Scientific Committee on Consumer Safety

SCCS

OPINION on

Sodium bromothymol blue (C186)

(CAS No. 34722-90-2, EC No. 252-169-7)

The SCCS adopted this document
during the plenary meeting on 21-22 March 2023

ACKNOWLEDGMENTS

SCCS members listed below are acknowledged for their valuable contribution to the finalisation of this Opinion.

For the preliminary and for the final version

SCCS members
Dr U. Bernauer
Dr L. Bodin
Prof. Q. Chaudhry (SCCS Chair)
Prof. P.J. Coenraads (SCCS Vice-Chair, Chairperson of the WG)
Prof. M. Dusinska
Dr J. Ezendam (Rapporteur)
Dr E. Gaffet
Prof. C. L. Galli
Prof. E. Panteri
Prof. V. Rogiers (SCCS Vice-Chair)
Dr Ch. Rousselle
Dr M. Stepnik
Prof. T. Vanhaecke
Dr S. Wijnhoven

SCCS external experts
Dr N. Cabaton
Dr A. Koutsodimou
Prof. W. Uter
Dr N. von Goetz

This Opinion has been subject to a commenting period of eight weeks after its initial publication (from 28 October to 23 December 2022). Comments received during this period were considered by the SCCS. For this Opinion, main changes occurred in the following sections: 3.1.5 (SCCS comment and impurity table), 3.1.9, 3.4.2 (NAM + last SCCS comment), respective discussion parts and conclusion number 2 accordingly.

All Declarations of Working Group members are available on the following webpage:
Register of Commission expert groups and other similar entities (europa.eu)

1. ABSTRACT

The SCCS concludes the following:

1. *In light of the data provided, does the SCCS consider Sodium Bromothymol Blue safe when used in non-oxidative hair colouring products up to a maximum on-head concentration of 0.5 %?*

Having considered the data provided, the SCCS is of the opinion that the safety of sodium bromothymol blue cannot be assessed because of the following reasons:

- The Applicant used TTC approach to justify the safety of sodium bromothymol blue, but the SCCS estimate of the SED indicates that it exceeds the TTC threshold for Cramer class III substances.

- The use of TTC on its own to justify the safety of the substances that are regulated under the EU Cosmetic Regulation is not sufficient to waive the information requirements on essential toxicological endpoints.

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

While the use of TTC is acceptable to justify the safety of impurities and cosmetic ingredients that are added to a final product at sufficiently low concentrations, it is not acceptable on its own for the substances that are regulated under the EU Cosmetic Regulation. Additional supporting data from NAMs that are scientifically-accepted for the purpose, and/or other acceptable in vivo data on systemic toxicity, are also required in an overall weight of evidence to assess safety.

Keywords: SCCS, scientific opinion, sodium bromothymol blue, C186, hair dye, Regulation 1223/2009, CAS No. 34722-90-2, EC No. 252-169-7

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on sodium bromothymol blue (C186) (CAS No. 34722-90-2, EC No. 252-169-7), preliminary version of 24-25 October 2022, final version of 21-22 March 2023, SCCS/1645/22

About the Scientific Committees

Two 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.

These Committees are: the Scientific Committee on Consumer Safety (SCCS) and the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) and they are made up of scientists appointed in their personal capacity.

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 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.).

Scientific Committee members

Contact

European Commission
Health and Food Safety
Directorate B: Public Health, Cancer and Health security
Unit B3: Health monitoring and cooperation, Health networks
L-2920 Luxembourg
SANTE-SCCS@ec.europa.eu

ISSN ISBN

Doi ND

The opinions of the Scientific Committees present the views of the independent scientists who are members of the committees. They do not necessarily reflect the views of the European Commission. The opinions are published by the European Commission in their original language only.

SCCS - Opinions (europa.eu)

TABLE OF CONTENTS

2. MANDATE FROM THE EUROPEAN COMMISSION

Background

The ingredient with the INCI name 'sodium bromothymol blue' and chemical name 'Sodium α-(3-bromo-5-isopropyl-4-oxo-2-methyl-2,5-cyclohexadienylidene)-2-(3-bromo-4-hydroxy-5-isopropyl-2-methylphenyl)toluenesulphonate' (CAS No. 34722-90-2, EC No. 252-169-7) is a weak acid in solution and thus exists in ionised and non-ionised forms that result in the appearance of different colours. Sodium bromothymol blue is intended to be used as a hair dye in non-oxidative hair colouring products with a final on-head concentration up to 0.5%.

In order to demonstrate the safety of sodium bromothymol blue, this dossier submission is established on a battery of non-animal methods and approaches, including *in silico* and *in vitro* methods, assessing inter alia irritation, skin sensitisation, genetic toxicity and percutaneous absorption, while following Good Laboratory Practice and OECD guidelines. In particular, notwithstanding the absence of data on repeated dose or reproductive/ developmental toxicity, the submitters have used the Toxicological Threshold of Concern (TTC) approach to support the safe use of this ingredient based on the very low consumer exposure estimate.

Terms of reference

1. *In light of the data provided, does the SCCS consider Sodium Bromothymol Blue safe when used in non-oxidative hair colouring products up to a maximum on-head concentration of 0.5 %?*

2. *Does the SCCS have any further scientific concerns with regard to the use of Sodium Bromothymol Blue 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

INCI name: Sodium bromothymol blue
Sodium 2-{[(1Z)-3-bromo-2-methyl-4-oxo-5-(propan-2-yl)cyclohexa-2,5-dien-1-ylidene][3-bromo-4-hydroxy-2-methyl-5-(propan-2-yl)phenyl]methyl}benzene-1-sulfonate (IUPAC ECHA)
Sodium;2-[(*E*)-(3-bromo-4-hydroxy-2-methyl-5-propan-2-ylphenyl)-(3-bromo-2-methyl-4-oxo-5-propan-2-ylcyclohexa-2,5-dien-1-ylidene)methyl]benzenesulfonate (IUPAC PubChem)

Ref.:
ECHA (https://echa.europa.eu/el/substance-information/-/substanceinfo/100.047.412);PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/Sodium-bromothymol-blue)

3.1.1.2 Chemical names

Chemical name: Sodium α-(3-bromo-5-isopropyl-4-oxo-2-methyl-2,5-cyclohexadienylidene)-2-(3-bromo-4-hydroxy-5-isopropyl-2-methylphenyl)toluenesulphonate [Ref. ECHA]
Phenol, 4,4'-(1,1-dioxido-3H-2,1-benzoxathiol-3-ylidene)bis(2-bromo-3-methyl-6-(1-methylethyl)-, monosodium salt [Ref PubChem]
Phenol, 4,4'-(3H-2,1-benzoxathiol-3-ylidene)bis(2-bromo-3-methyl-6-(1-methylethyl)-, S,S-dioxide, monosodium salt [Ref PubChem]
2-[alpha-(2-Methyl-3-bromo-5-isopropyl-4-oxo-2,5-cyclohexadiene-1-ylidene)-2-methyl-3-bromo-4-hydroxy-5-isopropylbenzyl]benzenesulfonic acid sodium salt
3’,3’’-dibromothymolsulfonephthalein sodium salt
Benzenesulfonic acid, 2-[[3-bromo-4-hydroxy-2-methyl-5-(1-methylethyl)phenyl][3-bromo-2-methyl-5-(1-methylethyl)-4-oxo-2,5-cyclohexadien-1-ylidene]methyl]-, sodium salt (1:1)

Other name: Bromothymol blue sodium salt

Ref.:
ECHA (https://echa.europa.eu/el/substance-information/-/substanceinfo/100.047.412);
PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/Bromothymol-Blue-sodium-salt);
EPA (https://comptox.epa.gov/dashboard/chemical/synonyms/DTXSID8067866)

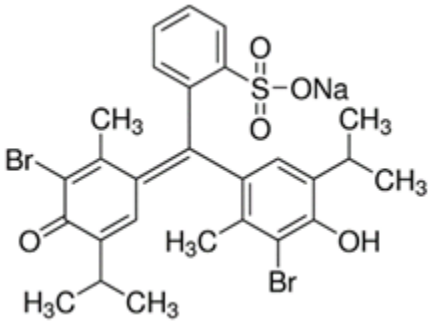
3.1.1.3 Trade names and abbreviations

Trade name: BROMOTHYMOL BLUE SODIUM SALT (Guanghua)
BROMOTHYMOL BLUE – 208690 (LOBA FEINCHEMIE GMBH)

3.1.1.4 CAS / EC number

CAS number: 34722-90-2
EC number: 252-169-7

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

Formula: $C_{27}H_{27}Br_2O_5S.Na$

3.1.2 Physical form

Dark yellowish green to light orange powder.

3.1.3 Molecular weight

646.369 g/mol

3.1.4 Purity, composition and substance codes

All toxicity studies submitted in the dossier were conducted using sodium bromothymol blue batch 20170531 which, according to the Applicant, was well characterised analytically. The summarised chemical characterisation of this batch is described in Table 1.
The purity of the (Guanghua) batch 20170531 was 90.05%. Two other batches (0583610001 and 0647670001) with a higher purity from a different supplier (Loba) were also characterised analytically and the results are presented in Table 1 as well.
HPLC purity of the 3 batches is above 95% (Relative purity, UV- area %). The purities of the different batches were estimated according to the formula: 100 – (Impurities contents + water + solvents +…) x UV purity of the main peak.
Sodium bromothymol blue E509991 (Guanghua) batch 20170531 is less pure than E512354 (Loba) batches 0583610001 and 0647670001. Several impurities were detected in E509991

(Guanghua) batch 20170531. The manufacturing processes of sodium bromothymol blue was different. Methyl alcohol is used for E509991 (Guanghua), isopropyl alcohol is used for E512354 (Loba). According to the Applicant, it can be concluded that Loba samples (E512354) were more pure.
The batch CFQ43741 of Bromothymol Blue Na Salt [^{14}C] (95.9% radiochemical purity) was used for the *in vitro* skin absorption study.

Table 1: Chemical characterisation

Analytical tests	E509991 (**Guanghua**) Batch 20170531	E512354 (**Loba**) Batch 0583610001	E512354 (**Loba**) Batch 0647670001
Appearance	Dark yellowish Green powder	Light orange powder	
Colour of solution at 0.05% in water/ACN (50/50) at 23°C	Dark green	Orange	
pH of solution (1% in water)	7.85	5.89	5.84
Infra-red spectrometry	In accordance with the proposed structure. Spectra of the three batches are comparable. The intensity of some bands is different between Guanghua batch and Loba batches.		
UV spectrometry	Compatible with the proposed structure. Spectra of the three batches are comparable but some differences between Guanghua batch and Loba batches are detected. (Identical wavelength, different absorbances)		
^{1}H NMR spectrometry	In accordance with the proposed structure		
Mass spectrometry	Compatible with the proposed structure		
HPLC UV Purity (% UV relative) (λ : 210 – 700 nm)	98.8 Detection of several impurities	99.9 All impurities <0.1 %	99.9 All impurities <0.1 %
Estimated titres *(%)	90.05	96.9	96.9
Impurities (content >0.1%)	Three impurities	-	-
Water content (g/100g)	3.6	< 2	< 2
Methyl alcohol determination by NMR	0.1 M/Mol 0.45 g/100g	Not used in the process	
Determination of isopropyl alcohol by NMR	Not used in the process	0.5 M/Mol 3 g/100g	0.5 M/Mol 3 g/100g
Elemental analysis: Conform with	3.6 % water content, 0.45 % methanol content and 4.8 % NaOH content	3 % isopropyl alcohol content	3 % isopropyl alcohol content
Estimated titre: 100 – (Impurities contents + water + solvents +…) x UV purity of the main peak.			

SCCS comment
The pH value of the batch E509991(Guanghua) is 7.85, while the pH values for the batches E512354 (Loba) and E512354 (Loba) are 5.89 and 5.84, respectively. These differences in pH might be an indication of different impurities due to the different manufacturing processes used.
The units for the methyl alcohol and isopropyl alcohol determination by NMR (M/Mol) provided by the Applicant in Table 1 seem to be incorrect.

3.1.5 Impurities / accompanying contaminants

Three impurities with a content above 0.1% were detected in E509991 (Guanghua) batch 20170531 (Table 2). In samples E512354 (Loba) batches 0583610001 and 0647670001 one minor impurity is detected (traces). No impurity with a content above 0.1% was detected in E512354 (Loba) batches. Corresponding area% of impurities in these batches are given in Table 2 below.

Table 2: Corresponding area % of impurities in E509991 (Guanghua) batch 20170531 and in E512354 (Loba) batches 0583610001 and 0647670001.

Impurities Retention time (min)	sodium bromothymol blue		
	E509991 (**Guanghua**) Batch 20170531	E512354 (**Loba**) Batch 0583610001	E512354 (**Loba**) Batch 0647670001
	Area% (λ = 210-700nm)		
1.07	0.08	ND*	ND*
1.19	0.05	ND*	ND*
1.22 Thymol Blue (imp 1)	**0.29**	ND*	ND*
1.686 (imp 2)	**0.33**	ND*	ND*
1.765 (imp 3)	**0.52**	ND*	ND*
2.18 (imp 4)	0.09	ND*	ND*
2.74	0.06	ND*	ND*

** ND: not detected; In bold, impurities to be quantified against sodium bromothymol blue, considered as the reference standard.*

Evaluation of impurities content in E509991 (Guanghua) batch:
Determination by High Performance Liquid Chromatography (HPLC) against sodium bromothymol blue E512354 (Loba) batch 0583610001 reference standard considered as 96.9% Pure. The separation was achieved by reversed phase LC and performed on a Waters Acquity UPLC system equipped with a photodiode array detector eλ (Waters). Detection process: λ = 420 nm. All samples and eluents were filtered through a 0.2 µm membrane filter (Pall Acrodisc GHP) prior to use. The dilution solvent is a mixture of water/acetonitrile 50/50 (v/v).

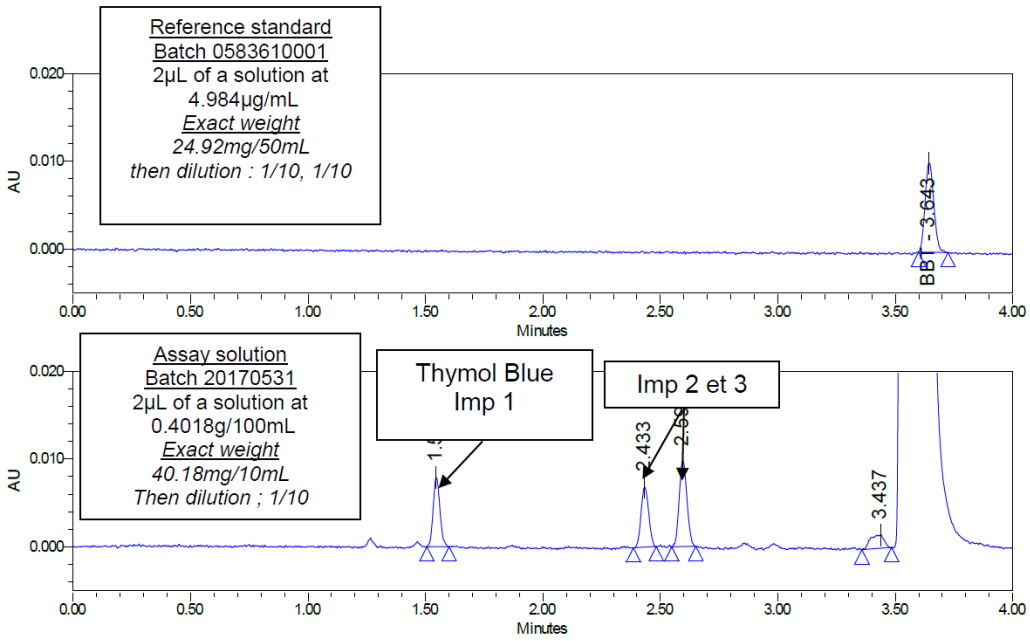


Figure 1. The contents of the impurities detected in E509991 (Guanghua) batch 2017531 with a level greater than 0.1% (area%) are given below:

Impurities	Content (**g/100g** expressed to sodium bromothymol blue)
Thymol Blue Imp 1	0.68 ± 0.04
Imp 2	0.67 ± 0.05
Imp 3	0.97 ± 0.08
Sum (g/100g)	**2.59 ± 0.17**

The identification of the structure of the three impurities with a content above 0.1% in E509991 (Guanghua) batch 20170531 was carried-out by High Resolution Mass Spectrometer (HRMS) coupled with a HPLC equipped with a Diode Array Detector (DAD). The analyses were performed on a 0.02 % solution in a water/acetonitrile mixture (50:50) of E509991 (Guanghua) batch 20170531. Based on these studies, the Applicant proposes the following structures and molecular formulas for the three quantified impurities.
Impurity 1 (HPLC-UV tR 1.22 min) corresponds to thymol blue: $C_{27}H_{30}O_5S$; The pK1 of thymol blue (CAS 76-61-9) is 1.7 and pK2=8.9. This impurity eluting at 2.35 minutes within the conditions of the HPLC method was detected in ESI- by the deprotonated ion [M-H]- at m/z 465.1741 Da indicating a $C_{27}H_{30}O_5S$ empirical formula and a molecular weight of 466.1814 Da. Based on the similarity of UV-Vis spectra and the fragmentation pattern of impurity 1 to that of BTB, the Applicant proposes the two structures below for impurity 1, corresponding to the thymol blue.

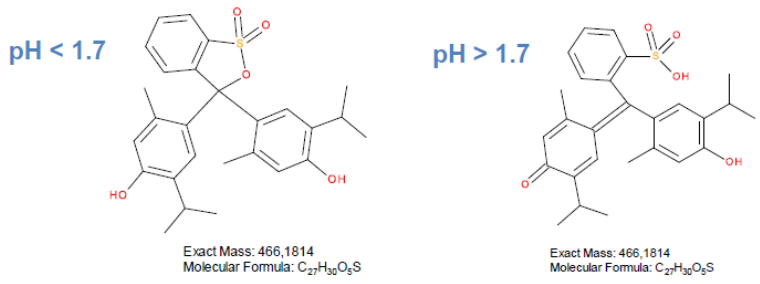


Figure 2a. Chemical structure of thymol blue

Impurity 2 (HPLC-UV tR 1.686 min) and impurity 3 (HPLC-UV tR 1.765 min) correspond to two isomers. $C_{27}H_{29}O_5BrS$, Exact mass =544,091908.
Impurity 2, eluted at 3.30 minutes within the conditions of the HPLC method, was detected in ESI- by the deprotonated ion [M-H]- at m/z 543.0847 Da indicating a $C_{27}H_{29}O_5BrS$ empirical formula and a molecular weight of 544.0919 Da. The [M-H]- ion is close to the fragment ion of BTB at m/z 542.0768 Da with a difference of 1 Da. The fragmentation pattern of this ion is characterised by the loss of one radical ion $Br^{\bullet}$ then consecutive losses of radical ions $CH_3^{\bullet}$ The product ions at m/z 315.0699 Da and m/z 155.9888 Da confirm that the structure of impurity 2 is similar to that of BTB. Furthermore, the chemical process and the similar fragmentation pattern as BTB led us to propose the four structural hypotheses below for impurity 2, depending on the pH of the solution:

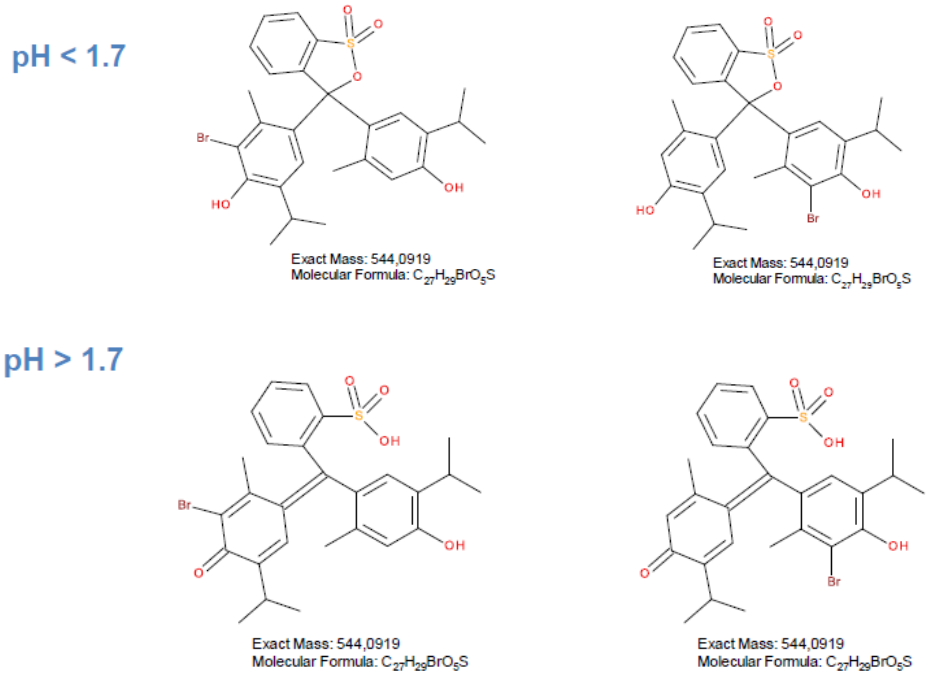


Figure 2b. Chemical structures of impurity 2 and impurity 3 (positional isomers)

Impurity 3 eluting at 3.45 minutes within the conditions of the HPLC method was detected in ESI- by the deprotonated ion [M-H]- at m/z 543.0847 Da indicating a $C_{27}H_{29}O_5BrS$ empirical formula and a molecular weight of 544.0919 Da. The fragmentation pattern of this ion shows the formation of the same product ions as those of impurity 2. Therefore impurity 3 is an isomer of impurity 2. This information led the Applicant, to propose for this third impurity the same above four structural hypothesis as for impurity 2. In an acid solution, impurity 2 corresponds to one of the two structures proposed (Br on one first cycle of thymol) while impurity 3 has its Br on the other cycle of thymol of the structure. The Applicant proposed that for the two structures proposed in a basic solution the same way can be proceeded.

Metals content:			
Metal	sodium bromothymol blue Experimental values (mg/kg)		
	E509991 (Guanghua synthesis) Batch 20170531	E512354 (Loba synthesis) Batch 0583610001	E512354 (Loba synthesis) 0647670001
Aluminium	<5	<5	<5
Antimony	<1	<1	<1
Arsenic	<1	<1	<1

Barium	<5	<5	<5
Calcium	<50	<50	<50
Cadmium	<5	<1	<1
Cobalt	<5	<5	<5
Chromium	<5	<5	<5
Copper	<5	<5	<5
Iron	27	11	25
Lead	<1	<1	<1
Mercury	<1	<1	<1
Manganese	<5	<5	<5
Molybdenum	<5	<5	<5
Nickel	<5	<5	<5
Potassium	<50	<50	<50
Palladium	<1	<1	<1
Phosphorus	<50	<50	<50
Selenium	<5	<5	<5
Titanium	<5	<5	<5
Tin oxide	<5	<5	<5
Vanadium	<5	<5	<5
Zinc	<5	<5	<5

SCCS comment

- Filtration of the samples through a 0.2 µm microfilter prior to the HPLC analysis of the test substance for impurity testing must be justified. If the samples of the test substance are not fully dissolved, impurities may remain in the filter. All compounds in the samples should be fully dissolved in the dilution solvent prior to the HPLC-PDA analysis.
- In view of the variability in the content of impurities within the Guangha and Loba batches, the Applicant must provide data on purity and impurities for at least five representative batches so as to better gauge the presence and nature of these impurities. The Applicant should also provide the specifications of the type of the batches intended to be used in the cosmetic products (whether Guangha or Loba).

TTC approach considered by the SCCS

As part of the evaluation, the SCCS applied a Threshold of Toxicological Concern (TTC) approach to assess whether the three impurities could be considered safe.

Impurity 1 (Thymol Blue)

- E product = 35ml (=35g) hair dye used per application which contains 0.5% of sodium bromothymol blue
- Concentration of impurity 1 per application: 0.72 g (mean + SED = 0.68+0.04) /100g sodium bromothymol blue = 0.72%
- As sodium bromothymol blue is present as 0.5% in the hair dye, the concentration of impurity 1 = 0.5/100 x 0.72%=0.0036% in hair dye product
- SED for (1) = Eprod. X Conc (1)/100 X DA/100= 35g/d X 0.0036/100 X 50/100 =0.00063 g/p/d
- Since not all hair dye is in contact with the skin a retention factor (Rt=0.1) is applied:
- - SED = 0.0000063 g/d = 63 µg/d or **1.05 µg/kg bw/d**

Impurity 2

- Concentration of impurity 2 per application: 0.72 g (mean +SD = 0.67+0.05) /100g sodium bromothymol blue = 0.72%
- As sodium bromothymol blue is present as 0.5% in the hair dye, the concentration of impurity 2 = 0.5/100 x 0.72%=0.0036% in hair dye product
- SED for (2) = Eprod. X Conc (2)/100 X DA/100= 35g/d X 0.0036/100 X 50/100 =0.00063 g/p/d
- Since not all hair dye is in contact with the skin a retention factor (Rt=0.1) is applied:

- SED = 0.0000063 g/d = 63 µg/d or **1.05 µg/kg bw/d**

Impurity 3

- Concentration of impurity 3 per application: 1.05 g (mean +SD = 0.97+0.08) /100g sodium bromothymol blue = 1.05%
- As sodium bromothymol blue is present as 0.5% in the hair dye, the concentration of impurity 3 = 0.5/100 x 1.05%=0.00525% in hair dye product
- - SED for (3) = Eprod. X Conc (3)/100 X DA/100= 35g/p/d X 0.00525/100 X 50/100 =0.00092 g/p/d
- Since not all hair dye is in contact with the skin a retention factor (Rt=0.1) is applied: SED = 0.000092 g/d = 92 µg/d or **1.53 µg/kg bw/d**

The nature of impurities identified in the Guanghua batch indicates that impurities 1, 2 and 3 belong to Cramer class III. The genotoxicity potential of these impurities is not known. However, considering the absence of genotoxic potential of sodium bromothymol blue (see 3.4.6), and structural similarity of these impurities to sodium bromothymol blue, these impurities can also be considered as potentially non genotoxic. Using the TTC threshold for Cramer class III for non-genotoxic substances (2.3 µg/kg bw/d), the levels of the impurities do not exceed the acceptable threshold. Therefore, the SCCS considers that the presence of these impurities is of no concern.

3.1.6 Solubility

Soluble in water, ethanol (chemical book)
Easily soluble in cold water (SDS spectrum chemical)
Water solubility (predicted): 0.653 mol/L (EPA)

Ref.: Chemical Book (https://www.chemicalbook.com/CASEN_34722-90-2.htm)
EPA (https://comptox.epa.gov/dashboard/chemical/properties/DTXSID8067866)

SCCS comment

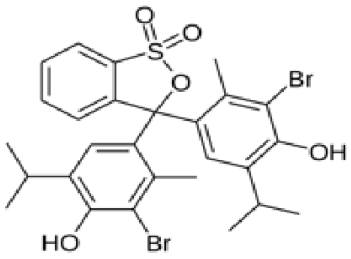
The Applicant needs to provide experimental data on the solubility of the test substance. The SCCS did retrieve predictive information in the public domain. It is not clear if these data are representative for the test substance used in the different toxicological tests.

3.1.7 Partition coefficient (Log Pow)

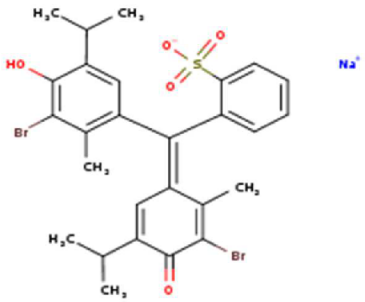
3.1.8 Additional physical and chemical specifications

Sodium bromothymol blue salt is a weak acid. The degree of ionisation of a chemical depends on its pKa and on the pH of the solution.
At a low pH, a weak organic acid such as sodium bromothymol blue salt is largely nonionised (cyclic form). At pH 7.1 corresponding to the pKa, exactly 50% of bromothymol blue is ionised (open ionised form) and 50% is nonionised. As the pH increases, sodium bromothymol blue salt continues to dissociate until almost all of it is in the ionised form. Thus, two possible forms exist based on pH as illustrated in Figure 3, the cyclic nonionised form mostly present at pH<pKa (7.1) and the open ionised form mostly present at pH>pKa (7.1). At pH 7.4 corresponding to the pH of the *in vitro* tests as well as to the pH of the formula tested in the skin penetration study, both the ionized and non-ionized forms are present. Considering that all *in vitro* safety data were performed in media at pH 7.4, and the formula tested in the skin

penetrations study and representing the use condition was at pH 7.4, we can consider that both the cyclic as well as the open ionised forms were assessed.



A) Cyclic nonionized form



B) Open ionized form

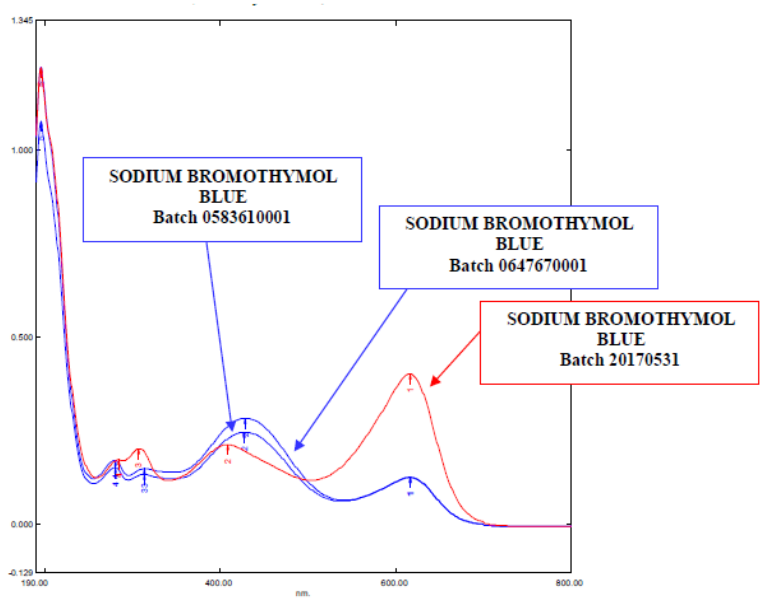
Figure 3: Ionised and non-ionised forms of sodium bromothymol blue according to pH

Organoleptic properties:	Characteristic odour (chemical book)
Melting point:	200-202°C (chemical book), ~349°C (SDS spectrum chemical)
Boiling point:	~900°C (SDS spectrum chemical) 662°C(predicted) (EPA)
Vapour pressure*:*	8.45e-10 (predicted) (EPA)
Density:	0.990 (chemical book) 1.66g/cm^3 (predicted) (EPA)
pKa at 25°C:	7.0, 7.1(at 25℃) (chemical book), 7.1 (David R. Line, Handbook of Chemistry and Physics, 84th edition (2003/2004), CRC Press, 2004)
pH (1% w/vol at 24°C)	7.85 (test item E509991 batch 20170531, supplier Guanghua**)** 5.89 (batch 0583610001, supplier Loba) 5.84 batch 0647670001, supplier Loba)
Refractive index:	1.63 (predicted) (EPA)

UV/visible light absorption spectrum:

	λ_{max} ε *	λ_{max} ε *	λ_{max} ε *	λ_{max} ε *	λ_{max} ε *
E509991 (Guanghua) batch 20170531 (0.0014g/100ml water)	616 18248.0	408 9668.7	307 9169.4	284 7853.0	196 55379.5
E512354(Loba) batch 058361000 (0.0013g/100ml water)	616 6281.2	426 12562.5	311 6843.0	281 7711.1	196 51999.3
E512354(Loba) batch 0647670001 (0.0013g/100ml water)	616 6264.2	426 14069.7	311 7407.7	281 8501.4	196 60753.2

* ε (L.mol^{-1}.cm^{-1})



According to the Applicant, the UV spectra of E509991 (Guanghua synthesis) batch 20170531, of E512354 (Loba synthesis) batches 0583610001 and 0647670001 were slightly different due to a difference in pH.

Ref.: SDS spectrum https://www.spectrumchemical.com/media/sds/BR155_AGHS.pdf
Chemical Book (https://www.chemicalbook.com/CASEN_34722-90-2.htm)
David R. Line, Handbook of Chemistry and Physics, 84th edition (2003/2004), CRC Press, 2004

3.1.9 Homogeneity and Stability

In the percutaneous absorption study, the HPLC analysis of the hair dye formulation performed following the dosing procedure and 24 hours post application was 96.4%, confirming that the formulation was stable for a 24-hour period.

Whitson, 2019

3.2 TOXICOKINETICS

3.2.1 Dermal / percutaneous absorption

In Vitro Percutaneous Absorption Study using Human Dermatomed Skin

Guideline:	OECD 428
Species/strain:	Frozen human dermatomed skin (200-500 µm)
Membrane integrity:	Checked by electrical resistance, at least 10.9 kW
Replicates:	12 intact skin samples (4 donors) per time point (24 in total)
Test substance:	Bromothymol Blue Na Salt
Batch:	Test batches 20170531 of Bromothymol Blue Na salt and CFQ43741 of Bromothymol Blue Na Salt [^{14}C]
Purity:	95.9% radiochemical pure

Test item:	Hair dye formulation containing a final on-head concentration of 0.5% (w/w) Bromothymol Blue Na salt
Dose applied:	Ca 20 mg/cm^2 of the test formulation (corresponding to a nominal dose rate of 100 µg/cm²))
Exposure area:	0.64 cm^2
Exposure period:	30 minutes
Sampling period:	24 and 72 hours
Receptor fluid:	Phosphate buffered saline containing polyoxyethylene 20 oleyl ether (PEG, 6%, w/v), sodium azide (0.01%, w/v), streptomycin (0.1 mg/mL) and penicillin (100 units/mL).
Solubility in receptor fluid:	96.01%
Mass balance analysis:	101.14 ± 4.04 of the applied dose at 24 h 101.30 ± 1.47% of the applied dose at 72 h
Tape stripping:	Yes (20)
Method of Analysis:	Liquid scintillation counting (LSC)
GLP:	In compliance
Study period:	January – March 2019

Test Procedure
Human abdominal skin samples were obtained from three different female and one male donors from a tissue bank. The membranes were stored frozen, at approximately -20°C, on foil until use.

Skin samples were dermatomed (200-500 µm in thickness) and mounted in diffusion cells. The receptor fluid chosen was phosphate buffered saline containing polyoxyethylene 20 oleyl ether (PEG, 6%, w/v), sodium azide (0.01%, w/v), streptomycin (0.1 mg/mL) and penicillin (100 units/mL). The pH of the receptor fluid was checked and adjusted to pH 7.37-7.40. Membrane integrity was determined by measurement of the electrical resistance across the skin membrane. Membranes with a measured resistance of <10.9 kΩ were regarded as having a lower integrity than normal and were excluded from subsequent absorption measurements. Twenty-four intact skin membranes (from four human donors) were used and skin was maintained at approximately 32°C.

A typical hair dye formulation at pH=7 containing 0.5% [^{14}C]-Bromothymol blue sodium salt was tested. About 20 mg/cm² of this formulation (corresponding to a nominal dose rate of 100 µg/cm²) was applied to the skin surface and left for 30 minutes. After this time period, the remaining formulation on the skin surface was removed using a standardized washing procedure, simulating use conditions. The percutaneous absorption of Bromothymol blue sodium salt was monitored for 24h and 72h (12 cells each) and estimated by measuring its concentration by liquid scintillation counting in the following compartments: skin washes, stratum corneum (isolated by tape strippings), living epidermis/dermis, unexposed skin and receptor fluid.

Results
A summary of the mean results, including the standard deviation (SD), is provided in Table 3. The same concentration and radiochemical purity were determined at 0 h and 24 h post dose, showing that the formulation was stable for a 24-hour period.

Table 3: Summary of the percutaneous absorption

Test Item	Bromothymol Blue Na Salt	
Number of Samples	12 for Group 1 and 11 for Group 2	
Analysis Type	Liquid Scintillation Counting	
Concentration of Test Item (mg/g)	4.94	
Applied Mass (mg/cm^2)	20.42	
Group	1 (24 h)	2 (72 h)
Distribution	% Applied Dose (Mean ± SD)	
Dislodgeable Dose 0.5 h	79.90 ± 4.71	79.19 ± 3.54
Dislodgeable Dose 24 h	79.99 ± 4.73	N/A
Dislodgeable Dose 72 h	N/A	79.28 ± 3.53
Stratum Corneum	19.71 ± 5.73	20.03 ± 3.03
Unexposed Skin	< 0.01 ± < 0.01	< 0.01 ± < 0.01
Total Unabsorbed	99.71 ± 4.02	99.31 ± 1.81
Epidermis	1.39 ± 1.60	1.90 ± 2.37
Dermis	0.03 ± 0.05	0.05 ± 0.05
Total Absorbed Dose	0.02 ± 0.02	0.04 ± 0.02
Dermal Delivery	1.44 ± 1.64	1.99 ± 2.40
Mass Balance	101.14 ± 4.04	101.30 ± 1.47
Distribution	µg equiv./cm^2 (Mean ± SD)	
Dislodgeable Dose 0.5 h	80.59 ± 4.75	79.87 ±3.57
Dislodgeable Dose 24 h	80.68 ± 4.77	N/A
Dislodgeable Dose 72 h	N/A	79.96 ±3.56
Stratum Corneum	19.88 ± 5.78	20.20 ± 3.05
Unexposed Skin	< 0.01 ± < 0.01	< 0.01 ± < 0.01
Total Unabsorbed	100.56 ± 4.06	100.16 ± 1.82
Epidermis	1.40 ± 1.62	1.92 ± 2.39
Dermis	0.03 ± 0.05	0.05 ± 0.05
Total Absorbed Dose	0.02 ± 0.02	0.04 ± 0.02
Dermal Delivery	1.45 ± 1.66	2.01 ± 2.42
Mass Balance	102.01 ± 4.07	102.18 ± 1.48

N/A = Not applicable
Dislodgeable dose 0.5 h = skin wash 0.5 h + tissue swab 0.5 h + pipette tip 0.5 h
Dislodgeable dose 24 h/72 h = dislodgeable dose 0.5 h + donor chamber wash
Stratum corneum = tape strips 1 to 20
Total unabsorbed dose = dislodgeable dose 24 h/72 h + *stratum corneum* + unexposed skin
Epidermis = epidermis + clingfilm
Total absorbed dose = cumulative receptor fluid + receptor chamber wash + receptor rinse
Dermal delivery = absorbed dose + epidermis + dermis
Mass balance = unabsorbed dose + dermal delivery

The 24-hour condition:
All diffusion cells yielded data that could be analysed, and the mean recovery rate was good at 101.14 ± 4.04%.
Most of the applied dose of Bromothymol blue sodium salt was rinsed off from the skin surface at 30 min post application, representing 79.9%. At 24h, 19.88 ± 5.78 µg/cm² (19.71 ± 5.73%) of Bromothymol blue sodium salt was recovered from the stratum corneum. This amount was not considered bioavailable. From the dermis 0.03 ± 0.05 µg/cm² (0.03 ± 0.05%) and from the epidermis 1.40 ± 1.62 µg/cm² (1.39 ± 1.60%) were recovered. A maximum amount of 0.02 ± 0.02 µg/cm² (0.02 ± 0.02%) Bromothymol blue sodium salt passed through the skin and was recovered in the receptor fluid during 24h exposure.

The 72-hour condition:
Eleven out of twelve diffusion cells yielded data that could be analysed, and the mean recovery rate was good at 101.30 ± 1.47%. For one cell, the absorption of the test item in the receptor fluid represented an outlier (> mean + 2SD) and thus the cell was excluded.
Most of the applied dose of Bromothymol blue sodium salt was rinsed off from the skin surface at 30 min post application, representing 79.19%. At 72h, 20.20 ± 3.05 µg/cm² (20.03 ± 3.03%) of Bromothymol blue sodium salt was recovered from the stratum corneum. This amount was not considered bioavailable. From the dermis 0.05 ± 0.05 µg/cm² (0.05 ± 0.05%) and from the epidermis 1.92 ± 2.39 µg/cm² (1.90 ± 2.37%) were recovered. A maximum amount of 0.04 ± 0.02 µg/cm² (0.04 ± 0.02%) Bromothymol blue sodium salt passed through the skin and was recovered in the receptor fluid during 72h exposure.

Most of the samples recovered from the receptor fluid at 24 and 72h were below the limit of reliable measurement (1.34 and 2.67 ng/cm², respectively). No movement of the dye from the skin reservoir to the receptor fluid, or from epidermis to dermis, occurred after 72 h. Therefore, a depot effect from the epidermis can be excluded. Under the described test conditions, the bioavailable amount corresponds to the sum of the amounts present in the receptor fluid and in the dermis. This equals 0.05 ± 0.07 µg/cm² (0.05 ± 0.07%) and 0.09 ± 0.07 µg/cm² (0.09 ± 0.07%) of sodium bromothymol blue at 24h and 72h, respectively.

Ref. Whitson, 2019

SCCS comment
Dermal absorption is measured using two different time-points, 24 and 72 hours. This methodology can be applied, according to the SCCS Notes of Guidance, when there is clearly no movement of chemical from the skin reservoir to the receptor fluid with 24h vs 72h (see Notes of Guidance SCCS/1628/21 section 3-3.5.1.1.).

After 24h, the total absorbed dose (which was defined by the Applicant as being the cumulative receptor fluid +receptor chamber wash +receptor rinse) was 0.02 ± 0.02 % and at 72h 0.04 ± 0.02%.
According to the Applicant, the absorption calculated throughout the course of the experiment was below the limit of reliable measurement for most samples. It was further clarified: "A limit of reliable measurement of 30 dpm above background has been instituted. Counts that are below 30 dpm above background represent a true value. This means that data are recorded with values that are less than the limit of reliable measurement."
As a consequence, different datapoints could not be accurately determined. Therefore, the SCCS is of the opinion that, based on the values 0.02 ± 0.02 % at 24h and 0.04 ± 0.02% at 72 h, it is not possible to state that there is no significant movement from the skin reservoir to the receptor fluid.
Since the 24-hour dermal absorption study is acceptable according to the SCCS Basic Criteria, the values defined by the Applicant as 'dermal delivery' (see Table 3 = absorbed dose+epidermis+dermis)) will be used. The dermal absorption of sodium bromothymol blue from this study is therefore 1.44% + 1.64 (Mean + 1SD) = 3.08 % and this value will be used in the SED calculation.

3.2.2 Other studies on toxicokinetics

/

3.3 EXPOSURE ASSESSMENT

3.3.1 Function and uses

The ingredient sodium bromothymol blue is used in non-oxidative hair colouring products at a maximum on-head concentration of 0.5%.

3.3.2 Calculation of SED/LED

Use in non-oxidative hair dye conditions

From the Applicant:

Exposure Assumptions

Percutaneous absorption (72h): 0.09 ± 0.07 µg/cm² *i.e.*, 0.16 µg/cm²

Surface area of exposed scalp: 580 cm^2

Average body weight: 60 kg

Systemic Exposure Calculation:

= percutaneous absorption x surface area of scalp exposed

= 0.16 µg/cm² x 580 cm^2 = 92.8 *i.e.*, 93 µg/ application

= 1.55 µg/kg/application (considering a body weight of 60 kg).

SCCS comment

After evaluation of the dermal absorption study, SCCS concluded that dermal absorption is 3.08%. This is higher than the dermal absorption value derived by the Applicant, based on the 72-hour exposure. Below, the SCCS has recalculated the SED using the value of 3.08% dermal absorption.

SED = percutaneous absorption x surface area of scalp exposed

20 mg product /cm² on the head; surface area scalp: 580cm²

= 20mg/cm² x 580cm² =11600 mg product on the head

Product contained 0.5% sodium bromothymol blue = 58 mg

Dermal absorption is 3.08%

Body weight is 60 kg

SED =58mg/60kgbw X 3.08/100= 0.02977 mg/ kg bw/d= 29.77 µg/kg bw/application
The SCCS will use **29.77 µg/kg bw/application** as the SED for calculating Margin of Safety.

3.4 TOXICOLOGICAL EVALUATION

3.4.1 Irritation and corrosivity

3.4.1.1 Skin irritation

***In vitro* Episkin^{SM} Skin Irritation Test**

Guideline:	OECD 439 (2015)
Test System:	Reconstructed human epidermis model Episkin (EPISKIN/S/13; 0.38 cm^2)
Replicates:	3 different tissue batches
Test substance:	Bromothymol blue sodium salt
Batch:	20170531
Purity:	98.9%
Test item:	Dark green powder
Dose level:	10±2 mg
Treatment period:	15 minutes at room temperature
Post-treatment incubation time:	42 ± 1 hour
Positive control:	5% aqueous solution of Sodium Dodecyl Sulfate
Negative control:	PBS^+ (Phosphate Buffer Saline with Ca^{2+} and Mg^{2+})
Solvent control:	Not done
Dead dermis negative control:	10 µL of PBS+ tested in duplicate on dead epidermis
Interaction with MTT:	Positive
Colouring of tissue:	Positive
GLP:	In compliance
Study period:	July 2020

The undiluted test item Bromothymol blue sodium salt, positive and negative control were tested in triplicate on the reconstructed human epidermis model.
The test item interacted with MTT and had a staining power. Therefore, 3 additional trials were therefore performed:

- MTT interaction on 2 dead epitheliums (negative control and test item)
- Staining power on 2 living epitheliums not incubated in MTT solution (test item)
- Staining power on 2 dead epitheliums not incubated in MTT solution (test item)

These tissues as well as the additional negative control were used as additional specific controls in order to quantify the Non Specific Color (NSC) and Non Specific MTT reduction (NSMTT) due to the colouring chemical interactions.

Five microliters of sterile water was spread on the surface of each epidermis and then 10±2 mg of powder test item was applied. Ten µL of different controls were applied onto the epidermis using a positive displacement pipette. After 15-minutes treatment period at room temperature, tissues were rinsed with PBS+, and then epidermis were transferred in 2 ml/well of fresh maintenance medium and incubated for 42 ± 1 hours at 37°C.

At the end of the 42±1 hours-treatment period, each epidermis unit was transferred to 12 well plates containing a dye solution (MTT) except for the negative control and the test item-treated epidermis without MTT which were transferred into a 12 well plate containing fresh medium. Plates were incubated for 3 hours ± 5 minutes at 37°C. At the end of the incubation period, a biopsy of the entire epidermis was taken. The epidermis was separated from the collagen matrix and both were transferred into a tube containing 500 µL of DMSO.
Formazan crystals were extracted and stirred to homogenize the solution and the optical density was measured at 570 nm.

Results
During the assay, interaction between the test item and the MTT has not been highlighted because the NSMTT was inferior to zero.
Similarly, the test item has a staining power, but during the assay, the latter has not been highlighted because the NSC was less than 5%.
The mean viability value for undiluted Bromothymol blue sodium salt was 84.3±4.8%.

Conclusion
Under the conditions of this study, undiluted Bromothymol blue sodium salt was considered to have no skin irritation potential.

Ref: Cannamela, 2020

3.4.1.2 Mucous membrane irritation / eye irritation

Bovine corneal opacity and permeability method (BCOP)

Guideline:	OECD 437 (2017)
Test material:	Bovine cornea
Replicates:	3 corneas per test condition
Test substance:	Bromothymol blue sodium salt
Batch:	20170531
Purity:	98.9%
Test item:	20% in 0.9% NaCl
Dose applied:	750µl
Treatment period:	4 hours
Post-treatment incubation time	/
Positive control:	Imidazole
Negative control:	0.9% NaCl
GLP:	In compliance
Study period:	July 2020

The test item Bromothymol blue sodium salt diluted at 20% (w/w) was applied onto the cornea (category: solid non surfactant) for 4 hours. Three corneas were used for each of the negative control, positive control and the test item. At the end of contact period, the corneas were rinsed and prepared for measurement of opacification (changes in light transmission) and for permeability (evaluation of transfer of fluorescein through the cornea by measuring the optical density of the media in the ocular posterior compartment). The corneal score, which is the combination of opacification and permeability, was then calculated.

Results
The score obtained for Bromothymol blue sodium salt diluted at 20% (w/w) in NaCl 0.9% after 4-hour contact was given as an indication (346.7 ± 3.5) because of the colouration of corneas and the optical density reading solution. The test item could not be classified according to the methodology OECD guideline 437.

Conclusion
Under the conditions of this study, the *in vitro* evaluation of the acute ocular irritation potential of Bromothymol blue sodium salt diluted at 20% in the BCOP was inconclusive.

Ref: Julienne, 2020

Episkin® Reconstructed human Cornea-Like Epithelium (HCE) model

Guideline:	OECD 492 (2019)
Test System:	Reconstructed human cornea-like epithelium Episkin model based on transformed human corneal keratinocytes - SkinEthic™HCE/Corneal Epithelium (HCE/S/5) (0.5 cm^2)
Replicates:	2 different tissue batches (No. 20-HCE-027 and ?)
Test substance:	Bromothymol blue sodium salt
Batch:	20170531
Purity:	98.9%
Test item:	Dark green powder
Dose level:	10±2 mg
Treatment period:	4 hours ± 5 minutes at 37°C in CO_2-incubator
Post-treatment incubation time:	18 hours ± 30 minutes at 37°C in CO_2-incubator
Positive control:	Methyl acetate
Negative control:	PBS^+ (Phosphate Buffer Saline with Ca^{2+} and Mg^{2+})
Solvent control:	Not done
Dead dermis negative control:	10 µL of PBS^+ tested in duplicate on dead epidermis
Interaction with MTT:	Positive
Colouring of tissue:	Positive
GLP:	Not in compliance*
Study period:	July 2020

* The *in vitro* ocular primary irritation test was not declared to be GLP-compliant according to the Contract Research Organization (although run under GLP conditions) due to the sticking of test item to the epithelium. According to the Applicant, this deviation from GLP has no impact on the safety evaluation.

The cell viability (MTT conversion test) is used as endpoint for the prediction of the eye irritation or serious eye damage potential, using the *in vitro* reconstructed Episkin® Human Cornea-like Epithelium (HCE) model.

In vitro Episkin® HCE eye irritation test method for solid chemicals was applied. Thirty (30) µL of phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS^-) were applied onto the reconstructed human corneal epithelium (2 epitheliums were used) to pre-moisten the tissue, and then 30 ± 2 mg of the test item were deposited. After a 4-hour treatment period at 37°C, tissues were thoroughly rinsed and incubated for an additional 30-minute in a maintenance medium (post-soak immersion) at room temperature. At the end of the 30-minute incubation period, the epitheliums were removed, dried with cotton bud and then incubated in one ml of maintenance medium for 18 hours ± 30 minutes at 37°C. Afterwards, the epitheliums were prepared for cell viability measurement (MTT conversion test).
Given that the test item has interacted with MTT and had a staining power, 3 additional trials were performed:

- MTT interaction on 2 dead epitheliums (negative control and test item)
- Staining power on 2 living epitheliums not incubated in MTT solution (test item)
- Staining power on 2 dead epitheliums not incubated in MTT solution (test item)

Negative (PBS^-) and positive controls (methyl acetate) as well as dead epitheliums were tested according to the same experimental conditions.
The reduction of cell viability in tissues treated with test chemicals is compared to tissues treated with negative control (100% viability) and expressed as a percentage.
The ocular irritancy potential of the test item, according to UN GHS, will be predicted by the mean viability percentage: if the mean viability value is above 50%, the test item is classified as No Category. If it is ≤ 50%, no prediction can be made.

Results
The results for the positive control were in conformity with the awaited data.

The mean cell viability value obtained for undiluted Bromothymol blue sodium salt was 2.0 ± 0.4%.

Test item	Epithelium 1: relative viability (%)	Epithelium 2: relative viability (%)	Mean relative viability (%)	Classification (according to UN GSH)
As supplied	1.7	2.3	2.0 ± 0.4	No conclusion

Conclusion
Under the conditions of the present study, no conclusion can be given for undiluted Bromothymol blue sodium salt on an Episkin® reconstructed Human Cornea-like Epithelium model after 4 hours of contact.

Ref: Maillet, 2020a

SCCS comment
The study report indicates that two different batches of the test system were used, but only one analysis certificate has been provided.

Episkin® Reconstructed human Cornea-Like Epithelium (HCE) model

Guideline:	OECD 492 (2019)
Test System:	Reconstructed human cornea-like epithelium Episkin model based on transformed human corneal keratinocytes - SkinEthic™HCE/Corneal Epithelium (HCE/S/5); (0.5 cm^2)
Replicates:	2 different tissue batches (No. 20-HCE-013 and 20-HCE-036)
Test substance:	Bromothymol blue sodium salt
Batch:	20170531
Purity:	98.9%
Test item:	Dark green powder
Dose level:	0.5 % (w/w) in sterile water
Treatment period:	30±2 minutes at 37°C in CO_2-incubator
Post-treatment incubation time:	30±2 minutes at 37°C in CO_2-incubator
Positive control:	Methyl acetate
Negative control:	PBS^+ (Phosphate Buffer Saline with Ca^{2+} and Mg^{2+})
Solvent control:	Not done
Dead dermis negative control:	10 µL of PBS^+ tested in duplicate on dead epidermis
Interaction with MTT:	Positive
Colouring of tissue:	Positive
GLP:	In compliance
Study period:	August 2020

The cell viability (MTT conversion test) is used as endpoint for the prediction of the eye irritation or serious eye damage potential, using the *in vitro* reconstructed Episkin® Human Cornea-like Epithelium (HCE) model.
In vitro Episkin® HCE eye irritation test method for liquids was applied. Ten (10) µL of phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS^-) were applied onto the reconstructed human corneal epithelium (2 epitheliums were used) to pre-moisten the tissue, and then 30 µl of the test item was deposited. After a 30-minute treatment period at 37°C, tissues were thoroughly rinsed and incubated for an additional 30-minute period in a maintenance medium (post-soak immersion) at room temperature. Afterwards, the epitheliums were prepared for cell viability measurement (MTT conversion test).
Given that the test item has interacted with MTT and had a staining power, 3 additional trials were performed:

- MTT interaction on 2 dead epitheliums (negative control and test item)
- Staining power on 2 living epitheliums not incubated in MTT solution (test item)
- Staining power on 2 dead epitheliums not incubated in MTT solution (test item)

Negative (PBS^-) and positive controls (methyl acetate) as well as dead epitheliums were tested according to the same experimental conditions.
The reduction of cell viability in tissues treated with test chemicals is compared to tissues treated with a negative control (100% viability) and expressed as a percentage.
The ocular irritancy potential of the test item, according to UN GHS, will be predicted by the mean viability percentage: if the mean viability value is above 60%, the test item is classified as No Category. If it is ≤ 60%, no prediction can be made.

Results
The results for the positive control were in conformity with the awaited data.
The mean cell viability value obtained for Bromothymol blue sodium salt diluted at 0.5% was 108.3 ± 4.7%.

Test item	Epithelium 1: relative viability (%)	Epithelium 2: relative viability (%)	Mean relative viability (%)	Classification (according to UN GSH)
0.5%	111.6	104.9	108.3 ± 4.7	No category

Conclusion
Under the conditions of the present study, Bromothymol blue sodium salt diluted at 0.5% on an Episkin® reconstructed Human Cornea-like Epithelium model after 30 minutes of contact is classified as 'No Category'.

Ref: Maillet, 2020b

SCCS overall conclusion
Based on the information provided, the SCCS considers sodium bromothymol blue salt non-irritant to the skin and the eyes at intended use concentration.

3.4.2 Skin sensitisation

Next Generation Risk Assessment (NGRA) for sodium bromothymol blue
Cosmetics industry has developed a NGRA framework to support the safety assessment of cosmetic ingredients without the generation of new animal data (Gilmour *et al.*, 2020). The Applicant followed the NGRA framework as illustrated below in Figure 4 to evaluate the skin sensitisation potential of sodium bromothymol blue.

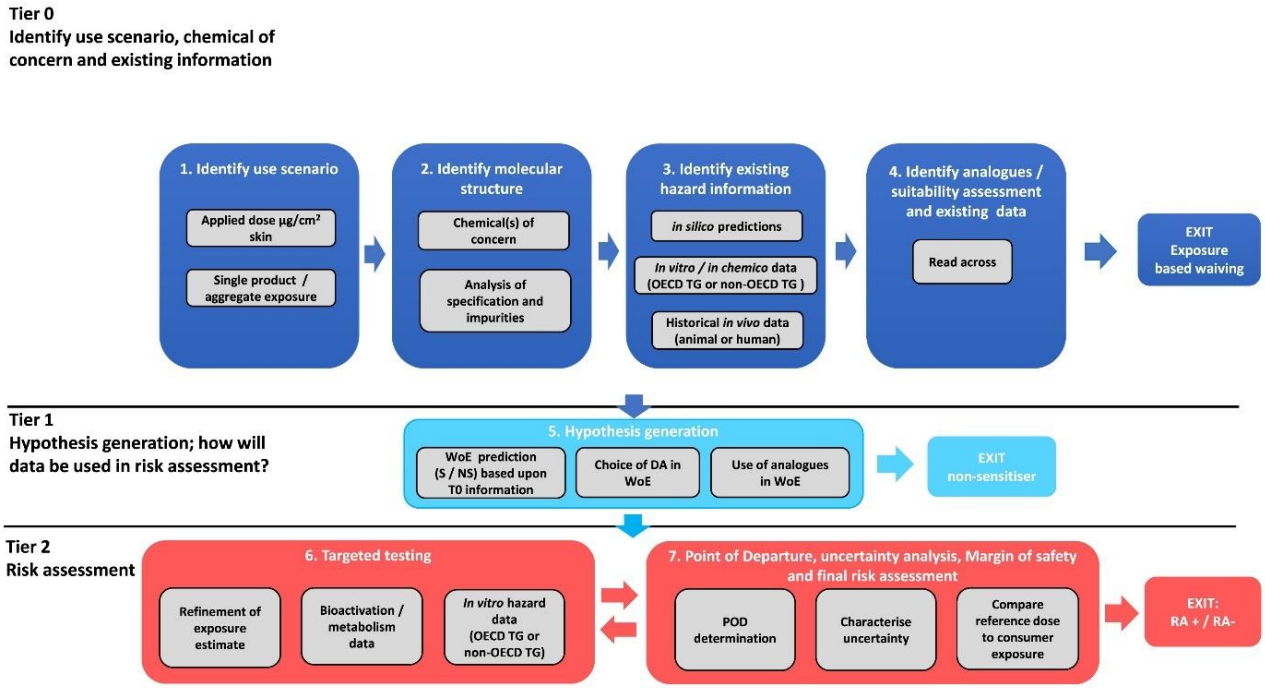


Figure 4: Next generation risk assessment (NGRA) framework for skin sensitisation (from Gilmour *et al.*, 2020)

TIER 0: identify use scenario, chemical of concern and existing information

II.a.1. Identify use scenario
Sodium bromothymol blue is intended to be used in non-oxidative hair colouring products at concentrations of up to 0.5% on-head.

II.a.2. Identify chemical of interest and molecular structure
Sodium bromothymol blue has been described in 3.1. Chemical and physical specifications.

II.a. Chemical Identity and Description and II.b. Typical Physical Properties
Summarised in the Table 3.

II.a.3. Identify existing hazard information
As a skin sensitisation hazard, characterisation should not be based on individual NAM as stand-alone, the collected information was listed in the table 4 below.

Table 4: Summary of parameters related to skin sensitisation and needed for the sequential testing strategy DA

Physicochemical properties	Molecular weight: 646.369 g/mol
	Volatility: non volatile[1]
	pH: 7.85[2]
TOXTREE protein binding (v2.6.13)	Reactive
TIMES-M/ TIMES-P (Times v2.29.1 - SS v23.28)	Inconclusive (out of domain)
DPRA	Reactive[3]
KeratinoSens™	Negative[3]
U-SENS™	Negative[3]

[1] Volatility class was calculated using a method by Spicer, 2002;

[2] pH was obtained for the batch 20170531 used *in vitro* tests (check analytical dossier)

[3] there is no reported limitation to the 3 *in vitro* assays. In addition, U-SENS™ assay was reported to correctly predict lipophilic tested chemicals (Alépée N, 2017).

II.a.4. Identify analogues/suitability assessment and existing data

The existing information were considered sufficient and read across was not deemed necessary, thus no search for analogues was conducted.

SCCS comment

The decisions made and data provided in Tier 0 give rise to several questions that need to be addressed by the Applicant.

- Table 4 includes not only hazard information, but also *in silico* predictions and physicochemical properties. It is recommended to describe the different sources of information in Tier 0 under the correct headings, similar to the boxes displayed in Figure 4.
- For *in silico* predictions, only Toxtree and TIMES-SS are used. The Applicant has not provided any substantiation why only these two programmes were used, and why further supporting evidence was not obtained by using other programmes, such as the OECD QSAR Toolbox.
- It is not clear why the NAM data are included in Tier 0 as existing data. The substance under evaluation is a new ingredient, and the tests were conducted in 2017 and were not submitted to the SCCS before. Hence, they should not be included in Tier 0 as the existing data, but should be described and evaluated under Tier 2 (targeted testing).
- The Applicant has deemed the existing information as sufficient. The SCCS however disagrees, especially given that the NAM data are newly generated and cannot be considered as existing data. It is also not clear why the Applicant did not search for any available data on other structural analogues of Sodium Bromothymol Blue to see if a read-across was possible. As more experience is still needed for the use of NGRA for skin sensitisation, the SCCS strongly urges the Applicant to carry out read-across to obtain further supporting information that can increase the overall weight of evidence.

TIER 1: hypothesis generation
Based on Gilmour et al, 2020, it is stated that "in the context of the framework, risk assessors will choose which DA to apply according to the risk assessment needs, data availability, accessibility and applicability of the DA, as well as their own expertise". Thus, the sequential testing strategy DA (Del Bufalo *et al.* 2018; Tourneix *et al.* 2019, 2020) was used for hypothesis generation considering the Applicant's expertise and high experience with the model. Thus, the collected information is integrated into the sequential testing strategy DA.

SCCS comment
The SCCS notes that no hypothesis is generated based on the information retrieved in Tier 0. The Applicant incorrectly included the newly generated NAM data in Tier 0. Clearly, for a new ingredient, there are no experimental data, but only *in silico* data. Therefore, based on the alert from Toxtree, it would have been possible for the Applicant to generate a hypothesis: e.g. sodium bromothymol blue has an alert for protein-reactivity and therefore there may be a concern for skin sensitisation and further testing may be required. In addition to this, the DA is described under Tier 1 in the dossier of the Applicant, but it belongs to Tier 2 (targeted testing).

From the Applicant
Background on the sequential testing strategy DA
The sequential testing strategy is constructed as a tiered approach with a decision point at the end of each tier, allowing stepwise and efficient information gathering (Figure 5). The first tier, which combines *in silico* predictions (TIMES-SS, ToxTree), Direct Peptide Reactivity Assay (DPRA), U-SENS™ and KeratinoSens™ as well as physicochemical parameters (pH, volatility), was built on 165 chemicals having a LLNA-based Sensitiser / Non-Sensitiser (S/NS) classification (Del Bufalo *et al.* 2018). The second tier, which combines DPRA, U-SENS™ and KeratinoSens™ data as well as physicochemical parameters (Molecular Weight, volatility and clogP), was built on 100 chemicals having a LLNA-based Sensitiser (UN GHS) cat. 1A/1B classification (Figure 5).
For each of those tiers, the combination of the different input parameters was achieved using a meta-model stacking five different statistical methods (Boosting, Naïve Bayes, Support Vector Machine (SVM), Sparse PLS-DA and Expert Scoring), providing a probability to belong to the group of interest ("to be a sensitizer" Tier 1, "to be a Cat. 1A" Tier 2).

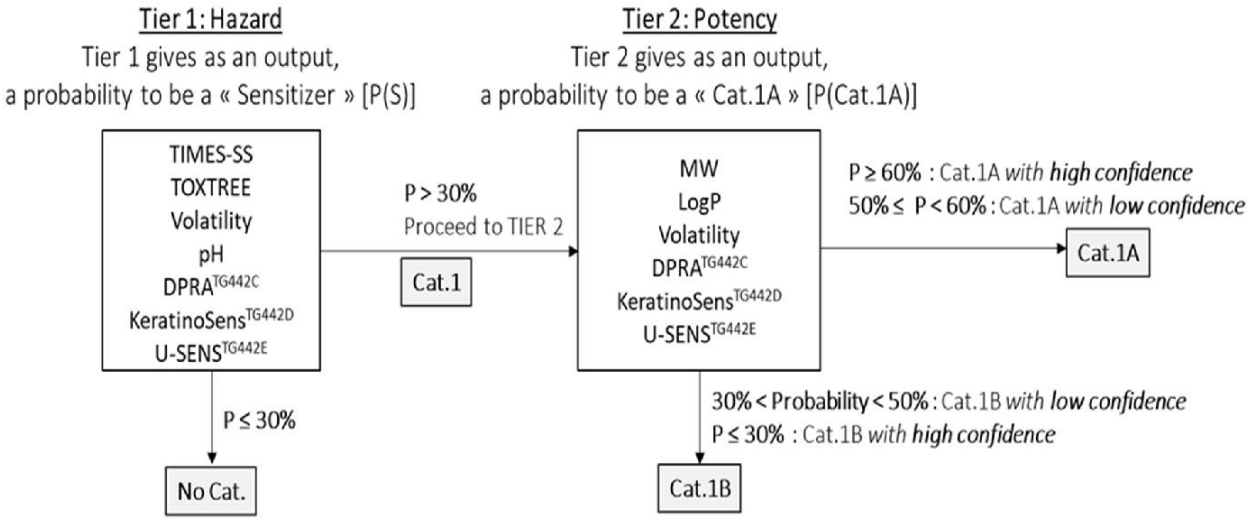


Figure 5: Schematic representation of sequential testing strategy for hazard identification (Tier 1) and potency (UN GHS cat. 1A/1B) categorisation (Tier 2) of skin sensitisation

This sequential testing strategy is constructed in such a way that it allows stepwise gathering of information using a tiered approach on skin sensitisation hazard (Tier 1) and potency (Tier 2). Both tiers integrate information describing the MIE (KE1), KE2 and KE3.
As such, the sequential testing strategy is based on intrinsic physicochemical properties of the chemical and descriptors of early innate immune cell responses key events, as described below.

1) Intrinsic physicochemical properties of the chemical
   - Descriptors that make it possible to integrate stability and/or bioavailability characteristics. As such, the measured pH and the calculated volatility, cLogP and MW were considered as relevant variables to combine with *in silico, in chemico* and *in vitro* methods, as defined in a splitting statistical analysis (Gomes *et al.*, 2012).
   - Chemical reactivity (which is directly linked to the initial key event *i.e.* haptenation of skin proteins): ToxTree skin sensitisation alerts (Aptula and Roberts, 2006). The Times-SS predictions also mainly take into account electrophilic binding to skin proteins either directly or following metabolism but it is not the only mechanism that is integrated (Patlewicz *et al.*, 2007). Finally, the *in chemico* DPRA (Gerberick *et al.*, 2004; OECD, 2019), related to AOP KE1, is a method giving a measurement of MIEs as cysteine and lysine peptides modifications by the chemical.

2) Descriptors of early innate immune cell responses:
   - KE2: *i.e.* keratinocyte activation, with the KeratinoSens™ assay assessing the induction of the Nrf-2 pathway (Emter *et al.*, 2010; OECD, 2018a).
   - KE3: *i.e.* dendritic cells activation, with the existing DC-surrogates based CD86 activation U-SENS™ assay (Piroird *et al.*, 2015; Alépée *et al.*, 2015; 2017).

For each of those tiers, the combination of the different input parameters was achieved using a stacking meta-model. From the large number of supervised classification models proposed in the literature, five different methods: Boosting, Naïve Bayes, Support Vector Machine (SVM), Sparse PLS-DA and Expert Scoring were selected (Gomes *et al.*, 2014; Nocairi *et al.* 2016). These methods have strong differences, but they all produce the posterior probability of belonging to the group of interest. Therefore, two stacking models (Tier 1 "to be a sensitizer" and Tier 2 "to be a UN GHS cat. 1A") were built independently on a proper training set (based on LLNA data). Instead of trying to choose a specific method, these methods were combined by the stacking methodology of Wolpert (1992) and Breiman (1996) to obtain a specific stacking meta-model for each tier.

Data from new approach methodology (NAM)
Sodium bromothymol blue was tested by the Applicant in individual *in chemico and in vitro* assays for skin sensitisation. The results of these assays were used together with other data in the sequential testing strategy DA, as described above.

Direct Peptide Reactivity Assay (DPRA)

Guideline:	OECD 442C: In Chemico Skin Sensitisation Assays addressing the AOP key event on covalent binding to proteins
Test System:	/
Replicates:	3
Test substance:	Bromothymol blue sodium salt
Batch:	20170531
Purity:	98.9%
Test item:	Greenish brown powder
Solvent:	1:1 mixture acetonitrile:milli-Q water
Dose level:	100 mM
Treatment period:	24 hours
Positive control:	Cinnamaldehyde (CAS No. 104-55-2)
Negative control:	/

Co-elution control samples:	test item incubated with each buffer used to dilute the peptides
Reference control samples:	A: check the accuracy of the calibration curve for peptide quantification B: check the stability of the peptide during analysis C: check that the solvent did not impact the percentage of peptide depletion.
GLP:	In compliance*
Study period:	November 2017

The objective of this study was to evaluate the reactivity of the Bromothymol blue sodium salt to synthetic cysteine and lysine peptides. This was evaluated *in chemico* by monitoring peptide depletion following a 24-hour contact between the test item and synthetic cysteine and lysine peptides. At the end of the incubation, the concentrations of residual peptides were evaluated by HPLC with Ultra-Violet detection at 220 nm.
Peptide reactivity was reported as the percent of depletion based on the peptide peak area of the replicate injection and the mean peptide peak area in the three relevant reference control C samples (in the appropriate solvent).

Results
The test item was dissolved at 100 mM in a 1:1 mixture acetonitrile:milliQ water after one minute of sonication. The acceptance criteria for the calibration curve samples, the reference and positive controls, as well as for the study samples were satisfied. The study was therefore considered to be valid.
Analysis of the chromatograms of the co-elution samples indicated that the test item did not co-elute with either the lysine or the cysteine peptides. As a result, the mean percent depletion values were calculated for each peptide using the formula described in the OECD guideline.

- for the cysteine peptide, the mean depletion value was 54.50%,
- for the lysine peptide, the mean depletion value was 97.33%.

The mean of the percent cysteine and percent lysine depletions was equal to 75.91%. Accordingly, the test item was considered to have a high peptide reactivity. Therefore, the DPRA prediction is considered as positive and the test item may have potential to cause skin sensitisation.

Conclusion
Under the experimental conditions of this study, the DPRA prediction is considered as positive and Bromothymol blue sodium was considered to have a high peptide reactivity.

Ref. Valin, 2018a

SCCS comment
SCCS agrees that under the conditions of this test sodium bromothymol blue has a high peptide reactivity. This is in line with the *in silico* prediction for protein reactivity generated by ToxTree. Hence, sodium bromothymol blue is able to bind to proteins and can initiate the molecular initiating event (MIE) of the skin sensitisation AOP.

Keratinosens™

Guideline:	OECD 442D *In vitro* skin sensitisation assays addressing the AOP key event on keratinocyte activation.
Test System:	KeratinoSens™ cell line
Replicates:	Two
Test substance:	Bromothymol blue sodium salt

Batch:	20170531
Purity:	98.9%
Test item:	Greenish brown powder
Solvent:	DMSO
Dose level:	First run: 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000 and 2000 µM in culture medium with 1% DMSO Second run: 4.57, 6.44, 9.08, 12.80, 18.05, 25.45, 35.9, 51, 71, 101, 142, and 200 µM in culture medium with 1% DMSO
Treatment period:	48 hours
Positive control:	Cinnamic aldehyde (CAS 14371-10-9)
Negative control:	culture medium with 1% DMSO
GLP:	In compliance*
Study period:	November-December 2017

The objective of this study was to evaluate the potential of the test item, Bromothymol blue sodium salt, to activate the Nrf2 transcription factor.
This *in vitro* test uses the KeratinoSens cell line, an immortalised and genetically modified human HaCaT keratinocyte cell line. The KeratinoSens cell line is stably transfected with a plasmid containing a luciferase gene under the transcriptional control of the SV40 origin of replication promoter. This promoter is fused with an ARE sequence. Sensitisers with electrophilic properties provoke the dissociation of Keap-1 from the transcription factor Nrf2. The free Nrf2 binds to the ARE sequence contained in the plasmid and therefore induces transcription of firefly luciferase.

The KeratinoSens cells were first plated on 96-well plates and grown for 24 hours at 37°C. Then the medium was removed and the cells were exposed to the vehicle control or to different concentrations of test item and of positive controls. The treated plates were then incubated for 48 hours at 37°C. At the end of the treatment, cells were washed and the luciferase production was measured. In parallel, the cytotoxicity was measured by a MTT reduction test and was taken into consideration in the interpretation of the sensitisation results. Two independent runs were performed.

Results
The test item was solubilised in DMSO at 200 mM for the first run and at 20 mM for the second run.

First run
All acceptance criteria were fulfilled for the positive and negative controls. The run was therefore considered to be valid.
This run was performed using the following concentrations: 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000 and 2000 µM in culture medium containing 1% DMSO.
At these tested concentrations:

- precipitate as well as medium coloration (due to test item coloration) were observed in treated wells at concentrations ≥ 62.5 µM,
- a decrease in cell viability (*i.e.* cell viability < 70%) was noted at concentrations ≥ 31.25 µM,
- the corresponding IC30 and IC50 were calculated to be 24.81 and 39.53 µM, respectively,
- no statistically gene-fold induction above the threshold of 1.5 was noted at non-cytotoxic concentrations. The Imax was 9.72 and the calculated EC1.5 was 29.25 µM. However, these values are not to take into consideration since they were obtained at cytotoxic concentrations.

Second run
All acceptance criteria were met for the positive and negative controls, this run was therefore considered to be valid.
This run was performed using the following concentrations: 4.57, 6.44, 9.08, 12.80, 18.05, 25.45, 35.9, 51, 71, 101, 142, and 200 µM in culture medium containing 1% DMSO.
At these tested concentrations:

- precipitate was observed in treated wells at concentrations ≥ 101 µM,
- medium colouration (due to test item colouration) was observed in treated wells at concentrations ≥ 35.9 µM,
- a decrease in cell viability (*i.e.* cell viability < 70%) was noted at concentrations ≥ 18.05 µM (with an exception at 25.45 µM for which cell viability was evaluated at 71%),
- the corresponding IC30 and IC50 were calculated to be 33.70 and 36.48 µM, respectively,
- no statistically gene-fold induction above the threshold of 1.5 was noted at non-cytotoxic concentrations,
- the Imax was 11.47 and the calculated EC1.5 was 25.81 µM. However, these values are not to take into consideration since they were obtained at cytotoxic concentrations.

The evaluation criteria for a negative response are met in both runs, the final outcome is therefore negative.
Since precipitate was only observed in test item-treated wells found cytotoxic (*i.e.* cell viability < 70%), this was considered not to have any impact on results obtained at non-cytotoxic concentrations.

Conclusion
Under the experimental conditions of this study, the test item, Bromothymol blue sodium salt, was negative in the KeratinoSens assay and therefore was considered to have no potential to activate the Nrf2 transcription factor.

SCCS comment
SCCS agrees that under the conditions of this test sodium bromothymol blue is negative in the Keratinosens assay.

Ref. Valin, 2018b

U-SENS™

Guideline:	OECD 442E *In Vitro* Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the AOP for Skin Sensitisation
Test System:	Human myeloid U937 cell line
Replicates:	Two
Test substance:	Bromothymol blue sodium salt
Batch:	20170531
Purity:	98.9%
Test item:	Greenish brown powder
Solvent:	Culture medium RPMI (at 50 mg/mL)
Dose level:	Exp. 1: 1, 10, 20, 50, 100 and 200 µg/mL Exp. 2: 50, 100, 120, 200 µg/mL
Treatment period:	45±3 hours
Positive control:	TNBS
Negative control:	Lactic Acid
GLP:	Under GLP-like conditions. It strictly followed the OECD guideline 442E and its associated SOP with respect to performance and documentation

Study period: November, 2017

The U-SENS™ method is an *in vitro* assay that quantifies changes of CD86 cell surface marker expression on a human myelomonocytic cell line, U937 cells, following exposure to the test chemical. CD86 is known to be a co-stimulatory molecule that may mimic monocytic activation, which plays a critical role in T-cell priming. The changes of CD86 cell surface marker expression are measured by flow cytometry following cell staining, typically with fluorescein isothiocyanate (FITC)-labelled antibodies. Cytotoxicity measurement is also conducted to assess whether upregulation of CD86 cell surface marker expression occurs at sub-cytotoxic concentrations.
The stimulation index (S.I.) of CD86 cell surface marker compared to solvent/vehicle control is calculated. The prediction model described in OECD TG 442E is used to decide if a substance is positive (P) or negative (N) (OECD, 2017).

The individual conclusion of a U-SENS™ run is considered

- Negative (N) if the S.I. of CD86 is less than 150% at all non-cytotoxic concentrations (cell viability ≥ 70%) and if no interference is observed (cytotoxicity, solubility or colour regardless of the non-cytotoxic concentrations at which the interference is detected).
- Positive (P) in all other cases: S.I. of CD86 higher or equal to 150% and/or interferences observed

An U-SENS™ prediction is considered negative or positive if at least two independent runs are negative or positive, respectively.

In the first experiment the stock was diluted to six test concentrations (1, 10, 20, 50, 100 and 200 µg/mL). As cytotoxicity was observed at the highest dose level of 200 µg/mL, a narrower dose-response analysis (50, 100, 120, 200 µg/mL) was performed in the second experiment to determine a sub-toxic dose-response of the test item.

In both experiments, the positive (TNBS) and negative (Lactic Acid) controls were considered valid. Both experiments passed the acceptance criteria.
Cytotoxicity was observed at the dose levels of 120 µg/mL and upwards. The CV70 value (corresponding to the concentration beyond which the molecule is considered as being cytotoxic) was calculated at 118 µg/mL. No induction of the CD86 activity was measured in two independent experiments and thus no EC150 value was applicable. The evaluation criteria for a negative response are met in both runs.

Conclusion
Under the conditions of this study, Bromothymol blue sodium salt was negative in the U-SENS™ assay and therefore was considered to have no potential to activate the CD86 marker in the U937 cell line.

Ref. Dreyfuss and Teluob, 2017

SCCS comment
The U-SENS assay was not conducted according to GLP. After evaluation of the dossier, SCCS noted that the assay was performed according to the OECD TG and therefore considered to be valid. The SCCS agrees with the Applicant that sodium bromothymol blue is negative in the U-SENS assay.

Data interpretation procedure applied
The respective input variables of each tier are entered into the model where they are run in 5 different supervised classification models (Boosting, Naïve Bayes, SVM, Sparse PLS-DA and Expert Scoring), each providing a probability of being a sensitiser (Tier 1) or a probability of being a UN GHS Cat. 1A (Tier 2). These intermediate probabilities that are evidently highly positively correlated (Gomes *et al.*, 2012, Nocairi *et al.* 2016) are then used in the stacking

meta-model that provides a final probability to be a sensitiser (Tier 1) or to be a UN GHS Cat. 1A (tier 2) (primary outcomes of the meta-models). Optimal predictive capacities were obtained by setting the following thresholds:

Tier 1 (Hazard):

- Chemicals with probability to be sensitiser ≥70% are predicted "Sensitiser"
- Chemicals with probability to be sensitiser ≤30% are predicted "Non-Sensitiser"
- Chemicals with probability between those two thresholds are predicted as "Equivocal Cat. 1" (>50%and <70%) or "Equivocal No Cat." (>30% and <50%) due to the uncertainty in the prediction.

Tier 2 (Potency):

- Chemicals with probability to be sensitiser ≥ 60% are predicted "UN GHS cat.1A"
- Chemicals with probability to be sensitiser ≤ 30% are predicted "UN GHS cat. 1B"
- Chemicals with probability between those two thresholds are predicted as "Equivocal Cat. 1A" (>50%and <60%) or "Equivocal Cat. 1B" (>30% and <50%) due to the uncertainty in the prediction.

Based on these predictions, the decision rules for a sequential testing strategy are the following: TIER 1:

- Chemicals with a probability to be sensitiser ≤ 30%, are classified "Non-Sensitiser". No further testing is needed.
- Chemicals with a probability > 30%, proceed to Tier 2.

Classification is based on expert judgment.

Results
The DA predicted sodium bromothymol blue as a GHS No Category (non-sensitiser) with a probability to be a skin sensitiser of 15%.
Sodium bromothymol blue is predicted as non-sensitiser and thus moves to TIER 2: risk assessment (Gilmour et al, 2020) is not needed.

SCCS comment
The sequential testing strategy as applied by the Applicant is described in several scientific papers. These papers, however, only describe only the rationale behind the prediction model of the first tier (hazard assessment) and not that of the second tier (potency subcategorization). The second tier is only mentioned in one publication, where it is used in a case study (Assaf-Van de Casteele, 2021). In this publication, no information is provided on how the prediction model of Tier 2 was developed.
The SCCS notes that the stacking method is an interesting state-of-the-art method that provides a probabilistic prediction. To be able to fully understand and evaluate this DA, it is essential that information is provided on how the model is built, e.g. training sets, rationale behind the data interpretation procedures in both tiers. The OECD has developed a reporting template that can be used for this purpose (provided as an Annex to OECD No. 256, 2016).
The SCCS is aware that this DA has been included in the case studies published in Annex I of OECD Guidance No. 256. It is, however, unclear if this DA was further developed after this publication, especially since Tier 2 was not included in this Annex document.
Therefore, the SCCS recommends the use of the reporting format of the OECD for DA that are not included in OECD Guidance No. 497 (OECD, 2021) to provide the SCCS with an accurate description of the DA used in the NGRA and to facilitate the evaluation.

To conclude, the NGRA was not performed according to the published framework (Gilmour *et al.*, 2020) and this has raised several questions. Furthermore, better reporting of the DA is needed before this can be properly evaluated by the SCCS. Despite these shortcomings in

reporting, the SCCS has evaluated all available data and applied the 2o3 DA as described in the OECD Guidance No. 497. In short, the prediction if a chemical is a skin sensitiser or not in the 2o3 DA is based on the results of NAMs mapping to KE1, 2 and 3. For sodium bromothymol blue, these were the DPRA, Keratinosens™ and U-SENS™. If the assays provide discordant results, as is the case for sodium bromothymol blue, the overall result is based on two concordant findings. Since the Keratinosens™ and U-SENS™ were negative, sodium bromothymol blue is not considered to be a skin sensitiser. This leads to the same conclusion, that based on the available data, sodium bromothymol blue is not a skin sensitiser.

3.4.3 Acute toxicity

3.4.3.1 Acute oral toxicity

No *in vivo* acute oral toxicity studies with sodium bromothymol blue were found in literature. *In silico* prediction with the OECD Toolbox for acute toxicity was not possible as the ingredient was out of domain of applicability of the Acute Oral Toxicity (AOT) Profiler model based upon a mechanistic structural category approach recommended by the OECD.

Therefore, no conclusion can be drawn on the acute toxicity profile. However, given that Bromothymol blue (CAS 76-59-5) is listed in Annex IV as part of the allowed colourants in cosmetic rinse-off products, and given the low exposure following at most one application every two weeks and the low skin penetration rate, the risk of acute toxicity via dermal route is expected to be very low.

3.4.3.2 Acute dermal toxicity

/

3.4.3.3 Acute inhalation toxicity

/

SCCS comment
It is not possible to conclude on the acute toxicity of sodium bromothymol blue in the absence of any data.

3.4.4 Repeated dose toxicity

<u>According to the Applicant:</u>
No *in vivo* toxicity data on repeated-dose toxicity studies using Bromothymol blue sodium salt were found in the literature. Since 2013, an animal testing ban has been in place for cosmetic ingredients and no assay could be performed on animals for the purpose of this submission. Therefore, no classical evaluation in repeated-dose animal studies could be performed. However, due to the low exposure estimate, the safety of sodium bromothymol blue can be supported by the application of the Toxicological Threshold of Concern (TTC).

SCCS comment
As expressed above, the SCCS considers the TTC a pragmatic tool that can be used for cosmetic ingredients that are intentionally added at low concentrations to a cosmetic product or are present as impurities of the ingredients in a cosmetic product (SCCP/1171/08, SCCS Notes of Guidance, 11th Revision). However, the TTC concept alone cannot be applied to justify the safety of cosmetic ingredients that have specific requirements for data, such as ANNEX substances (as is the case of sodium bromothymol blue) for their regulatory approval under the European Cosmetics Regulation. As indicated in the SCCS Notes of Guidance 11th Revision,

the use of TTC alone in this regard is currently not acceptable for ANNEX substances, and it can only be used in safety assessments in conjunction with other data as part of the overall weight of evidence.

3.4.5 Reproductive and developmental toxicity

According to the Applicant:
No *in vivo* toxicity data on reproductive/developmental toxicity studies using Bromothymol blue sodium salt, were found in the literature. Since 2013, an animal testing ban is in place for cosmetic ingredient and no assay can be performed on animals for the purpose of this submission. Therefore, no classical evaluation in repeated-dose animal studies can be performed. However, due to the low exposure estimate, the safety of sodium bromothymol blue can be supported by the application of the Toxicological Threshold of Concern (TTC).

Threshold of Toxicological Concern (TTC) is a toxicological concept which can be used for the safety evaluation of substances for which the toxicity dataset is limited.
In the absence of systemic toxicity data, the TTC approach was applied based on the thresholds proposed by the 'federated Yang *et al.* (2017)' dataset of 2.3 µg/kg bw/d and 46 µg/kg bw/d for Cramer classes III and I respectively, which are considered appropriate for use by the SCCS (SCCS, 2021).
Sodium bromothymol blue is a hair dye, a cosmetic class largely represented in COSMOS dataset with 122 hair dyes. In addition, halogenated dyes such as Tetrabromophenol blue are included in the dataset (Yang, 2017), supporting the idea that TTC values proposed by Yang *et al.* 2017 are suitable to cover the chemical space which sodium bromothymol blue belongs to.

SCCS comment
See comment above (3.4.4).

3.4.6 Mutagenicity / genotoxicity

3.4.6.1 Mutagenicity / genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD TG 471
Species/Strain:	*Salmonella typhimurium* (TA1535, TA1537, TA98, TA100 and TA102)
Replicates:	Triplicate plates in two separate experiments
Test substance:	Bromothymol blue sodium salt
Batch:	20170531
Purity:	98.88% (drying loss of 1.12%)
Solvent:	DMSO (stock solution at 50 mg/mL)
Positive controls:	Without S9 mix: sodium azide (TA1535; TA100), 9-Aminoacridine (TA 1537), 2-Nitrofluorene (TA98), Mitomycin C (TA102) With S9 mix: 2-Anthramine (TA1535, TA1537, TA98, TA102), Benzo(a)pyrene (TA100)
Concentrations:	Experiment 1: Direct plate incorporation method without S9 mix - for TA1535, TA1537, TA98, TA100: 61.73, 185.2, 555.6, 1667 and 5000 µg/plate - for TA102: 20.58, 61.73, 185.2, 555.6, 1667 µg/plate Direct plate incorporation method with S9 mix - for TA1535, TA1537, TA98, TA100, TA102: 61.73, 185.2, 555.6, 1667 and 5000 µg/plate

	Experiment 2: Direct plate incorporation method without S9 mix - for TA1535, TA98, TA100: 61.73, 185.2, 555.6, 1667 and 5000 µg/plate - for TA1537: 20.58, 61.73, 185.2, 555.6, 1667 and 5000 µg/plate - for TA102: 20.58, 61.73, 185.2, 555.6, 1667 µg/plate Pre-incubation method with S9 mix - for TA1535, TA98, TA100, TA102: 61.73, 185.2, 555.6, 1667 and 5000 µg/plate - for TA1537: 20.58, 61.73, 185.2, 555.6, 1667 and 5000 µg/plate
Treatment:	Direct plate incorporation incubated for 3 days protected from light without and with S9-mix. With the exception of the second test with S9 mix, which was performed according to the pre-incubation method
GLP:	In compliance
Study period:	October 2017 – February 2018

Methods
The test item Bromothymol blue sodium salt was evaluated in two independent experiments in the absence and presence of metabolic activation (S9 mix prepared from the livers of rats given Aroclor 1254).
A moderate toxicity was noted at 5000 µg/plate in all strains in the first experiment (using the direct plate incorporation method). In the second experiment (using the preincubation method), a moderate to strong toxicity was noted at dose levels ≥ 1667 µg/plate in the TA 1535, TA 1537 and TA 100 strains and at 5000 µg/plate in the TA 98 and TA 102 strains.

Results
All solvent and positive controls gave counts of revertants within expected ranges, and the experiments were therefore considered to be valid.
When compared to controls, no increases in the number of revertants were observed after treatment with Bromothymol blue sodium salt, either in the absence or presence of S9 mix.

Conclusion
Under the conditions of this study, Bromothymol blue sodium salt was not mutagenic in Salmonella typhimurium strains TA1535, TA1537, TA98, TA100 and TA102, either in the presence or absence of metabolic activation.

Ref. Sire, 2018

SCCS comment
The SCCS noticed that historical controls are old; they are reported from 2013 and 2014. The SCCS has also noted quite considerable cytotoxicity of sodium bromothymol blue at the highest concentration tested of 5000 µg/plate (in many cases the decrease in revertant counts was by 50%). However, considering that no other indications for increased mutagenic effects were observed (not a single increased count at any concentration in any strain in both experiments, using both direct plate and preincubation methods), the SCCS is of the opinion that the Ames test study is valid and sodium bromothymol blue has no mutagenic effect.

***In vitro* micronucleus test in cultured mouse lymphoma cells**

Guideline:	OECD TG 487
Cells:	L5178Y TK+/- Mouse Lymphoma Cells
Replicates:	Three independent experiments, two cultures per concentration
Test substance:	Bromothymol blue sodium salt
Batch:	20170531
Purity:	98.88% (drying loss of 1.12%)

Solvent:	DMSO (different concentrations of stock solutions in different experiments)
Positive controls:	With S9: cyclophosphamide (6 µg/mL) Without S9: mitomycin C (1 µg/mL), colchicine (0.5 µg/mL)
Concentrations:	See text below
Treatment:	See text below
GLP:	In compliance
Study period:	November 2017-November 2018

Methods
The test item Bromothymol blue sodium salt was evaluated in the absence and presence of metabolic activation (S9 mix prepared from the livers of Aroclor 1254-treated rats) in L5178Y TK+/- Mouse Lymphoma Cells. The highest concentration in each test condition was selected on the basis of cytotoxicity observed with the test item. Cytotoxicity was evaluated by determining the PD (Population Doubling) of cells.
Duplicate cultures were treated with each concentration of Bromothymol blue sodium salt or with known clastogens/aneugens in the presence or absence of S9. Solvent-treated cultures (DMSO) were used as negative controls.
For each main experiment (with or without S9 mix), micronuclei were analysed for the three dose levels of the test item, for the vehicle and the positive controls, in 1000 mononucleated cells per culture (total of 2000 mononucleated cells per dose).

Results

Cytotoxicity assessment
The following ranges of concentrations were selected for micronuclei frequency analysis:

3h-treatment in the absence of S9

- First preliminary cytotoxicity assessment: 1.56, 3.13, 6.25, 12.5, 25, 37.5, 50 and 100 µg/mL. A slight to severe cytotoxicity was observed at dose levels ≥ 50 µg/mL, as shown by a 27 to 100% decrease in the PD.
- **First micronucleus experiment: 12.5, 25 and 50 µg/mL,** the latter inducing only a 27% decrease in the PD, but with the higher dose level being too cytotoxic,
- Second preliminary cytotoxicity assessment: 3.75, 7.5, 15, 20, 30, 45, 60 and 100 µg/mL. A slight to severe cytotoxicity was observed at dose levels ≥ 30 µg/mL, as shown by a 29 to 100% decrease in the PD.
- **Second micronucleus experiment: 15, 30 and 45 µg/mL,** the latter inducing only a 41% decrease in the PD, but higher dose levels being too cytotoxic.

24h-treatment in the absence of S9

- First preliminary cytotoxicity assessment: 0.781, 1.56, 3.13, 6.25, 12.5, 18.75, 25 and 50 µg/mL. No noteworthy cytotoxicity was induced at any dose levels, as shown by the absence of notable decrease in the PD.
- Second preliminary cytotoxicity assessment: 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125 and 250 µg/mL. A marked to severe cytotoxicity was observed at dose levels ≥ 31.3 µg/mL, as shown by an 84 to 100% decrease in the PD.
- **First micronucleus experiment: 3.91, 7.81 and 15.6 µg/mL.** The latter concentration induced only a 17% decrease in the PD but with higher dose levels being too cytotoxic.
- Third preliminary cytotoxicity assessment: 5, 10, 15, 20, 30, 45, 60 and 100 µg/mL. A moderate to severe cytotoxicity was observed at dose levels ≥ 30 µg/mL, as shown by a 43 to 100% decrease in the PD.
- **Second micronucleus experiment: 10, 20 and 45 µg/mL.** The latter concentration induced only a 46% decrease in the PD but higher dose levels being too cytotoxic.

3h-treatment in the presence of S9

- First preliminary cytotoxicity assessment: 0.391, 0.781, 1.56, 3.13, 6.25, 9.38, 12.5 and 25 µg/mL. No noteworthy cytotoxicity was induced at any dose levels, as shown by the absence of notable decrease in the PD.
- Second preliminary cytotoxicity experiment: 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250 and 500 µg/mL. A slight to severe cytotoxicity was observed at dose levels ≥ 31.3 µg/mL, as shown by a 27 to 100% decrease in the PD.
- **First micronucleus experiment: 7.81, 15.6 and 31.3 µg/mL.** The latter concentration induced only a 27% decrease in the PD, but with higher dose levels being too cytotoxic.
- Third cytotoxicity experiment: 7.5, 15, 20, 30, 37.5, 45, 60 and 100 µg/mL. A slight to severe cytotoxicity was observed at dose levels ≥ 30 µg/mL, as shown by a 34 to 100% decrease in the PD.
- **Second micronucleus experiment: 15, 30 and 37.5 µg/mL.** The latter concentration induced only a 46% decrease in the PD, but with higher dose levels being too cytotoxic.

Micronuclei frequency assessment

The mean Population Doubling and the mean frequencies of micronucleated cells for the vehicle controls were as specified in the acceptance criteria. Also, positive control cultures showed clear statistically significant increases in the frequency of micronucleated cells. The study was therefore considered to be valid.

Under both short (3-h) and continuous (24-h) treatment conditions without metabolic activation, no statistically significant increase in the frequency of micronucleated cells was observed at any of the analysed dose levels relative to the vehicle control. No dose-response relationship was demonstrated by the linear regression and none of the analysed dose levels showed frequency of micronucleated cells of both replicate cultures above the corresponding vehicle control historical range. These results met the criteria of a negative response.

In the presence of metabolic activation, the second experiment showed no statistically significant increase in the frequency of micronucleated cells at any of the analysed dose levels relative to the vehicle control ($p>0.05$). However, a dose-response relationship was demonstrated by the linear regression ($p<0.05$) and the higher analysed dose level of 31.3 µg/mL showed frequency of micronucleated cells of both replicate cultures above the corresponding vehicle control historical range (*i.e.* 5‰ and 5‰ versus [0.0-3.5‰] for the historical data).

In the third experiment, no increase in the frequency of micronucleated cells was observed at any of the analysed dose levels relative to the vehicle control ($p>0.05$). A decreasing dose-response relationship was demonstrated by the linear regression ($p<0.05$) and none of the analysed dose levels showed frequency of micronucleated cells of both replicate cultures above the corresponding vehicle control historical range. These results met the criteria of a negative response.

Since the mean frequency of micronucleated cells observed in the second experiment (*i.e.* 5‰), remained of a relatively low magnitude when compared to the available historical data set (including all conditions) for the vehicle control and compared to the literature, and since the increase was not reproduced in the third experiment despite using a slightly higher (37.5 vs 31.3 µg/mL in the third and second experiment, respectively) and narrower range of dose levels, it was considered to be non-biologically relevant and the overall results were considered to show a negative response.

Conclusion

Under the conditions of the study, Bromothymol blue sodium salt did not induce any chromosome damage, or damage to the cell division apparatus, in cultured mammalian somatic cells, using L5178Y TK+/- mouse lymphoma cells, either in the presence or absence of a rat liver metabolizing system, and was therefore considered to have no clastogenic or aneugenic potential.

Ref. Sire, 2019

SCCS comment
The SCCS noted an increase (5‰) with a trend in micronuclei frequency above the historical vehicle control value (max 3.5‰) in cultures exposed to the test item at the highest concentration after 3 h in the presence of S9-mix. However, considering that the result was of low magnitude and was not present in the repeated experiment, it can be treated as not biologically meaningful.
The SCCS noted rather weak mutagenic effect of colchicine (24 h exposure: 11‰ in 1st and 16‰ in 2nd micronucleus experiment), which was close to minimum value in historical 24 h positive controls (10‰). Similarly, a very weak mutagenic effect below historical control values was observed for MMC in the first micronucleus experiment after 3h±24h (12‰ vs. min. 18‰ in historical controls). Moreover, the SCCS noted the rather wide range of the laboratory's historical positive control values for micronuclei frequency for mitomycin C: 3h±24: 18-306‰; 24h: 8-97.5‰, and for cyclophosphamide 3h±24: 14-251‰.
Based on the study results provided, the SCCS considers sodium bromothymol blue to have no aneugenic or clastogenic potential.

3.4.6.2 Mutagenicity / genotoxicity *in vivo*

/

The overall SCCS comment on mutagenicity
Based on the valid *in vitro* study results on gene mutations in bacteria (the Ames test) and micronucleus test in L5178Y TK+/- mouse lymphoma cells, the SCCS considers sodium bromothymol blue to be safe in regard to mutagenicity.

3.4.7 Carcinogenicity

/

3.4.8 Photo-induced toxicity

3.4.8.1 Phototoxicity / photo-irritation and photosensitisation

/

3.4.8.2 Photomutagenicity / photoclastogenicity

/

3.4.9 Human data

/

3.4.10 Special investigations

/

3.5 SAFETY EVALUATION (INCLUDING CALCULATION OF THE MOS)

According to the Applicant:
Based on structural profiling using the ToxTree tool (ToxTree v2.6.13), sodium bromothymol blue was allocated to Cramer Class III (refer to Appendix III). The TTC corresponding to Cramer Class III is thus applicable. Exposure per application of 1.55 µg/kg is well below the threshold of 2.3 µg/kg bw/day (Yang, 2017).
This calculation is conservative, as the intermittent use of hair colouring formulations containing sodium bromothymol blue further reduces consumer exposure.
Thus, the exposure to sodium bromothymol blue present in non-oxidative hair dye is considered safe for the consumer.

SCCS comment
Sodium bromothymol blue is an ANNEX III substance. As indicated in the Notes of Guidance, 11th Revision, the TTC can be used for cosmetic ingredients that are intentionally added at low concentrations to a cosmetic product or are present as impurities of the ingredients in a cosmetic product. However, the TTC concept alone cannot be applied to justify the safety of cosmetic ingredients that have specific data requirements, such as sodium bromothymol blue (SCCS Notes of Guidance, 11th Revision). In addition, the SED calculated by the SCCS (29.77 µg/kg bw/application) is much higher than the one calculated by the Applicant (1.55 µg/kg bw/application).

The absence of acceptable *in vivo* data on systemic, reproductive and developmental toxicity, and/or the availability of data from NAMs that have been developed and accepted for that purpose, does not allow safety assessment of sodium bromothymol blue. In this context, the TTC alone is not sufficient to justify the safety of sodium bromothymol blue.

The SCCS applied the TTC approach to assess safety of the three impurities that have been identified in Guanghua batch. These impurities were considered as potentially non-genotoxic and the threshold of 2.3 µg/kg bw/d for non-genotoxic Cramer class III substances was considered in the assessment. The levels of these three impurities exceeded the TTC threshold both individually and collectively. Therefore, the SCCS considers that the presence of these impurities is not safe and a batch of sodium bromothymol blue matching the impurity profile of Guanghua batch cannot be recommended for use in marketed products.

3.6 DISCUSSION

Physicochemical properties

- The pH value of the batch E509991(Guanghua) is 7.85, while the pH values for the batches E512354 (Loba) and E512354 (Loba) are 5.89 and 5.84, respectively. These differences in pH might be an indication of different impurities due to the different manufacturing processes used.
- Filtration of the samples through a 0.2 µm microfilter prior to the HPLC analysis of the test substance for impurity testing must be justified. If the samples of the test substance are not fully dissolved, impurities may remain in the filter. All compounds in the samples should be fully dissolved in the dilution solvent prior to the HPLC-PDA analysis.
- In view of the variability in the content of impurities within the Guangha and Loba batches, the Applicant must provide data on purity and impurities for at least five representative batches to better gauge the presence and nature of these impurities.

The Applicant should also provide the specifications of the type (Guangha or Loba) of the batches intended to be used in the cosmetic products.

- The TTC approach was used on three impurities. The nature of these impurities, identified in Guanghua batch, indicates that impurities 1, 2 and 3 belong to Cramer class III. The genotoxicity potential of these impurities is not known. However, considering the absence of genotoxic potential of sodium bromothymol blue (see 3.4.6), these impurities can also be considered as potentially non genotoxic. Using the threshold for Cramer class III for non-genotoxic substances, which is 2.3 µg/kg bw/d), the levels of the impurities do not exceed the acceptable threshold. Therefore, the SCCS considers that the presence of these impurities is of no concern.
-
- The Applicant needs to provide experimental data on the solubility of the test substance. The SCCS did retrieve predictive information in the public domain. It is unclear if these data are representative for the test substance used in the different toxicological tests.

Toxicokinetics

The methodology used to determine the dermal absorption is not the standard one according to the SCCS Basic Criteria in which the dermal absorption is measured over a 24-hour period. In this study, the 72-hour time point is also included. This methodology can be applied when there is clearly no movement of chemical from the skin reservoir to the receptor fluid with 24h vs 72h. After evaluating all data presented in this study, the SCCS concludes that different data points could not be accurately determined and that it is therefore not possible to state that there is no significant movement from the skin reservoir to the receptor fluid.

Since the 24-hour dermal absorption study is an acceptable study according to the SCCS Basic Criteria, the SCCS used the values from this timepoint. The dermal absorption is therefore 1.44% + 1.64 (Mean + 1SD) = 3.08 % and will be used in the SED calculation.

Exposure

The SED of sodium bromothymol blue is 29.77 µg/kg bw/d.

Toxicological Evaluation

Irritation and corrosivity

Based on the information provided, SCCS considers sodium bromothymol blue salt non-irritant to the skin and the eyes at intended use concentration.

Skin sensitisation

<u>NAM data:</u>

Sodium bromothymol blue has a high peptide reactivity in the DPRA. This is in line with the *in silico* prediction for protein reactivity generated by ToxTree. Hence, Sodium bromothymol blue is able to bind to proteins and can initiate the molecular initiating event (MIE) of the skin sensitisation AOP.

Under the conditions of the tests, sodium bromothymol blue is negative in the Keratinosens assay and in the U-SENS assay. Hence, sodium bromothymol blue did not activate test methods addressing KE2 and KE3 of the skin sensitisation AOP.

<u>DA and NGRA</u>

The NAM data were used in a sequential testing strategy DA that resulted in a prediction that sodium bromothymol blue is a non-sensitiser, with a 15% probability to be a skin sensitiser. The SCCS was not able to evaluate the DA, because essential information on the development of the DA and its prediction model was lacking. The Applicant did not report the NGRA according to the published framework which raised several questions for the SCCS.

Despite all these shortcomings in reporting the NGRA, the SCCS has evaluated all available data and applied the 2o3 DA as described in OECD Guidance No. 497. This

leads to the same conclusion, that based on the available data, sodium bromothymol blue is not a skin sensitiser.

Acute toxicity
In the absence of any data on acute toxicity, it is not possible to conclude on the acute toxicity of sodium bromothymol blue.

Repeated dose toxicity
No *in vivo* toxicity data from repeated dose toxicity studies using sodium bromothymol blue were provided. The Applicant applied the TTC approach to support the safety of this compound.
The TTC concept alone cannot be applied to justify the safety of chemicals with specific data requirements, such as ANNEX substances (e.g. sodium bromothymol blue) for their regulatory approval under the European Cosmetics Regulation. This is currently not acceptable, as indicated in the Notes of Guidance, 11th Revision.

Reproductive toxicity
No *in vivo* toxicity data from reproductive/developmental toxicity studies using sodium bromothymol blue were provided. The Applicant applied the TTC approach to support the safety of this compound.
The TTC concept alone cannot be applied to justify the safety of chemicals with specific data requirements, such as ANNEX substances (e.g. sodium bromothymol blue), for their regulatory approval under the European Cosmetics Regulation. This is currently not acceptable, as indicated in the Notes of Guidance, 11th Revision.

Mutagenicity / genotoxicity
Based on the valid *in vitro* study results on gene mutations in bacteria (the Ames test) and the micronucleus test in L5178Y TK+/- mouse lymphoma cells, the SCCS considers sodium bromothymol blue to be safe in terms of mutagenicity.

Carcinogenicity
No data provided

Photo-induced toxicity
No data provided

Human data
No data provided

Special investigation
No data provided

4. CONCLUSION

1. *In light of the data provided, does the SCCS consider Sodium Bromothymol Blue safe when used in non-oxidative hair colouring products up to a maximum on-head concentration of 0.5 %?*

Having considered the data provided, the SCCS is of the opinion that the safety of sodium bromothymol blue cannot be assessed because of the following reasons:

- The Applicant used TTC approach to justify the safety of sodium bromothymol blue, but the SCCS estimate of the SED indicates that it exceeds the TTC threshold for Cramer class III substances.

- The use of TTC on its own to justify the safety of the substances that are regulated under the EU Cosmetic Regulation is not sufficient to waive the information requirements on essential toxicological endpoints.

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

While the use of TTC is acceptable to justify the safety of impurities and cosmetic ingredients that are added to a final product at sufficiently low concentrations, it is not acceptable on its own for the substances that are regulated under the EU Cosmetic Regulation. Additional supporting data from NAMs that are scientifically-accepted for the purpose, and/or other acceptable *in vivo* data on systemic toxicity, are also required in an overall weight of evidence to assess safety.

5. MINORITY OPINION

/

6. REFERENCES

Alépée, N., Piroird, C., Nardelli, L., 2017. U-SENS™: A U937 cell line activation test for skin sensitization. In: Book Alternatives for Dermal Toxicity Testing. Springer, Cham, pp. 311–330.

Alépée, N., Piroird, C., Aujoulat, M., Dreyfuss, S., Hoffmann, S., Hohenstein, A., Meloni, M., Nardelli, L., Gerbeix, C., Cotovio, J., 2015. Prospective multicentre study of the U-SENS test method for skin sensitization testing. Toxicol in Vitro 30: 373-382.

Aptula A.O. and Roberts D.W., 2006. Mechanistic applicability domains for non-animal-based prediction of toxicological end points: general principles and application to reactive toxicity. Chem. Res. Toxicol. 19: 1097-1105.

Assaf Vandecasteele H, Gautier F, Tourneix F, van Vliet E, Bury D, Alépée N, 2021. Next generation risk assessment for skin sensitisation: A case study with propyl paraben. Regulatory Toxicology and Pharmacology 123, 104936.

Breiman, L., 1996. Stacked Regressions. Machine Training. 24, 49-64.

Bury D, Head J, Keller D, Klaric M, Rose J, 2021. The Threshold of Toxicological Concern (TTC) is a Pragmatic Tool for the Safety Assessment: Case Studies of Cosmetic Ingredients with Low Consumer Exposure. Reg Tox Pharm 123, 104964

Cannamela N (2020). E509991. Cutaneous Primary Irritation. Study Performed on an Episkin® Reconstructed Human Epidermis Model. MTT conversion assay. Evaluation of the interaction of the test item with MTT and staining power (According to the OECD guideline N°439 of 28 July 2015). IEC Report No. 200760RD.

Del Bufalo A., Pauloin T., Alepee N., Clouzeau J., Detroyer A., Eilstein J., Gomes C., Nocairi H., Piroird C., Rousset F., Tourneix F., Basketter D., Martinozzi Teissier S., 2018. Alternative Integrated Testing for Skin Sensitisation: Assuring Consumer Safety. Applied In Vitro Toxicology, 4(1):30-43 DOI: 10.1089/aivt.2017.0023

Dreyfuss S and Teluob S (2017). In Vitro Skin Sensitization U-SensTM Prediction of the Raw Material E509991. Episkin Study No. 17-ES-029

Emter, R., G. Ellis, and A. Natsch, 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. Toxicol. Appl. Pharmacol. 245: 281-290

Gerberick GF, Vassallo JD, Bailey RE, Chaney JG, Morrall SW, Lepoittevin JP., 2004. Development of a peptide reactivity assay for screening contact allergens. Toxicol Sci. 81: 332-343

Gilmour, N., Kern, P.S., Alépée, N., Boislève, F., Bury, D., Clouet, E., Hirota, M., Hoffmann, S., Kühnl, J., Lalko, J.F., Mewes, K., Miyazawa, M., Nishida, H., Osmani, A., Petersohn, D., Sekine, S., van Vliet, E., Klaric, M., 2020. Development of a next generation risk assessment framework for the evaluation of skin sensitisation of cosmetic ingredients. Toxicol. Pharmacol. 6, 104721.

Gomes, C., Noçairi, H., Thomas, M., Collin, J.F., Ibanez, F., Saporta, G., 2012. Stacking prediction for a binary outcome. COMPSTAT, 20th International Conference on Computational Statistics, Limassol, Cyprus.: 271-282

Gomes, C., H. Nocairi, H., Thomas, M., Collin J.F., Saporta, G., 2014. A simple and robust scoring technique for binary classification. Artificial Intelligence Research, vol. 3(1): 52-58

Julienne S (2020). E509991. Bovine Corneal Opacity and Permeability Test Method for Identifying Test Item Inducing Serious Eye Damage And Test Item Not Requiring Classification- OECD 437. Eurofins Evic Report No B20 0786 / 20-2111

Maillet S (2020a). E509991. Ocular Primary Irritation. Study Performed on an Episkin® Reconstructed Human Cornea-Like Epithelium Model. MTT conversion assay. Evaluation of the interaction of the test item with the MTT and staining power (According to the OECD guideline N°492 of 18 June 2019). IEC Report No. 200759RD.

Maillet S (2020b). E509991 diluted to 0.5%. Ocular Primary Irritation. Study Performed on an Episkin® Reconstructed Human Cornea-Like Epithelium Model. MTT conversion assay. Evaluation of the interaction of the test item with the MTT and staining power (According to the OECD guideline N°492 of 18 June 2019). IEC Report No. 201073RD.

Noçairi H., Gomes C., Thomas M., 2016. Improving Stacking methodology for Combining classifiers: application to cosmetic industry. Electronic Journal of Applied Statistical Analysis 9: 340-361

OECD, 2016. Guidance document on the reporting of defined approaches and individual information sources to be used within integrated approaches to testing and assessment (IATA) for skin sensitisation. Series on Testing & Assessment No. 256. ENV/JM/MONO(2016)29:
https://www.oecd-ilibrary.org/docserver/9789264279285-en.pdf?expires=1611241218&id=id&accname=guest&checksum=447DBF1ADCCCBF9A166D81C5A53304FB

OECD, 2018a. Test No. 442D: in vitro skin sensitisation:
https://www.oecd-ilibrary.org/content/publication/9789264229822-en

OECD, 2018b. Test No. 442E: in vitro skin sensitisation:
https://www.oecd-ilibrary.org/content/publication/9789264264359-en

OECD, 2019. Test No. 442C: in chemico skin sensitisation:
https://www.oecd-ilibrary.org/content/publication/9789264229709-en

OECD, 2021. Guideline on Defined Approaches for Skin Sensitisation No. 497. 14 June 2021.
https://www.oecd-ilibrary.org/docserver/b92879a4-en.pdf?expires=1624527401&id=id&accname=guest&checksum=411DA52B701F05C7CAF698D8781475BB

Patlewicz G, Dimitrov SD, Low LK, Kern PS, Dimitrova GD, Comber MI, Aptula AO, Phillips RD, Niemelä J, Madsen C, Wedebye EB, Roberts DW, Bailey PT, Mekenyan OG., 2007. TIMES-SS--a promising tool for the assessment of skin sensitization hazard. A characterization with respect to the OECD validation principles for (Q)SARs and an external evaluation for predictivity. Regul Toxicol Pharmacol. 48(2):225-239

Piroird, C., Ovigne, JM., Rousset, F., Martinozzo-Teissier, S., Gomes, C., Cotovio, J., Alépée, N., 2015. The Myeloid U937 Skin Sensitization Test (U-SENS) to address the Activation of Dendritic Cell Event in the Adverse Outcome Pathway for Skin Sensitization. Toxicology in Vitro 29: 901-916

SCCS. The SCCS Notes of Guidance for testing of cosmetic ingredients and their safety evaluation 11th revision. SCCS/1628/21

Sire G (2018). E509991: Bacterial Reverse Mutation Test. Citoxlab France Study No. 45717 MMO

Sire G (2019). E509991: In vitro Micronucleus Test in L5178Y TK+/- Mouse Lymphoma Cells. Citoxlab France Study No. 45714 MNV.

Tourneix, F., Alépée, N., Detroyer, A., Eilstein, J., Martinozzi Teissier, S., Nardelli, L., Noçairi, H., Pauloin, T., Piroird, C., Del Bufalo, A., 2019. Assessment of a defined approach based on a stacking prediction model to identify skin sensitization hazard. Toxicol. Vitro 60, 134–143.

Tourneix, F., Alépée, N., Detroyer, A., Eilstein, J., Ez-Zoubir, M., Martinozzi Teissier, S., Noçairi, H., Piroird, C., Basketter, D., Bufalo, A., 2020. Skin sensitisation testing in practice: applying a stacking meta model to cosmetic ingredients. Toxicol. Vitro 66, 104831.

Valin M (2018a). E509991: In Chemico Skin Sensitization: Direct Peptide Reactivity Assay (DPRA). Citoxlab Study No. 45716 TIR

Valin M (2018b). E509991: Keratinosens Test. An In Vitro Skin Sensitization Assay. Citoxlab Study No. 45715 TIK

Whitson S (2019). In Vitro Percutaneous Absorption of a Single Radiolabelled Test Item (Bromothymol Blue Na Salt) in Hair Dye Formulation Through Human Skin. Charles River, Report No 40963.

WHO, 1994. Assessing human health risks of chemicals: derivation of guidance values for health-based exposure limits. Environmental Health Criteria, No. 170. World Health Organization, Geneva.
https://apps.who.int/iris/bitstream/handle/10665/40675/9241571705-eng.pdf?sequence=1&isAllowed=y

Wolpert, D., 1992. Stacked Generalization. Neural Networks 5, 241-259.

Yang, C., Barlow, S.M., Muldoon Jacobs, K.L., Vitcheva, V., Boobis, A.R., Felter, S.P., Arvidson, K.B., Keller, D., Cronin, M.T.D., Enoch, S., Worth, A., Hollnagel, H.M., 2017. Thresholds of Toxicological Concern for cosmetics-related substances: new database, thresholds, and enrichment of chemical space. Food Chem. Toxicol. 109, 170–193.

Zuckerstätter V (2020). E509991. In vitro Skin Sensitisation: human Cell Line Activation Test (h-CLAT). Eurofins. Study No. STUGC20AA1590-3

7. GLOSSARY OF TERMS

See SCCS/1628/21, 11th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – from page 181

8. LIST OF ABBREVIATIONS

See SCCS/1628/21, 11th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – from page 181