



Scientific Committee on Consumer Safety
SCCS

OPINION ON
hair dye 1,2,4-trihydroxybenzene (1,2,4-THB)
COLIPA n° A33
(CAS 533-73-3)
Submission VI



The SCCS adopted this Opinion
at its plenary meeting on 20-21 June 2019

ACKNOWLEDGMENTS

Members of the Working Group are acknowledged for their valuable contribution to this Opinion. The members of the Working Group are:

For the Preliminary version

SCCS members:

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

SCCS external experts:

Dr A. Koutsodimou
Dr A. Simonnard

For the Final version

SCCS members:

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

SCCS external experts:

Dr A. Koutsodimou
Dr A. Simonnard
Dr. W. Uter

All Declarations of Working Group members are available on the following webpage:
http://ec.europa.eu/health/scientific_committees/experts/declarations/sccs_en.htm

This Opinion has been subject to a commenting period of a minimum eight weeks after its initial publication (from 02 July until 10 September 2018).

Comments received during this time period were considered by the SCCS. For this Opinion, comments received resulted in the following main changes: *physico-chem part, chapter 3.3.7 and its SCCS comment, chapter 3.3.11 and its SCCS comments as well as their respective parts under discussion and conclusion. References have been added.*

1. 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 the data available in open literature, the SCCS does not consider 1,2,4-trihydroxybenzene safe due to potential genotoxicity when used as an auto-oxidative hair dye component in permanent hair dye formulations.

The data provided by the Applicant indicate that consumers would be exposed to unreacted 1,2,4-THB and (semi)quinones during the use of a 1,2,4-THB containing hair dye formulation. The systemic availability of 1,2,4-THB raises the risk of intracellular generation of hydrogen peroxide, as well as potential genotoxicity via DNA adduct formation by 1,2,4-THB and/or (semi)quinones. These aspects have not been addressed in the studies provided in the submission.

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%?*

See above.

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?*

/

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

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion 1,2,4-trihydroxybenzene (1,2,4-THB) - A33 – Submission VI, preliminary version of 21-22 June 2018, final version of 20-21 June 2019, SCCS/1598/18

About the Scientific Committees

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

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

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

SCCS

The Committee shall provide Opinions on questions concerning 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.).

Scientific Committee members

Bernauer Ulrike, Bodin Laurent, Chaudhry Qasim, Coenraads Pieter-Jan, Dusinska Maria, Ezendam Janine, Gaffet Eric, Galli Corrado Lodovico, Granum Berit, Panteri Eirini, Rogiers Vera, Rousselle Christophe, Stępnik Maciej, Vanhaecke Tamara, Wijnhoven Susan

Contact

European Commission
Health and Food Safety
Directorate C: Public Health, Country Knowledge, Crisis Management
Unit C2 – Country Knowledge and Scientific Committees
L-2920 Luxembourg
SANTE-C2-SCCS@ec.europa.eu

© European Union, 2020

ISSN 1831-4767

ISBN 978-92-76-15249-1

Doi:10.2875/01768

EW-AQ-20-003-EN-N

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

http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

TABLE OF CONTENTS

| | |
|---|----|
| ACKNOWLEDGMENTS | 2 |
| 1. ABSTRACT | 4 |
| 2. MANDATE FROM THE EUROPEAN COMMISSION | 7 |
| 3. OPINION..... | 8 |
| 3.1 Chemical and Physical Specifications..... | 8 |
| 3.1.1 Chemical identity | 8 |
| 3.1.2 Physical form..... | 9 |
| 3.1.3 Molecular weight..... | 9 |
| 3.1.4 Purity, composition and substance codes | 9 |
| 3.1.5 Impurities / accompanying contaminants..... | 9 |
| 3.1.6 Solubility | 13 |
| 3.1.7 Partition coefficient (Log P _{ow})..... | 13 |
| 3.1.8 Additional physical and chemical specifications | 13 |
| 3.1.9 Homogeneity and Stability | 13 |
| 3.2 Function and uses..... | 15 |
| 3.3 Toxicological evaluation..... | 15 |
| 3.3.1 Acute toxicity | 16 |
| 3.3.2 Irritation and corrosivity | 16 |
| 3.3.3 Skin sensitisation..... | 16 |
| 3.3.4 Toxicokinetics..... | 18 |
| 3.3.5 Repeated dose toxicity | 21 |
| 3.3.6 Reproductive toxicity | 21 |
| 3.3.7 Mutagenicity / genotoxicity | 22 |
| 3.3.8 Carcinogenicity | 36 |
| 3.3.9 Photo-induced toxicity | 36 |
| 3.3.10 Human data | 36 |
| 3.3.11 Special investigations | 36 |
| 3.4 Safety evaluation (including calculation of the MoS)..... | 50 |
| 3.5 Discussion | 50 |
| 4. CONCLUSION..... | 53 |
| 5. MINORITY OPINION | 53 |
| 6. REFERENCES | 54 |
| 7. GLOSSARY OF TERMS | 56 |
| 8. LIST OF ABBREVIATIONS | 56 |

2. MANDATE FROM THE EUROPEAN COMMISSION

Background

Submission I on 1,2,4-trihydroxybenzene (1,2,4-THB) was submitted by COLIPA¹ 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 Applicant 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%?
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?

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

3. 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

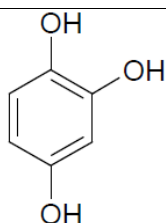
3.1.1.3 Trade names and abbreviations

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: C₆H₆O₃

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.

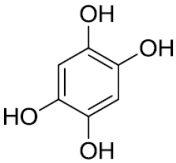
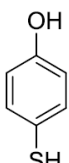
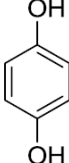
Purity: 97.8%
Impurities: <0.5%
Loss on Drying: <0.5%
Residue on Ignition: <0.5%

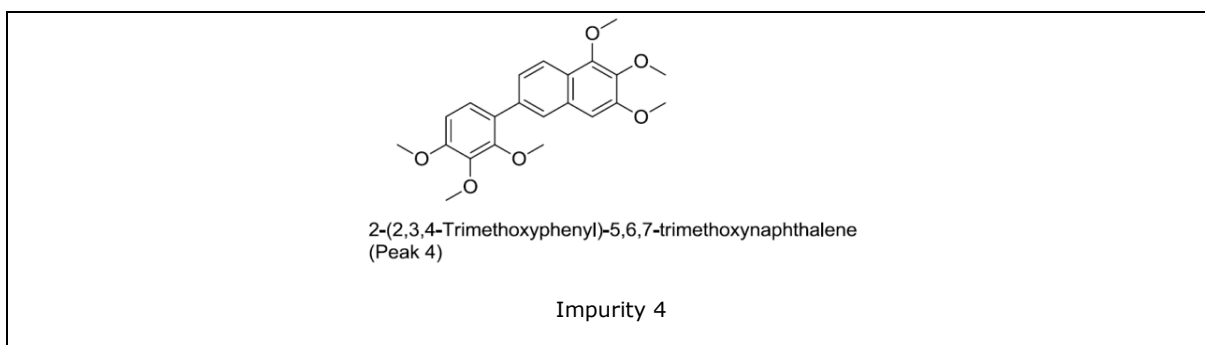
SCCS comment

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

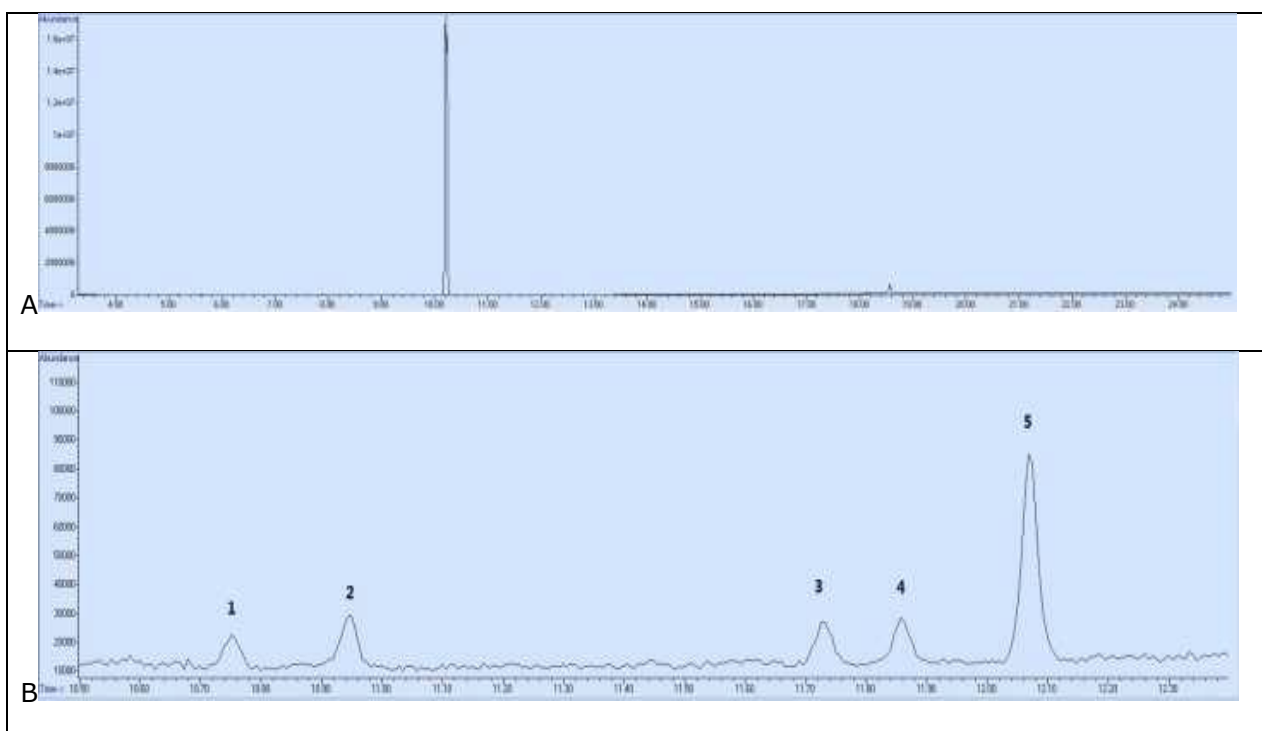
The impurities in three 1,2,4-trihydroxybenzene (1,2,4-THB) lots were identified by NMR spectroscopy (both ¹H and ¹³C) and GC-MS and were quantified by GC-FID.

| | | |
|---|--|--|
|  <p>benzene-1,2,4,5-tetraol (Peak 3 and 5 (isomers))</p> <p>Impurity 1</p> |  <p>4-mercaptophenol (Peak 1)</p> <p>Impurity 2</p> |  <p>hydroquinone (Peak 2)</p> <p>Impurity 3</p> |
|---|--|--|



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.

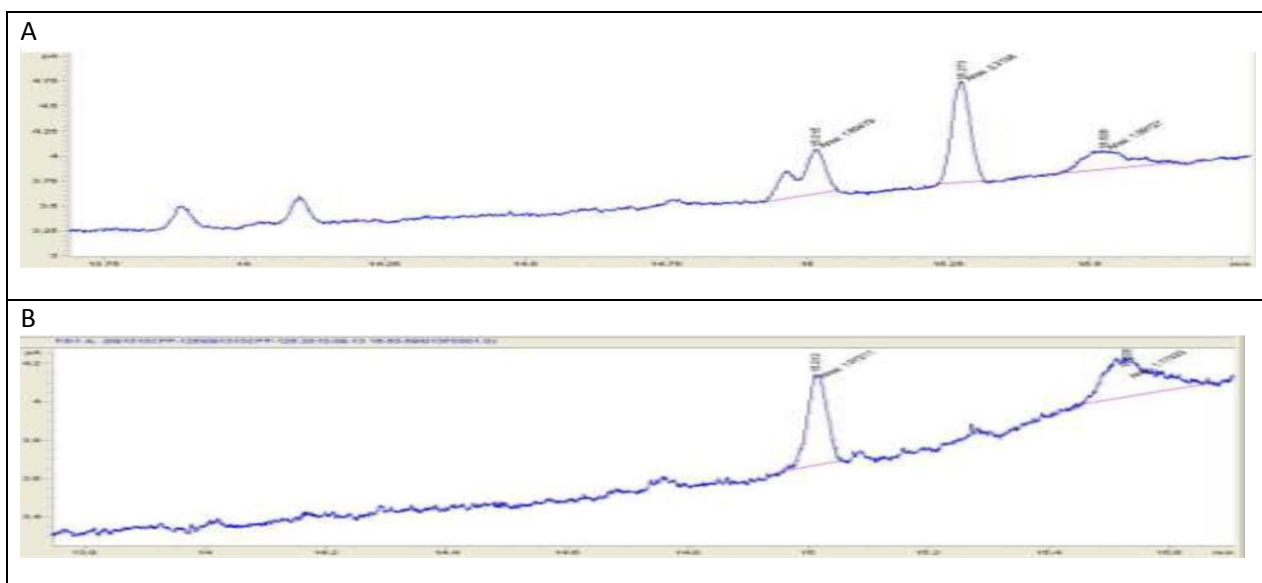
Figure 1. GC-MS chromatogram of THB0200112 (CO1787) with impurity peaks



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-trimethoxynaphthalene) 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

Solubility (g/100 ml - 22°C after 24 h)

- Water * : S = 20
- Ethanol : 1=S<10
- DMSO : 10=S<20

* Hydrosolubility according to OECD method A6 : 486 g/L at 20°C

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): | λ _{max} 291 nm |

Ref.: 1

3.1.9 Homogeneity and Stability**From submission V**

- 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.

Data provided in 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 on physicochemical 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 its level should be kept at a trace level.
- Quantification of the impurities as provided by the Applicant can be accepted when:
 - o 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 not readable.
 - o 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.
 - o 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%.

Data provided in 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

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).

Data provided in 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 μ M |
| Solvent: | DMSO in a final concentration of 1% in 1% DMEM |
| Positive control: | Cinnamic Aldehyde (4, 8, 16, 32 and 64 μ M) |
| 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 μ M
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 (μ M) | Mean IC ₅₀ (μ M) 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-phenylenediamine) 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 500 µM. 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, the SCCS considers 1,2,4-THB an extreme skin sensitiser based on the LLNA results (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/cm².

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 |

[¹⁴C]-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) [¹⁴C]-1,2,4-THB. The formulations were applied to the skin surface at a dose of 20 mg/cm² 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 [¹⁴C]-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 [¹⁴C]-1,2,4-THB and [¹⁴C]-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 n = 11 | 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 | |
|----------------------------|--|-------|--|-------|---|-------|--|-------|
| | 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 $1.13 \pm 0.58 \mu\text{g-eq } [^{14}\text{C}]\text{-1,2,4-THB/cm}^2$ (mean \pm 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 $1.94 + 1.76 \mu\text{g-eq } [^{14}\text{C}]\text{-1,2,4-THB/cm}^2$ (mean \pm 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 $1.13 \mu\text{g equivalents/cm}^2 + 0.58$ (1SD) or $1.71 \mu\text{g equivalents/cm}^2$. The systemically available dose of [^{14}C]-1,2,4-THB with PTD is $1.94 \mu\text{g equivalents/cm}^2 + 1.76$ (1SD) or $3.70 \mu\text{g equivalents/cm}^2$.

Ref.: 13

SCCS comment

The SCCS has 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 than without PTD, i.e. $1.94 + 1.76 = 3.7 \mu\text{g-eq}$ (with PTD) vs. $1.13 + 0.58 = 1.71 \mu\text{g-eq}$ (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 could be expected to be lower with PTD than without PTD. Based on the results, for 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. $1.94 \mu\text{g equivalents/cm}^2 + 1.76 (1\text{SD}) = 3.70 \mu\text{g equivalents/cm}^2$.

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*

Data provided in Submission VI

Gene mutation assay using bacteria

| | |
|-----------------|---|
| Guideline: | OECD 471 |
| Test system: | <i>Salmonella typhimurium</i> 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 <i>S. typhimurium</i> 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 (NaN₃): 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

Data provided in Submission VI with Supplemental Research, Data and Comments of February 19, 2019

As a follow-up to the comments submitted to the SCCS on September 7, 2018, the applicant performed the additional studies with Ames test with catalase and GSH.

Ames Bacterial Reverse Mutation with Catalase and GSH

| | |
|----------------------|--|
| Guideline: | OECD 471 (adopted July 21, 1997); partial since only one test strain was used |
| Species/strain: | Salmonella typhimurium TA1537 |
| Replicates: | Triplicate plates in the absence of metabolic activation and in the absence or presence of either catalase or glutathione reduced (GSH) |
| Test substance: | 1,2,4-Trihydroxybenzene (1,2,4-THB) |
| Solvent: | Deaerated water (N ₂ -purged) |
| Lot#: | THB0318002 |
| Purity: | 98.1% |
| Vehicle: | Deaerated water (N ₂ -purged) |
| Concentrations: | 100, 150, 175, 200, 250, 500 µg/plate without scavengers or metabolic activation. The same concentrations of 1,2,4-THB with 1000, 10,000 or 20,000 IU catalase or 5, 10 or 15 µM GSH |
| Assay modifications: | Standard Assay, i.e. 1,2,4-THB alone, where all components are added to a tube containing 2.0 mL of molten selective top agar at 45±2°C and Control Assay, i.e. 1,2,4-THB alone, where 2.0 mL of molten selective top agar is the last component added to the tube |
| Treatment: | plate incorporation, 48-72h incubation in the absence of metabolic activation |
| GLP: | in compliance |
| Study period: | Dec 2018; final report Jan 2019 |

The aim of the study

The purpose of this study was to evaluate the effect of radical scavengers, catalase and L-glutathione reduced (GSH) on the mutagenic response of 1,2,4-THB in *S. typhimurium* TA1537 without metabolic activation, that had been observed in a previous GLP study (SCCS 1598/18, Ref 14).

Material and methods

Dose solutions were prepared in deaerated water in a glove box where an inert environment was maintained with nitrogen gas in order to minimize the air oxidation of 1,2,4-THB prior to application. Six concentrations of 1,2,4-THB from 100-500 µg/plate were tested. The actual concentration of the test substance in the dose solutions was determined by UPLC-UV using a validated method. Both a concurrent vehicle (degassed water) and 9-aminoacridine positive control were used. Triplicate test plates were used per 1,2,4-THB concentration or per control. Toxicity was detected by a decrease in the number of revertants and clearing or reduction of the background lawn. Precipitation of the test item was recorded as applicable.

Standard Assay controls were conducted where the components were added to a tube already containing the molten top agar to verify the previously observed mutagenic response. In the treatments including scavengers, all the components were mixed before the molten top agar was added to provide exposure of the bacteria to the test article and ROS scavenger directly. Controls were conducted without scavengers following the same procedure where the top agar was added last.

Results

In both the Standard and Control Assays, the negative and positive controls met the acceptance criteria and the study was considered valid. Three-fold (minimum criteria for a positive response) or greater increases in mutation frequency in TA 1537 were observed at 150 and 175 µg/plate 1,2,4-THB (standard method) and at 150, 175 and 200 µg/plate (control method). Toxicity was observed at 250 and 500 µg/plate. No precipitate was observed. Treatments with 1,2,4-THB with GSH (5-10 µM) were negative whereas at 15 µM GSH, 175 and 200 µg/plate 1,2,4-THB were still positive (3.6 and 3.2-fold increase in mutations respectively). All concentrations of 1,2,4-THB were negative in the presence of catalase at all doses (1000, 10,000, 20,000 IU). Toxicity was also reduced in the presence of catalase but not with GSH.

Conclusion

Under the conditions of this study, the previous mutagenic effect of 1,2,4-THB in TA1537 in the absence of metabolic activation (GLP study # AE03RS.502.BTL, 2015) was reproduced. This mutagenic effect was eliminated in the presence of 5 µM and 10 µM GSH, and in the presence of 1,000, 10,000 and 20,000 Units of catalase. Catalase also reduced the toxicity of 1,2,4-THB.

SCCS comment

- In the comment to the study #14 SMVI (Bacterial Reverse Mutation Assay of 1,2,4-Trihydroxybenzene, BioReliance Corporation, Wagner, V.O., Report issued August 10, 2015) the SCCS indeed stated that the results of the study indicated a clear mutagenic effect of 1,2,4-THB in the absence of S9 mix in TA1537 strain. However, in the previous Opinion (SCCS 1452/11, Ref. 1) the SCCS stated that 1,2,4-THB 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. It is therefore not clear why the applicant performed the additional studies with antioxidant factors using *S. typhimurium* TA1537 strain only.

TA1537 strain is known for very low spontaneous revertant numbers (Mortelmans K., E. Zeiger. Mutation Research 455 (2000) 29–60, Ref. 21) hence detection of any inhibitory effect of an anti-oxidant on a relatively weak mutagenic effect of A33 is *a priori* more

difficult, compared to other strains (TA98 and TA100). It would therefore be more relevant to conduct the studies in the latter two strains.

- The effect of GSH on the mutagenic effect of A33 is not clear. The Applicant admitted that the results with GSH at 15 µM "are positive with a 3.6-fold, maximum increase. The results are dose-responsive, and the mean revertant count at the dose level where the maximum fold increase was observed is outside of the 95% HCL". The SCCS has performed additional statistical analysis of all the results, which indicate that: there was a significant effect of GSH at 5 µM when A33 was used at 150-250 µg/plate; no effect of GSH at 10 µM; and separated significant effects of GSH at 15 µM when A33 was used at 150 or 250 µg/plate. Overall, the effects of GSH seemed to be random and not concentration-dependent.

Ref.: 17

Data provided in Submission VI

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: | <p>Preliminary test (range-finder):</p> <p>±S9 mix (4 h exposure + 20 h): 0.126, 0.378, 1.26, 3.78, 12.6, 37.8, 126, 378, 1260 µg/mL</p> <p>-S9 mix (24 h exposure): 0.126, 0.378, 1.26, 3.78, 12.6, 37.8, 126, 378, 1260 µg/mL</p> <p>Exp1:</p> <p>-S9 mix (4 h exposure + 20 h): 1.26, 3.15, 6.3, 12.5, 25, 50, 85, 100, 125, 135, 150 µg/mL</p> <p>-S9 mix (24 h exposure): 0.1, 0.25, 0.5, 1.26, 12.5, 30, 40, 50, 85, 100 µg/mL</p> <p>+S9 mix (4 h exposure + 20 h): 12.5, 25, 50, 100, 125, 135, 150 µg/mL</p> |
| Solvent/negative control: | culture medium |
| Positive Controls: | <p>-S9 mix: Vinblastine (VB, 5, 7.5 and 10 ng/mL)</p> <p>+S9 mix: Cyclophosphamide (CP, 2.5, 5 and 7.5 µg/mL)</p> |
| 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 $\leq 5.0\%$ 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 $\leq 5.0\%$ 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 [$\geq 50\%$ cytokinesis-blocked proliferation index (CBPI) relative to the vehicle control] observed at dose levels $\geq 126 \mu\text{g/mL}$ 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 $\mu\text{g/mL}$. At the highest test concentration, 50 $\mu\text{g/mL}$, 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 $\mu\text{g/mL}$. At the highest test concentration, 100 $\mu\text{g/mL}$, 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 $\mu\text{g/mL}$. At the highest test concentration, 30 $\mu\text{g/mL}$, 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

Data provided in Submission VI with Supplemental Research, Data and Comments of February 19, 2019

3D Human Reconstructed Skin Micronucleus Assay using EpiDerm™

Guideline: Cosmetics Europe Genotoxicity Taskforce Protocol (Ref.: Dahl et al. The reconstructed skin micronucleus assay (RSMN) in EpiDerm™: detailed protocol and harmonized scoring atlas. Mutat Res., 720: 42-52, 2011)

Skin model: MatTek EpiDerm™

Replicates: One tissue model per concentration in dose range finding assay. Three tissue models per concentration in Experiments 1,2,3

Test Substance: 1,2,4-Trihydroxybenzene (1,2,4-THB)

Solvent: Deaerated acetone (N₂-purged)

Lot #: THB0318002

Purity: 98.1%

Concentrations: Dose range finding assay 48 hour exposure: 0.50-200 µg/cm²
Experiment 1: 48-hour total exposure: 1.5-200 µg/cm²
Experiment 2: 48-hour exposure: 12-224 µg/cm²
Experiment 3 (confirmatory trial): 72-hour exposure: 3-224 µg/cm²

Treatment: Experiment 1, 48-hour total exposure: repeated applications at 48 and 24 hours before cell isolation
Experiment 2, 72-hour total exposure: repeated applications at 72, 48 and 24 hours before cell isolation

Positive controls: mitomycin C (5 and 6 µg/mL), carbendazim (0.5 and 0.75 µg/mL), cyclophosphamide (50 and 60 mg/mL)

GLP: in compliance

Study Period: November 16, 2018 – March 27, 2019

Materials and methods

EpiDerm™ tissue from the MatTek Corporation (Ashland, MA, USA) was used in the experiments. The dose solutions of 1,2,4-THB were prepared fresh each day in deaerated acetone in a glove box where an inert environment was maintained with nitrogen gas in order to minimize the air oxidation of 1,2,4-THB prior to application. In the dose range finding study, nine concentrations from 0.50-200 µg/cm² were tested based on results of a previous 3D Comet Assay using Phenion® Reconstructed Human Skin (Submission VI, SCCS 1598/18 Ref. 16). Toxicity was observed at 50 µg/cm² (56% reduction in Cytochalasin B Proliferation Index, CBPI) and higher. In Experiment 1 and 2 with a 48 h exposure seven concentrations of 12-224 µg/cm² were tested.

Test article dosing solutions and 3 µg/mL cytoB in New Maintenance Medium (NMM) were prepared fresh on the day of use. The actual concentration of the test substance in the dose solutions was determined by UPLC-UV using a validated method.

The models were re-fed with fresh medium, warm (~37°C) NMM containing 3.0 µg/mL cytoB, and then dosed on the dermal surface with 10 µL of the test article dosing solution with appropriate target concentration, vehicle control, or positive control. The dosing solution was placed on the surface of the models, tilting the plate gently to help ensure that the surface of the model was covered by the dosing solution. After dosing, the models were incubated under standard conditions (37 ± 1°C in a humidified atmosphere of 5 ± 1% CO₂ in air).

After 24 hours (±3), the medium was carefully removed and the models were re-fed with fresh warm NMM containing 3 µg/mL cytoB, dosed again on the dermal surface with 10 µL dosing solution, and incubated under standard conditions for 24 hours, then harvested.

Since the result of the definitive micronucleus assay (Exp 1 and 2) was negative, a **confirmatory trial** was conducted with a 3-day treatment regimen using triplicate tissues (ten concentrations of 3-224 µg/cm² were tested).

At least two slides were prepared from each EpiDerm™ model, whenever possible (one slide was kept as back up). Slides were air dried at room temperature. The slides were stained with acridine orange. A minimum of 1000 binucleated cells with intact and red-stained cytoplasm were analyzed per tissue (minimum of 500 BN cells per tissue).

Cytotoxicity measurements included Cytochalasin B Proliferation Index (CBPI; at least 1,500 cells (500 cells per culture), if possible, were evaluated to determine the CBPI at each dose level and the control) and relative decrease in viable cell counts (RVCC; Trypan-blue exclusion method).

Results

Dose Formulation Analysis

The test article dose formulation analysis was conducted by the analytical chemistry laboratory at BioReliance and a copy of the analytical report is included in Appendix IV. The results of the analysis indicate that the actual mean concentrations of the analyzed formulation samples, 0.75 and 14 mg/mL, were 106.1 and 110.0% of target, respectively, with S/L ratios of > 0.925 in the first dose formulations from Experiment B2 and 96.5 and 110.1% of target, respectively, with a S/L ratio of > 0.925 for the high dose but < 0.925 for the low dose in the second dose formulations from Experiment B2.

The results of the analysis from the first, second and third doses in Experiment C1 indicate that the actual mean concentrations of the analyzed formulation samples, 0.1875 and 14 mg/mL, were 121.5 to 128.9% of target with S/L ratios of > 0.925. To verify the results observed, the original samples from the first dose were re-injected and the backup low dose and residual high dose formulations were also analyzed, and the results of the analysis confirm the original results observed (121.3 to 127.4% of target with S/L ratios of > 0.925 with the exception of the residual high dose sample which was < 0.925).

This indicates that the formulations were accurately prepared, except as indicated above. The actual concentrations of 0.1875 and 14 mg/mL samples in Experiment C1 were higher than the protocol-specified acceptance criterion, indicating that the test guideline recommended limit dose was exceeded. Therefore, the Study Director has concluded that the difference in nominal concentration at the high dose had no adverse impact on the study results or validity of the study conclusion. No test article was detected in the vehicle control samples.

Additionally, 1, 2, 4-Trihydroxybenzene in degassed acetone, at concentrations of 0.796 and 15.4 mg/mL, was stable on wet ice for at least 3.1 hours. Additional analysis indicated that 1, 2, 4-Trihydroxybenzene in degassed acetone, at concentrations of 17.0, 17.4 and 17.6 mg/mL, was stable on wet ice for at least 3.0 hours.

The preliminary toxicity test

The test was conducted using a 2-day dosing regimen (48-hour treatment) by exposing a single tissue per concentration to vehicle alone and nine concentrations (0.03 to 12.5 mg/mL) of the test article. Cytotoxicity [$\geq 50\%$ cytokinesis-blocked proliferation index (CBPI) relative to the vehicle control] was observed at concentrations ≥ 3.13 mg/mL. Cytotoxicity [$\geq 50\%$ reduction in viable cell count (RVCC) relative to the vehicle control] was observed at concentrations ≥ 6.25 mg/mL.

Colored material was visible (unaided eye) on top of the tissue at concentrations ≥ 0.78 mg/mL at the end of the first treatment and at concentrations ≥ 0.39 mg/mL at the end of the second treatment. At the end of first treatment, the color of the medium gradually became dark pink at concentrations ≥ 3.13 mg/mL and the skin coloration gradually became more dark and black. At the end of second treatment, the color of the medium gradually became dark pink at concentrations ≥ 1.56 mg/mL and the skin coloration gradually became more dark and black. Based on information from the Sponsor, the coloration on the skin was expected due to the nature of the test article (hair dye) and it was verified as indicated below this was not precipitation.

Experiment 1: The first micronucleus assay (48-hour treatment)

Based upon the results of the preliminary test, the micronucleus assay was conducted using triplicate tissues at concentrations ranging from 0.09 to 12.50 mg/mL (1.5-200 $\mu\text{g}/\text{cm}^2$).

In the test, cytotoxicity (50 to 60% CBPI and RVCC relative to the vehicle control) was observed at 12.5 mg/mL. The tissues were examined by phase contrast microscope to determine the nature of the skin coloration, and no visible precipitate was observed at the end of first or second treatments. At the end of the first treatment, the color of the tissues became gradually more dark black over the concentration range tested and color of the media became gradually pinker over concentrations of 3.13 to 12.5 mg/mL. At the end of second treatment, the color of the tissues became gradually more dark black over the concentration range tested and color of the media became gradually pinker over concentrations of 1.56 to 12.5 mg/mL.

Experiment 2: The repeat micronucleus assay (48-hour treatment)

Due to a possible technical error in dose formulation preparation which resulted in a shift in cytotoxicity ($>50\%$ at 12.5 mg/mL vs 3.13 mg/mL in preliminary toxicity test), the micronucleus assay was repeated at concentrations ranging from 0.75 to 14 mg/mL (12-224 $\mu\text{g}/\text{cm}^2$). In the test, cytotoxicity ($\geq 50\%$ CBPI relative to the vehicle control) was observed at concentrations ≥ 6.25 mg/mL. Cytotoxicity ($\geq 50\%$ RVCC relative to the vehicle control) was observed at concentrations ≥ 12.5 mg/mL. The tissues were examined by phase contrast microscope and no visible precipitate was observed at the end of first or second treatments. At the end of first treatment, the color of the tissues became gradually more dark black over the concentration range tested and color of the media became gradually pinker over concentrations of 1.5 to 14 mg/mL. At the end of second treatment, the color of the tissues became gradually more dark black and the color of media became gradually pinker over the concentration range tested. The concentrations selected for evaluation of micronuclei were 1.5, 3.125, and 6.25 mg/mL (59% toxicity at 6.25 mg/mL by CBPI).

No significant or dose-dependent increase in micronuclei induction was observed ($p > 0.05$; Fisher's Exact and Cochran-Armitage tests). A statistically significant increase in micronuclei induction was observed in the MMC and CBZ (positive controls) group ($p \leq 0.01$, Fisher's Exact test).

Experiment 3: Confirmatory trial (72-hour treatment)

Since the result of the micronucleus assay using a 2-day dosing regimen (48-hour treatment) was negative, a confirmatory assay was conducted with a 3-day dosing regimen (72-hour treatment) at concentrations ranging from 0.1875 to 14 mg/mL (3-224 µg/cm²) using triplicate tissues. In the confirmatory micronucleus assay, cytotoxicity (50 to 60% CBPI relative to the vehicle control) was observed at concentrations ≥ 6.25 mg/mL. Cytotoxicity (50 to 60% RVCC) was observed at concentrations ≥ 4.5 mg/mL. The concentrations selected for evaluation of micronuclei were 0.75, 1.5, and 4.5 mg/mL.

No significant or dose-dependent increases in micronuclei induction were observed at any concentration ($p > 0.05$; Fisher's Exact and Cochran-Armitage tests). A statistically significant increase in micronuclei induction was observed in the CP and CBZ (positive controls) group ($p \leq 0.01$, Fisher's Exact test).

Conclusion

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 reconstructed skin micronucleus assay (RSMN) in EpiDermTM.

Ref.: 18

SCCS comment

- For Historical Control Values MNBN 95% Control Limits are 0.01-0.22% (range 0.03-0.27%). According to Table 4 (p.29) 1,2,4-THB treatment for 48 h at 6.25 mg/mL (the highest concentration used) induced MNBN of 0.27%±0.12 with solvent control of 0.13%±0.06. This is a borderline value, but acceptable considering that the exposure to acetone (solvent) in Exp 3 after 72 h induced MNBN at 0.23%±0.06. Similar borderline value of MNBN (0.27%±0.15) was observed after 72 h exposure to 1,2,4-THB at 4.5 mg/mL (the highest concentration used).
- It is not clear why 95% Lower Control Limit for the historical positive control value is 0.00 (13. APPENDIX I: Historical Control Data, p.35, p.36 CBZ). It needs explanation, as it indicates that in some cultures the positive controls induced 0% MNBN, i.e. had no effect at all.

Data provided in Submission VI**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 $\mu\text{g}/\text{cm}^2$

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 $\mu\text{g}/\text{cm}^2$

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 $\mu\text{g}/\text{cm}^2$; aphidicolin (APC) was added to the culture medium 4 h prior to cell isolation at a final concentration of 5 $\mu\text{g}/\text{mL}$

Solvent/negative

control:

acetone degassed

Positive Controls:

Methyl methanesulfonate (MMS, 3h exposure at 3.16 mg/mL = 5 $\mu\text{g}/\text{cm}^2$)
Benzo[a]pyrene (BaP, 48 h, 24 h, 3 h exposure at 0.78 mg/mL = 12.5 $\mu\text{g}/\text{cm}^2$; with and without APC)

Vehicle:

acetone degassed with nitrogen (a clear, brown stock solution of A33 at 100 mg/mL; corresponding to 1600 $\mu\text{g}/\text{cm}^2$)

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 °C and ca. 5% CO₂. 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 $16 \mu\text{L}/\text{cm}^2$ 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 $1600 \mu\text{g}/\text{cm}^2$). 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 $50 \mu\text{g}/\text{cm}^2$ based on the AK assay and at $16 \mu\text{g}/\text{cm}^2$ based on LDH. As a result, $16 \mu\text{g}/\text{cm}^2$ 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 $16 \mu\text{g}/\text{cm}^2$ 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 $20 \mu\text{g}/\text{cm}^2$ 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 $20 \mu\text{g}/\text{cm}^2$ 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 $16 \mu\text{g}/\text{cm}^2$.

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 comments

- The *vitro* comet assay is currently under validation, and no OECD guideline is yet available.
- It is stated in the study report that 1,2,4-THB at a concentration of 16 µg/cm² is not cytotoxic. However, analysis of the data at 16 µg/cm² in all the cytotoxicity tests indicates that the values are already significantly increased compared to the control. Thus applying this concentration, and certainly a higher concentration (i.e. 20 µg/cm²), is not justified.
- A clear dose-related brown staining of the media and skin samples was observed, which 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.
- 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 studies on the 3D skin comet assay and 3D skin micronucleus test with negative results. In the *in vivo* micronucleus test provided by applicant (Submission V), the exposure of mice to 1,2,4-THB did not result in an increase in erythrocytes with micronuclei. However, it should be noted, that this test was not performed in accordance with the current OECD guideline (only one dose was tested) and thus has limited value.

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 using different endpoints under *in vitro*

conditions including direct DNA damaging effects, chromosomal aberrations and aneuploidy (see the list of references 22-40 attached to the Opinion).

The SCCS acknowledges the Applicant's reply that 1,2,4-THB is a genotoxin *in vitro*. It is strongly suggested by the Applicant that the increased levels of H₂O₂ after exposure to 1,2,4-THB would be due to the generation of superoxide anion which either enzymatically or non-enzymatically dismutates to H₂O₂. The SCCS is of the opinion that a major part of DNA damage (oxidative type) after exposure to 1,2,4-THB is induced by ROS as a result of H₂O₂ generation caused by 1,2,4-THB. However, it needs to be shown that the damage is exclusively ROS-related (preferably when generated intracellularly) and no other reactive radicals, such as semiquinone radicals, are generated, or that no mutations are induced in case they are formed. These aspects have not been addressed in the studies provided in the submission. Assuming a presence of not completely reacted 1,2,4-THB in final product, 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 additional studies.

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

3.3.8 Carcinogenicity

From Submission V

No conclusion with regard to carcinogenicity could 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

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

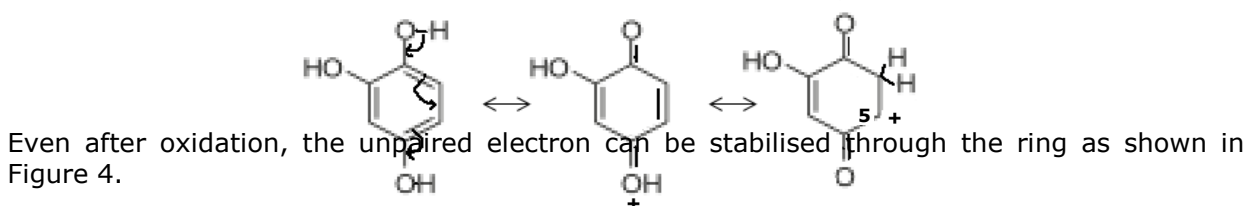
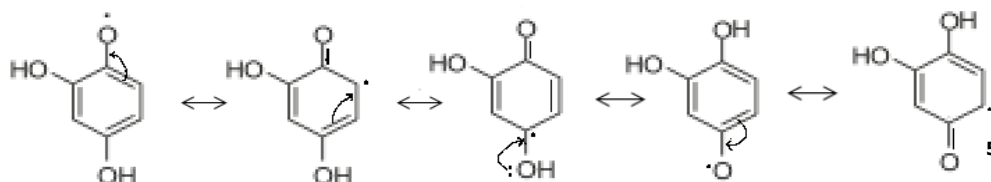


Figure 4. Some resonance structures for phenoxyl radical of 1,2,4-THB



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*-disubstituted 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.

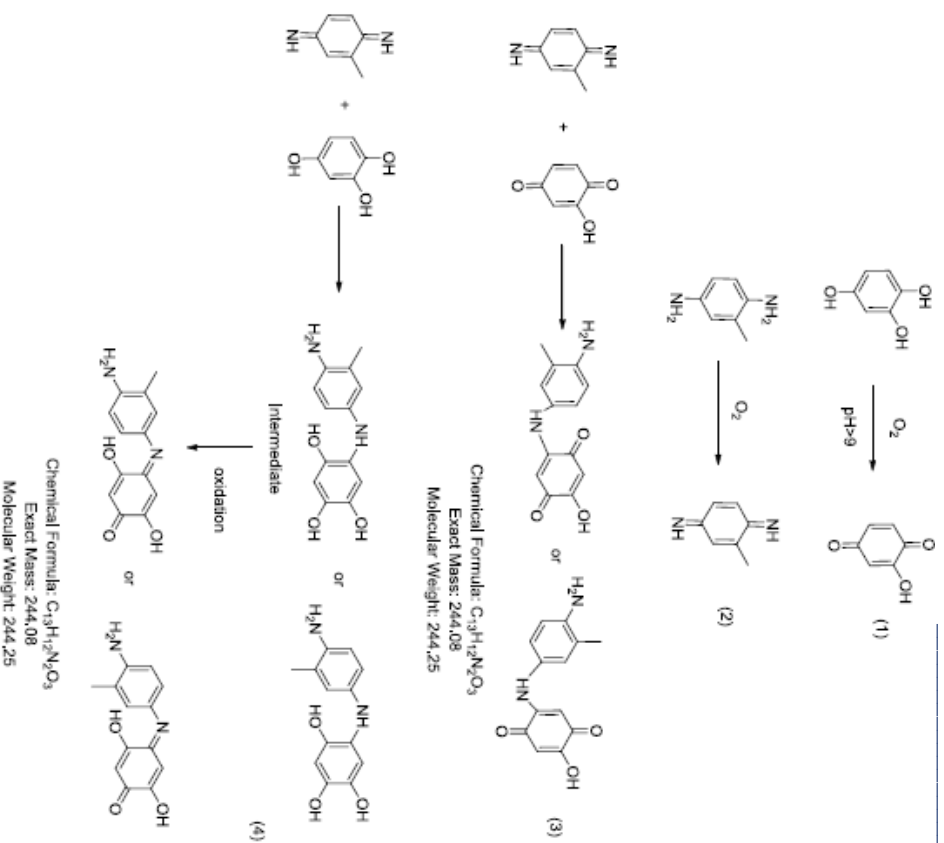


Figure 5a. Proposed reaction mechanism of A33 and A5.
(Ref. 7)

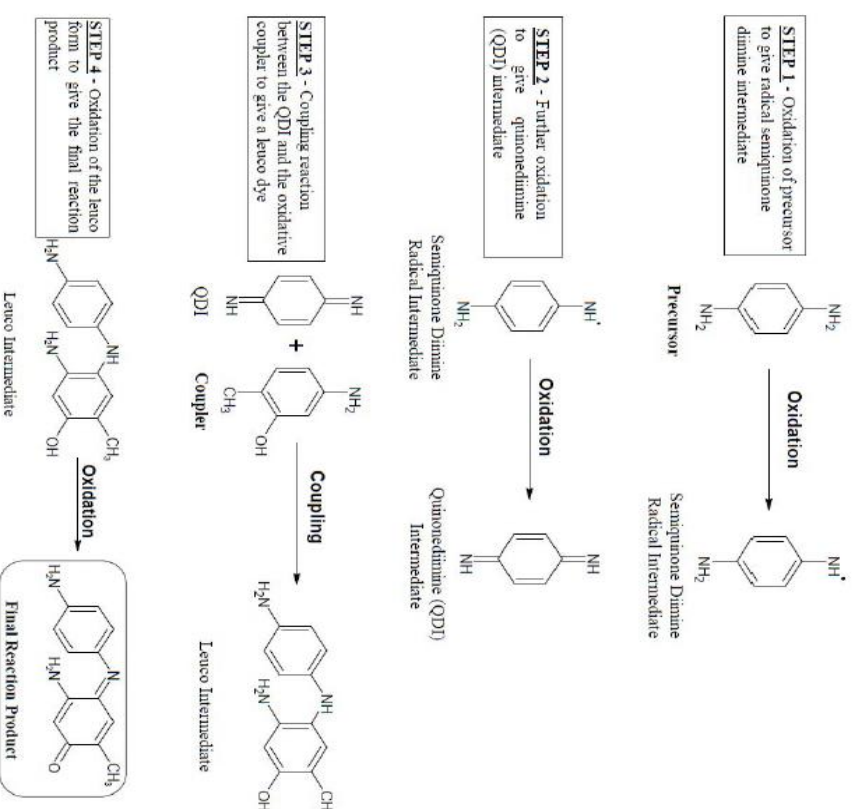
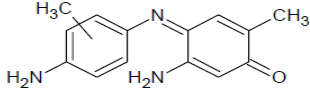
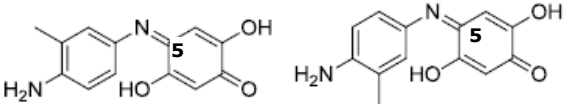
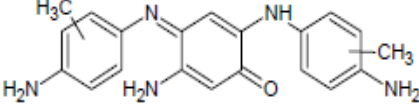
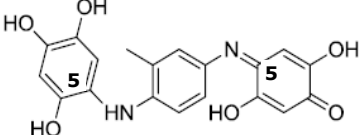


Figure 5b. Mechanism of oxidative hair dye formation, adapted from
(Ref. 11)

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 |
|--|--|
|  <p>Dimer A5-A27 PTD-AHT (4-Amino 2-Hydroxytoluene)</p> |  <p>Dimer A5-A33 PTD-1,2,4-THB</p> |
|  <p>Trimer A5-A15-A5 PTD-MAP (m-Aminophenol)</p> |  <p>Trimer A33-A5-A33 THB-PTD-THB</p> |

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:

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

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 tetraaminopyrimidine (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).

Data provided in Submission VI with Supplemental Research, Data and Comments on February 19, 2019**3.3.11.2 Hydrogen Peroxide Quantification by Fox Assay**

In order to quantitatively determine hydrogen peroxide formation from 1,2,4-THB in various solvent media, FOX assay was conducted by the applicant's in-house analytical group.

| | |
|--------------------------|--|
| Test Article: | 1,2,4-THB; Lot # THB 0318002; Purity: 98.1% |
| Control: | Hydrogen Peroxide solution: 30% (w/w); Sigma-Aldrich |
| Assay Kit: | National Diagnostic Hydrogen Peroxide Assay Kit; Catalog # CL-204 |
| Solvent Systems: | Deionized Water: DI Water; ≥ 18.0 m Ω , in-house Sodium Phosphate Monobasic Monohydrate (NaH ₂ PO ₄ •H ₂ O) \geq Sigma-Aldrich Phosphate-Buffer Saline (PBS); pH 7.4, Thermo-Fisher Gibco RPMI 1640: Thermo-Fisher, Catalog # 11875 Gibco Penicillin-Streptomycin, Thermo-Fisher, Catalog # 15070063 Fetal Bovine Serum (FBS): Heat Inactivated, Sigma-Aldrich # F4135 |
| Spectrophotometer: | Perkin Elmer 365 UV-Vis Spectrophotometer |
| Scan Range: | 400-700 nm |
| Scan Speed: | 200 nm/min |
| Resolution: | 1 nm |
| Wavelength of detection: | 560 nm |
| GLP compliance: | not stated |

Material and methods

The FOX Assay is a rapid, sensitive and quantitative method for the determination of hydrogen peroxide in chemical or biological systems. The assay is based on formation of a complex between Xylenol Orange and Ferric ion (Fe^{3+}), which is produced by the peroxide dependent oxidation of ferrous iron (Fe^{2+}) and is extensively used for measuring H_2O_2 (Halliwell, 2014). The Fe^{3+} ion will form a purple colored complex with Xylenol Orange, which absorbs at 560 nm. The formation of hydrogen peroxide was measured as a function of concentration and time in phosphate buffer at pH 7.4.

The quantitative generation of hydrogen peroxide from 1,2,4-THB was further evaluated in various solvents used for preparing dosing solutions commonly reported in literature for *in vitro* and *in vivo* evaluation of genotoxic effects of 1,2,4 THB. The time point of 30 minutes was assumed as the time between preparation of dosing solution to actual dosing.

Results

As seen in Table below, phosphate buffer-saline (PBS, pH 7.4) provided the most efficient matrix for the generation of hydrogen peroxide. In this solvent, 1 mole of 1,2,4 THB generates 0.9 moles of hydrogen peroxide. Deionized water was the least efficient solvent in generating hydrogen peroxide possibly due to lack of buffering capacity to maintain a pH.

Hydrogen peroxide formation as a function of solvent system

| Dosing Solvent* | 1,2,4-THB Concentration (μM) | Measured [H_2O_2] (μM)** |
|---|---|---|
| Deionized Water | 50 | 4.40 \pm 0.09 |
| | 100 | 4.80 \pm 0.14 |
| Deaerated Deionized Water, N_2 -filled glove box | 50 | 2.30 \pm 0.43 |
| | 100 | 3.40 \pm 0.58 |
| Phosphate Buffer (pH 7.4) | 50 | 25.00 \pm 0.46 |
| | 100 | 75.70 \pm 0.70 |
| Deaerated Phosphate Buffer (pH 7.4), N_2 -filled glove box | 50 | 16.00 \pm 0.40 |
| | 100 | 19.30 \pm 0.68 |
| Phosphate Buffer Saline (PBS, pH 7.4), | 50 | 44.80 \pm 0.42 |
| | 100 | 90.00 \pm 2.20 |
| Deaerated Phosphate Buffer Saline (PBS, pH 7.4), N_2 -filled glove box | 50 | 27.10 \pm 0.93 |
| | 100 | 21.80 \pm 0.40 |
| Serum-Free RPMI | 50 | 29.80 \pm 0.20 |
| | 100 | 81.90 \pm 0.40 |
| RPMI 1640 Complete Media | 50 | 2.50 \pm 0.10 |
| | 100 | 8.10 \pm 0.20 |

*TA at two representative concentrations in dosing solvents were incubated for 30 min at 25°C

** Average of three replicates

Conclusion

The data collected in this study confirm evidence in the literature and demonstrates that 1,2,4-THB is a spontaneous hydrogen peroxide releasing compound in 'cell-free' solution on exposure to atmospheric oxygen. The release of hydrogen peroxide is facile, quantitative and is time, solvent, pH and buffer, ionic salts and transition metal dependent. The

efficiency of the dosing solvents in catalyzing hydrogen peroxide generation from 1,2,4-THB may be depicted as follows: PBS > Serum Free RPMI > Phosphate buffer > RPMI > Water. It is apparent from these data that the formation of hydrogen peroxide in representative dosing solutions is significant, measureable and biologically relevant.

Ref.: 19

SCCS comment

- The effects of solvents and de-aeration on generation of hydrogen peroxide for 1,2,4-THB were studied after 30 min of incubation at 25°C. Data on longer incubation times (up to 48 h) were provided only for PBS and indicated rapid generation of H₂O₂ that sustained over a period of 48 h. The routinely used incubation times in genotoxicity studies on mammalian cells are 3 or 24 hrs. Moreover, the cell exposure is almost always conducted in complete culture media, i.e. with 10% serum. It is therefore unclear what would be the rate of generation of H₂O₂ in complete culture media within this time span (3 to 24 hrs). The information is particularly important considering that in RPMI 1640 complete medium the generation of H₂O₂ by 1,2,4-THB (both at 50 or 100 µM) was remarkably low.
- The applicant admitted that "the lack of generation of hydrogen peroxide in RPMI 1640 complete media is likely due to protein binding activity of 1,2,4 THB with serum proteins including albumin and prealbumin". Considering the above mentioned contrast between H₂O₂ generation by 1,2,4-THB in PBS and RPMI 1640 complete medium, the potential involvement of H₂O₂ in mediating the genotoxic effects of 1,2,4-THB becomes doubtful.
- The study provides some evidence of H₂O₂ generation in aqueous media, however still this is a proof for extracellular generation of H₂O₂. If 1,2,4-THB rapidly generates H₂O₂ extracellularly, then the molecule should be transformed into semiquinone radicals known to induce mutagenic effects. The applicant does not discuss this potential chemical reaction and its consequences for the cell.

3.3.11.3 Time dependent hair color simulating consumer exposure

Test Article: Representative Hair Color Formulation, with 2% 1,2,4-THB
Hair Swatches: 90% grey virgin hair swatches
Rinse: HPLC grade deaerated acidified water (formic acid); pH 3.1
HPLC: Shimadzu RD-LC 1 (HPLC 909 PDA)
Column: FluoroSep-RP Phenyl/HS 5µm 60A (250 x 4.60) mm
Mobile Phase: 80% Sodium Citrate Buffer pH 6.4, 20% MeOH
Oven: 40°C
Flow: 1.0 mL/min
Spectrophotometer: Minolta CM-600d

The aim

To estimate consumer exposure to 1,2,4-THB using simulated hair coloring conditions at various time points with a representative hair color formulation. Concentration of unreacted 1,2,4-THB over time is determined by HPLC assay.

Material and methods

A representative gel-cream based hair color formulation with 2% 1,2,4-THB at pH 9, was made in deaerated water purged with nitrogen. A placebo formulation was made under the same conditions without 1,2,4-THB. To measure concentrations of unreacted 1,2,4-THB at each time point, a reversed phase HPLC method was developed and a calibration curve was established using known concentrations of 1,2,4-THB (RT=4.7 min) and validated for

linearity, precision and accuracy. To measure the initial amount of 1,2,4-THB recovery from the formula, a ~2.0 gram aliquot of formulation was dispersed in 200 mL deaerated, acidified water (pH 3.1). Deaerated acidified water is used to arrest 1,2,4-THB oxidation during sample preparation for HPLC, to ensure accurate measurement of 1,2,4-THB concentrations. A sample (5 μ L) of this solution is injected into the HPLC and the concentration of 1,2,4-THB measured is designated as T0, prior to exposure to the hair swatch. For the simulated hair coloring, ~2 grams of formulation was applied to 1 gram of virgin hair using a brush to distribute the product. After a one minute exposure, the formulation was rinsed from the swatch using HPLC grade deaerated, acidified water to arrest the reaction for HPLC analysis. The rinsate was collected and sonicated for one minute and an aliquot was drawn, filtered through a 0.45 μ m HPLC syringe filter and injected into the HPLC to determine the level of 1,2,4-THB in the rinsate.

In this same manner, the additional time points of 5, 15, and 30 minutes were analyzed. This experiment was done in duplicate to verify the decrease in 1,2,4-THB concentration over time.

Results

The data in Figure below show that within the first five minutes more than 70% of the 1,2,4-THB has reacted and ~95% of the 1,2,4-THB has reacted by the end of 15 minutes and less than 3% remains at 30 minutes. This time course study shows that in a representative formulation when applied to hair, 1,2,4-THB undergoes rapid oxidation when exposed to the atmosphere.

Percent Concentration of 1,2,4-THB in the rinsate v Time (min)

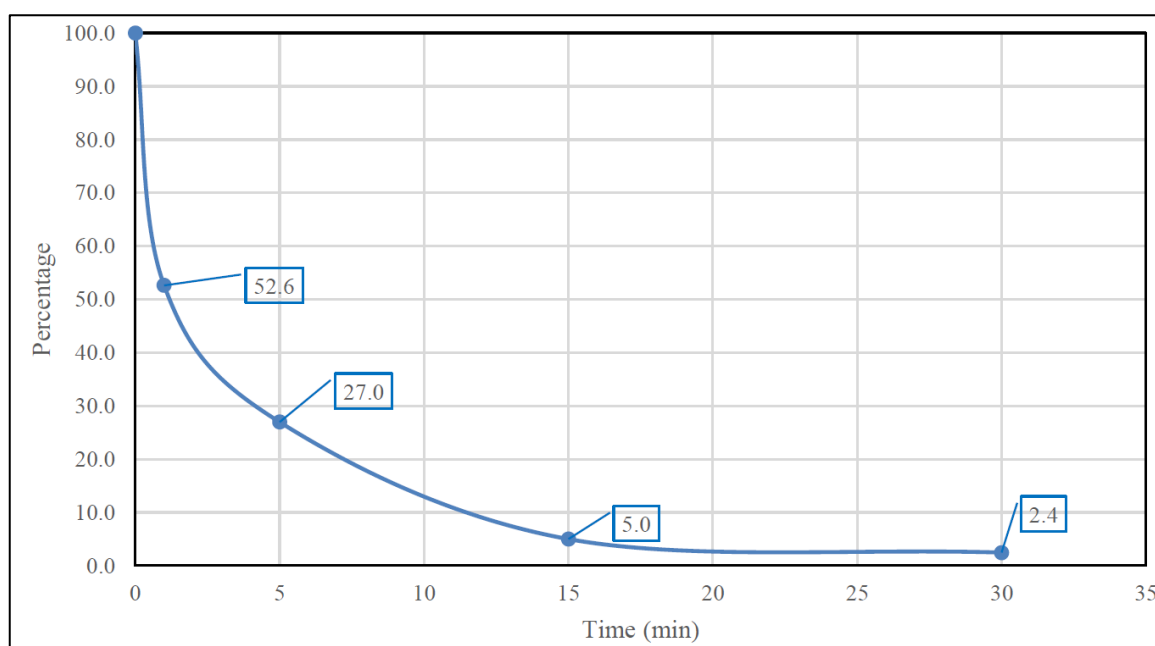


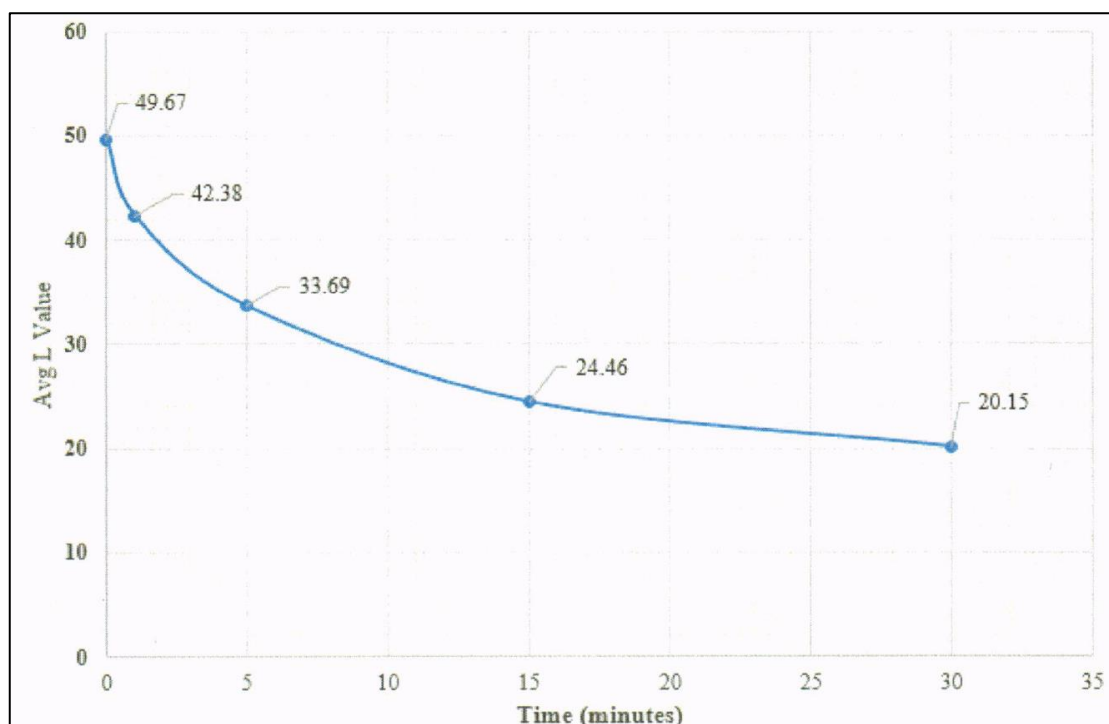
Figure below shows the corresponding color development in the hair swatches for each time point of exposure.

Color Development in hair at various exposure times with representative formulation.



Swatch color intensity, expressed as lightness or “L” values were also measured with Minolta Spectrophotometer from each trial and the average L value plotted versus time in Figure below. The lower “L” values correspond to darker hair. The decrease in L values as a function of time, which correspond to changes in 1,2,4-THB concentration, shows an increase in color intensity (or a decrease in lightness) as seen visually. Each value of L represents the average of two trials.

Average L values v. Time (min)



Conclusion

The data collected in this study are presented along with the HPLC chromatograms in the report. These data suggest that the expected consumer exposure to 1,2,4-THB is low, not only based on its limited skin permeability, but also based on the rapid decline in concentration in alkaline medium in the presence of hair under normal use conditions.

Ref.: 20

SCCS comments

The SCCS' concerns over potential consumer exposure are based on: 1) the provided data clearly indicate that a proportion of 1,2,4-THB remains unreacted in the final hair dye formulation, and 2) transformation of 1,2,4-THB also results in the generation of (semi)quinones.

- Figure 4 in the file 'Supplemental Research, Data and Comments' of February 19, 2019 shows the concentration of remaining 1,2,4-THB in the rinsate over time. It is notable that in the hair dye formulation studied, the amount of unreacted 1,2,4-THB after 5, 15 and 30 minutes was 27%, 5% and 2.4% respectively - which amounts to 5400, 1000 and 480 part per million respectively. These are quite considerable amounts, which means that by using a 1,2,4-THB containing hair dye formulation, the consumer would be exposed to considerable levels of 1,2,4-THB at the start and throughout a normal application period of 30 minutes. This, combined with the evidence for systemic exposure to by 1,2,4-THB through dermal route, raises the concern that generation of hydrogen peroxide and (semi)quinones may not only take place extracellularly but also inside the cells.
- The main emphasis of the kinetic study provided (Reference 7_Avomeen Applicant Interim Report 032514) is to study the kinetics of A33 and the coupler A5 and to detect dimer/trimers. However, it does provide evidence that 1,2,4-THB not only generates H₂O₂ but also (semi)quinones. The LS-MS data provided in Figure 6 shows a sizeable peak of hydroxyquinone (RT 5.18 min), alongside 1,2,4-THB (RT 3.33 min), even at time zero in the tested formulation. Regarding the reaction in the presence of ammonium, it notes "As shown in Figure 11, in 30 min, the production of the quinone and dimer of A33 is more significant compared with the ethanolamine reaction". At higher concentration, the quinone form of the dimer of A33 is observed as another product as shown in Figure 12.' It further points out that 'After 30 min, the dimers were observed in the solution as shown in Figure 19, but the concentration was low and the peaks of the reactants are still very significant.' Even after the dying process, it has noted that 'The MeOH extract contained mostly the A5 and quinone or dimer of A33' as shown in Figure 21.
- The Applicant has provided a mechanistic interpretation to regard the observed genotoxic effects due to the generation of reactive oxygen species during auto-oxidation of 1,2,4-THB. It is plausible that oxyradical generation plays a (major) part in the genotoxicity of 1,2,4-THB. However, this is unlikely to be the only mechanism as direct DNA adduct formation by (semi)quinones is also known. Therefore, it has to be underscored that in parallel to the decrease in concentrations of 1,2,4-THB in a freshly prepared formulation, generation of (semi)quinones increases. The Applicant has not discussed this aspect of the chemical reaction and its potential consequence for the consumer.

Opinion on hair dye 1,2,4-trihydroxybenzene (1,2,4-THB)- A33 - Submission VI

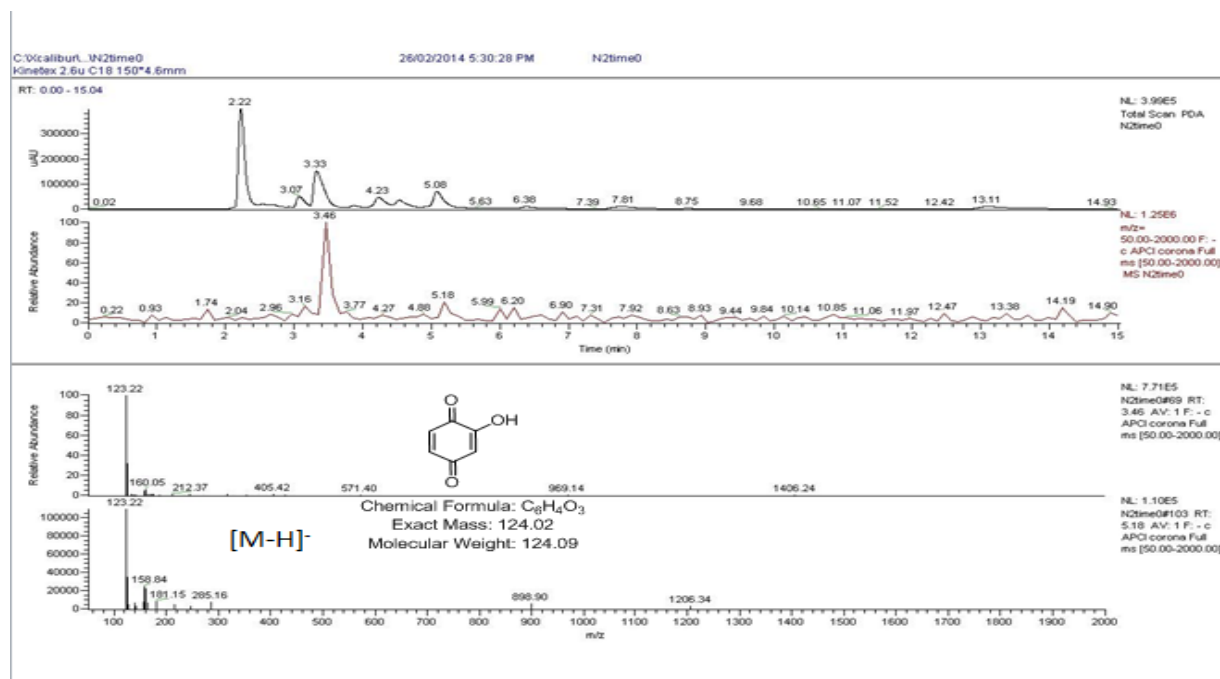


Figure 6. Ethanolamine RXN at time 0 (1/15 dilution) (Reference 7_Avomeen Applicant Interim Report 032514)

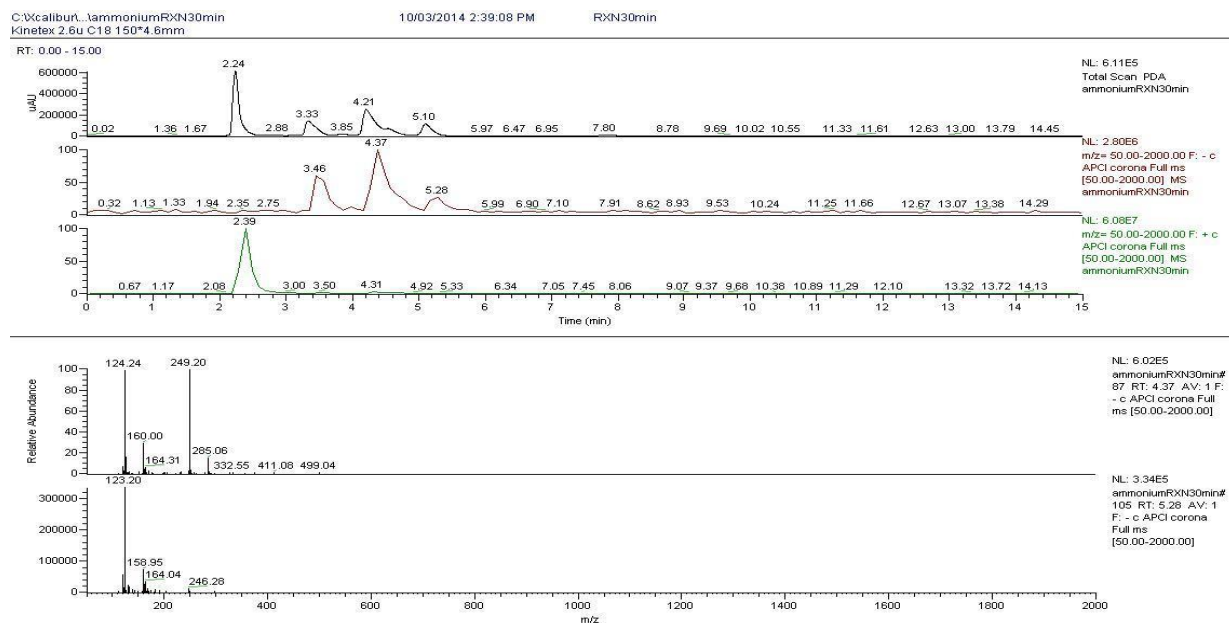


Figure 11. A33 and A5 reaction using ammonium to adjust pH (1/15 dilution) (Reference 7_Avomeen Applicant Interim Report 032514)

Opinion on hair dye 1,2,4-trihydroxybenzene (1,2,4-THB)- A33 - Submission VI

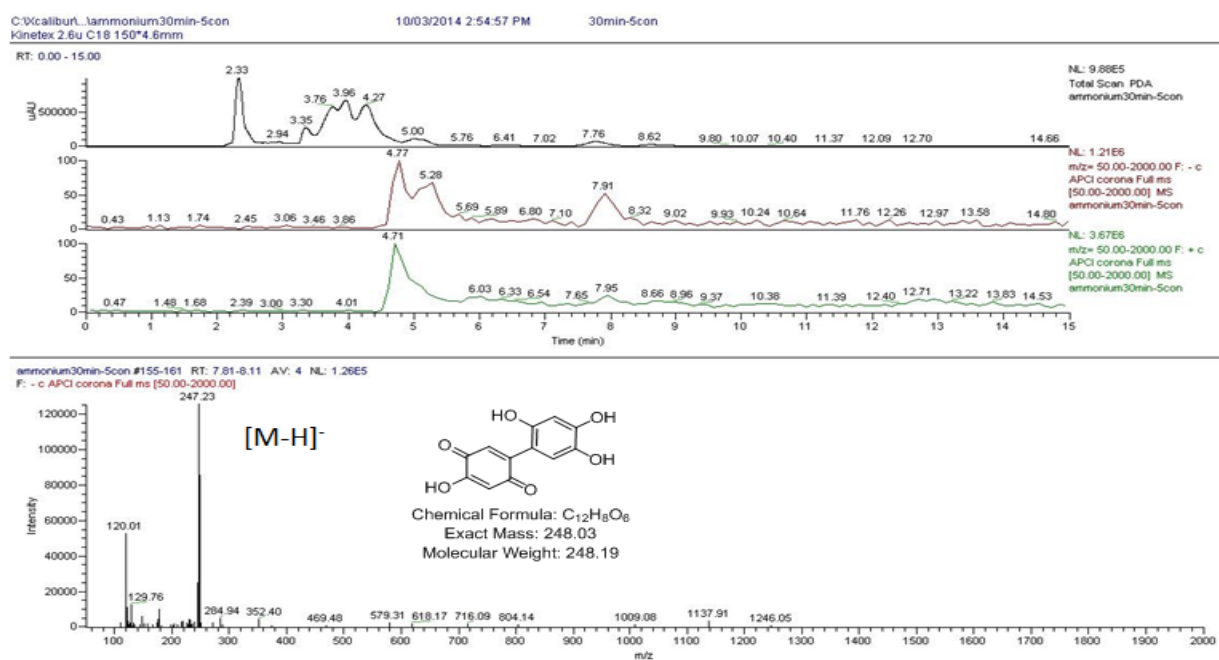


Figure 12. Quinone form of A33 dimer in ammonium RXN (1/3 dilution) (Reference 7_Avomeen Applicant Interim Report 032514)

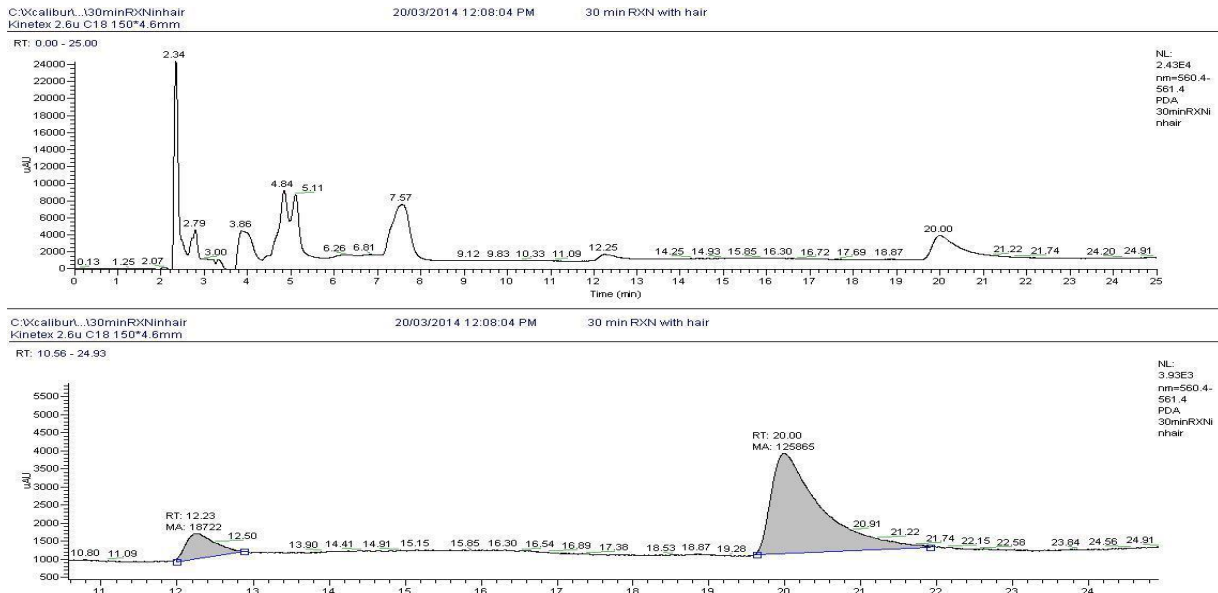


Figure 19. Integration of dimers observed in the presence of hair for 30 min (1/3 dilution) (Reference 7_Avomeen Applicant Interim Report 032514)

Opinion on hair dye 1,2,4-trihydroxybenzene (1,2,4-THB)- A33 - Submission VI

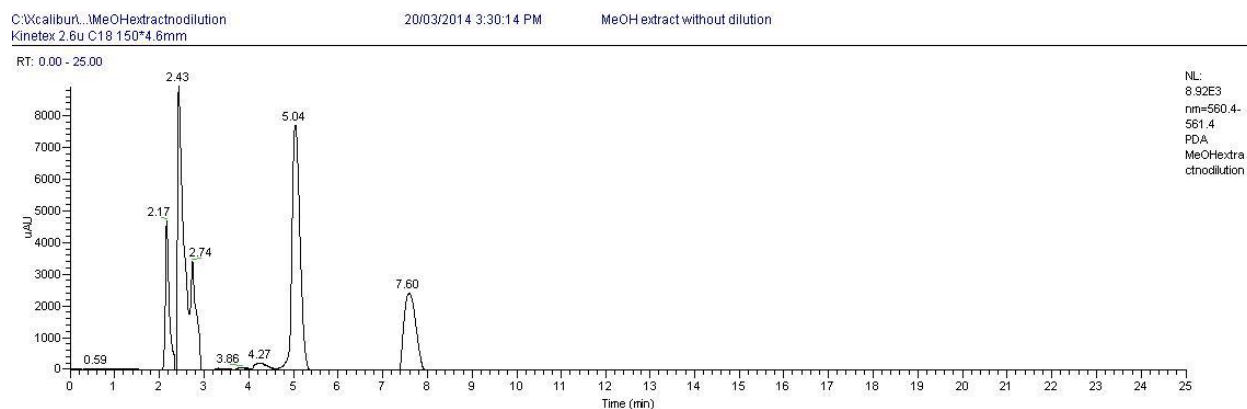


Figure 21. MeOH extract of hair after dying (Reference 7_Avomeen Applicant Interim Report 032514).

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.

Data and Comments' of February 19, 2019 shows that considerable levels of 1,2,4-THB would be present in a 1,2,4-THB containing hair dye formulation throughout the 30 minute application period (Figure 4, 'Supplemental Research, Data and Comments' of February 19, 2019).

Also, the kinetic study (Reference 7_Avomeen Applicant Interim Report 032514) shows that transformation of 1,2,4-THB would not only generate H₂O₂ but also (semi)quinones. Data provided in Ref. 7 (Avomeen Avomeen Applicant Interim Report 032514) shows sizeable levels of hydroxyquinone, alongside 1,2,4 THB, even at time zero in the tested formulation. After 30 min, the dimers were observed in the solution and the reactants (A33 and A5) are still very significant. Even after the dying process, it has been noted that the methanol extract contained mostly the A5 and quinone or dimer of A33'.

Based on the above, the SCCS has concerns over potential consumer exposure to both 1,2,4-THB that remains unreacted in the final hair dye formulation, and the (semi)quinones that are generated in the reaction.

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 sensitizer 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. [¹⁴C]-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) [¹⁴C]-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 could be expected to be lower with PTD than without PTD. Based on the results, for calculation of MoS the SCCS would suggest to take the highest value of the systemically available dose of [¹⁴C]-1,2,4-THB with PTD, i.e. 1.94 µg equivalents/cm² + 1.76 (1SD) or 3.70 µg equivalents/cm².

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 studies on the 3D skin comet assay and 3D skin micronucleus test with negative results. In the *in vivo* micronucleus test provided by applicant (Submission V), the exposure of mice to 1,2,4-THB did not result in an increase in erythrocytes with micronuclei. However, it should be noted, that this test was not performed in accordance with the current OECD guideline (only one dose was tested) and thus has limited value.

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 using different endpoints under *in vitro* conditions including direct DNA damaging effects, chromosomal aberrations and aneuploidy (see the list of references 22-40 attached to the Opinion).

The SCCS acknowledges the Applicant's reply that 1,2,4-THB is a genotoxin *in vitro*. It is strongly suggested by the Applicant that the increased levels of H₂O₂ after exposure to 1,2,4-THB would be due to the generation of superoxide anion which either enzymatically or non-enzymatically dismutates to H₂O₂. The SCCS is of the opinion that a major part of DNA damage (oxidative type) after exposure to 1,2,4-THB is induced by ROS as a result of H₂O₂ generation caused by 1,2,4-THB. However, it needs to be shown that the damage is exclusively ROS-related (preferably when generated intracellularly) and no other reactive radicals, such as semiquinone radicals, are generated, or that no mutations are induced in case they are formed. These aspects have not been addressed in the studies provided in the submission. Assuming a presence of not completely reacted 1,2,4-THB in final product, 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 additional studies.

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

Carcinogenicity

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

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

Special investigation

The SCCS has concerns over potential consumer exposure to both 1,2,4-THB that remains unreacted in the final hair dye formulation, and the (semi)quinones that are generated in the reaction.

4. CONCLUSION

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 the data available in open literature, the SCCS does not consider 1,2,4-trihydroxybenzene safe due to potential genotoxicity when used as an auto-oxidative hair dye component in permanent hair dye formulations.

The data provided by the Applicant indicate that consumers would be exposed to unreacted 1,2,4-THB and (semi)quinones during the use of a 1,2,4-THB containing hair dye formulation. The systemic availability of 1,2,4-THB raises the risk of intracellular generation of hydrogen peroxide, as well as potential genotoxicity via DNA adduct formation by 1,2,4-THB and/or (semi)quinones. These aspects have not been addressed in the studies provided in the submission.

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%?*

See above.

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?*

/

5. MINORITY OPINION

/

6. REFERENCES

From Submission VI

1. SCCS 1452/11, Opinion on 1,2,4-Trihydroxybenzene Colipa No. A33, December 11, 2012.
2. SCCS/1532/14, Addendum Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 2014.
3. SCCS/1564/15, The SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 9th revision, 2015.
4. 1,2,4-Trihydroxybenzene Impurity Identification and Quantification, Avomeen Analytical Services, Kolbert, A.C., Report issued September 24, 2013.
5. 1,2,4-Trihydroxybenzene Heavy Metal Testing by ICP-MS, Exova Labs, Report issued September 11, 2015.
6. Morel, O.J.X., and Christie, R.M. Current Trends in the Chemistry of Permanent Hair Dyeing, Chem. Rev., 111, 2537-2561, 2011.
7. 1,2,4-Trihydroxybenzene: Mechanism and Kinetics of A33 and A5, Avomeen Analytical Services, Kolbert, A.C., Reports issued March 27, 2014 and May 1, 2014.
8. SCCP 0941/05 Opinion on Exposure to reactants and reaction products of oxidative hair dye formulations, 2005.
9. SCCP 1004/06, Skin Penetration of oxidative hair dyes formed by the coupling of precursors and couplers under simulated conditions of hair dyeing. Update of the Annex to SCCP 0941/05, 2006.
10. SCCP 1198/08 Opinion on Intermediates and reaction products of oxidative hair dye ingredients formed during hair dyeing, 2009.
11. SCCS 1311/10, Opinion on reaction products of oxidative hair dye ingredients formed during hair dyeing processes, 2010.
12. Induction of Antioxidant-Response-Element Dependent Gene Activity and Cytotoxicity (using MTT) in the Kertinocyte ARE_Reporters Cell Line, KeratinoSens by 1,2,4-THB, Norman, K., Institute for *In Vitro* Sciences, Report issued June 8, 2016.
13. *In Vitro* Penetration of [¹⁴C]-1,2,4-Trihydroxybenzene through Human Dermatomed Skin, Dermal Technology Laboratories Ltd., Final Report issued 26 January 2015.
14. Bacterial Reverse Mutation Assay of 1,2,4-Trihydroxybenzene, BioReliance Corporation, Wagner, V.O., Report issued August 10, 2015.
15. *In Vitro* Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes (HPBL) of 1,2,4-Trihydroxybenzene, BioReliance Corporation, Roy, S., Report issued August 17, 2015.
16. 3D skin comet assay with 1,2,4-Trihydroxybenzene, using Phenion® full thickness skin models, Triskelion, Reus, A.A., Report issued January 2017.

From Submission VI with Supplemental Research, Data and Comments on February 19, 2019

17. Bacterial Reverse Mutation Assay with Catalase and GSH. Emily Dakoulas, 13 February 2019. BioReliance Corporation, Study Number AE03RS.502R.BTL.
18. *In Vitro* Micronucleus Test using Reconstructed skin Micronucleus (RSMN) assay in EpiDerm™. Shambhu Roy, 27 March 2019. BioReliance Study Number AE03RS.358.BTL.
19. Quantitative Determination of Hydrogen Peroxide from Autoxidation of 1,2,4-Trihydroxybenzene (1,2,4-THB) using Colorimetric FOX (Ferrous-Orange Xylenol) Assay. Applicant 2019.
20. Consumer Exposure Assessment to 1,2,4-Trihydroxybenzene (1,2,4-THB) using High-Performance Liquid Chromatography (HPLC). Applicant 2019.
21. Mortelmans K., E. Zeiger. The Ames Salmonella/microsome mutagenicity assay. *Mutat Res.* 2000 20, 455: 29-60.

References on mutagenic activity of 1,2,4-trihydroxybenzene found in the open literature:

22. Erexson, G.L., Wilmer, J.L., and Kligerman, A.D., Sister chromatid exchange induction in human-lymphocytes exposed to benzene and its metabolites *in vitro*. *Cancer Research* 45:2471–2477, 1985.
23. Pellack-Walker, P., Blumer, J.L., DNA damage in L5178YS cells following exposure to benzene metabolites. *Mol. Pharmacol.* 30(1):42-7, 1986.
24. Lewis J.G., Stewart W., and Adams D.O., Role of oxygen radicals in induction of DNA damage by metabolites of benzene. *Cancer Research* 48:4762-4765, 1988.
25. Kawanishi, S., Inoue, S., and Kawanishi, M., Human DNA damage induced by 1,2,4-benzenetriol, a benzene metabolite. *Cancer Research* 49:164-168, 1989.
26. Glatt, H.R., Padykula, R., Berchtold, G.A., Ludewig, G., Platt, K.L., Klein, J., and Oesch, F., Multiple activation pathways of benzene leading to products with varying genotoxic characteristics. *Environ Health Perspect.* 82:81–89, 1989.
27. Lee, E.W. and Garner, C.D., Effects of benzene on DNA strand breaks *in vivo* versus benzene metabolite-induced DNA strand breaks *in vitro* in mouse bone-marrow cells. *Toxicology and Applied Pharmacology*, 108:497–508, 1991.
28. Zhang, L., Robertson, M.L., Kolachana, P., Davison, A.J., and Smith, M.T., Benzene metabolite, 1,2,4-benzenetriol, induces micronuclei and oxidative DNA damage in human lymphocytes and HL-60 cells. *Environ. Mol. Mutagen.* 21:339–348, 1993.
29. Kolachana, P., Subrahmanyam, V.V., Meyer, K.B., Zhang, L., Smith, M.T., Benzene and its phenolic metabolites produce oxidative DNA damage in HL60 cells *in vitro* and in the bone marrow *in vivo*. *Cancer Res.* 53(5):1023-6, 1993.
30. Zhang, L., Venkatesh, P., Creek, M.L., Smith, M.T., Detection of 1,2,4-benzenetriol induced aneuploidy and microtubule disruption by fluorescence *in situ* hybridization and immunocytochemistry. *Mutat Res.* 320(4):315-27, 1994.

31. Anderson, D., Yu, T.W., Schmezer, P., An investigation of the DNA-damaging ability of benzene and its metabolites in human lymphocytes, using the comet assay. *Environ Mol Mutagen.* 26(4):305-14, 1995.
32. Andreoli, C., Leopardi, P., Crebelli, R., Detection of DNA damage in human lymphocytes by alkaline single cell gel electrophoresis after exposure to benzene or benzene metabolites. *Mutat Res.* 377(1):95-104, 1997.
33. Chung, H.W., Kang, S.J., and Kim, S.Y., A combination of the micronucleus assay and a FISH technique for evaluation of the genotoxicity of 1,2,4-benzenetriol. *Mutat Res.*, 516:49–56, 2002.
34. Pasquini, R., Villarini, M., Scassellati Sforzolini, G., Fatigoni, C., Moretti, M., Micronucleus induction in cells co-exposed *in vitro* to 50 Hz magnetic field and benzene, 1,4-benzenediol (hydroquinone) or 1,2,4-benzenetriol. *Toxicol In Vitro*, 17(5-6):581-6, 2003.
35. Moretti, M., Villarini, M., Simonucci, S., Fatigoni, C., Scassellati-Sforzolini, G., Monarca, S., Pasquini, R., Angelucci, M., Strappini, M., Effects of co-exposure to extremely low frequency (ELF) magnetic fields and benzene or benzene metabolites determined *in vitro* by the alkaline comet assay. *Toxicol Lett.* 157(2):119-28, 2005.
36. Zhang, L., Yang, W., Hubbard, A.E., Smith, M.T., Nonrandom aneuploidy of chromosomes 1, 5, 6, 7, 8, 9, 11, 12, and 21 induced by the benzene metabolites hydroquinone and benzenetriol. *Environ Mol Mutagen.* 45(4):388-96, 2005.
37. Pitarque, M., Creus, A., Marcos, R., Analysis of glutathione and vitamin C effects on the benzenetriol-induced DNA damage in isolated human lymphocytes. *Scientific World Journal* 6:1191-201, 2006.
38. Pandey, A.K., Gurbani, D., Bajpayee, M., Parmar, D., Ajmani, S., Dhawan, A., *In silico* studies with human DNA topoisomerase-II alpha to unravel the mechanism of *in vitro* genotoxicity of benzene and its metabolites. *Mutat Res.* 661(1-2):57-70, 2009.
39. Jia, H., Zhang, C., Glatt, H., Liu, Y., Role of exposure/recovery schedule in micronuclei induction by several promutagens in V79-derived cells expressing human CYP2E1 and SULT1A1. *Mutat Res Genet Toxicol Environ Mutagen.* 808:27-37, 2016.
40. Buick, J.K., Williams, A., Kuo, B., Wills, J.W., Swartz, C.D., Recio, L., Li, H.H., Fornace, A.J. Jr., Aubrecht, J., Yauk, C.L., Integration of the TGx-28.65 genomic biomarker with the flow cytometry micronucleus test to assess the genotoxicity of disperse orange and 1,2,4-benzenetriol in human TK6 cells. *Mutat Res.* 806:51-62, 2017.

7. GLOSSARY OF TERMS

See SCCS/1602/18, 10th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – from page 141

8. LIST OF ABBREVIATIONS

See SCCS/1602/18, 10th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – from page 141