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

CONCERNING

INDIGOFERA TINCTORIA

COLIPA n° C170

There has been a request to evaluate the dossier submitted on *Indigofera tinctoria*.

This safety assessment concerns only the review of the safety data provided and derived from natural indigo. However, additional information is available for synthetic indigo.

For completeness, a separate evaluation of the safety data derived from synthetic indigo is appended to the review of natural indigo.

adopted by the SCCNFP on 23 April 2004
by means of the written procedure

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 *Indigofera tinctoria* safe for use in cosmetic products as a hair dye ingredient?

* 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

Indigo (*Indigofera tinctoria, Fabaceae*) has been one of the most important dyestuffs; this dye was used as far back as 6000 years in China. Indigo produces an intense deep blue colour. The leaves and branches of the plant are harvested, placed in a vat, covered with water, and permitted to ferment. The sludge of partially rotted plant material, which settles to the bottom is collected and pressed into cakes. When dry, these produce a powder that makes a colourless solution in water. The colour only develops when an item is dipped into the solution, removed and then exposed to air.

2.1.1. Primary name

Indigofera tinctoria (INCI)

2.1.2. Chemical names

Indigofera tinctoria, dried and pulverised leafs of *Indigofera tinctoria L.*

2.1.3. Trade names and abbreviations

COLIPA n° : C170

2.1.4. CAS / EINECS number

CAS : /
EINECS : /

2.1.5. Structural formula

Complex chemical mixture.

2.1.6. Empirical formula

/

2.1.7. Purity, composition and substance codes

Raw material No. 19556 (Code DA 060492)

Purity
Indigofera tinctoria leaves collected at flowering time, irradiated with 11.5 kGy for disinfection. According to literature data (not presented), *Indigofera tinctoria* leaf powder contains 0.2-0.8 % Indican, which under oxidative conditions is transformed to indigo.

2.1.8. Physical properties

Appearance	:	Finely divided green dispersable powder
Melting point	:	/
Boiling point	:	/
Density	:	/
Rel. vap. dens.	:	/
Vapour Press.	:	/
Log P_{ow}	:	/

2.1.9. Solubility

Water	:	/
Ethanol	:	/

2.1.10 Stability

Stable at room temperature when stored in dark. *(No time limit available)*

General comments on analytical and physico-chemical characterisation

The absolute dye content is not known in any of the studies. The reported concentrations in all experiments reflect the amount of leaf powder present in the respective suspensions.

Indigo content in the batch no. V 196447 used for Micronucleus assay and Mammalian Cell Gene Mutation Assay is not reported

2.2. Function and uses

For a hair dye formulation, the pulverised plant material of *Indigofera tinctoria* is mixed with water, heated to 70 °C (1 part powder with 5 parts water). 30 to 150 g of this mush, depending on the hair length, are applied to the hair. Following an incubation time of 20 to maximum 40 minutes, the hair is rinsed thoroughly and washed with shampoo. The application is conducted every 4 to 6 weeks. Depending on the indigo content of the plant material, the final hair dye formulation contains up to 0.17 % natural indigo. This calculation is based on two worst case assumptions, i. e. that the formulation consists of 100% *Indigofera tinctoria* leaf powder, and that this powder contains 1% of the hair dye.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

Only the data submitted on natural indigo is considered in this evaluation.

2.3.1. Acute oral toxicity

No data.

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. Subchronic oral toxicity

No data

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	:	OECD 404 (1992)
Species	:	New Zealand white rabbits
Group size	:	3 females
Test substance	:	*Indigofera tinctoria* leaf powder
Batch No.	:	type 210741, 03.02.94.
Dose levels	:	approx. 80 mg/cm^2
Route	:	dermal
Exposure	:	4 h
GLP	:	in compliance

A cellulose patch with 0.5 g of *Indigofera tinctoria* powder was spread over approx. 6 cm^2 and fixed with a non-irritating tape. Animals were examined for erythema/eschar and oedema as well as for local and systemic signs: 1, 24, 48, and 72 h after patch removal.

Results
All animals revealed very slight oedema and erythema (score 1) after 1 h. Only one very slight erythema remained in 1 animal after 24 h, and scoring at all further time-points was negative for all parameters.

Conclusion
Indigo powder is considered as non-irritating to the skin in this test.

Ref.: 2

2.4.2. Irritation (mucous membranes)

Guideline	:	OECD 405 (1987)
Species	:	New Zealand white rabbits
Group size	:	3 females
Test substance	:	*Indigofera tinctoria* leaf powder
Batch No.	:	type 210741, 03.02.94.
Dose levels	:	47, 73, 71 mg, applied to animals 1, 2, and 3 respectively
Route	:	ocular
GLP	:	in compliance

0.1 ml of the diluted *Indigofera tinctoria* powder in water was administered to the conjunctival sac of the right eye, and the eye was held closed for about 1 second. The animals were examined at: 1, 24, 48, and 72 h using an otoscope-lamp.

Results
Redness (score 1 and 2) and chemosis (score 2 and 4) were observed in 3 animals each 1 h after exposure to indigo powder. No alterations of cornea and iris were observed at this time-point, but scoring at later time-points revealed corneal opacity in 2 animals. Redness and chemosis remained for several hours, and aggravated occasionally. Additional scoring after 6 and 8 days revealed no lesions of skin, but conjunctival redness and chemosis remained for 6 days in one animal.

Conclusion
The tested material induced significant damage to the rabbit eye and is considered to be irritating to the eye.

Ref.: 3

2.5. Sensitisation

Maximisation (Magnusson and Kligman) test, study 1

Guideline	:	Directive 92/69/EEC, method B.6. (1992)	
Species	:	Hartley guinea pigs	
Strain	:	Crl:(HA)BR	
Group size	:	main study: 15 females	
		preliminary study: 3 females	
Test substance	:	*Indigofera tinctoria* leaf powder	
Batch No.	:	type 210741, 03.02.94.	
Dose levels	:	intradermal induction:	0.1 % solution
		epicutaneous induction:	40 % solution
		epicutaneous challenge:	40 % solution
GLP	:	in compliance	

Indigofera tinctoria leaf powder, dissolved in physiological saline, was applied to 10 guinea pigs in the Magnusson and Kligman "maximisation test". 1,4-phenylenediamine was used as positive control substance.

Results
Following challenge exposure "well defined erythema" (score 2) or "severe erythema and / or oedema" (score 3) have been scored in all animals after 24 h and / or 48 h at visual examination.

Conclusion
Based on these results indigo powder is classified as a sensitizer following skin contact in this maximisation test.

Ref.: 4

Maximisation (Magnusson and Kligman) test, study 2

Guideline	:	OECD 406 (1992)	
Species	:	albino guinea pigs	
Strain	:	Dunkin Hartley	
Group size	:	10 males/dose group	
Test substance	:	*Indigofera tinctoria* leaf powder	
Batch No.	:	03.02.94 ? type: 21074, 03.02.94	
Dose levels	:	intradermal induction:	0.25 % solution
		epicutaneous induction:	12.5 % solution
		epicutaneous challenge:	12.5 % solution
GLP	:	in compliance	

The *Indigofera tinctoria* leaf powder, dissolved in distilled water, was applied to 10 guinea pigs in the Magnusson and Kligman "maximisation test".

Results
8 of 10 guinea pigs showed slight to intense erythema following challenge exposure after 24 h. 48 h after challenge, moderate to intense erythema were scored in 5 animals.

Conclusion
Based on these results, natural indigo is inducing delayed contact hypersensitivity.

Ref.: 5

Bühler study

Guideline	:	Directive 92/69/EEC, method B.6. (1992)
Species	:	Hartley guinea pigs
Strain	:	Crl:(HA)BR
Group size	:	test group: 20 females control group: 10 females
Test substance	:	*Indigofera tinctoria* leaf powder
Batch No.	:	type: 210741, 03.02.94.
Dose levels	:	epicutaneous induction (3x): 20 % solution epicutaneous challenge: 20 % solution
GLP	:	in compliance

The *Indigofera tinctoria* leaf powder, dissolved in distilled water, was applied to 20 guinea pigs in the Bühler sensitisation test. 1,4-phenylenediamine was used as positive control substance.

Results
No skin reactions were found after examination by eye and by histopathological examination following treatment with *Indigofera tinctoria* powder.

Conclusion
Indigofera tinctoria powder can be considered as non-sensitising following skin contact in this test.

Ref.: 6

Human dermal sensitisation

Guideline	:	/
Species	:	human
Group size	:	4 males and 49 females
Test substance	:	indigo dispersible powder, no further information
Batch No.	:	/
GLP	:	/

The original study is not available; it is reported in a review. Regarding the number of test persons and study design (e.g. only one patch application during the induction phase) the study is not responding to actual requirements and therefore is judged not to be suitable for the investigation of sensitising properties.

No report submitted. Data taken from review article (Ref 1)

2.6. Teratogenicity

No data

2.7. Toxicokinetics (incl. Percutaneous Absorption)

No information on the percutaneous absorption of indigo from *Indigofera tinctoria* leaf powder is available.

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Reverse Mutation Testing using Bacteria

Guideline	:	/
Species/strain	:	*S. typhimurium*: TA1535, TA1537, TA1538, TA98, TA100
Test item	:	Indigo, green leaves powder
Batch no.	:	DA 060492
Purity	:	/
Replicate	:	several replicates with different test material
Dose level	:	30, 100, 300, 1000 and 3000 µg/plate aqueous suspension, Centrifuged; water extracts at high temperatures; different types of ethanol extraction.
Metabolic Act.	:	Aroclor 1254 induced rat liver homogenate (S9)
Positive contr.	:	Sodium Azide; 9Aacr.; 2NF (-S9); 2AAntr.; 2AF (+S9)
GLP	:	/

Results

Mutagenicity: Powder has resulted non mutagenic. Some type of extract has been found mutagenic.

Conclusion
The extract is mutagenic on Salmonella.

Ref.: 9

***In vitro* Mammalian Chromosome Aberration Test**

Guideline	:	OECD 473 (1982)
Species/strain	:	Chinese hamster K1-BH(4) CHO
Test item	:	Indigo leaves powder
Batch	:	060492
Replicate	:	one experiment
Dose level	:	-S9: 125, 250, 500 µg/ml, 18 and 28 hours +S9: 125, 250 and 500 µg/ml, 3 hours.
Metabolic activ.	:	rat liver homogenate

Positive control	:	MMS (-S9); CPA (+S9)
GLP	:	In compliance

Results

Toxicity: in a preliminary cytotoxic experiment with 10 doses, 500 µg/ml resulted partially toxic. The mitotic index was reduced to ca.50 %.

Mutagenicity: MMS and CPA induced chromosome aberration with a percentage of 15-20 %.

The test item did not induce any type of chromosome aberrations.

Conclusion
In the test conditions, the test item (leaves powder) was not clastogenic to CHO cells treated *in vitro*.

Ref.: 16

_In_ vitro Mammalian Cell Gene Mutation Test

Guideline	:	OECD 476 (1997)
Species/strain	:	Mouse lymphoma L5178Y cells (Thymidine Kinase Locus)
Test item	:	19556 Indigoblatter
Batch no.	:	V 106447
Purity	:	green leaves' powder (powder dispersed in water, stirred, filtered and the filtrate tested
Replicate	:	2 experiments (4 hours treat.)
Dose level	:	5 to 1000 µg/ml +S9 5 to 2500 µg/ml - S9
Metabolic act.	:	Phenobarbital/Naphthoflavone induced rat liver homogenate
Positive control	:	EMS 0.7 mg/ml (-S9); BP 2.5 µg/ml (+S9)
GLP	:	In compliance

Results

Toxicity: a preliminary experiment with doses from 5 to 3000 µg/ml demonstrated that the dose of 1000 µg/ml had toxic effect.

Mutagenicity: BP induced 173.23 mutants per 10/6-342.51 per 10/6 cells (control: 57.70-53.72). EMS induced 1775.79- per 10/6 cells (control: 51.88).
The test item did not induce a significant increase of mutation frequency, compared to the untreated control.

Conclusion
The test item does not induce gene mutations in mammalian cells treated *in vitro*.

Ref.: 18

2.8.2 Mutagenicity/Genotoxicity *in vivo*

In vivo Mammalian Erythrocyte Micronucleus Test

Guideline	:	OECD 474 (1997)
Species/strain	:	NMRI mice, young adults (5animals/sex/group)
Test item	:	19556 Indigoblatter
Batch no.	:	V 106 447
Purity	:	green leaves powder, dispersed in water, stirred, filtered and the filtrate tested
Dose level	:	200, 1000 mg/Kg (24 hours treatment) 2000 mg/Kg (48 hours treatment)
Administration	:	i.p. one dose.
Positive control	:	CPA, 40 mg/kg i.p. (24 h)
GLP	:	in compliance

Results
Toxicity: In a preliminary experiment a dose of 2000 mg/kg induced lethargy at 6, 24 and 48 hours after the administration.

Mutagenicity: CPA, the positive control induced 2.96-2.22 % (M/F) of bone marrow cells (PCE) of MN (Control: 0.16-0.20):
The test item treated animals presented a percentage of PCE with MN in the range of the untreated animals. Cytotoxicity in the cells of animals treated with the test item was observed in relation to the dose.

Conclusion
The test item does not induce numerical/structural chromosome aberrations in mice *in vivo*.

Ref.: 19

Published paper

Natural Indigo suspended in 10 % of arabic gum aqueous solution was administered twice to mice by gavage at the doses of 0.1;0.5; and 2 g/kg. The animals were sacrificed 30 and 54 hours after the first administration; CPA was used as a positive control.
No increase in the percentages of PCEs containing MN was observed; no information is presented about the presence of the test item in the target cells.
The study is inadequate for the lack of information about the nature of the test item used and for the conditions of the assay.

Ref.: 10

2.9. Carcinogenicity

No data

2.10. Special investigations

No data

2.11. Safety evaluation

Not applicable

2.12. Conclusions

Toxicity
No data

Irritation and sensitisation
Indigo powder is considered as non-irritating to the skin in this test. The tested material induced significant damage to the rabbit eye and is considered to be irritating to the eye.
Indigo powder is classified as a sensitizer following skin contact in a maximisation test.
Indigofera tinctoria powder can be considered as non-sensitising following skin contact in a Buehler Test.

Percutaneous absorption
No data have been provided on natural indigo.

Mutagenicity/genotoxicity
The test item, as leaves powder (water extract) derived from the plant Indigo, has been tested on bacterial and mammalian cells *in vitro* for the induction of gene mutations in mammalian cells *in vitro* for the induction of chromosome aberrations and on mice in vivo for the induction of numerical/structural chromosome aberrations.
The test item (ethanol extract) has been found strongly positive for the induction of gene mutations in bacterial cells.
All the studies are inadequate because there is no characterisation of the material used as test item. No conclusion can be drawn about the potential mutagenicity/genotoxicity of the test item.

2.13. References

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3. Opinion of the SCCNFP

The SCCNFP is of the opinion that the information submitted is inadequate to assess the safe use of the substance.

Before any further consideration, a complete safety dossier on *Indigofera tinctoria* is required following the relevant SCCNFP-opinions and in accordance with the Notes of Guidance.

4. Other considerations

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5. Minority opinions

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Annex

Toxicological Evaluation and Characterisation on synthetic indigo, CI 73000

1.1. General

Synthetic indigo is listed as CI 73000 in Annex IV, part 1 – list of colouring agents allowed for use in cosmetic products – to Directive 76/768/EEC on cosmetic products; field of application 1: colouring agents allowed in all cosmetic products.

It was first produced in 1897.

1.1.1. Primary name

CI 73000 (INCI) is synthetic indigo

1.1.2. Chemical names

Chemical name	:	2-(1,3-dihydro-3-oxo-2H-indol-2ylidene)-1,2-dihydro-3H-indol-3-one
Other names	:	Pigment Blue 66, Vat Blue 1, indigo

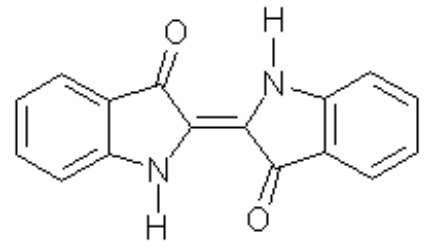
1.1.3. Trade names and abbreviations

Trade name	:	D&C Blue No. 6
COLIPA n°	:	C170

1.1.4. CAS / EINECS / COLOUR INDEX number

CAS	:	482-89-3
EINECS	:	207-586-9
Colour Index	:	CI 73000

1.1.5. Structural formula



1.1.6. Empirical formula

Emp. Formula	:	$C_{18}H_{10}N_2O_2$
Mol weight	:	262.3

1.1.7. Purity, composition and substance codes

Specification of FDA certified D&C Blue No.6 (recrystallised synthetic dye), 99% (colour content):

Total colour content	:	> 95%
Volatile matter (135°C)	:	< 3%
Insoluble matter in N,N'-dimethylformamide	:	< 1%
Isatin	:	< 0.3%
Anthranilic acid	:	< 0.3%
Indirubin	:	< 1%
Pb	:	10 ppm
As	:	3 ppm
Hg	:	1 ppm

1.1.8. Physical properties

Appearance	:	Dark blue powder
Melting point	:	Sublimation at approx. 300°C, decomposition at 390°C
Boiling point	:	/
Density	:	ca. 0.56g/cm^3
Rel. vap. dens.	:	/
Vapour Press.	:	/
Log P_{ow}	:	3.69

1.1.9. Solubility

Water	:	/
Ethanol	:	/

1.1.10 Stability

Stable at room temperature when stored in dark. *(No time limit available)*

General comments on analytical and physico-chemical characterisation

* FDA certificate of the test material(s) used in the experiments is not available.
* purity of the "textile grade" indigo used in some experiments is not reported.

1.2. Function and uses

CI 73000 is permitted for use in all types of cosmetic products. It is also used for dyeing fabrics.

TOXICOLOGICAL CHARACTERISATION

1.3. Toxicity

1.3.1. Acute oral toxicity

Species/strain	:	Rat, Sprague Dawley
Group size	:	5 males per group
Test substance	:	"Certified D&C Blue No.6"
Dose	:	31.6, 100, 316, 1000 and 3160 mg/kg bw
Vehicle	:	0.5% CMC aqueous solution
Results	:	no mortalities, LD_{50orl}> 3160 mg/kg bw

No report submitted. Data taken from review article (Ref 1)

Species/strain	:	Rat, Sprague Dawley
Group size	:	5 males and 5 females
Test substance	:	"Textile grade indigo dispersible powder"
Dose	:	5000 mg/kg bw
Vehicle	:	50% aqueous suspension
Results	:	no mortalities, LD_{50orl}> 5000 mg/kg bw

No report submitted. Data taken from review article (Ref 1)

Species/strain	:	Rat, Sprague Dawley
Group size	:	5 males and 5 females
Test substance	:	"Indigo 20% paste, textile grade"
Dose	:	5000 mg/kg bw
vehicle	:	6% sodium hydroxide solution
Results	:	2/5 males and 5/5 females died, mortality was comparable to that in rats receiving 6% sodium hydroxide vehicle and ascribed to the caustic nature of the substrate.

No report submitted. Data taken from review article (Ref 1)

Species/strain	:	Dog, mongrel
Group size	:	1 male and 1 female per group
Test substance	:	"Certified D&C Blue No.6"
Dose	:	31.6, 100, 316 and 1000 mg/kg bw
vehicle	:	not stated
Results	:	no mortalities.

No report submitted. Data taken from review article (Ref 1)

1.3.2. Acute dermal toxicity

Species/strain	:	New Zealand White Rabbits
Group size	:	5 males and 5 females

Test substance	:	"Textile grade 20% indigo"
Dose	:	2000 mg/kg bw
Vehicle	:	not stated
Results	:	eschar formation at treatment sites; no mortalities, LD_{50drm}> 2000 mg/kg bw

No report submitted. Data taken from review article (Ref 1)

1.3.3. Acute inhalation toxicity

Species/strain	:	Rat, Sprague Dawley
Group size	:	5 males and 5 females
Test substance	:	aerosol of "20% indigo textile grade"
Time weighted conc.	:	0.08 mg/l
Droplet size	:	74% < 10 microns (mean size 4.6 $\pm$ 3.3 microns)
Results	:	no mortalities

No report submitted. Data taken from review article (Ref 1)

Species/strain	:	Rat, Sprague Dawley
Group size	:	5 males and 5 females
Test substance	:	"indigo dispersible powder", not otherwise characterised
Time weighted conc.	:	0.76 mg/l
Droplet size	:	79% < 10 microns (mean size 2.1 $\pm$ 2.3 microns)
Results	:	bluish discoloration of lungs, no mortalities

No report submitted. Data taken from review article (Ref 1)

1.3.4. Repeated dose oral toxicity

No data

1.3.5 Repeated dose dermal toxicity

Guideline	:	/
Species/strain	:	Rabbits
Group size	:	1-4 males and females per group
Test substance	:	"Certified D&C Blue No.6"
Dose levels	:	500 mg/kg bw/day; at 0.1 or 1.0% in the vehicles; 5 days a week
Vehicles	:	white petrolatum and hydrophilic ointment
No. of applications:		15(3 weeks)or 64 (13 weeks)
Results	:	It was stated that the test substance was without effect on skin conditions or microscopic examination of a number of organs. No further details were, however, provided.

No report submitted. Data taken from review article (Ref 1)

Guideline	:	/
Species/strain	:	Swiss Webster Mice
Group size	:	50 males and 50 females per group
Test substance	:	"Certified D&C Blue No.6"
Dose levels	:	1 mg per animal at 0.1% in the vehicle; once a week
Vehicle	:	benzene
Exposure duration:		95 weeks

Results : It was stated that no evidence was found that repeated dermal application produced any test substance related effects. No further details were, however, provided.

No report submitted. Data taken from review article (Ref 1)

1.3.6. Repeated dose inhalation toxicity

No data

1.3.7. Subchronic oral toxicity

Guideline	:	/
Species/strain	:	rat, "Charles River albino"
Group size	:	10 males per group
Test substance	:	"Certified D&C Blue No.6"
Dose levels	:	0, 0.1, 0.23, 0.55, 1.29 and 3% in the diet
Exposure	:	6 weeks

Results

Clinical signs	:	No treatment related effects reported
Body weight	:	/
Food intake	:	/
Haematology	:	/
Clinical chemistry:		/
Organ weights	:	No treatment related effects reported
Macroscopy	:	Colour retention in fatty tissues, affected groups not stated
Histopathology	:	Degenerative centrilobular hepatocytic changes in the high-dose group

No report submitted. Data taken from review article (Ref 1)

1.3.8. Sub-chronic dermal toxicity

No data

1.3.9. Sub-chronic inhalation toxicity

No data

1.3.10. Chronic toxicity

Guideline	:	/
Species/strain	:	rat, "Charles River albino"
Group size	:	25 males and 25 females per group
Test substance	:	"Certified D&C Blue No.6"
Dose levels	:	0, 0.25, 1.0 and 3.0% in the diet
Exposure	:	2 years (interim sacrifice of 5 rats/sex/group after 1 year)

Results

Clinical signs	:	No treatment related effects
Survival	:	No treatment related effects
Body weight	:	/
Food intake	:	Decreased in the high-dose group during the first 6 months
Haematology	:	Consistently decreased haematocrit and haemoglobin in high-dose males
Clinical chemistry:		Not conducted
Urinalysis	:	Bilirubinuria in mid- and high-dose groups at 24 month stage
Macroscopy	:	No treatment related effects
Microscopy	:	No treatment related effects (in 10 rats of control and high-dose group both after 1 and 2 years)

No report submitted. Data taken from review article (Ref 1)

Guideline	:	/
Species/strain	:	dog, beagle
Group size	:	3 males and 3 females per test group; controls 10 dogs/sex/group
Test substance	:	"Certified D&C Blue No.6"
Dose levels	:	0, 0.25, 1.0 and 3.0% in the diet
Exposure	:	2 years

Results

Clinical signs	:	No treatment related effects
Haematology	:	No effects were reported
Clinical chemistry:		No effects were reported
Urinalysis	:	No effects were reported
Organ weights	:	No treatment related effects
Macroscopy	:	No treatment related effects
Microscopy	:	No treatment related effects

No report submitted. Data taken from review article (Ref 1)

1.4. Irritation & corrosivity

1.4.1. Irritation (skin)

No data

1.4.2. Irritation (mucous membranes)

No data

1.5. Sensitisation

No data

1.6. Teratogenicity

Three generation study

Guideline : /
Species/strain : rat, "Harlan Wistar"
Group size : 10 males and 20 females per group
Test substance : "Certified D&C Blue No.6"
Dose levels : 0, 5, 50 150 and 500 mg/kg diet

Results
It was concluded that no deleterious effects were associated with the inclusion of the test substance in the diet of rats for 3 generations. No further details were, however, provided.

No report submitted. Data taken from review article (Ref 1)

Teratogenicity studies

Guideline : /
Species/strain : rat, "Charles River CD"
Group size : 20 females (mated) per group
Test substance : "Certified D&C Blue No.6"
Dose levels : 50, 160 and 500 mg/kg bw/day
Vehicle : methyl cellulose suspensions
Treatment period : days 6-15 of gestation

Results
It was stated that the test substance was without effect on reproduction performance, maternal weight gain and foetal development. No further details were, however, provided.

No report submitted. Data taken from review article (Ref 1)

Guideline : /
Species/strain : New Zealand White rabbits
Group size : 10 females (mated) per group
Test substance : "Certified D&C Blue No.6"
Dose levels : 50, 160 and 500 mg/kg bw/day
Vehicle : methyl cellulose suspensions
Treatment period : days 6-18 of gestation

Results
It was stated that the test substance was without effect on reproduction performance, maternal weight gain and foetal development. No further details were, however, provided.

No report submitted. Data taken from review article (Ref 1)

1.7. Toxicokinetics (incl. Percutaneous Absorption)

No data

1.8. Mutagenicity/Genotoxicity

1.8.1 Mutagenicity/Genotoxicity *in vitro*

Reverse Mutation Testing using Bacteria

Published paper 1

Different batches of synthetic Indigo were tested on TA 98, TA 1538 and TA 100 of *S. typhimurium* in the presence and in the absence of Aroclor 1254 rat liver homogenate at the doses of 0,30, and 150 µl/plate of S9: the test items were found mutagenic on TA 98 and TA 1538 in the presence of increasing amounts of S9.
In the same conditions, purified synthetic Indigo and natural Indigo (nature not specified) were found non mutagenic.

Ref.: 10

Published paper 2

Natural Indigo (Ciba Geigy); Synthetic Indigo (Fluka).
S. typhimurium strains TA 1535, TA 100, TA 1538, TA 98
Metabolic activation: Aroclor induced rat livers (S9)
250 µg/plate and higher doses of natural Indigo and synthetic Indigo induced a strong increase in the number of mutant colonies in the strains TA 98 and TA 1538 in the presence of S9.

Ref.: 11

Published paper 3

Extracts of pure cotton and jeans fabrics dyed with synthetic Indigo.

Ref.: 12

Published paper 4

Synthetic Indigo was found mutagenic on TA 98 in the presence of S9 from various type of rat induction.

Ref.: 13

1.8.2 Mutagenicity/Genotoxicity *in vivo*

No data

1.9. Carcinogenicity

Oral administration, rat

A 2-year chronic feeding study of C.I. 73000 was carried out with 3 groups of 25 male and 25 female Charles River adult albino rats and a control group of 80 males and 80 females. The test material was incorporated in the basal diet at levels of 0.25, 1.0 and 3.0%. Throughout the 2-year study, observations were made daily for mortality and weekly for gross signs of toxicity. Autopsies were performed on all animals, which died during the study. At the termination of the study, histopathology was performed on all preserved tissues from 10 male and 10 female rats in the control group and on an equal number in the high dosage group. Appearance and behaviour of the test rats were generally comparable to those of the controls. At the 3% level, food consumption was significantly lower than controls for the first six months, but comparable to controls during the remainder of the study. Autopsies performed on the animals, which died during the second year of the study did not reveal any consistent gross related effects on the kidneys or other tissues in either sex. It is concluded that the study appeared to demonstrate that after a period of adjustment to higher dosage levels, the rats were able to tolerate up to 3% of synthetic indigo in their diet without serious effects (US FDA 1962-1973).

Skin painting, mice

C.I. 73000 was applied as a 1% solution, once per week for up to 95 weeks to 50 male and 50 female Swiss-Webster mice. 100 males and 100 females were used as negative controls. 50 males and 50 females received application of the vehicle (benzene). Autopsies were performed on all sacrificed animals in the absence of marked autolysis. Microscopic examination of the lungs, liver and skin on 10 negative controls, 10 vehicle controls and 10 compound-treated animals were performed at 75 weeks. At the terminal sacrifice (96 weeks), sections of lung liver and skin from 29 negative controls, 26 vehicle controls and 13 compound-treated animals were examined microscopically. Histopathology was also performed on most tissue masses and on grossly abnormal organs of the animals. No evidence was found that repeated dermal applications produced any effects attributable to synthetic indigo lesions. The tumours seen in treated mice were comparable to the tumours in the vehicle controls (US FDA 1962-1973).

Ref.: 1

Human studies

No data

1.10. Special investigations

No data

1.11. Safety evaluation

No applicable

1.12. Conclusions

Toxicity
Most of the data were derived from a review article (Ref 1) without sufficient details to enable evaluation by the SCCNFP.

Irritation and sensitisation
No data on have been provided on the irritation/sensitisation potential of CI 73000.

Percutaneous absorption
No data on dermal penetration have been provided on CI 73000. A skin permeation study should have been performed with CI 73000.

Mutagenicity/genotoxicity
The test item, as synthetic Indigo, has been tested on *Salmonella typhimurium* different strains and reported as strong mutagenic in the presence of S9 on TA 98 and TA 1538

Carcinogenicity
It is not possible to make any conclusions regarding carcinogenicity from the data available.

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