Scientific Committee on Consumer Safety

SCCS

SCIENTIFIC ADVICE ON

HC Red No. 18
(Colipa No. B124)

(CAS No. 1444596-49-9)

Submission II

The SCCS adopted this document by written procedure on 6 December 2024

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SCCS

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1. ABSTRACT

The SCCS concludes the following:

(1) In light of the data provided, does the SCCS consider HC Red 18 safe, when used as an ingredient at 1.5% in oxidative hair dye and at 0.5% in non-oxidative hair dye formulations?

In light of the data provided, the SCCS considers HC Red 18 safe when used as an ingredient at 1.5% in oxidative hair dye and at 0.5% in non-oxidative hair dye formulations.

(2) Does the SCCS have any further scientific concerns with regard to the use of HC Red 18 in cosmetic products?

/

Keywords: SCCS, scientific advice, hair dye, HC Red No. 18 (B124), CAS 1444596-49-9, submission II, Regulation 1223/2009

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), scientific advice on HC Red No. 18 (B124) (CAS 1444596-49-9) – submission II, preliminary version of 6 December 2024, SCCS/1673/24

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2. MANDATE FROM THE EUROPEAN COMMISSION

Background

The ingredient with the INCI name 'HC Red 18' and chemical name '2-chloro-4-{(E)-[3-(methylthio)-1,2,4-thiadiazol-5-yl]diazenyl}phenol' (CAS No. 1444596-49-9, EC No. -) may be used as an ingredient in oxidative hair dye formulations.

In 2015, the Commission services received a dossier from industry to support the safe use of 'HC Red 18' in cosmetic products. In its corresponding opinion (SCCS/1569/15)[1], the SCCS concluded that '*…the use of HC Red No. 18 (B124) as an ingredient at 1.5 % in oxidative hair dye formulations is safe'.* In addition, the SCCS stated that '*Neither the purity of HC Red No. 18 nor its impurities are quantified adequately. Data on purity and impurities of HC Red No. 18 (B124) should be provided, together with purity specifications of the substance intended for use in cosmetic products*'.

With the current submission (i.e., submission II), received in February 2024, the Applicant requests to assess the safety of 'HC Red 18' in view of the newly provided information on its purity, when used as an ingredient at 1.5% in oxidative hair dye formulations and at 0.5% in non-oxidative hair dye formulations.

Terms of reference

1. *In light of the data provided, does the SCCS consider HC Red 18 safe, when used as an ingredient at 1.5% in oxidative hair dye and at 0.5% in non-oxidative hair dye formulations?*

2. *Does the SCCS have any further scientific concerns with regard to the use of HC Red 18 in cosmetic products?*

[1] https://health.ec.europa.eu/publications/revision-opinion-hc-red-no-18-b124_en

3. SCIENTIFIC ADVICE

3.1 Physicochemical Specifications

3.1.1 Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Red No. 18 (INCI)

3.1.1.2 Chemical names

2-chloro-4-{(E)-[3-(methylthio)-1,2,4-thiadiazol-5-yl]diazenyl}phenol

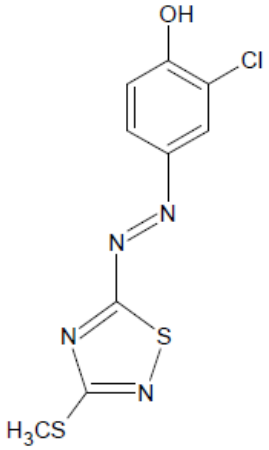
3.1.1.3 Trade names and abbreviations

FPK-245

3.1.1.4 CAS / EC number

CAS: 1444596-49-9
EC: /

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

$C_9H_7ClN_4OS_2$

3.1.2 Physical form

Red powder

3.1.3 Molecular weight

Molecular weight: 286.76 g/mol

3.1.4 Purity, composition and substance codes

From previous Opinion (SCCS/1569/15)

Table 1. Batches used in respective toxicological studies

WK120919	06041404	WK120914
- Eye irritation - Skin irritation - BCOP - *In vitro* dermal delivery of cream FPK-245 under oxidative conditions and non-oxidative conditions - Subchronic toxicity 90-day oral toxicity - DRF Teratogenicity - Teratogenicity - Gene mutation assay in Chinese Hamster V79 cells *in vitro* (V79/HPRT) - *In vitro* Micronucleus assay in human lymphocytes - Medium-term liver carcinogenesis assay - Toxicokinetics (non-labelled)	- Ames Assay - SHE Cell Trans-formation Assay	- Local Lymph Node Assay (LLNA) - DRF 14-day study

Additional data provided in submission II

According to the Applicant, the already high purity of HC Red 18 could be further improved by the manufacturer. Current commercial batches are of even better quality than the batches used in the previous submission, while existing toxicological data is still valid. This reflects in a further reduction in concentration and/or number of impurities in the current commercial batches of HC Red 18 compared to the batches used in the previous submission (WK120919, WK120914, 06041404). As these old batches do not represent the current commercial quality of the dye anymore and only the quality of the current commercial quality will be available on the market, the Applicant submitted the requested data by using three new commercial batches (Table 2).

Table 2. Overview of the batches used in previous submission ("Old Batches") and the batches used for providing the requested data ("New Commercial Batches").

Old batches	New commercial batches
WK120919	R18_18004_180 812 002
WK120914	R18_18005_181 108 284
06041404	R18_18006_190 220 121

Chemical characterisation of HC Red 18 was performed in three new commercial batches (Table 2) by H^1-NMR, ^{13}C-NMR and FT-IR.

^1H and ^{13}C NMR were obtained for all three HC Red 18 new commercial batches. The proton of the OH-group did not result in a sharp signal which indicates a proton-deuterium exchange. All 7 protons and all 9 carbon atoms could be detected in the NMR spectra.

R18_18004_180 812 002

^1H NMR (500 MHz, DMSO) δ 12.09 (s, 1H), 8.01 (d, *J* = 2.4 Hz, 1H), 7.90 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.20 (d, *J* = 8.8 Hz, 1H), 2.67 (s, 3H).

^{13}C NMR (126 MHz, DMSO) δ 194.41, 171.68, 160.62, 144.36, 127.13, 125.89, 122.11, 117.44, 14.32.

R18_18005_181 108 284

^{1}H NMR (500 MHz, DMSO) δ 12.10 (s, 1H), 8.01 (d, *J* = 2.4 Hz, 1H), 7.90 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.19 (d, *J* = 8.8 Hz, 1H), 2.67 (s, 3H).
^{13}C NMR (126 MHz, DMSO) δ 194.41, 171.68, 160.62, 144.36, 127.13, 125.88, 122.11, 117.44, 14.32.

R18_18006_190 220 121

^{1}H NMR (500 MHz, DMSO) δ 12.08 (s, 1H), 8.01 (d, *J* = 2.4 Hz, 1H), 7.90 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.19 (d, *J* = 8.8 Hz, 1H), 2.67 (s, 3H).
^{13}C NMR (126 MHz, DMSO) δ 194.41, 171.68, 160.62, 144.36, 127.13, 125.89, 122.11, 117.44, 14.32.

All ^{1}H and ^{13}C NMR spectra show the expected quantity of proton and carbon signals in DMSO-d6. The results of all three new commercial batches are comparable in terms of chemical shift, number of protons/carbon atoms and spin coupling.

IR spectra of HC Red 18 were measured in solid form on a Bruker Alpha II FT-IR spectrometer in the frequency range of 4000 to 400 cm^{-1} at room temperature (22 °C). All FT-IR spectra of HC Red 18 showed the same absorption bands for the unique fingerprint region of each compound between 1600 and 400 nm^{-1}.

New market specification

Table 3. Purity

Overall Purity (HPLC)	> 99.6 %
Water Content	< 1 %
Impurity 1B	≤ 0.4 %
Heavy Metal Content*	Arsenic < 5 ppm Antimony < 5 ppm Lead < 5 ppm Cadmium < 0.5 ppm Mercury < 0.3 ppm Iron< 20 ppm
Remaining solvent	Methanol ≤ 3000 ppm Acetonitrile ≤ 410 ppm Toluene ≤ 890 ppm Triethylamine ≤ 5000 ppm DMPU ≤ 800 ppm

*SCCS note: As, Pb, Cd, Hg and Fe were measured by ICP-MS after submission II
DMPU: N,N'-Dimethylpropyleneurea

3.1.5 Impurities / accompanying contaminants

Additional data provided in submission II

Purity and impurities of HC Red 18 were determined for all old and new commercial batches, using the same method and HPLC instrument at both 254 nm (Table 4) and 526 nm (absorption maximum of anionic HC Red 18, Table 5). In the UV-Vis spectra obtained by the analysis of the main peak in the HPLC spectrum at 13.20 min (HC Red 18), a single absorption maximum at about 526 nm can be found.

Table 4. Summary of the relative **area in %** obtained from the HPLC data of HC Red 18 **at** 254 nm

at 254 nm	Old Batches			New commercial batches		
	WK120919	WK120914	06041404	R18_18004	R18_18005	R18_18006
Impurity 1A 9.87 min	0.146	0.101				
HC Red 18 13.16-13.20 min	99.703	99.625	99.968	100.000	100.000	100.000
Impurity 2A 14.39 min	0.038	0.103				
Impurity 3A 14.60 min			0.032			
Impurity 4A 15.77 min	0.113	0.026				
Impurity 5A 16.02 min		0.145				
Total	100%	100%	100%	100%	100%	100%

Table 5. Summary of the relative area in % obtained from the HPLC data of HC Red 18 at 526 nm

At 526 nm	Old batches			New commercial batches		
	WK120919	WK120914	06041404	R18_18004	R18_18005	R18_18006
Impurity 1B 9.49-9.56 min	0.165	0.187	0.037	0.007	0.007	0.008
Impurity 2B 10.65 min	0.10	0.013	0.003			
Impurity 3B 11.42-11.43 min	0.031	0.040	0.016			
Impurity 4B 12.35-12.36 min	0.017	0.017				
Impurity 5B 12.70 min	0.013					
Impurity 6B 12.69 min		0.027				
HC Red 18 13.16-13.20 min	99.714	99.661	99.773	99.993	99.993	99.992
Impurity 7B 14.17 min	0.007					
Impurity 8B 14.38-14.39 min	0.013	0.029				
Impurity 9B 14.58 min	0.009	0.008	0.022			
Impurity 10B 16.99-17.04 min	0.021	0.016	0.013			
Impurity 11B 23.44 min			0.136			
Total	100%	100%	100%	100%	100%	100%

According to the Applicant, from the HPLC results obtained at 526 nm (Table 5), the already high purity of the old batches, greater than 99.66 %, could be further increased for the commercial batches to greater than 99.99 %. Additionally, the number of impurities could be reduced from 11 to one in the new commercial batches. The found impurity in the new commercial batches at ~9.5 min (Impurity 1B), is also present in the old batches but with an about 23-times lower maximum concentration for the new batches.
As Impurity 1B found in the new commercial batches at 526 nm is present with a noticeable lower concentration than in the old batches, the Applicant concluded that the toxicological data conducted with the old batches is also valid for the new commercial batches.
Data for water and heavy metal content, remaining solvent, and residue on ignition were taken from the specification sheet delivered for each batch by the dye manufacturer.
The water content was determined using Karl-Fischer method. Water and heavy metal content, remaining solvent, and residue on ignition are well below the specified maximum amount in all three batches (Table 6).

Table 6. Summary of the data available in the specification sheets of commercial batches of HC Red 18

Impurity	**Specification**	**R18_18004 180 812 002**	**R18_18005 181 108 284**	**R18_18006 190 220 121**
Total heavy metal	20 ppm or less	20 ppm or less	20 ppm or less	20 ppm or less
Iron	50 ppm or less	20 ppm or less	20 ppm or less	20 ppm or less
Arsenic	2 ppm or less	2 ppm or less	2 ppm or less	2 ppm or less
Remainig solvent Methanol	3000 ppm or less	188 ppm	161 ppm	161 ppm
Remaining solvent Acetonitrile	410 ppm or less	227 ppm	191 ppm	171 ppm
Remaining solvent Toluene	890 ppm or less	0 ppm	0 ppm	0 ppm
Remaining solvent Triethylamine	5000 ppm or less	173 ppm	175 ppm	227 ppm
Remaining solvent DMPU	800 ppm or less	0 ppm	0 ppm	0 ppm
Water content	1.0% or less	0.06%	0.02%	0.04%
Residue on ignition	1.0% or less	0.02%	0.04%	0.01%

The Applicant provided additional data on heavy metal impurities

Table 7: Heavy Metal Content of the 3 Batches of HC Red 18 by ICP-MS

Impurity	**LoQ Limit of Quantification**	**R 18-18004 180 812 002**	**R 18-18005 1 808 284**	**R 18-18006 190 220 121**
Arsenic	5 ppm	< 5 ppm	< 5 ppm	< 5 ppm
Lead	5 ppm	< 5 ppm	< 5 ppm	< 5 ppm
Cadmium	0,5 ppm	< 0,5 ppm	< 0,5 ppm	< 0,5 ppm
Mercury	0,3 ppm	< 0,3 ppm	< 0,3 ppm	< 0,3 ppm
Iron	20 ppm	< 20 ppm	< 20 ppm	< 20 ppm

Analytical report attached.

SCCS comments on purity and impurity
Upon request from the SCCS, the Applicant provided analytical data on the heavy metal content of three commercial batches of the test substance. The analysis was conducted using ICP-MS in line with DIN EN ISO 17294-2. Arsenic, lead, cadmium, mercury, and iron levels were found to be below the LOQ.
The SCCS agrees that the purity of the new commercial batches has been improved and only one impurity is detected at 526 nm (Impurity 1B). Based on additional data provided by the Applicant upon request on alkaline peroxide stability (see section 3.1.9), Impurity 1B was chemically characterised to be below 0.4% in the new commercial batches.

3.1.6 Solubility

From previous Opinion (SCCS/1569/15)

Water: insoluble (less than 0.001%)
DMSO: 12.5%
Ethanol: Less than 0.1%

Additional data provided in submission II

According to the Applicant, solubility of HC Red 18 for conducting toxicological studies was determined individually as a part of the respective study. Generally, the phenolic form of HC Red 18 is practically insoluble in pure water and shows a medium to good solubility in standard organic solvents like MeOH, EtOH, isoPrOH, DMSO, DMF. The more polar anionic of HC Red 18 shows good solubility in water above pH 9.

SCCS comment

In the previous submission, the study report of water solubility determination was not provided. It is not known whether the water solubility was determined by the EU Method A.6.

3.1.7 Partition coefficient (Log P_{ow})

From previous Opinion (SCCS/1569/15)

Log P (ACD): 0.90 ± 1.00 (monoanion); 4.05 ± 0.62 (neutral form)

Additional data provided in submission II

The logarithm of Partition Coefficient (log P_{ow}) was estimated using software "Molinspiration property engine v2018.10". This program was chosen as it can predict Log P_{ow} data of both phenolic and deprotonated, anionic dye form.

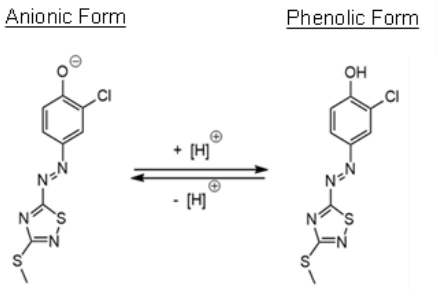


Figure 1: Protonated (phenolic) and deprotonated (anionic) form of HC Red 18

This method for Log P prediction developed at Molinspiration (miLogP2.2 - November 2005) is based on group contributions. These have been obtained by fitting calculated Log P with experimental Log P for a training set more than twelve thousand, mostly drug-like molecules. In this way hydrophobicity values for 35 small simple "basic" fragments have been obtained, as well as values for 185 larger fragments, characterizing intramolecular hydrogen bonding contribution to LogP and charge interactions. According to the Applicant, no standard deviation is given.

The calculated Log Pow values for HC Red 18 are shown below:
Log P_{ow}: 3.95 (phenolic form)
Log P_{ow}: 0.90 (anionic form)

The anionic form has a lower calculated Log Pow value as the phenolic form. This translates into a better water solubility for the anionic form, while the less polar phenolic form shows a

better solubility in unpolar octanol. This is confirmed by the Applicant's experience with the dye's solubility behaviour in water and organic solvents.

SCCS comment

In the previous submission, the study report for Log P_{ow} determination was not provided. It is not known whether the Log P_{ow} was determined by the EU Method A.8.

In the current submission, the Applicant provided calculated Log P_{ow} values by a specific software. The model calculated values are very close to the LogP_{ow} experimental values.

3.1.8 Additional physicochemical specifications

Additional data provided in submission II

Melting point:

Melting Point of HC Red 18 were determined for each batch. Melting points were determined for two heating cycles and summarized in Table 8. All samples showed comparable results; the crystalline needles sharply melted at 246 °C.

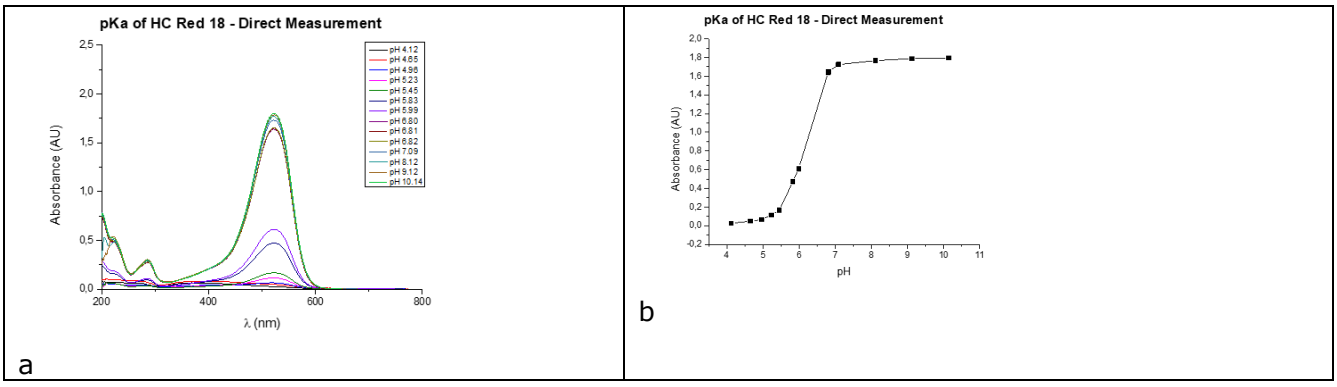
Table 8. Summary of the melting points of the three HC Red 18 new commercial batches

	Melting Point [°C]	
	Heating Cycle 1	**Heating Cycle 2**
R18_18004_180 812 002	245	246
R18_18005_181 108 284	246	246
R18_18006_190 220 121	245-246	246

Boiling point: /
Flash point: /
Vapour pressure: /
Density: /
Viscosity: /

pKa:

Method description for pKa determination: 0.004 g (0.02 %) of HC Red 18 were suspended in 200 mL of iso-PrOH, stirred at 40 °C for 1 hr and put into an ultra-sonic bath for 5 min. The solution was allowed to cool down to room temperature and was then filtered. 0.5 g of this dye stock solution was mixed with 19.5 g of buffer solution.
To achieve buffered pH solutions over a range from pH 4.5 to 11.0 the following buffers were used: pH 4.5 to 5.5 = sodium acetate/acetic acid; pH 5.8 to 8.0 = K_2HPO_4/KH_2PO_4 and pH 8.5 to 11.0 = $Na_2CO_3/NaHCO_3$.
After about 4 hrs, the pH of the sample was determined, and the sample measured using an Agilent Technologies Cary 8454 UV-Vis with an Agilent Peristaltic Pump.



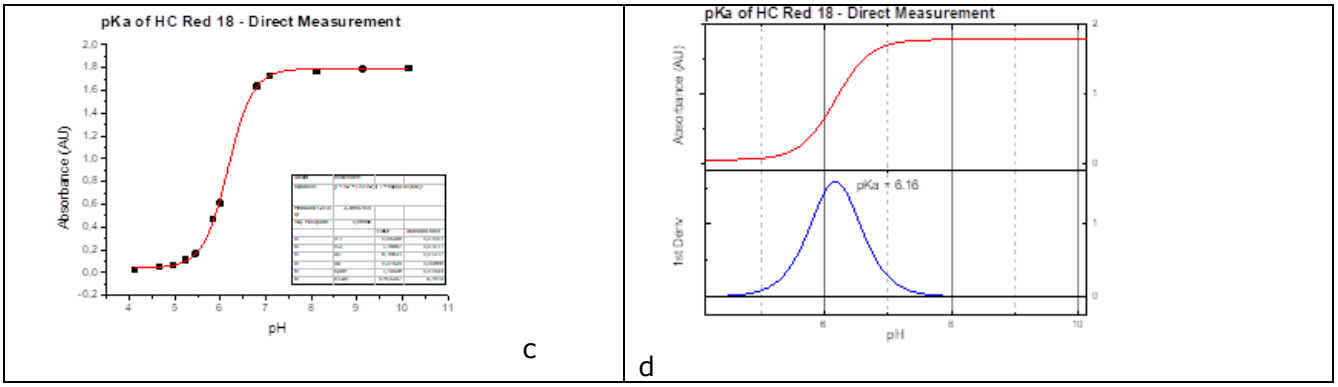


Figure 2: a) Display of the measured spectra in dependency of the pH; **b)** At the maximum absorbance at 533 nm, the absorbance was related to the corresponding pH; **c)** A sigmoidal Boltzman function was fitted to the data points, and **d)** the maximum of the first derivation is equal to the pKa value of HC Red 18.

The pKa of HC Red 18 in 2.5% iso-PrOH was found to be 6.16.

Refractive index: /
pH: HC Red 18 shows a pH of around 6.3 to 6.5 in deionized water.

UV-Vis spectrum (200 to 800 nm):

From previous Opinion (SCCS/1569/15)
λ_{max} ca. 545 nm in DMSO as diluent solvent (Ref 18), and λ_{max} ca. 526 nm in mobile phase (0.1% CH_3COOH, 0.1% TEA in methanol - 0.1% CH_3COOH, 0.1% TEA in water: 90:10, v/v).

Additional data provided in submission II
Depending on the pH of the solvent, the anionic, phenolic or a mixture of both forms might be present in the UV-Vis spectra of HC Red 18 (Figure 3).

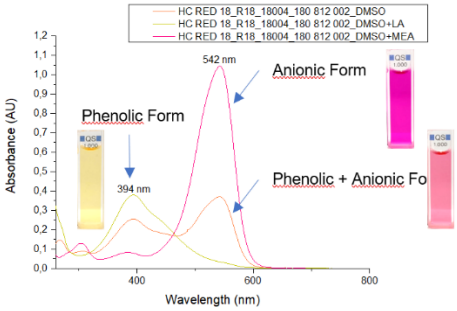


Figure 3: In pure DMSO (orange spectrum), both the anionic and phenolic form are present in the UV-Vis spectra

Table 9. Summary of solvents used for UV-Vis measurements of HC Red 18. A shift in absorption maxima is visible in dependence from the solvent.

Solvent		**Absorption Maximum [nm]**	
		Anionic Form	**Phenolic Form**
HPLC Solvents	0.3% (v/v) TEA and 0.3% (v/v) AcOH in MeOH (Solvent A)	524	386
	5% (v/v) iso-PrOH in water	529	393
	HPLC 50%(v/v) n-PrOH, 32% (v/v) AcCN, 8% (v/v) MeOH, 10% (v/v) H_2O	530	389
	DMSO	542	394

In its pink coloured anionic form, HC Red 18 shows an absorption maximum between 524 to 542 nm, depending on the solvent. In its protonated, phenolic form, the dye shows a (pale) yellow colour. For pure DMSO, both protonated and deprotonated form are present. For all other solvents, the absorption spectrum of the anionic form is found in the pure solvent mainly.

SCCS comment on additional physicochemical specifications:
The pKa value was calculated (spectrophotometric titration) by measuring the absorbance at 533 nm, over the pH values 4.12 to 10.14, given that at this analytical wavelength (533 nm) the ionized form of the test substance absorbs strongly, while the neutral form (phenolic form) absorbs relatively less.

3.1.9 Homogeneity and Stability

From previous Opinion (SCCS/1569/15)
A 0.5% FPK-245 solution (dissolved in MEA buffer pH 10, containing 4.5% monoethanolamine and 5% isopropanol in water) was shown to be stable for 45 min when mixed with 6% hydrogen peroxide in a ratio 1:1, v/v.
The HPLC chromatograms, taken at two different wavelengths, clearly show that FPK-245 is stable to alkaline peroxide over a 45 min period. There is no change in retention time, peak shape or peak area over 45 mins and no additional peaks (i.e. due to degradation products) can be detected. This is also confirmed in the following Table where the exact peak areas are shown to remain constant.

Table 10. Comparison of FPK-245 peak areas at time = 0 min and time = 45 min as quantification of stability

Sample Name	**Retention time**	**Area**	**Area**
	min	mAU*min	mAU*min
		530 nm	**254 nm**
FPK-245 0,5% MEA buffer 1:1 H_2O_2 6% t=0	12.767	649.9516	78,1203
FPK-245 0,5% MEA buffer 1:1 water (100%)	12.767	646.4885	77,5517
FPK-245 0,5% MEA buffer 1:1 H_2O_2 6% t=45	12.758	643.8065	77,6164
Stability at 45min		**99.1%**	**99.4%**

It is concluded that FPK-245 is stable in alkaline peroxide 6% over a 45-min period.

Additional data provided in submission II

Alkaline peroxide stability of HC Red 18 was determined using HPLC and UV-Vis, both using MEA buffer system at pH 10 and a 6 % peroxide solution. The stability was determined using the batch HC Red 18_18004_180 812 002 for both tests.

Stability of HC Red 18 in alkaline peroxide with HPLC:
Alkaline peroxide stability of HC Red 18 over 45 min at room temperature (22°C) was determined in a buffered alkaline solution (MEA buffer at pH 10). The sample was analyzed directly after mixing with 6 % peroxide solution (t=0) and after 45 minutes at room temperature (t=45 min). As Reference, the dye solution was mixed with water instead of 6 % peroxide solution. The data was evaluated for both 254 and 575 nm.

Method description: about 0.023 g of HC Red 18 was dissolved in 100 mL buffer (4.5% MEA, 5.0% iso-PrOH in water; pH adjusted to 10 with HCl). 4 g of the freshly prepared dye solution was mixed with 4 g of a 6% H_2O_2 solution in water (ratio = 1:1 w/w). The mixture was stirred intensely for 20 sec and the t=0 was measured using HPLC system Dionex Ultimate 3000/ P680 HPLC Pump. After 45 min of intense stirring at room temperature, the solution was measured again (t=45 min).
UV-Vis Baseline (Blank): Pure buffer solution without dye was mixed with 6% H_2O_2 solution and measured as background for t=0 and t=45 min.
Reference: To demonstrate that the dye is stable over 45 min in the MEA buffer at pH 10, the dye solution was mixed 1:1 w/w with deionized water measured as described above.

The results are summarized in Table 11.

Table 11. Alkaline peroxide stability of HC Red 18 over 45 min in MEA at pH 10 determined by HPLC at 254 and 575 nm

at 254 nm	**R_t in min**	**Area in mA*U**	
R18_18004 w.6% H_2O_2 t=0	12.08	30.9727	100.00%
R18_18004 w.6% H_2O_2 t=45 min	12.10	30.614	98.84% (-1.16%)
R18_18004 Reference t=0	12.09	30.063	100.00%
R18_18004 Reference t=45 min	12.10	30.863	100.45% (+0.45%)
at 575 nm	**R_t in min**	**Area in mA*U**	
R18_18004 w.6% H_2O_2 t=0	12.08	52.2590	100.00%
R18_18004 w.6% H_2O_2 t=45 min	12.11	52.0680	99.63% (-0.37%)
R18_18004 Reference t=0	12.09	52.0545	100.00%
R18_18004 Reference t=45 min	12.11	52.059	100.01% (+0.01%)

According to the Applicant, the stability of HC Red 18 in presence of alkaline peroxide over 45 minutes determined via HPLC, was found to be excellent with 98.84 % for 254 nm and 99.63 % for 575 nm. No new peaks were found in the HPLC data after 45 minutes for 254 and 575 nm under these conditions. According to the HPLC data, HC Red 18 can be considered stable under alkaline peroxide conditions.

UV stability
Alkaline peroxide stability of HC Red 18 over 45 min at room temperature (22°C) was determined in a buffered alkaline solution (MEA buffer at pH 10).

Table 12: Alkaline peroxide stability of HC Red 18 over 45 min in MEA at pH 10

	Absorbance @ 501 nm							
HC Red 18 – MEA pH 10	**t=0**		**t=15 min**		**t=30 min**		**t=45 min**	
Sample 1	1.77440	100%	1.76860	99.67%	1.75710	99.03%	1.74800	98.51%
Sample 2	1.77270	100%	1.76750	99.71%	1.75870	99.21%	1.74790	98.60%
Sample 3	1.76960	100%	1.76250	99.60%	1.75320	99.07%	1.74320	98.51%

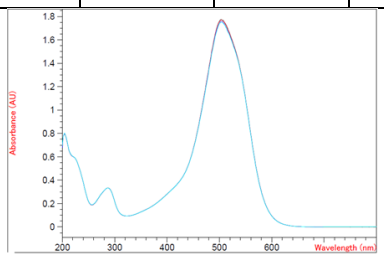


Figure 4: UV-Vis spectra of HC Red 18 in MEA buffer at pH 10 over 45 min to demonstrate stability in the alkaline medium over 45 min.

The Applicant provided further data with information of the peroxide stability:

The alkaline peroxide stability of HC Red 18 was studied using a typical colour formula for the dye buffer and solvent system as well as the peroxide system. This comprises an ammonia buffer mixed 1:1 with 6% hydrogen peroxide, with a final pH of 10 in the mixture.
The peroxide stability of HC Red 18 using the batch HC Red 18_18004_180 812 002 (from here on HC Red 18_18004) was measured using a dye concentration of 0.5 %. The results are summarized in Table 13.

Table 13. Alkaline peroxide stability of HC Red 18 over 45 min in ammonia buffer at pH 10 determined by HPLC at 254 and 526 nm

at 254 nm	**R_t in min**	**Area in mAU**	**% solution**
HC Red 18 – Stability with 6% H_2O_2			
t=0 min (start)	12.863	20.8053	100.00%
t=45 min	12.867	20.4444	98.27% (-1.73%)
Impurity 1P – Stability with 6% H_2O_2			
t = 0 min (start)	9.293	0.0412	0.20%
t= 45 min	9.287	0.1995	0.96 (+0.76%)
Reference HC Red 18 – Reference			
t = 0 min (start)	12.830	44.580	99.83%
t= 45 min	12.830	44.837	100.00%
Impurity 1P – Stability – Reference			
t = 0 min (start)	9.303	0.075	0.17%
t= 45 min	9.303	0.074	0.17%

at 526 nm	R_t in min	Area in mAU	% solution
HC Red 18 – Stability with 6% H_2O_2			
t=0 min (start)	12.863	194.1503	100.00%
t=45 min	12.867	191.4761	98.62% (-1.38%)
Impurity 1P – Stability with 6% H_2O_2			
t = 0 min (start)	9.297	1.0184	0.52%
t= 45 min	9.287	4.5189	2.33 (+1.80%)
Reference HC Red 18 – Reference			
t = 0 min (start)	12.830	408.372	99.72%
t= 45 min	12.830	422.592	103.19%
Impurity 1P – Stability – Reference			
t = 0 min (start)	9.300	1.163	0.28%
t= 45 min	9.303	1.219	0.30%

According to the Applicant, the stability of HC Red 18 in presence of alkaline peroxide over 45 minutes, determined via HPLC, was found to be excellent with 98.27% for 254 nm and 98.62% for 526 nm. During the measurement period of 45 minutes, one impurity was formed (Impurity 1P). The formed impurity is already present at the start of the measurement period at t = 0 minutes (Impurity 1B/1P). A total maximum of 2.33% of Impurity 1B/1P is found in the peroxide stability of HC Red 18 after 45 minutes using 6% peroxide solution. The reference, using the blank solution instead of the 6% peroxide solution, shows that the dye is stable in the given system. The slight changes are the result of manual integration of the peak area leading to slight changes in the concentration.

SCCS comment

Based on the new stability data provided for alkaline peroxide stability, upon SCCS request, HC Red 18, after 45 minutes in alkaline peroxide prepared using a typical colour formula, exhibits an impurity detected at both 526 nm and 254 nm, with percentage areas of 2.33% and 0.96%, respectively.

Analysis of Impurities Formed During Alkaline Peroxide Stability of HC Red 18 over 45 minutes using LC-MS

Impurity 1P was analyzed by LC-MS using an adapted version of the HPLC method to account for the technical differences of the two systems.

Table 14. Overview of the retention times between the HPLC and the LC-MS system due to technical difference

	Retention time HPLC	Retention time LC-MS
HC Red 18	13.3 min	15.6 min
Impurity 1P	9.3 min	11.6 min

Other than the shift in retention time, the LC-spectrum of the peroxide stability test of HC Red 18 after 0 and 45 minutes is comparable to the HPLC spectra, showing a single impurity "Impurity 1P".

Based on comparative analysis of sensitivity at both wavelengths used for the HPLC analysis (254 and 526 nm), it has been observed that both Impurity 1P and HC Red 18 exhibit the highest sensitivity when measured at an absorption wavelength of 526 nm, as opposed to 254 nm. Consequently, for optimal accuracy and reliability in detecting these substances, the Applicant will focus their analysis on the 526 nm wavelength.

Structural elucidation of Impurity 1P based on spectroscopic and mass spectrometric analysis

According to the Applicant, given that Impurity 1P exhibits an absorption maximum at 534 nm and a molecular ion peak at m/z 301 in negative ESI mode, which are comparable to the respective values for HC Red 18 (absorption maximum at 526 nm and molecular ion peak at m/z 285 in negative ESI mode), it is reasonable to hypothesize that Impurity 1P possesses a structure similarity to that of HC Red 18.

The mass difference of 16 amu between Impurity 1P (301 amu) and HC Red 18 (285 amu) is highly indicative of the addition of an oxygen atom. Oxygen has an atomic mass of approximately 16 amu, making it the most plausible explanation for the observed mass difference. This addition could be in the form of an oxo group (=O), resulting in the slight increase in molecular mass. Therefore, the addition of an oxygen atom is the most likely explanation for the mass difference between Impurity 1P and HC Red 18.

The found isotope pattern of the most likely molecular ion peak at m/z = 301 of the Impurity 1P is a good fit to the calculated molecular formula of a mono-oxidized HC Red 18 ($C_9H_7ClN_4O_2S_2$; HC Red18 + "O").

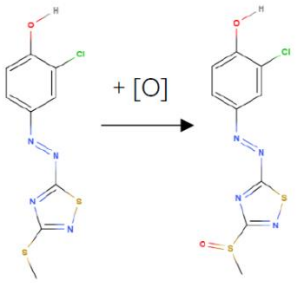


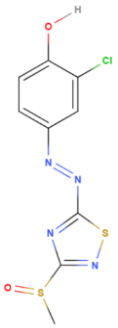
Figure 5: Oxidation of HC Red 18 to its sulfoxide derivative representing the most likely chemical structure for the Impurity 1B.

The oxidation of the dye, as depicted in Figure 5, is most likely resulting in the formation of a sulfoxide. This conclusion is supported by both mass spectrometry and UV-Vis spectroscopy data. Initially, the dye exhibits a molecular mass of 285 amu, which is consistent with its non-oxidized state. Upon oxidation, the molecular mass increases by 16 amu, resulting in a mass of 301 amu. This 16 amu increase is indicative of the addition of a single oxygen atom, characteristic of the transformation of a thioether (R-S-R') to a sulfoxide (R-S=O). The UV-Vis spectroscopy data further supports this conclusion. The non-oxidized dye has a maximum absorption peak at 526 nm. After oxidation, this peak shifts to 533 nm. This shift in absorption wavelength is consistent with changes in the electronic environment caused by the introduction of an oxygen atom, as expected upon the formation of a sulfoxide.

From a chemical perspective, the sulfur atom in a thioether is nucleophilic and electron-rich, rendering it particularly susceptible to oxidation. Under mild oxidative conditions, this typically leads to the formation of a sulfoxide rather than further oxidation to a sulfone, which would require more stringent conditions.

Based on the above, the Applicant concludes that the combination of the observed mass increase, the shift in the UV-Vis spectrum, and the chemical nature of thioethers strongly indicates that the dye undergoes oxidation to form a sulfoxide. This conclusion is well-supported by the analytical data and the inherent chemical properties of the dye.

Based on the analysis of the provided data, the Applicant suggested the structure presented in Figure 6, for Impurity 1P.



Formula	C9H7ClN4O2S2
Molecular weight	302.7529 u

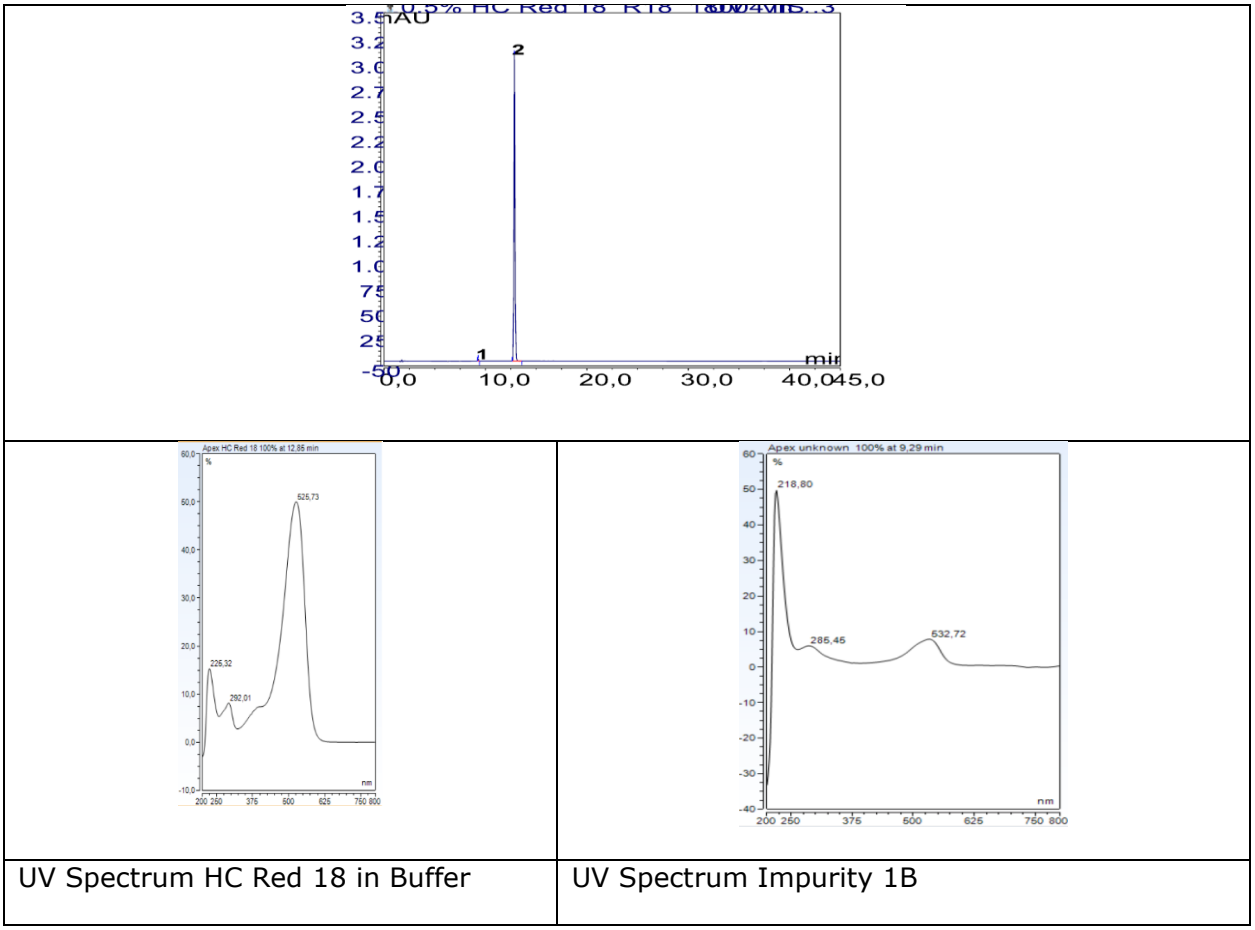
Figure 6: Structure suggested for Impurity 1P.

The Applicant provided further data on HPLC Characterization of HC Red 18

The Applicant, in line with the commitment to providing the most accurate and relevant data, includes an update for the characterisation data for HC Red 18 using the ammonia buffer system.

Table 15: Summary of the relative area in % obtained from the HPLC data of HC Red 18 at 524 nm and 526 nm.

at 254 nm	**R18_18004**	**18_28005**	**R18_18006**
Impurity 1B 9.29	0.158	0.098	0.254
HC Red 18 13.16-13.20	99.842	99.902	99.746
Total	100 %	100 %	100 %
at 526 nm	**R18_18004**	**18_28005**	**R18_18006**
Impurity 1B 9.29	0.375	0.142	0.403
HC Red 18 13.16-13.20	99.625	99.858	99.597
Total	100 %	100 %	100 %



UV Spectrum HC Red 18 in Buffer	UV Spectrum Impurity 1B

Figure 7: Chromatogram of new Commercial Batch: R18_18004_180 812 002 at 526 nm (top) and UV/Vis spectra of HC Red 18 in buffer and Impurity 1B.

The results of the characterization of HC Red 18 confirms the excellent purity of the dye, giving a purity of 99.7% for the analysis wavelength of 254 nm and 99.6% for the analysis wavelength of 526 nm. Only one impurity is present being Impurity 1B with the highest concentration of 0.4% at the analysis wavelength of 526 nm.

Suggested specifications by the Applicant based on the latest HPLC analysis of three commercial batches using the new buffer system:

HC Red 18: 99.6% at 526 nm
Impurity 1B: 0.4% at 526 nm

LC-MS Characterization of HC Red 18 using the latest buffer system

Updated LC-MS data for HC Red 18 were provided specifically addressing the found impurity, Impurity 1B, within the dye. A thorough impurity profile that is representative of the dye's use in commercial applications was also provided. Retention times between the HPLC and the LC-MS method will vary to some extend between the systems due to technical differences (Table 16). The analysis wavelength was chosen to be 526 nm as the dyes show best sensitivity at this wavelength.

Table 16: Overview of the retention times between the HPLC and the LC-MS system due to technical difference		
	Retention time HPLC	Retention time LC-MS
HC Red 18	12.8 min	15.8 min
Impurity 1B	9.3 min	11.8 min

Other than the shift in retention time, the LC-MS chromatogram of HC Red 18 is comparable to the HPLC chromatogram, showing a single impurity "Impurity 1B" (unknown 1).
The resulting TIC MS chromatogram of HC Red 18 is displayed below.

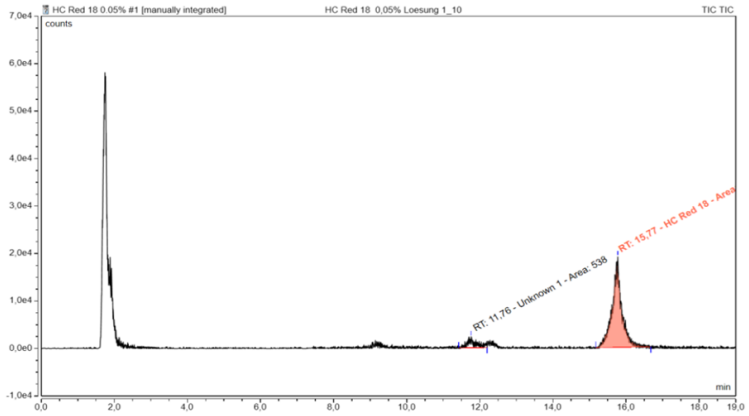
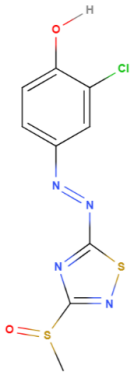


Figure 8: MS chromatogram (TIC) of HC Red 18 showing one impurity "Impurity 1B" with a retention time of 11.8 minutes.

Absorption spectra were taken by the UV-Vis detector of the LC-MS instrument. The peak with the retention time of 15.8 minutes shows an absorption maximum that fits the known spectrum of HC Red 18 with an absorption maximum of 526 nm, the spectrum of Impurity 1B shows a slightly red shifted absorption maximum to 534 nm compared to HC Red 18. This indicates that the chromophoric system of Impurity 1P must have a similar structure to that of HC Red 18.
MS analysis of the TIC peak with a retention time of 15.8 min (HC Red 18): In the ESI negative mode mass spectrum, the base peak which in this case is also the molecular ion peak, was observed at m/z 285 corresponding to the deprotonated molecule $[M\text{-}H]^-$. This fits to the expected calculated base peak of HC Red 18 with a molar mass of 386.75 g/mol.
MS analysis of peak with a retention time of 11.8 minutes – Impurity 1B: In the ESI negative mode mass spectrum, the most intense peak, identified as the base peak, was observed at m/z = 286. The Applicant proposes that this peak is likely a fragment ion due to its intensity and the nature of the mass spectrometric fragmentation patterns. Meanwhile, the peak observed at m/z = 301 is interpreted as the molecular ion peak $[M\text{-}H]^-$, corresponding to the deprotonated molecule of Impurity 1B. This assignment is supported by the mass difference of 16 amu relative to the molecular ion peak of HC Red 18, indicating the likely addition of an oxygen atom.

Structural Elucidation of Dye Impurity Based on Spectroscopic and Mass Spectrometric Analysis
Impurity 1B shows an absorption peak at 534 nm and a molecular ion peak at m/z = 301 in negative ESI mode as does Impurity 1P found in the data of the alkaline peroxide stability of HC Red 18. Again, these values are quite similar to those of HC Red 18, which has an absorption peak at 526 nm and a molecular ion peak at m/z = 285 in negative ESI mode. This similarity suggests that also Impurity 1B likely shares a structural resemblance with HC Red 18.
As with Impurity 1P found in the alkaline peroxide stability, the difference in mass between Impurity 1B (301 amu) and HC Red 18 (285 amu) is 16 amu. With an atomic mass of about 16 amu, again oxygen is the most likely candidate for this mass difference.

The calculated isotope pattern for the molecular formula $C_9H_7ClN_4O_2S_2$ (representing HC Red 18 plus one oxygen) closely matches the observed isotope pattern for the molecular ion peak at m/z = 301 for Impurity 1B; matching also Impurity 1P. This strong agreement supports the idea that Impurity 1B is a mono-oxidized form of HC Red 18 and is the same impurity as Impurity 1P from the alkaline peroxide stability.
According to the Applicant, the available data suggest that Impurity 1B in HC Red 18 and Impurity 1P formed under oxidative conditions during the alkaline peroxide test are of the same chemical structure. Both impurities showing the analogous retention times in HPLC and LC-MS, the same mass of 301 amu speaking for the addition of an oxygen to HC Red 18, and comparable absorption spectra with an absorption maximum of 534 nm.
Considering the oxidative conditions during the alkaline peroxide stability test, the expected chemical behavior of thioethers under such conditions, and the similarities between Impurity 1P and Impurity 1B, the Applicant suggests that the formation of a sulfoxide is the most likely structure for both impurities. This implies that the sulfoxide of HC Red 18 is already present in the dye as Impurity 1B and is further formed as Impurity 1P during the test.



Formula	$C_9H_7ClN_4O_2S_2$
Molecular weight	302.7529 u

Figure 9: Chemical structure of the sulfoxide of HC Red 18

SCCS comment
Based on the LC-MS data provided by the Applicant, the SCCS agrees that Impurity 1B, present in HC Red 18, and Impurity 1P, formed under oxidative conditions during the alkaline peroxide test, share the same chemical structure (Figure 9).

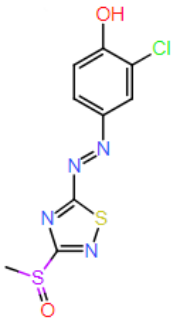
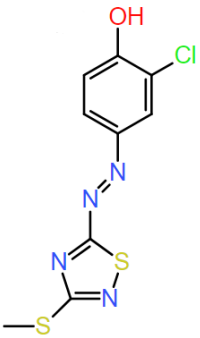
General SCCS comments to the physicochemical characterisation
- The SCCS agrees that the current commercial batches of the test substance are of better quality than the batches used in the previous submission, while the existing toxicological data are still valid.
- The SCCS agrees that Impurity 1B/1P was chemically characterised by the Applicant and matches the structure of the sulfoxide of HC Red 18 presented in Figure 9 (above).

Risk Assessment of the impurity 1B/1P in HC Red 18

New market specification:

According to the Applicant, relevant Impurity 1B/1P is only present at ≤ 0.4% in the raw material. During the alkaline peroxide stability, the highest concentration of Impurity 1B/1P is found after 45 minutes, with a maximum of 2.33%. This concentration is used as worst-case scenario for the risk assessment. The proposed structure of Impurity 1B/1P as explained above and comparison to HC Red 18 is given in the Table 17 below.

Table 17: Proposed structure of Impurity 1B/1P and comparison to HC Red 18

Impurity 1B/1P	**HC Red 18**
	
SMILES codes: Impurity 1B/1P: C(O)1=C(Cl)C=C(/N=N/C2SN=C(S(=O)C)N=2)C=C1 HC Red 18: C2=C(/N=N/C1=NC(SC)=NS1)C=CC(O)=C2Cl	

The only difference between the two structures HC Red 18 and Impurity 1B/1P is that Impurity 1B/P1 contains a monoxide at the thioether sulfur atom, forming a sulfoxide compared to HC Red 18. Since no toxicological data could be found for Impurity 1B/1P and only a low amount of this impurity was detected in the dye, the use of the TTC approach is indicated. By using NAMs, like state-of-the-art *in silico* predictions, and read-across the Applicant aimed to identify which TTC threshold is appropriate to use for the risk assessment.

First of all, the Applicant predicted the Cramer Class for both structures, HC Red 18 and Impurity 1B/1P, using the *in silico* tool Chemtunes.ToxGPS (https://mn-am.com/products/chemtunestoxgps/) developed by MN-AM. Both structures were classified as Cramer Class III based on three integrated models (Original (Munro *et al., 1996),* Extended and Revised (Yang *et al*., 2017; cosmetic-related); using Toxtree application (version 3.1.0.1851)).

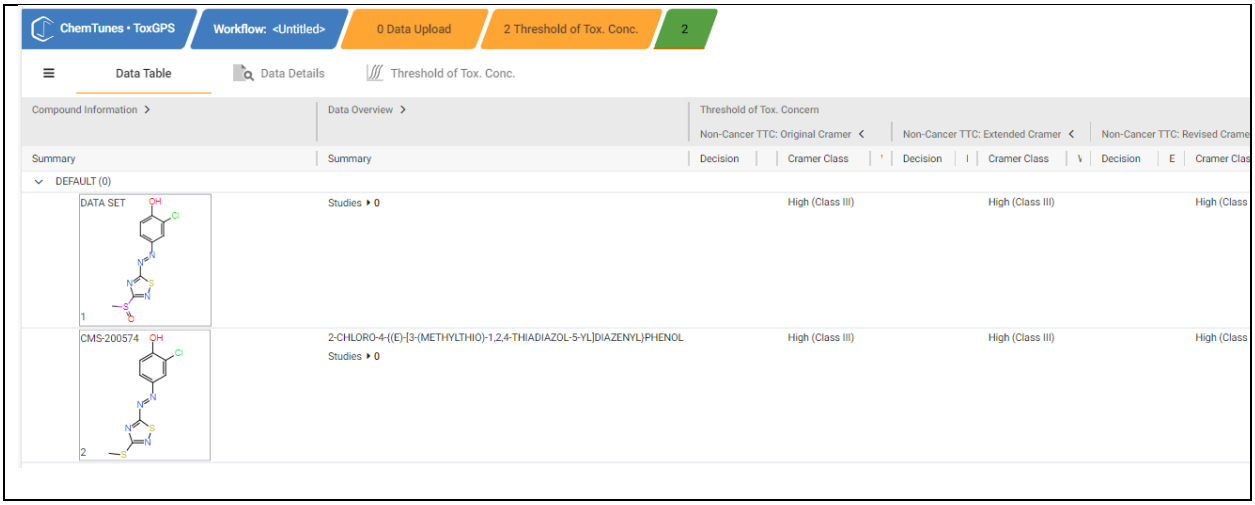


Figure 10: Cramer Class prediction of HC Red 18 and Impurity 1B/1P via Chemtunes.ToxGPS (Screenshot).

Structural similarity

In a first step, the Applicant calculated the pairwise similarity using the Tanimoto and Dice coefficient, and the chemical/molecular fingerprints incorporated in the *in silico* tools AMBIT, developed by Cefic-LRI (https://ambitlri.ideaconsult.net/tool2), and Chemtunes.ToxGPS (here RDKit Mol). The structural similarity was predicted to be very high with values greater than 80% or even greater than 90% (Figure 11).

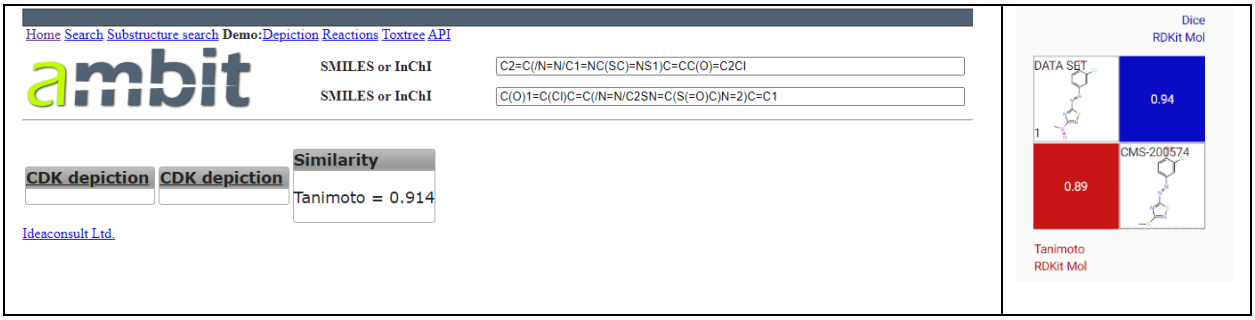


Figure 11: Determination of the structural similarity of HC Red 18 and Impurity 1B/1P via AMBIT and Chemtunes.ToxGPS (above, left: Impurity 1B/1P: below, right: HC Red 18) (Screenshots).

Biological/toxicological similarity

In a second step, the Applicant assessed the biological/toxicological similarity for the endpoint genotoxicity of both structures using chemotype predictions for DNA binders and toxicological endpoint predictions for Bacterial Reverse Mutagenicity, *in vitro* Chromosome Aberrration and *in vivo* Micronuclues made by Chemtunes.ToxGPS. The predictions made by the Applicant with Chemtunes.ToxGPS show similar results of Impurity 1B/1P compared to HC Red 18, indicating that there might be a similar (non)-genotoxicological activity. Both structures have the same two hits for DNA binders. In addition, the predicted negative outcome for the genotoxicity endpoint Bacterial Reverse Mutagenicity was also the same for Impurity 1B/1P and HC Red 18. Furthermore, even if the prediction for *in vitro* Chromosome Aberration was uncertain for Impurity 1B/1P (negative for HC Red 18), the prediction for the *in vivo* Micronuclues was negative compared to the positive outcome for HC Red 18. According to the Applicant, these results can be interpreted as evidence for a potential lower genotoxic activity of Impurity 1B/1P compared to HC Red 18.

Futhermore, the Applicant performed predictions for the same endpoints with two additional in silico tools, namely VEGA (Version 1.2.3) and OECD QSAR TB (Version 4.7). By that, the Applicant wanted to clarify if the predictions of Chemtunes.ToxGPS are reproducible and if a potential similar genotoxic activity of Impurity 1B/1P compared to HC Red 18 can be confirmed.

An overview on the predictions made with all three in silico tools (Chemtunes.ToxGPS, VEGA and OECD QSAR TB) as well as all available experimental *in vitro* and *in vivo* study results are listed in the Table below.

Table 18. *In silico* predictions for the endpoint genotoxicity as well as *in vitro* and *in vivo* study results for Impurity 1B/1P and HC Red 18 using different in silico models and *in vitro* and *in vivo* experimental models. Orange highlighted models indicate different outcomes, red highlighted outcomes indicate a potential genotoxic activity for a certain genotoxic endpoint, green highlighted outcomes indicate a low probability for a genotoxic activity for a certain endpoint. Reliability of a prediction is mentioned in brackets if available.

In silico QSAR Model		HC Red 18	Impurity 1B/1P
Chemtunes. ToxGPS	DNA Binders	2 Aromatic Azos 4 atoms Heterocyclic Azos 4 atoms	2 Aromatic Azos 4 atoms Heterocyclic Azos 4 atoms
	Bacterial reverse mutagenicity	negative	negative
	In vitro CA	negative	uncertain
	In vivo MN	positive	negative
VEGA	Mutagenicity (Ames test) CONSENSUS model 1.0.4	positive	positive
	Mutagenicity (Ames test) model (CAESAR) 2.1.14	positive (low reliability)	positive (low reliability)
	Mutagenicity (Ames test) model (ISS) 1.0.3	positive (low reliability)	positive (low reliability)
	Mutagenicity (Ames test) model (SarPy-IRFMN) 1.0.8	negative (moderate reliability)	negative (low reliability)
	Mutagenicity (Ames test) model (KNN-Read-Across) 1.0.1	positive (moderate reliability)	negative (low reliability)
	Mutagenicity (Ames test) model for aromatic amines (CONCERT/IRFMN) 1.0.0	negative (low reliability)	negative (low reliability)
	Chromosomal aberration model (CORAL) 1.0.1	positive (low reliability)	negative (low reliability)
	In vitro Micronucleus activity (IRFMN-VERMEER) 1.0.1	negative (low reliability)	negative (low reliability)
	In vivo Micronucleus activity (IRFMN) 1.0.2	negative (moderate reliability)	negative (moderate reliability)
OECD QSAR TB	DNA binding by OASIS	No alerts found	No alerts found
	DNA binding by OECD	SN1	SN1
	Carcinogenicity (genotox and nongenotox) alerts by ISS	Aromatic diazo (Genotox)	Aromatic diazo (Genotox)
	DNA Alerts for AMES, CA and MNT by OASIS	No alerts found	No alerts found
	In vitro mutagenicity (Ames test) alerts by ISS	Aromatic diazo	Aromatic diazo
	In vivo mutagenicity (Micronucleus) alerts by ISS	Aromatic diazo	Aromatic diazo
In vitro & *in vivo* experimental model		HC Red 18	Impurity 1B
OECD 471	Ames	negative	n/av
OECD 476	MLA *hprt*-locus	negative	n/av
OECD 487	MNT in Human Lyphocytes	negative	n/av
-	Mammalian Erythrocyte MNT integrated in a 14-day Toxicity Study	negative	n/av

According to the Applicant, when comparing the outcomes of the different genotoxicity predictions underlying various models of the different *in silico* tools, it becomes clear that a predominant proportion, namely 15 out of 19 predictions in total (= 79%) come to the same predicted outcome for both, HC Red 18 and Impurity 1B/1P. Four out of 19 predictions in total (= 21%) come to a difference, for Impurity 1B/1P mainly even better outcome. Of these four differently predicted outcomes, three can be interpreted as a potential lower genotoxic activity

of Impurity B1 compared to HC Red 18. These are the *in vivo* Micronuclues prediction from Chemtunes.ToxGPS, the Mutagenicity (Ames test) model (KNN-Read-Across) 1.0.1 from VEGA, and the Chromosomal aberration model (CORAL) 1.0.1 from VEGA. Only one prediction showed an uncertain outcome for Impurity 1B/1P (*in vitro* Chromosome Aberration from Chemtunes.ToxGPS). However, all other *in silico* results indicate that HC Red 18 and Impurity 1B/1P share a very similar genotoxicity activity profile, if not an even a better one for impurity 1B/1P compared to HC Red 18. This means that the biological/toxicological similarity of Impurity 1B/1P and HC Red 18, based on the above performed *in silico* predictions, can be concluded as perfectly high.

Analogue quality

According to the Applicant, the assessment of the structural and biological/toxicological similarity of Impurity 1B/1P and HC Red 18 via the *in silico* tools indicates a very similar genotoxicity activity profile. Some endpoints show an even better outcome (no genotoxicity) for Impurity 1B/1P. In this context, the read-across to the experimental genotoxicity results of HC Red 18, which are completely inconspicuous for all genotoxic endpoints (mutagenicity, clastogenicity & aneugenicity) and demonstrate no genotoxic activity of HC Red 18, would suggest also no genotoxic potential of Impurity 1B/1P. Thus, the Applicant considers high analogue quality.

Read-across conclusion

Based on the results above, the Applicant concluded that an alert for genotoxicity can be completely out ruled for Impurity 1B/1P.

SCCS comment

The SCCS agrees that both HC Red 18 and the impurity 1B belong to Cramer Class III. The SCCS calculated the similarity between HC Red 18 and Impurity 1B/1P and agrees that there is a close similarity between the structures. The results from Read-across and QSAR provided by the Applicant indicate that there is not an increased concern of genotoxic toxicity for impurity 1B. Hence, it can be considered that impurity 1B/1P has the same genotoxicological concern as HC Red 18.

3.2 Function and uses

New data

FPK-245, a non-reactive dye, is used as a direct hair colouring agent up to on-head concentration of 0.5% in non-oxidative as well as at 1.5% in oxidative hair dye formulations. According to the Applicant, FPK-245 is shown to be stable under conditions used in oxidative formulations and does not take part in the oxidative colour forming reaction.

The application procedure is described as follows:

Type of formulation	Application amount [mL]	Application time [min]	Rinse off	Mixing procedure	Frequency of use
Non oxidative	35-50	30	Yes	No	2/month
Oxidative	100	30	Yes	Yes, with H_2O_2	1/month

3.3 Dermal / percutaneous absorption

Taken from SCCS/1569/15

Experiment 1: oxidative conditions

Guideline:	OECD 428 (2004)
Test system:	Frozen dermatomed pig ear skin (430-450 µm)
Membrane integrity:	Electrical resistance barrier integrity
Replicates:	12 (6 donors)
Test substance:	FPK-245
Batch:	WK120919
Purity:	99.85%
Test item:	Cream (batch No. C1R2011003.04) with 3% FPK-245 mixed with hydrogen peroxide lotion 6% (batch No. B1E1995011.2) (mixing ratio:1/1)
Dose applied:	20 µL/cm^2 of the test item (approx. 300 µgFPK-245/cm^2)
Exposed area:	1 cm^2
Exposure period:	30 minutes
Sampling period:	24 hours
Receptor fluid:	20% EtOH/PBS
Solubility of test substance in receptor fluid:	Up to 2.20 µg/mL
Mass balance analysis:	Provided
Tape stripping:	Yes (4 pools of 5 strips each)
Method of Analysis:	LC-MS/MS
Positive control:	Benzoic acid
Negative control:	2-Ethylhexyl trans-4-methoxycinnamate
GLP:	In compliance
Study period:	February - March 2013

Two experiments were performed on thawed dermatomed pig skin samples under static and non-occluded conditions. The thickness of the skin used was 430-450 µm. The blank samples were collected immediately after filling the donor chambers at the maximal flow rate of the pump, but before the application of the test item. FPK-245 was not detected in the blank samples. The conductivity across the skin samples of each chamber was determined before treatment and after the last sampling as a measure of skin integrity. The integrity was given for all skin samples before and after the treatment

In each experiment 6 chambers were analysed and skin samples from 3 donors per experiment were used for the evaluation of each test preparation. A 20 µL aliquot of the test item, corresponding to approx. 300 µg FPK-245, were applied on each skin sample. The test item was left on the skin for 0.5 hours and was then washed off using 9 times (2x 1 mL H_2O warm + 5x1 mL 10% shampoo + 2x1 mL H_2O warm). The dermal delivery was monitored over 24 hours. The *stratum corneum* was separated by tape stripping from the remaining epidermis and dermis layers. The tape strips (5 strips per sample, 4 samples) were pooled and extracted for analysis. The remaining skin compartments were also extracted for their content of the test substance. The sample solutions from the skin dermal absorption assay were analysed by LC-MS/MS for the presence of FPK-245. The LOD was defined as 0.50 ng/mL in receptor solution and extraction solution. The LLOQ was 1.0 ng/mL in both, receptor solution and extraction solution.

Controls with benzoic acid (positive) and 2-ethylhexyl trans-4-methoxycinnamate (negative) on human skin were used to check the performance of the skin penetration system at least once a year.

Results

The integrity of the skin was demonstrated prior to application and after the last sampling. The conductivity prior to the experiment was in the acceptable range of < 900 µS/cm for all skin samples used. All 12 chambers met the acceptance criteria and could be used to calculate the dermal delivery.

Details of the results are summarised in the following Table:

Table 19. Results of the dermal absorption study under oxidative conditions

Cream with 3 % FPK-245 under oxidative conditions						
Amount of FPK-245	Expressed as µg/cm² of skin surface mean S.D. (n = 12)#			Expressed as % of dose mean S.D. (n = 12)#		
Applied Dose	220	±	10.8	100	±	4.90
Unabsorbed Dose	231	±	16.2	105	±	7.34
Adsorbed Dose *Stratum corneum* (isolated by stripping)	0.122	±	0.0762	0.0554	±	0.0346
Absorbable Dose Skin extracts (isolated after 24 hours)	0.328	±	0.202	0.148	±	0.0898
Absorbed dose	0.645	±	0.297	0.291	±	0.132
Recovery	233	±	16.3	105	±	4.00
Dermal Delivery (receptor fluid + epidermis + dermis)	0.973	±	0.381	0.439	±	0.168

Conclusion

It can be stated that under the experimental oxidative conditions reported, cream with 3% FPK-245 showed low penetration into the viable skin layers and into the receptor fluid with dermal delivery (receptor fluid, epidermis, dermis) of 0.97 ± 0.38 µg/cm² (0.44 ± 0.17 % of applied dose).

SCCS comment

In accordance with the SCCS Notes of Guidance, the mean + 1 SD i.e. 1.35 µg/cm² will be used for the MoS calculation under oxidative conditions at on-head concentrations of up to 1.5%.

Experiment 2: non-oxidative conditions

Guideline:	OECD 428 (2004)
Test system:	Frozen dermatomed pig ear skin (430-450 µm)
Membrane integrity:	Electrical resistance barrier integrity
Replicates:	12 (6 donors)
Test substance:	FPK-245
Batch:	WK120919
Purity:	99.85%
Test item:	Cream (batch No. C1R2011003.07) with 1.5% FPK-245
Dose applied:	20 µL/cm² of the test item (approx. 300 µg FPK-245/cm²)
Exposed skin area:	1 cm²
Exposure time:	30 minutes
Sampling period:	24 hours
Receptor fluid:	20% EtOH/PBS
Solubility of test substance in receptor fluid:	Up to 2.20 µg/mL
Mass balance analysis:	Provided
Tape stripping:	Yes (4 pools of 5 strips each)
Method of Analysis:	LC-MS/MS
Positive control:	Benzoic acid
Negative control:	2-Ethylhexyl trans-4-methoxycinnamate
GLP:	In compliance

Study period: February - March 2013

Two experiments were performed on thawed dermatomed pig skin samples under static and non-occluded conditions. The thickness of the skin used was 430 - 450 µm. The blank samples were collected immediately after filling the donor chambers at the maximal flow rate of the pump, but before the application of the test item. FPK-245 was not detected in the blank samples. The conductivity across the skin samples of each chamber was determined before treatment and after the last sampling as a measure of skin integrity. The integrity was given for all skin samples before and after the treatment.
In each experiment, 6 chambers were analysed and skin samples from 3 donors per experiment were used for the evaluation of each test preparation. A 20 µL aliquot of the test item, corresponding to approx. 300 µg FPK-245, was applied on each skin sample. The test item was left on the skin for 0.5 hours and was then washed off 9 times (2x 1 mL H_2O warm + 5x1 mL 10% shampoo + 2x1 mL H_2O warm). The dermal delivery was monitored over 24 hours. The *stratum corneum* was separated by tape stripping from the remaining epidermis and dermis layers. The tape strips (5 strips per sample, 4 samples) were pooled and extracted for analysis. The remaining skin compartments were also extracted for their content of the test substance. The sample solutions from the skin dermal absorption assay were analysed by LC-MS/MS for the presence of FPK-245. The LOD was defined as 0.50 ng/mL in receptor solution and extraction solution. The LLOQ was 1.0 ng/mL in both, receptor solution and extraction solution.
Controls with benzoic acid (positive) and 2-ethylhexyl trans-4-methoxycinnamate (negative) on human skin were used to check the performance of the skin penetration system at least once a year.

Results

The integrity of the skin was demonstrated prior to application and after the last sampling. The conductivity prior to the experiment was in the acceptable range of < 900 µS/cm for all skin samples used. Eleven chambers met the acceptance criteria and could be used to calculate the dermal delivery.
Details of the results are summarised in the following Table.

Table 20. Results of the dermal absorption study under non-oxidative conditions

Cream with 1.5 % FPK-245 under non-oxidative conditions						
Amount of FPK-245	Expressed as µg/cm^2 of skin surface mean S.D. (n = 11)#			Expressed as % of dose mean S.D. (n = 11)#		
Applied Dose	189	±	19.9	100	±	10.5
Unabsorbed Dose	193	±	13.7	102	±	7.22
Adsorbed Dose *Stratum corneum* (isolated by stripping)	0.136	±	0.154	0.0717	±	0.0815
Absorbable Dose Skin extracts (isolated after 24 hours)	1.21	±	0.956	0.637	±	0.460
Absorbed dose	1.43	±	0.984	0.793	±	0.580
Recovery	198	±	12.2	105	±	9.17
Dermal Delivery (receptor fluid + epidermis + dermis)	2.65	±	0.776	1.43	±	0.487
# only valid values with a recovery of > 85% were used. samples < LOD were replaced by value of LOD (0.50 ng/mL in matrix A and B) samples < LLOQ were replaced by value of LLOQ (1.00 ng/mL in matrix A and B)						

Conclusion
According to the Applicant, it can be stated that under the experimental non-oxidative conditions reported, cream with 1.5% FPK-245 showed a low penetration into the viable skin layers and into the receptor fluid with dermal delivery of FPK-245 of 2.65 ± 0.78 µg/cm^2 (1.43 ± 0.49 % of applied dose).

Ref. 11

SCCS comment
This study is reported in the previous SCCS Opinion, but it was not evaluated since it was conducted under non-oxidative conditions with a cream containing 1.5% FPK-245

New data
As FPK-245 is also intended as a hair dye ingredient in non-oxidative hair colouring products at on-head concentrations of up to 0.5%, an additional study was performed (Test Facility Study No. 794112).

Dermal Delivery Study under non-oxidative conditions

Guideline:	OECD 428 (2004)
Test system:	Frozen split-thickness human skin (stratum corneum) (320-400 µm)
Membrane integrity:	tritiated water barrier integrity test
Replicates:	12 samples (5 donors)
Test substance:	FPK-245
Batch:	WK120919
Purity:	99.85%
Test item:	Cream (batch No. WK120919) with 0.5% FPK-245
Dose applied:	20.09 mg/cm^2 (143 µg FPK245/cm^2)
Exposed skin area:	1.5 x 1.5 cm ^{2}
Exposure time:	30 minutes
Sampling period:	72 hours
Receptor fluid:	0.01 % w/v sodium azide in phosphate buffer saline
Solubility of test substance in receptor fluid:	-
Mass balance analysis:	Provided
Tape stripping:	Yes
Method of Analysis:	LC-MS/MS
Positive control:	no
Negative control:	no
GLP:	Yes
Study period:	11 March 2014 – 14 April 2014

An automated flow-through diffusion cell apparatus was used. The flow-through diffusion cells were placed in a manifold, heated via a circulating water bath, to maintain the skin surface temperature of 32 ± 1°C. The cells were connected to multi-channel peristaltic pumps. The surface area of exposed skin within the cells was 0.64 cm^2. Receptor fluid (phosphate buffered saline (PBS) supplemented with sodium azide (ca 0.01%, w/v)) was pumped underneath the skin at a flow rate of 1.5 mL/h ± 0.15 mL/h. A tritiated water barrier integrity test was performed and any human skin sample exhibiting absorption greater than 0.6% of the applied dose was excluded from subsequent absorption measurements.
Sections of split-thickness skin membrane, ca. 1.5 x 1.5 cm ^{2}, were cut and positioned on the receptor chamber of the diffusion cell. Test formulation was applied over the surface of the stratum corneum of human skin using a positive displacement pipette set to deliver ca. 12.8 mg (ca. 20 mg/cm2). The donor chambers were not occluded and were left open to the atmosphere. To accurately quantify the amount of test preparation applied to each skin sample, seven weighed aliquots (ca. 12.8 mg) were collected. The aliquots were mixed with

DMSO (10 mL) and sonicated until the test item was in solution. The aliquots were then analyzed by LC-MS/MS.
Absorption of FPK-245 was assessed by collection of 30 min fractions of receptor fluid from 0-1 h post dose, then hourly fractions from1-2 h post dose and then 2 h fractions from 2-72 h post dose. All samples were analyzed by LC-MS/MS. At 30 min post exposure, each skin was washed. A single aliquot of mild shampoo solution (2%, v/v) was applied to each skin. The water and shampoo solutions were pooled in a single pre-weighed skin wash vial per skin sample. All samples were analyzed by LC-MS/MS. At 72 h post dose the underside of the skin was rinsed with receptor fluid. The skin was washed and removed from the cell and placed on a piece of tissue to remove any remaining receptor fluid from the underside of the skin. The stratum corneum was removed with 20 successive tape strips (D-Squame® stratum corneum tape). The epidermis and dermis of the exposed skin were then separated. The skin was placed onto cling film, epidermis side up, and the cling film wrapped over the skin. The epidermis was then removed from the skin with a scalpel and forceps to peel the epidermis away from the dermis. The epidermis and dermis were then placed into individual vials. All samples were analyzed by LC-MS/MS. For any receptor fluid sample below the lower limit of quantification (LLOQ), where a peak was detected, the value was extrapolated. Where no peak was detected, the value was recorded as '0'.

Results

According to the Applicant, the absorption profiles were similar for all samples although the majority of the receptor fluid samples were below the limit of quantification. The mass balance for all samples was within 100 ± 15%. The results are, therefore, based on all 12 samples of human skin.
The mean mass balance was 96.90% (SD, 5.63%) of the applied dose. At 30 min post application, 91.52% of the applied dose was washed off. At 72 h post application, a further 2.86% of the applied dose was removed (donor chamber wash, 72 h skin wash, 72 h tissue swabs and 72 h pipette tips, contained 0.29%, 2.15%, 0.38% and 0.05% of the applied dose, respectively). Therefore, the total dislodgeable dose was 94.38% of the applied dose.
The mean total unabsorbed dose was 95.64% of the applied dose. This consisted of the dislodgeable dose, unexposed skin (0.05%) and the test item associated with the stratum corneum (1.20%).
The first five tape strips contained 0.45% of the applied dose. The epidermis and dermis contained 0.16% and 0.17% of the applied dose, respectively. The total absorbed dose (0.88%) was the sum of the receptor fluid (0.65%), receptor chamber wash (0.22%) and the receptor rinse (0.01%).
Most individual receptor fluid samples were less than the lower limit of quantification (LLOQ, 5 ng/mL). Where a receptor fluid value was below the LLOQ, 5 ng/mL has been used as the value for the sample. The epidermis, dermis and total absorbed dose were 0.23, 0.24 and 1.25 µg/cm2, respectively.
Exposure to the cream with 0.5% FPK-245 under non-oxidative conditions showed a low penetration into the viable skin layers and into the receptor fluid with dermal delivery of FPK-245 of 1.79 ± 0.5 µg/cm^2 (1.26 ± 0.35 % of applied dose).
A summary of the mean results is provided in the Table below:

Table 21. Results of the Dermal Delivery Study under non-oxidative conditions

Test Preparation	FPK-245	
Target concentration in test Preparation	0.5% (w/w)	
Actual Concentration in test Preparation	0.71% (w/w)	
Applied dose of Test Preparation	20.09 mg/cm^2	
Applied dose of Test Item	143 µg/cm^2	
Species	Human	
Number of samples	12	
Number of donors	5	
	(% Applied dose, mean ± SD)	(µg/cm^2, mean ± SD)
Dislodgeable Dose (30 min)	91.52 ± 5.69	130.44 ± 8.11
Total Dislodgeable Dose	94.38 ± 5.68	134.52 ± 8.10
Unabsorbed Dose	95.64 ± 5.64	136.32 ± 8.05
Absorbed Dose	0.88 ± 0.13	1.25 ± 0.18
Dermal delivery	1.26 ± 0.35	1.79 ± 0.5
Mass Balance	96.90 ± 5.63	138.11 ± 8.02

Dislodgeable dose (30 min) = skin wash (30 min) + tissue swab (30 min) + pipette tips (30 min)
Total dislodgeable dose = dislodgeable dose (30 min) + skin wash (72 h) + tissue swab (72 h) + pipette tips (72 h) + donor chamber wash
Total unabsorbed dose = total dislodgeable dose + *stratum corneum* + unexposed skin
Total absorbed dose = cumulative receptor fluid + receptor rinse + receptor chamber wash
Dermal Delivery = total absorbed dose + epidermis + dermis + cling film
Mass balance = total unabsorbed dose + epidermis + dermis + total absorbed dose

Conclusion

According to the Applicant, the dermal penetration of FSK-245 under non-oxidative conditions is very low. Most individual receptor fluid samples were less than the lower limit of quantification (LLOQ, 5 ng/mL) of FPK-245 equivalents. Exposure to the cream with 0.5% FPK-245 under non-oxidative conditions showed a low penetration into the viable skin layers and into the receptor fluid with dermal delivery of FPK-245 of 1.79 ± 0.5 µg/cm² (1.26 ± 0.35 % of applied dose). A value of 2.29 µg/cm² (1.79 µg/cm² plus 1SD 0.5 µg/cm²) with regards to the current SCCS procedure will be used for MoS calculation under non-oxidative conditions.

Ref: Craig S. 2014 (19); Test Facility study No 794112

SCCS comments

In accordance with the SCCS Notes of Guidance a dermal absorption value of 2.29 µg/cm² will be used for MOS calculations under non-oxidative conditions at on-head concentrations of up to 0.5%.

3.3 Special Investigation

TTC assessment on Impurity 1B/1P

The Applicant performed TTC assessment on impurity 1B/1P with the originally indicated Cramer Class III value of 90 µg/person/day. The Applicants' calculation is based on the method published by Kroes *et al.* (2007) and the SCCP/1171/08.

SCCS comment

The SCCS notes that, in the TTC calculations performed by the Applicant, the impurity levels were divided by the number of days, which is not an accurate approach. This is because the impurity is already present in the non-oxidative form and is further generated during the oxidation process. Consequently, the calculated exposure levels for the oxidative dye are too high when compared to the permissible threshold values. Furthermore, in the SCCS Notes of Guidance (12th revision) a TTC of 138 µg/p/d, is currently recommended by the SCCS for cosmetics-related substances.
The correct exposure values for both non-oxidative and oxidative dyes are, respectively, 7 and 30 times higher than initially estimated, resulting in exposure levels of 40.8 µg/person/day and 349.5 µg/person/day. For the non-oxidative dye, the exposure of 40.8 µg/person/day is below the Cramer Class III threshold of 138 µg per person/day, indicating safety. However, for the oxidative dye, the exposure of 349.5 µg/p exceeds the 138 µg per person/day threshold, suggesting it is not safe under these conditions. The Applicant, however, used a conservative dermal absorption (DA) value of 10% in their calculations, whereas the actual measured DA under oxidative conditions was 0.44% + 0.17 %, giving a total of 0.61%. If the measured DA had been applied, the exposure for the oxidative dye would be recalculated as: Adjusted exposure = (0.61x 349.5)/10 = 21.3 µg/person/day. This adjusted value of 21.3 µg/p/d is below the threshold of 138 µg/p/d, thus indicating safety for the oxidative dye.

The SCCS has recalculated the SED in accordance with the SCCS Notes of Guidance (12th revision). To confirm the safety of the impurity 1B/1P under both exposure conditions, the following points are also considered:
- In-depth analysis reveals that the impurity closely resembles the original dye, differing only by the addition of a single oxygen atom (sulfoxide versus original thioether).
- For non-oxidative dyes, the amount of impurity remains stable. In contrast, for oxidative dyes, small amounts of the impurity are generated as the original dye reacts with H_2O_2, however after 45 min of reaction impurity levels do not exceed 2.33%.

Based on the new calculations, the SCCS agrees that impurity 1B/1P does not pose a health risk to the consumer since exposure levels to this impurity are below the Cramer Class III level under non-oxidative as well as oxidative conditions.

3.3.1 Calculation of SED/LED

In its previous Opinion (SCCS/1569/15). the SCCS calculated SED for oxidative conditions as follows:

Oxidative conditions:
(3 % formulation, on-head concentration 1.5 %)

Absorption through the skin	A	=	1.35 µg/cm²
Skin Area surface	SAS	=	580 cm^2
Dermal absorption per treatment	SAS x A x 0.001	=	0.783 mg
Average human body weight		=	60 kg
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.013 mg/kg bw**

In this SCCS Opinion, the SCCS calculated the SED for non-oxidative conditions as follows:

Non-oxidative conditions:
(0.5% formulation, on head concentration 0.5%)

Absorption through the skin	A	=	2.29 µg/cm²
Skin Area surface	SAS	=	580 cm^2

Dermal absorption per treatment	SAS x A x 0.001	=	1.328 mg
Typical body weight of human		=	60 kg
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.022 mg/kg bw**

SCCS comment

The toxicological evaluation of HC Red 18 can be found in the SCCS/1569/15 Opinion; in this Opinion the NOAEL is established at 3 mg/kg bw/d.

3.3.2 Safety evaluation (including calculation of the MoS)

Calculation of the Margin of Safety

MOS calculations under oxidative conditions
(3 % formulation, on-head concentration 1.5 %)

Systemic exposure dose	SED	=	0.013 mg/kg bw
No observed adverse effect level (90-day, oral, rat)	NOAEL	=	3 mg/kg bw/d
Bioavailability 77%*		=	2.31 mg/kg bw/d
Margin of Safety	**adjusted NOAEL/SED**	**=**	**178**

* based on the toxicokinetic study (ref. 12)

MOS calculations under non-oxidative conditions
(0.5% formulation, on head concentration 0.5%)

Systemic exposure dose (SED)	SED	=	0.022 mg/kg bw
No observed adverse effect level (90-day, oral, rat)	NOAEL	=	3 mg/kg bw/d
Bioavailability 77%*		=	2.31 mg/kg bw/d
Margin of Safety	**adjusted NOAEL/SED**	**=**	**105**

* based on the toxicokinetic study (ref. 12)

3.3.3 Discussion

- Based on the additional physicochemical data provided by the Applicant, the SCCS agrees that the purity of the new commercial batches has been improved and only one impurity is detected at 526 nm (Impurity 1B).
- The test substance, HC Red 18, as indicated by the provided HPLC-PDA data, in the presence of alkaline peroxide for up to 45 minutes exhibits another Impurity 1P at 2.33%. Impurity 1P as indicated by the provided HPLC-PDA and LC-MS data, shares a close structural similarity with Impurity 1B. It was chemically characterised by the Applicant and matches the structure of the sulfoxide of HC Red 18 presented in Figure 9. The SCCS agrees that both HC Red 18 and the impurities 1B/1P belong to Cramer Class III. Read-across results indicate that these close analogues are negative for genotoxicity. Hence, it can be considered that impurities 1B/1P, like HC Red 18, are not of genotoxicological concern.

4 CONCLUSION

1. In light of the data provided, does the SCCS consider HC Red 18 safe, when used as an ingredient at 1.5% in oxidative hair dye and at 0.5% in non-oxidative hair dye formulations?

In light of the data provided, the SCCS consider HC Red 18 safe when used as an ingredient at 1.5% in oxidative hair dye and at 0.5% in non-oxidative hair dye formulations.

2. Does the SCCS have any further scientific concerns with regard to the use of HC Red 18 in cosmetic products?

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5 MINORITY OPINION

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6 REFERENCES

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7. GLOSSARY OF TERMS

See SCCS/1647/22, 12th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – Appendix 15 - from page 158

8. LIST OF ABBREVIATIONS

See SCCS/1647/22, 12th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – Appendix 15 - from page 158
