Scientific Committee on Consumer Safety

SCCS

OPINION ON

hair dye 1,2,4-trihydroxybenzene (1,2,4-THB) - A33 (CAS 533-73-3) Submission VI

The SCCS adopted this document
at its plenary meeting on 21-22 June 2018

ACKNOWLEDGMENTS

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http://ec.europa.eu/health/scientific_committees/experts/declarations/sccs_en.htm

ABSTRACT

The SCCS concludes the following:

1. *In light of the data provided, does the SCCS consider 1,2,4-trihydroxybenzene (1,2,4-THB) (A33) safe for use as an auto-oxidative hair dye in permanent hair dye formulations, not requiring the action of peroxide, with a maximum on-head concentration of 2.5%?*

   On the basis of all the data submitted by the Applicant, and data available in open literature, the SCCS considers that 1,2,4-trihydroxybenzene is not safe due to potential genotoxicity when used as an auto-oxidative hair dye in permanent hair dye formulations.

2. *In light of the data provided, does the SCCS consider 1,2,4-trihydroxybenzene (1,2,4-THB) (A33) safe for use as an auto-oxidative hair dye in hair colour shampoo formulations, not requiring the action of peroxide, with a maximum on-head concentration of 0.7%?*

   /

3. *Does the SCCS have any further scientific concerns with regard to the use of 1,2,4-trihydroxybenzene (1,2,4-THB) (A33) in cosmetic products?*

   No data have been provided to indicate the fate of 1,2,4-trihydroxybenzene in the finally applied mixtures and hence the extent of consumer exposure to 1,2,4-THB and transformation products is not known.

Keywords: SCCS, scientific opinion, 1,2,4-trihydroxybenzene (1,2,4-THB), A33, hair dye, CAS: 533-73-3, EC 208-575-1, SCCS/1598/18, Regulation 1223/2009

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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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MANDATE FROM THE EUROPEAN COMMISSION

Background

Submission I on 1,2,4-trihydroxybenzene (1,2,4-THB) was submitted by COLIPA[1] in August 1981. On 4 November 1991, the SCC deferred the assessment of that substance due to inadequate data.
Submission II was made by COLIPA in September 1994, and Submission III in August 2001.
Submission IV in 2003 provided additional updated scientific data on the above substance which is in line with the second step of the strategy on the evaluation of hair dyes.
Submission V was made to the SCCS in 2011.
In December 2012 the SCCS adopted an Opinion on 1,2,4-trihydroxybenzene (SCCS/1452/11)[2], with the following conclusion:

"The SCCS is of the opinion that the information submitted is inadequate to assess the safe use of the substance."
Before any further consideration, the following information is required:

- Proper characterisation and quantification of 1,2,4-Trihydroxybenzene as well as identification and quantification of impurities in all test batches.
- Characterisation of the oxidation reaction product(s) of 1,2,4-trihydroxybenzene to which the consumer is exposed, because of the reported instability of 1,2,4-trihydroxybenzene in aqueous systems. In the case of relevant exposure to the reaction products, further toxicity data might be required.
- In vivo testing would be required to explore the potential to induce gene mutations; such tests are no longer permitted. 1,2,4-Trihydroxybenzene was found to be an extreme skin sensitiser.'

Submission VI on the hair dye 1,2,4-trihydroxybenzene (1,2,4-THB) (A33) (CAS 533-73-3) was transmitted by Combe in October 2017.
According to the applicant, the current submission constitutes industry's response to the request for further information in last SCCS Opinion (SCCS/1452/11). In particular, the current submission is intended to support the use of 1,2,4-THB as an auto-oxidative dye in permanent hair dye formulations (not requiring the action of peroxide) at a maximum level of 2.5%, and in gradual hair colouring shampoos at a maximum level of 0.7%.

Terms of reference

1. *In light of the data provided, does the SCCS consider 1,2,4-trihydroxybenzene (1,2,4-THB) (A33) safe for use as an auto-oxidative hair dye in permanent hair dye formulations, not requiring the action of peroxide, with a maximum on-head concentration of 2.5%?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association, Cosmetics Europe
[2] https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_113.pdf

2. *In light of the data provided, does the SCCS consider 1,2,4-trihydroxybenzene (1,2,4-THB) (A33) safe for use as an auto-oxidative hair dye in hair colour shampoo formulations, not requiring the action of peroxide, with a maximum on-head concentration of 0.7%?*

3. *Does the SCCS have any further scientific concerns with regard to the use of 1,2,4-trihydroxybenzene (1,2,4-THB) (A33) in cosmetic products?*

OPINION

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

1,2,4-Trihydroxybenzene (INCI name)

3.1.1.2 Chemical names

1,2,4-Trihydroxybenzene
Benzene-1,2,4-triol
Hydroxyhydroquinone

Additional synonyms according to the applicant:
Benzene-1,2,4-triol
2-Hydroxyhydroquinone
1,2,4-Benzenetriol
4-Hydroxycatechol

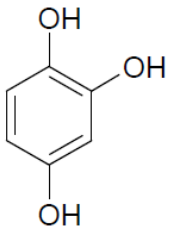
3.1.1.3 Trade names and abbreviations

Trade name: IMEXINE OAM
COLIPA n°: A33

3.1.1.4 CAS / EC number

CAS: 533-73-3
EC: 208-575-1

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

Formula: $C_6H_6O_3$

3.1.2 Physical form

Light-medium beige powder

3.1.3 Molecular weight

Molecular weight: 126.11 g/mol

3.1.4 Purity, composition and substance codes

The standard methodology to determine the purity of 1,2,4-THB is Gas Chromatography with a UV detector. The conditions were refined and validated both internally and transferred to the external analytical laboratory.

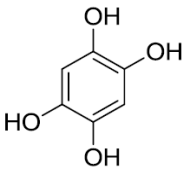
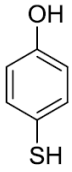
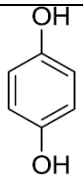
Purity:	97.8%
Impurities:	<0.5%
Loss on Drying:	<0.5%
Residue on Ignition:	<0.5%

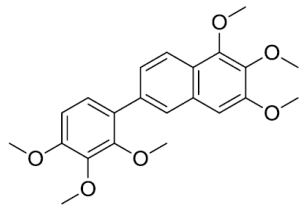
SCCS comment

Information on the purity for the batches THB0200112, THB0200212 and THB0200312, used to perform various studies, should be provided; along with information on the analytical methodology used providing analytical files with representative GC-UV chromatograms and UV spectrum of the test substance in the vapour phase.

3.1.5 Impurities / accompanying contaminants

The impurities in three 1,2,4-trihydroxybenzene (1,2,4-THB) lots were identified by NMR spectroscopy (both ^{1}H and ^{13}C) and GC-MS and were quantified by GC-FID.

 benzene-1,2,4,5-tetraol (Peak 3 and 5 (isomers) Impurity 1	 4-mercaptophenol (Peak 1) Impurity 2	 hydroquinone (Peak 2) Impurity 3

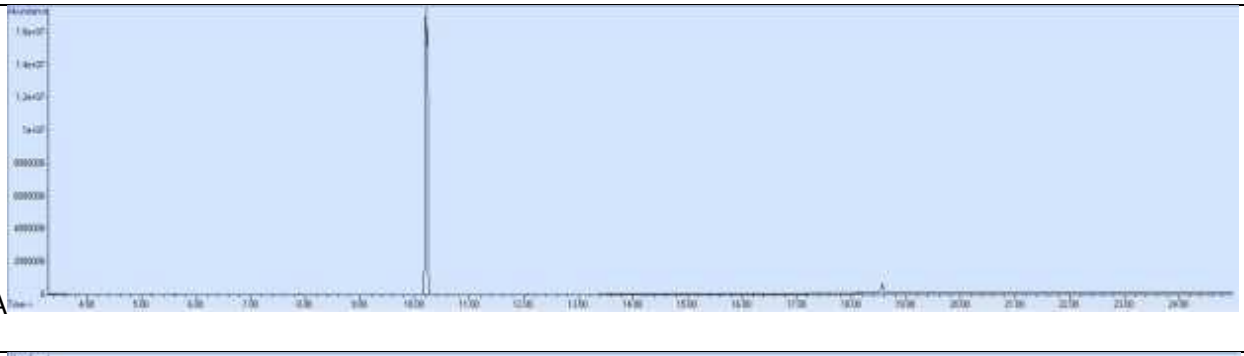


2-(2,3,4-Trimethoxyphenyl)-5,6,7-trimethoxynaphthalene
(Peak 4)

Impurity 4

The GC-MS chromatogram of THB0200112 (CO1787) derivative is shown in Figure 1A, silylated 1,2,4-trihydroxybenzene is eluted at RT=10.21 min. Enlargement of the chromatogram indicates additional peaks as shown in Figure 1B. Five impurity peaks were observed and the MS matches were shown: silylated 4-mercaptophenol at RT=10.751 min, silylated hydroquinone impurity at RT=10.944 min, silylated tetrahydroxybenzene impurity at RT=11.726 min, an impurity at RT=11.858 min and silylated tetrahydroxybenzene at RT=12.068 min, respectively. Column materials for CO 1787 are eluted at RT=18.564 min.

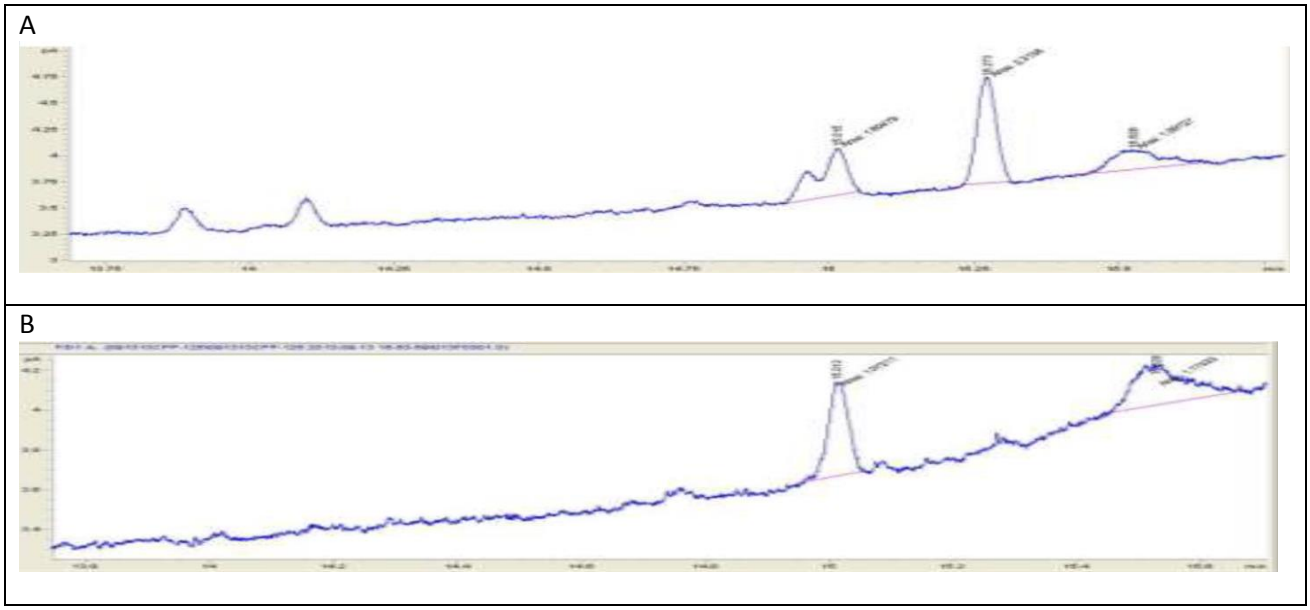
Figure 1. GC-MS chromatogram of THB0200112 (CO1787) with impurity peaks



GC-FID was further utilised to quantify the impurities that were identified by GC-MS. Silylated trihydroxybenzene elutes at 13.27 min. The impurity region was magnified and utilised for quantification and the impurities elute after silylated trihydroxybenzene. The GC-FID

chromatogram for THB0200112 (CO1787) is shown in Figure 2A and, once magnified, shows an interference peak at the impurity region. This peak was also observed in the sigma standard sample as shown in Figure 2B. The other peak corresponds to the impurity identified in the sigma standard.

Figure 2. A. GC-FID chromatogram of THB0200112 (CO1787) at impurity region; B. GC-FID for sigma standard at impurity region



Although five impurity peaks were identified in the GC-MS chromatogram, according to the GC-FID area counts, only the area of the peak corresponding to silylated tetrahydroxybenzene was >0.1% of silylated trihydroxybenzene. Therefore, only the isomer of tetrahydroxybenzene >0.1% was quantified using GC-FID. The area of silylated trihydroxybenzene and tetrahydroxybenzene for three samples were compared in Table 1.

Table 1. GC-FID Impurity quantification for tetrahydroxybenzene

Sample	Area(RT=13.27 min)	Area (RT=15.27 min)	Area%	Impurity %
THB0200112	1866.2	2.3	0.123	0.11
THB0200212	1881.5	2.4	0.128	0.11
THB0200312	2487.4	4	0.16	0.14

The percentage of tetrahydroxybenzene vs. trihydroxybenzene can then be calculated from the following equation:

$$\%\ \text{Impurity in Product} = \frac{(\text{Area}_{\text{tetra}})}{(\text{Area}_{\text{tri}})} * \frac{342.65/126.11}{430.83/142.11}$$

The impurities including 1,2,4,5-tetrahydroxybenzene, ethyl acetate and others were quantified by GC-FID and the results are shown in Table 2.

Table 2. Impurity profile of three 1,2,4-THB lots

Lot #	Analysis Data (%)				
	Ethyl acetate %	Impurity 1 (1,2,4,5-tetrahydroxybenzene) %	Impurity 2	Impurity 3	Impurity 4
THB0200112	0.15	0.11	<0.1		
THB0200212	0.15	0.11	<0.1		
THB0200312	0.15	0.14	<0.1		

Impurity 1 (1,2,4,5-tetrahydroxybenzene) accounts for 0.12% on average from these three lots. Combined, impurities 2 (4-mercaptophenol), 3 (hydroquinone) and 4 (2-(2,3,4-trimethoxyphenyl)-5,6,7-trimethoxynapthalene) account for less than 0.1% of total chemical composition of the material as supplied.

Ref.: 4

Heavy Metals: Determined by ICP-MS

Table 3. Summary of Heavy Metal Levels in Three Lots (values noted in ppm)

Lot #	As	Cd	Cr	Pb	Hg	Zn
THB0200112	0.33	ND	0.17	0.03	0.02	6.9
THB0200212	0.32	ND	0.12	0.05	0.03	7.1
THB0200312	0.20	ND	0.13	0.05	0.02	14
Detection Limit	0.01	0.01	0.02	0.01	0.01	0.03

* ND- not detected

Ref.: 5

SCCS comment

Quantification of the impurities cannot be accepted unless:

1. The analytical data is provided in a better resolution. The resolution of the GC-MS and GC-FID chromatograms presented in Ref. 4 is low with distorted x- and y-axis graphics and peak areas and retention times are impossible to read.
2. The Applicant explains the quantitation of the impurity that appears as a double-peak in the GC-FID chromatograms of the batches THB0200112, THB0200212 and THB0200312. GC-MS quantitation of the impurities should also be provided based on the GC-MS data presented in the report.

Hydroquinone content should be accurately quantified in each batch and be at unavoidable trace level in each batch.

3.1.6 Solubility

Water solubility according to OECD method A6: 486 g/L at 20°C
Solubility (g/100 mL, 22°C, 24 h)
Ethanol: 1<S<10
DMSO: 10<S<20

Ref.: 1

3.1.7 Partition coefficient (Log P_{ow})

Log Pow: 0.2 (calculated*)
* As the test item was not stable in water, the shake-flask method (EC A.6) was not applicable.

Ref.: 1

3.1.8 Additional physical and chemical specifications

Melting point:	139°C, 139.6°C and 144.5°C for 3 different batches
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm):	λmax 291 nm

Ref.: 1

3.1.9 Homogeneity and Stability

From submission V (from previous Opinion SCCS 1452/11)

- The identification and quantification of 1,2,4-trihydroxybenzene in the batches Op.29 and 0502124 was not sufficiently performed. A complete identification and quantification of 1,2,4-trihydroxybenzene in these batches, using state of art methods, is required. Identification and determination of impurities in these batches should also be performed.
- The content of 1,2,4-trihydroxybenzene, determined using Op.29 as reference standard, can only be considered as semi-quantitative determination.
- The stability testing of 1,2,4-trihydroxybenzene in solutions is inadequate, because it is performed after storage of test solutions in the dark and in an inert environment. The consumer is exposed to 1,2,4-trihydroxybenzene in ambient air.
- Stability of 1,2,4-trihydroxybenzene in typical hair dye formulations was not reported.

Additional information from Submission VI

Solutions of 1,2,4-THB were stable (variation <2%) after 2- and 4-hour storage at room temperature protected from light and in an inert gas atmosphere at the following concentrations:
- 50 mg/mL in purified water
- 2.5 mg/mL in DMF
- 10 mg/mL in DMF
- 250 mg/mL in DMF

Ref.: 1

Stability of 1,2,4-THB in solution depends on the amount of dissolved oxygen in the solution. If the solution has been thoroughly degassed and is stored in an inert atmosphere or in a sealed container, the material will be stable until oxygen exposure is allowed.

Once oxygen is available, the molecule will undergo oxidation and condense with itself to form dimers and oligomers in the absence of primary intermediates. Black particulate matter of high molecular weight precipitates out of the solution and settles to the bottom.

In view of the reactivity of 1,2,4-THB with oxygen, these hair dye formulations are filled into specialised, oxygen-barrier packaging for commercialisation. Stability tests are conducted under standard conditions (both room and elevated temperature) appropriate for cosmetic products. Final product stability meets standard requirements until the package is opened by the consumer for use. Upon dispensing and application, the combination of dyes (including 1,2,4-THB and precursors) in the formulation undergo oxidative reactions as is predicted by their chemical structure.

General SCCS comments to physico-chemical characterisation based on Submission V and VI

- Information on the purity for the batches THB0200112, THB0200212 and THB0200312, used to perform various studies, should be provided; along with information on the analytical methodology used providing analytical files with representative GC-UV chromatograms and UV spectrum of the test substance in the vapour phase. Hydroquinone content should be accurately quantified in each batch and their level should be kept at trace level.
- Quantification of the impurities as provided by the Applicant cannot be accepted unless:
    - The analytical data are provided in a better resolution. The resolution of the GC-MS and GC-FID chromatograms presented in Ref. 4 is low with distorted x- and y-axis graphics and peak areas and retention times are impossible to read.
    - The Applicant explains the quantitation of the impurity that appears as a double-peak in the GC-FID chromatograms of the batches THB0200112, THB0200212 and THB0200312.
    - GC-MS quantitation of the impurities is provided based on the GC-MS data presented in the report.
- Stability of 1,2,4-trihydroxybenzene in typical hair dye formulations was not reported except the reaction of 1,2,4-THB in the presence of the primary

intermediate PTD (A5) which was studied as a representative example of oxidative coupling with primary intermediates in general.

3.2 Function and uses

1,2,4-Trihydroxybenzene is an ingredient used in direct hair colouring products, i.e. without mixing with an oxidative agent, at a maximum on-head concentration of 3.0%.

Additional information from Submission VI

This submission is intended to support the use of 1,2,4-THB as an auto-oxidative dye in permanent hair dye formulations and in gradual hair colouring shampoos.
1,2,4-THB is an auto-oxidative dye used at a maximum formulation concentration of 2.5% in a permanent hair dye formulation and does not require peroxide to activate the oxidation and subsequent coupling reactions. The finished products are manufactured and filled in a strictly inert environment and are stable until activated once the package is opened and exposed to the air during usage.
1,2,4-THB is an auto-oxidative hair dye also used at a maximum formulation concentration of 0.7% in a gradual hair colouring shampoo that does not require hydrogen peroxide to activate the oxidation reaction and subsequent coupling reactions. The hair colour shampoo may be used to deposit small amounts of colour in a gradual fashion in the hair with each shampooing until the consumer achieves the desired colour and subsequently used 2-3 times in a week for maintenance.
We are not aware of any other industrial or functional uses for 1,2,4-THB other than oxidative hair dye use mentioned here.

In response to the request for clarification from the SCCS on uses of 1,2,4-THB, the Applicant confirmed that 1,2,4-THB is intended to be used in the presence of primary intermediates such as p-phenylenediamine (A7), p-toluenediamine (PTD)(A5), N,N bis–(2-hydroxyethyl) p-phenylenediamine (A50) and p-aminophenol (A16), just to name a few. This is done to achieve a range of desired permanent colours, and the particular primary intermediate or combination thereof will depend on the desired shade. It was also confirmed that the only study conducted in the presence of a primary intermediate was the dermal absorption work, which evaluated dermal absorption with the 1,2,4-THB in a simple formulation with PTD (A5). All other studies submitted for the safety evaluation of 1,2,4-THB were conducted with 1,2,4-THB alone.

3.3 Toxicological evaluation

In the following sections the Applicant provided studies which addressed concerns indicated in Opinion SCCS/1452/11, taking into account the testing strategy that the SCCS proposed in its 2014 Addendum to the Notes of Guidance (SCCS/1532/14, Ref. 2) and the updated Notes of Guidance (SCCS/1564/15, Ref. 3). In addition, the section provides data from a skin sensitisation study utilising the KeratinoSens™ test method and a new dermal absorption study.

3.3.1 Acute toxicity

From Submission V (from previous Opinion SCCS 1452/11)
The results of an acute dermal toxicity study in rats showed that the maximal non-lethal dose of 1,2,4-trihydroxybenzene was 2000 mg/kg bw.

3.3.1.1 Acute oral toxicity

No new data provided in Submission VI

3.3.1.2 Acute dermal toxicity

No new data provided in Submission VI

3.3.1.3 Acute inhalation toxicity

No new data provided in Submission VI

3.3.2 Irritation and corrosivity

From Submission V

A 3% dilution of 1,2,4-trihydroxybenzene was found to be slightly irritant to rabbit skin and to the rabbit eye.

3.3.2.1 Skin irritation

No new data provided in Submission VI

3.3.2.2 Mucous membrane irritation / eye irritation

No new data provided in Submission VI

3.3.3 Skin sensitisation

From Submission V

1,2,4-trihydroxybenzene was found to be an extreme skin sensitiser in mice in the Local Lymph Node Assay (LLNA).

Additional information from Submission VI

Although skin sensitisation was not identified as a data gap in the SCCS 1452/11 (Ref. 1), the following assay was conducted under OECD 442D and the following additional information was collected on this endpoint:

***In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method**

Guideline:	OECD 442D
Cell line:	HaCaT Keratinocytes
Test substance:	1,2,4-trihydroxybenzene (1,2,4-THB)
Purity:	97.8%
Lot:	THB0200312
Concentrations:	0.977, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500, 1000, 2000 µM
Solvent:	DMSO in a final concentration of 1% in 1% DMEM
Positive control:	Cinnamic Aldehyde (4, 8, 16, 32 and 64 µM)
GLP compliance:	in compliance
Study period:	08 Feb – 08 Jun 2016

The KeratinoSens™ test method is considered scientifically valid to be used to support the discrimination between skin sensitisers and nonsensitisers for the purpose of hazard classification and labelling. The ARE-Nrf2 luciferase test method makes use of an immortalised adherent cell line derived from HaCaT human keratinocytes stably transfected with a selectable plasmid. The cell line contains the luciferase gene under the transcriptional control of a constitutive promoter fused with an ARE element from a gene that is known to be up-regulated by contact sensitisers. A chemical is predicted to have potential to be a sensitiser if at least two of the following criteria are met:

1. the EC 1.5 value falls below 1000 µM
2. at the lowest concentration with a gene induction above 1.5, cellular viability is greater than 70%
3. there is an apparent overall dose-response which is similar between repetitions.

The test article, 1,2,4-THB, was tested in three definitive assays according the OECD protocol 442D.

Results

Table 4 summarises the data obtained in the assay for 1,2,4-THB. According to the current prediction model, and as expected based on existing data, 1,2,4-THB met the criteria to be classified a skin sensitiser.

Table 4. Summary of KeratinoSens™ data

Test Article	EC1.5 (µM)	Mean IC_{50} (µM) MTT	Maximal Induction (I_{max})[1]	Conc. For Maximal Gene Induction (CI_{max})[2]	Potential Sensitiser?
1,2,4-THB	374.31	992.11	3.50	500.0	Yes
Positive Control Cinnamic Aldehyde	10.37	> 64	NA	NA	Yes

Note: Where an EC1.5 or IC50 value was not obtained, the results were presented as greater than the highest value tested.

[1] Luciferase average is maximal fold induction as compared to DMSO solvent controls

[2] Concentration where average maximal fold induction occurred

Conclusions

The data confirmed that 1,2,4-THB has the potential to be a sensitiser but is not equivalent to the positive control, cinnamaldehyde, which is a potent sensitiser. The EC1.5 for 1,2,4-THB was determined to be 374.31 µM. In comparison, the EC1.5 value for PPD (p-phenylendiamine) is 11.6 µM in the KeratinoSens™ Assay, which is comparable to the positive control.

Ref.: 12

SCCS comment

In the Keratinosens™ Assay, 1,2,4-THB was positive at a concentration of 500 µM. Only average values without standard deviations were provided. Raw data of the three independent experiments was not provided. The dose-response curve shows a huge variation in gene induction at the 500 µM concentration. According to the prediction model of OECD TG442D, the Keratinosens™ assay is positive when gene induction is statistically significant from the solvent control in at least 2 out of 3 replicates. This statistical analysis is not provided. The results of this assay are therefore inconclusive. In addition, data from the Keratinosens™ assay cannot be used on their own to predict the potency of a test chemical. To conclude, 1,2,4-THB is an extreme skin sensitiser based on the LLNA (Ref. 1).

3.3.4 Toxicokinetics

3.3.4.1 Dermal / percutaneous absorption

From Submission V

The experiment was conducted with a direct dye formulation containing 2.78% 1,2,4-trihydroxybenzene and not 3%. The dose was slightly below that requested for use and stability in the receptor was not quantified. Therefore, the amount considered as being absorbed is the mean + 2SD. This is 0.03% of the applied dose or 0.17 µg/cm^2.

New data provided in Submission VI

Guideline: OECD 428 (2004); Guidance Document No. 28
Test System: frozen human dermatomed skin
Skin Integrity: checked by electrical resistance, at least 10 kΩ

Replicates:	12 per each test item; 6 female donors (age 60-84; 2 abdomen, 1 back, 1 buttock, 1 back/ buttock) for test item 1 and 4 female donors (age 63-74; 1 abdomen, 3 back) for test item 2
Method:	static glass diffusion cells (2.54 cm^2, approximately 4.5 mL receptor fluid volume)
Skin Temperature:	32±1°C
Test Substance:	1,2,4-Trihydroxybenzene (THB)
Batch:	THB0200312 (non-radiolabelled), 8188CEO008-2 ([^{14}C]-1,2,4-Trihydroxybenzene ([^{14}C]-1,2,4-THB)
Purity:	99.3% (non-radiolabelled); 98.3% (radio-labelled; 2.00 MBq/mg (6.81 mCi/mmol = 252 MBq/mol))
Test items:	1) 1,2,4-THB formulated at a level of 2.5% w/w in a hair dye vehicle at pH 7 2) 1,2,4-THB formulated at a level of 2.5% w/w in a hair dye vehicle also containing 2.25% p-Toluenediamine (PTD as the free base) at pH 7
Dose applied:	20 mg/cm^2 of test item (approx. 500 µg 1,2,4-THB/cm^2)
Exposed area:	2.54 cm^2
Exposure period:	30 minutes
Sampling period:	24 hours
Receptor fluid:	Phosphate buffered saline
Solubility in receptor fluid:	486 g/L (solubility in water)
Mass balance analysis:	provided
Tape stripping:	yes (up to 21 strips)
Method of Analysis:	liquid scintillation counting
GLP status:	in compliance
Study Period:	09 Sept 2014 - 26 Jan 2015

[^{14}C]-1,2,4-THB and unlabelled 1,2,4-THB were incorporated into hair dye formulations, with and without PTD to provide final concentrations of 2.5% (w/w) [^{14}C]-1,2,4-THB. The formulations were applied to the skin surface at a dose of 20 mg/cm^2 and after an exposure period of 30 minutes, the skin surface was washed with a mild soap solution. Following the washing procedure, the cells were returned to the water bath for the remainder of the 24 hour run time. At the end of the experiment, the distribution of [^{14}C]-1,2,4-THB in the test system was assessed by performing a mass balance procedure, which included a tape stripping and heat separation technique, and a 24-hour penetration profile was determined. All samples were analysed for radioactivity by Liquid Scintillation Counting (LSC). For both test items, one of the 12 dosed cells indicated membrane damage over the course of the 24 hour run and was therefore rejected and not included in the mean ± SD.

Results

Mass balances showed essentially complete recovery of radiolabel in each experiment. Mean recovery of the applied test from the formulation without and with PTD was 101% and 99.2%, respectively.

Table 5 represents the penetration and distribution of [^{14}C]-1,2,4-THB and [^{14}C]-1,2,4-THB with PTD from a hair dye formulation in the test system.

Table 5. Penetration and distribution of [^{14}C]-1,2,4-THB and [^{14}C]-1,2,4-THB with PTD from a hair dye formulation

Test Compartment	ug equivalents of [^{14}C]-1,2,4-THB/cm^2		% of applied dose [^{14}C]-1,2,4-THB/cm^2		ug equivalents of [^{14}C]-1,2,4-THB/cm^2 & PTD		% of applied dose [^{14}C]-1,2,4-THB/cm^2 & PTD	
n = 11	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Donor Chamber	0.203	0.265	0.041	0.053	0.301	0.284	0.061	0.058
Skin wash at 30 min	497	11.4	99.3	2.29	472	12.9	95.5	2.62
Skin Wash at 24 hrs	8.34	4.09	1.66	0.817	14.5	9.74	2.94	1.97
Stratum corneum	0.414	0.362	0.083	0.072	1.03	0.81	0.209	0.164
Epidermis	0.984	0.569	0.196	0.114	1.85	1.66	0.374	0.336
Dermis	0.101	0.097	0.02	0.019	0.069	0.082	0.014	0.017
Flange	0.169	0.236	0.034	0.047	0.258	0.17	0.052	0.034
Receptor Fluid	0.048	0.019	0.01	0.004	0.027	0.028	0.005	0.006
Total non-absorbed	506	9.38	101	1.87	488	5.32	98.8	1.08
Systemically available	1.13	0.581	0.226	0.116	1.94	1.76	0.393	0.357
Total recovered	508	9.46	101	1.89	490	4.66	99.2	0.943

Total non-absorbed = Σ donor chamber, skin wash (30 min + 24 hrs), flange and stratum corneum.
Systemically available = Σ epidermis, dermis, receptor fluid
Skin wash at 30 minutes = Σ 30 min pipette tips + 30 min sponge swabs + 30 min skin wash
Stratum corneum = Amount in tape strips
Epidermis = Tissue remaining after tape stripping and separated from the dermis plus final tape strip if the epidermis tore during tape stripping

The total systemically available dose (epidermis, dermis and receptor fluid) from the formulation containing [^{14}C]-1,2,4-THB alone was 1.13 ± 0.58 µg-eq [^{14}C]-1,2,4-THB/cm^2 (mean ± SD). The total systemically available dose of [^{14}C]-1,2,4-THB from the formulation containing 2.5% [^{14}C]-1,2,4-THB and PTD, was 1.94 +1.76 µg-eq [^{14}C]-1,2,4-THB/cm^2 (mean ± SD).

Conclusion

In accordance with the Notes of Guidance (SCCS/1564/15, Ref. 3) the mean +1SD absorption in an *in vitro* dermal absorption study is used to determine the systemically available dose for the purposes of the calculation of the margin of safety (MoS). The systemically available dose of [^{14}C]-1,2,4-THB alone is therefore 1.13 µg equivalents/cm^2 + 0.58 (1SD) or 1.71 µg equivalents/cm^2. The systemically available dose of [^{14}C]-1,2,4-THB with PTD is 1.94 µg equivalents/cm^2 + 1.76 (1SD) or 3.70 µg equivalents/cm^2.

Ref.: 13

SCCS comment

The SCCS noted that the thickness of the dermatomed skin has not been provided. Moreover, it is not clear why the dermal absorption of 1,2,4-THB with PTD was nearly double that without PTD, i.e. 1.94 + 1.76 = 3.7 µg-eq (with PTD) vs. 1.13 + 0.58 = 1.71 µg-eq (without PTD). Considering the reaction chemistry and formation of dimers (PTD-1,2,4-THB) and trimers (THB-PTD-THB) (the section Special investigation) it was expected to be lower than without PTD. Based on the results, for potential calculation of MoS, the SCCS would suggest to take the highest value of the systemically available dose of [^{14}C]-1,2,4-THB with PTD, i.e. 1.94 µg equivalents/cm^2 + 1.76 (1SD) or 3.70 µg equivalents/cm^2.

3.3.4.2 Other studies on toxicokinetics

No new data provided in Submission VI

3.3.5 Repeated dose toxicity

From Submission V

A No Observable Adverse Effect Level (NOAEL) of 50 mg/kg bw/day (90-day, oral, rat) was proposed by the applicant. The SCCP disagreed with this since the relative organ weight was increased significantly in the spleen of male rats treated with 50 mg/kg bw/day. This increase continued dose dependently in male rats treated with either 100 or 200 mg/kg bw/day. The absolute organ weight of the spleen also increased in male rats but this increase was not significant at the dose of 50 mg/kg bw/day. Therefore, the dose of 50 mg/kg bw/day was considered as Lowest Observed Adverse Effect Level (LOAEL).

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

No new data provided in Submission VI

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

No new data provided in Submission VI

3.3.5.3 Chronic (> 12 months) toxicity

No new data provided in Submission VI

3.3.6 Reproductive toxicity

From Submission V

No treatment related effects were seen in a prenatal developmental toxicity study on developmental toxicity parameters up to the highest tested dose of 300 mg/kg bw/day. At 300 mg/kg bw/day, a slight maternal toxicity was noted.

3.3.6.1 Fertility and reproduction toxicity

No new data provided in Submission VI

3.3.6.2 Developmental Toxicity

No new data provided in Submission VI

3.3.7 Mutagenicity / genotoxicity

From Submission V

Overall, the genotoxicity of 1,2,4-trihydroxybenzene is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. 1,2,4-trihydroxybenzene induced gene mutations in bacteria (a slight but reproducible mutagenic activity in *S. typhimurium* TA98 and TA100 without metabolic activation) but not in mammalian cells. 1,2,4-Trihydroxybenzene did not induce an increase in cells with chromosome aberrations, but the relevance of this test is questionable since the test conditions were considered to be insufficient by the SCCS. In an *in vitro* micronucleus test in combination with *in situ* hybridisation with specific centromeric probes for chromosomes 7 and 8, a concentration-dependent and statistically significant increase in the number of lymphocytes with micronuclei as well as in aneuploid cells was found. Moreover, 1,2,4-trihydroxybenzene induced an increase in sister chromatid exchanges in human peripheral blood lymphocytes and an induction of DNA single strand breaks in murine bone marrow cells.

The positive findings from the *in vitro* tests covering both chromosome aberrations and aneuploidy were not confirmed in an *in vivo* test. In an *in vivo* micronucleus test, 1,2,4-trihydroxybenzene exposure of mice did not result in an increase in erythrocytes with micronuclei. However, the positive finding in the gene mutation test in bacteria was not confirmed nor overruled with an *in vivo* test measuring the same genotoxic endpoint. Consequently, on the basis of these tests, 1,2,4-trihydroxybenzene has to be considered as an *in vitro* genotoxin. *In vivo* testing would be required to explore the potential to induce gene mutations; such tests are no longer permitted.

3.3.7.1 Mutagenicity / genotoxicity *in vitro*

New data provided in Submission VI

Gene mutation assay using bacteria

Guideline:	OECD 471
Test system:	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA102
Replicates:	two experiments, triplicate plates
Test substance:	1,2,4-Trihydroxybenzene
Batch:	THB0200312
Purity:	99.5% (provided by the Sponsor in the protocol) 97.8% (per Results Report)
Concentrations:	Experiment A (range finding) – Plate incorporation test: ±S9 mix: all *S. typhimurium* strains: 0, 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg/plate Experiment B1 +S9 mix plate incorporation test: all S. strains: 0, 15, 50, 150, 500, 1500 and 5000 µg/plate -S9 mix plate incorporation test: all S. strains: 0, 15, 50, 150, 500, 1500 and 5000 µg/plate

	Experiment B2 (retest assay with TA1537 strain) – Plate incorporation test: -S9 mix plate incorporation test: 0, 5.0, 15, 50, 150, 200, 300, 400, 500, 600, 750 and 1500 µg per plate
Vehicles:	water (stock solution of A33 at 50 mg/mL); degassed with nitrogen with calcium carbonate as trap
Positive Controls:	-S9 mix: 2-nitrofluorene (2NF): 1 µg/plate for TA98; sodium azide (NaN_3): 1 µg/plate for TA100, TA1535; 9-aminoacridine (AAC): 75 µg/plate for TA1537; mitomycin C (MMC): 1 µg/plate for TA102 +S9 mix: 2-Aminoanthracene (AAN): 1 µg/plate for TA98 and TA1535 or 2 µg/plate for TA100 and TA1537; sterigmatocystin: 15 µg/plate for TA102
Negative controls:	vehicle control
GLP:	in compliance
Study period:	22 Sep 2014 – 10 Aug 2015

Material and methods

A33 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 the Ames plate incorporation method 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).

Results

Dose formulation analysis

For the analysis of the dosing formulations, the submitted formulations were found to be accurately prepared. The vehicle control sample was free of test article. Additionally, 1,2,4-trihydroxybenzene in degassed deionized water, at concentrations of 0.253 and 43.7 mg/mL, was stable on wet ice for at least 2 hours.

Concentration analysis of the formulation samples collected prior to dosing indicated that the actual mean concentrations of the analysed low dose (0.50 mg/mL) and high dose (50 mg/mL) formulations in the initial mutagenicity assay were 86.5 and 88.2% of their respective target concentrations with < 5.0% relative standard deviation (RSD); and the actual mean concentrations of the analysed low dose (0.50 mg/mL) and high dose (15 mg/mL) formulations in the retest mutagenicity assay were 90.5 and 88.0% of their respective target concentrations with < 5.0% RSD.

For information purposes only, the samples used to dose the main study were analysed at the end of dosing. For Experiment B1, the 0.050 mg/mL low dose formulation submitted to the analytical laboratory after dosing was the incorrect sample and was below the validated range. Nevertheless, the result was found to be quantitative. The results of the analyses for Experiment B1 found the low and high doses to be 77.1% and 86.8% of target, respectively. The results of the analyses for Experiment B2 found the low and high doses to be 89.0% and

84.7% of target, respectively. This indicates that the test article concentrations decreased only slightly during the dosing period.

Preliminary Toxicity Assay

In this test, the maximum dose tested was 5000 µg per plate. This dose was achieved using a concentration of 50 mg/mL and a 100 µL plating aliquot. An increase in revertant counts (2.9-fold maximum increase) was observed only at 100 µg per plate with tester strain TA98 in the absence of S9 activation, with higher doses demonstrating toxicity. No precipitate was observed. Toxicity was observed beginning at 333, 667, 1000 or 3333 µg per plate. Based on the findings of the toxicity assay, the maximum dose tested in the mutagenicity assay was 5000 µg per plate.

Experiment B1

In this experiment, the dose levels tested were 15, 50, 150, 500, 1500 and 5000 µg per plate in the presence of S9 activation, and 5.0, 15, 50, 100, 150, 200, 500, 1500 and 5000 µg per plate in the absence of S9 activation. No positive mutagenic responses were observed with any of the tester strains (including TA98) in either the presence or absence of S9 activation.

An increase in revertant counts (3.5-fold at 100 µg per plate; 3.0-fold at 150 µg per plate; 4.0-fold maximum increase at 500 µg per plate that induced moderate toxicity) with tester strain TA1537 in the absence of S9 activation was observed. While this increase is an indicator of mutagenic activity, it was not evaluated as positive because of the variability in the individual revertant counts. This variability precluded demonstration of a definitive dose response, which is required for a positive evaluation. No precipitate was observed. Toxicity was observed beginning at 500 or at 5000 µg per plate.

To confirm the mutants, replicate plates were prepared for the following dose levels and the corresponding vehicle controls with tester strain TA1537 in the absence of S9 activation: 100, 150 and 500 µg per plate. The replicate plates confirmed that the correct colonies were evaluated as revertants.

Experiment B2 (retest assay with TA1537 strain)

Tester strain TA1537 in the absence of S9 activation was retested in Experiment B2 with an adjustment in dose levels to clarify the response observed.

In Experiment B2, the dose levels tested were 5.0, 15, 50, 150, 200, 300, 400, 500, 600, 750 and 1500 µg per plate. A positive mutagenic response (3.8-fold maximum increase at 150 µg per plate; 3.3-fold increase at 200 µg per plate) was observed with tester strain TA1537 in the absence of S9 activation. This response was evaluated as positive because the revertant counts demonstrated a dose response, yielding average revertant counts of 4 for the vehicle, 6 to 8 at 5.0 to 50 µg per plate and 13 to 15 at 150 to 200 µg per plate, with the maximum revertant counts being outside of the upper 95% control limit of 13. No precipitate was observed. Toxicity was observed beginning at 300 µg per plate. This repeat experiment meets the criteria for a positive response in TA1537 in the absence of S9 activation.

Conclusion

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, 1,2,4 trihydroxybenzene induced a positive mutagenic response with tester strain TA1537 in the absence of Aroclor-induced rat liver S9. Elevated revertant counts that exceeded the upper 95% control limit were observed in two trials. In one trial, there was a definitive dose response, and in the other trial, there was none. These increases were just over the minimum

3.0-fold increase required for evaluation as positive in TA1537, which suggests the presence of a weak or low-level mutagen.

SCCS comment

The results of the study indicate a clear mutagenic effect of 1,2,4-trihydroxybenzene in the absence of S9 mix in TA1537 strain.

The SCCS noted considerable width of range of revertant numbers in historical negative and positive controls (especially for TA1537 strain), with no information on the number of studies performed.

Ref.: 14

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

Guideline:	OECD 487 (draft approved April 2014)
Species/strain:	cultured human peripheral blood lymphocytes from one male volunteer
Replicates:	duplicate cultures, one experiment
Test substance:	1,2,4-Trihydroxybenzene
Batch:	THB0200312
Purity:	97.8% (per Results Report)
Concentrations:	Preliminary test (range-finder): ±S9 mix (4 h exposure + 20 h): 0.126, 0.378, 1.26, 3.78, 12.6, 37.8, 126, 378, 1260 µg/mL -S9 mix (24 h exposure): 0.126, 0.378, 1.26, 3.78, 12.6, 37.8, 126, 378, 1260 µg/mL Exp1: -S9 mix (4 h exposure + 20 h): 1.26, 3.15, 6.3, 12.5, 25, 50**,** 85, 100, 125, 135, 150 µg/mL -S9 mix (24 h exposure): 0.1, 0.25, 0.5, 1.26, 12.5, 30, 40, 50, 85, 100 µg/mL +S9 mix (4 h exposure + 20 h): 12.5, 25, 50, 100, 125, 135, 150 µg/mL
Solvent/negative control:	culture medium
Positive Controls:	-S9 mix: Vinblastine (VB, 5, 7.5 and 10 ng/mL) +S9 mix: Cyclophosphamide (CP, 2.5, 5 and 7.5 µg/mL)
Vehicle:	water (stock suspension of A33 at 50 mg/mL); degassed with nitrogen with calcium carbonate as trap
GLP:	in compliance
Study period:	25 Sept 2014 – 17 Aug 2015

Material and methods

In an *in vitro* micronucleus assay, A33 was tested using duplicate human lymphocyte cultures prepared from one male donor 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.
Treatments were conducted 48 hours following mitogen stimulation with Phytohaemagglutinin (PHA). Cells were exposed to the test item for 4 hours (followed by 20 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 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.

Results

Dose formulation analysis
The results of the analysis indicate that the actual mean concentrations of the analysed samples (0.25 and 1.5 mg/mL) were within the acceptance criteria of 85.0% to 115.0% of target with ≤ 5.0% relative standard deviation (RSD). No test article was detected in the vehicle control samples.
Concentration analysis of the dose formulation samples collected after dosing indicated that the actual mean concentrations of the analysed samples (0.25 and 1.5 mg/mL) were 91.8% and 77.6% of target with ≤ 5.0% RSD. The results of the post-dosing sample are reported but are not subject to the acceptance criteria specified in the protocol.
The results of stability of the dosing formulations indicated that 1,2,4-trihydroxybenzene in degassed deionized water, at concentrations of 0.253 and 43.7 mg/mL, was stable on wet ice for at least 2 hours.

Preliminary test
The results of the evaluation of CBPI and % cytotoxicity showed substantial cytotoxicity [≥ 50% cytokinesis-blocked proliferation index (CBPI) relative to the vehicle control] observed at dose levels ≥ 126 µg/mL in all three exposure groups.

Experiment 1 (the main study)
The dose levels selected for analysis of micronucleus test in the non-activated 4-hour exposure group were 12.5, 25, and 50 µg/mL. At the highest test concentration, 50 µg/mL, cytotoxicity was 52% relative to the vehicle control. The percentage of cells with micronuclei in the test article-treated group was not significantly increased relative to vehicle control at any dose level ($p > 0.05$, Fisher's Exact test).
The dose levels selected for analysis of micronucleus test in the S9-activated 4-hour exposure group were 25, 50, and 100 µg/mL. At the highest test concentration, 100 µg/mL, cytotoxicity was 54% relative to the vehicle control. The percentage of cells with micronuclei in the test article-treated group was not significantly increased relative to vehicle control at any dose level ($p > 0.05$, Fisher's Exact test). The percentage of micronucleated cells in the CP (positive control) group (1.1%) was statistically significant ($p \leq 0.01$, Fisher's Exact test).
The dose levels selected for analysis of micronucleus test in the non-activated 24-hour exposure group were 1.26, 12.5, and 30 µg/mL. At the highest test concentration, 30 µg/mL, cytotoxicity was 55% relative to the vehicle control. The percentage of cells with micronuclei in the test article-treated group was not significantly increased relative to vehicle control at any dose level ($p > 0.05$, Fisher's Exact test). The percentage of micronucleated cells in the VB (positive control) group (1.1%) was statistically significant ($p \leq 0.01$, Fisher's Exact test).

Conclusion

The positive and vehicle controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, 1,2,4-trihydroxybenzene was concluded to be negative for the induction of micronuclei in the non-activated and S9-activated test systems in the *in vitro* mammalian micronucleus test using human peripheral blood lymphocytes.

Ref.: 15

3D skin comet assay with 1,2,4-trihydroxybenzene, using Phenion® full thickness skin models

Guideline:	/
Species/strain:	skin sample from one volunteer
Test system:	Phenion® full thickness (FT) skin model
Replicates:	triplicate skin membranes per test group, two separate experiments
Test substance:	1,2,4-Trihydroxybenzene
Batch:	THB0200312
Purity:	97.8% (per Results Report)
Concentrations:	Preliminary test (dose range-finder): total exposure for 48h (3 repeated applications at 48h, 24h, 3h before cell isolation) (n=2): target concentrations: 0.1, 0.316, 1, 3.16, 10, 31.6, 100 mg/mL; corresponding to target applied doses: 1.6, 5, 16, 50, 160, 500, 1600 µg/cm^2 Exp1: total exposure for 48h (3 repeated applications at 48h, 24h, 3h before cell isolation) (n=3): 0.125, 0.25, 0.5, 1 mg/mL corresponding to target applied dose of 2, 4, 8, 16 µg/cm^2 Exp2: total exposure for 48h (3 repeated applications at 48h, 24h, 3h before cell isolation) (n=3): target concentrations: 0.25, 0.5, 1, 1.25 mg/mL; corresponding to target applied doses: 4, 8, 16, 20 µg/cm^2; aphidicolin (APC) was added to the culture medium 4 h prior to cell isolation at a final concentration of 5 µg/mL
Solvent/negative control:	acetone degassed
Positive Controls:	Methyl methanesulfonate (MMS, 3h exposure at 3.16 mg/mL = 5 µg/cm^2) Benzo[a]pyrene (BaP, 48 h, 24 h, 3 h exposure at 0.78 mg/mL = 12.5 µg/cm^2; with and without APC)
Vehicle:	acetone degassed with nitrogen (a clear, brown stock solution of A33 at 100 mg/mL; corresponding to 1600 µg/cm^2)
GLP:	in compliance
Study period:	08 Mar 2016 – 20 Jan 2017

Materials and methods

The Phenion® full thickness (FT) skin model consisted of human primary keratinocytes and fibroblasts from single donor origin and was obtained from Henkel AG & Co. KGaA, Düsseldorf, Germany.
Petri dishes, filter spacers, filter paper and air liquid interphase (ALI) medium without phenol red to be used for culture of the skin models were included in the shipment. Upon receipt, the skin models were individually cultured at an ALI according to the instructions for use provided by the supplier. In brief, a maximum of three small Petri dishes (35/10 mm) were placed in a large Petri dish (100/20 mm) and a filter spacer was placed in each small Petri dish together with ca. 5 mL ALI medium (further referred to as culture medium). Subsequently, a filter paper was placed on the top of the filter spacer and it was visually checked if the medium level and filter paper had the same level and if the filter paper was completely soaked with medium. If not, the medium level was adjusted. After transfer of the skin models from the transport agar to the filter paper, the lid of the large Petri dish was closed and the skin models were cultured at ca. 37 ºC and ca. 5% CO_2. Culture medium was refreshed one day after the start of the culture, i.e. shortly before the first application of the dose solutions. For this purpose, the culture medium was removed from each Petri dish and the skin models were supplied with fresh culture medium.

Two valid main experiments were performed. Prior to the main experiments, a dose range finding study was performed to select suitable dose levels for the comet assay. In all experiments, the total exposure time was 48 ± 3 h (repeated application at 48 ± 3 h, 24 ± 3 h and 3 h before cell isolation). Degassed acetone was used as the solvent for the test substance and dose solutions were prepared freshly on each day of application. The dose solutions were prepared in a nitrogen gas environment (glove box) to prevent reaction of the test substance with air. The application volume was 16 µL/cm^2 skin. Negative (degassed acetone) and positive controls (methyl methanesulfonate and benzo[a]pyrene, in the first and second main experiment, respectively) were run in parallel. In the dose-range finding study, the highest test concentration was the maximum concentration required in the assay of 100 mg/mL (corresponding to 1600 µg/cm^2). Six serial dilutions with 3.16-fold spacing in degassed acetone were prepared from the stock solution. Duplicate skin membranes per test concentration were used. In the first and second main experiment, the highest test concentration was limited by cytotoxicity as determined in the dose-range finding study. Four dose solutions with 2-fold spacing in degassed acetone were prepared from four individual weighed samples for each test substance, except for a 1.25-fold spacing between the highest and second highest test concentration in the second main experiment. The actual concentration of the test substance in the dose solutions was determined by UPLC-UV.
Cytotoxicity was determined in each experiment based on adenylate kinase (AK) and lactate dehydrogenase (LDH) leakage into the culture media and measurement of intracellular ATP. In the dose-range finding study, it was also taken into account if evaluation of the comet slides was feasible with regards to the number of cells and presence of ghost cells. In the main experiments, coded duplicate comet assay slides per skin membrane were evaluated (50 cells per slide) for both epidermal and dermal cells using Comet Assay IV software (Perceptive Instruments) and the fluorescent dye SYBR Gold to determine the genotoxic potential.

Results
In addition to dose-related brown staining of the culture medium, dose-related brown staining of the skin membrane was also observed at all concentrations. Both the staining in the skin

membrane and culture medium was more intense than the colour of the dose solutions that were applied, indicating the formation of coloured oxidative coupling reaction products during the conduct of the assay as expected with this test substance.

In the dose-range finding study, cytotoxicity was observed at and above 50 µg/cm^2 based on the AK assay and at 16 µg/cm^2 based on LDH. As a result, 16 µg/cm^2 was selected as the highest concentration in the first main experiment. The negative and positive controls met the acceptance criteria in both the epidermal and dermal fractions in this first experiment and the study was considered valid. No cytotoxicity was observed up to 16 µg/cm^2 and no statistically significant increase was observed at any of the test concentrations.
Since the results of the first main experiment were negative, a second main experiment with four concentrations up to 20 µg/cm^2 was performed using aphidicolin as inhibitor of DNA repair to increase the sensitivity of the assay. The negative and positive controls met the acceptance criteria in both the epidermal and dermal fractions in this second experiment and the study was considered valid. Cytotoxicity was observed at 20 µg/cm^2 based on measurement of intracellular ATP and therefore this concentration was excluded from genotoxicity assessment. No statistically significant increase was observed at any of the test concentrations up to 16 µg/cm^2.

Conclusion

The Applicant stated that based on the results obtained in this 3D skin comet assay using Phenion® full thickness skin models, under the conditions used in this study, 1,2,4-trihydroxybenzene is considered not to induce DNA damage to human skin cells after topical application.

SCCS comment

- The *vitro* comet assay is under validation yet, and no OECD guideline is available.
- In the study report it is stated that the 1,2,4-THB concentration of 16 µg/cm² is not cytotoxic. However, analysis of the data at 16 µg/cm² in all cytotoxicity tests done, indicates that the values are already significantly increased compared to the control. Thus applying this concentration and certainly higher (i.e. 20 µg/cm²) is not justified.
- A clear dose-related brown staining of the media and skin samples was observed, that might interfere with (some of) the cell viability measurements. Therefore, adapted controls should have been taken into consideration. Also possible interference of media and tissue colouration should be considered and the approach to avoid interference validated.
- In the study, cytotoxicity evaluation was not performed for the positive control groups (according to applicant: because these were treated as positive control groups for genotoxicity and not for cytotoxicity; therefore the dose levels of the positive control groups were the same as in the ongoing validation study of the 3D skin comet assay).
- In Experiment 2 for Group C: BaP + APC tail intensity was 26±11% for epidermis, which was below historical control range, i.e. 27-56%; for dermis the value of 27±3% was at the lower limit of historical control range, i.e. 27-57%.

Ref.: 16

3.3.7.2 Mutagenicity / genotoxicity *in vivo*

No new data provided in Submission VI

Overall SCCS comment on genotoxicity/mutagenicity

1,2,4-Trihydroxybenzene was clearly positive in the GLP-compliant Ames test studies submitted twice, i.e. in submission V and VI. The GLP-compliant *in vitro* mammalian gene mutations test (submission V) and micronucleus test (submission VI) were negative. Additionally, in submission VI the Applicant provided a study on the 3D skin comet assay model with negative results. The review conducted by the SCCS of a lot of supplementary data from the open literature indicates genotoxic/mutagenic effects of 1,2,4-THB detected using different endpoints under *in vitro* conditions including direct DNA damaging effects, chromosomal aberrations and aneuploidy (see the list of references attached to the Opinion). The positive literature findings from the *in vitro* tests covering both chromosome aberrations and aneuploidy were not confirmed in an *in vivo* test provided by applicant (*in vivo* micronucleus test, submission V), where the exposure of mice to 1,2,4-THB did not result in an increase in erythrocytes with micronuclei. It should be noted however, that this test was not performed in accordance with the current OECD guideline (only one dose was tested) and thus has limited value.

Based on the analysis, the SCCS is of the opinion that genotoxicity potential of 1,2,4-trihydroxybenzene cannot be excluded.

The SCCS notes that according to the Opinion SCCP/0971/06 "The potential to induce mutagenic / genotoxic effects has to be excluded for (i) the precursors and couplers, (ii) the formed products and (iii) the intermediates formed" (P.6, §2.4.2.). However, assuming a presence of not completely reacted 1,2,4-THB in final products, for which many positive genotoxic results have been described, the SCCS was able to conclude on genotoxic hazard of 1,2,4-THB without the need for conducting additional studies.

3.3.8 Carcinogenicity

From Submission V

No conclusion with regard to carcinogenicity can be made from the mice topical application carcinogenicity study submitted.

No new data provided in Submission VI

3.3.9 Photo-induced toxicity

3.3.9.1 Phototoxicity / photo-irritation and photosensitisation

No new data provided in Submission VI

3.3.9.2 Photomutagenicity / photoclastogenicity

No new data provided in Submission VI

3.3.10 Human data

No new data provided in Submission VI

3.3.11 Special investigations

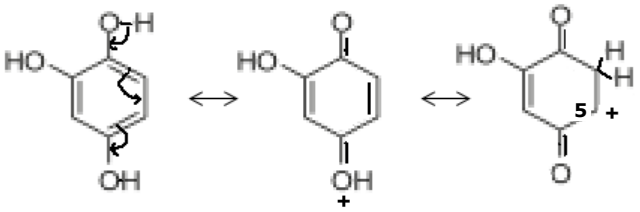
New data provided in Submission VI

In response to the SCCS's request in its Opinion (SCCS/1452/11, Ref. 1), the reaction products resulting from use of 1,2,4-THB in a typical hair dye formulation were explored with some necessary modifications in order to be able to isolate intermediate that indicate reaction progress from a mechanistic standpoint.

3.3.11.1 Reaction Chemistry

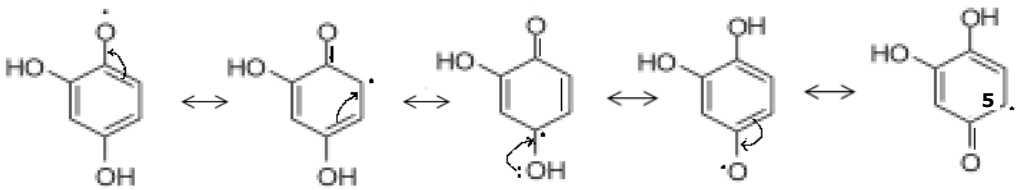
The substitution pattern of 1,2,4-THB has a significant effect on the reactivity and types of reactions in which it participates. The hydroxyl substituents are *ortho*, *para* directing in substitution reactions on the benzene ring. This makes the 5 position on the ring, as it is *ortho and para* to two of the three substituents, the most likely carbon to participate in any type of coupling reaction as shown below in Figure 3.

Figure 3. Some resonance structures for 1,2,4-THB



Even after oxidation, the unpaired electron can be stabilised through the ring as shown in Figure 4.

Figure 4. Some resonance structures for phenoxyl radical of 1,2,4-THB



1,2,4–THB reacts in the presence of oxygen and primary intermediates to form coupled products without requiring the action of peroxide. This knowledge regarding the reactivity of 1,2,4-THB has existed since the 1970s (Ref. 6).
In Opinion SCCS/1452/11, the SCCS expressed the view that submission V had failed to provide sufficient information on the characterisation of the oxidation reaction product(s) of 1,2,4-THB to which the consumer is exposed due to the reported instability of 1,2,4-trihydroxybenzene in aqueous systems. In order to address this concern, reaction kinetics studies were undertaken in the presence of a well-studied primary intermediate, 2,5-Diamino toluene (PTD, A5), in an aqueous medium at a molar ratio of 1.0 to 1.1 (0.35g PTD-2HCl: 0.25g 1,2,4-THB). The reaction was conducted at room temperature and pH was adjusted to 9.0 with ammonia or monoethanolamine. Aliquots were pulled at different time points, diluted and frozen to stop the reaction and to enable identification of intermediate coupling products. The self-coupled product (A33-A33) was only isolated by controlling the reaction conditions via dilution and temperature reduction and was not isolated under conditions of use of commercialised hair dye formulations. These aliquots were then analysed by LC-MS. To study the reaction in the presence of hair, each hair sample was weighed and the average weight recorded (~1.9 g). Hair samples were added to the reaction mixture. The hair samples were extracted with methanol at 40°C and the extract was studied without dilution by LC-MS.

The data showed that the reaction of 1,2,4-THB (coupler) with A5 (precursor/primary intermediate) in a basic aqueous medium proceeds according to the proposed reaction pathway in Figure 5a. Reaction intermediates that were isolated in the course of the study are identified by their exact mass (by LC-MS) and their molecular weight. This proposed reaction mechanism in Figure 5a is highly analogous to the one proposed in the SCCS Opinions on reaction products (SCCP/0941/05, SCCP/1004/06, SCCP/1198/08 and SCCS/1311/10 (Refs. 7-11)), and reproduced here as Figure 5b. Precursors/primary intermediates (1,4 or *para*-di-substituted benzenes) react with couplers (1,3 or *meta* di-substituted benzenes) in a very predictable manner. Likewise, 1,2,4-THB reacts in a very predictable manner.

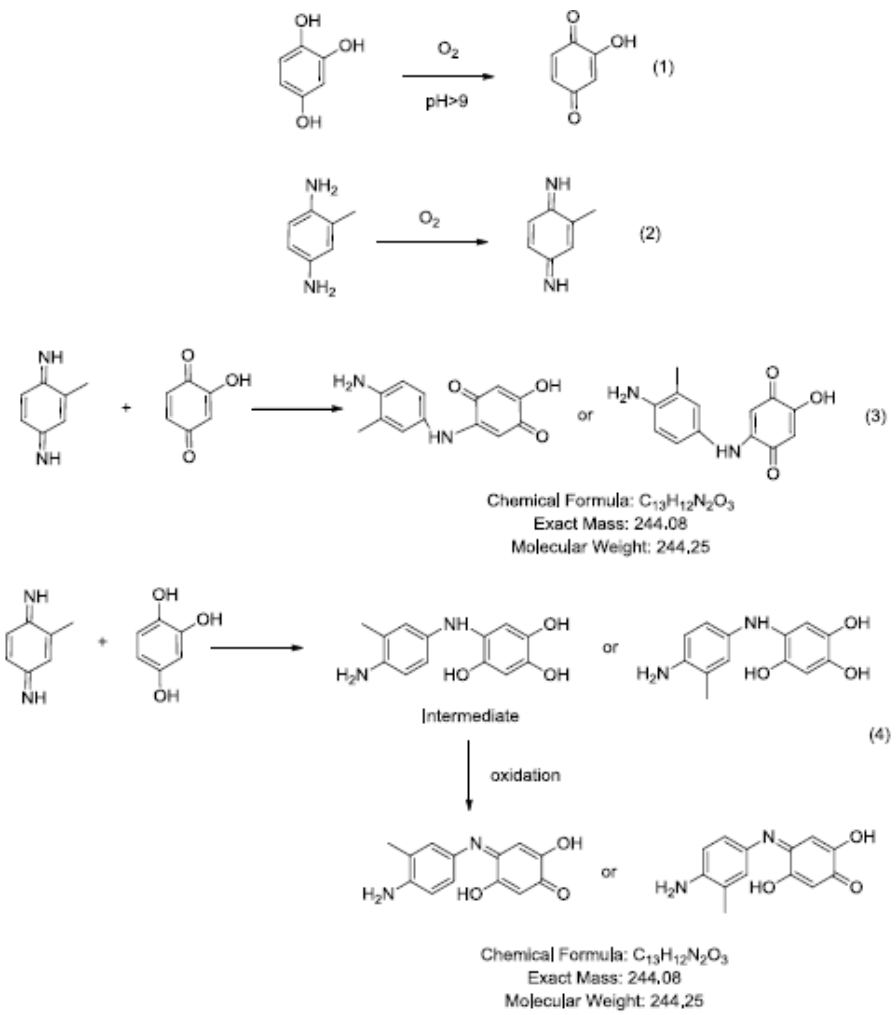


Figure 5a. Proposed reaction mechanism of A33 and A5. **(Ref. 7)**

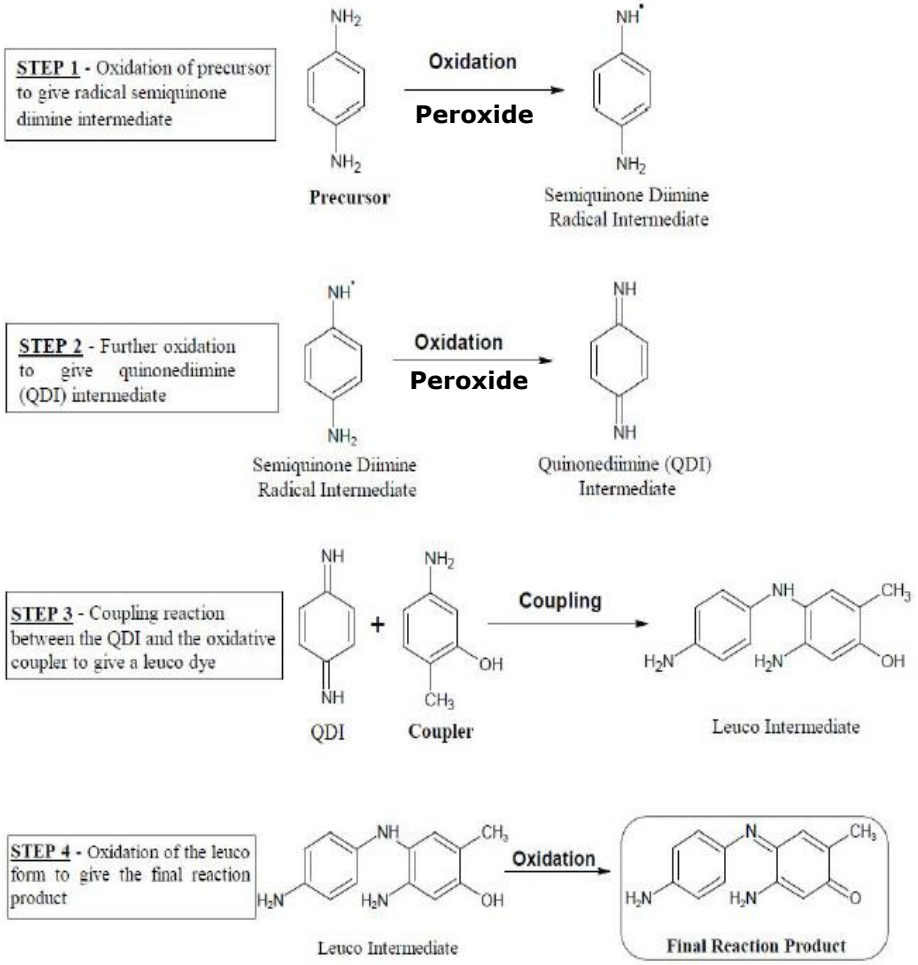
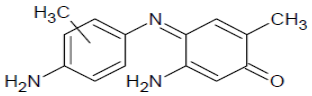
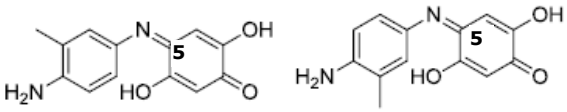
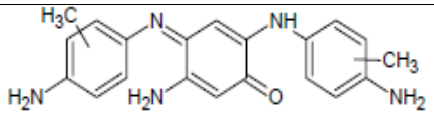
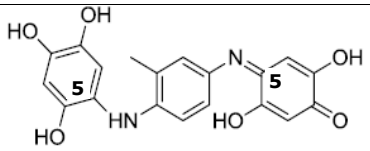


Figure 5b. Mechanism of oxidative hair dye formation, adapted from **(Ref. 11)**

Table 6 compares the reaction products of 1,2,4-THB with a primary intermediate such as PTD and historical data from Industry and summarised in SCCP 0941/05 (Ref. 8). As can be seen here the reaction product dimer (A5-A33) is analogous to the reaction product of PTD with the coupler 4-amino 2-hydroxytoluene (A27). The trimer that is formed (A33-A5-A33) is somewhat analogous to the product of A5 (PTD) with A15 (m-aminophenol).

Table 6. Comparison of reaction products of PTD and 1,2,4-THB with reaction products of PTD and A27 or A15

SCCP 0941/05	1,2,4-THB Reaction Products
 Dimer A5-A27 PTD-AHT (4-Amino 2-Hydroxytoluene)	 Dimer A5-A33 PTD-1,2,4-THB
 Trimer A5-A15-A5 PTD-MAP (m-Aminophenol)	 Trimer A33-A5-A33 THB-PTD-THB

The methanol extracts of hair samples did not yield any dimer or trimer products. This is likely due to the fact that, in the presence of hair, the reactions take place within the hair shaft and the reaction products are expected to be too large to be extracted with just a polar solvent such as methanol.

These data support the categorisation of 1,2,4-THB as an oxidative hair dye. The data further support that 1,2,4-THB has the versatility to react with primary intermediates as a coupler and also to react with coupled products to form trimers, like a primary intermediate. Given its ability to auto-oxidise in air, there is no need of a peroxide to initiate the reaction. All the reaction product studies were conducted in the absence of peroxide. Once the reaction mixture was exposed to air, the oxidative coupling of 1,2,4-THB with an available primary intermediate proceeds rapidly, slowed only by temperature and pH adjustment. Considering the half-volt potential of the molecule, this is only to be expected. The half wave potential of 1,2,4-THB is determined by polarography at pH=10 in a carbonate buffer:

$E_{l/2}$(A33) = -0,375 V/SCE $E_{l/2}$ (Ascorbic Acid) = -0,175 V/SCE (Ref. 1)

This means that 1,2,4-THB is much more oxidisable than ascorbic acid. Ascorbic acid, as is well known, is very easily oxidised. It is this facile oxidisability that allows the use of 1,2,4-THB in hair dye formulations without requiring a two-component system, where the dye

base would otherwise be mixed with a peroxide developer. Rates of reaction are controlled by concentration and pH just as in any other hair dye formulation.

Based on the data, reactions of 1,2,4-THB are atypical in that they do not require a source of reactive oxygen, such as hydrogen peroxide. In hair dye products, 1,2,4-THB performs in a manner that can be considered as a coupler in much the same way as 4-amino-2-hydroxytoluene. The reactions with other oxidative precursors (e.g. PPD, PAP etc.) are expected to yield products that follow established, predicted chemistries. These reaction products do not present any new concern from a toxicity perspective.

Ref.: 7-11

SCCS comment

In response to the SCCS's request for providing chemical structure(s) of any intermediates along with structure of the final products, in case 1,2,4-THB was not used alone in such products, the Applicant confirmed that the only study conducted in the presence of a primary intermediate was the dermal absorption work, which evaluated dermal absorption with the 1,2,4-THB in a simple formulation with PTD (A5). All other studies submitted for the safety evaluation of 1,2,4-THB were conducted with 1,2,4-THB alone.
The reaction of 1,2,4-THB in the presence of the primary intermediate PTD (A5) was studied as a representative example of oxidative coupling with primary intermediates in general. The products that were formed in the study of the reaction chemistry confirmed that the coupled products followed predictable patterns that have been well described in the literature and in the SCCP 1198/08. A relevant example may be found in the SCCP Opinion 1118/07 on tertraaminopyrimidine (A53) which was included in the reaction chemistry work outlined in SCCP/SCCS opinions.
The Applicant projected that the data from the reaction with PTD (A5) would be applicable to the other commonly used primary intermediates and the reaction products would be similarly predictable. In accordance with SCCS 1311/10, the Applicant considered that reactive intermediates formed from coupling of 1,2,4-THB and primary intermediates do not pose any additional safety risks given their short half-lives.
For those reasons, in its submission the Applicant only presented the structure of PTD (A5) (primary intermediate) and the structure of its reaction product with 1,2,4-THB (A33) (coupler).

3.4 Safety evaluation (including calculation of the MoS)

Based on analysis of data provided in all submissions as well as the data from the open literature, the SCCS is of the opinion that genotoxicity potential of 1,2,4-trihydroxybenzene cannot be excluded. Therefore, the SCCS concludes that 1,2,4-trihydroxybenzene is not safe when used as an auto-oxidative hair dye in permanent hair dye formulations.

3.5 Discussion

Physicochemical properties

1,2,4-Trihydroxybenzene is an ingredient used in direct hair colouring products, at a maximum use concentration of 2.5% in a permanent hair dye formulation, and does not require peroxide to activate the oxidation and subsequent coupling reactions. 1,2,4-THB is also used at a maximum formulation concentration of 0.7% in a gradual hair colouring shampoo that does not require hydrogen peroxide to activate the oxidation reaction and subsequent coupling reactions. 1,2,4-THB is intended to be used in the presence of primary intermediates such as p-phenylenediamine (A7), p-toluenediamine (PTD)(A5), N,N bis–(2-hydroxyethyl) p-phenylenediamine (A50) and p-aminophenol (A16), just to name a few.
Information on the purity for the batches THB0200112, THB0200212 and THB0200312, used to perform various studies, should be provided, along with information on the analytical methodology used providing analytical files with representative GC-UV

chromatograms and UV spectrum of the test substance in the vapour phase. Hydroquinone content should be accurately quantified in each batch and their level should be kept at trace level.
Quantification of the impurities as provided by the Applicant cannot be accepted unless:

- The analytical data are provided in a better resolution.
- The Applicant explains the quantitation of the impurity that appears as a double-peak in the GC-FID chromatograms of the batches THB0200112, THB0200212 and THB0200312.
- GC-MS quantitation of the impurities is provided based on the GC-MS data presented in the report.

Stability of 1,2,4-trihydroxybenzene in typical hair dye formulations was not reported except the reaction of 1,2,4-THB in the presence of the primary intermediate PTD (A5) which was studied as a representative example of oxidative coupling with primary intermediates in general.
No data have been provided to indicate the fate of 1,2,4-trihydroxybenzene in the finally applied mixture and hence the extent of consumer exposure to 1,2,4-THB is not known. From the information provided in SCCP/0941/05 Opinion, it is likely that some unreacted 1,2,4-THB will be present and hence, the consumer will be exposed to it.

Toxicological evaluation

All studies submitted for the safety evaluation of 1,2,4-THB (except the dermal absorption work in SM VI conducted in the presence of a primary intermediate, i.e. PTD (A5)) were conducted with 1,2,4-THB alone.

From the previous SCCS Opinion (SCCS 1452/11):
An acute dermal toxicity study in rats was performed, and the maximal non-lethal dose of 1,2,4-trihydroxybenzene was found to be 2000 mg/kg bw. A No Observable Adverse Effect Level (NOAEL) of 50 mg/kg bw/day (90-day, oral, rat) was proposed by the applicant. The SCCP disagreed with this since the relative organ weight was increased significantly in the spleen of male rats treated with 50 mg/kg bw/day. This increase continued dose dependently in male rats treated with either 100 or 200 mg/kg bw/day. The absolute organ weight of the spleen increased also in male rats but this increase was not significant at the dose of 50 mg/kg bw/day. Therefore, the dose of 50 mg/kg bw/day was considered as Lowest Observed Adverse Effect Level (LOAEL). No treatment related effects were seen in a prenatal developmental toxicity study on developmental toxicity parameters up to the highest tested dose of 300 mg/kg bw/day. At 300 mg/kg bw/day a slight maternal toxicity was noted.

Irritation and corrosivity

From the previous SCCS Opinion (SCCS 1452/11):
A 3% dilution of 1,2,4-trihydroxybenzene was found to be slightly irritant to rabbit skin and to the rabbit eye.

Skin sensitisation

From the previous SCCS Opinion (SCCS 1452/11) and Submission VI:
1,2,4-trihydroxybenzene was found to be an extreme skin sensitiser in mice in the Local Lymph Node Assay (LLNA).
In the Keratinosens™ Assay 1,2,4-THB was positive at the concentration of 500 µM. Only average values without standard deviations were provided. Raw data of the three independent experiments were not provided. The dose-response curve showed a huge variation in gene induction at the 500 µM concentration. According to the prediction model of OECD TG442D, the Keratinosens™ assay is positive when gene induction is statistically significant from the solvent control in at least 2 out of 3 replicates. This statistical analysis was not provided. The results of this assay are therefore inconclusive. In addition, data from the Keratinosens™ assay cannot be used on their own to predict the potency of a test chemical.

Percutaneous absorption

From the Submission VI:

The experiment was conducted with frozen human dermatomed skin. [^{14}C]-1,2,4-THB and unlabelled 1,2,4-THB were incorporated into hair dye formulations, with and without p-toluenediamine (PTD, 2.25%) to provide final concentrations of 2.5% (w/w) [^{14}C]-1,2,4-THB. The SCCS noted that the thickness of the dermatomed skin has not been provided. Moreover, it was not clear why the dermal absorption of 1,2,4-THB with PTD was nearly double than without PTD, i.e. 1.94 + 1.76 = 3.7 µg-eq (with PTD) vs. 1.13 + 0.58 = 1.71 µg-eq (without PTD). Considering the reaction chemistry and formation of dimers (PTD-1,2,4-THB) and trimers (THB-PTD-THB) (see the section Special Investigation) it was expected to be lower than without PTD. Based on the results, for potential calculation of MoS the SCCS would suggest to take the highest value of the systemically available dose of [^{14}C]-1,2,4-THB with PTD, i.e. 1.94 µg equivalents/cm^2 + 1.76 (1SD) or 3.70 µg equivalents/cm^2.

Mutagenicity / genotoxicity

1,2,4-Trihydroxybenzene was clearly positive in the GLP-compliant Ames test studies submitted twice, i.e. in Submission V and VI. The GLP-compliant *in vitro* mammalian gene mutations test (Submission V) and micronucleus test (Submission VI) were negative. Additionally, in Submission VI the Applicant provided a study on the 3D skin comet assay model with negative results. The review conducted by the SCCS of a lot of supplementary data from the open literature indicates genotoxic/mutagenic effects of 1,2,4-THB detected using different endpoints under *in vitro* conditions including direct DNA damaging effects, chromosomal aberrations and aneuploidy (see the list of references 17-35 attached to the Opinion). The positive literature findings from the *in vitro* tests covering both chromosome aberrations and aneuploidy were not confirmed in an *in vivo* test provided by applicant (*in vivo* micronucleus test, Submission V), where the exposure of mice to 1,2,4-THB did not result in an increase in erythrocytes with micronuclei. It should be noted however, that this test was not performed in accordance with the current OECD guideline (only one dose was tested) and thus has limited value.

Based on the analysis, the SCCS is of the opinion that genotoxicity potential of 1,2,4-trihydroxybenzene cannot be excluded.

The SCCS notes that according to the Opinion SCCP/0971/06 "The potential to induce mutagenic / genotoxic effects has to be excluded for (i) the precursors and couplers, (ii) the formed products and (iii) the intermediates formed". However, assuming a presence of not completely reacted 1,2,4-THB in final products, for which many positive genotoxic results have been described, the SCCS was able to conclude on genotoxic hazard of 1,2,4-THB without the need for conducting additional studies.

Carcinogenicity

From the previous SCCS Opinion (SCCS 1452/11):

No conclusion with regard to carcinogenicity can be made from the mice topical application carcinogenicity study submitted.

Special investigation

In response to the SCCS's request in it Opinion (SCCS/1452/11), the reaction products resulting from use of 1,2,4-THB in a typical hair dye formulation were explored with some necessary modifications in order to be able to isolate intermediate that indicate reaction progress from a mechanistic standpoint. The data obtained supported the categorisation of 1,2,4-THB as an oxidative hair dye. The data also supported that 1,2,4-THB has the versatility to react with primary intermediates as a coupler and also to react with coupled products to form trimers, like a primary intermediate. Given its ability to auto-oxidise in air, there was no need of a peroxide to initiate the reaction. All the reaction product studies were conducted in the absence of peroxide. Once the reaction mixture was exposed to air, the oxidative coupling of 1,2,4-THB with an available primary intermediate proceeded rapidly, slowed only by temperature and pH adjustment.

As already mentioned, no data have been provided to indicate the fate of 1,2,4-trihydroxybenzene in the finally applied mixtures and hence the extent of consumer exposure to 1,2,4-THB is not known. From the information provided in SCCP/0941/05 Opinion, it is likely that some unreacted 1,2,4-THB will be present and hence, the consumer will be exposed to it.

4. CONCLUSION

- *In light of the data provided, does the SCCS consider 1,2,4-trihydroxybenzene (1,2,4-THB) (A33) safe for use as an auto-oxidative hair dye in permanent hair dye formulations, not requiring the action of peroxide, with a maximum on-head concentration of 2.5%?*

  On the basis of all the data submitted by the Applicant, and data available in open literature, the SCCS considers that 1,2,4-trihydroxybenzene is not safe due to potential genotoxicity when used as an auto-oxidative hair dye in permanent hair dye formulations.

- *In light of the data provided, does the SCCS consider 1,2,4-trihydroxybenzene (1,2,4-THB) (A33) safe for use as an auto-oxidative hair dye in hair colour shampoo formulations, not requiring the action of peroxide, with a maximum on-head concentration of 0.7%?*

  /

- *Does the SCCS have any further scientific concerns with regard to the use of 1,2,4-trihydroxybenzene (1,2,4-THB) (A33) in cosmetic products?*

  No data have been provided to indicate the fate of 1,2,4-trihydroxybenzene in the finally applied mixtures and hence the extent of consumer exposure to 1,2,4-THB and transformation products is not known.

5. MINORITY OPINION

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6. REFERENCES

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7. GLOSSARY OF TERMS

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8. LIST OF ABBREVIATIONS

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