Scientific Committee on Consumer Safety

SCCS

OPINION ON

Acid Green 25

COLIPA n° C178

The SCCS adopted this opinion at its 18th plenary meeting

of 26 February 2013

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.).

Scientific Committee members

Jürgen Angerer, Ulrike Bernauer, Claire Chambers, Qasim Chaudhry, Gisela Degen, Elsa Nielsen, Thomas Platzek, Suresh Chandra Rastogi, Vera Rogiers, Christophe Rousselle, Tore Sanner, Jan van Benthem, Jacqueline van Engelen, Maria Pilar Vinardell, Rosemary Waring, Ian R. White

Contact
European Commission
Health & Consumers
Directorate D: Health Systems and Products
Unit D3 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-SCCS-Secretariat@ec.europa.eu

ISSN 1831-4767 ISBN 978-92-79-31189-5
Doi:10.2772/58467 ND-AQ-13-024-EN-N

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

ACKNOWLEDGMENTS

Prof. J. Angerer
Dr. C. Chambers
Dr. E. Nielsen (rapporteur)
Dr. W. Lilienblum (associated scientific advisor)
Prof. T. Platzek (chairman)
Dr. S.C. Rastogi
Dr. C. Rousselle
Prof. T. Sanner
Dr. J. van Benthem
Prof. M.P. Vinardell
Dr. I.R. White

External experts

Dr. Mona-Lise Binderup National Food Institute, Denmark

Keywords: SCCS, scientific opinion, hair dye, C178, Acid Green 25, directive 76/768/ECC, CAS 4403-90-1, EC 224-546-6

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on Acid Green 25, 26 February 2013

TABLE OF CONTENTS

1. BACKGROUND

Submission I for Acid Green 25, with the chemical name: 1,4-Di-((2-sulpho-4-methylphenyl)amino)-9,10-antracenedione disodium salt, was submitted in September 2003 by COLIPA[1]. Acid Green 25 is identical with CI 61570 also used as a colouring agent allowed in all cosmetic products.

The SCCP adopted at its 4th plenary meeting of 21 June 2005 an opinion (SCCP/0879/05) with the conclusion, *"that the information submitted is insufficient to assess the safe use of the substance. Before any further consideration, the following information is required:*

- *complete information on the >10% impurity;*
- *data on exposure from other cosmetic products;*
- *a percutaneous absorption study according to the Notes of Guidance;*
- *data on teratogenicity;*
- *an additional in vitro test specifically detecting chromosomal aberrations (preferably an in vitro micronucleus test which detects clastogenic and aneugenic effects) should be performed."*

According to the current submission II, submitted by COLIPA in July 2005, Acid Green 25 is proposed to be used as a non-reactive hair colouring agent ("direct dye") in semi-permanent hair dye formulations at a maximum on-head concentration of 0.3%. It is common practice to apply 35 to 50 g of the product over a period of 30 minutes followed by rinse off with water and shampoo. The application may be repeated at weekly intervals.

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

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Safety (SCCS) consider Acid Green 25 safe for use in non-oxidative hair dye formulations with a concentration of maximum 0.3% in the finished product taking into account the scientific data provided?*

2. *Does the SCCS recommend any restrictions with regard to the use Acid Green 25 in hair dye formulations?*

[1] COLIPA - 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

Acid Green 25 (INCI name)

3.1.1.2. Chemical names

Disodium 2,2'-(9,10-dioxoanthracene-1,4-diyldiimino)bis(5-methylsulphonate) (IUPAC)
Benzensulfonic acid, 2,2´-[(9,10-dihydro-9,10-dioxo-1,4-anthracenediyl) diimino]bis(5-methyl)-, disodium salt (CA Index name, 9CI)
1,4-di-[(2-sulfono-4-methylphenyl)amino]-9,10-anthracenedione, disodium salt

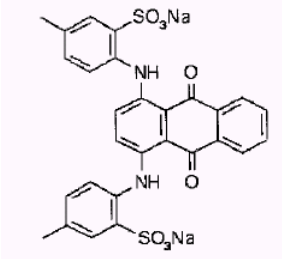
3.1.1.3. Trade names and abbreviations

Acid Green anthraquinone
Alizarin Cyanine Green F
Japan Green 201
D&C Green n° 5
Covacap Vert W 7103 (LCW)
Colour Index Number: CI 61570
COLIPA n° C178

3.1.1.4. CAS / EC number

CAS: 4403-90-1
EC: 224-546-6

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula : $C_{28}H_{22}N_2O_8S_2.2Na$

3.1.2. Physical form

Dark green powder

3.1.3. Molecular weight

Molecular weight: 622.58 g/mol

3.1.4. Purity, composition and substance codes

Batch tested: Batch 33 (FDA certified Lot AJ6720)
Identification: NMR* *(spectra not yet provided)*
Content determined by NMR: 86.1%
Purity determined by HPLC (qualitative)**: >80%

Detection wavelength	%peak area Acid Green 25	%peak area Isomeric double sulfonated 2,4-dinitro-1-naphthol
210 nm	83.5	13.2
254 nm	84.1	12.5
430 nm	84.7	13.3

Colour content
Citrate buffer titration: 94.8%
Spectrophotometric[a]: 94.5%
Water content: 8.2%
Volatile matter: 4.7% maximum
Sulfated ash content: 24.9%

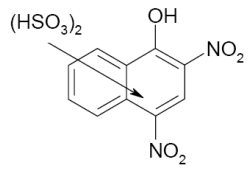
a The total colour content determined by spectrophotometry may also include other organic impurities present in the Acid Green 25 - the wavelength for the colour measurement is not reported.

* NMR spectrum not provided

Comment
According to FDA specifications Acid Green 25 also contains Ext. D&C Violet No.2 (1.11%)

3.1.5. Impurities / accompanying contaminants

Disulfonated 2,4-dinitro-1-naphthol: >10%



Double sulfonated 2,4-dinitro-1-naphthol

p-toluidine: <15 ppm 1,4-dihydroxyanthraquinone: <0.2 %
2-amino-5-methylbenzenesulfonic acid: <0.2 % 1,4-diaminoanthraquinone: /
Chloride content as sodium chloride: 4.8%
Sulfate content as sodium sulphate: 0.6%
Metal content: Pb: 0.05 ppm, As 0.1 ppm, Hg <0.1 ppm, Fe 13 ppm

3.1.6. Solubility

Water: 0.903 g/L (20°C, pH 9.5) EU Method A.6
Acetone: 0.2% (w/v)
DMSO: 5% (w/v)

3.1.7. Partition coefficient (Log Pow)

Log Pow : 5.71 ± 0.63 (acid, calculated)

Log Pow determined by EU Method A.8:
The Chromatogram of D&C Green n° 5 shows two peaks having same UV-spectra as for a reference standard of D&C Green n° 5
The partition coefficient of the test substances in the two HPLC peaks (isomers) was in the range
Log Pow: 0.61(peak 1) – 0.94 (peak 2)

pH 7.2, at room temperature

3.1.8. Additional physical and chemical specifications

Melting point:	decomposition at 255°C
Boiling point:	-/ not applicable
Flash point:	/
Vapour pressure:	<1.0 exp -7hPa (20°C, extrapolated)
Density:	1.507 g/ml (20°C)
Viscosity:	/
pKa:	-4.17 for R(Ar)-SO_3H (acidic)
Refractive index:	/
UV-Vis spectrum:	λmax 207.7nm, absorption at 254nm, 284nm, 414nm, 602nm and 645nm

3.1.9. Homogeneity and Stability

Stability testing in DMSO, water, water/acetone
The stability of the test substance (D&C Green n° 5; batch 33, lot AJ6720) in 3 different solvents was monitored over a total time period of seven days using HPLC/DAD at a detection wavelength of 640 nm. During the test procedure the test solutions were stored at ambient temperature in the absence of light. The indicated values were standardized on the initial value (t = 0).

Stability in DMSO (0.3% solution, w/v)
During the course of storage no decomposition of the substance could be observed.
Recovery at t = 0: 100.0%; t = 6h: 98.5%; t = 2d: 100.0%; t = 7d: 101.0%.
Stability in water (1.8% solution, w/v)
During the course of storage no decomposition of the substance could be observed.
Recovery at t = 0: 100.0%; t = 6h: 99.1%; t = 2d: 97.8%; t = 7d: 100.4%.
Stability in water/acetone (1:1, v/v; 2.7% solution, w/v)
During the course of storage no decomposition of the substance could be observed.
Recovery at t = 0: 100.0%; t = 6h: 99.0%; t = 2d: 100.7%; t = 7d: 101.0%.
Conclusion:
The solutions of the test substance (D&C Green n° 5; batch 33, lot AJ6720) in DMSO, Water/acetone 1:1 and Water can be regarded as stable during the test period of 7 days under the conditions described above.

General Comments on physico-chemical characterisation

- According to FDA specifications Acid Green 25 also contains Ext. D&C Violet No.2 (1.1%)

- Stability of Acid Green 25 in in typical hair dye formulations was not demonstrated.

3.2 Function and uses

Acid Green 25 is proposed for use in semi-permanent hair dye formulations as a direct dye at a maximum concentration of 0.3% in the finished cosmetic product.
Acid Green 25 is permitted for the use as a colorant in other cosmetic products according to Annex IV of the Cosmetics Directive.

3.3 Toxicological Evaluation

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

Taken from SCCP/0879/05

Rat
LD50 >3160 mg/kg bw
LD50 >10000 mg/kg bw

Dog
LD50 >1000 mg/kg bw

Taken from reference 2 of submission I (identical to reference 13 of submission II). No further details were provided.

Ref.: 2

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

Taken from SCCP/0879/05

No specific data from skin irritation tests in rabbits or in rodents like mice or rats are available or reported in the literature for Acid Green 25. In addition no data were noted in the literature with regard to human experience e.g. in patch tests.

An indication of the skin irritating potential of Acid Green 25 can, however, be deduced from the skin sensitisation test in guinea pigs (maximisation test, see point 3.3.3). In this test no skin irritating effects were noted when a 10% solution of Acid Green 25 in 1% CMC (carboxymethylcellulose) was applied to the back skin of guinea pigs under occlusive conditions for 24 h in both the pre-test and the main study. In the pre-test a 15% dilution produced only very mild irritation (score 1) that had completely vanished after 48 h.

Furthermore, an intradermal injection of up to 5% Acid Green 25 in CMC did not produce any irritation effects.

Although the guinea pig is not commonly used species for the determination of the skin irritation potential *in vivo* and is possibly less sensitive for skin irritation as the rabbit, the lack of any relevant skin irritation even when applied as 10% solution and above under severe test conditions (24 to 48 h occlusive patch) is a strong indication of a low irritating effect of Acid Green 25. At the intended use concentration in hair dye formulations of 0.3% skin irritating effects are not to be expected.

3.3.2.2 Mucous membrane irritation

Taken from SCCP/0879/05

Guideline:	/
Species/strain:	albino rabbit, strain not given
Group size:	6 or more animals
Test substance:	D&C Green n° 5
Batch:	not given, but specifications according to FDA requirements
Dosages:	20 mg (equal to 0.2 ml of a 10% aqueous solution)
GLP:	not in compliance

Together with several other colorants, Acid Green 25 was investigated with regard to its eye irritation and staining properties. 0.2 ml of a 10% aqueous solution was repeatedly applied to the conjunctival sac of one eye of each of the 6 or more animals per group for 4 weeks (twice daily on five days per week; 40 applications in total). One hour after each application, the eyes were scored for irritation according to the Draize system and for evidence of staining. In addition, scoring took also place the next day just prior to the first application of that day.

A 10% aqueous solution of Acid Green 25 did cause a slight or spotty discoloration in the orbital tissue of some animals under the described test conditions.
One hour after the application (figures are given for day 5) a mean irritation score of 2 was determined, indicating that immediately after application mild irritant effects were present. 24 hours after the applications of the 10% solution no indications for eye irritation were noted at any scoring throughout the entire study period.

Conclusion
Although the described study is not in line with currently recommended tests for the investigation of the eye irritating properties *in vivo* like OECD 405, the test allows an evaluation of the eye irritation potential of Acid Green 25.
As no irritation was noted under the described severe test conditions with a more than 30-times higher concentrated solution of Acid Green 25, no eye irritating effects are expected for the intended use concentrations of 0.3% in hair dyes.

Ref.: 3

3.3.3 Skin sensitisation

Taken from SCCP/0879/05

Maximisation Test (Magnusson and Kligman)

Guideline:	OECD 406 (1993)
Species/strain:	Ibm: GO HI SPF-quality female guinea pigs (Himalayan spotted)
Group size:	test group: 10 animals; control: 5 animals
Test substance:	D&C Green n° 5 (CI 61570)
Batch:	33 (lot No. AJ6720)

Concentrations: induction: 5% test substance in 1% carboxymethylcellulose (CMC), emulsified with Freund's complete adjuvant
Induction: 50% test substance in 1 % CMC, occluded
challenge: 10% test substance in 1 % CMC , occluded
GLP: in compliance

The dermal sensitisation potential of Acid Green 25 was evaluated by the Magnusson-Kligman Maximisation method in Ibm: GOHI SPF-quality female guinea pigs (Himalayan spotted). Based on a range-finding study, 10 animals were intradermally induced on day 1 with 0.1 ml of a 5% dilution (w/v) of Acid Green 25 in 1% CMC emulsified with Freund's complete adjuvant. On day 8, the animals were topically induced with a 50% dilution of Acid Green 25 in 1% CMC (about 0.3 g per animal). Animals were challenged on day 22 by application of about 0.2 ml of a 10% dilution in 1% CMC under occlusion. Approximately 24 and 48 hours after the challenge phase, the test sites were evaluated for signs of elicited sensitisation (readings after 72 hours and 7 days). The same procedures were carried out on a contemporaneous control group except that the solutions of the test article were replaced by 1% CMC (vehicle control). No contemporaneous positive control was found; however, a positive control substance is periodically tested in the laboratory in order to document the effect of a known sensitiser in this test system.

Results
Range-finding studies
1 animal, pre-treated with 4 intradermal injections of Freund's complete adjuvant, received 0.1 ml intradermal injections of concentrations of 1, 3 and 5% of Acid Green 25 in 1% CMC. The injection sites were assessed 24 hours later. No reactions were noted up to a concentration of 5%. However, a green discoloration at this dose level did not allow an evaluation for erythema.
Based on these results, a 5% dilution of Acid Green 25 in 1 % CMC was selected for intradermal induction in the main study.

A topical range-finding study with the concentrations listed in table 1 was conducted on 2 guinea pigs.

Table 1: Number of Animals Exhibiting Erythema in the Range-finding Test After Topical Application (24 and 48 hours after treatment)

Concentration	*50%*		*25%*		*15%*		*10%*	
Hours	24	48	24	48	24	48	24	48
Score	2/2	2/2	2/2	2/2	2/2	0/2	0/2	0/2

Based on the range-finding study, 50% in 1% CMC was found to produce dermal irritation and was selected for the topical induction, and 10% in 1% CMC did not produce dermal irritation and was selected as the challenge dose.

Main study
No dermal irritation was observed during the epidermal induction in the control group. As Acid Green 25 epidermally applied at 50% in 1% CMC causes black staining of the skin, erythema formation could not be evaluated. However, no oedema formation was noted.

Since a black discoloration was also observed after removal of the challenge patch, depilation was performed 3 hours prior to challenge reading. Skin reactions were observed neither in the control nor in the test group.
No test substance-related clinical signs of toxicity were observed.

Conclusion
Acid Green 25 had no skin sensitising effect under the conditions of the Maximisation test, in which the skin barrier is compromised. Based on these findings, Acid Green 25 is

evaluated not to be a skin-sensitiser. However, the concentration of the test substance used for induction (5%) appears to be too low as no erythema was observed.

Ref.: 5

3.3.4 Dermal / percutaneous absorption

New study, submission 2013

Guideline:	OECD 428
Tissue:	split-thickness human skin, 200-400 µm
Number of donors:	5
Method:	flow-through diffusion cells, 0.64 cm²
No. chambers:	12
Skin integrity:	tritiated water barrier integrity test
Test substance:	Acid Green 25 (C178) was tested incorporated in a typical hair dye formulation at 0.3% (w/w)
Batch:	33, Lot AJ6720 CFQ40842 (radio-labelled), 80.9 µCi/mg, 50.5 mCi/mmol
Purity:	84.2% (HPLC, 210 nm) 99.5% (radio-labelled)
Dose:	20 mg/cm²
Receptor fluid:	Minimum Essential Medium Eagle supplemented with polyethylene 20-oleyl ether (6%, w/v), glucose (1%, w/v), sodium azide (0.01%, w/v) and penicillin-streptomycin solution (100 units/mL and 0.1 mg/mL respectively)
Stability:	/
Analytic method:	liquid scintillation counting
GLP:	in compliance
Study period:	20 June 2011 – 1 March 2012

Dermal absorption of Acid Green 25 (C178) was studied following topical application to human skin under in-use conditions. Acid Green 25 (C178) was incorporated into a typical hair dye formulation at 0.3% (w/w).
Split-thickness human skin membranes were mounted into flow-through diffusion cells. Receptor fluid, Minimum Essential Medium Eagle supplemented with polyethylene 20-oleyl ether (6%, w/v), glucose (1%, w/v), sodium azide (0.01%, w/v) and penicillin-streptomycin solution (100 units/mL and 0.1 mg/mL respectively) was pumped underneath the skin at a flow rate of 1.5 ± 0.15 mL/h. The skin surface temperature was maintained at 32 ± 1°C throughout the experiment. A tritiated water barrier integrity test was performed and any human skin sample exhibiting absorption greater than 0.6% of the applied dose was excluded from subsequent absorption measurements.
Acid Green 25 (C178) was incorporated into formulations applied at an application rate of ca 20 mg/cm² to human split-thickness skin membranes mounted into flow through diffusion cells in vitro for 30 min.
Absorption was assessed by collecting receptor fluid in 30 min fractions from 0 to 1 h post dose, then hourly fractions from 1 to 6 h post dose and then in 2-hourly fractions from 6 to 72 h post dose.

Results

Table 1: summary of the mean results

Test Preparation	1	
Target Concentration (%, w/w)	0.30	
Concentration by Radioactivity (%, w/w)	0.31	
Total no. of Donors	5	
Total no. of Replicates Dosed	12	
Total no. of Replicates Contributing to Mean & SD Data	12	
Results	% Applied Dose	(µg equiv./cm^2)
30 min Dislodgeable Dose	96.84	64.70
72 h Dislodgeable Dose	1.38	0.92
Total Dislodgeable Dose	98.21	65.62
Stratum Corneum	1.94	1.29
Epidermis	1.22	0.82
Dermis	0.17	0.11
Unabsorbed Dose	100.15	66.92
Absorbed Dose	0.02	0.02
Mass Balance	101.57	67.86

30 min Dislodgeable Dose = 30 min Skin Wash + 30 min Tissue Swabs + 30 min Pipette Tips + Parafilm
72 h Dislodgeable Dose = 72 h skin wash + 72 h tissue swabs + 72 h Pipette Tips + Cell Donor Chamber Wash
Total Dislodgeable Dose = Dislodgeable Dose 30 min + Dislodgeable Dose 72 h
Unabsorbed Dose = Total Dislodgeable Dose + Stratum Corneum + Unexposed Skin
Absorbed Dose + Receptor Fluid + Cell Receptor Chamber Wash + Receptor Rinse

Table 2: Distribution of [^{14}C]-Acid Green 25 (% Applied Dose) at 72 h Post Dose Following Topical Application of the Test Preparation (0.3%, w/w) to Human Split-Thickness Skin

	Cell Number and Donor Number													
	Cell 22 0353	Cell 23 0353	Cell 24 0353	Cell 28 0323	Cell 29 0323	Cell 30 0323	Cell 32 0308	Cell 33 0308	Cell 40 0308	Cell 41 0314	Cell 42 0353	Cell 43 0318	Mean	SD
Skin Wash 30 min	97.51	97.49	96.40	86.77	98.93	95.53	97.08	91.15	95.52	97.91	94.61	91.85	95.06	3.51
Tissue Swab 30 min	1.04	0.49	1.50	2.91	0.70	3.28	1.23	2.18	0.70	0.99	1.21	2.29	1.54	0.91
Pipette Tips 30 min	0.12	0.21	0.27	0.29	0.10	0.60	0.27	0.14	0.13	0.09	0.15	0.27	0.22	0.14
Parafilm	*0.00	*0.00	0.00	*0.00	0.04	0.00	*0.00	*0.00	0.02	0.00	0.00	0.02	°0.01	°0.01
Dislodgeable Dose 30 min	98.68	98.19	98.18	89.98	99.76	99.42	98.58	93.48	96.36	98.99	95.97	94.43	96.84	2.94
Skin Wash 72 h	0.55	0.45	0.72	1.94	0.63	0.80	0.77	1.41	0.74	1.15	0.68	0.84	0.89	0.42
Tissue Swab 72 h	0.18	0.08	0.09	0.60	0.20	0.24	0.20	0.36	0.25	0.27	0.24	0.54	0.27	0.16
Pipette Tips 72 h	*0.02	0.03	*0.01	0.02	*0.01	0.13	0.01	0.03	0.01	*0.01	0.01	0.01	°0.02	°0.03
Donor Chamber Wash	*0.02	0.08	0.19	0.48	0.16	0.32	0.09	0.05	0.06	0.32	*0.04	0.50	°0.19	°0.17
Dislodgeable Dose 72 h	0.77	0.63	1.00	3.05	0.99	1.48	1.08	1.85	1.07	1.75	0.97	1.90	1.38	0.67
Total Dislodgeable Dose	99.44	98.82	99.18	93.02	100.75	100.90	99.66	95.33	97.43	100.73	96.94	96.33	98.21	2.45
Stratum Corneum 1-5	0.81	1.17	1.45	1.56	0.95	0.77	0.81	1.65	1.19	1.08	1.83	0.90	1.18	0.36
Stratum Corneum 6-10	0.40	0.51	0.34	0.28	0.20	0.27	0.29	0.42	0.35	0.47	0.49	0.46	0.37	0.10
Stratum Corneum 11-15	0.17	0.16	0.08	0.24	0.11	0.24	0.14	0.33	0.23	0.35	0.31	0.27	0.22	0.09
Stratum Corneum 16-20	0.13	0.20	0.16	0.19	0.13	0.11	0.10	0.32	0.15	0.20	0.09	0.16	0.16	0.06
Stratum Corneum	1.51	2.05	2.03	2.27	1.38	1.38	1.34	2.72	1.92	2.10	2.72	1.80	1.94	0.48
Unexposed Skin	*0.00	*0.00	0.04	0.00	0.01	0.00	*0.00	*0.00	0.00	0.01	0.02	0.00	°0.01	°0.01
Total Unabsorbed	100.95	100.87	101.25	95.30	102.14	102.28	101.00	98.06	99.36	102.84	99.68	98.13	100.15	2.17
Epidermis	1.60	1.18	0.72	1.91	0.79	0.68	1.42	1.75	0.86	0.46	1.62	1.67	1.22	0.50
Dermis	0.19	0.03	0.02	0.17	0.10	0.03	0.14	0.33	0.17	0.05	0.73	0.10	0.17	0.20
Receptor Fluid	*0.00	*0.01	*0.01	*0.01	*0.01	*0.00	*0.02	*0.00	*0.01	*0.00	*0.00	*0.01	°0.01	°0.00
Receptor Chamber Wash	*0.01	*0.00	0.12	*0.00	*0.01	*0.00	*0.00	*0.00	*0.02	*0.01	*0.00	*0.00	°0.02	°0.03
Receptor Rinse	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	°0.00	°0.00
Total Absorbed	0.01	0.02	0.14	0.01	0.02	0.01	0.02	0.01	0.03	0.01	0.00	0.02	0.02	0.04
Dermal Delivery	1.80	1.22	0.88	2.09	0.90	0.71	1.57	2.08	1.06	0.51	2.36	1.79	1.41	0.61
Mass Balance	102.75	102.09	102.13	97.39	103.04	102.99	102.57	100.14	100.41	103.35	102.05	99.92	101.57	1.76

*=Results 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

Table 3: Distribution of [^{14}C]-Acid Green 25 (µg equiv./cm2) at 72 h Post Dose Following Topical Application of the Test Preparation (0.3%, w/w) to Human Split-Thickness Skin

	Cell Number and Donor Number													
	Cell 22 0353	Cell 23 0353	Cell 24 0353	Cell 28 0323	Cell 29 0323	Cell 30 0323	Cell 32 0308	Cell 33 0308	Cell 40 0308	Cell 41 0314	Cell 42 0353	Cell 43 0318	Mean	SD
Skin Wash 30 min	65.15	65.14	64.41	57.98	66.10	63.83	64.87	60.91	63.82	65.42	63.21	61.37	63.52	2.35
Tissue Swab 30 min	0.70	0.33	1.00	1.94	0.47	2.19	0.82	1.46	0.47	0.66	0.81	1.53	1.03	0.61
Pipette Tips 30 min	0.08	0.14	0.18	0.20	0.06	0.40	0.18	0.09	0.08	0.06	0.10	0.18	0.15	0.09
Parafilm	*0.00	*0.00	0.00	*0.00	0.03	0.00	*0.00	*0.00	0.01	0.00	0.00	0.02	°0.01	°0.01
Dislodgeable Dose 30 min	65.93	65.61	65.60	60.12	66.66	66.43	65.87	62.46	64.39	66.14	64.13	63.09	64.70	1.96
Skin Wash 72 h	0.37	0.30	0.48	1.30	0.42	0.53	0.51	0.95	0.50	0.77	0.45	0.56	0.59	0.28
Tissue Swab 72 h	0.12	0.05	0.06	0.40	0.13	0.16	0.14	0.24	0.17	0.18	0.16	0.36	0.18	0.11
Pipette Tips 72 h	*0.01	0.02	*0.00	0.01	*0.00	0.09	0.01	0.02	0.00	*0.01	0.00	0.01	°0.02	°0.02
Donor Chamber Wash	*0.01	0.05	0.13	0.32	0.10	0.21	0.06	0.03	0.04	0.21	*0.03	0.34	°0.13	°0.12
Dislodgeable Dose 72 h	0.51	0.42	0.67	2.04	0.66	0.99	0.72	1.24	0.71	1.17	0.65	1.27	0.92	0.45
Total Dislodgeable Dose	66.44	66.03	66.27	62.15	67.32	67.42	66.59	63.70	65.10	67.31	64.77	64.36	65.62	1.64
Stratum Corneum 1-5	0.54	0.78	0.97	1.04	0.63	0.51	0.54	1.10	0.80	0.72	1.23	0.60	0.79	0.24
Stratum Corneum 6-10	0.26	0.34	0.23	0.19	0.13	0.18	0.19	0.28	0.23	0.31	0.33	0.31	0.25	0.07
Stratum Corneum 11-15	0.12	0.11	0.06	0.16	0.08	0.16	0.09	0.22	0.16	0.23	0.21	0.18	0.15	0.06
Stratum Corneum 16-20	0.09	0.13	0.11	0.13	0.08	0.07	0.07	0.21	0.10	0.13	0.06	0.11	0.11	0.04
Stratum Corneum	1.01	1.37	1.36	1.52	0.92	0.92	0.89	1.82	1.29	1.40	1.82	1.20	1.29	0.32
Unexposed Skin	*0.00	*0.00	0.02	0.00	0.01	0.00	*0.00	*0.00	0.00	0.00	0.01	0.00	°0.00	°0.01
Total Unabsorbed	67.45	67.40	67.65	63.67	68.25	68.34	67.48	65.52	66.39	68.71	66.60	65.56	66.92	1.45
Epidermis	1.07	0.79	0.48	1.28	0.53	0.45	0.95	1.17	0.58	0.30	1.08	1.12	0.82	0.33
Dermis	0.12	0.02	0.01	0.11	0.07	0.02	0.09	0.22	0.11	0.03	0.49	0.07	0.11	0.13
Receptor Fluid	*0.00	*0.01	*0.01	*0.01	*0.00	*0.00	*0.01	*0.00	*0.01	*0.00	*0.00	*0.01	°0.01	°0.00
Receptor Chamber Wash	*0.00	*0.00	0.08	*0.00	*0.01	*0.00	*0.00	*0.00	*0.01	*0.00	*0.00	*0.00	°0.01	°0.02
Receptor Rinse	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	°0.00	°0.00
Total Absorbed	0.01	0.01	0.09	0.01	0.01	0.00	0.01	0.00	0.02	0.01	0.00	0.01	0.02	0.02
Dermal Delivery	1.20	0.81	0.59	1.40	0.60	0.48	1.05	1.39	0.71	0.34	1.58	1.20	0.95	0.41
Mass Balance	68.65	68.21	68.24	65.07	68.85	68.82	68.54	66.91	67.09	69.05	68.18	66.76	67.86	1.18

*=Results 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

Following topical application of Test Preparation 1 (0.3% Acid Green 25, w/w) to human skin in vitro, the majority of the applied dose (96.84%) was removed by washing at 30 min post application. At 72 h post application, the total dislodgeable dose was 98.21% of the applied dose. The stratum corneum retained 1.94% of the applied dose; 1.18% was removed with the first 5 tape strips. The total unabsorbed dose was 100.15% of the applied dose. The absorbed dose that had penetrated through the skin and into the receptor fluid was 0.02 µg equiv./cm² (0.02%). The epidermis and dermis contained 1.22% (0.82 µg equiv./cm²) and 0.17% (0.11 µg equiv./cm²) of the applied dose, respectively.
The mass balance was complete with 101.57% (SD = 1.76%) of the applied dose recovered.
In conclusion, following topical application of the test preparation to human skin in vitro, the total absorbed dose that had penetrated through the skin into the receptor fluid was 0.02 µg equiv./cm² (0.02%). The epidermis contained 0.82 µg equiv./cm². The dermis contained 0.11 µg equiv./cm². The majority of the applied dose was removed by washing the skin; the total dislodgeable dose was 98.21%. The mass balance was complete (101.57%) for [^{14}C]-Acid Green 25 (C178).

Ref.: 1 (subm 2013)

Comment
The total dermal absorption of Acid Green 25 at 72 hours was 0.02 ± 0.02 µg equiv./cm². The mean + SD: 0.04 µg equiv./cm² may be used to calculate MoS.

Taken from SCCP/0879/05, revised

Guideline:	OECD 428 (Draft 1996)
Tissue:	Porcine ear skin (thickness: 300-400 µm)
Method:	diffusion glass chambers
Test substance:	D&C Green n° 5 (C.I. 61570) tested as such and as part of a commercial hair dye formulation.
Batch:	Lot AJ6720
Concentration:	5 mg/cm² (pure substance, dissolved in saline, pH 3 (adjusted), 5 mg/cm² (tested as part of a viscous hair dye formulation, pH 2.9-3.1)
No. of chambers:	6 per experiment
GLP:	in compliance

The skin absorption of Acid Green 25 was investigated on the outer skin of porcine ears (freshly obtained from the local slaughter-house, ca. 400 µm thick) with amounts corresponding to realistic use conditions. Two experiments were performed. In the first experiment 5 mg of the pure dye in 1 ml saline with an adjusted pH of 3 was applied to the skin. In the second experiment about 5 mg of the dye was applied to the skin as part of a commercial hair dye formulation (1 g hair colour gel).

The integrity of the skin was monitored throughout the study at 0, 0.5, 1, 2, 4, 6 8 and 24 h by measuring the conductivity across the skin. Intact skin usually ranges from 100-500 µS with aqueous solutions. The maximum conductivity with gross damage to the skin or without skin is 2-5 mS.

A glass diffusion chamber (donor chamber volume about 1.5 ml, skin surface 1 cm^2) was used. The receptor solution (0.9% NaCl-solution, pH 3) was pumped through the receptor chamber by a rate of 1 -2 ml/h. Six chambers per experimental group were investigated. The donor chambers were covered by Parafilm after adding the test substance to the chamber.

Before application of the test item, receptor fluid was added to the donor chamber to check the integrity of the skin by means of conductivity measurement and to obtain the blank samples for each chamber. Only intact skin samples were used for the study. 30 min after substance application, the test item was removed by washing the skin three-times with 1 ml washing solution (10% diluted shampoo-formulation). The washing solutions were combined and the amount of dye determined. For the remaining time of the experiment (24 h total) the donor chamber was filled with 1 ml saline solution.

Fractions of the receptor fluid were collected at 0, and for 0-0.5, 0.5-1, 1-2, 2-4, 4-6, 6-8 and 8-24 hours and stored at -20° C until analysis. The donor solution was also collected after 24 h and analysed. At termination of the experiment the skin samples (including the stratum corneum) were extracted and the dye content quantified by HPLC. Caffeine is used in the performing laboratory every 3 months as a positive control substance to demonstrate the validity of the used system.

Results
All samples/tissue extracts were analysed by HPLC, the limit of detection of the applied method was 0.15 µg/ml.

An increase of conductivity over time was observed in all chambers, but no major loss of barrier properties was noted as no abrupt change in conductivity was noted. However in the second experiment chambers 4 and 5 revealed relatively high conductivity already at begin of the study, which sharply increased and exceeded the historical control range from 1 h after application onwards. Therefore, it was concluded that the skin barrier was impaired in these two chambers after 1 h.

The mean recoveries of the test item for experiment I and II were 93.2% ± 4.02% and 84.6% + 14.18%. The higher variability of the recovery in the second experiment is most likely due to the viscous state of the formulation causing problems in application and removal of the test item. For this reason one chamber was excluded from the evaluation. The vast majority of the test item (> 99%) was determined in the combined washing solution. No measurable permeation through the skin was noted as no Acid Green 25 was detectable in the receptor fluid for any time interval investigated. For the calculation of the penetration rate it was therefore assumed as worst case that the maximum possible concentration in the receptor fluid was 0.15 µg/ml i.e. the detection limit of the analytical method. Based on this approach, an upper limit for the penetration rate of 6.1 µg/cm^2 (0.12% of the applied dose) and 5.8 µg/cm^2 (0.12% of the applied dose) in experiment I and II, respectively, can be calculated, taking into account the acceptor fluid alone. Taking

additionally into consideration the skin extracts (including the stratum corneum), penetration rates for Acid Green 25 of 7.9 µg/cm² (0.16% of the applied dose) for the pure substance and of 17.0 µg/cm² (0.34% of the applied dose) for the formulated product can be calculated from this study as worst case assumptions.

Conclusion
Under the described test conditions, a low skin penetration rate for Acid Green 25 was obtained as no detectable amounts were noted in the receptor fluid. In a worst case scenario, the penetration rate was therefore calculated based on the determined LOD of 0.15 µg/ml for the applied HPLC method. A penetration rate of 7.9 µg/cm² (about 0.16% of the applied amount) is obtained if the test item is applied as pure substance at pH 3 and the extract of the skin (including the stratum corneum) is considered to be absorbed. A slightly higher penetration rate of 17.0 µg/cm² (0.34% of the applied dose) is obtained if the dye is applied as part of a commercial hair colour gel.

Ref.: 6 (subm I); 18 (subm I, updated)

Comments
A dermal absorption study using a receptor fluid at pH 3, is not giving physiological data and is therefore not considered to be relevant here.

In conclusion, the study is inadequate.

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose (30 days) oral toxicity

No data submitted

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

Taken from SCCP/0879/05, revised

Guideline:	OECD 408
Species/strain:	Wistar rats, HanIbm: WIST(SPF)
Group size:	10 animals per sex and dose, no recovery group
Test Substance:	Acid Green 25 in bi-distilled water containing 1% carboxymethylcellulose
Batch:	33 (lot No. AJ 6720)
Purity:	95%
Dose level:	100, 300, 1000 mg/kg bw/day (0, 95, 285 and 950 mg active dye/kg bw/day)
Route:	Oral, gavage
Exposure period:	13 weeks
GLP:	in compliance

In a 14-day dose range finding study, no treatment-related effects were noted up to the limit dose of 1000 mg/kg bw/day, except discolouring of faeces, limbs, skin and testes (high dose group). Based on the results of this study, 100, 300 and 1000 mg/kg bw were chosen for the main study.

Ref.: 9 (subm I)

In the main study, Acid Green 25 dissolved in bi-distilled water containing 1% CMC was administered daily to groups of 10 males and 10 female Wistar HanIbm rats by gavage in a total volume of 10 ml/kg bw over a period of 13 consecutive weeks. In addition, an equally sized control group received the same dose volume of the vehicle. Homogeneity and the stability of the test solutions were evaluated.

Mortality was checked twice daily, clinical signs were recorded at least once daily. Detailed clinical observations, individual body weights and food consumption were recorded weekly. An ophthalmological examination was performed in all animals before treatment (all animals) and at week 13 (control and high dose group only).

During week 13 relevant parameters of a functional observational battery (modified Irwin screen test) were evaluated as well as grip strength and locomotor activity. Clinical laboratory investigations (haematology, blood/clinical biochemistry and urinalysis) were performed at the end of the treatment period. All animals were subjected to a detailed necropsy and a number of organs (adrenals, brain, heart, kidneys, liver, ovaries, testes, spleen, thyroid and thymus) were weighed and several tissues and organs were fixed and stored for further examinations (histopathology on all gross lesions from all animals and on all organs/tissues of all animals in the control and high-dose groups).

Results
No substance-related mortalities and significant clinical signs were noted in the treated animals. Discolouration of faeces was noted in all treated groups and discolouration of the animals was noted in all mid- and high-dose animals.
The functional observational battery as well as grip strength and locomotor activity measurements in week 13 did not reveal test item related effects.
No effects were noted with regard to body weight, body weight gain and food consumption during the study period. There were no differences in ophthalmoscopic findings between the treated groups and the control group.
The relative kidney weight was increased in males at 300 and 1000 mg/kg bw/day, being more pronounced at 300 mg/kg bw/day. The kidney/brain ratio revealed statistically significant increases at both doses (females) or at 300 mg/kg bw/day only (males). This finding was considered by the study authors to be test article related. Furthermore, the relative brain weights in females were slightly decreased compared to controls for all treated groups (statistically significant only in low- and mid-dose groups), without revealing a dose-response relationship. In high-dose males, the relative liver weight was significantly decreased. These findings were considered by the study authors to be fortuitous.

Statistically significant changes in haematology parameters included decreased red blood cell and platelet counts (high-dose females), decreased haematocrit (mid- and high-dose females), slightly increased mean corpuscular haemoglobin (high-dose females) and mean corpuscular haemoglobin concentration (high-dose males, and mid- and high-dose females), and increased prothrombin time (mid- and high-dose females). These findings were considered to be of no toxicological significance by the study authors. No test article related findings on clinical biochemical parameters were recorded. Urinalysis revealed a significant increase in urine volume in males of the high dose group; due to the increased kidney weights, this finding was considered to be test article related.

The macroscopical findings included bluish discolouration in the gastrointestinal tract and the testes in the low-dose group; in the mid- and high-dose groups the whole body was bluish discoloured. The microscopical findings were within the range of the spontaneous background lesions recorded in rats of this strain and age and therefore, not considered to be treatment-related.

Conclusion
The No Observed Adverse Effect Level (NOAEL) was 100 mg/kg bw/day (95 mg active dye/kg bw/day) based on the results in this study, especially the increased organ weight ratios of the kidneys.

Ref.: 4 (subm I; 14, subm II)

Comment
The SCCS agrees with the NOAEL as concluded by the study authors although it is noted that no abnormalities were noted in the histological investigation of the kidneys or with

regard to markers of kidney physiology except for the increase in urine volume in high-dose males. The SCCS also noted that this NOAEL is also the NOAEL for changes in haematological parameters.

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 gene mutation assay

Guideline:	OECD 471 (1997)	
Species/strain:	*S. typhimurium*, TA98, TA100, TA1535, TA1537; *E. coli*, WP2uvrA	
Replicates:	Two independent tests with and without metabolic activation	
Test substance:	D&C Green n° 5 (C.I. 61570)	
Batch:	33	
Purity:	95%	
Vehicle:	deionised water	
Concentrations:	33, 100, 333, 1000, 2500 and 5000 µg/plate without and with metabolic activation	
Treatment:	experiment I:	direct plate incorporation with at least 48 h incubation, without and with S9-mix
	experiment II:	pre-incubation method with 60 minutes pre-incubation and at least 48 h incubation, without and with S9-mix
GLP:	in compliance	
Study period:	25 August 1999 – 8 September 1999	

D&C Green n° 5 was investigated for the induction of gene mutation in *S. typhimurium* and *E. coli* (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 in a pre-experiment 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 spontaneous 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 the strains used, the pre-experiment is reported as part of experiment I. Since no relevant toxic effects were observed, 5000 µg/plate was chosen as the maximal concentration. Experiment I was performed with the direct 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, moderate toxic effects (reduced number of revertants) were seen in strain TA1535 at 5000 µg/plate without S9-mix and from 1000 up to 5000 µg/plate with S9-mix and in strain TA1537 at 2500 and 5000 µg/plate without S9-mix. A biologically relevant increase in the number of revertants in any of the tester strains was not observed following treatment with D&C Green n° 5 at any concentration level, neither in the absence nor presence of S9-mix.

Conclusion
Under the experimental conditions used D&C Green n° 5 was not mutagenic in this gene mutation tests in bacteria.

Ref.: 7 (subm I; 19, subm I updated 2005)

***In vitro* mammalian cell gene mutation test**

Guideline:	OECD 476 (1997)	
Cells:	L5178Y $tk^{+/-}$ mouse lymphoma cells	
Replicates:	duplicates in 2 independent tests	
Test substance:	D&C Green n° 5 (C.I. 61570)	
Batch:	33	
Purity:	95%	
Vehicle:	deionised water	
Concentrations:	Experiment I:	78.1, 156.3, 312.5, 625 and 1250 µg/ml without S9-mix 312.5, 625, 1250, 2000 and 2500 µg/ml with S9-mix
	Experiment II:	312.5, 625, 1250 and 2500 µg/ml without S9-mix
Treatment	Experiment I:	4 h treatment without and with S9-mix; expression period 72 h and selection period of 10-15 days
	Experiment II:	24 h treatment without S9-mix; expression period 48 h and selection period of 10-15 days
GLP:	in compliance	
Study period:	5 October 1999 – 22 November 1999	

D&C Green n° 5 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 with exposure up to the prescribed maximum concentration of 5000 µg/ml measuring relative suspension growth. In the main test, cells were treated for 4 h or 24 h (without S9-mix experiment II) followed by an expression period of 72 or 48 h (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 percentage relative total growth of the treated cultures relative to the total growth of the solvent control cultures. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-test and experiment II precipitation occurred at 2500 µg/ml and above at both treatment intervals without and with S9-mix; in experiment I at 2000 µg/ml and above. The appropriate level of toxicity (10-20% survival after the highest concentration) was not reached in experiment I without S9-mix.
In both experiment a reproducible, biologically relevant increase in the number of mutant colonies was not observed independent of the presence or absence of S9-mix. An isolated increase of the mutant frequency was observed in the second experiment at 1250 µg/ml in one out of two parallel cultures. As no increased mutant frequencies was observed at a higher concentration (2500 µg/ml) in the same test and no mutagenic effect was observed in the first experiment, this isolated positive effect was considered not biologically relevant.

Conclusion
Under the experimental conditions used, D&C Green n° 5 was not mutagenic in this mouse lymphoma assay using the *tk* locus as reporter gene.

Ref.: 8 (subm I; 20 (subm I updated 2005)

Induction of micronuclei in cultured human peripheral blood lymphocytes

Guideline:	In accordance with recommendations of IWTG workshop and accepted scientific/regulatory principles described in current guidelines for clastogenicity testing *in vitro*.

Species/strain:	human lymphocytes of 2 healthy, non-smoking, female volunteers	
Replicates:	duplicate cultures in 2 independent experiments	
Test item:	D&C Green n° 5 (WR 17086)	
Batch:	33 lot AJ6720	
Purity:	86.1%	
Vehicle:	purified water	
Concentrations:	experiment 1:	140.7, 274.9, 838.9 and 1311 µg/ml without S9-mix 671.1, 1049, 1638 and 3200 µg/ml with S9-mix
	experiment 2	175.9, 671.1 and 2560 µg/ml without S9-mix 429.5, 1311, 2560 and 4000 µg/ml with S9-mix
Treatment	experiment 1:	24 h PHA, 20 h treatment and 28 h recovery without S9-mix 24 h PHA, 3 h treatment and 45 h recovery with S9-mix
	experiment 2:	48 h PHA, 20 h treatment and 28 h recovery without S9-mix 48 h PHA, 3 h treatment and 45 h recovery with S9-mix
GLP:	in compliance	
Study period:	2 February – 18 March 2004	

D&C Green n° 5 has been investigated in 2 independent experiments in the absence and presence of metabolic activation for the induction of micronuclei in cultured human lymphocytes. Treatment periods were 24 h without S9-mix and 3 h with S9-mix. Harvest times were 72 hours (experiment 1) or 96 hours (experiments 2) after the beginning of culture. Approximately the final 28 h of incubation was in the presence of cytochalasin B (at a final concentration of 6 µg/ml). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. In every separate experiment various dilutions of D&C Green n° 5 were tested. However, only 3-4 concentrations were analyzed. Toxicity was determined by measuring the reduction in replication index (RI). The top concentration for analysis was to be the one with approximately 60% reduction in RI. The lower concentrations were chosen such that a range from maximum to little or none cytotoxicity is covered. Micronucleus preparations were stained with Giemsa and examined microscopically for RI and micronuclei. Negative and positive controls were included.

Results
Measurements on post-treatment media in the absence or presence of S9-mix indicated that D&C Green n° 5 had no marked effect on osmolarity or pH as compared to concurrent vehicle controls.
In both experiments without and with S9 metabolic activation D&C Green n° 5 did not induce a biological relevant increase in the number of cells with micronuclei compared to the concurrent untreated controls. The one exception was observed at 1049 µg/ml in experiment 1 for the 3 h treatment with S9-mix where a small but statistically significant increase was observed. Since there was no concentration response and only one of the 2 replicates marginally exceeded the historical control range, this increase was not considered biologically relevant.

Conclusion
Under the experimental conditions used, D&C Green n° 5 was not genotoxic (clastogenic and/or aneugenic) in human lymphocytes *in vitro*.

Ref.:21

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

No data submitted

3.3.7 Carcinogenicity

Updated submission, 2005

Mice

Albino mice were feed diet containing 0.05, 0.5, and 2.0% Acid Green 25 during a long-term study. All animals in the treatment groups had a green coloration and their feces were green because of their exposure. The mean body weight values of all the treated groups were slightly lower than that of the control groups. Significantly lower survival rates for mid and high-dose males and females compared to control groups. The study was terminated when the survival of a group within that sex reached ten animals. This occurred during week 88 in the mid-dose males and during week 97 in the low-dose females. There were no effects at any dose level with regard to the incidence or type of tumours observed.

Ref.: 23

Rats

Albino rats were exposed *in utero* at levels of 0.10, 0.25, and 1.0% Acid Green 25. The exposure was continued until the study was terminated at 130 weeks. All animals in the mid- and high-dose groups had a green coloration and their faeces were green because of their exposure. The mean body weight values of all the treated groups were similar to that of the control groups after week 50. Significantly greater survival rates for the low-dose females were observed while the survival rates for the other groups were similar to that of the control groups. No statistical differences of tumour incidence between the exposed animals and the controls were found.

Ref.: 24

Comment
Acid Green 25 has been studied in a long-term study with mice and with rats. No significant increase in tumour frequency was found. The studies are, however, incompletely reported and it is impossible to draw any conclusion from these studies.

3.3.8 Reproductive toxicity

3.3.8.1 Two generation reproduction toxicity

No data submitted.

3.3.8.2 Teratogenicity

Guideline:	OECD 414 (2001)
Species/strain:	rat, HanBrl:WIST (SPF)
Group size:	22 mated females per dose group
Test Substance:	benzenesulfonic acid, 2,2'-[(9,10-dihydro-9,10-dioxo-1,4-anthracenediyl)diimino]bis (5-methyl,) disodium salt
Batch:	33 lot AJ6720 (Goldwell)
Purity:	86.1 weight% (NMR)
Vehicle:	bi-distilled water containing 1% carboxymethylcellulose
Dose level:	0, 100, 300 and 1000 mg/kg bw/day (0, 86, 258, and 861 mg active dye/kg bw/day)
Dose volume:	10 ml/kg bw
Route:	oral, gavage
Administration:	once daily from day 6 (implantation) through to day 20 *post coitum*
GLP:	in compliance
Study period:	13 April – 26 August 2004

The test substance (in bi-destilled water containing 1% carboxymethylcellulose) was given daily at dose volumes of 10 ml/kg bw by oral gavage. Mortality, morbidity, signs of abortion, and clinical signs and/or symptoms was checked at least twice daily. Food consumption was recorded on 3-day intervals and body weights were recorded daily. The females were sacrificed on gestation day 21, subjected to macroscopic examination. The foetuses were removed by Caesarean section, sexed, weighed, examined for gross external abnormalities, killed, and allocated to either visceral or skeletal (about one half of the foetuses for each examination).

Results
No mortalities occurred. Clinical signs included darker faeces (all treated groups, from day 7-21 post coitum), and discolouration of skin, eyes and bedding (generally only in the high-dose group). The mean body weight gain was slightly decreased and the corrected body weight gain (corrected for gravid uterus weight) was marginally lower in high-dose females compared to control females. Food consumption was similar in treated and control groups. One low-dose female was not pregnant. The incidence of pre-implantation loss was slightly higher (statistically significant) in the high-dose group; this difference was considered as being incidental by the study authors because pre-implantation loss mainly occurred prior to onset of treatment. Post-implantation loss and number of foetuses per dam was not affected. At necropsy discolouration of intestinal contents, kidneys, fatty tissues, intrauterine fluids and foetuses was noted in all high-dose dams.
No effects on litter parameters or foetal weight were observed. Incidences of external, visceral and skeletal findings were similar for control and treated groups.

Conclusion
The No Observed Adverse Effect Level (NOAEL) for maternal toxicity is 300 mg/kg bw/day (258 mg active dye/kg bw/day) and the NOAEL for developmental toxicity is 1000 mg/kg bw/day (861 mg active dye/kg bw/day).

Ref.: 17 (subm I, updated)

3.3.9 Toxicokinetics

No data submitted

3.3.10 Photo-induced toxicity

3.3.10.1 Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2 Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11 Human data

No data submitted

3.3.12 Special investigations

No data submitted

3.3.13 Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(Acid Green 25)

(non-oxidative conditions)

Absorption through the skin	**A**	**=**	**0.04 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.023 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.0004 mg/kg bw/d**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**95 mg/kg bw/d**
50% bioavailability *		**=**	**48 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**120000**

* 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
Acid Green 25 is proposed for use in semi-permanent hair dye formulations as a direct dye at a maximum concentration of 0.3% in the finished cosmetic product.
Acid Green 25 is permitted for the use in other cosmetic products according to Annex IV of the Cosmetics Directive
Stability of Acid Green 25 in in typical hair dye formulations was not demonstrated.

Irritation / sensitisation
Acid Green 25 is evaluated not to be an irritant to the skin and eyes. It did not induce sensitisation in one Guinea pig experiment.

Dermal absorption
The total dermal absorption of Acid Green 25 at 72 hours was 0.02 ± 0.02 µg equiv./cm². The mean + SD: 0.04 µg equiv./cm² was used to calculate MoS.

General toxicity
The 13 week oral toxicity study in rats revealed a NOAEL of 100 mg/kg bw/day (95 mg active dye/kg bw/day) based on increased kidney weights and changes in haematological parameters at the two higher dose levels.
In the teratogenicity study, the NOAEL for maternal toxicity was 300 mg/kg bw/day (258 mg active dye/kg bw/day) and the NOAEL for developmental toxicity was 1000 mg/kg bw/day (861 mg active dye/kg bw/day).

Mutagenicity

Overall, the genotoxicity program on Acid Green 25 investigated the three endpoints of genotoxicity: gene mutations, structural chromosome aberrations and aneuploidy. Neither in bacteria nor in mammalian cells Acid Green 25 induced gene mutations. Exposure to Acid Green 25 did not result in an increase in human peripheral blood lymphocytes with micronuclei. This was confirmed by the absence of a shift in the ratio of small *versus* large colonies compared to the concurrent controls in the gene mutation test with mammalian cells. Consequently, Acid Green 25 can be considered to have no *in vivo* genotoxic potential and further testing is not essential.

Carcinogenicity
Acid Green 25 has been studied in a long-term study with mice and with rats. No significant increase in tumour frequency was found. The studies are, however, incompletely reported and it is impossible to draw any conclusion from these studies.

4. CONCLUSION

The SCCS is of the opinion that the use of Acid Green 25 as a non-oxidative hair dye with a maximum on head concentration of 0.3% does not pose a risk to the health of the consumer.

Acid Green 25 is also used as a colorant. However, this use has not been assessed in this opinion.

5. MINORITY OPINION

Not applicable

6. REFERENCES

Submission 2013

1. Blackstock C et al; (2011) The *In Vitro* Percutaneous Absorption of [14C]-Acid Green 25 (C178) in a Typical Hair Dye Formulation Through Human Skin. Test Facility Study No. 788659, Report No. 32833. Charles River, UK-Tranent, Edinburgh

Submission I, updated 2005

1. Rues, W. Determination of content of impurities of D&C Green No. 5; batch 33, lot AJ6720 and lot AK4027 (Acid Green 25, C178); WELLA AG; 2003
2. Damkröger, G. Determination of stability in vehicles and content of impurities of D&C Green 5; batch 33, lot AJ6720 (Acid Green 25, C178); WELLA AG; 2005
3. Lange, J. Partition coefficient (n-octanol f water) using High Performance Liquid Chromatography (HPLC); NOACK; 2004
4. Lange, J. Sieve analysis; NOACK; 2004
5. Lange, J. Water solubility (flask method); NOACK; 2004
6. Lange, J. Prediction of the pKa-value; NOACK; 2004
7. Smeykal, H. Melting point I boiling point; SIEMENS AXIVA GMBH A CO. KG; 2004
8. Lange, J. Determination of the density; NOACK; 2004
9. Franke, J. Vapour pressure; SIEMENS AXIVA GMBH & CO. KG, 2004
10. Lange, J. Surface tension; NOACK; 2004
11. Lange, J. Flammability of solids; NOACK; 2004
12. Smeykal, H. Auto-flammability (solids - determination of relative self-ignition temperature); SIEMENS AXIVA GMBH & CO. KG; 2004

13. Farbstoffkommission der Deutschen Forschungsgemeinschaft; CI 61570, Acid Green 25, Green 5; In: KOSMET FÄRBEMITTEL; 3, 553-556; 1991
14. Hamann, H,-J. D&C Green 5 (C.I. 61570): 13-week oral toxicity (gavage) study in the rat; MCC; 2000
15. Burnett, C. M. Opdyke, D. L.; Chronic eye irritation and staining properties of some organic colors and lakes; CTFA COSMET. J.; 3,17-22; 1971
16. Arcelin, G. Contact hypersensitivity in albino guinea pigs Maximization-test; ICC; 1999
17. Becker, H. Benzenesulfonic acid, 2,2'-[(9,10-dihydro-9,10-dioxo-1,4-anthracenediyl)diimino]bis[5-methyl-, disodium salt: Prenatal developmental toxicity study in the rat; RCC; 2005
18. Wollny, H.-E. Skin permeability in vitro absorption through porcine ear skin with D&C Green 5 (C.I. 61570); RCC; 2001
19. Wollny, H.-E. Salmonella Typhimurium and Escherichia Coli reverse mutation assay with D&C Green 5 (C.I. 61570); RCC; 2000
20. Wollny, H.-E. Cell mutation assay at the Thymidine Kinase locus (TK +/-) in mouse lymphoma L5178Y cells with D&C Green 5 (C.I. 61570); RCC; 2000
21. Whitwel, J.; WR 17086: Induction of micronuclei in outbred human peripheral Mood lymphocytes; COVANCE; 2004
22. Sigman C.C., Papa P.A., Doeltz M.K., Perry L.R., Twhigg A.M., Helmes C.T., A study of anthraquinone dyes for the selection of candidates for carcinogen bioassay; J. ENVIRON. SCI HEALTH; A20, 427-484; 1915
23. Final Report: Long-term feeding study in mice (summary); HAZLETON; 1981
24. Final Report: Long-term feeding study in rats exposed in utero (summary); HAZLETON; 1981
25. König P. Data base search for references for Acid Green 25, CI 61570: CAS 4403-90-1; Wella AG; 2005

Submission I, 2003

1. Otterstätter, G. (1995): Die Färbung von Lebensmitteln, Arzneimitteln, Kosmetika. Behr's Verlag
2. DFG (1991): Kosmetische Färbemittel, VCH Weinheim, page 553-556
3. Burnett, C.M. and Opdyke, D.L. (1971): Chronic eye irritation and staining properties of some organic colors and lakes. CTFA Cosmet. J. 3, 17-22
4. Hamann, H.-J. et al. (2000): D&C Green 5 (CI 61750): 13-week oral toxicity (gavage) study in rats. Project n° 740687, RCC Ltd, Switzerland
5. Arcelin, G. (1999): D&C Green 5 (CI 61570) contact hypersentivity in albino guinea pigs maximization-test. Project n° 740665, RCC Ltd, Switzerland
6. Wollny, E. and Röder A. (2002): Skin permeability - in vitro absorption through porcine ear skin with D&C Green 5 (CI 61570). Study N° 641303, RCC-Cytotest Cell Research GmbH, Germany
7. Wollny, H.-E. (2000): Salmonella typhimurium and Escherichia coli reverse mutation assay with D&C Green 5 (CI 61570). Study n° 641301, RCC-Cytotest Cell Research GmbH, Germany
8. Wollny H-E. (2002): Cell mutation assay at the thymidine kinase locus (TK+/-) in mouse lymphoma L5178 Y cells with D&C Green 5 (C.I. 61570). Study n° 641302, RCCCytotest Cell Research GmbH, Germany
9. Rosner, E. (1999): 14 day oral toxicity (gavage) study in the rat. Project n° 740676, RCC Ltd, Switzerland
10. LAN-Analysenbericht; Studien-nr A2002/342; Nov. 19, 2002. Wella AG; D-Darmstadt
11. LAN-Analysenbericht draft; Studien-nr A2003/311-001; Sep. 02, 2003. Wella AG; D-Darmstadt
12. Certificate of analysis by Hilton Davis; April 21, 1998