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

CONCERNING

ACID BLUE 62

COLIPA n° C67

Adopted by the SCCNFP during the 28th plenary meeting
of 25 May 2004

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 Acid Blue 62 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

Acid blue 62 is listed as CI 62045 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 4: colouring agents allowed exclusively in cosmetic products intended to come into contact only briefly with the skin.

2.1.1. Primary name

Acid Blue 62 (INCI)

2.1.2. Chemical names

Sodium 1-amino-4-(cyclohexylamino)-9,10-dihydro-9,10- dioxaanthracene-2-sulfonate (IUPAC);
2-Anthracenesulfonic acid, 1-amino-4-(cyclohexylamino)-9,10-dihydro-9,10-dioxo-, monosodium salt;
1-Amino-4-cyclohexylamino-anthraquinone-2-sulfonic acid, sodium salt

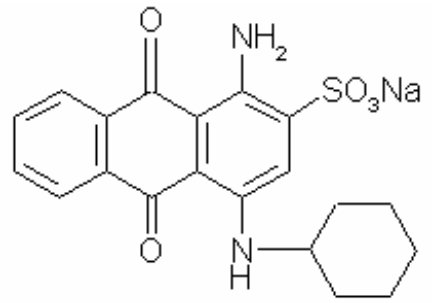
2.1.3. Trade names and abbreviations

Trade name : /
COLIPA n° : C67

2.1.4. CAS no. and EINECS no.

CAS n° : 4368-56-3
EINECS n° : 224-460-9
Colour Index no. : CI 62045

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula : $C_{20}H_{19}N_2NaO_5S$
Mol weight : 422.4

2.1.7. Purity, composition and substance codes

Purity

Batch No. 91100013 (Acid Blue 62 with dispersing agent)

Content of Acid Blue 62 determined by HPLC using batch 112 (considered as 100%) : 53.4 %
Loss on drying : 3.8%
Sulphated ash : 38.5%
Sulphate ions (as Na_2SO_4) : 4.2%
Chloride ions (as NaCl) : 22.9%
Residual solvents : <100 ppm (methanol, ethanol, isopropanol, n-propanol, acetone, ethylacetate, cyclohexane, methylethyl ketone and monochlorobenzene together) evaluated by Headspace-GC, FID

Batch No. 01515-200P-0 (Acid Blue 62 without dispersing agent)

Content of Acid Blue 62 determined by HPLC using batch 112 (considered as 100%) : 98.4 %

Impurity (reagents and intermediate products) : HPLC profile

Retention Time (min)	Batch No. 911003	Batch No. 01515-200P-0	λ_{max}	Comments
1.35	A	A	563 nm and 603 nm	
1.51	E		-	Trace amount
1.75	B	B	536 nm	
1.90		C	-	Trace amount
3.00		D	281 nm and 525 nm	Significant amount
7.94	F		530 nm	
9.43	G		250 nm and 281 nm	
12.70	H		245 nm and 281 nm	

Chemical compounds could not be characterised by LC-MS

2.1.8. Physical properties

Acid Blue 62 is described as a black powder containing 2-Anthracenesulfonic acid, 1-amino-4-(cyclohexylamino)-9,10-dihydro-9,10-dioxo-, monosodium salt (approximately 60%) together with dispersing agents (sodium salts)
Log Pow : -1.14

Batch No. 01515-200P-0 (Acid Blue 62 without dispersing agent)

Appearance : Dark Blue Powder, odourless
Melting point : /
Boiling point : /

Density (bulk density) : 400-600 kg/m^3
Rel. vap. Density : /
Vapour pressure : /
Log P_{ow} : 0.5 (calculated)

2.1.9. Solubility

Batch number 91100013 (Acid Blue 62 with dispersing agent)

Water : >0.1% (w/v)
Ethanol : <0.1% (w/v)

Batch number 01515-200P-0 (Acid Blue 62 without dispersing agent)

Water : Approximately 50 g/l at 20°C

2.1.10 Stability

It is reported that Acid blue 62 is stable for 7 years, when stored in a well-closed container protected from light and moisture.
Stability in aqueous solutions (23 mg/ml and 236 mg/ml) of Acid Blue 62: approximately 5% degradation in 8 days, measured by HPLC, detection at 230 nm.

General comments on analytical and physico-chemical characterisation

* It is considered that Acid Blue 62 containing about 50 % active ingredient will be used in hair dyeing formulations. The dispersing agents to be used in the dye are not described.
* The use of the dispersing agent is not clear because of the high water solubility of the compound.
* Acid Blue 62 is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content of the dye is not reported
* Stability of the dye in prototype formulations is not known. Inappropriate wavelength has been selected for checking the stability of the dye in aqueous solution by HPLC. The appropriate wavelengths for the detection are 575 nm or 637 nm.
* The purity of the dye used in some tests is not described.

2.2. Function and uses

Acid Blue 62 is used in semi-permanent and temporary hair colouring products at concentrations up to 0.5%

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	/
Species/strain	:	Sprague Dawley rat
Group size	:	10 animals (5 males & 5 females)
Observation period	:	14 days
Test substance	:	Acid Blue 62
Batch no	:	6120088
Purity of test substance	:	40%
Dose level	:	500, 1000, and 2000 mg/kg bw (in distilled water)
GLP statement	:	signed statement, no date

A preliminary study was performed using three groups of two male and female rats. The doses of 500, 1000 and 2000 mg/kg bw were administered. Because no deaths were noted during this study, the dose of 2000 mg/kg bw was chosen for the principal study. The purity of the dye used in these studies was unknown.
A single dose of 2000 mg/kg bw Acid Blue 62 (purity 40%) was administered in the principal study. Animals were observed 15 minutes, 1, 2 and 4 hours after compound administration, and daily thereafter for 14 days. Body weights were recorded on the day before treatment, day 1 prior to treatment, and days 8 and 15. Moribund animals were killed during the study and autopsied. All surviving animals were killed at the end of the study and examined grossly. The LD_{50} was calculated using the method of Bliss and that of Litchfield and Wilcoxon.

Results
One female rat died on day 2 of the study; no other compound-related mortalities were observed. Neither the cause of death, nor the possible relationship with substance administration, is described in the study report.
Blue coloration of urine and faeces was observed in all animals, from 2h after administration, till day 2.

Conclusion
Based on the results of this study, the performing laboratory concludes that the acute oral LD_{50} of Acid Blue 62 in rats was > 2000 mg/kg bw (equivalent to 800 mg active dye).

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

Guideline	:	OECD 407 (1981)
Species/strain	:	Sprague Dawley rat
Group size	:	12 animals (6 males & 6 females) / dose level
Observation period	:	14 days (no recovery group included)
Test substance	:	Acid Blue 62
Batch no	:	9110003
Purity of test substance	:	not stated in study report, 53.4% according to the summary (dye with dispersing agents)
Dose levels	:	0, 25, 100, 400 mg/kg bw/day (in water)
GLP statement	:	/

Three groups of six male and six female Sprague-Dawley rats received the test substance, Acid Blue 62 (53.4% pure), daily by oral gavage at doses of 25, 100 or 400 mg/kg bw/day (equivalent to 13, 53 or 214 mg/kg bw/day active dye) for 15 days. An additional group of six male and six female rats received the vehicle alone (water for injections) and served as the control. Animals were observed twice daily for mortality/morbidity and once daily for clinical abnormalities. Individual animal weights were recorded weekly. Body weight and food consumption were recorded twice weekly. Haematology, clinical chemistry and urinalysis evaluations were performed at the end of the study. At the end of the treatment period, all animals were killed and grossly examined. Selected organs were weighed. All animals were submitted to a complete macroscopic examination. Selected tissues and macroscopic lesions from animals in the control and high-dose groups were evaluated microscopically; only macroscopic lesions were evaluated in the low and intermediate dose groups.

Results
Test substance-related findings were limited to clinical signs of ptyalism and blue-coloured faeces at 100 and 400 mg/kg bw/day, and blue-coloured fur and/or tail and green-coloured urine at 400 mg/kg bw/day. Blue discoloration of the gastrointestinal tract and its contents as well as blue fur and blue tails were observed at necropsy at 100 and 400 mg/kg bw/day.

Conclusion
The No Observable Adverse Effect Level for this study (conducted with 53.4% pure Acid Blue 62) was considered to be 400 mg/kg bw/day (equivalent to 214 mg/kg bw/day active dye).

Ref.: 6

2.3.5 Repeated dose dermal toxicity

No data

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Subchronic oral toxicity

Guideline	:	OECD 408 (1981)
Species/strain	:	Sprague Dawley rat

Group size	:	20 animals (10 males & 10 females) / dose level
Observation period	:	90 days (no recovery group included)
Test substance	:	Acid Blue 62
Batch no	:	9110003
Purity of test substance	:	not stated in study report, 53.4% according to the summary (dye with dispersing agents)
Dose levels	:	0, 100, 300, 1000 mg/kg bw/day (in water)
GLP statement	:	/

Three groups of ten male and ten female Sprague Dawley rats received the test substance, Acid Blue 62 (53.4% pure), daily by oral gavage at doses of 100, 300 or 1000 mg/kg bw/day (equivalent to 53, 160 or 534 mg/kg bw/day active dye) for 13 weeks. An additional group of ten male and ten female rats received the vehicle alone (water for injections) and served as the control. Animals were observed twice daily for mortality/morbidity and once daily for clinical abnormalities. Individual animal weights were recorded weekly. Body weight and food consumption were recorded weekly; efficiency of food utilization was calculated weekly using these values. Ophthalmologic evaluations on control and high-dose animals were performed before the treatment period and at week 12. Haematology, clinical chemistry and urinalysis evaluations were performed once during week 13. At the end of the treatment period, all animals were killed and grossly examined. Selected organs were weighed. All animals were submitted to a complete macroscopic examination. All macroscopic lesions and required tissues from animals in the control and high-dose groups were evaluated microscopically; only macroscopic lesions and target tissues were evaluated in the low and intermediate dose groups.

Results
Mortalities were 2/10 animals (one male and one female) at 100 mg/kg bw/day, 4/10 females at 300 mg/kg bw/day and 1/10 females at 1000 mg/kg bw/day. Because the incidence was not dose-related and because death was attributed to regurgitation for most animals, the mortality in this study was not considered to be a primary effect of the test compound.

Test item-related findings were:

100 mg/kg bw/day :	ptyalism, regurgitation and loud and/or abnormal breathing, blue faeces, urine coloration and coloration of gastro-intestinal tract, urinary bladder content and lymph nodes.
300 mg/kg bw/day :	ptyalism, regurgitation and loud and/or abnormal breathing, blue faeces, green urine coloration and blue coloration of body extremities, coloration of gastro-intestinal tract, urinary bladder content and lymph nodes, slightly lowered glucose levels in males.
1000 mg/kg bw/day :	ptyalism, regurgitation and loud and/or abnormal breathing, blue faeces, green to dark blue urine coloration and blue coloration of body extremities, blue coloration of internal organs, slightly lowered glucose levels in males and females, increased urea blood concentration, albumin and cholesterol levels and alanine aminotransferase activity, elevated liver and kidney weights, correlated with centrilobular hepatocyte hypertrophy without nuclear or cytoplasmic degenerative or necrotic changes, and slight to marked tubular nephrosis, decreased body weight gain (not dose-related in severity).

The observed coloration of internal organs was not associated with any histopathological abnormalities. As a general comment, the performing laboratory states that the regurgitation and loud and/or abnormal breathing is due to the poor gastric tolerance to gavage of the animals.

Conclusion
The No Observable Adverse Effect Level for the study (conducted with 53.4% pure Acid Blue 62) was considered by the performing laboratory to be 300 mg/kg bw/day (160 mg/kg bw/day active dye).

Ref.: 7

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	:	/
Species	:	New Zealand White rabbits
Group size	:	6 males
Test substance	:	Acid Blue 62
Batch No.	:	AJ5004
Purity	:	/
Dose levels	:	1.5% in water on gauze pad 2 cm^2
Route	:	left flank skin and scarified right flank
Exposure	:	1 application for 23 hours
GLP	:	/

A group of six male New Zealand White rabbits (body weight: 2.5–3.5 kg) was used in this study.
The flanks of the rabbits were clipped 24 hours prior to administration of the test compound. The right flank of each rabbit was scarified. Acid Blue 62 (purity unknown) was administered at a concentration of 1.5% in water on two gauze pads, 2 cm^2 in area, with one pad placed on the left flank and the other on the scarified area of the right flank. These were immobilized by patches that were held in place by an adhesive bandage.
Twenty-three (23) hours later, the patches were removed, and 1 hour after this, the skin was evaluated for possible lesions. Skin biopsies were taken from the right flank of all animals at this time. Application sites were evaluated again 48 hours later (72 hours after application of the test substance), and skin biopsies from the left flank were taken at this time.

Results
There was no evidence of oedema at any of the patch test sites at the 24- and 72-hour observation periods. The compound discoloured the application sites such that erythema could not be evaluated; thus the skin biopsies were examined. There were no histopathological abnormalities noted in the skin biopsies.
Acid Blue 62 (purity unknown) was non-irritant to intact and scarified rabbit skin at the concentration of 1.5%.

Ref.: 3

2.4.2. Irritation (mucous membranes)

Guideline	:	/
Species	:	New Zealand White rabbits
Group size	:	6 (sex not stated)
Test substance	:	Acid Blue 62
Batch No.	:	AJ5004
Purity	:	/
Dose levels	:	0.1ml of 1.5% in water
Route	:	conjunctival sac
Exposure	:	1 application
GLP	:	/

A group of six New Zealand White rabbits (mean body weight: ~2.5 kg) was used in this study.

0.1 ml of a 1.5% concentration of Acid Blue 62 (purity unknown) in water was placed into the conjunctival sac of the right eye of each animal. The upper and lower lids were held closed for several seconds to avoid any loss of the test substance. The untreated left eye of each animal served as a control. Eyes were not rinsed after the product was administered. Evaluations were made 1 hour after compound administration and at 1, 2, 3, 4 and 7 days thereafter.
Based on the number of lesions observed in the conjunctiva, iris and cornea of each animal, an Individual Index of Ocular Irritation was calculated for each observation time. A mean score for each part of the eye examined was also calculated; the sum of these scores equalled the Mean Index of Ocular Irritation for each observation time. The largest mean index over the 7-day observation period was considered the Index of Acute Ocular Irritation; this was used to classify the test compound in terms of irritant potential.

Results
Conjunctival redness and iridal congestion were observed in all six rabbits 1 hour after compound administration. Chemosis of the conjunctiva was also observed in one rabbit at this time. The Mean Index of Ocular Irritation for this observation time was 7.33. Iridal congestion persisted in two rabbits at the 1-day observation period. The Mean Index of Ocular Irritation for this observation time was 1.67. No other clinical signs were observed during the study.

A 1.5% concentration of Acid Blue 62 (purity unknown) was found to be slightly irritant to the rabbit eye.

Ref.: 2

2.5. Sensitisation

Contact Sensitization in Guinea Pigs

Guideline	:	/
Species	:	Hartley albino guinea pigs
Group size	:	20 (10 females, 10 males)
Test substance	:	Acid Blue 62
Batch No.	:	/
Purity	:	/
Dose levels	:	Induction: 0.1ml Freund's Complete Adjuvant diluted to 50% in saline given intradermally on days 1 and 10 of the study (test substance not given subcutaneously). 0.5 ml of undiluted Acid Blue 62 appliedtopically three times a week a 2 day intervals under a 2 cm^2 moistened gauze.
Route	:	epicutaneously
GLP	:	/

Twenty (20) Hartley albino guinea pigs (body weight: 300-400 g) were used in the principal study.
Two preliminary studies were conducted to determine the challenge concentration of the test compound to be used in the principal study. The purity of the dye used for these studies was unknown.
The treatment region for each animal was clipped once a week. Ten male and ten female guinea pigs were administered a 0.1 ml intradermal injection of Freund's Complete Adjuvant (FCA) diluted to 50% in sterile isotonic saline on days 1 and 10 of the study. Beginning on day 1 of the study, 0.5 ml of undiluted Acid Blue 62 was applied topically to the treatment site (which was just above the injection site) three times per week at 2-day intervals and once at the beginning of the fourth week. It was applied using a 2 cm^2 square of gauze that was moistened with water and kept in place by an occlusive patch.

Treatment was suspended on day 24; the challenge application of 0.5 ml Acid Blue 62 at a concentration of 25% (w/w) was administered on untreated skin on the clipped left flank of the animals on day 36 of the study. This application was left on the skin for 48 hours under an occlusive patch. The skin was evaluated for evidence of sensitization, e.g. erythema and oedema, at 1, 6, 24 and 48 hours after removal of the patch. Treatment sites were biopsied 6-7 hours after patch removal due to staining of the skin that prevented evaluation of erythema at the treatment site.

Results
Four males and one female died during the course of the study; these deaths were not attributed to compound administration. No oedema was observed during the study. No abnormal histopathological findings were observed. Acid Blue 62 (purity unknown) at a concentration of 25% was non-sensitizing to the guinea pig under the conditions of this study.

Conclusion
This test is unacceptable.

Ref.: 4

Murine Local Lymph Node Assay

Guideline	:	OECD Draft 429 LLNA
Species	:	CBA/J mice
Group size	:	4 female
Test substance	:	Acid Blue 62
Batch No.	:	01515-2000P-0
Purity	:	98.4%
Dose levels	:	0.5% to 25%
Route	:	epicutaneously to ear
GLP	:	in compliance

Forty (40) female CBA/J mice were used for this study (mean body weight: 23.1 ± 1.1 g). Acid Blue 62 (98.4% pure) was prepared in the vehicle ethanol/water (50/50, v/v). The reference item (positive control), OR10432, was prepared in the same vehicle at 0.5%. The reagent used for the proliferation assay was [^{3}H]-methyl thymidine (^{3}H-TdR); this was diluted in 0.9% NaCl 3 days prior to injection.
Two separate experiments were performed; five groups of four females were used in each. In both experiments, Group 1 received the vehicle and Group 5 received the reference item, OR10432, at a concentration of 0.5%. In the first experiment, Groups 2, 3 and 4 received topical applications of concentrations of 0.25, 2.5 or 25% Acid Blue 62, respectively, on the dorsal surface of both ears. In the second experiment, Groups 2, 3 and 4 received topical applications of concentrations of 5, 10 or 25% Acid Blue 62, respectively, on the dorsal surface of both ears. Animals were treated for three consecutive days (days 1-3) in both experiments. All animals were lightly anesthetized to facilitate treatment.
Animals were observed once a day for clinical signs. Body weights were recorded on day 1 and day 6 of the study. On days 1 and 3 (before compound administration) and on day 6 (after killing), ear thickness measurements and local reactions were recorded to assess the level of irritation induced by the compound.
On day 6 of each experiment, all animals received a single intravenous injection of ^{3}H-TdR in 0.9% NaCl. Five hours later, they were killed by cervical dislocation and the auricular lymph nodes were removed. Nodes were pooled for each group. A single cell suspension of auricular lymph node cells was prepared from each group of nodes, and the amount of cell proliferation was assessed. The values obtained were used to calculate Stimulation Indices.

Results
None of the animals exhibited any adverse clinical signs or evidence of skin irritation during the study. Body weights were similar among treated and control groups. The mean Stimulation Indices for the test materials are presented below. Values ≥ 3 indicate sensitization.

Experiment 1			Experiment 2		
Treatment	Concentration (%)	Stimulation Index	Treatment	Concentration (%)	Stimulation Index
Acid Blue 62	0.25	1.83	Acid Blue 62	5	1.35
Acid Blue 62	2.5	2.46	Acid Blue 62	10	2.28

Acid Blue 62	25	1.66	Acid Blue 62	25	1.44
OR10432	0.5	11.81	OR10432	0.5	5.38

Under the conditions of the study, Acid Blue 62 (98.4% pure) did not induce delayed contact hypersensitivity in the LLNA.

Comments
The chemical identity of the positive control substance is not known.

Ref.: 5

2.6. Reproductive Toxicity / Teratogenicity

Dose-range finding prenatal development toxicity study

Guideline	:	/
Species/strain	:	Sprague Dawley rat
Group size	:	7 females / dose level
Observation period	:	20 days
Test substance	:	Acid Blue 62
Batch no	:	9110003
Purity of test substance	:	not stated in study report, 53.4% according to the summary (dye with dispersing agents)
Dose levels	:	0, 25, 100, 400 mg/kg bw/day (in water)
GLP statement	:	/

Three groups of seven mated female rats were administered Acid Blue 62 (53.4% pure) by oral gavage at doses of 25, 100 or 400 mg/kg bw/day (13, 53 or 214 mg/kg bw/day active dye) from day 6 through day 15 of gestation. An additional group of seven mated rats was administered the vehicle (water for injections) and served as a control group. The day of mating was designated as day 0 of pregnancy.
Animals were checked twice daily for mortality/morbidity, and once daily for clinical signs. Food consumption and body weight were recorded at designated intervals during pregnancy. On day 20 of pregnancy, the animals were killed and examined macroscopically. Foetuses were removed by Caesarean section. The following litter parameters were recorded: number of corpora lutea and implantation sites, number and distribution of early and late resorptions, and number and distribution of dead and live foetuses. Foetuses were weighed, sexed and submitted to external examinations. Calculations were made for pre- and post-implantation loss, observations in foetuses, and the total number of litters within each group containing foetuses with a particular observation.

Results
No mortality, resorptions or test substance-related clinical signs occurred in the dams during the study.
Litter data and foetal examinations from treated foetuses did not differ from those for control foetuses. No external malformations were observed.

Conclusion
Acid Blue 62 (53.4% pure) was neither maternotoxic, embryotoxic nor teratogenic at the doses of 25, 100 and 400 mg/kg bw/day (up to 214 mg/kg bw/day active dye).

Based on the results of this study, the doses for the prenatal developmental toxicity study were set on 0, 300, 1000 mg/kg bw/day.

Ref.: 11

Prenatal development toxicity study

Guideline	:	OECD 414 (1981)
Species/strain	:	Wistar rat
Group size	:	25 females / dose level
Observation period	:	20 days
Test substance	:	Acid Blue 62
Batch no	:	9110003
Purity of test substance	:	not stated in study report, 53.4% according to the summary
Dose levels:		0, 300, 1000 mg/kg bw/day (in water)
GLP statement	:	/

Two groups of 25 pregnant rats received Acid Blue 62 (53.4% pure) by oral gavage at doses of 300 or 1000 mg/kg bw/day (160 or 534 mg/kg bw/day active dye) from day 6 through day 15 of gestation. A third group of 25 pregnant rats received the vehicle only (water for injections) and served as a control group. The day of mating was designated as day 0 of pregnancy.

Animals were checked twice daily for mortality/morbidity, and once daily for clinical signs. Food consumption and body weight were recorded at designated intervals during pregnancy. On day 20 of pregnancy, the animals were killed and examined macroscopically. Foetuses were removed by Caesarean section. The following litter parameters were recorded: number of corpora lutea and implantation sites, number and distribution of early and late resorptions, and number and distribution of dead and live foetuses. Foetuses were weighed, sexed and submitted to external, soft tissue and skeletal examinations. Calculations were made for pre- and post-implantation loss, observations in foetuses, and the total number of litters within each group containing foetuses with a particular observation.

Results
No mortality occurred during the study. Ptyalism was noted after dosing in 6/25 females at 300 mg/kg bw/day and in all animals at 1000 mg/kg bw/day. On a single occasion, regurgitation was noted in two females and loud breathing was noted in one female at 1000 mg/kg bw/day. Food consumption and body weight gain were lower at 1000 mg/kg bw/day, with statistically significant differences in body weight gain being recorded on the first three days of treatment. Litter data and foetal examinations from treated foetuses did not differ from those for control foetuses. No anomalies or malformations of toxicological significance were observed.

Conclusion
Acid Blue 62 (53.4% pure) was noted to have maternotoxic effects at 1000 mg/kg bw/day, but was well tolerated at 300 mg/kg bw/day. It was not embryotoxic or teratogenic at any dose level. The No Observed Adverse Effect level was considered to be 300 mg/kg bw/day (160 mg/kg bw/day active dye) for the pregnant female rat and 1000 mg/kg bw/day (534 mg/kg bw/day active dye) for the foetus.

Ref.: 12

2.7. Toxicokinetics (incl. Percutaneous Absorption)

In Vitro Percutaneous Penetration Study using Human Dermatomed Skin

Guideline	:	/
Species	:	human
Group size	:	2 dermatomed samples from each of 4 donors
Test substance	:	Acid Blue 62
Batch No.	:	01515-2000P
Purity	:	98.4%
Dose levels	:	100 µg/cm^2 tested in a Hair Dye Formulation (473220). No details of formulation given
GLP	:	in compliance

Human skin samples from four donors were obtained from cosmetic surgery. They were kept frozen at about –18°C until they were used.
Two dermatomed skin samples per donor were used. Samples were mounted in static diffusion chambers with phosphate buffered saline as the receptor fluid. Their integrity was verified by measuring Trans-Epidermal Water Loss. Two separate experiments were performed.
Twenty (20) mg/cm² of the hair dye formulation 473220 containing 0.512 ± 0.016% (w/w) Acid Blue 62 (98.4% pure, corresponding to 101 ± 5 µg/cm² of the test substance) were applied to the skin surface and left for 30 minutes. After this time period, any of the formulation remaining on the skin was removed using a standardized washing procedure.
Twenty-four (24) hours after application, the percutaneous penetration of Acid Blue 62 was determined by measuring the concentration of the compound using HPLC and UV-Visible detection in the following compartments: skin excess, stratum corneum, epidermis + dermis, and receptor fluid.

Results
All eight samples tested yielded data that could be used. Most of the hair dye remaining on the skin after the application period was removed in the washing procedure. The cutaneous distribution of Acid Blue 62 (mean ± SD) was as follows:

Skin excess	92.20 ± 4.48 µg/cm² (91.32 ± 2.56%) of the applied dose
Stratum corneum	0.25 ± 0.12 µg/cm² (0.25 ± 0.13%) of the applied dose
Epidermis + dermis	0.20 ± 0.02 µg/cm² (0.20 ± 0.02%) of the applied dose
Receptor fluid	0.27 ± 0.04 µg/cm² (0.26 ± 0.05%) of the applied dose
Total recovery	92.04 ± 2.64% of the applied dose

The "total skin + receptor fluid" amount of Acid Blue 62 (stratum corneum + epidermis + dermis + receptor fluid) was 0.72 ± 0.15 µg/cm² (0.71 ± 0.18%) of the applied dose.
The "total absorbed" amount (epidermis + dermis + receptor fluid) was 0.47 ± 0.05 µg/cm² (0.46 ± 0.06%) of the applied dose. This represents the amount to be taken into consideration for the calculation of the safety factor.

The percutaneous penetration figure to be taken into consideration for calculation of the safety factor for Acid Blue 62 (98.4% pure) is 0.47 ± 0.06 µg/cm².

Ref.: 13

Comments
The wavelength (280 nm) used for the quantification of the dermally penetrated material is not appropriate. The quantification of the dermally absorbed dye should have been performed at its λ_{max}, 575 nm/ 637nm. The chemical nature of the material, determined at 280 nm, is not known. The material absorbing at 280 nm may also be a constituent, other than dye, of the formulation or it may be a biological material. The composition of formulation for percutaneous absorption study is not described.

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Assay

Guideline	:	OECD 471 (May 1983)
Species/strain	:	*S. typhimurium* TA 1535; TA 1537; TA 98; TA 100; TA 102
Test substance	:	Acid Blue 62
Batch number	:	/
Lot number	:	9110003
Purity	:	certificate of analysis
Concentrations	:	312.5–5000 µg/plate without S9 and in two tests with S9 (5 doses); 156.25–2500 µg/plate without S9
Replicates	:	3 plates/dose
Positive controls	:	according to guideline
Metabolic activ.	:	Aroclor induced rat liver homogenate (purchased). No control was made on it.
GLP	:	in compliance

Results
Toxicity study: a toxicity study was performed with the strain TA 100 with 6 doses (10–5000 µg/plate); a slight toxicity was observed with the maximum dose in the presence of S9.

Mutagenicity study: in the first experiment without S9 there was an extensive toxicity at three doses in the strains TA 1535, TA 1537; TA 102; this effect was not repeated in the second experiment. No toxicity was observed in the presence of S9 in the two experiments. The test item did not induce revertant colonies in a number higher than the control.
The positive controls gave the expected results

Conclusion
Acid Blue 62 was not mutagenic on *Salmonella typhimurium.*

Ref.: 8

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

Guideline	:	OECD 473 (May 1983)
Species/strain	:	Human lymphocytes from two healthy donors (M/F)
Test substance	:	Acid Blue 62
Batch number	:	/

Lot number	:	911003
Purity	:	certificate of analysis
Concentrations	:	3 doses:
		1st experiment –S9: 100, 200, 300 µg/ml (24 h)
		2nd experiment –S9: 125, 250, 500 µg/ml (24h)
		62.5, 125, 250 µg/ml (48h)
		1st experiment +S9: 3, 30, 100 µg/ml (24h)
		2nd experiment +S9: 25, 50, 100 µg/ml (2h)
Replicate	:	2 cultures/dose
Metabolic acti.	:	Aroclor induced rat liver homogenate (batch no.34)
Positive control	:	MMC: -S9; CPA: +S9
GLP	:	In compliance

Results
Toxicity study: in a preliminary study 6 concentrations were tested: a considerable toxicity at the concentrations higher than 625 µg /ml was recorded. The data are not included in the report.

Clastogenicity study: A reduction of the mitotic index was observed in some concentrations. The two positive controls gave the expected results. 200 metaphases/concentration were scored. No chromosome abnormalities were observed in all treated cultures, at all times and conditions. Under the condition of the assay, the test item did not induce chromosome aberrations

Conclusion
Acid Blue 62 was not clastogenic in the mammalian cells treated *in vitro*.

Ref.: 9

2.8.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus test

Guideline	:	OECD 474 (May 1983)
Species/strain	:	Swiss OF1/ICO: OF1 (IOPS Caw)
Test substance	:	Acid Blue 62
Batch number	:	/
Lot number	:	911003
Purity	:	Certificate of analysis
Dose levels	:	500, 1000, 2000 mg/kg (5M+5F/group)
Treatment time	:	administration by oral route, twice every 24 hours; animals sacrificed after the 2nd treatment
Positive control	:	CPA 50 mg/kg (one treatment; oral)
GLP	:	In compliance

Results
Toxicity study: 3 animals per sex were treated with a dose of 2000 mg/kg by oral route twice with an interval of 24 hours and observed for 48 hours. No clinical signs of toxicity were observed at the end of the observation.

Mutagenicity study:
Cyclophosphamide (CPA) induced 69.1/2000 MN with a ratio PE/NE of 0.4. The vehicle treated animals showed an induction of MN of 3.0/2000 with a ratio PE/NE of 0.9. The maximum dose

(2000 mg/kg) showed a ratio PE/NE of 0.6 indicating that the compound has reached the target cells.
The study is adequate and can be used for the evaluation. There was no increase of the number of MN cells in the bone marrow of the treated animals at all doses. The test item is not mutagenic in this assay.

Conclusion
The test item is not clastogenic neither aneugenic *in vivo* on mice treated orally.

Ref.: 10

2.9. Carcinogenicity

No data

2.10. Special investigations

No data

2.11. Safety evaluation

Not applicable

2.12. Conclusions

The commercial dye Acid blue 62 may contain 40-60% of dispersing agent(s). The dispersing agent(s) have not been reported. The purity of the dye used in several tests has not been provided. Acid blue 62 is a secondary amine, and thus it is prone to nitrosation. No information is provided on the nitrosamine content of the dye.

Acid Blue 62 (purity unknown) was non-irritant to intact and scarified rabbit skin at the concentration of 1.5%. A 1.5% concentration of Acid Blue 62 (purity unknown) was found to be slightly irritant to the rabbit eye. Acid Blue 62 (purity unknown) at a concentration of 25% was non-sensitizing to the guinea pig under the conditions of the study. However, the test does not conform to guinea pig maximisation test guideline.
Acid Blue 62 (98.4% pure) did not induce delayed contact hypersensitivity in the LLNA.

The available studies (acute oral toxicity, repeated dose toxicity and developmental toxicity) have all been performed with a test substance of a rather low purity.
Therefore, and especially since the composition of the tested batch is not known (purity is about 53%), it is not possible to give an indication of the NOAEL. The exact composition of the test substance must be provided.
Acid Blue 62 has been tested *in vitro* on bacterial cells for the induction of gene mutations and on mammalian cells for the induction of chromosome aberrations. It was found non mutagenic, neither clastogenic. The test item is neither clastogenic nor aneugenic *in vivo* on mice treated orally.

2.13. References

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3. Opinion of the SCCNFP

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

Before any further consideration, the following information is required:

* complete physico-chemical characterisation according to general comments under 2.1.10 and the information on the purity of test substance used in all studies and the composition of the formulation used for the percutaneous absorption study;

Additional information on acute oral toxicity in rats, eye irritation and skin irritation had been expected but was not submitted for evaluation.

4. Other Considerations

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5. Minority Opinions

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