Scientific Committee on Consumer Safety

SCCS

OPINION ON

Basic Blue 124 (3-Amino-7-(dimethylamino)-2-methoxyphenoxazin-5-ium chloride)

The SCCS adopted this opinion at its 10th plenary meeting

on 25 June 2015

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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1. BACKGROUND

Submission I on the new hair dye Basic Blue 124 with the chemical name 3-Amino-7-(dimethylamino)-2-methoxyphenoxazin-5-ium chloride (CAS No 67846-56-4) was transmitted by EFfCI in May 2014.

The new ingredient Basic Blue 124 is intended to be used as a direct hair dye in semi-permanent hair dye formulations at on-head concentrations in the range between 0.01% up to a maximum of 0.5% (w/v). The regular frequency of application is intended to be once per month up to once per 8 weeks.

2. TERMS OF REFERENCE

(1) In light of the data provided, does the SCCS consider Basic Blue 124 safe when used as a direct hair dye in semi-permanent hair dye formulations at on-head concentration up to a maximum of 0.5% (w/v)?

(2) Does the SCCS have any further scientific concerns with regard to the use of Basic Blue 124 in cosmetic products?

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Basic Blue 124 (INCI name)

3.1.1.2 Chemical names

3-Amino-7-(dimethylamino)-2-methoxyphenoxazin-5-ium chloride

3.1.1.3 Trade names and abbreviations

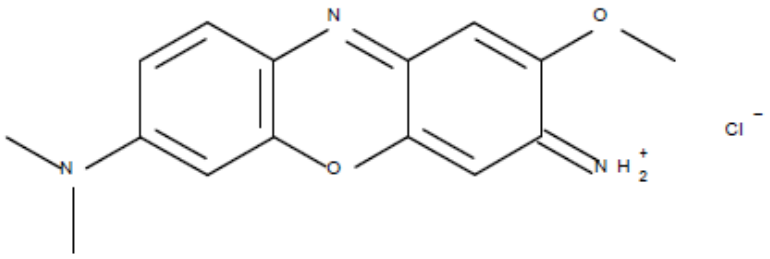
Basic Blue 124
C.I. Basic Blue 124
FAT31'048/I
FAT31'048/F
Maxilon Blau M -2G
Vibracolor Moonlight Blue

Blue MIP 2987 — See Dossier of Applicant
FAT31'048/G — Ref.: RCC Ltd. (1999)
Ref.: CIT (1995)

3.1.1.4 CAS / EC number

CAS: 67846-56-4; 89106-91-2
EC: 267-370-5

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

$C_{15}H_{16}N_3O_2Cl$

3.1.2 Physical form

Hair dye Basic Blue 124 is a solid in the form of a fine powder.

3.1.3 Molecular weight

Molecular weight: 305.8 g/mol
Exact mass: 305.09 g/mol

3.1.4 Purity, composition and substance codes

Basic Blue 124 was chemically characterised using combustion analysis, IR and NMR spectroscopy.

The applicant explains the use of the percent HPLC peak area as an appropriate method for determining approximate purity considering the specific chemical structure of this compound as follows:
To calculate the weight percentages (%(w/w)), the following approximation was used:
Assuming similar absorption coefficients in the UV (256 nm ± 2 nm), the %(w/w) was calculated as %(w/w) = %Area * (100%-∑ *inorganic constituents* + ∑ *solvents*).
(Abbreviations: %(w/w) = weight percentage [g/100 g]; %Area = detected peak area in HPLC at 256 nm ± 2 nm; HPLC: High performance liquid chromatography; inorganic constituents = *water* + *salts*; water: determined by Karl-Fischer-Titration; salts: determined by ion-chromatography; solvents: determined by Head-Space-Gas-Chromatography).

Batch LIA10G080
Purity from combustion analysis (%w/w):
Content on average 97.7% (C, H, N, O, Cl)
Main impurities detected were sodium 4.45%, water 2.30%, sulphur < 0.1%, Ca 14 ppm, K 10 ppm.Ref.: BASF Schweiz AG (2013a)

Lot 0009996997 94.3%, containing 3.41% NaCl and 2.25% water
Lot 0009997000 97.1%, containing 1.32 % NaCl and 1.55% water
Lot 0009997002 96.5%, containing 2.24 % NaCl and 1.20 % water
Potassium, sulfur and sulfate contents were <0.01, <0.1 and <0.1%, respectively.
Furthermore, the lots contained up to 1% organic impurities.
Ref.: BASF Schweiz AG (2014a, b, c)

HPLC analysis (% peak area):	254 nm	600 nm
Lot 0009996997	99.0%	99.2%
Lot0009997000	99.0	99.2%
Lot 0009997002	99.0	99.2%
Lot LIA09G044/2	99.7	99.7%

Ref.: BASF Schweiz AG (2014d)

Other batches or lots mentioned:

Lot LIA09G024/2
Purity 98.2% based on HLPC area, 240 nm; water and salt contents together about 4% resulting in about 94% purity Ref.: BASF Schweiz AG (2015a)
Purity 98.2-98.7% based on HPLC area, 240, 550, 620 nm
Ref.: Ciba Expert Services (2009)
In another report, purity 99.7%, based on HLPC area, was mentioned.
Ref.: BASF SE (2010f)

CGF-FO14192/0012 (purity >96%) Ref.: RCC Ltd. (1999)

LIA10G080 (purity >97.4 area-% (HPLC) Ref.: BASF AG 2013a; CIT (1995)

According to a declaration by the applicant, the evidence for the purity of the batches MIP 2987/4R-4 and CGF-FO14192/0012 is approximately covered by the comparable batch LIA09G024/2.

Ref.: BPCN E-EMC-QR (2015)

SCCS comments
Most analytical reports were not conducted under GLP.
The purity determination of Basic Blue 124 by HPLC was performed at wavelength of 256 nm, while the UV absorption of B124 according to UV spectrum occurs at 196, 242, 294, (451), 624 (max). Moreover, the documentation of ~100% recovery of the material applied on the column and the documentation of ~100% peak purity are both missing. The purity measurement should be performed at λmax 624 nm. Therefore, the purity determination cannot be accepted.

3.1.5 Impurities / accompanying contaminants

Lot 0009997000:

Metal contaminants	Content (mg/kg)	
	Required	Measured
Mercury	≤ 1	< 1
Arsenic	≤ 2	< 1
Cadmium	≤5	< 1
Nickel	≤5	< 1
Lead	≤10	< 1
Antimony	≤10	< 1

IPC-OES method BASF Schweiz AG (2014a)

Vibracolor Moonlight Blue:

Batch number	3-Dimethylaminophenol [mg/kg]	2,4-Diaminoanisol (freebase) [mg/kg]
0009996997	37.5	10.3
0009997000	12.3	11.4
0009997002	4.8	16.8

HPLC-GC/MS/MS method Intertek Expert Services 2013

The DMF content was determined to be < 50 mg/kg for all three test item batches.
GC-MS in selected ion monitoring (SIM) mode was used as method.

Ref.: BASF Schweiz AG (2013)

The total Nitrosamine content, calculated as NO, was determined to be < 0.04 mg/kg for all three test item batches.

Method: thermal energy analyser; the amount of NO formed was then determined by means of a chemiluminescence detector.

Ref.: BASF Schweiz AG (2014)

SCCS comment
The analytical report on Dimethylaminophenol and Diaminoanisol impurities (Intertek Expert Services (2013) is part of the analytical reports BASF Schweiz AG (2014a, b, c).

3.1.6 Solubility

Maxilon Blau M -2G

Solvent	Solubility [% w/w]	Conditions, comments
Water	ca. 15	at pH 4
Dimethylformamide (DMF)	ca. < 0.5	-
Tetrahydrofuran (THF)	ca. < 0.3	nearly insoluble
Methyl isobutyl ketone (MIBK)	none to weak	insoluble at 20-25 °C nearly insoluble at 80 °C
Methyl ethyl ketone (MEK)	none to weak	insoluble at 20-25 °C nearly insoluble at 80 °C
Isopropyl alcohol (iPrOH)	> 5	at 20-25 °C
Methanol	ca. 3	at 20-25 °C
	5.5 %	at boiling temperature
n-Butanol	> 4	at 20-25 °C
Acetone	none	insoluble at 20-25 °C and at boiling temperature

Ref.: BASF Schweiz AG (2014e)

Vibracolor Moonlight Blue

Solubility in propylene glycol (1,2-propandiol): 1.38%

Ref.: BASF Schweiz AG (2015)

SCCS comment
Water solubility has not been determined by EC Method A.6

3.1.7 Partition coefficient (Log P_{ow})

Log P_{ow}: -1.79 (pH 6.2, 23°C); 1-Octanol/water (shake flask method), OECD 107

Ref.: Allessa GmbH (2013)

3.1.8 Additional physical and chemical specifications

Melting point:	>225 °C (OECD 102)
Boiling point:	/
Flash point:	/
Vapour pressure:	≤ 0.066 Pa (20 °C) ;≤ 2.100 Pa (50 °C) (OECD 104)

Density:	1.39 (20 °C) (OECD 109)
Viscosity:	/
pKa:	/
Refractive index:	/
pH:	/

Ref.: Henkel 2013a, b, c

UV_Vis spectrum (nm):
196, 242, 294, (451), 624 (max) (pH 2; pH 7; pH 10)

Ref.: BASF Schweiz AG (2014a)

3.1.9 Homogeneity and Stability

The shelf-life of the hair dye Basic Blue 124 is 5 years. The stability was confirmed in a comparison of HPLC data including a test item batch from 2009 and current batches.

Stability of Basic Blue 124 in water was demonstrated at 0.2 mg/ml 0.6 mg/ml and 5.0 mg/ml (variation from original concentration <6%) for 6h at room temperature (Harlan Laboratories 2014). Basic Blue 124 in aqueous solutions at concentrations 0.8 mg/ml, 2.4 mg/ml and 8 mg/ml was also shown to be stable (variation from original concentration <6%) for 8 days at 5 ± 3 °C (Harlan Laboratories Ltd. (2013).

General Comments to physico-chemical characterisation
The purity determination of Basic Blue 124 by HPLC was performed at wavelength of 256 nm, while the UV absorption of B124 according to UV spectrum occurs at 196, 242, 294, (451), 624 (max). Moreover, the documentation of ~100% recovery of the material applied on the column and the documentation of ~100% peak purity are both missing. The purity measurement should be performed at λmax 624 nm. Therefore, the purity determination cannot be accepted.
- Basic Blue 124 is a tertiary amine. It should not be used together with nitrosating agents. Total nitrosamine content should be < 50 ppb.
- Stability of Basic Blue 124 in typical hair dye formulations has not been reported.

3.2 Function and uses

Basic Blue 124 is intended to be used as a direct hair dye in semi-permanent hair dye formulations at concentrations in the range between 0.01 % up to 0.5 % (w/v) at maximum (BASF SE (2014)). Following application, the hair dye formulations remain for about 30 minutes on the hair and will be rinsed off thereafter.

3.3 Toxicological Evaluation

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

Guideline:	OECD 425, up and down procedure
Species/strain:	Sprague-Dawley rats
Group size:	6 females
Test substance:	FAT31'048/I
Batch:	LIA09G044/2
Purity:	99.7 area% (HPLC)

Vehicle: distilled water
Dose levels: 175, 550, 2000 mg/kg bw in the main test (see below)
Administration: oral (gavage)
GLP: Yes
Study period: Experimental phase July 2009

Methods
In an acute oral toxicity study conducted according to the up and down procedure, 6 fasted (overnight) female Sprague-Dawley rats were given a single oral gavage administration of the test item FAT31'048/I, *i.e.* the hair dye Basic Blue 124 (purity: 99.7 area-% (HPLC, 254 ± 2 nm); batch LIA09G044/2), in distilled water (22.5 % w/w) at dose levels of 175 (2 rats), 550 (3 rats) or 2000 mg/kg bw (1 rat) in the main test. Based on the mortality noted in a pre-test in a single fasted (overnight) female at 2000 mg/kg bw, dosing in the main test was initiated at 175 mg/kg bw with doses spaced by a factor of 0.5 on a log dose scale. The animals were observed for mortality and clinical signs for the first several hours post-dosing and at least once daily thereafter. Bodyweights were determined once prior to the single treatment on day 0 and then on days 7 and 14 (termination). A macroscopic examination was performed in all animals killed on the day of scheduled necropsy and in all premature decedents. The LD_{50} and the confidence limit were calculated using the Acute Oral Toxicity (OECD 425) Statistical Programme (AOT425StatPgm).

Results
Oral administration of the test item resulted in 0/2, 2/3 and 2/2 deaths at 175, 550 and 2000 mg/kg bw, respectively (see Table). Clinical signs noted during the observation period at all dose levels included cyanosis, blue stain in the litter and blue staining (facial and/or ano-genital). In addition, hypo-activity was observed at 550 and 2000 mg/kg bw, and reduced faecal volume as well as blue staining of the region between stomach and tail was seen at 550 mg/kg bw. All surviving animals showed bodyweight gain throughout the observation period. Gross necropsy of decedents revealed blue discolouration of intestines, lungs and heart or diaphragm, as well as discharge from nose and mouth. No gross abnormalities were noted in animals necropsied at the end of the 14-day observation period. On the basis of the mortality incidence, the oral median lethal dose (LD50) in female rats was estimated to be 550 mg/kg bw, with a 95% profile likelihood-based confidence interval (CI) of 88.94 - 2430 mg/kg bw.

Dose level [mg/kg bw]	Toxicological result[a]	Time of clinical signs	Time of death	LD_{50} [mg/kg bw]
Female rats				
175	0/2/2	days 0-2	-	-
550	2/3/3	days 0-4, 6, 14	days 1 and 2	550[c]
2000[b]	2/2/2	days 0-1	days 0[c] and 1	-

LD: lethal dose
a: number of animals which died / number of animals with clinical signs / number of animals used
b: including the pre-test animal
c: the 95 % profile likelihood-based confidence interval was 88.94 - 2430 mg/kg bw.
Day 0 was the day of test item administration, day 14 was the day of scheduled necropsy.

Conclusion
Under the conditions of this acute oral toxicity study, the test item FAT31'048/I, i.e. the hair dye Basic Blue 124, was slightly to moderately toxic in female rats based on a LD50 of 550 mg/kg bw (95 % CI: 88.94 – 2430 mg/kg bw).

Ref.: Eurofins PSL (2010a)

SCCS comment
Based on an LD50 value of 550 mg/kg bw, the test item is considered as being of moderate acute oral toxicity.

3.3.1.2 Acute dermal toxicity

Guideline:	OECD 402
Species/strain:	Sprague-Dawley rats
Group size:	5 per sex (one dose level)
Test substance:	FAT31'048/I
Batch:	LIA09G044/2
Purity:	99.7 area% (HPLC)
Vehicle:	distilled water (60 % w/w mixture)
Dose levels:	2000 mg/kg bw as a limit dose
Administration:	topical
GLP:	Yes
Study period:	Experimental phase July 2009

Methods
Groups of 5 Sprague-Dawley rats/sex/dose levels were given a single topical application of the test item FAT31'048/I as a dry paste (60 % w/w mixture in distilled water) at a dose level of 2000 mg/kg bw (limit test). The test item was applied to a skin area corresponding to approximately 10% of the body surface area. The animals were exposed for a period of 24 hours under occlusive conditions and then observed for 14 days. Mortality and clinical signs were assessed at least once daily. Bodyweights were recorded once prior to application and again on days 7 and 14 (termination). Necropsies were performed on all animals at terminal sacrifice.

Results
Dermal administration of the test item resulted in no mortality at the limit dose level of 2000 mg/kg bw.
Apart from dermal irritation (erythema) noted at two dose sites between days 1 and 5 (2/10 rats) and a blue stain at all dose sites (10/10 rats), there were no other clinical findings recorded for any animal during the 14-day observation period. Although 2 females lost bodyweight by day 7, all animals gained bodyweight through the observation period. No gross lesions or abnormalities were noted for any of the animals when necropsied at the end of the observation period. Due to the lack of mortality, the dermal median lethal dose (LD_{50}) in male and female rats was > 2000 mg/kg bw.

Conclusion
Under the experimental conditions employed, the test item FAT31'048/I was practically non-toxic in male and female rats based on the dermal LD50 of > 2000 mg/kg bw and the lack of clinical signs of systemic toxicity.

Ref.: Eurofins PSL (2010b)

SCCS comment
Two females showed transient loss of bodyweight indicating signs of toxicity at 2000 mg/kg bw, suggesting that test substance might be absorbed by rat skin to some extent (see section 3.3.4). However, no discolouration of urine or faeces was reported.

3.3.1.3 Acute inhalation toxicity

No data.

3.3.1.4 Acute intraperitoneal toxicity

No data.

3.3.2 Irritation and corrosivity

3.3.2.1 Skin irritation

Guideline:	OECD 404
Species/strain:	New Zealand rabbits
Group size:	3 young adult females
Test substance:	FAT31'048/I
Batch:	LIA09G044/2
Purity:	99.7 area% (HPLC)
Vehicle:	distilled water (60% w/w mixture)
Dose level:	0.5 g
Dose volume:	0.83 g
Observation:	7 days
GLP:	Yes
Study period:	Experimental phase July 2009

Methods
In a primary dermal irritation study, 3 young adult female New Zealand rabbits were exposed via the dermal route to FAT31'048/I. Prior to application, the test item was moistened with distilled water to achieve a dry paste by preparing a 60% (w/w) mixture. Approximately 0.5 g of the test item (0.83 g of the dose formulation) was placed on a gauze pad and applied to one 6-cm² intact dose site on each animal. The pad and entire trunk of each animal were then wrapped with semi-occlusive 3-inch Micropore tape to avoid dislocation of the pad. Elizabethan collars were placed on each rabbit. After 4 hours of dermal exposure to the moistened test item, the pads and collars were removed and the test sites were gently cleansed of any residual test item. The animals were observed for 7 days. Dermal readings were taken at 30-60 min, and at 24, 48 and 72 hours after patch removal. Skin irritation was scored for each individual animal using the Draize scoring system. A primary dermal irritation index (PDII) was calculated by adding the average erythema and oedema scores for each scoring time point and dividing by the number of time points. The animals were observed for clinical signs of systemic toxicity at least once daily during the 7-day observation period.
In the study report, the classification system according to U.S. EPA Addendum 3 on data reporting to pesticide assessment guidelines, Dermal Irritation (January 1988), was used (with PDII 0: non-irritating, PDII > 0 – 2.0: slightly irritating, PDII 2.1 – 5.0: moderately irritating, PDII > 5.0: severely irritating).
For the purpose of the present submission, results are presented in line with EU requirements (see Table).

Results
The individual and mean skin irritation scores noted at 24, 48 and 72 hours after patch removal are presented in the table below. There was no oedema noted at any treated site during this study. Within 1 hour of patch removal, all treated sites exhibited very slight erythema (grade 1). The overall incidence and severity of irritation decreased during the observation period. All animals were free of dermal irritation by day 7. The PDII was calculated to be 0.6, indicating slight irritation.
All animals appeared active and healthy during the study. Apart from slight dermal irritation, there were no other signs of gross toxicity, adverse pharmacologic effects, or abnormal behaviour.
Light blue staining at all dose sites was noted at all observation time points.

Animal [number]	Erythema / Edema score[a]				Reversibility
	24 hours	48 hours	72 hours	mean	
Female rabbits					
3501	1 / 0	0 / 0	0 / 0	0.3 / 0	within 48 hours
3502	0 / 0	0 / 0	0 / 0	0 / 0	-
3503	1 / 0	1 / 0	1 / 0	1 / 0	within 7 days

a: the max. score was "4" for both erythema and edema.

Conclusion
Under the conditions of this study, the test item FAT31'048/I was not a skin irritant according to EU criteria.

Ref.: Eurofins PSL (2010b)

SCCS comment
Under the conditions of this study, the test substance is mildly irritating to rabbit skin; classification according to the CLP criteria is not warranted.

3.3.2.2 Mucous membrane irritation / Eye irritation

1.) Eye irritation study in rabbits

Guideline: OECD 405
Species/strain: New Zealand rabbits
Group size: 3 females
Test substance: FAT31'048/I
Batch: LIA09G044/2
Purity: 99.7 area% (HPLC)
Vehicle: distilled water (20% w/w mixture)
Dose level: 0.1 mL (0.02 g)
Dose volume: 0.83 g
Observation: 4 days
GLP: Yes
Study period: Experimental phase July 2009

Methods
Three female New Zealand White rabbits received a single application of 0.1 mL (0.02 g) of the test item FAT31'048/I into the conjunctival sac of the right eyelid. The untreated left eye of each animal served as negative control. Scoring of irritation effects was performed at 1, 24, 48 and 72 hours and at 4 days post-instillation for each individual animal according to the Draize scoring system by means of a high-intensity white light source (Mag Lite). A fluorescein dye evaluation procedure was used in the treated eye at 24 hours to verify the absence of corneal damage. The time interval with the highest mean score (maximum mean total score, MMTS) for all rabbits was used to classify the test item. Clinical signs of toxicity were recorded at least once daily during the test period, which was terminated 4 days after the instillation.
In the study report, the classification system according to Kay and Calandra (1962) was presented and used for evaluation. This system consists of 8 MMTS intervals covering a total MMTS score range from "0" to "110" ("non-irritant" to "maximally irritant"). A MMTS score of "21.3" as determined in the present study leads to classification as "mildly irritating" (MMTS interval "15.1" to "25.0").
For the purpose of the present submission, results are presented in line with EU requirements (see Table).

Results
Instillation of the test item (no washing) resulted in mild and transient irritant responses in all rabbits (readings at 24, 48 and 72 hours), which would not trigger classification according to EU criteria (see Table).
The test item was classified as a mild irritant based on a MMTS value of 21.3 determined for the 1-hour reading. At 1 hour after instillation, all three treated eyes exhibited iritis (score "1"), and conjunctivitis (score "2" or "3" for conjunctival redness, score "3" for discharge), and one treated eye showed corneal opacity (score "1" for both opacity degree and area involved).
The overall incidence and severity of irritation decreased with time. All animals were free of ocular irritation by day 4. Blue staining of the cornea was noted in a single rabbit at the 1-hour reading only. Blue staining in the fur around the entire eye due to blue ocular discharge was observed in all animals at all observation time points.

Parameter	Irritation score[a]				Reversibility
	24 hours	48 hours	72 hours	mean	
Rabbit 3401					
Corneal opacity	0	0	0	0	-
Iris findings	1	0	0	0.3	within 48 hours-
Conjunctival redness	2	2	1	1.7	within 4 days
Chemosis	1	1	0	0.7	within 72 hours
Rabbit 3402					
Corneal opacity	0	0	0	0	-
Iritis	0	0	0	0	-
Conjunctival redness	2	2	1	1.7	within 4 days
Chemosis	1	1	0	0.7	within 72 hours
Rabbit 3403					
Corneal opacity	0	0	0	0	-
Iris findings	1	0	0	0.3	within 48 hours-
Conjunctival redness	2	2	1	1.7	within 4 days
Chemosis	1	1	0	0.7	within 72 hours

a: the max. scores were "4" for corneal opacity (degree of density), "2" for iritis, "3" for conjunctival redness and "4" for chemosis.

Conclusion
In this *in vivo* study, the test item FAT31'048/I, i.e. the hair dye Basic Blue 124, was a mild irritant to the rabbit eye. Ocular irritation was fully reversed within 4 days after test item instillation.

Ref.: Eurofins PSL (2010c)

SCCS comment
Under the conditions of this study, the test substance is irritating to the rabbit eye (score 2 for conjunctival redness).

2.) Bovine corneal opacity and permeability test (BCOP test) in vitro

Guideline:	OECD 437, Regulation 1152/2010/EC, No B.47
Test system:	isolated corneas from the eyes of freshly slaughtered cattle
Endpoints:	opacity and permeability
Treatment period:	10 min followed by a 2-hours post-incubation period
Test substance:	Maxilon Blau M -2G

Batch: LIA09G044/2
Purity: 99.7 area-% (HPLC, 254 ± 2 nm)
Concentrations: 1% (w/v) in de-ionised water
Exposure conditions: single topical application of 750 µL
Positive control: 1 % (w/v) sodium hydroxide in deionised water
Negative control: de-ionised water
GLP status: Yes
Study period: March 2013

Methods
Due to the intense colour of the test item, the protocol with only 10-min exposure (for liquids) was selected for study conduct. Exposure to the test substance was finished by rinsing with deionised water. De-ionised water served as negative control item **(NC)** and 1% (w/v) Sodium hydroxide in deionised water served as positive control item **(PC)**, applied to three corneas each.
Corneal opacity was measured quantitatively as the amount of light transmission through the cornea using an opacitometer. Permeability was measured quantitatively as the amount of Sodium fluorescein dye that passed across the full thickness of the cornea by spectro-photometrical determination of the optical density at 490 nm (OD 490). Both measurements were used to calculate an *in vitro* irritancy score (IVIS) of the test item relative to the control corneas.
The IVIS value was calculated by means of the following formula:
IVIS = mean opacity value + (15 × mean OD490 value) (formula 1)
The evaluation criteria were as follows:
IVIS > 55 (ocular corrosive or severe irritant)
IVIS ≤ 55 (no ocular corrosive or severe irritant)

Group	Mean opacity [± SD, n = 3][a]	Mean permeability [± SD, n = 3][a]	IVIS [± SD, n = 3]
NC	1.5 ± 2.1	0.002 ± 0.002	1.6 ± 2.1
Basic Blue 124 (1 % w/v)	0.7 ± 0.7	0.001 ± 0.001	0.7 ± 0.7
PC	121.9 ± 1.2	2.274 ± 0.607	156.0 ± 10.1

n: number of samples, NC: negative control (deionised water), PC: positive control (1 % (w/v) Sodium hydroxide), SD: standard deviation

a: mean values of corrected individual values are presented; negative opacity change values were set to zero for further calculations.

Results
The results of corneal opacity and permeability determinations and the calculated IVIS values are summarised in the table above. The IVIS value of the hair dye Basic Blue 124 did not indicate a test item-related risk of serious damage to eyes. The positive control item demonstrated appropriate sensitivity of the test system.

Conclusion
Under the conditions of this study, the test item Maxilon Blau M -2G, Basic Blue 124, i.e. the hair dye Basic Blue 124, did not cause serious eye damage in the BCOP test at a concentration of 1% (w/v) in deionised water, which covers the maximum intended concentration of Basic Blue 124 in hair dye products. The applicant concluded that the result does not exclude an irritation potential of the test item at the tested concentration.

Ref.: BASF SE (2013a)

SCCS comment
Under the conditions of this study, serious eye damage was not observed; however, an eye irritation potential of the test substance cannot be excluded.

3.) EpiOcular™ eye irritation test in vitro

Guideline:	/
Test system:	Four EpiOcular™ tissue samples of a reconstructed three-dimensional human cornea model (EpiOcular™)
Endpoints:	Cell viability by measurement of dehydrogenase activity (MTT test)
Treatment period:	30 min followed by a 2-hours post-incubation period
Test substance:	Maxilon Blau M -2G
Batch:	LIA09G044/2
Purity:	99.7% (HPLC, 254 ± 2 nm)
Concentrations:	1% (w/v) in deionised water
Exposure conditions:	Single topical application of 50 µL
Positive control:	Methyl acetate at a volume of 50 µL
Negative control:	50 µL of de-ionised water
GLP:	Yes
Study period:	March 2013

Methods
Because of the test item colour, 4 instead of 2 tissue samples were treated with the test item. The 2 additional tissues served as a colour control **(CC)** which was required to allow for differentiation between Formazan production (as a measure of metabolic activity) and test item residues in the colorimetric test. Due to the test item colour, it was not possible to determine whether the test item is able to reduce a Tetrazolium salt (MTT, substrate for metabolic activity determination) directly. Therefore, an additional MTT reduction control (**KC**, freeze-killed control tissues) was introduced.

Tissue destruction was determined by measuring the metabolic activity of the tissue after exposure/post-incubation using a colorimetric test. The reduction of mitochondrial dehydrogenase activity, measured by reduced Formazan production after incubation with MTT, was chosen as endpoint.
The Formazan production of test item-treated epidermal tissues was assessed spectrophotometrically via the optical density at 570 nm (OD570) and compared to that of negative control (**NC**) tissues concurrently treated with 50 µL of de-ionised water. The quotient of the values indicated the relative tissue viability. In this *in vitro* test system, irritant potential of the test item was predicted from the mean relative tissue viabilities compared to the NC tissues. The test item was considered as irritant if the mean relative tissue viability of the test item-treated tissues was ≤ 50 %. At present no prediction is performed in this *in vitro* test if the mean relative tissue viability with a test item is > 50 and ≤ 60 %, as the cut off value is currently being evaluated within this range (see Table). Methyl acetate at a volume of 50 µL was used as positive control (**PC**) item.

Criteria for prediction of an irritant potential:

Mean tissue viability [% of NC]	Prediction
≤ 50	irritant
> 50 ≤ 60	no prediction
> 60	non-irritant

NC: negative control

Results
The mean viability of the test item-treated tissues determined after an exposure period of 30 min with about 2 hours post-incubation and corrected for colour interference was 67% as compared with the negative controls (see the table below). The test item was not able to reduce MTT directly. The value for inter-tissue variability of the test item was minimally out of the acceptance range of ≤ 20 %. Since all other quality criteria of the test were met and the viability values of both test item-treated tissues were well above the cut-off for eye irritation, this deviation was not considered to adversely affect the study result. The PC item demonstrated appropriate sensitivity (relative viability < 50 %, expected tissue viability of approximately 25 %) of the tissues used under test conditions.

Group	Relative viability	
	Mean (n = 2) [% of NC]	Inter-tissue variability [%]
NC	100	16.3
Basic Blue 124 (1 % w/v)a	67	20.3
PC	23	9.5

n: number of samples, NC: negative control (deionised water), PC: positive control (Methyl acetate)

a: the mean value for the test item was corrected by subtraction of the colour control (CC) mean value. CC tissues were test item-treated but not MTT incubated. The results of reduction controls (KC, freeze-killed control tissues) were not considered in viability calculation.

Conclusion
Under the experimental conditions employed, the test item Maxilon Blau M -2G, Basic Blue 124, i.e. the hair dye Basic Blue 124, did not cause eye irritation in the EpiOcularTM eye irritation test at a concentration of 1% (w/v) in deionised water, which covers the maximum intended concentration of Basic Blue 124 in hair dye products. On this basis and in view of the results of the BCOP test (BASF SE (2013a), aqueous Basic Blue 124 formulations at concentrations of up to 1% (w/v) are not considered as irritating to the eye.

Ref.: BASF SE (2013b)

SCCS comment
Based on the fact that only 2 tissues were used per condition, considerable tissue variability is observed. Raw data on OD measurements are not included and the subtraction of the different control values was described in a very confusing manner in the test report. For these reasons, the SCCS does not consider the results obtained from the EpiOcular test as valid. In principle, a new EpiOcular test would be needed, taking into account not only positive and negative controls, but also proper mild irritancy benchmarks.

General SCCS comment on eye irritation

The maximum intended concentration of Basic Blue 124 in a hair dye product is 0.5% (w/w) and the *in vivo* study indicates only a mild irritation potential of the test substance at 20% (w/w). No severe eye irritation potential was observed in the BCOP test for 1% (w/w) Basic Blue 124. Due to several shortcomings, the SCCS does not consider the results obtained from this test valid.

3.3.3 Skin sensitisation

Local Lymph Node Assay (LLNA)

First study

Guideline:	OECD 429; Commission Regulation (EC) No 440/2008, B.42; US EPA OPPTS 870.2600
Species/strain:	Female CBA/J mice
Group size:	
Test substance:	FAT 31'048/I Maxilon Blau M -2G
Batch:	LIA09G044/2
Purity:	99.7 area-% (HPLC)
Vehicle:	Propylene glycol
Concentrations:	1st run 0, 2.5, 5 and 10% (w/w); 2nd run 0, 0.1, 0.25 and 1 % (w/w)
Positive control:	α-Hexyl cinnamic aldehyde (HCA)
GLP:	Yes
Study period:	May-September 2010

A pre-test was performed to determine the highest test item concentration that does not induce local signs of skin irritation and/or systemic toxicity. Propylene glycol was used as the vehicle because good homogeneity of the preparation (suspension) was achieved. The highest test item concentration that could be technically used was 20 % (w/w). The highest dose tested (20%) induced skin irritation on the ears and bodyweight loss as a sign of systemic toxicity. This dose was not used in the main test. Dose levels of 2.5, 5 and 10% (w/w) were selected for the 1st run of the main test. Due to increases in lymph node parameters and ear weights in all test groups, further concentrations of 0.1, 0.25 and 1% (w/w) were tested in a 2nd run of the main test.

In the main tests, the test item was applied at concentrations of 2.5, 5 and 10% (w/w; 1st run) as well as 0.1, 0.25 and 1% (w/w, 2nd run). The vehicle Propylene glycol alone was used as a negative control. A concurrent positive control was not included; however, results of recent studies with the positive control item α-Hexyl cinnamic aldehyde (HCA) conducted in the same laboratory indicated appropriate sensitivity of the test system under the test conditions chosen.
The stimulation index (SI), i.e the increase above control, was calculated for each treated group for the endpoints cell count, ^{3}H-Thymidine incorporation, lymph node weight and ear weight. The increase of cell count by a factor of ≥ 1.5 and/or of ^{3}H-Thymidine incorporation by a factor of ≥ 3 as compared to the concurrent vehicle control group was considered to be indicative of the test item's sensitising potential.

The mean bodyweights of the animals treated at 5 and 10% (w/w) decreased moderately over the study period, indicating systemic toxicity. The test item caused a systemic discoloration as well as blue discoloured urine in all test group animals from day 1 up to termination on day 5.

A dose-dependent increase of lymphocyte proliferation was induced as noted by ^{3}H-Thymidine incorporation (see Table below). The threshold concentration for sensitisation induction was SI≥3 and this was used to calculate the EC3 value (estimated concentration that led to the SI of 3.0) by linear regression to be 1.2%.

Table: Stimulation indices (SI) on day 5

Group	**SI** **based on ^{3}H-Thymidine incorporation**
Vehicle	1

0.1%	1.34
0.25%	1.93*
1%	2.70**
2.5%	4.92**
5%	9.39**
10%	10.44**

Statistical comparisons with the concurrent vehicle control by means of the Wilcoxon test (*p ≤ 0.05; ** p ≤ 0.01)

Conclusion
Because the lymph node response observed in this study after dermal exposure to the test item FAT 31'048/I Maxilon Blau M -2G, i.e. the hair dye Basic Blue 124, was not fully attributable to the ear skin irritation observed, the test item suspended in Propylene glycol exhibited a skin sensitisation potential at concentrations of 2.5% (w/w) or above in the murine local lymph node assay under the test conditions chosen.

Ref.: BASF SE (2010f)

SCCS comment
Although the solubility in propylene glycol (1,2-propandiol) is low (1.38%), the vehicle is considered appropriate for the LLNA. Nominal concentrations of the test item solved might be much lower than the nominal concentrations in the suspension. However, the test item becomes bioavailable to some extent: application of 5%, 10% and 20% of the test substance dose-dependently caused body weight losses of 6%, 8% and 11%, respectively..
Analytically determined mean concentrations of the test item in the suspension were around 10% lower than the nominal concentrations. Accordingly, the corrected value for the EC3 is about 1.1%. Based on the conditions and results of this study, Basic Blue 124 can be considered to be a strong sensitiser (see SCCP 2005).

Second study

Guideline: / (similar to OECD 429;
Species/strain: Female CBA/J mice
Group size: 4 female mice per concentration
Test substance: FAT 31'048/F Maxilon Blau M -2G
Batch: LIA09G024/2
Purity: 99.7 area-% (HPLC)
Vehicle: Suspension in acetone/olive oil (4:1 v/v; AOO)
Concentration: 0%, 0.1%, 1%, 5%
Positive control: α-Hexyl cinnamic aldehyde (HCA) in AOO)
GLP: No
Study period: Experimental phase April-May 2009

According to the established evaluation criteria, the test item was considered to be non-irritant when applied at concentrations of up to and including 5 % (w/v) in AOO (see the table below). All dermal irritation scores of test item-treated mice were 0 for both erythema and oedema formation.

All mice were active and healthy during the study. All animals treated at the highest concentration of 5% (w/v) exhibited blue urine and blue staining in the ano-genital area, but were otherwise active and healthy throughout the study period. While all animals treated at 5% (w/v) lost bodyweight, all other animals gained weight over the entire 6-day study period. The bodyweight loss seen at 5% (w/v) was considered to be test item-related. There were no other clinical signs of toxicity observed in this study.

Treatment with the test item at concentrations of 0.1, 1 or 5 % (w/v) according to the LLNA protocol resulted in no stimulation of lymph node proliferation equal to or exceeding a factor of 3 as compared with concurrent vehicle controls. Treatment with the positive control item resulted in a SI value exceeding the cut-off value of 3, thus confirming sensitivity of the test system (see Table below). On this basis, the test item did not produce a skin sensitisation response in mice at concentrations up to and including 5 % (w/v) in AOO.

Table: Stimulation index (SI) and irritant response on day 6 (treatment on days 1-3)

Group	SI
Vehicle control	1.00
0.1%	0.64
1%	1.96
5%	2.68
Positive control	3.41

Conclusion
Based on the results of this study, the test item FAT 31'048/F, i.e. the hair dye Basic Blue 124, suspended in Acetone/olive oil (4:1 v/v) was not a skin sensitiser at concentrations up to and including 5% (w/v) in the murine local lymph node assay under the test conditions chosen. This covers the maximum intended concentration of Basic Blue 124 in hair dye products.

Ref.: Eurofins PSL (2009)

SCCS comment
The purity of batch LIA09G024/2 was not indicated in the study report but was determined elsewhere (BASF 2010f).
The mouse strain CBA/J was not indicated in the study report.
Given the solubility of the test substance in water and alcohols, the choice of the vehicle is not clear and has not been justified. Acetone/olive oil (4:1 v/v) is presumably a poor solvent for Basic Blue 124. Bioavailable concentrations of the test item might be much lower than the nominal concentrations in the suspension. Dermal absorption was demonstrated only at the highest concentration of 5% (discoloured urine and loss of body weight). The test item induced a dose-dependent increase of lymphocyte proliferation up to 2.7 in the high dose group (5%).
The stimulation index of the positive control (25 % (w/w) α-Hexyl cinnamic aldehyde (HCA) in AOO) is small (SI = 3.41) compared with historical data (SI = 11.86) (BASF SE (2010f)) suggesting low sensitivity of the test system in the test facility. For these reasons, the study is considered of limited value and a sensitisation potential of Basic Blue 124 cannot be excluded

Guinea pig maximization tests (GPMT)

First study

Guideline:	OECD 406; Directive 96/54/EEC, B.6
Species/strain:	Guinea pig
Group size:	5 control animals; 10 animals in the treatment group
Test substance:	Blue MIP 2987
Batch:	CGF-FO14192/0012
Purity:	>96 % (see SCCS comment)
Vehicle:	Bi-distilled water
Concentration:	5% (w/w) for intradermal induction; 50% and 3 % (w/w) for epicutaneous induction and challenge, respectively
Positive control:	2-Mercaptobenzothiazole
GLP:	Yes
Study period:	Experimental phase July-August 1999

In a pre-test, appropriate concentrations for the main test of 5, 50 and 3 % (w/w) were selected for intradermal induction, epicutaneous induction and epicutaneous challenge with the test item, respectively.

Appropriate sensitivity of the test system was demonstrated in a preceding GPMT, which was conducted with the positive control item 2-Mercaptobenzothiazole in the same laboratory shortly prior to the conduct of the main test.

On day 1, the intradermal induction of sensitization in the treatment group was performed with 5 % (w/w) test item or the vehicle control.
On day 8, the epicutaneous induction of sensitisation in the treatment group was conducted under occlusive conditions at a test item concentration of 50 % (w/w) or the vehicle control.
On day 22, the treated and control animals were challenged by epicutaneous application of patches saturated with the test item at 3 % (w/w) and with patches saturated with vehicle.
All skin reactions evaluated in this study were assessed and graded according to the scoring system by Magnusson and Kligman. An allergic reaction was defined by visible reddening of the challenge site. Based upon the percentage of animals sensitised (24- and 48-hour reading following patch removal, challenge exposure), the test item was assigned to 5 grades of allergenicity according to Magnusson and Kligman, ranging from weak to extreme.

Two animals of the treatment group were found dead on day 8. At necropsy, distended stomach, gray-white clay-coloured liver and dense parenchymal foci in the lungs were noted. The cause of death could not be established for these premature decedents. No clinical signs of toxicity were evident during the treatment or in the control animals during the study. Body weights were within the range commonly recorded for males of this strain and age.

Skin effects noted after the intradermal treatment on day 1 were FCA-related, and represented common and expected findings.
The epicutaneous treatment with the test item at 50 % (w/wwas initiated on day 8, and resulted in no oedema at 24 and 48 hours. Due to a dark-blue discolouration of the application sites produced by the test item, a possible erythema reaction could not be assessed at the reading time points. No treatment-related skin reactions (erythema, oedema) were noted in the controls at the 24- and 48-hour readings.

Challenge:
Following the epicutaneous challenge exposure to the test item at 3 % (w/w) there were no skin reactions in the treatment or control animals at the 24- and 48-hour readings. Blue discolouration of the application sites was noted directly after patch removal.
The concurrently conducted epicutaneous exposure of treatment and control animals to only the vehicle also failed to produce any skin reactions.

Conclusion
Under the conditions of this guinea pig maximisation test, the test item Blue MIP 2987 was not a skin sensitiser.

Ref.: RCC Ltd. (1999)

SCCS comment
No evidence for purity >96% has been provided for this batch.
Due to a blue discolouration of the application sites produced by the test item, a possible erythema reaction could not be assessed at the reading time points after induction. The same may be true for reading after challenge although the test sites were depilated three hours prior to the challenge readings in order to remove test item-related discolouration.
The value of the test is considered limited and a weak potential of sensitisation cannot be ruled out by this test.

Second study

Guideline:	OECD 406; Directive 92/69/EEC, B.6
Species/strain:	Guinea pig
Group size:	5 control animals/sex; 10 animals/sex in the treatment groups
Test substance:	FAT 31'048/G
Batch:	MIP 2987/4R-4
Purity:	>99 % (see SCCS comment)
Vehicle:	isotonic saline
Concentration:	1% (w/w) for intradermal induction; 20% and 10 % (w/w) for epicutaneous induction and challenge, respectively
Positive control:	2,4-Dinitro chlorobenzene
GLP:	Yes
Study period:	Experimental phase Nov-Dec 1994

In a pre-test the appropriate concentrations for the main test of 1, 20 and 10 % (w/w) were selected for intradermal induction, epicutaneous induction and epicutaneous challenge with the test item, respectively.
Appropriate sensitivity of the test system was demonstrated in a preceding GPMT, which was conducted with the positive control item 2,4-Dinitro chlorobenzene in the same laboratory shortly prior to the conduct of the main test.

The sensitisation potential of the test item was evaluated after a 10-day induction period, during which the animals were treated the test item or the vehicle. On day 1, the test item at 1 % (w/w) together with FCA was administered by intradermal injection to all animals of the treatment group, while the control animals received the vehicle only. All animals received epicutaneous treatments with Sodium Laurylsulfate on day 7 in order to create local irritation.
On day 8, the epicutaneous induction of sensitisation in the treatment group was conducted under occlusive conditions at a test item concentration of 20 % (w/w). Control animals were treated similarly with the vehicle only.

On day 22, the treatment and control animals were challenged by epicutaneous application of patches saturated with the test item at 10 % (w/w) and with patches saturated with vehicle.
All skin reactions evaluated in this study (pre-test and main test) were assessed and graded according to the Draize scoring system. A reaction was considered positive when macroscopic cutaneous reactions were clearly visible (erythema and/or edema score ≥ 2). Based upon the percentage of animals sensitised (24- and 48-hour reading following patch removal, challenge exposure), the test item was assigned to 5 grades of allergenicity, ranging from very weak to extreme.

One animal of the treatment group was found dead on day 17. The cause of death could not be established, but was considered not to be test item-related. No clinical signs of toxicity were noted. Bodyweight gains of the treated animals were normal as compared with the concurrent controls.

The epicutaneous treatment with the test item at 20% (w/w initiated on day 8 resulted in blue discolouration noted at 1 hour after patch removal. The application sites of test item-treated animals could not be reliably assessed for skin reactions. No signs of skin irritation were noted in the controls.

Following the epicutaneous challenge exposure to the test item at 10% (w/w), a blue discolouration was noted at the test item-treated site in all animals. This discolouration potentially masked very slight or well-defined erythema (grade 1 or 2) in 9/10 and 2/10 control animals as well as in 10/19 and 7/19 treated animals at the 24- and 48-hour reading, respectively. In the treatment group, well-defined erythema (grade 2) was observed in 2/19 animals 24 hours after patch removal. At the 48-hour reading, erythema persisted in both animals, and was only very slight (grade 1) in one of them (see Table below). No oedemas were noted after the challenge treatment in control and treated animals at the 24- and 48-hour reading.
The concurrently conducted epicutaneous exposure of treatment and control animals to the vehicle only resulted in no skin reactions (data not shown).

Table: Dermal responses following epicutaneous challenge in male and female guinea pigs (based on erythema)

Endpoint	Dermal reaction after patch removal[a]			
	Control group		Treatment group	
	24 hours	48 hours	24 hours	48 hours
Sensitization rate [%]	0	0	11	11
Incidence	0/10	0/10	2/19	2/19

a: challenge performed with 10 % (w/w) of the test item

Histopathologically, acanthosis and mononuclear cell infiltration were noted in the 2 control and 7 treatment animals examined. Both findings were often associated with mononuclear cell infiltration and vascular ectasia.
The microscopic findings noted were assessed as attributable to the preparation of the skin (shaving) in 1 control (1/2) and in 2 treated animals (2/7) and as attributable to irritation (test substance at 10 % (w/w) and/or shaving) in the remaining 1 control (1/2) and 5 treated animals (5/7).

Conclusion
The test item FAT 31'048/G, i.e. the hair dye Basic Blue 124, was a weak skin sensitiser under the conditions of this guinea pig maximisation test.

Ref.: CIT (1995)

SCCS comment
Purity was claimed to be >99%. However, a certificate of analysis was not provided.

SCCS general conclusion on sensitisation
Two LLNA tests in mice and two GPMT tests were performed. In the LLNA tests, Basic Blue 124 dissolved/suspended in propylene glycol revealed a strong sensitising potential whereas the second test performed with a suspension of the test substance in acetone/olive oil (4:1 v/v; AOO) was negative, probably due to the insolubility of Basic Blue 124 in this vehicle. The two GPMT tests also yielded different results: one was negative and the second one showed a weak sensitising potential. Masking of effects by coloured skin in the GPMT tests cannot be excluded. The SCCS considers the LLNA with propylene glycol as the most reliable test and used the EC3 value of this test to estimate the potency. The EC3 value of Basic Blue is 1.1.%; according to the definitions provided by the SCCS (2005) Basic Blue 124 is considered to have a strong sensitising potential.

3.3.4 Dermal / percutaneous absorption in vitro

First study

Guideline:	OECD 428, SCCS/1358/10
Species/strain:	Human skin
Membrane integrity:	Electrical resistance barrier integrity
Group size:	12 skin membranes from 5 human donors
Method:	Human dermatomed skin
Test substance:	Basic Blue 124
Batch:	1007-2006 (^{14}C-ring-labelled test item) LIA10G080, unlabelled test item
Purity:	98.8 area-% (RHPLC), radio-labelled test item 97.4 area-% (HPLC), unlabelled test item (ca. 2% impurity of an unidentified isomer)
Doses applied:	100 µg/cm^2 of test substance in 20 mg/cm^2 of the test formulation
Dose volume/amount:	254 µg of test substance in 50.4 mg of the test formulation
Receptor fluid:	Phosphate buffered saline
Method of Analysis:	Liquid scintillation counting (LSC)
GLP:	Yes
Study period:	Experimental phase June-July 2013

Methods
The test item Basic Blue 124 (nominal radiochemical purity: 98.8 area-% (RHPLC), batch 1007-2006 (radiolabelled test item) and purity: 97.4 area-% (HPLC, 254 ± 4 nm), batch LIA10G080 (unlabelled test item) was formulated in a typical hair dye product at a concentration of 0.5% (w/w). The penetration of Basic Blue 124 out of this hair dye formulation through human dermatomed skin over 24 hours (un-occluded) was then determined *in vitro* using static glass diffusion cells. The mass balance and distribution of Basic Blue 124 within the test system following the 24-hour run time was also determined.

An electrical resistance barrier integrity test was performed and any human skin sample exhibiting a resistance <10 kΩ was excluded from penetration measurements. Discs of intact human dermatomed skin membranes (12 skin membranes from 5 human donors)

were then mounted, dermal side down (exposed membrane area of 2.54 cm²), in diffusion cells and maintained at a constant temperature (32 ± 1 °C). The receptor chambers of the diffusion cells were filled with a recorded volume of degassed Phosphate buffered saline (PBS), which ensured free partition of the test item into the receptor fluid (adequate sink conditions).

The dose was applied for a period of 30 minutes at a nominal rate of 100 µg/cm² (nominal total dose of 254 µg) in terms of test item and 20 mg/cm² (nominal total dose of 50.8 mg) in terms of test item formulation. At the end of the exposure period, the skin was washed repeatedly with water and aqueous 2% (v/v) Sodium dodecyl sulfate (SDS) solution, and between each set of washes aspirated three times. After the final washes, which were performed with water only, the skin surface was dried using slightly moistened sponge swabs. At the end of the 24-hour run time, a mass balance procedure was performed. The skin was washed with sponges soaked in 3% (v/v) Teepol®L in water and with further sponges pre-wetted with water. The stratum corneum was removed by a tape-stripping process removing up to a maximum of 20 strips from each skin membrane. The flange skin was cut away from the dermis and the epidermis on the remaining skin disk, and the dermis was separated from the dermis using a heat separation technique.
The penetration process was monitored using [^{14}C]-radiolabelled Basic Blue 124, which was incorporated into the formulation prior to application. The distribution of Basic Blue 124 within the test system was measured and a 24-hour penetration profile was determined by collecting receptor fluid samples (0.5, 1, 2, 4, 8, 12, 24 hours after application). Receptor fluid samples, donor chamber wash, skin wash, stratum corneum, remaining epidermis (following tape stripping), dermis and flange were analysed for radioactivity contents by means of liquid scintillation counting (LSC). The systemic availability dose was considered to be the Basic Blue 124 detected in the sum of the remaining skin, dermis and receptor fluid.
Radioactivity content and homogeneity of the test item formulation were determined by LSC. The radiochemical purity, stability and concentration of Basic Blue 124 in the formulation were determined by means of a HPLC method.

Results
The mean penetration rate of the test item was 0.0001 µg/cm²/h between 0-30 minutes. Between 0-4 hours, the rate was 0.00006 µg/cm²/h, after which penetration through human skin slowed to 0.000006 µg/cm²/h between 4-24 hours. Between 0-24 hours, the penetration rate was, on average, 0.00001 µg/cm²/h.
The amounts of test item that penetrated through human skin at 4, 8 and 12 hours were 0.0002 ± 0.0001 µg/cm², 0.0002 ± 0.0002 µg/cm² and 0.0003 ± 0.0002 µg/cm², respectively. These respective amounts expressed as percentages of the applied dose were all 0.0003%. The mean amount penetrated over the entire 24-hour study period was 0.0004 ± 0.0002 µg/cm², corresponding to 0.0004% of the applied dose.

Three of the 12 dosed cells either had a mass balance outside the range of 85-115% (SCCS, 2010) or a penetration profile that indicated membrane damage and were therefore rejected and not included in the evaluations.
Mean recovery of the applied test item was 89.8 ± 3.33% (n = 9) (see Table below), with individual cell values ranging from 86.1 to 95.3%. The mean amount of remaining test item that was removed by washing the skin surface 30 minutes after application was 89.7 ± 3.33% (80.4 ± 2.98 µg/cm²). Washing at 24 hours removed a further amount of 0.033 ± 0.011% (0.030 ± 0.010 µg/cm²). The mean amount of the dose present in the outer layers of the stratum corneum was 0.009 ± 0.008 % of the applied dose (0.008 ± 0.007 µg/cm²) with 0.018 ± 0.018 % of the dose (0.016 ± 0.016 µg/cm²) present in the remaining epidermis. The mean amount recovered from the dermis was 0.0006 ± 0.0005% of the applied dose (0.0005 ± 0.0004 µg/cm²).
The proportion of the applied dose present in the receptor fluid after 24 hours was 0.0004 ± 0.0002% (0.0004 ± 0.0002 µg/cm²). The mean total non-absorbed dose (donor chamber, skin wash, stratum corneum and flange skin) represented 89.8 ± 3.33% (80.4 ± 2.98 µg/cm²) of the applied dose.

The mean total systemically available dose of Basic Blue 124 (remaining skin, dermis and receptor fluid) was 0.019 ± 0.018% of the applied dose (0.017 ± 0.016 µg/cm²) (see Table below).
LSC analysis of the test item formulation confirmed homogeneity prior to and following dosing. HPLC analysis demonstrated that the radiochemical purity of the labelled test item was 99.5% for a 24-hour period following application.

Table: Summary of Basic Blue 124 distribution in the *in vitro* test system (human epidermis)

Test compartment	Basic Blue 124 recovery (mean ± SD, n = 9)	
	[µg/cm²]	[% of applied dose]
Donor chamber	0.010 ± 0.011	0.011 ± 0.012
Skin wash at 30 minutes	80.4 ± 2.98	89.7 ± 3.33
Skin wash at 24 hours	0.030 ± 0.010	0.033 ± 0.011
Stratum corneum	0.008 ± 0.007	0.009 ± 0.008
Remaining epidermis	0.016 ± 0.016	0.018 ± 0.018
Dermis	0.0005 ± 0.0004#	0.0006 ± 0.0005#
Flange	0.002 ± 0.001	0.002 ± 0.002
Receptor fluid	0.0004 ± 0.0002#	0.0004 ± 0.0002#
Total non-absorbed[a]	80.4 ± 2.98	89.8 ± 3.33
Systemically available[b]	0.017 ± 0.016	0.019 ± 0.018
Total recovered	80.4 ± 2.99	89.8 ± 3.33

a.: sum of the applied dose retrieved in the donor chamber, skin wash (30-minute value plus 24-hour value), *stratum corneum* and flange skin

b: sum of the applied dose retrieved in the remaining epidermis, dermis and the receptor fluid[13]

#: value between the mean limit of detection (LOD) and the mean limit of quantification (LOQ)

SD: standard deviation; n: number of samples

Conclusion
Under the conditions of this study, the hair dye Basic Blue 124, formulated at the maximum in-use concentration of 0.5 % (w/w) in a typical hair dye product, penetrated through human dermatomed skin at an extremely slow rate. The extent of penetration was only 0.0004 ± 0.0002 µg/cm² (24 hours) following dermal application of a nominal test item dose rate of 100 µg/cm². The mean total systemically available dose of Basic Blue 124 was 0.017 ± 0.016 µg/cm².
According to the recommendations given by SCCS (2012), the mean value for dermal delivery plus 1 standard deviation (mean + 1 SD) is used for the systemic exposure dose (SED) calculation (please refer to section 5.9). This results in a dermal absorption value of 0.033 µg/cm² (0.017 µg/cm² + 0.016 µg/cm²).
This *in vitro* dermal absorption study conducted with human skin complies with the SCCS "Basic criteria" for dermal/percutaneous absorption testing as defined by SCCS (2012).

Ref.: Dermal Technology Laboratory Ltd. (2014)

SCCS comment
A systemically available dose of Basic Blue 124 of 0.017 + 0.016 µg/cm² = **0.033 µg/cm²** (mean + 1 SD) will be used for the calculation of the systemic exposure dose.

Second study

Guideline:	OECD 428
Species/strain:	Human skin
Membrane integrity:	Electrical resistance barrier integrity
Group size:	7 skin membranes/dose from 3 human donors
Method:	human split-thickness skin membranes
Test substance:	FAT 31'048/I, Maxilon Blau M -2G
Batch:	1007-2006 (radio-labelled test item) LIA09G044/2, unlabelled test item
Purity:	98.4 area-% (RHPLC), radio-labelled test item 99.7 area-% (HPLC), unlabelled test item
Doses applied:	0.01 mg/cm² and 0.1 mg/cm²
Vehicle	water
Dose volume:	500 µL
Receptor fluid:	Phosphate buffered saline
Method of Analysis:	Liquid scintillation counting (LSC)
GLP:	Yes
Study period:	Experimental phase December 2011-February 2012

Methods
The percutaneous penetration of the test item FAT 31'048/I Maxilon Blau M 2-G, i.e. the hair dye Basic Blue 124 (nominal radiochemical purity: 98.4 area-% (RHPLC); batch 1007-1037 (radiolabelled test item) and purity: 99.7 area-% (HPLC, 254 ± 2 nm); batch LIA09G044/2 (unlabelled test item), following topical application to split-thickness human skin was assessed in this study. The nominal target dose levels were 0.01 mg/cm² (low dose; corresponding to the test item concentration recommended for use) and 0.1 mg/cm² (high dose; corresponding to the maximum test item concentration which might result from use of a product containing the test item). The test item concentration in water was about 1 mg/mL and 10 mg/mL for the low dose treatment and high dose treatment, respectively.
Split-thickness human cadaver skin (upper leg dorsal from 3 individual Caucasian donors, 2 male and 1 female) was mounted in the diffusion cells between the donor and receptor chamber (7 cells per dose level) of an automated flow-through cell system. The skin integrity was assessed under occluded conditions (donor chamber covered with adhesive tape), and membranes with a permeability coefficient (Kp) >2.5 × 10-3 cm/h were excluded from the experiment. Following the test of skin membrane integrity, the cells were left under open conditions overnight (donor chamber uncovered) with saline (0.9 % NaCl w/v) flowing through the receptor chamber.
Then, a volume of 6 µL of dose formulation was applied to a skin area of 0.64 cm² on each membrane. Percutaneous absorption under non-occluded conditions (donor chamber covered with permeable tape) was then assessed by collecting the perfusates at 1-hour intervals during 0-8 hours post dose and at 2-hour intervals during 8-24 hours post dose. Exposure was terminated at 8 hours post dose by washing the skin surface. The surface of the skin membrane was thoroughly rinsed with a mild shower gel solution (1% in tap water, 3 times), gently brushed with cotton swabs wetted with the mild shower gel solution (2 times) and finally rinsed with water and dried with one cotton swab. The experimental period was terminated after 24 hours and the washing procedure repeated as described above. Loosely attached test item remaining in/on the application site was removed by tape stripping the application site twice.
Radioactivity was measured by Liquid Scintillation Counting (LSC) equipped for computing quench-corrected disintegrations per minute (dpm). Liquid specimens, i.e. perfusate and cell wash, were added directly to scintillation mixture Irgasafe for the measurement of radioactivity. Ethanol was added to each sample of skin membrane rinse. Aliquots were then mixed with Irgasafe prior to LSC. The radioactivity in tape strips and skin membranes was determined after digestion in Solvable tissue solubiliser. Aliquots of the digested specimens

were mixed with Irgasafe prior to LSC. Background values were measured with each specimen sequence using the respective scintillation mixture without any specimens.
The radiochemical purity of the test item in the dose formulations was assessed by HPLC with a UV detector and a radioactivity flow monitor.

Results
The amounts of dose recovered in the perfusates and tape-stripped skin membranes exclusive tape strips I and II (absorbed dose), and in the skin rinses, the tape strips I and II and the donor cell washes (unabsorbed dose) are summarised in the table below.
Within the exposure period of 8 hours, negligible low amounts of the applied radioactivity penetrated through the human skin membranes into the receptor fluid at both doses, i.e. less than 0.01% for each dose. The flux, which reflects the penetration rate under steady-state conditions, i.e. within the first 8 hours, was not calculated as most of the data were below the limit of determination.

At the end of the 8-hour exposure period, almost the entire applied dose could be removed from the application site by skin membrane rinse, which represented on average 95.63% and 102.61% of the applied low and high dose, respectively. An additional amount of 1.23% and 0.11% could be removed by the second skin membrane rinse at 24 hours for the low and high dose, respectively. After the rinsing procedure, small amounts could be recovered from the surface of the skin membranes by applying two tape strips, i.e. 0.30% of the low dose and 0.02% of the high dose. After tape stripping, 0.20% and less than 0.01% remained in the skin membrane for the low and high dose, respectively.

The totally absorbed test item was calculated based on the amount penetrated through the skin membrane (perfusate) and the amount measured in the remaining skin membrane after tape stripping. The total absorption was calculated to be on average 0.21% and less than 0.01% for the low and high dose, respectively. The mass balance was complete, with 97.40% (low dose) and 102.75% (high dose) of the applied dose recovered.
At the time of application, the radiochemical purity of the labelled test item in the dose formulations was determined to be 98.6% (low dose) and 98.9 % (high dose).

Conclusion
Under the conditions of this *in vitro* study, the test item FAT 31'048/I Maxilon Blau M 2-G , i.e. the hair dye Basic Blue 124, in an aqueous formulation reflecting in-use conditions penetrated through split-thickness human skin to a very low extent.

SCCS comment
SCCS "Basic criteria" for dermal/percutaneous absorption testing (2010) require 8 skin samples (per dose) from at least 4 individual human donors.
The total absorption was calculated to be on average 0.21% and less than 0.02% for the low and high dose, respectively. This corresponds on average to an absorbed amount of <0.001 µg-eq/ml and 0.001 µg-eq/ml for the low and high dose, respectively. Nearly all values were found below the limits of quantification (LOQ), which were 0.003 µg-eq/ml for the low dosage and in the range 0.0022-0.0030 µg-eq/ml for the high dosage, respectively. The use of a SD value is not considered useful in this case.

Table:
Amounts of test item dose recovered after application in a representative aqueous in-use dilution (0.01 mg/cm²) and in an aqueous formulation reflecting a "high dose exposure scenario" (0.1 mg/cm²) to human split-thickness skin

	Recovery [% of dose]	
Test system	Human skin membrane	
Nominal dose	0.01 mg/cm² [a]	0.1 mg/cm²
Applied dose	9.9 µg/cm²	92.4 µg/cm²
Perfusate		
0-8 hours	< 0.01	< 0.01
8-24 hours	< 0.01	< 0.01
Subtotal	< 0.01	< 0.01
Skin membrane (exclusive tape strips I and II)	0.20	< 0.01
Total absorption	**0.21**	**< 0.01**
Skin membrane rinse		
8 hours	95.63	102.61
24 hours	1.23	0.11
Subtotal	96.85	102.72
Tape strip I	0.23	< 0.01
Tape strip II	0.08	< 0.01
Subtotal	0.30	0.02
Dislodged dose	**97.15**	**102.74**
Donor cell wash	0.03	0.01
Recovery	**97.40**	**102.75**

a: mean data were calculated from 6 instead of 7 cells; data from the remaining cell were disregarded due to much higher penetration values as compared with the other cells.

Ref.: Harlan Laboratories Ltd. (2012)

General SCCS comment on dermal absorption

A systemically available dose of Basic Blue 124 of 0.017 + 0.016 µg/cm² = **0.033 µg/cm²** (mean + 1 SD) from study 1 will be used for the calculation of the systemic exposure dose (SED) in case of non-oxidative application.

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose oral toxicity

Guideline: /
Species/strain: Wistar rat
Group size: 3 females/dose level
Test substance: FAT 31'048/I Maxilon Blau M -2G
Batch: LIA09G044/2
Purity: 99.7 area-% (HPLC)
Vehicle: Water
Dose levels: 0, 10, 30, 100 and 250 mg/kg bw/day
Dose volume: 5 mL/kg bw
Route: Oral
Administration: Gavage
GLP: Yes

Study period: Experimental in-life phase, Aug-Sept 2010

Methods
In this dose range-finding toxicity study for a combined 28-day repeated dose toxicity study with the reproduction/developmental toxicity screening test of the hair dye Basic Blue 124, the test item FAT 31'048/I Maxilon Blau M -2G dissolved in water was administered to 3 female Wistar rats/dose level by single oral gavage at dose levels of 10, 30, 100 and 250 mg/kg bw/day for **10 days**. At 100 mg/kg bw/day, one female was killed in extremis on day 3 and replaced by one additional animal, which was treated with the test item for a total of 8 days.
During the treatment period, all animals were assessed repeatedly for mortality and clinical signs of toxicity. Body weights and food consumption were recorded at regular intervals. On the day of scheduled necropsy, blood samples were collected from all rats for the assessment of haematology and clinical chemistry parameters. At necropsy, all animals were examined macroscopically and selected organ weights (kidneys, liver, spleen) were determined.
The dose formulations prepared on day 10, with target concentrations of 2, 6 and 20 mg/mL (corresponding to dose levels of 10, 30 and 100 mg/kg bw/day, respectively), were analysed for test item concentration, homogeneity and stability (6 hours, at room temperature) by means of a HPLC method with UV detection.

Results
The dose formulations were formulated appropriately and remained within the concentration acceptance criterion (i.e., difference between analytically determined mean concentration and nominal concentration ≤ 15 %). The test item was homogenously distributed in the vehicle (i.e., coefficient of variation ≤ 10 %). All formulations analysed were stable when stored at room temperature under normal laboratory light conditions for at least 6 hours.

At 250 mg/kg bw/day, test item-related toxicity consisted of mortality (3/3 on day 4), preceded by severe clinical signs, body weight loss and changes in clinical pathology parameters. At 100 mg/kg bw/day, treatment with the test item resulted in mortality (1/4 on day 3), severe clinical signs, body weight loss/stasis, reduced food consumption, changes in clinical pathology parameters, enlargement of the spleen and increased spleen weight. At 30 mg/kg bw/day, test item-related findings included bodyweight loss/stasis, slightly reduced food consumption, minor changes in clinical pathology parameters and enlargement of the spleen. At 10 mg/kg bw/day, no test item-related toxicity was noted.

Conclusion
Under the conditions of this dose-range finding toxicity study, the no-observed-adverse-effect level (NOAEL) for the test item FAT 31'048/I Maxilon Blau M -2G was 10 mg/kg bw/day for female rats. Dose levels of 3, 10 and 30 mg/kg bw/day were selected for the subsequent 28-day repeated dose toxicity study with the reproduction/ developmental toxicity screening test.

Ref: NOTOX B.V. (2011a)

3.3.5.2 Repeated Dose (28 days) oral toxicity

Guideline:	OECD 422; US EPA OPPTS 870.3650
Species/strain:	Wistar Han rat
Group size:	10 animals/sex/dose level
Test substance:	FAT 31'048/I Maxilon Blau M -2G
Batch:	LIA09G044/2
Purity:	99.7 area-% (HPLC)
Vehicle:	Water

Dose levels:	0 (vehicle control), 3, 10 and 30 mg/kg bw/day
Dose volume:	5 mL/kg bw
Route:	Oral
Administration:	Gavage
GLP:	Yes
Study period:	In-life phase, September-November 2010

Methods
In a combined 28-day repeated dose toxicity study with the reproduction/developmental toxicity screening test, the test item FAT 31'048/I Maxilon Blau M -2G dissolved in water was administered by single oral gavage to 10 Wistar Han rats/sex/dose level at dose levels of 0 (vehicle control), 3, 10 and 30 mg/kg bw/day. Males were exposed for 29 days, i.e. 2 weeks prior to mating, during mating and up to termination. Females were exposed for 42-47 days, i.e. during 2 weeks pre-mating, during mating, during gestation and during at least 4 days of lactation. All parental animals were examined repeatedly for mortality, clinical signs (detailed observations), bodyweights and food consumption. From each group, 5 parental animals/sex were randomly selected for the conduct of functional observations (hearing ability, pupillary reflex, static righting reflex, grip strength) and assessment of locomotor activity. The assessments were performed towards the end of the treatment period, i.e. during week 4 of treatment and during the lactation period for males and females, respectively. General reproduction data were recorded. Pregnant females were examined to detect signs of prolonged parturition, and cage debris of pregnant females was examined for signs of abortion or premature birth. Any deficiencies in maternal care were assessed.

On the day of scheduled necropsy, blood samples were collected from the selected 5 parental animals/sex/group under Isoflurane anaesthesia for the assessment of haematology and clinical biochemistry parameters. The animals were deprived of food overnight prior to blood sampling. The selected 5 parental animals/sex/dose level were sacrificed and examined macroscopically with special attention being paid to the reproductive organs. Selected organ weights were determined. Organs/tissues of 5 selected high dose group and control group animals/sex were processed and examined microscopically for histopathological findings. Spermatogenesis staging was performed on testes slides of selected high-dose group and control-group males. All gross lesions of all parental animals were examined. In addition, the urinary bladder and the spleen of all selected 5 animals/sex of the low- and mid-dose groups were examined histopathologically due to changes in these organs noted in the high-dose group. Each litter was examined to determine mortality, clinical signs, bodyweights and sex of the pups. At scheduled necropsy, descriptions of all external abnormalities were recorded for all pups. The stomach was examined for the presence of milk.
Reproduction and mortality/viability indices were calculated.
Chemical analyses of dose formulations were conducted once during the study to assess accuracy of formulation (all concentrations), and homogeneity and 6-hour stability (lowest and highest concentration each).

Results
The reproductive and developmental results obtained in this study are described in **Section 3.3.8.2.** There were no toxicologically relevant findings in any reproductive or developmental parameters that were assessed.

Observations in parental animals:
Daily administration of the test item by oral gavage at dose levels of 3, 10 or 30 mg/kg bw/day resulted in no mortality, no clinical signs of toxicity, and no test item-related changes in functional observation parameters and locomotor activity. There was no test item-related effect on food consumption, bodyweights and bodyweight gain.

Blue discolouration/staining of the urine (from day 3 onward) and tail (from day 4-6 onward) of the test item without microscopic correlate was noted for all animals of all treatment groups. At 30 mg/kg bw/day, blue staining of faeces was noted in all female rats (from day 42 onward), and blue staining of the back was observed (from day 14 onward) for all animals. In addition, blue staining of the mouth was occasionally noted at 10 and 30 mg/kg bw/day.

Clinical laboratory investigations revealed test item-related changes in various erythrocyte parameters at 3 (females), 10 (males; not significant for females) and 30 mg/kg bw/day (both sexes), as well as increased total bilirubin levels (not statistically significant for females at 3 mg/kg bw/day). The changes seen in haematology parameters were indicative of an increased turnover of red blood cells, with more red blood cell production, and an increased proportion of immature cells in the circulation (see table below).

Results for the females at 3 mg/kg bw/day were considered as ambiguous by the study authors. However, the respective values were within the ranges of the historical controls; there was no dose-response relationship, and corresponding changes in haematology parameters were not observed in a sub-chronic oral repeated dose toxicity study in rats, i.e. in an assessment relying on a higher number of rats/dose level exposed for a longer period of time (see Section 3.3.5.3). On this basis, the changes in haematology parameters of females seen at 3 mg/kg bw/day are considered to be without toxicological relevance.

Macroscopically, enlarged spleen was noted for males and females at 30 mg/kg bw/day. Discoloration (black/dark red) was seen for both sexes at all dose levels, though the blue colour of the test item may have contributed to this finding. Enlarged spleen was associated with increased absolute and body weight-relative spleen weight at 30 mg/kg bw/day in male and female animals (see table below).

Microscopically, increased pigment (most likely haemosiderin pigment; both sexes), congestion (both sexes) and haematopoietic foci (males) of the spleen were noted for animals at 10 and 30 mg/kg bw/day. An increased incidence of minimal to slight haematopoietic foci was also seen for males at 3 mg/kg bw/day, but because this was also historically observed (up to a slight degree) for control animals, it was not considered to be an adverse effect at 3 mg/kg bw/day. Haemosiderin pigment, as a breakdown product of haemoglobin, is normally present in small quantities in the rat spleen. The increase in haemosiderin pigment and the presence of haematopoietic foci noted in this study were considered to be likely related to the increased red blood cell turnover, and to reflect the changes in erythrocyte parameters noted. The increased presence of red blood cells in the spleen, in the form of an increased congestion probably added to this. At 30 mg/kg bw/day, diffuse hyperplasia of urothelium (males and females) and inflammation (males) were recorded in the urinary bladder. These findings were considered to represent a test item-related adverse effect.

The concentrations analysed in the dose formulations for all dose levels were in agreement with the target concentrations (i.e. mean accuracies were between 85 % and 115 %). The test item was homogeneously distributed in low- and high-dose group formulations (i.e. coefficient of variation ≤ 10 %). Formulations at the entire concentration range were stable when stored at room temperature under normal laboratory light conditions for at least 6 hours.

Table: Haematology parameters after daily (sub-acute) oral gavage treatment

Hematology parameter[a] [mean ± SD]	Dose level [mg/kg bw/day]			
	0	3	10	30
Male rats				
RBC [T/L]	8.79 ± 0.45	8.28 ± 0.39	7.77 ± 0.46**	7.11 ± 0.47**
HB [mmol/L]	9.7 ± 0.3	9.3 ± 0.3	9.3 ± 0.3	8.8 ± 0.4**
HCT [rel. 1]	0.441 ± 0.016	0.415 ± 0.021	0.418 ± 0.020	0.400 ± 0.028*
MCV [fL]	50.2 ± 1.8	50.0 ± 0.8	53.8 ± 1.6**	56.3 ± 1.9**
MCH [fmol]	1.10 ± 0.03	1.13 ± 0.02	1.19 ± 0.05**	1.25 ± 0.03**
MCHC [mmol/L]	22.00 ± 0.49	22.54 ± 0.51	22.20 ± 0.64	22.13 ± 0.63
RETI [% RBC]	2.9 ± 0.1	3.1 ± 0.4	4.3 ± 0.4*	5.6 ± 0.9*
Female rats				
RBC [T/L]	7.13 ± 0.44	6.26 ± 0.48**	6.72 ± 0.34	6.00 ± 0.16**
HB [mmol/L]	8.5 ± 0.4	7.7 ± 0.4**	8.0 ± 0.3	7.6 ± 0.3**
HCT [rel. 1]	0.383 ± 0.014	0.351 ± 0.018*	0.366 ± 0.014	0.354 ± 0.017*
MCV [fL]	53.8 ± 2.2	56.3 ± 4.3	54.5 ± 1.0	58.9 ± 1.9*
MCH [fmol]	1.20 ± 0.06	1.23 ± 0.07	1.20 ± 0.05	1.26 ± 0.02
MCHC [mmol/L]	22.29 ± 0.66	21.85 ± 0.41	21.96 ± 0.40	21.49 ± 0.42*
RETI [% RBC]	6.9 ± 2.1	10.9 ± 7.1	7.3 ± 1.4	11.3 ± 1.6

All values are given as mean ± standard deviation (n = 5)

HB: hemoglobin, HCT: hematocrit, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, MCV: mean corpuscular volume, RBC: red blood cells, rel.: relative, RETI: reticulocytes, SD: standard deviation

Statistical comparisons with the concurrent control were conducted by means of the Dunnett test (RBC, HB, HCT, MCV, MCH, MCHC) and the Steel test (RETI) (* $p \leq 0.05$; ** $p \leq 0.01$)

a: hematology parameters which were assessed but not affected by the treatment with the test item are not shown

Table: Rat spleen weights after daily (sub-acute) oral gavage treatment

Parameter [mean ± SD]	Dose level [mg/kg bw/day]			
	0	3	10	30
Male rats				
Absolute weight [g]	0.657 ± 0.063	0.654 ± 0.109	0.698 ± 0.040	0.924 ± 0.141**
Body weight relative [%]	0.174 ± 0.011	0.182 ± 0.029	0.194 ± 0.014	0.260 ± 0.032**
Female rats				
Absolute weight [g]	0.613 ± 0.087	0.767 ± 0.295	0.707 ± 0.059	0.913 ± 0.122*
Body weight relative [%]	0.236 ± 0.028	0.309 ± 0.108	0.289 ± 0.027	0.390 ± 0.038**

All values are given as mean ± standard deviation (n = 5), SD: standard deviation

* significantly different from the control group value ($p \leq 0.05$), Dunnett's test

** significantly different from the control group value ($p \leq 0.01$), Dunnett's test

Conclusion

Under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for parental effects of the test item FAT 31'048/I Maxilon Blau M -2G was established at 3 mg/kg bw/day for male and female rats. For reproductive and/or developmental effects see Section 3.3.8.2.

Ref: NOTOX B.V. (2011b)

SCCS comment
Regarding haematological effects, the SCCS noted a dose-response relationship for the red blood cell count and the reticulocyte count in males. These changes were also observed in the 90-day study. In principle, such changes are considered to be of toxicological relevance.

3.3.5.3 Sub-chronic (90 days) toxicity (oral)

Guideline:	OECD 408; Directive 96/54/EC; B.26
Species/strain:	Wistar rat
Group size:	10 animals/sex/dose level
Test substance:	Maxilon Blau M -2G, Basic Blue 124
Batch:	LIA09G044/2
Purity:	99.7 area-% (HPLC)
Vehicle:	Purified water
Dose levels:	0 (vehicle control), 1, 3 and 25 mg/kg bw/day
Dose volume:	5 mL/kg bw
Route:	Oral
Administration:	Gavage
GLP:	Yes
Study period:	In-life phase, Nov 2012-Feb 2013. Study completion date 07 Feb 2014.

Methods
In this repeated dose toxicity study, the test item Maxilon Blau M -2G in purified water was administered by oral gavage to 10 Wistar rats/sex/dose level once a day at 0 (vehicle control), 1, 3 and 25 mg/kg bw/day, corresponding to concentrations of 0, 0.2, 0.6 and 5 mg/mL, respectively, applied in a dose volume of 5 mL/kg bw for 90/91 days (males/females).
During the acclimatisation (duration: 6 days) and treatment periods, all animals were observed twice daily for mortality. Daily general clinical observations were conducted throughout the acclimatisation and treatment periods. Detailed (behavioural) observations were performed on a weekly basis during acclimatisation and during weeks 1-12. In week 13, functional observation battery (FOB), locomotor activity and grip strength assessments were conducted. Bodyweights and food consumption were recorded weekly during the acclimatisation and treatment periods. Ophthalmological examinations were conducted on all animals once during the acclimatisation period and on control and high dose group animals in week 13.
For clinical pathology examinations including assessment of haematology and clinical chemistry parameters, blood samples were collected on the day of scheduled necropsy from all rats after an 18-hour fasting period. For the assessment of urinalysis parameters, urine samples were collected from all rats during the same 18-hour fasting period overnight from the last treatment day to the day of necropsy (day 90 for males, day 91 for females) using metabolism cages.
On the day of scheduled necropsy, all animals were examined macroscopically and the weights of selected organs were determined. Following histo-technological processing, full histopathology was performed on the preserved organs/tissues of the animals of the control and high dose groups. Due to lesions observed in high dose group animals, the spleen, the urinary bladder and the kidneys (both sexes) as well as the stomach (females only) were also examined microscopically in low- and mid-dose group animals. All gross lesions of all animals were examined.
The dose formulations used in this study were analysed spectrophotometrically for test item concentration, homogeneity and stability in the vehicle water. Analyses of concentration and homogeneity were conducted on samples collected after experimental start and during weeks 5and 13 (all dose groups). The 6-hour stability at room temperature was determined in samples collected after experimental start (all dose groups).

Results
Daily test item administration by single oral gavage to male and female Wistar rats for at least 90 days resulted in no mortality and in no test item-related adverse effects on clinical signs assessed during daily and detailed weekly examinations at dose levels of 1, 3 and 25 mg/kg bw/day. Similarly, no test item-related adverse effects were noted in functional observation battery parameters, grip strength, locomotor activity, food consumption, bodyweights and ophthalmological parameters at any dose level tested. Macroscopically, there were no test item-related adverse effects up to and including the high-dose level.

Several findings were considered to be related to the blue colour of the test item and not to be adverse. This included blue urine observed in all test item-treated groups, and dark faeces noted at 25 mg/kg bw/day. At the macroscopic examination, bluish discolouration was noted in the forestomach mucosa and in the urinary bladder of animals treated at 25 mg/kg bw/day. Microscopically, at the same dose level, focal blue/black pigment was observed.
Slight to moderate salivation was generally noted during test item administration at 25 mg/kg bw/day and considered to be a non-adverse effect related to the taste of the test item. Grip strength determinations during week 13 revealed decreased mean hind-limb grip strength in females at 3 and 25 mg/kg bw/day; however, the corresponding values were within the historical control values and thus considered as not adverse.

Test item-related changes of haematology parameters indicative of a slight regenerative anaemia were observed in both sexes at 25 mg/kg bw/day (decreased erythrocyte count (RBC), decreased haemoglobin concentration (HB) and haematocrit (HCT), increased mean corpuscular volume (MCV), decreased mean corpuscular haemoglobin concentration (MCHC) and increased reticulocyte count (RETI) (see table below). These changes were consistent with extramedullary haematopoiesis in the spleen and were regarded as adverse. In males treated with 1 or 3 mg/kg bw/day, there were slight decreases in HB and HCT as well as a minimal increase in RETI. These changes were not considered as adverse since all values were well within the range of the historical control data and there were no corresponding histological alterations in the spleen.
Assessment of clinical biochemistry parameters revealed slightly increased electrolyte concentrations (Sodium, Potassium, Chloride) in the plasma of males at 25 mg/kg bw/day. These increases were considered as potentially related to alterations in kidney function.
Treatment at 25 mg/kg bw/day resulted in increases in urinalysis parameters (urinary pH, protein content, bilirubin concentration and erythrocyte number) in males and females. However, it was noted by the study authors that due to the UV spectrum of Basic Blue 124 (peak between 500 and 700 nm, maximum at 675 nm) and possible interferences with the urinalysis measurements conducted at wavelengths of 470, 555 and 620 nm, the relevance of the urinalysis data generated in this study was regarded as questionable.
Despite of some questionable data, the picture of all these changes was considered to be attributable to test item-related effects on the kidney, the urinary bladder and/or the red blood cell count.

Table: Haematology parameters after daily (subchronic) oral gavage treatment

	Males				Females			
Group	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Dose Level (mg/kg bw/day)	**0**	**1**	**3**	**25**	**0**	**1**	**3**	**25**
Erythrocytes (RBC; T/l)	8.78	8.65	8.38	**7.69** (-12.4%)	7.90	7.74	7.67	**6.94** (-12.2%)
Hemoglobin (mmol/l)	10.2	**9.8*** (-3.9%)	**9.6**** (-5.9%)	**9.3**** (-8.8%)	9.5	9.2	9.4	**8.6**** (-9.5%)
Hematocrit (rel. 1)	0.46	**0.44** (-4.3%)	**0.44**** (-4.3%)	**0.43**** (-6.5%)	0.43	0.42	0.42	**0.40**** (-7.0%)
MCV (fl)	51.9	51.5	52.1	**56.6**** (+9.1%)	54.0	53.9	54.9	**57.3**** (+6.1%)
MCHC (mmol/l)	22.38	22.10	22.12	**21.44**** (-4.2%)	22.19	22.02	22.27	**21.74** (-2.0%)
Reticulocytes (rel. 1)	0.017	**0.019*** (+12%)	**0.022**** (+29%)	**0.041**** (+141%)	0.023	0.025	0.023	**0.047**** (+104%)
Reticulocytes (G/l)	138	**163** (+18%)	**174** (+26%)	**322**** (+133%)	187	180	174	**336**** (+80%)
L-RETI (rel. 1)	0.729	0.679	0.687	**0.639**** (-12.3%)	0.599	0.579	0.600	0.512
M-RETI (rel. 1)	0.247	0.292	0.285	**0.313**** (+27%)	0.372	0.381	0.351	0.404
H-RETI (rel. 1)	0.024	0.024	0.030	**0.053**** (+121%)	0.033	0.043	0.040	**0.075*** (+127%)

Abbreviations and Symbols: RBC, red blood cells; rel., relative; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; L-/M-/H-RETI, low/mid/high fluorescent reticulocytes; */**, statistically significant at 5% (*) or 1% (**). Changes considered test item-related are in bold print.

Mean absolute and relative spleen weights were increased in both sexes at 25 mg/kg bw/day (see Table below). This was considered to represent a test item-related finding. Increased spleen weights correlated with the test item-related histopathological finding of extra-medullary haematopoiesis in the spleen (higher incidence and severity as compared with controls), which was noted in both sexes at this dose level. The spleen weight changes and histopathological findings were consistent with the haematological changes noted at 25 mg/kg bw/day.

The only histopathological finding in the bone marrow was a minimally increased cellularity in one of the males at the highest dose but none in females.

Table: Rat spleen weights after daily (subchronic) oral gavage treatment

Parameter [mean ± SD]	**Dose level [mg/kg bw/day]**			
	0	**1**	**3**	**25**
Male rats				
Absolute weight [g]	0.74 ± 0.12	0.72 ± 0.09	0.72 ± 0.07	1.05 ± 0.20**
Body weight relative [%]	0.19 ± 0.03	0.18 ± 0.02	0.18 ± 0.02	0.25 ± 0.04**
Brain weight relative [%]	35.88 ± 5.73	35.08 ± 4.63	35.84 ± 4.02	50.74 ± 8.83**
Female rats				
Absolute weight [g]	0.54 ± 0.05	0.50 ± 0.08	0.50 ± 0.08	0.67 ± 0.08**
Body weight relative [%]	0.23 ± 0.01	0.22 ± 0.03	0.23 ± 0.04	0.29 ± 0.04**
Brain weight relative [%]	28.03 ± 2.81	26.19 ± 4.83	26.77 ± 4.08	35.62 ± 3.73**

All values are given as mean ± standard deviation (n = 10), SD: standard deviation
* significantly different from the control group value ($p \leq 0.05$), Dunnett's test
** significantly different from the control group value ($p \leq 0.01$), Dunnett's test

Increased mean absolute and brain weight-relative kidney weights were observed in males at 25 mg/kg bw/day and were considered as a test item-related effect with histopathological correlate (see Table below). Microscopically, the kidneys of high dose group males displayed a greater incidence and higher severity of intra-epithelial hyaline droplets as compared to control group males. This was associated with minimal single cell death of tubular epithelial cells in these males. In addition, brown pigment deposition in the renal tubular epithelium was noted in both sexes at 25 mg/kg bw/day.

Table: Rat kidney weights after daily (subchronic) oral gavage treatment

Parameter [mean ± SD]	Dose level [mg/kg bw/day]			
	0	1	3	25
Male rats				
Absolute weight [g]	2.20 ± 0.24	2.24 ± 0.25	2.12 ± 0.17	2.49 ± 0.20*
Body weight relative [%]	0.54 ± 0.04	0.56 ± 0.07	0.52 ± 0.06	0.59 ± 0.04
Brain weight relative [%]	106.5 ± 12.3	109.4 ± 12.8	105.0 ± 9.7	120.5 ± 9.8*
Female rats				
Absolute weight [g]	1.44 ± 0.10	1.54 ± 0.13	1.52 ± 0.16	1.42 ± 0.24
Body weight relative [%]	0.63 ± 0.05	0.69 ± 0.06	0.68 ± 0.07	0.62 ± 0.08
Brain weight relative [%]	75.5 ± 5.6	81.3 ± 7.1	81.1 ± 9.4	75.9 ± 11.2

All values are given as mean ± standard deviation (n = 10), SD: standard deviation
* significantly different from the control group value ($p \leq 0.05$), Dunnett's test

In addition to the test item-related microscopic findings noted in the spleen and the kidneys at 25 mg/kg bw/day, the histopathological examination revealed test item-related minimal to moderate diffuse hyperplasia of the transitional epithelium and minimal or mild chronic submucosal inflammation of the urinary bladder in both genders. These findings were associated in a few animals with submucosal haemorrhage, oedema, focal acute cystitis, arteritis, ulceration and/or blue/black pigment deposition. In addition, minimal diffuse epithelial hyperplasia was observed in the non-glandular stomach of the high dose group females, while no similar observation was made in the high-dose group males.

Analysis of the dose formulations used in this study revealed all actual concentrations were within the acceptance criterion of ±20 % of the nominal concentrations. All dose formulation samples met the acceptance criterion for homogeneity (≤15 %). The test item was found to be stable in dose formulations when kept for 6 hours at room temperature due to recoveries which met the variation limit of 10% from the time-zero (homogeneity) mean value.

Conclusion
Under the conditions of this subchronic repeated dose oral toxicity study, the no-observed-adverse-effect level (NOAEL) for the test item Maxilon Blau M -2G, Basic Blue 124 was established at 3 mg/kg bw/day for male and female rats. The spleen and the haematopoietic system as well as the kidneys and the urinary bladder were identified as targets of repeated oral exposure to Basic Blue 124.

Ref.: Harlan Laboratories Ltd. (2014)

SCCS comment
For dose formulations, the acceptance criterion of ±20% of the nominal concentrations of the test substance is considered ample. The study plan was not provided.
The SCCS does not consider an increase in reticulocyte count of a magnitude of 18-29% as minimal.

General SCCS comment on repeated dose toxicity
The oral studies consistently show that haematotoxicity occurs at doses of ≥10 mg/kg bw/day both in male and female rats. Furthermore, spleen and kidney are target organs of Basic Blue 124 at these doses. Moreover, at the doses of 3 or 1 mg/kg bw/day, single parameters of haematotoxicity parameters suggest mild effects. However, although some of these effects are statistically significant, they are variable between studies and genders and not consistent. Taken together, even when assuming mild haematotoxic effects or slight histopathological effects in spleen and kidney, these effects are not considered adverse at a dose of 3 mg/kg bw/day. Therefore, a NOAEL of 3 mg/kg bw/day can be used for the calculation of the MoS.

3.3.5.4 Chronic (> 12 months) toxicity

3.3.6 Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Reverse mutation assay using bacteria (Salmonella typhimurium and Escherichia coli)

Guideline:	OECD 471; Commission Regulation (EC) No 440/2008, B.13/B.14; US EPA OPPTS 870.5100
Species/strain:	*Salmonella typhimurium* TA1535, TA100, TA1537, TA98 and E. coli WP2 uvrA
Replicates:	triplicates in 2 individual experiments both in the presence and absence of S9-mix.
Test substance:	FAT 31'048/F
Solvent:	
Batch:	LIA09G024/2
Purity:	99.7 area-% (HPLC)
Concentrations:	0.158-5000 µg/plate without and with S9-mix in Experiment I 1.0-5000 µg/plate without and with S9-mix in Experiment II
Treatment:	Plate incorporation with at least 48 h incubation without and with S9-mix
GLP:	Yes
Study period:	2009

Methods
In a bacterial reverse mutation assay (Ames test), the test item FAT 31'048/F was tested for its mutagenic potential based on the ability to induce point mutations in selected loci of the bacterial strains S. typhimurium TA1535, TA100, TA1537, TA98 and E. coli WP2 uvrA. Two independent experiments (plate incorporation method) were conducted with and without a mammalian metabolic activation system (liver S9-mix from Phenobarbital and β-Naphthoflavone induced male Wistar rats) using concentration ranges of 0.158-5000 µg/plate (6-8 concentrations) and of 1.0-5000 µg/plate (7 concentrations) in Experiment I and II, respectively. The concentrations were selected based on a pre-experiment for toxicity.

In the main test, each experiment included concurrent negative controls (NC) in order to check for possible contaminants (NC with distilled water) and to determine the spontaneous mutation rate (solvent control with Dimethyl sulfoxide (DMSO)). Positive control (PC) items (with S9-mix: 2-Aminoanthracene (2-AA); without S9-mix: Sodium azide (NaN3), 4-Nitro-o-phenylenediamine (NOPD), Methyl methanesulfonate (MMS)) were used to check the

mutability of the bacteria and the activity of the S9-mix. In both independent experiments, 3 test plates (replicates) were used per test item concentration or per control.
Individual plate counts and the mean number of revertant colonies per plate were determined for mutagenicity assessment and a mutagenicity factor was calculated (mean revertants (test item) / mean revertants (solvent control)). Bacteriotoxicity was detected by a decrease in the number of revertants and clearing or diminution of the background lawn. Precipitation of the test item was recorded.

The test item was considered as mutagenic if a clear and concentration-related increase in the number of revertants occurred and/or a biologically relevant positive response (i.e. at least a 2-fold increase in the number of revertants for TA100, WP2 uvrA, at least a 3-fold increase in the number of revertants for TA1535, TA1537 and TA98) was noted as compared with the number of revertants in the solvent control. A test item producing neither a concentration-related increase in the number of revertants nor a reproducible biologically relevant positive response in any of the concentration groups was considered to be non-mutagenic in this system.

Results
No biologically-relevant increases in revertant colony numbers were noted in tester strains TA98, TA100, TA1535 and WP2 uvrA. Biologically relevant increases of revertant colony numbers were observed in tester strain TA1537 at 50 µg/plate in Experiment I (with S9-mix) and at 100 µg/plate in Experiment II (with S9-mix), both representing the highest evaluable concentration in the respective experiment. The threshold value of 3.0 was exceeded and a maximum mutation factor of 3.8 was reached at 50 µg/plate (with S9-mix) in Experiment I. A concentration-response relationship was found in tester strain TA 1537 in Experiment I and II (with S9-mix). The reference mutagens induced a distinct increase of revertant colonies indicating the validity of the experiments.

Toxic effects of the test item were noted in all tester strains used in Experiment I and II. In Experiment I, toxic effects of the test item were observed at 15.8 µg/plate and higher (with and without S9-mix), depending on the tester strain. In Experiment II, toxic effects of the test item were noted at 10 µg/plate and higher (without S9-mix) and at 25 µg/plate and higher (with S9-mix), depending on the tester strain. No test item precipitation was found with or without S9-mix.

Conclusion
Under the experimental conditions reported, the test item FAT31'0481F caused gene mutations by frameshifts in the genome of the tester strain TA1537. The test item did not induce mutations in any of the other tester strains (TA98, TA100, TA1535, WP2 uvrA), either with or without metabolic activation.
Accordingly, Basic Blue 124 was considered as mutagenic in this bacterial reverse mutation assay.

Ref: BSL Bioservice (2009)

SCCS comment
Frameshifts in the genome of the tester strain TA1537 are consistent with the flat aromatic structure of the Basic Blue 124 molecule.

In vitro gene mutation test in CHO cells (HPRT locus assay)

Guideline:	OECD 476
Cells:	Chinese hamster ovary (CHO) cells
Replicates:	duplicates in 3 independent experiments
Test substance:	FAT 31’048/I Maxilon Blau M -2G
Solvent:	culture medium (Ham's F12)
Batch:	LIA09G044/2

Purity:	99.7 area-% (HPLC)
Concentrations:	see text
Treatment	see text
GLP:	Yes
Study period:	2011

Methods
In an *in vitro* mammalian cell gene mutation test, the test item FAT 31'048/I Maxilon Blau M -2G, i.e. the hair dye Basic Blue 124 (purity: 99.7 area-% (HPLC, 254 ± 2 nm); batch LIA09G044/2), was assessed for its potential to induce gene mutations at the Hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus in Chinese hamster ovary (CHO) cells.

A pre-test for dose selection was conducted at test item concentrations ranging from 0.10-25.00 µg/mL (each 9 concentrations). Exposure durations were 4 hours (with and without the addition of a mammalian metabolic activation system (liver S9-mix from Phenobarbital and β-Naphthoflavone induced male Wistar rats)) or 24 hours (without S9-mix). Cytotoxicity, assessed as reduced relative cloning efficiency, was noted at 25 µg/mL after 4-hour exposure (with and without S9-mix) and at 3.13 µg/mL and above after 24-hour exposure (without S9-mix). Osmolarity and pH were not altered by the addition of the test item. There was no precipitation.

In the main assay, four independent experiments were carried out, with and without the addition of S9-mix. Due to the good solubility of the test item in water, culture medium (Ham's F12) was selected as vehicle.
No further reference is made here to the first of these experiments (Experiment I). This experiment was discontinued due to strong cytotoxicity, which occurred after the first subcultivation and resulted in an insufficient number of evaluable test concentrations. All relevant information on the discontinued Experiment I is given in the original report.

Based on cytotoxicity data, the following concentrations in the vehicle were tested. Concentrations given in bold face type were evaluated:

<u>Experiment II</u>
4-hour exposure (without S9-mix)
0, **0.06**, **0.12**, **0.23**, **0.47**, **0.94**, **1.88**, 3.75, 7.50, 15.00, 30.00 µg/mL
4-hour exposure period (with S9-mix)
0, **0.12**, **0.23**, **0.47**, **0.94**, **1.88**, **3.75**, 7.50, 15.00, 30.00, 60.00 µg/mL

<u>Experiment III</u>
24-hour exposure (without S9-mix)
0, 0.02, 0.05, **0.09**, **0.19**, **0.38**, **0.75**, **1.50**, **3.00**, 6.00 µg/mL
4-hour exposure period (with S9-mix)
0, 0.16, **0.31**, **0.63**, **1.25**, **2.50**, **5.00**, 10.00, 20.00 µg/mL

<u>Experiment IV</u>
24-hour exposure (without S9-mix)
0, **0.13**, **0.25**, **0.50**, **1.00**, 2.00, 4.00, 6.00 µg/mL

The stability of the test item at room temperature in water over a period of 4 hours was determined analytically in a separate study by means of a HPLC method with VIS-detection (BASF SE (2010g), study number 10L00201). Negative controls (NC) with and without S9-mix were treated in parallel to the test item groups, but only culture medium without test item was used. Appropriate reference mutagens (without S9-mix: Ethyl methanesulfonate (EMS), with S9-mix: Methylcholanthrene (MCA)) were concurrently used as positive control (PC) items to demonstrate the sensitivity of the test system and the activity of the S9-mix. Duplicate cultures were used for all experimental groups. After the exposure period, an

expression phase of about 7-9 days and a selection period of about 6-7 days followed. The colonies of each test group were fixed with Methanol, stained with Giemsa and counted. Cytotoxicity (cloning efficiency) and mutant frequency were assessed. In addition, as test-specific confounding factors, pH, osmolarity and solubility/precipitation were determined during the study. Cell morphology was assessed microscopically at the end of exposure for cell attachment evaluation.
The test item was considered as "positive" in this assay when there was (i) an increase of the corrected mutant frequency (i.e. mutant frequency corrected with the absolute cloning efficiency 2) both above the concurrent NC values and the laboratory's historical NC data range, (ii) evidence of reproducibility of any increase in mutant frequencies and (iii) a statistically significant ($p \leq 0.05$, Linear trend test) increase in mutant frequencies and evidence of a concentration-response relationship. The test item was considered non-mutagenic in this assay when the corrected mutant frequency in the dose groups was not statistically significant increased above the concurrent NC and was within the laboratory's NC data range.

Results
The test item did not induce a biologically relevant increase in the number of mutant colonies either with or without S9-mix in three valid experiments performed independently of each other. The mutant frequencies at any test item concentration were within or close to the range of the concurrent NC values and within or close to the range of the laboratory's historical NC data.

There was a weak statistically significant concentration-related increase in the number of mutant colonies observed in Experiment III after a 24-hour exposure without S9-mix. At the highest scorable concentration of 3 µg/mL, which induced clear cytotoxicity, the laboratory's historical NC data range was exceeded by the mean mutant frequency of both cultures treated in parallel. But, an increase was solely observed in one of the duplicate cultures. This single finding was not confirmed in the additional Experiment IV with its narrower concentration setting. Thus, this observation was regarded as a finding without biological relevance. The NCs gave mutant frequencies within the range expected for the CHO cell line. The increases in the frequency of mutant colonies induced by the PC substances EMS and MCA clearly demonstrated the sensitivity of the test method and the metabolic activity of the S9-mix employed.

In all experiments with or without S9-mix, at least the highest concentrations tested for gene mutations were clearly cytotoxic and revealed adversely influenced morphology and attachment of the cells. Osmolarity and pH values were not influenced by the test item treatment. No test item precipitation was noted in this study.

Conclusion
The test item FAT31'048/I Maxilon Blau M -2G was not mutagenic in the HPRT locus assay in CHO cells under the experimental in vitro conditions used, in the absence and the presence of a mammalian metabolic activation system.

Ref: BASF SE (2011)

In vitro chromosome aberration assay in V79 cells

Guideline:	OECD 473; Commission Regulation (EC) No 440/2008, B.10; US EPA OPPTS 870.5375
Replicates:	duplicate cultures
Cells:	V79
Test substance:	FAT 31'048/I Maxilon Blau M -2G
Solvent:	culture medium
Batch:	LIA09G044/2
Purity:	99.7 area-% (HPLC)

Concentrations:	see below
Treatment:	4-hour treatment and recovery time 18 hours after start of treatment both in the absence and presence of S9-mix
GLP:	Yes
Study period:	2010

Methods
In an *in vitro* chromosome aberration assay, the test item FAT 31'048/I Maxilon Blau M -2G was assessed for its potential to induce structural chromosome aberrations (clastogenic activity) and/or changes in the number of chromosomes (aneugenic activity) in V79 cells *in vitro* both in the absence and the presence of a mammalian metabolic activation system (liver S9-mix from Phenobarbital and β-Naphthoflavone induced male Wistar rats). The concentrations tested were based on the observations and the toxicity data of a preceding pre-test of a HPRT study.
Two experiments were conducted; no further reference is made here to the first of these experiments (Experiment I), which was discontinued due to severe cytotoxicity and partly poor metaphase quality. All relevant information on the discontinued Experiment I is given in the original report.
The following concentrations were tested and the test groups in bold type were evaluated:

Experiment II
4-hour exposure, 18-hour recovery (without S9-mix)

0, 0.08, **0.16**, **0.31**, **0.63**, 1.25, 2.50, 5.00 µg/mL

4-hour exposure, 18-hour recovery (with S9-mix)

0, 0.08, 0.16, **0.31**, **0.63**, **1.25**, 2.50, 5.00 µg/mL

The stability of the test item at room temperature in water over a period of 4 hours was determined analytically in a separate study by means of a HPLC method with VIS-detection (BASF SE (2010g), study number 10L00201). Concurrent negative control (NC) cultures with and without S9-mix were included in the test system. Appropriate reference mutagens (without S9-mix: Ethyl methanesulfonate (EMS), with S9-mix: Cyclophosphamide (CPP)) were employed as positive control (PC) items to demonstrate the sensitivity of the test system and the activity of the S9-mix. Duplicate cultures were used for all experimental groups.
Following an attachment period of seeded cells (24-30 hours), the medium was removed and medium only (NC), test item in medium or one of the PC items in medium was added, and the cells were incubated at 37 °C and 5 % (v/v) CO2 for 4 hours. At the end of the exposure period, medium was replaced and cultures were washed and incubated for the recovery period of 14 hours until harvest. About 2-3 hours prior to cell harvest, Colcemid was added to each culture in order to arrest mitosis in the metaphase. Subsequently, the culture medium was completely removed and the cells were fixed and stained (Giemsa) for microscopic analysis.

All cultures were prepared in duplicate, and 100 metaphases per culture were evaluated for the presence of structural chromosome aberrations. Aneuploid and polyploid cells were recorded separately. Due to clearly increased aberration rates (> 10 % aberrant cells exclusive gaps) for both PC cultures and for test item-treated cultures at 0.63 and 1.25 µg/mL (with S9-mix), the analysis of cytogenetic damage was restricted to each 50 metaphases in the respective cultures.
For the assessment of cytotoxicity, a mitotic index based on 1000 cells/culture was determined for all evaluated test groups in all cytogenetic experiments. At the end of the recovery period, cell counts were performed in single cell suspensions for all evaluated test groups (with and without S9-mix) to provide further information on cytotoxicity. In addition, as potential test-specific confounding factors, pH value, osmolarity and solubility (precipitation) were determined during the study. Cell morphology was assessed microscopically at the end of exposure for cell attachment evaluation.

The test item was considered as "positive" in this assay when there was a statistically significant ($p \leq 0.05$, $p \leq 0.01$, Fisher's exact test with Bonferroni-Holm correction), dose-related and reproducible increase in the number of cells with structural chromosome aberrations (exclusive gaps) and when the number of aberrant cells (exclusive gaps) exceeded both the concurrent NC value and the laboratory's historical NC data range. The test item was considered as "negative" when the number of cells with structural aberrations (exclusive gaps) in the dose groups showed no statistically significant increase above the concurrent NC value and was within the laboratory's historical NC data range.

Results
After 4-hour treatment and 18-hour recovery without S9-mix, a slight increase in the number of aberrant metaphases exclusive gaps was observed at the concentrations scored for cytogenetic damage (see table below). The values at 0.16 and 0.32 µg/mL slightly exceeded the historical NC data range (0-5.5% aberrant metaphases exclusive gaps). Additionally, the aberration rate (5.0% exclusive gaps) at the highest concentration scored for cytogenetic damage (0.63 µg/mL) was close to the concurrent NC group and within the historical NC data range.

After 4-hour treatment and 18-hour recovery with S9-mix, a statistically significant ($p \leq 0.01$) and dose-dependent increase in the number of chromosomally damaged cells was observed at 0.31, 0.63 and 1.25 µg/mL (10.5, 15.0 and 22.0 % aberrant metaphases exclusive gaps, respectively) (see table below). All values clearly exceeded the historical NC data range (0.0–5.5% aberrant metaphases exclusive gaps).
No relevant increase in the number of cells with changes in the number of chromosomes (aneugenicity) was noted either with or without S9-mix.

The increase in the frequencies of structural chromosome aberrations induced by the positive control substances EMS and CPP clearly demonstrated the sensitivity of the test system and of the metabolic activity of the S9-mix employed. The values were within the range of the historical PC data and thus fulfilled the acceptance criteria.

Evident cytotoxicity, when assessed as a decrease in the mitotic index, was not observed in the test groups scored for cytogenetic damage. However, higher concentrations were not scorable for the occurrence of mitotic cells as well as for cytogenetic damage. Cytotoxicity in terms of decreased cell counts was noted at 5.0 µg/mL (39.1% of NC) without S9-mix, while no clear cytotoxicity indicated by cell numbers below 50% of NC was observed up to 5.0 µg/mL with S9-mix (see table below).

Cell attachment as a further indicator of cytotoxicity was not influenced at any concentration evaluated for structural chromosome aberrations. Osmolarity and pH values were not influenced by test item treatment. There was no precipitation of the test item in the culture medium at any concentration tested.

Table: Chromosome aberrations and cytotoxicity (4-hour exposure, 18-hour recovery)

Treatment	Aberrant cells [%]		Cytotoxicity [%]	
	Inclusive gaps	Exclusive gaps	Cell number	Mitotic index
Without S9-mix				
NC	5.0	3.0	100.0	100.0
0.16 µg/mL[a]	11.5*	6.0	88.5	122.0
0.31 µg/mL[a]	9.0	6.5	81.7	79.9
0.63 µg/mL[a]	13.0*	5.0	74.2	72.3
PC (EMS)	29.0**	24.0**	n.d.	81.8
With S9-mix				
NC	8.0	3.0	100.0	100.0
0.31 µg/mL[a]	16.5**	10.5**	72.3	84.1
0.63 µg/mL[a]	21.0**	15.0**	64.2	52.4
1.25 µg/mL[a]	29.0**	22.0**	65.2	61.5
PC (CPP)	33.0**	25.0**	n.d.	91.3

CPP: Cyclophosphamide, EMS: Ethyl methanesulfonate, NC: negative control), n.d.: not determined, PC: positive control

Statistical comparisons with the concurrent NC were conducted by means of Fisher's exact test with Bonferroni-Holm correction (* $p \leq 0.05$, ** $p \leq 0.01$).

a: test item concentration

Conclusion
Under the experimental conditions chosen, the test item FAT 31'048/I Maxilon Blau M -2G was a chromosome-damaging (clastogenic, not aneugenic) substance *in vitro* for the V79 cell model in the absence and the presence of a mammalian metabolic activation system.

Ref: BASF SE (2010c)

3.3.6.2 Mutagenicity / Genotoxicity in vivo

Micronucleus test in bone marrow cells of the mouse

Guideline:	OECD 474; Commission Regulation (EC) No 440/2008, B.12; US EPA OPPTS 870.5395
Species/strain:	male NMRI mice
Group size:	Negative control and high dose group: 10 mice each, Both positive controls, low and mid dose group: 5 mice each
Test substance:	FAT 31'048/I Maxilon Blau M -2G
Batch:	LIA09G044/2
Purity:	99.7 area-% (HPLC)
Dose level:	0, 62.5, 125 and 250 mg/kg bw
Dose volume:	10 mL/kg bw
Route:	oral (gavage)
Vehicle:	de-ionised water
Sacrifice times:	24 h after treatment for low and mid dose group and both positive controls, 48 h for the vehicle control and high dose group
GLP:	Yes
Study period:	2010

Methods
The test item FAT 31'048/I Maxilon Blau M -2G was evaluated for its genotoxic potential (clastogenicity/aneugenicity) as measured by its ability to increase the incidence of micronucleated polychromatic erythrocytes (mnPCEs) in bone marrow cells of male NMRI mice after a single oral gavage administration. The test item in deionised water was administered to groups of 5 mice each at 62.5 and 125 mg/kg bw and to a group of 10 mice at 250 mg/kg bw. A concurrent vehicle control (VC) group of 10 mice was treated in the same manner with deionised water only. The positive control (PC) items, both dissolved in deionised water, were administered to each group of 5 mice once orally (Cyclophosphamide, CPP) or intraperitoneally (i.p.; Vincristine sulfate, VCR). A constant dose volume of 10 mL/kg bw was used for all animals. The animals were sacrificed 24 hours (VC group, low and mid dose group, both PC groups) and 48 hours (VC group, high dose group) after the treatment.

The dose levels for this micronucleus test were selected based on the outcome of a preceding acute oral toxicity pre-test, which showed mortality at 500 mg/kg bw and weak clinical signs of systemic toxicity but no mortality at 250 mg/kg bw, with no relevant differences between the sexes.

During the in-life period, clinical signs of toxicity were assessed repeatedly. Following necropsy and preparation of dried and stained bone marrow smears, the number of mnPCE was counted in 2000 PCEs for each animal (10000 PCEs per test group and sacrifice timepoint) using a microscope. The number of normochromatic erythrocytes (NCEs) with and without micronuclei was also recorded. The ratio of PCEs to NCEs was determined as an indicator for bone marrow exposure to the test item. Diameters of micronuclei (d) relative to cell diameters (D) were determined in order to distinguish between clastogenic effects ($d < D/4$) and spindle poison effects ($d \geq D/4$).

The test item was considered as "positive" in this assay when there was a statistically significant ($p \leq 0.05$, $p \leq 0.01$, one-sided Wilcoxon test) and dose-related increase in the number of mnPCEs and the number of mnPCEs exceeded both the concurrent VC value and the range of the laboratory's historical VC data. The test item was considered as "negative" when the number of cells containing micronuclei in the dose groups showed no statistically significant increase above the concurrent VC value and was within the range of the historical VC data.

Dose formulations used in this study were analysed for test item concentrations by means of a HPLC method with VIS detection (BASF SE (2010a), study number 10L00294). The homogeneity of the samples and the stability of the test item in the vehicle were confirmed indirectly based on the concentration analysis data.

Results
Treatment with the test item led to weak clinical signs of systemic toxicity. Hunched posture was noted 1 hour after the administration in all animals at 125 and 250 mg/kg bw. This transient finding was reversed within 2 hours in all affected animals. Discoloured faeces and urine as passive effects of the test item were observed in all treated animals at all observation time points. These findings indicated systemic exposure of the animals to the test item.
The single oral administration of the test item did not induce any biologically relevant increase in the number of PCE containing either small or large micronuclei above background (see Table below). All PC and NC values were within acceptable ranges and all criteria for a valid assay were met. No inhibition of erythropoiesis determined from the ratio of PCE to NCE was detected.
Based on the results of the dose formulation analysis, all test item formulations used in this study were prepared accurately and the test item was homogeneously distributed in the vehicle.

Table: Number of micronuclei (MN) in polychromatic erythrocytes (PCEs) of male mice

Treatment	Number of MN in PCEs[b]		Number of NCEs[e]
	Large[c] [‰]	Total[d] [‰]	
24 hour sacrifice interval			
NC	0.0	1.2	2962
62.5 mg/kg bw[a]	0.0	1.0	2929
125 mg/kg bw[a]	0.0	1.1	2764
250 mg/kg bw[a]	0.1	1.1	3503
PC (CPP)	0.1	16.9**	3147
PC (VCR)	18.0**	58.9**	4302
48 hour sacrifice interval			
NC	0.0	0.8	5112
250 mg/kg bw[a]	0.0	0.7	4065

CPP: Cycolphosphamide, MN: micronuclei, NC: negative control, NCEs: normochromatic erythrocytes, PC: positive control, PCEs: micronucleated polychromatic erythrocytes, VCR: Vincristine sulfate

a: test item dose level
b: 2000 PCEs/mouse scored in 5 male mice
c: large MN (indicator for spindle poison effects) $d \geq D/4$ (d: diameter of MN, D: cell diameter)
d: sum of small and large MN
e: number of NCEs observed when scoring 10000 PCEs

Statistical comparisons with the concurrent NC were conducted by means of the one-sided Wilcoxon test (*: $p \leq 0.05$, ** $p \leq 0.01$).

Conclusion
Under the conditions of this *in vivo* study, the test item FAT 31'048/I Maxilon Blau M -2G, i.e. the hair dye Basic Blue 124, was negative (no cytogenetic damage) in the bone marrow micronucleus test in male mice after administration of dose levels that produced effects of systemic toxicity.

Ref: BASF SE (2010e)

In vivo unscheduled DNA synthesis (UDS) assay in rat hepatocytes

Guideline:	OECD 486; Commission Regulation (EC) No 440/2008, B.39
Species/strain:	Wistar Han rats
Group size:	3-4 rats/dose level
Test substance:	FAT 31'048/I Maxilon Blau M -2G
Batch:	LIA09G044/2
Purity:	99.7 area-% (HPLC)
Dose level:	0, 125 and 250 mg/kg bw
Dose volume:	10 ml/kg bw
Route:	oral (gavage)
Vehicle:	de-ionised water
Sacrifice times:	3 hrs and 14 hrs (controls and all dosages), respectively
GLP:	Yes
Study period:	2010

Methods
The potential of the test item FAT 31'048/I Maxilon Blau M -2G or its metabolite(s) to induce Deoxyribonucleic acid (DNA) repair *in vivo* (unscheduled DNA synthesis (UDS)) was

assessed in rat hepatocytes at 3 hour and 14 hour sampling time. DNA repair was detected by the incorporation of ^{3}H-Thymidine into the DNA strand of hepatocytes and determined by autoradiography. Cells undergoing repair were identified by an increase in the number of silver grains overlying the nuclei (nuclear grain count (NGC)). The dose levels for this UDS assay were selected based on the outcome of a preceding acute oral toxicity pre-test, which showed mortality at 500 mg/kg bw and weak clinical signs of systemic toxicity but no mortality at 250 mg/kg bw, with no relevant differences between the sexes.

The test item dissolved in de-ionised water was administered by single oral gavage to 4 male rats (3 rats prepared)/dose level at 0 (negative (vehicle) control, NC), 125 or 250 mg/kg bw with 3-hour sampling time and at 0 (NC) or 125 mg/kg bw with 14-hour sampling time. Due to the unexpected death of 3 animals in this group, a total of 8 male rats (4 rats prepared) was treated at 250 mg/kg bw with 14 hour sampling time.

For both sampling times, concurrent positive control (PC) groups of each 4 male rats (3 rats prepared) received a single oral gavage treatment with 2-Acetylaminofluorene (2-AAF) suspended in corn oil at a dose level of 50 mg/kg bw. A constant dose volume of 10 mL/kg bw was used for all groups in this study. After the single treatment up to the time of sacrifice, the animals were examined twice for clinical signs of toxicity; the bodyweight was recorded once.

At 3 and 14 hours after treatment, all designated animals were anesthetized and the hepatocytes were harvested by *in situ* liver perfusion. After an attachment period of at least 2 hours, the cells were incubated for 4 hours with ^{3}H-Thymidine. After washing, the hepatocytes were cultivated overnight until fixation. Following autoradiography and staining, at least 100 cells per animal were scored microscopically for DNA repair activity (incorporation of ^{3}H-Thymidine) as assessed by the mean net nuclear grain count (NNGC; equal to nuclear grain count minus cytoplasmic grain count (NNGC = NGC – CGC)). Indicators for cytotoxicity like cell viability (vital staining, cell counting) after liver cell preparation were also determined.

The stability of the test item at room temperature in deionized water for 4 hours was verified analytically in a separate study by means of a HPLC method with VIS-detection (BASF SE (2010g), study number 10L00201). The test item concentration in the dose formulations used in the present study was also determined analytically using the same methodology (BASF SE (2010b), study number 10L00256). Since a solution was obtained with the vehicle, homogeneity was confirmed without analysis.

The test item was considered as "positive" in this assay when the NNGC exceeded zero in one of the dose groups and clearly exceeded the value of the concurrent NC group in one of the dose groups. A dose-related increase of the percentage of cells in repair (NNGC ≥ 5) with values of ≥ 20 %, and a dose-related increase in the mean number of NNGC of about zero were considered to be an indication for a marginal response that needs clarification. The test item was considered as "negative" when, in all dose groups, the mean NNGCs were close to the values of the concurrent NC group and within the range of the laboratory's historical NC data.

Results
There were 3 premature decedents in the high dose group (250 mg/kg bw) with 14-hour sampling time. The administration of the test item at 125 or 250 mg/kg bw led to distinct clinical signs of toxicity at both sacrifice intervals. Due to the observation of test item-related discolouration of the urine noted in all animals prepared at both dose levels, systemic exposure to the test item was confirmed.

No relevant cytotoxicity, as indication for test item-induced liver toxicity, was noted in this study. Cell viability was not influenced by the treatment with the test item. Also, the

number of primary hepatocytes was not reduced and there were no changes in cell morphology after test-item treatment in any case.

Treatment with the test item did not lead to a relevant increase in the mean NNGC or to a toxicologically relevant increase in the percentage of cells in repair (see table below).

All mean NNGC values of all dose groups were below zero, indicating that the test item was negative in the test system. In both parts of the study, the mean NNGC per animal of the test item-treated groups (-1.14 to -8.39) were close to the range of the respective NC values (-2.66 to -7.89) and close to the laboratory's historical NC data range (-1.96 to -7.92). The rate of cells in repair per animal was in the range from 0 - 1%, with the exception of the value of 18% cells in repair determined for a single animal treated at 250 mg/kg bw (14-hour sampling time), which was considered to represent an artificial finding (technical error in cell or slide preparation). The ranges of the concurrent and historical NC values were 0 – 3% and 0 – 1% cells in repair per animal, respectively. The induction of DNA repair (unscheduled DNA synthesis) by the PC substance clearly demonstrated the sensitivity of the test method applied.
The concentration analysis of dose formulations used in this study confirmed appropriate dosing of the animals.

Table: DNA repair activity

Treatment	NNGC [mean ± SD, n =3 or 4]	Cells in repair [%]
3 hour sacrifice interval		
NC	-4.63 ± 2.84	2
125 mg/kg bw[a]	-3.82 ± 0.52	1
250 mg/kg bw[a]	-4.97 ± 1.63	0
PC	4.84 ± 3.57	48
14 hour sacrifice interval		
NC	-5.40 ± 1.14	1
125 mg/kg bw[a]	-4.44 ± 0.63	0
250 mg/kg bw[a,b]	-4.79 ± 3.04	5
PC	10.54 ± 3.07	75

DNA: Deoxyribonucleic acid, n: number of animals, NC: negative control, NNGC: net nuclear grain count, PC: positive control, SD: standard deviation

a: test item dose level
b: 4 instead of 3 male rats were prepared; 400 instead of 300 cells were evaluated (100 cells/animal)

Statistical comparisons with the concurrent NC group were not performed.

Conclusion
Under the conditions of this *in vivo* study, the test item FAT 31'048/I Maxilon Blau M -2G was negative in the UDS test (no induction of DNA repair) in male rats after administration of dose levels that produced effects of systemic toxicity and mortality.

Ref: BASF SE (2010d)

SCCS conclusion on mutagenicity
Basic Blue 124 was tested for gene mutation in bacterial and mammalian cell systems up to cytotoxic concentrations with and without addition of a mammalian metabolic activation system. In the Ames test, Basic Blue 124 was mutagenic in bacterial strain TA1537. The frameshifts induced are consistent with the flat aromatic structure of the Basic Blue 124 molecule. No positive response was seen in the other tester strains and negative results were obtained in a mammalian cell gene mutation test. In addition, an *in vivo* UDS test demonstrated that the Basic Blue 124 is no inducer of DNA repair in mammalian liver cells.

The potential of the hair dye Basic Blue 124 to induce clastogenicity and/or aneugenicity was assessed *in vitro* in a chromosome aberration test with a positive test result and *in vivo* in a micronucleus test (BASF SE (2010), study number 26M0159/10M035) with a negative test result. The negative *in vivo* test result obtained overrules the positive *in vitro* finding, since a higher degree of reliability is attributed to *in vivo* testing data. The hair dye Basic Blue 124 is thus considered to be non-clastogenic and non-aneugenic.

SCCS is of the opinion that the negative result of the UDS test does not exclude a gene mutation potential as indicated in the positive Ames test.

No final conclusion on mutagenicity can be drawn without further studies to exclude gene mutation potential.

3.3.7 Carcinogenicity

No data

3.3.8 Reproductive toxicity

3.3.8.1 Two generation reproduction toxicity

No data

3.3.8.2 Other data on fertility and reproduction toxicity

Guideline:	OECD 422; US EPA OPPTS 870.3650
Species/strain:	Wistar rat
Group size:	10 animals/sex/dose level
Test substance:	FAT 31'048/I Maxilon Blau M -2G
Batch:	LIA09G044/2
Purity:	99.7 area-% (HPLC)
Vehicle:	Water
Dose levels:	0 (vehicle control), 3, 10 and 30 mg/kg bw/day
Dose volume:	5 mL/kg bw
Route:	Oral
Administration:	Gavage
GLP:	Yes
Study period:	In-life phase, September-November 2010

Methods
In a combined 28-day repeated dose toxicity study with the reproduction/developmental toxicity screening test, the test item FAT 31'048/I Maxilon Blau M -2G dissolved in water was administered by single oral gavage to 10 Wistar Han rats/sex/dose level at dose levels of 0 (vehicle control), 3, 10 and 30 mg/kg bw/day. Males were exposed for 29 days, i.e. 2 weeks prior to mating, during mating and up to termination. Females were exposed for 42-47 days, i.e. during 2 weeks pre-mating, during mating, during gestation and during at least 4 days of lactation. All parental animals were examined repeatedly for mortality, clinical signs (detailed observations), body weights and food consumption. From each group, 5 parental animals/sex were randomly selected for the conduct of functional observations (hearing ability, pupillary reflex, static righting reflex, grip strength) and assessment of locomotor activity. The assessments were performed towards the end of the treatment period, i.e. during week 4 of treatment and during the lactation period for males and females, respectively. General reproduction data were recorded. Pregnant females were examined to detect signs of prolonged parturition, and cage debris of pregnant females was

examined for signs of abortion or premature birth. Any deficiencies in maternal care were assessed.
On the day of scheduled necropsy, blood samples were collected from the selected 5 parental animals/sex/group under Isoflurane anaesthesia for the assessment of haematology and clinical biochemistry parameters. The animals were deprived of food overnight prior to blood sampling. The selected 5 parental animals/sex/dose level were sacrificed and examined macroscopically with special attention being paid to the reproductive organs. Selected organ weights were determined. Organs/tissues of 5 selected high-dose group and control-group animals/sex were processed and examined microscopically for histopathological findings. Spermatogenesis staging was performed on testes slides of selected high dose group and control group males. All gross lesions of all parental animals were examined. In addition, the urinary bladder and the spleen of all selected 5 animals/sex of the low- and mid-dose groups were examined histopathologically due to changes in these organs noted in the high dose group. Each litter was examined to determine mortality, clinical signs, body weights and sex of the pups. At scheduled necropsy, descriptions of all external abnormalities were recorded for all pups. The stomach was examined for the presence of milk.
Reproduction and mortality/viability indices were calculated.
Chemical analyses of dose formulations were conducted once during the study to assess accuracy of formulation (all concentrations), and homogeneity and 6-hour stability (lowest and highest concentration each).

Results
The test item-related findings in parental animals attributable to general repeated dose toxicity without primary relevance for reproduction are described in section 3.3.5.2. Treatment with the test item resulted in changes in haematology (erythrocyte) parameters, increased bilirubin levels, increased spleen weights, macroscopic and microscopic findings in the spleen (increased pigment, congestion and haematopoietic foci) and microscopic findings in the bladder (diffuse hyperplasia of urothelium and inflammation) at the mid- and/or high-dose levels.

Reproduction/developmental toxicity screening:
No test item-related changes were noted in any of the reproductive parameters (mating, fertility and conception indices, pre-coital time and numbers of corpora lutea and implantation sites) and developmental parameters (gestation index and duration, parturition, maternal care and early postnatal pup development including assessments of mortality, clinical signs body weights and examinations for macroscopic lesions) investigated in this study.

The concentrations analysed in the dose formulations for all dose levels were in agreement with the target concentrations (i.e. mean accuracies were between 85 % and 115 %). The test item was homogeneously distributed in low- and high-dose group formulations (i.e. coefficient of variation ≤ 10 %). Formulations at the entire concentration range were stable when stored at room temperature under normal laboratory light conditions for at least 6 hours.

Conclusion
Under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for parental effects of the test item FAT 31’048/I Maxilon Blau M -2G was established at 3 mg/kg bw/day for male and female rats. Due to the lack of adverse effects, the NOAEL for reproductive and/or developmental effects was set at the high dose level of 30 mg/kg bw/day.

NOTOX B.V. (2011b)

3.3.8.3 Developmental Toxicity

Guideline:	OECD 414; US EPA OPPTS 870.3700; MAFF 12 Nohsan 8147
Species/strain:	Han Wistar rat
Group size:	10 animals/sex/dose level
Test substance:	FAT 31'048/I Maxilon Blau M -2G
Batch:	LIA09G044/2
Purity:	99.7 area-% (HPLC)
Vehicle:	Water
Dose levels:	0 (vehicle control), 4, 12 and 40 mg/kg bw/day
Dose volume:	5 mL/kg bw
Route:	Oral
Administration:	Gavage
GLP:	Yes
Study period:	Experimental in-life phase, Dec 2012-Jan 2013, study completion date Oct 2013

Methods
The objective of this developmental toxicity study was to detect adverse effects of the test item Maxilon Blau M –G on pregnant female Han Wistar rats and development of the embryo and foetus consequent to exposure of the female from implantation to the day before Caesarean-sectioning (days 6-20 of presumed gestation, DGs 6-20).
The test item, suspended in purified water, was administered to each of the 37 pregnant female rats of the control- and mid-dose groups and to each of the 22 pregnant female rats of the low- and high-dose groups by single oral gavage at dose levels of 0 (vehicle control), 4, 12 and 40 mg/kg bw/day on DGs 6-20. Due to a high number of non-pregnant females in the control- and mid-dose groups, the number of females was increased from 22 to 37 animals per group by adding each further 15 females to the contro-l and mid-dose groups in a second delivery.

During the in-life period, all animals were observed twice daily for mortality and once daily for clinical signs of toxicity. Food consumption was assessed at regular intervals and bodyweights were recorded once daily. All female rats were sacrificed on DG 21. Blood samples were collected during necropsy from 5 animals per group for the assessment of selected haematology parameters (complete blood cell count). Post mortem examination, including gross macroscopic examination of all internal organs with emphasis on the uterus, uterine contents, corpora lutea count and position of foetuses in the uterus, was performed and data recorded. The uteri with contents of all females with live foetuses were weighed during necropsy to enable calculation of the corrected bodyweight gain.
Foetuses were removed from the uterus, sexed, weighed individually, examined for gross external abnormalities and sacrificed. At least one half of the foetuses from each litter was fixed and examined by a combination of serial sections of the head and micro-dissection of the thorax and abdomen (examinations included heart, major blood vessels and kidneys). The remaining foetuses were eviscerated, the skin removed and the carcasses processed for preservation and storage. The skeletons were examined and all abnormal findings and variations were recorded.
At treatment start and towards the end of the treatment period, dose formulation samples were collected for concentration, homogeneity and stability analysis by means of a UV-VIS spectrometer.

Results
Chemical analyses showed that the actual test item concentrations of the dose formulations were within the accepted range and that the test item was homogeneously distributed in the vehicle. The test item was found to be stable in the dose formulations when kept for 4 hours at room temperature and for 8 days in the refrigerator (5 ± 3 °C).

There was no mortality during the study. All females at 4 mg/kg bw/day had blue discoloured urine and all females at 12 and 40 mg/kg bw/day had blue discoloured urine

and faeces during treatment with the test item. These discolorations were considered to represent a passive effect of the test item and/or its metabolites.

There were no test item related adverse effects at the low- and mid-dose levels of 4 and 12 mg/kg bw/day in food consumption, bodyweight and haematology parameters. At the high dose level of 40 mg/kg bw/day, there was a reduction in food consumption during the entire treatment period associated with a slightly reduced bodyweight gain from DG 10 onwards. This resulted in reduced mean bodyweights and corrected bodyweight gain (see table below). At the same dose level, there were test item-related changes in several haematology parameters. The erythrocytes and haemoglobin levels and the haematocrit were statistically significantly decreased ($p \leq 0.05$ %, Dunnett test). Statistically significant increases were noted in the mean corpuscular volume and the red cell volume distribution width ($p \leq 0.01$ %, Dunnett test) and in the absolute and relative reticulocyte counts ($p \leq 0.01$ % and $p \leq 0.05$ %, respectively; Steel test). Except for the value for red cell volume distribution width, the values were outside the range of the laboratory's historical control data (see table below).

The relevant reproduction data (post-implantation loss and number of foetuses per dam) were not affected by treatment with the test item. No test item-related findings were observed at any dose level during macroscopic examination.

There were no test item-related effects on foetal body weight and foetal sex ratio at any dose level tested. No test item-related findings were noted in foetuses during the external and fresh visceral, skeletal and cartilage examinations. The treatment with the test item did not affect the ossification stages and the occurrence of supernumerary ribs

Table: Food consumption and bodyweight gain in pregnant rats treated by daily oral gavage from days of gestation 6 through 20 (DGs 6-20)

Parameter [mean ± SD]	Dose level [mg/kg bw/day]			
	0	4	12	40
Female rats				
Food consumption [g/rat/day][a]	23.3 ± 1.1	21.3 ± 1.2	22.7 ± 1.4	20.4 ± 1.3
Body weight gain [%][b]	45 ± 9	38 ± 14	42 ± 8	34 ± 10**
Corr. body weight gain [%][c]	9.8 ± 5.1	6.9 ± 7.6	10.7 ± 4.2	5.4 ± 3.4*

Corr.: corrected, SD: standard deviation
Statistical comparisons with the concurrent control were conducted by means of the Dunnett test (* $p \leq 0.05$; ** $p \leq 0.01$)

a: mean (n = 5) of means (n = 18 to 32) over the gestation period (DGs 6-21)
b: mean (n = 18 to 32) over the gestation period (DGs 6-21)
c: mean (n = 16 to 32) over the gestation period (DGs 6-21), corrected for the gravid uterus weight

Table: Haematology parameters in pregnant rats at necropsy following daily oral gavage treatment from days of gestation 6 through 20 (DGs 6-20)

Hematology parameter[a]	Dose level [mg/kg bw/day]			
	0	4	12	40
Female rats				
RBC [T/L]	6.39	6.11	6.09	4.78**
HB [mmol/L]	7.4	7.1	7.0	5.8**
HCT [rel. 1]	0.38	0.36	0.36	0.30**
MCV [fL]	58.6	59.0	59.8	62.7**
RDW [rel. 1]	0.118	0.117	0.116	0.135**
HDW [mmol/L]	1.20	1.24	1.17	1.31*
RETI [rel. 1]	0.035	0.042	0.051	0.093*
RETI [g/L]	240	247	296	440**

HB: hemoglobin, HCT: hematocrit, HDW: hemoglobin concentration distribution width, MCV: mean corpuscular volume, RBC: red blood cells, RDW: red cell volume distribution width, rel.: relative, RETI: reticulocytes

Statistical comparisons with the concurrent control were conducted by means of the Dunnett test (RBC, HB, HCT, MCV, RDW, HDW) and the Steel test (RETI) (* $p \leq 0.05$; ** $p \leq 0.01$)

a: hematology parameters which were assessed but not affected by the treatment with the test item are not shown

Conclusion
Based on the effects of the test item Maxilon Blau M -2G on food consumption, body weights and haematology parameters of pregnant females the maternal no-observed-adverse-effect level (NOAEL) was set at 12 mg/kg bw/day.
The NOAEL for developmental effects/teratogenicity was established at the high-dose level of 40 mg/kg bw/day due to the lack of relevant adverse embryo-foetal effects under the conditions of this study.

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3.3.9 Toxicokinetics

No data

3.3.9.1 Toxicokinetics in laboratory animals

3.3.9.2 Toxicokinetics in humans

3.3.10 Photo-induced toxicity

No data

3.3.10.1 Phototoxicity / photo-irritation and photosensitisation

3.3.10.2 Photomutagenicity / photoclastogenicity

3.3.11 Human data

No data

3.3.12 Special investigations

No data

3.3.13 Safety evaluation (including calculation of the MoS)

Not applicable.

3.3.14 Discussion

Physico-chemical properties
Basic Blue 124 is an organic salt and moderately soluble in water and several alcohols. Water solubility has not been determined by EC Method A.6

Purity has been described to be 95% and higher. Most analytical reports were not conducted under GLP. For Lots LIA09G024/2 and CGF-FO14192/0012, no evidence for purity has been provided.

The purity determination of Basic Blue 124 by HPLC was performed at a wavelength of 256 nm, while the UV absorption of B124 according to UV spectrum occurs at 196, 242, 294, (451), 624 (max)). Moreover, the documentation of ~100% recovery of the material applied on the column and the documentation of ~100% peak purity are both missing. The purity measurement should be performed at λmax 624 nm. Therefore, the purity determination cannot be accepted.

Stability of Basic Blue 124 was not reported in typical hair dye formulations.

Function and uses
Basic Blue 124 is intended to be used as a direct hair dye in semi-permanent hair dye formulations. The regular frequency of application is intended to be once per month up to once per 8 weeks.

Toxicological Evaluation

Acute toxicity
Based on an acute oral toxicity study conducted according to the up and down procedure, a LD50 value of 550 mg/kg bw was derived. The test item is therefore considered as being of moderately acute oral toxicity.

Dermal administration of Basic Blue 124 resulted in no mortality at the limit dose level of 2000 mg/kg bw. Apart from two females showing transient loss of bodyweight indicating signs of toxicity at the limit dose, no other clinical symptoms, gross lesions or other abnormalities indicating systemic toxicity were observed.

Local toxicity
Skin irritation:

In a dermal irritation study in rabbits (OECD TG 404), a 60% (w/w) mixture of approximately 0.5 g of Basic Blue 124 in water was placed on the skin of the animals. Under the conditions of this study, the test substance is mildly irritating to rabbit skin; classification according to the CLP criteria is not warranted.

Mucous membrane irritation, eye irritation:
In an eye-irritation study in rabbits according to OECD TG 405, instillation of a 20% mixture of Basic Blue 124 with water resulted in transient irritant responses. Under the conditions of this study, the test substance is irritating to the rabbit eye.
In a BCOP test *in vitro* according to OECD 437, Basic Blue 124, did not cause serious eye damage at a concentration of 1 % (w/v) in deionised water.
An EpiOcularTM eye-irritation test *in vitro* was conducted with 1% Basic Blue 124 in water. Due to several shortcomings, the SCCS does not consider the results obtained from this test as valid.
In vivo data in rabbits indicates only a mild eye irritation potential of the test substance at 20% (w/w) and no severe eye-irritation potential was observed in the BCOP test for 1% (w/w) Basic Blue 124.

Sensitisation
Two LLNA tests in mice and two GPMT tests were performed. In the LLNA tests, Basic Blue 124 dissolved/suspended in propylene glycol revealed a strong sensitising potential with an EC3 value of 1.1.%. The second LLNA performed with a suspension of the test substance in acetone/olive oil (4:1 v/v; AOO) induced a dose-dependent increase of lymphocyte proliferation, but did not reach the SI of 3, probably due to very low solubility of Basic Blue 124 in this vehicle.
The two GPMT tests also yielded different results: one was negative and the second one showed a weak sensitising potential. Masking of effects by coloured skin in the GPMT tests cannot be excluded.
The SCCS considers the LLNA with propylene glycol as the most reliable test and based on its results and according to the definitions provided by the SCCS (2005) Basic Blue 124 is considered to have a strong sensitising potential.
This is a new hair dye. As a potential allergen in humans, its capacity to induce allergic contact dermatitis should be evaluated when consumers are exposed to it.

Dermal absorption
An acute dermal toxicity study in rats (2000 mg/kg bw) and repeated applications in an LLNA test of 5%, 10% and 20%, Basic Blue 124 on the ears of mice revealed signs of systemic toxicity such as bodyweight loss indicating considerable dermal absorption in these species. However, discolouration of urine was only reported in the LLNA test.

Two dermal absorption studies *in vitro* with human skin conducted according to OECD TG 428 indicated very low dermal absorption of Basic Blue 124.
The substance was tested in a typical hair dye product at a concentration of 0.5 % (w/w) using dermatomed samples of human skin. A systemically available dose of Basic Blue 124 of 0.017 + 0.016 µg/cm² = **0.033 µg/cm²** (mean + 1 SD) resulted and can be used for the calculation of the systemic exposure dose SED.

In a second study *in vitro* according to OECD TG 428, samples of split-thickness human cadaver skin were exposed to about 1 mg/ml and 10 mg/ml in water, respectively. Only 7 skin membranes per dose from 3 human donors were used. An absorbed amount of <0.001 µg-eq/ml and 0.001 µg-eq/ml was calculated for the low and high dose, respectively. Nearly all concentrations determined were found below the LOQ.

Repeated dose toxicity

Data obtained were from a dose-range finding oral study in rats with 10 days exposure, a combined 28-day repeated dose oral toxicity study with the reproduction/developmental toxicity screening test according to OECD TG 422, a sub-chronic oral study according to TG 408 and a developmental toxicity study (OECD TG 414).
The oral studies consistently show that haematotoxicity occurs at doses of ≥10 mg/kg bw/day both in male and female rats. Furthermore, spleen and kidney are target organs of Basic Blue 124 at these doses. Moreover, at the doses of 3 or 1 mg/kg bw/day, single parameters of haematotoxicity parameters suggest mild effects. However, although some of these effects are statistically significant, they are variables between studies and genders and inconsistencies. Taken together, even when assuming mild haematotoxic effects or slight histopathological effects in spleen and kidney, these effects are not considered adverse at a dose of 3 mg/kg bw/day. Therefore, a NOAEL of 3 mg/kg bw/day can be used for the calculation of the MoS.

Mutagenicity
Basic Blue 124 was tested for gene mutation in bacterial and mammalian cell systems up to cytotoxic concentrations with and without addition of a mammalian metabolic activation system. In the Ames test, Basic Blue 124 was mutagenic in bacterial strain TA1537. The frameshifts induced are consistent with the flat aromatic structure of the Basic Blue 124 molecule. No positive response was seen in the other tester strains and negative results were obtained in a mammalian cell gene mutation test. In addition, an *in vivo* UDS test demonstrated that the Basic Blue 124 is no inducer of DNA repair in mammalian liver cells.

The potential of the hair dye Basic Blue 124 to induce clastogenicity and/or aneugenicity was assessed *in vitro* in a chromosome aberration test with a positive test result and *in vivo* in a micronucleus test with a negative test result. The negative *in vivo* micronucleus test result obtained overrules the positive *in vitro* finding, since a higher degree of reliability is attributed to *in vivo* testing data.

The SCCS is of the opinion that the negative result of the UDS test does not exclude a gene mutation potential as indicated in the positive Ames test.

No final conclusion on mutagenicity can be drawn without further studies to exclude gene mutation potential.

Carcinogenicity
No data available.

Reproductive toxicity
A screening test according to OECD TG 422 and a developmental toxicity study according to OECD TG 414 provided no evidence of toxicity to reproduction up to the highest dose levels tested (30 and 40 mg/kg bw/day, respectively).

Toxicokinetics
No data available.

Human data
No data available.

4. CONCLUSION

1. In light of the data provided, does the SCCS consider Basic Blue 124 safe when used as a direct hair dye in semi-permanent as well as in permanent (oxidative) hair dye formulations at on-head concentration up to a maximum of 0.5% (w/v)?

The safety of Basic Blue 124 cannot be assessed since no final conclusion on mutagenicity can be drawn without further studies to exclude gene mutation potential.

2. Does the SCCS have any further scientific concerns with regard to the use of Basic Blue 124 in cosmetic products?

The SCCS considers Basic Blue 124 as a strong skin sensitiser.

Basic Blue 124 is a tertiary amine. It should not be used together with nitrosating agents. Total nitrosamine content should be < 50 ppb.
The purity determination of Basic Blue 124 should be performed adequately.

5. MINORITY OPINION

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