Safety Assessment of 1,2,4-Trihydroxybenzene as Used in Cosmetics

Status:	Draft Report for Panel Review
Release Date:	November 9, 2023
Panel Meeting Date:	December 4-5, 2023

The Expert Panel for Cosmetic Ingredient Safety members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; David E. Cohen, M.D.; Curtis D. Klaassen, Ph.D.; Allan E. Rettie, Ph.D.; David Ross, Ph.D.; Thomas J. Slaga, Ph.D.; Paul W. Snyder, D.V.M., Ph.D.; and Susan C. Tilton, Ph.D. The Cosmetic Ingredient Review (CIR) Executive Director is Bart Heldreth, Ph.D., and the Senior Director is Monice Fiume. This safety assessment was prepared by Christina Burnett, M.S., Senior Scientific Analyst/Writer, CIR; Jinqiu Zhu, Ph.D., DABT, ERT, DCST, CIR Toxicologist; and Priya Cherian, M.S., Senior Scientific Analyst/Writer, CIR.

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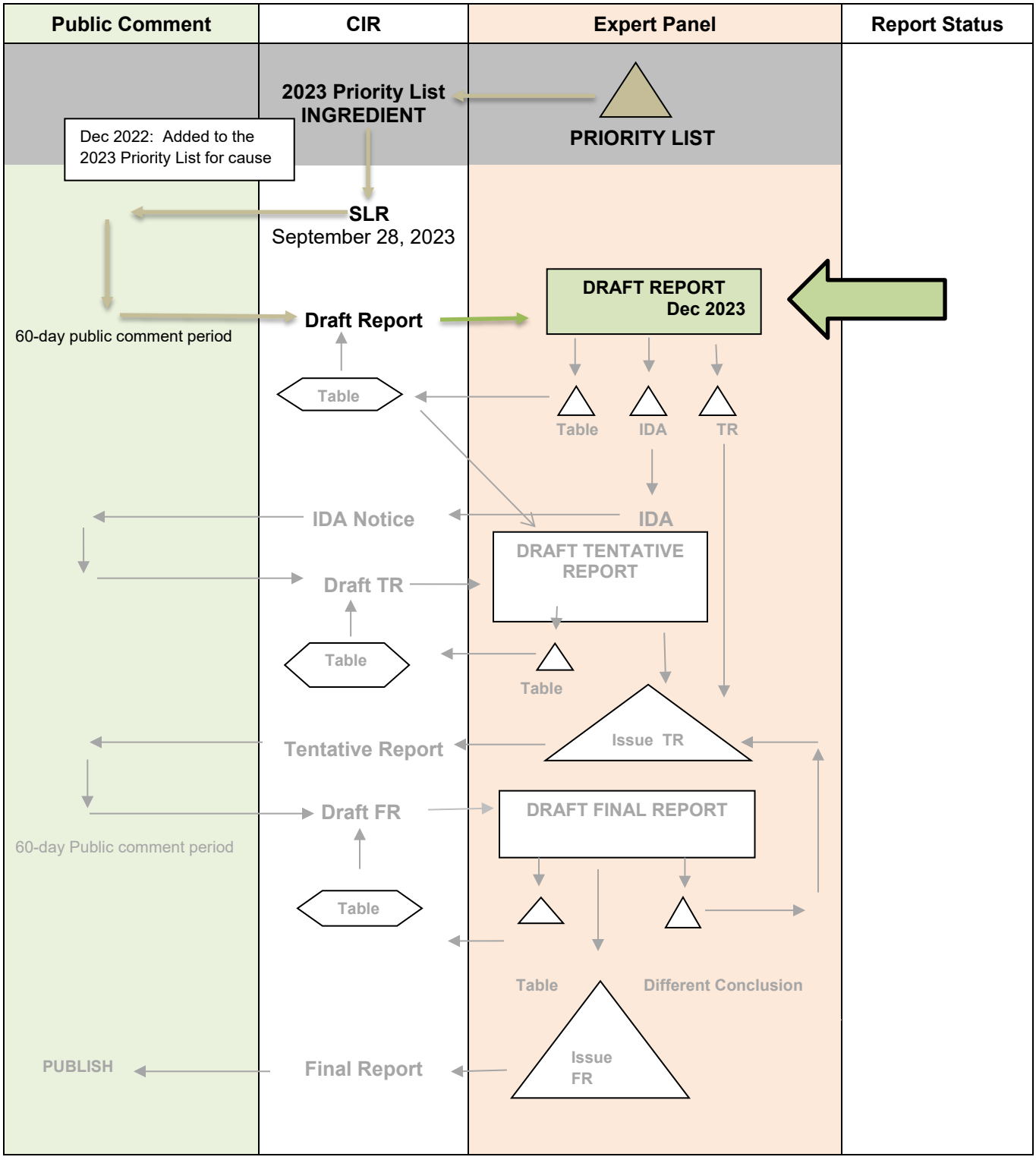
Public Comment
CIR
Expert Panel
Report Status
2023 Priority List
INGREDIENT
PRIORITY LIST
Dec 2022: Added to the 2023 Priority List for cause
SLR
September 28, 2023
DRAFT REPORT
Dec 2023
Draft Report
60-day public comment period
Table
Table
IDA
TR
IDA Notice
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DRAFT TENTATIVE REPORT
Draft TR
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Table
Issue TR
Tentative Report
Draft FR
DRAFT FINAL REPORT
60-day Public comment period
Table
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Different Conclusion
Issue FR
PUBLISH
Final Report

Cosmetic Ingredient Review

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Memorandum

To: Expert Panel for Cosmetic Ingredient Safety Members and Liaisons
From: Christina L. Burnett, M.S., Senior Scientific Analyst/Writer, CIR
Jinqiu Zhu, Ph.D., DABT, ERT, DCST, CIR Toxicologist
Date: November 9, 2023
Subject: Safety Assessment of 1,2,4-Trihydroxybenzene as Used in Cosmetics

Enclosed is the Draft Report on the Safety Assessment of 1,2,4-Trihydroxybenzene as Used in Cosmetics. (It is identified as *report_Trihydroxybenzene_122023* in the pdf document). The Scientific Literature Review (SLR) was issued by CIR on September 28, 2023. This ingredient is reported to function as a hair colorant in cosmetic formulations. It is an auto-oxidative dye used in permanent hair dye formulations and gradual hair coloring shampoos and does not require hydrogen peroxide to activate oxidation and subsequent coupling reactions.

According to 2023 VCRP survey data, 1,2,4-Trihydroxybenzene is reported to be used in 18 hair dye formulations and 1 hair shampoo (coloring). The results of the concentration of use survey conducted by the Council (*data1_Trihydroxybenzene_122023*) indicate 1,2,4-Trihydroxybenzene is used at up to 2.5% in hair dyes and colors.

Dr. Zhu has performed a margin of safety calculation for this ingredient (*data2_Trihydroxybenzene_122023*), which has been summarized in the report. Additionally, Combe has provided an in vivo mammalian erythrocyte micronucleus assay in mice (*data3_Trihydroxybenzene_122023*), a toxicological review (*data4_Trihydroxybenzene_122023*), expert analyses (*data5_Trihydroxybezene_122023* and *data6_Trihydroxybenzene_122023*), and a table of references from a literature review performed by Combe (*data7_Trihydroxybenzene_122023*). The micronucleus assay has been included with the genotoxicity section of the report, and the remaining information is for the Panel to review. Upon review, the Panel should provide comments and discussion where needed.

Comments provided by the Council on the SLR have been addressed (*PCPCcomments_Trihydroxybenzene_122023* and *response-PCPCcomments_Trihydroxybenzene_122023*). Other supporting documents for this report package include a flow chart (*flow_Trihydroxybenzene_122023*), report history (*history_Trihydroxybenzene_122023*), a search strategy (*search_Trihydroxybenzene_122023*), and a data profile (*dataprofile_Trihydroxybenzene_122023*).

If no further data are needed to reach a conclusion of safety, the Panel should formulate a Discussion and issue a Tentative Report. However, if additional data are required, the Panel should be prepared to identify those needs and issue an Insufficient Data Announcement.

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Alexandra Kowcz, MS, MBA
Industry Liaison to the CIR Expert Panel

DATE: October 10, 2023

SUBJECT: Scientific Literature Review: Safety Assessment of 1,2,4-Trihydroxybenzene as Used in Cosmetics (release date: September 28, 2023; comments due by November 27, 2023)

The Personal Care Products Council respectfully submits the following comments on the Scientific Literature Review Safety Assessment of 1,2,4-Trihydroxybenzene as Used in Cosmetics.

Abbreviations – If ^{3}H is going to be included in the abbreviation, e.g., ^{3}H-TTP, it should be defined (tritiated thymidine triphosphate).

Cosmetic Use – It would be helpful to state why the SCCS considered 1,2,4-Trihydroxybenzene to be unsafe.

Dermal Penetration – Usually units of ml/h is called a "rate" rather than "speed". In the second paragraph, please correct the spelling of "Trihydryoxybenzene". How many hair dye formulations containing 2.5% 1,2,4-Trihydroxybenzene were studied?

ADME – Is there any additional information about the disposition of 1,2,4-Trihydroxybenzene? For example, studies of benzene exposed workers have found that 1,2,3-Trihydroxybenzene is excreted in the urine.

Developmental and Reproductive Toxicity – The meaning of the following is not clear: "Exencephaly was already noted in fetuses coming from dams treated with a non-teratogenic substance." The SCCP (reference 2) also states that the historical incidence of exencephaly was 0.0%-1.0%. It would be much clearer if the CIR report just gave the historical rates and indicated that the incidence in the study of 1,2,4-Trihydroxybenzene was 1.2% which was slightly higher, but not a concern because all the fetuses with exencephaly came from the same dam.

Carcinogenicity, Dermal – It says that there was a sacrifice at 9 months, but later it says mice were necropsied at both 7 and 9 months. If there were two interim sacrifices this should be stated more clearly. The SCCP opinion cites this to a published reference which could be obtained to determine if there are any more details that should be included in the CIR report.

Hematotoxicity – Please correct “stomal” to “stromal”. “M” should be called a concentration rather than a dose.

1,2,4-Trihydroxybenzene - December 2023 – Christina Burnett	
Comment Submitter: Alexandra Kowcz, Personal Care Products Council **Date of Submission: October 10, 2023**	
Comment	**Response/Action**
Abbreviations – If 3H is going to be included in the abbreviation, e.g., 3H-TTP, it should be defined (tritiated thymidine triphosphate).	Definition expanded as suggested.
Cosmetic Use – It would be helpful to state why the SCCS considered 1,2,4-Trihydroxybenzene to be unsafe.	More detail added. Unsafe due to potential genotoxicity.
Dermal Penetration – Usually units of ml/h is called a "rate" rather than "speed". In the second paragraph, please correct the spelling of "Trihydryoxybenzene". How many hair dye formulations containing 2.5% 1,2,4-Trihydroxybenzene were studied?	"Rate" replaced "speed", correction to spelling made,
ADME – Is there any additional information about the disposition of 1,2,4-Trihydroxybenzene? For example, studies of benzene exposed workers have found that 1,2,3-Trihydroxybenzene is excreted in the urine.	Paragraph reorganized. No ADME data available for 1,2,4-Trihydroxybenzene; however, many studies available on ADME for benzene. Reference to metabolism of benzene into 1,2,4-Trihydroxybenzene is found under the Natural Occurrence subsection of Chemistry.
Developmental and Reproductive Toxicity – The meaning of the following is not clear: "Exencephaly was already noted in fetuses coming from dams treated with a non-teratogenic substance." The SCCP (reference 2) also states that the historical incidence of exencephaly was 0.0%-1.0%. It would be much clearer if the CIR report just gave the historical rates and indicated that the incidence in the study of 1,2,4-Trihydroxybenzene was 1.2% which was slightly higher, but not a concern because all the fetuses with exencephaly came from the same dam.	Clarification made as suggested.
Carcinogenicity, Dermal – It says that there was a sacrifice at 9 months, but later it says mice were necropsied at both 7 and 9 months. If there were two interim sacrifices this should be stated more clearly. The SCCP opinion cites this to a published reference which could be obtained to determine if there are any more details that should be included in the CIR report.	Burnett et al 1980 obtained to clarify the SCCP summary. Sacrifices were made at both 7 and 9 mo. Burnett et al added to the citation list but does not replace SCCP/SCCS citations as test material was not clearly defined in the original source.
Hematotoxicity – Please correct "stomal" to "stromal". "M" should be called a concentration rather than a dose.	Corrections made.

1,2,4-Trihydroxybenzene Ingredients History

September 28, 2023– The Scientific Literature Review was issued for public comment.

1,2,4-Trihydroxybenzene Data Profile* – December 2023 – Christina Burnett																																
				Toxicokinetics			Acute Tox			Repeated Dose Tox			DART		Genotox		Carci			Dermal Irritation			Dermal Sensitization				Ocular Irritation		Clinical Studies	
	Reported Use	Method of Mfg	Impurities	log P/log K_{ow}	Dermal Penetration	ADME	Dermal	Oral	Inhalation	Dermal	Oral	Inhalation	Dermal	Oral	In Vitro	In Vivo	Dermal	Oral	Other	In Vitro	Animal	Human	In Vitro	Animal	Human	Phototoxicity	In Vitro	Animal	Retrospective/ Multicenter	Case Reports
1,2,4-Trihydroxybenzene CAS No. 533-73-3	X	X	X	X	X	X	X	X			X			X	X	X	X	X			X		X	X				X			

* "X" indicates that data were available in a category for the ingredient

1,2,4-Trihydroxybenzene

Ingredient	CAS #	INCIpedia	PubMed	FDA	EU	ECHA	SCCS	SIDS	ECETOC	HPVIS	AICIS	NTIS	NTP	WHO	FAO	NIOSH	FEMA	Web
1,2,4-Trihydroxybenzene	533-73-3	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√

Search Strategy

PubMed

((1,2,4-trihydroxybenzene) OR (533-73-3[EC/RN Number]) = 215 hits, 44 relevant
((hydroxyhydroquinone) OR (533-73-3[EC/RN Number]) = 150 hits, 41 relevant (most the same as above)

ECHA

No dossier available.

Typical Search Terms

- INCI names
- CAS numbers
- chemical/technical names
- additional terms will be used as appropriate

LINKS

Search Engines

- Pubmed (- http://www.ncbi.nlm.nih.gov/pubmed)

appropriate qualifiers are used as necessary
search results are reviewed to identify relevant documents

Pertinent Websites

- wINCI - https://incipedia.personalcarecouncil.org/infobase/
- FDA databases http://www.ecfr.gov/cgi-bin/ECFR?page=browse
- FDA search databases: http://www.fda.gov/ForIndustry/FDABasicsforIndustry/ucm234631.htm;,
- Substances Added to Food (formerly, EAFUS): https://www.fda.gov/food/food-additives-petitions/substances-added-food-formerly-eafus
- GRAS listing: http://www.fda.gov/food/ingredientspackaginglabeling/gras/default.htm
- SCOGS database: http://www.fda.gov/food/ingredientspackaginglabeling/gras/scogs/ucm2006852.htm
- Indirect Food Additives: http://www.accessdata.fda.gov/scripts/fdcc/?set=IndirectAdditives
- Drug Approvals and Database: http://www.fda.gov/Drugs/InformationOnDrugs/default.htm
- FDA Orange Book: https://www.fda.gov/Drugs/InformationOnDrugs/ucm129662.htm
- (inactive ingredients approved for drugs: http://www.accessdata.fda.gov/scripts/cder/iig/
- HPVIS (EPA High-Production Volume Info Systems) - https://iaspub.epa.gov/oppthpv/public_search.html_page
- NIOSH (National Institute for Occupational Safety and Health) - http://www.cdc.gov/niosh/
- NTIS (National Technical Information Service) - http://www.ntis.gov/
    - technical reports search page: https://ntrl.ntis.gov/NTRL/

- NTP (National Toxicology Program) - http://ntp.niehs.nih.gov/
- Office of Dietary Supplements https://ods.od.nih.gov/
- FEMA (Flavor & Extract Manufacturers Association) GRAS: https://www.femaflavor.org/fema-gras
- EU CosIng database: http://ec.europa.eu/growth/tools-databases/cosing/
- ECHA (European Chemicals Agency – REACH dossiers) – http://echa.europa.eu/information-on-chemicals;jsessionid=A978100B4E4CC39C78C93A851EB3E3C7.live1
- ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) - http://www.ecetoc.org
- European Medicines Agency (EMA) - http://www.ema.europa.eu/ema/
- OECD SIDS (Organisation for Economic Co-operation and Development Screening Info Data Sets)- http://webnet.oecd.org/hpv/ui/Search.aspx
- SCCS (Scientific Committee for Consumer Safety) opinions: http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/index_en.htm
- AICIS (Australian Industrial Chemicals Introduction Scheme)- https://www.industrialchemicals.gov.au/
- International Programme on Chemical Safety http://www.inchem.org/
- FAO (Food and Agriculture Organization of the United Nations) - http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/
- WHO (World Health Organization) technical reports - http://www.who.int/biologicals/technical_report_series/en/
- www.google.com - a general Google search should be performed for additional background information, to identify references that are available, and for other general information

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ABBREVIATIONS

ARE	antioxidant responsive element
BrdU	bromodeoxyuridine
CIR	Cosmetic Ingredient Review
Council	Personal Care Products Council
CPSC	Consumer Product Safety Commission
Cu^{2+}	copper (II)
DMSO	dimethyl sulfoxide
DNCB	2,4-dinitrochlorobenzene
EC1.5	interpolated concentration resulting in a 1.5-fold luciferase induction
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EC_3	estimated concentrations of an SI of 3
ED_{50}	median effective dose
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
Fe^{3+}	iron (III)
GC	gas chromatography
HPLC	high-performance liquid chromatography
[^{3}H]TTP	tritiated thymidine triphosphate
IC_{50}	50% inhibitory concentration
IgE	immunoglobulin E
IU	international units
LLNA	local lymph node assay
LNC	lymph node cell
LOAEL	lowest-observed-adverse-effect-level
LPS	lipopolysaccharide
MOS	margin of safety
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
NOAEL	no-observed-adverse-effect-level
Nrf2	nuclear factor erythroid 2-related factor 2
OECD	Organisation for Economic Co-operation and Development
Panel	Expert Panel for Cosmetic Ingredient Safety
PBS	phosphate buffered saline
ROS	reactive oxygen species
SCCP	Scientific Committee on Consumer Products
SCCS	Scientific Committee on Consumer Safety
SCE	sister chromatid exchange
SED	systemic exposure dose
SI	stimulation index
TBARS	thiobarbituric acid-reactive substances
TG	test guideline
TGx	toxicogenomics
TMA	trimellitic anhydride
UV	ultraviolet light
VCRP	Voluntary Cosmetic Registration Program
Dictionary	web-based *International Cosmetic Ingredient Dictionary and Handbook* (wINCI)

INTRODUCTION

This assessment reviews the safety of 1,2,4-Trihydroxybenzene as used in cosmetic formulations. According to the web-based *International Cosmetic Ingredient Dictionary and Handbook (*wINCI; *Dictionary)*, this ingredient, also called 1,2,4-benzenetriol, 4-hydroxycatechol, or THB, is reported to function as a hair colorant.[1]

This safety assessment includes relevant published and unpublished data that are available for each endpoint that is evaluated. Published data are identified by conducting an extensive search of the world's literature; a search was last performed September 2023. A listing of the search engines and websites that are used and the sources that are typically explored, as well as the endpoints that the Expert Panel for Cosmetic Ingredient Safety (Panel) typically evaluates, is provided on the Cosmetic Ingredient Review (CIR) website (https://www.cir-safety.org/supplementaldoc/preliminary-search-engines-and-websites; https://www.cir-safety.org/supplementaldoc/cir-report-format-outline). Unpublished data are provided by the cosmetics industry, as well as by other interested parties.

Much of the data included in this safety assessment was found in the opinions of the Scientific Committee on Consumer Products (SCCP)[2] and Scientific Committee on Consumer Safety (SCCS).[3,4] Please note that these opinions provide summaries of information generated by industry, and it is those summary data that are reported in this safety assessment when the SCCP and SCCS are cited.

CHEMISTRY

Definition and Structure

According to the *Dictionary*, 1,2,4-Trihydroxybenzene (CAS No. 533-73-3) is the phenol that conforms to the structure in Figure 1.[1, CIR staff]

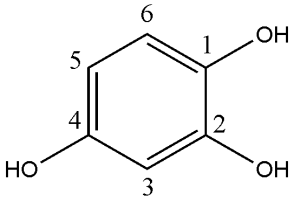

Figure 1. 1,2,4-Trihydroxybenzene

1,2,4-Trihydroxybenzene is an auto-oxidative dye used in permanent hair dye formulations and gradual hair coloring shampoos and does not require hydrogen peroxide to activate oxidation and subsequent coupling reactions.[4] This ingredient is intended to be used in the presence of primary intermediates such as *p*-phenylenediamine, *p*-toluenediamine, *p*-aminophenol, etc. However, if 1,2,4-Trihydroxybenzene is partially oxidized prior to coupling, the rates of active hair dye formation may be different from traditional oxidative hair dyes.

Reaction Chemistry

The hydroxyl substituent pattern of 1,2,4-Trihydroxybenzene affects its reactivity.[4] The hydroxyl groups direct the substitution reaction on the benzene ring, making the 5th carbon position on the ring (see Figure 1) most likely to participate in coupling reactions. 1,2,4-Trihydroxybenzene reacts in the presence of oxygen and primary intermediates to form coupled products, without peroxide. In formulation, once the reaction mixture is exposed to air, the oxidative coupling of 1,2,4-Trihydroxybenzene with an available primary intermediate proceeds rapidly, slowed only by temperature and pH adjustment.

1,2,4-Trihydroxybenzene, at physiological pH, can be oxidized spontaneously (auto-oxidation) or enzymatically.[5] Auto-oxidation is the "uncatalyzed" oxidation of a substance exposed to oxygen in air; however, these reactions are usually metal catalyzed, as redox active metals can serve as free radical initiators. 1,2,4-Trihydroxybenzene oxidation can be catalyzed by iron and copper salts, with copper (II) (Cu^{2+}) being a more active catalyst than iron (III) (Fe^{3+}). Of the metabolites of benzene, 1,2,4-Trihydroxybenzene is the most reactive toward molecular oxygen and rapidly auto-oxidizes to its corresponding quinone via semiquinone radical intermediates. If any such metal salts are present, any apparent "auto-oxidation" may actually be catalyzed oxidation.

The auto-oxidation of 1,2,4-Trihydroxybenzene produces reactive oxygen species (ROS) including superoxide, hydrogen peroxide, and hydroxyl radicals.[5] In the process, 1,2,4-Trihydroxybenzene is oxidized to 2-hydroxy-1,4-benzoquinone. (See Figure 2.)

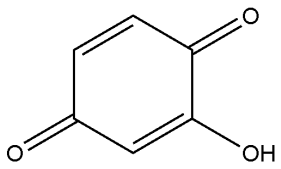

Figure 2. 2-hydroxy-1,4-benzoquinone

Chemical Properties

Chemical properties for 1,2,4-Trihydroxybenzene are summarized in Table 1. 1,2,4-Trihydroxybenzene is a light-medium beige powder with an estimated log P_{ow} of 0.2 and an ultraviolet light (UV) spectrum peak of 291 nm.[2-4]

Method of Manufacture

1,2,4-Trihydroxybenzene may be produced by mixing *p*-quinone with acetic anhydride and concentrated sulfuric acid at below 40° C.[6] The resulting compound is precipitated by water or alcohol to yield 1,2,4-triacetoxybenzene. This acetate is then mixed with cold, absolute alcohol and concentrated hydrochloric acid before heating in an inert gas stream on a water bath at 80° C. The solvent and acid are removed in a vacuum prior to solidifying with chloroform and nucleation (glass scratching).

Impurities

Total impurity content of 1,2,4-Trihydroxybenzene is reported to be < 2% in a batch analyzed by high-performance liquid chromatography (HPLC) and < 0.2% in a batch analyzed by potentiometry.[2] The four impurities detected by HPLC were tetrahydroxybenzene; 1,1'-biphenyl-2,2',4,4',5,5'-hexol; 2-hydroxybenzo-1,4-quinone; and 1,3,2-benzodioxathiole-5,6-diol 2,2-dioxide. Residual solvents were dichloromethane (300 µg/g) and n-propanol (1500 µg/g); isopropanol and ethyl acetate were below levels of detection. Heavy metal content was characterized as the following: aluminum (3 mg/kg), chromium (3 mg/kg), iron (11 mg/kg), nickel (2 mg/kg), zinc (2 mg/kg), and mercury (< 0.1 mg/kg).[3] Silver, arsenic, barium, bismuth, cadmium, cobalt, copper, manganese, molybdenum, lead, palladium, platinum, antimony, selenium, tin, titanium, vanadium were each < 1 mg/kg.

In the 2019 opinion by the SCCS, the purity of 1,2,4-Trihydroxybenzene, determined by gas chromatography (GC) with UV detector, was reported to be 97.8%.[4] Impurities identified by nuclear magnetic resonance (NMR) spectroscopy and GC-mass spectrometry (MS) of 3 lots of 1,2,4-Trihydroxybenzene were: benzene-1,2,4,5-tetraol; 4-mercaptophenol; hydroquinone; and 2-(2,3,4-trimethoxyphenyl)-5,6,7-trimethoxynaphthalene. The heavy metal content of the 3 lots of 1,2,4-Trihydroxybenzene was: arsenic (0.20 - 0.33 ppm), chromium (0.12 - 0.17 ppm), lead (0.03 - 0.05 ppm), mercury (0.02 - 0.03 ppm), and zinc (6.9 - 14 ppm). Cadmium was not detected.

Natural Occurrence

1,2,4-Trihydroxybenzene is a metabolite in biodegradation of many aromatic chemicals, including benzene.[7] Benzene is metabolized in the liver via benzene epoxide to phenol, which is then further hydroxylated to catechol, hydroquinone, and 1,2,4-Trihydroxybenzene.[5] Studies have detected 1,2,4-Trihydroxybenzene in the urine of humans and hamsters exposed to benzene, and in the urine of rats exposed to phenol, quinol, or catechol.[8,9] Metabolites of benzene, including 1,2,4-Trihydroxybenzene, may mediate the myelotoxicity and carcinogenicity of benzene.[5]

1,2,4-Trihydroxybenzene also occurs as a biodegradation product by fungi, yeast, and bacteria of catechin, resorcinol, and other aromatic chemicals.[10-12] Additionally, 1,2,4-Trihydroxybenzene is found in roasted coffee beans, with a typical cup of coffee containing 0.1-1.7 mg of the chemical.[13-16]

USE

Cosmetic

The safety of the cosmetic ingredient addressed in this assessment is evaluated based on data received from the US Food and Drug Administration (FDA) and the cosmetics industry on the expected use of this ingredient in cosmetics, and does not cover its use in airbrush delivery systems. Data were submitted by the cosmetic industry via the FDA's Voluntary Cosmetic Registration Program (VCRP) database (frequency of use) and in response to a survey conducted by the Personal Care Products Council (Council) (maximum use concentrations). The data are provided by cosmetic product categories, based on 21CFR Part 720. For most cosmetic product categories, 21CFR Part 720 does not indicate type of application and, therefore, airbrush application is not considered. Airbrush delivery systems are within the purview of the US Consumer Product Safety Commission (CPSC), while ingredients, as used in airbrush delivery systems, are within the jurisdiction of the FDA. Airbrush delivery system use for cosmetic application has not been evaluated by the CPSC, nor has the use of cosmetic ingredients in airbrush technology been evaluated by the FDA. Moreover, no consumer habits and practices data or particle size data are publicly available to evaluate the exposure associated with this use type, thereby preempting the ability to evaluate risk or safety.

According to 2023 VCRP survey data, 1,2,4-Trihydroxybenzene is reported to be used in 18 hair dye formulations and 1 hair shampoo (coloring).[17] The results of the concentration of use survey conducted by the Council indicate 1,2,4-Trihydroxybenzene is used at up to 2.5% in hair dyes and colors.[18]

Although products containing this ingredient may be marketed for use with airbrush delivery systems, this information is not available from the VCRP or the Council survey. Without information regarding the frequency and concentrations of use of this ingredient (and without consumer habits and practices data or particle size data related to this use technology), the data are insufficient to evaluate the exposure resulting from cosmetics applied via airbrush delivery systems.

Under European regulations for cosmetic ingredients, 1,2,4-Trihydroxybenzene, when used as a substance in hair and eyelash dye products, is categorized in Annex II, the list of substances prohibited in cosmetic products in Europe.[19] Due to

potential genotoxicity, the SCCS considers 1,2,4-Trihydroxybenzene unsafe when used as an auto-oxidative hair dye component in permanent hair dye formulations.[4]

TOXICOKINETIC STUDIES

Dermal Penetration

In Vitro

The dermal absorption/percutaneous penetration potential of 1,2,4-trihydroxy [U-^{14}C]benzene (93.5% radiochemical purity) through dermatomed human skin (~ 400 µm) was determined for a formulation containing 2.78% of the radiolabeled active dye.[2,3] The formulation also contained 50% PEG-6 and approximately 47% water. The study was performed in accordance with Organisation for Economic Co-operation and Development (OECD) test guideline (TG) 428 (draft). Using flow-through diffusion cells, 20 mg/cm^2 of the formulation, corresponding to 556 µg/cm^2, was applied for 30 min to 8 samples. The receptor fluid (phosphate buffered saline (PBS) containing 0.01% sodium azide) was pumped at a rate of approximately 1.5 ml/h. Application of the test material was terminated by rinsing with water (10x), 2% sodium dodecyl-sulfate solution, and water (10x) again. The washing solutions were combined, and the amount of radioactivity was determined. Post-exposure time was 23.5 h. Tape stripping was then performed to determine the 24-h penetration profile. The recovery of radioactivity was 105%. Most of the test material was recovered in the wash after 30 min of exposure. Virtually no penetration of radioactivity into the receptor fluid after 24 h was observed (0.0019 µg$_{eq}$/cm^2 or 0.0003% of the applied dose). The maximum absorption (dermal delivery) was 0.17 µg/cm^2 or 0.03% after being corrected by + 2 standard deviation from 0.07 $\pm$ 0.05 µg/cm^2 (0.01 $\pm$ 0.01%) due to correction of the concentration tested (original reported at 3% instead of 2.78%; the SCCP commented that degradation of circa 8% within 1 wk of the test substance (content 3%) in the test formulation was indicated, even though the test item was stored under an inert atmosphere.

In another dermal penetration study, [^{14}C]1,2,4-Trihydroxybenezene (98.3% pure; 2.00 MBq/mg) was incorporated at a final concentrations of 2.5% (w/w) into two hair dye formulations, one with and one without 2.25% *p*-toluenediamine.[4] The study was performed in accordance with OECD TG 428. The formulations were applied to dermatomed human skin (thickness not specified) in static glass diffusion cells at a dose of 20 mg/cm^2 of the test article; the dose of 1,2,4-Trihydryoxybenezene was approximately ~ 500 µg/cm^2. After 30 min, the skin samples were washed with a mild soap solution. After washing, the diffusion cells were returned to the water bath for the remaining 23.5 h. At the end of the experiment, the 24-h penetration profile was determined using tape stripping and a heat separation technique. The mean recovery of the applied test material without and with *p*-toluenediamine was 101% and 99.2%, respectively. The total systemically available dose (epidermis, dermis, and receptor fluid (PBS)) from the test formulation without *p*-toluenediamine was 1.13 $\pm$ 0.58 µg$_{eq}$/cm^2 or 0.226%. The total systemically available dose from the test formulation with *p*-toluenediamine was 1.94 $\pm$ 1.76 µg$_{eq}$/cm^2 or 0.393%.

Absorption, Distribution, Metabolism, and Excretion

No absorption, distribution, metabolism, or excretion studies were reported for 1,2,4-Trihydroxybenzene in the published literature and unpublished data were not submitted. Many studies are available on the absorption, distribution, metabolism, and excretion of benzene as a starting material, but not on 1,2,4-Trihydroxybenzene.

TOXICOLOGICAL STUDIES

Acute Toxicity Studies

Dermal

In an acute dermal toxicity study performed in accordance with OECD TG 402, 5 male and 5 female Sprague-Dawley rats received 2000 mg/kg bw 1% 1,2,4-Trihydroxybenzene (98.1% pure) in carboxymethylcellulose/water on skin (application site not described).[2,3] The test site was semi-occluded for 24 h. The rats were observed for a period of 14 d following the single application. No mortality was observed during the study period. Hypoactivity, piloerection, and dyspnea were observed in all female rats from days 2 through 8. One of the female rats had tremors. Overall body weight gain was comparable to historical control animals in all but 1 animal; one female had slightly reduced body weight gain during the second week of the study. A black coloration of the skin was noted in all animals from day 2 until study end. Erythema was observed in 2 males on day 2 and persisted in 1 animal until day 3. Edema was recorded between days 2 and 5 in 2 males and in all females between days 2 and 6. No apparent abnormalities were noted at necropsy in any animal.

Oral

In an acute oral toxicity study, groups of 5 male and 5 female OFA Sprague-Dawley-derived rats received 100, 250, 350, 500, or 1000 mg/kg bw 1% 1,2,4-Trihydroxybenzene in carboxymethylcellulose/water via gavage.[2,3] The animals were observed for 14 d following the single administration. No further details were provided. The LD_{50} for both sexes was between 350 and 500 mg/kg bw.

Short-Term Toxicity Studies

Oral

In an oral study, male BALB/c mice (number not reported) received diet of 400 g of normal feed mixed with 210 ml of water containing 8 g 1, 2, 4-Trihydroxybenzene (1.3% w/w) for 1 wk.[20] A control group received the feed mixed with untreated water. No observable toxic effects were observed. (No further details provided.)

In a 4-wk study, groups of 8 male KKAy mice received purified water or water with 100 or 500 mg/l 1,2,4-Trihydroxybenzene 5 times/wk.[21] Individual body weights were recorded weekly, feed intake was measured every 2-3 d, and water consumption was measured each time the water was replaced throughout the study. Metabolic rate was measured after 3 wk by indirect calorimetry. Blood, urine, and tissue samples were collected after 4 wk. Body and liver weights of the mice were not affected. Feed intake and water consumption decreased with increasing concentration of 1,2,4-Trihydroxybenzene, with consumption levels significantly lower ($p < 0.05$) in the high dose group than in controls. Blood glucose, serum triglyceride, and non-esterified fatty acid levels did not differ among the groups. Serum and urinary hydrogen peroxide levels increased with increasing 1,2,4-Trihydroxybenzne concentrations, with the 500 mg/l dose group significantly higher ($p < 0.01$) than the control group. Reduced blood nitric oxide metabolites ($p < 0.05$) and liver S-nitroslyated protein levels (not significant) and decreased whole-body fat utilization ($p < 0.001$) were observed following treatment with 1,2,4-Trihydroxybenzene, with the latter occurring in a dose-dependent manner. 1,2,4-Trihydroxybenzene treatment also decreased fatty acid oxidation in mouse primary liver cells.

Subchronic Toxicity Studies

Oral

In a 90-d gavage study, groups of 15 male and 15 female Han Wistar rats received 0, 50, 100, or 200 mg/kg bw/d 1,2,4-Trihydroxybenzene (purity not reported) in sterile water.[2,3] The study was performed in accordance with OECD TG 408. Clinical signs of toxicity, mortality, and water consumption were monitored daily. Examinations of individual animals for signs of reaction to the test material were performed daily immediately after dosing and approximately 1 and 3 h after dosing during the first 3 wk of the study; after this, observations were performed at approximately 15 min and 1 and 2 h after dosing until the end of the study. Prior to the commencement of treatment and weekly thereafter, each animal was subjected to a detailed clinical examination, including an evaluation of neurotoxicity. Body weight and feed consumption were recorded weekly. An ophthalmological examination was performed prior to the start of the study and in week 12. "Motor activity of the first 5 males and 5 females was measured once during week 12 of treatment." (No further details of this methodology were provided.) Hematology, blood clinical chemistry, and urinalysis were performed in week 13 of treatment. At study end, all surviving animals were killed and underwent macroscopic examination. Select organs (not described) were weighed and microscopic examination was performed of specified tissues and organs (lungs from all animals, other organs not described) from all rats that died during the study, all control and high-dose rats killed at the end of the study, and in animals where gross anomalies were noted.

During the study, 12 animals died: 1 male in each the control, low- and intermediate-dose groups and 5 males and 4 females of the high-dose group. Microscopic examination indicated that mis-dosing was the cause of death for the first 3 groups while the main cause of death for the high-dose rats was stomach ulcerations. Piloerection and salivation were observed in the 100 and 200 mg/kg bw/d dose groups. An overall slight reduction in body weight gain was observed in treated males when compared with controls from approximately 1 mo of treatment. A 14% decrease in feed consumption was observed at week 13 in high dose males, but this result was not observed in treated females. A statistically significant increase in mean red blood cell volume, mean corpuscular hemoglobin, and platelets and a statistically significant decrease in hematocrit, red blood cell count, and hemoglobin were observed in animals treated with 100 and 200 mg/kg bw/d, when compared to the controls; however, values remained within the normal range for this strain of rats. A statistically significant increase in bilirubin was observed in rats of the high dose group of both sexes; however, the color of the test compound may have interfered with the methodology used. No toxicological significance was given to the statistically significant increase in urea observed in treated females only. Statistically significant increases in the absolute weight and/or organ-to-body weight ratios were observed in treated males for the spleen (all dose levels), liver and kidney (100 and 200 mg/kg bw/d), and testes and heart (200 mg/kg bw/d). In the females, statically significant increase in the absolute weight and/or organ-to-body weight ratios were observed for the liver, spleen, and kidneys at 200 mg/kg bw/d. Ulcerations in the non-glandular gastric region were observed in 1/10 males and 1/11 females of the high dose group and in 1/14 males in the intermediate dose group at study end. The histopathological evaluation of the stomach in the remaining animals of the intermediate dose group did not reveal any further treatment-related gastric lesions. Dark-brown, microgranular pigmentation was clearly evident in single cells or in the lumen of renal cortical tubes of 10/15 males and 10/15 females in the high dose group and in 2/15 males and 1/15 females of the intermediate dose group. The no-observed-adverse-effect-level (NOAEL) was determined by the study authors to be 50 mg/kg bw/d. However, the SCCP concluded that no NOAEL could be derived in this study as the relative organ weight was increased significantly in the spleen of the 50 mg/kg bw/d male rats.[2,4] This increase continued in a dose-dependent manner in the male rats.[4] Absolute organ weight of the spleen was also increased in male rats, but the increase was not significant at 50 mg/kg bw/d. The value 50 mg/kg bw/d was considered to be the lowest-observed-adverse-effect-level (LOAEL) by the SCCP.

DEVELOPMENTAL AND REPRODUCTIVE TOXICITY STUDIES

Oral

The teratogenic potential of 1,2,4-Trihydroxybenzene (99.8% pure) was evaluated in groups of 25 mated female Sprague-Dawley (Crl CD (SD) BR) rats in accordance with OECD TG 414.[2,3] The rats received 0, 30, 100, or 300 mg/kg bw/d of the test material dissolved in water on gestation days 6 through 15. On day 20 of gestation, the rats were killed. The number of corpora lutea, resorptions, live and dead fetuses, and implantation sites were recorded. Live fetuses were weighed and examined externally. Half of the live fetuses per litter underwent skeletal examination and the remaining fetuses underwent soft tissue examination.

No clinical signs of toxicity or deaths occurred in the 0, 30, or 100 mg/kg bw/d groups. In the 300 mg/kg dose group, 3 females died or were killed in moribund conditions due to mis-dosing. Another female died without any clinical signs. At necropsy, gaseous dilatation of the stomach and intestine and congested lungs were noted. The mean body weight gain and feed consumption of females that survived to day 20 were similar to the control and other dose groups. In the 300 mg/kg dose group, the mean body weight gain was slightly lower than that of the controls between days 6 and 9 (not significantly) and the feed consumption was also slightly lower than that of the control females during the treatment period. No further details of these observations were provided. The litter parameters were comparable in the control and treated groups. No fetal external malformations were observed in the 0, 30, or 100 mg/kg bw/d groups; however, in the 300 mg/kg bw/d dose group, 4 fetuses from the same litter out of 325 fetuses had exencephaly associated with opened eyelids. Exencephaly was already noted in fetuses coming from dams treated with a non-teratogenic substance (mean incidence: 0.06%; range of incidence per study: 0.0 - 1.0%). This incidence was slightly higher (1.2%) than that of the historical data, but it was considered a congenital malformation due to the fetuses coming from the same dam, and no other malformations were noted in any other litters. The dam showed no sign of any toxicity. No other treatment-related fetal skeletal variations, anomalies, malformations, and/or fetal soft tissue anomalies or malformations were observed. 1,2,4-Trihydroxybenzene was maternotoxic at 300 mg/kg bw/d, but not embryotoxic or teratogenic.[2]

GENOTOXICITY STUDIES

1,2,4-Trihydroxybenzene is a compound known to induce DNA damage and is considered genotoxic. This compound can result in oxidative DNA damage, which is linked to the generation of ROS.[22-27] Additionally, 1,2,4-Trihydroxybenzene has been studied for its effects on cellular structures, inducing DNA strand breaks and mutations as well as potential epigenetic modifications, potentially leading to various diseases, including cancer.[28,29] 1,2,4-Trihydroxybenzene may inhibit mitochondrial DNA replication.[30] Furthermore, several studies have elucidated the role of 1,2,4-Trihydroxybenzene in causing chromosomal damage and subsequent biological consequences.[31-33]

In vitro and in vivo genotoxicity studies on 1,2,4-Trihydroxybenzene summarized here are detailed in Table 2. 1,2,4-Trihydroxybenzene was mutagenic in several Ames tests when tested at up to 5000 µg/plate.[2-4,34] Genotoxicity of 1,2,4-Trihydroxybenzene was observed in a gene mutation assay of mutants to 6-thioguanine (tested at up to a maximal concentration of 10 µM), DNA strand break tests (at up to 1000 µM), DNA synthetic activity inhibition assays (at up to 24 µM), sister chromatid exchange (SCE) assays (at up to 500 µM), and in a human lymphocyte study using fluorescence in situ hybridization of metaphase spreads (at up to 50 µM).[3,28,33-38] 1,2,4-Trihydroxybenzene was not genotoxic in a gene mutation test at the *hprt* locus (up to 240 µg/ml) or in a 3D skin comet assay (at up to 1250 µg/ml), and was not clastogenic in a chromosome aberration test (at up to 20 µg/ml).[2-4] Genotoxicity was not observed in micronucleus assays with human reconstructed skin tissue and human lymphocytes (at up to 224 µg/ml), but increases in micronucleus induction were observed in Chinese hamster V79 cells (at up to 25 µM) and human TK6 lymphoblastoid cells (at up to 30 µg/ml).[4,34,37] 1,2,4-Trihydroxybenzene was clastogenic and aneugenic in a cytokinesis-block micronucleus test with human lymphocytes at up to 100 µM.[3,39] In in vivo micronucleus tests in mice, 1,2,4-Trihydroxybenzene was not genotoxic when tested intraperitoneally at up to 50 mg/kg bw.[2,40]

Mechanism

Oxidant-mediated genotoxicity of 1,2,4-Trihydroxybenzene is a multifactorial process.[5] Redox reactions of 1,2,4-Trihydroxybenzene yield oxygen-derived active species and quinones. Copper salts stimulate oxidation of 1,2,4-Trihydroxybenzene, leading to 1,2,4-Trihydroxybenzene-induced genotoxicity. Copper salts change the mechanism of reaction from superoxide-propagated 1-election transfer to Cu^{2+}-mediated 2-electron transfer, which correlates with the changes in the pattern of 1,2,4-Trihydroxybenzene-induced micronucleus formation from kinetochore-positive to kinetochore-negative. (Kinetochore is a complex of proteins associated with the centromere of a chromosome during cell division, to which the microtubules of the spindle attach.)

CARCINOGENICITY STUDIES

Dermal

The dermal carcinogenicity potential of a semi-permanent hair dye formulation containing 0.5% 1,2,4-Trihydroxybenzene (purity not reported) was evaluated in groups of 50 male and 50 female Swiss Webster mice.[2,3,41] The test material (0.05 ml) was applied once weekly for 21 - 23 mo on a 1 cm^2 area of clipped skin on the interscapular region. Test sites were not occluded. The mice were observed daily for mortality and clinical signs of toxicity and were weighed

monthly. A weekly record was maintained for any skin lesions noted. After 7 and 9 mo of treatment, 10 males and 10 females per group were necropsied. The study was terminated after 23 mo. Skin and internal organs were evaluated histologically. Four males and 4 females that received 1,2,4-Trihydroxybenzene survived until study termination, while 3 males and 8 females survived until study termination in the control groups. There were no significant differences in absolute or relative liver or kidney weights in groups of 10 male and 10 female mice necropsied after 7 and 9 mo. There were no statistically significant differences in the distribution of tumors among treated and control groups. The SCCS determined that these data were insufficient to conclude on the carcinogenic potential of 1,2,4-Trihydroxybenzene.

Oral

In an oral study, 5 male and 5 female F344 rats received 1.5% 1,2,4-Trihydroxybenzene in diet continuously for 4 wk.[42] A control group of the same composition of animals received regular diet. At wk 4, the rats were injected intraperitoneally with 50 mg/kg bw bromodeoxyuridine (BrdU) and after 1 h, the rats were then sequentially killed. The stomachs were removed for histopathological and immunohistochemical examination. The number of cells incorporating BrdU into DNA per 2000 basal cells of the forestomach and the numbers of cells labeled with BrdU per 50 pyloric glands were counted. No deaths were observed during the treatment period and there were no clinical effects of toxicity observed, with the exception of a statistically significant reduction in weight gain in both sexes treated with 1,2,4-Trihydroxybenzene. This observation was associated with a decrease in feed consumption. No erosion or ulcer formation was observed in any of the treated animals or the controls. Significantly increased DNA synthesis ($p < 0.01$) was noted in both sexes of the treated animals when compared to the control animals. 1,2,4-Trihydroxybenzene was not associated with any hyperplasia changes in glandular stomach mucosa.

OTHER RELEVANT STUDIES

Cytotoxicity

The cytotoxicity of 1,2,4-Trihydroxybenzene was investigated in K562 erythroleukemia cells.[43] Cultured K562 cells were exposed to 0, 0.1, 0.2, 0.3, 0.4, or 0.5 mM 1,2,4-Trihydroxybenzene for 24 h. The K562 cells showed significant inhibition of viability ($p < 0.05$) in a concentration-dependent manner.

In another study, K562 cells were treated with 0, 0.01, 0.02, 0.04, or 0.08 mM 1,2,4-Trihydroxybenzene for 24 h.[44] Eighty-five percent (85%) of the total cells were viable after treatment at concentrations less than 0.08 mM. At 0.08 mM, cell viability was slightly greater than 60%.

Cytotoxicity was also investigated using HL-60 human promyelocytic leukemic cells incubated with 10 - 100 µM 1,2,4-Trihydroxybenzene, hydroquinone, and *p*-benzoquinone for 1 - 4 h.[45] The rank order of cytotoxicity of these benzene metabolites to the cells were determined to be *p*-benzoquinone > hydroquinone > 1,2,4-Trihydroxybenzene at any given time period. In further in vitro testing, cells were exposed to 50 µM of each metabolite for 2 h. Cell viability was more than 80% (no further details provided).

Hematotoxicity

In vitro studies using mouse bone marrow adherent stromal cells and K562 cells have been used to study the mechanisms of benzene hematoxicity.[44,46,47] 1,2,4-Trihydroxybenzene (3.1×10^{-6} to 500×10^{-6}) was studied for its effect on the ability of stromal cells to influence granulocyte/monocyte colony growth after incubation with the test material for 3 d.[46] 1,2,4-Trihydroxybenzene inhibited colony growth at concentrations $\geq 100 \times 10^{-6}$ M. K562 cells were used to determine the effects of 1,2,4-Trihydroxybenzene on erythroid differentiation.[44,47] The results of the studies on K562 cells indicated that 1,2,4-Trihydroxybenzne inhibited hemin-induced erythroid differentiation in concentration-dependent manner (tested at up to 40 µM).

Oxidative Stress

In an investigation on oxidative stress, groups of 6 mice received diet of 400 g of normal feed mixed with 210 ml of water containing 8 g 1, 2, 4-Trihydroxybenzene (1.3% w/w) for 1 - 2 wk, with or without exposure to air containing 10 ppm nitrogen dioxide.[20] Control groups received normal feed mixed with untreated water, with or without exposure to nitrogen dioxide. At the end of the exposure period, blood was collected and the heart, kidney, liver, and lungs were obtained from 1 mouse to measure thiobarbituric acid-reactive substances (TBARS) in assays with ethylenediaminetetraacetic acid (+ EDTA) and without EDTA (– EDTA). In the red blood cell membranes, there were no significant differences in the levels of TBARS in the 1,2,4-Trihydroyxbenzene or the control groups with the EDTA assay. In the organs, the level of TBARS with the - EDTA assay was significantly decreased by 1,2,4-Trihydroxybenzene in the kidney and liver. In the – and + EDTA assays of the lung, levels of TBARS from malonaldehyde derivatives plus alkadienal/alkenal derivatives were remarkably increased by 1,2,4-Trihydroxybenzene, but those from malonaldehyde derivatives alone were not. There were no effects of nitrogen dioxide inhalation on lung lipid peroxidation; lung lipid peroxidation was enhanced by 1,2,4-Trihydroxybenzene, but this effect was not greatly impacted in other tissues.

Neuroprotective Effects

1,2,4-Trihydroxybenzene (tested at 10 - 100 µM) was found to significantly inhibit lipopolysaccharide (LPS)-stimulated nitric oxide production in BV-2 microglia cells treated with 1 µg/ml LPS followed by the test material for 24 h.[48] 1,2,4-

Trihydroxybenzene also inhibited inducible nitric oxide synthase mRNA and protein expression (cells treated with test material and LPS for 4 h). Additionally, 1,2,4-Trihydroxybenzene significantly reduced the generation of ROS in hydrogen-peroxide-induced BV-2 cells (treated with 1 mM hydrogen peroxide followed by test material) and in hydrogen peroxide-cell free conditions (scavenging effect of test material assessed with 1 mM hydrogen peroxide or with 0.1 mg/ml ferrous sulfate heptahydrate). The neuroprotective effect of 1,2,4-Trihydroxybenzene (30 mg/kg) was observed in the ischemic male Sprague-Dawley rat brain under middle cerebral artery occlusion in an in vivo infarction assay.

Melanogenesis Inhibition

1,2,4-Trihydroxybenzene is reported to be a substrate of tyrosinase and a strong suicide substrate.[49] This may contribute to the depigmentation property of hydroquinone.

Immunomodulatory Effects

The effect of 1,2,4-Trihydroxybenzene on type IV and type I allergy responses was studied using male BALB/c mice.[20] Type IV allergy responses were investigated through contact sensitization responses induced by 2,4-dinitrochlorobenzene (DNCB). Groups of 3 mice received diet of 400 g of normal feed mixed with 210 ml of water containing 8 g 1, 2, 4-Trihydroxybenzene (1.3% w/w) for 1 - 2 wk, with or without exposure to air containing 10 ppm nitrogen dioxide. Control groups received normal feed mixed with untreated water, with or without exposure to nitrogen dioxide. The mice then underwent a local lymph node assay (LLNA) where both ears were treated with 25 µl of 1% (w/v) DNCB in acetone/olive oil (4:1 v/v). Lymph node weight was significantly lowered (p not reported) in 1,2,4-Trihydroxybenzene-treated mice, but total lymph node cell (LNC) number was unaffected. No difference in the weight and LNC number was noted between nitrogen dioxide exposed and non-exposed groups. The degree of DNCB-sensitized cell proliferation was increased approximately 2-fold by 1,2,4-Trihydroxybenzene in both nitrogen dioxide exposed and non-exposed groups.

Type I allergy responses were measured in serum immunoglobulin E (IgE) levels in the mice treated with the test material as described above. The mice were then sensitized with 50 µl of 1% DNCB or 25% (w/v) trimellitic anhydride (TMA) solution in acetone/olive oil on both shaved flanks. Controls were the same as described above. After 7 d, the mice received 25 µl of the same inducing agent on both ears. Seven days after the challenge, serum IgE was measured in an enzyme-linked immunosorbent assay (ELISA). 1,2,4-Trihydroxybenzene enhanced the serum IgE levels induced by DNCB and TMA, with no significant differences noted in the IgE levels between the nitrogen dioxide exposed and non-exposed groups. TMA sensitization was enhanced to a greater extent by 1,2,4-Trihydroxybenzene. Effects produced by nitrogen dioxide inhalation were observed only in control mice with TMA sensitization, but not in mice that received 1,2,4-Trihydroxybenzene. The authors concluded that 1,2,4-Trihydroxybenzene may have an adverse effect on both type IV and type I hypersensitivities.[20]

DERMAL IRRITATION AND SENSITIZATION STUDIES

Irritation

Animal

The irritation potential of 3% 1,2,4-Trihydroxybenzene (98.1% pure) in water was assessed in 3 male New Zealand White rabbits in accordance with OECD TG 404.[2,3] The test material (0.5 ml) was applied to 1 rabbit for durations of 3 min, 1 h, and 4 h. In the remaining 2 rabbits, the test material was applied for durations of 1 h and 4 h, each. The test material was placed on a dry gauze pad that was then applied to the clipped flanks of the animals with a semi-occluded dressing. The sites were clipped thereafter on several days up to day 9. Untreated skin served as the control. After 3 min, very slight or well-defined erythema (grade 1 or 2) was noted from day 2 to day 6. After the 1-h exposure in the same animal, a very slight or well-defined erythema (grade 1 or 2) was noted from day 1 to day 8. In the other 2 animals, discrete erythema was noted on day 1 and 2 in 1 animal and no erythema was observed in the other animal. After the 4-h exposure, a brown coloration of the skin was noted in all animals from day 1 to day 2, 6, or 9; this could have masked very slight or well-defined erythema. No other cutaneous reactions were recorded during the study. Based on the 1-h exposure, 3% 1,2,4-Trihydroxybenzene in water was slightly irritating to rabbit skin.

Sensitization

In Vitro

In an antioxidant responsive element-nuclear factor erythroid 2-related factor 2 (ARE-Nrf2) luciferase KeratinoSens™ test, HaCaT keratinocytes were exposed to 1,2,4-Trihydroxybenzene (97.8% pure) at concentrations ranging from 0.977-2000 µM in 1% dimethyl sulfoxide (DMSO) in 1% Dulbecco's modified Eagle medium.[4] The test was performed in accordance with OECD TG 442D. 1,2,4-Trihydroxybenzene was tested in 3 definitive assays, and the positive control was cinnamic aldehyde at concentrations ranging from 4 - 64 µM. The interpolated concentration resulting in a 1.5-fold luciferase induction (EC1.5) value for the test material was 374.31 µM, and is below the threshold value of 1000 µM. 1,2,4-Trihydroxybenzene has the potential to be a sensitizer, but is not equivalent to the potent sensitizer, cinnamic aldehyde. The SCCS noted that 1,2,4-Trihydroxybenzene was positive at a concentration of 500 µM, but the dose-response curve had a large variation in gene induction at 500 µM. According to test guidelines, the assay is positive when gene induction is statistically significant from the solvent control in at least 2 out of 3 replicates. Statistical analysis of the data for the results was not provided, and the SCCS determined the results of the assay inconclusive.

Animal

An LLNA was performed using 1,2,4-Trihydroxybenzene (98.1% pure) in accordance with OECD TG 429.[2,3] Female CBA mice were divided into groups of 4 and received 0.25, 0.5, 1, 2.5, or 5% (w/v) of the test material in dimethylformamide in experiment 1 and 0.01, 0.05, 0.1. 0.25, or 0.5% in experiment 2. The test material was applied to the ear surface (25 µl) once daily for 3 consecutive days. α-Hexylcinnamaldehyde (25% v/v) was used as the positive control. Five days after the first topical application, all animals were injected intravenously with [^{3}H]methyl thymidine and the proliferation of lymphocytes in the draining lymph nodes was measured.

No clinical signs or mortality related to treatment were observed. In experiment 1, dryness of the skin was noted on day 6 in 2/4 and 4/4 animals that received the test material at 1 and 2.5%, respectively. Additionally, a moderate increase in ear thickness (up to 45%) was observed at 2.5 and 5%, indicating irritation potential of the test material at these concentrations. No cutaneous reactions or noteworthy increases in ear thickness was observed in experiment 2.

In experiment 1, positive lymphoproliferative responses were observed at all tested concentrations, but without a clear dose-response relationship. Positive responses observed at concentrations of 0.25 and 0.5% were attributed to delayed contact hypersensitivity as there was no local irritation. The stimulation indices (SI) in experiment 1 ranged from 12.68 to 26.41 using concentrations from 0.25 to 5%. In experiment 2, a dose-related increase in SI (except for 0.1%) was noted and the threshold positive value of 3 was exceeded at 0.25%. The estimated concentration for an SI of 3 (EC_3) was calculated on the basis of the results in experiment 2 to be 0.08%. It was concluded that 1,2,4-Trihydroxybenzene induced delayed contact hypersensitivity, and based on the EC_3 value, should be categorized as an extreme sensitizer.[2,3]

OCULAR IRRITATION STUDIES

Animal

In an ocular irritation study performed in accordance with OECD TG 405, 3 male New Zealand rabbits received approximately 0.1 ml of a 3% dilution of 1,2,4-Trihydroxybenzene in water in the conjunctival sac of the left eye.[2,3] The right eyes served as the controls. The eyes were not rinsed after administration of the test material. Eyes were observed for reactions 1, 24, 48, and 72 h after instillation. Very slight chemosis and very slight redness of the conjunctiva were observed in all animals on day 1, which persisted in 2 of the 3 animals up to day 3. No other reactions were observed. It was concluded that 3% 1,2,4-Trhydroxybenzene was slightly irritating to rabbit eyes.

MARGIN OF SAFETY

CIR staff have calculated a margin of safety (MOS) for 2.5% 1,2,4-Trihydroxybenzene to be 1397.8 for a dermal exposure.[CIR Staff] This calculation is based on the NOAEL of 50 mg/kg bw/d from a 90-d oral rat study and a systemic exposure dose (SED) of 0.03577 mg/kg bw (skin area surface of 580 cm^2 x absorption through skin of 3.70 µg/cm^2 x 0.001 (unit conversion)/typical human bw of 60 kg).

The parameters that are used for the MOS calculation are listed below:

Systemically available dose: 3.70 $\mu g_{eq}/cm^2$ (derived from an in vitro study using frozen human dermatomed skin)[4]
Skin surface area for application: 580 cm^2 (½ area head)[50]
Dermal absorption per treatment: 3.70 μg_{eq} /cm^2 × 580 cm^2 = 2146 µg = 2.146 mg
Human body weight: 60 kg

SED: 2.146 mg ÷ 60 kg = 0.03577 mg/kg bw/d
NOAEL: 50 mg/kg bw/d (90-d, gavage, oral, rat)[2,3]

$$\text{MOS} = \frac{\text{NOAEL}}{\text{SED}} = \frac{\text{50 mg/kg bw/d}}{\text{0.03577 mg/kg bw/d}} = 1397.8$$

It should be noted the SCCS determined that the dose of 50 mg/kg bw/d cannot be viewed as the NOAEL because there was a significant increase in the relative spleen weight of male rats treated at this dose; instead, the SCCS considered 50 mg/kg bw/d as the LOAEL.[2,4] When an NOAEL cannot be established and only the LOAEL is available, an additional assessment factor might be applied. Typically, the maximum value of the assessment factor recommended for extrapolating an LOAEL to an NOAEL is 10; however, in some cases, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) suggests a default value of 3.[51] Consequently, using a dose of 50 mg/kg bw/d (presumed to be the LOAEL), the NOAEL can be deduced as either 16.7 mg/kg bw/d or 5 mg/kg bw/d, depending on whether an assessment factor of 3 or 10 is applied. As a result, the MOS values can be calculated to be 466.9 or 139.8, respectively. Both values are greater than 100, a figure generally accepted as the threshold for considering an ingredient safe to use.

SUMMARY

1,2,4-Trihydroxybenzene is reported to function as a hair colorant in cosmetics, according to the *Dictionary*. It is an auto-oxidative dye used in permanent hair dye formulations and gradual hair coloring shampoos; this ingredient does not require hydrogen peroxide to activate oxidation and subsequent coupling reactions.

1,2,4-Trihydroxybenzene is a metabolite in biodegradation of aromatic compounds, such as benzene. It also occurs as a biodegradation product of fungi, yeast, and bacteria of aromatic compounds, and is found in roasted coffee beans.

According to 2023 VCRP survey data, 1,2,4-Trihydroxybenzene is reported to be used in 18 hair dye formulations and 1 hair shampoo (coloring). The results of the concentration of use survey conducted by the Council indicate 1,2,4-Trihydroxybenzene is used at up to 2.5% in hair dyes and colors. Under European regulations for cosmetic ingredients, 1,2,4-Trihydroxybenzene, when used as a substance in hair and eyelash dye products, is categorized in Annex II, the list of substances prohibited in cosmetic products in Europe due to potential genotoxicity.

In a dermal penetration study, the maximum absorption of a formulation containing 2.78% 1,2,4-Trihydroxybenzene through dermatomed human skin (~ 400 μm) was 0.17 $\mu g/cm^2$ or 0.03% after being corrected by + 2 standard deviation from 0.07 ± 0.05 $\mu g/cm^2$ (0.01 ± 0.01%) due to correction of the concentration tested (originally reported at 3% instead of 2.78%). The total systemically available dose of a hair dye formulation containing 2.5% 1,2,4-Trihydroxybenzene was 1.94 $\mu g_{eq}/cm^2$ (0.393%) with 2.25% *p*-toluenediamine and 1.13 $\mu g_{eq}/cm^2$ (0.226%) without *p*-toluenediamine.

In an acute dermal toxicity study in rats, no mortality was observed following a dermal dose of 2000 mg/kg bw 1,2,4-Trihydroxybenzene. The LD_{50} in an acute oral toxicity rat study of 1,2,4-Trihydroxybenzene was between 350 and 500 mg/kg bw.

No observable toxic effects were observed in mice that received 1.3% (w/w) Trihydroxybenzene in feed mixed with water for 1 wk. In a 4-wk study in which mice received 100 or 500 mg/l 1,2,4-Trihydroxybenzene, feed intake and water consumption decreased, and serum and urinary hydrogen peroxide levels increased with increasing concentrations of the test material. Reduced blood nitric oxide metabolites and liver *S*-nitroslyated protein levels and decreased whole-body fat utilization were observed following treatment with 1,2,4-Trihydroxybenzene, with the latter occurring in a dose-dependent manner. 1,2,4-Trihydroxybenzene treatment also decreased fatty acid oxidation in mouse primary liver cells.

In a 90-d gavage study of 1,2,4-Trihydroxybenzene in rats, the NOAEL were determined to be 50 mg/kg bw/d, according to the researchers; this value was determined to be an LOAEL by the SCCP. Statistically significant increases in the absolute weight and/or organ-to-body weight ratios were observed in treated males for the spleen (all dose levels), liver and kidney (100 and 200 mg/kg bw/d), and testes and heart (200 mg/kg bw/d). In the females, statically significant increases in the absolute weight and/or organ-to-body weight ratios were observed for the liver, spleen, and kidneys at 200 mg/kg bw/d.

In a teratogenicity study in which gravid female Sprague-Dawley rats were dosed by gavage with up to 300 mg/kg bw/d of the test article, 1,2,4-Trihydroxybenzene was maternotoxic at 300 mg/kg bw/d. Embryotoxicity and teratogenicity were not observed in the fetuses.

1,2,4-Trihydroxybenzene is a compound known to induce DNA damage and is considered genotoxic. This compound can result in oxidative DNA damage, which is linked to the generation of ROS. 1,2,4-Trihydroxybenzene was mutagenic in several Ames tests when tested at up to 5000 μg/plate. Genotoxicity of 1,2,4-Trihydroxybenzene was observed in a gene mutation assay of mutants to 6-thioguanine (tested at a maximal concentration of 10 μM), DNA strand break tests (at up to 1000 μM), DNA synthetic activity inhibition assays (at up to 24 μM), SCE assays (at up to 500 μM), and in a human lymphocyte study using fluorescence in situ hybridization of metaphase spreads (at up to 50 μM). 1,2,4-Trihydroxybenzene was not genotoxic in a gene mutation test at the *hprt* locus (up to 240 μg/ml) or in a 3D skin comet assay (at up to 1250 μg/ml), and was not clastogenic in a chromosome aberration test (at up to 20 μg/ml). Genotoxicity was not observed in micronucleus assays with human reconstructed skin tissue and human lymphocytes (at up to 224 μg/ml), but increases in micronucleus induction were observed in Chinese hamster V79 cells (at up to 25 μM) and human TK6 lymphoblastoid cells (at up to 30 μg/ml). 1,2,4-Trihydroxybenzene was clastogenic and aneugenic in a cytokinesis-block micronucleus test with human lymphocytes at up to 100 μM. In in vivo micronucleus tests in mice, 1,2,4-Trihydroxybenzene was not genotoxic when tested intraperitoneally at up to 50 mg/kg bw.

No conclusion as to the carcinogenic potential of a hair dye formulation containing 0.5% 1,2,4-Trihydroxybenzene could be made in a 2-yr dermal study of mice. In a 4-wk oral dietary study, 1.5% 1,2,4-Trihydroxybenzene was not associated with any hyperplasia changes in glandular stomach mucosa.

1,2,4-Trihydroxybenzene was cytotoxic in a dose-dependent manner in K562 cells, but it was determined to be less cytotoxic than other benzene metabolites in HL-60 cells. 1,2,4-Trihydroxybenzene has been studied for its role in benzene hematotoxicity, oxidative stress, neuroprotective effects, and potential melanogenesis inhibition. 1,2,4-Trihydroxybenzene may have an adverse effect on both allergen-sensitized type IV and type I allergy responses.

In a dermal irritation study, 3% 1,2,4-Trihydroxybenzene was slightly irritating to rabbit skin. 1,2,4-Trihydroxybenzene was predicted to be a sensitizer in an ARE-Nrf2 luciferase KeratinoSens™ test, and was categorized as an extreme sensitizer

in an LLNA when tested at up to 5% in dimethylformamide and at up to 0.5% without dimethylformamide. In ocular studies, 3% 1,2,4-Trihydroxybenzene was slightly irritating to rabbit eyes.

An MOS for 2.5% 1,2,4-Trihydroxybenzene was determined to be 1397.8 for a dermal exposure. This calculation is based on the NOAEL of 50 mg/kg bw/d from a 90-d oral rat study and an SED of 0.03577 mg/kg bw. The MOS value is greater than 100, a figure generally accepted as the threshold for considering an ingredient safe to use.

DISCUSSION

To be developed.

CONCLUSION

To be determined.

TABLES

Table 1. Chemical properties

Property	Value	Reference
Physical Form	Light-medium beige powder	[4]
Molecular Weight (g/mol)	126.11	[2]
Melting Point (ºC)	139 - 150	[2,3]
Water Solubility (g/l @ 20 ºC)	486	[3]
Other Solubility (g/100 ml @ 22 ºC)	ethanol: > 1, < 10 DMSO: > 10, < 20	[3]
log P_{ow}	0.2 (estimated)	[2]
UV/Visible Spectrum (λ_{max}; nm)	291	[3]

Table 2. Genotoxicity studies

Ingredient	Concentration/Dose	Vehicle	Test System	Procedure	Results	Reference
			IN VITRO			
1,2,4-Trihydroxybenzene (purity not stated)	2.5 - 40 µg/plate (without metabolic activation); 1 – 2000 µg/plate (with metabolic activation)	not reported	*Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA104, TA1535	Bacterial reverse mutation test performed with and without metabolic activation	Mutagenic; test substance induced gene mutation in strain TA104 with metabolic activation	[34]
1,2,4-Trihydroxybenzene; 98.1% pure	6.25 - 4000 µg/plate	purified water	*S. typhimurium* strains TA98, TA100, TA102, TA1535, TA1537	Bacterial reverse mutation test in accordance with OECD TG 471; with and without S9 metabolic activation	Mutagenic; test material induced gene mutations in strains TA98 and TA100 without metabolic activation; toxic effects (i.e., reduction in the number of revertant colonies and/or thinning of the bacterial lawn) was observed at higher concentration with and without metabolic activation in nearly all strains used	[2,3]
1,2,4-Trihydroxybenzene; 97.8 - 99.5% pure	6.7 - 5000 µg/plate	degassed water	*S. typhimurium* strains TA98, TA100, TA102, TA1535, TA1537	Bacterial reverse mutation test in accordance with OECD TG 471; with and without S9 metabolic activation	Mutagenic; test material induced positive mutagenic response in strain TA1537 without metabolic activation	[4]
1,2,4-Trihydroxybenzene; 98.1% pure	100 - 500 µg/plate	degassed water	*S. typhimurium* strain TA1537	Bacterial reverse mutation test in accordance with OECD TG 471; without S9 metabolic activation; study evaluated the effect of radical scavengers, catalase (1000 - 20,000 IU) and L-glutathione (5 – 15 µM), with the test material	Mutagenic; effect observed in the study described above was repeated in test strain without metabolic activation; mutagenic effect was eliminated in presence of 5 and 10 µM L-glutathione and in the presence of 1000 - 20,000 IU of catalase	[4]
1,2,4-Trihydroxybenzene; 99.4% pure	Test 1: up to 20 µg/ml without metabolic activation and up to 160 µg/ml with metabolic activation Test 2: up to 22.5 µg/ml without metabolic activation and up to 240 µg/ml with metabolic activation	not reported	L5178Y mouse lymphoma cells	Mammalian cell gene mutation test at the *hprt* locus in accordance with OECD TG 476; with and without metabolic activation; appropriate negative and positive controls used	Not genotoxic; no statistically significant increase in mutant frequency was observed at any dose level tested, with or without metabolic activation; positive and negative controls yielded expected results	[2,3]
1,2,4-Trihydroxybenzene (purity not stated)	At least 5 concentrations used; optimal/maximal concentration of 10 µM; no further details provided	not reported	Chinese hamster V79 cells	Gene mutation assay; cells were incubated with test material for 24 h; frequency of mutants to 6-thioguanine (7 µg/ml) determined after an expression period of 6 d	Genotoxic; gene mutations induced at optimal/maximal concentration of 10 µM	[34]
1,2,4-Trihydoxybenzene; purity not reported	6, 12, or 24 µM	not reported	ICR mouse bone marrow cells	DNA strand break test; cells treated with test material for 1 h; alkaline DNA elution method (pH > 9.5) used; fractions collected every 24 min over a total of 120 min; cells exposed to test material also evaluated for protective effects of glutathione (350 µg/ml) and catalase (130 IU/ml) tested in parallel	Genotoxic; concentration-dependent increase in alkali-labile DNA single strand breaks observed, with a 42% increase at the highest concentration tested; double-strand breaks were not observed, but a significant pH-dependent increase in DNA elution rate was observed in treated cells when the elution pH increased from 9.6 to 12.6; DNA damage by test material was 53% blocked by glutathione and completely blocked by catalase	[3,35]

Table 2. Genotoxicity studies

Ingredient	Concentration/Dose	Vehicle	Test System	Procedure	Results	Reference
1,2,4-Trihydroxybenzene; 99.4% pure	Test 1: 1.25 - 5 µg/ml without metabolic activation and 3.75 - 15 µg/ml with metabolic activation Test 2: 2.5 - 7.5 µg/ml without metabolic activation and 10 - 20 µg/ml with metabolic activation	not reported	human lymphocytes	Mammalian chromosome aberration test in accordance with OECD TG 473, with and without S9 metabolic activation; appropriate negative and positive controls used	Not clastogenic; test material did not induce any significant increase in aberrant cell frequency, with or without metabolic activation; however, test concentrations did not induce required degree of cytotoxicity and an insufficient number of cells was evaluated in some cases	2
1,2,4-Trihydroxybenzene; purity not reported	24 µM	not reported	Crl:COBS CD-1 ICR BR mice bone marrow cells	DNA synthetic activity inhibition assay; DNA synthesis evaluated via the addition of [^{3}H]thymidine into DNA	Genotoxic; the test substance inhibited 64% of nuclear DNA synthetic activity; IC_{50} determined to be 19.4 µM	36
1,2,4-Trihydroxybenzene; purity not reported	0 - 24 µM	not reported	cell-free DNA assay system	DNA synthetic activity inhibition assay; test substance incubated with reaction mixture containing DNA polymerase (either DNA polymerase α or DNA polymerase I) for 30 min, followed by the addition of ^{3}H-TTP; filtered precipitated DNA evaluated for radioactivity	Genotoxic; when DNA polymerase α was used as the source of DNA polymerase, a dose-related inhibition of DNA synthesis was observed (IC_{50} = 15 µM); no inhibitory effect observed when DNA polymerase I was used	36
1,2,4-Trihydroxybenzene; purity not reported	1 - 1000 µM	not reported	L5178YS mouse lymphoma cells	DNA strand break test; cells exposed to test substance, followed by alkaline denaturation method, including hydroxylapatite chromatography, to separate single- and double-stranded DNA in order to examine DNA strand breaks	Genotoxic; the test substance produced DNA breaks in a dose-related fashion; ED_{50} values for induction of single-stranded DNA was 55µM	28
1,2,4-Trihydroxybenzene (purity not stated)	At least 5 concentrations used up to 25 µM, no further details provided	not reported	Chinese hamster V79 cells	Micronucleus assay; cells were incubated with test material for 24 h	Genotoxic; elevated frequencies of micronucleated cells observed at 25 µM	34
1,2,4-Trihydroxybenzene; 98.1% pure	Range finding: 0.50 - 200 µg/cm^2 Test 1: 1.5 - 200 µg/cm^2 Test 2: 12 – 224 µg/cm^2 Test 3: 3 - 224 µg/cm^2	degassed acetone	MatTek EpiDerm™ human reconstructed skin tissue	Micronucleus assay; 3 tissue models per concentration in Tests 1, 2, and 3; tissue was exposed to 10 µl of test material in solution and incubated for 24 h twice (48 h total) before harvesting in Tests 1 and 2; Test 3 was confirmatory and 72 h in duration	Not genotoxic; induction of micronuclei did not occur in reconstructed skin	4
1,2,4-Trihydroxybenzene; 97.8% pure	Range finding: 0.126 - 1260 µg/ml, with and without metabolic activation 4 h exposure + 20 h: 1.26 - 150 µg/ml, with and without metabolic activation 24 h exposure: 0.1 - 100 µg/ml without metabolic activation	degassed water	human lymphocytes	Micronucleus test in accordance with OECD TG 487, with and without S9 metabolic activation; appropriate negative and positive controls used	Not genotoxic; percentage of cells with micronuclei in treated group not significantly increased relative to vehicle control at any dose level, with or without metabolic activation; positive and negative controls yielded expected results	4

Table 2. Genotoxicity studies

Ingredient	Concentration/Dose	Vehicle	Test System	Procedure	Results	Reference
1,2,4-Trihydroxybenzene; purity not reported	15, 20, 30 µg/ml	DMSO and water	human TK6 lymphoblastoid cells	Flow cytometry assay used to measure relative survival, apoptotic/necrotic cells, and micronucleus induction in parallel with application of TGx-28.65 genomic biomarker; lymphoblastoid cell exposures to test substance occurred in the presence of 2% hepatic S9 fraction; vehicle used as negative control; benzo[*a*]pyrene used as positive control; TK6 cells exposed for 4 h, rinsed, re-suspended, and re-incubated for additional 3 - 4 h for gene expression analysis and 20 h for flow cytometry analysis note: TGx-28.65 genomic biomarkers were developed by the authors were previously based on a database of gene expression profiles derived from human TK6 cells exposed to 28 well-known compounds; the biomarker comprises 65 genes that can classify chemicals as DNA damaging or non-DNA damaging	Genotoxic; dose-dependent declines in relative survival and increase in apoptosis; strong significant increase in micronucleus induction at all concentrations; the test substance was considered to be genotoxic at all three test concentrations. Controls gave expected results. TGx-28.65 analysis classified the test substance as genotoxic.	[37]
1,2,4-Trihydoxybenezene; purity not reported	10, 25, 50 or 100 µM	not reported	human lymphocytes	Cytokinesis-block micronucleus test in combination with a fluorescence in situ hybridization technique with specific centromeric probes for chromosomes 7 and 8; lymphocytes were treated for 48 h with test material; 1000 binuclear cells scored for presence of micronuclei	Clastogenic and aneugenic; concentration-dependent and statistically significant increase in number of lymphocytes with micronuclei observed; concentration-dependent and statistically significant induction of aneuploidy of chromosomes 7 and 8 observed, with aneuploidy 8 being more frequent; non-disjunction of chromosomes 7 and 8 also observed	[3,39]
1,2,4-Trihydroxybenzene (purity not stated)	Optimal/maximal concentration was 10 µM, no further details provided	not reported	Chinese hamster V79 cells	SCE assay; cells were incubated with test material for 27 h; a total of 30 metaphases were scored for SCE per data point	Genotoxic; statistically significant ($p > 0.01$) number of SCE per cell above background at an optimal/maximal concentration of 10 µM	[34]
1,2,4-Trihydroxybenzene; 99% pure	5, 50, 70, 100, 300, or 500 µM	RPMI 1640 medium	human lymphocytes	SCE assay; lymphocytes treated with test material for 48 h in presence of BrdU	Genotoxic; concentration-dependent decrease of mitotic activity observed; concentration-dependent increase in SCE observed	[3,38]
1,2,4-Trihydroxybenzene; 97.8% pure	Range finding: 0.1 - 100 mg/ml or 1.6 - 1600 µg/cm^2 Test 1: 0.125 - 1 mg/ml or 2 - 16 µg/cm^2 Test 2: 0.25 - 1.25 mg/ml or 4 - 20 µg/cm^2	degassed acetone	Phenion® full thickness human skin model consisting of human primary keratinocytes and fibroblasts from single donor origin	3D skin comet assay; application volume was 16 µg/cm^2; total exposure time was 48 h; negative and positive controls were run in parallel	Not genotoxic; test material did not induce DNA damage to human skin cells after topical application; controls yielded expected results	[4]

Table 2. Genotoxicity studies

Ingredient	Concentration/Dose	Vehicle	Test System	Procedure	Results	Reference
1,2,4-Trihydroxybenzene; 99% pure	10, 25, and 50 µM	PBS	human peripheral blood	Blood exposed to test substance, and ploidy status of 9 different chromosomes evaluated using fluorescence in situ hybridization of metaphase spreads; 48-h chemical exposure	Genotoxic; test substance produced a dose-dependent increase in monosomy of chromosomes 5, 7, 8, and 9, but not of chromosomes 1 and 21; more profound effect observed on monosomy 5 and 7 compared to other chromosomes, and at low levels of exposure; the test substance also induced trisomy in all evaluated chromosomes in a dose-dependent manner	[33]
			IN VIVO			
1,2,4-Trihydroxybenzene; 99.4% pure	50 mg/kg bw	water	5 Swiss OF1 mice per sex	Mammalian erythrocyte micronucleus test in accordance with OECD TG 474; single intraperitoneal dose; appropriate negative and positive controls used	Not genotoxic; number of micronucleated polychromatic erythrocytes did not differ statistically from the vehicle control values; ratio of polychromatic to normochromatic erythrocytes decreased significantly ($p < 0.05$) 24 h after treatment and ($p < 0.001$) 48 h after treatment, indicating a toxic effect of the test material to bone marrow cells; SCCP noted test not in accordance with current OECD TG as only 1 dose was tested	[2]
1,2,4-Trihydroxybenzene; 99% pure	6.3, 12.5, or 25 mg/kg (low and mid-concentrations did not meet acceptance criterion for % of targe, actual values achieved were 5.32 and 8.15 mg/kg); range finding doses were 25, 50, and 100 mg/kg	degassed deionized water	groups of 6 Hsd:ICR (CD-1) male mice; range finding study used groups of 3 males and 3 females mice of same strain	Mammalian erythrocyte micronucleus test in accordance with OECD TG 474; mice received single intraperitoneal injection (5 ml/kg); blood samples taken 1-h post dosing in 3 mice/dose group; micronucleated cells scored at 24 and 48 h post-dosing; appropriate negative and positive controls used	Not genotoxic; no statistically significant increase in the incidence of micronucleated polychromatic erythrocytes observed at either time point relative to the vehicle control; positive control yielded expected results; no detectable presence of test article in blood samples; in range finding study, all but 1 female died in the high dose group and mice in mid-dose group had severe clinical signs of toxicity	[40]

REFERENCES

1. Nikitakis J, Kowcz A. Web-Based International Cosmetic Ingredient Dictionary and Handbook. https://incipedia.personalcarecouncil.org/winci/. Washington, DC: Personal Care Products Council. Last Updated 2023. Accessed 06/22/2023.

2. Scientific Committee on Consumer Products (SCCP). Opinion on 1,2,4-Trihydroxybenzene (COLIPA No. A33). 2006. SCCP/0962/05. https://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_041.pdf. Accessed 06/30/2023.

3. Scientific Committee on Consumer Safety (SCCS). Opinion on 1,2,4-Trihydroxybenzene (COLIPA No. A33). 2012. SCCS/1452/11. https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_113.pdf. Accessed 09/18/2023.

4. Scientific Committee on Consumer Safety (SCCS). Opinion on hair dye 1,2,4-trihydroxybenzene (1,2,4-THB), COLIPA No. A33 (CAS 533-73-3), Submission VI. 2019. SCCS/1598/18. https://health.ec.europa.eu/system/files/2021-08/sccs_o_222_0.pdf. Accessed 09/19/2023.

5. Zhang L, Bandy B, Davison AJ. Effects of metals, ligands and antioxidants on the reaction of oxygen with 1,2,4-benzenetriol. *Free Radic Biol Med*. 1996;20(4):495-505.

6. Jackson H. The oxidation of catechol and 1:2:4-trihydroxybenzene by polyphenol oxidase. *Biochem J*. 1939;33(9):1452-1459.

7. Medeiros AM, Bird MG, Witz G. Potential biomarkers of benzene exposure. *J Toxicol Environ Health*. 1997;51(6):519-539.

8. Inoue O, Seiji K, Nakatsuka H, et al. Excretion of 1,2,4-benzenetriol in the urine of workers exposed to benzene. *Br J Ind Med*. 1989;46(8):559-565.

9. Mathews JM, Etheridge AS, Matthews HB. Dose-dependent metabolism of benzene in hamsters, rats, and mice. *Toxicol Sci*. 1998;44(1):14-21.

10. Hopper W, Mahadevan A. Degradation of catechin by *Bradyrhizobium japonicum*. *Biodegradation*. 1997;8(3):159-165.

11. Arunachalam M, Raj MM, Mohan N, Mahadevan A. Biodegradation of catechin. *Proc Indian Natn Sci Acad B*. 2003;69(4):353-370.

12. Travkin VM, Solyanikova IP, Golovleva LA. Hydroxyquinol pathway for microbial degradation of halogenated aromatic compounds. *J Environ Sci Health B*. 2006;41(8):1361-1382.

13. Butt MS, MT S. Coffee and its consumption: Benefits and risks. *Crit Rev Food Sci Nutr*. 2011;51(4):363-373.

14. Kamae R, Nojima S, Akiyoshi K, et al. Hydroxyhydroquinone, a by-product of coffee bean roasting, increases intracellular Ca^{2+} concentration in rat thymic lymphocytes. *Food Chem Toxciol*. 2017;102:39-45.

15. Muller C, Lang R, Hofmann T. Quantitative precursor studies on di- and trihydroxybenzene formation during coffee roasting using "in bean" model experiments and stable isotope dilution analysis. *J Agric Food Chem*. 2006;54(26):10086-10091.

16. Ochiai R, Nagao T, Katsuragi Y, Tokimitsu I, Funatsu K, Nakamura H. Effects of hydroxyhydroquinone-reduced coffee in patients with essential hypertension. *Hypertens Res*. 2009;32(11):969-974.

17. U.S. Food and Drug Administration Center for Food Safety & Applied Nutrition (CFSAN). Voluntary Cosmetic Registration Program - Frequency of Use of Cosmetic Ingredients. College Park, MD. 2023. Obtained under the Freedom of Information Act from CFSAN; requested as "Frequency of Use Data" January 4, 2023; received February 2, 2023.

18. Personal Care Products Council. 2023. Concentration of use by FDA product category: 1,2,4-Trihydroxybenzene. Unpublished data submitted by Personal Care Products Council on February 22, 2023.

19. EUR-Lex. Commission Regulation (EU) 2020/1683 of 12 November 2020 amending Annexes II and III to Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products. (Text with EEA relevance). https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32020R1683&qid=1685047721061#ntr4-L_2020379EN.01003401-E0004. Last Updated 11/13/2020. Accessed 05/26/2023.

20. Mi H, Hiramoto K, Kujirai K, Ando K, Ikarashi Y, Kikugawa K. Effect of food reductones, 2,5-dimetyl-4-hydroxy-3(2H)-furanone (DMHF) and hydroxyhydroquinone (HHQ), on lipid peroxidation and Type IV and I allergy responses of mouse. *J Agric Food Chem*. 2001;49(10):4950-4955.

21. Ishida K, Misawa K, Yamamoto M, Shimotoyodome A. Hydroxyhydroquinone impairs fat utilization in mice by reducing nitric oxide availability. *J Physiol Sci*. 2018;68(6):855-864.

22. Kawanishi S, Inoue S, Kawanishi M. Human DNA damage induced by 1,2,4-benzenetriol, a benzene metabolite. *Cancer Res*. 1989;49(1):164-168.

23. Lee SF, Liang YC, Lin JK. Inhibition of 1,2,4-benzenetriol-generated active oxygen species and induction of phase II enzymes by green tea polyphenols. *Chem Biol Interact*. 1995;98(3):283-301.

24. Levay G, Bodell WJ. Potentiation of DNA adduct formation in HL-60 cells by combination of benzene metabolites. *Proc Natl Acad Sci*. 1992;89(15):7105-7109.

25. Lewis JG, Stewart W, Adams DO. Role of oxygen radicals in induction of DNA damage by metabolites of benzene. *Cancer Res*. 1988;48(17):4762-4765.

26. Shen Y, Shen H-M, Shi C-Y, Ong C-N. Benzene metabolites enhance reactive oxygen species generation in HL60 human leukemia cells. *Hum Exp Toxicol*. 1996;15(5):422-427.

27. Zhang L, Robertson ML, Kolachana P, Davison AJ, Smith MT. Benzene metabolite, 1,2,4-benzenetriol, induces micronuclei and oxidative DNA damage in human lymphocytes and HL60 cells. *Environ Mol Mutagen*. 1993;21(4):339-348.

28. Pellack-Walker P, Blumer JL. DNA damage in L5178YS cells following exposure to benzene metabolites. *Molecular Pharmacology*. 1986;30(1):42-47.

29. Nishikawa T, Miyahara E, Horiuchi M, et al. Benzene metabolite 1,2,4-benzenetriol induces halogenated DNA and tyrosines representing halogenative stress in the HL-60 human myeloid cell line. *Environ Health Perspect*. 2012;120(1):62-67.

30. Schwartz CS, Snyder R, Kalf GF. The inhibition of mitochondrial DNA replication in vitro by the metabolites of benzene, hydroquinone and *p*-benzoquinone. *Chem Biol Interact*. 1985;53(3):327-350.

31. Zhang L, Wang Y, Shang N, Smith MT. Benzene metabolites induce the loss and long arm deletion of chromosomes 5 and 7 in human lymphocytes. *Leuk Res*. 1998;22(2):105-113.

32. Zhang L, Venkatesh P, Creek MLR, Smith MT. Detection of 1,2,4-benzenetriol induced aneuploidy and microtubule disruption by fluorescence in situ hybridization and immunocytochemistry. *Mutat Res*. 1994;320(4):315-327.

33. Zhang L, Yang W, Hubbard AE, Smith MT. 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*. 2005;45(4):388-396.

34. Glatt H, Padykula R, Berchtold GA, et al. Multiple activation pathways of benzene leading to products with varying genotoxic characteristics. *Environ Health Perspect*. 1989;82:81-89.

35. Lee EW, Garner CD. Effects of benzene on DNA strand breaks *in vivo* versus benzene metabolite-induced DNA strand breaks *in vitro* in mouse bone marrow cells. *Toxicol Appl Pharmacol*. 1991;108(3):497-508.

36. Lee EW, Johnson JT, Garner CD. Inhibitory effect of benzene metabolites on nuclear DNA synthesis in bone marrow cells. *J Toxicol Environ Health*. 1989;26(3):277-291.

37. Buick JK, Williams A, Kuo B, et al. 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*. 2017;806:51-62.

38. Erexson GL, Wilmer JL, Kligerman AD. Sister chromatid exchange induction in human lymphocytes exposed to benzene and its metabolites *in vitro*. *Cancer Res*. 1985;45(6):2471-2477.

39. Chung HW, Kang SJ, Kim SY. A combination of the micronucleus assay and a FISH technique for evaluation of the genotoxicity of 1,2,4-benzenetriol. *Mutation Res*. 2002;516(1-2):49-56.

40. BioReliance Corporation. 2021. In Vivo Erythrocytes Micronucleus Assay in Mice. Unpublished data submitted by Combe on January 31, 2023.

41. Burnett C, Jacobs MM, Seppala A, Shubik P. Evaluation of the toxicity and carcinogenicity of hair dyes. *J Toxicol Environ Health*. 1980;6(2):247-257.

42. Shibata M-A, Hirose M, Yamada M, Tatematsu M, Uwagawa S, Ito N. Epithelial cell proliferation in rat forestomach and glandular stomach mucosa induced by catechol and analogous dihydroxybenzenes. *Carcinogenesis*. 1990;11(6):997-1000.

43. Wang Y, Zhang G-Y, Han Q-L, et al. Phenolic metabolites of benzene induced caspase-dependent cytotoxicities to K562 cells accompanied with decrease in cell surface sialic acids. *Environ Toxicol*. 2014;29(12):1437-1451.

44. Wu X-R, Xue M, Li X-F, et al. Phenolic metabolites of benzene inhibited the erythroid differentiation of K562 cells. *Toxicol Lett*. 2011;203(3):190-199.

45. Rao NR, Snyder R. Oxidative modifications produced in HL-60 cells on exposure to benzene metabolites. *J Appl Toxicol*. 1995;15(5):403-409.

46. Gaido K, Wierda D. In vitro effects of benzene metabolites on mouse bone marrow stromal cells. *Toxicol Appl Pharmacol*. 1984;76(1):45-55.

47. Yu C-H, Li Y, Zhao X, et al. Benzene metabolite 1,2,4-benzenetriol changes DNA methylation and histone acetylation of erythroid-specific genes in K562 cells. *Arch Toxicol*. 2019;93(1):137-147.

48. Hou R C-W, Chen Y-S, Chen C-H, Chen Y-H, Jeng K-C G. Protective effect of 1,2,4-benzenetriol on LPS-induced NO production by BV2 microglial cells. *J Biomed Sci*. 2006;13(1):89-99.

49. del Mar Garcia-Molina M, Munoz-Munoz JL, Berna J, Garcia-Ruiz PA, Rodriquez-Lopez JN, Garcia-Canovas F. Catalysis and inactivation of tyrosinase in its action on hydroxyhydroquinone. *IUBMB Life*. 2014;66(2):122-127.

50. Scientific Committee on Consumer Safety (SCCS). The SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation, 12th Revision. 2023. SCCS/1647/22. https://health.ec.europa.eu/document/download/9770133c-8120-47cf-81e6-5af997060724_en?filename=sccs_o_273.pdf. Accessed 10/25/2023.

51. European Centre for Ecotoxicology and Toxicolog Chemicals (ECETOC). Derivation of Assessment Factors for Human Health Risk Assessment. Brussels, Belgium 2003. Technical Report 86. https://www.ecetoc.org/wp-content/uploads/2014/08/ECETOC-TR-086.pdf. Accessed 10/25/2023.

Concentration of Use by FDA Product Category – 1,2,4-Trihydroxybenzene

Product Category	Maximum Concentration of Use
Hair dyes and colors	2.5%

Information collected in 2022-2023

Table prepared: February 22, 2023

Calculation of the Margin of Safety (MOS) for 1,2,4-Trihydroxybenzene

In a dermal absorption in vitro study using frozen human dermatomed skin, a direct dye formulation was used, resulting in final concentrations of 2.5% (w/w) [^{14}C]- 1,2,4-Trihydroxybenzene.[1] Given that 1,2,4-Trihydroxybenzene is intended to be used alongside primary intermediates such as p-toluenediamine (PTD), the test was also conducted both with or without p-PTD. According to the Council's 2023 survey, 1,2,4-Trihydroxybenzene is used at up to 2.5% in hair dyes and colors.[2] Thus, the test concentration in such in vitro study represents the maximum use concentration of 1,2,4-Trihydroxybenzene in cosmetics.

The parameters that are used for the MOS calculation are listed below:

The systemically available dose: 3.70 µg equivalents/cm^2 (the mean + 1 Standard Deviation (SD))[1]

Note: In the test, the systemically available dose of [^{14}C]-1,2,4- Trihydroxybenzene alone was 1.71 µg equivalents/cm^2 (1.13 µg equivalents/cm^2 + 0.58 (1SD)), while the systemically available dose of [^{14}C]-1,2,4- Trihydroxybenzene with p-PTD was 3.70 µg equivalents/cm^2 (1.94 µg equivalents/cm2 + 1.76 (1SD)). For a cautious risk estimation, the SCCS recommends using the maximum value of 3.70 µg equivalents/cm^2 in the risk assessment.

Skin surface area for application: 580 cm^2 (½ area head)[3]

Dermal absorption per treatment: 3.70 µg equivalents/cm^2 × 580 cm^2 = 2146 µg = 2.146 mg

Human body weight: 60 kg

Systemic exposure dose (SED): 2.146 mg ÷ 60 kg = 0.03577 mg/kg bw/d

No Observed Adverse Effect Level (NOAEL): 50 mg/kg bw/d (90-d, gavage, oral, rat)[4,5]

$$\textbf{MOS} = \frac{\textbf{NOAEL}}{\textbf{SED}} = \frac{50 \text{ mg/kg bw/d}}{0.03577 \text{ mg/kg bw/d}} = 1397.8$$

Note: the SCCS determined that the dose of 50 mg/kg bw/d can not be viewed as the NOAEL because there was a significant increase in the relative spleen weight of male rats treated at this dose; instead, the SCCS considered 50 mg/kg bw/d as the Lowest-observed-adverse-effect-level (LOAEL).[1,4] When an NOAEL cannot be established and only the LOAEL is available, an additional assessment factor might be applied. Typically, the maximum value of the assessment factor recommended for extrapolating an LOAEL to an NOAEL is 10; however, in some cases, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) suggests a default value of 3.[6] Consequently, using a dose of 50 mg/kg bw/d (presumed to be the LOAEL), the NOAEL can be deduced as either 16.7 mg/kg bw/d or 5 mg/kg bw/d,

depending on whether an assessment factor of 3 or 10 is applied. As a result, the MOS values can be calculated to be 466.9 or 139.8, respectively.

For comparison purpose, the in silico tool VERMEER Cosmolife (a software formerly known as SpheraCosmolife),[7] was further utilized to estimate an NOAEL for 1,2,4-Trihydroxybenzene. While VERMEER Cosmolife does not provide a precise SED, it suggests a predicted NOAEL of 107.52 mg/kg bw/d based on the IRFMN/Coral model.[7,8]

References:

1. Scientific Committee on Consumer Safety (SCCS). *Opinion on hair dye 1,2,4-trihydroxybenzene (1,2,4-THB), COLIPA No. A33 (CAS 533-73-3), Submission VI.* 2019. SCCS/1598/18.
2. Personal Care Products Council. Concentration of use by FDA product category: 1,2,4-Trihydroxybenzene. In:2023.
3. Scientific Committee on Consumer Safety (SCCS). *The SCCS's notes of guidance for the testing of cosmetic ingredients and their safety evaluation (12th Revision).* 2023. SCCS/1647/22.
4. Scientific Committee on Consumer Products (SCCP). *Opinion on 1,2,4-Trihydroxybenzene (COLIPA No. A33).* 2006. SCCP/0962/05.
5. Scientific Committee on Consumer Safety (SCCS). *Opinion on 1,2,4-Trihydroxybenzene (COLIPA No. A33).* 2012. SCCS/1452/11.
6. European Centre for Ecotoxicology and Toxicolog of Chemicals (ECETOC). Derivation of Assessment Factors for Human Health Risk Assessment - Technical Report No. 86. In:2003:1-90.
7. Selvestrel G, Robino F, Baderna D, et al. SpheraCosmolife: a new tool for the risk assessment of cosmetic products. *ALTEX.* 2021;38(4):565-579.
8. Toropov AA, Toropova AP, Pizzo F, Lombardo A, Gadaleta D, Benfenati E. CORAL: model for no observed adverse effect level (NOAEL). *Mol Divers.* 2015;19(3):563-575.

FINAL REPORT

Study Title

***In Vivo* Mammalian Erythrocyte Micronucleus Assay in Mice**

Test Article

1, 2, 4-Trihydroxybenzene

Author

Marie E. McKeon, MPhil

Study Completion Date

22 October 2021

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF98GS.123M012.BTL

Sponsor

Harris Beach PLLC
100 Wall Street
New York, NY 10005

1. STATEMENT OF COMPLIANCE

Study No. AF98GS.123M012.BTL was conducted in compliance with the following regulation: US FDA Good Laboratory Practice Regulations as published in 21 CFR Part 58. This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity and composition or other characteristics to define the test article were determined by Sigma-Aldrich (Steinheim, Germany). However, the characterization documents do not indicate the regulations under which the analyses were conducted.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test article as supplied.

2. The stability to define the test article has not been determined. The stability of the test article in vehicle was determined at BioReliance and was found to be stable on wet ice for at least 2 hours.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test article as supplied and on the results of the analysis of the formulations used for dosing.

Marie E. McKeon 22 October 2021

Marie E. McKeon, MPhil — Date
Study Director

2. QUALITY ASSURANCE STATEMENT

BioReliance® by SAFC

Quality Assurance Statement

Study Information

Number: AF98GS.123M012.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US FDA Good Laboratory Practices 21CFR 58

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
19-Nov-2019	19-Nov-2019	Protocol Review	19-Nov-2019	19-Nov-2019
06-Dec-2019	06-Dec-2019	Slide Preparation	06-Dec-2019	06-Dec-2019
09-Dec-2019	09-Dec-2019	Protocol Amendment Review	09-Dec-2019	09-Dec-2019
30-Dec-2019	03-Jan-2020	Data/Draft Report - Analytical Dose Sample Analysis	03-Jan-2020	03-Jan-2020
16-Jan-2020	16-Jan-2020	Final Report - Analytical Dose Sample Analysis	16-Jan-2020	16-Jan-2020
28-Jan-2020	30-Jan-2020	Data - Formulation	30-Jan-2020	30-Jan-2020
28-Jan-2020	30-Jan-2020	Data/Draft Report	30-Jan-2020	30-Jan-2020
18-Oct-2021	18-Oct-2021	Protocol Amendment Review	18-Oct-2021	18-Oct-2021
18-Oct-2021	18-Oct-2021	Protocol Amendment Review	18-Oct-2021	18-Oct-2021
18-Oct-2021	18-Oct-2021	Protocol Amendment Review	18-Oct-2021	18-Oct-2021
18-Oct-2021	19-Oct-2021	Final Report	19-Oct-2021	19-Oct-2021
18-Oct-2021	18-Oct-2021	Protocol Amendment Review	18-Oct-2021	18-Oct-2021
22-Oct-2021	22-Oct-2021	Protocol Amendment Review	22-Oct-2021	22-Oct-2021

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Carlos Bonilla 22-Oct-2021 6:12 pm GMT

Reason for signature: QA Approval

Printed by:Carlos Bonilla

Printed on:22-Oct-21

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4. STUDY INFORMATION

Study Conduct

Sponsor:	Harris Beach PLLC 100 Wall Street New York, NY 10005
Sponsor's Authorized Representative:	Judi Abbott Curry
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF98GS.123M012.BTL

Test Article

Identification:	1, 2, 4-Trihydroxybenzene
Synonym:	1,2,4-Benzenetriol
Lot No.:	S36884V
Storage Conditions:	Room temperature, protected from light and stored with desiccant under nitrogen. -10 to -30ºC**,** protected from light and stored with desiccant under nitrogen: updated per email on 13 November 2019).
Purity:	99%
Molecular Weight:	126.11 g/mol
Description:	Off-white powder
Special Handling:	Weighed inside glove box, purged with nitrogen after the first weighing and after each subsequent opening of the container.
Receipt Date:	13 November 2019

Study Dates

Study Initiation Date:	13 November 2019
Experimental Starting Date (First Day of Data Collection):	14 November 2019
Experimental Start Date (First Day of Dosing):	20 November 2019
Experimental Completion Date:	16 February 2020

Key Personnel

Study Directors:	Megan Young, PhD Marie E. McKeon, MPhil (Effective 07 Dec 2020)
Test Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Tiffany Blaylock, BS
Laboratory Manager (Dose Formulation Preparation):	Ann Noland, BS, RLATG, CMAR
Report Writer:	Joan Huynh, BS
Contributing Scientist (Analytical Chemistry Analysis):	Philip Atkins, MChem
Principal Investigator (BioAnalysis)	Tivadar Orban, PhD Senior Scientist and Manager
Analytical Test Site (BioAnalysis):	Frontage Laboratories Bioanalytical Services 10845 Wellness Way Concord, Ohio 44077

5. SUMMARY

The test article, 1, 2, 4-Trihydroxybenzene was evaluated for its clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocytes (PCEs) in mouse bone marrow. Degassed Deionized (DI) Water was used as the vehicle control. Test and/or vehicle control article formulations were administered once by intraperitoneal injection (IP) at a dose volume of 5 mL/kg/dose.

In the dose range-finding assay (DRF), the dose levels tested were 25, 50 and 100 mg/kg/dose in 3 animals/sex/group. Based upon the results demonstrating mortality at 100 mg/kg/dose, and severe clinical signs at 50 mg/kg/dose, the high dose for the definitive assay was 25 mg/kg/dose, which was estimated to be the maximum tolerated dose (MTD).

The definitive assay dose levels tested were 6.3, 12.5 and 25 mg/kg/dose in males only. Analysis of the formulations indicated that the high concentration was accurately prepared, however the low and mid concentrations did not meet the acceptance criterion for percent of target. Therefore, the actual low and mid dose levels achieved were 5.32 and 8.15 mg/kg instead of 6.3 and 12.5 mg/kg, respectively. The nominal dose levels of 6.3 and 12.5 mg/kg will be used throughout this report.

No statistically significant increase in the incidence of MnPCEs in the test article treated groups at either time point (24 or 28 hours) was observed relative to the vehicle control group. The positive control induced a statistically significant increase in the incidence of MnPCEs. The number of MnPCEs in the vehicle control group did not exceed the historical control range. There was no detectable presence of test article in blood samples taken 1 h post-dosing.

Under the conditions of this study, the administration of 1, 2, 4-Trihydroxybenzene at dose levels up to and including a dose level of 25 mg/kg was concluded to be negative in the micronucleus assay.

6. PURPOSE

The purpose of this study was to evaluate the test article for *in vivo* clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocytes in mouse bone marrow.

Historical control data are found in Appendix I. A copy of the study protocol and amendments is found in Appendix II.

The micronucleus assay is a short-term *in vivo* cytogenetic assay for detecting agents that induce chromosomal breakage or spindle malfunction. In this study, femoral bone marrow was microscopically evaluated for the presence of polychromatic erythrocytes (PCEs) containing micronuclei (MnPCEs). A clastogenic effect may be seen if micronuclei are formed during cell division as a result of chromosome breakage or malfunctioning of the mitotic spindle. In the case of chromosome breakage, acentric chromosome fragments may not be included in the nuclei of daughter cells forming single or multiple micronuclei in the cytoplasm. An aneugenic effect is seen if the test material interferes with the mitotic spindle apparatus; non-disjunction or lagging chromosome at anaphase may not be included in the nuclei of daughter cells forming single or multiple micronuclei in cytoplasm of the new cells. In this study, the incidence of micronucleated PCEs served as an indicator of test article genotoxicity.

7. CHARACTERIZATION AND PREPARATION OF TEST AND CONTROL ARTICLES

The supplier, Sigma-Aldrich (Steinheim, Germany) has determined the identity, strength, purity and composition or other characteristics to define the test article. Copy of the Certificate of Analysis is included in Appendix III, however stability of the test article has not been determined.

All unused Test Article was discarded prior to report finalization unless the test article is used on another study.

The following reagents were used in preparation of the vehicle:

Component	CAS Number	Supplier/Source	Lot/Batch Number	Expiration/Retest Date
Deionized (DI) water	N/A	Milli-Q IQ 7000	N/A	Day of use
Calcium carbonate (CO_3)	471-34-1	Sigma	SLB25250	05 Dec 21 & 30 Nov 22

Scoring positive control slides (fixed and unstained), generated from BioReliance Study No. AF65AY.123M012.BTL was included to verify scoring. These slides were generated from male rats treated once with cyclophosphamide monohydrate (CP) at 50 mg/kg, and the bone marrow harvested 24 hours after treatment.

The vehicle and positive control articles have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the vehicle and positive control articles and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

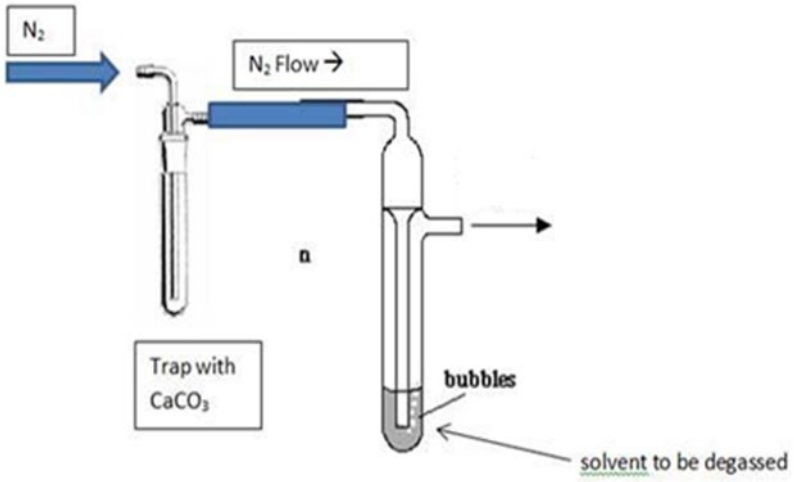
Stability of the Test Article in Vehicle

Stability of the dosing formulations was determined under BioReliance study number AE03RS.502.BTL. 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.

Preparation of Vehicle Control Article

The vehicle formulation was prepared prior to dose administration as follows:

Prior to use, DI was degassed for at least 30 minutes based on the Sponsor provided diagram, as indicated below. An appropriate amount of $CaCO_3$ was added to the first bubbler and appropriate amount of DI water was added second bubbler to reach final batch size. Nitrogen gas was flowed through the bubblers for at least 30 minutes until the presence of bubbles is visually confirmed. The container was sealed appropriately and use immediately or stored on wet ice and formulations were used within two hours of preparation.



Preparation of Test Article Dose Formulations

Dose formulations were prepared prior to dose administration as follows:

A suitably-sized amber glass vial with a PTFE stir bar was calibrated to the target batch size. All formulations were prepared on wet ice throughout the preparation whenever possible. The following steps were prepared inside a glove box purged with nitrogen gas. An appropriate amount of test article was placed within the calibrated vial. Approximately 70% of the total volume of vehicle was added while stirring and the remaining vehicle was added to achieve the final target volume. The formulations were stirred magnetically for 3 to 5 minutes and

vortexed (when needed) until uniform. Formulations were stored on wet ice and were used within two hours of preparation.

Dose Formulation Collection and Analysis

Dose formulation samples were collected on the day of preparation in an amber glass vial with crimp top:

Vehicle Sampling	
Number of Samples[A]	Volume
4[B]	0.5 mL

[A]On Day 1, two samples were used for analysis and the other samples served as backup. [B]Additional samples were needed for dosing; therefore, two additional samples were collected.

Solution Sampling		
Dose Level	Number of Samples[C]	Volume
High Dose	2	0.5 mL
Mid Dose	2	0.5 mL
Low Dose	2	0.5 mL

[C]One sample was used for analysis and the other sample served as backup

All samples collected for analysis or as backups were held on wet ice until delivered for analysis. Upon receipt, the samples designated for analysis were maintained at the conditions of receipt until the analysis was performed; however back-up samples were stored 2 to 8°C. After analysis, all samples and backups were stored at 2 to 8°C. Unused samples were discarded upon acceptance of the analytical results by the Study Director. A copy of the analytical report is included in Appendix IV.

8. MATERIALS AND METHODS

The assay was conducted according to established procedures (Heddle, 1973; Mavournin et al., 1990; Hayashi et al., 1994; OECD, 2016).

Animals were treated (on Day 1 at t = 0) with the test or control articles as described below.

The following procedures were used for the dose range-finding and definitive micronucleus assay as indicated.

Test System

Hsd:ICR (CD-1) mice were received from Envigo RMS, Inc., Frederick, MD on 14 November 2019 (DRF), 27 November 2019 (definitive assay).

The age at time of initiation, as well as the body weights and days of acclimation of the mice assigned to the study groups at randomization are indicated below:

Study	Sex	Body Weight Range at Randomization (grams)	Age at Initiation (weeks)	Days of Acclimation
DRF	Male	32.8 to 34.9	6	6
	Female	27.1 to 29.2	6	6
Definitive (Main)	Male	33.1 to 36.1	7	7
Definitive (TK)	Male	32.3 to 36.6	7	7

Justification for the Test System

This species has been routinely used as an animal model of choice for the mammalian bone marrow erythrocyte micronucleus assay. This strain is an outbred strain that maximizes genetic heterogeneity and therefore tends to eliminate strain-specific response to the test article.

Animal Welfare Provisions

This study is not duplicative or unnecessary. The number of animals, procedures, and design used for this study, has been reviewed and were approved by the BioReliance Institutional Animal Care and Use Committee. Procedures involving animals performed at BioReliance follow the specifications recommended in the most current version of *The Guide for the Care and Use of Laboratory Animals* adopted by BioReliance (National Academy Press, Washington, D.C., 2011).

Animal Receipt and Acclimation

Virus antibody-free (VAF) animals were acclimated as noted above and were judged to be healthy prior to utilization in the study.

Housing

Animals were housed in a controlled environment at 72 ± 3°F and 50 ± 20% relative humidity with a 12-hour light/dark cycle. The light cycle was not interrupted for study-related activities. The animal rooms were supplied with at least 10 changes of fresh HEPA-filtered air per hour. Animals were housed three per Micro-Barrier cage. Cages were placed on racks equipped with an automatic watering system and Micro-VENT full ventilation, HEPA filtered system.

Environmental Enrichment

Animals were provided with Nestlets™ as environmental enrichment.

Bedding, Food and Water

Heat treated hardwood chips were used for bedding to absorb liquids. A certified laboratory rodent chow (Envigo 2018C Teklad Global 18% Protein Rodent Diet) was provided *ad libitum*. The food was analyzed by the manufacturer for the concentrations of specified heavy metals, aflatoxin, chlorinated hydrocarbons, organophosphates and specified nutrients. Animals had free access to tap water, which met U.S. EPA drinking water standards [Washington Suburban Sanitary Commission (WSSC) Potomac Plant]. Drinking water was monitored at least annually for levels of specified microorganisms, pesticides, heavy metals, alkalinity and halogens. The results of bedding, food and water analyses are on file at BioReliance. There were no contaminants in the bedding, feed and water that were expected to interfere with the study.

Randomization and Identification

Animals were assigned to groups using a randomization procedure within Provantis™. At the time of randomization, the weight variation of animals did not exceed ±20% of the mean weight. Following randomization, animals were identified by sequentially numbered ear tags. The cage card contained, at least, the animal number(s), sex, study number, treatment group number, dose level, test article ID and route of administration. Cage cards were color coded by treatment group. Raw data records and specimens were also identified by the unique animal number.

Body Weights and Animal Observation

Body weights were recorded prior to the first dose for the purpose of dose volume calculations for all animals and once on the day of euthanasia excluding animals used for bioanalysis. Animals were observed once daily for signs of illness and poor health during the acclimation period. Once dosing was initiated, animals were observed for signs of illness or poor health or for clinical signs of toxicity as indicated below.

Procedure	Collection Timepoint
Moribundity and Mortality Check	Twice daily, beginning on the first day of dose administration
Detailed Hands-On Clinical Observations	Pre-dose on Day 1, excluding animals used for bioanalysis
Cage Side Observations	1 to 2 hours post-dose and at least once daily on non-dosing days, excluding animals used for bioanalysis.

Dose Administration

All dose formulations were administered once at a volume of 5 mL/kg via intraperitoneal injection (IP). This route has been routinely used and is widely-accepted for use in the mammalian bone marrow erythrocyte micronucleus assay. All Formulations were maintained on wet ice prior to and during dosing.

Dose Range-Finding Assay (DRF)

In the DRF assay, 3 animals/sex were exposed to 25, 50 and 100 mg/kg/dose of 1, 2, 4-Trihydroxybenzene. Mortality was observed in 3/3 males and 2/3 females at 100 mg/kg/dose. Clinical signs of decreased motor activity, hunched posture, labored breathing, piloerection rapid & shallow breathing and squinty eye were observed. Following the two day observation period, surviving animals were euthanized by carbon dioxide inhalation, and discarded without further examination. Due to mortality in both sexes and no differences in clinical observations were seen between the sexes, therefore only male rats were used in the definitive assay.

Definitive Micronucleus Assay

The high dose for the micronucleus assay was 25 mg/kg/dose, which is estimated to be the maximum tolerated dose (MTD).

Euthanasia Time (hrs after treatment)[A]				24	48
Group	Treatment*	Dose Level (mg/kg/dose)	Dose Volume[B] (mL/kg/dose)	Number of Male Animals	
1	Vehicle	0	5	6	6
2	1, 2, 4-Trihydroxybenzene	6.3	5	6	0
3	1, 2, 4-Trihydroxybenzene	12.5	5	6	0
4	1, 2, 4-Trihydroxybenzene	25	5	6	6

[A]Range(s): 24-27 hours and 45-48 hours, respectively

[B]Based upon individual body weight

* Analysis of the formulations indicated that the high concentration was accurately prepared, however the low and mid concentrations did not meet the acceptance criterion for percent of target. Therefore, the actual low and mid dose levels achieved were 5.32 and 8.15 mg/kg instead of 6.3 and 12.5 mg/kg, respectively.

Blood Collection and Sample Handling

Group	Treatment*	Dose Level (mg/kg)	Animals/ Timepoint	Collection Timepoint (hours after dose)	Total Number of Male Animals
6	Vehicle	0	3	1	3
7	1, 2, 4-Trihydroxybenzene	6.3	3	1	3
8	1, 2, 4-Trihydroxybenzene	12.5	3	1	3
9	1, 2, 4-Trihydroxybenzene	25	3	1	3

* Analysis of the formulations indicated that the high concentration was accurately prepared, however the low and mid concentrations did not meet the acceptance criterion for percent of target. Therefore, the actual low and mid dose levels achieved were 5.32 and 8.15 mg/kg instead of 6.3 and 12.5 mg/kg, respectively.

Frequency	1st day of dosing
Collection Site	Retro-orbital Sinus
Target Volume	0.5 mL of whole blood
Anesthesia	Animals were anesthetized prior to collection by 70% CO_2/30% O_2.
Anticoagulant	K_3EDTA
Sample Handling	Blood samples were maintained on wet ice until centrifugation.
Centrifugation	Blood samples were centrifuged for 5 minutes, 2-8°C, at 2000 g within 1 hour of collection, and plasma was harvested into two sets of approximately equal aliquots.
Sample Storage	Plasma samples were stored at ≤ -60ºC and was analyzed only as needed. One set was shipped on dry ice to the Principal Investigator for BioAnalysis and remaining samples were discarded.
Animal Disposition	Animals were sacrificed by CO_2 overdose after their last collection timepoint.

Bioanalysis (BioA)

Samples were shipped on dry ice by overnight courier to the Principal Investigator for BioAnalysis at the address below. Upon receipt, samples were stored at -60°C or below until required for method development qualification or analysis. Unused samples were discarded upon acceptance of the analytical results by the Study Director. A BioA contribution report with GLP Compliance Statement, signed by the Principal Investigator from the Test Site is included in Appendix V.

Shipment of the first set of samples were sent to:

Frontage Lab
Tivadar Orban, PhD
DMPK and Bioanalytical Services
Team Leader
10845 Wellness Way
Concord, Ohio 44077
Office Telephone: +1 (440) 357-3759
Email: torban@frontagelab.com

Notification of sample shipment by email to include:

Concord Sample Management
10845 Wellness Way
Concord, OH 44077
Office (440) 357-3039
ConcordSampleManagement@FrontageLab.com

Upon successful receipt of the first shipment, the second set of samples were shipped as above.

Tissue Sample Collection and Handling

Liver samples were collected from main study animals at approximately 24 or 48 hours after dose administration (at the time of bone marrow collection), as applicable. Liver (containing no gall bladder) samples were collected in duplicates of approximately 0.5g each for possible analysis. The liver samples were frozen on liquid nitrogen and stored at ≤ -60°C in pre-labeled plastic vials. Any unused samples were discarded prior to report finalization.

Bone marrow for possible analysis was collected from TK animals just following euthanasia after blood collection. The femurs were exposed, cut just above the knee, and the bone marrow was aspirated into an empty syringe. The bone marrow samples were frozen on liquid nitrogen and stored at ≤ -60°C in pre-labeled plastic vials. Any unused samples was discarded prior to report finalization.

Bone Marrow Collection and Slide Preparation

Animals were euthanized by carbon dioxide inhalation at approximately 24 or 48 hours after dose administration. Immediately following euthanasia, the femurs were exposed, cut just above the knee, and the bone marrow was aspirated into a syringe containing fetal bovine serum. The bone marrow was transferred to a centrifuge tube containing 1 mL fetal bovine serum, the cells were pelleted by centrifugation, and the supernatant was drawn off leaving a small amount of fetal bovine serum with the pellet. Cells were re-suspended and a small drop of the bone marrow suspension was spread onto a clean glass slide. Four slides were prepared from each animal, air dried and fixed by dipping in methanol. One set of two slides (including five positive control slides) were stained with acridine orange for microscopic evaluation. The other set of slides were kept as backup and were archived. Each slide was identified by the harvest date, study number, and animal number (or slide number for positive control slides). Slides were coded using a random number table by an individual not involved with the scoring process.

Scoring

Bone marrow was evaluated by fluorescent microscopy. The staining procedure permitted the differentiation by color of polychromatic and normochromatic erythrocytes (bright orange PCEs and ghost-like, dark green NCEs, respectively).

The criteria for the identification of micronuclei are those of Schmid (1975). Micronuclei are brightly stained bodies that generally are round and that generally are between 1/20 and 1/5 the size of the PCE. Scoring was based upon the micronucleated cell, not the micronucleus; thus, occasional cells with more than one micronucleus were counted as one micronucleated PCE (MnPCE), not two (or more) micronuclei.

4000 PCEs/animal were scored for the presence of micronuclei (MnPCEs). In addition, at least 500 total erythrocytes (PCEs + NCEs) were scored per animal to determine the proportion of PCEs as an index of bone marrow cytotoxicity.

Stained slides were discarded prior to report finalization.

Statistical Analysis

Statistical analysis was performed on the micronucleus frequency (%MnPCE) and %PCE using the animal as the unit. The mean and standard deviation of %MnPCE and %PCE were presented for each treatment group.

The use of parametric or non-parametric statistical methods in the evaluation of data was based on the variation between groups. The group variances for micronucleus frequency for the vehicle and test article groups at the respective sampling time were compared using Levene's test (significance level of $p \leq 0.05$). Since the variation between groups was found not to be significant in; a parametric one-way ANOVA was performed followed by a Dunnett's post-hoc analysis to compare each dose group to the concurrent vehicle control.

A linear regression analysis was conducted to assess dose responsiveness in the test article treated groups ($p \leq 0.01$ and $R^2 \geq 70\%$).

A pair-wise comparison (Student's T-test; $p \leq 0.05$) was used to compare the positive control group to the concurrent vehicle control group.

Criteria for Determination of a Valid Test

The group mean frequency of MnPCEs for the vehicle control group should ideally be within the 95% control limits of the distribution of the historical negative control database. If the concurrent negative control data fall outside the 95% control limits, they may be acceptable as long as these data are not extreme outliers (indicative of experimental or human error).

The frequency of MnPCEs for the scoring positive controls must be significantly greater than the concurrent vehicle control ($p \leq 0.05$) and should be compatible with those observed in the historical positive control data base.

At least three doses were tested for at least one sampling time. Six animals/group were available for analysis.

The maximum dose evaluated for micronucleus induction must:

a) be the MTD or MFD, or
b) demonstrate cytotoxicity in the bone marrow (reduction in the PCE/NCE ratio of more than 50% but not less than 20% of the control value), or
c) in the absence of cytotoxicity or MFD, a dose (limit dose) was used.

Evaluation of Test Results

A test article was considered to have induced a positive response if:

a) at least one of the test article doses exhibited a statistically significant increase when compared with the concurrent negative control ($p \leq 0.05$), and
b) when multiple doses were examined at a particular sampling time, the increase was dose-related ($p \leq 0.01$ and $R^2 \geq 70\%$), and
c) results of the group mean in at least one group were outside the 95% control limit of the historical negative control data.

A test article was considered to have induced a clear negative response if none of the criteria for a positive response were met and there was evidence that the bone marrow was exposed to the test article.

If the response is neither clearly positive nor clearly negative, or in order to assist in establishing the biological relevance of a result, the data will be evaluated by expert judgment and/or further investigations. Possible additional work may include scoring additional cells (where appropriate) or performing an additional experiment that could employ the use of modified experimental conditions. Such additional work will only be carried out following consultation with, and at the request of, the Sponsor.

In some cases, even after further investigations, the data set will preclude making a conclusion of positive or negative, at which time the response will be concluded to be equivocal. In such cases, the Study Director will use sound scientific judgment and report and describe all considerations.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included, but were not limited to, the following:

System	Purpose
LIMS Labware System	Test Article Tracking
Provantis™ (Instem)	Captures in-life toxicology, animal randomization and management data
Excel (Microsoft Corporation)	Calculations/Randomization
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting
Provantis™ Tables and Stats (Instem)	Generates in-life toxicology tables

Records and Archives

All raw data, the original signed protocol, amendments, and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

The raw data, reports, and other documents generated at locations other than BioReliance will be archived by the Test Site.

Deviations

The following deviation from the assay-method SOPs occurred during the conduct of this study.

Event No. 388465: On 04 Dec 201, one hour blood collection time point was completed for group 1 (vehicle) animals and was outside of SOP range (± 2 minutes). The three animals were collected 1-3 minutes outside the 2-minute window.

Study Director has concluded that this deviation had no adverse impact on the integrity of the data or the validity of the study conclusion. Since it occurred only on the vehicle animals and no compound was administered to these animals, therefore there is no degradation or metabolism of the compound occurring.

Event No. 395205: In the definitive study, formulation analysis was carried out on 04 December 2019. Batch size for group 2/7 was altered by technician based on the TA weigh out in order to meet a target concentration of 1.25 mg/ml, however, due to a calculation error; a final concentration of 1.437 mg/ml was obtained instead. A formulation of 1.437 mg/ml dosed at 5 ml/kg results in a dose level of 7.185 mg/kg rather than the 6.3 mg/kg value specified by the protocol. This is a protocol deviation.

While the calculation error resulted in a slightly high calculated concentration, the formulation analysis results for this concentration indicated that the formulation was actually only 84.4% of target. Further, the high concentration was within range for % of target, so the MTD for this study was achieved. The lower concentrations/dose levels are not as critical, so this deviation, overall, had no negative impact on the outcome of the study.

Deviations from Frontage

The reference standard used in the bioanalytical study phase was received at ambient temperature from the supplier and was stored at ambient temperature. This was a protocol deviation which states that storage conditions should be -10 to -30 °C.

The Certificate of Analysis for the reference standard used in the bioanalytical study phase did not list a storage temperature. The Material Safety Data Sheet (MSDS) added to the study suggests storage at refrigerated conditions. Documentation for reference standard storage conditions was requested from the study director. However, documentation for storage conditions other than what was listed in the protocol were not available. Though storage conditions are variable, the reference standard was detected at the expected lower limits of detection. Further, all concentrations from the evaluated samples fell below the lower limit of quantitation (BQL).

Control plasma used during the study was stored in a freezer maintained at approximately -20 C. During that time, the temperature of the freezer exceeded the allowable temperature range. On 11-15-2020 and 11-16-2020, the freezer exceeded the range for 6 hours 20 minutes and reached a temperature of approximately 0°C. This temperature excursion was deviation of SOP 04-C031 which only allows for temperature excursions up to 240 minutes.

These deviations overall, had no impact on the outcome of the study.

9. RESULTS AND DISCUSSION

Dose Range Finding Assay

Clinical signs are presented in Table 1 (Hands-On) and Table 2 (Cage side and Mortality). Mean group body weight data are found in Table 3.

Mortality was observed at the following concentrations:

Dose Level (mg/kg/day)	Males	Females
25	None	None
50	None	None
100	3/3	2/3

The following clinical signs were observed:

Dose Level (mg/kg/day)	Males	Females
25	Piloerection, decreased motor activity, hunched posture and squinty eye	Piloerection, decreased motor activity, hunched posture and squinty eye
50	Piloerection, hunched posture, and squinty eye	Hunched posture, piloerection, squinty eye, rapid & shallow breathing, and decreased motor activity
100	Decreased motor activity, hunched posture, labored breathing, piloerection and squinty eye	Decreased motor activity, hunched posture, labored breathing, piloerection and squinty eye

Mean body weight loss, relative to the Day 1 mean body weight, that was dose-related was noted in the surviving groups of males and females at 25 and 50 mg/kg/day.

Due to the mortality at 100 mg/kg/day, body weight loss, and/or clinical observations seen during the dose range finding assay at 50 mg/kg/day and higher, the maximum dose for the definitive micronucleus assay was set at 25 mg/kg in males only.

These results were similar in quality to a previous in vivo micronucleus study conducted in Swiss mice (OF1) with death after a single intraperitoneal injection at 100 mg/kg and higher (summarized in EC 2012; primary study report not available for review). In this previous study, administration of 50 mg/kg induced similar clinical signs of piloerection and hypokinesia (decreased motor activity) after treatment with piloerection in most mice at 24 and 48 hours after treatment. However, in this current study, additional observations of hunched posture, squinty eye and rapid and shallow breathing at 50 mg/kg were more severe and resulted in selection of 25 mg/kg as the high dose for the micronucleus assay.

Definitive Micronucleus Assay

Clinical signs are presented in Table 4 (Hands On), Table 5 (Cage side and Mortality. Mean group body weight data for the Main Cohort are found in Table 6.

No mortality was observed during the course of the definitive assay. There was a 3.5% loss on Day 3 relative to Day 1 mean body weight observed at 25 mg/kg/day.

The following clinical signs were observed:

Dose Level (mg/kg/day)	Males
0	None
6.3	Ruffled fur
12.5	Ruffled fur, squinty eye & hunched posture
25	Ruffled fur, squinty eye & hunched posture

Bone Marrow Analysis

The incidence of MnPCEs per 24,000 PCEs scored (4000 PCEs/animal) and the proportion of polychromatic erythrocytes per total erythrocytes are summarized and presented for each treatment group by sacrifice time in Table 7. Individual animal data are presented in Table 8.

The scoring results and a statistical analysis of data indicated the following:

- No appreciable reductions in the PCEs/NCE ratio was observed in the test article groups compared to the vehicle control group.

- Group variances for mean of the micronucleus frequency in the vehicle and test article groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance for %PCEs therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.

- No statistically significant increase in the incidence of MnPCEs was observed in the test article treated groups relative to the vehicle control groups (ANOVA followed by Dunnett's post-hoc analysis, $p > 0.05$).

- The positive control, CP, induced a statistically significant increase in the incidence of MnPCEs (Student's t test, $p \leq 0.05$).

- The number of MnPCEs in the vehicle control groups did not exceed the historical control range (Appendix I).

Based upon this, all criteria for a valid test were met as specified in the protocol. The Common Technical Document (CTD) Summary Table is included in Appendix VI.

Dose Formulation Analysis

Dose formulations were sent to the analytical chemistry laboratory at BioReliance for analysis. 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, 1.26, 2.5, and 5 mg/mL were 84.4, 65.2, and 109.6% of target, respectively, with S/L ratios of > 0.925. The 1.26 and 2.5 mg/mL formulations were found to be below the acceptable range for concentration (85.0 to 115.0% of target) but met the S/L ratio of > 0.925. Although the actual concentrations of the low and mid dose levels did not meet the acceptance criteria of 85.0% to 115.0%, the analysis indicates that the regulatory-required top dose level was achieved. Therefore, the Study Director has concluded that the observed departure had no adverse impact on the assessment of the test article and the results support the validity of the study conclusion. No test article was detected in the vehicle control sample.

Bioanalysis

Plasma samples were sent to Frontage Laboratories (Concord, Ohio) for analysis. A copy of the final report is included in Appendix V.

Plasma samples in mice were extracted and analyzed for the sole purpose of method development No test article was detectable in the samples to enable validation or analysis due to the instability of the test article in non-acidic medium.

10. CONCLUSION

Under the conditions of the assay described in this report, 1, 2, 4-Trihydroxybenzene was concluded to be negative for the induction of micronucleated polychromatic erythrocytes.

11. REFERENCES

Heddle J.A. A rapid in vivo test for chromosomal damage. Mutation Res. 18:187 190, 1973.

Hayashi M., Tice R.R., MacGregor J.T., Anderson D., Blakey D.H., Kirsch-Volders M., Oleson Jr. F.B., Pacchierotti F., Romagna F., Shimada H., Sutou S. and Vannier B. In vivo rodent erythrocyte micronucleus assay. Mutation Res. 312: 293-304, 1994.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Mavournin K.H., Blakey D.H., Cimino M.C., Salamone M.F. and Heddle J.A. The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene Tox Program. Mutation Res. 239:29-80, 1990.

National Research Council, Institute of Laboratory Animal Resources Commission on Life Sciences. Guide for the Care and Use of Laboratory Animals. National Academy Press. Washington, D.C., 2011.

OECD Guidelines for Testing of Chemicals, Guideline 474 (Genetic Toxicology: Mammalian Erythrocyte Micronucleus Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, Updated and Adopted 29 July 2016.

Schmid W. The micronucleus test. Mutation Res. 31:9-15, 1975.

European Commission, Health and Consumers Scientific Committee on Consumer Safety, Opinion on 1,2,4-Trihydroxybenzene, COLIPA no. A33, 11 December 2012.

12. DATA TABLES

Table 1: Dose Range-Finding Assay – Clinical Signs (Hands –On)

RTA001-02/01 Provantis (v.9.4.6.3) Date: 10/20/2021 15:13

Clinical Observations - Clinical Signs by Animal

AF98GS123DRF - AF98GS.123M012.BTL In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	1
1	m	481	No Abnormalities Detected	X
		482	No Abnormalities Detected	X
		483	No Abnormalities Detected	X
2	m	484	No Abnormalities Detected	X
		485	No Abnormalities Detected	X
		486	No Abnormalities Detected	X
3	m	487	No Abnormalities Detected	X
		488	No Abnormalities Detected	X
		489	No Abnormalities Detected	X
1	f	490	No Abnormalities Detected	X
		491	No Abnormalities Detected	X
		492	No Abnormalities Detected	X
2	f	493	No Abnormalities Detected	X
		494	No Abnormalities Detected	X
		495	No Abnormalities Detected	X
3	f	496	No Abnormalities Detected	X
		497	No Abnormalities Detected	X
		498	No Abnormalities Detected	X

Severity Codes: X = Present

Group 1 - 25 mg/kg /day Group 2 - 50 mg/kg /day Group 3 - 100 mg/kg /day

Table 2: Dose Range-Finding Assay – Clinical Signs (Cage Side and Mortality)

RTA001-02/01 Provantis (v.9.4.6.3) Date: 11/22/2019 10:57

Clinical Observations - Clinical Signs by Animal

AF98GS123DRF - AF98GS.123M012.BTL In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	1	2	3
1	m	481	No Abnormalities Detected	.	X	X
			Piloerection	X	.	.
		482	Decreased Motor Activity	.	.	X
			Hunched	X	.	.
			Piloerection	X	X	.
			Squinty Eye	X	.	X
		483	No Abnormalities Detected	.	X	X
			Hunched	X	.	.
			Piloerection	X	.	.
			Squinty Eye	X	.	.
2	m	484	Hunched	X	X	.
			Piloerection	X	X	X
			Squinty Eye	X	X	.
		485	Hunched	X	.	.
			Piloerection	X	X	X
			Squinty Eye	X	.	.
		486	Hunched	X	X	.
			Piloerection	X	X	X
3	m	487	Decreased Motor Activity	X	.	.
			Hunched	X	.	.
			Labored Breathing	S	.	.
			Observed Dead	F	.	.
			Piloerection	X	.	.
			Squinty Eye	X	.	.
		488	Decreased Motor Activity	X	.	.
			Hunched	X	.	.
			Labored Breathing	S	.	.
			Observed Dead	F	.	.
			Piloerection	X	.	.
			Squinty Eye	X	.	.
		489	Decreased Motor Activity	X	.	.
			Hunched	X	.	.
			Labored Breathing	S	.	.
			Observed Dead	F	.	.
			Piloerection	X	.	.
			Squinty Eye	X	.	.

Severity Codes: X = Present; S = Slight; F = Freezer

Group 1 - 25 mg/kg/day Group 2 - 50 mg/kg/day Group 3 - 100 mg/kg/day

Table 2 Cont.: Dose Range-Finding Assay – Clinical Signs (Cage Side and Mortality)

RTA001-02/01 Provantis (v.9.4.6.3) Date: 11/22/2019 10:57

Clinical Observations - Clinical Signs by Animal

AF98GS123DRF - AF98GS.123M012.BTL In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	1	2	3
1	f	490	No Abnormalities Detected	.	X	X
			Hunched	X	.	.
			Piloerection	X	.	.
		491	Decreased Motor Activity	.	.	X
			Hunched	X	X	X
			Piloerection	X	X	X
			Squinty Eye	X	.	.
		492	No Abnormalities Detected	.	X	X
			Hunched	X	.	.
			Piloerection	X	.	.
2	f	493	Hunched	X	X	X
			Piloerection	X	X	X
			Squinty Eye	.	X	.
		494	Hunched	X	X	X
			Rapid & Shallow	S	.	.
			Piloerection	X	X	X
			Squinty Eye	X	X	X
		495	Decreased Motor Activity	.	X	.
			Hunched	X	X	X
			Rapid & Shallow	S	.	.
			Piloerection	X	X	X
			Squinty Eye	X	X	X
3	f	496	Decreased Motor Activity	X	.	.
			Hunched	X	.	.
			Labored Breathing	S	.	.
			Piloerection	X	.	.
			Approved Moribund	F	.	.
			Squinty Eye	X	.	.
		497	Decreased Motor Activity	X	.	.
			Hunched	X	.	.
			Labored Breathing	S	.	.
			Observed Dead	F	.	.
			Piloerection	X	.	.
			Squinty Eye	X	.	.
		498	Decreased Motor Activity	X	.	.
			Hunched	X	.	.
			Labored Breathing	S	.	.
			Observed Dead	F	.	.
			Piloerection	X	.	.
			Squinty Eye	X	.	.

Severity Codes: X = Present; S = Slight; F = Freezer

Group 1 - 25 mg/kg/day Group 2 - 50 mg/kg/day Group 3 - 100 mg/kg/day

Table 3: Dose Range-Finding Assay – Group Mean Body Weights

RTA023-05/00 Provantis (v.9.4.6.3) Date: 11/22/2019 10:59

Bodyweights - Intergroup Comparison of Bodyweight Gains

AF98GS123DRF - AF98GS.123M012.BTL In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Body Weight Gain (Grams)

Day numbers relative to Start Date

Group	Sex	Base Weight Day 1		From: 1 To: 3	Abs Gain 1 3	% Gain 1 3
1	m	33.60	Mean	-1.47	-1.47	-4.37
		1.15	S.D.	0.42	0.42	1.25
2	m	33.93	Mean	-3.33	-3.33	-9.82
		0.64	S.D.	1.01	1.01	2.89
3	m	33.43	Mean	.	.	.
		0.25	S.D.	.	.	.
1	f	28.57	Mean	-1.47	-1.47	-5.06
		0.70	S.D.	1.42	1.42	4.78
2	f	28.27	Mean	-2.07	-2.07	-7.33
		0.60	S.D.	0.38	0.38	1.49
3	f	28.07	Mean	.	.	.
		0.72	S.D.	.	.	.

Statistical analysis not performed - Arithmetic mean values presented

Abs Gain = absolute bodyweight gain between base period and end of the analysis period
% Gain = percentage bodyweight gain between base period and end of the analysis period

Group 1 - 25 mg/kg/day Group 2 - 50 mg/kg/day Group 3 - 100 mg/kg/day

Table 4: Definitive Assay – Clinical Signs (Hands –On)

RTA001-02/01 Provantis (v.9.4.6.3) Date: 01/16/2020 9:20

Clinical Observations - Clinical Signs by Animal

AF98GS123DEF - AF98GS.123M012.BTL In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	1
1	m	951	No Abnormalities Detected	X
		952	No Abnormalities Detected	X
		953	No Abnormalities Detected	X
		954	No Abnormalities Detected	X
		955	No Abnormalities Detected	X
		956	No Abnormalities Detected	X
		957	No Abnormalities Detected	X
		958	No Abnormalities Detected	X
		959	No Abnormalities Detected	X
		960	No Abnormalities Detected	X
		961	No Abnormalities Detected	X
		962	No Abnormalities Detected	X
2	m	963	No Abnormalities Detected	X
		964	No Abnormalities Detected	X
		965	No Abnormalities Detected	X
		966	No Abnormalities Detected	X
		967	No Abnormalities Detected	X
		968	No Abnormalities Detected	X
3	m	969	No Abnormalities Detected	X
		970	No Abnormalities Detected	X
		971	No Abnormalities Detected	X
		972	No Abnormalities Detected	X
		973	No Abnormalities Detected	X
		974	No Abnormalities Detected	X
4	m	975	No Abnormalities Detected	X
		976	No Abnormalities Detected	X
		977	No Abnormalities Detected	X
		978	No Abnormalities Detected	X
		979	No Abnormalities Detected	X
		980	No Abnormalities Detected	X
		981	No Abnormalities Detected	X
		982	No Abnormalities Detected	X
		983	No Abnormalities Detected	X
		984	No Abnormalities Detected	X
		985	No Abnormalities Detected	X
		986	No Abnormalities Detected	X

Severity Codes: X = Present

Group 1 - 0 mg/kg/day Group 2 - 6.3 mg/kg/day Group 3 - 12.5 mg/kg/day Group 4 - 25 mg/kg/day

Table 5: Definitive Assay – Clinical Signs (Cage side and Mortality)

RTA001-02/01 Provantis (v.9.4.6.3) Date: 12/06/2019 22:26

Clinical Observations - Clinical Signs by Animal

AF98GS123DEF - AF98GS.123M012.BTL In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	1	2	3
1	m	951	No Abnormalities Detected	X	X	.
		952	No Abnormalities Detected	X	X	.
		953	No Abnormalities Detected	X	X	.
		954	No Abnormalities Detected	X	X	.
		955	No Abnormalities Detected	X	X	.
		956	No Abnormalities Detected	X	X	.
		957	No Abnormalities Detected	X	X	X
		958	No Abnormalities Detected	X	X	X
		959	No Abnormalities Detected	X	X	X
		960	No Abnormalities Detected	X	X	X
		961	No Abnormalities Detected	X	X	X
		962	No Abnormalities Detected	X	X	X
2	m	963	Ruffled Fur	X	X	.
		964	No Abnormalities Detected	.	X	.
			Ruffled Fur	X	.	.
		965	No Abnormalities Detected	.	X	.
			Ruffled Fur	X	.	.
		966	No Abnormalities Detected	X	X	.
		967	No Abnormalities Detected	.	X	.
			Ruffled Fur	X	.	.
		968	Ruffled Fur	X	X	.
3	m	969	No Abnormalities Detected	.	X	.
			Ruffled Fur	X	.	.
			Squinty Eye	X	.	.
		970	Ruffled Fur	X	X	.
		971	Ruffled Fur	X	X	.
		972	Ruffled Fur	X	X	.
			Hunched	X	X	.
			Squinty Eye	X	X	.
		973	Ruffled Fur	X	X	.
			Squinty Eye	X	X	.
		974	Ruffled Fur	X	X	.
			Hunched	X	X	.
			Squinty Eye	X	X	.

Severity Codes: X = Present

Group 1 - 0 mg/kg/day Group 2 - 6.3 mg/kg/day Group 3 - 12.5 mg/kg/day Group 4 - 25 mg/kg/day

Table 5 Cont.: Definitive Assay – Clinical Signs (Cage side and Mortality)

RTA001-02/01 Provantis (v.9.4.6.3) Date: 12/06/2019 22:26

Clinical Observations - Clinical Signs by Animal

AF98GS123DEF - AF98GS.123M012.BTL In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	1	2	3
4	m	975	No Abnormalities Detected	X	X	.
		976	Ruffled Fur	X	X	.
			Squinty Eye	X	X	.
		977	No Abnormalities Detected	X	X	.
		978	Ruffled Fur	X	X	.
			Hunched	X	X	.
			Squinty Eye	X	X	.
		979	Ruffled Fur	X	X	.
			Hunched	X	X	.
			Squinty Eye	X	X	.
		980	Ruffled Fur	X	X	.
			Hunched	X	X	.
			Squinty Eye	X	X	.
		981	Ruffled Fur	X	X	X
			Hunched	X	X	X
			Squinty Eye	X	X	X
		982	No Abnormalities Detected	X	X	.
			Ruffled Fur	.	.	X
		983	Ruffled Fur	X	X	X
			Hunched	X	X	X
			Squinty Eye	X	X	X
		984	Ruffled Fur	X	X	X
			Hunched	X	X	X
			Squinty Eye	X	X	.
		985	Ruffled Fur	X	X	X
			Hunched	X	X	X
			Squinty Eye	X	X	.
		986	Ruffled Fur	X	X	X
			Hunched	X	X	X
			Squinty Eye	X	X	.

Severity Codes: X = Present

Group 1 - 0 mg/kg/day Group 2 - 6.3 mg/kg/day Group 3 - 12.5 mg/kg/day Group 4 - 25 mg/kg/day

Table 6: Definitive Assay – Group Mean Body Weights

RTA023-05/00 Provantis (v.9.4.6.3) Date: 12/06/2019 22:27

Bodyweights - Intergroup Comparison of Bodyweight Gains

AF98GS123DEF - AF98GS.123M012.BTL In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Body Weight Gain (Grams)

Day numbers relative to Start Date

Group	Sex	Base Weight Day 1	From: To:	1 2	1 3	Abs Gain 1 3	% Gain 1 3
1	m	34.98	Mean	-0.15	0.10	0.10	0.27
		1.17	S.D.	0.55	0.35	0.35	0.99
2	m	34.77	Mean	-0.52	.	.	.
		0.72	S.D.	0.67	.	.	.
3	m	34.08	Mean	0.30	.	.	.
		1.60	S.D.	0.74	.	.	.
4	m	34.36	Mean	-1.67	-1.18	-1.18	-3.54
		1.79	S.D.	0.78	1.41	1.41	4.45

Statistical analysis not performed - Arithmetic mean values presented

Abs Gain = absolute bodyweight gain between base period and end of the analysis period
% Gain = percentage bodyweight gain between base period and end of the analysis period

Group 1 - 0 mg/kg/day Group 2 - 6.3 mg/kg/day Group 3 - 12.5 mg/kg/day Group 4 - 25 mg/kg/day

Table 7: Summary of Bone Marrow Micronucleus Analysis

Treatment	Gender	Time (Hrs)	Animals	%PCE (Mean +/- SD)	Toxicity (%)	% MnPCE (Mean +/- SD)	Number of MnPCE/PCE Scored
Vehicle							
0 mg/kg/dose	M	24	6	69.7 ± 9.5	---	0.02 ± 0.02	5 /24000
0 mg/kg/dose	M	48	6	57.6 ± 11.5	---	0.05 ± 0.03	11 /24000
1, 2, 4-Trihydroxybenzene							
6.3 mg/kg/dose	M	24	6	75.6 ± 7.2	9	0.01 ± 0.02	2 /24000
12.5 mg/kg/dose	M	24	6	69.5 ± 9.1	0	0.03 ± 0.02	6 /24000
25 mg/kg/dose	M	24	6	70.3 ± 8.6	1	0.02 ± 0.02	4 /24000
25 mg/kg/dose	M	48	6	64.7 ± 4.0	12	0.02 ± 0.02	5 /24000
CP							
50 mg/kg/dose	M	24	5	69.3 ± 4.4	-1	1.47 ± 0.44**	294 /20000

Vehicle = Degassed Deionized (DI) Water

PCE – Polychromatic erythrocytes; MnPCE – Micronucleated polychromatic erythrocytes

*p < 0.05 or **p < 0.01, One-Way ANOVA with Post-Hoc Dunnett's Test or T-Test

24 Hrs MnPCE Male GLM P-value = 0.485, R-sqr = 11.25%

Table 8: Individual Animal Bone Marrow Micronucleus Analysis (24 hours)

Treatment	Sex	Animal No.	%PCE	Micronucleus Frequency MnPCE	PCE	%
Vehicle	M	951	75.6	2	4000	0.05
0 mg/kg/dose		952	67.6	1	4000	0.03
		953	64.2	1	4000	0.03
		954	54.6	1	4000	0.03
		955	80.6	0	4000	0.00
		956	75.4	0	4000	0.00
1, 2, 4-Trihydroxybenzene	M	963	74.1	0	4000	0.00
6.3 mg/kg/dose		964	81.9	1	4000	0.03
		965	79.8	0	4000	0.00
		966	79.6	0	4000	0.00
		967	62.2	0	4000	0.00
		968	76.2	1	4000	0.03
1, 2, 4-Trihydroxybenzene	M	969	69.8	1	4000	0.03
12.5 mg/kg/dose		970	83.2	0	4000	0.00
		971	76.8	2	4000	0.05
		972	59.4	0	4000	0.00
		973	64.6	1	4000	0.03
		974	63.0	2	4000	0.05
1, 2, 4-Trihydroxybenzene	M	975	78.0	0	4000	0.00
25 mg/kg/dose		976	73.8	0	4000	0.00
		977	74.4	2	4000	0.05
		978	63.0	1	4000	0.03
		979	76.0	0	4000	0.00
		980	56.4	1	4000	0.03
CP	M	CP 289	70.4	78	4000	1.95
50 mg/kg/dose		CP 290	65.9	69	4000	1.73
		CP 291	76.0	37	4000	0.93
		CP 292	69.2	43	4000	1.08
		CP 293	65.0	67	4000	1.68

Vehicle = Vehicle = Degassed Deionized (DI) Water
PCE – Polychromatic erythrocytes; MnPCE – Micronucleated polychromatic erythrocytes

Table 8 Cont.: Individual Animal Bone Marrow Micronucleus Analysis (48 hours)

Treatment	Sex	Animal No.	%PCE	Micronucleus Frequency		
				MnPCE	PCE	%
Vehicle	M	957	69.5	0	4000	0.00
0 mg/kg/dose		958	46.4	3	4000	0.08
		959	40.8	1	4000	0.03
		960	65.8	2	4000	0.05
		961	64.6	3	4000	0.08
		962	58.5	2	4000	0.05
1, 2, 4-Trihydroxybenzene	M	981	66.8	0	4000	0.00
25 mg/kg/dose		982	61.0	2	4000	0.05
		983	70.7	0	4000	0.00
		984	66.6	1	4000	0.03
		985	60.6	1	4000	0.03
		986	62.2	1	4000	0.03

Vehicle = Vehicle = Degassed Deionized (DI) Water
PCE – Polychromatic erythrocytes; MnPCE – Micronucleated polychromatic erythrocytes

13. APPENDIX I: Historical Control

Mouse Micronucleus Test Historical Control Data 2016-2018

Historical Vehicle Control in Male Mice[1]

	Individual Animals		Studies	
	PCE%	**MN%**	**PCE%**	**MN%**
N	375	380	68	69
Mean[3]	52.4	0.08	52.4	0.08
SD	5.4	0.03	3.3	0.02
95% UCL	63.1	0.15	58.9	0.12
95% LCL	41.6	0.01	45.9	0.04
Max[4]	72.6	0.28	60.1	0.14
Min[4]	39.0	0.00	44.5	0.02

Historical Positive Control in Male Mice[2]

	Individual Animals		Studies	
	PCE%	**MN%**	**PCE%**	**MN%**
N	218	223	44	45
Mean[3]	44.3	2.28	44.3	2.27
SD	8.1	0.93	6.7	0.85
95% UCL	60.5	4.13	57.7	3.97
95% LCL	28.1	0.42	31.0	0.57
Max[4]	68.4	5.80	59.8	5.09
Min[4]	19.6	0.30	27.1	0.53

[1]Since no appreciable differences in the induction of MnPCEs by different vehicles and solvents (test article carriers) and different routes of administration were observed, this table contains data from carriers and routes of administration widely used during the conduct of contract studies at BioReliance.

Vehicles: water, water soluble vehicles (methylcellulose, carboxymethylcellulose, dextrose), saline, corn oil, and other vehicles.

Routes of administration: intraperitoneal (IP), intravenous (IV), oral gavage (PO), subcutaneous (SC).

Bone marrow collection time: approximately 24 and/or 48 hours post-final dose for Micronucleus studies; 3-4 hours post-final dose for the Micronucleus portion of combined Micronucleus/Comet studies.

[2]Positive control article: Cyclophosphamide monohydrate (CP); Doses: 50 mg/kg; Route of administration: PO.

[3]Average of the PCE ratio observed out of 500 or 1000 erythrocytes scored per animal for the total number of animals used; average of the number of MnPCE per 2000 or 4000 PCE for the total number of animals used; average of number of MnPCE/per group (containing 5-6 animals per group) for total number of groups used.

[4]Minimum and maximum range of PCE ratio observed out of 500 or 1000 erythrocytes scored per animal, the minimum and maximum range of MnPCE observed out of 2000 or 4000 PCE for the total number of animals used and the minimum and maximum range of MnPCE observed out of 10000 to 24000 PCE for the total number of groups used.

Formula: 95% control limit ranges = mean ± 2 x standard deviation

Note: This historical control data includes data from non-GLP studies.

Historical Vehicle Control in Female Mice[1]

	Individual Animals		Studies	
	PCE%	**MN%**	**PCE%**	**MN%**
N	155	160	28	29
Mean[3]	52.4	0.09	52.4	0.09
SD	7.3	0.04	5.1	0.02
95% UCL	67.0	0.17	62.6	0.12
95% LCL	37.8	0.01	42.2	0.05
Max[4]	72.4	0.28	66.8	0.12
Min[4]	12.0	0.00	42.9	0.05

Historical Positive Control in Female Mice[2]

	Individual Animals		Studies	
	PCE%	**MN%**	**PCE%**	**MN%**
N	10	10	2	2
Mean[3]	47.4	1.86	47.4	1.86
SD	9.5	0.48	6.8	0.04
95% UCL	66.3	2.82	60.9	1.94
95% LCL	28.4	0.90	33.9	1.79
Max[4]	71.6	2.58	52.2	1.89
Min[4]	39.4	0.75	42.6	1.84

[1]Since no appreciable differences in the induction of MnPCEs by different vehicles and solvents (test article carriers) and different routes of administration were observed, this table contains data from carriers and routes of administration widely used during the conduct of contract studies at BioReliance.

Vehicles: water, water soluble vehicles (methylcellulose, carboxymethylcellulose, dextrose), saline, corn oil, and other vehicles.

Routes of administration: intraperitoneal (IP), intravenous (IV), oral gavage (PO), subcutaneous (SC).

Bone marrow collection time: approximately 24 and/or 48 hours post-final dose for Micronucleus studies; 3-4 hours post-final dose for the Micronucleus portion of combined Micronucleus/Comet studies.

[2]Positive control article: Cyclophosphamide monohydrate (CP); Doses: 50 mg/kg; Route of administration: PO.

[3]Average of the PCE ratio observed out of 500 or 1000 erythrocytes scored per animal for the total number of animals used; average of the number of MnPCE per 2000 or 4000 PCE for the total number of animals used; average of number of MnPCE/per group (containing 5-6 animals per group) for total number of groups used.

[4]Minimum and maximum range of PCE ratio observed out of 500 or 1000 erythrocytes scored per animal, the minimum and maximum range of MnPCE observed out of 2000 or 4000 PCE for the total number of animals used and the minimum and maximum range of MnPCE observed out of 10000 to 24000 PCE for the total number of groups used.

Formula: 95% control limit ranges = mean ± 2 x standard deviation

Note: This historical control data includes data from non-GLP studies.

14. APPENDIX II: Study Protocol and Amendments

PROTOCOL AMENDMENT 1

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

1. Page 11, Section 9, Experimental Design and Methodology – Micronucleus Assay

   Effective: Date of Study Director signature on this amendment.

   Original:

Euthanasia Time (hours after treatment)[B]				24	48
Group	Treatment	Dose Level (mg/kg/dose)	Dose Volume[A] (mL/kg/dose)	Number of Male Animals	
1	Vehicle	0	5	6	6
2	TA	Low	5	6	0
3	TA	Mid	5	6	0
4	TA	High	5	6	6

[A]Based upon individual body weight
[B]Range(s): 24-27 hours and 45-48 hours, respectively

Change To:

Euthanasia Time (hours after treatment)[B]				24	48
Group	Treatment	Dose Level (mg/kg/dose)	Dose Volume[A] (mL/kg/dose)	Number of Male Animals	
1	Vehicle	0	5	6	6
2	TA	**6.3**	5	6	0
3	TA	**12.5**	5	6	0
4	TA	**25**	5	6	6

[A]Based upon individual body weight
[B]Range(s): 24-27 hours and 45-48 hours, respectively

Reason: To specify dose levels based on the outcome of the DRF assay.

PROTOCOL AMENDMENT 1

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

2. Page 11, Section 9, Experimental Design and Methodology – Blood Collection and Sample Hadling

Effective: Date of Study Director signature on this amendment.

Original:

Group	Treatment	Dose Level (mg/kg)	Animals/ Timepoint	Collection Timepoint (hours after last dose)	Total Number of Male Animals
6	Vehicle	0	3	1	3
7	TA	Low	3	1	3
8	TA	Mid	3	1	3
9	TA	High	3	1	3

Change To:

Group	Treatment	Dose Level (mg/kg)	Animals/ Timepoint	Collection Timepoint (hours after last dose)	Total Number of Male Animals
6	Vehicle	0	3	1	3
7	TA	**6.3**	3	1	3
8	TA	**12.5**	3	1	3
9	TA	**25**	3	1	3

Reason: To specify dose levels based on the outcome of the DRF assay.

PROTOCOL AMENDMENT 1

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Sponsor Approval:

Judi Abbott Curry
Sponsor Representative

11/26/19
Date

PROTOCOL AMENDMENT 1

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Study Director and Test Facility Management Approvals:

Megan Young, PhD
BioReliance Study Director

26 NOV 2019
Date

Ann Noland
BioReliance Study Management

26 Nov 2019
Date

PROTOCOL AMENDMENT 2

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

1. Page 1, Study Director

   Effective: Date of Study Director's signature on this amendment.

   Original:
   Study Director — Megan Young, PhD

   Change To:
   Study Director — **Marie E. McKeon, MPhil**

   Reason: Change in personnel.

2. Page 2, Section 1, Key Personnel – Test Facility Information, Study Director

   Effective: Date of Study Director's signature on this amendment.

   Original:
   Study Director — Megan Young, PhD
   BioReliance Corporation
   Phone: 301-610-2152
   Email: megan.young@milliporesigma.com

   Change To:
   Study Director — **Marie E. McKeon, MPhil**
   BioReliance Corporation
   Phone: 301-610-2152
   Email: marie.mckeon@external.milliporesigma.com

   Reason: Change in personnel.

3. Page 2, Section 1, Key Personnel – Test Site Information

   Effective: Date of Study Director signature on this amendment.

   Original:

Test Site Information:

Principal Investigator (BioAnalysis)	To be amended if needed
Test Site Quality Assurance Representative	To be amended if needed

Change To:
Test Site Information:

Principal Investigator (BioAnalysis)	**Tivadar Orban, PhD** **Frontage Lab** **DMPK and Bioanalytical Services** **Team Leader** **10845 Wellness Way** **Concord, Ohio 44077** **Office: +1 (440) 357-3759** **Email: torban@frontagelab.com**
Test Site Quality Assurance Representative	**Stephen B. Rogenthien, RQAP-GLP** **Senior Director, Quality Assurance** **10845 Wellness Way** **Concord, OH 44077** **Office (440) 357-3689** **Mobile (440) 382-7536** **Email: srogenthien@frontagelab.com**

Reason: Protocol completion with test site information.

4. Page 12, Section Bioanalysis (BioA)

   Effective: Date of Study Director signature on this amendment.

   Original:

PROTOCOL AMENDMENT 2

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Bioanalysis (BioA)
If conducted, a validated method will be used to analyze the concentrations of test article or metabolites in the plasma samples. Samples will be shipped on dry ice by overnight courier to the Principal Investigator for BioAnalysis. They will be sent on a non-holiday Monday, Tuesday or Wednesday that does not immediately precede a holiday. Upon receipt, samples will be stored at -60°C or below until required for analysis. Unused samples will be discarded upon acceptance of the analytical results by the Study Director. A BioA contribution report with GLP Compliance Statement, signed by the Principal Investigator from the Test Site, will be provided and included in the main micronucleus report, if applicable.

Change To:
Bioanalysis (BioA)
~~If conducted~~, A **qualified analytical** method will be used to analyze the concentrations of test article or metabolites in the plasma samples. Samples will be shipped on dry ice by overnight courier to the Principal Investigator for BioAnalysis **at the address below.** They will be sent on a non-holiday Monday, Tuesday or Wednesday that does not immediately precede a holiday. Upon receipt, samples will be stored at -60°C or below until required for **method development, qualification or analysis**. ***Unused samples will be discarded upon acceptance of the analytical results by the Study Director.*** A BioA contribution report, signed by the Principal Investigator from the Test Site, will be provided and included in the main micronucleus report, if applicable.

Shipment of the first set of samples will be sent to:

Frontage Lab
Tivadar Orban, PhD
DMPK and Bioanalytical Services
Team Leader
10845 Wellness Way
Concord, Ohio 44077
Office Telephone: +1 (440) 357-3759
Email: torban@frontagelab.com

Notification of sample shipment by email to include:

PROTOCOL AMENDMENT 2

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Concord Sample Management
10845 Wellness Way
Concord, OH 44077
Office (440) 357-3039
ConcordSampleManagement@FrontageLab.com

Upon successful receipt of the first shipment, the second set of samples will also be shipped as above.

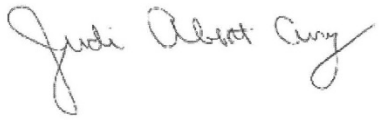
Reason: Protocol completion with information for bioanalysis sample shipment and reporting of results.

PROTOCOL AMENDMENT 2

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice



12/14/20

Judi Abbott Curry
Sponsor Representative

Date

PROTOCOL AMENDMENT 2

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Study Director and Test Facility Management Approvals:

Marie E. McKeon — 14 Dec 2020
Marie E. McKeon, MPhil — Date
BioReliance Study Director

V Johnson-Peltier — 14 Dec 2020
BioReliance Study Management — Date

PROTOCOL AMENDMENT 3

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

1. Protocol Amendment 2, Items 1 and 2, *Effective:*

   Effective: Date of Study Director's signature on this amendment.

   Original:

   Date of Study Director's signature on this amendment

   Change To:

   07 December 2020 per test facility management email

   Reason: Change in personnel was officially made at the time of management's reassignment of the study director.

2. Protocol Amendment 2, Items 1 and 2, *Effective:*

   Effective: Date of Study Director's signature on this amendment.

   Original:
   Bioanalysis (BioA)

   ~~If conducted~~, A qualified analytical method will be used to analyze the concentrations of test article or metabolites in the plasma samples. Samples will be shipped on dry ice by overnight courier to the Principal Investigator for BioAnalysis at the address below. They will be sent on a non-holiday Monday, Tuesday or Wednesday that does not immediately precede a holiday. Upon receipt, samples will be stored at -60°C or below until required for method development, qualification or analysis. *Unused samples will be discarded upon acceptance of the analytical results by the Study Director.* A BioA contribution report, signed by the Principal Investigator from the Test Site, will be provided and included in the main micronucleus report, if applicable

   Change To:
   Bioanalysis (BioA)

   Samples will be shipped on dry ice by overnight courier to the Principal Investigator for BioAnalysis at the address below. They will be sent on a non-holiday Monday, Tuesday or Wednesday that does not immediately precede a holiday. Upon receipt, samples will be stored at -60°C or below until required for method development. Unused samples will be

discarded upon acceptance of the analytical results by the Study Director. A summary of the method development results, signed by the Principal Investigator from the Test Site, will be provided and included in the main micronucleus report, if applicable.

Reason: Plasma samples were used for method development but the test article was not detectable in the samples to enable qualification or analysis.

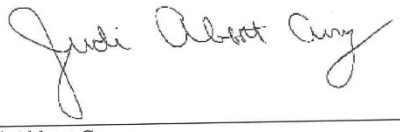
PROTOCOL AMENDMENT 3

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Sponsor Approval:



05/17/21

Judi Abbott Curry
Sponsor Representative

Date

PROTOCOL AMENDMENT 3

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Study Director and Test Facility Management Approvals:

Marie E. McKeon — 17 May 2021
Marie E. McKeon, MPhil — Date
BioReliance Study Director

V Johnson-Peltier — 17 May 2021
BioReliance Study Management — Date

PROTOCOL AMENDMENT 4

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

1. Protocol Amendment 2, item 2, and Protocol Page 2, Section 1, Key Personnel – Test Site Information

   Effective: Date of Study Director signature on this amendment.

   Original:

   Test Site Information:

Principal Investigator (BioAnalysis)	Tivadar Orban, PhD Frontage Lab DMPK and Bioanalytical Services Team Leader 10845 Wellness Way Concord, Ohio 44077 Office: +1 (440) 357-3759 Email: torban@frontagelab.com

   Change To:

   Test Site Information:

Principal Investigator (BioAnalysis)	Tivadar Orban, PhD **Frontage Laboratories** **Bioanalytical Services** **Senior Scientist and Manager** 10845 Wellness Way Concord, Ohio 44077 Office: +1 (440) 357-3759 Email: torban@frontagelab.com

   Reason: Title change for Principal Investigator. Title will not be changed in the sample shipment section because samples were already sent.

PROTOCOL AMENDMENT 4

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Sponsor Approval:

Judi Abbott Curry

6/9/2021

Judi Abbott Curry
Sponsor Representative

Date

PROTOCOL AMENDMENT 4

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Study Director and Test Facility Management Approvals:

Marie E. McKeon — 09 June 2021
Marie E. McKeon, MPhil — Date
BioReliance Study Director

09 Jun 2021
BioReliance Study Management — Date

PROTOCOL AMENDMENT 5

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

1. Protocol Amendment 3, Item 2:

   Effective: Date of Study Director's signature on this amendment.

   Original:
   Bioanalysis (BioA)

   Samples will be shipped on dry ice by overnight courier to the Principal Investigator for BioAnalysis at the address below. They will be sent on a non-holiday Monday, Tuesday or Wednesday that does not immediately precede a holiday. Upon receipt, samples will be stored at -60°C or below until required for method development. Unused samples will be discarded upon acceptance of the analytical results by the Study Director. A summary of the method development results, signed by the Principal Investigator from the Test Site, will be provided and included in the main micronucleus report, if applicable.

   Change To:
   Bioanalysis (BioA)

   Samples will be shipped on dry ice by overnight courier to the Principal Investigator for BioAnalysis at the address below. They will be sent on a non-holiday Monday, Tuesday or Wednesday that does not immediately precede a holiday. Upon receipt, samples will be stored at -60°C or below until required for method development. Unused samples will be discarded upon acceptance of the analytical results by the Study Director**. A BioA contribution report with GLP Compliance Statement, signed by the Principal Investigator from the Test Site, will be provided and included in the main micronucleus report.**

   Reason: The results of the plasma sample analysis will be provided in a GLP compliant report since the work was performed according to GLP.

PROTOCOL AMENDMENT 5

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Sponsor Approval:

Judi Abbott Curry

June 14, 2021

Judi Abbott Curry
Sponsor Representative

Date

PROTOCOL AMENDMENT 5

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Study Director and Test Facility Management Approvals:

Marie E. McKeon — 15 June 2021
Marie E. McKeon, MPhil — Date
BioReliance Study Director

15 June 2021
BioReliance Study Management — Date

PROTOCOL AMENDMENT 6

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

1. Protocol Amendment 3, Item 2, location of change

   Effective: Date of Study Director signature on this amendment.

   Original:
   Protocol Amendment 2, Items 1 and 2, *Effective:*

   Change To:
   Protocol Amendment 2, **Item 4**

   Reason: Correct the location of the change in Protocol Amendment 3 Item 2.

2. Protocol Amendment 4, Item 1, location of change

   Effective: Date of Study Director signature on this amendment.

   Original:
   Protocol Amendment 2, Item 2 and Protocol Page 2, Section 1, Key Personnel-Test Site Information

   Change To:
   Protocol Amendment 2, **Item 3** and Protocol Page 2, Section 1, Key Personnel-Test Site Information

   Reason: Correct the location of the change in Protocol Amendment 4 Item 1.

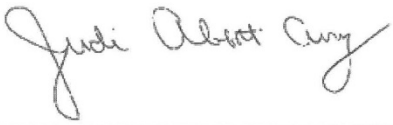
PROTOCOL AMENDMENT 6

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Sponsor Approval:



Judi Abbott Curry
Sponsor Representative

10/19/21
Date

PROTOCOL AMENDMENT 6

Sponsor: Harris Beach PLLC

BioReliance Study No.: AF98GS.123M012.BTL

Title: In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Study Director and Test Facility Management Approvals:

Marie E. McKeon — 19 October 2021
Marie E. McKeon, MPhil — Date
BioReliance Study Director

20 October 2021
BioReliance Study Management — Date

BioReliance®

Protocol

Study Title	*In Vivo* Mammalian Erythrocyte Micronucleus Assay in Mice
Study Director	Megan Young, PhD
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF98GS.123M012.BTL

1. KEY PERSONNEL

Sponsor Information:

Sponsor	Harris Beach PLLC 100 Wall Street New York, NY 10005
Sponsor's Authorized Representative	Judi Abbott Curry Harris Beach PLLC 100 Wall Street New York, NY 10005 Phone: 212-313-5404 Email: jcurry@harrisbeach.com

Test Facility Information:

Study Director	Megan Young, PhD BioReliance Corporation Phone: 301-610-2152 Email: megan.young@milliporesigma.com
Contributing Scientist (Analytical Chemistry Analysis)	Philip Atkins, MChem BioReliance Corporation Phone: 301-610-2114 Email: philip.atkins@milliporesigma.com
BioReliance Quality Assurance Representative	Wannie Madraymootoo, MS BioReliance Corporation Phone: 301-610-2267 Email: wannie.madraymootoo@milliporesigma.com

Test Site Information:

Principal Investigator (BioAnalysis)	To be amended if needed
Test Site Quality Assurance Representative	To be amended if needed

2. TEST SCHEDULE

Proposed Experimental Initiation Date	20 NOV 2019
Proposed Experimental Completion Date	13 JAN 2020
Proposed Report Date	30 JAN 2020

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US FDA Good Laboratory Practices 21 CFR Part 58

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

A Study Director-signed GLP Compliance Statement will be included in the final report. This statement will cite the GLP regulations with which this study is compliant and any exceptions to this compliance with an assessment of any effects on the quality or integrity of the data or study.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

For portions of the study conducted at BioReliance, the protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (if applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-TOX-TFM_SIAL@milliporesigma.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The objective of this study is to evaluate a test article for *in vivo* clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocyte cells in mouse bone marrow. This assay design is based on OECD Guideline 474 (OECD, 2016) and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST ARTICLE AND CONTROL ARTICLE INFORMATION

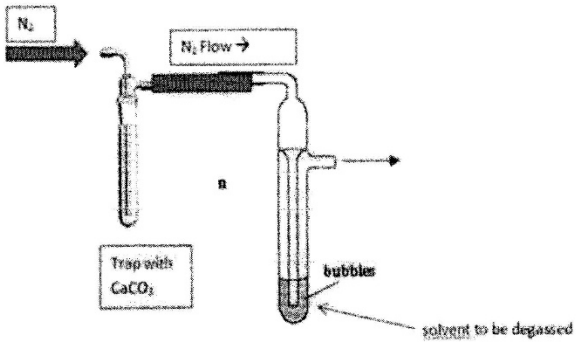
Test Article

Identification	1, 2, 4-Trihydroxybenzene
Synonym	1,2,4-Benzenetriol
Storage Conditions	-10 to -30°C Protect from light With nitrogen With desiccant
	The compound will be purged with nitrogen after the first weighing and after each subsequent opening of the container.
Purity	99% (no correction factor will be used for dose formulations)
Molecular Weight	126.11 g/mol
Special Handling	Sample will be weighed inside a glove box purged with nitrogen gas

Vehicle Control

Identification	Degassed Deionized Water

The Vehicle will be degassed for at least 30 minutes based on the Sponsor provided diagram, shown below. An appropriate amount of $CaCO_3$ will be added to the tube (indicated below), until the presence of bubbles is visually confirmed. The amount of $CaCO_3$ and the visual presence of bubbles will be documented in the raw data.



Positive Control

Identification Cyclophosphamide monohydrate (CP)

Characterization of Test Article
The test article will be characterized by the Certificates of Analysis provided by the Supplier(s).

Test Article Reserve Sample
Since the in-life portion of this study is less than four weeks in duration, a reserve sample will not be retained.

Characterization of Vehicle
The vehicle(s) used to prepare the test article formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Characterization of Dose Formulations
Dose formulations will be analyzed by BioReliance using a method validated under BioReliance study number AE03RS.GTCHEM.BTL.

Stability of Test Article in Vehicle
Formulations are stable at 2 to 8°C (or on wet ice) for up to two hours at concentrations of 0.25 and 50 mg/mL.

Preparation of Dose Formulations
The dose formulations will be prepared at least once on each day of use. Each concentration will be prepared by mixing an appropriate amount of the test article with the appropriate volume of the vehicle. If needed, the formulation will be vortexed, homogenized, sonicated, and/or stirred in order to achieve workable or soluble formulations. **Formulations will be prepared and maintained on wet ice or at 2 to 8°C.**

Positive Control
Scoring control slides (fixed and unstained), generated from a BioReliance study, will be included to verify scoring, and the study number will be documented in the raw data. These slides were generated from male mice treated once with cyclophosphamide monohydrate (CP) at 50 mg/kg and the bone marrow harvested 24 hours after the treatment.

Disposition of Test Article and Dose Formulations
All unused Test Article will be discarded prior to report finalization unless the test article is used on another study.

Residual dose formulations will be discarded after use.

Collection of Dose Formulation Samples
The dose formulations will be prepared in a glove box purged with nitrogen gas in order to minimize the air oxidation of the test substance. The dose solutions will be prepared freshly using degassed deionized water (see diagram in section 6) as the solvent.

Samples will be collected on the day of preparation as follows. Samples will not be collected for any portion of the assay used to assess only toxicity. The sampling plan will be:

Vehicle Sampling	
Number of Samples*	Volume
2	0.5 mL

* One sample will be used for analysis and the other will serve as the backup

Solution Sampling		
Dose Level	Number of Samples**	Volume
High Dose	2	0.5 mL
Mid Dose	2	0.5 mL
Low Dose	2	0.5 mL

** One sample will be used for analysis and the other will serve as the backup

Submitted samples that are below the validated range will not be analyzed. The lowest sample, within the validated range, will be analyzed instead. All samples collected for analysis or as backups will be held at 2 to 8°C or on wet ice, or under the conditions of use for the dosing formulations, until delivered for analysis. Upon receipt, the samples designated for analysis will be maintained at the conditions of receipt until the analysis is performed; however back-up samples may be stored 2 to 8°C. If the analysis is performed on subsequent days, all samples will be stored at 2 to 8°C until required for analysis. After analysis, all samples and backups will be stored at2 to 8°C. Unused samples will be discarded upon acceptance of the analytical results by the Study Director.

Stability
In the absence of confirmed stability in the vehicle at concentrations bracketing those used within this study, stability of the dose formulations will be confirmed by analyzing the appropriate samples from one set of dose formulations after **at least 2 hour storage at conditions that mimic the handling of the dose formulations during dosing (wet ice).** If the samples are not analyzed on the day of dosing, additional stability will be performed to cover the conditions and duration of storage, at a minimum. The concentrations obtained must be 90 to 110% of the original concentrations to be considered stable. Alternatively, the chemistry laboratory may establish stability on independently prepared samples.

Acceptance Criteria
For test article formulation samples that are solutions:

- The mean of each concentration must be 85.0% to 115.0% of nominal and the ratio of the small/large obtained concentrations for the duplicate dilutions of each solution must be > 0.925.

The vehicle control sample must confirm the absence of test article such that the concentration of the test article in the vehicle formulations must be below the Limit of Detection of the analytical method.

In the event that a sample is outside of the acceptable specification range, the Study Director will justify the acceptability of the results or suggest re-analysis of the backup samples or retest the affected portion of the study.

Data Collection and Analysis System
Data will be collected and analyzed using Agilent ChemStation.

Reporting
A draft report that summarizes the methods, analysis, and results carried out by the Analytical Chemistry laboratory will be provided to the Study Director and Sponsor and/or Authorized Representative. The final report will be included in the main study report as an appendix.

7. TEST SYSTEM

Species	Mouse
Strain	Hsd:ICR (CD-1)
Justification for Selection of Species and Strain	The mouse has been routinely used as an animal model of choice for the mammalian bone marrow erythrocyte micronucleus assay. This strain is an outbred strain that maximizes genetic heterogeneity and therefore tends to eliminate strain-specific response to test articles.
Source	Envigo RMS, Inc. (Frederick, MD or alternate Envigo location)
Animal Welfare Provisions	This study is not duplicative or unnecessary. The number of animals, procedures, and design used for this study, has been reviewed and were approved by the BioReliance Institutional Animal Care and Use Committee protocol number 10. All procedures involving animals performed at BioReliance follow the specifications recommended in the most current version of The Guide for the Care and Use of Laboratory Animals adopted by BioReliance.

Number and Sex of Animals

	DRF*	DEF**
Males (main/TK)	9	36/12
Females	9	--

*Additional or fewer animals may be used as needed to determine the maximum tolerated dose (MTD)
**Additional animals may be added to the top dose as possible replacements in the case of mortality

Body Weight at Randomization and Age of Animals on First Day of Dosing

	Age	Weight
Males	6-8 weeks	25.5-37.0 g
Females	6-8 weeks	19.5-31.0 g

Animals outside these body weight ranges may be used with written approval from the Study Director. The body weight ranges will be reported in the final study report.

Acclimation

The animals will be acclimated for at least five days. All animals will be judged to be healthy prior to use in the study. Prior to the first day of dose administration, animals will be observed at least once daily for signs of illness and poor health.

8. HUSBANDRY

Housing

Animals will be housed in a controlled environment at 72 ± 3°F and 50 ± 20% relative humidity with a 12-hour light/dark cycle. The light cycle may be temporarily interrupted for study related activities. The animal rooms will be supplied with at least 10 changes of fresh HEPA-filtered air per hour. Animals of the same sex may be housed up to five per Micro-Barrier cage. Cages will be placed on the racks equipped with an automatic watering system and Micro-VENT full ventilation, HEPA filtered system. If needed, an alternative housing system may be used.

Environmental Enrichment

Animals will be provided with environmental enrichment); the type of enrichment will be recorded in the raw data.

Food

A certified laboratory rodent chow (Teklad 2018C Global 18% Protein Rodent Diet) will be provided ad libitum. The food is analyzed by the manufacturer for the concentrations of specified heavy metals, aflatoxin, chlorinated hydrocarbons, organophosphates and specified nutrients.

Water

Animals will have free access to tap water, which meets U.S. EPA drinking water standards [Washington Suburban Sanitary Commission (WSSC) Potomac Plant]. Drinking water is monitored at least annually for levels of specified microorganisms, pesticides, heavy metals, alkalinity and

	halogens. If needed, animals may be given supplemental water, as tap water in a petri dish or Napa Nectar™ (Systems Engineering; Napa, CA) or an equivalent hydration gel.
Bedding	Heat treated hardwood chips will be used for bedding to absorb liquids.
Bedding, Food and Water Analysis	The results of bedding, food and water analyses are on file at BioReliance. Based on historical test results, there are no contaminants in the bedding, feed and water that are expected to interfere with the study.

9. EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be conducted according to established procedures (Heddle, 1973; Mavournin et al., 1990; Hayashi et al., 1994; OECD, 2016).

Randomization

The weight variation per sex of all animals assigned to study will not exceed ±20% of the mean weight without Study Director approval. Animals will be randomized to achieve random placement of animals throughout all groups.

Animal Identification

Following randomization, animals will be identified by sequentially numbered ear tags and/or programmed microchip. The cage card will contain, at least, the animal number(s), sex, study number, treatment group number, dose level and route of administration. Cage cards will be color coded by treatment group. Raw data records and specimens also will be identified by the unique animal number.

Dose Range-Finding Assay (DRF)

A DRF will be performed to assess test article toxicity and determine the maximum tolerated dose (MTD) or maximum feasible dose (MFD) using the same dosing regimen specified for the micronucleus assay. The MTD is defined as the dose that induces some signs of toxicity but is not expected to produce mortality or severe and prolonged clinical signs of toxicity. The MFD is defined by 1) physical properties that limit the dose formulation concentration, 2) limitations on volume that can be administered, 3) bioavailability of compound or 4) toxicity indicated by a reduction in PCE proportions of more than 50%, but to not less than 20% of the concurrent vehicle value. The maximum dose evaluated for non-toxic materials will be 2000 mg/kg/day (the limit dose for this assay).

The assay design will be as follows:

Group	Treatment	Dose Level (mg/kg/dose)	Dose Volume[A] (mL/kg/dose)	Animals/Sex[B]
1	TA	25	5	3
2	TA	50	5	3
3	TA	100	5	3

[A]Based upon individual body weight
[B]Based upon the results, additional groups of 1-3 animals/sex may be added by the Study Director in the DRF to determine the MTD.

Procedure*	Dose Range-Finder Animals
Body Weight	Prior to the first dose (for the purpose of all dose volume calculations) Once on the day of euthanasia
Moribundity and Mortality Check**	At least twice daily, beginning on the first day of dose administration
Detailed Hands-On Clinical Observations	Pre-dose on Day 1
Cage Side Observations	1 to 2 hours post-dose and at least once daily on non-dosing days
Dose Frequency	Once **Formulations will be held on wet ice prior to and during dosing**
Route of Administration***	Intraperitoneal injection (IP)

*Additional observations may be performed as needed.
**Moribund animals will be sacrificed immediately by CO_2 overdose. Animals found dead or sacrificed moribund will be discarded without necropsy.
***This route has been routinely used.

Following the two day observation period, all surviving animals will be euthanized by carbon dioxide inhalation, and discarded without further examination.

Micronucleus Assay
Animals will be dosed with the test article or vehicle control and euthanized at the appropriate time.

The high dose for the micronucleus assay will be the MTD/MFD, as determined in the DRF assay. Two additional doses will be evaluated, generally at one-half and one-fourth of the top dose. Actual dose levels used in the micronucleus assay will be documented in the raw data and report. If no significant differences in toxicity or mortality are observed in the DRF, only males may be used for the micronucleus assay.

The assay design will be as follows:

Euthanasia Time (hours after treatment)[B]				24	48
Group	Treatment	Dose Level (mg/kg/dose)	Dose Volume[A] (mL/kg/dose)	Number of Male Animals	
1	Vehicle	0	5	6	6
2	TA	Low	5	6	0
3	TA	Mid	5	6	0
4	TA	High	5	6	6

[A]Based upon individual body weight
[B]Range(s): 24-27 hours and 45-48 hours, respectively

Procedure*	Micronucleus Assay Animals
Body Weight	Prior to the first dose (for the purpose of all dose volume calculations) Once on the day of euthanasia, excluding animals used for bioanalysis
Moribundity and Mortality Check**	At least twice daily, beginning on the first day of dose administration
Detailed Hands-On Clinical Observations	Pre-dose on Day 1, excluding animals used for bioanalysis
Cage Side Observations	1 to 2 hours post-dose and at least once daily on non-dosing days, excluding animals used for bioanalysis
Dose Frequency	Once **Formulations will be held on wet ice prior to and during dosing**
Route of Administration***	Intraperitoneal injection (IP)

*Additional observations may be performed as needed.
**Moribund animals will be sacrificed immediately by CO_2 overdose. No additional data points or sample collection will be taken from moribund sacrificed or found dead animals. Animals found dead or sacrificed moribund will be discarded without necropsy.
***This route has been routinely used.

Blood Collection and Sample Handling

Group	Treatment	Dose Level (mg/kg)	Animals/ Timepoint	Collection Timepoint (hours after last dose)	Total Number of Male Animals
6	Vehicle	0	3	1	3
7	TA	Low	3	1	3
8	TA	Mid	3	1	3
9	TA	High	3	1	3

Frequency 1st day of dosing

Collection Site	Retro-orbital Sinus
Target Volume	0.5 mL of whole blood Any volume less than this will be documented in the raw data.
Anesthesia	Animals will be anesthetized prior to collection by 70% CO_2/30% O_2.
Anticoagulant	K_2EDTA
Sample Handling	Blood samples will be maintained on wet ice until centrifugation.
Centrifugation	Blood samples will be centrifuged for 5 minutes, 2-8°C, at 2000 g within 1 hour of collection, and plasma will be harvested into two sets of approximately equal aliquots.
Sample Storage	Plasma samples will be stored at ≤ -60°C and will be analyzed only as needed. If analysis is conducted, one set will be shipped on dry ice to the Principal Investigator for BioAnalysis.
Animal Disposition	Animals will be sacrificed by CO_2 overdose after their last collection timepoint.

Bioanalysis (BioA)

If conducted, a validated method will be used to analyze the concentrations of test article or metabolites in the plasma samples. Samples will be shipped on dry ice by overnight courier to the Principal Investigator for BioAnalysis. They will be sent on a non-holiday Monday, Tuesday or Wednesday that does not immediately precede a holiday. Upon receipt, samples will be stored at -60°C or below until required for analysis. Unused samples will be discarded upon acceptance of the analytical results by the Study Director. A BioA contribution report with GLP Compliance Statement, signed by the Principal Investigator from the Test Site, will be provided and included in the main micronucleus report, if applicable.

Tissue Sample Collection and Handling

Liver samples will be collected from main study animals at approximately 24 or 48 hours after dose administration (at the time of bone marrow collection), as applicable. Liver (containing no gall bladder) samples will be collected in duplicates of approximately 0.5g each for possible analysis. The liver samples will be frozen on liquid nitrogen and stored at ≤ -60°C in prelabeled plastic vials. If analysis will be conducted, it will be added by amendment. Any unused samples will be discarded prior to report finalization.

Bone marrow for possible analysis will be collected from TK animals just following euthanasia after blood collection. The femurs will be exposed, cut just above the knee, and the bone marrow will be aspirated into an empty syringe. The bone marrow samples will be frozen on liquid nitrogen and stored at ≤ -60°C in prelabeled plastic

vials. If analysis will be conducted, it will be added by amendment. Any unused samples will be discarded prior to report finalization.

Bone Marrow Collection
Animals will be euthanized by carbon dioxide inhalation approximately 24 or 48 hours after the final dose, as indicated. Immediately following euthanasia, the femurs will be exposed, cut just above the knee, and the bone marrow will be aspirated into a syringe containing fetal bovine serum.

Preparation of Micronucleus Slides
The bone marrow will be transferred to a centrifuge tube containing 1-3 mL fetal bovine serum, the cells will be pelleted by centrifugation, and the supernatant drawn off leaving a small amount of fetal bovine serum with the pellet. Cells will be re-suspended and a small drop of the bone marrow suspension will be spread onto a clean glass slide. At least four slides will be prepared from each animal, air dried and fixed by dipping in methanol. One set of two slides (including at least 5 positive control slides) will be stained with acridine orange for microscopic evaluation. The other set of slides will be kept as backup. Each slide will be identified by the harvest date, study number, and animal number (or slide number for positive control slides). Slides will be coded using a random number table by an individual not involved with the scoring process.

Scoring of Micronucleus Slides
Slides will be evaluated by fluorescent microscopy. The staining procedure permits the differentiation by color of polychromatic and normochromatic erythrocytes (bright orange PCEs and ghost-like, dark green NCEs, respectively).

The criteria for the identification of micronuclei are those of Schmid (1975). Micronuclei are brightly stained bodies that generally are round and that generally are between 1/20 and 1/5 the size of the PCE. The frequency of micronucleated cells will be recorded with cells containing one or more micronuclei counted as one micronucleated PCE (MnPCE).

At least 4000 PCEs/animal will be scored for the presence of micronuclei (MnPCEs) whenever possible. In addition, at least 500 total erythrocytes (PCEs + NCEs) will be scored per animal to determine bone marrow cytotoxicity.

Stained slides will be discarded prior to report finalization.

Statistical Analysis
Statistical analysis will be performed on the micronucleus frequency (%MnPCE) and %PCE using the animal as the unit. The mean and standard deviation of %MnPCE and %PCE will be presented for each treatment group.

The use of parametric or non-parametric statistical methods in evaluation of data will be based on the variation between groups. The group variances for micronucleus frequency for the vehicle and test article groups at the respective sampling time will

be compared using Levene's test (significance level of $p \leq 0.05$). If the variation between groups is found not to be significant, a parametric one-way ANOVA will be performed followed by a Dunnett's post-hoc analysis to compare each dose group to the concurrent vehicle control. If Levene's test indicates heterogeneous group variances (significance level of $p \leq 0.05$), the suitability of a transformation of the original data will be evaluated (e.g. using logarithm transformed values of the original data) in an attempt to meet the normality criteria. Afterwards, statistical analysis will be performed using the parametric tests described above. If parametric tests are not acceptable, non-parametric statistical methods (Kruskal Wallis and/or Mann Whitney test) may be used in evaluation of data.

A linear regression analysis will be conducted to assess dose responsiveness in the test article treated groups ($p \leq 0.01$ and $R^2 \geq 70\%$). Alternative statistical methods (e.g., Jonckheere's Test) may be used in the evaluation of data ($p \leq 0.01$).

A pair-wise comparison (Student's T-test; $p \leq 0.05$) will be used to compare the positive control group to the concurrent vehicle control group. If parametric tests are not acceptable, non-parametric statistical methods (Kruskal Wallis and/or Mann Whitney test) may be used in evaluation of data.

10. CRITERIA FOR DETERMINATION OF A VALID ASSAY

Vehicle Controls

The group mean frequency of MnPCEs should ideally be within the 95% control limits of the distribution of the historical negative control database. If the concurrent negative control data fall outside the 95% control limits, they may be acceptable as long as these data are not extreme outliers (indicative of experimental or human error).

Positive Controls

The frequency of MnPCEs for the scoring controls must be significantly greater than the concurrent vehicle control ($p \leq 0.05$) and should be compatible with those observed in the historical positive control data base.

Test Conditions

At least three doses will be tested for at least one sampling time. Five animals/group should be available for analysis. In the event of mortality, sufficient replacement animals will be added to the study to meet this criterion. If fewer than five animals/group are available for analysis, bone marrow will be collected and slides will be prepared from all surviving animals, but a decision about evaluation of the slides from the surviving animals will be made by the Study Director in consultation with the Sponsor.

Cell Analysis

At least 4000 PCEs/animal will be scored for the presence of micronuclei (MnPCEs) whenever possible. In addition, at least 500 total erythrocytes (PCEs + NCEs) will be scored per animal to determine the proportion of PCEs as an index of bone marrow

cytotoxicity. A reduction in the PCE proportions to less than 20% of vehicle control value will be considered excessively cytotoxic and the animal data will be excluded from evaluation.

Maximum Dose Evaluated
The maximum dose evaluated for micronucleus induction must

a) be the MTD or MFD, or
b) demonstrate cytotoxicity in the bone marrow (reduction in the PCE/NCE ratio of more than 50% but not less than 20% of the control value), or
c) in the absence of cytotoxicity or MFD, a dose of 2000 mg/kg/day (limit dose) is used.

11. EVALUATION OF TEST RESULTS

A test article will be considered to have induced a positive response if

a) at least one of the test article doses exhibits a statistically significant increase when compared with the concurrent negative control ($p \leq 0.05$), and
b) when multiple doses are examined at a particular sampling time, the increase is dose-related ($p \leq 0.01$ and $R^2 \geq 70\%$), and
c) results of the group mean or of the individual animals in at least one group are outside the 95% control limit of the historical negative control data.

A test article will be considered to have induced a clear negative response if none of the criteria for a positive response were met and there is evidence that the bone marrow was exposed to the test article (unless intravenous administration was used).

If the response is neither clearly positive nor clearly negative, or in order to assist in establishing the biological relevance of a result, the data will be evaluated by expert judgment and/or further investigations. Possible additional work may include scoring additional cells (where appropriate) or performing an additional experiment that could employ the use of modified experimental conditions. Such additional work will only be carried out following consultation with, and at the request of, the Sponsor.

In some cases, even after further investigations, the data set will preclude making a conclusion of positive or negative, at which time the response will be concluded to be equivocal. In such cases, the Study Director will use sound scientific judgment and report and describe all considerations.

12. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Article Tracking
Provantis™ (Instem)	Captures in-life toxicology, animal randomization and management data
Excel (Microsoft Corporation)	Calculations/Randomization
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting
Provantis™ Tables and Stats (Instem)	Generates in-life toxicology tables

13. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test article
- Vehicle
- Test Animals
- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations, 95% control limits)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test article, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- Location of archived material
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test article and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

14. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for

disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

The raw data, reports, and other documents generated at locations other than BioReliance, if applicable, will be archived by the Test Site.

15. REFERENCES

Heddle J.A. A rapid *in vivo* test for chromosomal damage. Mutation Res. 18:187-190, 1973.

Hayashi M., Tice R.R., MacGregor J.T., Anderson D., Blakey D.H., Kirsch-Volders M., Oleson Jr. F.B., Pacchierotti F., Romagna F., Shimada H., Sutou S. and Vannier B. *In vivo* rodent erythrocyte micronucleus assay. Mutation Res. 312: 293-304, 1994.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Mavournin K.H., Blakey D.H., Cimino M.C., Salamone M.F. and Heddle J.A. The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res. 239:29-80, 1990.

National Research Council, Institute of Laboratory Animal Resources Commission on Life Sciences. Guide for the Care and Use of Laboratory Animals. National Academy Press. Washington, D.C., 2011.

OECD Guidelines for Testing of Chemicals, Guideline 474 (Genetic Toxicology: Mammalian Erythrocyte Micronucleus Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, Updated and Adopted 29 July 2016.

Schmid W. The micronucleus test. Mutation Res. 31:9-15, 1975.

APPROVALS

Sponsor Approval

11-11-2019

Judi Abbott Curry
Sponsor Representative

Date

Study Director and Test Facility Management Approvals

BioReliance Study Director	13 NOV 2019 Date
BioReliance Study Management	15 NOV 2019 Date

15. APPENDIX III: Certificate of Analysis

Certificate of Analysis

SIGMA-ALDRICH

Product Name	1,2,4-Benzenetriol, *ReagentPlus*®, 99%
Product Number	173401
Product Brand	ALDRICH
CAS Number	533-73-3
Molecular Formula	$C_6H_3(OH)_3$
Molecular Weight	126.11

TEST	LOT S36884V RESULTS
QC Acceptance date	11-JUL-2006
APPEARANCE - COLOUR	OFF WHITE
APPEARANCE - STATE	POWDER
IR SPECTROSCOPY - FTIR SPECTRUM	CONFORMS TO STRUCTURE
MELTING POINT - COMMENT	139.9-142.4 DEG C
GAS CHROMATOGRAPHY - PURITY	98.8%

Claudia Mayer
Manager Quality Control
Steinheim, Germany

16. APPENDIX IV: Dose Formulation Analysis

FINAL ANALYTICAL REPORT

Study Title

***In Vivo* Mammalian Erythrocyte Micronucleus Assay in Mice**

Report Title

Determination of 1, 2, 4-Trihydroxybenzene in Degassed Deionized Water Dosing Formulations

Test Article

1, 2, 4-Trihydroxybenzene

Author

Philip Atkins, MChem

Final Analytical Report Date

04 February 2020

Analytical Laboratory

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF98GS.123M012.BTL

Sponsor

Harris Beach PLLC
100 Wall Street
New York, NY 10005

1. COMPLIANCE STATEMENT

This portion of the study, AF98GS.123M012.BTL, was conducted in compliance with the following regulation: US FDA Good Laboratory Practice Regulation as published in 21 CFR Part 58. The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

PAtkins — 04 Feb 2020

Philip Atkins, MChem
Contributing Scientist
BioReliance Corporation

Date

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3. ANALYTICAL CONDITIONS

The analysis of the test article formulations, 1, 2, 4-Trihydroxybenzene in Degassed Deionized Water, for study AF98GS.123M012.BTL, was performed by ultra-high performance liquid chromatography (UHPLC) using a method validated under BioReliance Study Number AE03RS.GTCHEM.BTL. The formulations were also analyzed in accordance with BioReliance SOPs "Dose Formulation Analysis". The analytical conditions used in this study are summarized in Table 1. The matrix standards were prepared per Table 2.

All stock formulations and standards were prepared on wet ice in a glove box under nitrogen gas.

Table 1: Analytical Conditions

Instrument:	Agilent 1290 UHPLC (in Standard Mode)
Detector:	UV 287 nm, bandwidth 4 nm, Reference off
Software:	Agilent ChemStation with Open Lab CDS
(MP) Mobile Phase A:	30:70 Acetonitrile: Deionized Water, pH 3.3
Diluent:	Deionized Water, pH 3.3
Vehicle:	Degassed deionized water
(SS) Stock Solution:	1024 µg/mL Test Article in Diluent
(WSS) Working Stock Solution:	200 µg/mL Test Article in Diluent
TA Correction Factor:	1.00
Column:	Phenomenex Luna C18 100A, 250 mm x 4.6 mm, 5µm particles
Column Temperature:	25°C
Autosampler Temperature:	4°C
Injection Volume:	5 µL
Flow Rate:	1 mL/min
Retention Time:	~3.167 minutes
Injections / Sample:	2
Run Time:	5 minutes
Calibration Curve:	y = Ax + B (not weighted)
Elution mode:	Isocratic

Table 2: Preparation of the Matrix Standard Solutions

Standard ID	WSS (mL)	Degassed deionized water (mL)	Final Volume with Diluent (mL)	Final TA Concentration (µg/mL)
M-0	0	1	10	0
M-1	1	1	10	20
M-2	2	1	10	40
M-3	3	1	10	60
M-4	4	1	10	80
M-5	5	1	10	100

4. DOSING FORMULATION ANALYSIS

Dosing formulations of, 1, 2, 4-Trihydroxybenzene in Degassed Deionized Water were collected and analyzed by UHPLC on the day of preparation to assess accuracy of the preparation (per Table 3). A sample of vehicle dosing solution was also analyzed to verify that it did not contain test article.

The dosing formulations were diluted to bring the test article concentration to a suitable level within the calibration range. The concentration of 1, 2, 4-Trihydroxybenzene was calculated by reference to the solvent standard solutions prepared (per Table 2) and analyzed concurrently with the dosing formulations. All matrix standard curves met the acceptance criteria (Table 4).

The 5 mg/mL dosing formulation analyzed met the acceptance criteria of 85.0 – 115.0% of target concentration and the ratio of the small/large (S/L) obtained concentrations for the duplicate dilutions of each solution was found to be > 0.925. The 1.26 mg/mL and 2.5 mg/mL dosing formulations were found to be below the acceptable range, but met the S/L ratio of > 0.925 (Table 5). No test article was detected in the vehicle control (VC) samples.

Table 3: Summary of the Dosing Formulations

Experiment No./Phase	Date of Preparation	Date of Analysis (Start/End)	Concentration (mg/mL)	Homogeneity Testing (Y) Yes or (N) No
Definitive Analysis	04 DEC 2019	04 DEC 2019/ 04 DEC 2019	Vehicle 0	N
			Vehicle 0(B)	N
			1.26	N
			2.5	N
			5	N

Table 4: Matrix Standards for the Definitive Dosing Formulation Analyses

Item	Value	Acceptance Criterion
Slope	6.925	NA
Intercept	-21.33	NA
Correlation Coefficient	0.99991	≥0.99
Recovery % (Range)	98.4-102.1	90-110

Table 5: Definitive Dosing Formulation Analysis

Formulation ID	Conc. of Form. (mg/mL)	Conc. of Sample (µg/mL)	TA Peak Area	Mean Peak Area	Mean Conc. (µg/mL)	Target (%)	x Dilution Factor	Final Mean Conc. (mg/mL)	S/L
VC (0)	0	0	ND	NA	NA	NA	2.5	NA	NA
VC (B)	0	0	ND	NA	NA	NA	2.5	NA	NA
1.26 A	1.26	50.4	280.1						
1.26 B	1.26	50.4	266.5	273.3	42.55	84.4	25	1.06	0.951
2.5 A	2.5	50	200.0						
2.5 B	2.5	50	208.7	204.4	32.60	65.2	50	1.63	0.958
5 A	5	50	359.4						
5 B	5	50	356.8	358.1	54.79	109.6	100	5.48	0.993

5. STABILITY OF 1,2,4-TRIHYDROXYBENZENE IN DEGASSED WATER DOSING FORMULATIONS

Stability of the dosing formulations was determined under BioReliance study number AE03RS.502.BTL. 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.

6. CONCLUSION

The results of the analysis indicate that the actual mean concentrations of the analyzed formulation samples, 1.26, 2.5, and 5 mg/mL were 84.4, 65.2, and 109.6% of target, respectively, with S/L ratios of > 0.925. The 1.26 and 2.5 mg/mL formulations were found to be below the acceptable range for concentration (85.0 to 115.0% of target) but met the S/L ratio of > 0.925. This indicates that the formulations were accurately prepared, except as indicated above. No test article was detected in the vehicle control sample. The impact will be assessed by the study director in the main report.

7. DEVIATIONS

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

8. ABBREVIATIONS AND CALCULATIONS

Calc. = Calculated
Conc. = Concentration
Form = Formulation
M = Matrix
NA = Not Applicable
ND =Not Detected
S/L = Small/Large
T = Time
TA = Test Article
UHPLC = Ultra-High Performance Liquid Chromatography
UV = Ultra-Violet
VC = Vehicle Control

The following formulas were used for the calculations:

1. Mean Concentration (µg/ mL) = (Mean Peak Area - Intercept) / Slope

   Intercept and slope calculated using linear regression analysis

2. Final Mean Conc. (mg/mL) = (Mean Conc. (µg/ mL) x Dilution Factor) / 1000

3. % of Target, % Recovery = $\frac{\text{Mean Calculated Concentration}}{\text{Concentration of Sample}} \times 100$

4. S/L = $\frac{\text{Small TA Peak Area}}{\text{Large TA Peak Area}}$

17. APPENDIX V: Bioanalysis

FRONTAGE
YOUR DRUG DEVELOPMENT PARTNER

Bioanalytical Method Development Summary Report: 038110

In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice

Test Site Study No.: 038110
Test Site: Frontage Laboratories, Inc., Concord OH
Testing Facility Study No.: AF98GS.123M012.BTL
Testing Facility: BioReliance Corporation
Sponsor: Harris Beach PLLC

Analysis Performed by:
Thomas Fellowes, Associate Scientist II

Report Written by:
Tivadar Orban, Ph.D., Principal Investigator (PI)

APPROVALS:

Reviewed by: James Formanik
James B. Formanik, M.S.
Principal Scientist
Bioanalytical Services
8-27-2021
Date

Approved by: (PI)
Tivadar Orban, Ph.D.
Senior Scientist & Manager
Bioanalytical Services
8-27-21
Date

Approved by: (Management) Jenny Minu for Jason Liu
Jason Liu, Ph.D.,
Concord Site Head, VP of
Preclinical Services
8/27/2021
Date

10845 Wellness Way, Concord OH 44077
Phone: 440-357-3200; Fax: 440-357-3800
Website: www.frontagelab.com

COMPLIANCE STATEMENT

Frontage Laboratories, Inc. study No. 038110 was conducted in accordance with protocol AF98GS.123M012.BTL, Food and Drug Administration, United States Code of Federal Regulations (CFR), Title 21, Part 58: Good Laboratory Practices and Frontage Laboratories, Inc. standard operating procedures (SOPs).

Principal Investigator

Tivadar Orban, Ph.D.
Senior Scientist & Manager
Bioanalytical Services

8-27-21
Date

QUALITY ASSURANCE UNIT STATEMENT

The Frontage Quality Assurance Unit has performed inspections on Frontage Project 038110, the bioanalytical phase of BioReliance study “In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice” study AF98GS.123M012.BTL. The results of these inspections, including any findings or observations, were reported to the Frontage Principal Investigator and Frontage Management for appropriate corrective actions, and to the Study Director/Management on the dates listed below:

Dates Inspected	Phase of Study Inspected	Dates Reported to Frontage Principal Investigator	Dates Reported to Frontage Management	Dates Reported to Study Director/Management
2/25/2021 and 3/05/2021	Data	3/5/2021	3/5/2021	3/5/2021
6/22/2021-6/23/2021	Data and Report	6/24/2021	6/24/2021	6/24/2021

Stephen Rogenthien, B.A., RQAP-GLP
Senior Director, Quality Assurance
Frontage Laboratories, Inc.

6/27/2021
Date

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LIST OF ABBREVIATIONS

BA	Bioanalytical
CFR	Code of Federal Regulations
CV	Coefficient of Variation
GLP	Good Laboratory Practices
THB	1,2,4-Trihydroxybenzene
HPLC	High Performance Liquid Chromatography
ID	Identification
IS	Internal Standard
K_2EDTA	Dipotassium Ethylenediamine Tetra acetic Acid
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
LIMS	Laboratory Information Management System
LLOQ	Lower Limit of Quantitation
mL	Milliliter
MS	Mass Spectrometry
NA	Not Applicable
No.	Number
ng/mL	nanogram per milliliter
QA	Quality Assurance
QAU	Quality Assurance Unit
QC	Quality Control
SD	Standard Deviation
SOP	Standard Operating Procedure
Std	Standard
TM	Test Method
ULOQ	Upper Limit of Quantitation
µL	Microliter
µm	Micrometer

STUDY INFORMATION

Sponsor	Harris Beach PLLC 100 Wall Street New York, NY 10005
Sponsor Representative	Judi Abbott Curry Harris Beach PLLC 100 Wall Street New York, NY 10005 Phone: 212-313-5404 Email: jcurry@harrisbeach.com
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Frontage Labs Scientists	Thomas Fellowes, B.S., Associate Scientist II
Test Site Study No.	038110

INTRODUCTION

The bioanalytical laboratory at Frontage Laboratories, Inc. used incurred plasma samples for the sole purpose to develop and qualify a quantitation method for the determination of 1,2,4-Tryhydroxybenzene in mouse K_2EDTA plasma. The study was initiated on 13-Nov-2019 and the study phase was initiated and completed on 16-Feb-2021. No peaks were detected during method development in any of the analyzed samples. Therefore, qualification was not performed. In addition, dosing additional animals and collecting samples was not planned or performed to be in compliance with animal testing legislation (see references 2, 3, and 4).

STORAGE TEMPERATURES

Temperatures designated throughout this report (e.g., -80°C, -20°C, refrigerator, room/ambient) were considered equivalent to the closest temperature conditions defined in SOP 00-C014. Storage temperatures listed throughout this document represent nominal values. The actual ranges for these temperatures are as follows:
A value of 5°C represents storage between 2°C and 8°C.
A value of -5°C represents storage between -15°C and 5°C.
A value of -20°C represents storage between -10°C and -30°C.
A value of -80°C represents storage between -70°C and -90°C.

EXPERIMENTAL

Bioanalytical Method

The draft bioanalytical method (BA-TM-0138-01/C) (Attachment 1) is a LC-MS/MS method developed for the determination of 1,2,4-Tryhydroxybenzene in mouse K_2EDTA plasma.

Materials

Reference Standard

Name:	1,2,4-Trihydroxybenzene (THB)
Frontage Reference ID:	MT00972
Source:	Sigma/Merck
Lot#:	S7832591
CAS#:	533-73-3
Purity:	99.0%
Expiration Date:	31-Aug-2024
Storage:	Ambient (5 °C)

Internal Standard

Name:	Hydroquinone D6

Frontage
Reference ID: MT01109
Source: Cambridge Isotope Laboratories, Inc.
Batch#: PR-26792
CAS#: NA
Purity: ≥98% (Assumed 100% for use as an IS)
Retest Date: 23-Jul-2021
Storage: Ambient (5 °C) dark and in desiccator

Refer to Attachment 2 for the respective Certificates of Analysis.

Biological Matrix

Blank K_2EDTA mouse plasma was purchased from BioIVT. The mouse plasma lots used were MSE328505 and MSE351858 which expire on 31-Aug-2021 and 31-May-2025, respectively. The above lots were acidified at Frontage Laboratories, Inc. and were used to prepare the calibration standards and QC samples. There were no analyte interferences detected in blanks. Matrix effects were not evaluated. The plasma was stored at a temperature of -20 ºC.

Stock and Spiking Solutions

The stock solution of analyte and internal standard were prepared in DMSO on 8-Feb-2021 and 9-Feb-2021, respectively. The spiking solutions were prepared in methanol on 9-Feb-2021, 10-Feb-2021, and 16-Feb-2021. The stock and spiking solutions were stored at -20 ºC. Stock and spiking solution stability was not determined.

Calibration Standards and QC Samples

Sets of eight non-zero calibration standards ranging from 50.0 ng/mL to 25,000 ng/mL for 1,2,4-Trihydroxybenzene were prepared on 16-Feb-2021. The QC samples, at concentrations (nominal, untreated) of 150 ng/mL, 7,500 ng/mL, and 18,800 ng/mL for 1,2,4-Trihydroxybenzene were prepared on 16-Feb-2021. The calibration standards and QC samples were stored at -80 ºC. Refer to draft method BA-TM-0138-01/C for the respective preparation procedures of the calibration standards and the QC samples.

Study Samples

Sample Source and Date of Receipt

Samples were received frozen on dry ice in good condition on 22-Dec-2020 and 3-Feb-2021.

Sample Storage

Study samples were stored in freezers at Frontage Laboratories, Inc., at a setpoint of -70°C for 56 and 13 days until analysis (see below).

Analyzed Samples

A total of 5 samples were extracted and analyzed for the sole purpose of method development (i.e., no concentrations will be reported). Details of the run are listed in Table 1.

Equipment

Column: Phenomenex Luna NH2 3 µm, 100 Å, 150 x 4.6 mm
HPLC System: Shimadzu Nexera UHPLC
Mass Spectrometer: AB Sciex 6500 TripleQuad
Sartorius ME 215P Balance
Eppendorf Centrifuge 5417R
VWR Scientific Multi-Tube Vortexer
Fisher Genie 2 Vortexer
2-mL micro-centrifuge tube with screw cap
Eppendorf automated pipettes
Gilson and Rainin adjustable pipettes
Autosampler vials
96-well plate with sealing mat
Disposable pipettes
Miscellaneous glassware for preparation of standard solutions and mobile phases

RESULTS

Concentrations of QC Samples

The results for the 1,2,4-Trihydroxybenzene QC samples in acidified mouse K_2EDTA plasma are summarized in Table 2. QC samples were considered acceptable if 66.7% (two-thirds) of the samples were within ±20.0% of the nominal concentration at each level and at least 50.0% of all QC samples per run were within ±20.0% of the nominal concentration. For the accepted run, the QC samples met the acceptance criteria.

Back-calculated Concentrations of Calibration Standards

The results for the calibration standards for 1,2,4-Trihydroxybenzene in acidified mouse K_2EDTA plasma are provided in Table 3. For a batch run to be acceptable, at least 75.0% of the total number of calibration standards or at least 6 calibration standards in the calibration range, including the LLOQ and ULOQ,

must not deviate by more than ±20.0% (±25.0% at the LLOQ) from their nominal values. The calibration standards met the acceptance criteria for the run.

Standard Curves

An example of the standard curve obtained for 1,2,4-Trihydroxybenzene in acidified mouse K_2EDTA plasma is presented in Figure 1. The curve parameters including the slope, intercept, and the coefficient of determination (r^2) are presented in Table 4. The curve parameter (r^2) met the acceptance criteria ($r^2 \geq 0.9701$) for the accepted run.

Study Sample Concentrations

Results for the analyzed samples used solely for method development are listed in Table 5. No concentrations are reported for any of the samples used during method development.

Repeat Analysis

Repeat analysis was not performed.

Incurred sample reanalysis

Incurred sample reanalysis was not performed.

Chromatograms

Serial chromatograms from Watson Run 1 are presented in Attachment 3 as representative examples.

DEVIATIONS

Protocol Deviation:
The reference standard used in the bioanalytical study phase was received at ambient temperature from the supplier and was stored at ambient temperature. This was a protocol deviation which states that storage conditions should be -10 to -30 °C.

The Certificate of Analysis for the reference standard used in the bioanalytical study phase did not list a storage temperature. The Material Safety Data Sheet (MSDS) added to the study suggests storage at refrigerated conditions. Documentation for reference standard storage conditions was requested from the study director. However, documentation for storage conditions other than what was listed in the protocol were not available. Though storage conditions are variable, the reference standard was detected at the expected lower limits of detection. Further, all concentrations from the evaluated samples fell below the lower limit of quantitation (BQL).

As reference standard degradation would be indicative of an even lower limit of quantitation, any potential degradation of the reference standard would not change the BQL outcome. Therefore, the deviation has no impact on this study phase.

ARCHIVES

Study data, including the report, study file, laboratory notebooks, instrument printouts, electronic data and other materials arising out of the study phase will be archived at Frontage Laboratories, Inc., in a secure off-site location for at least two years according to archive SOPs.

DATA ACQUISITION AND PROCESSING

Retention time and peak area were determined by *Analyst*® Data Acquisition/ Processing Software (Version 1.6.3). Analyte concentrations were obtained from a calibration curve constructed by plotting the peak area ratio versus the nominal concentration using Watson LIMS (Version 7.3). Watson LIMS and Microsoft Office Excel were used for statistical calculations. Watson LIMS (Version 7.3) and Analyst (Version 1.6.3) are validated systems. Concentrations were calculated using linear regression with $1/X^2$ weighting according to the following equation:

$$y = ax + b$$

Where:

y = peak area ratio of 1,2,4-Trihydroxybenzene /internal standard
a = slope of the corresponding standard curve
x = concentration of 1,2,4-Trihydroxybenzene
b = intercept of the corresponding standard curve

CONCLUSION

The K_2EDTA plasma samples collected in study "*In Vivo* Mammalian Erythrocyte Micronucleus Assay in Mice" were extracted and analyzed for the sole purpose of method development, therefore no concentrations will be reported. No test article was detectable in the samples to enable qualification or analysis.

REFERENCES

1 BA-TM-0138-01/C LC-MS/MS Assay for the Determination of 1,2,4-Trihydroxybenzene in Treated Mouse K2EDTA Plasma

2 California SB 1249

3 Illinois SB 0241

4 Nevada SB 197

TABLES

Table 1: Summary of Analytical Runs for 1,2,4-Trihydroxybenzene in Treated Mouse K_2EDTA Plasma

Watson Run ID	Extraction Date	Analysis Date	Purpose	Regression Status
1	16-Feb-2021	16-Feb-2021	Range finding	Accepted

Table 2: Performance of 1,2,4-Trihydroxybenzene QC Samples in Treated Mouse K_2EDTA Plasma

	Nominal Concentration		
Watson Run ID	QC Low 150 ng/mL	QC Mid 7500 ng/mL	QC High 18800 ng/mL
1 (16-Feb-2021)	138	6240	20600
	153	7750	19900
Mean	146	7000	20300
S.D.	10.6	1070	495
%CV	7.3	15.3	2.4
%Bias	-2.7	-6.7	8.0
n	2	2	2

Table 3: Back-calculated Concentrations of 1,2,4-Trihydroxybenzene in Treated Mouse K_2EDTA Plasma

	Nominal Concentrations							
Watson Run ID	Std-1 50.0 ng/mL	Std-2 100 ng/mL	Std-3 500 ng/mL	Std-4 2500 ng/mL	Std-5 10000 ng/mL	Std-6 15000 ng/mL	Std-7 20000 ng/mL	Std-8 25000 ng/mL
1 (16-Feb-2021)	48.1	107	466	2620	9780	13300	21900	24800
Mean.	48.1	107	466	2620	9780	13300	21900	24800
%CV	NA	NA	NA	NA	NA	NA	NA	NA
%Bias	-3.8	7.0	-6.8	4.8	-2.2	-11.3	9.5	-0.8
n	1	1	1	1	1	1	1	1

NA-Not applicable

Table 4: Calibration Curve Parameters for 1,2,4-Trihydroxybenzene in Treated Mouse K_2EDTA Plasma

Watson Run ID	A [a]	B [a]	C [a]	R-Squared
1 (16-Feb-2021)	-0.0000000114	0.00109	0.000934	0.9950

[a] = Quadratic Regression: y = Ax^2 + Bx + C where y is the peak area ratio of 1,2,4-Trihydroxybenzene to IS., x is the concentration of 1,2,4-Trihydroxybenzene, and A, B, and C are regression constants. Regression weighted 1/x^2.

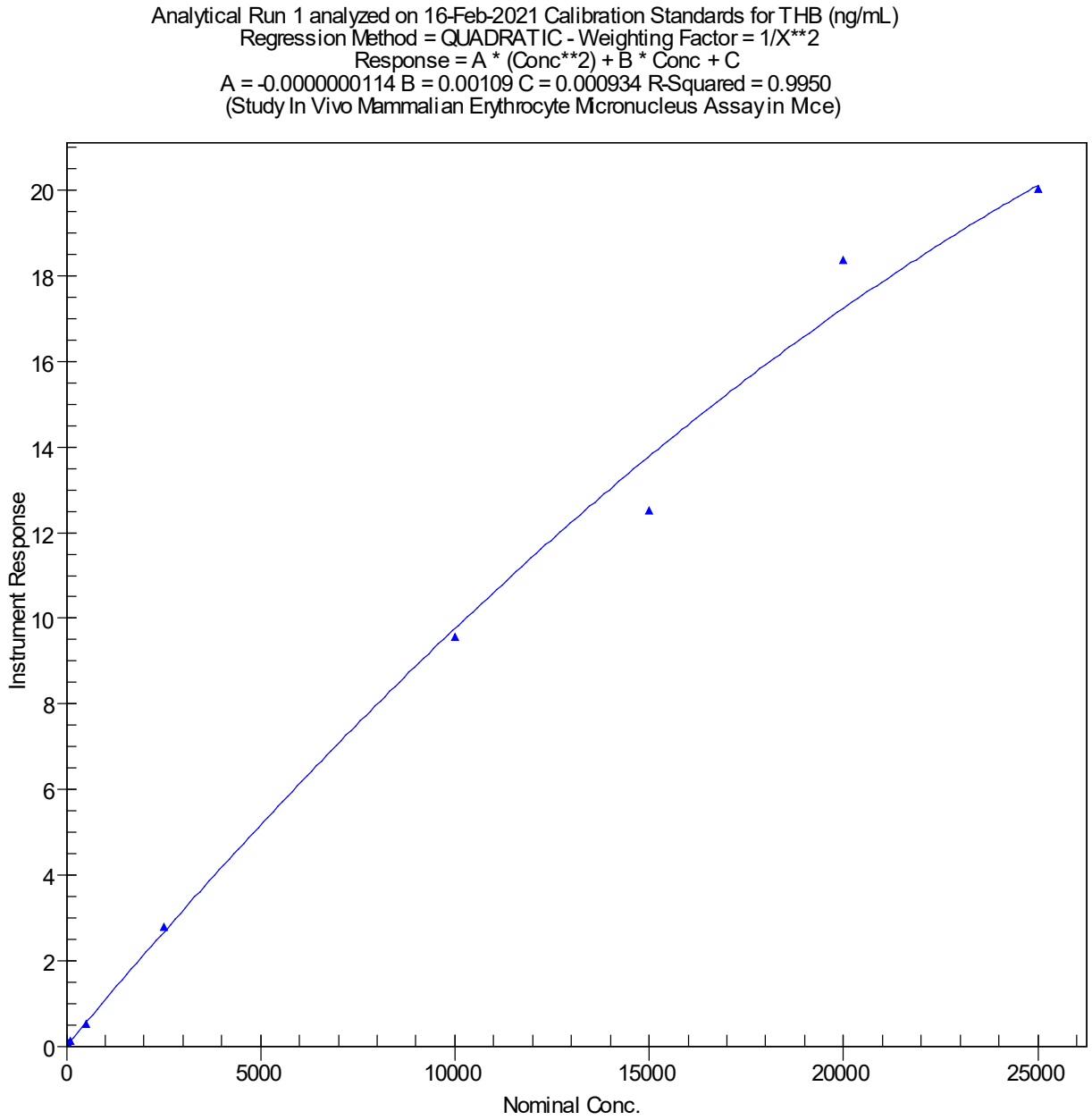
Table 5: Method Development Results of 1,2,4-Trihydroxybenzene in Treated Mouse K_2EDTA Plasma Samples

Subject	Result	Run
991	BQL(<50.0 ng/mL)	1
992	BQL(<50.0 ng/mL)	1
993	BQL(<50.0 ng/mL)	1
994	BQL(<50.0 ng/mL)	1
997	BQL(<50.0 ng/mL)	1

BQL = Below Quantitation Limit

FIGURES

Figure 1: Calibration Curve of 1,2,4-Trihydroxybenzene (THB) in Treated Mouse K_2EDTA Plasma



ATTACHMENTS

Attachment 1: Test Method BA-TM-0138-01/C

A. TITLE

LC-MS/MS Assay for the Determination of 1,2,4-Trihydroxybenzene in Treated Mouse K_2EDTA Plasma

B. INTRODUCTION

The purpose of this test method is to describe procedures to be employed for the analysis of 1,2,4-Trihydroxybenzene in treated Mouse K_2EDTA plasma using a protein precipitation procedure and analysis by LC-MS/MS.

C. SCOPE

The procedures provided in this test method are applicable for the assay of 1,2,4-Trihydroxybenzene in treated Mouse K_2EDTA plasma within the concentration range from 50.0-25,000 ng/mL using 0.025 mL sample volumes. The method will only be used in non-regulated studies

D. DEFINITIONS/ABBREVIATIONS

AQL	Above quantitation limit
Blank	Matrix with internal standard added and subjected to the extraction procedure
BQL	Below quantitation limit
Double blank	Matrix without analyte or internal standard subjected to extraction procedure
DIL	Dilution
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
LC	Liquid Chromatography
LLOQ	Lower Limit of Quantitation
m/z	Mass-to-charge ratio
MS	Mass Spectrometer or Mass Spectrometry
MRM	Multiple Reaction Monitoring
QC	Quality Control
QC-AQL	Dilution Quality Control prepared in plasma, also referred as QC-Dil
SST	System Suitability solution of analyte in plasma at LLOQ concentration level
ULOQ	Upper Limit of Quantitation
WIS	Working internal standard solution

Temperatures designated throughout this test method (e.g., -70 °C, -20 °C, refrigerator, room/ambient) were considered equivalent to the closest temperature conditions defined in SOP 00-C014. Ice indicates frozen water, not frozen carbon dioxide (dry ice).

E. MATERIALS AND EQUIPMENT

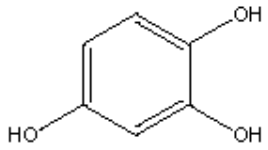
1. Reference Standard

Analyte Name: 1,2,4-Trihydroxybenzene

Formula: $C_6H_6O_3$

Molecular Weight: 126.11 (average), 126.03 (exact)

Structure:



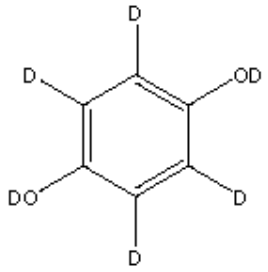
2. Internal Standard

IS Name: Hydroquinone D6

Formula: $C_6D_6O_2$

Molecular Weight: 116.15

Structure:



3. Reagents

Acetone	HPLC or Optima Grade, Sigma Aldrich or equivalent
Acetonitrile (ACN)	HPLC Grade, Sigma Aldrich or equivalent
Ascorbic Acid	98+%, Alfa Aesar or equivalent
Dimethyl Sulfoxide (DMSO)	Certified ACS Grade, Fisher Scientific or equivalent

Formic Acid (FA)	ACS Reagent, Sigma Aldrich or equivalent
Methanol (MeOH)	HPLC Grade, Sigma Aldrich or equivalent
2-Propanol	HPLC Grade, Sigma Aldrich or equivalent
Water (H_2O)	HPLC Grade, Sigma Aldrich or equivalent
Mouse Plasma	Mouse K_2EDTA plasma obtained from a commercial supplier or in-house animals
Mouse Whole Blood	Mouse K_2EDTA blood obtained from in-house animals or a commercial supplier

4. Equipment

HPLC Column: Phenomenex Luna NH_2

HPLC System: Shimadzu Nexera UHPLC, or equivalent

Mass Spectrometer: AB Sciex 6500

Sartorius ME 215P Balance or equivalent

Thermo Scientific Legend XTR Centrifuge or equivalent

Eppendorf Centrifuge 5417R or equivalent

VWR Scientific Multi-Tube Vortexer or equivalent

Fisher Genie 2 Vortexer or equivalent

2-mL micro-centrifuge tube with screw cap or equivalent

Eppendorf automated pipettes or equivalent

Gilson and Rainin adjustable pipettes or equivalent

Autosampler vials or equivalent

96-well plate with sealing mat

Disposable pipettes

Miscellaneous glassware for preparation of standard solutions and mobile phases

F. SOLUTIONS AND REAGENTS

1. Miscellaneous Solutions and Mobile Phase

The following solution preparation procedures may be adjusted proportionally to accommodate larger or smaller quantities of reagents needed. All changes will be documented. All these solutions will be stored at room or lower temperature and expiration for these solutions will be 3 months unless otherwise noted.

a) Mobile Phase A / Crashing Solution: 0.1% Formic Acid in H_2O

Combine and mix 1,000 mL water and 1.0 mL formic acid. Mix well. Store at room temperature. Prepare using a graduated cylinder.

b) Mobile Phase B: 0.1% Formic Acid in ACN

Combine and mix 1,000 mL ACN and 1.0 mL formic acid. Mix well. Store at room temperature. Prepare using a graduated cylinder.

c) Needle Wash: 2-Propanol/Acetone/Acetonitrile 33/33/33 + 1% Formic Acid

Combine and mix 330 mL 2-propanol, 330 mL acetone, 330 mL acetonitrile, and 10.0 mL formic acid. Mix well. Store at room temperature. Prepare using a graduated cylinder.

d) 1 M Ascorbic Acid in Water Solution

Combine approximately 0.352 g ascorbic acid and 2.0 mL water. Sonicate and mix well. Prepare fresh daily, discard after use.

e) Treated Plasma

Combine 18 mL Mouse plasma and 2 mL 1 M ascorbic acid in water solution. Mix well. Prepare fresh daily, discard after use.

Note: The preparations above solutions may be scaled up or down by adjusting the constituents proportionately.

2. Preparation of 1,2,4-Trihydroxybenzene Stock Solutions

Stock solutions for 1,2,4-Trihydroxybenzene will be prepared in duplicate in 20-mL amber glass vials using the appropriate weight for the lot purity correction. These solutions will be stored at freezer temperature conditions (nominally -20 °C). Solutions should be warmed to room temperature and mixed well before use (e.g. use vortex or sonication to mix). Unless longer stabilities have been determined, primary stock solutions for 1,2,4-Trihydroxybenzene will be assigned an expiration date of X days from date of preparation.

Solvents are to be dispensed using volumetric or calibrated pipettes. Dilutions will be generally made as described below. However, the weights, volumes, and stock solution concentrations may vary. Use of volumetric flasks may be substituted for dispensing solvents from volumetric glassware or calibrated pipettes. Any such changes will be documented.

a) 1,2,4-Trihydroxybenzene Primary Stock Solution (5,000 µg/mL) [SA01 and CA01]

Accurately weigh approximately 7.5 mg of 1,2,4-Trihydroxybenzene reference standard into an amber glass vial and record the actual weight (corrected for salt content, purity, water, and total volatiles as necessary). Dissolve the vial contents with 1.5 mL of DMSO. Vortex mix well. Calculate

the exact concentration of the stock solution.

Two separate primary stock solutions, SA01 and CA01, will be prepared. Once demonstrated that the two stocks are equivalent (within ±5% of mean of replicates), use SA01 for preparation of the secondary stock solution and subsequent preparation of calibration standards. Use solution CA01 for preparation of Quality Control samples. Secondary solutions made from the primary stock solution will account for the actual concentration of the primary stock solution to obtain the desired concentration for the secondary solution.

3. Internal Standard Stock Solutions and Working Stock Solutions

Store internal standard stock solutions and working internal standard stock solutions at freezer temperature conditions (nominally -20 °C). Unless longer stabilities have been determined, expiration dates of internal standard stock solutions will be assigned an expiration date of (TBD) days from date of preparation.

Solvents are to be dispensed using volumetric or calibrated pipettes. Dilutions will be generally made as described below. However, the weights, volumes, and stock solution concentrations may vary. Use of volumetric flasks may be substituted for dispensing solvents from volumetric glassware or calibrated pipettes. Any such changes will be documented.

In addition, 100% purity will be assumed when weighing the neat material for preparation of the internal standard stock solutions and subsequent spiking solutions.

a) Hydroquinone D6 Primary Stock Solution (1,000 µg/mL) [I01]

Accurately weigh approximately 2.0 mg of Hyrdroquinone D6 internal standard into an amber glass vial and record the actual weight (assume 100% purity for IS). Dissolve the vial contents with 2.0 mL of DMSO. Mix well. Calculate the exact concentration of the primary internal stock solution.

b) Working Internal Standard Solution (50 µg /mL) [WIS]

Transfer 1.0 mL of the above 1,000 µg/mL Hydroquinone D6 primary stock solution [I01] to a glass bottle and add 19.0 mL of 0.1% FA in ACN. Mix well. If the above primary solution is not exactly 1,000 µg/mL, the volume of the stock solution transferred should be adjusted to produce the desired concentration for the secondary solution.

Note: The preparations of the stock solutions may be scaled up or down by adjusting the constituents proportionately.

G. PREPARATION OF SYSTEM SUITABILITY TEST (SST)

The system suitability test (SST) will be a sample extracted using the extraction procedure at the LLOQ concentration level for 1,2,4-Trihydrozybenzene. The SST must be a different sample from the calibration standards, tested or bracketing QCs.

Preparation of a Mouse K_2EDTA plasma calibration standard is considered satisfactory if either its calculated concentration matches the nominal concentration when both values are rounded to three significant figures or is within ±0.5% of the nominal value. Use amber glass or plastic containers.

H. PREPARATION OF SPIKING SOLUTIONS

Spiking solutions are prepared according to the table below for 1,2,4-Trihydroxybenzene. If the stock solutions (SA01 and CA01) is not exactly 5,000 µg/mL, the volume of the stock solution or methanol solvent should be adjusted to produce the desired concentration for the spiking solution. Spiking solutions will be stored at freezer temperature conditions (nominally -20 °C) up to (TBD) days from date of preparation. Warm the solutions to room temperature, mix well before use (e.g. use vortex or sonication to mix).

Spiking Solutions

SS-Std ID	Source Solution ID	Source Solution (ng/mL)	MeOH Volume (mL)	Source Solution Aliquot Vol (mL)	Final Solution Volume (mL)	Nominal Conc. (ng/mL)
SS-8	SA01	5,000,000	0.525	0.175	0.700	1,250,000
SS-7	SS-8	1,250,000	0.100	0.400	0.500	1,000,000
SS-6	SS-7	1,000,000	0.125	0.375	0.500	750,000
SS-5	SS-6	750,000	0.150	0.300	0.450	500,000
SS-4	SS-5	500,000	0.375	0.125	0.500	125,000
SS-3	SS-4	125,000	0.400	0.100	0.500	25,000
SS-2	SS-3	25,000	0.400	0.100	0.500	5,000
SS-1/SS-SST	SS-2	5,000	0.200	0.200	0.400	2,500

Spiking solutions for QC's are listed below:

SS-QC ID	Source Solution ID	Source Solution (ng/mL)	1:1 DMSO:ACN (v/v), Vol (mL)	Source Solution Aliquot Vol (mL)	Final Solution Volume (mL)	Nominal Conc. (ng/mL)
SS-QC-AQL	CA01	5,000,000	0.375	0.225	0.600	1,875,000
SS-QC-High	CA01	5,000,000	4.875	1.125	6.000	937,500
SS-QC-Mid	SS-QC-High	937,500	0.360	0.240	0.600	375,000
SS-QC-Int	SS-QC-Mid	375,000	0.450	0.050	0.500	37,500
SS-QC-Low	SS-QC-Int	37,500	0.400	0.100	0.500	7,500
SS-QC-LLOQ	SS-QC-Low	7,500	0.200	0.100	0.300	2,500

Note: The preparation of the spiking solution may be scaled up or down by adjusting the constituents proportionately. Alternate source solutions may be used to generate the target concentrations.

I. PREPARATION OF PLASMA CALIBRATION STANDARDS

Calibration standards for 1,2,4-Trihydroxybenzene will be prepared in treated Mouse K_2EDTA plasma according to the table below. Calibration standards will be freshly prepared or stored up to (TBD) days (long-term stability for 1,2,4-Trihydroxybenzene) at ultralow freezer temperature conditions (nominally -70 °C). A control plasma sample (double blank) and a control plasma sample with fortification of the internal standard (blank) will also be prepared with each standard curve. Preparation of a treated Mouse K_2EDTA plasma calibration standard is considered satisfactory if either its calculated concentration matches the nominal concentration when both values are rounded to three significant figures or is within ±0.5% of the nominal value. The exact prepared concentrations each time may not be the same as the nominal values listed here. Use amber glass or plastic containers.

Plasma Standard Sample ID	Source Solution ID	Source Solution (ng/mL)	Mouse K_2EDTA Plasma Vol (mL)	Source Solution Aliquot Vol (mL)	Final Plasma Volume (mL)	Nominal Conc. in treated Plasma (ng/mL)	Nominal Conc. in untreated Plasma (ng/mL)
Std-8	SS-8	1,250,000	0.982	0.018	1.000	22,500	25,000
Std-7	SS-7	1,000,000	0.982	0.018	1.000	18,000	20,000
Std-6	SS-6	750,000	0.982	0.018	1.000	13,500	15,000
Std-5	SS-5	500,000	0.982	0.018	1.000	9,000	10,000
Std-4	SS-4	125,000	0.982	0.018	1.000	2,250	2,500
Std-3	SS-3	25,000	0.982	0.018	1.000	450	500
Std-2	SS-2	5,000	0.982	0.018	1.000	90.0	100
Std-1	SS-1	2,500	0.982	0.018	1.000	45.0	50.0
SST	SS-1	2,500	0.982	0.018	1.000	45.0	50.0

Note: The preparation of the calibration standards may be scaled up or down by adjusting the constituents proportionately. Alternate source solutions may be used to generate the target concentrations. During sample preparation, the nominal concentration values in treated plasma column are allowed to contain extra significant figure(s) and considered intermediate numbers. The nominal concentration values in treated plasma column in the test method and Watson are rounded up to 3 significant numbers.

J. PREPARATION OF QC and DILUTION QC SAMPLES

Quality Control samples (QC-LLOQ, QC-Low, QC-Mid, and QC-High) for 1,2,4-Trihydroxybenzene will be prepared in treated Mouse K_2EDTA plasma according to the table below. Quality Control samples will be freshly prepared or stored up to (TBD) days (long-term stability for 1,2,4-Trihydroxybenzene) at ultra low freezer temperature conditions (nominally -70 °C). QC samples will be prepared in at least two replicates. Preparation of a QC sample in treated plasma is considered satisfactory if either its calculated concentration matches the nominal concentration when both values are rounded to three significant figures or is within ±0.5% of the nominal value. The exact prepared concentrations each time may not be the same as the nominal values listed here. Use amber glass or plastic containers.

Quality control samples

Plasma QC Sample ID	Source Solution ID	Source Solution (ng/mL)	Mouse K_2 EDTA Plasma Vol (mL)	Source Solution Aliquot Vol (mL)	Final Plasma Volume (mL)	Nominal Conc. in treated Plasma (ng/mL)	Nominal Conc. in untreated Plasma (ng/mL)
QC-AQL (20X)	SS-QC-AQL	1,875,000	0.982	0.018	1.000	33,750	37,500
QC-High	SS-QC-High	937,500	0.982	0.018	1.000	16,875	18,750
QC-Mid	SS-QC-Mid	375,000	0.982	0.018	1.000	6,750	7,500
QC-Low	SS-QC-Low	7,500	0.982	0.018	1.000	135	150
QC-LLOQ	SS-QC-LLOQ	2,500	0.982	0.018	1.000	45.0	50.0

Note: The preparation of the QC samples may be scaled up or down by adjusting the constituents proportionately. Alternate source solutions may be used to generate the target concentrations. The QC samples may be freshly prepared for sample analysis or stored under various conditions for stability evaluation. Individual aliquoted QC samples will be stored and used for stability evaluations. The preparation of QC LLOQ is only for method qualification or as needed for sample analysis. During sample preparation, the nominal concentration values in treated plasma column are allowed to contain extra significant figure(s) and considered intermediate numbers. The nominal concentration values in treated plasma column in the test method and Watson are rounded up to 3 significant numbers.

Each QC-AQL dilution sample (QC-Dil) is prepared by addition of 20 µL of the QC-AQL sample to 380 µL of treated control plasma to make the 20-fold dilution. QC-AQL dilution samples will be prepared in at least two replicates. Extract each diluted sample according to the Extraction Procedure. Preparation of a plasma dilution QC sample is considered satisfactory if either its calculated concentration matches the nominal concentration when both values are rounded to three significant figures or is within ±0.5% of the nominal value.

20-fold Dilution: A minimum of two replicates will be processed according to the extraction procedure.

Note: The preparation of the dilution QC samples may be scaled up or down by adjusting the constituents proportionately.

K. EXTRACTION PROCEDURE

Include a control plasma sample (double blank) and a control plasma sample with internal standard (blank) sample with each run that includes a calibration curve.

1. Thaw treated control Mouse plasma, standards and QC samples, and treated incurred samples on benchtop. Vortex plasma/sample before taking aliquots.

2. For the double blank and blank, place 25 µL of treated control Mouse plasma into a 2-mL plastic tube. For SST, standards and QC samples, place 25 µL of the pre-spiked treated plasma into a 2-mL plastic tube. For incurred samples: place 25 µL of treated incurred sample plasma to 2 mL tubes.
3. Add 0.150 mL of 0.1% FA in ACN to the double blank.
4. Add 0.150 mL of WIS to all other samples.
5. Cap and vortex mix for approximately 2 minutes. Centrifuge the tubes at a setting of 14,000 rpm for 10 minutes at ~4 °C.
6. Transfer approximately 0.140 mL of the supernatant to a clean 96-well plate. Seal the plate.

The procedures will be documented on a sample preparation form. Any modification to the method will be documented.

L. PREPARATION FOR ANALYSIS (WATSON LIMS)

Perform the steps required in Watson LIMS to transfer sample information to the LC-MS/MS data system for sample analysis. It is not necessary to document completion of these steps.

Arrange the injection batch in a logical order for sample analysis. For example: system suitability test, blanks, Std-1, Std-3, Std-6, and Std-8, QC samples, incurred samples, QC samples followed by Std-2, Std-4, Std-5, and Std-7. If carryover needs to be addressed, position at least two double blank washes prior to samples predicted to be of low concentration as needed to reduce carryover from samples predicted to be of high concentration.

The maximum number of samples that may be analyzed in any one batch is (TBD for 1,2,4-Trihydroxybenzene. This includes double blanks, calibration standards, QC samples, and incurred samples.

M. LC-MS/MS OPERATIONS

The following are initial LC-MS/MS conditions that may be modified, if necessary, to optimize the results. However, all modifications will be documented in the raw data.

HPLC Conditions:

Guard Column:	KrudKatcher ULTRA HPLC 2.0 µm In-Line Filter or equivalent
Column:	Phenomenex Luna NH_2 3 µm , 100 Å, 150 x 4.6 mm
Column Temperature:	40 °C
Mobile Phase A:	0.1% FA in Water
Mobile Phase B:	0.1% FA in Acetonitrile
Needle Wash:	2-Propanol/Acetone/Acetonitrile 33/33/33 + 1% Formic Acid
Injection Volume:	10 µL

Flow Rate: 0.800 mL/min

Autosampler Temperature: 5 °C

Time (Minute)	0.25	3.00	4.49	4.50	5.00
%B	80	20	20	80	Stop

It is recommended to use a switch valve to divert the column flow to waste before and after peak elution.

MS/MS Conditions:

Instrument: AB Sciex 6500 or equivalent

Ionization Mode: Turbo IonSpray, Negative Ion Detection

Scan Mode: Multiple Reaction Monitoring (MRM)

Compound	Precursor → Product Ion Pair (*m/z*)
1,2,4-Trihydroxybenzene	125.03 → 107.20
Hydroquinone D6	113.15 → 112.00

MS parameters	1,2,4-TRIHYDROXY BENZENE	HYDROQUINONE D6
Curtain Gas (CUR)	30 psi	30 psi
Ion Source Gas 1 (GS1)	45 psi	45 psi
Ion Source Gas 2 (GS2)	40 psi	40 psi
Temperature (TEM)	500 °C	500 °C
IonSpray Voltage (IS)	-4200 V	-4200 V
Collision Gas (CAD)	12 V	12 V
Declustering Potential (DP)	-70 V	-50 V
Collision Energy (CE)	-20 V	-25 V
Entrance Potential (EP)	-10 V	-10 V
Collision Gas Exit Potential (CXP)	-5 V	-6 V

Mass settings for ions monitored may vary slightly from instrument to instrument as quadrupole mass spectrometers operate at unit mass resolution rendering minor differences in the tenths place of mass settings of no significance. Any modification will be documented in the raw data.

Reconstituted extracts may be diluted at the discretion of the LC-MS/MS analyst if it is suspected that LC-MS/MS sensitivity may be too high and will result in a standard

curve that exhibits MS detector saturation. Such dilution must be performed for all samples in a run and be documented.

N. CONDITIONING INJECTIONS

Before testing system suitability, inject the system suitability test (SST) sample a sufficient number of times to condition the system.

O. SYSTEM SUITABILITY

Before each analytical run, system suitability will be evaluated by at least three injections made prior to or at the start of the analytical batch. The system suitability test (SST) sample will be injected 3 times to evaluate the sensitivity (signal-to-noise ratio), chromatographic peak shape, and retention time of the analyte and internal standard.

The targets for system suitability performance are: the analyte signal-to-noise ratio of the LLOQ is at least 5:1.

The system suitability has no acceptance criteria. Therefore, the results of the system suitability analysis will not be used to accept or reject a run in place of criteria based on standard curve and QC samples.

System suitability injections are not necessary if a run is started immediately or soon after another run as the system is already considered as demonstrated suitable.

P. DATA PROCESSING

Conduct data processing and analysis that is generally consistent with the following procedure. It is not necessary to document completion of individual steps required to generate a report of bioanalytical analysis results. Briefly, chromatographic peak integrations are conducted with the LC MS/MS data system software (Analyst). The peak integrations are then transferred to Watson LIMS for regression analysis, calculation of sample concentrations, and acceptance or rejection of the analytical run. A more detailed description of the procedure is described below.

1) Once the run is complete, copy the Analyst data files from the LC-MS/MS data system computer to the corresponding LC/MS instrument folder on the company network. Use the Analyst quantitative analysis tools to integrate chromatographic peaks in compliance with SOP 04-K007. Save the results table file.

2) Import Analyst chromatographic peak integration results into Watson. A quadratic regression with $1/x^2$ weighting factor is used for analyte and r^2 must be ≥ 0.9701.

3) Check the calibration standards for acceptability. For a run to be acceptable, at least 75.0% of the calibration standards must be acceptable with an acceptable standard at both the LLOQ and ULOQ. For a typical 8-point standard curve, a maximum of two calibration standards may be deactivated. Back-calculated concentrations for acceptable calibration standards must be within ±20.0% (±25.0% at the LLOQ) of their respective nominal concentrations.

4) Check QC sample results for acceptability. For a run to be acceptable, at least 66.7% (two-thirds) of all the QC samples must be acceptable, with at least 50.0% of the QC samples acceptable at each concentration level. Calculated

concentrations for acceptable QC samples must be within ±20.0% (±25.0% at the LLOQ) of their respective nominal concentrations.

5) Check samples for AQL/BQL results. AQL and BQL samples will be flagged by Watson accounting for any dilution factors.

6) Accept/reject the run.

7) Report concentration results to three significant figures. Percentages such as accuracy, bias, relative error (RE), and coefficient of variation (CV) are both evaluated for acceptance and reported to one decimal place. Coefficient of determination (r^2) is reported to four decimal places.

Q. CHARACTERISTICS OF THE VALIDATED METHOD

Method Qualification Report:	038110-1 in progress
Matrix:	Treated Mouse K_2EDTA plasma
Compound:	1,2,4-Trihydroxybenzene
Internal Standard:	Hydroquinone D6
Extraction Volume:	0.025 mL
Calibration Range:	50.0 to 25,000 ng/mL in plasma for 1,2,4-Trihydroxybenzene with quadratic regression and weighting of $1/x^2$.
Standard Curve Acceptance Criteria:	Coefficient of determination for a quadratic fit, $r^2 \geq 0.9701$. At least 75.0% of the calibration standards must be acceptable with an acceptable standard at both the LLOQ and ULOQ. Back-calculated concentrations for acceptable calibration standards must be within ±20.0% (±25.0% at the LLOQ) of their respective nominal concentrations.
QC Sample Acceptance Criteria:	At least 66.7% (two-thirds) of all the calculated concentrations for all QC samples are within ±20.0% of the nominal value (±25.0% at the LLOQ) and a minimum of 50% of the calculated concentrations at each QC sample level are within ±20.0% of the theoretical value (±25.0% at the LLOQ).
Maximum Batch Size:	TBD (To be determined)
Dilution Integrity:	TBD
1,2,4-Trihydroxybenzene Stabilities	TBD

R. TM CHANGE HISTORY

BA-TM-0138-01/C: New test method.

Thomas Fellowes and Tivadar Orban

APPENDIX I

Batch and Reagent/Solution Preparation Forms

"Identification Form," "Methodology and Reagent List," "Instrument and Analytical Conditions and Reagents," and "Batch Acceptance" sheets in the test method appendix will be printed each day from the current signed test method, filled out recording raw data, and placed into batch folders to be stored with the study materials. Forms not included in the test method appendix may also be utilized if reviewed and approved by the Principal Investigator/Responsible Scientist.

Approval for batch paperwork not included in the test method should occur the day of and/or prior to the batch if possible, but this may not always be possible, in which case posterior approval will be acceptable.

During sample preparation, the nominal concentration values in treated plasma column are allowed to contain extra significant figure(s) and considered intermediate numbers. The nominal concentration values in treated plasma column in the test method and Watson are rounded up to 3 significant numbers.

FRONTAGE	Category: Concord/Test Methods Title: BA-TM-0138 Method Validation of an LC-MS/MS Assay for the Determination of 1,2,4-Trihydroxybenzene in Treated Mouse K2EDTA Plasma		
Version 01	State Draft	Effective Date	Document ID 441896

Project No.:	Run ID:

IDENTIFICATION FORM

1,2,4-Trihydroxybenzene QC Sample Identification

1,2,4-Trihydroxybenzene QC ID	1,2,4-Trihydroxybenzene Treated Plasma Nominal Concentration (ng/mL)	1,2,4-Trihydroxybenzene Untreated Plasma Nominal Concentration (ng/mL)	NB ID _____-___-see below
QC-High	16,875	18,750	
QC-Mid	6,750	7,500	
QC-Low	135	150	
QC-LLOQ	45.0	50.0	
QC-AQL (20x)	33,750	37,500	

1,2,4-Trihydroxybenzene Calibration Curve Identification

1,2,4-Trihydroxybenzene STD ID	1,2,4-Trihydroxybenzene Treated Plasma Nominal Concentration (ng/mL)	1,2,4-Trihydroxybenzene Untreated Plasma Nominal Concentration (ng/mL)	NB ID _____-___-see below
Std-8	22,500	25,000	
Std-7	18,000	20,000	
Std-6	13,500	15,000	
Std-5	9,000	10,000	
Std-4	2,250	2,500	
Std-3	450	500	
Std-2	90.0	100	
Std-1	45.0	50.0	
SST	45.0	50.0	

Procedure Performed by: _____ Date: _____

Reviewed by: _____ Date: _____

Project No.:	Run ID:

Sample Identification Continued

Sample ID	ID
Double blank	Treated Mouse plasma
Double blank wash	Treated Mouse plasma
Blank	Treated Mouse plasma with IS

Dilution QC or Study Samples Preparation only

Dilution Factor	Aliquot Volume of QC Sample or Study Sample	Aliquot Volume of Control Plasma	Aliquot Volume Used for Extraction	Equip. Used
_____ X	_____ mL	_____ mL	_____ mL	P_____ V_____

Procedure Performed by: _____ Date: _____

Reviewed by: _____ Date: _____

FRONTAGE	Category: Concord/Test Methods Title: BA-TM-0138 Method Validation of an LC-MS/MS Assay for the Determination of 1,2,4-Trihydroxybenzene in Treated Mouse K2EDTA Plasma		
Version 01	State Draft	Effective Date	Document ID 441896

Project No.:	Run ID:

METHODOLOGY AND REAGENT LIST

EXTRACTION PROCEDURE

Step	Description	Equipment or Pipettes Used	Step Completed (Check)
1	Thawtreated control Mouse plasma, standards and QC samples, and treated incurred samples on benchtop. Vortex plasma/sample before taking aliquots.	V_____	
2	For the Double blank and blank, place 25 µL of treated control Mouse plasma into a 2-mL plastic tube. For SST, standards and QC samples, place 25 µL of the pre-spiked treated plasma into a 2-mL plastic tube. For incurred samples: place 25 µL of treated incurred sample plasma to 2 mL tubes	P_____	
3	Add 0.150 mL of 0.1% FA in ACN to the double blank.	P_____	
4	Add 0.150 mL of WIS to all other samples.	P_____	
5	Cap and vortex mix for approximately 2 minutes. Centrifuge the tubes at 14,000 rpm for 10 minutes at ~4 °C.	V_____ C_____	
6	Transfer approximately 0.140 mL of the supernatant to a clean 96-well plate. Seal the plate.	P_____	

Samples ready for analysis:	Time: _____ AM / PM	
Extracted samples stored:	Benchtop location: _____Refrigerator ID: _____ Autosampler ID:_____ Room Temp / Refrigerated at time: _____ AM / PM	
Incurred samples removed from freezer:	Freezer ID:	at: AM / PM
Incurred samples returned to freezer:	Freezer ID:	at: AM / PM

Procedure Performed by: _____ Date: _____

Reviewed by: _____ Date: _____

Project No.:	Run ID:

METHODOLOGY AND REAGENT LIST (cont.)

Comments/Incidents noted during extraction procedure:

REAGENT LIST

Reagent Description	Notebook ID	Supplier	Grade	Lot #	Exp.
Treated Mouse K_2EDTA plasma		NA	NA	NA	
WIS (50,000 ng/mL)		NA	NA	NA	
0.1% FA in ACN		NA	NA	NA	

Procedure Performed by: _____ Date: _____

Reviewed by: _____ Date: _____

FRONTAGE	Category: Concord/Test Methods Title: BA-TM-0138 Method Validation of an LC-MS/MS Assay for the Determination of 1,2,4-Trihydroxybenzene in Treated Mouse K2EDTA Plasma		
Version 01	State Draft	Effective Date	Document ID 441896

Project No.:	Run ID:

INSTRUMENT ANALYTICAL CONDITIONS AND REAGENTS

LC ID: _____		MS ID: _____
Chromatography Column Information		
Manufacturer:	Phenomenex	
Type:	Luna NH_2	
Dimensions:	3 µm, 100 Å, 150 x 4.6 mm	
PN:	00F-4377-E0	
SN:	.0	
Guard column or prefilter:	KrudKatcher In-Line Filter	
Method Information		
Mobile Phase A (0.1% Formic Acid in Water):	ID:	Exp.:
Mobile Phase B (0.1% Formic Acid in Acetonitrile)	ID:	Exp.:
Needle Rinse 1: 2-Propanol/Acetone/ Acetonitrile 33/33/33 + 1% Formic Acid	ID:	Exp.:
Flow Rate:	mL/min	
Pump Pressure:	PSI	
Run Time:	Min	
Injection Volume:	µL	
Acquisition Method Filename:		
Ionization Source:	- ESI	
Column Oven Temp.:	°C	
Autosampler Tray Temp.:	°C	

Initials/Date: _____

Is system suitability acceptable? Yes, No NA
Is the peak shape adequate? Yes, No NA
Is the retention time consistent? Yes, No NA
Comments: _____

Procedure Performed by: _____ Date: _____

Reviewed by: _____ Date: _____

Project No.:	Run ID:

II. Batch Acceptance Form

Project Title:

Purpose:

Initials/Date of sample preparation scientist: _____
PI Approval (initial/date): _____

Batch Results

Integrations performed by: _____ Date: _____

Integrations reviewed by: _____ Date: _____

Please specify for each analyte

Standards met acceptance criteria: ☐ yes ☐ no

QC samples met acceptance criteria: ☐ yes ☐ no

Blanks met acceptance criteria: ☐ yes ☐ no

Batch accepted: ☐ yes ☐ no

Batch will be re-injected: ☐ yes ☐ no

Conclusion:

Performed by (initial/date): _____

Reviewed by (initial/date): _____

REVISION HISTORY

Version 01 is not yet approved or effective
Newtest method

DOCUMENT ELECTRONIC SIGNATURES

No workflows launched yet.

Attachment 2: Certificates of Analysis

Certificate of Analysis for 1,2,4-Trihydroxybenzene

MT 00972 MS 10-9-20

Certificate of Analysis

8.43791.0005 1,2,4-Trihydroxybenzene for synthesis
Batch S7832591

	Batch Values	
Assay (HPLC, area%)	99.0	% (a/a)
Melting range (lower value)	137	°C
Melting range (upper value)	138	°C
Identity (IR)	passes test	

Date of examination (DD.MM.YYYY) 13.08.2019
Minimum shelf life (DD.MM.YYYY) 31.08.2024

Dr. Jörg Bauer
Responsible laboratory manager quality control

This document has been produced electronically and is valid without a signature.

MS 10-9-20

Merck KGaA, Frankfurter Straße 250, 64293 Darmstadt (Germany): +49 6151 72-0
EMD Millipore Corporation - a subsidiary of Merck KGaA, Darmstadt, Germany
400 Summit Drive, Burlington, MA 01803, USA, Phone +1 (781) 533-6000

SALSA Version 869792 /990000675245// Date: 13.08.2019

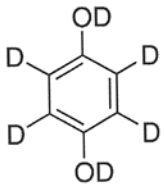
Certificate of Testing for Hydroquinone D6 (IS)

Cambridge Isotope Laboratories, Inc. **Certificate of Analysis**

Product Name: (Isotopic Label & Enrichment Specification)	HYDROQUINONE (D6, 98%)
Lot Number:	PR-26792
Catalog Number:	DLM-2306-0

Product Information

Chemical Purity Specification:	≥98%
MW*: * *For isotopically labeled compounds, MW listed is for the fully enriched product.*	116.15
Labeled CAS Number:	71589-26-9
Unlabeled CAS Number:	123-31-9
Chemical Formula:	DOC6D4OD
Storage:	Store at room temperature away from light and moisture.
Intended Use:	For Research Use Only. Not for use in diagnostic procedures.



Certification

Cambridge Isotope Laboratories, Inc. guarantees that this material meets or exceeds the specifications stated. Absolute identity as well as chemical and isotopic purities are assured by the use of unambiguous synthetic routes and multiple chemical analyses whenever possible. Results are representative of QC testing at time of release from Quality Control unless otherwise stated.

The retest date for this chemical has been designated based on CIL's experience in working with chemical standards for over 30 years, and includes review of actual analytical results and relevant literature references. The retest date is valid only for unopened vials or ampoules that have been stored as recommended.

Approved by: Sashi Sivendran-Basak

Sashi Sivendran-Basak, Ph.D., Quality Review

Quality Control Tests and Results

1H NMR for Isotopic Enrichment	98.6%
GC/MS for Chemical Purity	98.7%
Melting Point Range Determination	171-173°C

Additional Testing Information:
Retest/Review Date: 07/23/21

Hazard labels (DOT)	: 9 - Class 9 (Miscellaneous dangerous materials)

DOT Symbols	: G - Identifies PSN requiring a technical name
Packing group (DOT)	: III - Minor Danger
DOT Special Provisions (49 CFR 172.102)	: 8 - A hazardous substance that is not a hazardous waste may be shipped under the shipping description "Other regulated substances, liquid or solid, n.o.s.", as appropriate. In addition, for solid materials, special provision B54 applies. 146 - This description may be used for a material that poses a hazard to the environment but does not meet the definition for a hazardous waste or a hazardous substance, as defined in 171.8 of this subchapter, or any hazard class as defined in Part 173 of this subchapter, if it is designated as environmentally hazardous by the Competent Authority of the country of origin, transit or destination. 335 - Mixtures of solids that are not subject to this subchapter and environmentally hazardous liquids or solids may be classified as "Environmentally hazardous substances, solid, n.o.s," UN3077 and may be transported under this entry, provided there is no free liquid visible at the time the material is loaded or at the time the packaging or transport unit is closed. Each transport unit must be leak-proof when used as bulk packaging. A112 - Notwithstanding the quantity limits shown in Column (9A) and (9B) for this entry, the following IBCs are authorized for transportation aboard passenger and cargo-only aircraft. Each IBC may not exceed a maximum net quantity of 1,000 kg: a. Metal: 11A, 11B, 11N, 21A, 21B and 21N b. Rigid plastics: 11H1, 11H2, 21H1 and 21H2 c. Composite with plastic inner receptacle: 11HZ1, 11HZ2, 21HZ1 and 21HZ2 d. Fiberboard: 11G e. Wooden: 11C, 11D and 11F (with inner liners) f. Flexible: 13H2, 13H3, 13H4, 13H5, 13L2, 13L3, 13L4, 13M1 and 13M2 (flexible IBCs must be sift-proof and water resistant or must be fitted with a sift-proof and water resistant liner). B54 - Open-top, sift-proof rail cars are also authorized. IB8 - Authorized IBCs: Metal (11A, 11B, 11N, 21A, 21B, 21N, 31A, 31B and 31N); Rigid plastics (11H1, 11H2, 21H1, 21H2, 31H1 and 31H2); Composite (11HZ1, 11HZ2, 21HZ1, 21HZ2, 31HZ1 and 31HZ2); Fiberboard (11G); Wooden (11C, 11D and 11F); Flexible (13H1, 13H2, 13H3, 13H4, 13H5, 13L1, 13L2, 13L3, 13L4, 13M1 or 13M2). IP3 - Flexible IBCs must be sift-proof and water-resistant or must be fitted with a sift-proof and water-resistant liner. N20 - A 5M1 multi-wall paper bag is authorized if transported in a closed transport vehicle. T1 - 1.5 178.274(d)(2) Normal..... 178.275(d)(2) TP33 - The portable tank instruction assigned for this substance applies for granular and powdered solids and for solids which are filled and discharged at temperatures above their melting point which are cooled and transported as a solid mass. Solid substances transported or offered for transport above their melting point are authorized for transportation in portable tanks conforming to the provisions of portable tank instruction T4 for solid substances of packing group III or T7 for solid substances of packing group II, unless a tank with more stringent requirements for minimum shell thickness, maximum allowable working pressure, pressure-relief devices or bottom outlets are assigned in which case the more stringent tank instruction and special provisions shall apply. Filling limits must be in accordance with portable tank special provision TP3. Solids meeting the definition of an elevated temperature material must be transported in accordance with the applicable requirements of this subchapter.
DOT Packaging Exceptions (49 CFR 173.xxx)	: 155
DOT Packaging Non Bulk (49 CFR 173.xxx)	: 213
DOT Packaging Bulk (49 CFR 173.xxx)	: 240
DOT RQ	: 100 lbs.
Marine pollutant	: No.

14.3. Additional information

Other information	: No supplementary information available.

Overland transport

Packing group (ADR)	: III
Class (ADR)	: 9 - Miscellaneous dangerous substances and articles

HYDROQUINONE (D6, 98%) DLM-2306
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Hazard identification number (Kemler No.)	: 90
Classification code (ADR)	: M7
Hazard labels (ADR)	: 9 - Miscellaneous dangerous substances and articles

Orange plates	: 90 3077
Tunnel restriction code (ADR)	: E
Limited quantities (ADR)	: 5kg
EAC	: 2Z
Excepted quantities (ADR)	: E1

Transport by sea

DOT Vessel Stowage Location	: A - The material may be stowed "on deck" or "under deck" on a cargo vessel and on a passenger vessel.
MFAG-No	: 171

Air transport

DOT Quantity Limitations Passenger aircraft/rail (49 CFR 173.27)	: No limit
DOT Quantity Limitations Cargo aircraft only (49 CFR 175.75)	: No limit
Civil Aeronautics Law	: Miscellaneous dangerous substances & articles

14.4. Environmental hazards

Dangerous for the environment :

Other information : No supplementary information available.

14.5. Special precautions for user

14.6. Transport in bulk according to Annex II of MARPOL 73/78 and the IBC Code

Not applicable

SECTION 15: Regulatory information

15.1. US Federal regulations

HYDROQUINONE (D6, 98%) (71589-26-9)	
Listed on the United States SARA Section 302 Subject to reporting requirements of United States SARA Section 313	
SARA Section 311/312 Hazard Classes	Immediate (acute) health hazard Delayed (chronic) health hazard

15.2. International regulations

CANADA

HYDROQUINONE (D6, 98%) (71589-26-9)
Listed on the Canadian DSL (Domestic Substances List)

15.2.1. National regulations

No additional information available

Boiling point	: 285 °C (545 °F) at 1,013 hPa (760 mmHg)
Flash point	: 165 °C (329 °F) - closed cup
Auto-ignition temperature	: 515.56 °C (960.01 °F)
Decomposition temperature	: No data available
Flammability (solid, gas)	: No data available
Vapor pressure	: 1 hPa (1mmHg) at 132 °C (270 °F)
Relative vapor density at 20 °C	: 3.8 - (Air = 1.0)
Relative density	: No data available
Solubility	: Water: 50 g/l
Log Pow	: 0.59
Log Kow	: No data available
Viscosity, kinematic	: No data available
Viscosity, dynamic	: No data available
Explosive properties	: No data available
Oxidizing properties	: No data available
Explosion limits	: No data available

9.2. Other information

No additional information available

SECTION 10: Stability and reactivity

10.1. Reactivity

No additional information available

10.2. Chemical stability

One year after receipt of order if stored as stated in "Storage" section. Re-QC after one year.

10.3. Possibility of hazardous reactions

No additional information available

10.4. Conditions to avoid

Air, light.

10.5. Incompatible materials

Strong bases, Strong oxidizing agents.

10.6. Hazardous decomposition products

formed under fire conditions: Carbon oxides.

SECTION 11: Toxicological information

11.1. Information on toxicological effects

Acute toxicity : Oral: Harmful if swallowed.

HYDROQUINONE (D6, 98%) (71589-26-9)	
LD50 oral rat	367.3 mg/kg
LD50 dermal rabbit	> 2000 mg/kg

Skin corrosion/irritation	: Not classified No data available pH: 3.7 at 70 g/l
Serious eye damage/irritation	: Causes serious eye damage. No data available pH: 3.7 at 70 g/l
Respiratory or skin sensitization	: May cause allergic skin reaction. No data available
Germ cell mutagenicity	: Suspected of causing genetic defects (in contact with skin, if inhaled, if swallowed).
Carcinogenicity	: This product is or contains a component that has been reported to be possibly carcinogenic based on its IARC, ACGIH, NTP, or EPA classification. Limited evidence of carcinogenicity in animal studies.
Reproductive toxicity	: Not available
Specific target organ toxicity – single exposure	: Not classified No data available

HYDROQUINONE (D6, 98%) DLM-2306
Safety Data Sheet
according to Regulation (EC) No. 1907/2006 (REACH) with its amendment Regulation (EU) 2015/830 and according to Federal Register / Vol. 77, No. 58 / Monday, March 26, 2012 / Rules and Regulations

Specific target organ toxicity – repeated exposure	: Not classified No data available
Aspiration hazard	: Not classified
Potential Adverse human health effects and symptoms	: Absorption into the body leads to the formation of methemoglobin which in sufficient concentration causes cyanosis. Onset may be delayed 2 to 4 hours or longer. To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated. Liver - Irregularities - Based on Human Evidence.
IARC group	: 3
Symptoms/effects after inhalation	: May be harmful if inhaled. Causes respiratory tract irritation.
Symptoms/effects after skin contact	: May be harmful if absorbed through skin. Causes skin irritation.
Symptoms/effects after eye contact	: Causes eye irritation.
Symptoms/effects after ingestion	: Toxic if swallowed.

SECTION 12: Ecological information

12.1. Toxicity

HYDROQUINONE (D6, 98%) (71589-26-9)	
LC50 fish 1	0.04 - 0.1 mg/l Oncorhynchus mykiss (rainbow trout) - 96 h
EC50 Daphnia 1	0.13 mg/l Daphnia magna (Water flea) - 48 h
ErC50 (algae)	0.335 mg/l Pseudokirchneriella subcapitata (green algae) - 72 h

12.2. Persistence and degradability

HYDROQUINONE (D6, 98%) (71589-26-9)	
Persistence and degradability	Biotic/Aerobic. Biodegradability: Result - Readily biodegradable.

12.3. Bioaccumulative potential

HYDROQUINONE (D6, 98%) (71589-26-9)	
BCF fish 1	0.05 mg/l Leuciscus idus (Golden orfe) - 3d
Bioconcentration factor (BCF REACH)	40
Log Pow	0.59

12.4. Mobility in soil

HYDROQUINONE (D6, 98%) (71589-26-9)	
Ecology - soil	Not available.

12.5. Results of PBT and vPvB assessment

No additional information available

12.6. Other adverse effects

Other adverse effects : An environmental hazard cannot be excluded in the event of unprofessional handling or disposal. Very toxic to aquatic organisms.

SECTION 13: Disposal considerations

13.1. Waste treatment methods

Regional legislation (waste)	: Waste materials should be disposed of under conditions which meet Federal, State, and local environmental control regulations.
Product/Packaging disposal recommendations	: Dispose in a safe manner in accordance with local/national regulations.

SECTION 14: Transport information

In accordance with ADR / RID / IMDG / IATA / ADN

14.1. UN number

UN-No.(DOT)	: 3077
DOT NA no.	UN3077

14.2. UN proper shipping name

Proper Shipping Name (DOT)	: Environmentally hazardous substances, solid, n.o.s.
Class (DOT)	: 9 - Class 9 - Miscellaneous hazardous material 49 CFR 173.140

2.3. Other hazards

No additional information available

SECTION 3: Composition/Information on ingredients

3.1. Substances

Name	Product identifier	%	Classification according to Directive 67/548/EEC
HYDROQUINONE (D6, 98%)	(CAS-No.) 71589-26-9 (EC-No.) 204-617-8 (Unlabeled) (EC Index-No.) 604-005-00-4 (Unlabeled)	100	Xn; N; R22-36

Name	Product identifier	%	Classification according to Regulation (EC) No. 1272/2008 [CLP]
HYDROQUINONE (D6, 98%)	(CAS-No.) 71589-26-9 (EC-No.) 204-617-8 (Unlabeled) (EC Index-No.) 604-005-00-4 (Unlabeled)	100	Acute Tox. 4 (Oral), H302 Eye Dam. 1, H318 Skin Sens. 1, H317 Muta. 2, H341 Carc. 2, H351 Aquatic Acute 1, H400 Aquatic Chronic 1, H410

Full text of R- and H- phrases: see section 16

Name	Product identifier	%	GHS-US classification
HYDROQUINONE (D6, 98%) (Main constituent)	(CAS-No.) 71589-26-9	100	Acute Tox. 4 (Oral), H302 Eye Dam. 1, H318 Skin Sens. 1, H317 Muta. 2, H341 Carc. 2, H351 Aquatic Acute 1, H400 Aquatic Chronic 1, H410

Full text of H-phrases: see section 16

3.2. Mixtures

Not applicable

SECTION 4: First aid measures

4.1. Description of first aid measures

First-aid measures general : Move out of dangerous area. Consult a physician and show this safety data sheet.

First-aid measures after inhalation : If breathed in, move person to fresh air. If not breathing, give artificial respiration. Consult a physician.

First-aid measures after skin contact : Wash with soap and plenty of water. Consult a physician.

First-aid measures after eye contact : Flush eye with water for 15 minutes. Get medical attention.

First-aid measures after ingestion : Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

4.2. Most important symptoms and effects, both acute and delayed

Symptoms/effects after inhalation : May be harmful if inhaled. Causes respiratory tract irritation.

Symptoms/effects after skin contact : May be harmful if absorbed through skin. Causes skin irritation.

Symptoms/effects after eye contact : Causes eye irritation.

Symptoms/effects after ingestion : Toxic if swallowed.

4.3. Indication of any immediate medical attention and special treatment needed

No additional information available

SECTION 5: Firefighting measures

5.1. Extinguishing media

Suitable extinguishing media : Use water spray, alcohol-resistant foam, dry chemical, or carbon dioxide.

5.2. Special hazards arising from the substance or mixture

No additional information available

5.3. Advice for firefighters

Protection during firefighting : Do not enter fire area without proper protective equipment, including respiratory protection.

HYDROQUINONE (D6, 98%) DLM-2306
Safety Data Sheet
according to Regulation (EC) No. 1907/2006 (REACH) with its amendment Regulation (EU) 2015/830 and according to Federal Register / Vol. 77, No. 58 / Monday, March 26, 2012 / Rules and Regulations

SECTION 6: Accidental release measures

6.1. Personal precautions, protective equipment and emergency procedures

6.1.1. For non-emergency personnel

Emergency procedures : Use personal protective equipment. Avoid dust formation. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas. Avoid breathing dust.

6.1.2. For emergency responders

No additional information available

6.2. Environmental precautions

Prevent further leakage or spillage if safe to do so. Do not let product enter drains. Discharge into the environment must be avoided.

6.3. Methods and material for containment and cleaning up

For containment : Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

6.4. Reference to other sections

No additional information available

SECTION 7: Handling and storage

7.1. Precautions for safe handling

Additional hazards when processed : Avoid contact with skin and eyes. Avoid formation of dust and aerosols. Further processing of solid materials may result in the formation of combustible dusts. The potential for combustible dust formation should be taken into consideration before additional processing occurs. Provide appropriate exhaust ventilation at places where dust is formed.

Hygiene measures : Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

7.2. Conditions for safe storage, including any incompatibilities

Storage conditions : Store at room temperature away from light and moisture.

7.3. Specific end use(s)

No additional information available

SECTION 8: Exposure controls/personal protection

8.1. Control parameters

HYDROQUINONE (D6, 98%) (71589-26-9)		
Italy - Portugal - USA ACGIH	ACGIH TWA (mg/m³)	1 mg/m³ Eye irritation. Eye damage 2008 Adoption . Sensitizer.

8.2. Exposure controls

Personal protective equipment : Safety glasses. Gloves. Respiratory protection of the dependent type. Protective clothing.

Hand protection : Wear suitable protective clothing and gloves.

Eye protection : Wear safety glasses with side shields (or goggles) and a face shield.

Skin and body protection : Choose body protection according to the amount and concentration of the dangerous substance at the work place.

Respiratory protection : When appropriate, use NIOSH/CEN approved respirator.

SECTION 9: Physical and chemical properties

9.1. Information on basic physical and chemical properties

Physical state : Solid

Appearance : Crystalline.

Molecular mass : 116.15

Color : White to light brown.

Odor : No data available.

Odor threshold : No data available

pH : 3.7 at 70 g/l

Relative evaporation rate (butyl acetate=1) : No data available

Melting point : 172 - 175 °C (342 - 347 °F) - lit.

Freezing point : No data available

HYDROQUINONE (D6, 98%)

Safety Data Sheet

according to Regulation (EC) No. 1907/2006 (REACH) with its amendment Regulation (EU) 2015/830 and according to Federal Register / Vol. 77, No. 58 / Monday, March 26, 2012 / Rules and Regulations

Date of issue: 11/01/2011 Revision date: 10/08/2018 Supersedes: 30/01/2017 Version: 3.1

DLM-2306

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1. Product identifier

Product form	: Substance
Substance name	: HYDROQUINONE (D6, 98%)
EC Index-No.	: 604-005-00-4 (Unlabeled)
EC-No.	: 204-617-8 (Unlabeled)
CAS-No.	: 71589-26-9
Product code	: DLM-2306
Formula	: DOC6D4OD
Synonyms	: 1,4-Benzenediol ; 1,4-Dihydroxybenzene.

1.2. Relevant identified uses of the substance or mixture and uses advised against

1.2.1. Relevant identified uses

Main use category	: Professional use
Industrial/Professional use spec	: For professional use only

1.2.2. Uses advised against

No additional information available

1.3. Details of the supplier of the safety data sheet

Cambridge Isotope Laboratories, Inc.
50 Frontage Road
Andover, MA 01810
USA

USA: 1-800-322-1174 Int: 1-978-749-8000
cilsales@isotope.com www.isotope.com

Emergency telephone number

Emergency numbers:

Chemtrec: 1-800-424-9300 (24 hours)
International: 1-703-741-5970 (24 hours)

SECTION 2: Hazards identification

2.1. Classification of the substance or mixture

Classification according to Regulation (EC) No. 1272/2008 [CLP]

Acute Tox. 4 (Oral)	H302
Eye Dam. 1	H318
Skin Sens. 1	H317
Muta. 2	H341
Carc. 2	H351
Aquatic Acute 1	H400
Aquatic Chronic 1	H410

Full text of hazard classes and H-statements : see section 16

Classification according to Directive 67/548/EEC [DSD] or 1999/45/EC [DPD]

Xn; N; R22-36

Full text of R-phrases: see section 16

GHS-US classification

Acute Tox. 4 (Oral)	H302
Eye Dam. 1	H318
Skin Sens. 1	H317
Muta. 2	H341
Carc. 2	H351
Aquatic Acute 1	H400
Aquatic Chronic 1	H410

Full text of H statements : see section 16

Adverse physicochemical, human health and environmental effects

Blood, Liver, Kidney. Eyes.

2.2. Label elements

Labeling according to Regulation (EC) No. 1272/2008 [CLP]

Hazard pictograms (CLP) :

Signal word (CLP) : Danger

Hazard statements (CLP) :
H302 - Harmful if swallowed
H317 - May cause an allergic skin reaction
H318 - Causes serious eye damage
H341 - Suspected of causing genetic defects (in contact with skin, if inhaled, if swallowed)
H351 - Suspected of causing cancer (in contact with skin, if inhaled, if swallowed)
H410 - Very toxic to aquatic life with long lasting effects

Precautionary statements (CLP) :
P261 - Avoid breathing dust, mist, spray.
P264 - Wash both hands thoroughly after handling.
P270 - Do not eat, drink or smoke when using this product.
P272 - Contaminated work clothing should not be allowed out of the workplace.
P273 - Avoid release to the environment.
P280 - Wear eye protection, face protection, protective clothing, protective gloves.
P301+P312 - IF SWALLOWED: Call a doctor, a POISON CENTER if you feel unwell.
P302+P352 - IF ON SKIN: Wash with plenty of water.

GHS-US labeling

Hazard pictograms (GHS-US) :

Signal word (GHS-US) : Danger

Hazard statements (GHS-US) :
H302 - Harmful if swallowed
H317 - May cause an allergic skin reaction
H318 - Causes serious eye damage
H341 - Suspected of causing genetic defects (Dermal, Inhalation, oral)
H351 - Suspected of causing cancer (Dermal, Inhalation, oral)
H400 - Very toxic to aquatic life
H410 - Very toxic to aquatic life with long lasting effects

Precautionary statements (GHS-US) :
P201 - Obtain special instructions before use.
P202 - Do not handle until all safety precautions have been read and understood.
P261 - Avoid breathing dust, mist, spray.
P264 - Wash Both hands thoroughly after handling.
P270 - Do not eat, drink or smoke when using this product.
P272 - Contaminated work clothing must not be allowed out of the workplace
P273 - Avoid release to the environment.
P280 - Wear eye protection, face protection, protective clothing, protective gloves.
P301+P312 - If swallowed: Call a poison center or doctor if you feel unwell
P302+P352 - If on skin: Wash with plenty of water
P305+P351+P338 - If in eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
P308+P313 - If exposed or concerned: Get medical advice/attention.
P310 - Immediately call a poison center or doctor
P321 - Specific treatment (see Hazard pictograms (CLP) on this label)
P330 - Rinse mouth.
P333+P313 - If skin irritation or rash occurs: Get medical advice/attention.
P362+P364 - Take off contaminated clothing and wash it before reuse.
P391 - Collect spillage.
P405 - Store locked up.
P501 - Dispose of contents/container to Comply with applicable regulations

15.3. US State regulations

HYDROQUINONE (D6, 98%)(71589-26-9)	
U.S. - California - Proposition 65 - Carcinogens List	No
U.S. - California - Proposition 65 - Developmental Toxicity	No
U.S. - California - Proposition 65 - Reproductive Toxicity - Female	No
U.S. - California - Proposition 65 - Reproductive Toxicity - Male	No
State or local regulations	U.S. - Massachusetts - Right To Know List U.S. - Pennsylvania - RTK (Right to Know) List U.S. - New Jersey - Right to Know Hazardous Substance List This product does not contain any chemicals known to State of California to cause cancer, birth defects, or any other reproductive harm.

SECTION 16: Other information

Other information : This product is not radioactive. The data given for this product are those of the corresponding unlabeled compound, unless specifically indicated otherwise. Health and safety data for labeled compounds are generally not available, but are assumed to be similar or identical to the corresponding unlabeled compound.

Full text of R-, H- and EUH-phrases:

Acute Tox. 4 (Oral)	Acute toxicity (oral) Category 4
Aquatic Acute 1	Hazardous to the aquatic environment - Acute Hazard Category 1
Aquatic Chronic 1	Hazardous to the aquatic environment - Chronic Hazard Category 1
Carc. 2	Carcinogenicity Category 2
Eye Dam. 1	Serious eye damage/eye irritation Category 1
Muta. 2	Germ cell mutagenicity Category 2
Skin Sens. 1	Skin sensitization, Category 1
H302	Harmful if swallowed
H317	May cause an allergic skin reaction
H318	Causes serious eye damage
H341	Suspected of causing genetic defects
H351	Suspected of causing cancer
H400	Very toxic to aquatic life
H410	Very toxic to aquatic life with long lasting effects
Xn	Harmful

NFPA health hazard : 2 - Materials that, under emergency conditions, can cause temporary incapacitation or residual injury.

NFPA fire hazard : 1 - Materials that must be preheated before ignition can occur.

NFPA reactivity : 0 - Material that in themselves are normally stable, even under fire conditions.

Hazard Rating

Health : 2 Moderate Hazard - Temporary or minor injury may occur

Flammability : 1 Slight Hazard

Physical : 0 Minimal Hazard

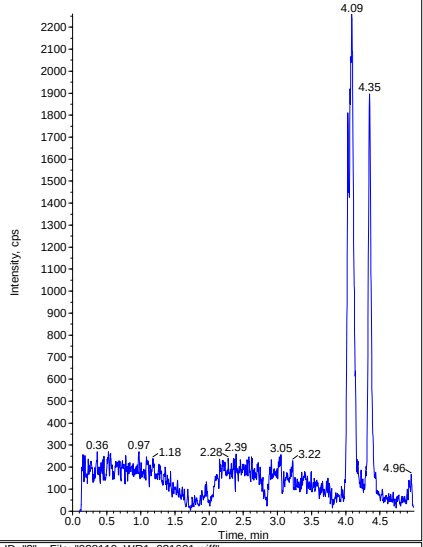
CIL Substance SDS

This information is based on our current knowledge and is intended to describe the product for the purposes of health, safety and environmental requirements only. It should not therefore be construed as guaranteeing any specific property of the product

Attachment 3: Representative Chromatograms (Watson Run 1)

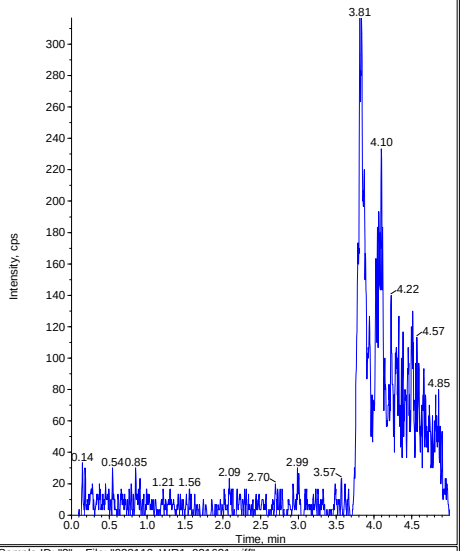
Sample Name: "1 001 038110 DB Wash 1" Sample ID: "1" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:        1
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc: No Intercept
Acq. Date:        2/16/2021
Acq. Time:        3:40:51 PM

Modified:            No
```



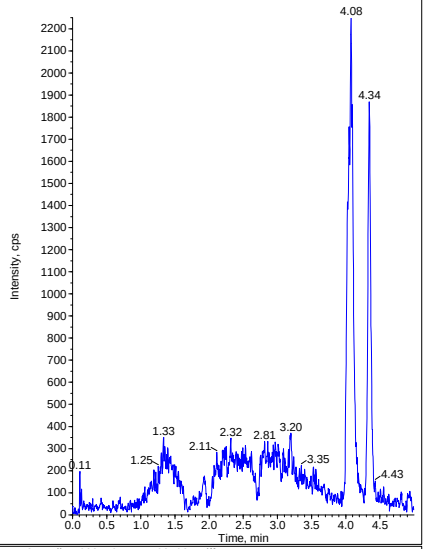
Sample Name: "1 001 038110 DB Wash 1" Sample ID: "1" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:        1
Sample Type:      Unknown
Concentration:      1.00     ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        3:40:51 PM

Modified:            No
```



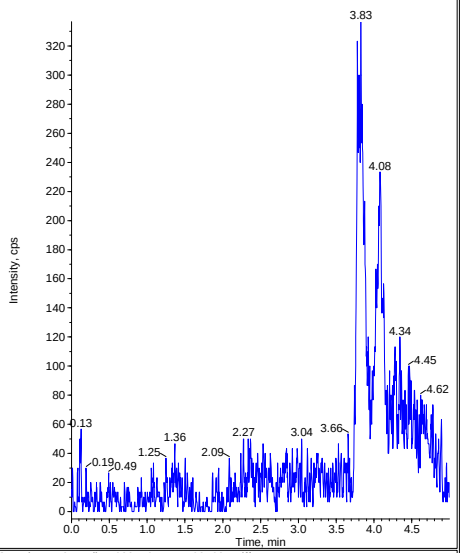
Sample Name: "1 002 038110 DB Wash 1" Sample ID: "2" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:        2
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc: No Intercept
Acq. Date:        2/16/2021
Acq. Time:        3:46:28 PM

Modified:            No
```



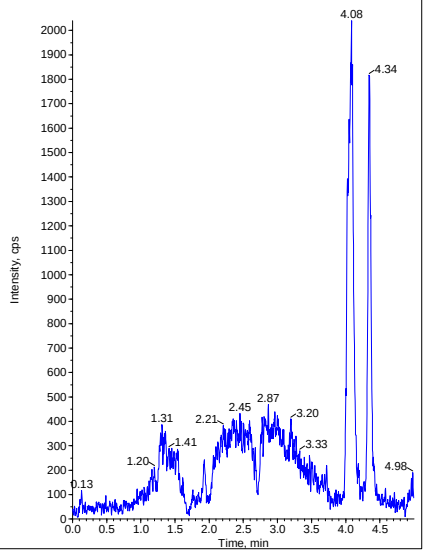
Sample Name: "1 002 038110 DB Wash 1" Sample ID: "2" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:        2
Sample Type:      Unknown
Concentration:      1.00     ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        3:46:28 PM

Modified:            No
```



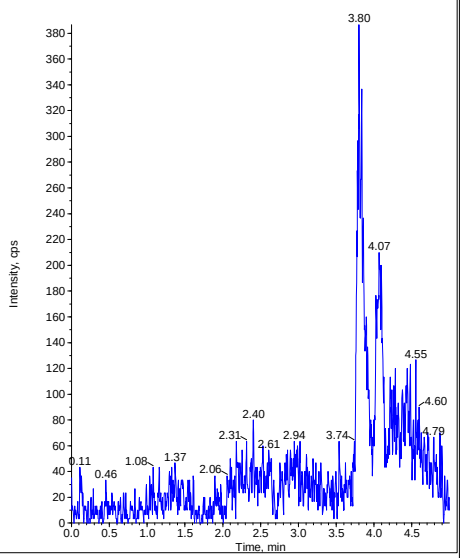
Sample Name: "1 003 038110 DB Wash 1" Sample ID: "3" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:        3
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc: No Intercept
Acq. Date:        2/16/2021
Acq. Time:        3:52:06 PM

Modified:            No
```



Sample Name: "1 003 038110 DB Wash 1" Sample ID: "3" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:        3
Sample Type:      Unknown
Concentration:      1.00     ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        3:52:06 PM

Modified:            No
```

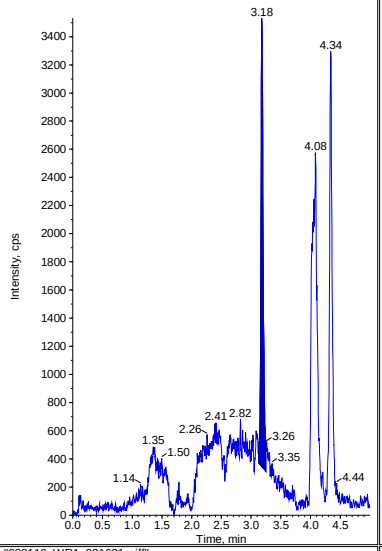


Sample Name: "1 004 038110 SST 1" Sample ID: "4" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

Sample Index: 4
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 48.5 ng/mL
Acq. Date: 2/16/2021
Acq. Time: 3:57:42 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 300.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 5.00 sec
Expected RT: 3.19 min
Use Relative RT: No

Int. Type: Valley
Retention Time: 3.18 min
Area: 8832. counts
Height: 3211.285 cps
Start Time: 3.12 min
End Time: 3.25 min

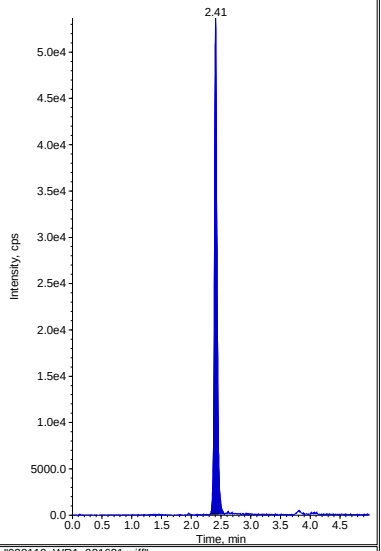


Sample Name: "1 004 038110 SST 1" Sample ID: "4" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

Sample Index: 4
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 3:57:42 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 500.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 10.0 sec
Expected RT: 2.41 min
Use Relative RT: No

Int. Type: Base To Base
Retention Time: 2.41 min
Area: 164900. counts
Height: 53608.388 cps
Start Time: 2.33 min
End Time: 2.56 min

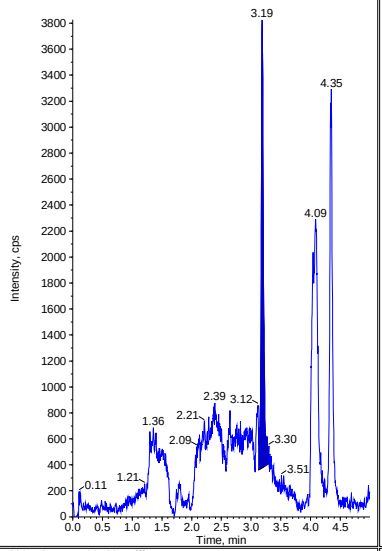


Sample Name: "1 005 038110 SST 1" Sample ID: "5" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

Sample Index: 5
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 53.7 ng/mL
Acq. Date: 2/16/2021
Acq. Time: 4:03:21 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 300.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 5.00 sec
Expected RT: 3.19 min
Use Relative RT: No

Int. Type: Valley
Retention Time: 3.19 min
Area: 9408. counts
Height: 3454.844 cps
Start Time: 3.13 min
End Time: 3.29 min

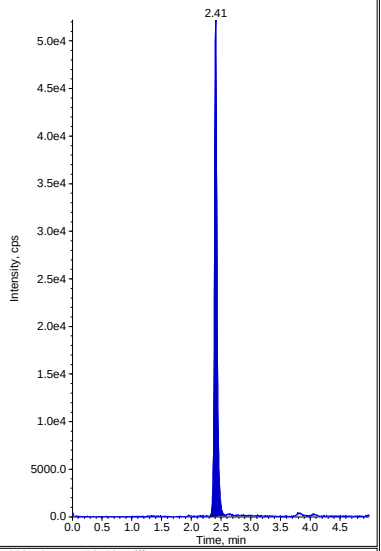


Sample Name: "1 005 038110 SST 1" Sample ID: "5" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

Sample Index: 5
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 4:03:21 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 500.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 10.0 sec
Expected RT: 2.41 min
Use Relative RT: No

Int. Type: Base To Base
Retention Time: 2.41 min
Area: 158900. counts
Height: 52389.660 cps
Start Time: 2.31 min
End Time: 2.57 min

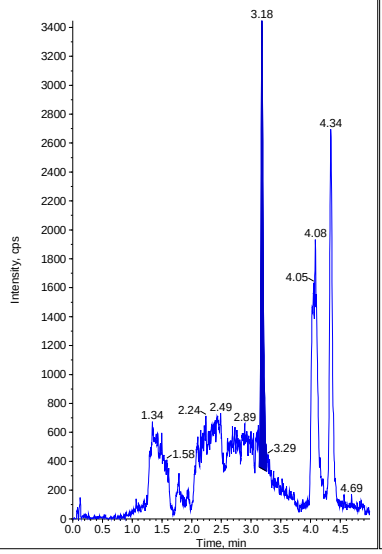


Sample Name: "1 006 038110 SST 1" Sample ID: "6" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

Sample Index: 6
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 47.4 ng/mL
Acq. Date: 2/16/2021
Acq. Time: 4:08:57 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 300.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 5.00 sec
Expected RT: 3.19 min
Use Relative RT: No

Int. Type: Base To Base
Retention Time: 3.18 min
Area: 8332. counts
Height: 3111.970 cps
Start Time: 3.14 min
End Time: 3.26 min

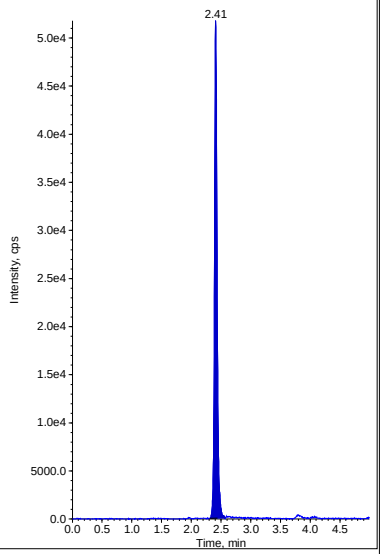


Sample Name: "1 006 038110 SST 1" Sample ID: "6" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

Sample Index: 6
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 4:08:57 PM

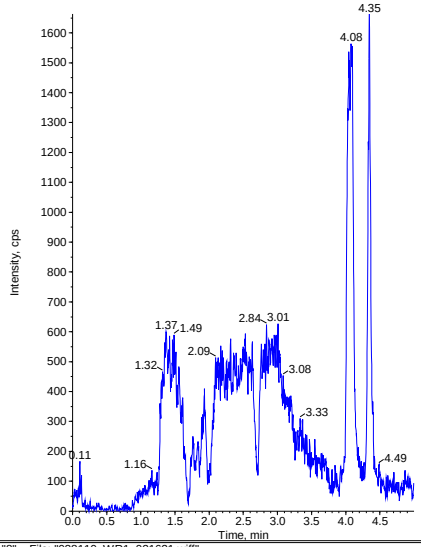
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 500.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 10.0 sec
Expected RT: 2.41 min
Use Relative RT: No

Int. Type: Base To Base
Retention Time: 2.41 min
Area: 159000. counts
Height: 51800.587 cps
Start Time: 2.31 min
End Time: 2.57 min



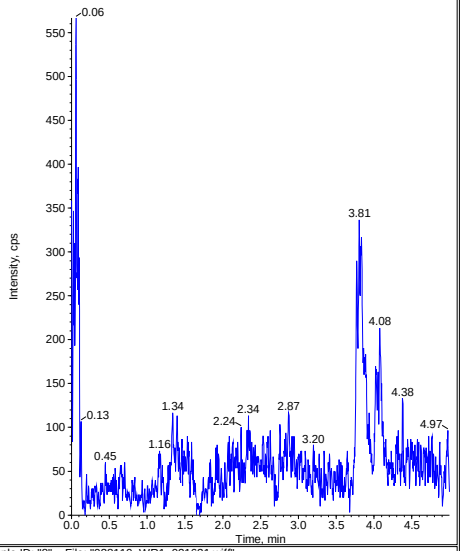
Sample Name: "1 007 038110 Double Blank 1" Sample ID: "7" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:        7
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc: No Intercept
Acq. Date:        2/16/2021
Acq. Time:        4:14:33 PM

Modified:            No
```



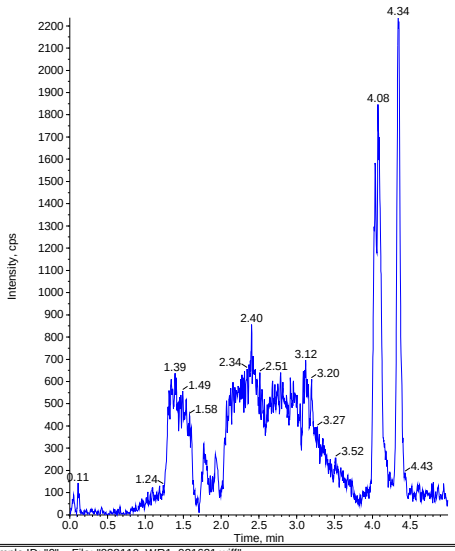
Sample Name: "1 007 038110 Double Blank 1" Sample ID: "7" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:        7
Sample Type:      Unknown
Concentration:      1.00     ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        4:14:33 PM

Modified:            No
```



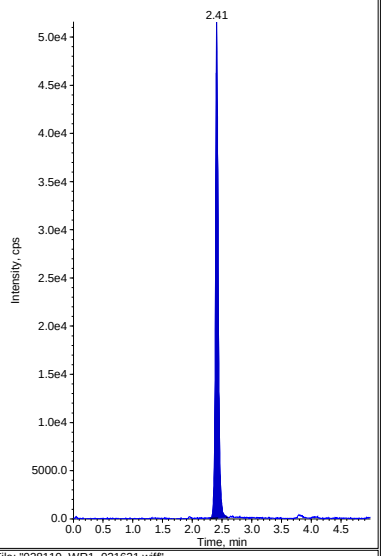
Sample Name: "1 008 038110 Blank 1" Sample ID: "8" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:        8
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc:    0.00     ng/mL
Acq. Date:        2/16/2021
Acq. Time:        4:20:09 PM

Modified:            No
```



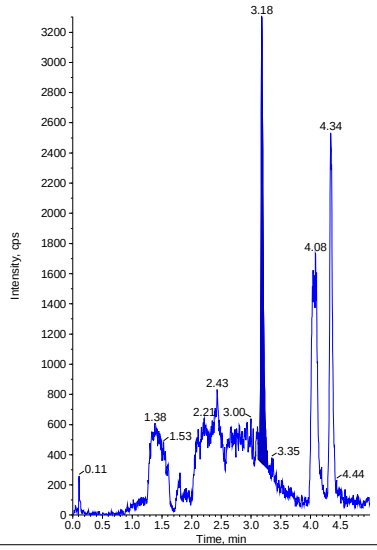
Sample Name: "1 008 038110 Blank 1" Sample ID: "8" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:        8
Sample Type:      Unknown
Concentration:      1.00     ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        4:20:09 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:          3      points
RT Window:          10.0    sec
Expected RT:          2.41   min
Use Relative RT:     No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:          160600.   counts
Height:         51597.109  cps
Start Time:          2.30   min
End Time:            2.60   min
```



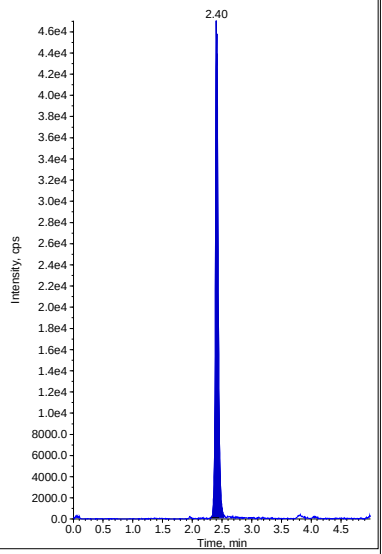
Sample Name: "1 009 038110 Std-1 1 1" Sample ID: "9" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:        9
Sample Type:      Standard
Concentration:      50.0     ng/mL
Calculated Conc:    48.4     ng/mL
Acq. Date:        2/16/2021
Acq. Time:        4:25:48 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    300.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:          3      points
RT Window:          5.00    sec
Expected RT:          3.19   min
Use Relative RT:     No

Int. Type:         Valley
Retention Time:     3.18   min
Area:            8035.   counts
Height:          2978.639  cps
Start Time:          3.12   min
End Time:            3.28   min
```



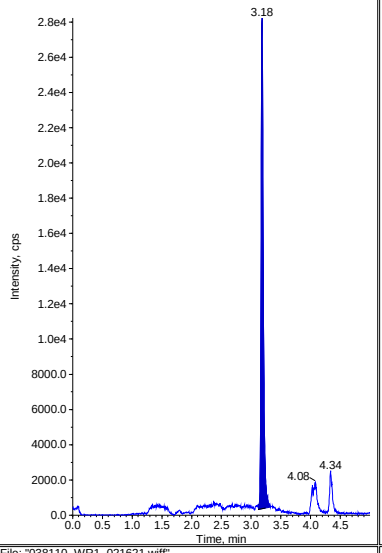
Sample Name: "1 009 038110 Std-1 1 1" Sample ID: "9" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:        9
Sample Type:      Standard
Concentration:      1.00     ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        4:25:48 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:          3      points
RT Window:          10.0    sec
Expected RT:          2.41   min
Use Relative RT:     No

Int. Type:         Valley
Retention Time:     2.40   min
Area:          150500.   counts
Height:         47043.308  cps
Start Time:          2.32   min
End Time:            2.52   min
```



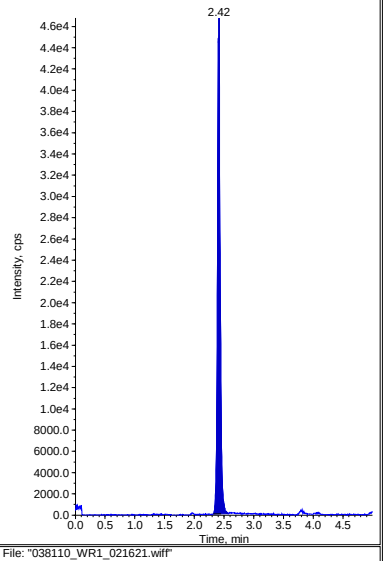
Sample Name: "1 010 038110 Std-3 1 1" Sample ID: "10" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:      10
Sample Type:     Standard
Concentration:      500.    ng/mL
Calculated Conc:    468.    ng/mL
Acq. Date:       2/16/2021
Acq. Time:       4:31:25 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    300.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          5.00    sec
Expected RT:        3.19    min
Use Relative RT:    No

Int. Type:        Valley
Retention Time:     3.18   min
Area:          74480.   counts
Height:         27953.739  cps
Start Time:         3.12   min
End Time:           3.31   min
```



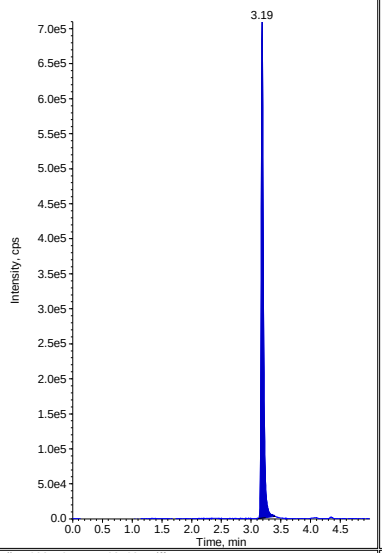
Sample Name: "1 010 038110 Std-3 1 1" Sample ID: "10" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:      10
Sample Type:     Standard
Concentration:      1.00    ng/mL
Calculated Conc:    N/A
Acq. Date:       2/16/2021
Acq. Time:       4:31:25 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          10.0    sec
Expected RT:        2.41    min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.42   min
Area:         147100.   counts
Height:         47463.259  cps
Start Time:         2.32   min
End Time:           2.56   min
```



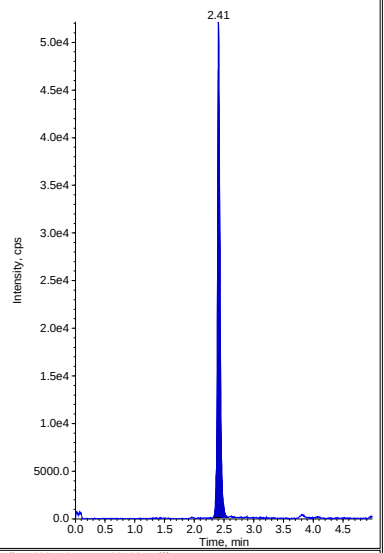
Sample Name: "1 011 038110 Std-6 1 1" Sample ID: "11" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:      11
Sample Type:     Standard
Concentration:    15000.    ng/mL
Calculated Conc:  13400.    ng/mL
Acq. Date:       2/16/2021
Acq. Time:       4:37:03 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    300.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          5.00    sec
Expected RT:        3.19    min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     3.19   min
Area:         1902000.   counts
Height:        708829.274  cps
Start Time:         3.12   min
End Time:           3.40   min
```



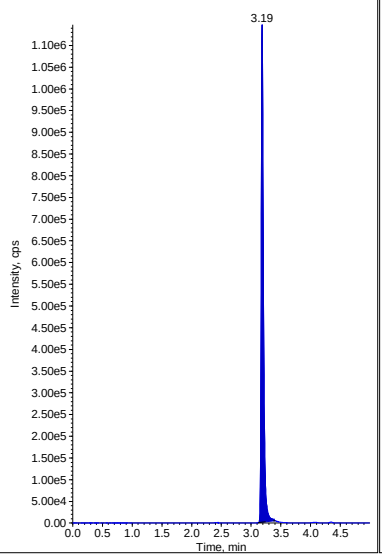
Sample Name: "1 011 038110 Std-6 1 1" Sample ID: "11" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:      11
Sample Type:     Standard
Concentration:      1.00    ng/mL
Calculated Conc:    N/A
Acq. Date:       2/16/2021
Acq. Time:       4:37:03 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          10.0    sec
Expected RT:        2.41    min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:          152000.   counts
Height:         52570.442  cps
Start Time:         2.32   min
End Time:           2.57   min
```



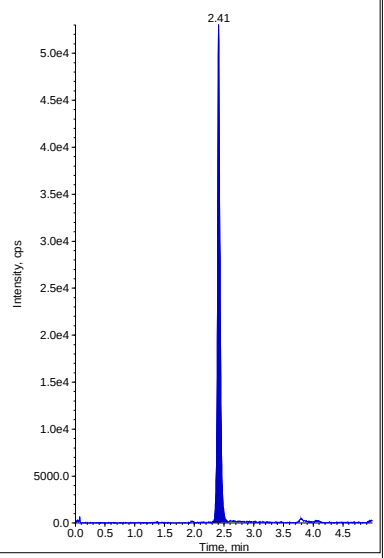
Sample Name: "1 012 038110 Std-8 1 1" Sample ID: "12" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:      12
Sample Type:     Standard
Concentration:    25000.    ng/mL
Calculated Conc:  25000.    ng/mL
Acq. Date:       2/16/2021
Acq. Time:       4:42:41 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    300.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          5.00    sec
Expected RT:        3.19    min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     3.19   min
Area:         3090000.   counts
Height:        1146610.732  cps
Start Time:         3.12   min
End Time:           3.41   min
```



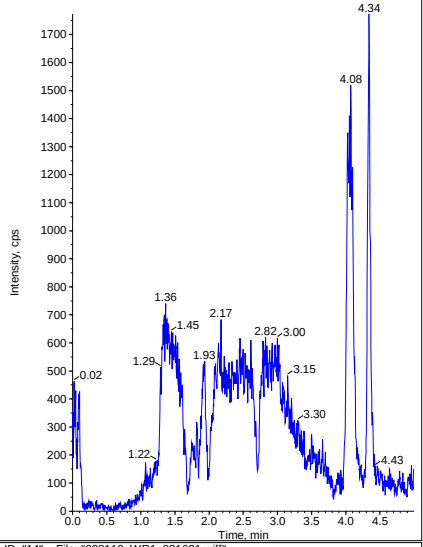
Sample Name: "1 012 038110 Std-8 1 1" Sample ID: "12" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:      12
Sample Type:     Standard
Concentration:      1.00    ng/mL
Calculated Conc:    N/A
Acq. Date:       2/16/2021
Acq. Time:       4:42:41 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          10.0    sec
Expected RT:        2.41    min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:          154200.   counts
Height:         52979.718  cps
Start Time:         2.32   min
End Time:           2.57   min
```



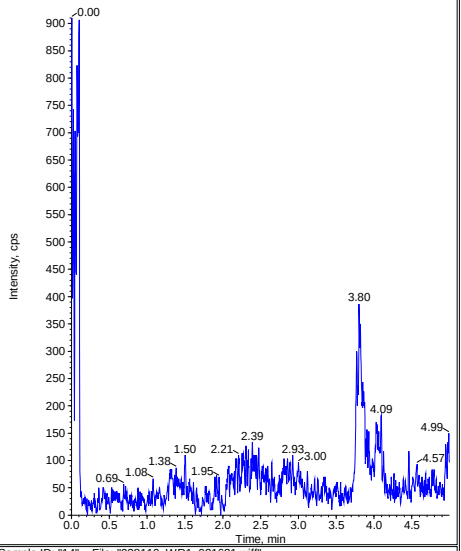
Sample Name: "1 013 038110 DB Wash 1" Sample ID: "13" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""
Sample Index: 13
Sample Type: Unknown
Concentration: N/A
Calculated Conc: No Intercept
Acq. Date: 2/16/2021
Acq. Time: 4:48:16 PM

Modified: No



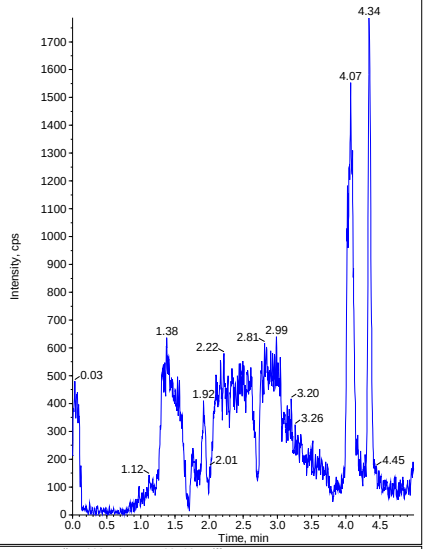
Sample Name: "1 013 038110 DB Wash 1" Sample ID: "13" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""
Sample Index: 13
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 4:48:16 PM

Modified: No



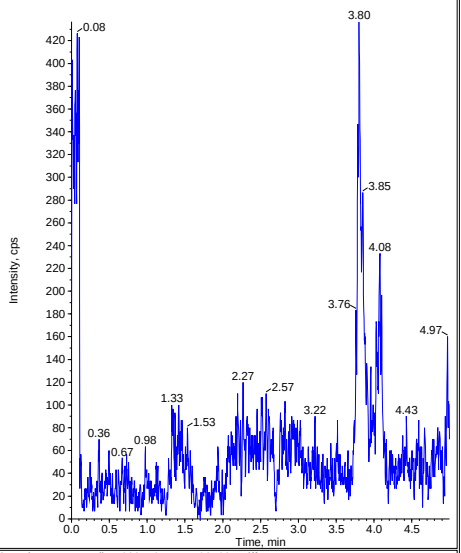
Sample Name: "1 014 038110 DB Wash 1" Sample ID: "14" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""
Sample Index: 14
Sample Type: Unknown
Concentration: N/A
Calculated Conc: No Intercept
Acq. Date: 2/16/2021
Acq. Time: 4:53:52 PM

Modified: No



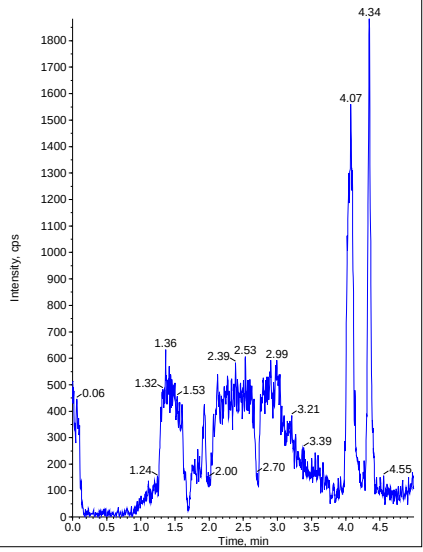
Sample Name: "1 014 038110 DB Wash 1" Sample ID: "14" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""
Sample Index: 14
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 4:53:52 PM

Modified: No



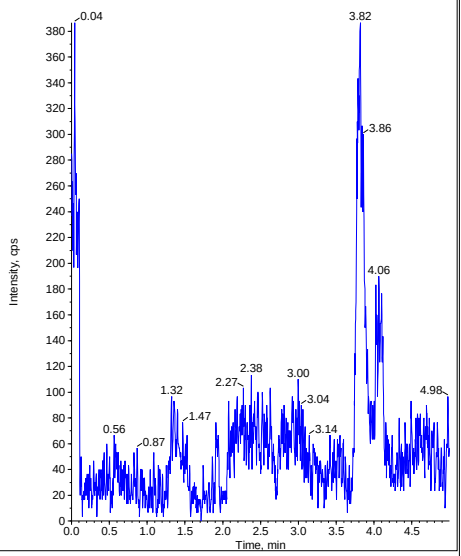
Sample Name: "1 015 038110 DB Wash 1" Sample ID: "15" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""
Sample Index: 15
Sample Type: Unknown
Concentration: N/A
Calculated Conc: No Intercept
Acq. Date: 2/16/2021
Acq. Time: 4:59:28 PM

Modified: No



Sample Name: "1 015 038110 DB Wash 1" Sample ID: "15" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""
Sample Index: 15
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 4:59:28 PM

Modified: No

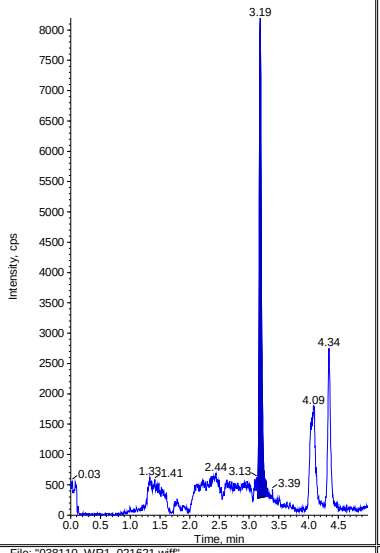


Sample Name: "1 016 038110 QC-Low 1 1" Sample ID: "16" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

Sample Index: 16
Sample Type: QC
Concentration: 150. ng/mL
Calculated Conc: 138. ng/mL
Acq. Date: 2/16/2021
Acq. Time: 5:05:04 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 300.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 5.00 sec
Expected RT: 3.19 min
Use Relative RT: No

Int. Type: Valley
Retention Time: 3.19 min
Area: 21590. counts
Height: 7963.757 cps
Start Time: 3.14 min
End Time: 3.32 min

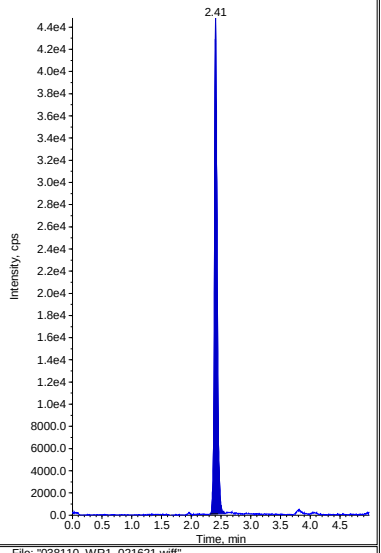


Sample Name: "1 016 038110 QC-Low 1 1" Sample ID: "16" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

Sample Index: 16
Sample Type: QC
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 5:05:04 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 500.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 10.0 sec
Expected RT: 2.41 min
Use Relative RT: No

Int. Type: Base To Base
Retention Time: 2.41 min
Area: 143300. counts
Height: 44709.985 cps
Start Time: 2.32 min
End Time: 2.56 min

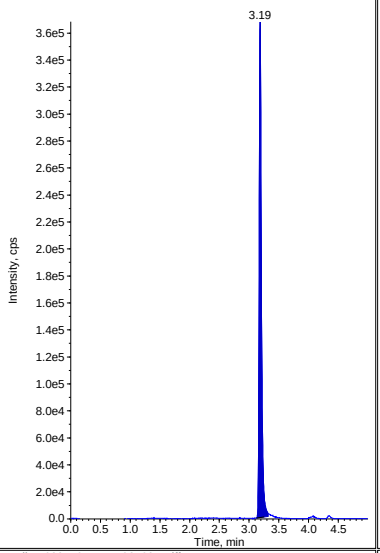


Sample Name: "1 017 038110 QC-Mid 1 1" Sample ID: "17" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

Sample Index: 17
Sample Type: QC
Concentration: 7500. ng/mL
Calculated Conc: 6270. ng/mL
Acq. Date: 2/16/2021
Acq. Time: 5:10:43 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 300.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 5.00 sec
Expected RT: 3.19 min
Use Relative RT: No

Int. Type: Valley
Retention Time: 3.19 min
Area: 970000. counts
Height: 367918.804 cps
Start Time: 3.13 min
End Time: 3.32 min

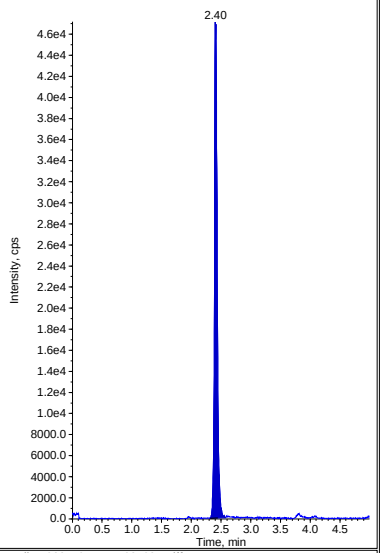


Sample Name: "1 017 038110 QC-Mid 1 1" Sample ID: "17" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

Sample Index: 17
Sample Type: QC
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 5:10:43 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 500.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 10.0 sec
Expected RT: 2.41 min
Use Relative RT: No

Int. Type: Base To Base
Retention Time: 2.40 min
Area: 152500. counts
Height: 47321.389 cps
Start Time: 2.31 min
End Time: 2.57 min

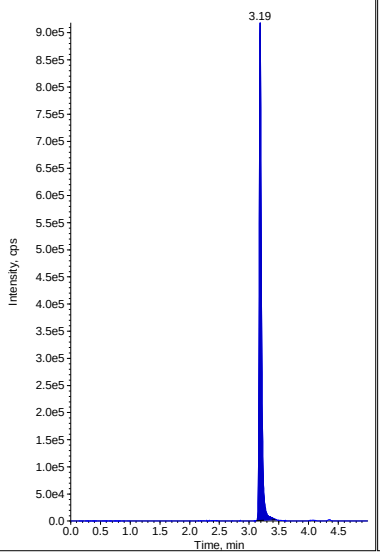


Sample Name: "1 018 038110 QC-High 1 1" Sample ID: "18" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

Sample Index: 18
Sample Type: QC
Concentration: 18800. ng/mL
Calculated Conc: 20800. ng/mL
Acq. Date: 2/16/2021
Acq. Time: 5:16:21 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 300.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 5.00 sec
Expected RT: 3.19 min
Use Relative RT: No

Int. Type: Base To Base
Retention Time: 3.19 min
Area: 2464000. counts
Height: 917776.963 cps
Start Time: 3.12 min
End Time: 3.44 min

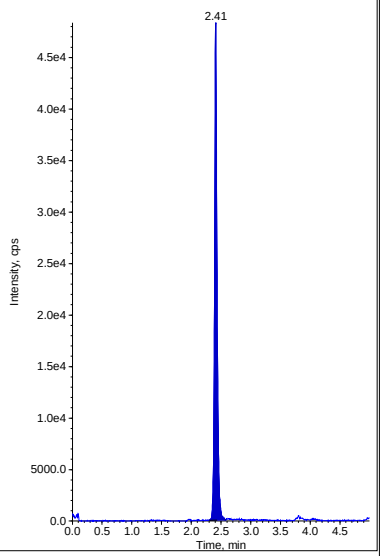


Sample Name: "1 018 038110 QC-High 1 1" Sample ID: "18" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

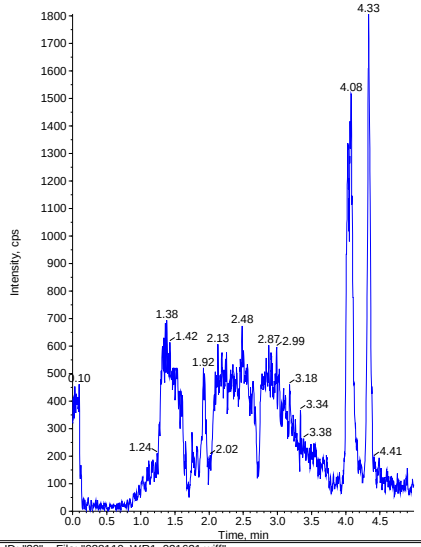
Sample Index: 18
Sample Type: QC
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 5:16:21 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 500.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 10.0 sec
Expected RT: 2.41 min
Use Relative RT: No

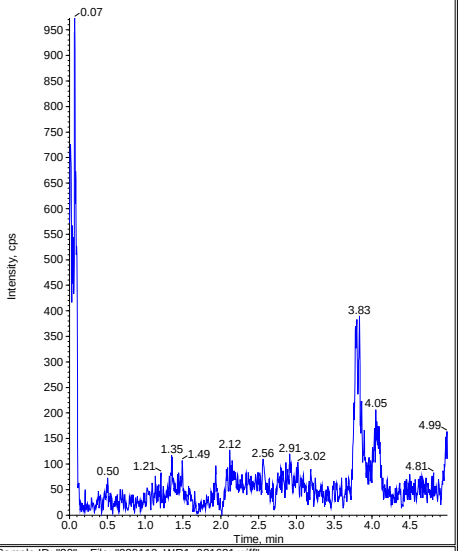
Int. Type: Base To Base
Retention Time: 2.41 min
Area: 139800. counts
Height: 48601.584 cps
Start Time: 2.30 min
End Time: 2.57 min



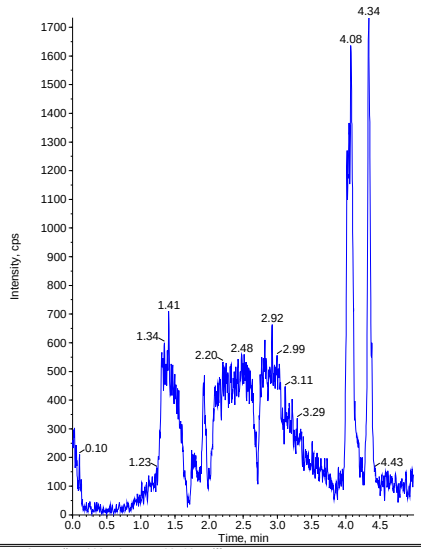
Sample Name: "1 019 038110 DB Wash 1" Sample ID: "19" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""
Sample Index: 19
Sample Type: Unknown
Concentration: N/A
Calculated Conc: No Intercept
Acq. Date: 2/16/2021
Acq. Time: 5:21:59 PM
Modified: No



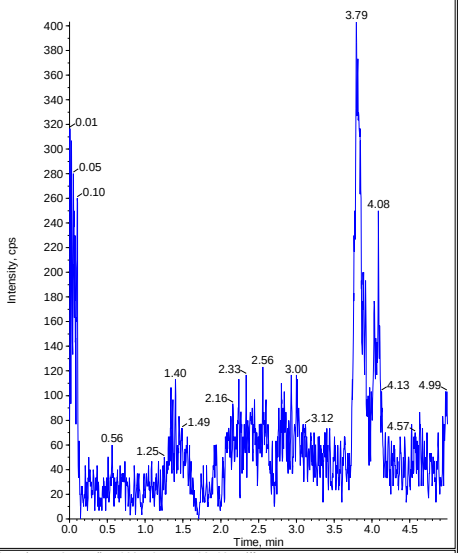
Sample Name: "1 019 038110 DB Wash 1" Sample ID: "19" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""
Sample Index: 19
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 5:21:59 PM
Modified: No



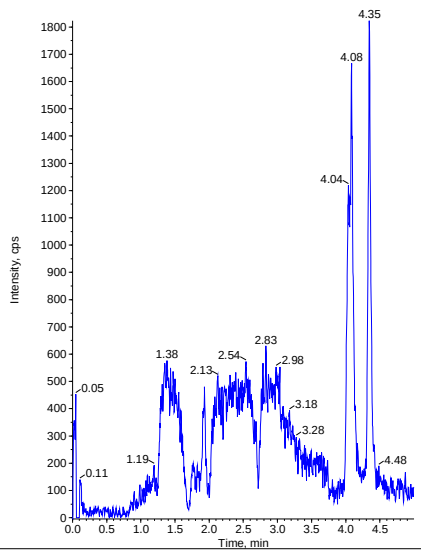
Sample Name: "1 020 038110 DB Wash 1" Sample ID: "20" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""
Sample Index: 20
Sample Type: Unknown
Concentration: N/A
Calculated Conc: No Intercept
Acq. Date: 2/16/2021
Acq. Time: 5:27:34 PM
Modified: No



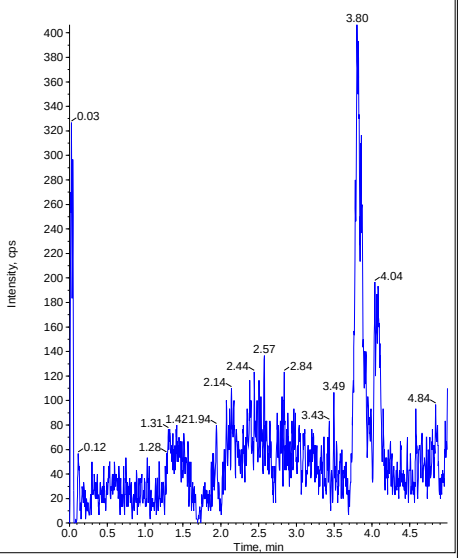
Sample Name: "1 020 038110 DB Wash 1" Sample ID: "20" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""
Sample Index: 20
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 5:27:34 PM
Modified: No



Sample Name: "1 021 038110 DB Wash 1" Sample ID: "21" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""
Sample Index: 21
Sample Type: Unknown
Concentration: N/A
Calculated Conc: No Intercept
Acq. Date: 2/16/2021
Acq. Time: 5:33:12 PM
Modified: No

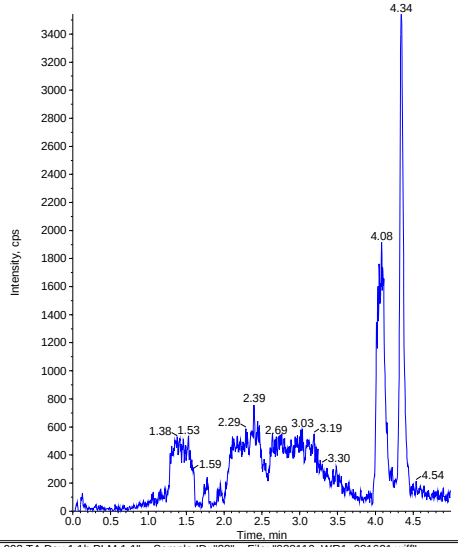


Sample Name: "1 021 038110 DB Wash 1" Sample ID: "21" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""
Sample Index: 21
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 5:33:12 PM
Modified: No



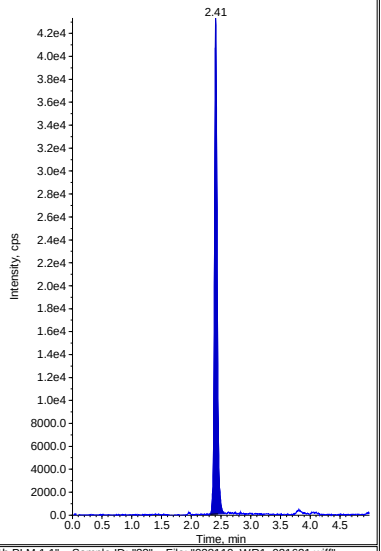
Sample Name: "1 022 038110 039700000005 991 TA Day 1 1h PLM-1 1" Sample ID: "22" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       22
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc:   0.00    ng/mL
Acq. Date:        2/16/2021
Acq. Time:        5:38:49 PM

Modified:           No
```



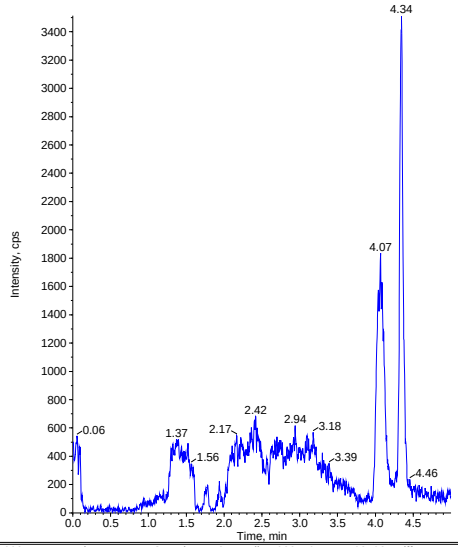
Sample Name: "1 022 038110 039700000005 991 TA Day 1 1h PLM-1 1" Sample ID: "22" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       22
Sample Type:      Unknown
Concentration:     1.00    ng/mL
Calculated Conc:   N/A
Acq. Date:        2/16/2021
Acq. Time:        5:38:49 PM

Modified:           No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          10.0    sec
Expected RT:         2.41   min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:          135900.   counts
Height:         43399.103  cps
Start Time:          2.32   min
End Time:            2.55   min
```



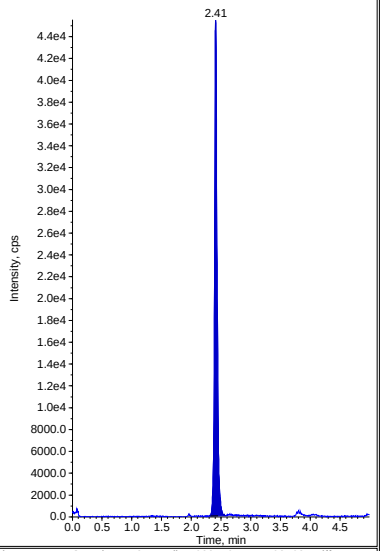
Sample Name: "1 023 038110 039700000006 992 TA Day 1 1h PLM-1 1" Sample ID: "23" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       23
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc:   0.00    ng/mL
Acq. Date:        2/16/2021
Acq. Time:        5:44:24 PM

Modified:           No
```



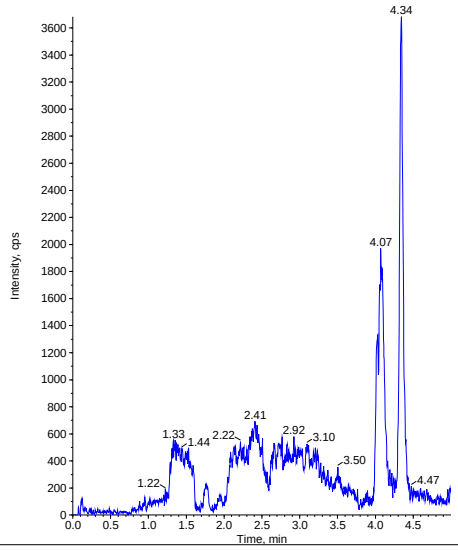
Sample Name: "1 023 038110 039700000006 992 TA Day 1 1h PLM-1 1" Sample ID: "23" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       23
Sample Type:      Unknown
Concentration:     1.00    ng/mL
Calculated Conc:   N/A
Acq. Date:        2/16/2021
Acq. Time:        5:44:24 PM

Modified:           No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          10.0    sec
Expected RT:         2.41   min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:          136300.   counts
Height:         45510.929  cps
Start Time:          2.32   min
End Time:            2.57   min
```



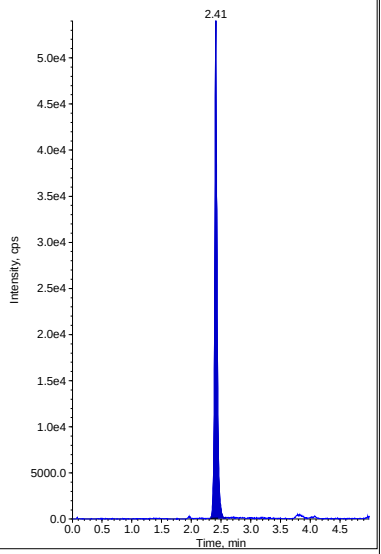
Sample Name: "1 024 038110 039700000007 993 TA Day 1 1h PLM-1 1" Sample ID: "24" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       24
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc:   0.00    ng/mL
Acq. Date:        2/16/2021
Acq. Time:        5:50:04 PM

Modified:           No
```



Sample Name: "1 024 038110 039700000007 993 TA Day 1 1h PLM-1 1" Sample ID: "24" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

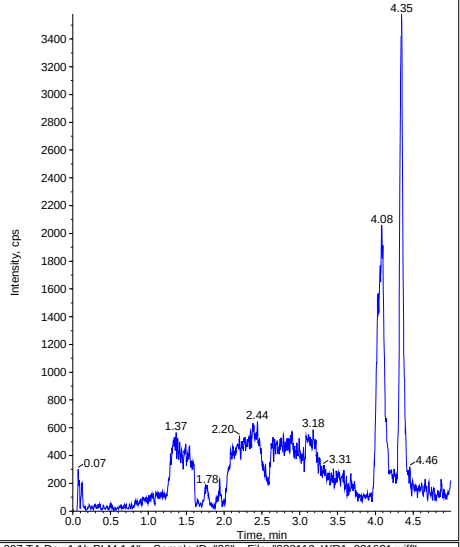
```
Sample Index:       24
Sample Type:      Unknown
Concentration:     1.00    ng/mL
Calculated Conc:   N/A
Acq. Date:        2/16/2021
Acq. Time:        5:50:04 PM

Modified:           No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          10.0    sec
Expected RT:         2.41   min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:          143500.   counts
Height:         53954.590  cps
Start Time:          2.32   min
End Time:            2.59   min
```



Sample Name: "1 025 038110 039700000008 994 TA Day 1 1h PLM-1 1" Sample ID: "25" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

Sample Index: 25
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 0.00 ng/mL
Acq. Date: 2/16/2021
Acq. Time: 5:55:41 PM

Modified: No

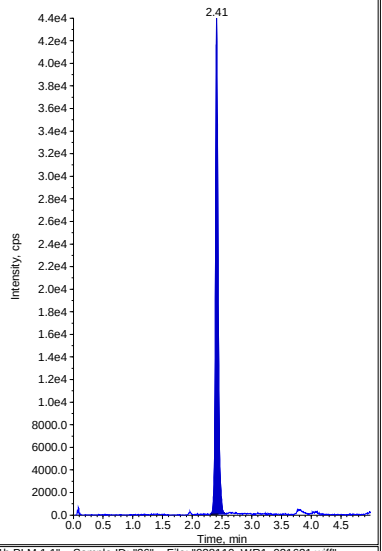


Sample Name: "1 025 038110 039700000008 994 TA Day 1 1h PLM-1 1" Sample ID: "25" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

Sample Index: 25
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 5:55:41 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 500.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 10.0 sec
Expected RT: 2.41 min
Use Relative RT: No

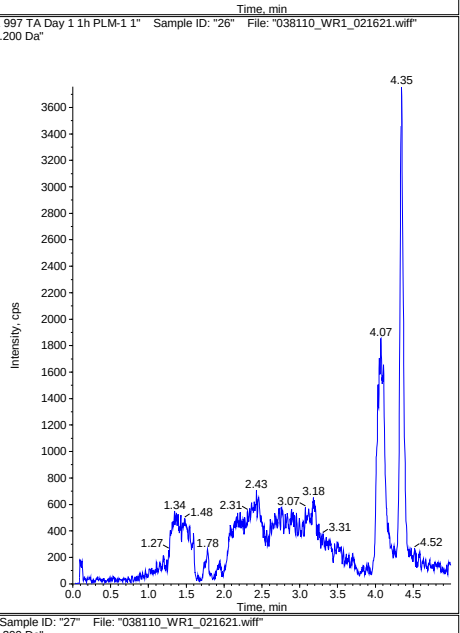
Int. Type: Base To Base
Retention Time: 2.41 min
Area: 132700. counts
Height: 43923.327 cps
Start Time: 2.31 min
End Time: 2.56 min



Sample Name: "1 026 038110 039700000011 997 TA Day 1 1h PLM-1 1" Sample ID: "26" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

Sample Index: 26
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 0.00 ng/mL
Acq. Date: 2/16/2021
Acq. Time: 6:01:19 PM

Modified: No

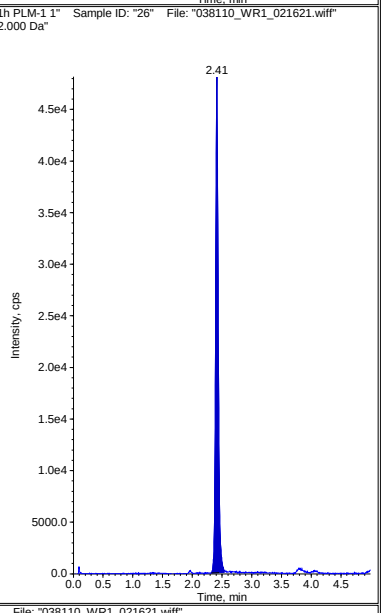


Sample Name: "1 026 038110 039700000011 997 TA Day 1 1h PLM-1 1" Sample ID: "26" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

Sample Index: 26
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 6:01:19 PM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 75
Base. Sub. Window: 0.25 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 500.00 cps
Min. Peak Width: 0.00 sec
Smoothing Width: 3 points
RT Window: 10.0 sec
Expected RT: 2.41 min
Use Relative RT: No

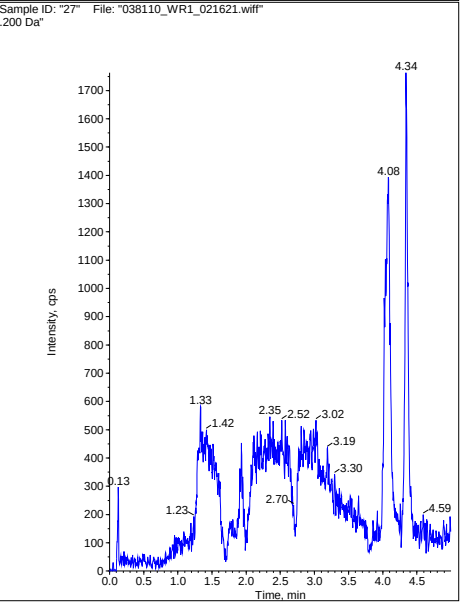
Int. Type: Valley
Retention Time: 2.41 min
Area: 145100. counts
Height: 48155.765 cps
Start Time: 2.32 min
End Time: 2.54 min



Sample Name: "1 027 038110 DB Wash 1" Sample ID: "27" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

Sample Index: 27
Sample Type: Unknown
Concentration: N/A
Calculated Conc: No Intercept
Acq. Date: 2/16/2021
Acq. Time: 6:06:57 PM

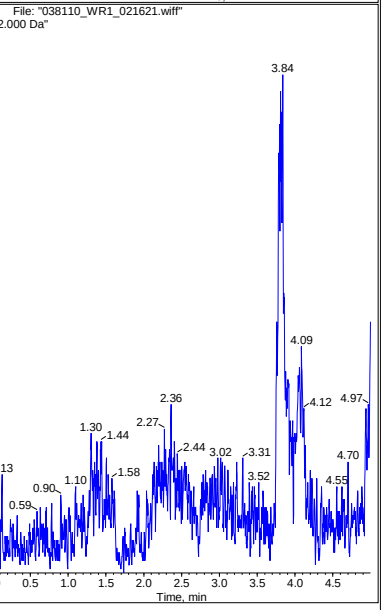
Modified: No



Sample Name: "1 027 038110 DB Wash 1" Sample ID: "27" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

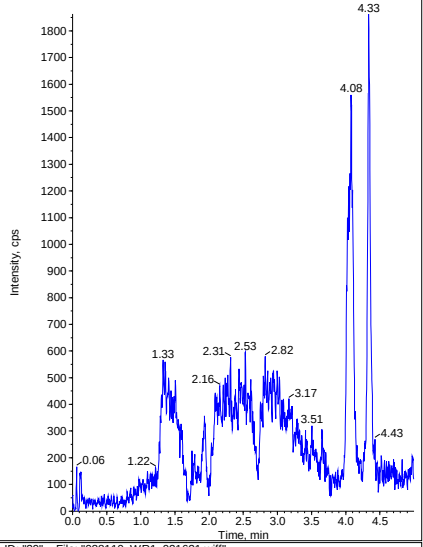
Sample Index: 27
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 2/16/2021
Acq. Time: 6:06:57 PM

Modified: No



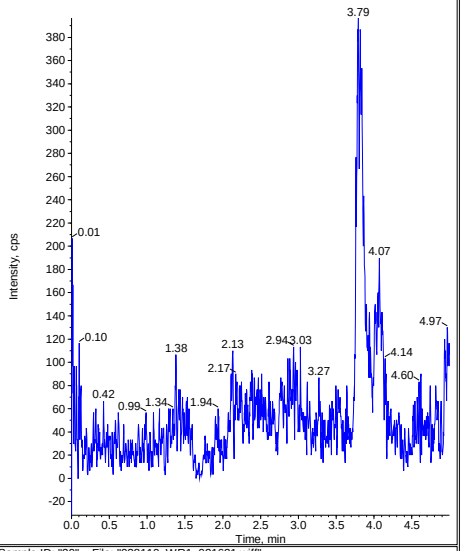
Sample Name: "1 028 038110 DB Wash 1" Sample ID: "28" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       28
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc: No Intercept
Acq. Date:        2/16/2021
Acq. Time:        6:12:35 PM

Modified:           No
```



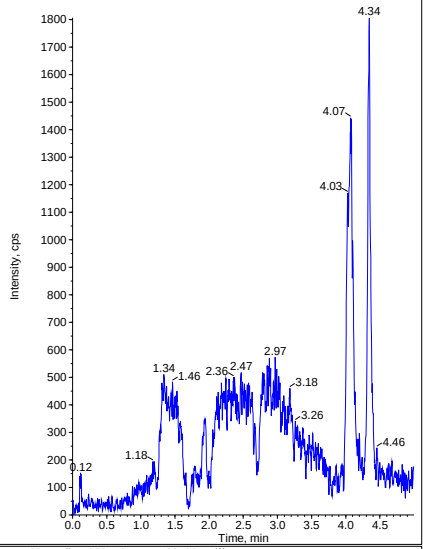
Sample Name: "1 028 038110 DB Wash 1" Sample ID: "28" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       28
Sample Type:      Unknown
Concentration:      1.00    ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        6:12:35 PM

Modified:           No
```



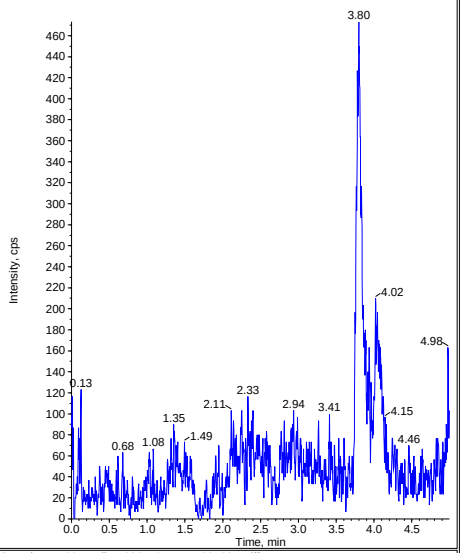
Sample Name: "1 029 038110 DB Wash 1" Sample ID: "29" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       29
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc: No Intercept
Acq. Date:        2/16/2021
Acq. Time:        6:18:10 PM

Modified:           No
```



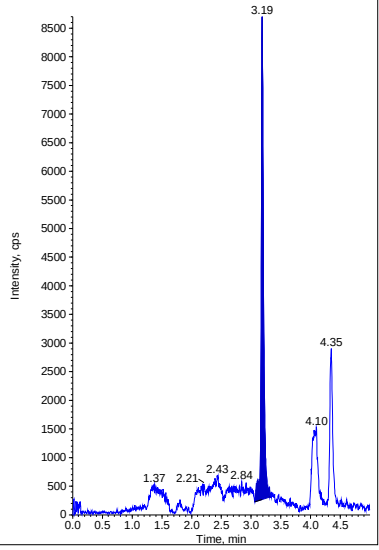
Sample Name: "1 029 038110 DB Wash 1" Sample ID: "29" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       29
Sample Type:      Unknown
Concentration:      1.00    ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        6:18:10 PM

Modified:           No
```



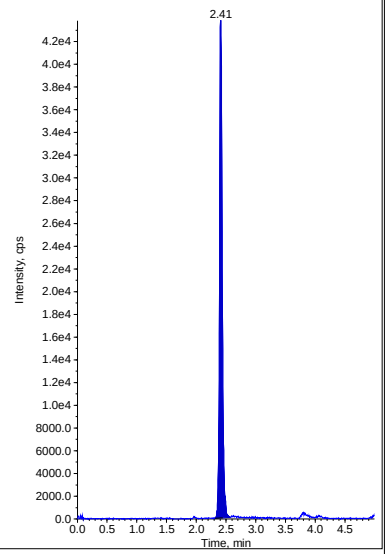
Sample Name: "1 030 038110 QC-Low 2 1" Sample ID: "30" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       30
Sample Type:        QC
Concentration:      150.    ng/mL
Calculated Conc:    154.    ng/mL
Acq. Date:        2/16/2021
Acq. Time:        6:23:50 PM

Modified:           No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:  Yes
Min. Peak Height:     300.00   cps
Min. Peak Width:        0.00   sec
Smoothing Width:        3      points
RT Window:            5.00    sec
Expected RT:          3.19    min
Use Relative RT:      No

Int. Type:        Base To Base
Retention Time:       3.19   min
Area:           23360.   counts
Height:          8474.451  cps
Start Time:           3.06   min
End Time:             3.32   min
```



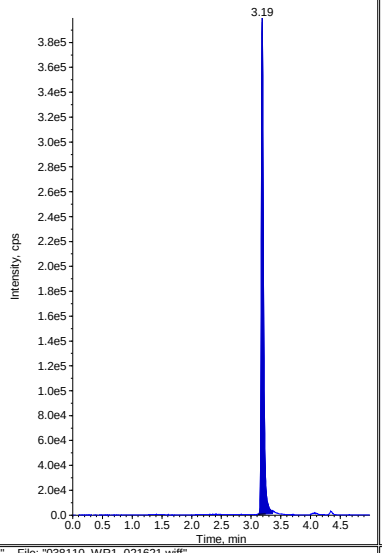
Sample Name: "1 030 038110 QC-Low 2 1" Sample ID: "30" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       30
Sample Type:        QC
Concentration:      1.00    ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        6:23:50 PM

Modified:           No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:  Yes
Min. Peak Height:     500.00   cps
Min. Peak Width:        0.00   sec
Smoothing Width:        3      points
RT Window:           10.0    sec
Expected RT:          2.41    min
Use Relative RT:      No

Int. Type:        Base To Base
Retention Time:       2.41   min
Area:          139100.   counts
Height:         43813.130  cps
Start Time:           2.32   min
End Time:             2.57   min
```



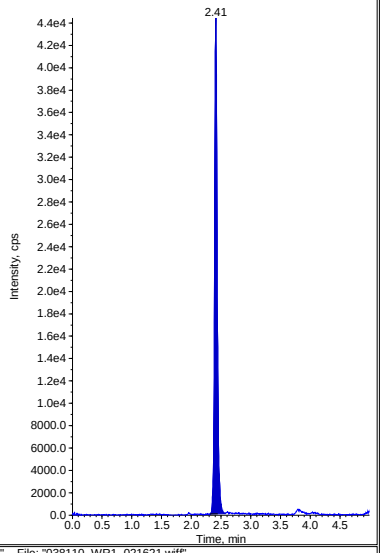
Sample Name: "1 031 038110 QC-Mid 2 1" Sample ID: "31" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       31
Sample Type:          QC
Concentration:     7500.    ng/mL
Calculated Conc:   7800.    ng/mL
Acq. Date:       2/16/2021
Acq. Time:       6:29:28 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    300.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          5.00    sec
Expected RT:        3.19    min
Use Relative RT:    No

Int. Type:        Valley
Retention Time:     3.19   min
Area:         1072000.   counts
Height:        400870.424  cps
Start Time:         3.12   min
End Time:           3.36   min
```



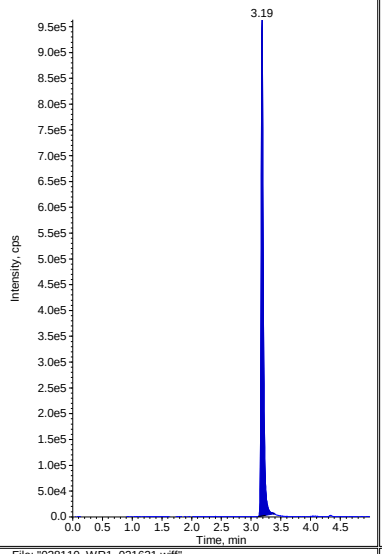
Sample Name: "1 031 038110 QC-Mid 2 1" Sample ID: "31" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       31
Sample Type:          QC
Concentration:     1.00    ng/mL
Calculated Conc:   N/A
Acq. Date:       2/16/2021
Acq. Time:       6:29:28 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          10.0    sec
Expected RT:        2.41    min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:          138000.   counts
Height:         44398.872  cps
Start Time:         2.32   min
End Time:           2.56   min
```



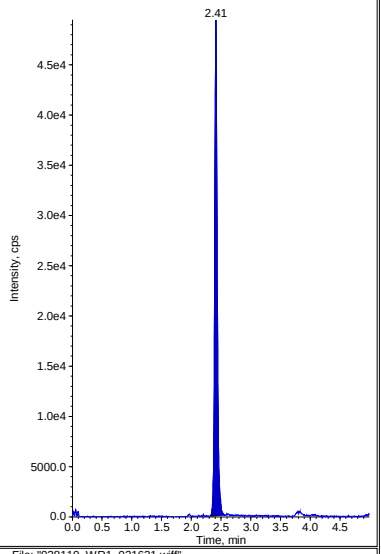
Sample Name: "1 032 038110 QC-High 2 1" Sample ID: "32" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       32
Sample Type:          QC
Concentration:    18800.    ng/mL
Calculated Conc:  20000.    ng/mL
Acq. Date:       2/16/2021
Acq. Time:       6:35:07 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    300.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          5.00    sec
Expected RT:        3.19    min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     3.19   min
Area:         2567000.   counts
Height:        962092.252  cps
Start Time:         3.12   min
End Time:           3.39   min
```



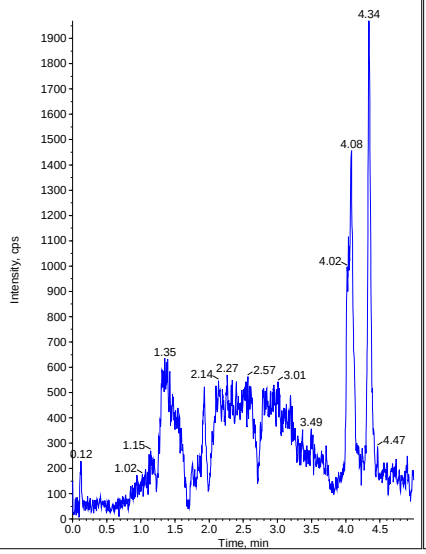
Sample Name: "1 032 038110 QC-High 2 1" Sample ID: "32" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       32
Sample Type:          QC
Concentration:     1.00    ng/mL
Calculated Conc:   N/A
Acq. Date:       2/16/2021
Acq. Time:       6:35:07 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          10.0    sec
Expected RT:        2.41    min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:          149600.   counts
Height:         49488.102  cps
Start Time:         2.32   min
End Time:           2.58   min
```



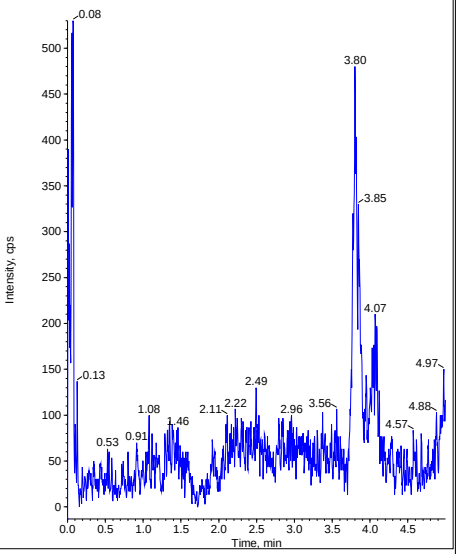
Sample Name: "1 033 038110 DB Wash 1" Sample ID: "33" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       33
Sample Type:     Unknown
Concentration:      N/A
Calculated Conc: No Intercept
Acq. Date:       2/16/2021
Acq. Time:       6:40:46 PM

Modified:            No
```



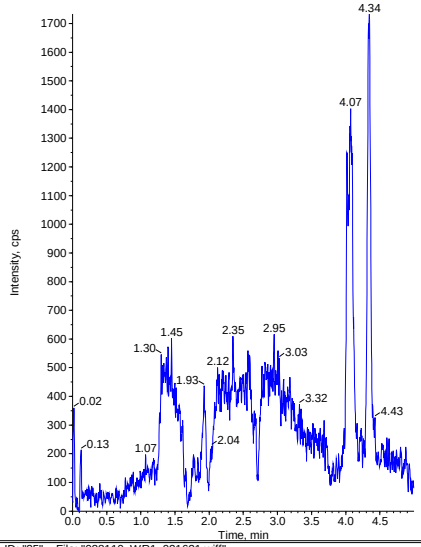
Sample Name: "1 033 038110 DB Wash 1" Sample ID: "33" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       33
Sample Type:     Unknown
Concentration:     1.00    ng/mL
Calculated Conc:   N/A
Acq. Date:       2/16/2021
Acq. Time:       6:40:46 PM

Modified:            No
```



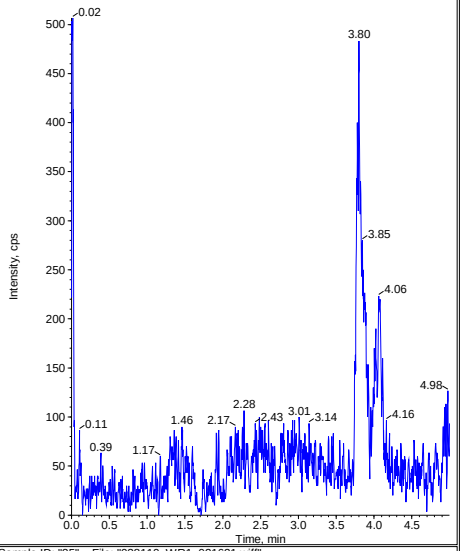
Sample Name: "1 034 038110 DB Wash 1" Sample ID: "34" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       34
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc: No Intercept
Acq. Date:        2/16/2021
Acq. Time:        6:46:23 PM

Modified:           No
```



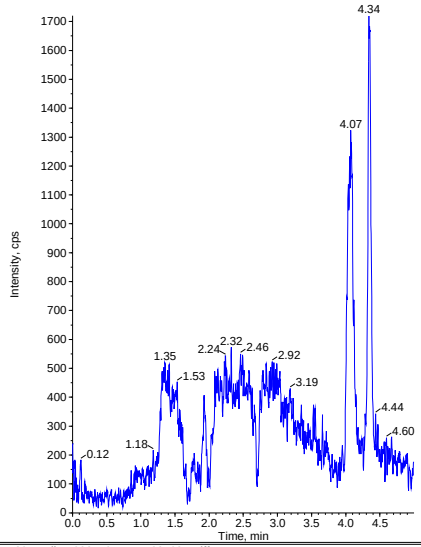
Sample Name: "1 034 038110 DB Wash 1" Sample ID: "34" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       34
Sample Type:      Unknown
Concentration:      1.00    ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        6:46:23 PM

Modified:           No
```



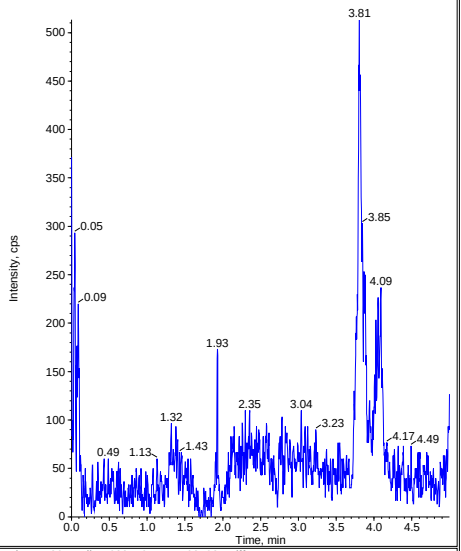
Sample Name: "1 035 038110 DB Wash 1" Sample ID: "35" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       35
Sample Type:      Unknown
Concentration:      N/A
Calculated Conc: No Intercept
Acq. Date:        2/16/2021
Acq. Time:        6:52:02 PM

Modified:           No
```



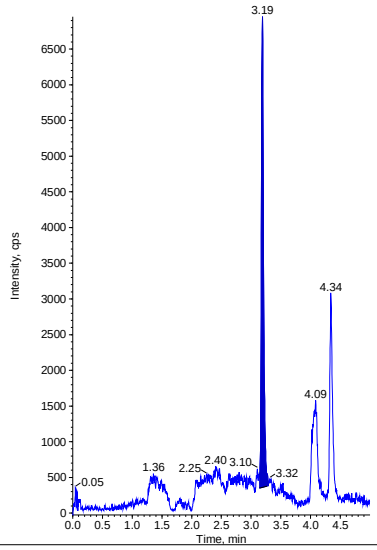
Sample Name: "1 035 038110 DB Wash 1" Sample ID: "35" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       35
Sample Type:      Unknown
Concentration:      1.00    ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        6:52:02 PM

Modified:           No
```



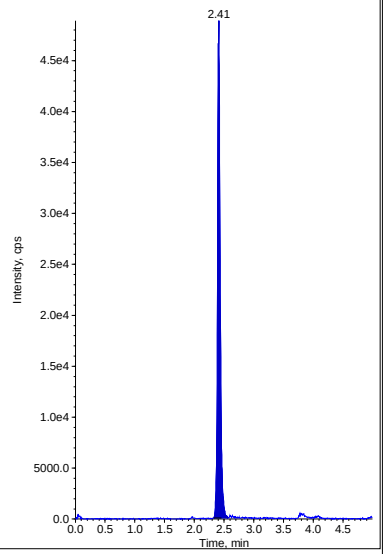
Sample Name: "1 036 038110 Std-2 1 1" Sample ID: "36" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:       36
Sample Type:      Standard
Concentration:      100.    ng/mL
Calculated Conc:    108.    ng/mL
Acq. Date:        2/16/2021
Acq. Time:        6:57:39 PM

Modified:           No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:  Yes
Min. Peak Height:    300.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3      points
RT Window:          5.00    sec
Expected RT:        3.19   min
Use Relative RT:    No

Int. Type:        Valley
Retention Time:     3.19   min
Area:          17530.   counts
Height:         6595.383  cps
Start Time:         3.14   min
End Time:           3.29   min
```



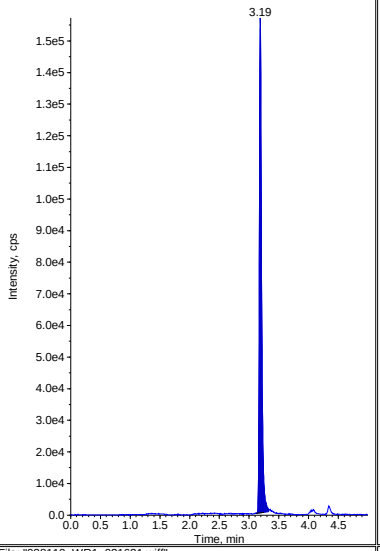
Sample Name: "1 036 038110 Std-2 1 1" Sample ID: "36" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:       36
Sample Type:      Standard
Concentration:      1.00    ng/mL
Calculated Conc:    N/A
Acq. Date:        2/16/2021
Acq. Time:        6:57:39 PM

Modified:           No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:  Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3      points
RT Window:         10.0    sec
Expected RT:        2.41   min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:         149000.   counts
Height:        48830.997  cps
Start Time:         2.33   min
End Time:           2.57   min
```



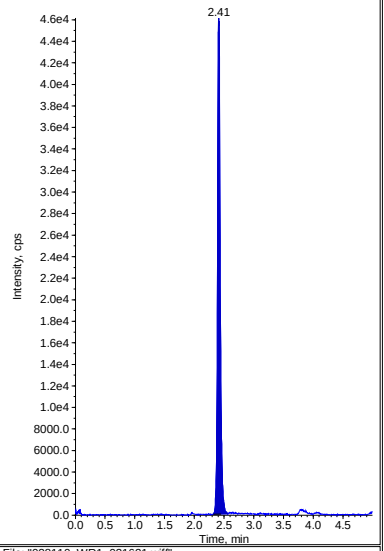
Sample Name: "1 037 038110 Std-4 1 1" Sample ID: "37" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:      37
Sample Type:     Standard
Concentration:     2500.    ng/mL
Calculated Conc:   2630.    ng/mL
Acq. Date:       2/16/2021
Acq. Time:       7:03:19 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    300.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          5.00    sec
Expected RT:        3.19    min
Use Relative RT:    No

Int. Type:        Valley
Retention Time:     3.19   min
Area:         410700.   counts
Height:        156646.244  cps
Start Time:         3.12   min
End Time:           3.33   min
```



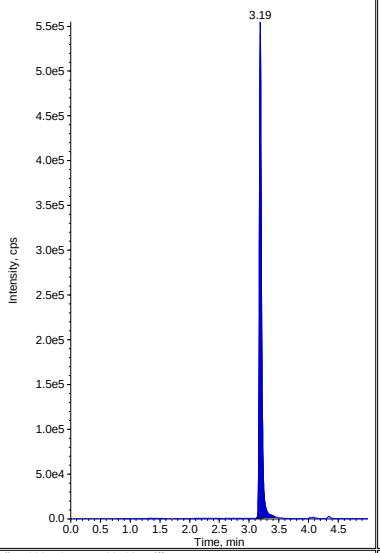
Sample Name: "1 037 038110 Std-4 1 1" Sample ID: "37" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:      37
Sample Type:     Standard
Concentration:     1.00    ng/mL
Calculated Conc:   N/A
Acq. Date:       2/16/2021
Acq. Time:       7:03:19 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:         10.0    sec
Expected RT:        2.41   min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:         147800.   counts
Height:        46212.141  cps
Start Time:         2.32   min
End Time:           2.56   min
```



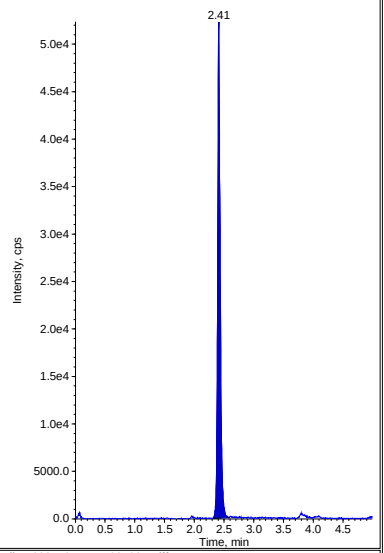
Sample Name: "1 038 038110 Std-5 1 1" Sample ID: "38" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:      38
Sample Type:     Standard
Concentration:    10000.    ng/mL
Calculated Conc:   9830.    ng/mL
Acq. Date:       2/16/2021
Acq. Time:       7:08:55 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    300.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          5.00    sec
Expected RT:        3.19    min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     3.19   min
Area:        1480000.   counts
Height:       554833.420  cps
Start Time:         3.12   min
End Time:           3.44   min
```



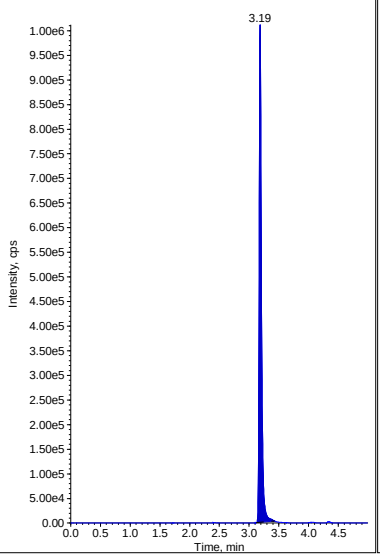
Sample Name: "1 038 038110 Std-5 1 1" Sample ID: "38" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:      38
Sample Type:     Standard
Concentration:     1.00    ng/mL
Calculated Conc:   N/A
Acq. Date:       2/16/2021
Acq. Time:       7:08:55 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:         10.0    sec
Expected RT:        2.41   min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:         154700.   counts
Height:        52328.619  cps
Start Time:         2.32   min
End Time:           2.59   min
```



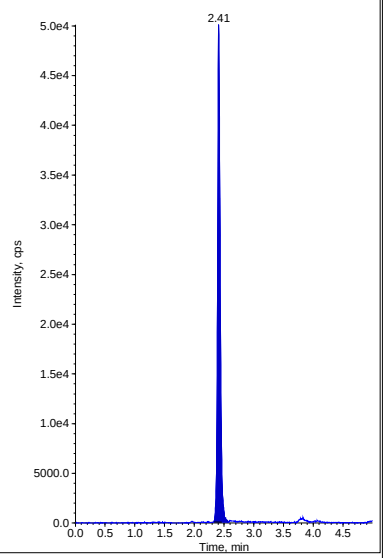
Sample Name: "1 039 038110 Std-7 1 1" Sample ID: "39" File: "038110_WR1_021621.wiff"
Peak Name: "THB" Mass(es): "125.030/107.200 Da"
Comment: "none" Annotation: ""

```
Sample Index:      39
Sample Type:     Standard
Concentration:    20000.    ng/mL
Calculated Conc:  22000.    ng/mL
Acq. Date:       2/16/2021
Acq. Time:       7:14:35 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    300.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:          5.00    sec
Expected RT:        3.19    min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     3.19   min
Area:        2746000.   counts
Height:       1011029.231  cps
Start Time:         3.12   min
End Time:           3.43   min
```



Sample Name: "1 039 038110 Std-7 1 1" Sample ID: "39" File: "038110_WR1_021621.wiff"
Peak Name: "Hydroquinone D6(IS)" Mass(es): "113.150/112.000 Da"
Comment: "none" Annotation: ""

```
Sample Index:      39
Sample Type:     Standard
Concentration:     1.00    ng/mL
Calculated Conc:   N/A
Acq. Date:       2/16/2021
Acq. Time:       7:14:35 PM

Modified:            No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage:     75
Base. Sub. Window:    0.25   min
Peak-Split. Factor:   2
Report Largest Peak:   Yes
Min. Peak Height:    500.00   cps
Min. Peak Width:       0.00   sec
Smoothing Width:       3       points
RT Window:         10.0    sec
Expected RT:        2.41   min
Use Relative RT:    No

Int. Type:       Base To Base
Retention Time:     2.41   min
Area:         149400.   counts
Height:        50308.679  cps
Start Time:         2.33   min
End Time:           2.57   min
```



18. APPENDIX VI: Common Technical Document (CTD) Table

Report Title: ***In Vivo*** **Mammalian Erythrocyte Micronucleus Assay in Mice**				**Test Article:** 1, 2, 4-Trihydroxybenzene	
Test for Induction of:	Bone marrow micronuclei	**Treatment Schedule:**	Once	**BioReliance Study No.:**	AF98GS.123M012.BTL
Species/Strain/Sex:	Hsd:ICR (CD-1) male mice	**Sampling Times:**	24 and 48 hours post-dose		
Age:	7 weeks old	**Route of Administration:**	Intraperitoneal injection (IP)	**GLP Compliance:**	Yes
Cells Evaluated:	Polychromatic erythrocytes (PCE)	**Vehicle for the Test Article Formulation:** **Positive control:**		Degassed Deionized (DI) Water Cyclophosphamide (CP)[a]	
No. of Cells Analyzed/Animal:	4000 PCE/animal	**Feeding Condition:**	*Ad libitum*	**Dates of Dosing:**	04 December 2019

Special Features:	Dose Formulation and BioAnalysis
Toxic/Cytotoxic Effects:	No mortality occurred at any dose level during the course of the definitive assay. Ruffled fur, squinty eye and hunched posture were noted in test article treated groups during clinical observations. No appreciable reductions in the PCEs/EC ratio was observed in the test article groups compared to the vehicle control group, indicating the test article did not induce cytotoxicity
Genotoxic Effects:	No statistically significant increase in the incidence of MnPCEs in the test article treated groups was observed relative to the vehicle control groups.
Evidence of Exposure:	Plasma samples in mice were extracted and analyzed for the sole purpose of method development, therefore no concentrations are reported. No test article was detectable in the samples to enable qualification or analysis.

[a]Scoring positive control slides (fixed and unstained), generated from BioReliance Study No. AF65AY.123M012.BTL were included to verify scoring.

Sampling Time	Controls/Test Articles	Dose Level mg/kg	Sex/No. of Animals/Group		%MnPCE (Mean± SD)	Number MnPCE/PCE scored
24 hrs post-dose	Degassed Deionized (DI) Wate	0	Males	6	0.02 ± 0.02	5 /24000
	1, 2, 4-Trihydroxybenzene	6.3	Males	6	0.01 ± 0.02	2 /24000
		12.5	Males	6	0.03 ± 0.02	6 /24000
		25	Males	6	0.02 ± 0.02	4 /24000
	Cyclophosphamide	50	Males	5	1.47 ± 0.44**	294 /20000
48 hrs post-dose	Degassed Deionized (DI) Wate	0	Males	6	0.05 ± 0.03	11 /24000
	1, 2, 4-Trihydroxybenzene	25	Males	6	0.02 ± 0.02	5 /24000

PCE: Polychromatic Erythrocytes; MnPCE: Micronucleated Polychromatic Erythrocytes

**$p < 0.01$, One-Way ANOVA with Post-Hoc Dunnett's Test (%PCE), Kruskal-Wallis test (%MnPCE) or T-Test (positive control)

1,2,4-TRIHYDROXYBENZENE

Toxicological Summary

January 31, 2023

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List of Exhibits:

Exhibit A: **SCCS 1598/18 Final Opinion on 1,2,4-Trihydroxybenzene**

Exhibit B: ***In Vivo*** **Mammalian Micronucleus Assay in CD-1 Mice Final Report**

Exhibit C: **Expert Analysis of Literature by Prof. H. Greim with Prof. B. Halliwell**

Exhibit D: **Expert Review of Genotoxicity of 1,2,4-THB by Dr. Marilyn J. Aardema**

Exhibit E: **Literature Review - 2018**

Executive Summary

Overview

This submission is intended to assist the CIR Expert Panel in analyzing the safety of 1,2,4-Trihydroxybenzene (1,2,4-THB) for its use in cosmetic hair dye formulations. Among other things, this submission addresses comments expressed by the European Scientific Committee for Consumer Safety (SCCS) in its Opinion SCCS 1598/18 (the "SCCS Final Opinion") (**Exhibit A**), as a result of inconsistent *in vitro* findings. The SCCS Final Opinion was based on the SCCS Opinion of 1452/11 of December 11, 2012 ("SCCS 1452/11") (***Ref. 1),*** which concluded that the inconsistent *in vitro* data could only be rebutted through *in vivo* testing, but since such animal testing on cosmetic products is not allowed in the EU, it was not possible for a final safety determination to be made.

This submission provides critical evidence that was generated in animals after the SCCS Final Opinion. Specifically, it provides new results from a Micronucleus Assay in mice (see **Exhibit B**). This *in vivo* genotoxicity testing data demonstrates that 1,2,4-THB is not genotoxic *in vivo*.

While it is possible that 1,2,4-THB may appear genotoxic in some *in vitro* tests because it generates reactive oxygen species (ROS) and hydrogen peroxide, these effects are eliminated *in vivo* by mammalian biological antioxidant systems such as catalase and superoxide dismutase. These enzymes decompose ROS to water and oxygen, preventing any damage from ROS.

It is critical to note that the SCCS Final Opinion concluded that genotoxicity could not be ruled out largely on the basis of a "weight of evidence" of the open literature, which consisted mainly of *in vitro* studies. The new and compelling data from the *in vivo* Micronucleus Assay showing that 1,2,4-THB is not genotoxic *in vivo*, which the SCCS was unable to review due to the EU prohibition on animal testing of cosmetic products, will help inform the CIR Expert Panel about the safe use of 1,2,4-THB in cosmetic hair dye formulations.

In addition to the new data from the *in vivo* Micronucleus Assay, this submission also summarizes the comprehensive set of *in vitro* studies and results supporting the safety of 1,2,4-THB. The *in vitro* assays described in these studies include Bacterial Reverse Mutation Assays, a Mammalian (Human) Cell Micronucleus Assay, a 3D Comet Assay using Phenion® Full Thickness Skin Model, a 3D Human Reconstructed Skin Micronucleus Assay, a KeratinoSens™ Assay, and Dermal Absorption Assays. In addition, this submission summarizes reaction chemistry information and a simulated consumer exposure study, demonstrating that exposure to 1,2,4-THB from hair dye is low due to its rapid air oxidation and self-coupling reaction, which results in a rapid decline in 1,2,4-THB concentration after application.

This report also includes a review of the broader literature on the safety and mechanism of 1,2,4-THB as well as exposure calculations. Among other things, the literature shows that two cups of coffee contain approximately 1.14 to 3.4 mg of 1,2,4-THB. In contrast, the use of 2.5% of 1,2,4-THB as a hair dye would provide a maximum systemic exposure of 2.1 mg, comparable to 2 cups of coffee a day. And while many people consume more than two cups of coffee every day, hair dyes are used only occasionally. Although we understand that the CIR Expert Panel will base its opinion on the scientific evidence supporting the safety of 1,2,4-THB, the exposure data reveal that the potential exposure from hair dye is not large relative to a consumer's regular exposure from other sources, a further practical consideration supporting its safety.

The totality of the evidence in this submission, including the new *in vivo* genotoxicity data, demonstrate that 1,2,4-THB is not harmful *in vivo* and can continue to be used safely in cosmetic hair dye formulations.

Input, Guidance and Support

The findings and conclusions advanced in this document have been developed under the guidance and with significant input from Prof. Helmut Greim and Prof. Barry Halliwell, world-renowned subject matter experts in medical toxicology and the management and disposition of ROS in biological systems, respectively. Both Professors Greim and Halliwell fully support the findings and conclusions presented in this document. An expert analysis by Dr. Greim with input from Prof. Barry Halliwell is attached as **Exhibit C**. In addition, we engaged Dr. Marilyn J. Aardema for her expert guidance in the conduct and review of the critical genotoxicity endpoints. Her independent analysis of the genotoxicity data is provided in **Exhibit D**.

CHEMICAL AND TOXICOLOGICAL EVALUATION

1.0 Chemical and Physical Specifications

This section summarizes available information on the identification and quantification of 1,2,4-THB, including stability testing. Some of the data is reproduced from the SCCS Final Opinion and SCCS 1452/11 ***(Ref. 1)***. Additional data collected is referenced appropriately.

Primary Name or INCI Name

INCI Name: 1,2,4-Trihydroxybenzene

Chemical Name

Synonyms: Benzene-1,2,4-triol
2-Hydroxyhydroquinone
1,2,4-Benzenetriol
4-Hydroxycatechol

Trade Names and Abbreviations

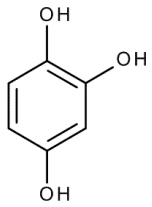
Trade Names: Jarocol THB
Rodol THB

CAS/EC Number

CAS No: 533-73-3

EINECS No: 208-575-1

Structural Formula/Chemical Structure:



Empirical formula

Empirical Formula: $C_6H_6O_3$

1.1 Physical form

Physical Form: light – medium beige powder

1.2 Molecular weight

Molecular Weight: 126.11 g/mol

1.3 Purity composition

The standard methodology to determine the purity of 1,2,4-THB is Gas Chromatography (GC). 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%

Additional information on purity is summarized in ***(Ref. 1***).

1.6 Solubility

Note: Data provided in Section 1.6 – 1.9 are reproduced from ***(Ref. 1).***

Water solubility according to OECD method A6: 486 g/L at 20°C

Solubility (g/100 ml, 22°C, 24 h)

Ethanol: 1< S <10

DMSO: 10< S <20

1.7 Partition Coefficient (Log Pow)

Log $P_{o/w}$: 0.2 (calculated*)

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

1.8 Additional physical chemical specifications

Melting Point:	134-141ºC
Boiling Point:	/
Flash Point:	/
Vapor Pressure:	/
UV-Vis λ_{max}:	291 nm

1.9 Stability

Solutions of 1,2,4-THB were stable (variation <2%) after 2 and 4-hour storage at room temperature protected from light and under 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

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 self-coupling 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, hair dye formulations are filled into specialized, oxygen barrier packaging for commercialization. Stability tests are conducted under standard conditions (both room and elevated temperatures) 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 other precursors) in the formulation undergo oxidative coupling reactions as predicted by their chemical structure.

2.0 Function and Uses

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 coloring shampoos.

1,2,4-THB is an auto-oxidative dye used at a maximum concentration of 2.5% in a permanent hair dye formulation and does not require hydrogen peroxide to activate the oxidation and subsequent coupling reactions. The finished products are manufactured and filled in a strict inert environment and are stable until the package is opened and exposed to the air during usage.

We are not aware of any other industrial or functional uses for 1,2,4 THB other than the auto-oxidative hair dye use mentioned here. However, 1,2,4-THB is naturally present in many organisms and in food, such as coffee.

3.0 Toxicological Evaluation

The following data was collected in support of the safety of 1,2,4-THB, utilizing the testing strategy described in SCCS Notes of Guidance available at the time (SCCS/1602/18, ***Ref. 2***).

3.1 Acute Toxicity

Acute oral toxicity was assessed in a 1977 study by oral gavage of 1,2,4-THB using 10 Sprague-Dawley rats (5 male and 5 female) at the following doses: 100, 250, 350, 500 and 1000 mg/kg-bw. The animals were observed during the first hours after intubation and then for the following 14 days. Mortality was checked during the 14-day observation period. Body weights were presented for the beginning of the study only. Death occurred within 24 hrs. of administration. The LD50 is between 350 and 500 mg/kg-bw. This value was calculated for both sexes. (See ***Ref. 1)***

3.2 Irritation and Corrosivity

In vivo Skin irritation in New Zealand White Rabbits was assessed in a study conducted in 2004 in compliance with OECD 404 and GLP, using 3 male rabbits. 0.5 ml of 3% 1,2,4-THB on a dry gauze square was applied to the flanks of the animals; for 3 minutes (one animal), 1 hour (three animals) and 4 hours (three animals). Due to the skin coloration by 1,2,4-THB after a 4-hour exposure, it was not possible to definitely conclude on the irritant potential. Based on the results obtained with the 1-hour exposure, 1,2,4-THB at 3% in water was slightly irritant for the rabbit skin. (See ***Ref. 1***)

3.3 Skin Sensitization

Although skin sensitization was not identified as a data gap in SCCS 1452/11, these data were submitted and are included in the SCCS Final Opinion. The following assay was conducted under OECD 442D and the following additional information was collected on this endpoint in effort to add to the existing LLNA data which concluded that 1,2,4-THB was an extreme sensitizer.

Guideline:	OECD 442D
Species/strain:	HaCaT Keratinocytes
Test substance:	1,2,4-THB
Purity:	97.8%
Lot:	THB0200312
Concentrations:	0.977, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500, 1000, 2000 µM
Test Conducted:	February 16-19, 2016

The KeratinoSens™ test method is considered scientifically valid to be used to support the discrimination between skin sensitizers and non-sensitizers for the purpose of hazard classification and labelling. The ARE-Nrf2 luciferase test method makes use of an immortalized 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 sensitizers. A chemical is predicted to have potential to be a sensitizer if at least two of the following criteria are met:

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

The test article, 1,2,4-THB, was tested in three definitive assays according to the OECD protocol 442D (i.e., EC1.5, Mean IC_{50} and Maximal Induction).

Results:
Table 3 summarizes 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 sensitizer.

Table 3: Summary of KeratinoSens™ data

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

Note: *Where an IC_{50} 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.*

Conclusion:
The EC1.5 for 1,2,4-THB was determined to be 374.31 µM and the material is classified as a potential sensitizer. ***(Ref. 3)***

Note: The SCCS Final Opinion noted that the SCCS noted that KeratinoSens Assay is not intended to provide information with regard to potency. We agree with the SCCS's view.

The SCCS also noted the variability of data raised questions regarding statistical treatment of data. A KeratinoSens is considered positive when the criteria noted above are met. The value of I_{max} must be statistically significantly different when compared to solvent/vehicle control as determined by a two-tailed, unpaired Student T-test. The data was provided a response on the statistical treatment of the data which was in turn submitted to SCCS in September of 2018. ***(Ref. 4)***.

3.4 Dermal Absorption

Guideline:	OECD 428 (2004); Guidance Document No. 28
Test System:	Human skin samples 12 replicates per test item
Test Item 1:	6 female donors, age 60-84 (2 abdomen, 1 back, 1 buttock, 1 back/ buttock)
Test Item 2:	4 female donors, age 63-74 (1 abdomen, 3 back)
Diffusion Cell:	Static glass diffusion cells (2.54 cm^2, approximately 4.5 ml receptor fluid volume)
Group size:	2 experiments, each with 12 diffusion cells
Skin Temperature:	32±1°C
Skin Integrity:	Determined by measurement of the electrical resistance across the sample. Skin with a measured resistance of <10 kΩ were regarded as having a lower integrity than normal and not used for exposure to the test materials.

Test Substance:	1,2,4- THB
Batch:	THB0200312
Purity:	99.3%
Radio-labeled 1,2,4-THB:	[^{14}C]-1,2,4-Trihydroxybenzene ([^{14}C]-1,2,4-THB)
Radiochemical purity:	98.3%,
	2.00MBq/mg (6.81mCi/mmol = 252 MBq/mol),
Molecular Weight:	126.33 g/mole
Batch:	8188CEO008-2
Test items:	1) 1,2,4-THB formulated at a level of 2.5% in a hair dye vehicle at pH 7
	2) 1,2,4-THB formulated at a level of 2.5% in a hair dye vehicle also containing 2.25% p-Toluenediamine (PTD as the free base) at pH 7
Receptor fluid:	Phosphate buffered saline
Solubility receptor fluid:	486 g/L (solubility in water)
Stability receptor fluid:	Not determined, permeation expressed on the basis of µg equivalents 1,2,4-trihydroxybenzene
Method of Analysis:	Liquid scintillation counting
Study Period:	October 2014-January 2015

[^{14}C]-1,2,4-THB and unlabeled 1,2,4-THB were incorporated into hair dye formulations, with and without *p*-toluene diamine (PTD) to provide final concentrations of 2.5% (w/w) [^{14}C]-1,2,4-THB. The formulations were applied to the skin surface at a dose of 20 mg/cm^2 and after an exposure period of 30 minutes the skin surface was washed with a mild soap solution. Following the washing procedure, the cells were returned to the water bath for the remainder of the 24-hour run time. At the end of the experiment, the distribution of [^{14}C]-1,2,4-THB in the test system was assessed by performing a mass balance procedure, which included a tape stripping and heat separation technique, and a 24-hour penetration profile was determined. All samples were analyzed for radioactivity by Liquid Scintillation Counting (LSC).

Results:
The total systemically available dose (epidermis, dermis and receptor fluid) from the formulation containing [^{14}C]-1,2,4-THB alone was 1.13 ± 0.58 ug-eq [^{14}C]-1,2,4-THB/cm^2 (mean ± SD). The total systemically available dose of [^{14}C]-1,2,4-THB from the formulation containing 2.5% [^{14}C]-1,2,4-THB and PTD, was 1.94 $\pm$1.76 ug-eq [^{14}C]-1,2,4-THB/cm^2 (mean ± SD) as shown in ***Table 4*** below.

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

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

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

Mass balances showed essentially complete recovery of radiolabel in each experiment.

Conclusion:
In accordance with the SCCS Notes of Guidance available at the time (SCCS 1602/18), the mean +1SD absorption in an *in vitro* dermal absorption study is used to determine the systemically available dose for the purposes of the calculation of the margin of safety (MoS). The systemically available dose of [^{14}C]-1,2,4-THB alone is therefore 1.13 µg equivalents/cm^2+ 0.58 (1SD) or 1.71 µg equivalents/cm^2. The systemically available dose of [^{14}C]-1,2,4-THB with PTD is 1.94 µg equivalents/cm^2+ 1.76 (1SD) or 3.70 µg equivalents/cm^2.

The mean total systemically available dose (epidermis, dermis and receptor fluid) of 1,2,4-THB was 0.226% of the applied dose. As a comparison, one may consider the percutaneous absorption of PTD alone which was determined to be approximately 3.5% of the applied dose (SCCS 1479/12). PTD is absorbed at approximately 15x the percentage of 1,2,4-THB. ***(Ref. 5)***

Note: The SCCS Final Opinion noted that the thickness of the dermatomed skin was not reported in the final report. However, we determined that the thickness of the dermatomed skin is 400 µm. This thickness of the dermatomed skin was identified in the original Study Plan from Dermal Technology Laboratories Ltd (DTL) for the Percutaneous Absorption Study and confirmed in a follow-up communication from DTL. We provided this information back to SCCS in September of 2018 in response to the Draft Final Opinion.

The SCCS also expressed concern regarding increased dermal absorption determined for the formulation with PTD and 1,2,4-THB. However, percutaneous absorption studies typically show variability with respect to both donors and replicates within donors. Therefore, any inferences about the relative systemically available amounts for 1,2,4- THB alone and 1,2,4-THB + PTD must be made with caution. Inspection of the data shows that the largest contribution to the calculated systemically available amounts was from the epidermal compartment, which contributed 86.7% for 1,2,4-THB alone and 95.3% for 1,2,4-THB + PTD. The amounts found in this compartment were higher for 1,2,4-THB + PTD than for 1,2,4-THB alone, whereas this was not the case for the dermis or receptor fluid. Moreover, the variability (SD's added to the mean values) of the epidermal amounts was greater for the combination than for 1,2,4-THB alone. Reactive chemistry forming larger molecular weight species occurred under both exposure scenarios and noted as skin staining, given the variability inherent to *in vitro* percutaneous absorption studies, the apparent difference between treatments could reflect chance alone.

3.5 Subchronic Toxicity

Using OECD 408 and in compliance with GLP, a 90-day subchronic oral toxicity study was conducted. 15 males and 15 female Wistar rats were dosed with 50, 100 and 200 mg/kg-bw by oral gavage. The investigators deduced a No Observed Adverse Effect Level of 50 mg/kg-bw/day. The SCCS concluded however that this value should more appropriately be considered a Lowest Observed Adverse Effect Level (LOAEL). This study was conducted in 2001. (See ***Ref. 1***) This value was treated as a LOAEL in our exposure assessment.

3.6 Reproductive Toxicity

Under OECD 414, and in compliance with GLP, three groups of 25 mated Sprague Dawley rats received 1,2,4-THB by oral gavage at doses of 30, 100, 300 mg/kg-bw/day from Day 6 to 15 of gestation. The control group received only vehicle. 1,2,4-THB administered by oral route to pregnant female rats was not materno-toxic, embryotoxic or teratogenic at 30 and 100 mg/kg-bw/day dose. The highest dose of 300 mg/kg-bw/day was materno-toxic but not embryotoxic or teratogenic. The study was conducted in 1991. (See ***Ref. 1)***

3.7 Mutagenicity/Genotoxicity

This section reports in detail on studies that comply with current testing requirements (OECD guideline compliant testing), as well as additional studies that are currently in the validation process.

1,2,4-THB was tested for gene mutations in accordance with the Ames Bacterial Reverse Mutation Test (OECD 471) and for both structural and numerical chromosome aberrations with the *in vitro* Mammalian Cell Micronucleus Test (OECD 487). As the Ames test was positive and the *in vitro* micronucleus test was negative, 1,2,4-THB was also tested with a 3D Comet Assay using Phenion® Reconstructed Human Skin.

3.7.1 Mutagenicity/Genotoxicity In Vitro

Ames Bacterial Reverse Mutation Assay (Salmonella typhimurium)

Guideline:	OECD 471 (adopted July 21, 1997)
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA102
Replicates:	Single plates in the preliminary toxicity test in the presence and absence of metabolic activation. Triplicate plates in the presence and absence of metabolic activation in Experiments 1 and 2
Test substance:	1,2,4-THB
Solvent:	Degassed water
Lot#:	THB0200312
Purity:	97.8%
Concentrations:	6.7-5000 µg/plate in the presence and absence of metabolic activation in the preliminary toxicity assay; Experiment 1: 5.0-5000 µg/plate, all strains, in the presence and absence of metabolic activation; Experiment 2: 5.0-1500 µg/plate TA1537 only in the absence of metabolic activation.
Treatment:	Plate incorporation, 48-72h incubation in the presence and absence of metabolic activation
GLP:	Yes
Study period:	2014-2015

In the Ames bacterial reverse mutation assay, the test article, 1,2,4-THB, was evaluated for its mutagenic potential based on the ability to induce point mutations in selected loci of the bacterial strains *S. typhimurium* strains TA98, TA100, TA1535, TA1537, TA102. Experiment 1 was conducted with and without addition of an exogenous metabolic activation system using concentrations ranging from 5.0-5000 µg /plate (9 concentrations) in the absence of metabolic activation and 15-5000 µg /plate (6 concentrations) in the presence of metabolic activation based on results from a preliminary toxicity assay. Experiment 2 was conducted without metabolic activation using concentrations ranging from 5.0-1500 µg/plate (11 concentrations) in TA1537 only.

Experiments 1 and 2 included concurrent vehicle controls (degassed water) and positive controls as per OECD guidelines. In both independent experiments, 3 test plates (replicates) were used per test article concentration or per control. Toxicity was detected by a decrease in the number of revertants and clearing or reduction of the bacterial background lawn. Precipitation of the test item was recorded as applicable.

Results:
In Experiment 1, no biologically relevant increases in revertant colonies were observed at concentrations up to 5000 µg/plate in TA98, TA100, TA1535 or TA102 with or without metabolic activation or in TA1537 with metabolic activation. An increase (3.5 fold at 100 µg/plate, 3.0 fold at 150 µg/plate, 2.3 fold at 200 µg/plate, and 4.0 fold at 500 µg/plate) in revertant colonies was observed in tester strain TA1537 without metabolic activation. Mutants were confirmed by replica plating tester strain TA1537 in the absence of S9 activation at 100, 150 and 500 µg/plate.

Toxicity was noted in all tester strains. Without metabolic activation, toxicity was observed at 500 µg/plate and higher in all strains except TA102 where toxicity was observed at 5000 µg/plate. In

the presence of metabolic activation, toxicity occurred at 5000 µg/plate. No precipitation was found in any experiment. The positive control mutagens induced an increase in revertant colonies indicating the validity of the experiments.

Since the background revertants in TA1537 without metabolic activation (4 +/-2) was on the lower side of the historical control values (mean 7 +/-4; 0-28 range) and the dose response was not clear-cut (e.g. 200 µg/plate did not exceed the 3 fold increase), the biological relevance of this result was investigated in Experiment 2 conducted with TA1537 without metabolic activation.

In Experiment 2, an increase (3.8-fold at 150 µg/plate; 3.3-fold at 200 µg/plate) in revertant colonies was observed in TA1537 without metabolic activation. The dose levels tested were 5.0, 15, 50, 150, 200, 300, 400, 500, 600, 750 and 1500 µg/plate in TA1537 without metabolic activation. Toxicity was observed beginning at 300 µg/plate. No precipitation was observed. This repeat experiment meets the criteria for a positive response in TA1537 in the absence of metabolic activation.

Conclusion:
Under the conditions of this study, 1,2,4-THB was concluded to be positive in the reverse mutation assay in bacteria. Increased revertant counts that exceeded the minimum 3.0-fold increase considered positive for TA1537 were observed in two trials. In one trial there was a definitive dose response, whereas there was no definitive dose response in the other trial. Since these increases were just over the minimum 3.0-fold increase required for evaluation as positive in TA1537, it was concluded that 1,2,4-THB induces a weak mutagenic response. ***(Ref. 6)***

Ames Bacterial Reverse Mutation Assay in presence of Catalase and GSH

In response to the SCCS determination that the Ames Bacterial Reverse Mutation Assay (Salmonella typhimurium) demonstrated a mutagenic effect, this modified Ames test with catalase and GSH was undertaken to include ROS scavenging material to demonstrate that whatever effect was seen in vitro was due to ROS, and not to any inherent genotoxicity of 1,2,4-THB, and moreover was mitigated by normal ROS scavenging mechanisms available in vivo.

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-THB
Solvent:	De-aerated water (N_2-purged)
Lot#:	THB0318002
Purity:	98.1%
Vehicle:	De-aerated water (N_2-purged)
Concentrations:	Vehicle, 100 µg, 150 µg, 175 µg, 200 µg, 250 µg, 500 µg 1,2,4-THB without scavengers or metabolic activation. Same 1,2,4-THB concentrations with 20,000 IU, 10,000 IU or 1000 IU Catalase. Repeat 1,2,4-THB concentrations with 15, 10 or 5 µM GSH.

Standard Assay: (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 (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, 48-72 hrs. incubation in the absence of metabolic activation

GLP: Yes

Study Period: Dec 2018; Final Report Jan 2019

The purpose of this study was to evaluate the effect of ROS scavengers, catalase and GSH, on the slight mutagenic response of 1,2,4-THB in *Salmonella typhimurium* TA1537 without metabolic activation, that had been observed in the previous GLP study. Dosing solutions were prepared in de-aerated 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 bacterial 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 15µM 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 presences of catalase at all doses (1,000, 10,000, 20,000 U). Toxicity was also reduced in the presence of catalase but not with GSH.

Conclusion:

Under the conditions of this study, the previous weak 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. ***(Ref. 7)***

Note: In SCCS 1452/11 (see ***Ref 1***), the SCCS stated that "1,2,4-THB induced gene mutations in bacteria […] but not in mammalian cells." In the SCCS Final Opinion, the SCCS's comment to the modified Ames test was to note that it would be more relevant to conduct the study with the two strains TA98 and TA100, which in previously conducted studies showed a slight but reproducible effect. The modified Ames test above was conducted with TA1537, rather than TA98 and TA100 strains, as that was the only strain that demonstrated a mutagenic effect in the most recent Ames Assay. Neither TA 98 nor TA100 showed a mutagenic effect in the recent study (which seems to call into question the validity of the earlier study), so there was little additional information to be gained with reproducing a clear negative when the TA1537 result needed exploration.

In Vitro Mammalian Cell Micronucleus Test in Human Peripheral Blood Lymphocytes

Guideline:	OECD guideline no. 487 (draft April 2014; final Sept. 2014)
Species/strain:	Human peripheral blood lymphocytes from a male donor
Replicates:	One culture per concentration in the presence and absence of metabolic activation in preliminary toxicity test. Two cultures per concentration in the presence and absence of metabolic activation Experiment 1
Test substance:	1,2,4-THB
Solvent:	Degassed water
Lot#:	THB0200312
Purity:	97.8%
Concentrations:	Preliminary Toxicity: 0.126-1260 µg/ml in the presence and absence of metabolic activation. Main Experiment: 1.26-150 µg/ml 4 hrs. treatment in the absence of metabolic activation; 12.5-150 µg/ml 4 hrs. treatment in the presence of metabolic activation; 0.1-100 µg/ml 24 hrs. treatment in the absence of metabolic activation.
Treatment:	Treatment initiated 44-48 hrs. after mitogen stimulation. 4 hrs. treatment in the presence and absence of metabolic activation harvested 20 hrs. later (24 hrs. from start of treatment); 24 hrs. treatment in the absence of metabolic activation harvested immediately.
GLP:	Yes
Study Period:	2014-2015

In an *in vitro* micronucleus test in human peripheral blood lymphocytes, the test article, 1,2,4-THB, was evaluated for its clastogenic and aneugenic potential. The study was conducted with and without addition of an exogenous metabolic activation system. Concentrations ranged from 1.26-150 µg/ml, 4 hrs. treatment in the absence of metabolic activation; 12.5-150 µg/ml 4 hrs. treatment in the presence of metabolic activation; 0.1-100 µg/ml, 24 hrs. treatment in the absence of metabolic activation based on results from a preliminary toxicity assay.

Experiments included concurrent vehicle controls (degassed water) and positive controls as per OECD guidelines. Two replicate cultures were used per test article concentration or per control. Toxicity was measured by the cytokinesis-blocked proliferation index (CBPI). Precipitation of the test item was recorded.

Results:
The percentage of cells with micronucleated/binucleated cells in the test article-treated groups was not statistically significantly increased relative to vehicle control at any dose level ($p > 0.05$, Fisher's Exact test). The results for the positive and negative controls indicate that all criteria for a valid assay were met.

Toxicity of the test article was observed. Substantial cytotoxicity (55 ± 5% or greater reduction in CBPI relative to the vehicle control) was observed at dose levels ≥ 50 µg/mL in the non-activated 4 hrs. treatment group, at dose levels ≥ 100 µg/ml in the S9-activated 4 hrs. treatment group, and at dose levels ≥ 30 µg/ml in the non-activated 24 hrs. treatment group. The highest dose analyzed under each treatment condition produced 55 ± 5% reduction in CBPI which met the dose limit as recommended by testing guidelines for this assay. A minimum of 2000 binucleated cells total were scored for the presence of micronuclei.

Conclusion:
Under the conditions of this study, 1,2,4-THB was concluded to be negative for the induction of micronuclei in human peripheral blood lymphocytes in both the presence and absence of metabolic activation. ***(Ref. 8)***

3D Comet Assay using Phenion® Reconstructed Human Skin

Guideline:	Cosmetics Europe Genetoxicity Taskforce Standard Operating Procedure (date 16 January 2015).
Species/Strain:	Phenion® Full thickness human skin model
Replicates:	One tissue model per concentration in dose range finding assay. Three tissue models per concentration in Experiments 1 and 2.
Test Substance:	1,2,4,-THB
Solvent:	Degassed acetone
Lot #:	THB0200312
Purity:	97.8%
Concentrations:	Dose range finding assay: 0.1-100 mg/ml (0.16-1600 µg/cm^2). Experiment 1: 0.125-1.0 mg/ml (2.0-16 µg/cm^2). Experiment 2: 0.25-1.25 mg/ml (4-20 µg/cm^2).
Treatment:	Total exposure time was 48 ± 3 h (repeated application at 48 ± 3 hrs., 24 ± 3 hrs. and 3 hrs. before cell isolation)
GLP:	Yes
Study Period:	2016

For all experiments, the dose solutions were prepared in a glove box. The environment in the glove box was maintained with nitrogen gas in order to minimize the air oxidation of the test article prior to application. On each day of application, the dose solutions were prepared fresh using degassed acetone as the solvent. In the dose range finding study, the highest test concentration was 100 mg/mL (corresponding to 1600 µg/cm^2), the maximum required concentration for this asay. Six serial dilutions with 3.16-fold spacing in degassed acetone were prepared from the stock solutions. In the first and second main experiments, 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 individually weighed samples for each test substance.

The actual concentration of the test substance in the dose solutions was determined by UPLC-UV using a validated method. Triplicate skin membranes per test group were used in both the first and second main experiment. The positive control MMS was freshly prepared in acetone on the day of dosing at a concentration of 0.316 mg/mL (corresponding to 5 µg/cm^2). The positive control B[a]P was freshly prepared in acetone on each day of dosing at a concentration of 0.78 mg/mL (corresponding to 12.5 µg/cm^2).

A volume of 25 µL of the dose solutions was applied to the skin model surface which corresponded to an application volume of 16 µL/cm^2. The total exposure time was 48 ± 3 hrs. (repeated application at 48 ± 3 hrs., 24 ± 2 hrs. and 3 hrs. prior to cell isolation) for the test substance and B[a]P, and 3 hrs. (single application) for MMS. During exposure to the study substances, the skin models were maintained at *ca.* 37 ºC, *ca.* 5% CO_2 and *ca.* 95% humidity.

Cytotoxicity measurements included % lactate dehydrogenase (LDH) release, % adenylate kinase (AK) release and % intracellular adenosine triphosphate (ATP) measurements.

Results:
In the dose range finding study, cytotoxicity was observed at and above 50 µg/cm^2 based on the AK assay and at 16 µg/cm^2 based on LDH. As a result, 16 µg/cm^2 was selected as the highest concentration in the first main experiment. 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 statistically significant increase in tail intensity was observed at any of the test concentrations up to 16 µg/cm^2.

As per the current (at the time of testing) Cosmetics Europe SOP for the 3D Comet assay and validation studies, a second experiment was performed using aphidicolin as an inhibitor of DNA repair to increase the sensitivity of the assay. Four concentrations up to 20 µg/cm^2 were tested. The negative and positive controls in this second experiment met the acceptance criteria in both the epidermal and dermal fractions and the study was considered valid. Cytotoxicity was observed at 20 µg/cm^2 based on measurement of intracellular ATP and therefore this concentration was excluded from genotoxicity assessment. No statistically significant increase in tail intensity was observed at any of the test concentrations up to 16 µg/cm^2.

Conclusion:
Under the conditions of the test, 1,2,4-THB is considered not to induce DNA damage to human skin cells after topical application. ***(Ref. 9)***

Note: The SCCS Final Opinion noted that this protocol was still in validation and no OECD guideline was available. This was already cited and acknowledged in the submission that the SCCS reviewed. In our view, the SCCS's comment was odd as lack of compliance with a valid OECD guideline did not deter the SCCS from accepting open literature to assess 1,2,4-THB.
The SCCS also commented that doses 16 and 20 µ/cm^2 were too high and not justified. We note that the dose range finding study informed the choice of the doses and that these studies were

conducted based on the best recommendations of the principal investigator. The two doses cited were the highest of each experiment and represented the limit of cytotoxicity included.

The SCCS further noted that staining would potentially interfere with some of the cytotoxicity measurements and adapted controls should have been taken into consideration. In effect, the dose-related brown staining could certainly interfere with colorimetric-based ATP measurements which was why additional LDH and Adenylate Kinase measurements were added to assess cytotoxicity. However, we consider that this skin staining also demonstrated that reactive intermediates of concern (quinones and semiquinones) had formed and reacted without causing an adverse effect.

Finally. the SCCS also commented that in Experiment 2, for Group C, the positive control BaP +APC the tail intensity was 26±11% for the epidermis (which was slightly below the historical control range of 27-56%) and 27±3% for the dermis (which was within the lower limit of the historical control range of 27-57%). We note that the principal investigator of the study concluded that this slight discrepancy for the epidermis did not affect the validity of the experiment. In effect, the experiments were done using triplicate samples to mitigate such variability.

3D Human Reconstructed Skin Micronucleus Assay using EpiDerm™

Guideline:	Cosmetics Europe Genotoxicity Taskforce Protocol (***Ref. 10***)
Species/Strain:	MatTek EpiDerm™
Replicates:	One tissue model per concentration in dose range finding assay. Three tissue models per concentration in Experiments 1 and 2.
Test Substance:	1,2,4-THB
Solvent:	De-aerated acetone (N_2-purged)
Lot #:	THB0318002
Purity	98.1%
Concentrations:	Dose range finding assay 48 hour exposure: 0.50- 200 μg/cm^2 Experiment 1: 48-hour total exposure: 12- 224 μg/cm^2 Experiment 2: 72-hour exposure: 12- 224 μg/cm^2
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.
GLP:	Yes
Study Period:	December 2018-February 2019

For all experiments, dose solutions of 1,2,4-THB were prepared fresh each day in de-aerated 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/cm^2 were tested based on results of a previous 3D Comet Assay using Phenion® Reconstructed Human Skin. Toxicity was observed at 50 μg/cm^2 (56% reduction in Cytochalsin B Proliferation Index, CBPI) and higher. In Experiment 1, with a 48 hour exposure, and Experiment 2, with a 72 hour exposure, seven concentrations of 12-224 μg/cm^2 were tested.

The actual concentration of the test substance in the dose solutions was determined by UPLC-UV using a validated method.

Triplicate skin models per test group were used in Experiments 1 and 2. In Experiment 1 (48 hour exposure), the negative and positive controls met the acceptance criteria and the study was considered valid. The highest concentration for analysis of micronuclei was 100 µg/cm^2 selected based on induction of a 59% reduction in Cytochalsin B Proliferation Index (CBPI). No statistically significant increase in micronuclei was observed at any concentration of 24, 50 or 100 µg/cm^2 1,2,4 THB. In Experiment 2 (72 hour exposure),, the negative and positive controls met the criteria for a valid study. Cytotoxicity (50 to 60% CBPI relative to the vehicle control) was observed at concentrations ≥ 100 µg/cm^2. Cytotoxicity as measured by RVCC of 50 to 60% was observed at concentrations ≥ 72 µg/cm^2. The concentrations selected for evaluation of micronuclei were 12, 24 and 72 µg/cm^2 (51% toxicity based on RVCC). No significant or dose-dependent increases in micronuclei induction were observed at any concentration ($p > 0.05$; Fisher's Exact and Cochran-Armitage tests).

Results*:*
In the dose range finding study, cytotoxicity was observed at and above 50 µg/cm^2 (56% reduction in the CBPI at 50 µg/cm^2). In Experiment 1 (48 hour exposure), the negative and positive controls met the acceptance criteria and the study was considered valid. The highest concentration for analysis of micronuclei was 100 µg/cm^2 selected based on induction of a 59% reduction in CBPI. No statistically significant increase in micronuclei was observed at any concentration of 24, 50 or 100 µg/cm^2 1,2,4 THB.

Conclusion:
Under the conditions of the assay, 1,2,4-THB was concluded to be negative for the induction of micronuclei in the reconstructed skin micronucleus assay (RSMN) in EpiDerm™ in both the 48-hour and 72-hour treatment. ***(Ref. 11)***

Note: In the SCCS Final Opinion, the SCCS commented that the 95% Lower Control Limit for the historical positive control value was 0.0 in Appendix I and expressed that this required an explanation. Our principal investigator offered the following explanation:

> *Calculation of 95% confidence limits (Mean ±2SD) can yield a lower confidence limit of "0." This is a phenomenon of the calculation and does not reflect a "0" value for the positive control.*

A summary of all completed *in vitro* assays can be found in Section 7 in Table 13: Summary of GLP Genotoxicity Testing.

3.7.2 ***Mutagenicity/Genotoxicity In Vivo***

This section describes the results of a new ***in vivo*** study, which was conducted in compliance with OECD 474 (see **Exhibit B**). The SCCS Final Opinion was based on the conclusion of SCCS 1452/11 which stated that:

> *"In vivo testing would be required to explore the potential to induce gene mutations; such tests are no longer permitted."* (***Ref. 1)***

This new *in vivo* study was conducted after publication of the SCCS Final Opinion. It demonstrated conclusively what was suggested by the *in vitro* evidence: 1,2,4-THB is not mutagenic/genotoxic *in vivo*.

In vivo Micronucleus Assay in CD-1 Mice

Guideline:	OECD 474 (OECD 2016)
Species/Strain:	Mouse/CD-1(Hsd:ICR); Envigo RMS Inc.
Test Substance:	1,2,4-THB
Solvent:	De-aerated acetone (N_2-purged)
Lot #:	S36884V (Sigma-Aldrich)
Purity:	98.1%
Frequency:	1st day of dosing
Collection Site:	Retro-orbital Sinus
Target Volume:	0.5 mL of whole blood
Anesthesia:	Animals were anesthetized prior to collection by 70% CO_2/30% O_2.
Anticoagulant:	K_3EDTA
Sample Handling:	Blood samples were maintained on wet ice until centrifugation.
Centrifugation:	Blood samples were centrifuged for 5 minutes, 2-8°C, at 2000 g within 1 hour of collection, and plasma was harvested into two sets of approximately equal aliquots.
Sample Storage:	Plasma samples were stored at ≤ -60ºC and will be analyzed only as needed to confirm a negative response.
Animal Welfare:	The number of animals, procedures, and design used for this study, has been reviewed and were approved by the BioReliance Institutional Animal Care and Use Committee protocol number 10. All procedures involving animals performed at BioReliance follow the specifications recommended in the most current version of The Guide for the Care and Use of Laboratory Animals adopted by BioReliance

Animal Disposition: Animals were sacrificed by CO_2 overdose after their last collection timepoint.

The micronucleus assay is a short-term *in vivo* cytogenetic assay for detecting agents that induce chromosomal breakage or spindle malfunction. In this study, femoral bone marrow was microscopically evaluated for the presence of polychromatic erythrocytes (PCEs) containing micronuclei (MnPCEs). A clastogenic effect may be seen if micronuclei are formed during cell division as a result of chromosome breakage or malfunctioning of the mitotic spindle. In the case of chromosome breakage, acentric chromosome fragments may not be included in the nuclei of daughter cells forming single or multiple micronuclei in the cytoplasm. An aneugenic effect is seen if the test material interferes with the mitotic spindle apparatus; non-disjunction or lagging chromosome at anaphase may not be included in the

nuclei of daughter cells forming single or multiple micronuclei in cytoplasm of the new cells. In this study, the incidence of MnPCEs served as an indicator of test article genotoxicity.

The test article, 1,2,4-THB, was evaluated for its clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in PCEs in mouse bone marrow. Degassed deionized water was used as the vehicle control. Test and/or vehicle control article formulations were administered once by intraperitoneal injection (IP) at a dose volume of 5 mL/kg. The use of IP administration means that large quantities of the drug can be absorbed quickly.

In the dose range-finding assay, the dose levels tested were 25, 50 and 100 mg/kg in 3 animals/sex. Based upon the results, the high dose for the definitive assay was 25 mg/kg, which was estimated to be the maximum tolerated dose (MTD).

Results:
The definitive assay dose levels tested were 6.3, 12.5 and 25 mg/kg in males only, as it was determined in the dose range finding study that there was no gender-based response discernible. According to analytical testing done as part of the study, the 25 mg/kg high dose met all acceptance criteria. The other two doses were actually tested at levels slightly lower than the nominal dose (i.e., 5.32 and 8.15 mg/kg); the nominal dose levels of 6.3 and 12.5 mg/kg were used in the report. ***Table 5*** below summarizes the micronucleus analysis.

Table 5: Summary of Bone Marrow Micronucleus Analysis

Treatment	Gender	Time (Hrs)	Animals	%PCE (Mean +/- SD)	Toxicity (%)	% MnPCE (Mean +/- SD)	Number of MnPCE/PCE Scored
Vehicle							
0 mg/kg/dose	M	24	6	69.7 ± 9.5	---	0.02 ± 0.02	5 /24000
0 mg/kg/dose	M	48	6	57.6 ± 11.5	---	0.05 ± 0.03	11 /24000
1, 2, 4-Trihydroxybenzene							
6.3 mg/kg/dose	M	24	6	75.6 ± 7.2	9	0.01 ± 0.02	2 /24000
12.5 mg/kg/dose	M	24	6	69.5 ± 9.1	0	0.03 ± 0.02	6 /24000
25 mg/kg/dose	M	24	6	70.3 ± 8.6	1	0.02 ± 0.02	4 /24000
25 mg/kg/dose	M	48	6	64.7 ± 4.0	12	0.02 ± 0.02	5 /24000
CP							
50 mg/kg/dose	M	24	5	69.3 ± 4.4	-1	1.47 ± 0.44**	294 /20000

Positive Control = Cyclophosphamide (CP); Negative Control = vehicle

Conclusion:
No statistically significant increase in the incidence of MnPCEs in the test article treated groups at either time point (24 or 28 hours) was observed relative to the vehicle control group. The positive control induced a statistically significant increase in the incidence of MnPCEs. The number of MnPCEs in the vehicle control group did not exceed the historical control range.

Thus, the administration of 1,2,4-THB at dose levels up to and including a dose level of 25 mg/kg was concluded to be negative in the *in vivo* micronucleus assay. This demonstrates conclusively that 1,2,4-THB is not mutagenic/genotoxic *in vivo.* ***(Ref. 12)***

4.0 Mechanistic Investigations

The additional studies summarized in this section were undertaken to address the questions that the SCCS Final Opinion raised with regard to mode of action, formation of reaction products, and most importantly consumer exposure over time.

4.1 Hydrogen Peroxide Formation

Hydrogen Peroxide Quantification by FOX Assay

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 mΩ, in–house
	Sodium Phosphate Monobasic Monohydrate (NaH2P04. H20) ≥ Sigma-Aldrich
	Phosphate-Buffer Saline (PBS); pH 7.4, Thermo-Fisher
	Gibco Roswell Park Memorial Institute Media **(**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
$\lambda_{detection}$:	560 nm

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.

Results:
The quantitative generation of hydrogen peroxide from 1,2,4-THB was 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. As seen in ***Table 6***, 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.

Table 6: Hydrogen peroxide formation as a function of solvent system

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

*TA at two representative concentrations in dosing solvents were incubated for 30 min at 25^0C
** Average of three replicates

Conclusion:
The data collected in this study confirms 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 and quantitative. The rate and amount of peroxide is dependent on a number of parameters: time, solvent, pH, buffer, ionic salts and transition metal. 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, measurable and biologically relevant. The formation of hydrogen peroxide in aqueous media owing to 1,2,4-THB supports the parallel to hydrogen peroxide in genotoxicity studies; namely, **that effects *in vitro* do not predict *in vivo* effects. *(Ref. 13)***

4.2 Consumer Exposure

A primary consideration in risk assessment with regard to the use of any hair dye is the question of consumer exposure. The main question is often how long the consumer is exposed to the hair dye material and how quickly the 1,2,4-THB is consumed. It has been amply demonstrated in this submission, as well as in the literature, that 1,2,4-THB generates hydrogen peroxide in solution, which is accelerated with increasing pH. At alkaline pH, the reaction proceeds so quickly that measurement of 1,2,4-THB can be a challenge (Clapp et. al. 1990). The study summarized here was conducted internally using the protocol summarized in SCCP 1198/08 and SCCS SCCP 1311/10 ***(Ref. 14, 15)*** as a guidance.

Time Dependent Hair Color Simulating Consumer Usage

Test Article:	Representative Hair Color Formulation, with 2% 1,2,4-THB
Hair Swatches:	90% grey virgin hair swatches
Rinse:	HPLC grade de-aerated acidified water (formic acid); pH 3.10
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

A representative gel-cream based hair color formulation with 2% 1,2,4-THB at pH 9, was made in de-aerated water purged with nitrogen. A placebo or control 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 reverse phase HPLC method was developed and a calibration curve was established using known concentrations of 1,2,4-THB (R.T= 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 gr aliquot of formulation was dispersed in 200 mL de-aerated, acidified water (pH 3.1). De-aerated 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 µL) of this solution is injected into the HPLC and the concentration of 1,2,4-THB measured is designated as T_0, 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 de-aerated, 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 0.45µm 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, as shown below in ***Figure 2.***

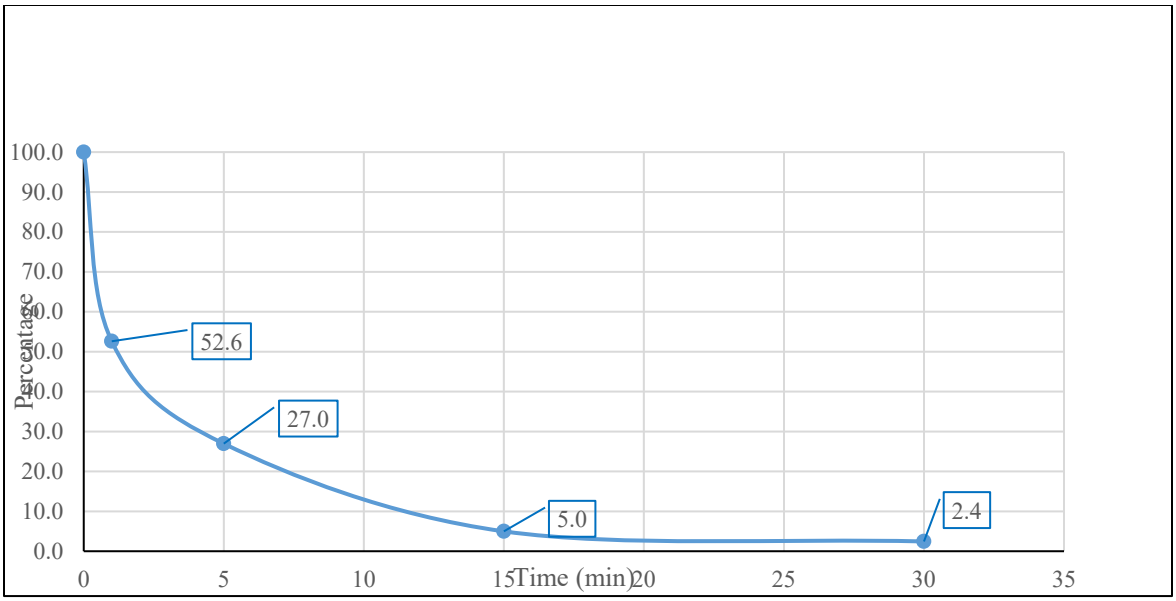


Figure 2: % Concentration of 1,2,4-THB in the rinsate v Time (min)

The data shows that more than 70% of the 1,2,4-THB has reacted within the first five minutes and ~95% of the 1,2,4-THB has reacted by the end of 15 minutes and less than 3% of the original 2% formulation of 1,2,4-THB 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. ***Figure 3*** shows the corresponding color development in the hair swatches for each time point of exposure.

Figure 3: 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 below in ***Figure 4***. 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.

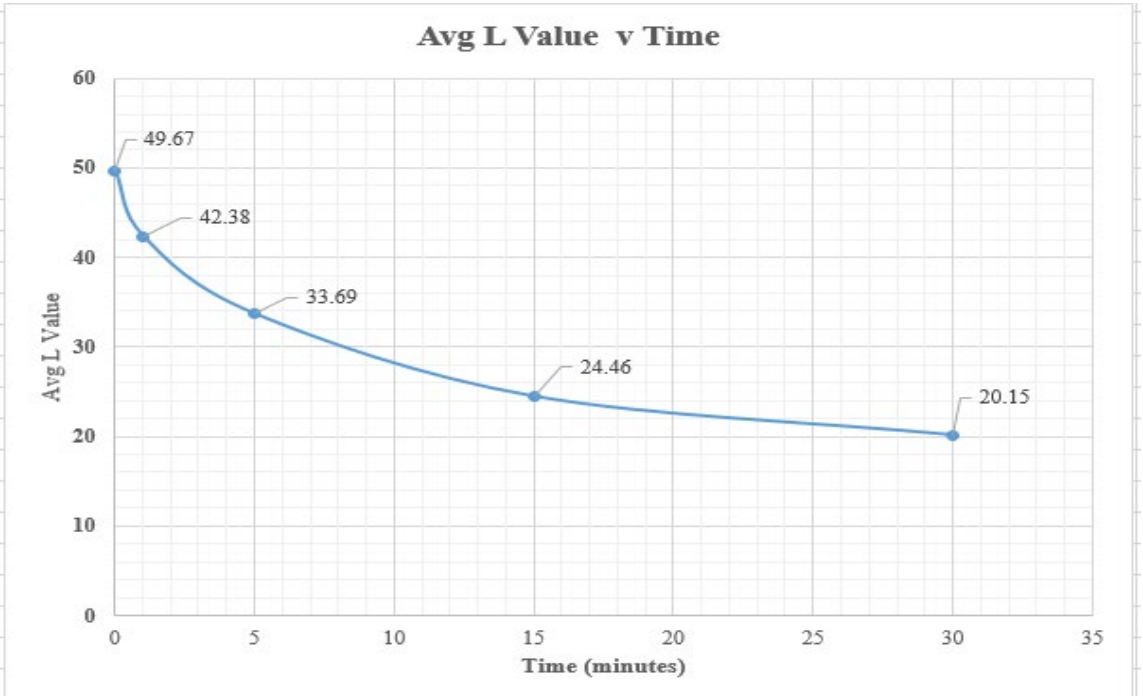


Figure 4: Average L values v. Time (min)

The data collected in this study are presented along with the HPLC chromatograms in the report. These data suggest that the consumer exposure to 1,2,4-THB is expectedly 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. 16)***

4.3 Reaction Chemistry Investigation

1,2,4 –THB reacts in the presence of oxygen and primary intermediates to form coupled products without requiring the action of hydrogen peroxide. This knowledge regarding the reactivity of 1,2,4-THB has existed since the 1970s ***(Ref. 17 and Ref. 30)***.

In opinion SCCS/1452/11 (see ***Ref. 1***), the SCCS expressed the view that the submission had failed to provide sufficient information on the characterization 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-THB in aqueous systems. In order to address this concern, reaction kinetics studies were undertaken in the presence of a well-studied primary intermediate, *para*-Toluene Diamine (PTD) 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 (1,2,4-THB – 1,2,4-THB) was only isolated by controlling the reaction conditions via dilution and temperature reduction and was not isolated under conditions of use of commercialized hair dye formulations. These aliquots were then analyzed 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. ***(Ref. 18)***

The data showed that the reaction of 1,2,4-THB (coupler) with PTD (precursor/primary intermediate) in a basic aqueous medium proceeds according to the proposed reaction pathway, as shown in ***Figure 5a*** below. 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 ***(Ref. 19)***, SCCP/1004/06 ***(Ref. 20)***, SCCP/1198/08 and SCCS/1311/10) and reproduced below as ***Figure 5b***. Precursors/primary intermediates (1,4 or *para*-di-substituted benzenes) react with couplers (1,3 or *meta* di-substituted benzenes) in a very predictable manner. Likewise, 1,2,4-THB reacts in a very predictable manner.

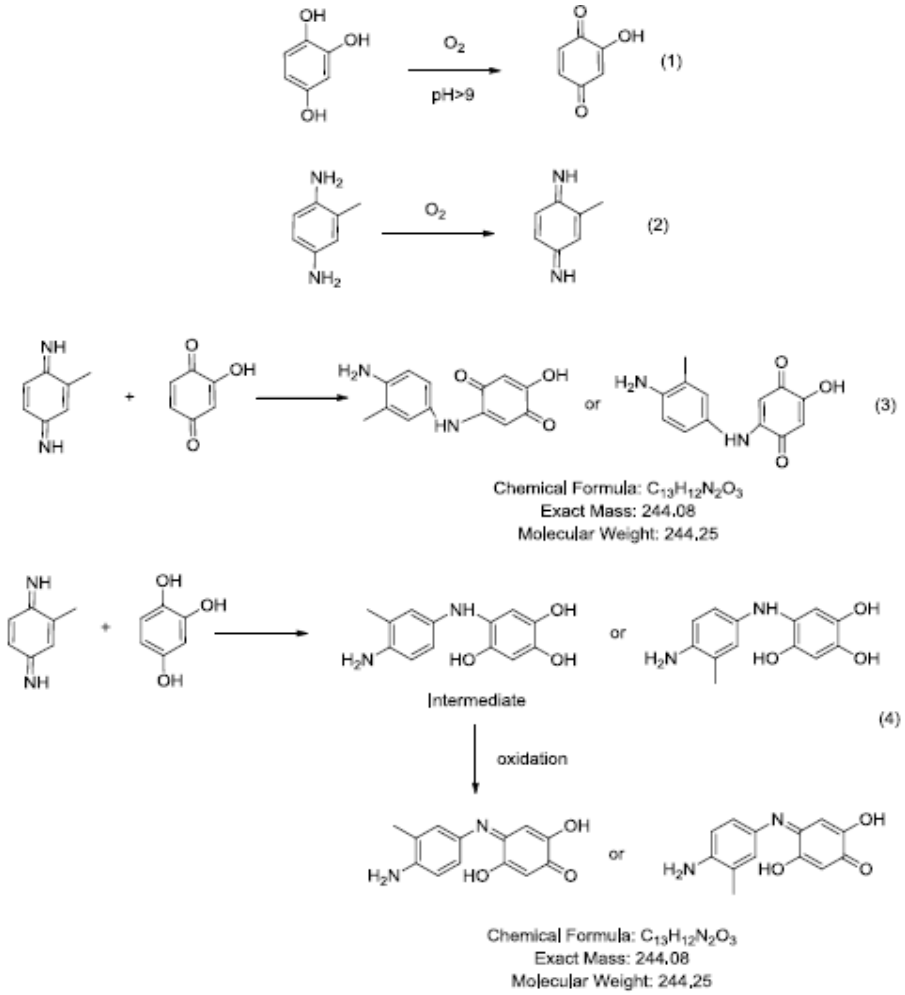


Figure 5a: Proposed reaction mechanism of 1,2,4-THB and PTD. (Ref. 18)

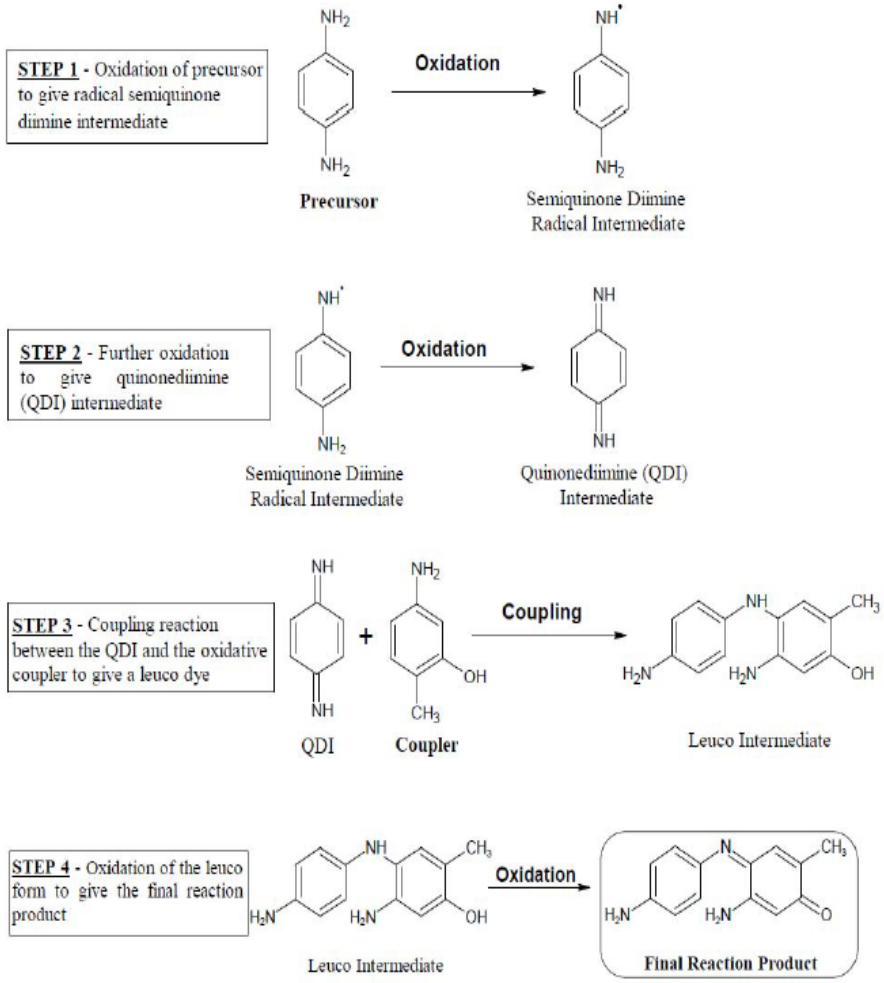
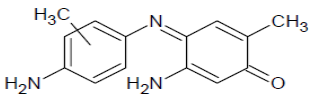
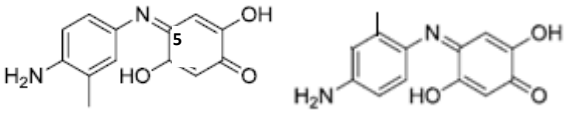
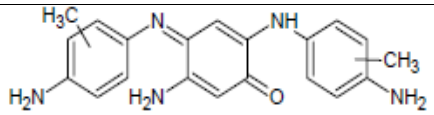
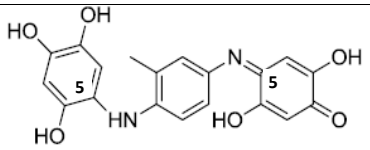


Figure 5b: Mechanism of oxidative hair dye formation, adapted from (Ref. 20)

Table 7 below compares the reaction products of 1,2,4-THB with a primary intermediate such as PTD and historical data from Industry and summarized in SCCP 0941/05. We see the reaction product dimer PTD-THB is analogous to the reaction product of PTD with the coupler 4-Amino 2-Hydroxytoluene (AHT). The trimer that is formed is somewhat analogous to the product of PTD with m-Aminophenol.

Table 7: Comparison of Reaction Products of PTD and 1,2,4-THB with Reaction Products of PTD and AHT and MAP

SCCP 0941/05	1,2,4-THB Reaction Products
 Dimer PTD-AHT	 Dimer PTD-THB
 Trimer PTD-MAP-PTD	 Trimer THB-PTD-THB

The methanol extracts of hair samples did not yield any dimer or trimer products. This is possibly because the coupling reactions may be forming oligomers within the hair shaft, and the reaction products are expected to be too large to be extracted even with a polar solvent such as methanol.

The data further support that 1,2,4-THB has the versatility to react with primary intermediates acting as a coupler and also, to react with coupled products to form trimers, like a primary intermediate such as PPD. Given its ability to auto-oxidize in air, there is no need of a peroxide developer component to initiate the reaction. All the reaction product studies were conducted in the absence of hydrogen 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.

5.0 Literature Review

The SCCS Final Opinion cited a number of articles in the open literature relating to the mutagenic/genotoxic effects of 1,2,4-THB, primarily *in vitro.*

In order to understand the scope of information available on 1,2,4-THB more thoroughly, we conducted a broad literature review. This review highlights that, while 1,2,4-THB can cause genotoxic effects *in vitro* due to hydrogen peroxide formation, this genotoxic effect is prevented by the inclusion of ROS scavengers normally available in living systems. Thus, the literature supports the conclusion of the negative *in vivo* micronucleus study and the negative DNA adduct study that **1,2,4-THB is not genotoxic *in vivo*.**

We conducted two literature searches. We first conducted an in-house search in the PubMed database on 5 October 2018. Alternative synonyms, such as trihydroxybenzene and hydroxyhydroquinone, were used as search terms and found to generate subsets of the search as described below.

Table 8: Literature Search Terms and Numeric Results

Search Terms	Search Details	# of Articles
1,2,4-benzenetriol	"hydroxyhydroquinone"[Supplementary Concept] OR "hydroxyhydroquinone"[All Fields] OR "1,2,4 benzenetriol"[All Fields]	208

The search results were tabulated and the abstracts included and reviewed for each entry. Two of the 208 articles were noted as having been retracted owing to concerns of data integrity and therefore removed from consideration. An additional ten articles were noted as having no abstract available. These were therefore excluded.

Thereafter, we commissioned with NERAC Inc. (a research and advisory firm) an additional and more focused search on DNA damage/genotoxicity. The following search terms and criteria were included:

> *"1,2,4-Benzenetriol, Hydroxyhydroquinone, 1,2,4 trihydroxybenzene, 4-Hydroxycatechol, 2,5-Dihydroxyphenol, Oxyhydroquinone, DNA Damage, Genotoxicity, DNA Repair, Mutagenicity, Mutagenic, Gene Mutation, Genotoxicity in vivo, AMES, DNA Damage, Aneuploidy, Clastogenicity."*

These search results were further curated by NERAC, which reported a list of 48 articles that met the search criteria. This output was compared to the results of the broader PubMed search, resulting in a final list of 209 encompassing publication dates from 1984 to October 2018. The output of the literature search is provided in **Exhibit E** as an EXCEL spreadsheet. All the references are numbered, organized and displayed both in their entirety and by category.

Based on the summary information available in the abstract, each article was sorted into the broad categories identified below.

Table 9: Summary of Categories and Statistics

Categories	Description	Count
1,2,4-THB Dermal Absorption	Substitution pattern related to dermal penetration	1
1,2,4-THB Food/Beverage/Nature	Detection/Measurement of 1,2,4-THB in foods or beverages	20
1,2,4-THB Genotoxicity	Description of genotoxicity studies including 1,2,4-THB	59
1,2,4-THB Toxicity	Studies regarding 1,2,4-THB effects on other tissues/systems	27
Analytical Methods	Methods of detection	7
Catechol & Ferulic Acid	Primarily concerned with catechol/ferulic acid and its action	3
Chemistry	Description of reaction chemistry; electrochemistry; photochemistry	11
Degradation Pathway	Identification of 1,2,4-THB as an intermediate in biodegradation of various phenolics and pesticides by aerobic and anaerobic microbes	77
Synthesis	Synthesis in laboratory	4
Grand Total		**209**

Some of the articles were designated as "not selected for detailed review" (NSDR) owing to inapplicability of the information to the use of 1,2,4-THB as hair dye formulations. The categories that were considered to provide more immediate and relevant additional information on 1,2,4-THB use or exposure in cosmetic hair dyes are as follows:

- 1,2,4-THB Dermal Absorption
- 1,2,4-THB in Food/Beverages
- 1,2,4-THB Toxicity
- 1,2,4-THB Genotoxicity

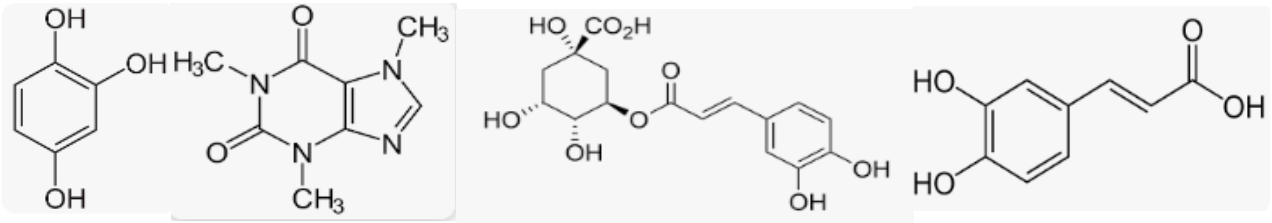
These four categories comprise 107 publications and are summarized below to highlight information that is available to further inform an overall assessment of the safety of 1,2,4-THB. A separate section is devoted to various aspects of the Mode of Action (see Section 5.5), which is focused on genotoxicity of 1,2,4-THB.

5.1 1,2,4-THB Dermal Absorption

Dermal penetration of substituted phenols was investigated by du Plessis et al. (***Ref. 21***). The effect of substitution pattern on the dermal penetration of various di- and tri-hydroxybenzenes was calculated using diffusion coefficients. Main factors influencing diffusion were identified to be dipolar and hydrogen bonding capabilities quantified by the Hansen partial solubility parameters δ_p and δ_h. These in turn are affected by the degree of symmetry of the molecule. When symmetry is absent, as in 1,2,4-THB, the –OH groups cause a very slow diffusion. These data support the historical dermal penetration data that show low percutaneous adsorption and therefore low bioavailability. These data align with our experimental findings (Section 3.4) of 3.70 μg/cm^2 used in the exposure determination and represents the actual value of 1.94 μg/cm^2 (this represents about 0.4% of the applied dose) plus one standard deviation, demonstrating some concordance in dermal exposure assessments.

5.2 1,2,4-THB in Food/Beverages/Nature

Publications in this category were concerned with detection of 1,2,4-THB in foods, plants and beverages. A few of these were subjected to a more in-depth review since, in general, it was felt essential to understand that exposure to 1,2,4-THB is not limited to occupational exposure to benzene or to its use in hair dye formulations. The literature shows that coffee is a significant source of exposure to 1,2,4-THB in Europe and globally ***(Ref. 22, 23)***. Coffee as a dietary anti-cancer aid has been proposed multiple times and some identify polyphenolic components including 1,2,4-THB (***Ref. 24, 25***).



1,2,4-THB Caffeine Chlorogenic Acid Caffeic Acid

Figure 6: Chemical structures of coffee components
*(adapted from Butt & Sultan; see **Ref. 25**)*

Ochiai et al., in 2009 determined the amount of 1,2,4-THB in a cup of roasted coffee as being 0.1 – 1.7 mg (***Ref. 26***). So, 2 cups of coffee contain a maximum of 3.4 mg 1,2,4-THB. More recently, Katada et al., estimated that each cup of coffee may contain 0.57 mg of 1,2,4-THB (***Ref. 27***) which yields 1.14 mg for 2 cups of coffee. In our dermal absorption studies, we estimated conservatively that 2.5% of 1,2,4-THB (580 cm^2 x 3.70 $\mu g/cm^2$), a maximal use formulation of hair color, would provide a systemic exposure of 2.1 mg, which is comparable to 2 cups of coffee a day (roughly the midpoint between 1.14 mg and 3.4 mg found in these studies). However, exposure via 1,2,4-THB in hair dyes can be considered occasional based on their frequency of use versus the regular, daily exposure from coffee. The SCCS failed to consider the effects of a consumer's daily exposure to 1,2,4-THB from coffee consumption as compared to occasional hair dye exposure.

5.3 1,2,4-THB Toxicity

The potential non-genotoxic damage to tissues when exposed to 1,2,4-THB *in vitro*, largely owing to oxidative stress, has also been extensively investigated as a consequence of the considerable interest in benzene metabolism. A significant number of the articles in this section describe effects that were traced back to reactive oxygen formation. The citations identified in this search do not add significant new information, as they are limited to the potential toxicity of 1,2,4-THB *in vitro*.

5.4 1,2,4-THB Genotoxicity

In total, 59 publications were identified to have described the investigation of potential genotoxic effects of 1,2,4-THB and other similar compounds. These publications also include the 19 cited by SCCS in SCCS 1598/18. Some 13 others were identified as multiple publications from the same research laboratory, having the same primary investigators and overlapping objectives. Of the 59, ten publications made no overt attempt to identify a mode of action.

The bulk of the literature on genotoxicity describes *in vitro* investigations conducted to understand the effects of benzene metabolism. The consensus in the literature is that 1,2,4-THB is genotoxic *in vitro* most likely as a result of reactive oxygen species (ROS) production.

Special attention is given in this literature review to those studies that looked at potential *in vivo* effects. Numerous *in vivo* and *in vitro* studies have shown that benzene is primarily metabolized to phenol and after additional hydroxylation, hydroquinone, catechol and 1,2,4-trihydroxybenzene. It is understood that the majority of these phenolic metabolites are excreted in urine as glucuronide and sulfate conjugates. Typical dosing of benzene in these investigations could be classified as acute (e.g., 100, 200, 440, 880 mg/kg or 1760 mg/kg). Effects were measured as early as one-hour post dosing for example by Kolachana et al, (***Ref. 28***; SCCS 1598/18 Ref. 24); and at 1, 24 and 36 hours by Lee and Garner (***Ref. 29***, SCCS 1598/18 Ref. 22). Kolachana et al., in 1993, in recognition of the mode of action, measured the formation of 8-hydroxy-2-deoxyguanosine (8-OHdG) as an indicator of oxidative DNA damage. While they detected an increase in 8-OHdG at the one-hour post benzene dosing, this level drops over the remainder of the study period. This decline in 8-OHdG levels during the time course study of benzene exposure was likely to be the result of normal DNA repair mechanisms (see ***Ref. 56***).

Lee and Garner (see ***Ref. 29***), in their study, measured DNA single-strand breaks (SSB) for benzene, *in vivo*, and benzene metabolites, *in vitro*. The group repeated the *in vitro* studies with the glutathione, catalase and superoxide dismutase. They found that the ability of 1,2,4-THB *in vitro* to induce SSB was completely blocked by the action of catalase whereas glutathione decreased single strand breaks by 53%. Previously, Reddy et al., (cited by both these studies, ***Ref. 30)*** were not able to detect DNA adduct formation after IP administration of 200 and 500 mg/kg benzene over a ten-week dosing period in rats using a ^{32}P-postlabeling assay.

The additional articles beyond the 19 articles cited by SCCS that were identified by this broader literature search do not provide any new information with regard to potential *in vitro* genotoxicity of 1,2,4-THB. One additional *in vivo* DNA adduct study was identified and it showed no increase in oxidative DNA damage. This is discussed below in *Section 5.5 Mode of Action*.

5.5 Mode of Action

A breakdown of the number of papers per category/mode of action is given in ***Table 10***.

Table 10: Mode of Action

Category/ MoA	ROS Formation	Topoisomerase Inhibition	No Mechanism Proposed	No Effect	Total
1,2,4-THB Dermal Absorption	-	-	1	-	1
1,2,4-THB Food/Beverage/Nature	6	-	13	-	19
1,2,4-THB Genotoxicity	42	3	10	4	59
1,2,4-THB Toxicity	12	-	14	1	27
Grand Total	**60**	**3**	**39**	**5**	**107**

The following discussion focuses on mode of action of genotoxicity of 1,2,4-THB.

Role of ROS/Hydrogen Peroxide Formation in in vitro Genotoxicity of 1,2,4-THB

The auto-oxidation of 1,2,4-THB was first reported by Corbett in 1970 ***(Ref. 31)***. It is apparent that the ability of 1,2,4-THB to reduce molecular oxygen to form hydrogen peroxide is independent of biological systems and can occur in cell-free solutions. The ability of 1,2,4-THB to generate hydrogen peroxide in cell-free solutions was measured by Hiramoto et al., ***(Ref. 32)*** in 2001 and confirmed by Akagawa et al in 2003 ***(Ref. 33)***. Hydrogen peroxide formation was also measured as a function of pH as one of an array of polyphenols studied by Clapp et al., (1990) ***(Ref. 34)***. They note that at pH 8, **the reaction was too fast** to be studied. While Akagawa et al. (2002) and Hiramoto et al. (2001), measured peroxide generation of 0.6 mole H_2O_2/mole 1,2,4-THB, for Clapp et al. (1990), the yield of peroxide was 0.37 mole H_2O_2/1mole 1,2,4-THB. These data are key to understanding the mechanism associated with *in vitro* genotoxicity reported in the literature.

An oxidative bacterial mutagenic mechanism was also confirmed by Martinez et al., in 2000 ***(Ref. 35)***, in an *E.coli* tester strain. The WP2 Mutotoxitest assay was used to classify 80 chemicals. In their assessment, 1,2,4-THB was an oxidative mutagen. The mutagenic/genotoxic effects of 1,2,4-THB *in vitro* due to the generation of reactive oxygen were shown to be reduced with ROS scavengers readily available in living systems.

While not directly identifying/examining ROS involvement, Buick et al. (SCCS 1598/18 Ref. 35, ***Ref. 36***) evaluates the *in vitro* gene expression signatures as predictors of toxicological responses. In this study, they applied the TGx-28.65 genomic biomarker in parallel with the *in vitro* micronucleus assay. No information regarding the sample preparation or any control measures to limit oxygen exposure prior to dosing was reported. 1,2,4-THB was determined to induce micronuclei at all concentrations. The TGx-28.65 genomic biomarker analysis classified 1,2,4-THB as genotoxic at the three concentrations studied. In analyzing the pathway, most prevalent were genes involved in p53 signaling, cell cycle G2/M checkpoint response to DNA damage, apoptosis, oxidative stress, cell cycle. The data showing that these genes, sensitive to oxidative stress, were upregulated would be expected given the mode of action. The researchers acknowledge that these data, while potentially useful, must be confirmed with *in vivo* data.

Given the strong data supporting the generation of hydrogen peroxide in cell-free solutions by 1,2,4-THB, demonstrated in the open literature and reproduced in the current study summarized in Section 4.1 (see ***Ref. 14***), a parallel may be drawn between 1,2,4-THB and hydrogen peroxide. In a similar manner to hydrogen peroxide, the positive *in vitro* data for 1,2,4-THB is not believed to be indicative of an *in vivo* effect. This is relevant and analogous to the negative *in vivo* effect demonstrated with 1,2,4-THB. Accordingly, Lee and Garner (1991, see ***Ref 29***) caution against overreliance on *in vitro* data as predictor of *in vivo* effects.

Through the literature search, some 42 papers clearly identify ROS as the putative cause of the demonstrated genotoxic effect of 1,2,4-THB. The overwhelming majority of these papers are *in vitro* studies. Thus, the weight of evidence is overwhelmingly in favor of an ROS mechanism.

Topoisomerase Inhibition by 1,2,4-THB

Three articles identify the potential effect on DNA topoisomerase by 1,2,4-THB. Of these three articles, Pandey et al. (SCCS 1598/18 Ref.33; ***Ref. 37***) was cited by SCCS. Frantz et al. (***Ref. 38***) noted that only 1,4-benzoquinone and hydroquinone were directly inhibitory whereas 1,2,4-THB required activation by peroxidase. Another paper by Chen et al. (***Ref. 39***) described inhibitory effects of 1,2,4-THB at high concentrations (500 μM) and note that inhibition was seen at lower concentrations with peroxidase activation. Additionally, two articles by Lindsey et al. (***Ref. 40***) were categorized under the "no effect" since in their work 1,2,4-THB showed little inhibition of the enzyme. It seems clear that the inhibition is reliant on peroxidase and peroxide.

Lopez-Lazaro et al. described the ability of hydrogen peroxide, generated from residues of epigallocatechin 3-gallate (EGCG), to form complexes with both topoisomerase I and II (***Ref. 41***). EGCG is a major polyphenolic component of green tea and identified as undergoing clinical trials for efficacy as an anti-cancer therapy. It was also identified as a DNA topoisomerase "poison," which is then thought to lead to apoptosis of cancer cells; the cancer cells being more sensitive to topoisomerase inhibition. The group studied the ability of hydrogen peroxide to bind with these enzymes as a test article. The data showed that hydrogen peroxide was able to form topoisomerase complexes, which correlated well to subsequent apoptosis. The result of this investigation highlights again the importance of hydrogen peroxide as the putative cause for these biological effects and seems likely the cause where topoisomerase complex formation with 1,2,4-THB was observed.

No effect

Of the 59 publications categorized as focused on DNA damage/genotoxicity, four of the publications noted no genotoxic effect attributed to 1,2,4-THB. Importantly, one of these publications addresses *in vivo* effects of 1,2,4-THB. Yasuhara et al. (***Ref. 42***) in 2002, described a feeding study of 2% w/w of 1,2,4-THB in male Wistar rats. After one week of feeding, organ sampling showed that levels of 8-OHdG production were in line with levels measured for the control group. While some oxidative stress, as measured by phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide levels was seen in the lungs and heart, it was not seen in the liver, which is known to expresses high levels of catalase. The authors related this to differences in the endogenous enzymes that remove ROS. Lastly, as an additional measure of oxidative stress, thiobarbituric acid reactive substances (TBARS) were measured in the organ homogenates with and without EDTA. TBARS levels of the heart were elevated in rats on the 1,2,4-THB supplemented diet but not in the lungs and liver. The data collected by Yasuhara et al. showing the lack of 8-OHdG induction by 1,2,4-THB does not confirm the increase in adducts reported by Kolachana et al., though it is recognized that the study designs were different.

The second publication showing the lack of genotoxic effect of 1,2,4-THB is Sommers et al. (***Ref. 43***). The DEL assay yeast, in *Saccharomyces cerevisiae,* is considered to be sensitive in detecting intra-chromosomal recombination events resulting in deletions and DNA strand breaks. The results of the work showed that 2% solutions of benzene and its metabolites including phenol, catechol, *p*-benzoquinone and hydroquinone in acetone induced DEL recombination while 1,2,4-THB was observed to be negative as reported in the abstract. A third article by Lindsey et al. is discussed above in the topoisomerase inhibition section.

The last article which is by Hou (see ***Ref. 57***), summarizes work that while mentioning literature data on genotoxicity owing to ROS, the group does not look at genotoxicity per se. They are more concerned with potential radical scavenging capability of 1,2,4-THB. The literature is divided on the subject of a "pro-oxidant" effect of 1,2,4-THB *in vitro* and its potential radical scavenging ability. This is analogous to the overall split seen in the literature on polyphenols as described by Halliwell in his review (***Ref. 44***). Of the literature described here, 15 articles addressing a reactive oxygen mechanism in relation to 1,2,4-THB also discuss its ability to function as a free radical scavenger which is considered characteristic of polyphenols occurring in nature.

5.6 Discussion of Literature

The literature search returned a large number of publications that mention 1,2,4-THB. Support for an ROS mediated mechanism of toxicity, including genotoxicity for 1,2,4-THB, dominates the discussion. This is in agreement with the measured physico-chemical properties and the demonstrated capacity of 1,2,4-THB to generate hydrogen peroxide in neutral and alkaline pH solutions. **Hydrogen peroxide, like 1,2,4-THB, is genotoxic *in vitro* but not *in vivo*. Careful sample preparation of 1,2,4-THB with exclusion of oxygen or supplementation with a radical scavenging system removes these *in vitro* effects.** This effect is well-described in the literature and in new data described in Section 3.7 above.

Relevance of ROS in vivo:

Catalase and superoxide dismutase are two of the key endogenous enzymes that modulate the potential effects of ROS in living systems. For example, the effects seen by Pathak et al. (***Ref. 45***) could be related to inter-strain and inter-organ variability in murine catalase activity as described by Ito et al. in 1982 (***Ref. 46***). The study by Pathak et al. extended studies already conducted by Reddy et al., on benzene, which involved i.p. dosing over a 10-week period, but in B6C3F1 mice rather than rats. Pathak et al. noted that once daily dosing of a range of benzene doses i.p failed to result in DNA adduct formation. DNA adducts were only detected in a twice daily dosing of 100–440 mg/kg of benzene and after three days. They identified a minor adduct (at 250 mg/kg benzene) as correlating to DNA adducts formed with 1,2,4-THB in HL-60 cells. These results were assessed by Whysner, et al. (***Ref. 47***). They note that the low level of adduct formation at a twice a day dosing scheme yields a covalent binding index (CBI) of 0.001 which is comparable to the CBI of saccharin (CBI <0.005), a non-hepatocarcinogen. Whysner et al. observe that if ^{32}P-postlabeling proves to be the more accurate measure of true covalent binding, then this suggest that these low levels of DNA adducts may not play a significant role in benzene-induced carcinogenesis.

B6C3F1 mice used by Kolachana et al. show a reduced catalase activity as compared to other murine strains and significant variability based on tissue sampled. This variation in catalase activity noted by Ito et al. was correlated to duodenal tumor incidence owing to hydrogen peroxide exposure and used to support the use of hydrogen peroxide in tooth whitening products. The catalase measurements are summarized in ***Table 11***, reproduced from Ito et al. and cited in SCCP 0844/04 (***Ref. 48***).

Table 11: Catalase Activity in Different Organs & Strains of Mice

Strain	***Number of mice***	***Mean Catalase activity (10^{-4} k/mg protein)***		
		Duodenum	***Blood***	***Liver***
C3H/HeN	11	5.3 ± 1.4	7.8 ± 0.4	75.3 ± 3.8
B6C3F1	12	1.7 ± 0.2, (32.1%)	7.7 ± 0.1, (97.4%)	62.8 ± 9.8, (83.4%)
C57BL/6N	8	0.7 ± 0.3, (13.2%)	5.1 ± 0.2, (65.4%)	40.7 ± 4.0, (54.1%)
C3H/C	7	0.4 ± 0.1, (7.5%)	0.4 ± 0.2, (5.1%)	33.3 ± 2.6, (44.2%)

Note: Percent values expressed based on C3H/HeN mice

While catalase in murine bone marrow was not determined by Ito et al., this has been investigated by others. It has been shown by Gupta et al. that culturing rat bone marrow with catalase shows dramatic protection from hydrogen peroxide induced hematopoietic effects (***Ref. 49).*** Subramanyam et al. in their review (***Ref. 50***) have considered the differing metabolic capability of the two types of cells in bone marrow (macrophages and fibroblastoid stromal cells) to account for discrepancies in the responses that had been noted for hydroquinone. Hydrogen peroxide-dependent bioactivation of hydroquinone occurred readily in bone marrow macrophages, but not in fibroblastoid stromal cells, indicating macrophages contain an active peroxidase. Similarly, variability in the levels of superoxide dismutase, interspecies (among mammals including humans) and between organs within species has been explored by Marklund (***Ref. 51, 52***) and others.

The levels of catalase and superoxide dismutase in human stratum corneum have been measured by Hellemans et al. in 2003 (***Ref. 53***). This study was conducted to understand the effects of UV exposure on antioxidant capability in the skin. It demonstrates that under normal/baseline conditions, the skin is very competent to manage exposure to reactive oxygen species, i.e., under normal hair dye exposure conditions.

Halliwell and Whiteman reviewed in detail the problems inherent to measuring oxidative damage in general and specifically using 8-OHdG as the sole indicator, or "biomarker" of oxidative DNA damage (***Ref. 54, 55***). They concluded (emphasis added):

> *"…Thus it is **intrinsically unreliable** to measure any single reaction product as an index of oxidative DNA damage, but that is what is usually done, as 8-hydroxy-2-deoxyguanosine (8OHdG)…The **major problems arise from artifactual oxidative damage to DNA and consequent 8OHdG formation during isolation of DNA,** its preparation for analysis and the analysis itself. In general, there is no agreement even on basal levels of 8OHdG in cellular DNA, nor does an agreement seem close*."

Additionally, Halliwell observed that the comet assay can be a good option for studying chemicals that act via oxidative mechanism as it can measure not only DNA strand breaks but also DNA damage during repair. All of these considerations demonstrate that results of *in vitro* and even some *in vivo* studies, when the endpoint is not a clear biomarker, may not be conclusive. This is especially the case when dealing with a material that is auto-oxidative and acts via ROS since the data are very dependent on variables that may not have been adequately addressed. The data from the *in vivo* study in the literature by Kolachana et al. are not considered reliable since there are no additional measures of DNA damage save the 8-OHdG. The level measured could be an artifact, driven by the isolation process and resolved by normal repair mechanisms. This could explain the decrease in 8-OHdG levels over time for benzene and 1,2,4-THB. Repair of 8-OHdG damage in hydrogen peroxide treated cells is very fast as described by Halliwell and Gutteridge (***Ref. 56***) and shown in ***Figure 7***.

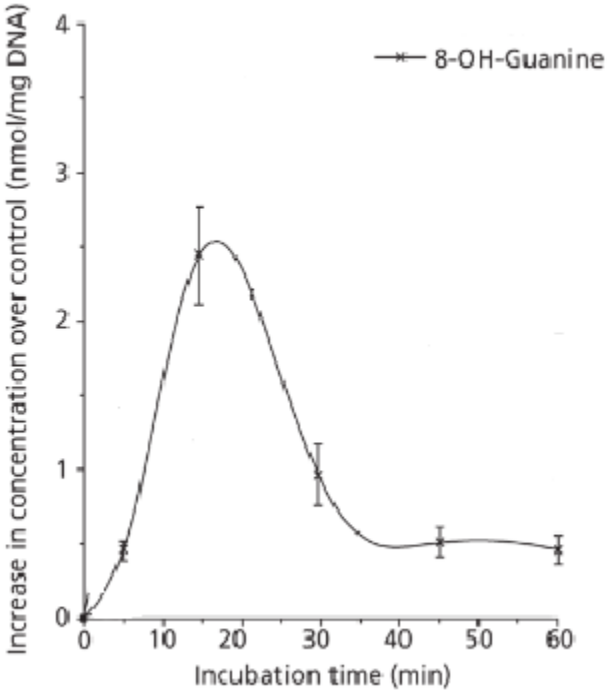


Figure 7: Rates of repair of oxidative DNA base damage.
*Adapted from Halliwell & Gutteridge page 243 (see **Ref. 56**). From data shared by Dr. Jeremy Spencer.*

Importantly, Yasuhara et al. (see ***Ref. 42***) reported no increase in oxidative DNA adducts in Wistar rats. These data do not corroborate the oxidative DNA damage described by Kolachana et al. (SCCS 1598/18 Ref. 24; see ***Ref. 28***) in B6C3F1. However, it should be noted that the latter study was conducted using an i.p. administration.

Many of the publications cited by SCCS in SCCS 1598/18 note an attenuation of effect when an ROS scavenger is added to the test system. This diminution of effect is, for example documented by:

- Lewis et al. (SCCS 1598/18 Ref. 19),
- Pitarque et al. (SCCS 1598/18 Ref. 32),
- Anderson et al., (SCCS 1598/18 Ref. 26),
- Lee and Garner (SCCS 1598/18 Ref. 22),
- Kawanishi et al. (SCCS 1598/18 Ref. 20).

Additional Considerations:

The following additional details should be taken into account in an overall summary of published data:

- Like others in the polyphenolic class of materials, 1,2,4-THB has been shown to function as an antioxidant. Hou et al. (***Ref. 57***) studied *in vitro* as well as *in vivo* (using Sprague-Dawley rats) 1,2,4-THB action as a radical scavenger and have shown some neuroprotective effects as well.

- In order to compare exposures from coffee to exposure from a hair dye formulation, coffee consumption in various countries was reviewed. Depending on the region, the definition of moderate coffee consumption varies. In the UK, moderate coffee consumption was identified as being 3-4 cups/day (***Ref. 58)***. Gunter et al. noted intakes of coffee by daily volume consumed, were highest in Denmark (median 900 mL/day or 4.8 cups/day) and the Netherlands (approx. 3 cups/day) and were lowest in Italy and Spain with 1 cup/day (***Ref. 59)***. In Brazil, consumption was 1-3 cups/day (***Ref. 60);*** and Japanese and US coffee consumption was 1-3 cups per day (***Ref. 61***,***62***). For this comparison, a moderate intake of two cups of coffee per day was assumed.

- Thus, 1,2,4-THB would yield an oral exposure of approximately 2 mg (assuming 2 cups of coffee per day; average of 1.0 mg/cup of coffee; and assuming 100% absorption). An aqueous hair dye solution at neutral pH containing a maximum concentration 2.5% of 1,2,4-THB (580 cm^2 x 3.70 $\mu g/cm^2$) would provide a systemic exposure of 2.1 mg, which is comparable to 2 cups of coffee. However, exposure via 1,2,4-THB in hair dyes can be considered occasional versus the regular daily exposure from coffee. The SCCS failed to consider the effects of a consumer's daily exposure to 1,2,4-THB from coffee consumption as compared to occasional hair dye exposure.

- The formation of 1,2,4-THB *in vivo* owing to the metabolism of benzene is considered minor based on analysis by Inoue et al. in their study of industrial exposure to benzene and their follow-up studies in rats and rabbits (***Ref. 63***). The level of 1,2,4-THB in the urine of workers was measured and found to correspond to ~0.47% of benzene absorbed. Based on separate dosing of hydroquinone and catechol to rats and rabbits, Inoue et al. postulated that 1,2,4-THB arises only from further hydroxylation of hydroquinone but not catechol.

- The exposure to exogenous 1,2,4-THB, as would be the case from a cosmetic application, is inherently different from *in vivo* exposure to benzene. There is general agreement that overall aim of enzymatic hydroxylation *in vivo* is to improve partitioning of the benzene metabolite into water so that it can be excreted. Because of its high solubility in water, 1,2,4-THB does not readily cross the protein-lipid bilayer of the skin. Multiple dermal exposure studies have confirmed that 1,2,4-THB is poorly absorbed through the skin (SCCS 1598/18; SCCS 1452/11).

- Overall, as observed by Halliwell, hydrogen peroxide formation *in vivo* is a fundamental reality of normal cellular function (1974, ***Ref. 64***). Indeed, the management of oxygen has long been considered a key driving force behind the evolution of life (e.g., ***Ref. 65***).

Based on the data in the published literature, specific to 1,2,4-THB genotoxicity and the relevant clarifying publications cited here, 1,2,4-THB can cause genotoxic effects *in vitro* owing to hydrogen peroxide formation. However, this genotoxic effect is prevented by the presence of ROS scavengers normally available in living systems. Therefore, this review of the literature supports the conclusion, based on the negative *in vivo* micronucleus study and the negative DNA adduct study, that **1,2,4-THB is not genotoxic *in vivo*.**

6.0 Expert Analysis of Historical Literature

In this section, the published literature concerning 1,2,4-THB and cited by the SCCS, was reviewed by Dr. Helmut Greim with input from Prof. Barry Halliwell. The review is attached hereto as **Exhibit C** and is reproduced in large part below. Dr. Greim's report has not been updated to include the new *in vivo* study data set forth in this submission. Emphasis in formatting was at Dr. Greim's discretion. This assessment is organized as follows:

1. Evaluation of the weight of evidence to support an absence of genotoxicity *in vivo*.
2. Consideration of the underlying mechanism to evaluate whether 1,2,4-THB is a hydrogen peroxide releasing compound with a mode of action similar to hydrogen peroxide.
3. Comparison of 1,2,4-THB exposure from hair dye formulations vs. estimated dietary exposure.

6.1 Weight of Evidence (WoE) to support an absence of 1,2,4-THB genotoxicity in vivo

The following review of the relevant literature supports the absence of 1,2,4-THB genotoxicity *in vivo*.

- **In a cell-free system,** 1,2,4-THB (purity not specified) was tested for its ability to generate $O_2^{\bullet-}$ radicals at physiological pH by measuring the SOD (superoxide dismutase)-inhibitable reduction of cytochrome c. The results show that 1,2,4-THB is auto-oxidised and produced significant quantities of $O_2^{\bullet-}$ radicals. At a concentration of 1 mM, the rate of $O_2^{\bullet-}$ radical formation was 7.5 nmol/min. The addition of ferric iron 10 nM $FeCl_3$ enhanced the rate of cytochrome c reduction by 1,2,4-THB to 17.4 nmol/min. DNA strand breaks induction was investigated in supercoiled ØX-174 DNA, which was exposed to 1,2,4-THB with and without scavengers of reactive oxygen, namely, benzoate, catalase or SOD. 1,2,4-THB was very efficient at inducing DNA strand breaks and caused a complete DNA degradation at 5.5×10^{-6} M. The ED50 was 7×10^{-7} M. Catalase and benzoate inhibited this effect. Taken together, **these data support the view that 1,2,4-THB induce DNA damage through the production of oxygen radicals** (Lewis et al. 1988; SCCS 1598/18 Ref. 19, ***Ref. 66***).

- **In a reaction mixture of DNA fragments,** 1,2,4-THB induced DNA damage, most likely by hydroxyl radical $OH^{\bullet-}$, which was produced by auto-oxidation of 1,2,4-THB. The DNA damage was inhibited by scavengers and increased by Cu(II) (Kawanishi et al. 1989, SCCS 1598/18 Ref. 20, ***Ref. 67***). In this investigation, a DNA sequencing technique using ^{32}P5'-end-labeled DNA fragments obtained from human c-Ha-ras-1 proto-oncogene was used. The reaction mechanism was studied by UV-visible and electron-spin resonance spectroscopies, the extent of DNA damage was estimated by gel electrophoretic analysis. 1,2,4-THB (purity not specified) caused strong DNA

damage even without alkali treatment, when exposed 90 minutes in a concentrations of 5 mM with the reaction mixture of [^{32}P]DNA fragments in buffer. DNA damage induced by 1,2,4-THB was inhibited completely by catalase, superoxide dismutase (SOD) and methional, but not by sodium formate, another hydroxyl radical scavenger. The addition of bathocuproine, a Cu(I)-specific chelating agent, inhibited DNA damage. The effect was accelerated by the addition of Cu(II). Fe(III) did not have any significant effects on DNA damage induced by 1,2,4-THB, even though the addition of Fe(III) increased hydroxyl radical production during the auto-oxidation of 1,2,4-THB. The addition of Cu(II) did not increase hydroxyl radical production, but copper ions were shown to be necessary for the induction of DNA damage by 1,2,4-THB.

- ***In vitro*,** 1,2,4-THB (200 µM) resulted in DNA adduct levels of 0.21 x 10^{-7} in HL-60 cells. Addition of 50–250 µM H_2O_2 to HL-60 cells had no effect on adduct formation by 1,2,4-THB. DNA adducts were measured by [^{32}P]-postlabeling. **Significant increases of cellular H_2O_2 levels were observed after exposure of HL-60 cells to 1,2,4-THB.** The authors previously reported induction of relative DNA adducts *in vitro* in peroxidase containing cells such as promyelocytic HL-60 cells, mouse bone marrow macrophages and human bone marrow cells treated with benzene metabolites. No DNA adducts were observed in cells lacking detectable peroxidase activity. 1,2,4-THB is known to be a reducing co-substrate for peroxidase enzymes and **the formation of DNA adducts in cells treated with 1,2,4-THB was dependent on peroxidase activity. However, the addition of H_2O_2 to HL-60 cells did not influence the DNA adduct levels produced by 1,2,4-THB treatment** (Lévay and Bodell 1996, ***Ref. 68***).

  1,2,4-THB (10 µM) increases 8-hydroxy-2-deoxyguanosine (8-OHdG), **a biomarker for oxidative DNA damage** in HL-60 cells, a human leukemia cell line, which, like the bone marrow, contains high levels of myeloperoxidase. The increase of 8-OHdG was 3.8-fold within 0.5 hours (Zhang et al. 1993, ***Ref. 69***; Kolachana et al 1993***)***.

- ***In vivo*,** in a mouse bone marrow micronucleus test conducted according to OECD guideline 474 (1983), Swiss OF1 mice with 5 per sex per dose were exposed to 0 or 50 mg/kg 1,2,4-THB (purity: 99.4%) i.p (SCCP 962/05). Bone marrow cells were collected 24 and 48 hours after treatment. Dose selection was based on the results of preliminary assays, in which two mice per dose received i.p. doses between 50 and 2000 mg/kg-bw. Mortality and clinical signs were observed over a period of 48 hours. Doses of 500 mg/kg bw and higher were lethal for all animals immediately after treatment, 100 mg/kg-bw caused sedation after 6 hours and lethality of all mice 24 hours after treatment. The dose of 50 mg/kg-bw induced piloerection and hypokinesia after 6 hours and piloerection 24 and 48 hours after treatment. Based on these results, 50 mg/kg-bw is considered a maximum tolerated dose and was used in the micronucleus assay. The same clinical signs were observed in the micronucleus test. One mouse died and was replaced from a supplementary treated group. The ratio of polychromatic to normochromatic erythrocytes (PCE/NCE) decreased in a statistically significant manner. Thus, toxicity and exposure of the target cells has been proven. **1,2,4-THB exposure of male and female Swiss OF1 mice did not result in an increase in the number of erythrocytes with micronuclei.** Thus, in this test system, 1,2,4-THB is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

However, SCCS noted that this test has not been performed in accordance with the current OECD guideline because only one dose was tested and thus has limited value. This is formally correct. However, as indicated above, the single selected dose showed toxicity at the target organ (decrease in the PCE/NCE ratio) and showed clinical signs and mortality (one animal died). Therefore, there is the clear conclusion that this was a well-defined highest dose. Considering the underlying mechanisms and the negative *in vivo* data for hydrogen peroxide, it is not plausible that lower doses will give positive results. This is further supported by the study of Lee and Garner (1991; SCCS 1598/18; ***Ref. 28***). *In vitro,* GSH and catalase scavenged the DNA damaging effect of the benzene metabolite 1,2,4-THB, whereas an i.p. application of 1760 mg/kg benzene to male and female mice did not induce DNA strand breaks in bone marrow cells.

In the bone marrow of B6C3F1-mice, 1,2,4-THB has been tested (Kolachana et al. 1993) for its possible induction of oxidative DNA damage as determined by 8-hydroxy-2'-deoxyguanosine (8-OHdG). The detection of 8-OHdG provides a direct measure of genotoxic effects of active oxygen. Three mice per group were given i.p. injections of 1,2,4-THB (purity not specified) doses of 25, 50, or 75 mg/kg bodyweight, dissolved in PBS. A fourth group of 3 mice served as control and received an equivalent amount of PBS alone. All mice were euthanized 1 hour after treatment. **1,2,4-THB significantly increased oxidative DNA damage (8-OHdG) approximately 2.5-fold at the dose of 25 mg/kg bodyweight, whereas the effect at 50 and 75 mg/kg bodyweight was not significantly different from controls.** The authors provided no explanation for the peak in DNA-damage at 25 mg/kg bodyweight.

This is in contrast to the study in female Swiss OF1 mice. A single dose of 50 mg/kg did not induce micronuclei (see above). It was also not corroborated by Yasuhara et al. (2002) in their 1-week feeding study in Wistar rats. Organ sampling showed that levels of 8-OHdG production were in line with levels measured for the control group. While some oxidative stress as measured by phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide levels was seen in the lungs and heart, it was not seen in the liver (which is known to expresses high levels of catalase). The authors related this to differences in the endogenous enzymes that remove reactive oxygen species (ROS). Lastly, as an additional measure of oxidative stress, thiobarbituric acid reactive substances (TBARS) were measured in the organ homogenates with and without EDTA. TBARS levels of the heart were elevated in rats on the 1,2,4-THB supplemented diet, but not in the lungs and liver. The data collected by Yasuhara et al. showing the lack of 8-OHdG induction by 1,2,4-THB does not confirm the increase in adducts reported by Kolachana et al. (1993), though it is recognized that the study designs were different.

- **The effects of antioxidants:** To investigate the involvement of reactive oxygen species, several authors introduced scavengers of reactive oxygen species to their test systems. Anderson et al. (1995) showed that catalase abolished DNA damage in the Comet assay; Pitarque et al. (2006) reported the abolishing effects of GSH and vitamin C, Lee and Garner (1991) that of GSH and catalase, Kawanishi et al. (1989) that of catalase, superoxide dismutase and methional. The involvement of reactive oxygen species is further supported by the specific sensitivity of HL-60 cells, which as a bone marrow derived cell line contains high levels of myeloperoxidase (Kolachana

et al 1993, Zhang et al 1993). Further, the increase of micronuclei in HL-60 cells in presence of Cu^{2+}, as Cu2+ catalyses the formation of reactive oxygen species (Zhang et al 1993).

6.2 1,2,4-THB as a hydrogen peroxide releasing compound

6.2.1 Genotoxic Mechanisms of 1,2,4-THB and Hydrogen Peroxide

According to Annex III to Regulation 1223/2009 on Cosmetic Products, as last amended, hydrogen peroxide can be used in hair color products, **either present or released,** at concentrations up to 12%. In hair color products, hydrogen peroxide is used to form reactive oxygen species to oxidise the primary intermediate in hair dye formulations to the quinone imine. Similarly, 1,2,4-THB releases hydrogen peroxide (Akagawa et al. 2003 and Hiramoto et al. 2001), which also forms reactive oxygen species. If the oxidative mechanisms of H_2O_2 and 1,2,4-THB are the same, 1,2,4-THB can be considered as a hydrogen peroxide releasing substance.

6.2.2 1,2,4-THB

While appreciating the overall conclusion of the SCCS regarding the genotoxicity of 1,2,4-THB *in vitro*, it must be noted that most of the tests on which the conclusion lies, have deficiencies. Moreover, it should be noted that the majority of the studies on which the SCCS relied were performed to investigate the mode of action of benzene toxicity. In most cases, **the authors conclude that 1,2,4-THB is not genotoxic, per se**. Specifically, the following studies noted that 1,2,4-THB triggers interaction with DNA via the production of reactive oxygen species:

- Anderson et al. 1995,
- Andreoli et al. 1997,
- Hiramoto et al 2001,
- Kawanishi et al. 1989,
- Kolachana et al. 1993,
- Lewis et al. 1988,
- Jia et al. 2016,
- Moretti et al. 2005;
- Pellack-Walker and Blumer 1986,
- Pitarque et al. 2006;
- Zhang et al 1993.

Pellack-Walker and Blumer (1986) describe two mechanisms for the activation of 1,2,4-THB. First, is its oxidation to the corresponding quinone by sequential one-electron or direct two-electron oxidations, which might form covalent adducts with nucleophilic sites in DNA. Second is the formation of reactive oxygen species during its auto-oxidation (Greenlee et al. 1981; ***Ref. 70***). 1,2,4-THB can be oxidized by molecular oxygen to semiquinone with the concomitant generation of superoxide radicals. Alternatively, it can be viewed as the sequential two-electron reduction of oxygen to form hydrogen peroxide. In cellular systems, the semiquinone can be enzymatically reduced by NADPH-cytochrome P-450 reductase back to 1,2,4-THB or further oxidized to the quinone, again accomplished with

superoxide formation. In the presence of transition metal ions, superoxide radicals may further react with hydrogen peroxide to yield hydroxyl radicals (see ***Figure 8*** below). Since the studies by Zhang et al. 1993, Pitarque et al. 2006 clearly demonstrated oxidative DNA damage and Kolachana et al. (1993) in the formation of 8-OHdG, the genotoxicity of 1,2,4-THB can be seen as preferentially induced by oxidative DNA damage.

Formation of reactive oxygen species is further confirmed by the scavenging effects of antioxidants such as:

- Catalase (Andersen et al. 1995, as above)
- Catalase and benzoate, both scavengers of reactive oxygen (Lewis et al. 1988, as above)
- Catalase, superoxide dismutase (Hiramoto et al 2001, as above)
- Superoxide dismutase and methional (Kawanishi et al. 1989, as above)
- Vitamin C (Pitarque et al. 2006, as above)
- DMSO, a hydroxyl radical scavenger (Kolachana et al. 1993, as above)

All of these can be considered to clearly prevent *in vivo* genotoxicity of 1,2,4-THB.

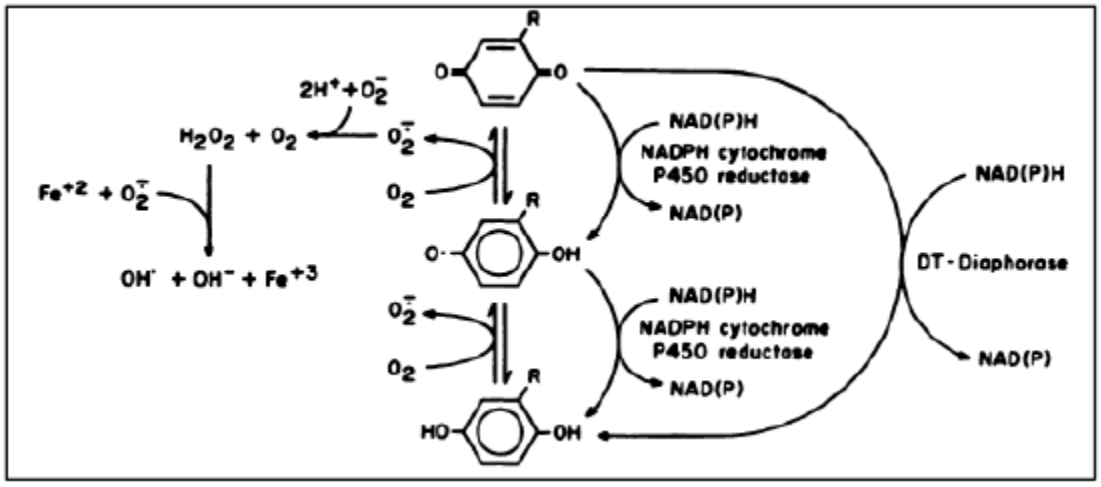


Figure 8: Potential toxification and/or detoxification pathways
For benzoquinone and benzenetriol via redox cycling between the quinone and its hydroxylated counterpart. Reaction scheme also demonstrates the concomitant production of reactive oxygen species. Benzoquinone: R = H; benzenetriol: R = OH (reproduced from Pellack-Walker and Blumer 1986)

6.2.3 Hydrogen Peroxide

In its opinion concerning hydrogen peroxide and hydrogen peroxide-releasing substances used in oral care products, adopted by the Scientific Committee on Cosmetic Products and Non-Food products intended for Consumers of 23 June 1999, the committee described the many positive *in vitro* genotoxicity tests and the mostly negative *in vivo* studies for genotoxicity of H_2O_2 and referenced the European Chemicals Bureau on the mutagenicity of hydrogen peroxide (ECB 2003):

"In conclusion, the available studies are not in support of a significant genotoxicity/mutagenicity for hydrogen peroxide under in vivo conditions. A wider database in vitro of genotoxicity and mutagenicity observations on other relevant target tissues in direct contact with hydrogen peroxide is, however, desirable. Mechanistic studies suggest that cells are adapted to repair DNA damage caused by oxidants; on the other hand, there is some evidence that the hydrogen peroxide may inhibit repair of DNA lesions inflicted by other types of reactive chemicals (Chung et al 1995, Pero et al 1990, Hu et al 1995). According to the principles followed by the EU, hydrogen peroxide is not classified as a mutagen."

The SCCP endorsed the conclusions of the European Chemicals Bureau on hydrogen peroxide. In its opinion on "Oxidative hair dye substances and hydrogen peroxide used in products to colour eye lashes" (SCCS/1475/12) of December 2012, it referred to the previous evaluation of hydrogen peroxide again supporting this previous conclusion (SCCP/1129/07).

The opinion describes the activation of H_2O_2 to reactive oxygen species as follows:

"The oxidative reactivity of hydrogen peroxide with biological molecules such as carbohydrates, proteins, fatty acids or nucleic acids is not pronounced in the absence of transition metals, except for a few nucleophilic reactions. In the organism, the highly reactive (and thus toxic) hydroxyl radical can be produced non-enzymatically from superoxide anion and hydrogen peroxide through catalysis by transition metal ions like Fe2+ and Cu+ (the so-called Haber-Weiss- and Fenton reactions):

$$H_2O_2 + O_2^{-\bullet} \rightarrow OH\bullet + OH^- + O_2 \text{ (Haber-Weiss reaction)}$$
$$H_2O_2 + Fe^{2+}/Cu^{+} \rightarrow OH\bullet + OH^- + Fe^{3+}/Cu^{2+} \text{ (Fenton reaction)}$$

In all likelihood the "full" Haber-Weiss reaction (i.e., the reduction of H_2O_2 by $O_2^{-}\bullet$) is as follows (showing that the Fenton reaction is representing one particular part of the Haber-Weiss reaction):

$$O_2^{-}\bullet + Fe^{3+}/Cu^{2+} \rightarrow O_2 + Fe^{2+}/Cu^{+}$$
$$H_2O_2 + Fe^{2+}/Cu^{+} \rightarrow OH\bullet + OH^- + Fe^{3+}/Cu^{2+}$$"

According to Pellack-Walker and Blumer (1986), the same reaction occurs with 1,2,4-THB (see above and ***Figure 8***). Formation of hydrogen peroxide from 1,2,4-THB in various solvents was determined and confirmed to be present in biologically relevant amounts by means of additional studies performed (See Section 3.7).

6.3 Comparison of 1,2,4-THB exposure from hair dye formulations vs. estimated dietary exposure

1,2,4-THB is a component of coffee. Based on the concentrations determined by Ochiai et al (2009) and Katada et al (2018), 2 cups of coffee contain 3.4 mg (Ochiai) and 1.14 mg (Katada) of 1,2,4-THB, respectively. Assuming a 100% absorption, the daily exposure to 1,2,4-THB of a 60 kg adult is 0.056 and 0.018 mg/kg-bw, respectively. In Submission VI, the 1,2,4-THB exposure from a typical

hair dyeing process was estimated. The application of a hair dye aqueous solution containing 2.5% of 1,2,4-THB would result in a systemic exposure of 0.035 mg/kg (or 2.1 mg for 60 kg person), which is in the range of the exposure from 2 cups of coffee.

Overall, considering that the application of a hair dye formulation is occasional, whereas the exposure to coffee is a daily occurrence for much of the population, 1,2,4-THB exposure from a hair dye formulation can be considered negligible. The SCCS failed to consider the effects of a consumer's daily exposure to 1,2,4-THB from coffee consumption as compared to occasional hair dye exposure.

6.4 Conclusions

Based on the data available in the literature and from previous submissions to the SCCS with respect to the safety of 1,2,4-THB, the following can be concluded:

1. *In vitro* 1,2,4-THB induces sister chromatid exchanges (SCEs), micronuclei, chromosomal aberration and gene mutations (6-thioguanine resistance) in mammalian cells. The substance induces DNA strand breaks, which are elevated by addition Cu^{2+} or Fe^{3+}. 1,2,4-THB interferes with microtubule assembly. **All of these effects are considered to be the result of the formation of reactive oxygen species.** DNA damage induced by 1,2,4-THB is inhibited by superoxide dismutase, catalase and benzoate and other hydroxyl radical scavengers. The addition of S9-mix greatly reduced the effects, which suggests that the compound is negative *in vivo* due to rapid inactivation. This is supported by a negative *in vivo* micronucleus test in mice. Although not completely meeting the requirements of the current OECD 474 test guideline, which requires several doses, the single dose used meets the requirements of the highest tolerated dose. It induces slight bone marrow toxicity as indicated by the reduction of polynucleated erythrocytes but did not result in increased micronuclei in erythrocytes. It is not plausible that lower concentrations would show genotoxic effects and higher test doses would only demonstrate toxicity. In addition, in 2002, a feeding study by Yasuhara et al. showed no oxidative DNA damage in rats.

2. As explained above, 1,2,4-THB, like hydrogen peroxide, triggers the formation of reactive oxygen species via identical mechanisms. According to Annex III to Regulation 1223/2009 on Cosmetic Products, as last amended, hydrogen peroxide can be used in hair color products, **either present or released,** at concentrations up to 12%. In hair color products, hydrogen peroxide is used to form reactive oxygen species to oxidise the primary intermediate, which forms the quinone imine, the reactive intermediate in oxidative coupling. Similarly, 1,2,4-THB releases hydrogen peroxide, which also forms reactive oxygen species. Since there is strong evidence that the oxidative mechanisms of H_2O_2 and 1,2,4-THB are the same, this allows for the conclusion that 1,2,4-THB can be considered as a hydrogen peroxide releasing compound. In analogy to hydrogen peroxide and due to potent anti-oxidative mechanisms, 1,2,4-THB can be considered negative *in vivo*.

3. The application of a hair dye aqueous solution containing 2.5% of 1,2,4-THB would result in an exposure of 0.035 mg/kg-bw, which is in the range of the exposure from 2 cups of coffee which is between 0.018 mg/kg-bw and 0.056 mg/kg-bw. Furthermore, the exposure via 1,2,4-THB in hair dyes can be considered occasional and therefore, much less significant versus the regular and often

times repeated daily exposure from coffee (see Section 7 below for the Margin of Safety (MoS) calculation).

7.0 General Discussion and Conclusion

This section summarizes all the data collected from the recent investigations. For this submission as well as submissions to SCCS, the use of QSAR was not considered helpful in that it provided no new insight. The key programs in use for *in silico* assessments, such as DEREK, are knowledge-based systems which rely on published literature to populate their structure activity assessments. We know from our literature review that the literature provides evidence of some genotoxic effects for polyhydroxybenzenes as a class of chemicals. We understand from fundamental organic chemistry that the substitution pattern for benzene and indeed any aromatic substance drives reactivity and moving one substituent on a benzene ring can dramatically alter the behavior of the molecule. In recognition of this, we have not included *in silico* findings in the overall risk assessment and have confined the review to actual data collected using the latest validated methods on the molecule in question.

Dermal Absorption:

The percutaneous absorption of 1,2,4-THB was determined using human dermatomed skin. A large proportion of the applied 1,2,4-THB (mean of 99.3%) was washed off the surface of the skin 30 minutes after application. The proportions of the applied dose that were recovered from the stratum corneum, epidermis and dermis were 0.083%, 0.196% and 0.020%, respectively. The proportion recovered from the flange was 0.034% of the applied dose. The mean total non-absorbed dose (donor chamber, skin wash at 30 minutes and 24 hours, stratum corneum and flange) of 1,2,4-THB represented 101% of the applied dose.

The results obtained in this study indicate that the total systemically available dose of 1,2,4-THB was 1.71 µg equivalents/cm^2 (Mean + 1SD) when formulated without PTD and 3.70 µg equivalents/cm^2 (Mean + 1SD) when formulated with PTD. The margin of safety was calculated using the systemically available dose of 1,2,4-THB with PTD of 3.70 µg equivalents/cm^2.

The mean total systemically available dose (epidermis, dermis and receptor fluid) of 1,2,4-THB was 0.226% of the applied dose. This indicates a material that is ultimately very poorly absorbed and therefore not bioavailable to an appreciable level after application to the skin; even less to incidental skin contact. The dermal absorption of 1,2,4-THB makes sense when the high solubility in water and small Log Po/w. Compared to dermal absorption for another commonly used hair dye, such as PTD, dermal absorption represents a critical data point around which any considerations of toxicity or genotoxicity should be centered.

Sensitization:

Several *in vitro* methodologies to test sensitization have been validated and an OECD protocol published since opinion SCCS/1452/11. The sensitization potential of 1,2,4-THB was further investigated even if the SCCS did not identify it as a data gap in opinion SCCS/1452/11 (***Ref. 1***). The KeratinoSens™ Assay

showed an EC1.5 = 374.31 µM. Based on these results, 1,2,4-THB was predicted to be a skin sensitizer which agrees with historical data collected on this ingredient.

Reaction Chemistry:

The investigations, both in the literature and in the laboratory for this submission, support that the chemistry fits well within the existing knowledge framework of hair dye chemistry. Foundationally, these are coupling reactions that rely on the formation of a highly reactive oxidized transient species which then reacts to form colored molecules. As noted in Section 5.5 - Role of ROS/Hydrogen Peroxide Formation, the auto-oxidation reaction in room air is pH dependent, almost non-existent in acidic medium and slow at neutral pH. In alkaline pH, the reaction is too fast to be measured, as noted by Clapp et.al. (see ***Ref. 34***). The reaction products under normal consumer exposure scenarios can be predicted and have been determined. Moreover, the genotoxicity studies undertaken can be considered to assess not only the molecule in question but also air oxidation products that may be formed in a typical exposure.

The SCCS Final Opinion expressed concerns that quinones and semi-quinones that are considered to be formed as reactive intermediates were not adequately studied or characterized and could be generated intracellularly to cause DNA damage. It must be noted that products of auto-oxidative coupling, as well as the transient reactive intermediates (such as semi-quinones and quinones) were available to the tissues upon dosing, in all of the assays, to cause any genotoxic effect during the conduct of the assays. The special condition required to allow the auto-oxidation to proceed is **room air**. This is the reason that particular attention was given to the preparation of dosing solutions in carefully de-aerated solvents after weighing and mixing in Nitrogen gas-purged glove boxes. Stabilizing the 1,2,4-THB can be accomplished by excluding oxygen (and lowering pH).

The evidence of the generation of these intermediates was observed in every assay conducted, via staining of the media or skin as the case may be. No adducts or micronuclei were noted in the either the Full Thickness Phenion® Comet Assay or the 3D Skin Micronucleus Assay as a result of these exposures, even though color formation was noted as a clear signal that auto-oxidation had occurred.

Lastly, the SCCS concern about these reactive intermediates being generated intracellularly may be pre-supposing an environment like in the *in* vitro assay where free oxygen is readily available intracellularly and normal compensatory mechanisms are not available. This is not the case *in vivo* where oxygen is bound to heme protein and compensatory enzymes such as catalase and superoxide dismutase are circulating to manage intracellular ROS. In addition, 1,2,4-THB has been identified as an end product of Phase I metabolism (a primary goal of this metabolic pathway is improved water solubility) of benzene which in then excreted out. The extreme water solubility of 1,2,4-THB is critical characteristic to keep in mind as it tends to minimize cross-membrane diffusion and makes rapid excretion more likely. Another key assumption to allow for intracellular quinone formation is bioavailability. This hinges on dermal absorption of 1,2,4-THB which is only poorly absorbed as noted above.

Consumer Exposure:

A study simulating consumer hair color application using virgin human hair swatches with 90% grey to estimate consumer exposure to 1,2,4-THB was conducted. After each color application at various time points, the rinsates were analyzed by HPLC. In the first minute, the rate of 1,2,4-THB oxidation appears to be greater than the rate of diffusion into the hair as seen in the relatively limited color development in the hair swatch at T = 1 min. Subsequently, the diffusion into the hair increases, and color development visibly increases. The color intensity measurements of the swatches confirmed that 1,2,4-THB was penetrating the cuticle of the hair and forming larger, colored dye molecules which did not rinse out of the hair. The rinsate at 5 minutes contained roughly 27% of the starting concentration of 1,2,4-THB. The consumer exposure at 15 minutes corresponded to about 5% of the starting 1,2,4-THB level (which translates to 0.1% in a formula). This indicates that under normal exposure conditions, the reaction is rapid and consumes the available 1,2,4-THB such that at 30 minutes the consumer is being exposed to less than 3% of the material not absorbed into the hair. The amount of 1,2,4-THB remaining in the rinsate is considered reflective of the consumer exposure. Products using this technology in the marketplace are intended to be left on the hair for 10 minutes or less. For context, it is useful to recall that the amounts of unreacted primary intermediates (precursors) and couplers was determined in the Ring Study conducted by the industry consortium published in 2005. Table 12 below represents a partial reproduction from SCCP 0941/05 (see ***Ref 19,*** page 16) in that only the first three combinations are reproduced for simplicity. The other entries were omitted as they demonstrate the same pattern as the first three and these data are representative of the whole.

Table 12: Amounts of unreacted precursors and couplers after hair dyeing for 30 min

Precursor & Coupler Combination	Total µmole applied to hair	Unreacted precursor/coupler recovered after 30 min hair dyeing		
		µmole in formulation	µmole in methanol extracts	Total unreacted (%)
PTD	92- 125	9 - 28	2 - 30	12 - 46
AHT	88 -121	6 - 21	7 - 32	15 - 42
PPD	110 - 132	22 - 42	0 - 40	30 - 54
AHT	107 - 143	16 - 28	13 - 56	30 - 58
PAP	103	19	33	51
AHT	91	11	31	46

Note: the first three combinations were the ranges from studies conducted in 5 different laboratories. The last entry reports the results from one laboratory. PTD is p-toluenediamine; AHT is 4-Amino 2-Hydroxytoluene, PPD is p-phenylenediamine; PAP is p-Aminophenol.

The last column, which summarizes the total unreacted precursors and couplers in terms of %, is most relevant here. We see from Table 12 that, in traditional oxidative hair dyeing systems, the unreacted precursor and coupler are available at a far higher percentage after thirty minutes than what was determined for 1,2,4-THB, where only 2.4% remained after 30 minutes development time (see Figure 2).

Mutagenicity / Genotoxicity:

In Vitro Studies: In the Bacterial Reverse Mutation Assay data, 1,2,4-THB caused a weak mutagenic response limited to TA 98, TA 100 (2004 study; SCCS 0962/05) and in TA1537 (2015 study). Since the study conducted in 2015 demonstrated no genotoxic effect with TA98 and TA100, these tester strains were not included in the repeat Ames work with ROS scavengers that was conducted in 2019. The latest repeat assay using TA 1537 with the test article 1,2,4-THB confirmed the results from study conducted in 2015. The number of revertants was slightly increased (3.2 and 3.6-fold which are essentially the same as 3.8-fold from 2015) over the background but was less than the positive control. In parallel, 1,2,4-THB was combined with 5, 10, and 15 µM glutathione (GSH) and 1,000, 10,000 and 20,000 IU catalase with the tester strain TA 1537 without S9. Both ROS scavengers reduced the greater than 3-fold increase in revertants so as to be statistically insignificant and were seen as substantially equivalent to the controls. In addition to preventing the increase in revertants, catalase reduced the overall toxicity of the 1,2,4-THB. The results of this experiment support that the weak mutagenic effect demonstrated in previous studies is attributable to reactive oxygen species, specifically hydrogen peroxide, given the superior efficacy of catalase over glutathione. The *in vitro* Micronucleus assay of 1,2,4-THB was concluded to be negative for the induction of micronuclei in human peripheral blood lymphocytes in both the presence and absence of metabolic activation. DNA damage was assessed under conditions relevant to dermal exposures using the 3D Phenion® Full-Thickness Human Skin Model Comet assay. Results of the 3D Skin Comet assay were negative adding to the weight of evidence for lack of mutagenicity of 1,2,4-THB. The 3D Reconstructed Skin Micronucleus (RSMN) was conducted and showed no statistically significant increase in micronuclei formation.

The new 3D skin methods offer a highly relevant model to assess genotoxicity of cosmetic ingredients. For an auto-oxidative material such as 1,2,4-THB, not only was the potential to cause a genotoxic response assessed for the subject material but also for the oxidation intermediates and products. This is a rationale that has been used and accepted in past reviews by SCCS.

The entire testing genotoxicity battery for GLP/OECD studies conducted to support 1,2,4-THB as a hair dye, including the *in vivo* study, can be summarized as follows in the ***Table 13*** below:

Table 13: Summary of GLP Genotoxicity Testing

OECD #	Study Date	Description	Result
471 (1997)	Mar 2004	Ames Bacterial Gene Mutation Assay	**Weak Positive** (TA98, TA100)
471 (1997)	Aug 2015	Ames Bacterial Gene Mutation Assay	**Weak Positive** (TA1537)
471 (1997) *mod*	Dec. 2018	Ames Bacterial Gene Mutation Assay with ROS Scavengers	**Negative** (TA1537)
474 (1983)	Jan-1993	*In Vivo* Micronucleus Test (murine erythrocytes)	**Negative** (50 mg/kg-bw)
473 (1983)	1995	*In vitro* Mammalian Chromosome Aberration Test	**Negative**
476 (1997)	Nov 2004	*In Vitro* Mammalian Cell Gene Mutation Test (hprt locus)	**Negative**
487 (2014)	Aug-2015	*In Vitro* Micronucleus Test (HPBL)	**Negative**
NA	Jan-2017	3D Skin Comet Assay	**Negative**
NA	Jan 2019	3D Reconstructed Skin Micronucleus Assay	**Negative**
474 (2016)	Dec 2019	*In vivo* Micronucleus Assay in Mice	**Negative**

NA = OECD Guideline not yet issued

Mechanistic studies on hydrogen peroxide formation from 1,2,4-THB in various types of solvent systems, support the view previously reported in the literature that positive *in vitro* results are likely due to generation of hydrogen peroxide during preparation of the dosing solution or during the conduct of the assay.

In Vivo Studies*:* Following the conclusion of SCCS 1452/11 that animal data is required to explore the potential to induce gene mutations, and also Buick et. al. (2018) who expressed that the *in vitro* results must be confirmed with *in vivo* studies, an *in vivo* micronucleus assay was conducted. The aim was to repeat the study completed in 1983 but in compliance with current OECD guidelines. The dose range finding study set the Maximum Tolerated Dose (MTD) at 25 mg/kg not 50 mg/kg as indicated in the earlier study; the two lower doses of 12.5 and 6.3 mg/kg representing approximately half the MTD and one quarter the MTD were included. No statistically significant increase in the incidence of MnPCEs in the test article treated groups at either time point (24 or 28 hours) was observed relative to the vehicle control group. The positive control induced a statistically significant increase in the incidence of MnPCEs. The number of MnPCEs in the vehicle control group did not exceed the historical control range. Under the conditions of this study, the administration of 1,2,4-THB at dose levels up to and including a dose level of 25 mg/kg was concluded to be negative in the micronucleus assay. These data add to the *in vivo* data collected earlier at 50 mg/kg which also showed no genotoxicity. These additional animal data, which could not be conducted for inclusion in a submission to the SCCS, are included in ***Table 13*** above, with a full report attached hereto as **Exhibit B**, provide additional conclusive evidence to support the absence of genotoxicity.

It should also be noted that the negative *in vivo* micronucleus assay from 1993, while not in compliance with current OECD guidelines (indeed that would not be possible as it was not in place at the time), was based on the use of a single MTD which was considered acceptable under the version of the guidance in use at the time. It was considered to add useful evidence in support of safety, but the SCCS Final Opinion did not consider it relevant. The doubt cast by the SCCS necessitated the new *in vivo* study conducted in

compliance with current OECD 474. The SCCS expressed concern for a potential "downturn" effect, as was sometimes seen in historical benzene studies (e.g., Kolachana et. al who saw increased 8-OHdG at their low dose and no change with higher doses). This so-called "downturn" effect was not seen in the new *in vivo* murine micronucleus assay where the highest dose was half the MTD in the 1983 study and the two lower doses at one half and one quarter the highest doses showed no micronuclei.

Lastly, it is interesting and imperative to note the inherent contradictions of the SCCS Final Opinion as it welcomed "open literature" data, and indeed emphasized it over and above data collected using validated OECD protocols and in compliance with GLP, in direct opposition to the historical position of the SCCS. In SCCS 1452/11, the SCCS provided comments on three sets of genotoxicity data (see ***Ref. 1,*** pages 20-21), which read as follows:

> *In vitro* micronucleus test – SCCS comment: *"The data are from a publication in the open literature. The test was not conducted in compliance with GLP and OECD guidelines. The purity of the 1,2,4-benzenetriol was not reported."*
>
> *In vitro* sister chromatid exchange test – SCCS Comment: *"The data are from publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines. The purity of 1,2,4-benzenetriol was not reported. The test has only limited value and can at most be used for confirmation purposes."*
>
> *In vitro* DNA strand break test – SCCS Comment: *"The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines. The purity of the 1,2,4-benzenetriol was not reported. The test has only limited value and can at most be used for confirmation purposes."*

The SCCS Final Opinion cited these same three open literature articles, which were dismissed in SCCS 1452/11, to demonstrate genotoxicity of concern (SCCS 1598/18 – Erexson et al., 1985; Lee & Garner, 1991; and Chung et al., 2002).

In this submission, all of the genotoxicity work was conducted in partnership with well-known, highly respected contract research organizations, using OECD validated methods where available and in compliance with GLP. Also, in compliance with GLP, the purity of the test article was well characterized, and the dosing solutions were analyzed to confirm exposure.

All of these recent data support the conclusion that 1,2,4-THB is not genotoxic in vivo and therefore does not present a genotoxic hazard to the consumer in a cosmetic hair dye formulation.

Margin of Safety (MoS) Calculation

In a previous repeat dose toxicity study in rats, a No Adverse Effect Level (NOAEL) of 50 mg/kg/d was proposed by the researchers (see SCCS 1452/11). However, the SCCS suggested that this value be set as the Lowest Observed Adverse Effect Level (LOAEL). Therefore, an assessment factor of 3 was applied in the calculation of the MoS. A dermal absorption of 1.94 $\mu g/cm^2$ for 1,2,4-THB formulated with PTD was

determined experimentally; the addition of one standard deviation (SD = 1.76) gives a maximal value of 3.70 µg/cm^2 at neutral pH.

In an auto-oxidative permanent hair dye solution containing 2.5% w/w 1,2,4-THB at pH 7.4, the margin of safety is calculated as follows:

Exposure Assumptions:

Experimental percutaneous absorption:	3.70 µg/cm^2 (mean + 1SD)	(***SCCS 1598/18***)
Surface area of scalp exposed:	580 cm^2	
Average body weight:	60 kg	
LOAEL (90 day oral toxicity study, rat):	50 mg/kg	***(SCCS 1452/11)***
Assessment factor:	3	

Exposure Calculation and Margin of Safety:

$$\text{Consumer Exposure (SED)} = \frac{\text{(percutaneous absorption) x (surface of scalp exposed)}}{\text{Body weight}}$$

$$= \frac{(3.70\ \mu g/cm^2) \times (580\ cm^2)}{60\ kg}$$

$$= 3.58 \times 10^{-2}\ mg/kg$$

$$\textbf{Margin of Safety (MoS)} = \frac{\text{LOAEL/3}}{\text{SED}}$$

$$= \frac{16.67\ mg/kg}{3.58 \times 10^{-2}\ mg/kg}$$

$$= \textbf{466}$$

The margin of safety for cosmetic hair dye ingredients requires a safety factor of 100; in this instance we have a safety factor of 466 which affords a robust greater than 4.5-fold protection to the consumer over what is expected or required.

Conclusion

Overall, the weight of the evidence from the existing published data, and the extensive additional investigations and results, particularly the negative *in vivo* micronucleus study and the negative DNA adduct study, support the conclusion that 1,2,4-THB is not genotoxic. Thus, 1,2,4-THB does not pose a risk to the health of consumers when used in hair dye formulations at a maximum concentration of 2.5%.

References

1 SCCS 1452/11, Opinion on 1,2,4-Trihydroxybenzene Colipa No. A33, December 11, 2012

2 SCCS 1602/18 Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation, 10th Revision, October 2018

3 Induction of Antioxidant-Response-Element Dependent Gene Activity and Cytotoxicity (using MTT) in the Kertinocyte ARE_Reporter Cell Line, KeratinoSens by 1,2,4-THB, Norman, K., Institute for In Vitro Sciences, Report June 8, 2016.

4 Statistical Treatment for KeratinoSens

5 In Vitro Penetration of [14C]-1,2,4-Trihydroxybenzene through Human Dermatomed Skin, Dermal Technology Laboratories Ltd., Final Report, 26 January 2015.

6 Ames Bacterial Reverse Mutation Assay of 1,2,4-Trihydroxybenzene, BioReliance Corporation, Wagner, V.O., Report, August 2015

6 Modified Ames Bacterial Reverse Mutation Assay with Catalase and GSH Final Report, BioReliance, 2019.

8 *In Vitro* Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes (HPBL) of 1,2,4-Trihydroxybenzene, BioReliance Corporation, Roy, S., Report, August 2015.

9 3D Skin Comet Assay with 1,2,4-Trihydroxybenzene, using Phenion® full thickness skin models, Triskelion, Reus, A.A., Report January 2017.

10 Dahl, E.L., Curren, R., Barnett, B.C., Khambatta, Z., Reisinger, K., Ouedraogo, G., Faquet, B., Ginestet, A.C., Mun, G., Hewitt, N.J., Carr, G., Pfuhler, S., Aardema, M.J., *The reconstructed skin micronucleus assay (RSMN) in EpiDerm™: detailed protocol and harmonized scoring atlas*. Mutat Res., 720(1-2):42-52, 2011.

11 3D Reconstructed Skin Micronucleus Assay Final Report, BioReliance 2019. (EXHIBIT B)

12 *In Vivo* Mammalian Micronucleus Assay in CD-1 Mice, BioReliance, Report 2021.

13 Hydrogen Peroxide Quantification by Fox Assay_15Feb2019

14 SCCP 1198/08, *Opinion on Intermediates and reaction products of oxidative hair dye ingredients formed during hair dyeing*, 2009

15 SCCP 1311/10, *Opinion on reaction products of oxidative hair dye ingredients formed during hair dyeing processes*, 2010.

16 Time Dependent Hair Color Simulating Consumer Usage_09Feb2009

17 Morel, O.J.X., and Christie, R.M. Current Trends in the Chemistry of Permanent Hair Dyeing, Chem. Rev., 111, 2537-2561, 2011.

18 1,2,4-Trihydroxybenzene: Mechanism and Kinetics of A33 and A5, Avomeen Analytical Services, Kolbert, A.C., Reports March 2014 and May 2014.

19 SCCP 0941/05 Opinion on Exposure to reactants and reaction products of oxidative hair dye formulations, 2005.

20 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.

21 du Plessis, J., Pugh, W.J., Judefeind, A., Hadgraft, J., *Physico-chemical determinants of dermal drug delivery: effects of the number and substitution pattern of polar groups*. Eur J Pharm Sci., 16(3):107-12, 2002.

22 European Coffee Federation: https://www.ecf-coffee.org/about-coffee#history-of-coffee ;

23 British Coffee Association: http://www.britishcoffeeassociation.org/about_coffee/history_of_coffee/

24 Gaascht, F., Dicato, M., Diederich, M., *Coffee provides a natural multi-target pharmacopeia against the hallmarks of cancer*. Genes Nutr., 10(6):5 2015.

25 Butt, M.S., Sultan, M.T., *Coffee and its consumption: benefits and risks.* Crit Rev Food Sci Nutr., 51(4):363-73, 2011.

26 Ochiai, R., Chikama, A., Kataoka, K., Tokimitsu, I., Maekawa, Y., Ohishi, M., Rakugi, H., Mikami, H.,. *Effects of hydroxyhydroquinone-reduced coffee on vasoreactivity and blood pressure*. Hypertens Res., 32(11):969-74, 2009.

27 Katada, S., Watanabe, T., Mizuno, T., Kobayashi, S., Takeshita, M., Osaki, N., Kobayashi, S., Katsuragi, Y., *Effects of Chlorogenic Acid-Enriched and Hydroxyhydroquinone-Reduced Coffee on Postprandial Fat Oxidation and Antioxidative Capacity in Healthy Men: A Randomized, Double-Blind, Placebo-Controlled, Crossover Trial.* Nutrients., 10(4) 2018.

28 Kolachana, P., Subrahmanyam, V.V., Meyer, K.B., Zhang, L., Smith, M.T., *Benzene and its phenolic metabolites produce oxidative DNA damage in HL-60 cells in vitro and in the bone marrow in vivo*, Cancer Res., 53(5):1023-6, 1993.

29 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.* Toxicol Appl Pharmacol., 108(3):497-508, 1991.

30 Reddy, M.V., Blackburn, G.R., Schreiner, C.A., Mehlman, M.A., Mackerer, C.R.. *32P analysis of DNA adducts in tissues of benzene-treated rats*, Environ Health Perspect., 82:253-7, 1989.

31 Corbett, J.F. *The Chemistry of Hydroxyl-quinones. Part VI: Formation of 2-hydroxy-semiquinones during Auto-oxidation of Benzene-1,2,4-triol in Alkaline Solution.* J. Chem. Soc.,(C), 0, 2101-2106, 1970.

32 Hiramoto, K., Mochizuki, R., Kikugawa, K., *Generation of Hydrogen Peroxide from Hydroxyhydroquione and its Inhibition by Superoxide Dismutase,* J. Oleo Sci., 50(1), 21-28, 2001.

33 Akagawa, M., Shigemitsu, T., Suyama, K., *Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions*. Biosci Biotechnol Biochem, 67(12):2632-40, 2003.

34 Clapp, P.A., Ngoc, D., and Evans, D.F., *Thermal and Photochemical Production of Hydrogen Peroxide from Dioxygen and Tannic Acid, Gallic Acid and other related Compounds in Aqueous Solution*. J. Chem. Soc. Faraday Trans, 1990, 86(14), 2587-2592.

35 Martinez, A., Urios, A., Blanco, M., *Mutagenicity of 80 chemicals in Escherichia coli tester strains IC203, deficient in OxyR, and its oxyR+ parent WP2 uvrA/pKM101: detection of 31 oxidative mutagens,* Mutat Res. 467, 41-53, 2000.

36 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

37 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.

38 Frantz, C.E., Chen, H., Eastmond, D.A., *Inhibition of human topoisomerase II in vitro by bioactive benzene metabolites,* Environ Health Perspect,.104 Suppl 6:1319-23, 1996.

39 Chen, H., Eastmond, D.A. *Topoisomerase inhibition by phenolic metabolites: a potential mechanism for benzene's clastogenic effects*, Carcinogenesis. 16(10):2301-7, 1995.

40 Lindsey, R.H., Bender, R.P., Osheroff, N., *Stimulation of topoisomerase II-mediated DNA cleavage by benzene metabolites,* Chem Biol Interact. 153-154:197-205, 2005.

41 Lopez-Lazaro, M., Calderón-Montaño, J.M., Burgos-Morón, E., Austin, C.A., *Green tea constituents (-)-epigallocatechin-3-gallate (EGCG) and gallic acid induce topoisomerase I- and topoisomerase II-DNA complexes in cells mediated by pyrogallol-induced hydrogen peroxide.* Mutagenesis, 26(4):489-98, 2011.

42 Yasuhara, Y., and, K., Koyama, K., Hiramoto, K.., and Kikugawa, K., *Effect of Supplementation of a reductone in coffee Hydroxyhydroquinone, on lipid peroxidation and DNA Damage*, J. Oleo Sci. 51(10), 669-675, 2002.

43 Sommers, C.H., Schiestl, R.H., *Effect of benzene and its closed ring metabolites on intrachromosomal recombination in Saccharomyces cerevisiae*, Mutat Res., 593(1-2):1-8, 2006.

44 Halliwell, B., *Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies?*, Arch Biochem Biophys., 476(2):107-12, 2008.

45 Pathak, D.N., Lévay, G., Bodell, W.J., *DNA adduct formation in the bone marrow of B6C3F1 mice treated with benzene*, Carcinogenesis, 6(8):1803-8, 1995.

46 Ito, A., Naito, M., Naito, Y., Watanaee, H., *Induction and characterization of gastroduodenal lesions in mice given continuous oral administration of hydrogen peroxide*. Gann 73: 315-322, 1982.

47 Whysner, J., Reddy, M.V., Ross, P.M., Mohan, M., Lax, E.A., *Genotoxicity of benzene and its metabolites.* Mutat Res., 566(2):99-130, 2004

48 SCCP 0844/04, *Opinion on Hydrogen Peroxide in Tooth Whitening Products*, 2005.

49 Gupta, R., Karpattkin, S., Basch, R.S., *Hematopoiesis and stem cell renewal in long-term bone marrow cultures containing catalase*. Blood, 107(5):1837-46, 2006.

50 Subrahmanyam, V.V., Ross, D., Eastmond, D.A., Smith, M.T., *Potential role of free radicals in benzene-induced myelotoxicity and leukemia*. Free Radic Biol Med. ;11(5):495-515, 1991.

51. Marklund, S., *Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species*, Biochem J., 222(3): 649–655, 1984.

52. Marklund, S., *Extracellular superoxide dismutase in human tissues and human cell lines*, J Clin Invest. 74(4):1398-403, 1984.

53 Hellemans, L., Corstjens, H., Neven, A., Declercq, L., Maes, D., *Antioxidant enzyme activity in human stratum corneum shows seasonal variation with an age-dependent recovery*, J Invest Dermatol., 120(3):434-9, 2003.

54 Halliwell, B., and Whiteman, M., *Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean?* Br J Pharmacol.,142(2):231-55, 2004.

55 Halliwell, B., *Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come?* Am J Clin Nutr.,72(5):1082-7, 2000.

56 Halliwell, B., Gutteridge, J.M.C., Free Radicals in Biology and Medicine, 5th Edition, Chapter 5, *Oxidative stress and redox regulation: adaptation, damage, repair, senescence, and death,* 2015.

57 Hou, R.C., Chen, Y.S., Chen, C.H., Chen, Y.H., Jeng, K.C., *Protective effect of 1,2,4-benzenetriol on LPS-induced NO production by BV-2 microglial cells*, J Biomed Sci.13(1):89-99, 2006.

58 Poole, R., Kennedy,O.J., Roderick,P., Fallowfield, J.A., Hayes, P.C., Parkes, J., *Coffee consumption and health: umbrella review of meta-analyses of multiple health outcomes,* BMJ, 359:j5024, 2017.

59 Gunter, M.J. et al., *Coffee Drinking and Mortality in 10 European Countries: A Multinational Cohort Study*. Ann Intern Med., 167(4):236-247, 2017.

60 Miranda, A.M., Steluti, J., Fisberg, R.M., Marchioni, D.M., *Association between Coffee Consumption and Its Polyphenols with Cardiovascular Risk Factors: A Population-Based Study*, Nutrients, 9(3) 2017.

61 Sado, J., Kitamura, T., Kitamura, Y., Sobue, T., Nishino, Y., Tanaka, H., Nakayama, T., Tsuji, I., Ito, H., Suzuki, T., Katanoda, K., Tominaga, S.; Three-Prefecture Cohort Study Group., *Association between coffee consumption and all-sites cancer incidence and mortality*, Cancer Sci. 108(10):2079-2087, 2017.

62 Park, S.Y., Freedman, N.D., Haiman, C.A., Le Marchand, L., Wilkens, L.R., Setiawan, V.W., *Association of Coffee Consumption With Total and Cause-Specific Mortality Among Nonwhite Populations.* Ann Intern Med., 167(4):228-235, 2017.

63 Inoue O, Seiji K, Nakatsuka H, Watanabe T, Yin S, Li GL, Cai SX, Jin C, Ikeda M., *Excretion of 1,2,4-benzenetriol in the urine of workers exposed to benzene*, Br J Ind Med. 1989 Aug;46(8):559-65.

64 Halliwell, B., *Superoxide dismutase, catalase and glutathione peroxidase: solutions to the problem of living with oxygen,* Volume73(6), 1075-1086, 1974

65 Reinhard, C.T., Planavsky, N.J., Olson, S.L., Lyons, T.W., Erwin, D.H., *Earth's oxygen cycle and the evolution of animal life,* Proc Natl Acad Sci U S A., 113(32):8933-8, 2016.

66 Lewis, J.G., Stewart, W., Adams, D.O.. *Role of oxygen radicals in induction of DNA damage by metabolites of benzene.* Cancer Research 48, 4762-4765, 1988.

67 Kawanishi, S., Inoue, S., Kawanishi, M., *Human DNA damage induced by 1,2,4-benzenetriol, a benzene metabolite.* Cancer Res.;49(1):164-8, 1989.

68 Lévay, G., Bodell, W.J. *Potentiation of DNA adduct formation in HL-60 cells by combinations of benzene metabolites*., Proc Natl Acad Sci U S A.; 89(15):7105-9, 1992.

69 Zhang, L., Robertson, M.L., Kolachana, P., Davison, A.J., Smith, M.T.*, Benzene metabolite, 1,2,4-benzenetriol, induces micronuclei and oxidative DNA damage in human lymphocytes and HL60 cells*. . Environ Mol Mutagen.;21(4):339-48, 1993.

70 Greenlee, W.F., Sun, J.D/, us, J.S., *A proposed mechanism of benzene toxicity: formation of reactive intermediates from polyphenol metabolites*., Toxicol Appl Pharmacol.;59(2):187-95, 1981.

EXHIBIT C

Expert Analysis of Literature

Prepared by Prof. Helmut Greim
with input from Prof Barry Halliwell

Introduction

In its opinion on hair dye 1,2,4-trihydroxybenzene (1,2,4-THB) – A33 (CAS 533-73-3) Submission VI, adopted at its plenary meeting on 21-22 June 2018, the SCCS had been requested to respond to the following question:

> *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%?*

SCCS responded as follows:

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

To further explain this conclusion the SCCS stated:

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

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

To respond to this conclusion, the following points are discussed:

1. Evaluation of the weight of evidence to support an absence of genotoxicity *in vivo*.

2. Consideration of the underlying mechanism to evaluate whether 1,2,4-THB is a hydrogen peroxide releasing compound with a mode of action similar to hydrogen peroxide.
3. Comparison of 1,2,4-THB exposure from hair dye formulations vs. estimated dietary exposure.

1. Weight of Evidence (WoE) to support an absence of 1,2,4-THB genotoxicity *in vivo*

The following review of the relevant literature considered by the SCCS supports the absence of 1,2,4-THB genotoxicity *in vivo*.

- **In a cell-free system,** 1,2,4-THB (purity not specified) was tested for its ability to generate $O_2^{\bullet-}$ radicals at physiological pH by measuring the SOD (superoxide dismutase)-inhibitable reduction of cytochrome c. The results show that 1,2,4-THB is auto-oxidised and produced significant quantities of $O_2^{\bullet-}$ radicals. At a concentration of 1 mM, the rate of $O_2^{\bullet-}$ radical formation was 7.5 nmol/min. The addition of ferric iron 10 nM $FeCl_3$ enhanced the rate of cytochrome c reduction by 1,2,4-THB to 17.4 nmol/min. DNA strand breaks induction was investigated in supercoiled ØX-174 DNA, which was exposed to 1,2,4-THB with and without scavengers of reactive oxygen, namely, benzoate, catalase or SOD. 1,2,4-THB was very efficient at inducing DNA strand breaks and caused a complete DNA degradation at 5.5×10^{-6} M. The ED50 was 7×10^{-7} M. Catalase and benzoate inhibited this effect. Taken together, **these data support the view that 1,2,4-THB induce DNA damage through the production of oxygen radicals** (Lewis et al. 1988; #55 Annex 1; SCCS 1598/18 Ref. 19, ***Ref. 1***).

- **In a reaction mixture of DNA fragments,** 1,2,4-THB induced DNA damage, most likely by hydroxyl radical $OH^{\bullet-}$, which was produced by auto-oxidation of 1,2,4-THB. The DNA damage was inhibited by scavengers and increased by Cu(II) (Kawanishi et al. 1989, #53 Annex 1; SCCS 1598/18 Ref. 20, ***Ref. 2***). In this investigation, a DNA sequencing technique using ^{32}P5'-end-labeled DNA fragments obtained from human c-Ha-ras-1 proto-oncogene was used. The reaction mechanism was studied by UV-visible and electron-spin resonance spectroscopies, the extent of DNA damage was estimated by gel electrophoretic analysis. 1,2,4-THB (purity not specified) caused strong DNA damage even without alkali treatment, when exposed 90 minutes in a concentrations of 5 mM with the reaction mixture of [^{32}P]DNA fragments in buffer. DNA damage induced by 1,2,4-THB was inhibited completely by catalase, superoxide dismutase (SOD) and methional, but not by sodium formate, another hydroxyl radical scavenger. The addition of bathocuproine, a Cu(I)-specific chelating agent, inhibited DNA damage. The effect was accelerated by the addition of Cu(II). Fe(III) did not have any significant effects on DNA damage induced by 1,2,4-THB, even though the addition of Fe(III) increased hydroxyl radical production during the auto-oxidation of 1,2,4-THB. The addition of Cu(II) did not increase hydroxyl radical production, but copper ions were shown to be necessary for the induction of DNA damage by 1,2,4-THB.

- **In vitro,** 1,2,4-THB (200 µM) resulted in DNA adduct levels of 0.21×10^{-7} in HL-60 cells. Addition of 50–250 µM H_2O_2 to HL-60 cells had no effect on adduct formation by 1,2,4-THB. DNA adducts were measured by [^{32}P]-postlabeling. **Significant increases of cellular H_2O_2 levels were observed after exposure of HL-60 cells to 1,2,4-THB.** The authors previously reported

induction of relative DNA adducts *in vitro* in peroxidase containing cells such as promyelocytic HL-60 cells, mouse bone marrow macrophages and human bone marrow cells treated with benzene metabolites. No DNA adducts were observed in cells lacking detectable peroxidase activity. 1,2,4-THB is known to be a reducing co-substrate for peroxidase enzymes and **the formation of DNA adducts in cells treated with 1,2,4-THB was dependent on peroxidase activity. However, the addition of H_2O_2 to HL-60 cells did not influence the DNA adduct levels produced by 1,2,4-THB treatment** (Lévay and Bodell 1996, ***Ref. 3***).

1,2,4-THB (10 μM) increases 8-hydroxy-2'-deoxyguanosine (8-OHdG), **a biomarker for oxidative DNA damage** in HL-60 cells, a human leukemia cell line, which, like the bone marrow, contains high levels of myeloperoxidase. The increase of 8-OHdG was 3.8-fold within 0.5 hours (Zhang et al. 1993, ***Ref. 4***; SCCS 1598/18 Ref 2; Kolachana et al 1993; SCCS 1598/18 Ref. 24***)***.

- **In vivo,** in a mouse bone marrow micronucleus test conducted according to OECD guideline 474 (1983), Swiss OF1 mice with 5 per sex per dose were exposed to 0 or 50 mg/kg 1,2,4-THB (purity: 99.4%) i.p (SCCP 962/05). Bone marrow cells were collected 24 and 48 hours after treatment. Dose selection was based on the results of preliminary assays, in which two mice per dose received i.p. doses between 50 and 2000 mg/kg-bw. Mortality and clinical signs were observed over a period of 48 hours. Doses of 500 mg/kg bw and higher were lethal for all animals immediately after treatment, 100 mg/kg-bw caused sedation after 6 hours and lethality of all mice 24 hours after treatment. The dose of 50 mg/kg-bw induced piloerection and hypokinesia after 6 hours and piloerection 24 and 48 hours after treatment. Based on these results, 50 mg/kg-bw is considered a maximum tolerated dose and was used in the micronucleus assay. The same clinical signs were observed in the micronucleus test. One mouse died and was replaced from a supplementary treated group. The ratio of polychromatic to normochromatic erythrocytes (PCE/NCE) decreased in a statistically significant manner. Thus, toxicity and exposure of the target cells has been proven. **1,2,4-THB exposure of male and female Swiss OF1 mice did not result in an increase in the number of erythrocytes with micronuclei.** Thus, in this test system, 1,2,4-THB is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

  However, SCCS noted that this test has not been performed in accordance with the current OECD guideline because only one dose was tested and thus has limited value. This is formally correct. However, as indicated above, the single selected dose showed toxicity at the target organ (decrease in the PCE/NCE ratio), and showed clinical signs and mortality (one animal died). Therefore, there is the clear conclusion that this was a well-defined highest dose. Considering the underlying mechanisms and the negative *in vivo* data of hydrogen peroxide, it is not plausible that lower doses will give positive results. This is further supported by the study of Lee and Garner (1991, SCCS 1598/18 Ref 22). *In vitro,* GSH and catalase scavenged the DNA damaging effect of the benzene metabolite 1,2,4-THB, whereas an i.p. application of 1760 mg/kg benzene to male and female mice did not induce DNA strand breaks in bone marrow cells.

  In the bone marrow of B6C3F1-mice, 1,2,4-THB has been tested (Kolachana et al. 1993) for its possible induction of oxidative DNA damage as determined by 8-hydroxy-2'-deoxyguanosine (8-OHdG). The detection of 8-OHdG provides a direct measure of genotoxic effects of active oxygen.

Three mice per group were given i.p. injections of 1,2,4-THB (purity not specified) doses of 25, 50, or 75 mg/kg bodyweight, dissolved in PBS. A fourth group of 3 mice served as control and received an equivalent amount of PBS alone. All mice were euthanized 1 hour after treatment. **1,2,4-THB significantly increased oxidative DNA damage (8-OHdG) approximately 2.5-fold at the dose of 25 mg/kg bodyweight, whereas the effect at 50 and 75 mg/kg bodyweight was not significantly different from controls.** The authors provided no explanation for the peak in DNA-damage at 25 mg/kg bodyweight.

This is in contrast to the study in female Swiss OF1 mice. A single dose of 50 mg/kg did not induce micronuclei (see above). It was also not corroborated by Yasuhara et al. (2002) in their 1-week feeding study in Wistar rats. Organ sampling showed that levels of 8-OHdG production were in line with levels measured for the control group. While some oxidative stress as measured by phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide levels was seen in the lungs and heart, it was not seen in the liver (which is known to expresses high levels of catalase). The authors related this to differences in the endogenous enzymes that remove reactive oxygen species (ROS). Lastly, as an additional measure of oxidative stress, thiobarbituric acid reactive substances (TBARS) were measured in the organ homogenates with and without EDTA. TBARS levels of the heart were elevated in rats on the 1,2,4-THB supplemented diet, but not in the lungs and liver. The data collected by Yasuhara et al. showing the lack of 8-OHdG induction by 1,2,4-THB does not confirm the increase in adducts reported by Kolachana et al. (1993), though it is recognized that the study designs were different.

- **The effects of antioxidants:** To investigate the involvement of reactive oxygen species, several authors introduced scavengers of reactive oxygen species to their test systems. Anderson et al. (1995; #38 Annex 1) showed that catalase abolished DNA damage in the Comet assay; Pitarque et al. (2006; #8 Annex 1) reported the abolishing effects of GSH and vitamin C, Lee and Garner (1991) that of GSH and catalase, Kawanishi et al. (1989) that of catalase, superoxide dismutase and methional. The involvement of reactive oxygen species is further supported by the specific sensitivity of HL-60 cells, which as a bone marrow derived cell line contains high levels of myeloperoxidase (Kolachana et al 1993, Zhang et al 1993). Further, the increase of micronuclei in HL-60 cells in presence of Cu^{2+}, as Cu2+ catalyses the formation of reactive oxygen species (Zhang et al 1993).

2. 1,2,4-Trihydroxybenzene (1,2,4-THB) as a hydrogen peroxide releasing compound

3.1 Genotoxic Mechanisms of 1,2,4-THB and Hydrogen Peroxide

According to, Annex III to Regulation 1223/2009 on Cosmetic Products, as last amended, hydrogen peroxide can be used in hair color products, **either present or released,** at concentrations up to 12%. In hair color products, hydrogen peroxide is used to form reactive oxygen species to oxidise the primary intermediate in hair dye formulations to the quinone imine. Similarly, 1,2,4-THB releases hydrogen peroxide (Akagawa et al. 2003 and Hiramoto et al. 2001;), which also forms reactive oxygen species. If

the oxidative mechanisms of H_2O_2 and 1,2,4-THB are the same, 1,2,4-THB can be considered as a hydrogen peroxide releasing substance.

3.2 1,2,4-THB

While appreciating the overall conclusion of the SCCS regarding the genotoxicity of 1,2,4-THB, it must be noted that most of the tests on which the conclusion lies, have deficiencies (see Applicant's Comments on SCCS Opinion 1598/18). Moreover, it should be noted that the majority of the studies on which the SCCS relied were performed to investigate the mode of action of benzene toxicity. In most cases, **the authors conclude that 1,2,4-THB is not genotoxic, per se**. Specifically, the following studies noted that 1,2,4-THB triggers interaction with DNA via the production of reactive oxygen species:

- Anderson et al. 1995, SCCS 1598/18 Ref. 26
- Andreoli et al. 1997, SCCS 1598/18 Ref. 27
- Hiramoto et al 2001,
- Kawanishi et al. 1989, SCCS 1598/18 Ref. 20;
- Kolachana et al. 1993, SCCS 1598/18 Ref. 24;
- Lewis et al. 1988, SCCS 1598/18 Ref. 19;
- Jia et al. 2016, SCCS 1598/18 Ref. 34
- Moretti et al. 2005; SCCS1598/18 Ref. 30
- Pellack-Walker and Blumer 1986, SCCS 1598/18 Ref. 18
- Pitarque et al. 2006; SCCS 1598/18 Ref. 32
- Zhang et al 1993, SCCS 1598/18 Ref. 23;

Pellack-Walker and Blumer (1986; SCCS 1598/18 Ref. 18) describe two mechanisms for the activation of 1,2,4-THB. First, is its oxidation to the corresponding quinone by sequential one-electron or direct two-electron oxidations, which might form covalent adducts with nucleophilic sites in DNA. Second is the formation of reactive oxygen species during its auto-oxidation (Greenlee et al. 1981; ***Ref. 5***). 1,2,4-THB can be oxidized by molecular oxygen to semiquinone with the concomitant generation of superoxide radicals. Alternatively, it can be viewed as the sequential two-electron reduction of oxygen to form hydrogen peroxide. (See below in section 1.5, ROS/Hydrogen Peroxide in Genotoxicity of 1,2,4 THB). In cellular systems, the semiquinone can be enzymatically reduced by NADPH-cytochrome P-450 reductase back to 1,2,4-THB or further oxidized to the quinone, again accomplished with superoxide formation. In the presence of transition metal ions, superoxide radicals may further react with hydrogen peroxide to yield hydroxyl radicals (see Figure 1). Since the studies by Zhang et al. 1993, Pitarque et al. 2006 clearly demonstrated oxidative DNA damage and Kolachana et al. (1993) in the formation of 8-OHdG, the genotoxicity of 1,2,4-THB can be seen as preferentially induced by oxidative DNA damage.

Formation of reactive oxygen species is further confirmed by the scavenging effects of antioxidants such as:

- Catalase (Andersen et al. 1995, as above)

- Catalase and benzoate, both scavengers of reactive oxygen (Lewis et al. 1988, as above)
- Catalase, superoxide dismutase (Hiramoto et al 2001, as above)
- Superoxide dismutase and methional (Kawanishi et al. 1989, as above)
- Vitamin C (Pitarque et al. 2006, as above)
- DMSO, a hydroxyl radical scavenger (Kolachana et al. 1993, as above)

All of these can be considered to clearly prevent *in vivo* genotoxicity of 1,2,4-THB.

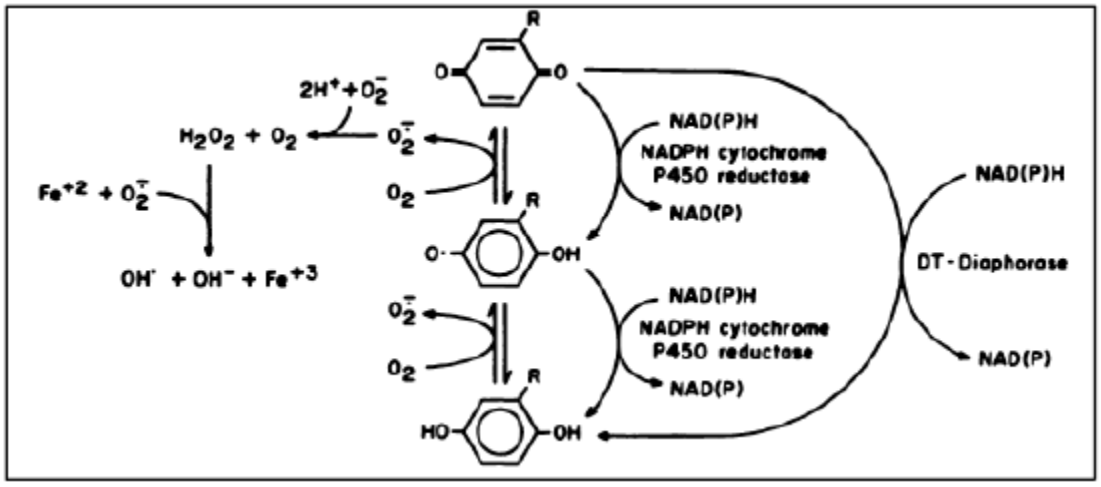


Figure 1: Potential toxification and/or detoxification pathways
For benzoquinone and benzenetriol via redox cycling between the quinone and its hydroxylated counterpart. Reaction scheme also demonstrates the concomitant production of reactive oxygen species. Benzoquinone: R = H; benzenetriol: R = OH (reproduced from Pellack-Walker and Blumer 1986)

3.3 Hydrogen Peroxide

In its opinion concerning hydrogen peroxide and hydrogen peroxide releasing substances used in oral care products, adopted by the Scientific Committee on Cosmetic Products and Non-Food products intended for Consumers of 23 June 1999, the committee described the many positive *in vitro* genotoxicity tests and the mostly negative *in vivo* studies for genotoxicity of H_2O_2 and referenced the European Chemicals Bureau on the mutagenicity of hydrogen peroxide (ECB 2003):

> *"In conclusion, the available studies are not in support of a significant genotoxicity/mutagenicity for hydrogen peroxide under in vivo conditions. A wider database in vitro of genotoxicity and mutagenicity observations on other relevant target tissues in direct contact with hydrogen peroxide is, however, desirable. Mechanistic studies suggest that cells are adapted to repair DNA damage caused by oxidants; on the other hand, there is some evidence that the hydrogen peroxide may inhibit repair of DNA lesions inflicted by other types of reactive chemicals (Chung et al 1995, Pero et al 1990, Hu et al 1995). According to the principles followed by the EU, hydrogen peroxide is not classified as a mutagen."*

The SCCP endorsed the conclusions of the European Chemicals Bureau on hydrogen peroxide. In its opinion on "Oxidative hair dye substances and hydrogen peroxide used in products to colour eye lashes" (SCCS/1475/12) of December 2012, it referred to the previous evaluation of hydrogen peroxide again supporting this previous conclusion (SCCP/1129/07).

The opinion describes the activation of H_2O_2 to reactive oxygen species as follows:

> *"The oxidative reactivity of hydrogen peroxide with biological molecules such as carbohydrates, proteins, fatty acids or nucleic acids is not pronounced in the absence of transition metals, except for a few nucleophilic reactions. In the organism, the highly reactive (and thus toxic) hydroxyl radical can be produced non-enzymatically from superoxide anion and hydrogen peroxide through catalysis by transition metal ions like Fe2+ and Cu+ (the so-called Haber-Weiss- and Fenton reactions):*
>
> $H_2O_2 + O_2^{-\bullet} \rightarrow OH\bullet + OH^- + O_2$ (Haber-Weiss reaction)
> $H_2O_2 + Fe^{2+}/Cu^{+} \rightarrow OH\bullet + OH^- + Fe^{3+}/Cu^{2+}$ (Fenton reaction)
>
> *In all likelihood the "full" Haber-Weiss reaction (i.e., the reduction of H_2O_2 by $O_2^{-}\bullet$) is as follows (showing that the Fenton reaction is representing one particular part of the Haber-Weiss reaction):*
>
> $O_2^{-}\bullet + Fe^{3+}/Cu^{2+} \rightarrow O_2 + Fe^{2+}/Cu^{+}$
> $H_2O_2 + Fe^{2+}/Cu^{+} \rightarrow OH\bullet + OH^- + Fe^{3+}/Cu^{2+}$"

According to Pellack-Walker and Blumer (1986; #57 Annex 1, SCCS 1598/18 Ref. 18), the same reaction occurs with 1,2,4-THB (see above and Figure 1). Formation of hydrogen peroxide from 1,2,4-THB in various solvents was determined and confirmed to be present in biologically relevant amounts by means of additional studies performed by the applicant (See Section 2.2).

3. Comparison of 1,2,4-THB exposure from hair dye formulations vs. estimated dietary exposure

1,2,4-THB is a component of coffee. Based on the concentrations determined by Ochiai et al (2009) and Katada et al (2018), 2 cups of coffee contain 3.4 mg (Ochiai) and 1.14 mg (Katada) of 1,2,4-THB, respectively. Assuming a 100% absorption, the daily exposure to 1,2,4-THB of a 60 kg adult is 0.056 and 0.018 mg/kg-bw, respectively. In Submission VI, the 1,2,4-THB exposure from a typical hair dyeing process was estimated. The application of a hair dye aqueous solution containing 2.5% of 1,2,4-THB would result in a systemic exposure of 0.035 mg/kg (see SCCS 1598/18 Ref. 13), which is in the range of the exposure from 2 cups of coffee.

Overall, considering that the application of a hair dye formulation is occasional, whereas the exposure to coffee is a daily occurrence for much of the population, 1,2,4-THB exposure from a hair dye formulation can be considered negligible.

4. Conclusions

Based on the data available in the literature and from previous submissions to the SCCS with respect to the safety of 1,2,4-THB, the following can be concluded:

1. *In vitro* 1,2,4-THB induces sister chromatid exchanges (SCEs), micronuclei, chromosomal aberration and gene mutations (6-thioguanine resistance) in mammalian cells. The substance induces DNA strand breaks, which are elevated by addition Cu^{2+} or Fe^{3+}. 1,2,4-THB interferes with microtubule assembly. **All of these effects are considered to be the result of the formation of reactive oxygen species.** DNA damage induced by 1,2,4-THB is inhibited by superoxide dismutase, catalase and benzoate and other hydroxyl radical scavengers. The addition of S9-mix greatly reduced the effects, which suggests that the compound is negative *in vivo* due to rapid inactivation. This is supported by a negative *in vivo* micronucleus test in mice. Although not completely meeting the requirements of the current OECD 474 test guideline, which requires several doses, the single dose used meets the requirements of the highest tolerated dose. It induces slight bone marrow toxicity as indicated by the reduction of polynucleated erythrocytes, but did not result in increased micronuclei in erythrocytes. It is not plausible that lower concentrations would show genotoxic effects and higher test doses would only demonstrate toxicity. In addition, in 2002, a feeding study by Yasuhara et al. (see ***Ref. 27***) showed no oxidative DNA damage in rats.

2. As explained above, 1,2,4-THB, like hydrogen peroxide, triggers the formation of reactive oxygen species via identical mechanisms. According to Annex III to Regulation 1223/2009 on Cosmetic Products, as last amended, hydrogen peroxide can be used in hair color products, **either present or released,** at concentrations up to 12%. In hair color products, hydrogen peroxide is used to form reactive oxygen species to oxidise the primary intermediate, which forms the quinone imine, the reactive intermediate in oxidative coupling. Similarly, 1,2,4-THB releases hydrogen peroxide, which also forms reactive oxygen species. Since there is strong evidence that the oxidative mechanisms of H_2O_2 and 1,2,4-THB are the same, this allows for the conclusion that 1,2,4-THB can be considered as a hydrogen peroxide releasing compound. In analogy to hydrogen peroxide and due to potent anti-oxidative mechanisms, 1,2,4-THB can be considered negative *in vivo*.

3. The application of a hair dye aqueous solution containing 2.5% of 1,2,4-THB would result in an exposure of 0.035 mg/kg-bw, which is in the range of the exposure from 2 cups of coffee which is between 0.018 mg/kg-bw and 0.056 mg/kg-bw. Furthermore, the exposure via 1,2,4-THB in hair dyes can be considered occasional and therefore, much less significant versus the regular and often times repeated daily exposure from coffee (See Section 4 for the Margin of Safety (MoS) calculation).

[1] Lewis, J.G., Stewart, W., Adams, D.O.. *Role of oxygen radicals in induction of DNA damage by metabolites of benzene.* Cancer Research 48, 4762-4765, 1988.

[2] Kawanishi, S., Inoue, S., Kawanishi, M., *Human DNA damage induced by 1,2,4-benzenetriol, a benzene metabolite.* Cancer Res.;49(1):164-8, 1989.

[3] Lévay, G., Bodell, W.J. *Potentiation of DNA adduct formation in HL-60 cells by combinations of benzene metabolites*., Proc Natl Acad Sci U S A.; 89(15):7105-9, 1992.

[4] Zhang, L., Robertson, M.L., Kolachana, P., Davison, A.J., Smith, M.T*., Benzene metabolite, 1,2,4-benzenetriol, induces micronuclei and oxidative DNA damage in human lymphocytes and HL60 cells*. . Environ Mol Mutagen.;21(4):339-48, 1993.

[5] Greenlee, W.F., Sun, J.D/, us, J.S., *A proposed mechanism of benzene toxicity: formation of reactive intermediates from polyphenol metabolites*., Toxicol Appl Pharmacol.;59(2):187-95, 1981.

Marilyn Aardema Consulting, LLC

EXHIBIT D

Expert Review of Genotoxicity of 1,2,4-Trihydroxybenzene (1,2,4-THB)

Dr. Marilyn J. Aardema

Executive Summary

A comprehensive weight-of-evidence assessment of the genotoxicity of 1,2,4-Trihydroxybenzene (1,2,4-THB), an auto-oxidative hair dye ingredient in permanent hair dye formulations, has been conducted by the sponsor. The assessment includes data from state-of-the-art studies that meet the latest regulatory guidelines. In addition, mechanistic investigations have been conducted to support the overall assessment of the genotoxicity of 1,2,4-THB. I have reviewed the recent genotoxicity testing on 1,2,4-THB and in my opinion, these state-of-the-art studies provide an extremely thorough assessment of genotoxicity of 1,2,4-THB, and overall, the results support the conclusion that 1,2,4-THB does not pose a genotoxic risk.

Introduction

Recent genotoxicity testing and assessment of 1,2,4-THB as a hair dye ingredient has been conducted. This included state-of-the-art, GLP assays to meet current regulatory guidelines along with novel assays to directly address genotoxicity in skin, the tissue of greatest relevance and exposure to 1,2,4-THB. The test battery consisted of standard *in vitro* assays to measure gene mutation in bacteria [Ames test], cytogenetic effects [*in vitro* micronucleus assay in human peripheral blood lymphocytes], and an *in vivo* mouse bone marrow micronucleus test. In addition, *in vitro* assays in 3D skin models were conducted to assess DNA damage [Comet in Phenion® Full Thickness Skin Model] and cytogenetic effects [micronucleus in Epiderm™ 3D skin models]. Experienced contract testing facilities were used for each assay to insure the generation of the highest quality data, including for the conduct of the novel 3D skin genotoxicity tests that were conducted at laboratories involved in the development and validation of these tests (Triskelion, 3D Comet; BioReliance, 3D micronucleus). Robust study designs were used in all assays including repeat studies with closely spaced concentrations (as needed), verification of mutant colonies by replica plating (Ames assay), among other items detailed below. Importantly, in all assays, dose solutions were carefully prepared in deaerated solvent where an inert environment was maintained with nitrogen gas in order to minimize the air oxidation of 1,2,4-THB prior to application. Table 1 summarizes the recent genotoxicity studies on 1,2,4-THB (2015-2021).

Previous studies conducted for 1,2,4-THB (published and unpublished) have been summarized in past SCCS submissions/opinions and are outside the scope of this review which is focused on the recent OECD/guidance compliant, GLP genotoxicity studies in Table 1. This review follows the approach of various expert groups where the greatest weight for a genotoxicity assessment is provided by studies that are guideline compliant, conducted according to GLP standards (insuring detailed documentation of experimental conditions, test article purity, dosing solution analysis, and data), unbiased analysis of coded samples, with detailed reporting of data, among other features (Brusick et al, 2016; Kirkland et al, 2021). For completeness, Table 2 provides a summary of all previous GLP, OECD compliant assays (discrepancies from OECD are listed) included in past SCCS opinions, though these studies are unpublished and reports are not available for review

of the actual data. As such, these studies provide less weight than the recent studies to the overall assessment of genotoxicity of 1,2,4-THB, but are summarized in Table 2 as supporting data in the overall weight of evidence on genotoxicity. Where appropriate, results from previous assays are mentioned below.

In brief, previous studies on the genotoxicity of 1,2,4-THB indicate that 1,2,4-THB can induce genotoxicity *in vitro,* and the preponderance of data indicate this is most likely a result of reactive oxygen species (ROS) production. In contrast, 1,2,4-THB is negative in *in vivo* genotoxicity tests.

Results

Table 1 provides a summary of the recent assays reviewed in this assessment. Detailed summaries from these recent genotoxicity tests conducted for 1,2,4-THB are provided in Appendix 1.

Table 1: Overview of Recent Genotoxicity Testing Of 1,2,4-THB (2015-2021)

OECD	Assay (report date)	Result
OECD 471	Bacterial Reverse Mutation Assay, 2015 -with & without ROS scavengers (2019)	Weak positive Negative with catalase and 2 conc of GSH
OECD 487	*In vitro* Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes, (2015)	Negative
N.A.	*In vitro* 3D Comet Assay using Phenion® Full Thickness Skin Model (2017)	Negative
N.A.	*In vitro* 3D Human Reconstructed Skin Micronucleus Assay (2019)	Negative
OECD 474	*In vivo* Mouse Bone Marrow Micronucleus assay (2021)	Negative

Bacterial Reverse Mutation Test, “Ames” assay

1,2,4-THB produced a weak, but reproducible positive result in a GLP, OECD compliant Ames test (plate incorporation) only in TA1537 in the absence of metabolic activation. All conditions for a valid test were met. Concentrations up to 5000 ug/plate were tested with toxicity observed at concentrations of 300 ug/plate and higher.

Elevated revertant counts that were just over the minimum 3.0-fold increase considered as a positive effect in TA1537 were observed. These increases exceeded the upper 95% control limit in 2 trials. In the first trial, variability in the individual revertant counts was observed that precluded demonstration of a dose response. Mutant induction in TA1537 was verified by replica plating of colonies. In the second trial, more closely spaced concentrations were evaluated in TA1537 (without metabolic activation) and a similar increase in mutants over 3.0-fold occurred (3.3-fold increase in mutants at 200 ug/plate, 3.8-fold increase in mutants at 150 ug/plate) with evidence of a dose response.

In an investigative study, the presence of catalase (CAT, 1000, 10,000, 20,000 IU) eliminated the increase in mutation frequency in TA1537. Elimination of mutation induction was also observed in the presence of reduced glutathione (GSH, 5,10 uM) but a 3.2-3.6X increase mutants was observed with 15 uM GSH. Overall, results with GSH were variable whereas induction of mutations was eliminated with all concentrations of CAT. The results of this study support the conclusion that the weak mutagenic effect demonstrated in previous studies in the literature and unpublished, can be attributed to reactive oxygen species, specifically hydrogen peroxide. This is consistent with supplemental information on the chemistry of 1,2,4-THB (Appendix 2).

In vitro Micronucleus Assay

1,2,4-THB was negative in a GLP, OECD compliant assay for the induction of micronuclei in human peripheral blood lymphocytes in both the presence and absence of metabolic activation. All conditions for a valid test were met. Concentrations were analyzed up to those that induced the required 55 ± 5% toxicity as measured by reduction in cytokinesis-blocked proliferation index relative to control [50 ug/ml, 4h exposure without metabolic activation, 52% toxicity; 100 ug/ml, 4 h exposure with metabolic activation, 54% toxicity), 30 ug/ml, 24 h exposure without metabolic activation, 55% toxicity].

In Vitro 3D Comet Assay using Phenion® Reconstructed Human Skin

Based on the positive result in the Ames test, 1,2,4-THB was evaluated in the novel, *in vitro* 3D Comet Assay using Phenion® Reconstructed Human Skin. This follow-up testing is aligned with current expert guidance (International Workshop on Genotoxicity Testing, IWGT, Pfuhler et al 2020), SCCS notes of guidance in place at the time (SCCS/1602/18) as well as the most recent SCCS notes of guidance (SCCS/1628/21). This GLP study was conducted according to expert guidelines of the Cosmetics Europe Genetoxicity Taskforce Standard Operating Procedure (date 16 January 2015) since there is not an OECD guideline for this assay.

Negative results for induction of DNA damage in both epidermis and dermis were obtained at concentrations up to 16 ug/cm^2, the top concentration based on toxicity. Toxicity was assessed by AK release, LDH, and intracellular ATP measures. The presence of brown staining (colored reaction products of 1,2,4-THB) of the tissue and

underlying media was observed at high concentrations which interfered with the AK assay but the other measurements verified that appropriate levels of toxicity were obtained. In a second study, aphidicolin was used as an inhibitor of DNA repair to increase the sensitivity of the assay. Negative results were again observed up to 16 ug/cm^2.

In Vitro 3D Human Reconstructed Skin Micronucleus (RSMN) Assay using EpiDerm™

Since dermal exposure is the most relevant route for 1,2,4-THB, 3D skin genotoxicity assays are considered the most appropriate tools for addressing biological relevance of positive results in standard *in vitro* genotoxicity assays. Though 1,2,4-THB was negative in a state-of-the-art GLP, OECD compliant *in vitro* micronucleus assay in human peripheral blood lymphocytes, previous studies (published literature, SCCS opinions) have reported increases in micronuclei *in vitro*. To address this, and add further strength to the assessment of the genotoxicity of 1,2,4-THB, an in vitro micronucleus assay in 3D skin was conducted. The Cosmetics Europe Genotoxicity Taskforce Protocol (Dahl et al 2011) which is aligned with the standard *in vitro* micronucleus assay (OECD 487), was used in the conduct of the assay since there is no OECD guideline for this study at this time. Both 48 h and 72 h exposures were used

Negative results in the RSMN assay were obtained at concentrations up to 100 ug/cm^2 (48 h exposure) that induced a 59% reduction in Cytochalsin B Proliferation Index, CBPI and up to 72 ug/cm^2 that induced 51% cytotoxicity based on reduction in RVCC.

In Vivo Micronucleus Assay

1,2,4-THB was negative for induction of micronuclei in a GLP, OECD compliant mouse bone marrow assay up to 25 mg/kg (single intraperitoneal injection) at both 24h and 48h. Results for positive and negative controls met the criteria for a valid test. According to OECD 474, the anticipated route of human exposure should be considered in the design of the invivo micronucleus assay but topical exposures (most relevant for 1,2,4-THB) have not been validated for this test. Though i.p. dose administration is generally not recommended since it is not an intended route of exposure for most test articles, it can be used with scientific justification. In the case of 1,2,4-THB, i.p. administration was selected to try and maximize exposure and to further address unpublished negative results previously reported for 1,2,4-THB in a GLP compliant *in vivo* mouse micronucleus assay conducted with a single dose of 50 mg/kg i.p. (SCCP/0962/05, modified SCCS/1452/11, primary report not available.)

In the dose range-finding assay (DRF), dose levels tested were 25, 50 and 100 mg/kg in 3 animals/sex. Mortality was observed in 3/3 males and 2/3 females at 100 mg/kg. Based on clinical signs and loss of body weight gain at 50 mg/kg, the high dose for the definitive assay was 25 mg/kg, which was estimated to be the maximum tolerated dose (MTD). The definitive assay dose levels tested were 6.3, 12.5 and 25 mg/kg in males as it was determined in the dose range finding study that there was no difference in toxicity between

males and females. Since there were fewer clinical signs in males at 25 mg/kg, it could be argued that 50 mg/kg was a more appropriate MTD for males. As discussed below, 50 mg/kg i.p. was negative in a previous *in vivo* mouse micronucleus assay.

No statistically significant increase in micronucleated PCE was observed at any dose level of 1,2,4-THB up to 25 mg/kg at 24 h or 48 h. Further, there was no increase in individual animal micronucleated PCE compared to solvent controls and all values were within the historical control 95% confidence limits (mouse historical control data from testing facility provided separately due to error in including rat historical control data in final report). There was no significant reduction in PCE/NCE ratio in the test article treated groups indicating lack of toxicity to the bone marrow. Clinical signs were observed in all test article treatment groups. Systemic exposure based on blood samples could not be assessed due to instability of the test article in the non-acidic medium used to prepare the plasma samples (ascorbic acid required to be added to plasma to achieve stability). In the absence of measured systemic exposure, and lack of bone marrow toxicity, it is unclear the extent of exposure to the bone marrow. It is important to note that i.p. administration has been used historically to maximize exposure in *in vivo* genotoxicity assays, therefore serves as a “worse-case” hazard identification approach with systemic exposure occurring through diffusion of test articles into the blood vessel filled peritoneal cavity (reviewed in Kirkland et al 2019). In contrast, current *in vivo* genotoxicity testing is now directed at addressing biologically relevant routes of exposure. Thus, i.p. administrations can be considered to provide information on high dose genotoxicity hazard, therefore the negative i.p. *in vivo* micronucleus assay for 1,2,4-THB helps support the conclusion of a lack of genotoxicity *in vivo*.

Discussion

I reviewed the recent testing program of the genotoxicity of 1,2,4-THB, Table 1. The recent studies are GLP compliant, state-of-the-art assays that meet the latest regulatory guidelines and expert assay guidelines. Test article purity and precise preparation of the dosing solutions in deaerated solvents where an inert environment was maintained with nitrogen gas in order to minimize the air oxidation of 1,2,4-THB prior to application. The data generated in these studies are of high quality from laboratories with extensive experience.

Overall, there is sufficient data to conclude that 1,2,4-THB can induce genotoxicity *in vitro* (Ames positive) which is eliminated by catalase, and to some degree by GHS. This supports a conclusion that the mutagenic effects induced by 1,2,4-THB are most likely a result of reactive oxygen species (ROS) production, more specifically hydrogen peroxide. This conclusion is supported by an extensive assessment of existing published literature on 1,2,4-THB including reaction chemistry and hydrogen peroxide generation (published genotoxicity studies with expert review by Halliwell and Griem. In this review of the published literature, the induction of genotoxicity in other *in vitro* assays including the *in vitro* micronucleus test is summarized. Of particular note as related to SCCS Notes of Guidance (2021) discussion of toxicogenomics analysis as a useful follow-up approach

to address relevance of positive results in *in vitro* genotoxicity assay, is the publication of a toxicogenomics analysis by Buick et al (2017). In this study, an increase in micronucleus induction *in vitro* with 1,2,4-THB was observed and the TGx-28.65 genomic biomarker analysis classified 1,2,4-THB as inducing pathways of genes involved in p53 signaling, cell cycle G2/M checkpoint response to DNA damage; apoptosis, oxidative stress, cell cycle. This is consistent with the proposed mechanism of genotoxicity induction involving reaction oxygen species.

In an overall weight-of-the evidence assessment of genotoxicity, *in vivo* results are considered of high weight. The negative results in the recent *in vivo* micronucleus assay (i.p. administration up to 25 m/kg) indicate that 1,2,4-THB does not pose a genotoxic hazard *in vivo*. This is consistent with the previous GLP *in vivo* micronucleus assay, i.p. administration at a single, yet higher dose of 50 mg/kg (SCCS/1452/11, unpublished data, report not available) where negative results were reported. Since this previous study only employed one dose which was not a limit dose, the assay is not OECD compliant and has less weight when compared to the high weight, recent OECD, GLP-compliant *in vivo* micronucleus assay described in this review. However, due to the high weight of *in vivo* data on an overall genotoxicity assessment, it is important to not discount these data. In fact, the 50 mg/kg dose in this previous study appears to be a well-defined high dose based on observation of clinical signs, morality of 1 animal, and bone marrow toxicity (reduction in PCE/NCE) that establishes bone marrow exposure. Thus, the negative results observed in this previous study are useful in the overall weight of evidence.

While the i.p. *in vivo* micronucleus assays are of high value in hazard identification and support the conclusion that 1,2,4-THB does not pose a genotoxic risk *in vivo*, the *in vitro* 3D skin genotoxicity tests can also be considered of high weight for assessing the biological relevance of dermal exposures to 1,2,4-THB. As such, the negative results in the *in vitro* Comet and micronucleus assays in 3D human skin models for 1,2,4-THB add significantly to the conclusion of the lack of genotoxicity of 1,2,4-THB specifically for dermal exposures.

As summarized in Table 2, the complete list of GLP, OECD compliant (except noted otherwise) genotoxicity studies for 1,2,4-THB adds further support to the conclusion that 1,2,4-THB does not pose a genotoxic risk. Though the older studies in Table 2 do not meet current regulatory guidelines and detailed reports are not available for review, the overall weight of evidence supports a conclusion that 1,2,4-THB can induce weak effects in the Ames assay, but mutagenicity/genotoxicity is not observed in other *in vitro* mammalian cell assays nor in *in vivo* assays. Importantly, the genotoxicity of 1,2,4-THB from dermal exposures is most definitively addressed in the 3D skin genotoxicity assays, where negative results were obtained both for DNA damage (Comet) and micronuclei. Based on the preponderance of data showing a ROS mechanism for genotoxicity of 1,2,4-THB, negative results *in vivo* and in the 3D skin assays are consistent with the presence of more biologically relevant ROS detoxification.

Overall, the data set are comprehensive and the results support the conclusion that 1,2,4-

THB does not pose a genotoxic risk.

Table 2: Summary of All, OECD, GLP Genotoxicity Testing of 1,2,4-THB (gray rows are studies from Table 1)

OECD #	Study Date	Assay	Result
474 (1983)	1993	*In vivo* Mouse Micronucleus Test, i.p. administration Note: not OECD compliant based on use of 1 dosage only	Negative
471 (1997)	2004	Ames Bacterial Gene Mutation Assay	Weak Positive (TA98, TA100)
471 (1997)	2015	Ames Bacterial Gene Mutation Assay	Weak Positive (TA1537)
473 (1983)	1995	*In vitro* Mammalian Chromosome Aberration Test	Negative
471 (1997) *modified for investigation of mechanism*	2019	Ames Bacterial Gene Mutation Assay with ROS Scavengers	Negative
476 (1997)	2004	*In vitro* Mammalian Cell Gene Mutation Test (hprt locus)	Negative
487 (2014)	2015	*In vitro* Micronucleus Test (HPBL)	Negative
NA	2017	*In vitro* 3D Skin Comet Assay	Negative
NA	2019	*In vitro* 3D Reconstructed Skin Micronucleus Assay	Negative
474 (2016)	2021	*In vivo* Mouse Micronucleus Test, i.p. administration	Negative

Citations

Brusick, D., et al., 2016. Genotoxicity Expert Panel review: weight of evidence evaluation of the genotoxicity of glyphosate, glyphosate-based formulations, and aminomethylphosphonic acid. Crit Rev Toxicol. 46**,** 56-74.

Buick, J.K., et al, 2017. 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.

Dahl, E.L. et al, 2011. The reconstructed skin micronucleus assay (RSMN) in EpiDerm™: detailed protocol and harmonized scoring atlas. Mutat Res., 720, 42-52

Kirkland et al, 2019. *In vivo* genotoxicity testing strategies: Report from the 7th International workshop on genotoxicity testing (IWGT), Mutat Res., 847, 403035

Pfuhler, S. et al, 2020. Use of in vitro 3D tissue models in genotoxicity testing: Strategic fit, validation status and way forward. Report of the working group from the 7th International Workshop on Genotoxicity Testing (IWGT). Mutat Res., 850–851, 503135

SCCS, 2021. The SCCS notes of guidance for the testing of cosmetic ingredients and their safety evaluation, 11th revision, March 2021 SCCS/1628/21.

Appendix 1
Detailed Summary of Mutagenicity/Genotoxicity Studies on 1,2,4-THB

Ames Bacterial Reverse Mutation Assay (Salmonella typhimurium)

Guideline:	OECD 471 (adopted July 21, 1997)
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA102
Replicates:	Single plates in the preliminary toxicity test in the presence and absence of metabolic activation. Triplicate plates in the presence and absence of metabolic activation in Experiments 1 and 2
Test substance:	1,2,4-Trihydroxybenzene (1,2,4-THB)
Solvent:	Deaerated water (N_2-purged)
Lot#:	THB0200312
Purity:	97.8%
Concentrations:	6.7-5000 µg/plate in the presence and absence of metabolic activation in the preliminary toxicity assay; Experiment 1: 5.0-5000 µg/plate, all strains, in the presence and absence of metabolic activation; Experiment 2: 5.0-1500 µg/plate TA1537 only in the absence of metabolic activation.
Treatment:	Plate incorporation, 48-72h incubation in the presence and absence of metabolic activation
GLP:	Yes
Study period:	2014-2015

In a bacterial reverse mutation assay (Ames test), the test article, 1,2,4-THB, was evaluated for its mutagenic potential based on the ability to induce point mutations in selected loci of the bacterial strains *S. typhimurium* strains TA98, TA100, TA1535, TA1537, TA102. Experiment 1 was conducted with and without addition of an exogenous metabolic activation system using concentrations ranging from 5.0-5000 µg /plate (9 concentrations) in the absence of metabolic activation and 15-5000 µg /plate (6 concentrations) in the presence of metabolic activation based on results from a preliminary toxicity assay. Experiment 2 was conducted without metabolic activation using concentrations ranging from 5.0-1500 µg/plate (11 concentrations) in TA1537 only. Experiments 1 and 2 included concurrent vehicle controls (degassed water) and positive controls (PC) as per OECD guidelines. In both independent experiments, 3 test plates (replicates) were used per test article 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.

Results

In Experiment 1, no biologically relevant increases in revertant colonies were observed at concentrations up to 5000 µg/plate in TA98, TA100, TA1535 or TA102 with or without metabolic activation or in TA1537 with metabolic activation. An increase (3.5 fold at 100 µg/plate, 3.0 fold at 150 µg/plate, 2.3 fold at 200 µg/plate, and 4.0 fold at 500 µg/plate) in revertant colonies was observed in tester strain TA1537 without metabolic activation. Mutants were confirmed by replica plating tester strain TA1537 in the absence of S9 activation at 100, 150 and 500 µg/plate.

Toxicity was noted in all tester strains. Without metabolic activation, toxicity was observed at 500 µg/plate and higher in all strains except TA102 where toxicity was observed at 5000 µg/plate. In the presence of metabolic activation, toxicity occurred at 5000 µg/plate. No precipitation was found in any experiment. The positive control mutagens induced an increase in revertant colonies indicating the validity of the experiments.
Since the background revertants in TA1537 without metabolic activation (4 +/-2) was on the lower side of the historical control values (mean 7 +/-4; 0-28 range) and the dose response was not clear-cut (e.g. 200 µg/plate did not exceed the 3 fold increase), the biological relevance of this result was investigated in Experiment 2 conducted with TA1537 without metabolic activation.
In Experiment 2, an increase (3.8-fold at 150 µg/plate; 3.3-fold at 200 µg/plate) in revertant colonies was observed in TA1537 without metabolic activation. The dose levels tested were 5.0, 15, 50, 150, 200, 300, 400, 500, 600, 750 and 1500 µg/plate in TA1537 without metabolic activation. Toxicity was observed beginning at 300 µg/plate. No precipitation was observed. This repeat experiment meets the criteria for a positive response in TA1537 in the absence of metabolic activation.

Conclusion
Under the conditions of this study, 1,2,4-THB was concluded to be positive in the reverse mutation assay in bacteria. Increased revertant counts that exceeded the minimum 3.0-fold increase considered positive for TA1537 were observed in two trials. In one trial there was a definitive dose response; whereas, there was no definitive dose response in the other trial. Since these increases were just over the minimum 3.0-fold increase required for evaluation as positive in TA1537, it is concluded that 1,2,4-THB induces a weak mutagenic response.

<u>Ames Bacterial Reverse Mutation Assay with Catalase and GSH</u>

Guideline:	OECD 471 (adopted July 21, 1997); modified for investigation of mechanisms; 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)
Lot#:	THB0318002
Purity:	98.1%
Vehicle:	Deaerated water (N_2-purged)
Concentrations:	Vehicle, 100 µg, 150 µg, 175 µg, 200 µg, 250 µg, 500 µg 1,2,4-THB without scavengers or metabolic activation. Same 1,2,4-THB concentrations with 20,000 IU, 10,000 IU or 1000 IU Catalase. Repeat 1,2,4-THB concentrations with 15, 10 or 5 µM GSH.
Standard Assay:	(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 (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:	Yes
Study Period:	Dec 2018; Final Report Jan 2019

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 *Salmonella typhimurium* TA1537 without metabolic activation, that had been observed in a previous GLP study (SCCS 1598/18 Ref 14). 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 15µM 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 presences of catalase at all doses (1000, 10,000, 20,000 U). 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.

In Vitro Mammalian Cell Micronucleus Test in Human Peripheral Blood Lymphocytes

Guideline:	OECD guideline no. 487 (draft April 2014; final Sept. 2014)
Species/strain:	Human peripheral blood lymphocytes from a male donor
Replicates:	One culture per concentration in the presence and absence of metabolic activation in preliminary toxicity test. Two cultures per concentration in the presence and absence of metabolic activation Experiment 1
Test substance:	1,2,4-Trihydroxybenzene (1,2,4-THB)
Solvent:	Deaerated water (N_2-purged)
Lot#:	THB0200312
Purity:	97.8%
Concentrations:	Preliminary Toxicity: 0.126-1260 ug/ml in the presence and absence of metabolic activation. Main Experiment: 1.26-150 ug/ml 4h treatment in the absence of metabolic activation; 12.5-150 ug/ml 4h treatment in the presence of metabolic activation; 0.1-100 ug/ml 24h treatment in the absence of metabolic activation.
Treatment:	Treatment initiated 44-48h after mitogen stimulation. 4h treatment in the presence and absence of metabolic activation harvested 20h later (24h from start of treatment); 24h treatment in the absence of metabolic activation harvested immediately.
GLP:	Yes
Study Period:	2014-2015

In an *in vitro* micronucleus test in human peripheral blood lymphocytes, the test article, 1,2,4-THB, was evaluated for its clastogenic and aneugenic potential. The study was conducted with and without addition of an exogenous metabolic activation system. Concentrations ranged from 1.26-150 µg/ml 4h treatment in the absence of metabolic activation; 12.5-150 µg/ml 4h treatment in the presence of metabolic activation; 0.1-100 µg/ml 24 h treatment in the absence of metabolic activation based on results from a preliminary toxicity assay.

Experiments included concurrent vehicle controls (degassed water) and positive controls (PC) as per OECD guidelines. Two replicate cultures were used per test article concentration or per control. Toxicity was measured by the cytokinesis-blocked proliferation index, CBPI). Precipitation of the test item was recorded.

Results

The percentage of cells with micronucleated/binucleated cells in the test article-treated groups was not statistically significantly increased relative to vehicle control at any dose level ($p > 0.05$, Fisher's Exact test). The results for the positive and negative controls indicate that all criteria for a valid assay were met.

Toxicity of the test article was observed. Substantial cytotoxicity (55 ± 5% or greater reduction in CBPI relative to the vehicle control) was observed at dose levels ≥ 50 µg/mL in the non-activated 4h treatment group, at dose levels ≥ 100 µg/ml in the S9-activated 4h treatment group, and at dose levels ≥ 30 µg/ml in the non-activated 24h treatment

group. The highest dose analyzed under each treatment condition produced 55 ± 5% reduction in CBPI which met the dose limit as recommended by testing guidelines for this assay. A minimum of 2000 binucleated cells total were scored for the presence of micronuclei.

Conclusion
Under the conditions of this study, 1,2,4-THB was concluded to be negative for the induction of micronuclei in human peripheral blood lymphocytes in both the presence and absence of metabolic activation.

In Vitro 3D Comet Assay using Phenion® Reconstructed Human Skin

Guideline:	Cosmetics Europe Genotoxicity Taskforce Standard Operating Procedure (date 16 January 2015).
Species/Strain:	Phenion® Full thickness human skin model
Replicates:	One tissue model per concentration in dose range finding assay. Three tissue models per concentration in Experiments 1 and 2.
Test Substance:	1,2,4,-Trihydroxybenzene
Solvent:	Deaerated acetone (N_2-purged)
Lot #:	THB0200312
Purity:	97.8%
Concentrations:	Dose range finding assay: 0.1-100 mg/ml (0.16-1600 ug/cm^2). Experiment 1: 0.125-1.0 mg/ml (2.0-16 ug/cm^2). Experiment 2: 0.25-1.25 mg/ml (4-20 ug/cm^2).
Treatment:	Total exposure time was 48 ± 3 h (repeated application at 48 ± 3 h, 24 ± 3 h and 3 h before cell isolation)
GLP:	Yes
Study Period:	2016

For all experiments, the dose solutions were prepared in a glove box (Cleatech LLC). The environment in the glove box was maintained with nitrogen gas in order to minimize the air oxidation of the test article prior to application. On each day of application, the dose solutions were prepared fresh using degassed acetone as the solvent. In the dose range finding study, the highest test concentration was 100 mg/mL (corresponding to 1600 µg/cm^2), the maximum required concentration for this assay. Six serial dilutions with 3.16-fold spacing in degassed acetone were prepared from the stock solutions. In the first and second main experiments, 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 individually weighed samples for each test substance. The actual concentration of the test substance in the dose solutions was determined by UPLC-UV using a validated method. Triplicate skin membranes per test group were used in both the first and second main experiment. The positive control MMS was freshly prepared in acetone on the day of dosing at a concentration of 0.316 mg/mL (corresponding to 5 µg/cm^2). The positive control B[a]P was freshly prepared in acetone on each day of dosing at a concentration of 0.78 mg.mL^{-1} (corresponding to 12.5

µg/cm^2).

A volume of 25 µL of the dose solutions was applied to the skin model surface which corresponded to an application volume of 16 µL/cm^2. The total exposure time was 48 ± 3 h (repeated application at 48 ± 3 h, 24 ± 2 h and 3 h prior to cell isolation) for the test substance and B[a]P, and 3 h (single application) for MMS. During exposure to the study substances, the skin models were maintained at *ca.* 37 ºC, *ca.* 5% CO_2 and *ca.* 95% humidity.

Cytotoxicity measurements included % lactate dehydrogenase (LDH) release, % adenylate kinase (AK) release and % intracellular adenosine triphosphate (ATP) measurements.

Results:
In the dose range finding study, cytotoxicity was observed at and above 50 µg/cm^2 based on the AK assay and at 16 µg/cm^2 based on LDH. As a result, 16 µg/cm^2 was selected as the highest concentration in the first main experiment. 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 statistically significant increase in tail intensity was observed at any of the test concentrations up to 16 µg/cm^2.

As per the current Cosmetics Europe SOP for the 3D Comet assay and validation studies, a second experiment was performed using aphidicolin as an inhibitor of DNA repair to increase the sensitivity of the assay. Four concentrations up to 20 µg/cm^2 were tested. The negative and positive controls in this second experiment met the acceptance criteria in both the epidermal and dermal fractions and the study was considered valid. Cytotoxicity was observed at 20 µg/cm^2 based on measurement of intracellular ATP and therefore this concentration was excluded from genotoxicity assessment. No statistically significant increase in tail intensity was observed at any of the test concentrations up to 16 µg/cm^2.

Conclusion:
Under the conditions of the test, 1,2,4-THB is considered not to induce DNA damage to human skin cells after topical application.

<u>3D Human Reconstructed Skin Micronucleus Assay using EpiDerm™</u>

Guideline:	Cosmetics Europe Genotoxicity Taskforce Protocol
Species/Strain:	MatTek EpiDerm™
Replicates:	One tissue model per concentration in dose range finding assay. Three tissue models per concentration in Experiments 1 and 2.
Test Substance:	1,2,4-Trihydroxybenzene (1,2,4-THB)
Solvent:	Deaerated acetone (N_2-purged)
Lot #:	THB0318002

Purity	98.1%
Concentrations:	Dose range finding assay 48 hour exposure: 0.50- 200 µg/cm^2 Experiment 1: 48-hour total exposure: 12- 224 µg/cm^2 Experiment 2: 72-hour exposure: 12- 224 µg/cm^2
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.
GLP:	Yes
Study Period:	December 2018-February 2019

For all experiments, 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/cm^2 were tested based on results of a previous 3D Comet Assay using Phenion® Reconstructed Human Skin. Toxicity was observed at 50 µg/cm^2 (56% reduction in Cytochalsin B Proliferation Index, CBPI) and higher. In Experiment 1 with a 48 h exposure and Experiment 2 with a 72 hour exposure, seven concentrations of 12-224 µg/cm^2 were tested. The actual concentration of the test substance in the dose solutions was determined by UPLC-UV using a validated method.

Triplicate skin models per test group were used in Experiments 1 and 2. For Experiment 1, 48-hour exposure, positive controls mitomycin C (5 and 6 µg/ml) and carbendazim (0.5 and 0.75 µg/ml) were freshly prepared in acetone each day of dosing. For Experiment 2, a 72-hour exposure was used in a confirmatory study. Cytotoxicity measurements included Cytochalsin B Proliferation Index (CBPI) and relative decrease in viable cell counts (RVCC)

Results*:*
In the dose range finding study, cytotoxicity was observed at and above 50 µg/cm^2 (56% reduction in the CBPI at 50 µg/cm^2). In Experiment 1, 48 hour exposure, the negative and positive controls met the acceptance criteria and the study was considered valid. The highest concentration for analysis of micronuclei was 100 µg/cm^2 selected based on induction of a 59% reduction in CBPI. No statistically significant increase in micronuclei was observed at any concentration of 24, 50 or 100 µg/cm^2 1,2,4 THB. In Experiment 2, the negative and positive controls met the criteria for a valid study. Cytotoxicity (50 to 60% CBPI relative to the vehicle control) was observed at concentrations ≥ 100 µg/cm^2. Cytotoxicity as measured by RVCC of 50 to 60% was observed at concentrations ≥ 72 µg/cm^2. The concentrations selected for evaluation of micronuclei with the 72h exposure were 12, 24, 72 µg/cm^2 (51% toxicity based on RVCC). No significant or dose-dependent increases in micronuclei induction were observed at any concentration ($p > 0.05$; Fisher's Exact and Cochran-Armitage tests).

Conclusion:
Under the conditions of the assay, 1,2,4-THB was concluded to be negative for the induction of micronuclei in the reconstructed skin micronucleus assay (RSMN) in EpiDerm™ in both the 48-hour and 72-hour treatment.

In vivo Micronucleus Assay in Bone Marrow of CD-1 Mice

Guideline:	OECD 474 (OECD 2016)
Species/Strain:	Mouse/CD-1(Hsd:ICR); Envigo RMS Inc.
Group Size:	Dose range finding: 3 males and 3 females per test group; Micronucleus assay: 6 males/test group; Bioanalysis: 3 males/test group
Test Substance:	1,2,4-Trihydroxybenzene (1,2,4-THB)
Solvent:	Deaerated water (N_2-purged)
Lot #:	S36884V (Sigma-Aldrich)
Purity:	99%
Dose Level:	0, 6.3,12.5, 25 mg/kg
Administration:	One, intraperitoneal injection (IP) at a dose volume of 5 mL/kg
Sacrifice Times:	24h and 48h (high dose only) after injection
Blood collection for Bioanalysis:	Retro-orbital Sinus, 0.5 mL of whole blood, 1h post dose
GLP:	in compliance

The test article, 1,2,4-THB was evaluated for its clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocytes (PCEs) in mouse bone marrow. Degassed deionized (DI) Water was used as the vehicle control. Test and/or vehicle control article formulations were administered once by intraperitoneal injection (IP) at a dose volume of 5 mL/kg. A single i.p. dose administration was selected to further address negative results previously reported for 1,2,4-THB in an in vivo mouse micronucleus assay conducted with a single dose of 50 mg/kg i.p. (SCCP/0962/05, primary report not available). For each dose group, 4000 PCE were analyzed per animal, 24,000 PCE for each test article group of 6 animals.

Results:
In the dose range-finding assay (DRF), dose levels tested were 25, 50 and 100 mg/kg in 3 animals/sex. Mortality was observed in 3/3 males and 2/3 females at 100 mg/kg. Based on clinical signs and loss of body weight gain at 50 mg/kg, the high dose for the definitive assay was 25 mg/kg, which was estimated to be the maximum tolerated dose (MTD). The definitive assay dose levels tested were 6.3, 12.5 and 25 mg/kg in males only as it was determined in the dose range finding study that there was no difference in toxicity between males and females. Analysis of the formulations indicated that the high concentration was accurately prepared, however the low and mid concentrations did not meet the acceptance criterion for percent of target. Therefore, the actual low and mid dose levels achieved were 5.32 and 8.15 mg/kg instead of 6.3 and 12.5 mg/kg, respectively. The nominal dose levels of 6.3 and 12.5 mg/kg were used in the report.

No statistically significant increase in micronucleated PCE was observed at any dose level of 1,2,4-THB at 24 h or 48 h. Further, there was no increase in individual animal micronucleated PCE compared to solvent controls and all values were within the historical control 95% confidence limits (mouse historical control data from testing facility provided separately due to error in including rat historical control data in final report). There was no significant reduction in PCE/NCE ratio in the test article treated groups indicated lack of toxicity to the bone marrow. Clinical signs were observed in all test article treatment groups. The study met the criteria for a valid assay based on the positive control that induced a statistically significant increase in the incidence of MnPCEs and the number of MnPCEs in the vehicle control group did not exceed the historical control range. No test article was detectable in the blood samples collected 1 h post dosing due to the instability of the test article in non-acidic medium. Based on this, systemic exposure to the test article could not be evaluated.

Conclusion

Under the conditions of this study, the administration of 1,2,4-THB at dose levels up to and including a dose level of 25 mg/kg i.p. was concluded to be negative in the micronucleus assay.

Searxh Terms
"hydroxyhydroquinone"[Supplementary Concept] OR "hydroxyhydroquinone"[All Fields] OR "1,2,4 benzenetriol"[All Fields]
Search Conducted: 5-Oct-18

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
1	Buick JK, Williams A, Kuo B, Wills JW, Swartz CD, Recio L, Li HH, Fornace AJ Jr, Aubrecht J, Yauk CL.	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. 2017 Dec;806:51-62. doi: 10.1016/j.mrfmmm.2017.09.002. Epub 2017 Sep 13.	In vitro gene expression signatures to predict toxicological responses can provide mechanistic context for regulatory testing. We previously developed the TGx-28.65 genomic biomarker from a database of gene expression profiles derived from human TK6 cells exposed to 28 well-known compounds. The biomarker comprises 65 genes that can classify chemicals as DNA damaging or non-DNA damaging. In this study, we applied the TGx-28.65 genomic biomarker in parallel with the in vitro micronucleus (MN) assay to determine if two chemicals of regulatory interest at Health Canada, disperse orange (DO: the orange azo dye 3-[[4-[(4-Nitrophenyl)azo]phenyl] benzylamino]propanenitrile) and 1,2,4-benzenetriol (BT: a metabolite of benzene) are genotoxic or non-genotoxic. Both chemicals caused dose-dependent declines in relative survival and increases in apoptosis. A strong significant increase in MN induction was observed for all concentrations of BT; the top two concentrations of DO also caused a statistically significant increase in MN, but these increases were <2-fold above controls. TGx-28.65 analysis classified BT as genotoxic at all three concentrations and DO as genotoxic at the mid and high concentrations. Thus, although DO only caused a small increase in MN, this response was sufficient to induce a cellular DNA damage response. Benchmark dose modeling confirmed that BT is much more potent than DO. The results strongly suggest that follow-up work is required to assess whether DO and BT are also genotoxic in vivo. This is particularly important for DO, which may require metabolic activation by bacterial gut flora to fully induce its genotoxic potential. Our previously published data and this proof of concept study suggest that the TGx-28.65 genomic biomarker has the potential to add significant value to existing approaches used to assess genotoxicity.	Genotoxicity of THB in vitro TK-6 cells. Need follow-up to confirm in vivo effects (if any)	THB Genotoxicity	
2	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	The standard procedure for the micronucleus test in cell lines requires a short exposure (≤0.5 cell cycle) to the test compounds followed by a long recovery (≥1.5 cell cycle), and in case of negative or equivocal results, a second test with extended exposure (≥2 cell cycles) without or with a recovery time. In general the two procedures are advantageous for detecting clastogens and aneugens, respectively. However, whether the recommended procedures apply to micronucleus tests with promutagens in cell lines genetically engineered for expressing biotransformation enzymes has not been identified. In this study, several promutagens dependent on cytochrome P450 (CYP) 2E1 and/or sulfotransferase (SULT) 1A1 were used in the micronucleus test in a Chinese hamster V79-derived cell line expressing human CYP2E1 and SULT1A1 (V79-hCYP2E1-hSULT1A1), with varying exposure/recovery schedules: 3h/21h, 6h/18h, 12h/12h, 18h/6h, and 24h/0h, in comparison with known clastogens and aneugens in V79 control cells. The results showed peaked micronuclei induction by mitomycin C and bleomycin (clastogens) at the 12h/12h schedule, while colchicine and vinblastine (aneugens) showed the strongest effect at 24h/0h. Catechol and trihydroxybenzene (activated by CYP2E1) induced micronuclei most strongly at 6h/18h, whereas somewhat longer exposures were optimal for hydroquinone, another compound activated by CYP2E1. 1-Hydroxymethylpyrene (activated by SULT1A1) and 1-methylpyrene (activated sequentially by CYP2E1 and SULT1A1) produced the highest response with the 18h/6h treatment regimen. Moreover, mitotic arrest by 1-hydroxymethylpyrene was observed in V79-hCYP2E1-hSULT1A1 cells but not in V79 cells, and 1-methylpyrene arrested mitosis in V79-hCYP2E1-hSULT1A1 more strongly than in V79 cells. Our study suggests that intracellular bioactivation of promutagens may not delay the induction of micronuclei in the present model, and 1-methylpyrene and 1-hydroxymethylpyrene may be activated to mitosis-arresting metabolites.	Benzene metabolites and micronuclei formation in vitro V79 Chinese Hamster cell line	THB Genotoxicity	ROS formation
3	Hossain MZ, Gilbert SF, Patel K, Ghosh S, Bhunia AK, Kern SE.	Biological clues to potent DNA-damaging activities in food and flavoring.	Food Chem Toxicol. 2013 May;55:557-67. doi: 10.1016/j.fct.2013.01.058. Epub 2013 Feb 8.	Population differences in age-related diseases and cancer could stem from differences in diet. To characterize DNA strand-breaking activities in selected foods/beverages, flavorings, and some of their constituent chemicals, we used p53R cells, a cellular assay sensitive to such breaks. Substances testing positive included reference chemicals: quinacrine (peak response, 51×) and etoposide (33×); flavonoids: EGCG (19×), curcumin (12×), apigenin (9×), and quercetin (7×); beverages: chamomile (11×), green (21×), and black tea (26×) and coffee (3-29×); and liquid smoke (4-28×). Damage occurred at dietary concentrations: etoposide near 5μg/ml produced responses similar to a 1:1000 dilution of liquid smoke, a 1:20 dilution of coffee, and a 1:5 dilution of tea. Pyrogallol-related chemicals and tannins are present in dietary sources and individually produced strong activity: pyrogallol (30×), 3-methoxycatechol (25×), gallic acid (21×), and 1,2,4-benzenetriol (21×). From structure-activity relationships, high activities depended on specific orientations of hydroxyls on the benzene ring. Responses accompanied cellular signals characteristic of DNA breaks such as H2AX phosphorylation. Breaks were also directly detected by comet assay. Cellular toxicological effects of foods and flavorings could guide epidemiologic and experimental studies of potential disease risks from DNA strand-breaking chemicals in diets.	Polyphenols in foods and flavors and potential DNA dmaage	THB Genotoxicity	
4	North M, Tandon VJ, Thomas R, Loguinov A, Gerlovina I, Hubbard AE, Zhang L, Smith MT, Vulpe CD.	Genome-wide functional profiling reveals genes required for tolerance to benzene metabolites in yeast.	PLoS One. 2011;6(8):e24205. doi: 10.1371/journal.pone.0024205. Epub 2011 Aug 30.	Benzene is a ubiquitous environmental contaminant and is widely used in industry. Exposure to benzene causes a number of serious health problems, including blood disorders and leukemia. Benzene undergoes complex metabolism in humans, making mechanistic determination of benzene toxicity difficult. We used a functional genomics approach to identify the genes that modulate the cellular toxicity of three of the phenolic metabolites of benzene, hydroquinone (HQ), catechol (CAT) and 1,2,4-benzenetriol (BT), in the model eukaryote Saccharomyces cerevisiae. Benzene metabolites generate oxidative and cytoskeletal stress, and tolerance requires correct regulation of iron homeostasis and the vacuolar ATPase. We have identified a conserved bZIP transcription factor, Yap3p, as important for a HQ-specific response pathway, as well as two genes that encode putative NAD(P)H:quinone oxidoreductases, PST2 and YCP4. Many of the yeast genes identified have human orthologs that may modulate human benzene toxicity in a similar manner and could play a role in benzene exposure-related disease.	THB generate oxidative stress in yeast strains **Same lab (Smith & Zhang)**	THB Genotoxicity	ROS formation
5	Nishikawa T, Miyahara E, Horiuchi M, Izumo K, Okamoto Y, Kawai Y, Kawano Y, Takeuchi T.	Benzene metabolite 1,2,4-benzenetriol induces halogenated DNA and tyrosines representing halogenative stress in the HL-60 human myeloid cell line.	Environ Health Perspect. 2012 Jan;120(1):62-7. doi: 10.1289/ehp.1103437. Epub 2011 Aug 22.	BACKGROUND: Although benzene is known to be myelotoxic and to cause myeloid leukemia in humans, the mechanism has not been elucidated. OBJECTIVES: We focused on 1,2,4-benzenetriol (BT), a benzene metabolite that generates reactive oxygen species (ROS) by autoxidation, to investigate the toxicity of benzene leading to leukemogenesis. METHODS: After exposing HL-60 human myeloid cells to BT, we investigated the cellular effects, including apoptosis, ROS generation, DNA damage, and protein damage. We also investigated how the cellular effects of BT were modified by hydrogen peroxide (H_2O_2) scavenger catalase, hypochlorous acid (HOCl) scavenger methionine, and 4-aminobenzoic acid hydrazide (ABAH), a myeloperoxidase (MPO)-specific inhibitor. RESULTS: BT increased the levels of apoptosis and ROS, including superoxide ($O_2^{\bullet-}$), H_2O_2, HOCl, and the hydroxyl radical (•OH). Catalase, ABAH, and methionine each inhibited the increased apoptosis caused by BT, and catalase and ABAH inhibited increases in HOCl and •OH. Although BT exposure increased halogenated DNA, this increase was inhibited by catalase, methionine, and ABAH. **BT exposure also increased the amount of halogenated tyrosines; however, it did not increase 8-oxo-deoxyguanosine**. CONCLUSIONS: We suggest that BT increases H_2O_2 intracellularly; this H_2O_2 is metabolized to HOCl by MPO, and this HOCl results in possibly cytotoxic binding of chlorine to DNA. Because myeloid cells copiously express MPO and because halogenated DNA may induce both genetic and epigenetic changes that contribute to carcinogenesis, halogenative stress may account for benzene-induced bone marrow disorders and myeloid leukemia.	THB increases intracellular peroxide which is inhibted by catalase; did not increase the 8-oxodguanosine	THB Genotoxicity	ROS formation

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
6	Pandey AK, 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. 2009 Feb 10;661(1-2):57-70. doi: 10.1016/j.mrfmmm.2008.11.006. Epub 2008 Nov 18. Erratum in: Mutat Res. 2009 Dec 1;671(1-2):100.	Exposure of humans to benzene present in environment may lead to adverse chronic effects-even at the genetic level. However, the mechanism of its genotoxicity is not well understood. In the present study, in vitro genotoxicity of benzene (BZ) and its major metabolites [p-benzoquinone (BQ), hydroquinone (HQ), catechol (CT), 1,2,4-benzenetriol (BT) and trans-trans muconic acid (MA)] at concentrations 0.5-50 microM, was assessed in Chinese hamster ovary (CHO) cells employing the alkaline Comet assay, cytokinesis blocked micronucleus (CBMN) assay, flow cytometric analysis of micronucleus (flow MN) and chromosome aberration (CA) test. The data revealed significant (P<0.05) concentration-dependent response in all end points. HQ was found to be the most potent DNA damaging metabolite in the Comet assay followed by BQ>BT>CT>BZ>MA. Both CBMN and flow MN assays revealed a good correlation in their results, where BQ and MA exhibited maximum and minimum micronucleus induction respectively. Significant chromosomal aberrations were induced mainly by BQ, BT and HQ, with moderate response shown by CT and BZ and least by MA. The results demonstrated the utility of sensitive techniques like Comet assay and flow cytometry for determination of MN, to quantify in vitro genotoxicity at low levels and also suggested that partly non-repaired DNA damage could cause adverse health effects in human population exposed to benzene. In silico studies using different endpoints of genotoxicity and molecular docking studies with human topoisomerase-II alpha, a major DNA repair enzyme were also conducted. These corroborated the results obtained from the in vitro data, pointing to a direct relationship of the observed genotoxicity with the structural properties and various interactions of metabolites with the enzyme. This comprehensive study demonstrated that genotoxicity of benzene in mammalian cells is mainly due to the inhibition of topoisomerase by the metabolites.	Benzene metabolite genotoxicity via Comet assay via DNA topoisomerase mechanism	THB Genotoxicity	Topoisomerase
7	Pandey, AK.; Bajpayee, M; Parmar, D; Kumar, R; Rastogi, SK.; Mathur, N; Thorning, P; de Matas, M; Shao, Q; Anderson, D; Dhawan, A	Multipronged evaluation of genotoxicity in Indian petrol-pump workers	Environ Mol Mutagen, 49(9), 695-707, (2008)	Petrol (gasoline) contains a number of toxicants. This study used human biomonitoring to evaluate the genotoxic effects of exposure to benzene in petrol fumes in 100 Indian petrol-pump workers (PPWs) and an equal number of controls. The study was corroborated with in silico assessments of the Comet assay results from the human biomonitoring study. An in vitro study in human lymphocytes was also conducted to understand the genotoxicity of benzene and its metabolites. In a subset of the population studied, higher blood benzene levels were detected in the PPWs (n = 39; P < 0.01) than the controls (n = 18), and 100-250 ppb benzene was also detected in air samples from the petrol pumps. PPWs had higher levels of DNA damage than the controls (P < 0.01). In addition, the micronucleus assay was performed on lymphocytes from a subset of the subjects, and the micronucleus frequency for PPWs was significantly higher (n = 39; 14.79 +/- 3.92 per thousand) than the controls (n = 18; 7.54 +/- 3.00 per thousand). Human lymphocytes were treated in vitro with benzene and several of its metabolites and assayed for DNA damage with the Comet assay. Benzene and its metabolites produced significant (P < 0.05) levels of DNA damage at and above concentrations of 10 microM. The metabolite, p-benzoquinone, produced the greatest amount of DNA damage, followed by hydroquinone > benzene > catechol > 1,2,4,-benzenetriol > muconic acid. This study demonstrates that, using sensitive techniques, it is possible to detect human health risks at an early stage when intervention is possible. possible.	Indian petrol workers were biomonitored to assess BZ expsoure. Benzene and metabolites; THB is among least effective metabolites causing DNA damage **Same lab: #6**	THB Genotoxicity	
8	Pitarque M, Creus A, Marcos R.	Analysis of glutathione and vitamin C effects on the benzenetriol-induced DNA damage in isolated human lymphocytes.	ScientificWorldJournal. 2006 Sep 25;6:1191-201.	The alkaline single-cell gel electrophoresis (or Comet) assay was applied to evaluate the eventual DNA damage induced by the triphenolic metabolite of benzene, 1,2,4-benzenetriol (BT), in isolated human lymphocytes. Prior to BT treatment, ranging from 5 to 50 microM, a supplementation with glutathione (GSH, 350 microg/ml) was carried out to assess whether GSH may have a modulating effect on the Comet response. The effect of a fixed dose of BT was also evaluated in the presence of the exogenous antioxidant vitamin C (40 and 200 microM). Additionally, we investigated whether the polymorphism of glutathione S-transferase T1 (GSTT1) gene may affect the individual level of BT-induced DNA damage in vitro. For all donors included in the present study, BT produced a significant dose-response relationship. No clear effect of GSH preincubation was seen on the BT-induced response. On the contrary, a significant reduction of DNA damage was observed in the presence of vitamin C (at least at 200 microM). Although our data suggest some individual differences according to the GSTT1 genotype in the outcome of the Comet assay, a large number of individuals should be studied in further investigations to obtain reliable conclusions.	THB induced DNA damage via oxidative mechanism reduced by quenching with Vit C	THB Genotoxicity	ROS Formation
9	Hou RC, Chen YS, Chen CH, Chen YH, Jeng KC.	Protective effect of 1,2,4-benzenetriol on LPS-induced NO production by BV2 microglial cells.	J Biomed Sci. 2006 Jan;13(1):89-99. Epub 2005 Nov 25.	Hydroxyhydroquinone or 1,2,4-benzenetriol (BT) detected in the beverages has a structure that coincides with the water-soluble form of a sesame lignan, sesamol. We previously showed that sesame antioxidants had neuroprotective abilities due to their antioxidant properties and/or inducible nitric oxide synthase (iNOS) inhibition. However, studies show that BT can induce DNA damage through the generation of reactive oxygen species (ROS). Therefore, we were interested to investigate the neuroprotective effect of BT in vitro and in vivo. The results showed that instead of enhancing free radical generation, BT dose-dependently (10-100 microM) attenuated nitrite production, iNOS mRNA and protein expression in lipopolysaccharide (LPS)-stimulated murine BV-2 microglia. BT significantly reduced LPS-induced NF-kappaB and p38 MAPK activation. It also significantly reduced the generation of ROS in H2O2-induced BV-2 cells and in H2O2-cellfree conditions. The neuroprotective effect of BT was further demonstrated in the focal cerebral ischemia model of Sprague-Dawley rat. Taken together, the inhibition of LPS-induced nitrite production might be due to the suppression of NF-kappaB, p38 MAPK signal pathway and the ROS scavenging effect. These effects might help to protect neurons from the ischemic injury.	**In vivo** neuroprotective effects of THB; free radical scavenging in Sprague-Dawley rats	THB Genotoxicity	No effect
10	Sommers CH, Schiestl RH.	Effect of benzene and its closed ring metabolites on intrachromosomal recombination in Saccharomyces cerevisiae.	Mutat Res. 2006 Jan 29;593(1-2):1-8. Epub 2005 Sep 9.	Genome rearrangements, such as DNA deletions, translocations and duplications, are associated with cancer in rodents and humans, and clastogens are capable of inducing such genomic rearrangements. The clastogen benzene and several of its toxic metabolites have been shown to cause cancer in animals. Benzene is associated with leukemia and other blood related disorders in humans. Benzene and metabolites tested negative in short-term bacterial mutation assays such as the Salmonella Mutagenicity Test and the Escherichia coli Tryptophan Reversion Assay. These assays, while reliable for the detection of point-mutagenic carcinogens, are incapable of detecting DNA strand break inducing xenobiotics. The yeast DEL assay is based on intrachromosomal recombination events resulting in deletions and is very sensitive in detecting DNA strand breaks. In previous results the DEL assay detected 17 Salmonella positive as well as 25 Salmonella negative carcinogens [Bishop, Schiestl, Hum. Mol. Genet. 9 (2000) 2427-2434]. The carcinogen benzene and its metabolites including phenol, catechol, p-benzoquinone and hydroquinone induced DEL recombination. The benzene metabolite 1,2,4-benzenetriol was negative. Interestingly, p-benzoquinone induced DEL recombination at a dose 300-fold lower than any of the other metabolites, suggesting that it might be responsible for much of benzene's genotoxicity. In addition, an excision repair deficient strain was used, but no difference was detected compared to the wildtype, indicating that DNA adducts subject to excision repair were not formed by benzene or its metabolites.	The yeast DEL assay is based on intrachromosomal recombination events resulting in deletions and is very sensitive in detecting DNA strand breaks. The benzene metabolite THB was negative. Interestingly, p-benzoquinone induced DEL recombination at a dose 300-fold lower than any of the other metabolites, suggesting that it might be responsible for much of benzene's genotoxicity	THB Genotoxicity	No effect
11	Lindsey RH, Bender RP, Osheroff N.	Stimulation of topoisomerase II-mediated DNA cleavage by benzene metabolites.	Chem Biol Interact. 2005 May 30;153-154:197-205. Epub 2005 Apr 25.	Benzene is a human carcinogen that induces hematopoietic malignancies. It is believed that benzene does not initiate leukemias directly, but rather generates DNA damage through a series of phenolic and quinone-based metabolites, especially 1,4-benzoquinone. Since the DNA damage induced by 1,4-benzoquinone is consistent with that of topoisomerase II-targeted drugs, it has been proposed that the compound initiates specific types of leukemia by acting as a topoisomerase II poison. This hypothesis, however, was not supported by initial in vitro studies. While 1,4-benzoquinone inhibited topoisomerase II catalysis, increases in enzyme-mediated DNA cleavage were not observed. Because of the potential involvement of topoisomerase II in benzene-induced leukemias, we re-examined the effects of benzene metabolites (including 1,4-benzoquinone, 1,4-hydroquinone, catechol, **1,2,4-benzenetriol,** 2,2'-biphenol, and 4,4'-biphenol) on DNA cleavage mediated by human topoisomerase IIalpha. In contrast to previous reports, we found that 1,4-benzoquinone was a strong topoisomerase II poison and was more potent in vitro than the anticancer drug etoposide. Other metabolites displayed considerably less activity. DNA cleavage enhancement by 1,4-benzoquinone was unseen in previous studies due to the presence of reducing agents and the incubation of 1,4-benzoquinone with the enzyme prior to the addition of DNA. Unlike anticancer drugs such as etoposide that interact with topoisomerase IIalpha in a noncovalent manner, the actions of 1,4-benzoquinone appear to involve a covalent attachment to the enzyme. Finally, 1,4-benzoquinone stimulated DNA cleavage by topoisomerase IIalpha in cultured human cells. These findings are consistent with the hypothesis that topoisomerase IIalpha plays a role in the initiation of some benzene-induced leukemias.	DNA cleavage mediated by human topoisomerase IIalpha. In contrast to previous reports, we found that 1,4-benzoquinone was a strong topoisomerase II poison and was more potent in vitro than the anticancer drug etoposide. **Other metabolites displayed considerably less activity**. DNA cleavage enhancement by 1,4-benzoquinone was unseen in previous studies due to the presence of reducing agents and the incubation of 1,4-benzoquinone with the enzyme prior to the addition of DNA	THB Genotoxicity	No effect

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
12	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. 2005 Jun 17;157(2):119-28.	In the present study, we investigated in vitro the possible genotoxic and/or co-genotoxic activity of 50 Hz (power frequency) magnetic fields (MF) by using the alkaline single-cell microgel-electrophoresis (comet) assay. Sets of experiments were performed to evaluate the possible interaction between 50 Hz MF and the known leukemogen benzene. Three benzene hydroxylated metabolites were also evaluated: 1,2-benzenediol (1,2-BD, catechol), 1,4-benzenediol (1,4-BD, hydroquinone), and 1,2,4-benzenetriol (1,2,4-BT). MF (1 mT) were generated by a system consisting of a pair of parallel coils in a Helmholtz configuration. To evaluate the genotoxic potential of 50 Hz MF, Jurkat cell cultures were exposed to 1 mT MF or sham-exposed for 1h. To evaluate the co-genotoxic activity of MF, the xenobiotics (benzene, catechol, hydroquinone, and 1,2,4-benzenetriol) were added to Jurkat cells subcultures at the beginning of the exposure time. In cell cultures co-exposed to 1 mT (50 Hz) MF, benzene and catechol did not show any genotoxic activity. However, co-exposure of cell cultures to 1 mT MF and hydroquinone led to the appearance of a clear genotoxic effect. Moreover, co-exposure of cell cultures to 1 mT MF and 1,2,4-benzenetriol led to a marked increase in the genotoxicity of the ultimate metabolite of benzene. The possibility that 50 Hz (power frequency) MF might interfere with the genotoxic activity of xenobiotics has important implications, since human populations are likely to be exposed to a variety of genotoxic agents concomitantly with exposure to this type of physical agent.	Benzene metabolites were evaluated for ability to induce DNA damage via Comet assay.	THB Genotoxicity	ROS Formation
13	Zhang L, Yang W, Hubbard AE, Smith MT.	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. 2005 May;45(4):388-96.	The loss and gain of whole chromosomes (aneuploidy) is common in the development of leukemia and other cancers. In acute myeloid leukemia, the loss (monosomy) of chromosomes 5 and 7 and the gain (trisomy) of chromosome 8 are common clonal chromosomal abnormalities. Here, we have tested the hypothesis that metabolites of the human leukemogen benzene cause a higher rate of gain and loss among the chromosomes involved in leukemogenesis and, as such, are nonrandom and selective in their effects. Human peripheral blood was exposed to two metabolites of benzene, namely, hydroquinone (HQ) and benzenetriol (BT), and the ploidy status of nine different chromosomes (1, 5, 6, 7, 8, 9, 11, 12, and 21) was examined using fluorescence in situ hybridization of metaphase spreads. Poisson regression was used to provide interpretable incidence rate ratios and corresponding P values for all nine chromosomes. Statistically significant differences were found between the sensitivity of the nine chromosomes to gain or loss. Chromosomes 5 and 7 were highly sensitive to loss following HQ and BT exposure, whereas chromosomes 7, 8, and 21 were highly sensitive to gain in comparison to other chromosomes. Significant support for the a priori hypothesis that chromosomes 5 and 7 are more sensitive to loss induced by HQ and BT than the other seven chromosomes was also obtained. These data support the notion that benzene metabolites affect the ploidy status of specific chromosomes more than others and may initiate or promote leukemia induction through these specific effects.	Aneuploidy in vitro resulting from exposure of Human Peripheral blood to benzene metabolites.	THB Genotoxicity	ROS Formation
14	Scassellati Sforzolini G, Moretti M, Villarini M, Fatigoni C, Pasquini R.	[Evaluation of genotoxic and/or co-genotoxic effects in cells exposed in vitro to extremely-low frequency electromagnetic fields].	Ann Ig. 2004 Jan-Apr;16(1-2):321-40. Italian.	During the last two decades, concerns have arisen regarding a possible association between extremely-low frequency (ELF) electromagnetic fields (EMF) exposure and cancer incidence (e.g. childhood acute leukaemia, cancer of the nervous system, and lymphomas). In 1979, Wertheimer and Leeper firstly reported an excess of cancer mortality among children living in homes located near power lines and presumably exposed to elevated magnetic fields. Subsequently, a large number of epidemiological studies investigated the possible association between residential or occupational exposure to ELF-EMF and cancer. Several in vivo and in vitro models have been investigated with the effort to determine a link, if any, between such fields and mutagenesis and to determine the possible mechanism of cancer risk. However, a causal relationship between exposure to ELF-EMF and cancer has been suggested but has not been unequivocally demonstrated. In 1998, following an analysis of the results retrieved in the literature, the U.S. National Institute of Environmental Health Sciences proposed to apply a "possible human carcinogen" category (Group 2B) to ELF-EMF. More recently, in 2002, the same classification for ELF-MF was proposed by the International Agency for Research on Cancer. In this in vitro approach, to test the genotoxic and/or co-genotoxic potency of ELF-MF, we used the alkaline single-cell microgel-electrophoresis (comet) assay and the cytokinesis block micronucleus test. Co-exposure assays were performed in the presence of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), 4-nitroquinoline N-oxide (4NQO), benzene, 1,4-benzenediol (1,4-BD), or 1,2,4-benzenetriol (1,2,4-BT). An ELF-MF (50 Hz, 5 mT) was obtained by a system composed of capsulated induction coils. ELF-MF alone was unable to cause direct primary DNA damage. Whereas, an increased extent of DNA damage was observed in cells co-exposed to ELF-MF and MNNG, 1,4-BD, or 1,2,4-BT. An opposite trend was observed in cells treated with 4NQO and co-exposed to ELF-MF. Moreover, the frequency of micronucleated cells in ELF-MF-exposed cells was higher than in control cultures. Our findings suggest that the tested ELF-MF (50 Hz, 5 mT) possess genotoxic (micronucleus test) and co-genotoxic (comet assay) capabilities. The possibility that ELF-MF might interfere with the genotoxic activity of xenobiotics has important implications, since human populations are likely to be exposed to a variety of genotoxic agents concomitantly with exposure to this type of physical agent.	An increased extent of DNA damage was observed in cells co-exposed to ELF-MF and MNNG, 1,4-BD, or 1,2,4-BT. An opposite trend was observed in cells treated with 4NQO and co-exposed to ELF-MF. Moreover, the frequency of micronucleated cells in ELF-MF-exposed cells was higher than in control cultures. **Same lab as SCCS cited reference (15)**	THB Genotoxicity	ROS Formation
15	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.	The generation, transmission (e.g. power lines, transformers, service wires, and electrical panels), and use (e.g. home appliances, such as electric blankets, shavers, and televisions) of electrical energy is associated with the production of weak electric and magnetic fields (EMF) which oscillate 50 (Europe) or 60 (USA) times per second (power-line frequency), falling in the extremely-low frequency (ELF) region of the electromagnetic spectrum. Epidemiological reports suggest a possible association between exposure to ELF-EMF and an increased risk of cancer (e.g. childhood acute leukaemia). Benzene is an established human leukomogen. This xenobiotic, which is unlikely to be the ultimate carcinogen, is metabolized in the liver to its primary metabolite phenol, which is hydroxylated to hydroquinone (1,4-benzenediol) and 1,2,4-benzenetriol. In this in vitro approach, to test the genotoxic and / or co-genotoxic potency of ELF-EMF, the cytokinesis block micronucleus (MN) method with Jurkat cells has been used. A 50 Hz magnetic field (MF) of 5 mT field strength was applied for different length of time (from 1 to 24 h), either alone or with benzene, 1,4-benzenediol, or 1,2,4-benzenetriol. Our preliminary results show that, after 24 h exposure, the frequency of micronucleated cells in MF-exposed cultures is 1.9 fold higher than in sham-exposed (control) cultures. Benzene exposure does not show any cytogenetic activity, whereas 1,4-benzenediol or 1,2,4-benzenetriol alone significantly affect the number of MN in Jurkat cells, as compared to untreated cultures. Moreover, co-exposure to ELF-MF does not seem to affect the frequency of micronuclei induced by benzene, 1,4-benzenediol, or 1,2,4-benzenetriol.	THB increased MN formation in vitro independent of low dose ELF-MF co-dosing.	THB Genotoxicity	ROS Formation
16	Yasuhara, Y., and, K., Koyama, K., Hiramoto, K., and Kikugawa, K	Effect of Supplementation of a reductone in coffee Hydroxyhydroquinone, on lipid peroxidation and DNA Damage	J. Oleo Sci. 51(10), 669-675 (2002)	The present study was undertaken in order to know the effect of supplementation of a reductone in coffee, hydroxyhydroquinone (HHQ), on lipid peroxidation and DNA damage of rat organs. Wistar make rats were fed a diet of HHQ at 2.0% w/w for 1 week. The extents of lipid peroxidation of lung and heart were increased by HHQ supplementation but that of the liver was not, when assessed by the levels of phospholipid hydroperoxides and thiobarbituric acid-reactive substances. In contrast, the extents of DNA damage of lung, heart and liver were not increased as assessed by 8-hydroxy-2'-deoxyguanisine levels. The results indicate that supplementation of HHQ to rats caused oxidative stress in lung and heart but did not mediate DNA damage in those organs. The effect of HHQ on oxdative stress and the relationship between lipid peroxidation and DNA damage are discussed.	The study showed that THB, a powerful DNA damaging compound **in vivo** did not induce DNA damage in rat lung, heart and liver. [Study limitation N=3 per group]	THB Genotoxicity	No effect

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
17	Chung HW, Kang SJ, Kim SY.	A combination of the micronucleus assay and a FISH technique for evaluation of the genotoxicity of 1,2,4-benzenetriol.	Mutat Res. 2002 Apr 26;516(1-2):49-56.	The cytokinesis-block micronucleus (CBMN) assay has emerged as one of the preferred methods for assessing chromosome damage. Micronuclei (MN) are small, extranuclear bodies that are formed in mitosis from acentric chromosomal fragments or chromosomes that are not included in each daughter nucleus. Thus, MN contain either chromosomal fragments or whole chromosomes. The CBMN assay, together with a fluorescence in situ hybridization (FISH) technique using specific centromeric probes for chromosomes 7 and 8, were employed in mitogen-stimulated human lymphocytes pretreated with the benzene metabolite, 1,2,4-benzenetriol (BT). Treatment of human lymphocytes resulted in the induction of MN in a dose-dependent manner. The frequency of MN in control lymphocytes was 4.5 per 1000 binucleated (BN) cells and this increased to 9.5, 14, 28 and 40 per 1000 BN cells at 10, 25, 50 and 100 microM BT, respectively. The frequency of aneuploidy 7 and 8 in BN cells also increased at each concentration. Aneuploidy 8 was more frequent than aneuploidy 7, suggesting that chromosome 8 is more sensitive to aneuploidy induction by BT. The frequency of MN containing centromere positive signals for chromosomes 7 and 8 increased with the concentration of BT. The frequency of MN with centromere positive signals was higher for chromosome 8 than for chromosome 7, also suggesting a greater sensitivity of chromosome 8 to this agent. These results suggest that combined application of the CBMN assay with a FISH technique, using chromosome-specific centromeric probes, would allow the detection of aneuploidy in human lymphocytes and identify the mechanistic origin of MN induced by a clastogen or aneugen.	Micronuclei formation in vitro on treatment with benzene metabolites using CBMN and FISH.	THB Genotoxicity	ROS Formation
18	Chung HW, Kim SY.	Detection of chromosome-specific aneusomy and translocation by benzene metabolites in human lymphocytes using fluorescence in situ hybridization with DNA probes for chromosomes 5, 7, 8, and 21.	J Toxicol Environ Health A. 2002 Mar;65(5-6):365-72.	Benzene is a widespread human carcinogen, inducing leukemia and hematotoxicity. Exposure of human lymphocytes to benzene metabolites has been shown to cause genetic damage, including aneusomy and chromosome aberrations. In order to detect the specific chromosomal changes in chromosomes 5, 7, 8, and 21 induced by benzene metabolites, 1,2,4-benzenetriol (BT), hydroquinone (HQ), and trans,trans-muconic acid (t,t-MA), fluorescence in situ hybridization (FISH) procedure in the metaphase spread of human lymphocytes was employed. Treatment with BT, HQ and tt-MA resulted in the induction of monosomy 5, 7, 8, and 21 in human lymphocytes in a concentration-dependent manner. All of these metabolites also induced trisomy 5, 7, 8, and 21, but no correlation between frequencies of trisomy and concentration was found. Translocations between chromosome 8 and another unidentified chromosome [t(8;?)] and between chromosome 21 and another unidentified chromosome [t(21;?)] were found. However, translocation between chromosome 8 and 21 [t(8;2 1)] was not found. Results indicate that the benzene metabolites BT, HQ and t,t-MA induce chromosome-specific numerical and structural aberrations, and the fluorescence in situ hybridization (FISH) approach may be a useful and powerful technique for detection of aneuploidy.	In vitro chromosomal damage detected by FISH; **Same lab as SCCS cited reference (17)**	THB Genotoxicity	ROS Formation
19	Li AS, Bandy B, Tsang S, Davison AJ.	DNA breakage induced by 1,2,4-benzenetriol: relative contributions of oxygen-derived active species and transition metal ions.	Free Radic Biol Med. 2001 May 1;30(9):943-56.	We report here the relative roles of metals and selected reactive oxygen species in DNA damage by the genotoxic benzene metabolite 1,2,4-benzenetriol, and the interactions of antioxidants in affording protection. 1,2,4-Benzenetriol induces scission in supercoiled phage DNA in neutral aqueous solution with an effective dose (ED(50)) of 6.7 microM for 50% cleavage of 2.05 microg/ml supercoiled PM2 DNA. In decreasing order of effectiveness: catalase (20 U/ml), formate (25 mM), superoxide dismutase (20 U/ml), and mannitol (50 mM) protected, from 85 to 28%. Evidently, H(2)O(2) is the dominant active species, with O(2)(*)(-) and *OH playing subordinate roles. Desferrioxamine or EDTA inhibited DNA breakage by 81-85%, despite accelerating 1,2,4-benzenetriol autoxidation. Consistent with this suggestion of a crucial role for metals, addition of cupric, cuprous, ferric, or ferrous ions enhanced DNA breakage, with copper being more active than iron. Combinations of scavengers protected more effectively than any single scavenger alone, with implications for antioxidants acting in concert in living cells. Synergistic combinations were superoxide dismutase with *OH scavengers, superoxide dismutase with desferrioxamine, and catalase with desferrioxamine. Antagonistic (preemptive) combinations were catalase with superoxide dismutase, desferrioxamine with *OH scavengers, and catalase with *OH scavengers. The most striking aspect of synergism was the extent to which metal chelation (desferrioxamine) acted synergistically with either catalase or superoxide dismutase to provide virtually complete protection. Concluding, 1,2,4-benzenetriol-induced DNA damage occurs mainly by site-specific, Fenton-type mechanisms, involving synergism between several reactive intermediates. Multiple antioxidant actions are needed for effective protection.	THB induced DNA damage occurs mainly by site-specific, Fenton-type mechanisms, involving synergism between several reactive intermediates. Multiple antioxidant actions are needed for effective protection.	THB Genotoxicity	ROS Formation
20	Li AS, Bandy B, Tsang SS, Davison AJ.	DNA-breaking versus DNA-protecting activity of four phenolic compounds in vitro.	Free Radic Res. 2000 Nov;33(5):551-66.	Given the paradoxical effects of phenolics in oxidative stress, we evaluated the relative pro-oxidant and antioxidant properties of four natural phenolic compounds in DNA nicking. The phenolic compounds differed dramatically in their ability to nick purified supercoiled DNA, with the relative DNA nicking activity in the order: 1,2,4-benzenetriol (100% nicking) > gallic acid > caffeic acid > gossypol (20% nicking). Desferrioxamine (0.02 mM) decreased DNA strand breakage by each phenolic, most markedly with gallate (85% protection) and least with caffeic acid (26% protection). Addition of metals accelerated DNA nicking, with copper more effective (approximately 5-fold increase in damage) than iron with all four phenolics. Scavengers revealed the participation of specific oxygen-derived active species in DNA breakage. Hydrogen peroxide participated in all cases (23-90%). Hydroxyl radicals were involved (32-85%), except with 1,2,4-benzenetriol. Superoxide participated (81-86%) with gallic acid and gossypol, but not with caffeic acid or 1,2,4-benzenetriol. With 1,2,4-benzenetriol, scavengers failed to protect significantly except in combination. Thus, in the presence of desferrioxamine, catalase or superoxide dismutase inhibited almost completely. When DNA breakage was induced by Fenton's reagent (ascorbate plus iron) the two catechols (caffeic acid and gossypol) were protective, whereas the two triols (1,2,4-benzenetriol and gallic acid) exacerbated damage.	DNA damage caused by THB. Scavengers failed to protect significantly except in combination. Thus, for THB in the presence of desferrioxamine, catalase or superoxide dismutase inhibited almost completely. **Same lab as #19**	THB Genotoxicity	ROS Formation
21	Martínez A, Urios A, Blanco M.	Mutagenicity of 80 chemicals in Escherichia coli tester strains IC203, deficient in OxyR, and its oxyR(+) parent WP2 uvrA/pKM101: detection of 31 oxidative mutagens.	Mutat Res. 2000 Apr 13;467(1):41-53.	Strain IC203, deficient in OxyR, and its oxyR(+) parent WP2 uvrA/pKM101 (denoted IC188) are the basis of a new bacterial reversion assay, the WP2 Mutoxitest, which has been used in the evaluation of 80 chemicals for oxidative mutagenicity. The following 31 oxidative mutagens were recognized by their greater mutagenic response in IC203 than in IC188: (1) peroxides: hydrogen peroxide (HP), t-butyl hydroperoxide (BOOH) and cumene hydroperoxide (COOH); (2) benzoquinones (BQ): 2-methyl-1,4-BQ, 2,6-dimethyl-1,4-BQ and 2,3,5,6-tetramethyl-1,4-BQ; (3) naphthoquinones (NQ): 1,4-NQ, 2-methyl-1, 4-NQ and 2-hydroxy-1,4-NQ; (4) phenol derivatives: catechol, hydroquinone, pyrogallol, 1,2,4-benzenetriol, t-butylhydroquinone, gallic acid and 4-aminophenol; (5) catecholamines: DL- and L-dopa, DL- and L-epinephrine, dopamine and L-norepinephrine; (6) thiols: L-cysteine methyl ester, L-cysteine ethyl ester, L-penicillamine and dithiothreitol; (7) diverse: 3,4-dihydroxyphenylacetic acid, hypoxanthine and xanthine, both in the presence of xanthine oxidase, L-ascorbic acid plus copper (II) and phenazine methosulfate. Among these oxidative mutagens, 25 were found to be uniquely positive in IC203. With the exception of BOOH and COOH, mutagenesis by all oxidative mutagens was inhibited by catalase present in rat liver S9, indicating that it is mediated by HP generation, probably in autoxidation reactions. These catalase-sensitive oxidative mutagens were poor inducers of mutations derived from 8-oxoguanine lesions, whereas such mutations were efficiently induced by organic hydroperoxides. The results support the usefulness of incorporating IC203 in the bacterial battery for testing of chemicals. The well-characterized oxidative mutagens available with the use of the WP2 Mutoxitest may serve as a reference in studies on the genotoxicity of oxidative stress.	in vitro mutagenicity (E. coli) for 80 screened chemicals show THB is an oxidative mutagen	THB Genotoxicity	ROS Formation
22	Wiemels J, Wiencke JK, Varykoni A, Smith MT.	Modulation of the toxicity and macromolecular binding of benzene metabolites by NAD(P)H:Quinone oxidoreductase in transfected HL-60 cells.	Chem Res Toxicol. 1999 Jun;12(6):467-75.	Benzene is oxidized in the liver to produce a series of hydroxylated metabolites, including hydroquinone and 1,2,4-benzenetriol. These metabolites are activated to toxic and genotoxic species in the bone marrow via oxidation by myeloperoxidase (MPO). NAD(P)H:quinone oxidoreductase (NQO1) is an enzyme capable of reducing the oxidized quinone metabolites and thereby potentially reducing their toxicities. We introduced the NQO1 gene into the HL-60 cell line to create a high MPO-, high NQO1-expressing cell line, and tested its response in assays of benzene metabolite toxicity. NQO1 expression reduced a class of hydroquinone- and benzenetriol-induced DNA adducts by 79-86%. The cytotoxicity and apoptosis caused by hydroquinone were modestly reduced, while protein binding was unchanged and the rate of glutathione depletion increased. NQO1's activity in reducing a class of benzene metabolite-induced DNA adducts may be related to its known activities in maintaining membrane-bound endogenous antioxidants in reduced form. Alternatively, NQO1 activity may prevent the formation of adducts which result from polymerized products of the quinones. In either case, this protection by NQO1 may be an important mechanism in the observation that a lack of NQO1 activity affords an increased risk of benzene poisoning in exposed individuals [Rothman, N., et al. (1997) Cancer Res. 57, 2839-2842].	Benzene Metabolites DNA adduct formation in vitro	THB Genotoxicity	ROS Formation

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
23	Hiramoto K, Li X, Makimoto M, Kato T, Kikugawa K.	Identification of hydroxyhydroquinone in coffee as a generator of reactive oxygen species that break DNA single strands.	Mutat Res. 1998 Nov 9;419(1-3):43-51.	A component in instant coffee that caused DNA single strand breaks was isolated by successive ethyl acetate:ethanol extraction, silica gel column chromatography and high performance liquid chromatography using a reversed phase column. The active component was identified as hydroxyhydroquinone (HHQ). Incubation of supercoiled pBR 322 DNA with HHQ at 0.1 mM in phosphate buffer (pH 7.4) at 37 degreesC for 1 h caused single strand breaks, and reactive oxygen species, hydrogen peroxide and hydroxyl radical, were involved in DNA breaking by HHQ. Genotoxic effects of HHQ including DNA breaking activity through generation of reactive oxygen species have been well-demonstrated because the component is considered to be an important genotoxic intermediate metabolite of benzene. Occurrence of HHQ in coffee must have an important significance to consider genotoxicity of coffee.	THB causes DNA damage via ROS and is in coffee maybe coffee causes genotoxocity	THB Genotoxicity	ROS Formation
24	Jankowiak, R.; Zamzow, D.; Stack, D. E.; Todorovic, R.; Cavalieri, E. L.; Small, G. J.	Spectral characterization of fluorescently labeled catechol estrogen 3,4-quinone-derived N7 guanine adducts and their identification in rat mammary gland tissue.	Chem Res Toxicol., 11 (11) 1339-1345 (1998)	The oxidation of carcinogenic 4-hydroxycatechol estrogens (CE) of estrone (E1) and estradiol (E2) to catechol estrogen 3,4-quinones (CE-3,4-Q) results in electrophilic intermediates that covalently bind to DNA to form depurinating adducts [Cavalieri et al. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 10937]. These DNA adducts, 4-OHE1-1-N7Gua and 4-OHE2-1-N7Gua, are nonfluorescent. To utilize laser-excited fluorescence methods, the catechol estrogen-derived metabolites and adducts were labeled with a fluorescent marker. The 4-OHEi-1-N7Gua adduct standards (i = 1, 2) and 4-OHEi metabolites have been derivatized with 1-pyrenesulfonyl chloride and investigated by low-temperature spectroscopy under non-line-narrowing and line-narrowing conditions. Molecular modeling studies assisted in interpretation of the fluorescence spectra; energetically favored structures of the 4-OHE2-1-N7Gua-dipyrene adduct and 4-OHE2-dipyrene metabolite reveal unique conformations which, in agreement with fluorescence data, show a significant pi-pi interaction of pyrene labels with guanine and/or the aromatic ring of catechol estrogen. The conformation obtained for the 4-OHE2-1-N7Gua-dipyrene adduct appears to be conducive to mixing of its pipi state with pyrene-guanine charge-transfer states, consistent with the experimentally observed strong electron-phonon coupling. Non-line-narrowed and line-narrowed spectra obtained at 77 and 4.2 K, respectively, are shown to distinguish 4-OHE2-1-N7Gua-dipyrene adducts from 4-OHE2-dipyrene metabolites. These standards have subsequently been used for the spectroscopic identification of depurinating DNA adducts formed in a tissue culture experiment where rat mammary gland tissue was treated with the estrogen quinone E2-3,4-Q. The depurinating adduct formed is 4-OHE2-1-N7Gua.	reference to catechol but not THB? Only in NERAC	THB Genotoxicity	ROS formation
25	Zhang L, Wang Y, Shang N, Smith MT.	Benzene metabolites induce the loss and long arm deletion of chromosomes 5 and 7 in human lymphocytes.	Leuk Res. 1998 Feb;22(2):105-13.	Two of the most common cytogenetic changes in therapy- and chemically-related leukemia are the loss and long (q) arm deletions of chromosomes 5 and 7 (i.e. -5, -7, del(5q) and del(7q)). We have used a novel fluorescence in situ hybridization (FISH) procedure to determine if the benzene metabolites hydroquinone (HQ) and 1,2,4-benzenetriol (BT) can induce these specific changes in human lymphocytes cultured as whole blood. Metaphase spreads were prepared and hybridized with centromeric probes for chromosomes 1, 5 and 7 and sequence specific probes for 5q31 and 7q36-qter. HQ and BT significantly increased monosomy 5 and 7 by 3-5 fold ($p < 0.0001$). Both HQ and BT also significantly increased the rate of del(5q) and del(7q) by 8-12 fold ($p < 0.0001$). Chromosome 7 was especially susceptible to aneusomy induction by HQ and BT at low doses. These results show that metabolites of benzene are highly effective in inducing changes in chromosomes 5 and 7 that are involved in the development of myeloid leukemia.	THB causes sytogenetic effect; **Same lab as SCCS cited reference**	THB Genotoxicity	ROS Formation
26	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.	The alkaline single cell gel electrophoresis (Comet) assay was applied to study the occurrence of DNA damage in peripheral lymphocytes of human subjects with occupational exposure to low levels of benzene (twelve gasoline station attendants, with average benzene exposure of 0.3 mg/m3, 8 h TWA). The results obtained show a significant excess of DNA damage in lymphocytes of exposed workers, compared to matched unexposed controls ($p = 0.028$, Mann-Whitney U-test). Averaged tail moment values, based on 100 cells/individual, were 1.900 microns in the exposed and 0.936 micron in the unexposed group. In addition, exposed subjects showed a clearcut excess of heavily damaged cells, with tail moments > 90th percentile of the overall distribution (13.5 vs. 6.5%, $p = 0.013$, Mann-Whitney U-test). No correlation was found between the extent of DNA damage and the ages or smoking habits of the subjects. In order to assess the plausibility of the involvement of benzene in the results of the ex vivo study, further experiments were performed treating in vitro peripheral lymphocytes from unexposed donors with benzene metabolites hydroquinone, benzoquinone and benzenetriol. In these experiments, all benzene metabolites exerted a marked effect on resting lymphocytes, the lowest effective concentrations being below 1 microgram/ml. Conversely, far greater concentrations were required for the induction of significant DNA damage in parallel experiments with hydroquinone on mitogen stimulated lymphocytes. Addition of the DNA repair inhibitor cytosine arabinoside (Ara-C, 1-10 micrograms/ml) partially restored the sensitivity of stimulated cells to hydroquinone, an indication of the active processing of induced DNA lesions in growing cells. These results are discussed also in relation to the role of peripheral lymphocytes as target tissue in the biomonitoring of human exposure to genotoxic agents.	Benzene metabolite induce DNA damage via Comet assay in vitro using human whole blood obtained from petrol station workers in Italy	THB Genotoxicity	ROS Formation
27	Zhang L, Rothman N, Wang Y, Hayes RB, Bechtold W, Venkatesh P, Yin S, Wang Y, Dosemeci M, Li G, Lu W, Smith MT.	Interphase cytogenetics of workers exposed to benzene.	Environ Health Perspect. 1996 Dec;104 Suppl 6:1325-9.	Fluorescence in situ hybridization (FISH) is a powerful new technique that allows numerical chromosome aberrations (aneuploidy) to be detected in interphase cells. In previous studies, FISH has been used to demonstrate that the benzene metabolites hydroquinone and 1,2,4-benzenetriol induce aneuploidy of chromosomes 7 and 9 in cultures of human cells. In the present study, we used an interphase FISH procedure to perform cytogenetic analyses on the blood cells of 43 workers exposed to benzene (median = 31 ppm, 8-hr time-weighted average) and 44 matched controls from Shanghai, China. High benzene exposure (> 31 ppm, n = 22) increased the hyperdiploid frequency of chromosome 9 ($p < 0.01$), but lower exposure (< or = 31 ppm, n = 21) did not. Trisomy 9 was the major form of benzene-induced hyperdiploidy. The level of hyperploidy in exposed workers correlated with their urinary phenol level ($r = 0.58$, $p < 0.0001$), a measure of internal benzene dose. A significant correlation was also found between hyperdiploidy and decreased absolute lymphocyte count, an indicator of benzene hematotoxicity, in the exposed group ($r = -0.44$, $p = 0.003$) but not in controls ($r = -0.09$, $p = 0.58$). These results show that high benzene exposure induces aneuploidy of chromosome 9 in nondiseased individuals, with trisomy being the most prevalent form. They further highlight the usefulness of interphase cytogenetics and FISH for the rapid and sensitive detection of aneuploidy in exposed human populations.	Aneuploiday as a result of exposure to benzene metabolites in vitro **Same lab as SCCS cited reference**	THB Genotoxicity	ROS Formation
28	Frantz CE, Chen H, Eastmond DA.	Inhibition of human topoisomerase II in vitro by bioactive benzene metabolites.	Environ Health Perspect. 1996 Dec;104 Suppl 6:1319-23.	Benzene is a clastogenic and carcinogenic agent that induces acute myelogenous leukemia in humans and multiple of tumors in animals. Previous research has indicated that benzene must first be metabolized to one or more bioactive species to exert its myelotoxic and genotoxic effects. To better understand the possible role of individual benzene metabolites in the leukemogenic process, as well as to further investigate inhibition of topoisomerase II by benzene metabolites, a series of known and putative benzene metabolites, phenol, 4,4'-biphenol, 2,2'-biphenol, hydroquinone, catechol, 1,2,4-benzenetriol, 1,4-benzoquinone, and trans-trans-muconaldehyde were tested for inhibitory effects in vitro on the human topoisomerase II enzyme. With minor modifications of the standard assay conditions, 1,4-benzoquinone and trans-trans-muconaldehyde were shown to be directly inhibitory, whereas all of the phenolic metabolites were shown to inhibit enzymatic activity following bioactivation using a peroxidase activation system. The majority of compounds tested inhibited topoisomerase II at concentrations at or below 10 microM. These results confirm and expand upon previous findings from our laboratory and indicate that many of the metabolites of benzene could potentially interfere with topoisomerase II. Since other inhibitors of topoisomerase II have been shown to induce leukemia in humans, inhibition of this enzyme by benzene metabolites may also play a role in the carcinogenic effects of benzene.	Benzene metabolite inhibition of DNA Topoisomerase II. Only BQ and HQ were directly inhibitory while THB inhibition required action of a peroxidase activation system.	THB Genotoxicity	Topoisomerase
29	Lévay G, Bodell WJ.	Role of hydrogen peroxide in the formation of DNA adducts in HL-60 cells treated with benzene metabolites.	Biochem Biophys Res Commun. 1996 May 6;222(1):44-9.	We have investigated the influence of peroxides on DNA adduct formation in HL-60 cells treated with polyphenolic metabolites of benzene. Treatment of HL-60 cells with 50 microM hydroquinone (HQ), 500 microM catechol (CAT) or 200 microM 1,2,4-benzenetriol (BT) resulted in adduct levels of 0.27, 0.21 and 0.21 x 10(-7), respectively. Addition of 50-250 microM H2O2 or 250 microM cumene hydroperoxide to HL-60 cells increased DNA adduct formation 2.7-10-fold following treatment with HQ or CAT but had no effect on adduct formation by BT. Treatment of HL-60 cells with the combinations of HQ plus either BT or phorbol myristate acetate (PMA) potentiated DNA adduct formation by 2.5-4-fold. Significant elevations of cellular H2O2 levels occurred after treatment of HL-60 cells with either PMA, CAT or BT. These results indicate that cellular levels of H2O2 regulate the peroxidase dependent activation of benzene metabolites to form DNA adducts.	THB causes oxidative DNA damage in vitro **Same lab as other**	THB Genotoxicity	ROS Formation

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
30	Sze CC, Shi CY, Ong CN.	Cytotoxicity and DNA strand breaks induced by benzene and its metabolites in Chinese hamster ovary cells.	J Appl Toxicol. 1996 May-Jun;16(3):259-64.	The cytotoxicity of benzene (BZ) and its major metabolites phenol (PHE), hydroquinone (HQ), catechol (CAT), 1,4-benzoquinone (BQ), 1,2,4-benzenetriol (BT), trans,trans-muconic acid (ttMA) and S-phenylmercapturic acid (S-PMA) was assessed by exposing Chinese Hamster Ovary (CHO) cells to these compounds. Benzene was the least toxic (LD50 = 20 mM), while BQ showed the highest potency (LD50 = 10 microM), followed by HQ (LD50 = 40 microM). It was found that the trend of cytotoxicity was: BQ > HQ > > CAT > ttMA > BT > S-PMA > > PHE > BZ. 1,4-Benzoquinone and HQ also demonstrated considerable ability to induce DNA strand breaks in CHO cells, which was assayed using the fluorimetric analysis of DNA unwinding. The other metabolites were unable to cause DNA strand breaks. When HQ was administered in combination with other metabolites, no synergism was observed in the induction of DNA strand breaks. From these results, it can be seen that BQ and HQ are the most bioreactive species among the benzene metabolites when tested on CHO cells. Differences between the results obtained in our study and other studies were discussed.	DNA damage in vitro (CHO) BQ and HQ caused DNA strand breaks but **not the others.**	THB Genotoxicity	
31	Shen Y, Shen HM, Shi CY, Ong CN.	Benzene metabolites enhance reactive oxygen species generation in HL60 human leukemia cells.	Hum Exp Toxicol. 1996 May;15(5):422-7.	Benzene is myelotoxic and leukemogenic in humans. The mechanisms leading to these effects, however have not been fully elucidated. One of the underlying mechanisms is believed to be the oxidative damage caused by its metabolites. A comparative study was undertaken to examine the relationships between reactive oxygen species (ROS) production, lipid peroxidation and subsequent cytotoxicity induced by five major benzene metabolites. The generation of ROS by benzene metabolites was demonstrated by the significant and dose-dependent increase of intracellular ROS formation in HL60 human promyelocytic leukemia cells in vitro. 1,4-Benzoquinone (BQ) was found to be the most potent metabolite in induction of ROS formation, followed by 1,2,4-benzenetriol (BT) and to a lesser extent, phenol (PH) and trans, trans-muconaldehyde (MD). No significant effect was observed when the cells were treated with trans, trans-muconic acid (MA). The enhancement of ROS production by BQ was effectively inhibited by the addition of catalase, deferoxamine (DFO) and dimethyl sulfoxide (DMSO), but unchanged by superoxide dismutase (SOD), suggest that hydrogen peroxide (H2O2) and hydroxyl radicals (OH) are the two major forms of ROS involved. The results also demonstrate that the ability of benzene metabolites in enhancing ROS generation is closely correlated to their capacity in causing lipid peroxidation and subsequent cytotoxicity. These findings together with earlier parallel observations on DNA damage suggest that ROS play an important role in the mechanism of carcinogenesis induced by benzene metabolites.	THB causes oxidative DNA damage in vitro	THB Genotoxicity	ROS Formation
32	Zhang L, Bandy B, Davison AJ.	Effects of metals, ligands and antioxidants on the reaction of oxygen with 1,2,4-benzenetriol.	Free Radic Biol Med. 1996;20(4):495-505.	1,2,4-Benzenetriol is an active metabolite of the human leukemogen benzene that reacts rapidly with molecular oxygen (O2). The mechanism of autoxidation of benzenetriol is scantily characterized, and little is known of the effects of metals, metal chelators, radical scavengers, and antioxidants on the rate of reduction of O2. Here, we report that catalytic amounts of Cu2+ and Fe3+ accelerated the oxidation of benzenetriol (250 mu M) in a dose-dependent manner. Fe3+ (50 mu M) increased the rate of autoxidation by 91%, and Cu2+ (10 mu M) increased it 11-fold. In the absence of added metals, superoxide dismutase inhibited and desferrioxamine stimulated the autoxidation. In the Cu2+ -catalyzed reaction, superoxide dismutase neither inhibited nor stimulated, while desferrioxamine abolished the catalysis by Cu2+. In the presence of Fe3+, superoxide dismutase slowed the reaction, but desferrioxamine, surprisingly, did not. The presence of both superoxide dismutase and desferrioxamine blocked the autoxidation, either in the presence or absence of metals. We conclude: (1) superoxide is a propagator of sequential one-electron transfer reactions in the absence of added metals; (2) addition of Cu2+, unlike Fe3+, removes the dependence of the reaction on propagation by superoxide, presumably changing the radical-propagated chain reaction to a concerted two-electron transfer; (3) the further addition of desferrioxamine restores superoxide-dependent propagation. Taken with our previous data on the genotoxicity of benzenetriol, these findings have implications regarding a role for transition metals in the carcinogenicity of benzene.	Same lab as SCCS cited literature	THB Genotoxicity	ROS Formation
33	Hedli CC, Rao NR, Reuhl KR, Witmer CM, Snyder R.	Effects of benzene metabolite treatment on granulocytic differentiation and DNA adduct formation in HL-60 cells.	Arch Toxicol. 1996;70(3-4):135-44.	Reactive metabolites of benzene (BZ) play important roles in BZ-induced hematotoxicity. Although reactive metabolites of BZ covalently bind to DNA, the significance of DNA adduct formation in the mechanism of BZ toxicity is not clear. These studies investigated the covalent binding of the BZ metabolites hydroquinone(HQ) and 1,2,4-benzenetriol(BT) using the DNA [32P]postlabeling method and explored the potential relationship between DNA adduct formation and cell differentiation in human promyelocytic leukemia (HL-60) cells, a model system for studying hematopoiesis. Maturation of HL-60 cells to granulocytes, as assessed by light and electron microscopy, was significantly inhibited in cells that were pretreated with HQ or BT prior to inducing differentiation with retinoic acid (RA). The capacity of RA-induced cells to phagocytose sheep red blood cells (RBC) and to reduce nitroblue tetrazolium (NBT), two functional parameters characteristic of mature, differentiated neutrophils, was also inhibited in cells pretreated with HQ or BT. These BZ metabolite treatments induced DNA adduct formation in HQ- but not in BT-treated cells. These results indicate that whereas HQ and BT each block granulocytic differentiation in HL-60 cells, DNA adducts were observed only following HQ treatment. Thus DNA adduct formation may be important in HQ but not in THB Toxicity.	These BZ metabolite treatments induced DNA adduct formation in HQ- **but not in BT-treated cells.**	THB Genotoxicity	
34	Lee SF, Liang YC, Lin JK.	Inhibition of 1,2,4-benzenetriol-generated active oxygen species and induction of phase II enzymes by green tea polyphenols.	Chem Biol Interact. 1995 Dec 22;98(3):283-301.	Autooxidation of polyphenolic metabolites of benzene, such as hydroquinone (HQ), catechol (CT), 1,2,4-benzenetriol (BT) and pyrogallol (PG), produced several kinds of active oxygen species (AOS). BT and PG induced DNA breaks in the absence of metal ions, especially when producing AOS such as H2O2, O2-, HO. or 1 delta gO2. HQ and CT did not result in double-strand DNA breaks, except when ferrous ion was added, indicating the participation of the Fenton reaction. Polyphenolic fractions isolated from green tea (GTP) exerted inhibitory effects on the autooxidation of BT and suppressive effects on H2O2 or HO. generated from phenolic metabolites of benzene in the presence of S9 or an in vivo system. Additionally, although the activities of antioxidant and phase II enzymes were elevated by both GTP and phenolic metabolites of benzene, GTP counteracted the lowering GSH caused by phenolic metabolites of benzene in rat liver. The above results suggest that GTP and phenolic metabolites of benzene are antagonistic in their response to AOS, especially hydroxyl radical.	Polyphenols in green tea cause genetox in vitro by ROS	THB Genotoxicity	ROS Formation
35	Chen H, Eastmond DA.	Topoisomerase inhibition by phenolic metabolites: a potential mechanism for benzene's clastogenic effects.	Carcinogenesis. 1995 Oct;16(10):2301-7.	Exposure to benzene, a human and animal carcinogen, results in the formation of structural chromosomal aberrations in the bone marrow and blood cells of animals and humans. The mechanisms underlying these clastogenic effects are unknown. Inhibition of enzymes involved in DNA replication and repair, such as topoisomerase enzymes, by the metabolites of benzene represents a potential mechanism for the formation of chromosomal aberrations. To test this hypothesis, the inhibitory effects of various phenolic and quinone metabolites of benzene on the activity of human topoisomerases I and II were studied in vitro. No inhibition of topoisomerase I was seen with any of the tested metabolites. Inhibitory effects on topoisomerase II were not observed for hydroquinone, phenol, 2,2'-biphenol, 4,4'-biphenol and catechol at concentrations as high as 500 microM. 1,4-Benzoquinone and 1,2,4-benzenetriol inhibited topoisomerase II at relatively high 500 and 250 microM concentrations, respectively. However following bioactivation using a peroxidase/H2O2 system, inhibitory effects were seen at concentrations as low as 50 microM for both phenol and 2,2'-biphenol and 10 microM for 4,4'-biphenol. The addition of reduced glutathione (GSH) to the 4,4'-biphenol and horseradish peroxidase reaction system protected topoisomerase II from inhibition suggesting that diphenoquinone or another oxidation product formed from 4,4'-biphenol might be the reactive species. These in vitro results indicate that inhibition of topoisomerase II may contribute to the clastogenic and carcinogenic effects of benzene. In addition, metabolites formed from these phenolic compounds appear to represent several new types of topoisomerase II-inhibiting compounds.	THB inhibited Topoisomerse II at high concentrations	THB Genotoxicity	Topoisomerase

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
36	Rao NR, Snyder R.	Oxidative modifications produced in HL-60 cells on exposure to benzene metabolites.	J Appl Toxicol. 1995 Sep-Oct;15(5):403-9.	We have studied the effects of the benzene metabolites hydroquinone, p-benzoquinone or 1,2,4-benzenetriol on cytotoxicity, active oxygen formation, hydrogen peroxide (i.e. hydroperoxide) production and nitric oxide formation in HL-60 cells. We also examined the effects of these compounds on antioxidant enzymes and intracellular antioxidants in these cells. The cytotoxicity of benzene metabolites to HL-60 cells was found to be of the order of p-benzoquinone>hydroquinone>benzenetriol. No appreciable changes in the basal levels of either superoxide anion production or nitric oxide formation were observed following exposures to the benzene metabolites, but significant increases in superoxide were seen on stimulation with TPA for each metabolite, whereas hydroquinone and p-benzoquinone, but not 1,2,4-benzenetriol, increased nitric oxide production under these conditions. Following exposure to the benzene metabolites, HL-60 cells showed significant rises in hydrogen peroxide formation compared to controls. The study of antioxidant enzymes and intracellular antioxidants suggested that the benzene metabolites inhibit or reduce the levels of different antioxidant mechanisms and, thereby, cause the accumulation of free radicals in these cells predisposing them for oxidative damage.	Increase in H2O2 production in HL-60 cells after expsoure to benzene metabolites.	THB Genotoxicity	ROS Formation
37	Pathak DN, Lévay G, Bodell WJ.	DNA adduct formation in the bone marrow of B6C3F1 mice treated with benzene.	Carcinogenesis. 1995 Aug;16(8):1803-8.	We used P1-enhanced 32P-postlabeling to investigate DNA adduct formation in the bone marrow of B6C3F1 mice treated intraperitoneally with benzene (BZ). No adducts were detected in the bone marrow of controls or mice treated with various doses of BZ once a day. After twice-daily treatment with BZ, 440 mg/kg, for 1 to 7 days, one major and two minor DNA adducts were detected. The relative adduct levels ranged from 0.06-1.46 x 10(-7). In vitro treatment of bone marrow from B6C3F1 mice with various doses of hydroquinone (HQ) for 24 h also produced three DNA adducts. These adducts were the same as those formed after in vivo treatment of bone marrow with BZ. Co-chromatography experiments indicated that the principal DNA adduct detected in the bone marrow of B6C3F1 mice was the same as that detected in HL-60 cells treated with HQ. This finding suggests that HQ may be the principal metabolite of BZ leading to DNA adduct formation in vivo. DNA adduct 2 corresponds to the DNA adduct formed in HL-60 cells treated with 1,2,4-benzenetriol. DNA adduct 3 remains unidentified. After a 7-day treatment with BZ, 440 mg/kg twice a day, the number of cells per femur decreased from 1.6 x 10(7) to 0.85 x 10(7), indicating myelotoxicity. In contrast, administration of BZ once a day produced only a small decrease in bone marrow cellularity. These studies demonstrate that metabolic activation of BZ leads to the formation of DNA adducts in the bone marrow. Further investigation is required to determine the role of DNA adducts and other forms of DNA damage in the myelotoxic effects of exposure to BZ.	DNA adduct formed with treatment of THB which crrelated to adduct 2 formed after multiple twice daily treatments IP of benzene in B6C3F1 mice Same lab as other #29	THB Genotoxicity	
38	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.	Benzene and five of its known metabolites--muconic acid, hydroquinone, catechol, p-benzoquinone, and benzentriol--were examined for DNA damage in human lymphocytes using the alkaline Comet assay, and conditions were optimised to determine responses. Metabolic activation (S-9 mix) was included in the assay for varying times to try to enhance effects. In addition, the effects of catalase were investigated as it is known to be present in S-9 mix reducing oxidative damage, and some benzene metabolites are known to react through oxygen radical mechanisms. Effects were also examined in cycling cells to determine whether they were more sensitive to damage then noncycling cells. Comets were measured either by eye or by image analysis. Data have been presented according to length of treatments. When Comets were measured by eye after treatment with hydrogen peroxide (H2O2), the positive control, and each compound for 0.5 hr, only H2O2 and benzenetriol induced pronounced DNA damage without metabolic activation. The effect of catechol was moderate compared with that of benzenetriol. There was a very weak effect of benzene in the absence of rat liver S-9 mix. In the presence of S-9 mix, benzene was not activated. The effect of benzenetriol was greatly reduced by the external metabolising system, but p-benzoquinone became activated to some extent. Catalase abolished the effect of benzenetriol, suggesting that H2O2 formed during autoxidation may be responsible for the DNA-damaging ability of this metabolite. The presence of catalase in S-9 mix may explain the detoxification of benzenetriol and the failure to detect consistent benzene responses. Mitogen-stimulated cycling cells were less sensitive to H2O2 and benzenetriol than unstimulated G0 lymphocytes. When comets were measured by image analysis, a 0.5-hr treatment with H2O2 and benzenetriol and catechol confirmed results analysed by eye, with S-9 mix greatly reducing responses. When treatments were increased to 1 hr in the presence and absence of S-9 mix, benzene at a 5-fold increased dose produced a significant positive response but not at the lower dose. When treatment times were increased to 2 and 4 hr, doses were also increased, and muconic acid, hydroquinone, catechol, and benzoquinone in the presence of S-9 mix showed positive time and dose-related responses, and at the highest dose of benzoquinone the morphology of the nucleus was affected. Effects tended to become more pronounced at high doses and after longer exposures, although this was not always consistent from experiment to experiment. In conclusion, benzene and all metabolites investigated gave positive responses. Where altered responses were observed, they were significantly different from the corresponding controls.	Benzene metabolites cause DNA damage in vitro Comet assay. Presence of S9 explains the detoxification of THB	THB Genotoxicity	ROS Formation
39	Laskin JD, Rao NR, Punjabi CJ, Laskin DL, Synder R.	Distinct actions of benzene and its metabolites on nitric oxide production by bone marrow leukocytes.	J Leukoc Biol. 1995 Mar;57(3):422-6.	Benzene is a widely used industrial solvent known to cause bone marrow depression. This is associated with increased production of reactive oxygen metabolites and nitric oxide by bone marrow phagocytes, which have been implicated in hematotoxicity. Benzene metabolism to phenolic intermediates appears to be an important factor in bone marrow toxicity. In the present studies, we compared the effects of benzene and several of its metabolites on nitric oxide production by murine bone marrow leukocytes. Bone marrow cells readily produced nitric oxide in response to the inflammatory mediators lipopolysaccharide (LPS) and interferon-gamma (IFN-gamma). Treatment of mice with benzene (800 mg/kg), or its metabolites hydroquinone (100 mg/kg), 1,2,4-benzenetriol (25 mg/kg), or p-benzoquinone (2 mg/kg), at doses that impair hematopoiesis, sensitized bone marrow leukocytes to produce increased amounts of nitric oxide in response to LPS and IFN-gamma. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) augmented bone marrow leukocyte production of nitric oxide induced by inflammatory mediators. Benzene, as well as its metabolites, markedly increased the sensitivity of the cells to both GM-CSF and M-CSF. Cells from hydroquinone- or 1,2,4-benzenetriol-treated mice were significantly more responsive to the inflammatory cytokines and growth factors than cells isolated from benzene- or p-benzoquinone-treated mice, suggesting that the phenolic metabolites of benzene are important biological reactive intermediates. Because nitric oxide suppresses cell growth and can be metabolized to mutagens and carcinogens, the ability of benzene and its metabolites to modulates its production in the bone marrow may be important in their mechanism of action.		THB Genotoxicity	
40	Li Y, Trush MA.	Reactive oxygen-dependent DNA damage resulting from the oxidation of phenolic compounds by a copper-redox cycle mechanism.	Cancer Res. 1994 Apr 1;54(7 Suppl):1895s-1898s.	Recently, copper has been shown to be capable of mediating the activation of several xenobiotics producing reactive oxygen and other radicals. Since copper exists in the nucleus and is closely associated with chromosomes and DNA bases, in this study we have investigated whether the activation of 1,4-hydroquinone (1,4-HQ) and a variety of other phenolic compounds by copper can induce strand breaks in double-stranded phi X-174 RF I DNA (phi X-174 relaxed form I DNA). In the presence of micromolar concentrations of Cu(II), DNA strand breaks were induced by 1,4-HQ and other phenolic compounds including 4,4'-biphenol, catechol, 1,2,4-benzenetriol, 2-methoxyestradiol, 2-hydroxyestradiol, diethylstilbestrol, butylated hydroxytoluene, butylated hydroxyanisole, tert-butylhydroquinone, ferulic acid, caffeic acid, chlorogenic acid, eugenol, 2-acetamidophenol, and acetaminophen. Structure-activity analysis shows that in the presence of Cu(II), the DNA cleaving activity for phenolic compounds with a 1,4-hydroquinone structure, such as 1,2,4-benzenetriol and tert-butylhydroquinone is greater than those with a catechol group (catechol, 2-hydroxyestradiol and caffeic acid). Those compounds having one phenol group, such as eugenol, 2-acetamidophenol, and acetaminophen, are the least reactive. In addition, the induced DNA strand breaks could be inhibited by bathocuproinedisulfonic acid, a Cu(I)-specific chelator, or catalase indicating that a Cu(II)/Cu(I) redox cycle and H2O2 generation are two major determinants involved in the observed DNA damage. Using reactive oxygen scavengers, it was observed that the DNA strand breaks induced by the 1,4-HQ/Cu(II) system could not be efficiently inhibited by hydroxyl radical scavengers, but could be protected by singlet oxygen scavengers, suggesting that either singlet oxygen or a singlet oxygen-like entity, possibly a copper-peroxide complex, but not free hydroxyl radical probably plays a role in the DNA damage. The above results would suggest that macromolecule-associated copper and reactive oxygen generation may be important factors in the mechanism of 1,4-HQ and other phenolic compound-induced DNA damage in target cells	THB causes oxidative DNA damage in vitro	THB Genotoxicity	ROS Formation

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
41	Lee SF, Lin JK.	Generation of Hydrogen Peroxide, Superoxide Anion and the Hydroxyl Free Radical from Polyphenols and Active Benzene Metabolites: Their Possible Role in Mutagenesis.	J Biomed Sci. 1994 Mar;1(2):125-130.	Benzene is strongly suspected of being an animal and human carcinogen, but the mechanisms by which it induces tumors of lymphoid and hematopoietic organs are unknown. Production of active oxygen species from benzene metabolites [hydroquinone (HQ), catechol and 1,2,4-benzenetriol (1,2,4-BT) and related polyphenols (resorcinol, pyrogallol and phloroglucinol)] are investigated. Pyrogallol and 1,2,4-BT can produce H(2)O(2), O(-)(2) and (.)OH simultaneously, and have powerful mutagenic potential. Resorcinol and phloroglucinol cannot produce all of the active oxygen species, and show no mutagenic effects. Catechol can produce H(2)O(2), but cannot produce O(-)(2) and (.)OH, and has no mutagenic activity. These data strongly support the hypothesis that benzene metabolites can cause mutagenicity via the generation of oxygen radicals. Although HQ produces H(2)O(2) only, and less than produced by pyrogallol and 1,2,4-BT, the mutagenicity of HQ is higher. The results indicate that HQ may act via another mechanism to cause mutagenicity. In the presence of trace metal ions, the reactivity of polyphenols is increased. The biological significance of these phenomena are investigated and discussed. Copyright 1994 S. Karger AG, Basel.	Role of ROS in mutagenesis Same lab as other	THB Genotoxicity	ROS Formation
42	Zhang L, Venkatesh P, Creek ML, Smith MT.	Detection of 1,2,4-benzenetriol induced aneuploidy and microtubule disruption by fluorescence in situ hybridization and immunocytochemistry.	Mutat Res. 1994 Mar;320(4):315-27.	Fluorescence in situ hybridization (FISH) is becoming increasingly used to detect chromosomal changes in cancer cytogenetics. Here, we report its use in human HL60 cells to detect aneuploidy induced by the benzene metabolite, 1,2,4-benzenetriol (BT). Human centromeric probes specific for chromosomes 9 and 7 were used. Untreated HL60 cells were 0.72 +/- 0.29% hyperdiploid for chromosome 9. Treatment with 5 microM BT increased this level 3-fold to 2.20 +/- 0.87% and 50 microM increased it 4-fold to 2.96 +/- 0.74%. Similar results were obtained with the chromosome 7 probe. The induction of aneuploidy by BT is therefore not chromosome-specific nor is it artifactual. Immunocytochemical staining with anti-tubulin antibodies also showed that BT disrupted microtubule organization at these concentrations. Thus, mitotic spindle disruption probably plays an important role in BT-induced aneuploidy. Trisomy and not tetrasomy accounted for the majority of the hyperdiploidy induced by BT in the two C-group chromosomes 7 and 9. Since trisomy of C-group chromosomes is commonly observed in leukemia, BT-induced aneuploidy may be involved in benzene-induced leukemia.	Benzene metabolites cause DNA damage in vitro	THB Genotoxicity	ROS Formation
43	Li Y, Trush MA.	DNA damage resulting from the oxidation of hydroquinone by copper: role for a Cu(II)/Cu(I) redox cycle and reactive oxygen generation.	Carcinogenesis. 1993 Jul;14(7):1303-11.	The myelotoxicity, including leukemia, associated with benzene exposure has been attributed to the further activation of benzene-derived metabolites. In a previous study, we observed that (Cu(II) strongly mediates the oxidation of hydroquinone (HQ) producing benzoquinone (BQ) and H2O2 through Cu(II)/Cu(I) redox mechanism. Since copper exists in the nucleus and is closely associated with chromosomes and DNA, in this study we investigated whether this chemical--metal redox system induces strand breaks in phi X-174 RFI plasmid DNA. In the presence of micromolar concentrations of Cu(II) and HQ, both single and double strand breaks were induced, whereas HQ, Cu(II), H2O2 or BQ alone at the employed concentrations elicited no significant damage to DNA. The HQ/Cu(II) system was at least twice as efficient as a H2O2/Cu(II) system at inducing DNA strand breaks. Of Cu(II), Fe(III), Mn(II), Cd(II) and Zn(II), only HQ/Cu(II) induced extensive DNA strand breaks. Among HQ, 1,2,4-benzenetriol (BT), catechol and phenol, HQ/Cu(II) and BT/Cu(II) were the two most efficient DNA cleaving systems. The presence of bathocuproinedisulfonic acid (BCS) or catalase prevented the HQ/Cu(II)-induced DNA strand breaks. In addition, the HQ/Cu(II)-induced DNA strand breaks could be completely blocked by reduced glutathione and dithiothreitol, but not by L-cysteine. The interaction of L-cysteine with copper in the absence of HQ induced significant DNA strand breaks with the same pattern of DNA strand breaks as that of HQ/Cu(II) plus L-cysteine. In contrast to the HQ/Cu(II) system, a HQ/myeloperoxidase (MPO)/H2O2 system did not induce any DNA strand breaks, and furthermore, the presence of MPO inhibited the HQ/Cu(II)-induced DNA strand breaks. When DNA pretreated with Cu(II) was exposed to HQ, DNA strand breaks were formed that could be prevented by BCS or catalase, indicating that DNA-bound copper can undergo redox cycling in the presence of HQ, generating H2O2. Similar to the H2O2/Cu(II) system, the HQ/Cu(II)-induced DNA strand breaks could not be efficiently inhibited by hydroxyl radical scavengers but could be protected by singlet oxygen scavengers, indicating that the localized generation of singlet oxygen or a singlet oxygen-like entity, possibly a copper-peroxide complex, rather than free hydroxyl radical probably plays a role in the HQ/Cu(II)-induced DNA strand breaks. The above results suggest that macromolecule-associated copper and reactive oxygen generation may be important factors in the mechanism of HQ-induced DNA damage in target cells.	Benzene metabolite cause oxidative DNA damage	THB Genotoxicity	ROS Formation
44	Kolachana P, Subrahmanyam VV, Meyer KB, Zhang L, Smith MT.	Benzene and its phenolic metabolites produce oxidative DNA damage in HL60 cells in vitro and in the bone marrow in vivo.	Cancer Res. 1993 Mar 1;53(5):1023-6.	Benzene, an important industrial chemical, is myelotoxic and leukemogenic in humans. It is metabolized by cytochrome P450 2E1 to various phenolic metabolites which accumulate in the bone marrow. Bone marrow contains high levels of myeloperoxidase which can catalyze the further metabolism of the phenolic metabolites to reactive free radical species. Redox cycling of these free radical species produces active oxygen. This active oxygen may damage cellular DNA (known as oxidative DNA damage) and induce genotoxic effects. Here we report the induction of oxidative DNA damage by benzene and its phenolic metabolites in HL60 cells in vitro and in the bone marrow of C57BL/6 x C3H F1 mice in vivo utilizing 8-hydroxy-2'-deoxyguanosine as a marker. HL60 cells (a human leukemia cell line) contain high levels of myeloperoxidase and were used as an in vitro model system. Exposure of these cells to phenol, hydroquinone, and 1,2,4-benzenetriol resulted in an increased level of oxidative DNA damage. An increase in oxidative DNA damage was also observed in the mouse bone marrow in vivo 1 h after benzene administration. A dose of 200 mg/kg benzene produced a 5-fold increase in the 8-hydroxydeoxyguanosine level. Combinations of phenol, catechol, and hydroquinone also resulted in significant increases in steady state levels of oxidative DNA damage in the mouse bone marrow but were not effective when administered individually. Administration of 1,2,4-benzenetriol alone did, however, result in a significant increase in oxidative DNA damage. This represents the first direct demonstration of active oxygen production by benzene and its phenolic metabolites in vivo. The conversion of benzene to phenolic metabolites and the subsequent production of oxidative DNA damage may therefore play a role in the benzene-induced genotoxicity, myelotoxicity, and leukemia.	Benzene metabolites cause oxidative DNA damage	THB Genotoxicity	ROS Formation
45	Zhang L, Robertson ML, Kolachana P, Davison AJ, Smith MT.	Benzene metabolite, 1,2,4-benzenetriol, induces micronuclei and oxidative DNA damage in human lymphocytes and HL60 cells.	Environ Mol Mutagen. 1993;21(4):339-48.	The triphenolic metabolite of benzene, 1,2,4-benzenetriol (BT), is readily oxidized to its corresponding quinone via a semiquinone radical. During this process, active oxygen species are formed that may damage DNA and other cellular macromolecules. The ability of BT to induce micronuclei (MN) and oxidative DNA damage has been investigated in both human lymphocytes and HL60 cells. An antikinetochore antibody based micronucleus assay was used to distinguish MN containing kinetochores and potentially entire chromosomes (kinetochore-positive, K+) from those containing acentric chromosome fragments (kinetochore-negative, K-). BT increased the frequency of MN formation twofold in lymphocytes and eightfold in HL60 cells with the MN being 62% and 82% K+, respectively. A linear dose-related increase in total MN, mainly in K(+)-MN, was observed in both HL60 cells and lymphocytes. Addition of copper ions (Cu2+) potentiated the effect of BT on MN induction threefold in HL60 cells and altered the pattern of MN formation from predominantly K+ to K-. BT also increased the level of 8-hydroxy-2'-deoxyguanosine (8-OH-dG), a marker of active oxygen-induced DNA damage. Cu2+ again enhanced this effect. Thus, BT has the potential to cause both numerical and structural chromosomal changes in human cells. Further, it may cause point mutations indirectly by generating oxygen radicals. BT may therefore play an important role in benzene-induced leukemia.	Benzene metabolites cause oxidative DNA damage in vitro	THB Genotoxicity	ROS Formation
46	Lin JK, Lee SF.	Enhancement of the mutagenicity of polyphenols by chlorination and nitrosation in Salmonella typhimurium.	Mutat Res. 1992 Oct;269(2):217-24.	The hydrolytic products of lignins, humic acids and industrial waste including hydroquinone, catechol, resorcinol, pyrogallol and 1,2,4-benzenetriol are widely distributed in water sources. These polyphenols can interact with chlorine or nitrite to yield new derivatives. Generally, these new products possess more mutagenic potential than their original compounds. Furthermore, the mutagenicity of these polyphenols and their derivatives can be dramatically reduced by rodent liver microsomal enzymes (S9). The mutagenicity of polyphenols is in this order: hydroquinone greater than 1,2,4-benzenetriol greater than pyrogallol, while catechol, resorcinol and phloroglucinol are non-mutagenic. The ultimate product of chlorination or nitrosation of hydroquinone has been identified to be p-benzoquinone. The formation of active oxygen species including superoxide anion and hydrogen peroxide by polyphenols has been demonstrated and this may contribute partly to the molecular mechanisms of polyphenol mutagenicity.	In vitro genetox Ames	THB Genotoxicity	ROS Formation

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
47	Lévay G, Bodell WJ.	Potentiation of DNA adduct formation in HL-60 cells by combinations of benzene metabolites.	Proc Natl Acad Sci U S A. 1992 Aug 1;89(15):7105-9.	We have investigated the influence of peroxides on DNA adduct formation in HL-60 cells treated with polyphenolic metabolites of benzene. Treatment of HL-60 cells with 50 microM hydroquinone (HQ), 500 microM catechol (CAT) or 200 microM 1,2,4-benzenetriol (BT) resulted in adduct levels of 0.27, 0.21 and 0.21 x 10(-7), respectively. Addition of 50-250 microM H2O2 or 250 microM cumene hydroperoxide to HL-60 cells increased DNA adduct formation 2.7-10-fold following treatment with HQ or CAT but had no effect on adduct formation by BT. Treatment of HL-60 cells with the combinations of HQ plus either BT or phorbol myristate acetate (PMA) potentiated DNA adduct formation by 2.5-4-fold. Significant elevations of cellular H2O2 levels occurred after treatment of HL-60 cells with either PMA, CAT or BT. These results indicate that cellular levels of H2O2 regulate the peroxidase dependent activation of benzene metabolites to form DNA adducts.	DNA adduct formed with in vitro treatment of THB **Same lab as #29 and 37**	THB Genotoxicity	ROS Formation
48	Lee EW, Garner CD.	Effects of benzene on DNA strand breaks in vivo versus benzene metabolite-induced DNA strand breaks in vitro in mouse bone marrow cells.	Toxicol Appl Pharmacol. 1991 May;108(3):497-508.	Previously, we identified p-benzoquinone (BQ) and 1,2,4-benzenetriol (BT) as toxic metabolites of benzene on the basis of their inhibitory effect on DNA synthesis. In the present study, the capability of benzene and the two metabolites to induce DNA strand breaks was investigated in either the in vivo or the in vitro system by comparing the DNA elution rate on a fine membrane filter at alkaline pH. In the in vitro system were bone marrow cells were reacted with test chemicals for 60 min, both BQ and BT induced a dose-related increase in alkali-labile DNA single-strand breaks (SSBs) of bone marrow cells. However, when glutathione (350 micrograms/ml) was added to the same reaction system, the DNA damaging effect of BQ (24 microM) and BT (24 microM) was blocked by 100 and 53%, respectively. Catalase (130 units/ml) completely blocked the DNA damaging effect of BT, while no protection was afforded with BQ. Consistent with these observations, no induction of alkali-labile DNA SSBs was observed in the in vivo system by an anesthetic dose of benzene (1760 mg/kg, ip or po) at 1, 24, and 36 hr postadministration in both male and female ICR mice. These results suggest that benzene exposure would not induce direct DNA strand breaks in vivo under realistic work-related or accidental exposure conditions and also indicate that caution should be exercised in the interpretation of in vitro data for whole-body toxicity evaluation.	Benzene metabolites cause oxidative DNA damage	THB Genotoxicity	ROS Formation
49	Subrahmanyam VV, Ross D, Eastmond DA, Smith MT.	Potential role of free radicals in benzene-induced myelotoxicity and leukemia.	Free Radic Biol Med. 1991;11(5):495-515. Review.	Occupational exposure to benzene, a major industrial chemical, has been associated with various blood dyscrasias and increased incidence of acute myelogenous leukemia in humans. It is established that benzene requires metabolism to induce its effects. Benzene exposure in humans and animals has also been shown to result in structural and numerical chromosomal aberrations in lymphocytes and bone marrow cells, indicating that benzene is genotoxic. In this review we have attempted to compile the available evidence on the role of increased free radical activity in benzene-induced myelotoxic and leukemogenic effects. Benzene administration to rodents has been associated with increased lipid peroxidation in liver, plasma, and bone marrow, as shown by an increase in the formation of thiobarbituric-acid reactive products that absorb at 535 nm. Benzene administration to rodents also results in increased prostaglandin levels indicating increased arachidonic acid peroxidation. Other evidence includes the fact that bone marrow cells and their microsomal fractions isolated from rodents following benzene-treatment have a higher capacity to form oxygen free radicals. The bone marrow contains several peroxidases, the most prevalent of which is myeloperoxidase. The peroxidatic metabolism of the benzene metabolites, phenol and hydroquinone, results in arachidonic acid peroxidation and oxygen activation to superoxide radicals, respectively. These metabolites, upon co-administration also produce a myelotoxicity similar to that observed with benzene. Recently, we have found that exposure of human promyelocytic leukemia (HL-60) cells (a cell line rich in myeloperoxidase), to the benzene metabolites, hydroquinone and 1,2,4-benzenetriol results in increased steady-state levels of 8-hydroxydeoxyguanosine a marker of oxidative DNA damage. Peroxidatic metabolism of benzene's phenolic metabolites may therefore be responsible for the increased free radical activity and toxicity produced by benzene in bone marrow. We thus hypothesize that free radicals contribute, at least in part, to the toxic and leukemogenic effects of benzene.		THB Genotoxicity	ROS Formation
50	Rao GS, Pandya KP.	Release of 2-thiobarbituric acid reactive products from glutamate or deoxyribonucleic acid by 1,2,4-benzenetriol or hydroquinone in the presence of copper ions.	Toxicology. 1989 Nov;59(1):59-65.	Cytotoxic effects of various quinone compounds are thought to be due to the formation of semiquinone free radicals. Hydroquinone and 1,2,4-benzenetriol in the presence of copper ions release from glutamate or DNA aldehydic products capable of reacting with 2-thiobarbituric acid (TBA). The formation of TBA reactive products (TBAR) was greater in the presence of 1,2,4-benzenetriol in comparison with hydroquinone. Complete inhibition of formation of TBAR from glutamate by 1,2,4-benzenetriol and copper was observed in the presence of catalase, thiourea and mannitol. Albumin and superoxide dismutase offered substantial protection. Complete protection of formation of TBAR from DNA was observed in the presence of catalase and thiourea. Presence of albumin, mannitol and superoxide dismutase caused only partial inhibition. The formation of TBAR from glutamate or DNA is dependent on copper ion concentration. The present data indicate that hydroquinone and 1,2,4-benzenetriol in the presence of copper ions can lead to the formation of reactive hydroxyl radicals which can release TBAR from glutamate or DNA.		THB Genotoxicity	ROS Formation
51	Lee EW, Johnson JT, Garner CD.	Inhibitory effect of benzene metabolites on nuclear DNA synthesis in bone marrow cells.	J Toxicol Environ Health. 1989;26(3):277-91.	Effects of endogenously produced and exogenously added benzene metabolites on the nuclear DNA synthetic activity were investigated using a culture system of mouse bone marrow cells. Effects of the metabolites were evaluated by a 30-min incorporation of [3H]thymidine into DNA following a 30-min interaction with the cells in McCoy's 5a medium with 10% fetal calf serum. Phenol and muconic acid did not inhibit nuclear DNA synthesis. However, catechol, 1,2,4-benzenetriol, hydroquinone, and p-benzoquinone were able to inhibit 52, 64, 79, and 98% of the nuclear DNA synthetic activity, respectively, at 24 microM. In a cell-free DNA synthetic system, catechol and hydroquinone did not inhibit the incorporation of [3H]thymidine triphosphate into DNA up to 24 microM but 1,2,4-benzenetriol and p-benzoquinone did. The effect of the latter two benzene metabolites was completely blocked in the presence of 1,4-dithiothreitol (1 mM) in the cell-free assay system. Furthermore, when DNA polymerase alpha, which requires a sulfhydryl (SH) group as an active site, was replaced by DNA polymerase I, which does not require an SH group for its catalytic activity, p-benzoquinone and 1,2,4-benzenetriol were unable to inhibit DNA synthesis. Thus, the data imply that p-benzoquinone and 1,2,4-benzenetriol inhibited DNA polymerase alpha, consequently resulting in inhibition of DNA synthesis in both cellular and cell-free DNA synthetic systems. The present study identifies catechol, hydroquinone, p-benzoquinone, and 1,2,4-benzenetriol as toxic benzene metabolites in bone marrow cells and also suggests that their inhibitory action on DNA synthesis is mediated by mechanism(s) other than that involving DNA damage as a primary cause.	**Same lab as SCCS cited reference**	THB Genotoxicity	ROS Formation
52	Glatt, H.R., Padykula, R., Berchtold, G.A., Ludewig, G., Platt, K.L., Klein, J.,	Multiple activation pathways of benzene leading to products with varying genotoxic characteristics	Environ Health Perspect. 82:81–89, 1989	Benzene and 13 potential metabolites were investigated for genotoxicity in Salmonella typhimurium and V79 Chinese hamster cells. In the presence of NADPH-fortified hepatic postmitochondrial fraction (S9 mix), benzene reverted his- S. typhimurium strains. The effect was strongest in strain TA1535. Among the potential metabolites, only the trans-1,2-dihydrodiol, in the presence of S9 mix, and the diol epoxides, in the presence and absence of S9 mix, proved mutagenic in this strain. The anti-diol epoxide was more potent than the syn-diastereomer. Both enantiomers of the anti-diastereomer showed similar activities. S9 mix did not appreciably affect the mutagenicity of the anti-diol epoxide. However, detoxification was observed when purified rat liver dihydrodiol dehydrogenase (EC 1.3.1.20) was used at concentrations comparable to that present in the liver. The (1S)-anti-diol epoxide was a much better substrate than the (1R)-enantiomer, as was true also for (1S)-versus (1R)-trans-1,2-dihydrodiol. The anti-diol epoxide reverted all six strains of S. typhimurium used and induced all four genotoxic effects studied in V79 cells (sister chromatid exchange greater than acquisition of 6-thioguanine resistance, acquisition of ouabain resistance, micronuclei). However, other potential benzene metabolites showed genotoxic effects in V79 cells, as well: sister chromatid exchange was induced by the syn-diol epoxide, 1,2,4-trihydroxybenzene, hydroquinone, catechol, and 1,2,3-trihydroxybenzene. Elevated frequencies of micronucleated cells were observed after treatment with hydroquinone, 1,2,4-trihydroxybenzene, catechol, phenol, 1,2,3-trihydroxybenzene, and quinone. Mutations to 6-thioguanine resistance were induced by quinone, hydroquinone, 1,2,4-trihydroxybenzene, catechol, and the trans-1,2-dihydrodiol.	Benzene metabolites cause oxidative DNA damage	THB Genotoxicity	ROS Formation

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
53	Kawanishi S, Inoue S, Kawanishi M.	Human DNA damage induced by 1,2,4-benzenetriol, a benzene metabolite.	Cancer Res. 1989 Jan 1;49(1):164-8.	Reactivities of benzene metabolites (phenol, catechol, hydroquinone, 1,4-benzoquinone, 1,2,4-benzenetriol) and related polyphenols (resorcinol, pyrogallol, phloroglucinol) with DNA were investigated by a DNA sequencing technique using 32P 5'-end-labeled DNA fragments obtained from human c-Ha-ras-1 protooncogene, and the reaction mechanism was studied by UV-visible and electron-spin resonance spectroscopies. 1,2,4-Benzenetriol caused strong DNA damage even without alkali treatment. Alkali-labile sites induced by 1,2,4-benzenetriol were base residues of guanine and adjacent thymine. Catalase, superoxide dismutase and methional inhibited the DNA damage completely, but sodium formate did not inhibit it. 1,2,4-Benzenetriol-induced DNA damage was inhibited by the addition of a Cu(I)-specific chelating agent, bathocuproine, and was accelerated by the addition of Cu(II). The addition of Fe(III) did not create any significant effects on 1,2,4-benzenetriol-induced DNA damage. Electron-spin resonance studies using spin traps demonstrated that addition of Fe(III) increased hydroxyl radical production during the autoxidation of 1,2,4-benzenetriol, whereas the addition of Cu(II) did not. The results suggest that DNA damage was caused by an unidentified active species which was produced by the autoxidation of 1,2,4-benzenetriol in the presence of Cu(II), rather than by hydroxyl radicals. The possibility that 1,2,4-benzenetriol-induced DNA damage is one of the primary reactions in carcinogenesis induced by benzene is discussed.	Benzene metabolites cause oxidative DNA damage	THB Genotoxicity	ROS Formation
54	Koike N, Haga S, Ubukata N, Sakurai M, Shimizu H, Sato A.	[Mutagenicity of benzene metabolites by fluctuation test].	Sangyo Igaku. 1988 Nov;30(6):475-80. Japanese.	Mutagenicity of five benzene metabolites was examined by a fluctuation test modified by Gatehouse. The test was performed by using Salmonella typhimurium TA100 with or without a metabolic activation system. Catechol showed mutagenic activity with and without a metabolic activation system in the fluctuation test. Hydroxyhydroquinone, phenol, hydroquinone and p-benzoquinone were positive only with a metabolic activation system in this test. The Ames' plate method and preincubation method are widely used as a screening test for the mutagenic compounds. However, the fluctuation test is very useful in detecting mutagenic activity of chemicals which are negative in the Ames test though being a mutagen or carcinogen.	THB showed Ames positive with S9 using a fluctuation test (using S. typhimurium TA 100)	THB Genotoxicity	
55	Lewis JG, Stewart W, Adams DO.	Role of oxygen radicals in induction of DNA damage by metabolites of benzene.	Cancer Res. 1988 Sep 1;48(17):4762-5.	Benzene is strongly suspected of being an animal and human carcinogen, but the mechanisms by which benzene induces tumors of lymphoid and hematopoietic organs are unknown. Binding studies in vivo suggest a very low level of covalent binding to the DNA of bone marrow elements. Since several metabolites of benzene have the potential to undergo autooxidation and thereby generate reactive oxygen intermediates, we have tested the hypothesis that benzene metabolites can induce DNA damage through the generation of oxygen radicals. Hydroquinone (HQ), benzoquinone (BQ), catechol, and 1,2,4-benzenetriol (BT) were first tested for their ability to generate O2-. at a physiological pH. BT, and to a lesser extent HQ, were autooxidized and produced significant quantities of O2-.. No detectable O2-. was produced by catechol or BQ. Similarly, BT was very efficient at degrading DNA, and this degradation was inhibited by scavengers of O2-., H2O2 and .OH. HQ did not degrade DNA but did induce single- and double-strand breaks. In contrast to the action of BT, the breakage of DNA by HQ was not inhibited by scavengers of reactive oxygen intermediates. The metabolites which did not produce O2-. (catechol and BQ) did not induce significant breakage of DNA. Taken together, the data support the hypothesis that certain benzene metabolites can induce DNA damage through the production of oxygen radicals; they further suggest that other metabolites may act, via another mechanism, to damage DNA.	Benzene metabolites cause oxidative DNA damage in vitro	THB Genotoxicity	ROS Formation
56	Ciranni R, Barale R, Marrazzini A, Loprieno N.	Benzene and the genotoxicity of its metabolites. I. Transplacental activity in mouse fetuses and in their dams.	Mutat Res. 1988 May;208(1):61-7.	Benzene and some of its metabolites (hydroquinone, phenol, catechol, 1,2,4-benzenetriol, p-benzoquinone, o,o'-biphenol, p,p'-biphenol) have been tested for their capability to induce micronuclei in bone marrow cells of pregnant mice and, transplacentally, in fetal liver cells. Dams are scarcely sensitive to the genotoxic activity of benzene and its metabolites while the latter are able to produce only evident toxic effects. Benzene and hydroquinone transplacentally induce micronuclei in fetal liver cells while all other metabolites show weak or negative genotoxicity, although they produce severe cellular toxicity.	**In vivo** study showed benzene and hydroquinone transplacentally induce micronuclei in fetal liver cells while all other metabolites show weak or negative genotoxicity, although they produce severe cellular toxicity.	THB Genotoxicity	
57	Pellack-Walker P, Blumer JL.	DNA damage in L5178YS cells following exposure to benzene metabolites.	Mol Pharmacol. 1986 Jul;30(1):42-7.	Because DNA modification may be a prerequisite for chemical carcinogenesis, the DNA-damaging potential of benzene and its metabolites was examined in order to identify the proximate DNA-damaging agent associated with benzene exposure. A DNA synthesis inhibition assay previously identified p-benzoquinone as the most potent overall cellular toxin and inhibitor of DNA synthesis, but failed to discriminate among the hydroxylated metabolites. Therefore, the ability of benzene and its metabolites to induce DNA strand breaks in the mouse lymphoma cell line, L5178YS, was examined in order to provide a more accurate indication of the DNA damage associated with benzene and its metabolites. Cells were exposed to benzene, hydroquinone, catechol, phenol, 1,2,4-benzenetriol, or p-benzoquinone over a 1000-fold concentration range (1.0 microM-1.0 mM). Concentrations of benzene, phenol, or catechol as high as 1.0 mM did not increase the percentage of single-stranded DNA observed. Concentrations of hydroquinone as high as 0.1 mM were also ineffective. In contrast, both p-benzoquinone and 1,2,4-benzenetriol produced DNA breaks in a dose-related fashion. Of the two, benzoquinone proved to be more potent with an ED50 of approximately equal to 2.5 microM compared with 55.0 microM for benzenetriol. The DNA damage induced by 6.0 microM benzoquinone was maximal within 3 min of exposure and yielded approximately 70% single-stranded DNA after alkaline denaturation. By contrast, the single-stranded DNA observed after benzenetriol exposure required 60 min of exposure to achieve the same extent of damage as that found with benzoquinone. These results suggest that the benzene metabolites, benzenetriol and benzoquinone, may cause DNA damage and that the mechanisms responsible for the damage associated with these two compounds may be different.	Benzene metabolites cause in vitro DNA damage	THB Genotoxicity	ROS Formation
58	Erexson GL, Wilmer JL, Kligerman AD.	Sister chromatid exchange induction in human lymphocytes exposed to benzene and its metabolites in vitro.	Cancer Res. 1985 Jun;45(6):2471-7.	Previous in vivo studies have shown that low-dose benzene exposure (10 to 28 ppm for 4 to 6 h) in mice can induce sister chromatid exchange (SCE) in peripheral blood B-lymphocytes and bone marrow as well as micronuclei in bone marrow polychromatic erythrocytes. Because benzene is metabolized to a variety of intermediate compounds and two of these, catechol and hydroquinone, have been reported to be potent SCE-inducers, it is possible that other known and proposed metabolites could have chromosome-damaging effects in lymphocytes. Induced SCE frequencies, mitotic indices, and cell cycle kinetics were quantitated in human peripheral blood T-lymphocytes exposed to benzene, phenol, catechol, 1,2,4-benzenetriol, hydroquinone, 1,4-benzoquinone, or trans,trans-muconic acid. Three proposed metabolites of phenol, 4,4'-biphenol, 4,4'-diphenoquinone, and 2,2'-biphenol, which can be generated by a phenol-horseradish peroxidase-hydrogen peroxide system were also examined. Benzene, phenol, catechol, 1,2,4-benzenetriol, hydroquinone, and 1,4-benzoquinone induced significant concentration-related increases in the SCE frequency, decreases in mitotic indices, and inhibition of cell cycle kinetics. Based on the slope of the linear regression curves for SCE induction, the relative potencies were as follows: catechol greater than 1,4-benzoquinone greater than hydroquinone greater than 1,2,4-benzenetriol greater than phenol greater than benzene. On an induced SCE per microM basis, catechol was approximately 221 times more active than benzene at the highest concentrations studied. trans,trans-Muconic acid had no significant effect on the cytogenetic parameters analyzed. 2,2'-Biphenol induced a significant increase in SCE only at the highest concentration analyzed, and 4,4'-biphenol caused a significant increase in SCE frequency that was not clearly concentration related. However, both 2,2'- and 4,4'-biphenol caused significant cell cycle delay and mitotic inhibition. 4,4'-Diphenoquinone caused only a significant decrease in mitotic activity. These data indicate that in addition to phenol, di- and trihydroxybenzene metabolites play important roles in SCE induction. Furthermore, the results suggest either that benzene alone can induce SCE or, a more likely possibility, that mononuclear leucocytes have a limited capability to activate benzene.	Benzene metabolites cause in vitro SCE	THB Genotoxicity	ROS Formation

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
59	Rushmore T, Snyder R, Kalf G.	Covalent binding of benzene and its metabolites to DNA in rabbit bone marrow mitochondria in vitro.	Chem Biol Interact. 1984 Apr;49(1-2):133-54.	Rabbit bone marrow mitochondria, stripped of their outer membrane (mitoplasts), have been shown to carry out the NADPH-dependent bioactivation of radiolabelled benzene in vitro to metabolites capable of covalently binding to mtDNA, thereby inhibiting transcription. The metabolites of benzene produced in bone marrow cells by the microsomal cytochrome P-450 are thought to be phenol, catechol, hydroquinone and p-benzoquinone (Andrews et al., Life Sci., 25 (1979) 567; Irons et al., Chem.-Biol. Interact., 30 (1980) 241). Incubation of mitoplasts from rabbit bone marrow cells in vitro with varying concentrations of the putative microsomal metabolites showed a concentration-dependent inhibition of RNA synthesis. The 50% inhibitory molar concentration (IC50) for each metabolite was determined to be: 1,2,4- benzenetriol , 6.3 X 10(-7); p-benzo-quinone, 2 X 10(-6); phenol, 2.5 X 10(-5); hydroquinone, 5 X 10(-5); catechol, 2 X 10(-3); benzene, 1.6 X 10(-2). DNA, isolated from rabbit bone marrow cell or rat liver mitoplasts prelabelled in DNA with [3H]dGTP and exposed to [14C]benzene in vitro, was enzymatically hydrolyzed to nucleosides which were chromatographed on a Sephadex LH-20 column to separate free nucleosides from nucleoside-adducts. The elution profiles indicated that rat liver mtDNA contained six guanine nucleoside-adducts and rabbit bone marrow cell mtDNA contained seven guanine nucleoside-adducts. Incubation of bone marrow mitoplasts in vitro in the presence of benzene and the hydroxyl radical scavenger, mannitol, resulted in the inhibition of formation of four of the guanosine-adducts. When [3H]dATP was substituted as the prelabelled precursor nucleotide, the LH-20 column profile indicated that two adenine nucleoside-adducts were also formed from benzene in vitro. Furthermore, a comparison of the Sephadex LH-20 column profiles of purine adducts derived from [14C]benzene- and [3H]dGMP-labelled mtDNA with profiles generated by individually incubating each of the putative unlabelled metabolites with bone marrow mitoplasts in vitro has indicated that p-benzoquinone, phenol, hydroquinone and 1,2,4- benzenetriol form adducts with guanine. One of the two adenosine-adducts may arise from hydroquinone; the compound forming the other adduct is unknown at the present time. Exposure of mitoplasts to catechol in vitro resulted in the formation of a guanine nucleoside-adduct that was present in rat liver mtDNA but absent from the DNA isolated from rabbit bone marrow cell mitoplasts exposed to [14C]benzene in vitro. This suggests that catechol is probably not a major metabolite of benzene formed in bone marrow cell mitochondria.	THB forms adducts with guanine in vitro rabbit bone marrow. **Same lab as #204**	THB Genotoxicity	
60	Yu CH, Li Y, Zhao X, Yang SQ, Li L, Cui NX, Rong L, Yi ZC.	Benzene metabolite 1,2,4-benzenetriol changes DNA methylation and histone acetylation of erythroid-specific genes in K562 cells.	Arch Toxicol. 2018 Oct 16. doi: 10.1007/s00204-018-2333-6. [Epub ahead of print]	1,2,4-Benzenetriol (BT) is one of the phenolic metabolites of benzene, a general occupational hazard and ubiquitous environmental air pollutant with leukemogenic potential in humans. Previous studies have revealed that the benzene metabolites phenol and hydroquinone can inhibit hemin-induced erythroid differentiation in K562 cells. We investigated the roles of DNA methylation and histone acetylation in BT-inhibited erythroid differentiation in K562 cells. When K562 cells were treated with 0, 5, 10, 15 or 20 μM BT for 72 h, hemin-induced hemoglobin synthesis decreased in a concentration-dependent manner. Both 5-aza-2′-deoxycytidine (5-aza-CdR, DNA methyltransferase inhibitor) and trichostatin A (TSA, histone deacetylases inhibitor) could prevent 20 μM BT from inhibiting hemin-induced hemoglobin synthesis and the mRNA expression of erythroid genes. Exposure to BT changed DNA methylation levels at several CpG sites of erythroid-specific genes, as well as the acetylation of histone H3 and H4, chromatin occupancy of GATA-1 and recruitment of RNA polymerase II at α-globin and β-globin gene clusters after hemin induction. These results demonstrated that BT could inhibit hemin-induced erythroid differentiation, where DNA methylation and histone acetylation also played important roles by down-regulating erythroid-specific genes. This partly explained the mechanisms of benzene hematotoxicity.	Benzene metabolites and DNA polymerase inhibition	THB Toxicity	
61	Tang KY, Yu CH, Jiang L, Gong M, Liu WJ, Wang Y, Cui NX, Song W, Sun Y, Yi ZC.	Long-term exposure of K562 cells to benzene metabolites inhibited erythroid differentiation and elevated methylation in erythroid specific genes.	Toxicol Res (Camb). 2016 Jun 30;5(5):1284-1297. doi: 10.1039/c6tx00143b. eCollection 2016 Sep 1.	Benzene is a common occupational hazard and a widespread environmental pollutant. Previous studies have revealed that 72 h exposure to benzene metabolites inhibited hemin-induced erythroid differentiation of K562 cells accompanied with elevated methylation in erythroid specific genes. However, little is known about the effects of long-term and low-dose benzene metabolite exposure. In this study, to elucidate the effects of long-term benzene metabolite exposure on erythroid differentiation, K562 cells were treated with low-concentration phenol, hydroquinone and 1,2,4-benzenetriol for at least 3 weeks. After exposure of K562 cells to benzene metabolites, hemin-induced hemoglobin synthesis declined in a concentration- and time-dependent manner, and the hemin-induced expressions of α-, β- and γ-globin genes and heme synthesis enzyme porphobilinogen deaminase were significantly suppressed. Furthermore, when K562 cells were continuously cultured without benzene metabolites for another 20 days after exposure to benzene metabolites for 4 weeks, the decreased erythroid differentiation capabilities still remained stable in hydroquinone- and 1,2,4-benzenetriol-exposed cells, but showed a slow increase in phenol-exposed K562 cells. In addition, methyltransferase inhibitor 5-aza-2′-deoxycytidine significantly blocked benzene metabolites inhibiting hemoglobin synthesis and expression of erythroid genes. Quantitative MassARRAY methylation analysis also confirmed that the exposure to benzene metabolites increased DNA methylation levels at several CpG sites in several erythroid-specific genes and their far-upstream regulatory elements. These results demonstrated that long-term and low-dose exposure to benzene metabolites inhibited the hemin-induced erythroid differentiation of K562 cells, in which DNA methylation played a role through the suppression of erythroid specific genes.	Benzene metabolite effect on erythroid differentiation owing to DNA methylation in vitro **Same lab as #4 in vitro K562 cell study**	THB Toxicity	
62	Miyahara E, Nishikawa T, Takeuchi T, Yasuda K, Okamoto Y, Kawano Y, Horiuchi M.	Effect of myeloperoxidase inhibition on gene expression profiles in HL-60 cells exposed to 1,2,4,-benzenetriol.	Toxicology. 2014 Mar 20;317:50-7. doi: 10.1016/j.tox.2014.01.007. Epub 2014 Feb 12.	While it is known that benzene induces myeloid leukemia in humans, the mechanism has yet to be clarified. Previously, we suggested that myeloperoxidase (MPO) was the key enzyme because it promotes generation of powerful oxidant hypochlorous acid (HOCl) which, reacting with DNA, causes leukemogenesis. In this study, using a whole-human-genome oligonucleotide microarray to clarify the relationships between myelotoxicity of benzene and MPO, we analyzed the genome-wide expression profiles of HL-60 human promyelocytic cell lines exposed to 1,2,4-benzenetriol (BT) with or without MPO inhibition. The microarray analysis revealed that short (1 h) and longer (4 h) exposure to BT changed the expression in HL-60 cells of 1,213 or 1,214 genes associated with transcription, RNA metabolic processes, immune response, apoptosis, cell death, and biosynthetic processes (|Z-score|> 2.0), and that these changes were dramatically lessened by MPO-specific inhibition. The presence of functionally important genes and, specifically, genes related to apoptosis, carcinogenesis, regulation of transcription, immune responses, oxidative stress, and cell-cycle regulation were further validated by real-time RT-PCR. Gene expression profiles along with Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation analysis suggest that BT-induced DNA halogenation by MPO is a primary reaction in the leukemogenesis associated with benzene.	THB induced DNA halogenation by myeloperoxidase in leukemogenesis with benzene	THB Toxicity	ROS formation
63	Wang Y, Zhang GY, Han QL, Wang J, Suriguga, Li Y, Yu CH, Li YR, Yi ZC.	Phenolic metabolites of benzene induced caspase-dependent cytotoxicities to K562 cells accompanied with decrease in cell surface sialic acids.	Environ Toxicol. 2014 Dec;29(12):1437-51. doi: 10.1002/tox.21874. Epub 2013 Jun 17.	Benzene-induced erythropoietic depression has been proposed to be due to the production of toxic metabolites. Presently, the cytotoxicities of benzene metabolites, including phenol, catechol, hydroquinone, and 1,2,4-benzenetriol, to erythroid progenitor-like K562 cells were investigated. After exposure to these metabolites, K562 cells showed significant inhibition of viability and apoptotic characteristics. Each metabolite caused a significant increase in activities of caspase-3, -8, and -9, and pretreatment with caspase-3, -8, and -9 inhibitors significantly inhibited benzene metabolites-induced phosphatidylserine exposure. These metabolites also elevated expression of Fas and FasL on the cell surface. After exposure to benzene metabolites, K562 cells showed an increase in reactive oxygen species level, and pretreatment with N-acetyl-l-cysteine significantly protected against the cytotoxicity of each metabolite. Interestingly, the control K562 cells and the phenol-exposed cells aggregated together, but the cells exposed to other metabolites were scattered. Further analysis showed that hydroquione, catechol, and 1,2,4-benzenetriol induced a decrease in the cell surface sialic acid levels and an increase in the cell surface sialidase activity, but phenol did not cause any changes in sialic acid levels and sialidase activity. Consistently, an increase in expression level of sialidase Neu3 mRNA and a decrease in mRNA level of sialyltransferase ST3GAL3 gene were detected in hydroquione-, catechol-, or 1,2,4-benzenetriol-treated cells, but no change in mRNA levels of two genes were found in phenol-treated cells. In conclusion, these benzene metabolites could induce apoptosis of K562 cells mainly through caspase-8-dependent pathway and ROS production, and sialic acid metabolism might play a role in the apoptotic process.	Benzene metabolites induce apoptosis of K562 cells in part by ROS formation	THB Toxicity	ROS formation

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
64	Suzuki K, Kori S, Morikawa M, Takagi A, Namiki H.	Oxidative stress-mediated bimodal regulation of polymorphonuclear leukocyte spreading by polyphenolic compounds.	Int Immunopharmacol. 2010 Nov;10(11):1448-55. doi: 10.1016/j.intimp.2010.08.020. Epub 2010 Sep 15.	Pyrogallol-bearing polyphenolic compounds induce spreading of polymorphonuclear leukocytes (PMNL), although their optimal concentrations for induction of spreading are quite different (2000, 200, and 2 μM for pyrogallol, (-)-epigallocatechin gallate (EGCG), and tannic acid (TA), respectively), and TA tends to inhibit spreading at higher concentrations. In this study, we examined the involvement of oxidative stress in the regulation of PMNL spreading by these compounds. All three compounds in solution generated H(2)O(2) to a similar extent. Adsorption of the polyphenols to cell surfaces and their accumulation within cells were assessed by detection of the H(2)O(2) precursor O(2)(-) produced by the compounds through reduction of cytochrome c and p-nitro-blue tetrazolium, respectively. TA showed the highest degree of adsorption. EGCG adhered only to PMNL pre-fixed by paraformaldehyde, whereas pyrogallol did not adhere. None of the compounds caused intracellular O(2)(-) generation. A non-pyrogallic compound, 1,2,4-benzenetriol (BT), also produced H(2)O(2); it had no stimulatory effect on PMNL spreading, but inhibited spreading induced by other stimuli. BT did not adhere to PMNL but accumulated within them, and generated O(2)(-) in the presence of glycine. Thiol antioxidants abrogated all of the above spreading-regulatory effects of the polyphenolic compounds. We conclude that H(2)O(2)-generating polyphenols bimodally regulate the spreading of PMNL by subjecting them to oxidative stress. The ability of polyphenol to adhere to, or accumulate within, PMNL may govern the nature of the oxidative stress and determine the optimal concentration of each compound for induction of spreading, as well as whether spreading is promoted or inhibited.	THB showed peroxide formation Thiol antioxidants reversed the spreading-regulatory effects of Polyphenols	THB Toxicity	ROS formation
65	Kim K, Kim SH, Lépine F, Cho YH, Lee GR.	Global gene expression analysis on the target genes of PQS and HHQ in J774A.1 monocyte/macrophage cells.	Microb Pathog. 2010 Oct;49(4):174-80. doi: 10.1016/j.micpath.2010.05.009. Epub 2010 Jun 2.	We have previously shown that PQS and HHQ, two quorum sensing molecules, can down-regulate host the innate immune responses and that this is mediated through the NF-kappaB pathway. In this study, to search for a comprehensive set of genes regulated by these quorum sensing molecules, we performed a global gene expression analysis using DNA microarray in J774A.1 monocyte/macrophage cells line. The expression of these genes was confirmed by RT-PCR. We found that PQS and HHQ down-regulated the expression of genes involved in immune responses and transcription as well as other functions, some of which are downstream of NF-kappaB pathway consistent with our previous results. PQS and HHQ inhibited LPS-induced morphological change and nitric oxide production, suggesting that they inhibit macrophage activation. However, PQS and HHQ did not affect apoptosis, suggesting that their effects on immune system are not from general alteration of cell function. This study provides insight how the quorum sensing molecules influence host cells.	THB effects in down-regulation of innate immune response	THB Toxicity	
66	Gillis B, Gavin IM, Arbieva Z, King ST, Jayaraman S, Prabhakar BS.	Identification of human cell responses to benzene and benzene metabolites.	Genomics. 2007 Sep;90(3):324-33. Epub 2007 Jun 15.	Benzene is a common air pollutant and confirmed carcinogen, especially in reference to the hematopoietic system. In the present study we analyzed cytokine/chemokine production by, and gene expression induction in, human peripheral blood mononuclear cells upon their exposure to the benzene metabolites catechol, hydroquinone, 1,2,4-benzenetriol, and p-benzoquinone. Protein profiling showed that benzene metabolites can stimulate the production of chemokines, the proinflammatory cytokines TNF-alpha and IL-6, and the Th2 cytokines IL-4 and IL-5. Activated cells showed concurrent suppression of anti-inflammatory cytokine IL-10 expression. We also identified changes in global gene expression patterns in response to benzene metabolite challenges by using high-density oligonucleotide microarrays. Treatment with 1,2,4-benzenetriol resulted in the suppression of genes related to the regulation of protein expression and a concomitant activation of genes that encode heat shock proteins and cytochrome P450 family members. Protein and gene expression profiling identified unique human cellular responses upon exposure to benzene and benzene metabolites.	Benzene metabolite stimulate proinflammatory cytokines	THB Toxicity	
67	Lindsey RH Jr, Bender RP, Osheroff N.	Effects of benzene metabolites on DNA cleavage mediated by human topoisomerase II alpha: 1,4-hydroquinone is a topoisomerase II poison.	Chem Res Toxicol. 2005 Apr;18(4):761-70.	Although benzene induces leukemias in humans, the compound is not believed to generate chromosomal damage directly. Rather, benzene is thought to act through a series of phenolic- and quinone-based metabolites, especially 1,4-benzoquinone. A recent study found that 1,4-benzoquinone is a potent topoisomerase II poison in vitro and in cultured human cells [Lindsey et al. (2004) Biochemistry 43, 7363-7374]. Because benzene is metabolized to multiple compounds in addition to 1,4-benzoquinone, we determined the effects of several phenolic metabolites, including catechol, 1,2,4-benzenetriol, 1,4-hydroquinone, 2,2'-biphenol, and 4,4'-biphenol, on the DNA cleavage activity of human topoisomerase II alpha. Only 1,4-hydroquinone generated substantial levels of topoisomerase II-mediated DNA scission. DNA cleavage with this compound approached levels observed with 1,4-benzoquinone (approximately 5- vs 8-fold) but required a considerably higher concentration (approximately 250 vs 25 microM). 1,4-Hydroquinone is a precursor to 1,4-benzoquinone in the body and can be activated to the quinone by redox cycling. It is not known whether the effects of 1,4-hydroquinone on human topoisomerase II alpha reflect a lower reactivity of the hydroquinone or a low level of activation to the quinone. The high concentration of 1,4-hydroquinone required to increase enzyme-mediated DNA cleavage is consistent with either explanation. 1,4-Hydroquinone displayed attributes against topoisomerase II alpha, including DNA cleavage specificity, that were similar to those of 1,4-benzoquinone. However, 1,4-hydroquinone consistently inhibited DNA ligation to a greater extent than 1,4-benzoquinone. This latter result implies that the hydroquinone may display (at least in part) independent activity against topoisomerase II alpha. The present findings are consistent with the hypothesis that topoisomerase II alpha plays a role in the initiation of specific types of leukemia that are induced by benzene and its metabolites.	Of benzene metabolites only 1,4-hydroquinone generated substantial levels of topoisomerase II-mediated DNA scission. DNA cleavage with this compound approached levels observed with 1,4-benzoquinone (approximately 5- vs 8-fold) but required a considerably higher concentration (approximately 250 vs 25 microM). 1,4-Hydroquinone is a precursor to 1,4-benzoquinone in the body and can be activated to the quinone by redox cycling. **Same lab as #83**	THB Toxicity	No effect
68	Wiemels J, Smith MT.	Enhancement of myeloid cell growth by benzene metabolites via the production of active oxygen species.	Free Radic Res. 1999 Feb;30(2):93-103.	In low concentrations, benzene and its metabolite hydroquinone are known to have diverse biological effects on cells, including the synergistic stimulation with GM-CSF of hematopoietic colony formation in vitro, stimulation of granulocytic differentiation in vitro and in vivo, and general suppression of hematopoiesis in vivo. These chemicals are also known to be active in the induction of active oxygen species. We used several assays to determine the effects of benzene metabolites (hydroquinone, benzenetriol, benzoquinone) and active oxygen species (xanthine/xanthine oxidase) on cell growth and cell cycle kinetics of the human myeloid cell line HL-60. HL-60 cells treated with these chemicals for 2 h in PBS showed increased growth over untreated controls in a subsequent 18h growth period in complete media. Incorporation of 3H-thymidine was also increased proportionately by these treatments. Catalase treatment abrogated the increased cell growth of all chemicals, suggesting an oxidative mechanism for the effect of all treatments alike. Cell cycle kinetics assays showed that the growth increase was caused by an increased recruitment of cells from G0/G1 to S-phase for both hydroquinone and active oxygen, rather than a decrease in the length of the cell cycle. Benzene metabolite's enhancement of growth of myeloid cells through an active oxygen mechanism may be involved in a number of aspects of benzene toxicity, including enhanced granulocytic growth and differentiation, stimulation of GM-CSF-induced colony formation, apoptosis inhibition, and stimulation of progenitor cell mitogenesis in the bone marrow. These effects in sum may be involved in the benzene-induced "promotion" of a clonal cell population to the fully leukemic state.	Benzene metabolites effects on hematopoiesis owing to ROS formation. Catalase shown to diminish all effects in support of oxidative mode of action.	THB Toxicity	ROS Formation
69	Yang Y, Quitschke WW, Brewer GJ.	Upregulation of amyloid precursor protein gene promoter in rat primary hippocampal neurons by phorbol ester, IL-1 and retinoic acid, but not by reactive oxygen species.	Brain Res Mol Brain Res. 1998 Sep 18;60(1):40-9.	The APP gene promoter has multiple regulatory sequences, some of which may contribute to the neuropathology of Alzheimer's disease (AD). In this study, we investigated the effects of phorbol ester (PMA), IL-1, retinoic acid and reactive oxygen species on APP promoter activity in primary hippocampal neurons. We transfected neurons with either of two APP promoter constructs, a -2.8 kb and a shorter -488 bp upstream fragment fused to the chloramphenicol transferase (CAT) reporter gene. We demonstrated that phorbol 12-myristate-13 acetate (PMA), retinoic acid and IL-1 all stimulated both APP promoter constructs in hippocampal neurons after 24 h treatment. PMA and IL-1 treatments led to 2-fold increases of APP promoter activity. Retinoic acid induced a 3-fold increase. In addition, the magnitude of APP promoter responses to PMA and IL-1 treatment was similar between APP -2.8 kb and -488 bp plasmid transfected neurons. This suggests that the AP-1 sequence at -350 to -344 in the APP promoter may mediate the stimulatory effects of PMA and IL-1, as previously observed in endothelial and HeLa cells. In contrast, hydrogen peroxide, which was shown to activate NF-kappaB in primary neurons, failed to stimulate APP promoter activity, suggesting that the regulatory elements in the APP promoter may not respond to reactive oxygen species. Overall, these data indicate that APP expression in primary neurons can be modulated by PMA, IL-1 and retinoic acid. However, the contribution of reactive oxygen to Alzheimer's disease may not be directly related to the activation of the APP gene promoter but instead to neuronal damage associated with oxidative stress. Since elevated levels of IL-1 have been observed in AD brain, IL-1 could contribute to development of Alzheimer's disease by stimulating APP synthesis in primary neurons.		THB Toxicity	ROS formation

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
70	Wilmer JL, Simeonova PP, Germolec DR, Luster MI.	Benzene and Its Principal Metabolites Modulate Proinflammatory Cytokines and Growth Factors in Human Epidermal Keratinocyte Cultures.	In Vitro Toxicol. 1997 Dec;10(4):429-436.	Benzene is an established leukemogen and hematotoxin in humans. However, the finding that benzene is a multiple-site carcinogen in rodents raises the possibility that other tissues could be susceptible to benzene-induced carcinogenicity, especially since a significant excess of squamous cell carcinomas and papillomas arise from epidermal and oral keratinocytes in benzene-exposed rats. Since inflammation and sustained hyperplasia are two integral components in carcinogenesis, the elaboration of proinflammatory cytokines and growth factors by keratinocytes might provide a mechanistic link between tumor initiation and promotion in benzene-induced cancers. We observed that the principal benzene metabolites, represented by hydroquinone, 1,4-benzoquinone, phenol, 1,2,4-benzenetriol, and catechol, significantly alters the production of transforming growth factor of (TGF)-α and interleukin (IL)-8 in human epidermal keratinocyte cultures. These cytokines represent the primary growth promoting factor and neutrophil chemotactant in the skin, respectively. Cytokine secretion correlated with the known redox potential of individual benzene metabolites and antioxidants, including dimethyl sulfoxide, 1,1,3,3-tetramethylthiourea, and N-acetylcysteine, attenuated the response. Binary combinations of selected benzene metabolites synergized in the induction of IL-8, while benzene, by itself, induced about a three-fold increase in IL-8 production. Taken together, our studies suggest that benzene and many of its phase I metabolites induce inflammatory cytokines and growth factors and this occurs through direct covalent binding or the generation of reactive oxygen species by autooxidation and reduction. The elaboration of proinflammatory cytokines and growth factors by keratinocytes in response to benzene and its principal metabolites may participate in benzene-induced skin carcinogenesis.	Benzene metabolites induce proinflammatory cytokine	THB Toxicity	ROS Formation
71	Moran JL, Siegel D, Sun XM, Ross D.	Induction of apoptosis by benzene metabolites in HL60 and CD34+ human bone marrow progenitor cells.	Mol Pharmacol. 1996 Sep;50(3):610-5.	Two cell types, HL60 human promyelocytic leukemia cells and CD34+ human bone marrow progenitor cells, were used as model systems to explore a possible role for apoptosis in the myelotoxicity of the phenolic metabolites of benzene. HL60 cells were treated with either phenol, catechol, hydroquinone, or 1,2,4-benzenetriol and then stained with Hoechst 33342 and propidium iodide and subjected to fluorescent microscopy. Cells with nuclear condensation and fragmentation were scored as apoptotic, and etoposide (40 microM) was used as a positive control. Catechol, 1,2,4-benzenetriol, and hydroquinone induced marked time- (0-24 hr) and concentration- (25-100 microM) dependent apoptosis, whereas phenol (750 microM) did not. Under these conditions, no significant necrosis was observed. The induction of apoptosis was confirmed by internucleosomal cleavage of DNA, assessed by agarose gel electrophoresis. CD34+ cells treated with etoposide (40 microM) or hydroquinone (50 microM) for 18 hr were stained and subjected to fluorescent microscopy as above. The percentage of cells exhibiting nuclear condensation and/or fragmentation as well as high intensity staining significantly increased in both cases. The induction of apoptosis was confirmed using a terminal deoxynucleotidyl transferase assay. These data show that apoptosis can be induced in both HL60 and CD34+ human bone marrow progenitor cells by benzene metabolites. The ability of phenolic metabolites of benzene to induce apoptosis in human bone marrow progenitor cells may contribute to benzene myelotoxicity.	In vitro study of catechol, 1,2,4-benzenetriol, and hydroquinone induced marked time- (0-24 hr) and concentration- (25-100 microM) dependent apoptosis, whereas phenol (750 microM) did not. Under these conditions, no significant necrosis was observed. The induction of apoptosis was confirmed by internucleosomal cleavage of DNA, assessed by agarose gel electrophoresis.	THB Toxicity	
72	Kuo PC, Abe KY.	Interleukin 1-induced production of nitric oxide inhibits benzenetriol-mediated oxidative injury in rat hepatocytes.	Gastroenterology. 1995 Jul;109(1):206-16.	BACKGROUND & AIMS:Nitric oxide modifies free radical-mediated cell processes in multiple in vivo and in vitro systems. The aim of this study was to determine the role of hepatocyte production of NO in oxidative injury. METHODS: Rat hepatocytes in primary culture were incubated with 1,2,3-benzenetriol, a source of superoxide. Interleukin (IL) 1 was added to induce NO synthesis. Injury was determined by aspartate aminotransferase (AST), malondialdehyde (MDA), and glutathione (GSH) levels.RESULTS: Benzenetriol-induced injury increased AST and MDA levels and decreased GSH levels in control and IL-1-treated cells. Inhibition of NO synthesis in IL-1-treated cells significantly increased AST and MDA production while enhancing GSH depletion. In the presence of superoxide dismutase or S-nitroso-albumin, an exogenous source of NO, injury was decreased or abolished. NO production was significantly increased with oxidative stress. In benzenetriol-induced injury in IL-1-stimulated hepatocytes, reverse-transcription polymerase chain reaction showed significantly increased levels of inducible NO synthase messenger RNA, whereas immunoblot analysis showed similarly increased levels of inducible NO synthase protein. CONCLUSIONS: In this rat hepatocyte model of IL-1/benzenetriol-mediated injury, NO, derived from endogenous synthesis or an exogenous donor, is protective. Oxidative stress may have a role in the transcriptional control of NO synthesis.	Liver injury related to oxidative stress in rat hepatocytes in vitro	THB Toxicity	ROS Formation
73	Manning BW, Adams DO, Lewis JG.	Effects of benzene metabolites on receptor-mediated phagocytosis and cytoskeletal integrity in mouse peritoneal macrophages.	Toxicol Appl Pharmacol. 1994 Jun;126(2):214-23.	Exposure to benzene can induce a number of hematotoxicities and decrease host resistance to microorganisms and tumors. Several studies have shown that metabolism of benzene to reactive intermediates is required for myelotoxicity. Since receptor-mediated phagocytosis by macrophages is an important host defense, we have examined the effects of benzene metabolites on receptor-mediated phagocytosis in cultured murine peritoneal macrophages. 1,4-Benzoquinone (BQ) was the most potent of the metabolites examined. Ten-minute exposures to a 12.5 microM concentration inhibited Fc and complement receptor-mediated phagocytosis by > or = 90%. Macrophage viability was largely unaffected by BQ treatment. Exposure to 50 and 100 microM 1,2,4-benzenetriol (BT) inhibited Fc receptor-mediated phagocytosis by 70 and 95%, respectively. Hydroquinone (HQ) elicited a major decrease (50%) only at 100 microM. The comparative inhibitory potencies of BT and HQ correlate with previously published data on their relative facility for autooxidation to quinones at physiological pH. Catechol had no effect at the concentrations employed. Macrophages treated with BQ and BT failed to recover their Fc receptor-mediated phagocytic capacity when incubated overnight in the absence of the xenobiotics. Only small differences in the inhibition of Fc receptor-mediated phagocytosis were observed between macrophages exposed to BQ at 4 versus 37 degrees C. BQ also had little effect on the Fc receptor binding of target cells. Fluorescent digital imaging microscopy demonstrated that BQ treatment markedly decreased the filamentous actin content of macrophages. However, BQ bound in low amounts to purified actin and did not affect its assembly. Our findings suggest that a mechanism for inhibition of Fc receptor-mediated phagocytosis by BQ is disruption of filamentous actin via an effect(s) other than the direct alkylation of actin by BQ.	Benzene metabolites effects in vitro on receptor mediated phagocytosis in murine macrophages. Exposure to 50 and 100 microM THB inhibited Fc receptor-mediated phagocytosis by 70 and 95%, respectively.	THB Toxicity	
74	Kawabe M, Takaba K, Yoshida Y, Hirose M.	Effects of combined treatment with phenolic compounds and sodium nitrite on two-stage carcinogenesis and cell proliferation in the rat stomach.	Jpn J Cancer Res. 1994 Jan;85(1):17-25.	The effects of combined treatment with NaNO2 and phenolic compounds on N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) stomach carcinogenesis were investigated in F344 rats. In the first experiment, groups of 15-20 male rats were treated with an intragastric dose of 150 mg/kg body weight of MNNG, and starting 1 wk later, were given 2.0% butylated hydroxyanisole, 0.8% catechol, 2.0% 3-methoxycatechol or basal diet either alone or in combination with 0.2% NaNO2 in the drinking water until they were killed at week 52. All three antioxidants significantly enhanced forestomach carcinogenesis without any effect of additional NaNO2 treatment. However, in the absence of MNNG pretreatment, the grade of forestomach hyperplasia in the catechol and 3-methoxycatechol groups was significantly increased by the combined treatment with NaNO2. In a second experiment, the combined effects of various phenolic compounds and NaNO2 on cell proliferation in the upper digestive tract were examined. Groups of 5 rats were given one of 24 phenolic compounds or basal diet either alone or in combination with 0.3% NaNO2 for 4 weeks and then killed. Particularly strong enhancing effects in terms of thickness of the forestomach mucosa were seen with t-butylhydroquinone (TBHQ), catechol, gallic acid, 1,2,4-benzenetriol, dl-3-(3,4-dihydroxyphenyl)-alanine and hydroquinone in combination with NaNO2. In the glandular stomach, similar enhancing effects were evident in 11 cases, and in the esophagus with phenol, TBHQ and gallic acid. These results demonstrate that NaNO2 can augment cell proliferation induced in the stomach epithelium by various phenolic compounds.	Exposure to various phenolic compounds in combination with sodium nitrite showed enhancing effects in terms of thickness of stomach mucosa in an invivo feeding study. Results demonstrate ability of sodium nitrite to enhance cell proliferation effects of phenolics, including THB, on the stomach epithelium.	THB Toxicity	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
75	Rao GS.	Hematin catalysed autooxidation of hydroquinone or 1,2,4-benzenetriol.	Chem Biol Interact. 1991;80(3):339-47.	Autooxidation of hydroquinone (HQ) or 1,2,4-benzenetriol (BT), catalysed by hemin in the presence of dithiothreitol was studied in phosphate buffered saline. Inclusion of glutamate in the above reaction mixture resulted in the formation of thiobarbituric acid reactive products (TBAR) only in an aerobic atmosphere and was linear up to 2 h. Oxygen consumption was noticed during the reaction process. The formation of TBAR was linear with the increase in concentration of heme (1-4 microM), dithiothreitol (0.2-2 mM) or BT (0.17-0.85 mM). Linearity of TBAR formation from glutamate for up to 2 h was observed during the autooxidation of BT in the presence of heme. Besides glutamate, heme concentration dependent formation of TBAR from deoxyuridine or DNA was also observed. Almost complete inhibition of TBAR formation from glutamate, deoxyuridine or DNA was observed in the presence of catalase or superoxide dismutase (SOD). The presence of thiourea or mannitol in the reaction mixture caused substantial diminution of TBAR formation. Albumin or dimethyl sulfoxide also caused partial inhibition. Complete to partial inhibition observed in the presence of oxyradical scavengers in this study indicates that hemin catalysed autooxidation of BT results in the formation of reactive oxygen radicals.		THB Toxicity	ROS Formation
76	Wallin H, Morgenstern R.	Activation of microsomal glutathione transferase activity by reactive intermediates formed during the metabolism of phenol.	Chem Biol Interact. 1990;75(2):185-99.	The activity of microsomal glutathione transferase was increased 1.7-fold in rat liver microsomes which carried out NADPH dependent metabolism of phenol. Known phenol metabolites were therefore tested for their ability to activate the microsomal glutathione transferase. The phenol metabolites benzoquinone and 1,2,4-benzenetriol both activated the glutathione transferase in microsomes 2-fold independently of added NADPH. However, NADPH was required to activate the enzyme in the presence of hydroquinone. Catechol did not activate the enzyme in microsomes. The purified enzyme was activated 6-fold and 8-fold by 5 mM benzenetriol and benzoquinone respectively. Phenol, catechol or hydroquinone had no effect on the purified enzyme. When microsomal proteins that had metabolized [14C]phenol were examined by SDS polyacrylamide gel electrophoresis and fluorography it was found that metabolites had bound covalently to a protein which comigrated with the microsomal glutathione transferase enzyme. We therefore suggest that reactive metabolites of phenol activate the enzyme by covalent modification. It is discussed whether the binding and activation has general implications in the regulation of microsomal glutathione transferase and, since some reactive metabolites might be substrates for the enzyme, their elimination through conjugation.	THB activates GSH transferase in rat liver microsomes	THB Toxicity	
77	Lode HN, Bruchelt G, Rieth AG, Niethammer D.	Release of iron from ferritin by 6-hydroxydopamine under aerobic and anaerobic conditions.	Free Radic Res Commun. 1990;11(1-3):153-8.	6-hydroxydopamine (6-OHDA) proved to be a very effective agent for iron release from ferritin. Iron release was enhanced in the presence of SOD, catalase and under anaerobic conditions. Ascorbic acid, a well known agent able to release iron from ferritin, increased the amount of released iron in more than an additive manner when used in combination with 6-OHDA. Similar to 6-OHDA, 6-hydroxydopa (Topa) and 1,2,4-benzenetriol were also able to release iron in large amounts; in contrast, catecholamines and other benzenediols were comparatively ineffective.		THB Toxicity	ROS Formation
78	Rao GS, Siddiqui SM, Pandya KP, Shanker R.	Relative toxicity of metabolites of benzene in mice.	Vet Hum Toxicol. 1988 Dec;30(6):517-20.	Repeated ip administration of hydroquinone (10 mg/kg/day), benzoquinone (2 mg/kg/day) or benzenetriol (6.25 mg/kg/day) to rats for 6 weeks produced significant decreases in RBC and bone marrow cell counts and hemoglobin content, together with relative changes in organ weights. In addition, benzoquinone and benzenetriol elicited histological injuries in liver, thymus, spleen, kidney and peripheral lymph nodes which warrant further investigation.	**in vivo** THB ip administration caused in jury to liver, thymus, spleen, kidney and peripheral lymph nodes.	THB Toxicity	
79	Lewis JG, Odom B, Adams DO.	Toxic effects of benzene and benzene metabolites on mononuclear phagocytes.	Toxicol Appl Pharmacol. 1988 Feb;92(2):246-54.	Benzene is a potent bone marrow toxin in animals and man. Animal studies have shown that exposure to benzene can alter T lymphocyte functions and decrease the resistance of animals to Listeria monocytogenes and transplanted tumor cells. Mononuclear phagocytes participate in host resistance to Listeria and tumor cells. The purpose of the studies presented here was to determine the effects of benzene and benzene metabolites on macrophage functions and the ability of macrophages to be activated for functions which are important in host defense. Benzene had no effects on macrophage function or activation for any of the functions tested. Conversely, metabolites of benzene, catechol (CAT), hydroquinone (HQ), benzquinone (BQ), and 1,2,4-benzenetriol (BT) had potent and varied effects on macrophage function and activation. BQ inhibited the broadest range of functions including release of H2O2, Fc receptor-mediated phagocytosis, interferon gamma priming for tumor cell cytolysis, and bacterial lipopolysaccharide (LPS) triggering of cytolysis. BQ was also the most potent metabolite causing inhibition at lower concentrations than the other metabolites. HQ inhibited H2O2 release and priming for cytolysis and BT inhibited phagocytosis and priming for cytolysis. CAT only inhibited the release of H2O2. None of the compounds tested inhibited the induction of class II histocompatibility antigens on the cell surface. All of the effects measured occurred using concentrations of compounds which did not disrupt the cell integrity or inhibit general functions such as protein synthesis. Taken together these data suggest that benzene metabolites alter macrophage function through several mechanisms including inhibition of output enzymes and disruption of signal transduction systems.	THB cause changes to macrophage function and activation **Same lab as SCCS cited reference**	THB Toxicity	ROS Formation
80	Pellack-Walker P, Walker JK, Evans HH, Blumer JL.	Relationship between the oxidation potential of benzene metabolites and their inhibitory effect on DNA synthesis in L5178YS cells.	Mol Pharmacol. 1985 Dec;28(6):560-6.	The effects of benzene and its metabolites on the rate of DNA synthesis were measured in the mouse lymphoma cell line, L5178YS. The direct toxicity of benzene could be distinguished from that of its metabolites since bioactivation of benzene in L5178YS cells was not observed. Cells were exposed to benzene, phenol, catechol, hydroquinone, p-benzoquinone, or 1,2,4-benzenetriol over the range of 1.0 X 10(-7) to 1.0 X 10(-2) M for 30 min, and the rate of DNA synthesis was measured at various times after chemical washout. Cell viability and protein synthesis were determined by trypan blue dye exclusion and [3H]leucine incorporation, respectively. Effects were designated as "DNA specific" when DNA synthesis was inhibited in the absence of discernible effects on cell membrane integrity and protein synthesis. Concentrations of benzene as high as 1 mM had no effect on DNA synthesis. Comparison of the effects at the maximum nontoxic dose for each compound showed that catechol and hydroquinone were the most effective, inhibiting DNA synthesis by 65%. Phenol, benzoquinone, and benzenetriol inhibited DNA synthesis by approximately 40%. Maximum inhibition was observed 60 min after metabolite washout in each case. Benzoquinone was the most potent inhibitor of DNA synthesis, followed by hydroquinone, benzenetriol, catechol, and phenol with ED50 values of 5 X 10(-6), 1 X 10(-5), 1.8 X 10(-4), 2.5 X 10(-4), and 8.0 X 10(-4), respectively. Cyclic voltammetric experiments were performed on the hydroxylated metabolites of benzene to assess the possible involvement of a redox-type mechanism in their inhibition of DNA synthesis. The ease of oxidation of these metabolites correlated with their ED50 values for inhibition of DNA synthesis (r = 0.997). This suggests that oxidation of phenol or one of its metabolites may be necessary for production of the species involved in inhibition of DNA synthesis.	**Same lab as SCCS cited reference**	THB Toxicity	ROS Formation
81	Schwartz CS, Snyder R, Kalf GF.	The inhibition of mitochondrial DNA replication in vitro by the metabolites of benzene, hydroquinone and p-benzoquinone.	Chem Biol Interact. 1985 May;53(3):327-50.	Rat liver mitochondria incubated with the metabolites of benzene, p-benzoquinone or 1,2,4-benzenetriol, showed a dose-dependent inhibition of [3H]dTTP incorporation into mtDNA with median inhibitory concentrations of 1 mM for each compound. Benzene and the metabolites phenol, catechol and hydroquinone did not inhibit at concentrations up to 10 mM. Similarly, incubation of p-benzoquinone or hydroquinone with rabbit bone marrow mitochondria showed a dose-dependent inhibition of mtDNA synthesis with 50% inhibition at 1 mM and 10 mM, respectively. That these metabolites inhibit mitochondrial replication was evidenced by the fact that [3H]dTTP incorporation into characteristic 38S, 27S and 7S mitochondrial replication intermediates was decreased by the quinones, as analyzed on 5-20% neutral sucrose velocity gradients. p-Benzoquinone, hydroquinone and 1,2,4-benzenetriol inhibited the activity of partially purified rat liver mtDNA polymerase gamma using either activated calf thymus DNA or poly(rA) X p(dT)12-18 as primer/template, with 50% inhibitory concentrations of 25 microM, 25 microM and 180 microM, respectively. Preincubation of the metabolites with polymerase gamma or primer/template, followed by removal of the unreacted metabolite by gel filtration, indicated that inhibition resulted from interaction of the metabolites with the enzyme, rather than with the template. Binding appeared to involve a sulfhydryl residue on the enzyme since the binding of [14C]hydroquinone was prevented by N-ethylmaleimide. The ability of hydroquinone or p-benzoquinone to inhibit binding of [14C]hydroquinone to the enzyme suggests that the compounds bind to a common site or are converted to a common intermediate. Inhibition of, or changes in, replication in mitochondria of bone marrow cells by hydroquinone and p-benzoquinone may explain the changes in the mitochondrial genome observed in marrow stem cells in acute myelogenous leukemia and may suggest a mechanism for benzene leukemogenesis.	Benzene metabolite BQ and HQ showed dose dependent inhibition of tritiated dTTP into mDNA.	THB Toxicity	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
82	Kalf GF, Snyder R, Rushmore TH.	Inhibition of RNA synthesis by benzene metabolites and their covalent binding to DNA in rabbit bone marrow mitochondria in vitro.	Am J Ind Med. 1985;7(5-6):485-92.	Mitoplasts from rabbit bone marrow cells in vitro incubated with benzene metabolites showed a concentration-dependent inhibition of mitochondrial RNA synthesis. The 50% molar inhibitory concentration for each metabolite was determined to be p-benzoquinone, 2 X 10(-6); phenol, 2.5 X 10(-5); hydroquinone, 5 X 10(-5); catechol, 2 X 10(-3); benzene, 1.6 X 10(-2). Activated metabolites of radiolabeled benzene covalently bound to mitochondrial DNA in vitro. Labeled deoxynucleosides, enzymatically produced from DNA isolated from mitoplasts prelabeled in DNA with [3H]dGTP and exposed to [14C]benzene in vitro, were chromatographed on a Sephadex LH-20 column to obtain deoxynucleoside-adducts. The elution profiles indicated that mtDNA contained seven deoxyguanosine-adducts. A similar experiment in which the mtDNA was prelabeled with [3H]dATP indicated that two deoxyadenosine-adducts of mtDNA were formed from benzene in vitro. Tentative identification of several deoxyguanosine-adducts indicates that p-benzoquinone, hydroquinone, phenol, and 1,2,4-benzenetriol produced from benzene form adducts with guanine.	THB forms adducts with guanine in vitro rabbit bone marrow.	THB Toxicity	
83	Gaido K, Wierda D.	In vitro effects of benzene metabolites on mouse bone marrow stromal cells.	Toxicol Appl Pharmacol. 1984 Oct;76(1):45-55.	Benzene exposure can result in bone marrow myelotoxicity. We examined the effects of benzene metabolites on bone marrow stromal cells of the hemopoietic microenvironment. Male B6C3F1 mouse bone marrow adherent stromal cells were plated at 4 X 10(6) cells per 2 ml of DMEM medium in 35-mm tissue culture dishes. The growing stromal cell cultures were exposed to log 2 doses of five benzene metabolites: hydroquinone, benzoquinone, phenol, catechol, or benzenetriol for 7 days. The dose which caused a 50% decrease in colony formation (TD50) was 2.5 X 10(-6) M for hydroquinone, 17.8 X 10(-6) M for benzoquinone, 60 X 10(-6) M for benzenetriol, 125 X 10(-6) M for catechol, and 190 X 10(-6) M for phenol. We next examined the effect of benzene metabolites on the ability of stromal cells to influence granulocyte/monocyte colony growth (G/M-CFU-C) in a coculture system. Adherent stromal cells were plated and incubated for 14 days and then exposed to a benzene metabolite. After 3 days the medium and metabolite were removed and an agar:RPMI layer containing 10(6) fresh bone marrow cells was placed over the stromal layer. After incubation for 7 days the cultures were scored for G/M colony formation. Hydroquinone and benzoquinone were most toxic, while catechol and benzenetriol inhibited colony growth only at high doses. These results indicate that injured bone marrow stromal cells may be a significant factor in benzene-induced hemotoxicity.	Benzene metabolite effects on murine bone marrow stromal cells. Hydroquinone and benzoquinone were most toxic, while catechol and benzenetriol inhibited colony growth only at high doses. These results indicate that injured bone marrow stromal cells may be a significant factor in benzene-induced hemotoxicity.	THB Toxicity	
84	Post GB, Snyder R, Kalf GF.	Inhibition of mRNA synthesis in rabbit bone marrow nuclei in vitro by quinone metabolites of benzene.	Chem Biol Interact. 1984 Jul;50(2):203-11.	mRNA synthesis by rabbit bone marrow nuclei has been shown to be inhibited by the quinone metabolites of benzene, hydroquinone and p-benzoquinone, in a concentration-dependent manner with 50% inhibitory concentration (IC50[M]) for both compounds of 6 X 10(-6) M. Catechol and 1,2,4-benzenetriol also showed a concentration-dependent inhibition of synthesis, however, 50% inhibition was not reached by 10(-4) M. Phenol did not inhibit mRNA synthesis even at 10(-3) M. It is possible that myelotoxicity from benzene might result from such an inhibition of mRNA synthesis by quinone metabolites in pluripotent and/or committed bone marrow stem cells.	**Damage results from quinone metabolites** **Oxidative damage** **Same lab as #204**	THB Toxicity	ROS Formation
85	Pfeifer RW, Irons RD.	Effect of benzene metabolites on phytohemagglutinin-stimulated lymphopoiesis in rat bone marrow.	J Reticuloendothel Soc. 1982 Feb;31(2):155-70.	Chronic exposure of animals to benzene results in lymphocytopenia and bone marrow depression. A highly sensitive microculture system was developed to amplify and characterize marrow mitogen response which depended on the isolation of cells by Percoll continuous density gradient centrifugation and strict adherence to optimal culture conditions. Maximal mitogen responses to phytohemagglutinin (PHA) and concanavalin A (con A), as assessed by (3H)-thymidine uptake, occurred at later times in rat bone marrow cultures (days 5-7 of culture) compared with spleen and thymus (days 2-3). Compared with spleen and thymus, the PHA: con A stimulation ratio was inverted for marrow, yet the responsive cells were morphologically identical to those of peripheral lymphoid organs. Populations enriched in lymphoid precursors were inhibited from responding to PHA stimulation at noncytotoxic concentration (less than microM) of the benzene metabolites, p-benzoquinone or 1, 2, 4-benzenetriol. Pretreatment with less than microM concentrations of metabolite resulted in a modulation of lectin-induced responses such the blastogenesis was induced at higher concentrations of lectin relative to untreated cells. These changes indicate that known metabolites of benzene induce a concentration dependent modulation of differentiation and proliferation in lectin-stimulated cells from rat bone marrow in vitro.	Data indicate that known metabolites of benzene induce a concentration dependent modulation of differentiation and proliferation in lectin-stimulated cells from rat bone marrow in vitro.	THB Toxicity	
86	Ahmad S, Rao GS.	Complexation of 1,2,4-benzenetriol with inorganic and ferritin-released iron in vitro.	Biochem Biophys Res Commun. 1999 May 27;259(1):169-71.	The reactive metabolite(s) responsible for the expression of benzene toxicity is not clearly known despite extensive information on the metabolism and hematotoxicity of benzene. It is now widely believed that hematotoxicity of benzene is due to the concerted action of several metabolites which arise from multiple pathways of benzene. In our earlier study, we proposed iron polyphenol chelates as possible toxic metabolites of benzene due to their prooxidant activity. In continuation, we demonstrate the formation of an iron and 1,2,4-benzenetriol (BT) complex, when added together in an acetate buffer, 0.1 M, pH 5.6, by sephadex G-10 column chromatography. It was also observed that iron released from ferritin in the presence of BT formed a complex with BT.		Degradation Pathway	ROS Formation
87	Ahmad S, Singh V, Rao GS.	Release of iron from ferritin by 1,2,4-benzenetriol.	Chem Biol Interact. 1995 May 19;96(2):103-11.	Release of iron from ferritin in the presence of polyhydroxy metabolites of benzene i.e., hydroquinone (HQ) or 1,2,4-benzenetriol (BT) was studied in acetate buffer, pH 5.6. The release of iron from ferritin was quantitated by monitoring the formation of iron-ferrozine complex. The presence of hydroquinone (330 microM) did not result in the release of iron from ferritin, whereas the same concentration of BT resulted in the release of significant amounts of iron (3.2 microM/min) from ferritin. BT concentration-dependent increase in iron release from ferritin was observed although the increase was not linear with the concentration of BT. Under a N2 atmosphere the presence of BT resulted in the release of iron (2.1 microM/min) from ferritin. The presence of oxyradical scavengers i.e., albumin, catalase or superoxide dismutase significantly inhibited iron release from ferritin by BT. The iron released from ferritin by BT enhanced lipid peroxidation in rat brain homogenate and released aldehydic products from bleomycin-dependent degradation of DNA. Addition of BT to bone marrow lysate resulted in an increase of iron release as a function of time. These studies indicate that BT is a potent reductant of ferric iron of ferritin and also mobilizes and releases iron from ferritin core. The release of iron from bone marrow lysate by BT may be of toxicological significance as this could lead to disruption of intracellular iron homeostasis in bone marrow cells.		Degradation Pathway	ROS Formation
88	Singh V, Ahmad S, Rao GS.	Prooxidant and antioxidant properties of iron-hydroquinone and iron-1,2,4-benzenetriol complex. Implications for benzene toxicity.	Toxicology. 1994 Mar 25;89(1):25-33.	Bleomycin-dependent degradation of DNA in bone marrow cells was studied in vitro in the presence of iron or iron polyphenol chelates which are formed during biotransformation of benzene. Iron polyphenol chelates markedly enhanced bleomycin-dependent DNA degradation in comparison to iron alone. About 1.5 and 2.5-fold increase was observed in the presence of iron hydroquinone (HQ) chelate and iron 1,2,4-benzenetriol (BT) chelate, respectively. Endogenous iron chelators such as glutamate, citrate, aspartate, glycine, cysteine, dithiothreitol, AMP, ADP and ATP did not enhance iron-catalysed bleomycin-dependent degradation of DNA. By bleomycin assay, the recovery of iron polyphenol chelate added externally to bone marrow lysate was complete. However, the presence of iron polyphenol chelate resulted in less thiobarbituric acid reactive products from glutamate or brain homogenate than with iron alone. The optical spectra of BT were modified in the presence of ferrous sulphate, revealing a new absorption peak at 259 nm indicating complexation with iron. Thus, the iron polyphenol chelate, on one hand, is a more potent DNA cleaving agent in the presence of bleomycin, and on the other hand, it is a less effective free radical generator as compared to iron alone.		Degradation Pathway	
89	Ahmad S, Singh V, Rao GS.	Antioxidant potential in serum and liver of albino rats exposed to benzene.	Indian J Exp Biol. 1994 Mar;32(3):203-6.	Release of iron from ferritin in the presence of polyhydroxy metabolites of benzene i.e., hydroquinone (HQ) or 1,2,4-benzenetriol (BT) was studied in acetate buffer, pH 5.6. The release of iron from ferritin was quantitated by monitoring the formation of iron-ferrozine complex. The presence of hydroquinone (330 microM) did not result in the release of iron from ferritin, whereas the same concentration of BT resulted in the release of significant amounts of iron (3.2 microM/min) from ferritin. BT concentration-dependent increase in iron release from ferritin was observed although the increase was not linear with the concentration of BT. Under a N2 atmosphere the presence of BT resulted in the release of iron (2.1 microM/min) from ferritin. The presence of oxyradical scavengers i.e., albumin, catalase or superoxide dismutase significantly inhibited iron release from ferritin by BT. The iron released from ferritin by BT enhanced lipid peroxidation in rat brain homogenate and released aldehydic products from bleomycin-dependent degradation of DNA. Addition of BT to bone marrow lysate resulted in an increase of iron release as a function of time. These studies indicate that BT is a potent reductant of ferric iron of ferritin and also mobilizes and releases iron from ferritin core. The release of iron from bone marrow lysate by BT may be of toxicological significance as this could lead to disruption of intracellular iron homeostasis in bone marrow cells.		Degradation Pathway	ROS Formation

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90	Qu Q, Melikian AA, Li G, Shore R, Chen L, Cohen B, Yin S, Kagan MR, Li H, Meng M, Jin X, Winnik W, Li Y, Mu R, Li K.	Validation of biomarkers in humans exposed to benzene: urine metabolites.	Am J Ind Med. 2000 May;37(5):522-31.	BACKGROUND: The present study was conducted among Chinese workers employed in glue- and shoe-making factories who had an average daily personal benzene exposure of 31+/-26 ppm (mean+/-SD). The metabolites monitored were S-phenylmercapturic acid (S-PMA), trans, trans-muconic acid (t,t-MA), hydroquinone (HQ), catechol (CAT), 1,2, 4-trihydroxybenzene (benzene triol, BT), and phenol. METHODS: S-PMA, t,t-MA, HQ, CAT, and BT were quantified by HPLC-tandem mass spectrometry. Phenol was measured by GC-MS. RESULTS: Levels of benzene metabolites (except BT) measured in urine samples collected from exposed workers at the end of workshift were significantly higher than those measured in unexposed subjects (P < 0.0001). The large increases in urinary metabolites from before to after work strongly correlated with benzene exposure. Concentrations of these metabolites in urine samples collected from exposed workers before work were also significantly higher than those from unexposed subjects. The half-lives of S-PMA, t,t-MA, HQ, CAT, and phenol were estimated from a time course study to be 12.8, 13.7, 12.7, 15.0, and 16.3 h, respectively. CONCLUSIONS: All metabolites, except BT, are good markers for benzene exposure at the observed levels; however, due to their high background, HQ, CAT, and phenol may not distinguish unexposed subjects from workers exposed to benzene at low ambient levels. S-PMA and t,t-MA are the most sensitive markers for low level benzene exposure.		Degradation Pathway	
91	Inoue O, Seiji K, Nakatsuka H, Watanabe T, Yin S, Li GL, Cai SX, Jin C, Ikeda M.	Excretion of 1,2,4-benzenetriol in the urine of workers exposed to benzene.	Br J Ind Med. 1989 Aug;46(8):559-65.	Urine samples were collected from 152 workers (64 men, 88 women) who had been exposed to benzene, 53 workers (men only) exposed to a mixture of benzene and toluene, and 213 non-exposed controls (113 men, 100 women). The samples were analysed for 1,2,4-benzentriol (a minor metabolite of benzene) by high performance liquid chromatography. The time weighted average solvent exposure of each worker was monitored by diffusive sampling technique. The urinary concentration of 1,2,4-benzentriol related linearly to the intensity of exposure to benzene both in men and women among workers exposed to benzene, and was suppressed by toluene co-exposure among male workers exposed to a mixture of benzene and toluene. A cross sectional balance study in men at the end of the shift of a workday showed that only 0.47% of benzene absorbed will be excreted into urine as 1,2,4-benzenetriol, in close agreement with previous results in rabbits fed benzene. The concentration of 1,2,4-benzenetriol in urine was more closely related to the concentration of quinol than that of catechol. The fact that phenol and quinol, but not catechol, are precursors of 1,2,4-benzentriol in urine was further confirmed by the intraperitoneal injection of the three phenolic compounds to rats followed by urine analysis for 1,2,4-benzenetriol.	1,2,4-THB is identified as a minor metabolite of benzene; from further oxidation of HQ but not catechol	Degradation Pathway	
92	Luo YJ, Zuo YM, Zhang ZL, Cai MT, Luo GM.	[Study on chemical constituents of Gardenia jasminoides(III)].	Zhong Yao Cai. 2014 Jul;37(7):1196-9. Chinese.	OBJECTIVE: To investigate the chemical constituents of Gardenia jasminoides fruits. METHODS: Various column chromatography were used in the isolation and purification, and physiochemical constant determination and spectral analysis were adopted to determine the chemical structures. RESULTS: Twelve compounds were isolated from Gardenia jasminoides including jasminoside I (1), gardenoside (2), gardaloside (3), 3-hydroxy-urs-12-ene-11-ketone(4), 5, 4'-dihydroxyl-7, 3', 5'-trimethoxyflavone (5), 5, 7, 3', 4', 5'-pentamethoxyflavone(6), 3, 5, 6, 4'-tetrahydroxy-3', 5'-dimethoxyflavone (7), shikimic acid (8), 1, 2, 4-benzenetriol (9), 3, 4-dimethoxy-benzoic acid (10), dibutyl phthalate (11) and diisobutyl phthalate (12). CONCLUSION: Compounds 4 - 7 and 9 -10 were isolated from this plant for the first time.	Detection of THB in gardenia jasminoides fruit for the first time	THB Food/Beverage/Nature	
93	Katada S, Watanabe T, Mizuno T, Kobayashi S, Takeshita M, Osaki N, Kobayashi S, Katsuragi Y.	Effects of Chlorogenic Acid-Enriched and Hydroxyhydroquinone-Reduced Coffee on Postprandial Fat Oxidation and Antioxidative Capacity in Healthy Men: A Randomized, Double-Blind, Placebo-Controlled, Crossover Trial.	Nutrients. 2018 Apr 23;10(4). pii: E525. doi: 10.3390/nu10040525.	Chlorogenic acids (CGAs) reduce blood pressure and body fat, and enhance fat metabolism. In roasted coffee, CGAs exist together with the oxidant component hydroxyhydroquinone (HHQ). HHQ counteracts the antihypertensive effects of CGA, but its effects on CGA-induced fat oxidation (FOX) are unknown. Here we assessed the effects of CGA-enriched and HHQ-reduced coffee on FOX. Fifteen healthy male volunteers (age: 38 ± 8 years (mean ± SD); BMI: 22.4 ± 1.5 kg/m²) participated in this crossover study. Subjects consumed the test beverage (coffee) containing the same amount of CGA with HHQ (CGA-HHQ(+)) or without HHQ (CGA-HHQ(−)) for four weeks. Postprandial FOX and the ratio of the biological antioxidant potential (BAP) to the derivatives of reactive oxygen metabolites (d-ROMs) as an indicator of oxidative stress were assessed. After the four-week intervention, postprandial FOX and the postprandial BAP/d-ROMs ratio were significantly higher in the CGA-HHQ(−) group compared with the CGA-HHQ(+) group (4 ± 23 mg/min, group effect: p = 0.040; 0.27 ± 0.74, group effect: p = 0.007, respectively). In conclusion, reducing the amount of HHQ facilitated the postprandial FOX effects of CGA in coffee. Our findings also suggest that the mechanism underlying the inhibition of FOX by HHQ is related to postprandial oxidative stress.	THB reduction in coffee reduces oxidative stress markers	THB Food/Beverage/Nature	ROS formation
94	Ishida K, Misawa K, Yamamoto M, Shimotoyodome A.	Hydroxyhydroquinone impairs fat utilization in mice by reducing nitric oxide availability.	J Physiol Sci. 2018 Mar 23. doi: 10.1007/s12576-018-0605-9. [Epub ahead of print]	Habitual consumption of chlorogenic acid compounds (CGAs) from coffee increases fat catabolism and reduces body fat; however, the contribution of roasted coffee remains unclear. Hydroxyhydroquinone (HHQ) impairs the vasodilatory and antihypertensive effects of CGAs by reducing nitric oxide (NO) bioavailability. Since HHQ also reduces fat catabolism, we hypothesized that HHQ does so by decreasing NO availability. Therefore, we investigated the effect of HHQ on energy metabolism in KKAy mice. In HHQ-treated mice, fat oxidation was significantly low and dose-dependent, serum and urinary hydrogen peroxide were high, and plasma NO metabolites and S-nitrosylated liver proteins were low. In HHQ-treated mouse hepatocytes, the palmitate-induced increase in cellular oxygen consumption was negatively affected, and HHQ or L-NAME reduced cellular fatty acid utilization. In conclusion, HHQ can impair fat utilization by reducing NO availability in mice. Protein S-nitrosylation reduction in liver cells after HHQ consumption may be associated with impaired fatty acid oxidation.	THB reduction in coffee reduces oxidative stress markers	THB Food/Beverage/Nature	ROS formation
95	Wada H, Oyama K, Kamae R, Masuda T, Kanemaru K, Yokoigawa K, Oyama Y.	Zinc-dependent and independent actions of hydroxyhydroquinone on rat thymic lymphocytes.	Drug Chem Toxicol. 2018 Feb 9:1-6. doi: 10.1080/01480545.2018.1429462. [Epub ahead of print]	Coffee contains hydroxyhydroquinone (HHQ). HHQ is one of the by-products released during bean roasting. Therefore, it is important to elucidate the bioactivity of HHQ to predict its beneficial or adverse effects on humans. We studied zinc-dependent and independent actions of commercially procured synthetic HHQ in rat thymocytes using flow cytometric techniques with propidium iodide, FluoZin-3-AM, 5-chloromethylfluorescein diacetate, and annexin V-FITC. HHQ at 1050 μM elevated intracellular Zn2+ levels by releasing intracellular Zn2+. HHQ at 10 μM increased cellular thiol content in a zinc-dependent manner. However, HHQ at 30-50 μM reduced cellular thiol content. Although the latter actions of HHQ (30-50 μM) were suggested to increase cell vulnerability to oxidative stress, HHQ at 0.3-100 μM significantly protected cells against oxidative stress induced by H2O2. **The process of cell death induced by H2O2 was delayed by HHQ,** although both H2O2 and HHQ increased the population of annexin V-positive living cells. However, HHQ at 10-30 μM promoted cell death induced by A23187, a calcium ionophore. HHQ at 10-30 μM exerted contrasting effects on cell death caused by oxidative stress and Ca2+ overload. Because HHQ is considered to possess diverse cellular actions, coffee with reduced amount of HHQ may be preferable to avoid potential adverse effects.	THB at 0.3-100 μM had an anti-oxidant or radical scavenging effect	THB Food/Beverage/Nature	ROS formation
96	Kajikawa M, Maruhashi T, Hidaka T, Nakano Y, Kurisu S, Matsumoto T, Iwamoto Y, Kishimoto S, Matsui S, Aibara Y, Yusoff FM, Kihara Y, Chayama K, Goto C, Noma K, Nakashima A, Watanabe T, Tone H, Hibi M, Osaki N, Katsuragi Y, Higashi Y.	Coffee with a high content of chlorogenic acids and low content of hydroxyhydroquinone improves postprandial endothelial dysfunction in patients with borderline and stage 1 hypertension.	Eur J Nutr. 2018 Jan 12. doi: 10.1007/s00394-018-1611-7. [Epub ahead of print]	PURPOSE: The purpose of this study was to evaluate acute effects of coffee with a high content of chlorogenic acids and different hydroxyhydroquinone contents on postprandial endothelial dysfunction. METHODS: This was a single-blind, randomized, placebo-controlled, crossover-within-subject clinical trial. A total of 37 patients with borderline or stage 1 hypertension were randomized to two study groups. The participants consumed a test meal with a single intake of the test coffee. Subjects in the Study 1 group were randomized to single intake of coffee with a high content of chlorogenic acids and low content of hydroxyhydroquinone or coffee with a high content of chlorogenic acids and a high content of hydroxyhydroquinone with crossover. Subjects in the Study 2 group were randomized to single intake of coffee with a high content of chlorogenic acids and low content of hydroxyhydroquinone or placebo coffee with crossover. Endothelial function assessed by flow-mediated vasodilation and plasma concentration of 8-isoprostanes were measured at baseline and at 1 and 2 h after coffee intake. RESULTS: Compared with baseline values, single intake of coffee with a high content of chlorogenic acids and low content of hydroxyhydroquinone, but not coffee with a high content of chlorogenic acids and high content of hydroxyhydroquinone or placebo coffee, significantly improved postprandial flow-mediated vasodilation and decreased circulating 8-isoprostane levels. CONCLUSIONS: These findings suggest that a single intake of coffee with a high content of chlorogenic acids and low content of hydroxyhydroquinone is effective for improving postprandial endothelial dysfunction.	Reduction of THB in coffee improves postprandial effects	THB Food/Beverage/Nature	
97	Soga S, Ota N, Shimotoyodome A.	Reduction in hydroxyhydroquinone from coffee increases postprandial fat utilization in healthy humans: a randomized double-blind, cross-over trial.	Biosci Biotechnol Biochem. 2017 Jul;81(7):1433-1435. doi: 10.1080/09168451.2017.1301802. Epub 2017 Mar 21.	The present study aimed to clarify the effect of reduction in hydroxyhydroquinone (HHQ) from roasted coffee on energy utilization in humans. Indirect calorimetry showed that one-week ingestion of HHQ-reduced coffee led to significantly higher postprandial fat utilization than that of HHQ-containing coffee. This finding indicates that reduction in HHQ from coffee increases postprandial fat utilization.	THB reduction in coffee increases postprandial fat utilization	THB Food/Beverage/Nature	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
98	Kamae R, Nojima S, Akiyoshi K, Setsu S, Honda S, Masuda T, Oyama Y.	Hydroxyhydroquinone, a by-product of coffee bean roasting, increases intracellular Ca2+ concentration in rat thymic lymphocytes.	Food Chem Toxicol. 2017 Apr;102:39-45. doi: 10.1016/j.fct.2017.01.025. Epub 2017 Jan 30.	Hydroxyhydroquinone (HHQ) is generated during coffee bean roasting. A cup of coffee contains 0.1-1.7 mg of HHQ. The actions of HHQ on mammalian DNA were examined because HHQ is a metabolite of benzene, which causes leukemia. Currently, information on the cellular actions of HHQ is limited. We examined the effects of sublethal levels of HHQ on the concentration of intracellular Ca2+ in rat thymic lymphocytes by using a flow cytometric technique with fluorescent probes. HHQ at 10 μM or more significantly elevated intracellular Ca2+ levels by increasing the membrane permeability of divalent cations, resulting in hyperpolarization via the activation of Ca2+-dependent K+ channels. HHQ-induced changes in the intracellular Ca2+ concentration and membrane potential may affect the cell functions of lymphocytes. HHQ-reduced coffee may be preferable in order to avoid the possible adverse effects of HHQ.	THB level in a cup of coffee ranges 0.1 - 1.7 mg	THB Food/Beverage/Nature	
99	Andreu-Navarro A, Fernández-Romero JM, Gómez-Hens A.	Determination of polyphenolic content in beverages using laccase, gold nanoparticles and long wavelength fluorimetry.	Anal Chim Acta. 2012 Feb 3;713:1-6. doi: 10.1016/j.aca.2011.11.049. Epub 2011 Dec 1.	An enzymatic fluorimetric method for the determination of polyphenol compounds in beverages is described, which is based on the temporal inhibition caused by these compounds on the oxidation of the long wavelength fluorophor indocyanine green (λ(ex) 764 nm, λ(em) 806 nm), in the presence of the enzyme laccase and positively charged gold nanoparticles (AuNPs). The oxidation of the dye gives rise to a fast decrease in its fluorescence, but it is delayed by the polyphenol, obtaining a time period directly proportional to its concentration, which has been used as the analytical parameter. The behaviour of several benzenediols and benzenetriols in the system and the modification of the activity of the enzyme by its interaction with AuNPs have been studied. The system has been optimized using gallic acid as a polyphenol model, but the dynamic ranges of the calibration graphs and the detection limits for several of the polyphenols assayed were obtained (μmol L(-1)): gallic acid (0.13-5, 0.04), catechol (0.08-5, 0.01), hydroquinone (0.05-2, 0.01), hydroxyhydroquinone (0.09-5, 0.03), pyrogallol (0.17-5, 0.04). Most of the values of the regression coefficients were 0.999 and the precision of the method, expressed as RSD% and checked at two concentration levels of each analyte, ranged between 1.8 and 5.6%. The method has been applied to the determination of polyphenol content in several foodstuff samples and the results compared with those obtained with the standard Folin-Ciocalteu method.	Analytical method for detection of polyphenols in beverages. THB 0.09 - 5 μmol/L	THB Food/Beverage/Nature	
100	Butt MS, Sultan MT.	Coffee and its consumption: benefits and risks.	Crit Rev Food Sci Nutr. 2011 Apr;51(4):363-73. doi: 10.1080/10408390903586412. Review.	Coffee is the leading worldwide beverage after water and its trade exceeds US $10 billion worldwide. Controversies regarding its benefits and risks still exist as reliable evidence is becoming available supporting its health promoting potential; however, some researchers have argued about the association of coffee consumption with cardiovascular complications and cancer insurgence. The health-promoting properties of coffee are often attributed to its rich phytochemistry, including caffeine, chlorogenic acid, caffeic acid, hydroxyhydroquinone (HHQ), etc. Many research investigations, epidemiological studies, and meta-analyses regarding coffee consumption revealed its inverse correlation with that of diabetes mellitus, various cancer lines, Parkinsonism, and Alzheimer's disease. Moreover, it ameliorates oxidative stress because of its ability to induce mRNA and protein expression, and mediates Nrf2-ARE pathway stimulation. Furthermore, caffeine and its metabolites help in proper cognitive functionality. Coffee lipid fraction containing cafestol and kahweol act as a safeguard against some malignant cells by modulating the detoxifying enzymes. On the other hand, their higher levels raise serum cholesterol, posing a possible threat to coronary health, for example, myocardial and cerebral infarction, insomnia, and cardiovascular complications. Caffeine also affects adenosine receptors and its withdrawal is accompanied with muscle fatigue and allied problems in those addicted to coffee. An array of evidence showed that pregnant women or those with postmenopausal problems should avoid excessive consumption of coffee because of its interference with oral contraceptives or postmenopausal hormones. This review article is an attempt to disseminate general information, health claims, and obviously the risk factors associated with coffee consumption to scientists, allied stakeholders, and certainly readers.	Chlorogenic acid (3-3,4-Dihydroxycinnamoyl quinic acid), caffeic acid (3,4-Dihydroconnamic acid), and hydroxyhydroquinone (1,2,4-Trihydroxybenzene) are potent antioxidants and impart several health benefits like protecting the body from the hazardous effects of free radicals.	THB Food/Beverage/Nature	
101	Ochiai R, Chikama A, Kataoka K, Tokimitsu I, Maekawa Y, Ohishi M, Rakugi H, Mikami H.	Effects of hydroxyhydroquinone-reduced coffee on vasoreactivity and blood pressure.	Hypertens Res. 2009 Nov;32(11):969-74. doi: 10.1038/hr.2009.132. Epub 2009 Aug 28.	Recent studies suggest that chlorogenic acids, which are the main components of the polyphenol class in coffee, decrease blood pressure, and that hydroxyhydroquinone (HHQ), which is generated by roasting coffee beans, inhibits the antihypertensive effect of chlorogenic acids in brewed coffee. Here, we examined the vasoreactivity and antihypertensive effects of HHQ-reduced coffee in mild hypertension. The study design was a double blind, randomized, placebo-controlled intervention study, with a 4-week run-in period, followed by an 8-week test beverage ingestion period. The subjects were Japanese men and women with mild hypertension and vascular failure, who were not taking any antihypertensive drugs. During the test beverage ingestion period, the subjects ingested either active or placebo HHQ-reduced coffee (chlorogenic acids per 184 ml of coffee: active, 300 mg; and placebo, 0 mg) daily. Subjects were randomly divided into two groups: active group (n=9) and placebo group (n=12). In the active beverage group, endothelium-dependent, flow-mediated vasodilation impairment was significantly ameliorated and systolic blood pressure was significantly decreased from the baseline, but not in the placebo group. There were no test beverage consumption-related changes in other parameters that may influence blood pressure, such as pulse, cardiac output, body weight or 24-h urine volume. Ingestion of the active beverage significantly decreased urinary isoprostane levels, suggesting a reduced oxidative stress. These findings indicate that HHQ-reduced coffee decreased blood pressure in subjects with mild hypertension. The decreased blood pressure was associated with improved vascular endothelial function.	THB reduction in coffee resulted in decrease of antihypertensive effect of CGA	THB Food/Beverage/Nature	
102	Suzuki A, Fujii A, Jokura H, Tokimitsu I, Hase T, Saito I.	Hydroxyhydroquinone interferes with the chlorogenic acid-induced restoration of endothelial function in spontaneously hypertensive rats.	Am J Hypertens. 2008 Jan;21(1):23-7.	BACKGROUND: Coffee is a rich source of antioxidative polyphenols, but epidemiological studies and interventional trials have failed to demonstrate any clear beneficial effects of coffee consumption on hypertension. The interaction between hydroxyhydroquinone (HHQ) and 5-caffeoylquinic acid (CQA) was examined, in an attempt to understand the controversial effects of coffee on hypertension. METHODS: Male Wistar Kyoto (WKY) rats or spontaneously hypertensive rats (SHRs, 14 weeks old) were divided into the following four groups; those on a control diet, 0.005% HHQ diet, 0.5% CQA diet, and HHQ plus CQA diet. The rats were fed the above diets for 8 weeks, and the tail arterial blood pressure was monitored in conscious rats at 2-week intervals. Urinary nitric oxide (NO) metabolites and hydrogen peroxide (H(2)O(2)) excretion were measured 8 weeks after the start of the experiment. Endothelium-dependent and -independent vasorelaxant responses and immunohistochemical staining for nitrotyrosine were examined in aortas. RESULTS: HHQ inhibited the CQA-induced improvement in hypertension, urinary NO metabolites or H(2)O(2) excretion, endothelial dysfunction, and nitrotyrosine deposits in aortas in SHR. However, the administration of HHQ alone had little effect on either strain. CONCLUSIONS: Based on the content ratio of HHQ and chlorogenic acids in coffee, HHQ interfered with the CQA-induced improvement in blood pressure and endothelial function in SHR. The results explain, at least in part, the conflicting action of coffee drinking on hypertension and vascular reactivity.	THB reduction in coffee resulted in decrease of antihypertensive effect of CGA	THB Food/Beverage/Nature	
103	Yamaguchi T, Chikama A, Mori K, Watanabe T, Shioya Y, Katsuragi Y, Tokimitsu I.	Hydroxyhydroquinone-free coffee: a double-blind, randomized controlled dose-response study of blood pressure.	Nutr Metab Cardiovasc Dis. 2008 Jul;18(6):408-14. Epub 2007 Oct 22.	BACKGROUND AND AIM: Coffee is rich in chlorogenic acids (CGA), whose metabolites may have beneficial effects such as anti-hypertensive effects. However, trial results concerning the effects of coffee on blood pressure (BP) are not consistent. A recent study suggested that hydroxyhydroquinone (HHQ), produced by the roasting of green coffee beans, inhibits the effect of CGA. In the present study, the dose-response for CGA in HHQ-free coffee on BP were investigated in mildly hypertensive men and women. METHODS AND RESULTS The trial design was a double-blind, randomized controlled trial, with five study groups including, control, zero-dose, low-dose, middle-dose and high-dose. The control beverage was identical to ordinary coffee. The others contained reduced HHQ levels, compared to ordinary coffee, and the CGA were adjusted in target concentration. A total of 203 subjects were randomly allocated. Each subject drank one cup of coffee per day. The study involved a screening and a baseline observation period of 6 weeks and an intervention period of 4 weeks. BP response showed CGA has an anti-hypertensive effect in a dose-dependent manner in HHQ-free coffee, and ordinary coffee showed almost no effect. As a result, a significant correlation between BP change and the three dose-response patterns was observed ($p<0.001$). CONCLUSIONS: This study demonstrates a dose-dependent decrease in BP for CGA in HHQ-free coffee.	THB reduction in coffee resulted in decrease of antihypertensive effect of CGA	THB Food/Beverage/Nature	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
104	Müller C, Hofmann T.	Quantitative studies on the formation of phenol/2-furfurylthiol conjugates in coffee beverages toward the understanding of the molecular mechanisms of coffee aroma staling.	J Agric Food Chem. 2007 May 16;55(10):4095-102. Epub 2007 Apr 18.	To gain a more comprehensive knowledge of the contribution of recently identified phenol/thiol conjugates to the storage-induced degradation of odorous thiols, the concentrations of the sulfury-roasty smelling key odorant 2-furfurylthiol and the concentrations of the putative thiol-receptive di- and trihydroxybenzenes pyrogallol (1), hydroxyhydroquinone (2), catechol (3), 4-ethylcatechol (4), 4-methylcatechol (5), and 3-methylcatechol (6), as well as of the phenol/thiol conjugates 3-[(2-furylmethyl)sulfanyl]catechol (7), 3-[(2-furylmethyl)sulfanyl]-5-ethylcatechol (8), 4-[(2-furylmethyl)sulfanyl]hydroxyhydroquinone (9), and 3,4-bis[(2-furylmethyl)sulfanyl]hydroxyhydroquinone (10) were quantitatively determined in fresh and stored coffee beverages by means of stable isotope dilution analyses (SIDA). Although 2 was found to be the quantitatively predominant trihydroxybenzene in freshly prepared coffee brew, this compound exhibited a very high reactivity and decreased rapidly during coffee storage to generate the conjugates 9 and 10. After only 10 min, about 60% of the initial amount of 2-furfurylthiol in a coffee beverage reacted with 2 to give 9 and 10. In contrast, conjugate 7 was found to be exclusively formed during coffee roasting because its initial concentration as well as the amount of its putative precursor, phenol 3, was not affected by storage. It is interesting to note that the concentration of 8 was increased with increasing incubation time, but its putative precursor 4 was not affected, thus indicating another formation pathway most likely via the chlorogenic acid degradation product 4-vinylcatechol. This study demonstrates for the first time that the loss of 2-furfurylthiol during coffee storage is mainly due to the oxidative coupling of the odorant to hydroxyhydroquinone (2), giving rise to the conjugates 9 and 10.	THB was found to be the quantitatively predominant trihydroxybenzene in freshly prepared coffee brew, this compound exhibited a very high reactivity and decreased rapidly during coffee storage to generate the conjugates	THB Food/Beverage/Nature	
105	Müller C, Lang R, Hofmann T.	Quantitative precursor studies on di- and trihydroxybenzene formation during coffee roasting using "in bean" model experiments and stable isotope dilution analysis.	J Agric Food Chem. 2006 Dec 27;54(26):10086-91.	The objective of this study was to investigate the potential of various raw bean components as precursors of pyrogallol (1), hydroxyhydroquinone (2), catechol (3), 4-ethylcatechol (4), 4-methylcatechol (5), and 3-methylcatechol (6) under quasi "natural" roasting conditions by using the recently developed "in bean" model roast experiments. Freeze-dried, fully extracted bean shells were loaded with aqueous solutions of either single coffee compounds or fractions isolated from the raw bean solubles. After freeze-drying, these reconstituted beans were roasted, aqueous coffee brews were prepared, and the target phenols were quantified by means of a stable isotope dilution assay with LC-MS/MS detection. On the basis of the quantitative data, it can be concluded that upon coffee bean roasting, catechol (3) is primarily formed by degradation of caffeoylquinic acids from both the caffeic acid and the quinic acid moiety of the molecule, as well as from Maillard-type reactions from carbohydrates and amino acids. In contrast, pyrogallol (1) and hydroxyhydroquinone (2) are efficiently generated from carbohydrates and amino acids and, in addition, from free or chlorogenic acid bound quinic acid moieties. 4-Ethylcatechol (4) is exclusively generated upon thermal breakdown of caffeic acid moieties. 3-Methylcatechol (6) is formed primarily from the Maillard reactions and, to a minor extent, also from various phenolic precursors, whereas 4-methylcatechol (5) is produced in trace amounts only from all of the different precursors investigated. On the basis of this precursor study, reaction routes explaining the formation of the target phenols are proposed.	THB is generated from carbohydrates, CGA and amino acids in coffee roasting process	THB Food/Beverage/Nature	
106	Müller C, Hemmersbach S, Slot GV, Hofmann T.	Synthesis and structure determination of covalent conjugates formed from the sulfury-roasty-smelling 2-furfurylthiol and di- or trihydroxybenzenes and their identification in coffee brew.	J Agric Food Chem. 2006 Dec 27;54(26):10076-85.	Recent investigations demonstrated that the reaction of odor-active thiols such as 2-furfurylthiol with thermally generated chlorogenic acid degradation products is responsible for the rapid aroma staling of coffee beverages. To get a clear understanding of the molecular mechanisms underlying this aroma staling, the existence of putative phenol/thiol conjugates needs to be verified in coffee. The aim of the present study was therefore to synthesize such conjugates for use as reference substances for LC-MS screening of coffee. To achieve this, catechol, 3-methyl-, 4-methyl-, and 4-ethylcatechol, pyrogallol, hydroxyhydroquinone, 5-O-caffeoylquinic acid, and caffeic acid, respectively, were reacted with 2-furfurylthiol in the presence of iron(III) chloride and air oxygen. After purification, the structures of 25 phenol/thiol conjugates were identified by means of LC-MS/MS and 1D/2D NMR experiments. Using these compounds as reference materials, four conjugates, namely, 3-((2-furylmethyl)sulfanyl)catechol, 3-((2-furylmethyl)sulfanyl)-5-ethylcatechol, 4-((2-furylmethyl)sulfanyl)hydroxyhydroquinone, and 3,4-bis((2-furylmethyl)sulfanyl) hydroxyhydroquinone, were identified for the first time in coffee brew by means of HPLC-MS/MS(MRM). These findings clearly demonstrate catechol, 4-ethylcatechol, and hydroxyhydroquinone as the primary thiol trapping agents involved in the aroma staling of coffee beverages.	THB functions as a thiol trapping agent shown by reaction with 2-furfurylthiol in the presence of Lewis acid Ferric Chloride and air	THB Food/Beverage/Nature	
107	Suzuki A, Fujii A, Yamamoto N, Yamamoto M, Ohminami H, Kameyama A, Shibuya Y, Nishizawa Y, Tokimitsu I, Saito I.	Improvement of hypertension and vascular dysfunction by hydroxyhydroquinone-free coffee in a genetic model of hypertension.	FEBS Lett. 2006 Apr 17;580(9):2317-22. Epub 2006 Mar 24.	Chlorogenic acid, a polyphenol found in coffee, has antihypertensive actions, but epidemiologic data on the effects of coffee on blood pressure are controversial. Specific coffee components that inhibit the hypotensive effect of chlorogenic acid and the physiologic mechanisms underlying the effects of coffee without these components were investigated. One component, hydroxyhydroquinone (HHQ), inhibited the hypotensive effects of chlorogenic acid in spontaneously hypertensive rats (SHR). The attenuation of hypertension by HHQ-free coffee was associated with nitric oxide, the suppression of mRNA expression of NAD(P)H oxidase, and the improvement in endothelium-dependent vasodilation in the aorta. Thus, HHQ-free coffee might regulate vascular tone by improving the bioavailability of nitric oxide in SHR.	THB reduction in coffee resulted in decrease of antihypertensive effect of CGA	THB Food/Beverage/Nature	
108	Halliwell B, Long LH, Yee TP, Lim S, Kelly R.	Establishing biomarkers of oxidative stress: the measurement of hydrogen peroxide in human urine.	Curr Med Chem. 2004 May;11(9):1085-92.	Hydrogen peroxide (H(2)O(2)) can be detected in freshly-voided human urine from healthy subjects and has been proposed as a "biomarker" of oxidative stress. This paper summarizes our studies to examine the extent to which urinary H(2)O(2) measurement fulfils the criteria for the "ideal biomarker". Levels of H(2)O(2), standardised for creatinine, varied widely between subjects. In most subjects, levels also varied considerably when measurements were made at different times and on different days. A reproducible increase in urinary H(2)O(2) was detected in all subjects examined after drinking coffee, a beverage rich in H(2)O(2). By contrast, green tea decreased urinary H(2)O(2) levels. We conclude that the H(2)O(2) in coffee is not excreted into urine. Instead, hydroxyhydroquinone from coffee is absorbed, excreted and oxidises in urine to produce H(2)O(2). No other confounders of urinary H(2)O(2) have been identified to date. Work is underway to compare H(2)O(2) levels with variations in other biomarkers of oxidative damage, to test the possibility that there are daily or other periodic variations in oxidative damage rates.	Peroxide detected in fresh human urine owing to THB content in coffee	THB Food/Beverage/Nature	ROS formation
109	Akagawa M, Shigemitsu T, Suyama K.	Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions.	Biosci Biotechnol Biochem. 2003 Dec;67(12):2632-40.	To investigate the ability of the production of H(2)O(2) by polyphenols, we incubated various phenolic compounds and natural polyphenols under a quasi-physiological pH and temperature (pH 7.4, 37 degrees C), and then measured the formation of H(2)O(2) by the ferrous ion oxidation-xylenol orange assay. Pyrocatechol, hydroquinone, pyrogallol, 1,2,4-benzenetriol, and polyphenols such as catechins yielded a significant amount of H(2)O(2). We also examined the effects of a metal chelator, pH, and O(2) on the H(2)O(2)-generating property, and the generation of H(2)O(2) by the polyphenol-rich beverages, green tea, black tea, and coffee, was determined. The features of the H(2)O(2)-generating property of green tea, black tea, and coffee were in good agreement with that of phenolic compounds, suggesting that polyphenols are responsible for the generation of H(2)O(2) in beverages. From the results, the possible significances of the H(2)O(2)-generating property of polyphenols for biological systems are discussed	Peroxide formation by various polyphenols found in green tea, black tea and coffee was measured using the FOX Assay. THB was able to produce hydrogen peroxide in a pH dependent manner.	THB Food/Beverage/Nature	ROS formation
110	Hiramoto K, Kida T, Kikugawa K.	Increased urinary hydrogen peroxide levels caused by coffee drinking.	Biol Pharm Bull. 2002 Nov;25(11):1467-71.	Experiments with volunteers in Singapore have demonstrated that coffee drinking increases urinary hydrogen peroxide levels (Long, Halliwell, Free Rad. Res., 32, 463-467 (2000)). We re-examined the effect of coffee drinking of healthy Japanese subjects on urinary hydrogen peroxide levels. A cup of brewed or canned coffee commercially available in Japan generated 120-420 micro mol hydrogen peroxide in incubation in a neutral medium at 37 degrees C for 6 h. The increased levels were higher than those obtained from a cup of green tea extract or a glass of red wine. After the subject drank a cup of coffee, apparent hydrogen peroxide levels (micro mol/g creatinine) in urine collected 1-2 h after coffee drinking increased 3-10-fold compared to the levels before coffee drinking. The increased urinary hydrogen peroxide levels are likely derived mainly from 1,2,4-benzenetriol excreted in urine, because the major component that generates hydrogen peroxide is found to be 1,2,4-benzenetriol, and storing urine collected after coffee drinking increased hydrogen peroxide levels in a time-dependent fashion. Total hydrogen peroxide equivalent levels excreted in 3 h-urine after coffee drinking were estimated to be 0.5-10% that of coffee consumed. A residual amount of hydrogen peroxide may be retained or consumed in human bodies.	The increased urinary hydrogen peroxide levels are likely derived mainly from 1,2,4-benzenetriol excreted in urine, because the major component that generates hydrogen peroxide is found to be 1,2,4-benzenetriol, and storing urine collected after coffee drinking increased hydrogen peroxide levels in a time-dependent fashion.	THB Food/Beverage/Nature	ROS formation

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
111	Mi H, Hiramoto K, Kujirai K, Ando K, Ikarashi Y, Kikugawa K.	Effect of food reductones, 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF) and hydroxyhydroquinone (HHQ), on lipid peroxidation and type IV and I allergy responses of mouse.	J Agric Food Chem. 2001 Oct;49(10):4950-5.	The effect of long-term supplementation of food reductones, 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF) (2%, w/w), detected in many foodstuffs including soy sauce, and hydroxyhydroquinone (1,2,4-benzenetriol) (HHQ) (1.2%, w/w), detected in coffee, on mouse lipid peroxidation and type IV and I allergy responses was investigated. The effect of supplementation of these reductones combined with NO(2) inhalation (5-6 ppm) was also investigated. Levels of thiobarbituric acid-reactive substances in lung were remarkably increased, and those in kidney and liver were slightly decreased by supplementation of DMHF or HHQ. The degree of 2,4-dinitrochlorobenzene (DNCB)-sensitized lymph node cell proliferation as assessed by lymph node assay was remarkably enhanced by supplementation of DMHF or HHQ. Both the DNCB-sensitized and the trimellitic anhydride-sensitized increases in IgE levels of mice were enhanced to greater extent by supplementation of DMHF or HHQ. In no cases were additive effects of NO(2) inhalation observable. Allergen-sensitized type IV and I allergy responses of mice may be enhanced by supplementation of food reductones, DMHF or HHQ.	Reductone Supplementation of THB and substituted furanone in food showed an increase in IgE in DNCB-sensitized and trimetallic anhydrife sensitized mice combination with nitrous oxide inhalation did not show an additive effect.	THB Food/Beverage/Nature	
112	du Plessis J, Pugh WJ, Judefeind A, Hadgraft J.	Physico-chemical determinants of dermal drug delivery: effects of the number and substitution pattern of polar groups.	Eur J Pharm Sci. 2002 Aug;16(3):107-12.	The aim of this study was to investigate the effect of number and substitution pattern of -OH groups of a set of phenols on the in vitro permeation of heat-separated human epidermis. The diffusion was calculated from Log(D/x)=logk(p)-0.59logK(oct)+0.024 (D, diffusion coefficient; x, pathlength; k(p), permeability coefficient (cm/h); and K(oct), octanol-water partition coefficient). The main factors reducing D were the dipolar and hydrogen bonding capabilities of the permeants quantified as their Hansen partial solubility parameters delta(p) and delta(h). These parameters are significantly reduced by the degree of symmetry of the molecule, so that phloroglucinol (1,3,5-benzenetriol), with three -OH groups, diffuses more rapidly that phenol. When symmetry is absent, as in 1,2,4-benzenetriol, the number of -OH groups results in very slow diffusion. D/x (cm/h) was related to the combined solubility parameter delta(a) defined as radical(delta(p)(2)+delta(h)(2)) by: (D/x)=0.0024-0.000065delta(a) (n=7, R(2)=0.70, P=0.012).	Effect of hydroxyl substitution on dermal penertration. When symmetry is absent as in THB diffusion across epidermis is very slow.	Dermal Absorption	
113	Min J, Wang J, Chen W, Hu X.	Biodegradation of 2-chloro-4-nitrophenol via a hydroxyquinol pathway by a Gram-negative bacterium, Cupriavidus sp. strain CNP-8.	AMB Express. 2018 Mar 20;8(1):43. doi: 10.1186/s13568-018-0574-7.	Cupriavidus sp. strain CNP-8 isolated from a pesticide-contaminated soil was able to utilize 2-chloro-4-nitrophenol (2C4NP) as a sole source of carbon, nitrogen and energy, together with the release of nitrite and chloride ions. It could degrade 2C4NP at temperatures from 20 to 40 °C and at pH values from 5 to 10, and degrade 2C4NP as high as 1.6 mM. Kinetics assay showed that biodegradation of 2C4NP followed Haldane substrate inhibition model, with the maximum specific growth rate (μmax) of 0.148/h, half saturation constant (Ks) of 0.022 mM and substrate inhibition constant (Ki) of 0.72 mM. Strain CNP-8 was proposed to degrade 2C4NP with hydroxyquinol (1,2,4-benzenetriol, BT) as the ring-cleavage substrate. The 2C4NP catabolic pathway in strain CNP-8 is significant from those reported in other Gram-negative 2C4NP utilizers. Enzymatic assay indicated that the monooxygenase initiating 2C4NP catabolism had different substrates specificity compared with previously reported 2C4NP monooxygenations. Capillary assays showed that strain CNP-8 exhibited metabolism-dependent chemotactic response toward 2C4NP at the optimum concentration of 0.5 mM with a maximum chemotaxis index of 37.5. Furthermore, microcosm studies demonstrated that strain CNP-8, especially the pre-induced cells, could remove 2C4NP rapidly from the 2C4NP-contaminated soil. Considering its adaptability to pH and temperature fluctuations and great degradation efficiency against 2C4NP, strain CNP-8 could be a promising candidate for the bioremediation of 2C4NP-contaminated sites.	Bacterial biodegradation of nitrophenols with THB as a substrate	Degradation Pathway	
114	Zhao L, Huang Y, Keller AA.	Comparative Metabolic Response between Cucumber (Cucumis sativus) and Corn (Zea mays) to a Cu(OH)2 Nanopesticide.	J Agric Food Chem. 2018 Jul 5;66(26):6628-6636. doi: 10.1021/acs.jafc.7b01306. Epub 2017 May 17.	Due to their unique properties, copper-based nanopesticides are emerging in the market. Thus, understanding their effect on crop plants is very important. Metabolomics can capture a snapshot of cellular metabolic responses to a stressor. We selected maize and cucumber as model plants for exposure to different doses of Cu(OH)2 nanopesticide. GC-TOF-MS-based metabolomics was employed to determine the metabolic responses of these two species. Results revealed significant differences in metabolite profile changes between maize and cucumber. Furthermore, the Cu(OH)2 nanopesticide induced metabolic reprogramming in both species, but in different manners. In maize, several intermediate metabolites of the glycolysis pathway and tricarboxylic acid cycle (TCA) were up-regulated, indicating the energy metabolism was activated. In addition, the levels of aromatic compounds (4-hydroxycinnamic acid and 1,2,4-benzenetriol) and their precursors (phenylalanine, tyrosine) were enhanced, indicating the activation of shikimate-phenylpropanoid biosynthesis in maize leaves, which is an antioxidant defense-related pathway. In cucumber, arginine and proline metabolic pathways were the most significantly altered pathway. Both species exhibited altered levels of fatty acids and polysaccharides, suggesting the cell membrane and cell wall composition may change in response to Cu(OH)2 nanopesticide. Thus, metabolomics helps to deeply understand the differential response of these plants to the same nanopesticide stressor.	THB (and others) levels enhanced by exposure to Copper nanopesticides indicating activation of shikimate biosynthesis pathway	Degradation Pathway	
115	Pacheco-Sánchez D, Molina-Fuentes Á, Marín P, Medina-Bellver JI, González-López Ó, Marqués S.	The Azoarcus anaerobius 1,3-Dihydroxybenzene (Resorcinol) Anaerobic Degradation Pathway Is Controlled by the Coordinated Activity of Two Enhancer-Binding Proteins.	Appl Environ Microbiol. 2017 Apr 17;83(9). pii: e03042-16. doi: 10.1128/AEM.03042-16. Print 2017 May 1.	The anaerobic resorcinol degradation pathway in Azoarcus anaerobius is unique in that it uses an oxidative rather than a reductive strategy to overcome the aromatic ring stability in degradation of this compound, in a process that is dependent on nitrate respiration. We show that the pathway is organized in five transcriptional units, three of which are inducible by the presence of the substrate. Three σ54-dependent promoters located upstream from the three operons coding for the main pathway enzymes were identified, which shared a similar structure with conserved upstream activating sequences (UASs) located at 103 to 111 bp from the transcription start site. Expression of the pathway is controlled by the bacterial enhancer-binding proteins (bEBPs) RedR1 and RedR2, two homologous regulators that, despite their high sequence identity (97%), have nonredundant functions: RedR2, the master regulator which also controls RedR1 expression, is itself able to promote transcription from two of the promoters, while RedR1 activity is strictly dependent on the presence of RedR2. The two regulators were shown to interact with each other, suggesting that the natural mode of activation is by forming heterodimers, which become active in the presence of the substrate after its metabolization to hydroxybenzoquinone through the pathway enzymes. The model structure of the N-terminal domain of the proteins is composed of tandem GAF and PAS motifs; the possible mechanisms controlling the activity of the regulators are discussed.IMPORTANCEAzoarcus anaerobius is a strict anaerobe that is able to use 1,3-dihydroxybenzene as the sole carbon source in a process that is dependent on nitrate respiration. We have shown that expression of the pathway is controlled by two regulators of almost identical sequences: the bEBPs RedR1 and RedR2, which share 97% identity. These regulators control three promoters with similar structure. Despite their sequence identity, the two bEBPs are not redundant and are both required for maximum pathway expression. In fact, the two proteins function as heterodimers and require activation by the pathway intermediate hydroxyhydroquinone. The structure of the domain sensing the activation signal resembles that of regulators that are known to interact with other proteins.	Bacterial biodegradation of resorcinol	Degradation pathway	
116	Min J, Zhang JJ, Zhou NY.	A Two-Component para-Nitrophenol Monooxygenase Initiates a Novel 2-Chloro-4-Nitrophenol Catabolism Pathway in Rhodococcus imtechensis RKJ300.	Appl Environ Microbiol. 2015 Nov 13;82(2):714-23. doi: 10.1128/AEM.03042-15. Print 2016 Jan 15.	Rhodococcus imtechensis RKJ300 (DSM 45091) grows on 2-chloro-4-nitrophenol (2C4NP) and para-nitrophenol (PNP) as the sole carbon and nitrogen sources. In this study, by genetic and biochemical analyses, a novel 2C4NP catabolic pathway different from those of all other 2C4NP utilizers was identified with hydroxyquinol (hydroxy-1,4-hydroquinone or 1,2,4-benzenetriol [BT]) as the ring cleavage substrate. Real-time quantitative PCR analysis indicated that the pnp cluster located in three operons is likely involved in the catabolism of both 2C4NP and PNP. The oxygenase component (PnpA1) and reductase component (PnpA2) of the two-component PNP monooxygenase were expressed and purified to homogeneity, respectively. The identification of chlorohydroquinone (CHQ) and BT during 2C4NP degradation catalyzed by PnpA1A2 indicated that PnpA1A2 catalyzes the sequential denitration and dechlorination of 2C4NP to BT and catalyzes the conversion of PNP to BT. Genetic analyses revealed that pnpA1 plays an essential role in both 2C4NP and PNP degradations by gene knockout and complementation. In addition to catalyzing the oxidation of CHQ to BT, PnpA1A2 was also found to be able to catalyze the hydroxylation of hydroquinone (HQ) to BT, revealing the probable fate of HQ that remains unclear in PNP catabolism by Gram-positive bacteria. This study fills a gap in our knowledge of the 2C4NP degradation mechanism in Gram-positive bacteria and also enhances our understanding of the genetic and biochemical diversity of 2C4NP catabolism.	Degradation pathway of nirtophenols	Degradation Pathway	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
117	Molina-Fuentes Á, Pacheco D, Marín P, Philipp B, Schink B, Marqués S.	Identification of the Gene Cluster for the Anaerobic Degradation of 3,5-Dihydroxybenzoate (α-Resorcylate) in Thauera aromatica Strain AR-1.	Appl Environ Microbiol. 2015 Oct;81(20):7201-14. doi: 10.1128/AEM.01698-15. Epub 2015 Aug 7.	Thauera aromatica strain AR-1 degrades 3,5-dihydroxybenzoate (3,5-DHB) with nitrate as an electron acceptor. Previous biochemical studies have shown that this strain converts 3,5-DHB to hydroxyhydroquinone (1,2,4-trihydroxybenzene) through water-dependent hydroxylation of the aromatic ring and subsequent decarboxylation, and they suggest a pathway homologous to that described for the anaerobic degradation of 1,3-dihydroxybenzene (resorcinol) by Azoarcus anaerobius. Southern hybridization of a T. aromatica strain AR-1 gene library identified a 25-kb chromosome region based on its homology with A. anaerobius main pathway genes. Sequence analysis defined 20 open reading frames. Knockout mutations of the most relevant genes in the pathway were generated by reverse genetics. Physiological and biochemical analyses identified the genes for the three main steps in the pathway which were homologous to those described in A. anaerobius and suggested the function of several auxiliary genes possibly involved in enzyme maturation and intermediate stabilization. However, T. aromatica strain AR-1 had an additional enzyme to metabolize hydroxyhydroquinone, a putative cytoplasmic quinone oxidoreductase. In addition, a specific tripartite ATP-independent periplasmic (TRAP) transport system was required for efficient growth on 3,5-DHB. Reverse transcription-PCR (RT-PCR) analysis showed that the pathway genes were organized in five 3,5-DHB-inducible operons, three of which have been shown to be under the control of a single LysR-type transcriptional regulator, DbdR. Despite sequence homology, the genetic organizations of the clusters in T. aromatica strain AR-1 and A. anaerobius differed substantially.	Bacterial degradtion pathway of resorcinol	Degradation Pathway	
118	Chen XJ, Dai YZ, Wang XY, Guo J, Liu TH, Li FF.	Synthesis and characterization of Ag3PO4 immobilized with graphene oxide (GO) for enhanced photocatalytic activity and stability over 2,4-dichlorophenol under visible light irradiation.	J Hazard Mater. 2015 Jul 15;292:9-18. doi: 10.1016/j.jhazmat.2015.01.032. Epub 2015 Jan 13.	A series of visible-light responsive photocatalysts prepared using Ag3PO4 immobilized with graphene oxide (GO) with varying GO content were obtained by an electrostatically driven method, and 2,4-dichlorophenol (2,4-DCP) was used to evaluate the performance of the photocatalysts. The composites exhibited superior photocatalytic activity and stability compared with pure Ag3PO4. When the content of GO was 5%, the degradation efficiency of 2,4-DCP could reach 98.95%, and 55.91% of the total organic (TOC) content was removed within 60 min irradiation. Meanwhile, the efficiency of 91.77% was achieved for 2,4-DCP degradation even after four times of recycling in the photocatalysis/Ag3PO4-GO (5%) system. Reactive species of O2(˙-), OH˙ and h(+) were considered as the main participants for oxidizing 2,4-DCP, as confirmed by the free radical capture experiments. And some organic intermediates including 4-chlorophenol (4-CP), hydroquinone (HQ), benzoquinone (BZQ), 2-chlorohydroquinone and hydroxyhydroquinone (HHQ) were detected by comparison with the standard retention times from the high performance liquid chromatography (HPLC). In short, the enhanced photocatalytic property of Ag3PO4-GO was closely related to the strong absorption ability of GO relative to 2,4-DCP, the effective separation of photogenerated electron-hole pairs, and the excellent electron capture capability of GO.	Detection of THB in the photocatalyzed degradation of dichlorophenol	Degradation Pathway	
119	Gérecová G, Neboháčová M, Zeman I, Pryszcz LP, Tomáška Ľ, Gabaldón T, Nosek J.	Metabolic gene clusters encoding the enzymes of two branches of the 3-oxoadipate pathway in the pathogenic yeast Candida albicans.	FEMS Yeast Res. 2015 May;15(3). pii: fov006. doi: 10.1093/femsyr/fov006. Epub 2015 Mar 4.	The pathogenic yeast Candida albicans utilizes hydroxyderivatives of benzene via the catechol and hydroxyhydroquinone branches of the 3-oxoadipate pathway. The genetic basis and evolutionary origin of this catabolic pathway in yeasts are unknown. In this study, we identified C. albicans genes encoding the enzymes involved in the degradation of hydroxybenzenes. We found that the genes coding for core components of the 3-oxoadipate pathway are arranged into two metabolic gene clusters. Our results demonstrate that C. albicans cells cultivated in media containing hydroxybenzene substrates highly induce the transcription of these genes as well as the corresponding enzymatic activities. We also found that C. albicans cells assimilating hydroxybenzenes cope with the oxidative stress by upregulation of cellular antioxidant systems such as alternative oxidase and catalase. Moreover, we investigated the evolution of the enzymes encoded by these clusters and found that most of them share a particularly sparse phylogenetic distribution among Saccharomycotina, which is likely to have been caused by extensive gene loss. We exploited this fact to find co-evolving proteins that are suitable candidates for the missing enzymes of the pathway.	Cellular response to oxidative stress is upregulation of cellular antioxidant systems (catalase).	Degradation Pathway	
120	Yang H, An B, Wang S, Li L, Jin W, Li L.	Destruction of 4-phenolsulfonic acid in water by anodic contact glow discharge electrolysis.	J Environ Sci (China). 2013 Jun 1;25(6):1063-70.	Destruction of 4-phenolsulfonic acid (4-PSA) in water was carried out using anodic contact glow discharge electrolysis. Accompanying the decay of 4-PSA, the amount of total organic carbon (TOC) in water correspondingly decreased, while the sulfonate group of 4-PSA was released as sulfate ion. Oxalate and formate were obtained as minor by-products. Additionally, phenol, 1,4-hydroquinone, hydroxyquinol and 1,4-benzoquinone were detected as primary intermediates in the initial stages of decomposition of 4-PSA. A reaction pathway involving successive attacks of hydroxyl and hydrogen radicals was assumed on the basis of the observed products and kinetics. It was revealed that the decay of both 4-PSA and TOC obeyed a first-order rate law. The effects of different Fe ions and initial concentrations of 4-PSA on the degradation rate were investigated. It was found that the presence of Fe ions could increase the degradation rate of 4-PSA, while initial concentrations lower than 80 mmol/L had no significant effect on kinetic behaviour. The disappearance rate of 4-PSA was significantly affected by pH.	THB detected as intermediate in degradation of phenolsulfonic acid	Degradation Pathway	
121	Hayes RP, Lewis KM, Xun L, Kang C.	Catalytic mechanism of 5-chlorohydroxyhydroquinone dehydrochlorinase from the YCII superfamily of largely unknown function.	J Biol Chem. 2013 Oct 4;288(40):28447-56. doi: 10.1074/jbc.M113.499368. Epub 2013 Aug 16.	TftG, 5-chloro-2-hydroxyhydroquinone (5-CHQ) dehydrochlorinase, is involved in the biodegradation of 2,4,5-trichlorophenoxyacetate by Burkholderia phenoliruptrix AC1100. It belongs to the YCII superfamily, a group of proteins with largely unknown function. In this work, we utilized structural and functional studies, including the apo-form and 2,5-dihydroxybenzoquinone binary complex crystal structures, computational analysis, and site-directed mutagenesis, to determine the dehydrochlorination mechanism. The His-Asp dyad, which initiates catalysis, is strongly conserved in YCII-like proteins. In addition, other catalytically important residues such as Pro-76, which orients the His-Asp catalytic dyad; Arg-17 and Ser-56, which form an oxyanion hole; and Asp-9, which stabilizes the oxyanion hole, are among the most highly conserved residues across the YCII superfamily members. The comprehensive characterization of TftG helps not only for identifying effective mechanisms for chloroaromatic dechlorination but also for understanding the functions of YCII superfamily members, which we propose to be lyases.	Bacterial metabolism of 5-chloro-HHQ	Degradation Pathway	
122	Philipp B, Schink B.	Different strategies in anaerobic biodegradation of aromatic compounds: nitrate reducers versus strict anaerobes.	Environ Microbiol Rep. 2012 Oct;4(5):469-78. doi: 10.1111/j.1758-2229.2011.00304.x. Epub 2011 Nov 27.	Mononuclear aromatic compounds are degraded anaerobically through pathways that are basically different from those used in the presence of oxygen. Whereas aerobic degradation destabilizes the aromatic π-electron system by oxidative steps through oxygenase reactions, anaerobic degradation is most often initiated by a reductive attack. The benzoyl-CoA pathway is the most important metabolic route in this context, and a broad variety of mononuclear aromatics, including phenol, cresols, toluene, xylenes and ethylbenzene, are channelled into this pathway through various modification reactions. Multifunctional phenolic compounds are metabolized via the reductive resorcinol pathway, the oxidative resorcinol pathway with hydroxyhydroquinone as key intermediate, and the phloroglucinol pathway. Comparison of the various pathways used for modification and degradation of aromatics in the absence of oxygen indicates that the strategies of breakdown of these compounds are largely determined by the redox potentials of the electron acceptors used, and by the overall reaction energetics. Consequently, nitrate reducers quite often use strategies for primary attack on aromatic compounds that differ from those used by sulfate-reducing, iron-reducing or fermenting bacteria.	Anaerobic degradation of aromatic compounds determined by redox potentials	Degradation Pathway	
123	Rabaaoui N, Saad Mel K, Moussaoui Y, Allagui MS, Bedoui A, Elaloui E.	Anodic oxidation of o-nitrophenol on BDD electrode: variable effects and mechanisms of degradation.	J Hazard Mater. 2013 Apr 15;250-251:447-53. doi: 10.1016/j.jhazmat.2013.02.027. Epub 2013 Feb 20.	The electrochemical oxidation of pesticide, o-nitrophenol (ONP) as one kind of pesticide that is potentially dangerous and biorefractory, was studied by galvanostatic electrolysis using boron-doped diamond (BDD) as anode. The influence of several operating parameters, such as applied current density, supporting electrolyte, and initial pH value, was investigated. The best degradation occurred in the presence of Na2SO4 (0.05 M) as conductive electrolyte. After 8h, nearly complete degradation of o-nitrophenol was achieved (92%) using BDD electrodes at pH 3 and at current density equals 60 mA cm(-2). The decay kinetics of o-nitrophenol follows a pseudo-first-order reaction. Aromatic intermediates such as catechol, resorcinol, 1,2,4-trihydroxybenzene, hydroquinone and benzoquinone and carboxylic acids such as maleic glycolic, malonic, glyoxilic and oxalic, have been identified and followed during the ONP treatment by chromatographic techniques. From these anodic oxidation by-products, a plausible reaction sequence for ONP mineralization on BDD anodes is proposed.	Degradation of o-nitrophenol involves hydroxybenzenes	Degradation Pathway	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
124	Li T, Zhao K, Huang Y, Li D, Jiang CY, Zhou N, Fan Z, Liu SJ.	The TetR-type transcriptional repressor RolR from Corynebacterium glutamicum regulates resorcinol catabolism by binding to a unique operator, rolO.	Appl Environ Microbiol. 2012 Sep;78(17):6009-16. doi: 10.1128/AEM.01304-12. Epub 2012 Jun 15.	The rol (designated for resorcinol) gene cluster rolRHMD is involved in resorcinol catabolism in Corynebacterium glutamicum, and RolR is the TetR-type regulator. In this study, we investigated how RolR regulated the transcription of the rol genes in C. glutamicum. The transcription start sites and promoters of rolR and rolHMD were identified. Quantitative reverse transcription-PCR and promoter activity analysis indicated that RolR negatively regulated the transcription of rolHMD and of its own gene. Further, a 29-bp operator rolO was located at the intergenic region of rolR and rolHMD and was identified as the sole binding site for RolR. It contained two overlapping inverted repeats and they were essential for RolR-binding. The binding of RolR to rolO was affected by resorcinol and hydroxyquinol, which are the starting compounds of resorcinol catabolic pathway. These two compounds were able to dissociate RolR-rolO complex, thus releasing RolR from the complex and derepressing the transcription of rol genes in C. glutamicum. It is proposed that the binding of RolR to its operator rolO blocks the transcription of rolHMD and of its own gene, thus negatively regulated resorcinol degradation in C. glutamicum.	Role of THB in resorcinol catabolism	Degradation Pathway	
125	Wu XR, Xue M, Li XF, Wang Y, Wang J, Han QL, Yi ZC.	Phenolic metabolites of benzene inhibited the erythroid differentiation of K562 cells.	Toxicol Lett. 2011 Jun 24;203(3):190-9. doi: 10.1016/j.toxlet.2011.03.012. Epub 2011 Mar 23.	Benzene is a common occupational hazard and a ubiquitous environmental pollutant. Benzene exposure at the levels even below 1ppm still showed hematotoxicity. It is widely accepted that the metabolites of benzene play important roles in the benzene toxicity to the hematopoietic system, but little is known about the effects of benzene metabolites on erythropoiesis. In present study, erythroid progenitor-like K562 cells were used to determine the effects of phenolic metabolites of benzene, including phenol, hydroquinone and 1,2,4-benzenetriol, on the erythroid differentiation. After the treatment with these benzene metabolites at the concentrations with no obvious cytotoxicity, the hemin-induced hemoglobin synthesis in K562 cells decreased in a concentration- and time-dependent manner, and the expression of CD71 and GPA protein on the surface of K562 cells was also inhibited. The reverse transcription-PCR was used to determine the mRNA level of the erythroid related genes in the K562 cells that were treated with benzene metabolites. The hemin-induced expression of globin genes, including α-, β- and γ-globin genes, and the gene encoding the heme synthesis enzyme porphobilinogen deaminase was inhibited by benzene metabolites. When the K562 cells were pretreated with benzene metabolites, the hemin-induced expression of two transcription factor genes GATA-1 and NF-E2 was distinctly reduced, and the pre-treatment with benzene metabolites promoted the decrease of the mRNA level of transcription factor gene GATA-2 by hemin. These results indicated that benzene metabolites inhibited the hemin-induced erythroid differentiation through affecting the transcription of the erythroid related genes.	Benzene metabolites inhibit erythroid differentiation	THB Toxicity	
126	Yamamoto K, Nishimura M, Kato D, Takeo M, Negoro S.	Identification and characterization of another 4-nitrophenol degradation gene cluster, nps, in Rhodococcus sp. strain PN1.	J Biosci Bioeng. 2011 Jun;111(6):687-94. doi: 10.1016/j.jbiosc.2011.01.016. Epub 2011 Mar 10.	4-Nitrophenol (4-NP) is a toxic compound formed in soil by the hydrolysis of organophosphorous pesticides, such as parathion. We previously reported the presence of the 4-NP degradation gene cluster (nphRA1A2) in Rhodococcus sp. strain PN1, which encodes a two-component 4-NP hydroxylase system that oxidizes 4-NP into 4-nitrocatechol. In the current study, another gene cluster (npsC and npsRA2A1B) encoding a similar 4-NP hydroxylase system was cloned from strain PN1. The enzymes from this 4-NP hydroxylase system (NpsA1 and NpsA2) were purified as histidine-tagged (His-) proteins and then characterized. His-NpsA2 showed NADH/FAD oxidoreductase activity, and His-NpsA1 showed 4-NP oxidizing activity in the presence of His-NpsA2. In the 4-NP oxidation using the reconstituted enzyme system (His-NpsA1 and His-NpsA2), hydroquinone (35% of 4-NP disappeared) and hydroxyquinol (59% of 4-NP disappeared) were detected in the presence of ascorbic acid as a reducing reagent, suggesting that, without the reducing reagent, 4-NP was converted into their oxidized forms, 1,4-benzoquinone and 2-hydroxy-1,4-benzoquinone. In addition, in the cell extract of recombinant Escherichia coli expressing npsB, a typical spectral change showing conversion of hydroxyquinol into maleylacetate was observed. These results indicate that this nps gene cluster, in addition to the nph gene cluster, is also involved in 4-NP degradation in strain PN1.	THB detected in degradation of pNP via NP hydroxylase	Degradation Pathway	
127	Liu Y, Wang D, Sun B, Zhu X.	Aqueous 4-nitrophenol decomposition and hydrogen peroxide formation induced by contact glow discharge electrolysis.	J Hazard Mater. 2010 Sep 15;181(1-3):1010-5. doi: 10.1016/j.jhazmat.2010.05.115. Epub 2010 Jun 1.	Liquid-phase decomposition of 4-nitrophenol (4-NP) and formation of hydrogen peroxide (H(2)O(2)) induced by contact glow discharge electrolysis (CGDE) were investigated. Experimental results showed that the decays of 4-NP and total organic carbon (TOC) obeyed the first-order and pseudo-first-order reaction kinetics, respectively. The major intermediate products were 4-nitrocatechol, hydroquinone, benzoquinone, hydroxyhydroquinone, organic acids and nitrite ion. The final products were carbon dioxide and nitrate ion. The initial formation rate of H(2)O(2) decreased linearly with increasing initial concentration of 4-NP. Addition of iron ions, especially ferric ion, to the solution significantly enhanced the 4-NP removal due to the additional hydroxyl radical formation through Fenton's reaction. A reaction pathway is proposed based on the degradation kinetics and the distribution of intermediate products.	THB etc detected in degradation of pNPand formation of peroxide	Degradation Pathway	
128	Wei M, Zhang JJ, Liu H, Zhou NY.	para-Nitrophenol 4-monooxygenase and hydroxyquinol 1,2-dioxygenase catalyze sequential transformation of 4-nitrocatechol in Pseudomonas sp. strain WBC-3.	Biodegradation. 2010 Nov;21(6):915-21. doi: 10.1007/s10532-010-9351-2. Epub 2010 Apr 2.	Pseudomonas sp. strain WBC-3 utilizes para-nitrophenol (PNP) as a sole source of carbon, nitrogen and energy. PnpA (PNP 4-monooxygenase) and PnpB (para-benzoquinone reductase) were shown to be involved in the initial steps of PNP catabolism via hydroquinone. We demonstrated here that PnpA also catalyzed monooxygenation of 4-nitrocatechol (4-NC) to hydroxyquinol, probably via hydroxyquinone. It was the first time that a single-component PNP monooxygenase has been shown to catalyze this conversion. PnpG encoded by a gene located in the PNP degradation cluster was purified as a His-tagged protein and identified as a hydroxyquinol dioxygenase catalyzing a ring-cleavage reaction of hydroxyquinol. Although all the genes necessary for 4-NC metabolism seemed to be present in the PNP degradation cluster in strain WBC-3, it was unable to grow on 4-NC as a sole source of carbon, nitrogen and energy. This was apparently due to the substrate's inability to trigger the expression of genes involved in degradation. Nevertheless, strain WBC-3 could completely degrade both PNP and 4-NC when PNP was used as the inducer, demonstrating its potential in bioremediation of the environment polluted by both 4-NC and PNP.	Bacterial degradation of pNP via THB intermediate	Degradation Pathway	
129	Chauhan A, Pandey G, Sharma NK, Paul D, Pandey J, Jain RK.	p-Nitrophenol degradation via 4-nitrocatechol in Burkholderia sp. SJ98 and cloning of some of the lower pathway genes.	Environ Sci Technol. 2010 May 1;44(9):3435-41. doi: 10.1021/es9024172.	Microbial degradation studies have pointed toward the occurrence of two distinct PNP catabolic pathways in Gram positive and Gram negative bacteria. The former involves 4-nitrocatechol (4-NC), 1,2,4-benzenetriol (BT), and maleylacetate (MA) as major degradation intermediates, whereas the later proceeds via formation of 1,4-benzoquinone (BQ) and hydroquinone (HQ). In the present study we identified a Gram negative organism viz. Burkholderia sp. strain SJ98 that degrades PNP via 4NC, BT, and MA. A 6.89 Kb genomic DNA fragment of strain SJ98 that encompasses seven putatively identified ORFs (orfA, pnpD, pnpC, orfB, orfC, orfD, and orfE) was cloned. PnpC is benzenetriol dioxygenase belonging to the intradiol dioxygenase superfamily, whereas PnpD is identified as maleylacetate reductase, a member of the Fe-ADH superfamily showing NADH dependent reductase activity. The in vitro activity assays carried out with purified pnpC and pnpD (btd and mar) gene products transformed BT to MA and MA to beta-ketoadipate, respectively. The cloning, sequencing, and characterization of these genes along with the functional PNP degradation studies ascertained the involvement of 4-NC, BT, and MA as degradation intermediates of PNP pathway in this strain. This is one of the first conclusive reports for 4-NC and BT mediated degradation of PNP in a Gram negative organism.	Bacterial degradation of pNP via THB intermediate	Degradation Pathway	
130	Zheng Y, Liu D, Liu S, Xu S, Yuan Y, Xiong L.	Kinetics and mechanisms of p-nitrophenol biodegradation by Pseudomonas aeruginosa HS-D38.	J Environ Sci (China). 2009;21(9):1194-9.	The kinetics and mechanisms of p-nitrophenol (PNP) biodegradation by Pseudomonas aeruginosa HS-D38 were investigated. PNP could be used by HS-D38 strain as the sole carbon, nitrogen and energy sources, and PNP was mineralized at the maximum concentration of 500 mg/L within 24 h in an mineral salt medium (MSM). The analytical results indicated that the biodegradation of PNP fit the first order kinetics model. The rate constant kPNP is 2.039 x 10(-2)/h in MSM medium, KPNP+N is 3.603 x 10(-2)/h with the addition of ammonium chloride and KPNP+C is 9.74 x 10(-3)/h with additional glucose. The addition of ammonium chloride increased the degradation of PNP. On the contrary, the addition of glucose inhibited and delayed the biodegradation of PNP. Chemical analysis results by thin-layer chromatography (TLC), UV-Vis spectroscopy and gas chromatography (GC) techniques suggested that PNP was converted to hydroquinone (HQ) and further degraded via 1,2,4-benzenetriol (1,2,4-BT) pathway.	Bacterial degradation of pNP via THB intermediate	Degradation Pathway	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
131	Zhong Q, Zhang H, Bai W, Li M, Li B, Qiu X.	Degradation of aromatic compounds and degradative pathway of 4-nitrocatechol by Ochrobactrum sp. B2.	J Environ Sci Health A Tox Hazard Subst Environ Eng. 2007 Dec;42(14):2111-6.	The potential capacity of a soil methyl parathion-degrading bacterium strain, Ochrobactrum sp. B2, for degrading various aromatic compounds were investigated. The results showed B2 was capable of degrading diverse aromatic compounds, but amino-substituted benzene compounds, at a concentration up to 100 mg L(-1) in 4 days. B2 could use 4-nitrocatechol (4-NC) as a sole carbon and energy source with release of nitrite ion. The pathway for 4-NC degradation via 1,2,4-benzenetriol (BT) and hydroquinone (HQ) formation in B2 was proposed based on the identification and quantification of intermediates by gas chromatography-mass spectrometry (GC-MS), and high performance liquid chromatography (HPLC). Degradation studies carried out on a plasmid-cured derivative showed that the genes for 4-NC degradative pathway was plasmid-borne in B2, suggesting that B2 degrades both p-nitrophenol and 4-NC by enzymes encoded by genes on the same plasmid.	Bacterial degradation of pNP via THB intermediate	Degradation pathway	
132	Darley PI, Hellstern JA, Medina-Bellver JI, Marqués S, Schink B, Philipp B.	Heterologous expression and identification of the genes involved in anaerobic degradation of 1,3-dihydroxybenzene (resorcinol) in Azoarcus anaerobius.	J Bacteriol. 2007 May;189(10):3824-33. Epub 2007 Mar 16.	Azoarcus anaerobius, a strictly anaerobic, gram-negative bacterium, utilizes resorcinol as a sole carbon and energy source with nitrate as an electron acceptor. Previously, we showed that resorcinol degradation by this bacterium is initiated by two oxidative steps, both catalyzed by membrane-associated enzymes that lead to the formation of hydroxyhydroquinone (HHQ; 1,2,4-benzenetriol) and 2-hydroxy-1,4-benzoquinone (HBQ). This study presents evidence for the further degradation of HBQ in cell extracts to form acetic and malic acids. To identify the A. anaerobius genes required for anaerobic resorcinol catabolism, a cosmid library with genomic DNA was constructed and transformed into the phylogenetically related species Thauera aromatica, which cannot grow with resorcinol. By heterologous complementation, a transconjugant was identified that gained the ability to metabolize resorcinol. Its cosmid, designated R(+), carries a 29.88-kb chromosomal DNA fragment containing 22 putative genes. In cell extracts of T. aromatica transconjugants, resorcinol was degraded to HHQ, HBQ, and acetate, suggesting that cosmid R(+) carried all of the genes necessary for resorcinol degradation. On the basis of the physiological characterization of T. aromatica transconjugants carrying transposon insertions in different genes of cosmid R(+), eight open reading frames were found to be essential for resorcinol mineralization. Resorcinol hydroxylase-encoding genes were assigned on the basis of sequence analysis and enzyme assays with two mutants. Putative genes for hydroxyhydroquinone dehydrogenase and enzymes involved in ring fission have also been proposed. This work provides the first example of the identification of genes involved in the anaerobic degradation of aromatic compounds by heterologous expression of a cosmid library in a phylogenetically related organism.	Anaerobic bacterial degration of resorcinol forms THB as an intermediate	Degradation pathway	
133	Travkin VM, Solyanikova IP, Golovleva LA.	Hydroxyquinol pathway for microbial degradation of halogenated aromatic compounds.	J Environ Sci Health B. 2006;41(8):1361-82. Review.	Several peripheral metabolic pathways can be used by microorganisms to degrade toxic aromatic compounds that are known to pollute the environment. Hydroxyquinol (1,2,4-trihydroxybenzene) is one of the central intermediates in the degradative pathway of a large variety of aromatic compounds. The present review describes the microorganisms involved in the degradative pathway, the key enzymes involved in the formation and splitting of the aromatic ring of (chloro)hydroxyquinol as well as the central intermediates formed. An attempt was also made to provide some estimation for genetic basis of the hydroxyquinol pathway.	THB identified as a primary intermediate in bacterial degradation pathway of aromatics	Degradation Pathway	
134	Pakala SB, Gorla P, Pinjari AB, Krovidi RK, Baru R, Yanamandra M, Merrick M, Siddavattam D.	Biodegradation of methyl parathion and p-nitrophenol: evidence for the presence of a p-nitrophenol 2-hydroxylase in a Gram-negative Serratia sp. strain DS001.	Appl Microbiol Biotechnol. 2007 Jan;73(6):1452-62. Epub 2006 Oct 17.	A soil bacterium capable of utilizing methyl parathion as sole carbon and energy source was isolated by selective enrichment on minimal medium containing methyl parathion. The strain was identified as belonging to the genus Serratia based on a phylogram constructed using the complete sequence of the 16S rRNA. Serratia sp. strain DS001 utilized methyl parathion, p-nitrophenol, 4-nitrocatechol, and 1,2,4-benzenetriol as sole carbon and energy sources but could not grow using hydroquinone as a source of carbon. p-Nitrophenol and dimethylthiophosphoric acid were found to be the major degradation products of methyl parathion. Growth on p-nitrophenol led to release of stoichiometric amounts of nitrite and to the formation of 4-nitrocatechol and benzenetriol. When these catabolic intermediates of p-nitrophenol were added to resting cells of Serratia sp. strain DS001 oxygen consumption was detected whereas no oxygen consumption was apparent when hydroquinone was added to the resting cells suggesting that it is not part of the p-nitrophenol degradation pathway. Key enzymes involved in degradation of methyl parathion and in conversion of p-nitrophenol to 4-nitrocatechol, namely parathion hydrolase and p-nitrophenol hydroxylase component "A" were detected in the proteomes of the methyl parathion and p-nitrophenol grown cultures, respectively. These studies report for the first time the existence of a p-nitrophenol hydroxylase component "A", typically found in Gram-positive bacteria, in a Gram-negative strain of the genus Serratia.	Bacteria utilize THB as carbon source from various sources including pNP catechol	Degradation Pathway	
135	Qiu XH, Bai WQ, Zhong QZ, Li M, He FQ, Li BT.	Isolation and characterization of a bacterial strain of the genus Ochrobactrum with methyl parathion mineralizing activity.	J Appl Microbiol. 2006 Nov;101(5):986-94.	AIMS: To isolate and characterize a methyl parathion (MP)-mineralizing bacterium, and to elucidate the degradative pathway of MP and localize the responsible degrading genes. METHODS AND RESULTS: A bacterial strain, designated B2, capable of mineralizing MP was isolated from the MP-polluted soil. Analysis of the 16S rRNA gene sequence and phenotypic analysis suggested that strain B2 had a close relationship with Ochrobactrum anthropi. B2 could totally degrade MP and four metabolites [p-nitrophenol (PNP), 4-nitrocatechol (4-NC), 1,2,4-benzenetriol (BT) and hydroquinone (HQ)] were identified by HPLC and gas chromatography-mass spectrometry analyses. Plasmid curing of strain B2 resulted in the loss of ability of B2 to degrade PNP, but not the ability to hydrolyse MP. CONCLUSIONS: Ochrobactrum sp. B2 can mineralize MP rapidly via PNP, 4-NC, BT and HQ pathway. B2 harbours a plasmid encoding the ability to degrade PNP, while MP-hydrolysing activity is encoded on the bacterial chromosome. SIGNIFICANCE AND IMPACT OF THE STUDY: This new bacterial strain (B2) capable of mineralizing MP will be useful in a pure-culture remediation process of organophosphate pesticides and their metabolites such as nitroaromatics.	Bacterial degradation of pNP via THB intermediate	Degradation Pathway	
136	Boll M.	Dearomatizing benzene ring reductases.	J Mol Microbiol Biotechnol. 2005;10(2-4):132-42. Review.	The high resonance energy of the benzene ring is responsible for the relative resistance of aromatic compounds to biodegradation. Nevertheless, bacteria from nearly all physiological groups have been isolated which utilize aromatic growth substrates as the sole source of cell carbon and energy. The enzymatic dearomatization of the benzene nucleus by microorganisms is accomplished in two different manners. In aerobic bacteria the aromatic ring is dearomatized by oxidation, catalyzed by oxygenases. In contrast, anaerobic bacteria attack the aromatic ring by reductive steps. Key intermediates in the anaerobic aromatic metabolism are benzoyl-CoA and compounds with at least two meta-positioned hydroxyl groups (resorcinol, phloroglucinol and hydroxyhydroquinone). In facultative anaerobes, the reductive dearomatization of the key intermediate benzoyl-CoA requires a stoichiometric coupling to ATP hydrolysis, whereas reduction of the other intermediates is readily achieved with suitable electron donors. Obligately anaerobic bacteria appear to use a totally different enzymology for the reductive dearomatization of benzoyl-CoA including selenocysteine- and molybdenum- containing enzymes.	THB is one of key intermediates in anaerobic bacterial degradation of aromatic carbon sources	Degradation Pathway	
137	Zhang L, Kanki T, Sano N, Toyoda A.	Pathways and kinetics on photocatalytic destruction of aqueous phenol.	Environ Monit Assess. 2006 Apr;115(1-3):395-403. Epub 2006 Apr 16.	In this study, the TiO2 photocatalytic decomposition process of aqueous phenol was investigated. The intermediate products generated in the elementary reaction steps in the mineralization process were experimentally identified as hydroquinone, catechol and hydroxyhydroquinone. The concentration variations of these intermediate products with time passage were traced by high performance liquid chromatograph. The pathways of the decomposition process were given. Based on Langmuir isothermal theory and Langmuir-Hinshelwood mechanism, the multi-compounds competition kinetic model was established. In this model, the observed time-dependent concentrations of phenol and the intermediate products were simulated.	THB one of intermediates in phenol degradation	Degradation Pathway	
138	Nordin K, Unell M, Jansson JK.	Novel 4-chlorophenol degradation gene cluster and degradation route via hydroxyquinol in Arthrobacter chlorophenolicus A6.	Appl Environ Microbiol. 2005 Nov;71(11):6538-44.	Arthrobacter chlorophenolicus A6, a previously described 4-chlorophenol-degrading strain, was found to degrade 4-chlorophenol via hydroxyquinol, which is a novel route for aerobic microbial degradation of this compound. In addition, 10 open reading frames exhibiting sequence similarity to genes encoding enzymes involved in chlorophenol degradation were cloned and designated part of a chlorophenol degradation gene cluster (cph genes). Several of the open reading frames appeared to encode enzymes with similar functions; these open reading frames included two genes, cphA-I and cphA-II, which were shown to encode functional hydroxyquinol 1,2-dioxygenases. Disruption of the cphA-I gene yielded a mutant that exhibited negligible growth on 4-chlorophenol, thereby linking the cph gene cluster to functional catabolism of 4-chlorophenol in A. chlorophenolicus A6. The presence of a resolvase pseudogene in the cph gene cluster together with analyses of the G+C content and codon bias of flanking genes suggested that horizontal gene transfer was involved in assembly of the gene cluster during evolution of the ability of the strain to grow on 4-chlorophenol.	Bacterial degradation pathway show THB as an intermediate in phenol	Degradation Pathway	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
139	Srokol Z, Bouche AG, van Estrik A, Strik RC, Maschmeyer T, Peters JA.	Hydrothermal upgrading of biomass to biofuel; studies on some monosaccharide model compounds.	Carbohydr Res. 2004 Jul 12;339(10):1717-26.	During the hydrothermal upgrading of biomass, hydrolysis to glucose is an important step. To elucidate some of the reaction pathways that follow this initial hydrolysis, the hydrothermal treatment (340 degrees C, 27.5 MPa, 25-204 s) of dilute (50 mM) solutions of D-glucose and some other monosaccharides were studied. As a result of the increase of Kw under subcritical conditions, both acid and base catalysed reactions occur. The acid catalysed reactions are mainly dehydrations leading initially to 5-hydroxymethylfurfural. Important base catalysed reactions result in glycolaldehyde and glyceraldehyde. Further fragmentations and dehydrations lead to a variety of low molecular weight compounds such as formic acid, acetic acid, lactic acid, acrylic acid, 2-furaldehyde and 1,2,4-benzenetriol. Important pathways leading to a decrease of the O-content of the liquid reaction products start from the intermediate glyceraldehyde, which forms pyruvaldehyde, which in its turn is converted into formic acid and acetaldehyde. The latter compound can also be formed via isomerisation of glyceraldehyde into lactic acid followed by decarbonylation.	Hydrothermal treatment of D-glucose investigated to show THB as one of reaction products	Degradation Pathway	
140	Solyanikova IP, Golovleva LA.	Bacterial degradation of chlorophenols: pathways, biochemica, and genetic aspects.	J Environ Sci Health B. 2004 May;39(3):333-51. Review.	Chlorophenols belong to the group of toxic and persistent to microbial attack xenobiotics. Nevertheless, due to the adaptation microorganisms acquire the ability to use chlorophenols as the sole source of carbon and energy. The present review describes the diversity of aerobic pathways for the utilization of halogenated phenols by bacteria with the emphasis on the main reactions and intermediates formed, enzymes responsible for these reactions and their genetic basis. Taking into account (i) the fact that enzymes degrading chlorophenols are similar to the ones involved in the conversion of other (chloro)aromatic compounds and (ii) that present numerous publications describing the properties of separated enzymes or encoding their genes are published, this review was planned as the attempt to present both, the most general and specific aspects in chlorophenols degradation with the emphasis on the literature of the last ten years.	Bacterial degradation of chlorophenol as a carbon source for some strains	Degradation Pathway	
141	Rao NN, Dubey AK, Mohanty S, Khare P, Jain R, Kaul SN.	Photocatalytic degradation of 2-chlorophenol: a study of kinetics, intermediates and biodegradability.	J Hazard Mater. 2003 Aug 1;101(3):301-14.	The kinetics of photocatalytic (TiO(2)/UV) degradation of 2-chlorophenol (2-CP), characterization of intermediates and induction of biodegradability in treated chlorophenol solutions is reported. Approximately 95% of the 2-CP is removed in approximately 2h at pH 5 and 0.2g TiO(2)l(-1) when the 2-CP concentration is < or =100mgl(-1); the pseudo-first-order rate constant (k) is estimated to be 0.0183 min(-1). GC-MS analyses detected phenol, catechol, hydroxyhydroquinone (HHQ), and chlorohydroquinone (CHQ) intermediates during the short irradiation time (<1h); however two other higher carbon intermediates 2-hydroxy-benzaldehyde (HB) and [1.1'-biphenyl]-2,2'-diol (BPD) are found as major intermediates over longer irradiation times. The biochemical oxygen demand (BOD) of treated 2-CP solutions improved substantially. A tentative mechanistic pathway to explain formation of higher carbon intermediates is presented.	Chlorophenol biodegradation shows THB as an intermediate in addition to catechol and HQ.	Degradation Pathway	
142	Vogna D, Marotta R, Napolitano A, D'Ischia M.	Advanced oxidation chemistry of paracetamol. UV/H(2)O(2)-induced hydroxylation/degradation pathways and (15)N-aided inventory of nitrogenous breakdown products.	J Org Chem. 2002 Aug 23;67(17):6143-51.	The advanced oxidation chemistry of the antipyretic drug paracetamol (1) with the UV/H(2)O(2) system was investigated by an integrated methodology based on (15)N-labeling and GC-MS, HPLC, and 2D (1)H, (13)C, and (15)N NMR analysis. Main degradation pathways derived from three hydroxylation steps, leading to 1,4-hydroquinone/1,4-benzoquinone, 4-acetylaminocatechol and, to a much lesser extent, 4-acetylaminoresorcine. Oxidation of the primary aromatic intermediates, viz. 4-acetylaminocatechol, 1,4-hydroquinone, 1,4-benzoquinone, and 1,2,4-benzenetriol, resulted in a series of nitrogenous and non-nitrogenous degradation products. The former included N-acetylglyoxylamide, acetylaminomalonic acid, acetylaminohydroxymalonic acid, acetylaminomaleic acid, diastereoisomeric 2-acetylamino-3-hydroxybutanedioic acids, 2-acetylaminobutenedioic acid, 3-acetylamino-4-hydroxy-2-pentenedioic acid, and 2,4-dihydroxy-3-acetylamino-2-pentenedioic acid, as well as two muconic and hydroxymuconic acid derivatives. (15)N NMR spectra revealed the accumulation since the early stages of substantial amounts of acetamide and oxalic acid monoamide. These results provide the first insight into the advanced oxidation chemistry of a 4-aminophenol derivative by the UV/H(2)O(2) system, and highlight the investigative potential of integrated GC-MS/NMR methodologies based on (15)N-labeling to track degradation pathways of nitrogenous species.	Oxidstion of paraacetomol with UV and peroxide explored.	Degradation Pathway	
143	Philipp B, Kemmler D, Hellstern J, Gorny N, Caballero A, Schink B.	Anaerobic degradation of protocatechuate (3,4-dihydroxybenzoate) by Thauera aromatica strain AR-1.	FEMS Microbiol Lett. 2002 Jun 18;212(1):139-43.	The denitrifying bacterium Thauera aromatica strain AR-1 grows anaerobically with protocatechuate (3,4-dihydroxybenzoate (DHB)) as sole energy and carbon source. This bacterium harbors two distinct pathways for degradation of aromatic compounds, the benzoyl-coenzyme A (CoA) pathway for benzoate degradation and the hydroxyhydroquinone (HHQ) pathway for degradation of 3,5-DHB. In order to elucidate whether protocatechuate is degraded via the benzoyl-CoA or the HHQ pathway, induction experiments were carried out. Dense suspensions of cells grown on protocatechuate or benzoate readily degraded benzoate and protocatechuate but not 3,5-DHB. Dense suspensions of 3,5-DHB-grown cells degraded 3,4- and 3,5-DHB at similar rates, but benzoate was not degraded. 3,5-DHB hydroxylating activity was found only in cells grown with this substrate. HHQ dehydrogenase activity was found in extracts of cells grown with 3,5-DHB and at a low rate also in protocatechuate-grown cells, but not in extracts of cells grown with benzoate. Activities of protocatechuyl-CoA synthetase and protocatechuyl-CoA reductase leading to 3-hydroxybenzoyl-CoA were found in extracts of cells grown with protocatechuate. There was no repression of the HHQ pathway by the presence of protocatechuate, unlike by degradation of benzoate. We conclude that protocatechuate is not degraded via the HHQ pathway because there was no evidence of a hydroxylation reaction involved in this process. Instead, our results strongly suggest that protocatechuate is degraded via a pathway which connects to the benzoyl-CoA route of degradation.	Bacterial degradation of dihydroxybenzoate and THB. THB dehydration pathway is described in this strain.	Degradation Pathway	
144	Bhushan B, Chauhan A, Samanta SK, Jain RK.	Kinetics of biodegradation of p-nitrophenol by different bacteria.	Biochem Biophys Res Commun. 2000 Aug 11;274(3):626-30.	Three bacterial species, i.e., Ralstonia sp. SJ98, Arthrobacter protophormiae RKJ100, and Burkholderia cepacia RKJ200, have been examined for their efficiency and kinetics behavior toward PNP degradation. All the three bacteria utilized PNP as the sole source of carbon, nitrogen, and energy. The rates of radiolabeled [U-(14)C]PNP degradation by all the bacteria were higher in the nitrogen-free medium compared to the medium with nitrogen. The apparent K(m) values of PNP degradation by SJ98, RKJ100, and RKJ200 were 0.32, 0.28, and 0.23 mM, respectively, as determined from the Michaelis-Menten curves. The maximum rates of PNP degradation (V(max)) according to Lineweaver-Burk's plots were 11.76, 7.81, and 3.84 micromol PNP degraded/min/mg dry biomass, respectively. The interpretation drawn from the Lineweaver-Burk's plots showed that the PNP degradation by SJ98 was stimulated by 4-nitrocatechol and 1, 2,4-benzenetriol. Benzoquinone and hydroquinone inhibited PNP degradation by RKJ100 noncompetitively and competitively, respectively, whereas in the case of RKJ200, benzoquinone and hydroquinone inhibited PNP degradation in an uncompetitive manner. beta-Ketoadipate did not affect the rate of PNP degradation in any case.	Bacterial degradation of p-nitrophenol	Degradation pathway	
145	Philipp B, Schink B.	Two distinct pathways for anaerobic degradation of aromatic compounds in the denitrifying bacterium Thauera aromatica strain AR-1.	Arch Microbiol. 2000 Feb;173(2):91-6.	Mononuclear aromatic compounds are degraded anaerobically through pathways that are basically different from those used in the presence of oxygen. Whereas aerobic degradation destabilizes the aromatic π-electron system by oxidative steps through oxygenase reactions, anaerobic degradation is most often initiated by a reductive attack. The benzoyl-CoA pathway is the most important metabolic route in this context, and a broad variety of mononuclear aromatics, including phenol, cresols, toluene, xylenes and ethylbenzene, are channelled into this pathway through various modification reactions. Multifunctional phenolic compounds are metabolized via the reductive resorcinol pathway, the oxidative resorcinol pathway with hydroxyhydroquinone as key intermediate, and the phloroglucinol pathway. Comparison of the various pathways used for modification and degradation of aromatics in the absence of oxygen indicates that the strategies of breakdown of these compounds are largely determined by the redox potentials of the electron acceptors used, and by the overall reaction energetics. Consequently, nitrate reducers quite often use strategies for primary attack on aromatic compounds that differ from those used by sulfate-reducing, iron-reducing or fermenting bacteria.	Bacterial degradation of aromatics edtermined by redox potentials	Degradation pathway	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
146	Johnson GR, Jain RK, Spain JC.	Properties of the trihydroxytoluene oxygenase from Burkholderia cepacia R34: an extradiol dioxygenase from the 2,4-dinitrotoluene pathway.	Arch Microbiol. 2000 Feb;173(2):86-90.	Burkholderia cepacia R34 mineralizes 2,4-dinitrotoluene via an oxidative pathway. The initial steps in the degradative pathway lead to formation of 2,4,5-trihydroxytoluene, which serves as the substrate for the ring cleavage dioxygenase. The trihydroxylated substrate differs from the usual substituted catechols found in pathways for aromatic compound degradation. To determine whether the characteristics of the trihydroxytoluene oxygenase reflect the unusual ring cleavage substrate of the 2,4-dinitrotoluene pathway, the gene encoding trihydroxytoluene oxygenase (dntD) was cloned and sequenced, and ring cleavage activity determined from recombinant bacteria carrying the cloned gene. The findings were compared to the trihydroxytoluene oxygenase from Burkholderia sp. strain DNT and to other previously described ring cleavage dioxygenases. The comparison revealed that only 60% identity was shared by the two trihydroxytoluene oxygenases, but the amino acid residues involved in cofactor binding, catalysis, and protein folding were conserved in the DntD sequence. The enzyme catalyzed meta-fission of trihydroxytoluene as well as the substrate analogues 1,2,4-benzenetriol, catechol, 3-methylcatechol, 4-methylcatechol, 3-chlorocatechol, 4-chlorocatechol and 2,3-dihydroxybiphenyl. However, results from enzyme assays indicated a strong preference for trihydroxytoluene, implying that it was the native substrate for the enzyme. The apparent enzyme specificity, its similarity to the trihydroxytoluene oxygenase from Burkholderia sp. strain DNT, and the distant genetic relationship to other ring cleavage enzymes suggest that dntD evolved expressly to carry out trihydroxytoluene transformation.	THT oxygenase catalysis of degradation THT, THB, MeCatechol coparison between different bacterial strains	Degradation pathway	
147	Chauhan A, Samanta SK, Jain RK.	Degradation of 4-nitrocatechol by Burkholderia cepacia: a plasmid-encoded novel pathway.	J Appl Microbiol. 2000 May;88(5):764-72.	Pseudomonas cepacia RKJ200 (now described as Burkholderia cepacia) has been shown to utilize p-nitrophenol (PNP) as sole carbon and energy source. The present work demonstrates that RKJ200 utilizes 4-nitrocatechol (NC) as the sole source of carbon, nitrogen and energy, and is degraded with concomitant release of nitrite ions. Several lines of evidence, including thin layer chromatography, gas chromatography, 1H-nuclear magnetic resonance, gas chromatography-mass spectrometry, spectral analyses and quantification of intermediates by high performance liquid chromatography, have shown that NC is degraded via 1,2, 4-benzenetriol (BT) and hydroquinone (HQ) formation. Studies carried out on a PNP- derivative and a PNP+ transconjugant also demonstrate that the genes for the NC degradative pathway reside on the plasmid present in RKJ200; the same plasmid had earlier been shown to encode genes for PNP degradation, which is also degraded via HQ formation. It is likely, therefore, that the same sets of genes encode the further metabolism of HQ in NC and PNP degradation	Nitrocatechol is degraded vai THB and HQ formation	Degradation Pathway	
148	Chauhan A, Chakraborti AK, Jain RK.	Plasmid-encoded degradation of p-nitrophenol and 4-nitrocatechol by Arthrobacter protophormiae.	Biochem Biophys Res Commun. 2000 Apr 21;270(3):733-40.	Arthrobacter protophormiae strain RKJ100 is capable of utilizing p-nitrophenol (PNP) as well as 4-nitrocatechol (NC) as the sole source of carbon, nitrogen and energy. The degradation of PNP and NC by this microorganism takes place through an oxidative route, as stoichiometry of nitrite molecules was observed when the strain was grown on PNP or NC as sole carbon and energy sources. The degradative pathways of PNP and NC were elucidated on the basis of enzyme assays and chemical characterization of the intermediates by TLC, GC, (1)H NMR, GC-MS, UV spectroscopy, and HPLC analyses. Our studies clearly indicate that the degradation of PNP proceeds with the formation of p-benzoquinone (BQ) and hydroquinone (HQ) and is further degraded via the beta-ketoadipate pathway. Degradation of NC involved initial oxidation to generate 1,2,4-benzenetriol (BT) and 2-hydroxy-1,4-benzoquinone; the latter intermediate is then reductively dehydroxylated, forming BQ and HQ, and is further cleaved via beta-ketoadipate to TCA intermediates. It is likely, therefore, that the same set of genes encode the further metabolism of HQ in PNP and NC degradation. A plasmid of approximately 65 kb was found to be responsible for harboring genes for PNP and NC degradation in this strain. This was based on the fact that PNP(-) NC(-) derivatives were devoid of the plasmid and had simultaneously lost their capability to grow at the expense of these nitroaromatic compounds.	THB is identified as an intermediate in thedegradation of PNP and Nitro catechol	Degradation Pathway	
149	Reichenbecher W, Philipp B, Suter MJ, Schink B.	Hydroxyhydroquinone reductase, the initial enzyme involved in the degradation of hydroxyhydroquinone (1,2,4-trihydroxybenzene) by Desulfovibrio inopinatus.	Arch Microbiol. 2000 Mar;173(3):206-12.	The recently isolated sulfate reducer Desulfovibrio inopinatus oxidizes hydroxyhydroquinone (1,2,4trihydroxybenzene; HHQ) to 2 mol acetate and 2 mol CO2 (mol substrate)-1, with stoichiometric reduction of sulfate to sulfide. None of the key enzymes of fermentative HHQ degradation, i.e. HHQ-1,2,3,5-tetrahydroxybenzene transhydroxylase or phloroglucinol reductase, were detected in cell-free extracts of D. inopinatus, indicating that this bacterium uses a different pathway for anaerobic HHQ degradation. HHQ was reduced with NADH in cell-free extracts to a nonaromatic compound, which was identified as dihydrohydroxyhydroquinone by its retention time in HPLC separation and by HPLC-mass spectrometry. The compound was identical with the product of chemical reduction of HHQ with sodium borohydride. Dihydrohydroxyhydroquinone was converted stoichiometrically to acetate and to an unknown coproduct. HHQ reduction was an enzymatic activity which was present in the cell-free extract at 0.25-0.30 U (mg protein)-1, with a pH optimum at 7.5. The enzyme was sensitive to sodium chloride, potassium chloride, EDTA, and o-phenanthroline, and exhibited little sensitivity towards sulfhydryl group reagents, such as copper chloride or p-chloromercuribenzoate.	Oxidation of THB to acetate and Carbon dioxide with reduction of sulfate to sulfide.	Degradation pathway	
150	Schink B, Philipp B, Müller J.	Anaerobic degradation of phenolic compounds.	Naturwissenschaften. 2000 Jan;87(1):12-23. Review.	Mononuclear aromatic compounds are degraded anaerobically through three main pathways, the benzoyl-CoA pathway, the resorcinol pathway, and the phloroglucinol pathway. Various modification reactions channel a broad variety of mononuclear aromatics including aromatic hydrocarbons into either one of these three pathways. Recently, a further pathway was discovered with hydroxyhydroquinone as central intermediate through which especially nitrate-reducing bacteria degrade phenolic compounds and some hydroxylated benzoates. Comparison of the various strategies taken for the degradation of aromatics in the absence of oxygen demonstrates that the biochemistry of breakdown of these compounds is determined largely by the overall reaction energetics and, more precisely, by the redox potentials of the electron acceptor systems used. Nitrate reducers differ in their strategies significantly from those used by sulfate-reducing or fermenting bacteria.	Nitrate reducing bacteria anaerobically degrade phenolics and hydroxybenzoates governed by redox potentials	Degradation pathway	
151	Claussen M, Schmidt S.	Biodegradation of phenylbenzoate and some of its derivatives by Scedosporium apiospermum.	Res Microbiol. 1999 Jul-Aug;150(6):413-20.	Scedosporium apiospermum, a recently isolated phenol-degrading hyphomycete, was shown to be able to productively utilise the diaryl ester phenylbenzoate as its sole source of carbon and energy. The characterisation of degradation intermediates together with the detection of the corresponding catabolic enzymes in crude extracts enabled us to propose a pathway for the degradation of this diaryl ester. According to our results, an inducible esterase initiated the biodegradation of phenylbenzoate by hydrolysing the ester bond to yield stoichiometric amounts of phenol and benzoate. While phenol was catabolised via catechol and hydroxyhydroquinone, the benzoate was further degraded via the protocatechuate branch of the ortho-pathway. In addition, the fungus utilised p-tolylbenzoate and 4-chlorophenylbenzoate by employing similar catabolic sequences.	Biodegradation of hydroxybenzoates differing from phenols	Degradation pathway	
152	Armengaud J, Timmis KN, Wittich RM.	A functional 4-hydroxysalicylate/hydroxyquinol degradative pathway gene cluster is linked to the initial dibenzo-p-dioxin pathway genes in Sphingomonas sp. strain RW1.	J Bacteriol. 1999 Jun;181(11):3452-61.	The bacterium Sphingomonas sp. strain RW1 is able to use dibenzo-p-dioxin, dibenzofuran, and several hydroxylated derivatives as sole sources of carbon and energy. We have determined and analyzed the nucleic acid sequence of a 9,997-bp HindIII fragment downstream of cistrons dxnA1A2, which encode the dioxygenase component of the initial dioxygenase system of the corresponding catabolic pathways. This fragment contains 10 colinear open reading frames (ORFs), apparently organized in one compact operon. The enzymatic activities of some proteins encoded by these genes were analyzed in the strain RW1 and, after hyperexpression, in Escherichia coli. The first three ORFs of the locus, designated dxnC, ORF2, and fdx3, specify a protein with a low homology to bacterial siderophore receptors, a polypeptide representing no significant homology to known proteins, and a putative ferredoxin, respectively. dxnD encodes a 69-kDa phenol monooxygenase-like protein with activity for the turnover of 4-hydroxysalicylate, and dxnE codes for a 37-kDa protein whose sequence and activity are similar to those of known maleylacetate reductases. The following gene, dxnF, encodes a 33-kDa intradiol dioxygenase which efficiently cleaves hydroxyquinol, yielding maleylacetate, the ketoform of 3-hydroxy-cis,cis-muconate. The heteromeric protein encoded by dxnGH is a 3-oxoadipate succinyl coenzyme A (succinyl-CoA) transferase, whereas dxnI specifies a protein exhibiting marked homology to acetyl-CoA acetyltransferases (thiolases). The last ORF of the sequenced fragment codes for a putative transposase. DxnD, DxnF, DxnE, DxnGH, and DxnI (the activities of most of them have also been detected in strain RW1) thus form a complete 4-hydroxysalicylate/hydroxyquinol degradative pathway. A route for the mineralization of the growth substrates 3-hydroxydibenzofuran and 2-hydroxydibenzo-p-dioxin in Sphingomonas sp. strain RW1 thus suggests itself.	Aromatics (phenolics) as carbon sources for bacteria S. sp strain	Degradation pathway	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
153	Leung KT, Campbell S, Gan Y, White DC, Lee H, Trevors JT.	The role of the Sphingomonas species UG30 pentachlorophenol-4-monooxygenase in p-nitrophenol degradation.	FEMS Microbiol Lett. 1999 Apr 1;173(1):247-53.	Pentachlorophenol-4-monooxygenase is an aromatic flavoprotein monooxygenase which hydroxylates pentachlorophenol and a wide range of polyhalogenated phenols at their para position. The PCP-degrading Sphingomonas species UG30 was recently shown to mineralize p-nitrophenol. In this study, the UG30 pcpB gene encoding the pentachlorophenol-4-monooxygenase gene was cloned for use to study its potential role in p-nitrophenol degradation. The UG30 pcpB gene consists of 1614 bp with a predicted translational product of 538 amino acids and a molecular mass of 59,933 Da. The primary sequence of pentachlorophenol-4-monooxygenase contained a highly conserved FAD binding site at its N-terminus associated with a beta alpha beta fold. UG30 has been shown previously to convert p-nitrophenol to 4-nitrocatechol. We observed that pentachlorophenol-4-monooxygenase catalyzed the hydroxylation of 4-nitrocatechol to 1,2,4-benzenetriol. About 31.2% of the nitro substituent of 4-nitrocatechol (initial concentration of 200 microM) was cleaved to yield nitrite over 2 h, indicating that the enzyme may be involved in the second step of p-nitrophenol degradation. The enzyme also hydroxylated p-nitrophenol at the para position, but only to a very slight extent. Our results confirm that pentachlorophenol-4-monooxygenase is not the primary enzyme in the initial step of p-nitrophenol metabolism by UG30.	4-NC conversion to THB via monoxygenase in Sphingomonas Sp.	Degradation Pathway	
154	Claussen M, Schmidt S.	Biodegradation of phenol and p-cresol by the hyphomycete Scedosporium apiospermum.	Res Microbiol. 1998 Jun;149(6):399-406.	Scedosporium apiospermum, a recently isolated phenol-degrading hyphomycete, was shown to be able to productively utilise the diaryl ester phenylbenzoate as its sole source of carbon and energy. The characterisation of degradation intermediates together with the detection of the corresponding catabolic enzymes in crude extracts enabled us to propose a pathway for the degradation of this diaryl ester. According to our results, an inducible esterase initiated the biodegradation of phenylbenzoate by hydrolysing the ester bond to yield stoichiometric amounts of phenol and benzoate. While phenol was catabolised via catechol and hydroxyhydroquinone, the benzoate was further degraded via the protocatechuate branch of the ortho-pathway. In addition, the fungus utilised p-tolylbenzoate and 4-chlorophenylbenzoate by employing similar catabolic sequences.	Biodegradation of phenols via catechol and THB variations in pathway from phenol and hydroxylbenzoates	Degradation Pathway	
155	Briganti F, Mangani S, Pedocchi L, Scozzafava A, Golovleva LA, Jadan AP, Solyanikova IP.	XAS characterization of the active sites of novel intradiol ring-cleaving dioxygenases: hydroxyquinol and chlorocatechol dioxygenases.	FEBS Lett. 1998 Aug 14;433(1-2):58-62.	The intradiol cleaving dioxygenases hydroxyquinol 1,2-dioxygenase (HQI,2O) from Nocardiodes simplex 3E, chlorocatechol 1,2-dioxygenase (CIC1,2O) from Rhodococcus erythropolis ICP, and their anaerobic substrate adducts (hydroxyquinol-HQ1,2O and 4-chlorocatechol-CIC1,2O) have been characterized through X-ray absorption spectroscopy. In both enzymes the iron(III) is pentacoordinated and the distance distribution inside the Fe(III) first coordination shell is close to that already found in the extensively characterized protocatechuate 3,4-dioxygenase. The coordination number and the bond lengths are not significantly affected by the substrate binding. Therefore it is confirmed that the displacement of a protein donor upon substrate binding has to be considered a general step valid for all intradiol dioxygenases.	Bacterial dioxygenases characterized by x-ray absorption spectroscopy	Degradation Pathway	
156	Philipp B, Schink B.	Evidence of two oxidative reaction steps initiating anaerobic degradation of resorcinol (1,3-dihydroxybenzene) by the denitrifying bacterium Azoarcus anaerobius.	J Bacteriol. 1998 Jul;180(14):3644-9.	Mononuclear aromatic compounds are degraded anaerobically through pathways that are basically different from those used in the presence of oxygen. Whereas aerobic degradation destabilizes the aromatic π-electron system by oxidative steps through oxygenase reactions, anaerobic degradation is most often initiated by a reductive attack. The benzoyl-CoA pathway is the most important metabolic route in this context, and a broad variety of mononuclear aromatics, including phenol, cresols, toluene, xylenes and ethylbenzene, are channelled into this pathway through various modification reactions. Multifunctional phenolic compounds are metabolized via the reductive resorcinol pathway, the oxidative resorcinol pathway with hydroxyhydroquinone as key intermediate, and the phloroglucinol pathway. Comparison of the various pathways used for modification and degradation of aromatics in the absence of oxygen indicates that the strategies of breakdown of these compounds are largely determined by the redox potentials of the electron acceptors used, and by the overall reaction energetics. Consequently, nitrate reducers quite often use strategies for primary attack on aromatic compounds that differ from those used by sulfate-reducing, iron-reducing or fermenting bacteria.	Aerobic degradation pathways inherently different using oxygenase reactions v reductuve pathway.	Degradation Pathway	
157	Sottofattori E, Martelli A, Brambilla G.	The effect of benzenediols and benzenetriols on the nitrosation of propranolol depends on the position of hydroxyl groups on the benzene ring.	Mutat Res. 1998 Feb 26;398(1-2):75-82.	Nitrosation of propranolol under the standard conditions recommended by the World Health Organization (10 mM propranolol hydrochloride, 40 mM sodium nitrite, pH 3.5) was carried out in the absence and in the presence of phenol, benzenediols and benzenetriols added to the nitrosation mixture in concentrations ranging from 2 to 40 mM. The yield of N-nitrosopropranolol (NOP) was reduced, with potency decreasing in the following order, by 1,2-benzenediol > 1,2,3-benzenetriol > 1,4-benzediol; their inhibiting effect was dose-dependent. 1,2,4-Benzenetriol displayed a significant inhibitory activity only at 20-40 mM concentrations. The maximum reduction of NOP formation (7% of the yield obtained under control conditions) was produced at 120 min by 40 mM 1,2-benzenediol. In contrast, the yield of NOP was increased by 1,3-benzenediol and 1,3,5-benzenetriol, but this effect was inversely related to the concentration. The effect of the various phenols on the time course of propranolol nitrosation was dependent on both the test phenol and its concentration. 1,2-Benzenediol and 1,3-benzenediol displayed on the nitrosation of proline effects qualitatively of the same type, but quantitatively different, as compared with those observed on the nitrosation of propranolol. Taken as a whole, the results of this study indicate that depending on the positions of hydroxyl groups on the benzene ring benzenediols and benzenetriols may inhibit or hasten nitrosation reactions.	Nitrosation of propranalol in presence and absence of phenolics. Phenolics exerted an inhibitory effect on NOP formation.	Chemistry	
158	Gallus C, Schink B.	Anaerobic degradation of alpha-resorcylate by Thauera aromatica strain AR-1 proceeds via oxidation and decarboxylation to hydroxyhydroquinone.	Arch Microbiol. 1998 Apr;169(4):333-8.	Anaerobic degradation of alpha-resorcylate (3,5-dihydroxybenzoate) was studied with the denitrifying strain AR-1, which was assigned to the described species Thauera aromatica. alpha-Resorcylate degradation does not proceed via the benzoyl-CoA, the resorcinol, or the phloroglucinol pathway. Instead, alpha-resorcylate is converted to hydroxyhydroquinone (1,2,4-trihydroxybenzene) by dehydrogenative oxidation and decarboxylation. Nitrate, K3[Fe(CN)6], dichlorophenol indophenol, and the NAD+ analogue 3-acetylpyridine adeninedinucleotide were suitable electron acceptors for the oxidation reaction; NAD+ did not function as an electron acceptor. The oxidation reaction was strongly accelerated by the additional presence of the redox carrier phenazine methosulfate, which could also be used as sole electron acceptor. Oxidation of alpha-resorcylate with molecular oxygen in cell suspensions or in cell-free extracts of alpha-resorcylate- and nitrate-grown cells was also detected although this bacterium did not grow with alpha-resorcylate under an air atmosphere. alpha-Resorcylate degradation to hydroxyhydroquinone proceeded in two steps. The alpha-resorcylate-oxidizing enzyme activity was membrane-associated and exhibited maximal activity at pH 8.0. The primary oxidation product was not hydroxyhydroquinone. Rather, formation of hydroxyhydroquinone by decarboxylation of the unknown intermediate in addition required the cytoplasmic fraction and needed lower pH values since hydroxyhydroquinone was not stable at alkaline pH.	THB as an intermediate n the dihydroxybenzoates	Degradation Pathway	
159	Reichenbecher W, Schink B.	Desulfovibrio inopinatus, sp. nov., A new sulfate-reducing bacterium that degrades hydroxyhydroquinone (1,2,4-trihydroxybenzene)	Arch Microbiol. 1998 Jan;169(1):88. No abstract available.	No Abstract Available		Degradation Pathway	
160	Reichenbecher W, Schink B.	Desulfovibrio inopinatus, sp. nov., a new sulfate-reducing bacterium that degrades hydroxyhydroquinone.	Arch Microbiol. 1997 Oct;168(4):338-44. Erratum in: Arch Microbiol 1998 Jan;169(1):88.	No Abstract Available		Degradation Pathway	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
161	Meulenberg R, Pepi M, de Bont JA.	Degradation of 3-nitrophenol by Pseudomonas putida B2 occurs via 1,2,4-benzenetriol.	Biodegradation. 1996 Aug;7(4):303-11.	Growth of Pseudomonas putida B2 in chemostat cultures on a mixture of 3-nitrophenol and glucose induced 3-nitrophenol and 1,2,4-benzenetriol-dependent oxygen uptake activities. Anaerobic incubations of cell suspensions with 3-nitrophenol resulted in complete conversions of the substrate to ammonia and 1,2,4-benzenetriol. This indicates that P. putida B2 degrades 3-nitrophenol via 1,2,4-benzenetriol, via a pathway involving a hydroxylaminolyase. Involvement of this pathway in nitroaromatic metabolism has previously only been found for degradation of 4-nitrobenzoate. Reduction of 3 nitrophenol by cell-free extracts was strictly NADPH-dependent. Attempts to purify the enzymes responsible for 3-nitrophenol metabolism were unsuccessful, because their activities were extremely unstable. 3-Nitrophenol reductase was therefore characterized in cell-free extracts. The enzyme had a sharp pH optimum at pH 7 and a temperature optimum at 25 degrees C. At 30 degrees C, reductase activity was completely destroyed within one hour, while at 0 degrees C, the activity in cell-free extracts was over 100-fold more stable. The Km values for NADPH and 3-nitrophenol were estimated at 0.17 mM and below 2 microM, respectively. The substrate specificity of the reductase activity was very broad: all 17 nitroaromatics tested were reduced by cell-free extracts. However, neither intact cells nor cell-free extracts could convert a set of synthesized hydroxylaminoaromatic compounds to the corresponding catechols and ammonia. Apparently, the hydroxylaminolyase of P. putida B2 has a very narrow substrate specificity, indicating that this organism is not a suitable biocatalyst for the industrial production of catechols from nitroaromatics.	Bacterial degradation	Degradation Pathway	
162	Bock C, Kroppenstedt RM, Schmidt U, Diekmann H.	Degradation of prochloraz and 2,4,6-trichlorophenol by environmental bacterial strains.	Appl Microbiol Biotechnol. 1996 Mar;45(1-2):257-62.	Eight bacterial isolates from enrichment with 2,4,6-trichlorophenol (TCP) as sole carbon source were tested for their potential to degrade prochloraz. None of them could grow on prochloraz. Strain C964, identified as Aureobacterium sp., effectively reduced the fungitoxic activity of prochloraz in a bioassay and degradation was confirmed by HPLC. Two other isolates, strain C611 and C961, using TCP as a carbon source, belong to the beta subclass of the proteobacteria and presumely degrade TCP via 2,4-dichlorohydroquinone and hydroxyhydroquinone as indicated by oxygen-consumption tests.	Halophenols degraded via THB and halo HQ	Degradation Pathway	
163	Uotila JS, Kitunen VH, Coote T, Saastamoinen T, Salkinoja-Salonen M, Apajalahti JH.	Metabolism of halohydroquinones in Rhodococcus chlorophenolicus PCP-1.	Biodegradation. 1995 Jun;6(2):119-26.	The actinomycete Rhodococcus chlorophenolicus PCP-1 metabolizes pentachlorophenol into ultimate inorganic end products via tetrachloro-p-hydroquinone. This intermediate was further dehalogenated in the cytoplasm requiring reductant in the cell free system. Tetrafluoro-p-hydroquinone and tetrabromo-p-hydroquinone were also dehalogenated. Chlorophenol analogs, thiol blocking agents and molecular oxygen inhibited the activity. The dehalogenating reactions led to 1,2,4-trihydroxybenzene, which was further metabolized into maleic acid.	Halo-hydroquinones dehalogenated to THB then to maleic acid	Degradation Pathway	
164	Nadeau LJ, Spain JC.	Bacterial degradation of m-nitrobenzoic acid.	Appl Environ Microbiol. 1995 Feb;61(2):840-3.	Pseudomonas sp. strain JS51 grows on m-nitrobenzoate (m-NBA) with stoichiometric release of nitrite. m-NBA-grown cells oxidized m-NBA and protocatechuate but not 3-hydroxybenzoate, 4-hydroxy-3-nitrobenzoate, 4-nitrocatechol, and 1,2,4-benzenetriol. Protocatechuate accumulated transiently when succinate-grown cells were transferred to media containing m-NBA. Respirometric experiments indicated that the conversion of m-NBA to protocatechuate required 1 mol of oxygen per mol of substrate. Conversions conducted in the presence of 18O2 showed the incorporation of both atoms of molecular oxygen into protocatechuate. Extracts of m-NBA-grown cells cleaved protocatechuate to 2-hydroxy-4-carboxymuconic semialdehyde. These results provide rigorous proof that m-NBA is initially oxidized by a dioxygenase to produce protocatechuate which is further degraded by a 4,5-dioxygenase.	Nitrobenzoate metabolism via dioxygenases	Degradation Pathway	
165	Hofrichter M, Bublitz F, Fritsche W.	Cometabolic degradation of o-cresol and 2,6-dimethylphenol by Penicillium frequentans Bi 7/2.	J Basic Microbiol. 1995;35(5):303-13.	o-Cresol induced glucose-grown resting mycelia of Penicillium frequentans Bi 7/2 (ATCC-number: 96048) immediately oxidized o-cresol and other phenols. After precultivation on glucose and phenol degradation started after a lag-phase of 24 hours. Metabolites of o-cresol metabolism were methylhydroquinone, methyl-p-benzoquinone, 2-methyl-5-hydroxyhydroquinone and 2-methyl-5-hydroxy-p-benzoquinone. The initial reaction is probably catalyzed by a NADPH dependent hydroxylase which is specific for o-cresol. The metabolism of 2,6-dimethylphenol (2,6-xylenol) occurred via 2,6-dimethylhydroquinone, 2,6-dimethyl-p-benzoquinone, 2,6-dimethyl-3-hydroxyhydroquinone, 2,6-dimethyl-3-hydroxy-p-benzoquinone and 3-methyl-2-hydroxybenzoic acid.	Phenol degradation in penicillium strains	Degradation Pathway	
166	Zaborina O, Latus M, Eberspächer J, Golovleva LA, Lingens F.	Purification and characterization of 6-chlorohydroxyquinol 1,2-dioxygenase from Streptomyces rochei 303: comparison with an analogous enzyme from Azotobacter sp. strain GP1.	J Bacteriol. 1995 Jan;177(1):229-34.	The enzyme which cleaves the benzene ring of 6-chlorohydroxyquinol was purified to apparent homogeneity from an extract of 2,4,6-trichlorophenol-grown cells of Streptomyces rochei 303. Like the analogous enzyme from Azotobacter sp. strain GP1, it exhibited a highly restricted substrate specificity and was able to cleave only 6-chlorohydroxyquinol and hydroxyquinol and not catechol, chlorinated catechols, or pyrogallol. No extradiol-cleaving activity was observed. In contrast to 6-chlorohydroxyquinol 1,2-dioxygenase from Azotobacter sp. strain GP1, the S. rochei enzyme had a distinct preference for 6-chlorohydroxyquinol over hydroxyquinol (kcat/Km = 1.2 and 0.57 s-1.microM-1, respectively). The enzyme from S. rochei appears to be a dimer of two identical 31-kDa subunits. It is a colored protein and was found to contain 1 mol of iron per mol of enzyme. The NH2-terminal amino acid sequences of 6-chlorohydroxyquinol 1,2-dioxygenase from S. rochei 303 and from Azotobacter sp. strain GP1 showed a high degree of similarity.	Halophenols degraded via THB and halo HQ	Degradation Pathway	
167	Rani NL, Lalithakumari D.	Degradation of methyl parathion by Pseudomonas putida.	Can J Microbiol. 1994 Dec;40(12):1000-6.	Pseudomonas putida utilized methyl parathion as sole carbon and (or) phosphorus source. The bacterium elaborated the enzyme organophosphorus acid anhydrase, which hydrolyzed methyl parathion to p-nitrophenol. p-Nitrophenol was further degraded to hydroquinone and 1,2,4-benzenetriol. The final ring compound, 1,2,4-benzenetriol, was cleaved by benzenetriol oxygenase to maleyl acetate.	Bacterial degradation of parathion to PNP and then to THB then to maleyl acetate	Degradation Pathway	
168	Jain RK, Dreisbach JH, Spain JC.	Biodegradation of p-nitrophenol via 1,2,4-benzenetriol by an Arthrobacter sp.	Appl Environ Microbiol. 1994 Aug;60(8):3030-2.	The degradation of p-nitrophenol (PNP) by Moraxella and Pseudomonas spp. involves an initial monooxygenase-catalyzed removal of the nitro group. The resultant hydroquinone is subject to ring fission catalyzed by a dioxygenase enzyme. We have isolated a strain of an Arthrobacter sp., JS443, capable of degrading PNP with stoichiometric release of nitrite. During induction of the enzymes required for growth on PNP, 1,2,4-benzenetriol was identified as an intermediate by gas chromatography-mass spectroscopy (GC-MS) and radiotracer studies. 1,2,4-Benzenetriol was converted to maleylacetic acid, which was further degraded by the beta-ketoadipate pathway. Conversion of PNP to 1,2,4-benzenetriol is catalyzed by a monooxygenase system in strain JS443 through the formation of 4-nitrocatechol, 4-nitroresorcinol, or both. Our results clearly indicate the existence of an alternative pathway for the biodegradation of PNP.	PNP degradation via monooxygenases to THB intermediate	Degradation Pathway	
169	Stolz A, Knackmuss HJ.	Degradation of 2,4-dihydroxybenzoate by Pseudomonas sp. BN9.	FEMS Microbiol Lett. 1993 Apr 1;108(2):219-24.	The aerobic degradation of 2,4-dihydroxybenzoate by Pseudomonas sp. BN9 was studied. Intact cells of Pseudomonas sp. BN9 grown with 2,4-dihydroxybenzoate oxidized 2,4-dihydroxybenzoate but not salicylate. Cell-free extracts of Pseudomonas sp. BN9 converted 2,4-dihydroxybenzoate after the addition of NAD(P)H. A partially purified protein fraction converted 2,4-dihydroxybenzoate with NADH to 1,2,4-trihydroxybenzene. 1,2,4-Trihydroxybenzene was converted by a 1,2-dioxygenase to maleylpyruvate, which was reduced by a NADH-dependent enzyme to 3-oxoadipate. 2,4-Dihydroxybenzoate 1-monooxygenase, 1,2,4-trihydroxybenzene 1,2-dioxygenase and maleylpyruvate reductase were induced in Pseudomonas sp. BN9 after growth with 2,4-dihydroxybenzoate.	Degradation of hydroxybenzoates via THB and dioxygenases	Degradation pathway	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
170	Middelhoven WJ.	Catabolism of benzene compounds by ascomycetous and basidiomycetous yeasts and yeastlike fungi. A literature review and an experimental approach.	Antonie Van Leeuwenhoek. 1993 Feb;63(2):125-44. Review.	A literature review is given on growth of yeasts on benzene compounds and on the catabolic pathways involved. Additionally, a yeast collection was screened for assimilation of phenol and 3-hydroxybenzoic acid. Fifteen ascomycetous and thirteen basidiomycetous yeast species were selected and were tested for growth on 84 benzene compounds. It appeared that 63 of these compounds supported growth of one or more yeast species. The black yeast Exophiala jeanselmei assimilated 54 of these compounds. The catechol branch of the 3-oxoadipate pathway and its hydroxyhydroquinone variant were involved in phenol and resorcinol catabolism of ascomycetes as well as of basidiomycetes. However, these two groups of yeasts showed characteristic differences in hydroxybenzoate catabolism. In the yeastlike fungus E. jeanselmei and in basidiomycetes of the genera Cryptococcus, Leucosporidium and Rhodotorula, the protocatechuate branch of the 3-oxoadipate pathway was induced by growth on 3- and 4-hydroxybenzoic acids. In three Trichosporon species and in all ascomycetous yeasts tested, 4-hydroxybenzoic acid was catabolyzed via protocatechuate and hydroxyhydroquinone. These yeasts were unable to cleave protocatechuate. 3-Hydroxybenzoic and 3-hydroxycinnamic acids were catabolized in ascomycetous yeasts via the gentisate pathway, but in basidiomycetes via protocatechuate. Incomplete oxidation of phenol, some chlorophenols, cresols and xylenols was observed in cultures of Candida parapsilosis growing on hydroquinone. Most compounds transformed by the growing culture were also converted by the phenol monooxygenase present in cell-free extracts of this yeast. They did not support growth. The relationship between the ability of ascomycetous yeasts to assimilate n-alkanes, amines and benzene compounds, and the presence of Coenzyme Q9 is discussed.	Phenolics as a carbon source for certain yeasts	Degradation Pathway	
171	Middelhoven WJ, Coenen A, Kraakman B, Sollewijn Gelpke MD.	Degradation of some phenols and hydroxybenzoates by the imperfect ascomycetous yeasts Candida parapsilosis and Arxula adeninivorans: evidence for an operative gentisate pathway.	Antonie Van Leeuwenhoek. 1992 Oct;62(3):181-7.	The imperfect ascomycetous yeasts Candida parapsilosis and Arxula adeninivorans degraded 3-hydroxybenzoic acid via gentisate which was the cleavage substrate. 4-Hydroxybenzoic acid was metabolized via protocatechuate. No cleavage enzyme for the latter was detected. In stead of this NADH- and NADPH-dependent monooxygenases were present. In cells grown at the expense of hydroquinone and 4-hydroxybenzoic acid, enzymes of the hydroxyhydroquinone variant of the 3-oxoadipate pathway were demonstrated, which also took part in the degradation of 2,4-dihydroxybenzoic acid by C. parapsilosis.	Hydroxybenzoate degradationA	Degradation Pathway	
172	Brune A, Schnell S, Schink B.	Sequential Transhydroxylations Converting Hydroxyhydroquinone to Phloroglucinol in the Strictly Anaerobic, Fermentative Bacterium Pelobacter massiliensis.	Appl Environ Microbiol. 1992 Jun;58(6):1861-8.	The recently isolated fermenting bacterium Pelobacter massiliensis is the only strict anaerobe known to grow on hydroxyhydroquinone (1,2,4-trihydroxybenzene) as the sole source of carbon and energy, converting it to stoichiometric amounts of acetate. In this paper, we report on the enzymatic reactions involved in the conversion of hydroxyhydroquinone and pyrogallol (1,2,3-trihydroxybenzene) to phloroglucinol (1,3,5-trihydroxybenzene). Cell extracts of P. massiliensis transhydroxylate pyrogallol to phloroglucinol after addition of 1,2,3,5-tetrahydroxybenzene (1,2,3,5-TTHB) as cosubstrate in a reaction identical to that found earlier with Pelobacter acidigallici (A. Brune and B. Schink, J. Bacteriol. 172:1070-1076, 1990). Hydroxyhydroquinone conversion to phloroglucinol is initiated in cell extracts without an external addition of cosubstrates. It involves a minimum of three consecutive transhydroxylation reactions characterized by the transient accumulation of two different TTHB isomers. Chemical synthesis of the TTHB intermediates allowed the resolution of the distinct transhydroxylation steps in this sequence. In an initial transhydroxylation, the hydroxyl group in the 1-position of a molecule of hydroxyhydroquinone is transferred to the 5-position of another molecule of hydroxyhydroquinone to give 1,2,4,5-TTHB and resorcinol (1,3-dihydroxybenzene) as products. Following this disproportionation of hydroxyhydroquinone, the 1,2,4,5-isomer is converted to 1,2,3,5-TTHB, an enzymatic activity present only in hydroxyhydroquinone-grown cells. Finally, phloroglucinol is formed from 1,2,3,5-TTHB by transfer of the 2-hydroxyl group to either hydroxyhydroquinone or resorcinol. The resulting coproducts are again cosubstrates in earlier reactions of this sequence. From the spectrum of hydroxybenzenes transhydroxylated by the cell extracts, the minimum structural prerequisites that render a hydroxybenzene a hydroxyl donor or acceptor are deduced.	Bacterial degradation of THB as a carbon source for certain strains.	Degradation pathway	
173	Spain JC, Gibson DT.	Pathway for Biodegradation of p-Nitrophenol in a Moraxella sp.	Appl Environ Microbiol. 1991 Mar;57(3):812-9.	A Moraxella strain grew on p-nitrophenol with stoichiometric release of nitrite. During induction of the enzymes for growth on p-nitrophenol, traces of hydroquinone accumulated in the medium. In the presence of 2,2'-dipyridyl, p-nitrophenol was converted stoichiometrically to hydroquinone. Particulate enzymes catalyzed the conversion of p-nitrophenol to hydroquinone in the presence of NADPH and oxygen. Soluble enzymes catalyzed the conversion of hydroquinone to gamma-hydroxymuconic semialdehyde, which was identified by high-performance liquid chromatography (HPLC)-mass spectroscopy. Upon addition of catalytic amounts of NAD, gamma-hydroxymuconic semialdehyde was converted to beta-ketoadipic acid. In the presence of pyruvate and lactic dehydrogenase, substrate amounts of NAD were required and gamma-hydroxymuconic semialdehyde was converted to maleylacetic acid, which was identified by HPLC-mass spectroscopy. Similar results were obtained when the reaction was carried out in the presence of potassium ferricyanide. Extracts prepared from p-nitrophenol-growth cells also contained an enzyme that catalyzed the oxidation of 1,2,4-benzenetriol to maleylacetic acid. The enzyme responsible for the oxidation of 1,2,4-benzenetriol was separated from the enzyme responsible for hydroquinone oxidation by DEAE-cellulose chromatography. The results indicate that the pathway for biodegradation of p-nitrophenol involves the initial removal of the nitro group as nitrite and formation of hydroquinone. 1,4-Benzoquinone, a likely intermediate in the initial reaction, was not detected. Hydroquinone is converted to beta-ketoadipic acid via gamma-hydroxymuconic semialdehyde and maleylacetic acid.	Enzymatic oxidation of THB to maleylacetate from nitrophenols	Degradation Pathway	
174	Shibata MA, Hirose M, Yamada M, Tatematsu M, Uwagawa S, Ito N.	Epithelial cell proliferation in rat forestomach and glandular stomach mucosa induced by catechol and analogous dihydroxybenzenes.	Carcinogenesis. 1990 Jun;11(6):997-1000.	An investigation of antioxidant-induced lesions in stomach epithelia was performed using F344 rats of both sexes. Histopathological changes, levels of DNA synthesis and pepsinogen isozyme 1 altered pyloric gland (PAPG) induction were assessed following 4 weeks oral administration of catechol (CC) or analogs such as hydroxyhydroquinone (HHQ), protocatechuic acid (PCA), protocatechualdehyde (PCAH), dopamine and DL-dopa. While epithelial hyperplasia of the forestomach was only observed in the groups given CC, DNA synthesis in this epithelium was increased in groups of both sexes treated with CC, HHQ or dopamine. In the glandular stomach, CC induced submucosal growth of pyloric mucosal cells and an increase in crypt height associated with an elevation of DNA synthesis and numbers of PAPG. In contrast, dopamine brought about significant reduction in DNA synthesis in the pyloric mucosa of both sexes. The other CC analogs did not exert any obvious influence on glandular stomach mucosa. Since cell proliferation is well correlated to tumor promotion, the results suggested that HHQ and dopamine may have promoting potential for rat two-stage forestomach carcinogenesis in common with CC, while dopamine might be expected to inhibit glandular stomach carcinogenesis.	Stomach epithelial DNA synthesis is increased	Degradation Pathway	
175	Brune A, Schink B.	Pyrogallol-to-phloroglucinol conversion and other hydroxyl-transfer reactions catalyzed by cell extracts of Pelobacter acidigallici.	J Bacteriol. 1990 Feb;172(2):1070-6.	Permeabilized cells and cell extracts of Pelobacter acidigallici catalyzed the conversion of pyrogallol (1,2,3-trihydroxybenzene) to phloroglucinol (1,3,5-trihydroxybenzene) in the presence of 1,2,3,5-tetrahydroxybenzene. Pyrogallol consumption by resting cells stopped after lysis by French press or mild detergent (cetyltrimethylammonium bromide [CTAB]) treatment. Addition of 1,2,3,5-tetrahydroxybenzene to the assay mixture restored pyrogallol consumption and led to stoichiometric phloroglucinol accumulation. The stoichiometry of pyrogallol conversion to phloroglucinol was independent of the amount of tetrahydroxybenzene added. The tetrahydroxybenzene concentration limited the velocity of the transhydroxylation reaction, which reached a maximum at 1.5 mM tetrahydroxybenzene (1 U/mg of protein). Transhydroxylation was shown to be reversible. The equilibrium constant of the reaction was determined, and the free-energy change (delta G degree') of phloroglucinol formation from pyrogallol was calculated to be -15.5 kJ/mol. Permeabilized cells and cell extracts also catalyzed the transfer of hydroxyl moieties between other hydroxylated benzenes. Tetrahydroxybenzene and hydroxyhydroquinone participated as hydroxyl donors and as hydroxyl acceptors in the reaction, whereas pyrogallol, resorcinol, and phloroglucinol were hydroxylated by both donors. A novel mechanism deduced from these data involves intermolecular transfer of the hydroxyl moiety from the cosubstrate (1,2,3,5-tetrahydroxybenzene) to the substrate (pyrogallol), thus forming the product (phloroglucinol) and regenerating the cosubstrate.	THB functions as a hydroxyl donor and acceptor	Degradation Pathway	
176	Inoue O, Seiji K, Ikeda M.	Pathways for formation of catechol and 1,2,4-benzenetriol in rabbits.	Bull Environ Contam Toxicol. 1989 Aug;43(2):220-4. No abstract available.	No abstract available		Degradation Pathway	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
177	Barkovskiĭ AL, Shub GM.	[Acinetobacter calcoaceticus strain with a wide spectrum of utilizing aromatic compounds and carrying a plasmid for resorcin degradation].	Mikrobiologiia. 1986 Mar-Apr;55(2):237-40. Russian.	A bacterial strain was isolated from soil and identified as Acinetobacter calcoaceticus var. lwoffii. The strain can utilize a wide spectrum of aromatic compounds. It carries a transmissive plasmid pBSW13 which determines resorcin utilization via the ortho pathway including the following steps: resorcin-hydroxyhydroquinone-maleylacetate-beta-ketoadipi c acid. The plasmid has been transferred by conjugation into the recipient strains of A. calcoaceticus 5734 CCM rifr, Escherichia coli J-53 met-pro-rifr and Klebsiella sp. Plasmid DNA with a molecular mass close to that of phage gamma was detected by electrophoresis in the donor and recombinant strains. The degradation of other substrates is not a phenotypic expression of the genes of this plasmid.	Bacterial degradation of a range of aromatic compounds from resorcinol to THB to maleylacetate betaketoadipate	Degradation Pathway	
178	Krug M, Ziegler H, Straube G.	Degradation of phenolic compounds by the yeast Candida tropicalis HP 15. I. Physiology of growth and substrate utilization.	J Basic Microbiol. 1985;25(2):103-10.	The yeast Candida tropicalis HP 15 was able to utilize phenol up to concentrations of 2.5 g/l as a sole carbon and energy source. Phenol was metabolized via the beta-ketoadipate pathway by an inducible enzyme system. Besides phenol, resorcinol, quinol, hydroxyquinol, catechol, and to a lesser extend 4-chlorocatechol, protocatechuate, p-cresol, m-chlorophenol, and p-chlorophenol were metabolized by the yeast. A total of 30 aromatic compounds were tested as substrates.	Phenolics degradation in yeast strains	Degradation Pathway	
179	Sawahata T, Neal RA.	Biotransformation of phenol to hydroquinone and catechol by rat liver microsomes.	Mol Pharmacol. 1983 Mar;23(2):453-60.	Hepatic microsomal biotransformation of phenol to hydroquinone and catechol has been investigated with special reference to the covalent binding to microsomal protein of reactive metabolites formed during microsomal metabolism of phenol. Incubation of [14C]phenol with microsomes from phenobarbital-treated rat liver in the presence of an NADPH-generating system resulted in the formation of hydroquinone and catechol in the ratio of 20:1. No significant formation of 1,2,4-benzenetriol was observed. The biotransformation of phenol to both hydroquinone and catechol required NADPH and molecular oxygen. NADH was much less effective than NADPH as an electron donor and exhibited no significant synergistic effect when used together with NADPH. The biotransformation was inhibited by typical cytochrome P-450 inhibitors such as carbon monoxide, SKF 525-A, and metyrapone. These results indicated the involvement of cytochrome P-450 in the microsomal hydroxylation of phenol at both the ortho- and para-positions. Covalent binding of radioactivity to microsomal protein was observed when [14C]phenol was incubated with rat liver microsomes in the presence of an NADPH-generating system. The covalent binding was also found to require NADPH and molecular oxygen. Inclusion of cytochrome P-450 inhibitors in the incubation mixture resulted in a decrease in the covalent binding. These results indicated that at least one step in the metabolic activation of phenol to the metabolites responsible for covalent binding to microsomal protein was mediated by cytochrome P-450. Inclusion of N-acetylcysteine in the incubation mixture resulted in the complete inhibition of the covalent binding of radioactivity derived from [14C]phenol to microsomal protein, and there was a concomitant formation of N-acetylcysteine adducts of hydroquinone and catechol. These results indicated that hydroquinone and catechol were both precursors to reactive metabolites responsible for the covalent binding.	THB is not formed in the degradation of phenol in rat liver	Degradation Pathway	
180	Gaal A, Neujahr HY.	Metabolism of phenol and resorcinol in Trichosporon cutaneum.	J Bacteriol. 1979 Jan;137(1):13-21.	Trichosporon cutaneum was grown with phenol or resorcinol as the carbon source. The formation of beta-ketoadipate from phenol, catechol, and resorcinol was shown by a manometric method using antipyrine and also by its isolation and crystallization. Metabolism of phenol begins with o-hydroxylation. This is followed by ortho-ring fission, lactonization to muconolactone, and delactonization to beta-ketoadipate. No meta-ring fission could be demonstrated. Metabolism of resorcinol begins with o-hydroxylation to 1,2,4-benzenetriol, which undergoes ortho-ring fission yielding maleylacetate. Isolating this product leads to its decarboxylation and isomerization to trans-acetylacrylic acid. Maleylacetate is reduced by crude extracts to beta-ketoadipate with either reduced nicotinamide adenine dinucleotide or reduced nicotinamide adenine dinucleotide phosphate as a cosubstrate. The enzyme catalyzing this reaction was separated from catechol 1,2-oxygenase, phenol hydroxylase, and muconate lactonizing enzyme on a diethyl-aminoethyl-Sephadex A50 column. As a result it was purified some 50-fold, as was the muconate-lactonizing enzyme. Methyl-, fluoro-, and chlorophenols are converted to a varying extent by crude extracts and by purified enzymes. None of these derivatives is converted to maleylacetate, beta-ketoadipate, or their derivatives. Cells grown on resorcinol contain enzymes that participate in the degradation of phenol and vice versa.	Metabolism of resorcinol begins with hydroxylation to THB which then undergoes ring fission to maleylacetate	Degradation pathway	
181	Haigler BE, Johnson GR, Suen WC, Spain JC.	Biochemical and genetic evidence for meta-ring cleavage of 2,4, 5-trihydroxytoluene in Burkholderia sp. strain DNT.	J Bacteriol. 1999 Feb;181(3):965-72.	2,4,5-Trihydroxytoluene (THT) oxygenase from Burkholderia sp. strain DNT catalyzes the conversion of THT to an unstable ring fission product. Biochemical and genetic studies of THT oxygenase were undertaken to elucidate the mechanism of the ring fission reaction. The THT oxygenase gene (dntD) was previously localized to the 1.2-kb DNA insert subcloned in the recombinant plasmid designated pJS76 (W. C. Suen and J. C. Spain, J. Bacteriol. 175:1831-1837, 1993). Analysis of the deduced amino acid sequence of DntD revealed the presence of the highly conserved residues characteristic of the catechol 2,3-dioxygenase gene family I. The deduced amino acid sequence of DntD corresponded to a molecular mass of 35 kDa. The native molecular masses for the THT oxygenase estimated by using gel filtration chromatography and nondenaturing gel electrophoresis were 67.4 and 77.8 kDa, respectively. The results suggested that the native protein consists of two identical subunits. The colorless protein contained 2 mol of iron per mol of protein. Stimulation of activity in the presence of ferrous iron and ascorbate suggested a requirement for ferrous iron in the active site. The properties of the enzyme are similar to those of the catechol 2,3-dioxygenases (meta-cleavage dioxygenases). In addition to THT, the enzyme exhibited activity towards 1,2,4-benzenetriol, catechol, 3- and 4-methylcatechol, and 3- and 4-chlorocatechol. The chemical analysis of the THT ring cleavage product showed that the product was 2, 4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid, consistent with extradiol ring fission of THT.	Bacterial THT Dioxygenase exhibits activity to THB and other phenolics.	Degradation Pathway	
182	Kumalaputri AJ, Randolph C, Otten E, Heeres HJ, Deuss PJ.	Lewis Acid Catalyzed Conversion of 5-Hydroxymethylfurfural to 1,2,4-Benzenetriol, an Overlooked Biobased Compound.	ACS Sustain Chem Eng. 2018 Mar 5;6(3):3419-3425. doi: 10.1021/acssuschemeng.7b03648. Epub 2018 Jan 30.	5-Hydroxymethylfurfural (HMF) is a platform chemical that can be produced from renewable carbohydrate sources. HMF can be converted to 1,2,4-benzenetriol (BTO) which after catalytic hydrodeoxygenation provides a route to cyclohexanone and cyclohexanol. This mixture, known as KA oil, is an important feedstock for polymeric products such as nylons which use benzene as feedstock that is obtained from the BTX fraction produced in oil refineries. Therefore, the conversion of HMF to BTO provides a renewable, alternative route toward products such as nylons. However, BTO is usually considered an undesired byproduct in HMF synthesis and is only obtained in small amounts. Here, we show that Lewis acid catalysts can be utilized for the selective conversion of HMF to BTO in subsuper critical water. Overall, up to 54 mol % yield of BTO was achieved at 89% HMF conversion using ZnCl2. ZnCl2 and similarly effective Zn(OTf)2 and Fe(OTf)2 are known as relatively soft Lewis acids. Other Lewis acid like Hf(OTf)4 and Sc(OTf)3 gave increased selectivity toward levulinic acid (up to 33 mol %) instead of BTO, a well-known HMF derivative typically obtained by acid catalysis. Catalytic hydrodeoxygenation of BTO toward cyclohexanone in water was achieved in up to 45% yield using 5 wt % Pd on Al2O3 combined with AlCl3 or Al(OTf)3 as catalysts. Additionally, a mild selective oxygen induced dimerization pathway of BTO to 2,2',4,4',5,5'-hexahydroxybiphenyl (5,5'-BTO dimer) was identified.	HMF convertion to THB in lab	Synthesis	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
183	Kimura H, Nakahara M, Matubayasi N.	Solvent effect on pathways and mechanisms for D-fructose conversion to 5-hydroxymethyl-2-furaldehyde: in situ 13C NMR study.	J Phys Chem A. 2013 Mar 14;117(10):2102-13. doi: 10.1021/jp312002h. Epub 2013 Mar 4.	Noncatalytic reactions of D-fructose were kinetically investigated in dimethylsulfoxide (DMSO), water, and methanol as a function of time at temperatures of 30-150 °C by applying in situ (13)C NMR spectroscopy. The products were quantitatively analyzed with distinction of isomeric species by taking advantage of site-selective (13)C labeling technique. In DMSO, D-fructose was converted first into 3,4-dihydroxy-2-dihydroxymethyl-5-hydroxymethyltetrahydrofuran having no double bond in the ring, subsequently into 4-hydroxy-5-hydroxymethyl-4,5-dihydrofuran-2-carbaldehyde having one double bond through dehydration, and finally into 5-hydroxymethyl-2-furaldehyde (5-HMF) having two double bonds. No other reaction pathways were involved, as shown from the carbon mass balance. In water, 5-HMF, the final product in DMSO, was generated with the precursors undetected and furthermore transformed predominantly into formic and levulinic acids and slightly into 1,2,4-benzenetriol accompanied by polymerization. D-glucose was also produced through the reversible transformation of the reactant D-fructose. In methanol, some kinds of anhydro-D-fructoses were generated instead of 5-HMF. The reaction pathways can thus be controlled by taking advantage of the solvent effect. The D-fructose conversion reactions are of the first order with respect to the concentration of D-fructose and proceed on the order of minutes in DMSO but on the order of hours in water and methanol. The rate constant was three orders of magnitude larger in DMSO than in water or methanol.	Solvent effects on 5-HMF transformation into THB then polymerized	Synthesis	
184	Hu X, Lievens C, Larcher A, Li CZ.	Reaction pathways of glucose during esterification: effects of reaction parameters on the formation of humin type polymers.	Bioresour Technol. 2011 Nov;102(21):10104-13. doi: 10.1016/j.biortech.2011.08.040. Epub 2011 Aug 18.	The formation of humin-type polymers and other products during exposure of glucose to methanol/water mixtures with methanol/water mass ratios from 10 to 0.22 in the presence of the acid catalyst Amberlyst 70 was investigated. In water-rich medium (methanol/water mass ratio: 0.22), dehydration of glucose produced 5-(hydroxymethyl)furfural (HMF), furfural, and substantial amounts of polymer. In methanol-rich medium (methanol/water mass ratio: 10), the hydroxyl and carbonyl groups of glucose, HMF or furfural were protected via etherification and acetalisation. These protections stabilized these reactive compounds and significantly lowered the polymer formation (1.43% of the glucose loaded). The polymerization of glucose and HMF was also favored at high temperatures and long residence times. Conversely, high catalyst dosage mainly accelerated the conversion of glucose to methyl levulinate. Thus, the polymerization of glucose and HMF can be suppressed in methanol/water mixtures with high methanol ratios, at low temperatures and short residence times.	Humin type polymers from glucose and 5HMF (dehydration)	Synthesis	
185	Hansen CA, Frost JW.	Deoxygenation of polyhydroxybenzenes: an alternative strategy for the benzene-free synthesis of aromatic chemicals.	J Am Chem Soc. 2002 May 29;124(21):5926-7.	New synthetic connections have been established between glucose and aromatic chemicals such as pyrogallol, hydroquinone, and resorcinol. The centerpiece of this approach is the removal of one oxygen atom from 1,2,3,4-tetrahydroxybenzene, hydroxyhydroquinone, and phloroglucinol methyl ether to form pyrogallol, hydroquinone, and resorcinol, respectively. Deoxygenations are accomplished by Rh-catalyzed hydrogenation of the starting polyhydroxybenzenes followed by acid-catalyzed dehydration of putative dihydro intermediates. Pyrogallol synthesis consists of converting glucose into myo-inositol, oxidation to myo-2-inosose, dehydration to 1,2,3,4-tetrahydroxybenzene, and deoxygenation to form pyrogallol. Synthesis of pyrogallol via myo-2-inosose requires 4 enzyme-catalyzed and 2 chemical steps. For comparison, synthesis of pyrogallol from glucose via gallic acid intermediacy and the shikimate pathway requires at least 20 enzyme-catalyzed steps. A new benzene-free synthesis of hydroquinone employs conversion of glucose into 2-deoxy-scyllo-inosose, dehydration of this inosose to hydroxyhydroquinone, and subsequent deoxygenation to form hydroquinone. Synthesis of hydroquinone via 2-deoxy-scyllo-inosose requires 2 enzyme-catalyzed and 2 chemical steps. By contrast, synthesis of hydroquinone using the shikimate pathway and intermediacy of quinic acid requires 18 enzyme-catalyzed steps and 1 chemical step. Methylation of triacetic acid lactone, cyclization, and regioselective deoxygenation of phloroglucinol methyl ether affords resorcinol. Given the ability to synthesize triacetic acid lactone from glucose, this constitutes the first benzene-free route for the synthesis of resorcinol.	New synthetic pathway connections between glucose and polyphenols described	Synthesis	
186	Choi S, Il Kim H, Hag Park S, Jung Lee M, Yeoul Jun J, Lee Kim H, Hoon Chung J, Ho Yeum C.	Endothelium-dependent vasodilation by ferulic acid in aorta from chronic renal hypertensive rats.	Kidney Res Clin Pract. 2012 Dec;31(4):227-33. doi: 10.1016/j.krcp.2012.09.001. Epub 2012 Sep 21.	BACKGROUND: Ferulic acid (FA) is a naturally occurring nutritional compound. Although it has been shown to have antihypertensive effects, its effects on vascular function have not been established. The aim of this study was to assess the vasoreactivity of FA in chronic two-kidney, one-clip (2K1C) renal hypertensive rats. METHODS: Hypertension was induced in 2K1C rats by clipping the left renal artery and age-matched rats that received a sham treatment served as a control. Thoracic aortas were mounted in tissue baths to measure isometric tension. The effects of FA on vasodilatory responses were evaluated based on contractile responses induced by phenylephrine in the aortic rings obtained from both 2K1C and sham rats. Basal nitric oxide (NO) bioavailability in the aorta was determined by the contractile response induced by NO synthase inhibitor N (G)-nitro-l-arginine methyl ester (l-NAME). RESULTS: FA induced concentration-dependent relaxation responses which were greater in 2K1C hypertensive rats than in sham-clipped control rats. This relaxation induced by FA was partially blocked by the removal of endothelium or by pretreating with l-NAME. l-NAME-induced contractile responses were augmented by FA in 2K1C rats, while no significant differences were noted in sham rats. FA improved acetylcholine-induced endothelium-dependent vasodilation in 2K1C rats, but not in sham rats. The simultaneous addition of hydroxyhydroquinone significantly inhibited the increase in acetylcholine-induced vasodilation by FA. CONCLUSION: These results suggest that FA restores endothelial function by altering the bioavailability of NO in 2K1C hypertensive rats. The results explain, in part, the mechanism underlying the vascular effects of FA in chronic renal hypertension.	THB reduced vasodilation of Ferulic Acid; mechanistic hypothesis on action of FA	Ferulic Acid	
187	Suzuki A, Yamamoto M, Jokura H, Fujii A, Tokimitsu I, Hase T, Saito I.	Ferulic acid restores endothelium-dependent vasodilation in aortas of spontaneously hypertensive rats.	Am J Hypertens. 2007 May;20(5):508-13.	BACKGROUND: Ferulic acid (FA), a phytochemical constituent, has antihypertensive effects, but a detailed understanding of its effects on vascular function remains unclear. The vasoreactivity of FA was assessed using aortic rings isolated from normotensive Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR). METHODS: The effects of FA (10(-5) to 10(-3) mol/L) on vasodilatory responses were evaluated based on contractile responses induced by phenylephrine (10(-6) mol/L) in thoracic aortic rings from male WKY rats and SHR. Basal nitric oxide (NO) bioavailability in the aorta was determined from the contractile response induced by the NO synthase inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME, 10(-4) mol/L). The effects of FA on the production of NADPH-dependent superoxide anion were examined in SHR aortas. The impact of hydroxyhydroquinone, a generator of superoxide anions, on the FA-induced enhancement in acetylcholine-stimulated vasodilation was also investigated. RESULTS: The FA (10(-3) mol/L)-induced relaxation was partially blocked by removal of the endothelium or by pretreating SHR aortas with L-NAME. FA increased NO bioavailability, and decreased NADPH-dependent superoxide anion levels in SHR aortas. Ferulic acid improved acetylcholine-induced endothelium-dependent vasodilation in SHR, but not in WKY. Furthermore, the simultaneous addition of hydroxyhydroquinone significantly inhibited the increase in acetylcholine-induced vasodilation by FA. CONCLUSIONS: Ferulic acid restores endothelial function through enhancing the bioavailability of basal and stimulated NO in SHR aortas. The results explain, in part, the mechanisms underlying the effects of FA on blood pressure (BP) in SHR.	Ferulic Acid	Ferulic Acid	
188	Kong C, Huang H, Xue Y, Liu Y, Peng Q, Liu Q, Xu Q, Zhu Q, Yin Y, Zhou X, Zhang Y, Cai M.	Heterologous pathway assembly reveals molecular steps of fungal terreic acid biosynthesis.	Sci Rep. 2018 Feb 1;8(1):2116. doi: 10.1038/s41598-018-20514-x.	Terreic acid is a potential anticancer drug as it inhibits Bruton's tyrosine kinase; however, its biosynthetic molecular steps remain unclear. In this work, the individual reactions of terreic acid biosynthesis were determined by stepwise pathway assembly in a heterologous host, Pichia pastoris, on the basis of previous knockout studies in a native host, Aspergillus terreus. Polyketide synthase AtX was found to catalyze the formation of partially reduced polyketide 6-methylsalicylic acid, followed by 3-methylcatechol synthesis by salicylate 1-monooxygenase AtA-mediated decarboxylative hydroxylation of 6-methylsalicylic acid. Our results show that cytochrome P450 monooxygenase AtE hydroxylates 3-methylcatechol, thus producing the next product, 3-methyl-1,2,4-benzenetriol. A smaller putative cytochrome P450 monooxygenase, AtG, assists with this step. Then, AtD causes epoxidation and hydroxyl oxidation of 3-methyl-1,2,4-benzenetriol and produces a compound terremutin, via which the previously unknown function of AtD was identified as cyclooxygenation. The final step involves an oxidation reaction of a hydroxyl group by a glucose-methanol-choline oxidoreductase, AtC, which leads to the final product: terreic acid. Functions of AtD and AtG were determined for the first time. All the genes were reanalyzed and all intermediates and final products were isolated and identified. Our model fully defines the molecular steps and corrects previous	Synthesis using 3-methyl THB	Chemistry	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
189	Villota N, Lomas JM, Camarero LM.	Effect of ultrasonic waves on the water turbidity during the oxidation of phenol. Formation of (hydro)peroxo complexes.	Ultrason Sonochem. 2017 Nov;39:439-445. doi: 10.1016/j.ultsonch.2017.05.024. Epub 2017 May 16.	Analysis of the kinetics of aqueous phenol oxidation by a sono-Fenton process reveals that the via involving ortho-substituted intermediates prevails: catechol (25.0%), hydroquinone (7.7%) and resorcinol (0.6%). During the oxidation, water rapidly acquires color that reaches its maximum intensity at the maximum concentration of p-benzoquinone. Turbidity formation occurs at a slower rate. Oxidant dosage determines the nature of the intermediates, being trihydroxylated benzenes (pyrogallol, hydroxyhydroquinone) and muconic acid the main precursors causing turbidity. It is found that the concentration of iron species and ultrasonic waves affects the intensity of the turbidity. The pathway of (hydro)peroxo-iron(II) complexes formation is proposed. Operating with 20.0-27.8mgFe2+/kW rates leads to formation of (hydro)peroxo-iron(II) complexes, which induce high turbidity levels. These species would dissociate into ZZ-muconic acid and ferrous ions. Applying relationships around 13.9mgFe2+/kW, the formation of (hydro)peroxo-iron(III) complexes would occur, which could react with carboxylic acids (2,5-dioxo-3-hexenedioic acid). That reaction induces turbidity slower. This is due to the organic substrate reacting with two molecules of the (hydro)peroxo complex. Therefore, it is necessary to accelerate the iron regeneration, intensifying the ultrasonic irradiation. Afterwards, this complex would dissociate into maleic acid and ferric ions.	Kinetics of Phenol oxidation	Chemistry	ROS formation
190	Thavasi V, Bettens RP, Leong LP.	Temperature and solvent effects on radical scavenging ability of phenols.	J Phys Chem A. 2009 Apr 2;113(13):3068-77. doi: 10.1021/jp806679v.	In this work we have demonstrated the free radical scavenging ability of two-hydroxy (catechol, hydroquinone, resorcinol) and three-hydroxy (phloroglucinol, pyrogallol, 1,2,4-benzenetriol) phenols against the diphenylpicrylhydrazyl radical at various temperatures (15-40 degrees C) and in different solvent media. Kinetic measurements, made by the stopped-flow method, showed that the phenols with OH groups in the ortho positions have the largest rate coefficients compared to those with OH groups in the meta and para positions at all temperatures and in all solvent media. Among the ortho-structured phenols catechol, pyrogallol, and 1,2,4-benzenetriol, pyrogallol (three OH groups ortho to each other) had the greatest radical scavenging ability. This suggested that intramolecular hydrogen bonding in phenols controlled the rate of radical scavenging ability. The radical scavenging ability of phenols was fastest in methanol and slowest in THF, which emphasized the importance of the interactive behavior of the phenolic OH with the solvent. We concluded from our kinetic data together with our theoretically calculated OH bond dissociation enthalpies of phenols that the OH position played a crucial role in addition to the temperature and nature of the medium in determining the rate of the radical scavenging ability of polyphenols.	THB shown to function as free radical scavenger. Efficiency varies with substitution pattern and temperature	Chemistry	ROS formation
191	West LM, Faulkner DJ.	Acanthosulfate, a sulfated hydroxyhydroquinone sesterterpenoid from the sponge Acanthodendrilla sp.	J Nat Prod. 2008 Feb;71(2):269-71. doi: 10.1021/np070443h. Epub 2008 Feb 8.	The marine sponge Acanthodendrilla sp. contains the proteasome inhibitor acanthosulfate (4), a disulfated merosesterterpene having a scalarane-type skeleton. The structure of acanthosulfate (4), which possesses an unusual configuration, was elucidated by interpretation of spectroscopic data.	Detection of a sulfaetd THB in marine sponge	Chemistry	
192	Ammar S, Mahjoub MA, Charfi N, Skandarani I, Chekir-Ghedira L, Mighri Z.	Mutagenic, antimutagenic and antioxidant activities of a new class of beta-glucoside hydroxyhydroquinone from Anagallis monelli growing in Tunisia.	Chem Pharm Bull (Tokyo). 2007 Mar;55(3):385-8.	A new skeleton of an O-heteroside natural substance named zinolol, the first representative of a new class of aminated hydroxyhydroquinone, has been isolated from the whole plant Anagallis monelli. Its structure has been established by one and two dimensional NMR spectroscopic procedures. Antioxidant, mutagenic, antimutagenic activities were realised and positive results were recorded.	THB glucoside isolated in Anagallis monelli	Chemistry	
193	Sirés I, Arias C, Cabot PL, Centellas F, Garrido JA, Rodríguez RM, Brillas E.	Degradation of clofibric acid in acidic aqueous medium by electro-Fenton and photoelectro-Fenton.	Chemosphere. 2007 Jan;66(9):1660-9. Epub 2006 Aug 30.	Acidic aqueous solutions of clofibric acid (2-(4-chlorophenoxy)-2-methylpropionic acid), the bioactive metabolite of various lipid-regulating drugs, have been degraded by indirect electrooxidation methods such as electro-Fenton and photoelectro-Fenton with Fe(2+) as catalyst using an undivided electrolytic cell with a Pt anode and an O(2)-diffusion cathode able to electrogenerate H(2)O(2). At pH 3.0 about 80% of mineralization is achieved with the electro-Fenton method due to the efficient production of oxidant hydroxyl radical from Fenton's reaction between Fe(2+) and H(2)O(2), but stable Fe(3+) complexes are formed. The photoelectro-Fenton method favors the photodecomposition of these species under UVA irradiation, reaching more than 96% of decontamination. The mineralization current efficiency increases with rising metabolite concentration up to saturation and with decreasing current density. The photoelectro-Fenton method is then viable for treating acidic wastewaters containing this pollutant. Comparative degradation by anodic oxidation (without Fe(2+)) yields poor decontamination. Chloride ion is released during all degradation processes. The decay kinetics of clofibric acid always follows a pseudo-first-order reaction, with a similar rate constant in electro-Fenton and photoelectro-Fenton that increases with rising current density, but decreases at greater metabolite concentration. 4-Chlorophenol, 4-chlorocatechol, 4-chlororesorcinol, hydroquinone, p-benzoquinone and 1,2,4-benzenetriol, along with carboxylic acids such as 2-hydroxyisobutyric, tartronic, maleic, fumaric, formic and oxalic, are detected as intermediates. The ultimate product is oxalic acid, which forms very stable Fe(3+)-oxalato complexes under electro-Fenton conditions. These complexes are efficiently photodecarboxylated in photoelectro-Fenton under the action of UVA light.	THB is one of intermediate detected in the electro-Fenton Reaction of clofibric acid to end in oxalic acid which complexes with Fe3+	Chemistry	ROS formation
194	Thavasi V, Leong LP, Bettens RP.	Investigation of the influence of hydroxy groups on the radical scavenging ability of polyphenols.	J Phys Chem A. 2006 Apr 13;110(14):4918-23.	Recently, O-H bond dissociation enthalpies (BDEs) have been successfully used to express the free radical scavenging ability of polyphenolic antioxidants. In this work, the BDEs of phenol, catechol, resorcinol, hydroquinone, pyrogallol, phloroglucinol, 1,2,4-benzenetriol, and 5-hydroxypyrogallol have been calculated at B3LYP/6-311G++(3df, 3pd) and used to elucidate the effect of OH groups. Increasing the number of OH groups in the adjacent (vicinal) position decreases the BDE of phenols. Increasing the number of O-H groups in the alternative position C(1,3) as in resorcinol and C(1,3,5) as in phloroglucinol does not show any notable change in the BDEs when compared to that of OH in C(1) as in phenol. 5-Hydroxypyrogallol has the smallest BDE (250.3 kJ mol(-1)) followed by pyrogallol (289.4 kJ mol(-1)), then 1,2,4-benzenetriol (294.8 kJ mol(-1)), and then catechol (312.8 kJ mol(-1)). Overall, our results indicated that the presence of ortho and para hydroxy groups reduces the BDEs. An intramolecular hydrogen bond (IHB) develops due to the ortho arrangement of OH's and plays a dominant role in decreasing the BDEs. This key study on phenols showed that the reactive order of OH position in the benzene ring is the following: 5-hydroxypyrogallol > pyrogallol > 1,2,4-benzenetriol > catechol > hydroquinone >> phenol approximately resorcinol approximately phloroglucinol.	Enthalpies of dissociation are related to substitution pattern THB being lower than catechol and HQ	Chemistry	ROS formation
195	Poitras M, Bernier S, Boulay G, Fournier A, Guillemette G.	Interaction of benzene 1,2,4-trisphosphate with inositol 1,4,5-trisphosphate receptor and metabolizing enzymes.	Eur J Pharmacol. 1993 Feb 15;244(3):203-10.	In a wide variety of cells, inositol 1,4,5-trisphosphate (InsP3) is an important second messenger involved in the regulation of intracellular Ca2+ concentration. InsP3 interacts with specific receptors and triggers the release of sequestered Ca2+ from an internal store. We have synthesized a structural analogue of InsP3 by phosphorylation of the free hydroxyl groups of 1,2,4-benzenetriol with dibenzylphosphorochloridate. The product benzene 1,2,4-trisphosphate (BzP3) was shown to interact with InsP3 receptor and InsP3 metabolizing enzymes of bovine adrenal cortex. BzP3 competitively blocked InsP3 binding to adrenal cortex microsomes with a half-maximal efficiency at 34 microM. This affinity was about 10,000 times lower than that of InsP3 for its receptor. The Ca2+ releasing activity of BzP3 on the same microsomal preparation was monitored with the fluorescent indicator fura-2. BzP3 had no agonistic effect on this activity but it was able to inhibit InsP3-induced Ca2+ release in a dose-dependent manner. The activity of InsP3 phosphatase was also studied. BzP3 inhibited the activity of the phosphatase with a half-maximal efficiency of 32 microM. BzP3 was also able to inhibit the activity of the cytosolic InsP3 kinase with a half-maximal efficiency of 6.1 microM. These results show that BzP3 is interacting with the three specific recognition sites for InsP3 in the bovine adrenal cortex. The inhibitory effect of this compound is relatively more potent on the metabolizing enzymes than on the Ca(2+)-mobilizing receptor.	Triphosphate derivative of THB studied as an inhibitor of InsP3 receptor and phosphatase	Chemistry	
196	Ewers J, Rubio MA, Knackmuss HJ, Freier-Schröder D.	Bacterial metabolism of 2,6-xylenol.	Appl Environ Microbiol. 1989 Nov;55(11):2904-8.	Strain DM1, a Mycobacterium sp. that utilizes 2,6-xylenol, 2,3,6-trimethylphenol, and o-cresol as sources of carbon and energy, was isolated. Intact cells of Mycobacterium strain DM1 grown with 2,6-xylenol cooxidized 2,4,6-trimethylphenol to 2,4,6-trimethylresorcinol. 4-Chloro-3,5-dimethylphenol prevents 2,6-xylenol from being totally degraded; it was quantitatively converted to 2,6-dimethylhydroquinone by resting cells. 2,6-Dimethylhydroquinone, citraconate, and an unidentified metabolite were detected as products of 2,6-xylenol oxidation in cells that were partially inactivated by EDTA. Under oxygen limitation, 2,6-dimethylhy-droquinone, citraconate, and an unidentified metabolite were released during 2,6-xylenol turnover by resting cells. Cell extracts of 2,6-xylenol-grown cells contained a 2,6-dimethylhydroquinone-converting enzyme. When supplemented with NADH, cell extracts catalyzed the reduction of 2,6-dimethyl-3-hydroxyquinone to 2,6-dimethyl-3-hydroxyhydroquinone. Since a citraconase was also demonstrated in cell extracts, a new metabolic pathway with 2,6-dimethyl-3-hydroxyhydroquinone as the ring fission substrate is proposed.		Chemistry	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
197	Sekiguchi J, Katayama S, Yamada Y.	6-Methyl-1,2,4-benzenetriol, a new intermediate in penicillic acid biosynthesis in Penicillium cyclopium.	Appl Environ Microbiol. 1987 Jul;53(7):1531-5.	Penicillic acid-negative mutants were obtained from a color mutant derived from Penicillium cyclopium NRRL 1888 through N-methyl-N'-nitro-N-nitrosoguanidine treatment. One mutant (SK2N6) accumulated 6-methyl-1,2,4-benzenetriol, which was not previously known to be a metabolite of P. cyclopium, in addition to orsellinic acid and orcinol. The radioactivity of [1-14C]acetic acid was rapidly incorporated into 6-methyl-1,2,4-benzenetriol in a culture of P. cyclopium SK2N6. Moreover, the radioactivity of [14C]6-methyl-1,2,4-benzenetriol was efficiently incorporated into penicillic acid in a culture of P. cyclopium NRRL 1888. These data indicate that 6-methyl-1,2,4-benzenetriol is a precursor for penicillic acid biosynthesis. The results on the addition of 1,4-dihydroxy-6-methoxy-2-methylbenzene, 6-methoxy-2-methylbenzoquinone(1,4), and 1-O-methylorcinol to a culture of P. cyclopium SK2N6 indicated that only the former two compounds are converted to penicillic acid. Thus, a new portion of the penicillic acid biosynthetic pathway is proposed.		Chemistry	
198	Li XF, Wu XR, Xue M, Wang Y, Wang J, Li Y, Suriguga, Zhang GY, Yi ZC.	The role of DNA methylation in catechol-enhanced erythroid differentiation of K562 cells.	Toxicol Appl Pharmacol. 2012 Nov 15;265(1):43-50. doi: 10.1016/j.taap.2012.09.018. Epub 2012 Sep 27.	Catechol is one of phenolic metabolites of benzene in vivo. Catechol is also widely used in pharmaceutical and chemical industries. In addition, fruits, vegetables and cigarette smoke also contain catechol. Our previous study showed that several benzene metabolites (phenol, hydroquinone, and 1,2,4-benzenetriol) inhibited erythroid differentiation of K562 cells. In present study, the effect of catechol on erythroid differentiation of K562 cells was investigated. Moreover, to address the role of DNA methylation in catechol-induced effect on erythroid differentiation in K562 cells, methylation levels of erythroid-specific genes were analyzed by Quantitative MassARRAY methylation analysis platform. Benzidine staining showed that exposure to catechol enhanced hemin-induced hemoglobin accumulation in K562 cells in concentration- and time-dependent manners. The mRNA expression of erythroid specific genes, including α-globin, β-globin, γ-globin, erythroid 5-aminolevulinate synthase, erythroid porphobilinogen deaminase, and transcription factor GATA-1 genes, showed a significant concentration-dependent increase in catechol-treated K562 cells. The exposure to catechol caused a decrease in DNA methylation levels at a few CpG sites in some erythroid specific genes including α-globin, β-globin and erythroid porphobilinogen deaminase genes. These results indicated that catechol improved erythroid differentiation potency of K562 cells at least partly via up-regulating transcription of some erythroid related genes, and suggested that inhibition of DNA methylation might be involved in up-regulated expression of some erythroid related genes.	Catechol as a benzene metabolite inhibition of erythroid differntiation; DNA methylation effects on K562 cells	Catechol	
199	Bajorowicz B, Kowalska E, Nadolna J, Wei Z, Endo M, Ohtani B, Zaleska-Medynska A.	Preparation of CdS and Bi2S3 quantum dots co-decorated perovskite-type KNbO3 ternary heterostructure with improved visible light photocatalytic activity and stability for phenol degradation.	Dalton Trans. 2018 Oct 15. doi: 10.1039/c8dt03094d. [Epub ahead of print]	Hydroxyhydroquinone (HHQ) was characterized kinetically as a tyrosinase substrate. A kinetic mechanism is proposed, in which HHQ is considered as a monophenol or as an o-diphenol, depending on the part of the molecule that interacts with the enzyme. The kinetic parameters obtained from an analysis of the measurements of the initial steady state rate of 2-hydroxy p-benzoquinone formation were kcatapp=229.0±7.7 s(-1) and KMapp,HHQ=0.40±0.05 mM. Furthermore, the action of tyrosinase on HHQ led to the enzyme's inactivation through a suicide inactivation mechanism. This suicide inactivation process was characterized kinetically by λmaxapp (the apparent maximum inactivation constant) and r, the number of turnovers made by 1 mol of enzyme before being inactivated. The values of λmaxapp and r were (8.2±0.1)×10(-3) s(-1) and 35,740±2,548, respectively.	Kinetics of THB binding with tyrosinase	Degradation Pathway	
200	García-Molina Mdel M, Muñoz Muñoz JL, Martinez-Ortiz F, Martinez JR, García-Ruiz PA, Rodriguez-López JN, García-Cánovas F.	Tyrosinase-catalyzed hydroxylation of hydroquinone, a depigmenting agent, to hydroxyhydroquinone: A kinetic study.	Bioorg Med Chem. 2014 Jul 1;22(13):3360-9. doi: 10.1016/j.bmc.2014.04.048. Epub 2014 May 4.	Hydroquinone (HQ) is used as a depigmenting agent. In this work we demonstrate that tyrosinase hydroxylates HQ to 2-hydroxyhydroquinone (HHQ). Oxy-tyrosinase hydroxylates HQ to HHQ forming the complex met-tyrosinase-HHQ, which can evolve in two different ways, forming deoxy-tyrosinase and p-hydroxy-o-quinone, which rapidly isomerizes to 2-hydroxy-p-benzoquinone or on the other way generating met-tyrosinase and HHQ. In the latter case, HHQ is rapidly oxidized by oxygen to generate 2-hydroxy-p-benzoquinone, and therefore, it cannot close the enzyme catalytic cycle for the lack of reductant (HHQ). However, in the presence of hydrogen peroxide, met-tyrosinase (inactive on hydroquinone) is transformed into oxy-tyrosinase, which is active on HQ. Similarly, in the presence of ascorbic acid, HQ is transformed into 2-hydroxy-p-benzoquinone by the action of tyrosinase; however, in this case, ascorbic acid reduces met-tyrosinase to deoxy-tyrosinase, which after binding to oxygen, originates oxy-tyrosinase. This enzymatic form is now capable of reacting with HQ to generate p-hydroxy-o-quinone, which rapidly isomerizes to 2-hydroxy-p-benzoquinone. The formation of HHQ during the action of tyrosinase on HQ is demonstrated by means of high performance liquid chromatography mass spectrometry (HPLC-MS) by using hydrogen peroxide and high ascorbic acid concentrations. We propose a kinetic mechanism for the tyrosinase oxidation of HQ which allows us the kinetic characterization of the process. A possible explanation of the cytotoxic effect of HQ is discussed.	THB as a product of hydroquinone hydroxylation	Degradation Pathway	
201	del Mar Garcia-Molina M, Muñoz-Muñoz JL, Berna J, García-Ruiz PA, Rodriguez-Lopez JN, Garcia-Canovas F.	Catalysis and inactivation of tyrosinase in its action on hydroxyhydroquinone.	IUBMB Life. 2014 Feb;66(2):122-7. doi: 10.1002/iub.1250. Epub 2014 Feb 27.	Hydroxyhydroquinone (HHQ) was characterized kinetically as a tyrosinase substrate. A kinetic mechanism is proposed, in which HHQ is considered as a monophenol or as an o-diphenol, depending on the part of the molecule that interacts with the enzyme. The kinetic parameters obtained from an analysis of the measurements of the initial steady state rate of 2-hydroxy p-benzoquinone formation were kcatapp=229.0±7.7 s(-1) and KMapp,HHQ=0.40±0.05 mM. Furthermore, the action of tyrosinase on HHQ led to the enzyme's inactivation through a suicide inactivation mechanism. This suicide inactivation process was characterized kinetically by λmaxapp (the apparent maximum inactivation constant) and r, the number of turnovers made by 1 mol of enzyme before being inactivated. The values of λmaxapp and r were (8.2±0.1)×10(-3) s(-1) and 35,740±2,548, respectively.	Tyrosinase inactivation by THB	Degradation Pathway	
202	Muñoz JL, García-Molina F, Varón R, Rodriguez-Lopez JN, García-Cánovas F, Tudela J.	Calculating molar absorptivities for quinones: application to the measurement of tyrosinase activity.	Anal Biochem. 2006 Apr 1;351(1):128-38. Epub 2006 Jan 26.	The molar absorptivities of the quinones produced from different o-diphenols, triphenols, and flavonoids were calculated by generating the respective quinones through oxidation with an excess of periodate. Oxidation of these substrates by this reagent was analogous to oxidation by tyrosinase with molecular oxygen, although the procedure showed several advantages over the enzymatic method in that oxidation took place almost immediately and quinone stability was favored because no substrate remained. The o-diphenols studied were pyrocatechol, 4-methylcatechol, 4-tert-butylcatechol, 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylethylamine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid, and caffeic acid; the triphenols studied were pyrogallol, 1,2,4-benzenetriol, 6-hydroxydopa, and 6-hydroxydopamine; and the flavonoids studied were (+)catechin, (-)epicatechin, and quercetin. In addition, the stability of the quinones generated by oxidation of the compounds by [periodate]0/[substrate]0 << 1 was studied. Taking the findings into account, tyrosinase could be measured by following o-quinone formation in rapid kinetic studies using the stopped-flow method. However, measuring o-quinone formation could not be useful for steady-state studies. Therefore, several methods for following tyrosinase activity are proposed, and a kinetic characterization of the enzyme's action on these substrates is made.	Polyphenols as precursors of quinones; then molar absortivity was measured	Degradation Pathway	
203	Xie H, Duan K, Xue M, Du Y, Wang C.	Photoelectrocatalytic analysis and electrocatalytic determination of hydroquinone by using a Cu2O-reduced graphene oxide nanocomposite modified rotating ring-disk electrode.	Analyst. 2016 Aug 7;141(15):4772-81. doi: 10.1039/c6an00545d. Epub 2016 Jun 14.	Reduced graphene oxide (rGO)-based Cu2O nanocomposites were prepared by a facile one-pot reaction process. The surface morphology, structure and chemical composition of Cu2O-rGO nanocomposites were characterized by transmission electron microscopy, X-ray photoelectron spectroscopy and X-ray diffraction. The Cu2O-rGO modified Pt rotating ring-disk electrode (RRDE) was successfully fabricated for the photoelectrocatalytic analysis of hydroquinone (HQ). The photoelectrochemical behaviors of HQ were investigated by the hydrodynamic differential pulse voltammetry technique, using the Cu2O-rGO modified Pt RRDE as the working electrode. The effects of pH values, rotation rates, illumination time and applied bias potential have been discussed. The possible electroactive intermediate product, namely hydroxyhydroquinone, was obtained through the photoelectrocatalytic degradation of HQ on the Cu2O-rGO modified Pt disk electrode, which was compulsively transported and could only be detected at the bare Pt ring electrode at around +0.02 V with an oxidation signal. We found that the peak current at +0.02 V had a good linear relationship with the HQ concentration in the range from 5.0 × 10(-6) to 1.0 × 10(-3) M, with a low limit of detection and excellent reproducibility. The present work has demonstrated that Cu2O-rGO nanocomposites have enhanced photoelectrocatalytic ability for the degradation of organic pollutants and this modified RRDE technique can be potentially applied for the in situ determination of organic pollutants.	Photoelectric determination of THB	Analytical Methods	

#	Authors	Title	Reference	Abstract	Short Summary	Category	MoA
204	Fan SL, Zhang LK, Lin JM.	Post-column detection of benzenediols and 1,2,4-benzenetriol based on acidic potassium permanganate chemiluminescence.	Talanta. 2006 Jan 15;68(3):646-52. doi: 10.1016/j.talanta.2005.05.004. Epub 2005 Jun 13.	Based on the sensitizing effect of formic acid on the chemiluminescence (CL) reaction of polyhydroxylbenzenes with acidified potassium permanganate and the combination technique of high-performance liquid chromatography (HPLC), a sensitive, selective and simple post-column CL detection method for simultaneously determining catechol, resorcinol, hydroquinone and 1,2,4-benzenetriol is described. The optimal conditions for the CL detection and HPLC separation were carried out. The linear ranges were: 6.0 x 10(-3)-1.5 mg/L for hydroquinone, 8.0 x 10(-3)-1.5 mg/L for 1,2,4-benzenetriol, 1.0 x 10(-2)-2.0 mg/L for resorcinol and 1.0 x 10(-2)-2.5 mg/L for catechol, respectively. The detection limits are: 3.2 x 10(-3) mg/L for hydroquinone, 3.9 x 10(-3) mg/L for 1,2,4-benzenetriol, 4.7 x 10(-3) mg/L for resorcinol and 5.2 x 10(-3) mg/L for catechol, respectively. Combining with solid phase extraction, the proposed method has been successfully applied to the determination of the polyhydroxylbenzenes in river water. The recoveries for three benzentriols were 92.1-95.4% and 82.0% for 1,2,4-benzenetriol, respectively.	HPLC determination of polyphenols	Analytical Methods	
205	Lei Y, Mulchandani P, Chen W, Wang J, Mulchandani A.	Whole cell-enzyme hybrid amperometric biosensor for direct determination of organophosphorous nerve agents with p-nitrophenyl substituent.	Biotechnol Bioeng. 2004 Mar 30;85(7):706-13.	In this paper, we reported the construction of a hybrid biosensor for direct, highly selective, sensitive, and rapid quantitative determination of organophosphate pesticides with p-nitrophenyl substituent using purified organophosphorus hydrolase (OPH) for the initial hydrolysis and Arthrobacter sp. JS443 for subsequent p-nitrophenol oxidation. The biocatalytic layer was prepared by co-immobilizing Arthrobacter sp. JS443 and OPH on a carbon paste electrode. OPH catalyzed the hydrolysis of organophosphorus pesticides with p-nitrophenyl substituent such as paraoxon and methyl parathion to release p-nitrophenol that was oxidized by the enzymatic machinery of Arthrobacter sp. JS443 to carbon dioxide through electroactive intermediates 4-nitrocatechol and 1,2,4-benzenetriol. The oxidization current of the intermediates was measured and correlated to the concentration of organophosphates. The best sensitivity and response time were obtained using a sensor constructed with 0.06 mg dry weight of cell and 965 IU of OPH operating at 400 mV applied potential (vs. Ag/AgCl reference) in 50 mM citrate-phosphate pH 7.5 buffer at room temperature. Using these conditions, the biosensor measured as low as 2.8 ppb (10 nM) of paraoxon and 5.3 ppb (20 nM) of methyl parathion without interference from phenolic compounds, carbamate pesticides, triazine herbicides, and organophosphate pesticides that do not have the p-nitrophenyl substituent. The biosensor had excellent operational life-time stability with no decrease in response for more than 40 repeated uses over a 12-h period when stored at room temperature, while its storage life was approximately 2 days when stored in the operating buffer at 4 degrees C.	A biosensor for organophosphorous pesticides with pNP substituents is described	Analytical Methods	
206	Ong CN, Lee BL.	Determination of benzene and its metabolites: application in biological monitoring of environmental and occupational exposure to benzene.	J Chromatogr B Biomed Appl. 1994 Oct 3;660(1):1-22. Review.	Methods for the biological monitoring of benzene and its metabolites in exhaled air, blood and urine are reviewed. Analysis of benzene in breath can be carried out by using an exhaled-air collection tube and direct analysis by GC or GC-MS; however, this technique is less reliable when compared to analysis using blood or urine. For the determination of non-metabolized benzene in blood and urine, GC head-space analysis is recommended. Phenol, the major metabolite of benzene can be monitored by either HPLC or GC methods. However, urinary phenol has proved to be a poor biomarker for low-level benzene exposure. Recent studies have shown that trans,trans-muconic acid, a minor metabolite of benzene can be determined using HPLC with UV detection. This biomarker can be used for detection of low-level benzene exposure. Urinary S-phenylmercapturic acid is another sensitive biomarker for benzene, but it can be detected only by GC-MS. Hydroquinone, catechol and 1,2,4-benzenetriol can be measured using HPLC with either ultraviolet or fluorimetric detection. Nevertheless, their use for low-level assessment requires further studies. Eventually, for the assessment of health risks caused by benzene, biological-exposure reference values need to be established before they can be widely used in a field setting.		Analytical Methods	
207	Hofrichter M, Scheibner K.	Utilization of aromatic compounds by the Penicillium strain Bi 7/2.	J Basic Microbiol. 1993;33(4):227-32.	The Penicillium strain Bi 7/2 utilized phenol, catechol, resorcinol, hydroquinone, pyrogallol, hydroxyhydroquinone, phloroglucinol, m- and p-cresol, orcinol, 4-methylcatechol, 4-methoxyphenol, 4-aminophenol, benzyl alcohol, benzoic acid, 2-, 3- and 4-hydroxybenzoic acid, anthranilic acid, protocatechuic acid and gallic acid as sole sources of carbon and energy. The central metabolites catechol, protocatechuic acid and hydroxyquinone could be determined by HPLC with diode-array detection. Pathways for the degradation of aromatic substances were proposed.		Analytical Methods	
208	Krumholz LR, Bryant MP.	Characterization of the pyrogallol-phloroglucinol isomerase of Eubacterium oxidoreducens.	J Bacteriol. 1988 Jun;170(6):2472-9.	Cell extracts of Eubacterium oxidoreducens, in the presence of dimethyl sulfoxide, catalyzed the conversion of pyrogallol to phloroglucinol with methyl sulfide as a product. The isomerization reaction also proceeded when 1,2,3,5-benzenetetrol was present rather than dimethyl sulfoxide. An assay to quantitate this activity was developed. The assay followed the disappearance of 1,2,4-benzenetriol as determined colorimetrically after incubation with sodium molybdate at neutral pH. The products of this reaction were resorcinol and 2,6-dihydroxyquinone. The enzyme(s) catalyzing this reaction was purified fivefold from cells grown on gallate plus H2. The purification procedure involved treatment with 40% acetone, precipitation with ammonium sulfate, DEAE-cellulose chromatography, concentration by ultrafiltration (molecular weight cutoff, greater than 100,000), and hydroxylapatite chromatography. This preparation had a specific activity of 14.7 mumol/min per mg of protein and a pH optimum of about 7.3. It was strongly inhibited by p-chloromercuribenzoate. The mechanism of the reaction involved oxidation of the pyrogallol followed by introduction of water. The benzenetetrol intermediate was then reduced and dehydrated to phloroglucinol.		Analytical Methods	
209	Greenlee WF, Chism JP, Rickert DE.	A novel method for the separation and quantitation of benzene metabolites using high-pressure liquid chromatography.	Anal Biochem. 1981 Apr;112(2):367-70. No abstract available.	A method for the separation of benzene metabolites using reverse-phase high-pressure liquid chromatography is described. The antoxidant, ascorbic acid is added to an aqueous mixture of 1,2,4-benzenetriol, hydroquinone, catechol, and phenol, to prevent autooxidation. The eluting solvents are equilibrated with nitrogen, degassed, and maintained under a nitrogen atmosphere during the analysis. A highly resolved and reproducible profile of the metabolites is achieved under these conditions. This method should prove useful in a number of pharmacokinetic studies where the biotransformation of the parent compound to autooxidizable species such as polyphenols and quinones precludes analysis under aerobic conditions.		Analytical Methods	