OPINION OF THE SCIENTIFIC COMMITTEE ON COSMETIC PRODUCTS AND NON-FOOD PRODUCTS INTENDED FOR CONSUMERS

CONCERNING

HC GREEN N° 1

COLIPA n° C129

adopted by the SCCNFP during the 23rd plenary meeting
of 18 March 2003

1 Terms of reference

1.1 Context of the question

The adaptation to technical progress of the Annexes to Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products.

1.2 Request to the SCCNFP

The SCCNFP is requested to answer the following questions :

* Is HC Green n° 1 safe for use in cosmetic products?

* Does the SCCNFP propose any restrictions or conditions for its use in cosmetic products?

1.3 Statement on the toxicological evaluation

The SCCNFP is the scientific advisory body to the European Commission in matters of consumer protection with respect to cosmetics and non-food products intended for consumers.

The Commission's general policy regarding research on animals supports the development of alternative methods to replace or to reduce animal testing when possible. In this context, the SCCNFP has a specific working group on alternatives to animal testing which, in co-operation with other Commission services such as ECVAM (European Centre for Validation of Alternative Methods), evaluates these methods.
The extent to which these validated methods are applicable to cosmetic products and its ingredients is a matter of the SCCNFP.

SCCNFP opinions include evaluations of experiments using laboratory animals; such tests are conducted in accordance with all legal provisions and preferably under chemical law regulations. Only in cases where no alternative method is available will such tests be evaluated and the resulting data accepted, in order to meet the fundamental requirements of the protection of consumer health.

2. Toxicological Evaluation and Characterisation

2.1. General

2.1.1. Primary name

HC Green n° 1 (INCI-name)

2.1.2. Chemical names

Chemical name	:	2-(β-Hydroxyethylamino)-5-(4'-bis(β-hydroxyethyl)amino)-anilino-1,4-benzoquinone
CAS name	:	2,5-cyclohexadiene-1,4-dione,2-[[4-[bis(-2-hydroxyethyl)amino]phenyl] amino]-5-[(2-hydroxyethyl)amino]-
Synonyms	:	2-[[4-[bis(2-hydroxyethyl)amino]phenyl]amino]-5-[(2-hydroxyethyl) amino-2,5-cyclohexadiene-1,4-dione

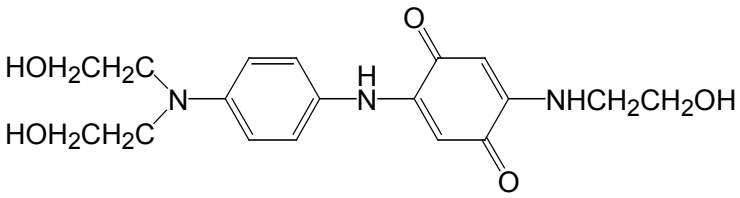
2.1.3. Trade names and abbreviations

Trade name	:	IMEXINE® IK
COLIPA n°	:	C129

2.1.4. CAS no. /EINECS No.

CAS n°	:	52136-25-1
EINECS n°	:	257-687-7

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula	:	$C_{18}H_{23}N_3O_5$
Mol weight	:	361.4

2.1.7. Purity, composition and substance codes

All analytical data relate to batch op.7.

Purity (potentiometric titration against perchloric acid) : 97.9%

Water content	:	1.52%
Ash content	:	< 0.1%
Heavy metals	:	< 10 ppm
Chloride ions	:	0.06%
Hydrogen peroxide (as peroxide value)	:	1
acetone	:	< 50 ppm
toluene	:	< 50 ppm

HPLC analysis of C129 revealed five impurities (reagents, and intermediates):

A) 2-[(2-hydroxy-ethyl)-(4-nitroso-phenyl)-amino]-ethanol : < 100 ppm
B) 3,4-dihydroxy-2H-benzo[1,4]oxazin-6-ol : < 100 ppm
C) 2-amino-5-{4-[bis-(2-hydroxy-ethyl)-amino]-phenylamino}-[1,4] benzoquinone
D) Present in insufficient concentration for establishing the chemical structure.
E) (4-{4-[bis-(2-hydroxy-ethyl)-amino]-phenylamino}3,6-dioxo-cyclohexa-1,4-dienylamino)-acetaldehyde

It is reported that C and E are suggested chemical structures elucidated by HPLC-MS analysis, and that these compounds could not be quantified in the absence of the reference compounds.

Batch-to-batch declaration : The industrial batches used for the safety studies were obtained using the same process. They are representative of manufacturing and commercial batches put onto the market since their analytical results complied with the data for the specification in force at the date of their manufacture.

2.1.8. Physical properties

Subst. Code	:	COLIPA C129
Appearance	:	Black powder with dark green coloured highlights
Melting point	:	198°C
Boiling point	:	-
Density (apparent):		0.2 g/cm^3
Rel. vap. dens.	:	-
Vapour Press.	:	-
Log P_{ow}	:	-0.9 (calculated)
pH	:	6.4, at 0.1% in deionised water

2.1.9. Solubility

<0.1% in water
Soluble in 95% ethanol at 0.1%

General comments on analytical and physico-chemical characterisation

* The purity of C129 has been determined by alkalinity (content of amines). Thus, the impurities A, C and E are also included in the reported 97.9% purity of the C129. Thus, the purity information is inadequate.
* Chromatographic purity of C129 has not been reported. However, it is assessed from the submitted HPLC chromatogram that C129 may contain 1-2% C and 10-20% E.

* Information on solubility the test material is insufficient. The substance is poorly soluble in water, but the solubility of the test material in physiological saline (the receptor fluid used in percutaneous absorption study) is not reported.
* Stability of the test material in a hair dye formulation is not known.

2.2. Function and uses

COLIPA C129 will be incorporated in semi-permanent hair dye formulations at a maximum concentration of 0.1%. It is common practice for 35ml of undiluted formulation to be applied for a period of 30 minutes before washing. It assumed that application may be repeated weekly.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

The study reported is disregarded, because the identification of the test substance did not match with any of the listed codes or synonyms of COLIPA C129.

Ref. : 1

2.3.2. Acute dermal toxicity

No data

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

No data

2.3.5. Repeated dose dermal toxicity

No data

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Sub-chronic oral toxicity

Guideline : OECD 408 (1981)
Species/strain : Crl: CD (SD) BR strain rat

Group size	:	10 male + 10 female
Test substance	:	IMEXINE IK in aqueous suspension
Batch no	:	op.7 (purity 100.7%)
Dose levels	:	0, 100, 300 and 1000 mg/kg bw/day, 7 days/week by gavage
Exposure period	:	13 weeks
GLP	:	in compliance

Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 0, 100, 300 and 1000 mg/kg bw/day, 7 days/week for 13 weeks. The dosing solutions were analysed before the start of the study for stability, and during weeks 1, 4, 8 and 12 for verification of concentration. During the study, the animals were observed daily for clinical signs and mortality, and weekly for bodyweight and food consumption. In week 13 urine was collected overnight for urinalysis, and blood was sampled from the orbital sinus for haematology and blood biochemistry. At the end of the treatment periods, a full autopsy was conducted with recording of weights, and macroscopic and microscopic examination of major organs. Ophthalmological examination was conducted before the start of the study and at the end of the treatment period on controls and high dose animals.

Results
There were two mortalities (one female dosed at 300 mg/kg/day and one male dosed at 1000 mg/kg/day, both without prior clinical signs and they were considered to be probably due to dosing errors. There were no clinical signs of treatment except for soft consistency of the faeces in 1/10 males and 1/10 females treated at 300 mg/kg bw/day and 4/10 males and 7/10 females at 1000 mg/kg bw/day in week 13. Coloration of the urine and faeces was noted at all dose levels and also of the fur and tail in some animals, and this was attributed to the staining properties of the substance. The mean food consumption of the female rats treated at 1000 mg/kg bw/day was slightly, but not significantly, lower than in controls throughout the dosing period. This was associated with a decreased body weight gain (83% of control over the 13 weeks), although body weights did not differ significantly. Food consumption and weight for other dose groups were comparable to control. No abnormalities were noted during ophthalmological examinations.
There were no treatment-related changes in haematological parameters. A dose-related decrease in blood potassium was significant from control in females at 300 and 1000 mg/kg bw/day (-12% and –20% respectively and in males at 1000 mg/kg bw/day (-13%). The authors concluded that this effect was treatment-related at the high dose, but noted that the individual values for mid-dose females were within the historical control range and therefore not of importance.
Other slight differences in biochemical parameters were minor, not dose-related and not considered to be of toxicological significance. The mean urinary volume of males treated at 1000 mg/kg bw/day was significantly lower than for controls (7mL vs 17mL). A decrease was also apparent for the high dose females (5mL vs 9mL), but this was not statistically significant. Other urinary parameters showed no difference between controls and test groups.
Relative kidney weights were significantly increased in male rats at 300 and 1000 mg/kg bw/day (110% and 109% respectively). Relative liver weights were significantly increased in males at 1000 mg/kg bw/day (110% of control) and in females at 300 and 1000 mg/kg bw/day (108% and 107% respectively). Spleen weight were also increased in females (relative weights: 115% and 128% at 300 and 1000 mg/kg bw/day; absolute weight: 120% at 1000 mg/kg bw/day). The study authors considered that these weight changes were not of toxicological significance because they were not associated with histopathological changes.
At autopsy, staining was reported in various regions of the gastro-intestinal tract. Thickening of the mucosae of the forestomach was apparent in 3/10 males and 2/10 females at 1000 mg/kg bw/day. In most cases these macroscopic observations were accompanied by submucosal

oedema and/or inflammatory cell aggregation. The study authors considered these observations to be consistent with spontaneous occurrence and not of toxicological importance. Other findings were similar for control and treated animals.
Based upon the decreased food consumption and weight gain in females, hypokalemia in both sexes, and oliguria in males, the authors concluded that the NOAEL was 300 mg/kg bw/day.
The dose-related hypokalemia was statistically significant in female rats treated at 300 and 1000 mg/kg bw/day. A NOEL of 100 mg/kg bw/day should therefore be assumed.

Ref. : 5

2.3.8. Sub-chronic dermal toxicity

No data

2.3.9. Sub-chronic inhalation toxicity

No data

2.3.10. Chronic toxicity

No data

2.4. Irritation & corrosivity

2.4.1. Irritation (skin)

Guideline	:	Journal Officiel de la République Française of 21/2/82
Species/strain	:	New Zealand albino rabbit
Group size	:	6 males
Test substance	:	IMEXINE IK at 0.5% in water/propyleneglycol, 50/50
Batch no	:	op.3 (purity not stated in study report)
Dose	:	0.5 ml
GLP	:	not in compliance

The substance was suspended in water/propyleneglycol, 50/50, at a concentration of 0.5%, and 0.5 ml was applied to 4cm^2 of intact skin on the left flank and scarified skin on the right flank. Occlusive patches were applied and left in place for 24 hours. Cutaneous reactions were evaluated 30 min and 48 hours after removal of the patches.

Results
Green staining due to the dye interfered with evaluation of erythema. No cutaneous reactions were reported and the substance was classified as non-irritating to intact or scarified rabbit skin at a concentration of 0.5% in water/propyleneglycol.
The test was conducted under extreme conditions in which an irritant substance would be expected to produce cutaneous responses that could be observed in the presence of skin staining. It therefore seems unlikely that this substance would be irritant under conditions of use.

Ref. : 3

2.4.2. Irritation (mucous membranes)

Guideline	:	OECD 405 (1987)
Species/strain	:	New Zealand albino rabbit
Group size	:	3 male
Test substance	:	IMEXINE IK at 0.5% in 0.5% carboxymethylcellulose
Batch no	:	op.5 (purity 94.9%)
Dose	:	0.1 ml
GLP	:	not in compliance

The test substance was suspended in 0.5% carboxymethylcellulose at a concentration of 0.5% and 0.1 ml was applied once to the left eye of 3 male rabbits, without rinsing. The right eye served as control and was untreated. Ocular reactions were recorded at 1, 24, 48 and 72 hours after instillation.

Results
No reactions were reported in any of the rabbits and the substance was classified as non-irritating to the rabbit eye at a concentration of 0.5%.

Ref. : 2

2.5. Sensitisation

Epicutaneous maximisation test

Guideline	:	AFNOR : FD n° TO3-300	
Species/strain	:	Albino Hartley guinea pig	
Group size	:	10 male + 10 female (no control group)	
Test substance	:	IMEXINE IK	
Batch no	:	op.3 (purity not stated)	
Concentrations	:	intradermal induction :	0.1 ml 50% Freund's complete adjuvant (FCA), on day 1
		topical induction :	0.2 g neat test substance for 48 hours occluded, on 7 occasions
		challenge :	0.2 g neat test substance for 24 hours, occluded
GLP	:	not in compliance	

Induction consisted of an intradermal injection of FCA on day 1 and 7 topical applications of 0.2g of the neat test substance under occlusive patch to the shoulder for 48 hours 3 times per week for two weeks and once at the start of the third week. An interval of 12 days was allowed after induction and then the animals were challenged by a single 0.2g topical application of the neat test substance under occlusive patch on the flank for 48 hours. The skin was examined 1, 6, 24 and 48 hours after removal of the challenge patches for erythema and oedema.

Results
Green staining of the skin due to the hairdye prevented macroscopic assessment of erythema. Very slight oedema was observed in one male rabbit at 1 and 6 hours after removal of the challenge patch, and in a second animal at 1 hour only.
The test did not conform with OECD guideline 406.

Ref. : 4

2.6. Teratogenicity

Guideline	:	OECD 414 (1981)
Species/strain	:	Sprague-Dawley rat, Crl: CD (SD) BR strain
Group size	:	24 or 25 females (mated)
Test substance	:	IMEXINE IK in aqueous suspension
Batch no	:	op.7 (purity 100.7%)
Dose levels	:	0, 100, 300 and 1000 mg/kg bw/day
Treatment period	:	Days 6 to 15 of pregnancy, inclusive
GLP	:	in compliance

Groups of 24 or 25 female rats were dosed with the test substance by gavage at 0, 100, 300 and 1000 mg/kg bw/day on days 6 to 15 after mating. The dams were observed daily for clinical signs and mortality, bodyweight and food consumption were recorded on days 0, 2, 6, 9, 12, 15 and 20. The dams were sacrificed on day 20 of pregnancy, and examined for number of corpora lutea, number and distribution of live and dead foetuses, of early or late resorptions and of implantation sites, and for macroscopic observations. The foetuses were examined for bodyweight, sex and macroscopic external observations, and for skeletal and visceral abnormalities (half for each endpoint).

Results
There were two mortalities: one at 300 mg/kg/day on day 19, and one at 1000 mg/kg/day on day 9. The causes of death were not established but they were not considered to be treatment-related because there were no signs of toxicity prior to death. There were no abortions. Green coloration of the urine and faeces was noted in all mid and high dose animals from day 7/8 to 16. The only other clinical sign was of piloerection and dyspnea in one dam treated at 1000 mg/kg/day, from day 9 to 12. The authors considered that this observation was not treatment-related, but failed to explain their reasoning for this judgement. Food consumption and bodyweight gains were comparable for all dose groups.
No maternal abnormalities were noted at the scheduled autopsy. The mean numbers of corpora lutea, sex distribution and the mean foetal bodyweights were comparable for control and treated groups. The numbers of resorptions and dead foetuses (from the same litters) were slightly higher in the low dose group (100 mg/kg/day) but comparable to control in the mid and high dose groups. Because of this absence of dose-response, this observation was considered to be a chance occurrence.
There were no dose-related increases in foetal abnormalities or malformations. Some individual significant differences were observed for abnormalities of a very low incidence, which were considered to be spontaneous. In the high dose group, the only abnormal observation was of 2/166 foetuses with fused ribs. In absence of other abnormalities, and because the two foetuses were from the same litter, this was not considered to have toxicological significance.
The highest dose, 1000 mg/kg bw/day, was a NOAEL for the pregnant female rat and for the foetus.

Ref. : 11

2.7. Toxicokinetics (incl. Percutaneous Absorption)

2.7.1. Percutaneous Absorption *in vitro*

Guideline	:	/
Tissue	:	Human mammary epidermis, heat-separated

Method	:	Franz diffusion cell (static)
Test substance	:	IMEXINE IK, 0.1% in formulation
Batch no	:	op.3 (purity not stated in study report)
Dose levels	:	c. 40mg formulation in the presence/absence of 10 mg hair
Replicate cells	:	9 cells without hair and 8 cells with hair
GLP	:	not in compliance

The skin penetration of COLIPA C129 was evaluated in a static Franz diffusion cell system. Human epidermis was prepared by heat-separation from previously frozen mammary skin. The test substance was prepared at a concentration of 0.1% in a formulation. Approximately 40 mg of the mixture was applied to 2cm^2 of epidermal membrane with and without addition of 10 mg finely chopped bleached hair for 30 minutes and then excess washed off with 2% sodium lauryl sulphate solution and dried. Four hours later the levels of substance were measured in the receptor fluid (physiological saline) using HPLC. Integrity of the epidermal membrane was checked by microscopy before the study, and by means of addition of Chinese ink at the end of the study. Any cells showing penetration of the ink were eliminated from the analysis.

Results
The amounts of test substance penetrating through the epidermis to the receptor fluid were close to or below the limits of detection of the assay for all cells. Penetration was calculated to be 0.045% of applied dose in the presence of hair and 0.035% in the absence of hair.
This study did not include determination of recovery of the test substance. Physiological saline was used as the receptor fluid which is inappropriate for a substance of poor water solubility. A longer period should be allowed for permeation of substance from the epidermis into the receptor fluid.
The study is considered inadequate (see SCCNFP Notes of Guidance).

Ref. : 12

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline	:	OECD 471 (1983)
Species/strain	:	*Salmonella typhimurium*, TA98, TA100, TA1535, TA1537 E*scherichia coli* WP2uvrA
Replicates	:	2 independent tests
Test substance	:	IMEXINE IK in DMSO
Batch no	:	op.5 (purity of 98.3%)
Concentrations	:	1.6 - 1000 μg/plate with and without metabolic activation
GLP	:	in compliance

COLIPA C129 has been investigated for gene mutation in *Salmonella typhimurium* and *Escherichia coli* with the plate incorporation protocol. Liver S9 fraction from rats pretreated with β-naphthoflavone and phenobarbitone was used as the exogenous metabolic activation system. Negative and positive controls were in accordance with the OECD guideline.

Results

1st experiment

Precipitate occurred at 500 µg/plate
Toxicity as evidenced by a reduction in background lawn was noted at 1000 µg/plate
- S9 : positive in TA 1535 tester strains at 40 µg/plate
+ S9 : negative in all tester strains

2nd experiment

- S9 : positive in TA 1535 tester strains at 40 µg/plate, statistical trend for dose-response.
+ S9 : negative in all tester strains

Conclusions
Based on the reversion rate, it is concluded that the test agent C 129 shows reproducible evidence of mutagenic activity in the absence of activation system in *Salmonella typhimurium* tester strain (TA1535).

Ref. : 6

***In Vitro* Mammalian Cell Gene Mutation Test**

Guideline	:	OECD 476 (1984)
Species/strain	:	Mouse lymphoma L5178Y $TK^{+/-}$ cells
Replicates	:	2 independent tests
Test substance	:	IMEXINE IK in DMSO solution
Batch no	:	op.5 (purity 98.3%)
Concentr. scored	:	3 – 400 µg/ml with and without metabolic activation
Exposure time	:	3 hours
GLP	:	in compliance

Liver S9 fraction from rats pre-treated with Aroclor 1254 was used as the exogenous metabolic activation system.

Results

1st experiment

Heavy precipitate occurred at 1000 µg/ml and a moderate precipitate was noted at 300 µg/ml. However, the Cloning Efficiency (indicator of cytotoxicity) was acceptable at those concentrations.
Viability at day 2 was similar to that of the controls.
At 300 µg/ml in the absence and in the presence of activation system, the compound shows statistically significant positive effects.

2nd experiment

Precipitate occurred at doses of 200 to 400 µg
The Cloning Efficiency (indicator of cytotoxicity) was acceptable at those concentrations.

A statistically significant increase in mutant frequencies has been observed at 300 µg/ml in the absence of activation system, for lower doses; a trend for positivity was noted.
A statistically significant increase in mutant frequencies has been observed at 400 µg/ml in the presence of activation system, for lower doses; a trend for positivity was noted.

Conclusions
From the results generated in 2 independent assays, it may be concluded that C 129 give positive results at top doses but with a trend for positivity in dose lower than the positive ones. Moreover, acceptability of studies are questionable because the experiments were not performed at the same dates.

Preliminary	November 1994
1st test – S9 mix	4 January 1995
1st test + S9 mix	10 January 1995
2nd test – S9 mix	15 February 1995
2nd test M+ S9 mix	12 April 1995

This study is not adequate

Ref. : 7

***In Vitro* Mammalian Chromosome Aberration Test**

Guideline	:	OECD 473 (1983 and draft of 1991)
Species/strain	:	Chinese Hamster Ovary (CHO) cells
Replicates	:	Duplicate cultures, two independent tests
Test substance	:	IMEXINE IK in DMSO solution
Batch no	:	op.5 (purity of 98.3%)
Concentrations	:	25 - 200 µg/ml without metabolic activation 200 to 2500 µg/ml with metabolic activation
Harvest times	:	24 and 48 hours
Exposure	:	3 h + S9 mix, 24 h and 48 H – S9 mix.
GLP	:	in compliance

Liver S9 fraction from rats pretreated with β-naphthoflavone and phenobarbitone was used as the exogenous metabolic activation system.
200 cells with 19-23 chromosomes were scored for chromosome aberrations. (2n=22).
No positive control for aneuploidy has been included. Therefore, in this assay, aneugenicity means near tetraploid cells.

Results

1st experiment

With or Without S9 mix, no statistically significant increase in the number of cells with structural chromosomal aberrations was observed but a positive trend was noted.

2nd experiment

Increased number of cells with structural chromosomal aberrations was observed at the following concentration 1000 and 2500µg/ml with S9 mix. Significant increases were also observed at 24 h and 48h harvest with activation. Increase in poliploid cells was also observed at 24 h in the presence of S9 mix.

Conclusions
The study provided gives positive results, at different dose, harvest times. Moreover, aneugenicity has been observed. The test substance is considered as clastogen and aneugen.

Ref. : 8

2.8.2. Mutagenicity/Genotoxicity *in vivo*

In Vivo Mammalian Erythrocyte Micronucleus Test

Guideline	:	OECD 474 (1981)
Species/strain	:	Mouse, CD1
Group size	:	5 male + 5 female/dose/harvest time
Test substance	:	IMEXINE IK in 0.5% aqueous carboxymethylcellulose
Batch no	:	op.5 (purity of 98.3)
Dose levels	:	0, 375, 750 and 1500 mg/kg bw, i.p.
Sacrifice times	:	24, 48 and 72 hours
Adminstration	:	Single intraperitoneal injection
GLP	:	in compliance

After determination of the Maximum tolerated dose (2000 mg/kg), COLIPA C 129 has been investigated for induction of micronuclei in the bone marrow cells of CD-1 mice.. The substance was administered once by single intraperitoneal injection at 375, 750 & 1500 mg/kg bw and the bone marrow harvested after 24 h, 48 h and 72 h post dosing. Negative and positive controls were in accordance with the OECD guideline.

Results

Mean values of micronucleated PCE

A statistically significant increase in the incidence of micronucleated polychromatic cells over the concurrent vehicle control values were observed at 48 h in the 750 mg/kg dosage group. The authors consider this results without biological significance due to the fact that the frequencies observed are within the historical control values and that no dose-response was noted.

Remark
A very large number of NCE were necessary to achieve to score 1000 PCE, this indicate a cytotoxicity as evidenced by a decrease in the PCE numbers. In addition there is a large inter-individual variation in those haematological parameters.
At 72 hours, a large cytotoxicity was observed and no incidence of increased micronucleated erythrocytes was observed. However, due to the cytotoxic properties of the test agent, it is also possible that, due to mitotic delay (as observed in CA in CHO cells), the heavily damaged cells may not be present after 72 h so that only “normal” micronucleated PCE frequencies could be found.

NCE/PCE ratio
Groups of mice treated with COLIPA C 129 did exhibit a time and dose-related decrease in the of the NCE/PCE ratio ; this constitutes an indication that the test substance has reached the bone marrow.

Conclusions
Under the conditions of the test, it can be concluded that there was no evidence of induced chromosomal or other damage leading to the micronucleus formation in polychromatic erythrocytes treated mice. The results are, however, equivocal (see Remark).

Ref. : 9

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

Guideline	:	draft OECD guideline 486 (1991)
Species/strain	:	Wistar rat, HanIbm: WST (SPF) strain
Group size	:	4 male/dose
Test substance	:	IMEXINE IK in 1% aqueous carboxymethylcellulose
Batch no	:	op.7 (purity 97.9%)
Dose levels	:	0, 200 and 2000 mg/kg bw, by gavage
Sacrifice times	:	16 hours: all dose groups; 2h: high dose group
GLP	:	in compliance

COLIPA C 129 has been investigated for induction of unscheduled DNA synthesis in Wistar rats hepatocytes at 2 doses 200 and 2000 mg/kg.
The pre-experiment for the Maximum Tolerated dose is acceptable. Urine were blue-green coloured 24 h after administration of the test agent.
Positive controls are in accordance with OECD guideline and UDS analyzed by autoradiography.

Results
No difference in viability of treated rats hepatocytes has been observed as compared to controls.
No evidence of UDS induced by the test agent was observed.

Conclusions
This study is adequate and the results are negative.

Ref. : 10

2.9. Carcinogenicity

No data

2.10. Special investigations

No data

2.11. Safety evaluation

NOT APPLICABLE

CALCULATION OF THE MARGIN OF SAFETY

HC Green n° 1
(Semi-permanent)

Based on a usage volume of 35 ml, containing at maximum … %

Maximum absorption through the skin	A ($\mu g/cm^2$)	=	$\mu g/cm^2$
Typical body weight of human		=	60 kg
Skin Area surface	SAS (cm^2)	=	cm^2
Dermal absorption per treatment	SAS x A x 0.001	=	mg
Systemic exposure dose (SED)	SAS x A x 0.001/60	=	mg/kg
No observed adverse effect level (mg/kg) (species, study)	NOAEL	=	mg/kg

Margin of Safety	NOAEL / SED =

2.12. Conclusions

Three batches of C129, i.e op.3, op.5 and op.7 have been used for various studies. The purity reported for op.7 is 100.7 % and 97.9%; op.5 has been reported to be 94.9% and 98.3% pure, while no purity for op.3 has been reported. The purity description reflects quality control problems in the analytical laboratory.

The purity of C129 has been determined by alkalinity. HPLC analysis of C129 revealed the presence of 5 impurities (reagents and intermediates), of which impurities C and E (*cf.* 2.1.9) appear to be in the range 1-2% and 10-20% respectively. The structure of these compounds should be established, they should be quantified, and the major toxic properties of these compounds should be elucidated

A 13-week oral study in rats provided evidence of toxicity in both sexes at 1000 mg/kg bw/day, and minimal signs in females only at 300 mg/kg bw/day. The NOEL was 100 mg/kg bw/day.

It was not irritant to the rabbit skin or eye but should be regarded as a potential sensitiser.

Percutaneous penetration of 0.045% is indicated from the available information, but the study conditions were not adequate and this value could be a considerable underestimation.

C 129 has been tested in procaryotic cells for gene mutation in two experiments that gave positive results.

The *in vitro* test for mammalian gene mutation assay is accepted as being positive.

The *in vitro* test for clastogenicity on CHO cells is clearly positive, C 129 is considered as a clastogen and an aneugen.

UDS *in vivo/in vitro* on rats hepatocytes is negative (different batch).

The *in vivo* micronucleus test in mice gave equivocal results; evidence that the bone marrow was reached by the test agent was noted.

When strictly considering the results presented, C 129 is only clearly negative in the UDS assay. Taken together the clastogenicity and mitotic delay in CHO and the results observed at 48 h and not in 72 hours, the substance could be considered as a mutagen, clastogen, aneugen under *in vitro*. It produced equivocal results in the *in vivo* studies.

2.13. References

1. Colorant Capillaire ref. IFD 398.82. Essai de Toxicity Aigue par Voie Orale chez le Rat. Institut Francais de Toxicologie (IFREB), Saint Germain-sur-l'Arbresle, France Rapport n° 302250, 22 February 1983
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3. Opinion of the SCCNFP

HC Green n° 1 is considered to be a mutagen, clastogen and aneugen *in vitro*. It produced equivocal results *in vivo*. Accordingly, the SCCNFP considers that it is not possible to assess the safe use of the substance.

Consequently, and before any further consideration, the following are required :

data on the genotoxicity/mutagenicity following the SCCNFP-opinion "Proposal for a Strategy for Testing Hair Dye Cosmetic Ingredients for their Potential of Genotoxicity / Mutagenicity", doc. n° SCCNFP/0566/02 of 4 June 2002, and in accordance with the Notes of Guidance, regularly updated by the SCCNFP (doc. n° SCCNFP/0321/00).

4. Other considerations

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5. Minority opinions

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