	Cell Number and Animal Number													
	Cell 1 P026	Cell 2 P026	Cell 3 P026	Cell 4 P027	Cell 5 P027	Cell 6 P027	Cell 7 P028	Cell 8 P028	Cell 9 P028	Cell 10 P029	Cell 11 P029	Cell 12 P029	Mean	SD
Skin Wash 30min	54.83	67.20	60.52	73.10	78.95	80.36	53.46	45.58	66.82	75.49	69.02	76.79	66.84	11.13
Tissue Swab 30min	21.09	10.34	7.74	6.84	4.04	3.45	13.14	16.71	6.63	5.77	8.15	6.55	9.20	5.30
Pipette Tip 30min	1.46	0.39	0.09	0.14	0.03	0.04	0.88	1.40	0.20	0.19	1.07	0.21	0.51	0.54
Dislodgeable Dose 30min	77.38	77.93	68.35	80.09	83.02	83.85	67.49	63.70	73.64	81.45	78.24	83.55	76.56	6.80
Skin Wash 24h	2.45	2.91	1.40	2.59	1.72	1.52	1.99	3.87	1.28	2.27	2.28	1.31	2.13	0.77
Tissue Swab 24 h	0.13	0.60	0.08	0.62	0.29	0.09	0.76	0.36	0.22	0.21	0.17	0.09	0.30	0.23
Pipette Tip 24h	0.01	0.02	*0.00	0.01	*0.00	*0.00	0.00	0.02	0.00	0.01	0.00	*0.00	*0.01	*0.01
Donor Chamber Wash	*0.04	0.21	*0.01	0.14	*0.05	0.10	0.11	0.19	0.10	*0.07	*0.07	*0.07	*0.10	*0.06
Total Dislodgeable Dose	80.00	81.67	69.84	83.45	85.09	85.55	70.36	68.14	75.26	84.01	80.77	85.02	79.09	6.49
Unexposed Skin	0.10	0.18	0.12	0.05	0.07	0.04	0.25	0.22	0.15	0.07	0.04	0.22	0.13	0.08
Total Unabsorbed	80.09	81.85	69.96	83.50	85.16	85.59	70.61	68.36	75.41	84.08	80.80	85.24	79.22	6.44
Stratum Corneum 1-5	0.46	1.35	0.70	1.18	0.84	0.77	1.21	1.20	1.19	0.06	0.63	0.06	0.81	0.45
Stratum Corneum 6-10	0.28	0.21	0.27	0.26	0.43	0.22	0.63	0.53	0.45	0.02	0.08	0.02	0.28	0.19
Stratum Corneum 11-15	0.39	0.06	0.35	0.19	0.25	0.12	0.33	0.48	0.39	*0.01	0.08	*0.02	*0.22	*0.16
Stratum Corneum 16-20	0.10	0.07	0.06	0.10	0.13	0.07	0.13	0.34	0.23	*0.03	0.11	*0.06	*0.12	*0.09
Dermal Adsorption (Total Stratum Corneum)	1.23	1.69	1.38	1.72	1.66	1.17	2.30	2.55	2.26	0.12	0.90	0.16	1.43	0.78
Dermal Absorption (Exposed Skin)	2.50	2.74	5.35	2.79	2.80	3.03	3.71	6.24	3.97	5.00	4.16	3.17	3.79	1.20
Receptor Fluid	*8.08	*8.31	17.66	*7.07	*5.45	*6.36	16.13	15.43	15.77	*4.85	*9.73	*7.18	*10.17	*4.69
Receptor Chamber Wash	*0.07	*0.05	0.10	*0.03	*0.06	*0.05	*0.09	*0.09	0.14	*0.05	*0.06	*0.05	*0.07	*0.03
Percutaneous Penetration	8.15	8.36	17.76	7.11	5.51	6.42	16.22	15.52	15.92	4.90	9.79	7.22	10.24	4.72
Dermal Bioavailability	10.65	11.10	23.11	9.90	8.30	9.45	18.92	21.77	19.89	9.90	13.95	10.40	14.03	5.50
Mass Balance	91.97	94.64	94.45	95.12	95.12	96.21	92.83	92.68	97.55	94.11	95.65	95.80	94.68	1.60

*=Results directly calculated from data less than 30 d.p.m. above background

*=Mean includes results calculated from data less than 30 d.p.m above background

The dermal absorption of A94 at 2% concentration in a hair dye formulation (1% final applied concentration) under non-oxidative condition and oxidative conditions were 23.52 ± 8.22 µg/cm² and 14.05 ± 4.00 µg/cm² respectively. The dermal absorption of A94 at 1% concentration in a hair dye formulation (0.5% final applied concentration) under non-oxidative condition was 14.03 ± 5.50 µg/cm².

Conclusions

In conclusion, following topical application of radio-diluted [^{14}C]-A094 in a typical hair dye formulation without hydrogen peroxide to pig skin in vitro, the dermal bioavailability of

[^{14}C]-A094 was 13.86%, 11.36% and 9.21% of the applied dose for Test Preparations 1, 3 and 5, respectively. The majority of the dose was removed by washing the skin with water and mild shampoo solution.
Following topical application of radio-diluted [^{14}C]-A094 in a typical hair dye formulation with hydrogen peroxide to pig skin in vitro, the dermal bioavailability of [^{14}C]-A094 was 8.51%, 7.00% and 5.04% of the applied dose for Test Preparations 2, 4 and 6, respectively. The majority of the dose was removed by washing the skin with water and mild shampoo solution.

The dermal absorption of 5-amino-6-chloro-o-cresol at 1% on head final concentration in a hair dye formulation under non-oxidative condition and oxidative conditions was 23.52 ± 8.22 µg/cm² and 14.05 ± 4.00 µg/cm² respectively. The dermal bioavailability of 5-amino-6-chloro-o-cresol at 0.5% on head final concentration in a hair dye formulation under non-oxidative condition was 14.03 ± 5.50 µg/cm².

Ref.: 4 (subm III)

Comments
The mean + SD = 31.74 and 18.05 µg/cm² under non oxidative and oxidative conditions respectively will be used to calculate MoS for a 2% formulation.
The mean + SD = 19.53 under non oxidative conditions will be used to calculate MoS for a 1% formulation.

Submission II, 2005

Guideline:	OECD 428 (2004)
Tissue:	dermatomed pig skin, 2 animals (1 male and 1 female)
Group size:	16 skin samples, 8 per experiment
Diffusion cells:	Static Franz diffusion cells, 1.0 cm² application area
Skin integrity:	transcutaneous electrical resistance (TER of at least 7kΩ)
Test substance:	A094 SAT 040556 5-amino-6-chloro-o-cresol [ring -U-^{14}C]-, 13.02 MBq/mg
Batch:	5A6COCD0131 SEL/1842 (labelled)
Purity:	> 99.6 area% (HPLC) > 99% (HPLC and ^{1}H-NMR) (labelled)
Test item:	experiment A: cream formulation TM 0031-1a containing 4% A094, mixed with developer but without hydrogen peroxide (final concentration A094 2.1%) Experiment B: cream formulation TM 0031-1a containing 4% A094, mixed with developer and with 6% hydrogen peroxide (final concentration A094 2.1%)
Doses:	20 mg/cm² or 0.42 mg A094/cm²
Receptor fluid:	Dulbecco's phosphate buffered saline
Solubility receptor fluid:	assumed to be close to that in water i.e., 2.5g/L
Stability:	/
Method of Analysis:	liquid scintillation counter
GLP:	in compliance
Study period:	15 -28 November 2005

The composition of the basic cream and the developer mix with and without hydrogen peroxide is shown in the tables below.

Ingredient of basic cream	Concentration in %
5-Amino-6-chloro-o-cresol (COLIPA A094)	4.00
Toluene-2,5-diamine (COLIPA A 005)	3.10
Hydrenol D	9.35
Texapon NSO-UP	15.00
Dehyton K	12.50
Lorol techn.	2.20
Eumulgin B2	0.75
Sodium sulphite	0.20
Ammonium sulfate	0.40
Ascorbic acid	0.20
Citric acid	for pH adjustment
Ammonia	for pH adjustment
Water	ad 100
	pH 9.62

Ingredient of developer mix	with H_2O_2 in %	without H_2O_2 in %
Dipicolinic acid	0.10	0.10
Sodium pyrophosphate, acid	0.03	0.03
Turpinal SL	1.50	1.50
Texapon NSO-UP	2.00	2.00
Ammonia, 25%	for pH adjustment	for pH adjustment
Tartaric acid	for pH adjustment	for pH adjustment
Aculyn 33	15.00	15.00
Hydrogen peroxide (50% H_2O_2 solution)	12.00	-
Water	ad 100	ad 100
	pH 3.82	pH 3.91

Shortly before topical application to skin the basic cream was mixed with the developer mix with and without hydrogen peroxide as study A and B, respectively. The resulting formulations were then traced with [^{14}C] radio-labelled A094 shortly before application.
The nominal concentration of A094 in the two final application formulations was 2.1%.
The dermal absorption/percutaneous penetration of the test substance was investigated for the open application of about 20 mg formulation per cm² pig skin. Therefore the resulted nominal dose of the test substance was approx. 0.42 mg/cm² skin. Skin discs of 1.0 cm² were exposed to the formulations for 30 minutes, terminated by gently rinsing with a 0.01% Tween 80 solution and water.

Experiment A without hydrogen peroxide

	Amount recovered (µgeq/cm²)									
Sample number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**mean**	**SD**
Donor	28	28	28	28	29	29	29	29		
Adsorbed after 48h	0.78	0.86	0.97	2.76	0.40	0.26	0.29	0.31	***0.83***	0.83
Absorbed after 48h	12.68	6.81	10.89	11.21	7.66	9.59	4.94	7.62	***8.92***	2.60
Penetration 0-18h	49.43	38.66	49.37	48.62	44.47	39.95	41.99	46.74	***44.90***	4.31
Rinsings skin	533.6	321.8	351.8	318.4	398.9	279.2	380.4	406.4	***373.8***	77.9
Bioavailable after 48h	**62.11**	**45.47**	**60.26**	**59.83**	**52.13**	**49.54**	**46.93**	**54.36**	***53.83***	6.38
Balance	/	/	/	/	/	/	/	/	/	/

	Amount recovered (% of dose)									
Sample number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**mean**	**SD**
Donor	28	28	28	28	29	29	29	29		
Adsorbed after 48h	0.14	0.15	0.17	0.49	0.09	0.06	0.06	0.07	***0.15***	0.14
Absorbed after 48h	2.27	1.94	2.17	2.57	1.71	2.40	1.04	1.47	***1.95***	0.51
Penetration 0-18h	8.86	10.99	9.85	11.15	9.91	9.99	8.86	9.02	***9.83***	0.90
Rinsings skin	95.62	91.51	70.19	73.04	88.93	69.78	80.29	78.45	***80.98***	10.00
Bioavailable after 48h	**11.13**	**12.93**	**12.02**	**13.73**	**11.62**	**12.38**	**9.91**	**10.49**	***11.78***	1.26
Balance	107	105	83	88	101	83	91	89	93	10

Experiment B with hydrogen peroxide

	Amount recovered (µgeq/cm²)									
Sample number	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**mean**	**SD**
Donor	28	28	28	28	29	29	29	29		
Adsorbed after 48h	2.05	0.98	1.89	8.34	2.41	3.00	2.26	1.73	***2.83***	2.30
Absorbed after 48h	12.62	5.84	8.93	5.20	7.71	6.76	10.60	18.59	***9.53***	4.42
Penetration 0-18h	24.72	12.98	16.19	16.19	25.52	19.62	19.59	30.62	***20.68***	5.86
Rinsings skin	474.5	424.5	531.4	486.1	456.1	493.1	497.5	564.4	***491.0***	43.1
Bioavailable after 48h	**37.34**	**18.82**	**25.12**	**21.39**	**33.23**	**26.38**	**30.19**	**49.21**	***30.21***	9.78
Balance	/	/	/	/	/	/	/	/	/	/

	Amount recovered (% of Dose)									
Sample number	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**mean**	**SD**
Donor	28	28	28	28	29	29	29	29		
Adsorbed after 48h	0.41	0.19	0.37	1.65	0.52	0.65	0.49	0.37	***0.58***	0.45
Absorbed after 48h	2.49	1.40	1.84	1.03	1.67	1.39	2.00	3.10	***1.86***	0.67
Penetration 0-18h	4.88	3.10	3.34	3.21	5.52	4.03	3.70	5.11	***4.11***	0.94
Rinsings skin	93.70	101.5	109.5	96.39	98.67	101.2	93.95	94.26	***98.65***	5.39
Bioavailable after 48h	**7.37**	**4.50**	**5.18**	**4.24**	**7.19**	**5.41**	**5.70**	**8.22**	***5.98***	1.45
Balance	102	106	115	102	107	107	100	103	105	5

Conclusion
In this *in vitro* dermal penetration study, the amount of A094 considered systemically available from a standard cream formulation with or without hydrogen peroxide (final concentration of A094 2.1%) was found to be:

30.21 ± 9.78 (range 18.82-49.21) µg/cm² or 5.98 ± 1.45 (range 4.24-8.22)% with peroxide, and

53.8 ± 6.38 (range 45.47-62.1) µg/cm² or 11.78 ± 1.26 (range 9.91-13.73)% without peroxide.

Ref.: 15

Comment
Under the condition of the experiments in which hair dye formulations contained a final concentration of 2.1% A94, the amounts considered absorbed were 30.21 ± 9.78 (A_{max} 49.21) µg/cm² with peroxide and 53.8 ± 6.38 (A_{max} 62.1) µg/cm² without peroxide.

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose (28 days) oral toxicity

No data submitted

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

Guideline:	OECD 408 (1998)
Species/strain:	rat: Wistar Crl:(WI) BR (outbred, SPF-Quality)
Group size:	12 per sex per dose
Recovery group:	5 per sex (control and high dose)
Test substance:	A094
Batch:	5A6COCD0131
Purity:	> 99.6 FL-% (HPLC)
Dose:	0, 100, 300 and 600 mg/kg bw
Vehicle:	propylene glycol
Stability:	The formulations used for application were analysed to check homogeneity and accuracy on days 5, 40 and 84.

Dosing:	10 ml/kg bw
Administration:	daily by oral gavage
Exposure:	90-day + 28 day recovery period
GLP:	in compliance
Study period:	24 June - 21 October 2004

Doses (as determined in a 28-day dose-finding-study) were 0, 100, 300 and 600 mg/kg/d.

COLIPA A094 (batch 5A6COC-DO131, purity > 99.6%, according to fluorescence HPLC) was administered in a single dose in propylene glycol as the vehicle by gavage (dose volume 10ml/kg bw). The control animals received the vehicle alone.

Clinical signs were recorded for all animals pretest and at least once daily. All symptoms were recorded and graded according to fixed scales. Body weight and food consumption were recorded weekly. During week 12-13 the following tests were performed on all animals of the recovery group (n= 10) and on 4 animals of each of the 3 dosed groups and of the controls. The recovery group was observed up to 28 days after treatment.

Hearing ability, pupillary reflexes, static righting reflex, grip strength and motor activity were tested. Ophthalmologic examinations of both eyes were examined pretest in all animals and during the test phase in week 13 in 4 controls and 4 animals of the high dose group.

Blood samples were taken prior to post mortem examination for the examination of haematology, clotting potential and clinical biochemistry.

All animals were killed and examined post mortem. Organ weights of several organs were recorded. Histopathology of organs from all control, high dose animals, animals of all groups which died during the experiment and all gross lesions from all animals was performed. In addition lungs, liver and kidneys of all animals of all dose groups were investigated for histopathological effects. Moreover the stomach from all rats of the intermediate dose group and of the recovery group was examined.

Results
Most obvious observations were perturbations of the liver function. Histopathology revealed centrilobular hypertrophy of the liver at 100, 300 and 600 mg/kg bw/day. These findings were clearly dose related and occurred primarily in males. High dose (600): 12/12 males; 6/12; females; medium dose (300): 11/12 males, 1/12 females; low dose (100): 1/12 males, 0/12 females. This finding persisted in all high dose males (5/5) at the end of recovery (although at slight severity; no recovery groups for medium and low dose). Based on these findings (under exclusion of the one incidence at the low dose) the authors concluded "Hepatocytic hypertrophy […] occurred in the absence of any other supportive functional or morphological changes. Centrilobular hypertrophy of hepatocytes is frequently an adaptive change in response to xenobiotics and at low incidence and severity considered not to be adverse."). The authors of the study establish a NOAEL of 100 mg/kg/day.

Other microscopic findings were only related to the high dose group (and confined to the males), like cortical tubular basophilia in the kidneys (8/12 males), limiting ridge hyperplasia of the forestomach (12/12 males) with squamous hyperplasia of the main stomach (2/12 males).

At the high dose (600) histopathological findings were accompanied by increased relative and absolute liver weights (persisting through the recovery period) and effects on a number of clinical biochemistry parameters (like increased bilirubin and potassium levels, reduced urea levels, increased cholesterol levels and increased alanine aminotransferase activity).

Several statistically significant deviations in haematological parameters were observed in the high dose group (some of them also in the low dose group, but none in the medium dose group, except MCHC in all doses for females), like reduced blood cell counts in males, reduced platelet counts in males, increased mean corpuscular volume in males and females (both in the 100 and 600 group), increased mean corpuscular Hb level in females, reduced mean corpuscular Hb concentration in males (in the 100 and 600 group) and in all groups (100, 300 and 600) of treated females; increased haematocrit values in males (in the low dose only). In the females some of these effects persisted through the recovery period. Opposite to the perturbations of the liver function (predominantly in males), effects on haematological parameters seemed to be more pronounced in females (down to the low dose group) and persisted in the recovery group (MCH and MCV).

Three deaths occurred, one in the low and one in the high dose group (on days 54 and 48 respectively), and one death in the control recovery group at week 4 of the recovery period).

All dose groups showed a brown discoloration of the urine, all animals in the medium and high dose group showed salivation from week 3 of treatment onwards.

Ref.: 12

Comment
The liver and the haematopoetic system are the targets of systemic toxicity of 5-amino-6-chloro-o-cresol. Based on the haematological changes, in particular the reduced mean corpuscular Hb concentration in all dose groups of female rats, the SCCS considers 100 mg/kg/day (the lowest dose tested) as an LOAEL.
This LOAEL is supported by the obvious dose-dependency of centrilobular hypertrophy of the liver which in males was observed down to the low dose. In the high dose recovery group, this effect persisted until termination of the study.

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 (1997)	
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537.	
Replicates:	triplicate in two independent experiments	
Test substance:	A094	
Batch:	5A6COCD0131	
Purity:	99.6% (area%, HPLC)	
Solvent:	DMSO	
Concentrations:	Experiment I:	33, 100, 333, 1000, 2500 and 5000 µg/plate, with and without S9-mix
	Experiment II:	10, 33, 100, 333, 1000, 2500 and 5000 µg/plate, with and without S9-mix
Treatment:	Experiment I:	plate incorporation test, with and without S9-mix
	Experiment II:	pre-incubation method was used with 60 minutes pre-incubation and at least 48h incubation, without and with S9-mix.
GLP:	in compliance	
Study period:	13 April – 2 June 2004	

A094 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity and mutation in a pre-experiment with strains TA98 and TA100 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 thinning of the bacterial background lawn. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in both strains used the pre-experiment is reported as experiment I. Experiment I was performed with the plate incorporation method, experiment II with the pre-incubation method. Negative and positive controls were in accordance with the OECD guideline.

Results
In experiment I toxic effects evident as reduced background growth were observed for TA98 at 100 µg/plate without S9-mix and at 333 µg/plate with S9-mix: in experiment II toxic effects were seen at 5000 µg/plate for TA98 without S9-mix and for TA102 with S9-mix. Toxic effects evident as a reduction in the number of revertants were observed in strains TA 1537 (experiment I) and TA 98 (experiment II) without S9-mix and in TA 102 (experiment I and II) with and without S9-mix.
In experiment I in the presence of S9-mix the number of colonies did not reach the lower limit of the historical control data in the negative controls for TA98 and TA100. Since these deviations are rather small these results are judged to be not detrimental for the outcome of the study.
In both experiments, A094 treatment did not result in a biologically relevant increase in revertant colonies in any of the five tester strains neither in the absence nor in the presence of S9-mix.

Conclusion
Under the experimental conditions used A094 was not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 8

In vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476 (1997)	
Species/strain:	L5178Y $tk^{+/-}$ mouse lymphoma cells	
Replicates:	duplicate cultures in two independent experiments	
Test substance:	5-amino-6-chloro-o-cresol	
Batch:	5A6 COCD 0131	
Purity:	99.6% (area%, HPLC)	
Solvent:	DMSO	
Concentrations:	Experiment I:	100, 200, 400, 600, 800 and 1000 µg/ml without S9-mix 1.56, 3.13, 6.25, 12.5 and 18.9µg/ml with S9-mix
	Experiment II:	25, 50, 100, 200 and 400 µg/ml without S9-mix 8, 10, 12, 14, 16 and 18 µg/ml with S9-mix
Treatment	Experiment I:	4h with and without S9-mix; 72h expression period
	Experiment II:	24h without S9-mix; 48h expression period 4 hours with S9-mix; 72h expression period
GLP:	in compliance	
Study period:	14 April – 2 August 2004	

5-amino-6-chloro-o-cresol 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 pre-test on toxicity measuring relative suspension growth. In the main test, cells were treated for 4 h or 24 h (without S9

experiment II) followed by an expression period of 72 or 48 h (without S9 experiment II) to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Toxicity was measured in the main experiments as relative total growth compared to the relative total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
Exclusively, in both experiments 5-amino-6-chloro-o-cresol was tested up to the required toxicity (10-20 % survival compared to the concurrent negative controls). However, in experiment I in the presence of S9-mix and in experiment II in the absence of S9-mix the highest dose (18.9 µg/ml and 400 µg/ml respectively) outreached the required level of toxicity.
No reproducible increase in the mutant frequency was observed in both experiments without metabolic activation. A dose related increase in the mutant frequency was only observed in experiment I with S9-mix. These increases were due to an increase in the number of small colonies indicating chromosomal aberrations. However, as the increases were not reproducible in experiment II, it was considered not biologically relevant.

Conclusion
Under the experimental conditions used, 5-amino-6-chloro-o-cresol did not induce gene mutations in this gene mutation test in mammalian cells.

Ref.: 9

In vitro Mammalian Chromosome Aberration Test

Guideline:	OECD 473 (1983)
Species/strain:	Chinese hamster V79 cells
Replicates:	two parallel cultures
Test substance:	RO 543
Batch:	2665/196
Purity:	approximately 85%
Concentrations:	10, 500, 800 and 1100 µg/ml
Solvent:	culture medium without foetal calf serum, pH 7
Treatment:	4 h treatment and harvest times 7 h (1100 µg/ml), 18 h (10, 500 and 800 µg/ml) and 28 h (1100 µg/ml) after start of treatment both in the absence and presence of S9-mix
GLP:	in compliance
Study period:	25 July 1988 – 16 February 1989

RO 543 has been investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in V79 cells. Test concentrations were based on the results of a range finding pre-experiment on toxicity and colony forming ability with 7, 18 and 28 h treatment both with and without S9-mix. The highest dose in the pre-test was 1100 µg/. In the main test cells were treated for 4 h and harvested 7, 18 and 28 h after the start of treatment both with and without S9-mix. 2 h (7 h harvest time) or 2.5 h (18 and 28 h harvest time) before harvest, each culture was treated with colcemid (final concentration 0.2 µg/ml) to block cells at metaphase of mitosis. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was determined by measuring the decrease in the mitotic index. Chromosome (metaphase) preparations were stained with Giemsa and examined microscopically for chromosomal aberrations and mitotic index. Negative and positive controls were in accordance with the OECD guideline.

Results
At the 18 and 28 h harvest time a decrease in mitotic indices up to the required 50% decrease was observed indicating toxic effects.

Both in the absence and presence of S9-mix RO 543 induced a statistically significant increase in the number of cells with chromosomal aberrations except after 4 h treatment with 18 and 28 h harvest times. At 18 h harvest time this increase was dose related.

Conclusion
Under the experimental conditions used the increase in cells with structural chromosomal aberrations indicates a genotoxic (clastogenic) activity of RO 543 in V79 cells *in vitro*.

Ref.: 10

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474 (1997)
Species/strain:	NMRI mice
Group size:	5 mice/sex/group
Test substance:	A094
Batch:	5A6COC-D0131
Purity:	99.9% (area%, HPLC)
Dose level:	100, 200 and 400 mg/kg bw
Route:	i.p.
Vehicle:	aqueous DMSO (30%)
Sacrifice times:	24 h and 48 h (high dose only) after the treatment.
GLP:	in compliance
Study period:	9 August 2004 – 30 June 2005

A094 has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on the acute toxicity in a pre-test with 2 animals per sex/group, measuring toxicity at various intervals around 1 to 48 h after treatment. In the main experiment mice were exposed to single i.p. doses of 0, 100, 200 and 400 mg/kg bw. At 24 h or 48 h (highest dose only) after dosing bone marrow cells were collected. The animals of the highest dose group were examined for acute toxic symptoms 1, 2-4, 6 and 24 h after start of treatment.
Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/TE). Satellite groups of 3 male mice per sampling time (1 h and 4 h after start of treatment) treated with 400 mg/kg bw were included for determination of blood concentrations of A094.
Bone marrow preparations were stained with May-Grünwald/Giemsa and examined microscopically for the PCE/TE ratio and micronuclei. Five mice/sex/group were analysed; a remaining 6th animal of each group were only evaluated in case a mouse died spontaneously. Negative and positive controls were in accordance with the OECD guideline.

Results
In the main experiment none of the animal died. Treatment with A094 did not result in substantially decreased PCE/TE ratios compared to the untreated controls indicating that A094 did not have cytotoxic properties in the bone marrow. In contrast, clinical signs like reduction in spontaneous activity, abdominal position, eyelid closure and ruffled fur indicating to systemic toxicity were observed in almost all animals treated with the highest dose (400 mg/kg bw) which was first observed 2 h but was lost 48 h after treatment. At 200 mg/kg bw reduction in spontaneous activity and ruffled fur was observed 2 h after treatment. The animals treated with 100 mg/kg bw did not express any toxic reaction.
The analysis of the blood samples of the males treated with 400 mg/kg bw showed that the test item could be quantified in the blood confirming the bioavailability of A094.
Biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found for bone marrow collected at both 24 and 48 h following treatment with A094.

Conclusion
Under the experimental conditions used A094 did not induce micronuclei in bone marrow cells of treated mice and, consequently, A094 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 11

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

Guideline:	OECD 414 (1981)
Species/strain:	rat, Wistar/HAN (Kfm: WIST, Outbred, SPF Quality)
Group size:	100 females, 25 per group
Test substance:	Ro 543
Batch:	2665/196
Purity:	90%
Dose:	0, 30, 90 and 270 mg/kg bw
Vehicle:	distilled water
Stability in vehicle:	2h at least
Dosing:	10 ml/kg bw with daily adjustment to the actual body weight
Administration:	once daily by oral gavage
Exposure:	from day 6 through day 15 *post coitum*
GLP:	in compliance
Study period:	7 March – 5 April 1988

The test substance was administered orally, once daily by gavage, from day 6 to 15 of gestation (volume: 10 ml/kg bw) to groups of 25 pregnant rats (184 – 234 g) at dose levels of 0, 30, 90 and 270 mg/kg bw/d. The control group received the vehicle (distilled water) only.
The animals were checked twice daily for mortality, symptoms of ill health and signs of treatment. Food consumption was recorded on d a y s 6, 11, 16 and 21 post coitum. Body weights were recorded daily from day 0 until day 21 post coitum.
On day 21 *post coitum*, the females were killed by CO_2 asphyxiation and the foetuses were removed by Caesarean section.
Post mortem examinations included gross macroscopic examination of all internal organs, with emphasis on the uterus, uterine contents, position of foetuses in the uterus and number of Corpora lutea. The foetuses were removed from the uterus, sexed, weighed individually and examined for gross external abnormalities One half of the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and one half was evaluated for visceral alterations.

Results
At terminal necropsy, no treatment–related macroscopic changes could be observed. The corrected body weight gain in dams was reduced in group 3 (90 mg/kg bw/d) and group 4 (270 mg/kg bw/d) compared to the control group and slight effects on food consumption were reported for these groups.

Beyond normal range of variation there were no differences concerning reproduction between controls and dose groups. This applies to the number of pregnant females, mean numbers of implantations and post implantation losses.
Foetal data did not show differences in sex ratios and body weights between groups. External examination of foetuses did not show abnormal findings in controls and in the low dose group. In one foetus in group 3 hind limbs were malrotated and one foetus of group 4 was a runt with palatoschisis. In the judgement of the authors these two cases were not related to treatment.
Skeletal examinations showed dumbbell formed thoracic vertebral bodies in 1, 3, 2, 4 foetuses of groups 1 to 4. The difference between controls and dosed groups were significant. According to the authors these cases are within the normal range of abnormal findings in this strain of rats.
There was no further statistical evidence for differences between the treated groups and the control group.

Conclusion
The study authors considered the NOAEL for this study was 90 mg/kg bw/d for maternal toxicity and the NOEL for developmental toxicity was 270 mg/kg bw/day.

Ref.: 14

Comment
The SCCS considers 30 mg/kg bw/d (24 mg/kg bw/d calculated as free base) as the NOAEL of maternal toxicity in this study due to reduced maternal body weight gain. The NOAEL of embryo-foetal toxicity was 270 mg/kg bw/d (219 mg/kg bw/d calculated as free base), the highest dose tested. There are significant, but not dose-related differences in the number of skeletal abnormalities between controls and dosed groups down to a concentration of 30 mg/kg (24 mg/kg bw/d calculated as free base). These findings are considered as variations of no toxicological relevance.

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

(5-Amino-6-chloro-o-cresol)

(non-oxidative conditions)
(2% formulation, 1% on head)

Absorption through the skin	**A**	**=**	**31.74 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**18.41 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.31 mg/kg bw**
Lowest observed effect level (90-day, oral, rat)	**LOAEL**	**=**	**100 mg/kg bw**
Adjustment factor of 3 for LOAEL	**adjusted LOAEL**	**=**	**33 mg/kg bw**
Bioavailability 50%		**=**	**16.5 mg/kg bw/d**
Margin of Safety	**adjusted LOAEL / SED**	**=**	**53**

(oxidative conditions)
(2% formulation, 1% on-head)

Absorption through the skin	**A**	**=**	**18.05 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**10.47 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.17 mg/kg bw**
Lowest observed adverse effect level (90-day, oral, rat)	**LOAEL**	**=**	**100 mg/kg bw**
Adjustment factor of 3 for LOAEL	**adjusted LOAEL**	**=**	**33 mg/kg bw/d**
Bioavailability 50%		**=**	**16.5 mg/kg bw/d**
Margin of Safety	**adjusted LOAEL/SED**	**=**	**97**

Since the margin of safety for a 2% formulation under non-oxidative conditions was only 53, the margin of safety was recalculated for an 1% formulation as is shown below

(non-oxidative conditions)
(1% formulation, 0.5% on head)

Absorption through the skin	**A**	**=**	**19.53 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**11.33 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.19 mg/kg bw**
Lowest observed effect level (90-day, oral, rat)	**LOAEL**	**=**	**100 mg/kg bw**
Adjustment factor of 3 for LOAEL	**adjusted LOAEL**	**=**	**33 mg/kg bw**
Bioavailability 50%*		**=**	**16.5 mg/kg bw/d**
Margin of Safety	**adjusted LOAEL / SED**	**=**	**87**

*standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.14 Discussion

Physico-chemical properties
5-Amino-6-chloro-o-cresol and it salts is used as a precursor for hair dyeing products. The final concentration of 5-amino-6-chloro-o-cresol on head can be up to 1.0% (calculated for the free base).
For most animal experiments batch 5A6COCD 0131 of 5-amino-6-chloro-o-cresol has been used. This material contains up to 1% of non-identified impurities.
Batch 2665/196 which is used for teratogenicity and chromosome aberration testing contains 7% and 3% respectively of 2 substances which have a lower toxic potential than 5-amino-6-chloro-o-cresol.
5-Amino-6-chloro-o-cresol is stable at room temperature. The solutions of batches of 5-amino-6-chloro-o-cresol which have been used for the examination of subchronic toxicity and teratogenicity are stable within the time between preparation and application. Their concentrations complied with the intended ones.

General toxicity
In an acute oral toxicity study in rats, the acute median lethal dose (LD50) and its 95% confidence limits to rats of 2-chloro-6-methyl-3-aminophenol were calculated to 1.36 (1.21-1.54) g/kg bw.
The liver and the haematopoietic system are the targets of systemic toxicity of 5-amino-6-chloro-o-cresol. Based on the haematological changes, in particular the reduced mean corpuscular Hb concentration in all dose groups of female rats, the LOAEL is 100 mg/kg/day (the lowest dose tested). This LOAEL is supported by the obvious dose-dependency of centrilobular hypertrophy of the liver which in males was observed down to the low dose. In the high dose recovery group, this effect persisted until termination of the study.

In a teratogenicity study, the NOAEL of maternal toxicity was 30 mg/kg bw/d (24 mg/kg bw/d calculated as free base) due to reduced maternal body weight gain; the NOAEL for developmental toxicity was 270 mg/kg bw/d.
For the margin of safety calculation, the LOAEL of the 90-day study was adjusted with a factor of 3, resulting in 33 mg/kg bw/day. This is similar to the NOAEL for maternal toxicity in the teratogenicity study. This value was corrected for 50% bio-availability (16.5 mg/kg bw/d).

Irritation / sensitisation
5-Amino-6-chloro-o-cresol was not irritant to rabbit skin. Under the conditions of the study, the undiluted test material was irritating to the rabbit eye.
The discrepancy between the two skin sensitisation experiments using a 50% concentration of 5-Amino-6-chloro-o-cresol is unexplained. 5-amino-6-chloro-o-cresol is considered to be a non-sensitiser.

Dermal absorption
The dermal absorption of A94 at 1% on head final concentration in a hair dye formulation under non oxidative condition and oxidative conditions were 23.52 ± 8.22 µg/cm² and 14.05 ± 4.00 µg/cm² respectively.
The mean values + SD = 31.74 and 18.05 µg/cm² under non oxidative conditions and oxidative conditions, respectively, are used for calculating the MOS.
Because the MoS under non oxidative conditions was only 53, the dermal absorption at 0.5% on head final concentration in a hair dye formulation under non oxidative conditions was considered for calculating the MoS, then the mean values + SD = 19.53 µg/cm² under non oxidative conditions are used for calculating the MOS.

Mutagenicity / genotoxicity
Overall, the genotoxicity of 5-amino-6-chloro-o-cresol is sufficiently investigated for the three endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy.
5-Amino-6-chloro-o-cresol was not genotoxic *in vitro* in gene mutation tests with either bacteria or mammalian cells, although in mammalian cells indications were found for a clastogenic effect of 5-amino-6-chloro-o-cresol (increase in small colonies in 1 of 2 experiments). 5-Amino-6-chloro-o-cresol was genotoxic (clastogenic) in an *in vitro* chromosome aberration test.
The positive *in vitro* results could not be confirmed in *in vivo* experiments covering the same endpoints. 5-Amino-6-chloro-o-cresol was negative in a mouse bone marrow micronucleus tests.
As the clastogenic effects found *in vitro* were not confirmed in *in vivo* tests, 5-amino-6-chloro-o-cresol itself can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No data submitted

The calculation of the Margin of Safety resulted in a MOS of 97 for oxidative conditions and in a MoS of 87 for non-oxidative conditions, which are considered sufficient because of the intermittent exposure to hair dyes.

4. CONCLUSION

The SCCS is of the opinion that the use of 5-amino-6-chloro-o-cresol as oxidative hair dye with a concentration on head of maximum 1.0% and as non-oxidative hair dye with a concentration on head of maximum 0.5% does not pose a risk to the health of the consumer.

5. MINORITY OPINION

Not applicable

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