Scientific Committee on Consumer Safety

SCCS

OPINION ON

hair dye *Indigofera tinctoria* (C170)
CAS 84775-63-3

Submission III

The SCCS adopted this document
by written procedure on 3 April 2020

ACKNOWLEDGMENTS

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https://ec.europa.eu/transparency/regexpert/index.cfm

This Opinion has been subject to a commenting period of a minimum eight weeks after its initial publication (from 13 January until 13 March 2020). Comments received during this time period are considered by the SCCS. For this Opinion, no comment was received. Therefore, the final version is unchanged except for a minor correction in SCCS comment on submission III, page 13 (section 3.1.5).

1. ABSTRACT

The SCCS concludes the following:

(1) In light of the data provided, does the SCCS consider Indigofera tinctoria (C170) safe when used in non-oxidative conditions hair colouring products at on-head concentrations of up to 25%?

In light of the data provided, the SCCS considers that *Indigofera tinctoria* is safe when used in non-oxidative condition hair colouring products at on-head concentrations of up to 25%.

(2) Does the SCCS have any further scientific concerns with regard to the use of Indigofera tinctoria (C170) in cosmetic products?

A weak skin sensitisation potential cannot be excluded for *Indigofera tinctoria*.

Keywords: SCCS, scientific opinion, *Indigofera tinctoria*, C170, hair dye, Regulation 1223/2009, CAS 84775-63-3, EC 283-892-6

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on hair dye *Indigofera tinctoria* (C170), Submission III, preliminary version of 10 January 2020, final version of 3 April 2020, SCCS/1615/20

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

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2. MANDATE FROM THE EUROPEAN COMMISSION

Background

Submission I for *Indigofera tinctoria* (C170) (CAS 84775-63-3) was submitted in October 2003.

On the 23 April 2004, the Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) adopted, by written procedure, an opinion (SCCNFP/0790/04) according to which..."a complete safety dossier on *Indigofera tinctoria* is required".

Following the Submission II (2006) the SCCS adopted an opinion (SCCS/1439/11) at its 16th plenary meeting of 18 September 2012 on *Indigofera tinctoria* (C170) with the following conclusion:
The SCCS is of the opinion that the safety of Indigofera tinctoria used as a hair dye at a maximum concentration on the head of 25% cannot be assessed due to incomplete information.
The safety of Indigo (CI 73 000) used as a colorant should be re-assessed.

Submission III on the hair dye *Indigofera tinctoria* (C170) was transmitted by Cosmetics Europe in November 2017.

According to the applicant, the current submission supports the safe use of *Indigofera tinctoria* in non-oxidative conditions hair colouring products at on-head concentrations of up to 25%.

Terms of reference

(1) In light of the data provided, does the SCCS consider Indigofera tinctoria (C170) safe when used in non-oxidative conditions hair colouring products at on-head concentrations of up to 25%?

(2) Does the SCCS have any further scientific concerns with regard to the use of Indigofera tinctoria (C170) in cosmetic products?

3. OPINION

After requests for new information from the SCCS, an amended Submission III was transmitted in April 2018 followed by a Submission IV in May 2019. These two submissions are referred to as Submission III in this Opinion.

3.1 CHEMICAL AND PHYSICAL SPECIFICATIONS

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Common name: *Indigofera tinctoria*

INCI:
Indigofera tinctoria leaf
Indigofera tinctoria leaf powder
Indigofera tinctoria leaf extract
Indigofera tinctoria extract

COLIPA code: C170

3.1.1.2 Chemical names

Indigofera tinctoria, dried and pulverised leaves of *Indigofera tinctoria L*.

3.1.1.3 Trade names and abbreviations

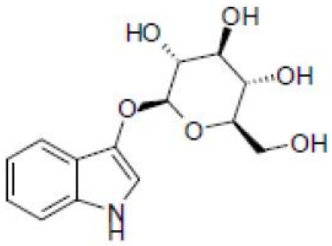
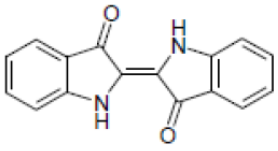
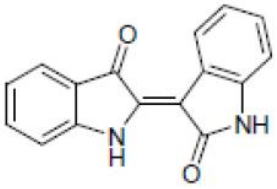
HQ Indigo

3.1.1.4 CAS / EC number

CAS: 84775-63-3
EC: 283-892-6

3.1.1.5 Structural formula

Complex chemical mixture.
Major components:

 indican (1)	 indigo (2)	 indirubin (3)
IUPAC name: (2R,3S,4S,5R)-2-(hydroxymethyl)-6-(1H-indol-3-yloxy)oxane-3,4,5-triol Molecular formula: $C_{14}H_{17}NO_6$	IUPAC name: (2E)-2-(3-oxo-1H-indol-2-ylidene)-1H-indol-3-one Molecular formula: $C_{16}H_{10}N_2O_2$	IUPAC name: (3E)-3-(3-oxo-1H-indol-2-ylidene)-1H-indol-2-one Molecular formula: $C_{16}H_{10}N_2O_2$

Ref: 1-3

3.1.1.6 Empirical formula

/

3.1.2 Physical form

Finely divided greenish dispersible powder.

Ref: 1

3.1.3 Molecular weight

M.W.:
Indican: 295.29 g/mol
Indigo; Indigotin: 262.26 g/mol
Indirubin: 262.26 g/mol

Ref: 1

3.1.4 Purity, composition and substance codes

The analytical study of *Indigofera tinctoria* (E213339 and E502657) was performed on four batches:
- E213339 batch SM/KS/24/8kGy/2
- E502657 batch INP 161038
- E502657 batch KAN/IN/25 KGY/1216/01
- E502657 batch KAN/IN/25 KGY/1216/03

Upon clarification sought by the SCCS, the Applicant stated that a complete analytical description of the test material E213339 is not available due to shortage of one batch (SM/KS/24/8kGy/2). However, the two batch codes of *Indigofera tinctoria* E502657 and E213339 correspond to the same raw material i.e. *Indigofera tinctoria* leaf powder originating from the same geographical area in south of India, cultivated and harvested in Tindivanam, in the Tamil Nadu area. The batches were prepared using the same process, as evidenced by similar contents of the tracer molecule Indican (3.40% versus 3.68-3.75%). The only difference between the two codes is the level of irradiation to avoid bacteria contamination where the codes E213339 and E502657 were irradiated at 8 and 25 kGy, respectively. This has no impact on the powder composition. For the sake of clarification, Indirubin is highly expected to be present at the same level in the batches from both codes

E213339 and E502657. This is corroborated by the measurements done on many batches of *Indigofera tinctoria* where the level of Indirubin was always found in the same range as in the described batches.
Purity of the three major components (Indican, Indigo and Indirubin) was tested by a UPLC/DAD method using external standard calibration against commercial reference standards presented in the Tables below. Detection wavelengths were set at 190-700 nm (extract profile) and 288 nm (quantification).

Reference standards					
Molecule	**CAS**	**Supplier**	**Product-batch**	**Aspect & color**	**Purity**
Indican (Indoxyl *β* -D-glucoside)	487-60-5 1328-73-0	Sigma	MKBW4665V	white powder	98 %
Indigo	482-89-3	edQm	Indigo CRS batch 1	blue purple powder	98.2%
Indirubin	479-41-4 906748-38-7	Sigma	116M4603V	Dark purple powder	98.4%

Content of the three major components (Indican, Indigo and Indirubin)				
	Indigofera tinctoria (E213339) Batch:	*Indigofera tinctoria* (E502657) Batches:		
	SM/KS/24/8kGy/2 *(g/100g)*	INP 161038 *(g/100g)*	KAN/IN/25 KGY/ 1216/01 *(g/100g)*	KAN/IN/25 KGY/ 1216/03 *(g/100g)*
Indican	3.4	3.69	3.68	3.75
Indigo	0.02	0.07	0.08	0.06
Indirubin		0.02	0.02	0.04

Analytical data on additional six different batches of the code E502657 are summarised in the Table below, which shows that irradiation at 25 kGy does not result in significant changes in Indican, Indirubin and Indigo contents when compared to the same non-irradiated batches.

Analytical data on individual batches												
Batch number	KAN/INCONTRO/1216/01		KAN/INCONTRO/1216/02		KAN/INCONTRO/1216/03		KAN/INCONTRO/1216/04		KAN/INCONTRO/1216/05		KAN/INCONTRO/1216/06	
	Non-irradiated	Irradiated (25 kGy)	Non-irradiated	Irradiated (25 kGy)	Non-irradiated	Irradiated (25 kGy)	Non-irradiated	Irradiated (25 kGy)	Non-irradiated	Irradiated (25 kGy)	Non-irradiated	Irradiated (25 kGy)
Indican (%)	3.9	3.7	4.0	3.7	4.1	3.8	4.1	3.9	4.1	3.9	4.1	3.9
Indigo (%)	0.065	0.080	0.067	0.057	0.073	0.057	0.068	0.057	0.068	0.057	0.070	0.057
Indirubin (%)	0.014	0.024	0.013	0.023	0.016	0.035	0.014	0.022	0.014	0.027	0.015	0.035
Enzyme activity*10 (UA/g)	Not available	11.29	Not available	6.99	Not available	6.92	Not available	10.03	Not available	7.62	Not available	8.28

Ref: 4-5

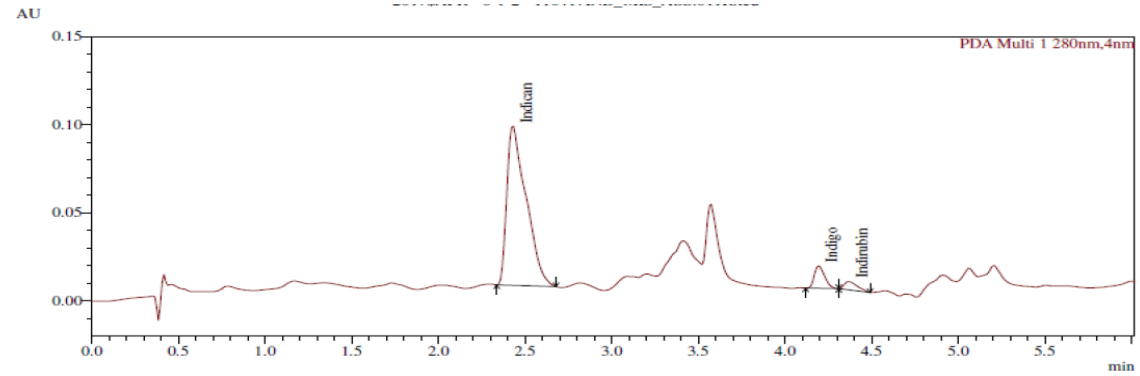
SCCS comment on Submission III
The additional data on the analysis of six different batches of the code E502657 demonstrate that there is no significant batch-to-batch variation in the content of Indican, Indigo and Indirubin.

In the irradiated batches of the code E502657, the content of the Indican is reduced and the content of Indirubin is increased in relation to the non-irradiated ones, while Indigo is increased only in KAN/IN/25 KGY/1216/01, and decreased in the rest of the batches.
The composition analysis refers only to 4% of the components for the test material E213339 that was used in 67% of the studies conducted for submission III.

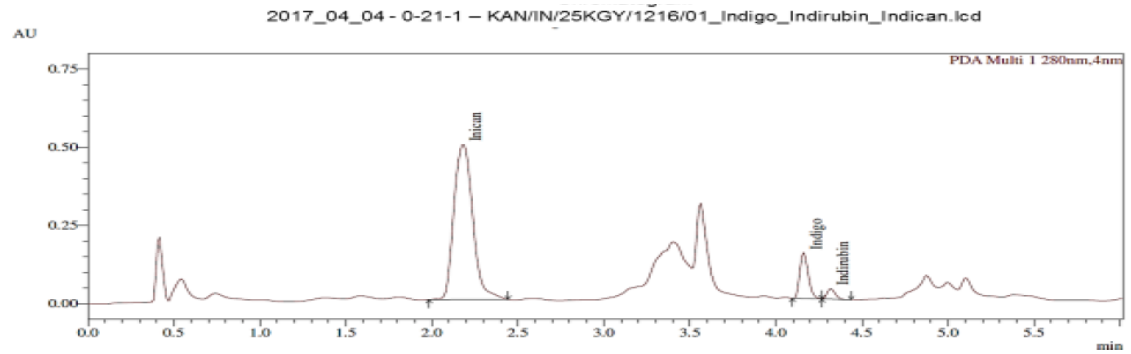
3.1.5 Impurities / accompanying contaminants

Submission III

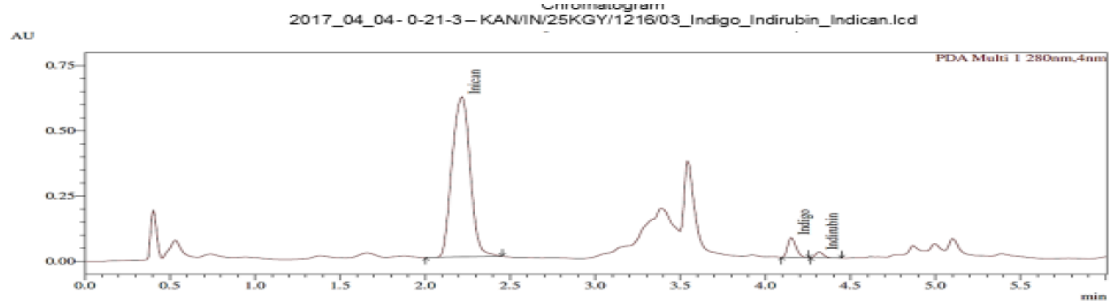
UPLC-PDA analysis
Approximately 100 mg of the leaf powder (accurately weighed) were transferred in a 10 mL volumetric flask (sample content depending). Samples were dissolved in DMSO and make up to volume with the same solvent. Samples were magnetically stirred for 30 min at 700 rpm. The final concentration of the solution is 1 g/100 mL (10 mg/mL). All samples were filtered through a 0.2 µm membrane filter (Acrodisc GHP -Pall) prior to the UPLC-PDA analysis. The separation was achieved on an Acquity BEH Phenyl analytical column (particle size 1.7 µm). Wavelengths were set to 190-700 nm (extract profile) and 288 nm (quantification).



Indigofera Tinctoria (E502657) batch INP 161038 – Characteristic chromatogram



Indigofera Tinctoria (E502657) batch KAN/IN/25 KGY/1216/01
Characteristic chromatogram



Indigofera Tinctoria (E502657) batch KAN/IN/25 KGY/1216/03
Characteristic chromatogram

Chemical characterisation by IR and ^{1}H-NMR

Additionally, batches KAN/IN/25KGY/1216/01 and KAN/IN/25KGY/1216/03 were characterised analytically by IR and ^{1}H-NMR. The samples were prepared and analysed in Dimethylsulfoxide-d6 (DMSO-d6). The samples were not totally dissolved in DMSO-d6. The spectra were recorded on the soluble part of sample obtained after filtration. The chemical characterization is described in the Table below.

According to the Applicant, the main results of the four batches were similar and are presented in the Table below.

Taking into account that the four batches of *Indigofera tinctoria* leaf powder originate from the same geographical area, that they are produced using the same production process, that their contents of the tracer molecules Indican and Indigo are comparable and that the IR spectra and the ^{1}H NMR spectra of these four batches are identical, the Applicant considers that the composition analysis can be read across these four analysed batches.

In conclusion, the composition of the three batches that underwent extensive analytical characterisation is considered to be fully representative of the composition of batch E213339 SM/KS/24/8kGy/2 and of the material that will be used in hair-colouring products.

	E213339	**E502657**		
	SM/KS/24 /8kGy/2	**INP 161038**	**KAN/IN/25 KGY/1216**	
			01	**03**
^{1}H NMR spectrometry	Similar ^{1}H NMR spectra			
IR Spectrometry	Similar IR spectra			
Mineral matter *(%w/w)*		13.4	11.9	12.3
Ashes *(%w/w)*		4.6	3.1	3.0
Lipids *(%w/w)*		6.8	8.0	6.8
Soluble sugars *(%w/w)*		19.6	21.8	22.5
Polyphenols *(%w/w)*		3.7	3.6	3.1
Loss on drying *(%w/w)*		4.1	5.3	5.5
Crude fiber *(%w/w)*		3.5	8.1	7.8
Indican *(%w/w)*	3.40	3.69	3.68	3.75
Indigo *(%w/w)*	0.02	0.07	0.08	0.06
Indirubin *(%w/w)*		0.02	0.02	0.04
Amino acids (free) *(%w/w)*		1.0	0.9	0.9
Total protein content		20.5	19.1	15.1

(%w/w)				
Fragrance allergens (µg/g)		<10		
Heavy metals		Analysis performed Please refer to analytical file		
Pesticides		Rotenoids < 100 ppm (Not detected) Other pesticides < 50 ppb		

The heavy metal test was performed by ICP-MS. Micro-wave assisted acid digestion using nitric acid and hydrogen peroxide was used for sample preparation.

Indigofera tinctoria* (E502657) batches			
	INP 161038 *(µg/g)*	KAN/IN/25 KGY/1216/01 *(µg/g)*	KAN/IN/25 KGY/1216/03 *(µg/g)*
Cr	3.1	1.1	1.1
Co	0.6	0.3	0.3
Ni	4.2	4.0	3.1
Cu	9.5	9.5	9.4
Zn	35.0	40.4	40.2
As	0.1	<0.1	<0.1
Se	0.1	<0.1	<0.1
Cd	<0.1	<0.1	<0.1
Sb	<1.0	<1.0	<1.0
Ba	109.3	87.0	86.0
Nd	0.9	0.4	0.4
Hg	<0.1	<0.1	<0.1
Pb	79.1	8.4	8.2

* Contents of heavy metals are controlled by quality specifications for the industrial quality.

Protein matter:
1D gel electrophoresis profiles of the three batches of *Indigofera tinctoria* (E502957) are comparable and highlight the presence of several protein bands between 6 and 188 kDa. The two main detected bands correspond to the following molecular weights: 14 and 49 kDa.
Total protein matter and free amino-acids contents using ion exchange chromatography:
Total protein matter:
- E502657 batch INP 161038: 20.5 (% w/w)
- E502657 batch KAN/IN/25 KGY/1216/01 : 19.1 (% w/w)
- E502657 batch KAN/IN/25 KGY/1216/03 : 15.1 (% w/w)

Free amino-acids:
- E502657 batch INP 161038 : 1.0 (% w/w)
- E502657 batch KAN/IN/25 KGY/1216/01 : 0.9 (% w/w)
- E502657 batch KAN/IN/25 KGY/1216/03 : 0.9 (% w/w)

Fragrance level
Fragrance allergens level in the 3 batches: < 10 µg/g.

Pesticides content
The determination of pesticides was performed by LC/MS/MS or GC/MS/MS on 3 batches of the code E502657. Each sample is prepared using a QuEChERS extraction before injection in the analytical system. The absence of pesticides was checked in each *Indigofera tinctoria* sample by an adduct corresponding to the limit of quantification.
In the 3 batches of the code E502657, the 316 tested pesticides content was:
- E502657 batch INP 161038 : < 0.05 µg/g
- E502657 batch KAN/IN/25 KGY/1216/01 : < 0.05 µg/g
- E502657 batch KAN/IN/25 KGY/1216/03 : < 0.05 µg/g

Rotenoides (tephrosin, deguelin, rotenone. dehydrodeguelin)
Rotenone, deguelin, tephrosin and dehydrodegeulin were measured by UPLC with UV detection at 270nm and were not found in any of the 3 batches of the code E502657 (INP 161038, KAN/IN/25/KGY 1216/01 and KAN/IN/25/KGY 1216/03) with a limit of quantification at 100 mg/kg (100 ppm).

Ref: 1, 6

SCCS comment on Submission III
A number of unknown peaks are apparent in the UPLC-PDA chromatograms of leaf powder for E502657; while the runtime of the provided chromatograms is short (it should be at least 5xtR of the latest eluted main compound (~ 25 min)). The analysis should provide chemical characterisation and quantification of all major impurities based on the % area content calculated at λ_{max} of each of the major components.
In all of the analysed batches, the amount of lead (Pb) (listed in Annex II, EC1223/2009) is above the acceptable limit set to 2 mg/kg. Heavy metal impurities must be kept below the acceptable limits. For heavy metal impurities > trace levels, the valence state of the ion and the salt form should be provided.

Ref: 7

3.1.6 Solubility

The raw material is a finely divided greenish dispersible powder made of dried and crushed leaves of *Indigofera tinctoria* containing insoluble matter such as cellulose fibres. For this reason, *Indigofera tinctoria* is not soluble, but dispersible. The test samples are prepared extemporaneously by making a homogeneous suspension of powder in the different vehicles (water, dimethylformamide or DMSO). This homogeneous suspension was used in the safety tests.
Indigo is sparsely soluble in organic solvents. The solvent of choice for its extraction is the DMSO. DMF is also able to solubilise Indigo, but this solvent conducts to the leuco form of Indigo.

Ref: 1

Solubility results of code E213339, based on visual inspection:

Table 1 – Results of the Solubility Test

Vehicle Tested	Test item Concentration	Solubility Check	Comments
AOO	10%	Unstable suspension	Not OK
DMF	10%	Unstable suspension	Not OK
MEK	10%	Unstable suspension	Not OK
PG	10%	Unstable suspension	Not OK
DMSO	10%	Homogeneous suspension	OK
DMSO	25%	Homogeneous suspension	OK
DMSO	50%	Non-homogeneous paste	Not OK

Ref: 8

Quantification of Indican, Indigo and Indirubin under the safety tests conditions
Upon clarification sought by the SCCS, the Applicant analysed three safety tests conditions in one batch (E502657 batch KAN/IN/25KGY/1216/01). Indican, Indigo and Indirubin levels were measured at 5 and 180 minutes (3 h) after sample preparation, the latter time point corresponding to the duration of test systems exposure in mutagenicity and genotoxicity studies. The results are presented in the Table below.

Safety tests conditions-% of Indican, Indigo and Indirubin

E502657 batch KAN/IN/25KGY/1216/01		*Indican (%)*	*Indigo (%)*	*Indirubin (%)*
Systemic toxicity test (water)_condition 1	*5 min*	2.03	0.09	0.02
	180 min	0.07	0.21	0.04
Micronucleus/mammalian mutation tests (DMF then dilution in water)_condition 2	*5 min*	3.69	0.05	0.04
	180 min	1.11	0.46	0.03
Ames test (DMF then dilution in water)_condition 3	*5 min*	3.10	0.04	0.02
	180 min	0.91	0.44	0.02

The levels of Indican, Indigo and Indirubin in the cataplasm at 60 min, the application time on-head, were compared to the levels obtained under the different toxicological tests conditions at 180 min, the exposure duration in the mutagenicity/genotoxicity tests and results are presented in the Table below.

Comparison of the levels of Indican, Indigo and Indirubin in cataplasm (at 60 min) versus the 3 toxicological tests conditions (at 180 min)

	Indican (%)	*Indigo (%)*	*Indirubin (%)*
Cataplasm (60 min)	0.11	0.38	0.04
Systemic toxicity test condition (180 min)_condition 1	0.07	0.21	0.04
Micronucleus test condition (180 min)_condition 2	1.11	0.46	0.03
Ames test condition (180 min)_ condition 3	0.91	0.44	0.02

Overall, Indican was less hydrolysed under conditions 2 and 3 i.e. in the presence of DMF compared to the cataplasm (1.11, 0.91 and 0.11%, respectively), however the levels of Indigo and Indirubin were overall similar. Under condition 1, Indican was hydrolysed similarly as in the cataplasm yielding comparable levels of Indigo and Indirubin. In conclusion, these results suggest that the toxicological test systems were exposed to comparable levels of Indigo and Indirubin present in the cataplasm i.e. under on-head use conditions.

In the absence of full-characterisation of the hydrolysis products of Indican, the comparison of the chromatograms of the cataplasm versus the condition 1 (i.e. systemic toxicity test) for instance show overlapping profiles, corroborating the fact that the compounds present in the cataplasm on-head were also evaluated in the toxicological tests.

In brief, it is the Applicant's view that, taken together, the results presented above support the hydrolysis of Indican in the aqueous media used in the toxicological tests conditions, whether the tested material had been pre-suspended in DMF, in hot or room temperature water, and that both Indigo and Indirubin were generated therein at similar levels to which the consumer would be exposed to under on-head use conditions.

3.1.7 Partition coefficient (Log P_{ow})

3.1.8 Additional physical and chemical specifications

Where relevant:
- organoleptic properties (colour, odour, taste if relevant)
- melting point
- boiling point
- flash point
- vapour pressure
- density
- viscosity
- pKa
- pH: pH determination of code E502657 was performed at 25°C of an aqueous solution at 25% w/w:
- INP 161 038, KAN/IN/25KGY/1216/01 and KAN/IN/25KGY/1216/03: pH = 7.1
- Refractive index
- UV/visible light absorption spectrum

Storage conditions: Ambient temperature (20°C) and shielded from humidity.

Particles size distribution was determined using Static Light Scattering particle sizing (SLS) method.

	Indigofera Tinctoria (E502657) batches		
	INP 161038	KAN/IN/25 KGY/1216/01	KAN/IN/25 KGY/1216/03
Volume results			
D50 (µm)	36.4	45.3	51.4
Distribution width (µm)	0.7 to 424.0	0.8 to 625.0	0.8 to 639
Number results			
D50 (µm)	1.2	1.4	1.4
Distribution width (µm)	0.6 to 45	0.7 to 53.5	0.7 to 57.3

Ref: 9

SCCS comment Submission III
The pH of the code E213339 was not provided for the 25% formulation. According to the applicant, this code is not available anymore and it is not possible to supply further analysis.

3.1.9 Homogeneity and Stability

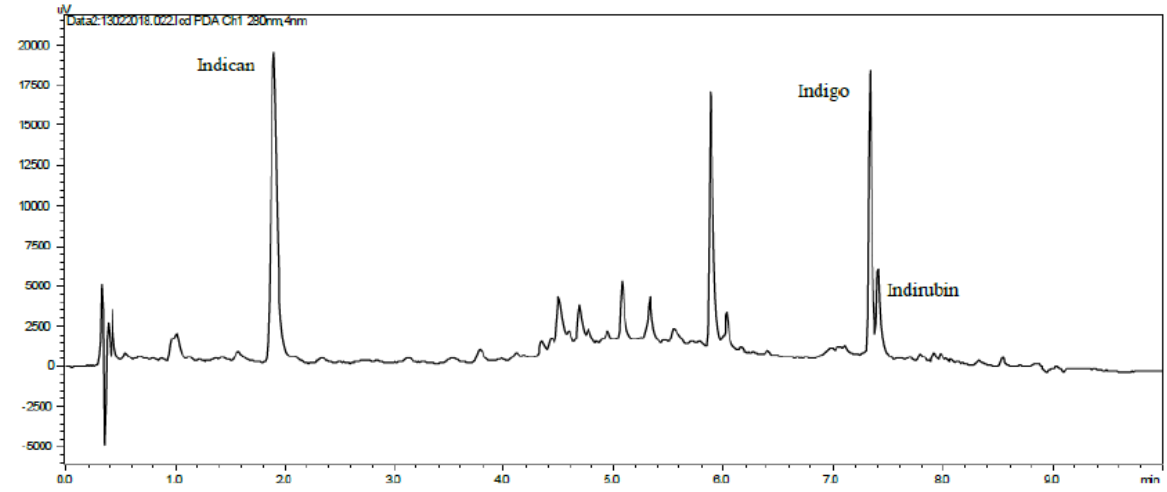
Submission III
Stability of Indican in *Indigofera tinctoria* Cataplasm

Test Substance: *Indigofera tinctoria* (code E502657) and batches KAN/IN/25 KGY/1216/01 and KAN/IN/25 KGY/1216/03

Method

For the kinetics, 5 g of the batches were added to three different mixing bowls with 15 g of water at appropriate temperature (25, 50 and 100 °C). A uniform cataplasm was then obtained by mixing with a flat-ended spatula. At every time point, a weighed amount of the cataplasm was transferred to a 5 mL volumetric flask and dissolved in DMSO and sonicated for 5 min. At T5 minutes, the first sample was collected and dissolved in DMSO. Sample weights were chosen in the range of 12 to 22 mg/mL. All samples were filtered through a 0.2 µm membrane filter (GHP) prior to use.

HPLC-UV: gradient elution – external standard calibration against commercial reference standards, λ = 280 and 288 nm (quantification)



Indigofera Tinctoria (E502657) batch KAN/IN/25 KGY/1216/01

Kinetics Data:

Indigofera Tinctoria (E502657) batches KAN/IN/25Kgf/1216/01 and KAN/IN/25Kgf/1216/03 – Data in %

				Room temperature (25±2°C)			50 °C			100 °C		
				Time in minutes								
	Enzyme (UA/g)	g/100 mL of	Content in dry biomass	T0	T30	T60	T0	T30	T60	T0	T30	T60
				%								
KAN/IN/25Kgf/1216/01	**11.29**	Indican	3.93	0.495	0.097	0.031	0.249	0.053	0.028	0.064	0.025	0.020
		Indigo	0.07	0.047	0.106	0.193	0.050	0.114	0.216	0.057	0.112	0.168
		Indirubin	0.05	0.019	0.024	0.032	0.020	0.025	0.031	0.021	0.027	0.033
KAN/IN/25Kgf/1216/03	**6.92**	Indican	3.78	0.60	0.07	0.03	0.38	0.05	0.03	0.12	0.03	0.02
		Indigo	0.07	0.05	0.11	0.18	0.05	0.10	0.18	0.05	0.10	0.16
		Indirubin	0.04	0.02	0.02	0.03	0.02	0.02	0.03	0.02	0.02	0.03

Conclusions

Two batches of *Indigofera tinctoria* (E502657 batch KAN/IN/25KGY/1216/01 and E502657 batch KAN/IN/25KGY/1216/03) containing Indican at 3.93% and 3.78%, respectively, were analysed. Stability of Indican in an *Indigofera tinctoria* cataplasm was tested.

- Rate of conversion of Indican is very fast in aqueous medium ~ 84 to 87 % at T5 minutes.
- Significant conversion of Indican and formation of Indigo and Indirubin is achieved at T30 minutes itself.
- Significant conversion of Indican is seen at room temperature. Although it is observed that higher temperatures result in faster rates of change.
-The results suggest that Indican is immediately hydrolysed after the addition of water, whatever its temperature (RT, 50° or 100°C), though at a slower pace at RT. No significant differences were noted after 1 hour as the levels of the 3 markers (Indican, Indigo and Indirubin) were similar between the three conditions evaluated (i.e. at RT, 50°C and

100°C). Approximately 0.02-0.03% of Indican was left, with a slight increase in Indigo reaching approximately 0.2% at 1h, whatever the initial water temperature, the powder final temperature reaching RT shortly after mixing. Indirubin level remains practically the same as in the dry powder.
- These results are in agreement with the literature data. The low levels of Indigo and Indirubin after one hour are not surprising, as they are related to the complexity of the reactions in the leaf powder as well as the short kinetic of 1h (maximum application time under use conditions). According to the existing knowledge, one hour is not sufficient to allow the complete transformation of Indican into the coloured components Indigo and Indirubin. The Indigo plants used to dye the textiles are generally covered with water and left to macerate for at least 10, or more, usually 16-22 hours, until the hydrolysis of the Indican into Indoxyl is as complete as possible. Then Indoxyl will be oxidized to Indigo (Cardon, 2007). Thus, it is highly likely that the Indigo and Indirubin levels will increase over time if the analysis was performed 24 h later.

Ref: 1, 10

SCCS comments Submission III
It is well known that both Indigo and Indirubin can be formed after hydrolysis of Indican (Indoxyl glucoside) to Indoxyl and further oxidation. According to the Applicant, a significant conversion of Indican and formation of Indigo is observed after 30 min in all the tested conditions (25, 50 and 100°C). A number of unknown peaks are apparent in the provided HPLC-UV chromatograms (λ=280 nm) for *Indigofera tinctoria* code E502657 batch KAN/IN/25 KGY/1216/01, while Indigo and Indirubin are not well resolved so as to be accurately quantitated. However, the chromatograms of the cataplasm versus the conditions used for systemic toxicity studies show overlapping profiles, indicating that the compounds present in the cataplasm on-head were also evaluated in the toxicological tests.

3.2 FUNCTION AND USES

The ingredient *Indigofera tinctoria* is used in non-oxidative hair colouring products at a maximum on-head concentration of 25% (i.e. 100 g of *Indigofera tinctoria* mixed with 300 mL of hot water between 50°C and 100°C).

Ref: 1

3.3 TOXICOLOGICAL EVALUATION

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

Submissions I and II
No data submitted.

Submission III
No acute oral toxicity study was performed with *Indigofera tinctoria*. However, *Indigofera tinctoria* can be considered to be non-toxic following a single administration by the oral route on the basis of the data from a 13-week toxicity study in rats. In this study, no deaths were observed at dose levels of up to the limit dose level of 1000 mg/kg/day.

Ref: 11

3.3.1.2 Acute dermal toxicity

Submissions I and II
No data submitted.

Submission III
No data submitted.

3.3.1.3 Acute inhalation toxicity

Submissions I, II and III
No data submitted.

3.3.2 Irritation and corrosivity

3.3.2.1 Skin irritation

SCCS comments Submission I
Indigo powder is considered as non-irritating to the skin in this test (OECD 404 (1992)).

SCCS comments Submission II
DA 060492, applied 'as is' under the conditions of this test, caused mild transient irritation to rabbit skin.

Submission III

Guideline:	OECD 439 (July 2015), ECVAM Standard Operating Procedure version 1.2: Episkin™ Skin irritation test 42 hours Determination of IL-1α concentration in the culture medium (September 2005)
Test system:	Human reconstructed epidermis (Episkin™ tissues; 0.38 cm²)
Replicates:	Triplicate tissues for each item
Test substance:	E502657
Batch:	INP 161038
Purity:	/
Vehicle:	water
Concentration:	25% (liquid) and 100 % (solid)
Route:	dermal
Exposure:	Single application, 10 µl (25% concentration) and 11 mg ±1 mg (100% concentration)
Exposure duration:	15 minutes
Post-treatment incubation time:	42 hours
Negative control:	Dulbecco's Phosphate-Buffered Saline (D-PBS)
Positive control:	Sodium Dodecyl Sulphate (SDS) at a 5% (w/v) aqueous solution

Direct interaction with MTT: Positive
Colouring potential test item: Positive

GLP:	Yes
Study period:	6. March 2017 – 2. May 2017

A solubility test was performed prior to the preliminary tests in order to evaluate the solubility of the test item in water at the preselected concentration (i.e. 25% (w/v)). The solubility of the test item in water was evaluated by visual inspection.

Both undiluted and diluted *Indigofera tinctoria* at 25% were evaluated on 3 different batches of reconstructed human epidermis model. *Indigofera tinctoria*, the negative control (D-PBS), and the positive control (5% aqueous solution of Sodium Dodecyl Sulfate) were tested in triplicate. One additional negative control (which followed the same treatment as the negative control, except the MTT incubation period) was added.

As the neat test item and the test item dilution were found to have both direct MTT reduction and colouring potential in the preliminary test, a third set of controls for non-specific colour in killed tissues was required. In this additional control, the test item (at both concentrations) was applied to two water-killed tissues and compared to two untreated water-killed tissues, which undergo the entire testing procedure but were incubated with medium instead of MTT solution during the MTT incubation step.

10 mg +/- 2 mg of neat *Indigofera tinctoria* and 10 µL of the 25% (w/v) dilution in water and different controls were applied onto the epidermis using a positive displacement pipette. After a 15-minute treatment period (13 minutes for the neat test item) at room temperature, tissues were rinsed with D-PBS, and then epidermis were transferred in 2 ml/well of fresh maintenance medium and incubated for 42 hours +/- 1 hour at 37°C.
The fact that the test item exhibits tissue colouring potential and an ability to directlyinteract with MTT was reflected in the performed calculations. The percentage of OD due to non-specific MTT reduction (NSMTT) as well as the non-specific OD due to the residual chemical colour (NSC_{living} and NSC_{killed}) were calculated. These allowed to calculate the true relative mean viability of the tissues treated with *Indigofera tinctoria*.
In addition, the concentration of the inflammatory mediator IL-1α was evaluated in the culture medium retained following the 42-hour recovery period. This quantification, based on an ELISA assay, was performed because the mean relative viability of the test item-treated tissues was > 50% following the MTT reduction assay.

The study authors consider the study valid. The true mean viability value for *Indigofera tinctoria* at the concentrations of 100% and 25% w/v in water was 77% and 70%, with a mean IL-1α concentration of 9.8 and 9 pg/mL, respectively.

The study authors conclude that, under the conditions of this study, *Indigofera tinctoria* at 100% and at 25% w/v in water is potentially non-irritating.

Ref: 12

SCCS overall conclusion on skin irritation
Based on all available data from Submissions I-III, the SCCS considers *Indigofera tinctoria,* in the form of a dispersion or powder, to be non-irritating to the skin.

3.3.2.2 Mucous membrane irritation / eye irritation

SCCS comments Submission I
The tested material induced significant damage to the rabbit eye and is considered to be irritating to the eye.

SCCS comments Submission II
DA 060492 is considered to be irritating to the eye.

Submission III

Study number 1

Guideline/method:	OECD 437 (July 2013)
GLP:	Yes
Test system:	Corneas obtained from freshly slaughtered calves
Replicates:	Triplicate tissues
Test substance:	E502657 (100% *Indigofera tinctoria* Leaf powder)
Test item:	20% (w/w) in 0.9% (w/v) NaCl in water
Batch:	INP 161038
Vehicle:	0.9% (w/v) NaCl in water

Dose applied:	750 ± 8 µL
Negative control:	0.9% (w/v) NaCl in water
Positive control:	20% (w/w) imidazole solution in 0.9% (w/v) NaCl in water
Exposure:	Single application
Exposure duration:	4 hours ± 10 min
Study period:	11. June 2015 – 22. March 2017

Bovine eyes (from cattle less than 12 months old) were collected at slaughterhouses and prepared within 4 hours of collection. Eyes that were too big or were presenting defects were rejected. After the pre-incubation and equilibration period of the corneas at 32 ± 1°C for 1h ± 10 minutes, 750 ± 8 µL of the test item *Indigofera tinctoria* (category: solid non surfactant) diluted at 20% (w/w) in NaCl 0.9% and controls were applied onto the corneas. The treatment period of 4 hours ± 10 minutes was followed by a 3 x rinsing step and visual examination of rinsing efficiency. Then, the corneal opacity and permeability were measured. Corneas were incubated with 0.5% fluorescein solution for 90 ± 5 minutes at 32 ± 1°C; corneal permeability was performed by measuring optical density at 490 nm.

The IVIS score obtained for *Indigofera tinctoria* diluted at 20% (w/w) in NaCl 0.9% after 4-hour contact was 31.2±9.9. These scores, specifically the opacity, were probably overestimated as the corneas were coloured in a very light green by the test item.

The study authors conclude that, under the conditions of this study, no conclusion can be drawn regarding the acute ocular irritation potential of *Indigofera tinctoria* diluted at 20% (w/w).

Ref: 13

Study number 2

Guideline/method:	No
GLP:	Yes
Test system:	Corneas obtained from freshly slaughtered calves
Replicates:	Triplicate tissues
Test substance:	E502657
Test item:	25% (w/w) in distilled water
Batch:	INP 161038
Vehicle:	Distilled water
Dose applied:	750 ± 8 µL
Negative control:	Nutritive medium
Positive control:	0.5% (w/w) cetyl trimethylammonium bromide (CTAB) in water
Exposure duration:	10 and 30 minutes
Study period:	23 December 2014 – 2. March 2017

The test item *Indigofera tinctoria* at the concentration of 25% (w/w) in water was applied onto the cornea. The test item remained in contact with the isolated cornea for 10 ± 1 minutes and 30 ± 5 minutes. At the end of the contact period, the corneas were rinsed and prepared for measurement of opacification (changes in light transmission) and 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. Negative (nutritive medium) and positive control substances (hexadecyl trimethylammonium bromide at 0.5% in water) were tested according to the same experimental conditions.
The following validity criteria were applied in the study:

- initial opacity of the corneas: between 0 and 10
- O.D. of the negative control (nutritive medium) corneas: lower than 0.100
- O.D. of the fluorescein solution to 5 µg/mL: between 0.850 and 0.940
- score CTAB to 0.5%:
  - 30 minutes: between 85.0 and 105.0

- 10 minutes: between 25.0 and 40.0.

The study fulfilled the validity criteria stated in the study report.
The score obtained for *Indigofera tinctoria* at the concentration of 25% (w/w) in water after 10- and 30-minutes contact was 9.5±0.6 and 10.7±0.5, respectively; indicating classification as moderately irritant. However, the study authors state that the score and classification are given as a rough guide since the corneas were coloured lightly green and the opacity probably has been overestimated.

The study authors conclude that, under the conditions of this study, no conclusion can be given for the test item diluted at 25% (w/w) in distilled water.

Ref: 14

SCCS comments Submission III
The applicants provided two new BCOP studies, one following the OECD guideline and one according to an in-house protocol. The exposure time of 10 to 30 minutes is too short compared to 4 hours recommended in the OECD guideline. In both studies, colouring of the cornea occurred, indicating potential interference with the opacity and permeability measurement endpoints, which may lead to false estimates of the eye irritation potential. SCCS agrees with the study authors that both BCOP tests are inconclusive.

SCCS notes that in ECHA C&L inventory *Indigofera tinctoria*, ext. (extractives and their physically modified derivatives such as tinctures, concretes, absolutes, essential oils, oleoresins, terpenes, terpene-free fractions, distillates, residues, etc., obtained from *Indigofera tinctoria*, Leguminosae) with CAS 84775-63-3, in liquid form is self-classified by notifiers as eye irritant 2, H319: Causes serious eye irritation.

Ref: 15

SCCS overall conclusion on eye irritation
Based on animal eye irritation studies from the previous submissions, the SCCS considers *Indigofera tinctoria* to be irritating to the eye. It should however be noted that since *Indigofera tinctoria* is a dispersion, mechanical damage to the eye may have been caused by the leaf powder.

3.3.3 Skin sensitisation

SCCS comments Submission I
Indigo powder is classified as a sensitizer following skin contact in a maximisation test.
Indigofera tinctoria powder can be considered as non-sensitising following skin contact in a Buehler Test.

SCCS comments Submission II
Two Magnusson & Kligman studies show that DA 060492 is a contact allergen in this model and may be considered a strong contact allergen.

Submission III

Local Lymph Node Assay (LLNA)

Guideline/method:	OECD 429 (July 2010)
GLP:	Yes
Test system:	Local Lymph Node Assay (LLNA)
Species/strain:	Female mice, CBA/JRj, 8 weeks old

Group size:	2 (preliminary test) 4 per dose group (main test)
Test substance:	E213339
Batch:	SM/KS/24/8kGy/2
Vehicles:	Dimethyl sulfoxide (DMSO), 100% (w/v) Acetone/olive oil (AOO), 4:1 (v/v)
Concentration:	Preliminary test: 10% (w/v) in DMSO Main test: 0.5%, 1%, 2.5%, 5% and 10% (w/v) in DMSO
Route:	Application to the dorsal surface of both ears on days 1, 2 and 3
Positive control:	25% alpha-hexylcinnamaldehyde (HCA) in AOO
Negative control no. 1:	DMSO, 100% (W/v)
Negative control no. 2:	AOO, 4:1 (v/v)
Study period:	26. November 2012 – 26. December 2013 (in life phase ended February 2013)

Test material E213339 batch SM/KS/24/8kGy/2 (100% pure) was used in this study. Test concentrations used in the main study were selected based on a pre-screen test, in which 10% w/v of E213339 was considered as the maximum suitable test concentration. Four female CBA/J mice per group were used in the main study.

Animals were treated with test solutions (25 µL per ear) at the concentrations of 0.5%, 1%, 2.5%, 5% and 10% w/v in DMSO for three consecutive days by topical application on the dorsum of both ears. Negative control group received the vehicle alone (DMSO). Since a positive control groups was treated with HCA in AOO, a concurrent vehicle control group was treated with AOO alone. After 2 days of resting, all animals received a single intravenous injection of tritiated methyl thymidine (^{3}H-TdR). After five hours, the draining auricular lymph nodes were excised and pooled per group to assess the proliferation of lymph node cells by measuring the incorporation of ^{3}H-TdR. The values obtained were used to calculate stimulation indices (SI). The irritant potential of the test item was assessed by measuring ear thickness on days 0, 2 and 5.

No mortality or significant body weight losses (related to controls) or clinical signs of toxicity were observed in any of the treated groups. No erythema was induced by the test item. Slight ear thickness increase (+12.7%) was induced by the test item at a 10% concentration. As these ear thickness increases did not reach +25% on day 6 compared to day 1, they were not considered as excessive local irritation. Cell viability indexes were considered satisfactory in all groups. Calculated SI values were 0.9, 1.1, 1.5, 1.9 and 2.3 for the test concentrations of 0.5%, 1%, 2.5%, 5%, and 10%, respectively. The SI value for positive control was 11.4.

Under the conditions of this study, *Indigofera tinctoria* did not induce delayed contact hypersensitivity and was therefore considered to be devoid of sensitising potential. Based on these results, no EC3 value was calculated.

Ref: 8

SCCS comments Submission III

Under the conditions of this study, *Indigofera tinctoria* was negative in the LLNA. SCCS notes that there is a dose-dependent increase in lymphocyte proliferation and that at the highest concentration tested (10%) a 2.3 fold increase was induced. This increase may be indicative for a sensitising potential at higher concentrations. It was, however, not possible to test *Indigofera tinctoria* at higher doses, because at 25% excessive local irritation in the prescreen test was noted.

Previous results of two independent Guinea pig maximisation tests showed that *Indigofera tinctoria* clearly induced contact allergy (Submission II, SCCS/1439/11). The Buehler test was negative, but erythema may have been masked by the green skin staining caused by the test chemical. Hence taken all data together, a weak skin sensitising potential cannot be excluded.

3.3.4 Toxicokinetics

3.3.4.1 Dermal / percutaneous absorption

Submission I
No data submitted.

SCCS comments Submission II
These were well performed experiments. Therefore, the mean+1SD may be used in calculating the MOS (1.34 µg/cm² or 0.85% of the applied dose of Indoxy ß-D-glucoside).

Submission III
No data submitted

3.3.4.2 Other studies on toxicokinetics

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3.3.5 Repeated dose toxicity

3.3.5.1 Repeated dose (28 days) oral / dermal / inhalation toxicity

Submissions I, II, III
No data submitted.

3.3.5.2 Sub-chronic (90 days) oral / dermal / inhalation toxicity

Submissions I and II
No data submitted.

Submission III

Guideline:	OECD 408
Species/strain:	Rat (*Rattus norvegicus*)/Wistar Hannover
Group size:	10 /sex/group
Test substance:	E213339 (natural complex substances)
Batch:	SM/KS/24/8kGy/2
Purity:	100% *Indigofera tinctoria* plant powder
Vehicle:	Deionized water
Dose levels:	0, 30, 100, 300 or 1000 mg/kg bw/day
Dose volume:	10 mL/kg bw
Route:	Oral
Administration:	Gavage
Duration:	90 days
GLP:	In compliance
Study period:	14. November 2012 – 22. April 2014 (in life phase ended February 2013)

The test material E213339 (natural complex substances) batch SM/KS/24/8kGy/2 (100% *Indigofera tinctoria* plant powder) was used in this study. Fifty (50) Wistar rats per sex were used in this study. The subchronic toxicity of *Indigofera tinctoria* was investigated in Wistar rats (10/sex/group) after daily oral gavage at 0, 30, 100, 300 or 1000 mg/kg/day in water (10 mL/kg) for 13 weeks. Evaluations and measurements included mortality checks, daily clinical observations, weekly body weight and food intake, ophthalmoscopy prior to dosing and at the end of the treatment period, neurotoxicological evaluation during week 12,

haematology, blood clinical chemistry and urinalysis (week 13). At the end of treatment period, surviving animals were killed and subjected to macroscopic examination; selected organs were weighed and a wide range of organs/tissues were preserved. Microscopic examination was performed for specified tissues/organs from control and high-dose rats killed at the end of the dosing period, as well as for any gross anomaly.

The test item did not induce any relevant treatment-related changes with respect to survival, clinical signs, ophthalmological examinations as well as haematology, coagulation, and clinical chemistry parameters evaluated. No test item-related changes were detected regarding absolute and relative organ weights, gross and microscopic examination. No toxicologically significant test item-related neurological abnormalities were observed. There were no test item-related changes in body weights, body weight gain and food consumption at any of the dose levels.

The only findings consisted of some changes in urinary parameters observed in females at 300 and 1000 mg/kg/day that were collectively indicative of urinary concentration. This higher urine concentration is also probably responsible for the associated higher incidence of detection of proteins in females at the same dose levels. These effects, which relationship to treatment remains doubtful in the absence of similar changes in males, were not considered to be adverse in the absence of any associated biological, histopathological or kidney weight changes.
A higher incidence of presence of urinary ketone bodies was observed in the high dose in males and at 300 and 1000 mg/kg in females. The ketone values in the urine were of minimal degree. Ketonuria is not generally an indicator of impaired renal function but is rather related to a deficiency in carbohydrate metabolism, whereas there were no associated biochemical or pathological changes in this study. False-positive reactions with the detection of urinary ketone bodies is not uncommon in rat toxicity studies, specifically with indole chemicals oxidised to quinones known to produce such false positive reactions. Since the test item contains the indole compound "Indican", it is highly likely that the positive reactions observed are related to the urinary elimination of the test item *Indigofera tinctoria*. Based on all the above, these minor isolated findings were considered of no toxicological relevance.
Thus, under the conditions of the study, the NOAEL (No Observed Adverse Effect Level) of this 90-day oral toxicity study on *Indigofera tinctoria* was 1000 mg/kg/day in Wistar rats.

Ref: 11

3.3.5.3 Chronic (> 12 months) toxicity

Submissions I, II and III
No data submitted.

3.3.6 Reproductive toxicity

Submissions I, II and III
No data submitted.

3.3.6.1 Fertility and reproduction toxicity

Submissions I, II and III
No data submitted.

3.3.6.2 Developmental Toxicity

Submissions I and II
No data submitted.

Submission III

Guideline:	OECD 414
Species/strain:	Rat (*Rattus norvegicus*)/Wistar Hannover
Group size:	25 pregnant female rats/group (a total of 125 rats)
Test substance:	E213339 (natural complex substances)
Batch:	SM/KS/24/8kGy/2
Purity:	100% *Indigofera tinctoria* plant powder
Vehicle:	Deionized water
Dose levels:	0, 30, 100, 300 and 1000 mg/kg bw/day on GDs 6-19
Dose volume:	10 mL/kg bw
Route:	Oral gavage
Exposure period:	From gestation day 6 to gestation day 19
GLP:	In compliance
Study period:	4. January 2013 – 21. November 2014 (in life phase ended February 2013)

The test material E213339 (natural complex substances) batch SM/KS/24/8kGy/2 (100% *Indigofera tinctoria* plant powder) was used in this study. One hundred twenty-five (125) mated female Wistar rats were used. The potential effects of *Indigofera tinctoria* on pregnant rats and embryo-foetal development were evaluated through daily oral gavage in which mated Wistar female rats (25/group) were dosed at 0, 30, 100, 300 or 1000 mg/kg/day during the sensitive period of organogenesis from gestation day 6 to day 19 [the day of mating was designated as Gestation Day 0 (GD 0)]. The test item was dissolved in deionized water and given at 10 mL/kg. Maternal evaluations and measurements included daily clinical signs and body weight/food intake measured at designated intervals. The dams were killed on GD 20 and subjected to macroscopic examination. Usual litter parameters were recorded and foetuses were sexed, weighed and submitted to external examination. About one half of the foetuses were also examined for soft tissue anomalies, and remaining foetuses were examined for skeletal anomalies.

The administration of *Indigofera tinctoria* to pregnant female Wistar rats over the organogenesis period produced no mortality or gross necropsy findings. No statistically or biologically significant differences were observed in mean body weight, body weight gain, and corrected body weight gain and food intake when compared to controls. No changes in maternal parameters were noted in any group.

There were no fetal external and visceral findings attributed to the administration of the test item. Slight fetal skeletal malformations were observed at all the doses except the high dose of 1000 mg/kg. Since these changes were of moderate magnitude with no dose-response, they were not considered to be treatment-related. Even though some delayed skeletal ossification and/or incomplete ossification in the high-dose groups showed a statistically significant increase, these changes were isolated, of minimal to slight magnitude, and with incidences that overall remained within the historical control range. Therefore, they were considered as not related to the test item.

On the basis of the results obtained in the present study, the No Observed Adverse Effect Level (NOAEL) for maternal and developmental toxicity of *Indigofera tinctoria* was set at 1000 mg/kg/day. *Indigofera tinctoria* was considered to have no teratogenic potential.

Ref: 16

SCCS comments Submission III
The SCCS has noted the bell-shaped dose-response curve regarding the delay of ossification, which may be considered a transitory morphological alteration rather than a malformation. The SCCS considers that such a phenomenon is unlikely to be associated with the treatment. Nevertheless, the SCCS has considered the lowest dose, at which the effects on the litter were seen (30 mg/kg/day), as LOAEL and this value has been used for calculating the MoS.

3.3.7 Mutagenicity / genotoxicity

3.3.7.1 Mutagenicity / genotoxicity *in vitro*

Overall SCCS comments on mutagenicity/genotoxicity taken from Submission I

The test item, as leaves powder (water extract) derived from the plant Indigo, has been tested on bacterial and mammalian cells *in vitro* for the induction of gene mutations, in mammalian cells *in vitro* for the induction of chromosome aberrations and on mice *in vivo* for the induction of numerical/structural chromosome aberrations.
The test item (ethanol extract) has been found strongly positive for the induction of gene mutations in bacterial cells. All the studies are inadequate because there is no characterisation of the material used as test item. No conclusion can be drawn about the potential mutagenicity/genotoxicity of the test item.

Overall SCCS comments on mutagenicity/genotoxicity taken from Submission II
Overall, the genotoxicity of *Indigofera tinctoria* is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. Under *in vitro* conditions, *Indigofera tinctoria* did not generally induce gene mutations in bacteria nor in mammalian cells. In one gene mutation test in bacteria with limited value due to a bad performance of the test, an induction of the number of revertants was observed. This positive finding was considered not biologically relevant. *Indigofera tinctoria* induced an increase in the number of cells with chromosome aberrations in one chromosome aberration test with limited value whereas in a well-performed test it was not genotoxic.
The positive finding in the single *in vitro* chromosome aberration test was not confirmed in a well-performed *in vivo* experiment. *Indigofera tinctoria* exposure of mice did not result in an increase in erythrocytes with micronuclei.
Consequently, based on the present reports, *Indigofera tinctoria* can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Submission III

Bacterial Reverse Mutation Test

Guideline:	OECD 471
Test system:	Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, TA102
Replicates:	Two experiments, triplicate plates
Test substance:	E213339
Batch:	SM/KS/24/8kGy/2 (purity: composition was stated as 100% of *Indigofera tinctoria* plant powder)
Concentrations:	Experiment 1 – Plate incorporation test:

	±S9-mix: all S. typhimurium strains: 0, 5, 15.81, 50, 158.1, 500, 1581 and 5000 µg/plate Experiment 2 ±S9-mix pre-incubation test: all S. strains: 0, 78.13, 156.3, 312.5, 625, 1250, 2500, 5000 µg/plate Additional plate incorporation test for TA102 S. strain +S9 mix: 0, 78.13, 156.3, 312.5, 625, 1250, 2500, 5000 µg/plate
Vehicles:	Dimethylformamide, DMF (stock suspension at 100 mg/mL); volume additions for the Experiment 2 pre-incubation treatments were reduced to 0.05 mL
Positive Controls:	-S9 mix: 2-nitrofluorene (2NF): 5 µg/plate for TA98; Sodium azide (NaN_3): 2 µg/plate for TA100, TA1535; 9-Aminoacridine (AAC): 50 µg/plate for TA1537; Mitomycin C (MMC): 0.2 µg/plate for TA102 +S9 mix: Benzo[a]pyrene (B[a]P): 10 µg/plate for TA98; 2-Aminoanthracene (AAN): 5 µg/plate for TA100, TA1535 and TA1537, or 20 µg/plate for TA102
Negative controls:	Vehicle control
GLP:	In compliance
Study period:	09 Nov 2012 – 25 April 2013

E213339 was tested for mutagenicity in the reverse mutation assay with and without metabolic activation in S. typhimurium strains TA1535, TA1537, TA98, TA100 and TA102, using both the Ames plate incorporation and pre-incubation methods at up to seven dose levels, in triplicate, in two separate experiments, both with and without the addition of a rat liver homogenate metabolising system (induced with Aroclor 1254, 10% liver S9 in standard co-factors).

Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of S9-mix, using final concentrations of E213339 at 5, 15.81, 50, 158.1, 500, 1581 and 5000 µg/plate, plus negative (vehicle) and positive controls. Following these treatments, no evidence of toxicity was observed. Experiment 2 treatments of all the tester strains were performed in the absence and in the presence of S9-mix. The maximum test concentration of 5000 µg/plate was retained for all strains. Narrowed concentration intervals were employed covering the range 78.13 – 5000 µg/plate. In addition, all treatments in the presence of S9-mix were further modified by the inclusion of a pre-incubation step. An additional plate incorporation treatment of strain TA102 in the presence of S9-mix was included in order to assess the reproducibility of results from Experiment 1. Following these treatments, no evidence of toxicity was observed.
Precipitation, in the form of suspension particles, was observed on the test plates at concentrations of 1581 µg/plate and above in Experiment 1 and from 312.5 µg/plate and above in Experiment 2.
The positive control chemicals all induced increases in revertant numbers of ≥2.0-fold (in strains TA98, TA100 or TA102) or ≥3.0-fold (in strains TA1535 or TA1537) in the concurrent vehicle control, confirming discrimination between different strains, and an active S-9 preparation.

Following Experiment 1 treatments of strain TA100 in the absence of S-9 and strain TA102 in the absence and presence of S9-mix, increases in revertant numbers were observed that were statistically significant when the data were analysed at the 1% level using Dunnett's test. The increases observed were small in magnitude, did not display a clear concentration relationship and were not reproduced in a subsequent experiment. These increases are considered therefore to be chance occurrences and not indicative of mutagenic activity. No other increases in revertant numbers were observed that were statistically significant when the data were analysed at the 1% level using Dunnett's test.

It was concluded by the authors of the study that E213339 did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of Salmonella typhimurium when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S9-mix).

Ref: 17

SCCS comments

SCCS agrees that the results of the study indicate no mutagenic effect of *Indigofera tinctoria* in the absence or presence of S9 mix in all bacterial strains used. The SCCS noted that in Experiment 1, C170 has shown to increase the revertant number in three strains of S. typhimurium (TA100, -S9-mix, TA1537, +S9-mix and TA102, both ±S9-mix) at several concentrations, however, the increases were not greater than 2 fold compared to the control values and the results were not reproducible in Experiment 2.

Mammalian Cell Gene Mutation Test in Mouse Lymphoma Cells (*Hprt* locus)

Guideline:	OECD 476 (the Microtitre^{R} fluctuation technique)
Test system:	L5178Y mouse lymphoma cell line *tk*$^{+/-}$ (3.7.2C), (*hprt*) locus
Replicates:	Two independent experiments, each two parallel cultures
Test substance:	E213339
Batch:	SM/KS/24/8kGy/2 (purity: composition was stated as 100% of *Indigofera tinctoria* plant powder)
Concentrations:	Preliminary test (range-finder): ±S9 mix (3 h exposure) and -S9 mix (3 h exposure): 31.25, 62.5, 125, 250, 500, 1000 µg/mL Main test: Experiment I: +S9-mix (3 h exposure) and -S9-mix (3 h exposure): 10, 20, 40, 60, 80, 90, 100, 110, 125, 250, 500, 1000 µg/mL Experiment II: +S9-mix (3 h exposure) and -S9-mix (3 h exposure): 7.5, 15, 30, 60, 90, 115, 125, 250, 500, 1000 µg/mL
Vehicle:	Dimethylformamide (DMF) (stock suspension at 100 mg/mL)
Negative control:	Culture medium and DMF diluted 100-fold in the treatment medium
Positive controls:	-S9-mix: 4-nitroquinoline 1-oxide (NQO), 0.15 and 0.2 µg/mL +S9-mix: benzo[a]pyrene (B[a]P), 2 and 3 µg/mL
GLP:	Yes
Study period:	08 Nov 2012 – 29 April 2013

The *in vitro* mammalian cell gene mutation assay was conducted to investigate the potential of C170 dissolved in DMF to induce gene mutations at the *hprt* locus of the L5178Y mouse lymphoma cell line. Prior to the main study, a preliminary toxicity test was performed on cell cultures using a 3-hour exposure time both with and without metabolic activation (S9, liver post mitochondrial supernatant of rats treated with Aroclor 1254). The dose range used was 31.25 to 1000 µg/mL (the maximum practicable concentration, limited by the solubility in the primary vehicle). The test article was formulated as a doseable suspension in DMF, therefore all concentrations may be regarded as nominal.

The following main study was performed in two independent experiments, using two parallel cultures each. In the first experiment of the main study, C170 treatments were performed for 3 h in duplicate (A + B) both with and without metabolic activation (S9-mix) at 12 dose

levels of the test item (10 - 1000 µg/mL), vehicle and positive controls. In the second experiment of the main study, the dose range of the test item was 7.5 - 1000 µg/mL in the presence and absence of S9-mix.

In the cytotoxicity **Range-Finder Experiment**, upon addition of the test article to the cultures, precipitate/undissolved test article was observed at the highest three concentrations tested in the absence and presence of S9-mix (250 to 1000 µg/mL). Following the 3 hour treatment incubation period, precipitate/undissolved test article was observed at the highest four concentrations in the absence and presence of S9-mix (125 to 1000 µg/mL). The lowest concentration at which precipitate/undissolved test article was observed at the end of the treatment incubation period in the absence and presence of S9-mix was retained and higher concentrations were discarded. The highest concentration analysed (125 µg/mL) gave minimal toxicity, such that relative survival (RS) values were 112% and 76% in the absence and presence of S9-mix, respectively.

In **Experiment 1,** twelve concentrations, ranging from 10 to 1000 µg/mL, were tested in the absence and presence of S9-mix. No precipitate/undissolved test article was observed at the time of treatment but following the 3 hour treatment incubation period, precipitate/undissolved test article was observed at the highest three concentrations in the absence and presence of S9-mix (250 to 1000 µg/mL). The lowest concentration at which precipitate/undissolved test article was observed at the end of the treatment incubation period in the absence and presence of S9-mix was retained and higher concentrations were discarded. Seven days after treatment, concentrations of 10 and 80 µg/mL in the absence and presence of S9-mix were not selected as there were sufficient non-toxic concentrations. All other concentrations were selected in the absence and presence of S9-mix. The highest concentration analysed was 250 µg/mL, which gave 61% and 68% RS in the absence and presence of S9-mix, respectively.

In **Experiment 2,** ten concentrations, ranging from 7.5 to 1000 µg/mL, were tested in the absence and presence of S9-mix. No precipitate/undissolved test article was observed at the time of treatment but following the 3 hour treatment incubation period, precipitate/undissolved test article was observed at the highest six concentrations in the absence of S9-mix (90 to 1000 µg/mL) and the highest four concentrations in the presence of S9-mix (125 to 1000 µg/mL). The lowest concentrations at which precipitate/undissolved test article was observed at the end of the treatment incubation period in the absence and presence of S9-mix were retained and higher concentrations were discarded. The highest concentrations analysed were 90 and 125 µg/mL, which gave 81% and 62% RS in the absence and presence of S9-mix, respectively.

Negative (vehicle) and positive control treatments were included in each Mutation Experiment in the absence and presence of S9-mix. Mutant frequencies (MF) in vehicle control cultures fell within acceptable ranges and clear increases in mutation were induced by the positive control chemicals 4-nitroquinoline 1-oxide (without S9-mix) and benzo(a)pyrene (with S9-mix). Therefore the study was accepted as valid.

In Experiments 1 and 2, no statistically significant increases in **mutant frequency** were observed following treatment with E213339 at any concentration tested in the absence and presence of S9-mix and there were no significant linear trends. It was, however, noted that a significant difference in MF was observed for the Untreated Control (UTC) compared to the vehicle control MF in Experiment 1 in the presence of S9-mix. However, as the UTC does not form part of the endpoint analysis and the vehicle control MF (5.34) was within three times the historical MF (5.75 at the time of Experiment 1) and therefore meets the acceptance criteria, this observation was considered to have had no adverse effect on the integrity of the experiment.

It is concluded that E213339 did not induce mutation at the hprt locus of L5178Y mouse lymphoma cells when tested up to the limit of solubility of the test article in culture medium

in two independent experiments, in the absence and presence of a rat liver metabolic activation system (S9-mix).

Ref: 18

SCCS comments

The SCCS agrees that the study indicates no mutagenic effect of *Indigofera tinctoria* in the *Hprt* locus in mouse lymphoma L5178Y cells.
The SCCS noted that in the study, a rather low MF was observed for the positive control substance. The ratio C/H was 0.337-0.531 for NQO at 0.2 µg/mL, while for NQO at 0.1 µg/mL MF was generally similar to historical control (ratio C/H: 0.836-0.908).

***In vitro* Micronucleus Test in human lymphocytes**

Guideline:	OECD 487 (2010)
Species/strain:	Cultured human peripheral blood lymphocytes from two male volunteers (pooled blood)
Replicates:	Duplicate cultures, one experiment
Test substance:	E213339
Batch:	SM/KS/24/8kGy/2 (purity: composition was stated as 100% *Indigofera tinctoria* plant powder)
Concentrations:	Preliminary test (range-finder): ±S9 mix (3 h exposure + 21 h) and -S9 mix (24 h exposure): 3.628, 6.047, 10.08, 16.80, 27.99, 46.66, 77.76, 129.6, 216, 360, 600, 1000 µg/mL Exp1: ±S9-mix (3 h exposure + 21 h): 175, 200, 300, 400, 600 µg/mL -S9-mix (24 h exposure): 150, 175, 200, 300, 400, 600 µg/mL
Solvent/negative control:	Culture medium and dimethylformamide (DMF)
Positive Controls:	-S9-mix: Mitomycin C (MMC, 0.6 and 0.8 µg/mL), Vinblastine (VIN, 0.02, 0.03 and 0.04 µg/mL) +S9-mix: Cyclophosphamide (CPA, 6.25 and 12.5 µg/mL)
Vehicle:	DMF (stock suspension at 100 mg/mL)
GLP:	In compliance
Study period:	09 Nov 2012 – 24 April, 2013

In an *in vitro* micronucleus assay, C170 was tested using duplicate human lymphocyte cultures prepared from the pooled blood of two male donors in one experiment for clastogenicity and aneugenicity assessment. The maximum concentrations analysed were determined following a preliminary cytotoxicity experiment. Cytotoxicity was assessed as reduction in the replication index (RI). Suitable maximum concentrations for analysis were selected.
The test article was formulated as a doseable suspension in dimethyl formamide (DMF), therefore all concentrations may be regarded as nominal. The highest concentrations analysed in the Micronucleus Experiment were limited by the appearance of precipitate and/or undissolved test article and were determined following a preliminary cytotoxicity Range-Finder Experiment.
Treatments were conducted 48 hours following mitogen stimulation with Phytohaemagglutinin (PHA). Cells were exposed to the test item in the vehicle DMF for 3 hours (followed by 21 hours recovery) in the absence and the presence of a mammalian metabolic activation system (S9-mix from the liver of Aroclor 1254 induced rats. In addition, cells were exposed for 24 hours (equivalent to approximately 1.5 to 2 times the average generation time of cultured lymphocytes from the panel of donors used in this laboratory) in the absence of S9-mix.
Negative and positive controls were in accordance with the OECD guideline.

All cultures were sampled 24 hours after the beginning of treatment (*i.e.* 72 hours after culture initiation). A total of 1000 binucleate cells from each culture (2000 cells /concentration) was analysed for micronuclei.

Treatment of cells with E213339 in the absence and presence of S-9 resulted in frequencies of MNBN cells that were similar to (and not significantly higher than) those observed in concurrent vehicle controls at all concentrations analysed under all treatment conditions. The MNBN cell frequency of all E213339 treated cultures fell within the normal ranges.

It is concluded that E213339 did not induce micronuclei in cultured human peripheral blood lymphocytes when tested up to precipitating concentrations for 3 hours in the absence and presence of a rat liver metabolic activation system (S9-mix) and for 24 hours in the absence of S9-mix.

Ref: 19

SCCS comments
The SCCS agrees that the study indicates no mutagenic effect of *Indigofera tinctoria* in the micronucleus test in human lymphocytes.
The SCCS noted that the frequency of MNBN cells in vehicle controls fell within the normal ranges with the exception of one culture following 24+0 hour treatment in the absence of S9-mix (1.7 vs observed range of 0.00-1.5, 95% reference range of 0.1-1.1 in historical control).

3.3.7.2 Mutagenicity / genotoxicity *in vivo*

Submission III
No additional data submitted.

Overall SCCS comments on *in vitro* and *in vivo* mutagenicity/genotoxicity based on Submission I, II and III
The genotoxicity of *Indigofera tinctoria* was investigated with valid *in vitro* genotoxicity tests for the three endpoints of genotoxicity: gene mutations (in both bacterial and mammalian test systems), chromosome aberrations and aneuploidy (in the micronucleus test), with negative results. In an *in vivo* experiment, *Indigofera tinctoria* exposure of mice did not result in an increase in erythrocytes with micronuclei.
Based on the provided studies, *Indigofera tinctoria* can be considered to have no *in vivo* genotoxic potential.

3.3.8 Carcinogenicity

Submissions I, II and III
No data submitted.

3.3.9 Photo-induced toxicity

3.3.9.1 Phototoxicity / photo-irritation and photosensitisation

Submissions I, II and III
No data submitted.

3.3.9.2 Photomutagenicity / photoclastogenicity

Submissions I, II and III
No data submitted.

3.3.10 Human data

Submissions I, II and III
No data submitted.

SCCS comments on Submission III
There are no published studies that show the existence of contact allergy induced by Indigo. Indirubin has anti-inflammatory anti-cell proliferative properties. It is currently under investigation as a topical treatment for skin diseases such as psoriasis.

Ref: 20-22

3.3.11 Special investigations

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3.3.12 Safety evaluation (including calculation of the MOS)

CALCULATION OF THE MARGIN OF SAFETY

(on-head concentrations of up to 25%)

A LOAEL of 30 mg/kg bw/day derived from the developmental toxicity study is taken as a Point of Departure (PoD) for the MoS-calculations. Since the POD is based on a LOAEL, an assessment factor of 3 is added in the calculation of the MoS.

Absorption through the skin	A	=	1.34 µg/cm²
Skin Surface Area	SSA	=	580 cm^2
Dermal absorption per treatment	SSA x A x 0.001	=	0.78 mg
Typical body weight of human	bw	=	60 kg
Systemic exposure dose (SED)	SSA x A x 0.001/bw	=	0.013 mg/kg
Lowest observed adverse effect level	LOAEL	=	30 mg/kg bw/d
NOAEL (Adjusted LOAEL)	NOAEL	=	10 mg/kg bw/d
Bioavailability 50%*	$NOAEL_{syst}$	=	5 mg/kg bw/d
Margin of Safety	**$NOAEL_{syst}$/SED**	**=**	**385**

* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.13 Discussion

Physicochemical properties

The composition analysis refers only to 4% of the components for the test material E213339 that was used in the 2/3 of the studies conducted.
Indigofera tinctoria is a complex chemical mixture. All impurities should be chemically characterised and quantified based on the % area content calculated at λmax of the major components.

Heavy metal impurities such as lead must be kept below the acceptable limits. For heavy metal impurities > trace levels, the valence state of the ion and the salt form should be provided.

Function and uses
The ingredient *Indigofera tinctoria* is used in non-oxidative hair colouring products at a maximum on-head concentration of 25% (i.e. 100 g of *Indigofera tinctoria* mixed with 300 mL of hot water between 50°C and 100°C).

Toxicological Evaluation

Acute toxicity
No acute oral toxicity study was performed with *Indigofera tinctoria*. However, on the basis of the data from a 13-week toxicity study in rats, no deaths were observed at any dose level up to the limit dose level of 1000 mg/kg/day.

Irritation and corrosivity
Based on all available data from Submissions I-III, the SCCS considers *Indigofera tinctoria,* in the form as a dispersion or powder, to be non-irritating to the skin. Based on animal eye irritation studies from the previous submissions, the SCCS considers *Indigofera tinctoria* to be irritating to the eye. It should however be noted that since *Indigofera tinctoria* is a dispersion, mechanical damage to the eye may have been caused by the leaf powder. The SCCS notes that in ECHA C&L inventory *Indigofera tinctoria* extract in liquid form is self-classified by notifiers as eye irritant 2, H319: Causes serious eye irritation.

Skin sensitisation
Under the conditions of the provided study, *Indigofera tinctoria* was negative in the LLNA. The SCCS notes the highest dose tested (10%) induced a 2.3 fold increase in lymphocyte proliferation. This increase may be indicative for a sensitising potential at higher concentrations. It was, however, not possible to test *Indigofera tinctoria* at higher doses, because at 25% excessive local irritation in the pre-screen test was noted. Previous results of two independent Guinea pig maximisation tests showed that *Indigofera tinctoria* induced contact allergy (Submission II, SCCS/1439/11). Taking the data together, a weak skin-sensitising potential cannot be excluded. It is also noted that there are no indications from human data that *Indigofera tinctoria* causes allergic contact dermatitis.

Toxicokinetics
The mean+1SD is used in calculating the margin of safety (MOS), *i.e*. 1.34 µg/cm² or 0.85% of the applied dose of Indican.

Repeated dose toxicity
A NOAEL of the 90-day oral toxicity study on *Indigofera tinctoria* was 1000 mg/kg/day in rats.

Reproductive toxicity
The SCCS has noted the bell-shaped dose response curve regarding the delay of ossification, which may be considered a transitory morphological alteration rather than a malformation. The SCCS considers that such a phenomenon is unlikely to be associated with the treatment. Nevertheless, the SCCS has considered the lowest dose at which the effects on litter was seen (30 mg/kg/day) as LOAEL and this value has been used for calculating the MoS

Mutagenicity / genotoxicity
The genotoxicity of *Indigofera tinctoria* was investigated with valid *in vitro* genotoxicity tests for the three endpoints of genotoxicity: gene mutations (in both bacterial and mammalian test systems), chromosome aberrations and aneuploidy (in the micronucleus test), with negative results. In an *in vivo* experiment, *Indigofera tinctoria* exposure of mice did not

result in an increase in erythrocytes with micronuclei. Based on the provided studies, *Indigofera tinctoria* can be considered to have no *in vivo* genotoxic potential.

Carcinogenicity
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Photo-induced toxicity
/

Human data
There are no published studies that show the existence of contact allergy induced by Indigo. Indirubin has anti-inflammatory and anti-proliferative properties.

Special investigation
/

4. CONCLUSION

1. *In light of the data provided, does the SCCS consider Indigofera tinctoria (C170) safe when used in non-oxidative conditions hair colouring products at on-head concentrations of up to 25%?*

   In light of the data provided, the SCCS considers that *Indigofera tinctoria* is safe when used in non-oxidative condition hair colouring products at on-head concentrations of up to 25%.

2. *Does the SCCS have any further scientific concerns with regard to the use of Indigofera tinctoria (C170) in cosmetic products?*

   A weak skin sensitisation potential cannot be excluded for *Indigofera tinctoria*.

5. MINORITY OPINION

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6. REFERENCES

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7. GLOSSARY TERMS

See SCCS/1564/15, 9th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – from page 144

8. LIST OF ABBREVIATIONS

See SCCS/1564/15, 9th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – from page 144.