EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

Acid Blue 62

COLIPA N° C 67

Adopted by the SCCP
during the 4th plenary meeting of 21 June 2005

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1. BACKGROUND

Submission I of Acid Blue 62 (COLIPA[1] n° C 67) was received in April 2003.

In its opinion SCCNFP/0782/04 of 25 May 2004, the SCCNFP[2] asked for additional studies. Submission II was submitted as an updated dossier in line with the second step of the strategy on the evaluation of hair dyes:
http://pharmacos.eudra.org/F3/cosmetic/doc/HairDyeStrategyInternet.pdf.

2. TERMS OF REFERENCE

1. On the basis of currently available information, the SCCP is asked to assess the risk to consumers of Acid Blue 62, when used in hair dye formulations.

2 Does the SCCP recommend any further restrictions with regard to the use of Acid Blue 62 in hair dye formulations?

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Acid Blue 62 (INCI name)

3.1.1.2. Chemical names

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

3.1.1.3. Trade names and abbreviations

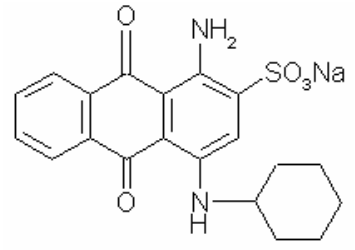
Trade name : Acid Blue 62
Colour Index : CI 62045

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] SCCNFP- Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers

3.1.1.4. CAS / EINECS number

CAS : 4368-56-3
EINECS : 224-460-9

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula : $C_{20}H_{19}N_2NaO_5S$

3.1.2. Physical form

Dark blue powder, odourless

3.1.3. Molecular weight

Molecular weight : 422.43

3.1.4. Purity, composition and substance codes

Acid Blue 62 contains 2-Anthracenesulfonic acid, 1-amino-4-(cyclohexylamino)-9,10-dihydro-9,10-dioxo-, monosodium salt (approximately 60%) together with dispersing agents (22.9% sodium chloride and made up to 100% with sodium lignosulphonate).

Batch No.	**9110003**	**01515-200P-0**	**502**
IR spectrum	In accordance with the proposed structure		
UV spectrum	UV spectra are similar		
Mass spectrum	Compatible	-	Compatible
^1H and ^{13}C NMR spectrum	In accordance with the proposed structure		
Purity[1], HPLC titre	53.4 g/100 g	98.4 g/100 g	98.7 g/100 g
Water content (K.F. method)	-	-	4.7 g/100 g
Loss on drying	3.8 g/100 g	4.5 g/100 g	4.5 g/100 g
Sulfate ion content (as Na_2SO_4)	4.2 g/100 g	-	-
Chloride ion content (as NaCl)	22.9 g/100 g	-	-
Impurities:			
A-A´ (HPLC t_R 2.0-8.5 min)[2]		+	+
B[3]	+	+	+ (<0.1%)[6]

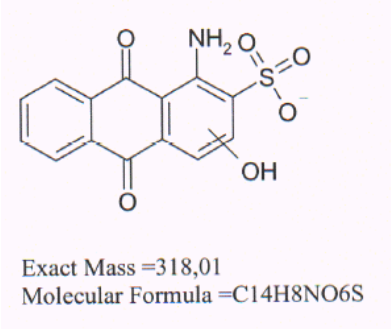
Batch No.	9110003	01515-200P-0	502
C[4]	+	+	+ (0.1%)[6]
D[3]	n.d	+	n.d.
E[5]	n.d.	n.d.	+(0.8%)[6]
F[3]	+	n.d.	n.d.
G[3]	+	n.d.	n.d.
H[3]	+	n.d.	n.d.
Residual solvents	n.d.	n.d.	Acetone (<100 ppm)

1 Determined using a primary standard of Acid Blue 62 (batch 112), which was considered as 100% pure

2 Proposed chemical structure: chemical structure similar to polyethylene glycol containing amino functional groups

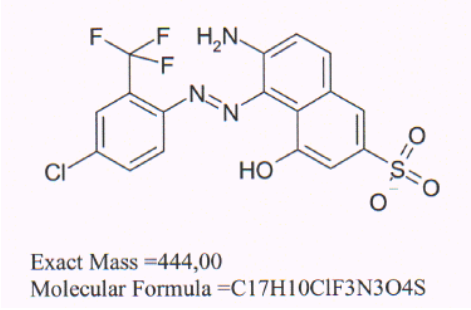
3 Chemical structure could not be established by LC-MS analysis

4 The proposed possible structure is:



Exact Mass =318,01
Molecular Formula =C14H8NO6S

5 The proposed possible structure is an azo dye (Acid Red 266)



Exact Mass =444,00
Molecular Formula =C17H10ClF3N3O4S

6 Content expressed as relative HPLC-peak area

\+ detected but not quantified

n.d. not detected

\- not done or not applicable

The difference in impurities in Acid Blue 62 of different batches is considered due to different routes of synthesis of this dye.

Acid Blue 62 test materials (declared by industry)

Four studies were conducted using a test batch of unknown purity (period 1978-1987, Ref. 1, 3, 5, 7). However, all of these studies were considered valid, contained collateral evidence results, and were repeated with well characterized analytically test batches.

All further studies were conducted using a test batch well characterised analytically, i.e.:

Batch 9110003 (53.4% pure) was used for all studies performed in the period 1993-1995 (Ref. 9-15). In this batch, the main excipients were dispersing agents, i.e. 22.9% sodium chloride and qsp 100% sodium lignosulphonate (according to information given by the supplier).
Batch 01515-200P-0 (98.4% pure) was used for the skin sensitisation study performed in 2002 (Ref. 8), and for the in vitro percutaneous absorption study conducted in 2001 (Ref. 13).
Batch 502 (98.7% pure) was used for studies conducted in 2004 (Ref. 14, 15, 16).

3.1.5.	Impurities / accompanying contaminants

See point 3.1.4. "Purity, composition and substance codes"

3.1.6.	Solubility

Pure dye (without dispersing agent)

Water	:	50 mg/l (according to EC method n° A6, Directive 67/548/EEC)
Ethanol	:	<1% (w/v)
DMSO	:	>1% - < 10 (w/v)

3.1.7.	Partition coefficient (Log P_{ow})

Log P_{ow} : 3.66

3.1.8.	Additional physical and chemical specifications

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

Organoleptic properties	:	/
Melting point	:	> 100 °C
Boiling point	:	/
Flash point	:	/
Vapour pressure	:	/
Density	:	400-600 kg/m³
Viscosity	:	/
pKa	:	/
Refractive index	:	/

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.
Homogeneity and stability of the solutions of Acid Blue 62

Homogeneity
The homogeneity of the test item in purified water (15 mg/ml and 200 mg/ml) is demonstrated.

Stability
The aqueous solutions of Acid Blue 62 (up to 200 mg/ml) were stable, when stored up to 4-hours at room temperature protected from light and under inert gas atmosphere.

General comments on analytical and physico-chemical characterisation

- Acid Blue 62 is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content of the dye is not reported

3.2. Function and uses

Acid Blue 62 is used in semi-permanent and temporary hair colouring products at concentrations up to 0.5%. Acid Blue 62 is also permitted for the use as colorant in other cosmetic products, which have short contact with the skin (Product category 4, according to Annex 4 of the Cosmetics Directive).

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

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

Guideline	:	OECD 420
Species strain	:	Sprague-Dawley rats (mean body weight 206 g)
Group size	:	4 female rats
Observation	:	up to 15 days
Test substance	:	Acid Blue 62
Batch no	:	502
Purity	:	98.7%
Dose level	:	2000 mg/kg
GLP	:	signed statement, no date

A preliminary study was performed using one female rat. The dose of 2000 mg/kg was administered. Because no death was noted during this study, the same dose was chosen for the principal study.
A fixed dose of 2000 mg/kg Acid Blue (purity 98.7%) was administered in the principal study. Animals were observed 30 minutes, 1, 2, 4 and 5 hours after compound administration, and daily thereafter for 15 days. Body weights were recorded on day 1 prior to treatment and days 8 and 15. All surviving animals were sacrificed at the end of the study and a gross macroscopic examination of the main organ was performed.

Results
No clinical signs and no mortality observed at 2000 mg/kg bw. A blue coloration of the fur and tail was noted in all animals from day 1 up to day 15.

Conclusion
The acute oral maximum non lethal dose of Acid Blue 62 (purity 98.7%) in rats was > 2000 mg/kg bw.

Ref.: 14

3.3.1.2. Acute dermal toxicity

/

3.3.1.3. Acute inhalation toxicity

/

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline	:	/
Species	:	New Zealand White rabbits
Group size	:	6 males
Test substance	:	Acid Blue 62
Batch No.	:	AJ5004
Purity	:	/
Dose levels	:	0.5 ml of 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	:	not in compliance

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 applied (0.5 ml) at a concentration of 1.5% in water on two gauze pads, 2 cm² 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

Guideline	:	OECD 404 (2002)
Species strain	:	New Zealand White rabbits
Group size	:	3 male rabbits
Observation	:	up to 6 days
Test substance	:	Acid Blue 62
Batch no	:	502
Purity	:	98.7%
Dose level	:	0.5 ml 1.5% concentration in purified water

0,5 ml of Acid Blue 62 (purity 98.7%, pH 5.75, osmolarity 45 mOsm/l) was firstly administered to a single animal at a concentration of 1,5% in purified water on semi-occlusive patches applied

for 3 minutes on the anterior left flank, 1 hour on the anterior right flank and 4 hours on the posterior right flank. The untreated skin served as a control.
Since the dosage form was not severely irritant in the first animal, it was then applied for 4 hours to two other animals.
The results obtained were evaluated in conjunction with the nature (erythema and/or oedema) and the reversibility of the findings observed at 24, 48 and 72 hours after removal of the dressing. Since there were persistent discolouring reactions at 72 hours in one animal, the observation period was extended up to their complete reversibility (day 6).

Results
After the 3-minute or 4-hour exposure (one animal), only a blue colouration of the skin was observed from day 1 up to day 4. After a 4-hour exposure (three animals), there was no evidence of erythema or oedema at any of the patch test sites at the 24-, 48- and 72 hour observation periods. The compound discoloured the application site in one animal such that erythema could not be evaluated up to day 5: thus the mean score over 24, 48 and 72 hours for erythema was not calculable for 1/3 animal. For the two other animals, it was 0.0 and 0.0. For oedema, mean scores over 24, 48 and 72 hours for each animal were 0.0.

Conclusion
Acid Blue 62 (purity 98.7%) was found to be non-irritant to rabbit skin at the concentration of 1.5% in purified water.

Ref.: 16

3.3.2.2. Mucous membrane irritation

Study 1

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	:	not in compliance

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 (range 0 to 100) for this observation time was 7.33 (5 to 15 slight irritation). 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 following days (2 to 7) of the study.

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

Ref.: 2

Study 2

Guideline	:	OECD 405 (2002)
Species strain	:	New Zealand White rabbits
Group size	:	3 male rabbits
Observ. Period	:	up to 3 days
Test substance	:	Acid Blue 62
Batch no	:	502
Purity	:	98.7%
Dose level	:	0.1 ml of 1.5% in purified water
GLP	:	signed statement, no date

0.1 ml of a 1,5% concentration of Acid Blue 62 (purity 98.7%, pH 5.75, osmolarity 45 mosm/l) in purified water was placed into the conjunctival sac of the left eye one animal, after gently pulling the lower lid away from the eyeball. The upper and lower lids were held closed for about one second to avoid any loss of the compound. The untreated right eye 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, and 3 days thereafter.
Since it was not severely irritant in the first animal, the compound was then evaluated on two other animals in the same experimental conditions. The study was ended on day 4 in the absence of persistent ocular lesions.
Conjunctival reactions (chemosis, redness, discharge), iris lesions and corneal opacification were evaluated daily for each animal. For the evaluation or corneal opacification, examination was realized (on day 2, and repeated thereafter whenever necessary) under a UV lamp after instillation of one drop of 0.5 % sodium fluorescein solution.
The compound is considered irritant for the eyes if significant or severe ocular lesions are caused within 72 hours after exposure and which persist for 24 hours or more after treatment with the compound. The scores at each reading time (24h, 48h and 72h) and for an effect are used for calculating the respective mean values.

Results
Conjunctival redness and/or chemosis were observed in all three rabbits 1 and 24 hours after compound administration. Conjunctival redness was also observed in 1 animal at 48h. No other clinical signs were observed during the study. Mean scores calculated for each animal over 24,

48 and 72 hours were 0.3 for conjunctival redness and/or chemosis and 0.0, 0.0 and 0.0 for corneal opacity.

Conclusion
A 1.5% concentration of Acid Blue 62 (purity 98.7%) was found to be slightly irritant to the rabbit eye.

Ref.: 15

3.3.3.	Skin 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 applied topically (10 times) three times a week a 2 day intervals under a 2 cm^2 moistened gauze. Challenge: 0.5 ml aqueous solution of Acid Blue 62 at 25% (w/w)
Route	:	epicutaneously
GLP	:	not in compliance

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 cm² 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 a 0.5 ml aqueous solution of 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) 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	Concentration (%)	Stimulation Index
Acid Blue 62	0.25	1.83	5	1.35
Acid Blue 62	2.5	2.46	10	2.28
Acid Blue 62	25	1.66	25	1.44
OR10432	0.5	11.81	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

3.3.4. Dermal / percutaneous absorption

***In Vitro* Percutaneous Penetration Study**

Guideline	:	draft OECD 428, 2000
Tissue	:	human abdominal skin
Tissue integrity	:	transepidermal water loss measurement
Method	:	static diffusion cells (6 ml volume)
Test substance	:	Acid Blue 62 commercial formulation 0.512 % ± 0.016 %
Dose applied	:	formulation 20 mg/cm², i.e. 100 µg of Acid Blue 62/cm²
Assay	:	HPLC
Contact	:	30 minutes, then washing of the skin surface, monitoring of the diffusion during 24 hours
Batch no	:	01515-200P, purity 98.4 %
Stability	:	1 month
Replicate cells	:	8 cells (4 donors)
GLP	:	study in compliance

The skin penetration of Acid Blue 62 was evaluated in a static diffusion cell. Human abdominal skin was obtained from 4 different donors and dermatomed to a controlled thickness (329 ± 39 µm). The receptor fluid in the dermal compartment was a phosphate buffer containing 0.9 % NaCl (Acid Blue 62 is soluble in the medium 29 µg/ml. Under the experimental conditions the skin temperature was 31.9°C ± 0.5°C. The hair dye was applied (20 mg of formulation/cm² - 0.512 % i.e. 100 µg/cm² Acid Blue 62) over 2 cm² during 30 minutes, the skin was washed with 2% sodium lauryl sulphate solution and dried. The diffusion was monitored during 24 hours. The mass balance of the study was calculated (92 %)
Results
The absorbed amounts of Acid Blue 62 were as follows (sum of amounts contained in epidermis, dermis and receptor fluid):

- 0.47 ± 0.05 µg/cm²
- 0.46 ± 0.06 % of the applied dose

The dermal absorption of Acid Blue 62 contained at 0.512 % in hair dye formulation was estimated by the applicant to be at most 0.47 μg/cm^2 (0.46 % of the applied dose).

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.
The study is unacceptable.

Ref.: 13

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (14 days) oral toxicity

Guideline	:	OECD 407 (1981)
Species/strain	:	Sprague Dawley rat
Group size	:	12 animals (6 males & 6 females) / dose level
Observation	:	14 days (no recovery group included)
Test substance	:	Acid Blue 62
Batch no	:	9110003
Purity	:	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	:	not in compliance

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
No treatment-related abnormalities were observed at the end of two week among the haematological parameters, blood biochemistry and urinalysis. Test substance-related findings were limited to clinical signs of ptyalism (salivation) 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

3.3.5.2. Sub-chronic (90 days) oral toxicity

Guideline	:	OECD 408 (1981)
Species/strain	:	Sprague Dawley rat
Group size	:	20 animals (10 males & 10 females) / dose level
Observation	:	90 days (no recovery group included)
Test substance	:	Acid Blue 62
Batch no	:	9110003
Purity	:	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	:	not in compliance

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 examined grossly. 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

3.3.5.3. Chronic (> 12 months) toxicity

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3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. 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	:	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
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	:	911003	
Purity	:	certificate of analysis	
Concentrations	:	3 doses:	
		1st experiment, –S9:	100, 300, 500 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 (2h; preparation 24h)
		2nd experiment, +S9:	25, 50, 100 µg/ml (2h; preparation 24h)
Replicate	:	2 cultures/dose	
Metabolic activ.	:	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 stated. 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 increased frequencies of 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*. However, in some trials toxicity of the test substance (reduction of the mitotic index) was less than 50% for the highest concentration tested.

Ref.: 9

3.3.6.2 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	:	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 24 hours after the 2nd treatment
Positive control	:	CPA 50 mg/kg (one treatment; oral)
GLP	:	In compliance

Results
3 animals per sex were treated with a dose of 2000 mg/kg by oral route twice with an interval of 24 hours and evaluated 24h after the second treatment. No clinical signs of toxicity were observed at the end of the observation period.

Cyclophosphamide (CPA) induced 69.1/2000 MN with a ratio PCE/NCE of 0.4. The vehicle treated animals showed an incidence of MN of 3.0/2000 with a ratio PCE/NCE of 0.9. The maximum dose (2000 mg/kg) showed a ratio PCE/NCE 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 in the frequency of MNPCE 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 or aneugenic *in vivo* in mice treated orally.

Ref.: 10

3.3.7. Carcinogenicity

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3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

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3.3.8.2. 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	:	not in compliance

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 to 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	:	not stated in study report, 53.4% according to the summary
Dose levels	:	0, 300, 1000 mg/kg bw/day (in water)
GLP statement	:	not in compliance

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

3.3.9. Toxicokinetics

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3.3.10. Photo-induced toxicity

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3.3.11. Human data

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3.3.12. Special investigations

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3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Regarding the criticism formulated on the percutaneous absorption study, 100 % absorption is considered. The Margin of Safety will be identical independent of the mode of calculation (percentage versus μg/cm^2). Only the calculation based on usage data is shown.

Based on the usage data

Based on a usage volume of 35 ml, containing at maximum 0.5 %

Maximum amount of ingredient applied	I (mg)	=	175 mg
Typical body weight of human		=	60 kg
Maximum absorption through the skin	A	=	1.00
Retention factor	R	=	0.1
Dermal absorption per treatment	I x A x R	=	17.5 mg
Systemic exposure dose (SED)	I x A x R / 60 kg	=	0.29 mg/kg
No observed adverse effect level (mg/kg) (rat, subchronic, oral)	NOAEL	=	160 mg/kg
Margin of Safety	**NOAEL / SED**	**=**	**552**

3.3.14. Discussion

Acid Blue 62 is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content of the dye is not reported.

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).

Acid Blue 62 (53.4% pure) was noted to have materno-toxic 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.

Acid Blue 62 was non-irritant to intact and scarified rabbit skin at the concentration of 1.5%. A 1.5% concentration of Acid Blue 62 was found to be slightly irritant to the rabbit eye.
Acid Blue 62 was non-sensitizing to the guinea pig under the conditions of the study. However, the study is unacceptable.
Acid Blue 62 (98.4% pure) did not induce delayed contact hypersensitivity in the LLNA. The stimulation indexes were < 3, indicating that the substance is a non-sensitiser.

The dermal absorption of Acid Blue 62 contained at 0.512 % in hair dye formulation was estimated by the applicant to be at most 0.47 µg/cm^2 (0.46 % of the applied dose). Regarding the criticism formulated on the percutaneous absorption study, 100 % absorption was considered.

Acid Blue 62 is not mutagenic in bacteria and does not induce chromosome aberrations in mammalian cells *in vitro*. The test item is neither clastogenic nor aneugenic *in vivo* in the bone marrow of mice treated orally.

4. CONCLUSION

The SCCP is of the opinion that the use of Acid Blue 62 as a hair colouring agent ('direct' dye) in semi-permanent and temporary hair dye formulations at a maximum concentration of 0.5% in the finished cosmetic product does not pose a risk to the health of the consumer.
However, its nitrosamine content should not exceed 50 ppb.

Acid Blue 62 is also permitted for the use as a colorant (CI 62045) in other cosmetic products, which have short contact with the skin (Product category 4, according to Annex 4 of the Cosmetics Directive 76/768/EEC).

This assessment pertains only to its use as a direct hair dye. There has been no assessment of the safety of the substance as a colorant for other purposes.

5. MINORITY OPINION

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