Scientific Committee on Consumer Safety

SCCS

OPINION ON

Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes (nano)

The SCCS adopted this document
at its plenary meeting on 21-22 March 2023

ACKNOWLEDGMENTS

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

The SCCS members:
Dr U. Bernauer (Chairperson)
Dr L. Bodin
Prof. Q. Chaudhry
Prof. P.J. Coenraads
Prof. M. Dusinska
Dr E. Gaffet
Prof. E. Panteri (Rapporteur)
Dr M. Stepnik
Dr S. Wijnhoven

The SCHEER members
Dr W.H. de Jong

External experts
Dr N. von Goetz

All Declarations of Working Group members are available on the following webpage:
Register of Commission expert groups and other similar entities

1. ABSTRACT

The SCCS concludes the following:

1. In view of the above, and taking into account the scientific data provided, does the SCCS consider Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes safe when used in cosmetic products according to the maximum concentrations and specifications as reported via CPNP, taking into account reasonably foreseeable exposure conditions?

Having assessed the information provided by the Notifiers, and the information available from published literature, the SCCS has not been able to conclude on the safety of fullerenes and (hydrated) hydroxylated forms of fullerenes due to a number of uncertainties and data gaps in regard to physicochemical, toxicokinetic and toxicological aspects. These uncertainties and data gaps have been indicated in relevant sections of the Opinion and must be addressed by the Notifiers to enable a conclusion on the safety of the materials for use in cosmetic products.

In particular, the SCCS has not been able to conclude on the genotoxicity potential of fullerenes (C60 and C70). The available evidence indicates that hydrated forms of hydroxylated fullerenes are genotoxic and hence SCCS considers them as not safe for use in cosmetic products. In view of equivalence as discussed before (see section 3.1.1.5), the same concerns over genotoxicity potential also apply to hydroxylated fullerenes.

2. Based on the currently available scientific literature and SCCS' expert judgement, the SCCS is requested to assess any further scientific concerns with regard to the use of Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes in cosmetic products and whether a potential risk to human health can be identified according to Article 16(6) Reg.1223/2009.

In Annex-1 of this Opinion, the SCCS has noted the basis for concerns over risks that the use of fullerenes, hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes in cosmetic products may pose to the consumer. In brief, the SCCS has a concern in regard to:

- the potential presence of impurities, heavy metals, accompanying contaminants and/or organic solvents in the notified nanomaterials. Lack of data on stability of hydroxylated fullerenes and their hydrated forms.
- the potential ability of fullerenes and derivatives to induce production of free oxyradicals when used in cosmetic products.
- phototoxicity of hydroxylated fullerenes – with similar concerns for the hydrated forms of hydroxylated fullerenes.
- sensitising potential of hydroxylated fullerenes.
- dermal absorption and systemic availability of the nanoparticles after use in cosmetic products.
- distribution of systemically available fullerenes to various organs in the body and potential accumulation of the nanoparticles in certain organs – such as lungs and liver.
- the available information does not allow the SCCS to exclude genotoxic/carcinogenic potential of any of the materials assessed in this Opinion.

Keywords: SCCS, scientific opinion, Fullerenes, Hydroxylated Fullerenes, hydrated forms of Hydroxylated Fullerenes, nano, CAS/EC No. 99685-96-8/628-630-7, 11538-22-7/-, 182024-42-6/-, Regulation 1223/2009

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Contact
European Commission
Health and Food Safety
Directorate B: Public Health, Cancer and Health security
Unit B3: Health monitoring and cooperation, Health networks
L-2920 Luxembourg
SANTE-SCCS@ec.europa.eu

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TABLE OF CONTENTS

ACKNOWLEDGMENTS 2
1. ABSTRACT 3
2. MANDATE FROM THE EUROPEAN COMMISSION 7
3. OPINION 9
3.1 CHEMICAL AND PHYSICAL SPECIFICATIONS 9
3.1.1 Chemical identity 9
3.1.2 Physical form 13
3.1.3 Molecular weight 14
3.1.4 Purity, composition, and substance codes 14
3.1.5 Impurities / accompanying contaminants 19
3.1.6 Solubility 20
3.1.7 Partition coefficient (Log P_{ow}) 23
3.1.8 Additional physical and chemical specifications 24
3.1.9 Particle size 25
3.1.10 Crystal structure 30
3.1.11 UV absorption 30
3.1.12 Surface characteristics 31
3.1.13 Droplet size in formulations 32
3.1.14 Homogeneity and stability 32
3.1.15 Other parameters of characterisation 32
3.1.16 Summary on supplementary physicochemical characterisation 33
3.2 TOXICOKINETICS 34
3.2.1 Dermal / percutaneous absorption 34
3.2.2 Other studies on toxicokinetics 41
3.3 EXPOSURE ASSESSMENT 44
3.3.1 Function and uses 44
3.4 TOXICOLOGICAL EVALUATION 46
3.4.1 Acute toxicity 47
3.4.2 Irritation and corrosivity 51
3.4.3 Skin sensitisation 54
3.4.4 Repeated dose toxicity 56
3.4.5 Mutagenicity/genotoxicity 61
3.4.6 Carcinogenicity 70
3.4.7 Reproductive toxicity 71
3.4.8 Photo-induced toxicity 71
3.4.9 Human data 73
3.5 SAFETY EVALUATION (INCLUDING CALCULATION OF THE MOS) 74
3.6 DISCUSSION 74
4. CONCLUSION 75
5. MINORITY OPINION 76
6. REFERENCES 77
Annex 1 81

2. MANDATE FROM THE EUROPEAN COMMISSION

Background

Article 2(1)(k) of Regulation (EC) No. 1223/2009 (Cosmetics Regulation) states that "nanomaterial" means an insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm. In addition, the Commission Recommendation of 2011 on the definition of nanomaterial specifically addressed the issue of Fullerenes by stating: 'By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials'.

The nanomaterials definition covers materials in the nano-scale that are intentionally made and are insoluble/partially-soluble or biopersistent (e.g. metals, metal oxides, carbon materials, etc.). It does not cover those that are soluble or degradable/non-persistent in biological systems (e.g. liposomes, emulsions, etc.). Article 16 of the Cosmetics Regulation requires cosmetic products containing nanomaterials other than colorants, preservatives and UV-filters and not otherwise restricted by the Cosmetics Regulation to be notified to the Commission six months prior to being placed on the market. Article 19 of this Regulation requires nano-scale ingredients to be labelled (name of the ingredient, followed by 'nano' in brackets). If there are concerns over the safety of a notified nanomaterial, the Commission shall refer it to the Scientific Committee on Consumer Safety (SCCS) for a full risk assessment.

The Commission services received 19 notifications under Article 16 of the Cosmetics Regulation via the Cosmetic Product Notification Portal (CPNP) for cosmetic products containing Fullerenes, Hydroxylated Fullerenes (CAS/EC No.: 99685-96-8/628-630-7, 11538-22-7/-, 182024-42-6/-0), and hydrated forms of Hydroxylated Fullerenes (for example CAS / EC No.: 2803976-74-9/-)

According to the notifications submitted via the CPNP, Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes are used in cosmetic products with different concentration and specifications. These ingredients are reported in CosIng database with the function of 'antimicrobial' and 'skin conditioning-miscellaneous' and in the open literature as 'antioxidants' (scavenging ability against free radicals). Currently, Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes are not regulated under the Cosmetic Regulation (EC) No. 1223/2009.

The Commission has concerns on the use of Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes because of the potential for nanoparticles to be absorbed dermally or across a mucous membrane and to enter cells. Therefore, we request the SCCS to carry out a safety assessment of Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes reported in the notifications.

Terms of reference

1. In view of the above, and taking into account the scientific data provided, does the SCCS consider Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes safe when used in cosmetic products according to the maximum concentrations and specifications as reported via CPNP, taking into account reasonably foreseeable exposure conditions?

2. Based on the currently available scientific literature and SCCS' expert judgement, the SCCS is requested to assess any further scientific concerns with regard to the use of Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes in cosmetic products and whether a potential risk to human health can be identified according to Article 16(6) Reg.1223/2009.

3. OPINION

Preamble

The information provided by the Notifiers through CPNP on the materials considered in this Opinion (Fullerenes, Hydroxylated fullerenes and Hydrated forms of hydroxylated fullerenes) was assessed by the SCCS, and further clarifications were requested where necessary. Additionally, a call for information was made and a literature search was performed by the Commission to obtain further information from other sources. In developing this Opinion, the SCCS has therefore also considered the responses received from the Notifiers, the information received from the Commission's call for information, and the results of the literature search.

It needs to be emphasised that the safety evaluations carried out by the SCCS are limited to cosmetic ingredients, and not formulations. Two of the notified materials, Radical Sponge® and Lipofullerene® are formulations and are therefore out of scope for assessment in this Opinion. Radical Sponge® is a water-soluble polymer-enwrapped fullerene (PVP/C60 fullerene), and LipoFullerene® is an oil soluble fullerene in which fullerenes are dissolved in olive squalane. Only the fullerenes present in these formulations (Radical Sponge® and LipoFullerene®) can be considered as basic cosmetic ingredients that are covered in this assessment as ingredients but not as part of a formulation.

The SCCS has not evaluated safety of fullerene materials via inhalation exposure because application in sprayable or products that could lead to inhalation exposure of the consumer is not supported by the Notifiers.

3.1 CHEMICAL AND PHYSICAL SPECIFICATIONS

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Fullerenes:
IUPAC name:
(C60-Ih) [5,6] fullerene

Hydroxylated fullerenes: C60(OH)x [where x has been reported to range 24-60]

Hydrated forms of Hydroxylated Fullerenes:
INCI name: Hydroxylated Fullerene (and) Aqua, also termed as Hyperharmonized Fullerenol/Water Complex (HFWC).

Ref: 281_safety_file_2020-3-12-18-44-18.pdf

3.1.1.2 Chemical names

Fullerenes:
Fullerene (C60),
Fullerene(C70)

Ref: NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2

Hydroxylated fullerenes: /

Hydrated forms of Hydroxylated Fullerenes: /

3.1.1.3 Trade names and abbreviations

Fullerenes: /

Hydroxylated fullerenes: /

Hydrated forms of Hydroxylated Fullerenes:
Product name: 3HFWC, (or) HFWC

Ref: 281_spec_file_2020-2-28-19-37-53
Ref: PRODUCT INFORMATION DOSSIER-Radical Sponge_VC60 - V9

SCCS comment
Trade names were not provided for fullerene (C60 and C70) and hydroxylated fullerenes.

3.1.1.4 CAS / EC number

Fullerenes:
Fullerene C60: 99685-96-8/628-630-7
Fullerene C70: 115383-22-7/-

Ref: PRODUCT INFORMATION DOSSIER-Radical Sponge_VC60 - V9; NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2; https://pubchem.ncbi.nlm.nih.gov/compound/Buckminsterfullerene#section=Related-CAS

Hydroxylated fullerenes: 182024-42-6/-

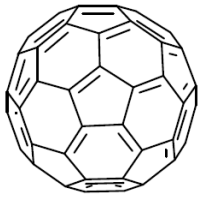
Hydrated forms of Hydroxylated Fullerenes: 2803976-74-9/-

3.1.1.5 Structural formula

Fullerenes:
A polyhedral carbon structure composed of around 60-80 carbon atoms in pentagon and hexagon configuration. They are named after Buckminster Fuller because of structural resemblance to geodesic domes. Fullerenes can be made in high temperatures, such as arc discharge in an inert atmosphere.

Fullerene C60 and Fullerene C70:
The molecular structures, shape and size of Fullerene C60 and Fullerene C70 are shown in Table 1, as given by the notifier.

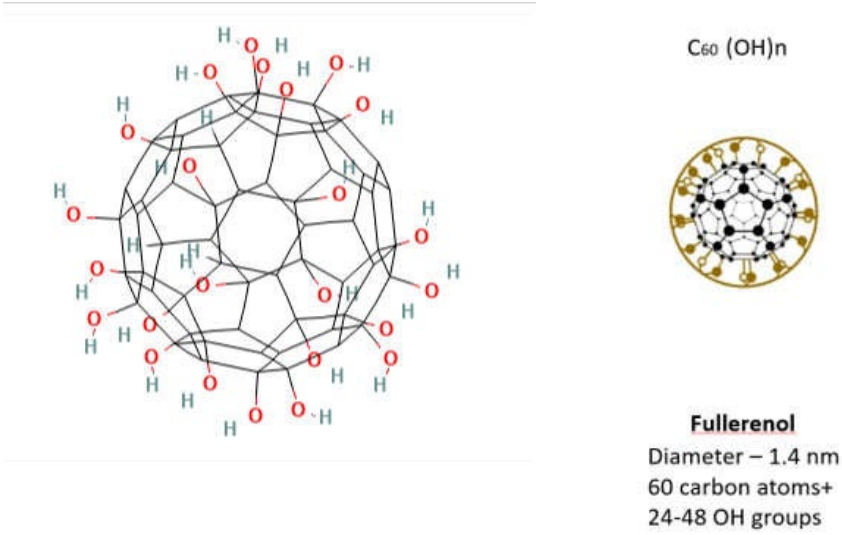
Table 1: Molecular structure, shape and size of Fullerenes C60, C70

	Fullerene C60	Fullerene C70
Molecular structure		
Shape	sphere	rugby-ball shape
Size	0.7 nm	Major-Axis 0.8 nm and minor-Axis 0.7nm

Fullerene C60 molecule is the most common fullerene with a spherical structure – a truncated icosahedron, like a football, and a molecular size of about 0.7 nm. Fullerene C70 has a short axis diameter of 0.7 nm like C60, but its long axis diameter is 0.8 nm, making it like a rugby ball.

Ref: https://pubchem.ncbi.nlm.nih.gov/compound/123591; NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2; Risk_Assessment_-_Fullerenes_NEDO_Oct_16_2009

Hydroxylated fullerenes:



a **b**

Figure 1: a) Structure of Hydroxylated fullerenes, and **b)** Hydroxylated fullerenes (Fullerenol) structure with numbers of OH groups, as given by the notifier.

Ref: 06 HF Number of OH groups

Hydrated forms of Hydroxylated Fullerenes:

According to one of the Notifiers, the ingredient used in the intended cosmetic formulation contains additionally functionalised fullerenol, an ingredient which qualifies as a nanomaterial according to the EU legislation. Polyhydroxylated fullerenes, known as fullerenols, are a class of fullerenes that have many hydroxyl groups, formed by the chemical modification of covalent C–O bonds, on their spherical surfaces. In recent years, they have gained a lot of

attention due to their unique properties, their ability to bio-physically interact with biological systems and their excellent antioxidant efficacy. Fullerenol and Harmonised Fullerenol-Water Complex (HFWC) substance are derived from the same spherical molecule (fullerene C60) with icosahedral symmetry, consisting of 60 carbon atoms. The addition of hydroxyl groups (OH group) to the surface of the fullerene sphere creates a hydroxylated fullerene [C60(OH)x] or fullerenol (Figure 2). The fullerenol molecule itself is in the form of powder and unlike fullerene, it is soluble in water and polar solvents. By additionally functionalising fullerenols by means of water molecules and energy that oscillates according to icosahedral symmetry, a Hyperharmonised Fullerenol-Water Complex (HFWC) is formed.

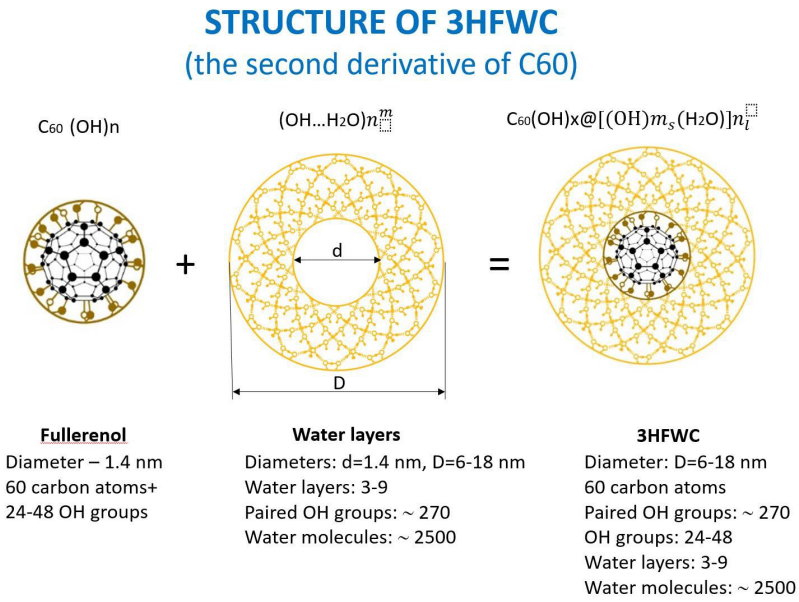


Figure 2: Structure, generated for 3HFWC by ACD/ChemSketch

This harmonised particle can be best described chemically as [C60(OH)x * (H2O)n], where x describes the number of covalently bound hydroxy groups (x = 36±12) and n the number of water molecules surrounding the fullerenol and held in place and stabilised with hydrogen bonds under the influence of an oscillating magnetic field, according to the Fibonacci law (n = 144-2528). Through the harmonisation process, water layers, bound by hydrogen bonds, are formed around the fullerenol and have properties similar to liquid crystals (Figure 2).

3HFWC: 3H – hyper-harmonized-hydroxylated, F – fullerene core C60, W-water, C- complex stabilized with hydrogen bonds under the influence of oscillating magnetic field according to the Fibonacci law (F/f): [C60(OH)x@(H_2O)y]F/f.

3HFWC/HFWC substance is a nanomaterial, without any covalent chemical modification, which is entirely based on hydroxylated fullerene (fullerenol) and water. Hydroxylated fullerene is based in the core of the substance, surrounded by water layers in the form of liquid crystalline. The substance retains as a particle in the formulation.

Ref: 281_safety_file_2020-3-12-18-44-18.pdf; 281_spec_file_2020-2-28-19-37-53

SCCS comment

Despite a few exchanges of queries and clarifications between the SCCS and the Notifier, the basis for regarding 3HFWC as being a discretely different entity from other hydrated forms of hydroxylated fullerenes in terms of chemical identity/composition and physicochemical

properties remains unclear. Therefore, for the purpose of this safety assessment, the SCCS has considered 3HFWC a hydrated form of hydroxylated fullerene - similar to other hydroxylated fullerenes dispersed in aqueous media, for the following reasons:

1. From the Notifier's feedback, the SCCS understands that the linkage between water molecules and hydroxylated fullerene (the starting material used in the synthesis of 3HFWC) is hydrogen bonding in nature. Thus, in terms of chemical nature, there is little difference between 3HFWC and other hydroxylated fullerenes dispersed in water, except that higher number of water molecules are claimed to be surrounding the core hydroxylated fullerene in 3HFWC in a coordinated structure.
2. The reported range of the number of surrounding (claimed to be coordinated) water molecules is very large (144-2528). This, in the absence of a reasonable scientific explanation for the nature of bonding involved (other than hydrogen bonding), casts further uncertainty over the exact chemical composition of this material and the necessity to regard it as a discrete entity that is different from a hydroxylated fullerene dispersed in aqueous media.
3. Other possible reactions/transformations of the starting material (hydroxylated fullerene) from the formation of -OH or other oxyradicals on reaction with the added hydrogen peroxide and exposure to strong magnetic field during the manufacturing process of 3HFWC are currently not known.

3.1.1.6 Empirical formula

Fullerenes:
C_{60}, C_{70}

Hydroxylated fullerenes:
$C_{60}(OH)_{24-48}$

According to one for the Notifiers, the manufacturer specification for the empirical formula of hydroxylated fullerene is $C_{60}(OH)_{30-50}$

Hydrated forms of Hydroxylated Fullerenes:
3HFWC**:** $C_{60}(OH)_{36\pm12}@(H_2O)_{144-2528}$

According to one for the Notifiers, the empirical formula of 3HFWC is given as $C_{60}(OH)_{30-50}@(H2O)_{144-2528}$

Ref: 281_spec_file_2020-2-28-19-37-53; 06 HF Number of OH groups; 10 Characterization 3HFWC

SCCS comment
The exact degrees of hydroxylation for hydroxylated fullerenes and their hydrated forms must be specified.

3.1.2 Physical form

Fullerenes:
Morphology: solid
Agglomeration/aggregation state: aggregate

Ref: NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2

Hydroxylated fullerenes:
Physical form: Hydroxylated fullerene is a clear flowable liquid, as given by the Notifier. It is nearly colourless with a yellow shine, not comparable with RAL colour.

Ref: 02 Colour, odour and physical state HF

Hydrated forms of Hydroxylated Fullerenes:
Physical form: 3HFWC is a clear flowable liquid, as given by the Notifier. It is nearly colourless with a yellow shine, not comparable with RAL colour.

Ref: 02 Colour, odour and physical state HFWC

3.1.3 Molecular weight

Fullerenes:
C60: 720.60
C70: 840.77

Ref: Risk_Assessment_-_Fullerenes_NEDO_Oct_16_2009

Hydroxylated Fullerenes: /

Hydrated forms of Hydroxylated Fullerenes: 3,826 – 47,126 g/mol

Ref: 10 Characterization 3HFWC

SCCS comment
Molecular weights of hydroxylated fullerenes were not provided. From the empirical formulae, these could be calculated to range between 1128.60 to 1536.60 g/mol of $C_{60}(OH)_{24-48}$, and 1248.77 to 1656.77 g/mol of $C_{70}(OH)_{24-48}$.

3.1.4 Purity, composition, and substance codes

Fullerenes:

Purity:

Fullerene (C60) [65%], Fullerene(C70): /

According to one of the Notifiers, the appearance of Fullerene C60 (Lot 040406) was as a black powder. The IR spectrum of the Fullerene C60 had absorbance at 526.5, 576.7, 673.1, 794.6, 1182,3 and 1427.2 cm^{-1}. The purity of the Fullerene C60 was 66.4 ± 0.78 %, and C.V. 1.2%.

The content of Fullerene C60 in three batches of the raw fullerene powder is described in Table 2. Fullerene C60 was dissolved in toluene and the sample solution was analysed by HPLC with UV detection at 285 nm. The Fullerene C60 content of raw fullerene powder was quantified under the same conditions and on the same day.

Table 2: Purity of Fullerene C60 in three batches

Sample	Amount of sample (mg)	Average peak area	C60 content (%)
standard	10.47	90.19	-

Lot 170529	10.04	62.17	71.9
Lot 190806	11.34	78.7	80.5
Lot 190701	11.41	75.09	76.2

Ref: C60 content of raw fullerene powder for cosmetics

According to one of the Notifiers, the raw fullerene powder is a mixture of C60 and C70, and the content of C60 measured by HPLC-UV in five batches ranges approximately from 70 to 80%.

Ref: NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2; Appendix 3 Manufacturing Process, Composition and Properties of Raw Fullerene Powder; B040337_Characteristics Analysis Study of Fullerene

Hydroxylated fullerenes:

According to the Notifier, the purity of the test item was determined as 99.9% by chromatography.

Ref: 10 Characterization HF

IR spectroscopy

According to one of the Notifiers, IR spectroscopy was used to calculate the number of -OH groups for hydroxylated Fullerene C60(OH)30-50, batch no 20H0229A according to the method of the DIN EN ISO 4629-2:2016 (hydroxyl value) standards. This method can be applied to resins, binders for coating materials, primary alcohols, glycols and fats. The results are given in mg KOH/g sample. The number of hydroxyl groups was determined as ≈ 40. IR main absorbance bands with structural assignments are presented in Table 3.

Table 3: IR main absorbance bands with structural assignments of the solid test item, as given by the Notifier

Wavenumber (cm^{-1})	Transmission (%T)	Structural Assignment Vibrations
3357.68	92.89	ν OH
1582.01	69.55	ν C-C
1323.91	71.06	δ OH, ν C-O
777.72	75.20	
513.04	66.27	

As concluded by the Notifier, all the typical vibrations such as ν OH water and ν C-C were found in the IR. The observed absorption bands correlate excellent with the existing reference spectrum of Fullerenol.

Ref: 10 Characterization HF

Elemental Analysis

Elemental analysis data, as reported by the Notifier, are presented in Table 4.

Table 4: Elemental analysis data

Parameter	Experimental value	Calculated on: C60 (C60(OH)30-50)
C	50.45 50.40	51.44 %
H	2.02 2.05	2.87 %
O	48.6 48.7	45.68 %

Ref: 06 HF Number of OH groups

Gel permeation Chromatography (GPC)

According to the Notifier, the peak-area report of GPC shows three different separated peaks.

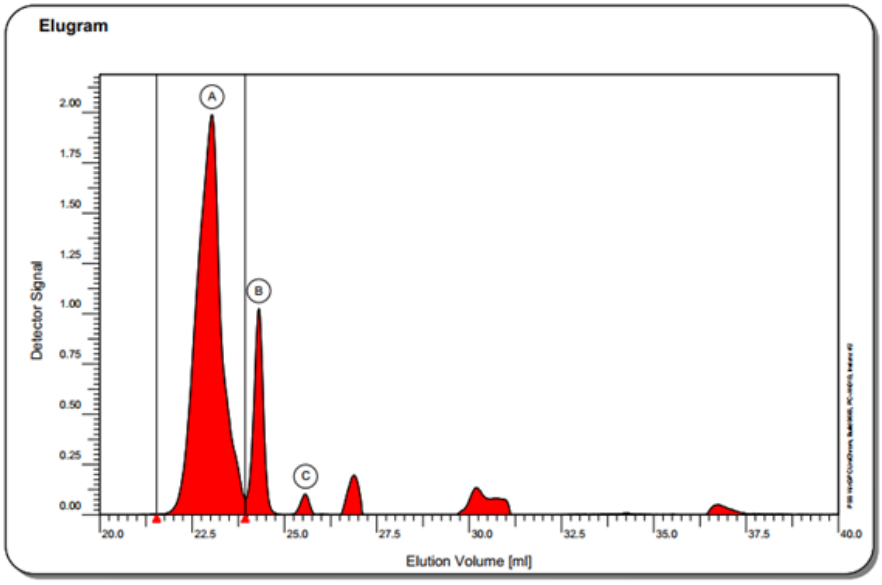


Figure 3: Elugram of Hydroxylated Fullerene

The relative content of each "species" and the molar mass at the peak maximum, Mp, is listed in the table below.

Table 5. Gel permeation chromatography data for hydroxylated fullerene

	Molar mass at the peak maximum (Mp), Da / Peak Area %
Peak A	48819/ 82.04%
Peak B	22462/ 16.43%
Peak C	10232/ 1.53%
Peak D	/

Ref: 10 Characterization HF

LC-MS
According to the Notifier, the LC-MS showed one peak for the test item, hydroxylated Fullerene. MS spectra were extracted from TIC chromatograms obtained by using ESI (+) and ESI (-) ion mode. The LC-MS observes and detects a fragmentation about m/z 68 which correlate with 4 OH-groups. The range of measurements of LC-MS is limited at m/z 2000, therefore, no further OH-groups can or could be observed.

Ref: The Regulatory Company – 3HFWC data submission main document; 10 Characterization HF

Hydrated forms of Hydroxylated Fullerenes:
According to the Notifier, the amount of water was determined as 99.5% by the Karl-Fischer method and the concentration of 3HFWC in water as 0.5%. The purity of this concentration of 0.5% in water was determined as >99.9 by chromatography, no further impurities were observed.

Ref: 10 Characterization 3HFWC

The composition information of HFWC is presented in Table 6.

Table 6. Composition information of HFWC, as given by the Notifier

Components	**Chemical formula**	**%**
Hydroxylated Fullerenes	C60(OH)24-48	0.015
Ultra-pure water	H2O	99.985

Ref: 281_spec_file_2020-2-28-19-37-53

Determination of the content of active ingredient in five batches of 3HFWC by HPLC
According to the Notifier, the test item, 3HFWC, is a fullerene with a 30 – 50 covalently attached hydroxyl groups and further coordinated with 144 – 2528 water molecules. The composition of the test item is hydroxylated fullerene C60 0.015 % and ultra-pure water (0.055 µS/cm) 99.985 %. The SANCO 3030/99 rev. 5 guideline requires an analytical method which is specific for the active ingredient. Method development was performed for the dry active ingredient (hydroxylated fullerene) using HPLC coupled to UV and mass spectrometric detection. Column types ranging from C18 (separation based on hydrophobic interaction) to HILIC (separation based on hydrophilic interaction) were tested. Under no tested conditions was retention achieved. The test item showed one peak at or even before the dead time of the tested column. This indicated that, due to the large aqueous solvation shell, the target molecule is not able to interact with the column material and/or is too large to enter the pores of the column materials. Experiments were then performed by gel permeation chromatography.

Ref: 09 Active ingredient 3HFWC HPLC

Gel- Permeation Chromatography (GPC)
According to the Notifier, fullerenes are expected to exhibit UV activity and calculations of molar mass distributions have only been carried out for the UV active species. Peak areas of RI signal of side components have been analysed as well. Analysis of the UV signal at 250 nm revealed that the sample solutions contain a UV active main component and different side components, that eluted in the relevant elution volume area for GPC analysis. Three peaks and their observed masses are within the calibration curve, and one is out (peak D) of the calibration curve.

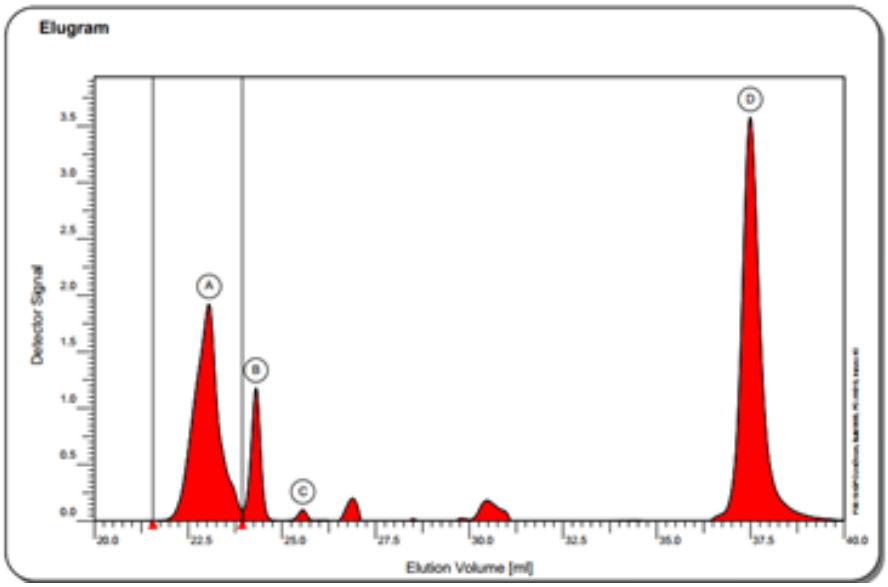


Figure 4: Elugram of 3HFWC

According to the Notifier, the mass of the peak D cannot be determined, but it should be more hydrophilic than the test item "3HFWC". Peaks at elution volumes of appr. 27 and 31 mL are presumably system peaks. The test item 3HFWC contains an additional, UV active component which elutes outside of the GPC calibrated range (appr. 37.5 mL). Hence, no molar mass result has been calculated for the substance. Size distribution was found to be corresponding to the range 10314 to 48213 g/mol (Table 7). Due to very broad peaks in this methodology, it is not suitable for sensitive quantitative analysis.

Table 7. Gel permeation chromatography data for 3HFWC

	Molar mass at the peak maximum (Mp), Da / Peak Area %
Peak A	48213/ 35.77%
Peak B	22409/ 9.02%
Peak C	10314/ 0.71%
Peak D	Out of calibration range / 53.49%

Ref: 09 Active ingredient 3HFWC HPLC; Ref: The Regulatory Company – 3HFWC data submission main document; 10 Characterization HFWC

NMR data

According to the Notifier, the recording of ^{1}H-NMR spectra was only feasible with water suppression. The first spectra were performed with tetramethylsilane as standard, but the broad signal of TMS made it difficult to separate the peaks for the integrations. Therefore, the recording of the NMR spectra was repeated without TMS, which is the log signal, in the hope that more precise signals might be received for the integration. The chemical shift at about 8.5 ppm in the ^{1}H-NMR can be assigned as Fullerenol C60 with more hydroxylic groups. This conclusion is based on the high value of the integral which increased from 3.57 (3HFWC) to 15.38 OH-groups. The factor of the integral can be assumed as approximately 5. Through the high symmetry and the water layers, only one signal was observed, at about 8.458 ppm of the hydroxylic groups in 3HFWC.

The difference between 3HFWC and Hydroxylated Fullerene are marked in Table 8. The integral high at about 8.5 ppm was determined in 3HFWC as 3.57 and in Hydroxylated Fullerene as 15.38. The sum of all other protons was determined in 3HFWC as 96.44 and in Hydroxylated Fullerene as 84.41. The total sum of all kinds of protons was determined as approximately 100.

Table 8: NMR data of 3HFWC and Hydroxylated Fullerene with and without Tetramethylsilan (TMS)

^{1}H-NMR data with TMS

3HFWC with TMS chemical shift / ppm	3HFWC with TMS integral	Hydroxylated Fullerene with TMS chemical shift / ppm	Hydroxylated Fullerene with TMS integral
		10.027	0.02
8.458	0.22	8.770 – 5.490	6.04
3.774 – 3.741	15.25	4.385 – 0.868	93.80
3.123	3.95		
2.849	2.18		
2.612 – 2.158	10.04		
1.921 – 2.158	44.63		
1.280 – 1.039	4.18		
-0.050 - -0.060	19.55	-0.007 – -0.055	0.14

^{1}H-NMR data without TMS

3HFWC without TMS chemical shift / ppm	3HFWC without TMS integral	Hydroxylated Fullerene C60 without TMS chemical shift / ppm	Hydroxylated Fullerene C60 without TMS integral
		10.027	0.05
8.459	**3.57**	8.765 – 4.811	**15.38**
3.123	**11.81**	4.537 – 1.108	**84.41**
2.851 – 2.075	**44.59**		
1.921	**22.70**		
1.342 – 0.899	**17.34**		
Sum of protons (marked)	**96.44**		**84.41**
Sum total of protons	100.01		99.79

LC-MS data

MS spectra were extracted from TIC chromatograms obtained using ESI (+) and ESI (-) ion mode. According to the Notifier, the LC-MS observes and detects a fragmentation about m/z 68 which correlate with 4 OH-groups. The range of measurements of LC-MS is limited at m/z 2000, therefore, non-further OH-groups can or could be observed.

Ref: 10 Characterization 3HFWC

SCCS comment

According to the Notifiers, raw fullerene powder is a mixture of fullerenes C60 and C70, and the content of Fullerene C60 measured in five batches ranges approximately from 70 to 80%. Data on the exact content of fullerene C70 were not provided.
According to the Notifier, under no tested conditions was retention achieved for 3HFWC by HPLC, and the GPC method is not suitable for sensitive quantitative analysis.
The composition of 3HFWC is provided in Table 6 by measuring the content of hydroxylated fullerenes and water; this supports further the conclusion of the SCCS that, in terms of chemical composition, 3HFWC is a hydrated form of hydroxylated fullerene - similar to other hydroxylated fullerenes dispersed in aqueous media.

3.1.5 Impurities / accompanying contaminants

Fullerenes:

Fullerene (C60) [65%],

Fullerene(C70): /
Coatings or surface moieties: None
Doping material: None
Encapsulating materials: None
Processing chemicals: None
Dispersing agents: None
Stabilizers: None
Other additives: None

According to the Notifier, the concentration of other fullerenes such as C82 and oxygenated fullerene was less than 1% in 5 batches of raw fullerene powder, and no impurities derived from raw fullerene powder were detected with liquid chromatography. Since toluene is used in the extraction of raw fullerene powder, the residual amount of toluene was also measured and the values were much lower than the residual tolerance, i.e. 890 ppm, specified in the ICH guideline.
It is stated by the Notifier(s) that in Fullerenes formulations, heavy metals should be not more than 20 ppm, arsenic should be not more than 2 ppm, and the residue on ignition should be not more than 0.1%.

Ref: NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2; Appendix 3 Appendix 3 Manufacturing Process, Composition and Properties of Raw Fullerene Powder

Hydroxylated fullerenes
According to the Notifier, the amount of water (moisture) was determined as 99.1% by the Karl-Fischer method.

Ref: 10 Characterization HF

Hydrated forms of Hydroxylated Fullerenes
According to the Notifier, the amount of water was determined as 99.5% by the Karl-Fischer method. Based on the data submitted by the Notifier and as reported in the purity section of this Opinion, no further impurities were observed.

Ref: 10 Characterization 3HFWC

SCCS comment
The Notifiers should provide detailed information on the levels of impurities, heavy metals, accompanying contaminants and organic solvents, along with detailed information on the methods of manufacturing (synthesis route, solvent removal and any co-synthesised by-products) for fullerenes (C60 and C70), hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes.

3.1.6 Solubility

Fullerenes:
It is stated by the Notifier(s), that Fullerene is a strong hydrophobic substance which is insoluble in aqueous media.

Ref: NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2

Data of the solubility of fullerenes C60 and C70 in various solvents are presented in Table 9, as submitted by the Notifier(s).

Table 9. Solubility of Fullerenes C60 and C70

	References
SOLUBILITY OF C_{70} AND C_{60} IN ORGANIC SOLVENTS MF : Mole Fraction*10^4 n : Refractive Index ** : Solubility not measured SP: Hildebrand's Solubility Parameter (δ) ($J^{1/2}.cm^{-3/2}$)	Sivaranam *et al.,* 2006
Solubility of C60 fullerene (mg/L) Brassica methyl ester (biodiesel) 187 mg/L Sunflower triglyceride 116 mg/L Soybean triglyceride 134 mg/L Linseed triglyceride 91 mg/L Olive triglyceride 173 mg/L	Cataldo *et al.,* 2007

	C-70		C-60		
Solvent	µg/ml	MF	µg/ml	SP	n
1. Pentane	2	0.00268	4	14.52	1.358
2. Hexane	13	0.02074	40	14.85	1.380
3. Heptane	47	0.08258	**	15.10	1.387
4. Octane	42	0.08037	25	15.45	1.392
5. Isooctane	**	**	26	14.17	1.398
6. Decane	53	0.12208	70	15.81	1.411
7. Dodecane	98	0.26399	91	16.07	1.422
8. Tetradecane	**	**	126	16.24	1.428
9. Cyclohexane	80	0.1030	51	16.77	1.426
10. Acetone	1.9	0.0017	**	20.00	1.359
11. Isopropanol	2.1	0.0020	**	23.70	1.377
12. Dioxane	**	**	41	20.50	1.423
13. CCl_4	121	0.1390	447	17.59	1.460
14. p-Xylene	3985	5.8127	**	18.00	1.496
15. Mesitylene	1472	2.4373	997	18.04	1.498
16. Toluene	1406	1.7785	2150	18.20	1.497
17. Benzene	1300	1.3829	1440	18.82	1.501
18. CS_2	9875	7.065	5160	20.50	1.627
19. Dichloro-methane	80	0.0610	254	20.00	1.424
20. o-Dichloro-benzene	36210	48.286	**	20.50	1.550

Solubility of C_{60} in various solvents

Solvent	$[C_{60}]$ (mg mL^{-1})	Mole fraction ($\cdot 10^4$)	n
n-Pentane	0.005	0.008	1.36
n-Hexane	0.043	0.073	1.38
Cyclohexane	0.036	0.059	1.43
n-Decane	0.071	0.19	1.41
Decalines	4.6	9.8	1.48
Dichloromethane	0.26	0.27	1.42
Carbon disulfide	7.9	6.6	1.63
Dichloromethane	0.26	0.27	1.42
Chloroform	0.16	0.22	1.45
Tetrachloromethane	0.32	0.40	1.46
Tetrahydrofuran	0.000	0.000	1.41
Benzene	1.7	2.1	1.50
Toluene	2.8	4.0	1.50
Tetraline	16	31	1.54
Benzonitrile	0.41	0.71	1.53
Anisole	5.6	8.4	1.52
Chlorobenzene	7.0	9.9	1.52
1,2-Dichlorobenzene	27	53	1.55
1-Methylnaphthalene	33	68	1.62
1-Chloronaphthalene	51	97	1.63
Acetone	0.001	0.001	1.36
Methanol	0.000	0.000	1.33

A. Hirsch and M. Brettreich, 2005

Ref: Appendix 2 Physicochemical Properties of Fullerenes C60 and C70

Hydroxylated fullerenes

Water solubility: Since the test item, hydroxylated fullerene (batch no. 20H0229A) is the dry material for an aqueous formulation, the solubility of the test item in water was performed using a simplified flask method. In this case it was not possible to weigh the fivefold saturation concentration of the test item in water to perform a main test following OECD 105. The results of the main test indicate that Hydroxylated Fullerenes is miscible with water in all proportions. The calculated concentration of the test item in the test solutions corresponds to the nominal load of the test item 150 mg/L (146.4 – 157.6 mg/L). In the flasks 4 and 5, higher concentrations were measured as the determination of DOC is less precise in the low range (< 10 mg/L).

Ref: 08 Water solubility HF

Hydrated forms of Hydroxylated Fullerenes:

Solubility/dissolution (in relevant solvents): /

Water solubility: Since the test item hyperharmonized hydroxylated fullerene water complex (3HFWC) (batch 01-2021-10-14) is an aqueous formulation, the solubility of the test item in water was performed using a simplified flask method. In this casem it was not possible to weigh the fivefold saturation concentration of the test item in water to perform a main test following OECD 105. The results of the main test indicate that hyperharmonized hydroxylated fullerene water complex (3HFWC) is miscible with water in all proportions. The calculated concentration of the test item in the test solutions corresponds to the nominal concentration of the test item 150 mg/L (141.3 – 161.6 mg/L). In the flask, 5 higher concentration was measured as the determination of DOC is less precise in the low range (< 5 mg/L).

N-Octanol (mg/L): n.a.

Ref: 281_spec_file_2020-2-28-19-37-53; 08 Water solubility 3HFWC

Additional solubility data – SCCS literature survey

The results of the SCCS literature survey have indicated that fullerenes are practically insoluble in water, whereas hydroxylated fullerenes are soluble in water. Fullerenes are also virtually insoluble in acetone, ethers, alcohols (Taylor, 2001) and other polar solvents, sparingly soluble in alkanes, while appreciably soluble in aromatic solvents and in carbon disulfide. The solubility of fullerene C60 in a number of solvents ranges from 0.0 g/L in methanol and tetrahydrofuran, to 41 g/L in 1-chloronaphthalene (Cadek *et al.,* 1999).
Ruoff *et al*. (2003) have determined room temperature solubility of pure fullerene C60 in 47 solvents. These range from 0.01 g/L in methanol to 50 g/L in l-chloronaphthalene. The solubilities in CS2, toluene, and hexane, three of the commonly employed solvents, are 7.9, 2.8, and 0.04 g/L, respectively.

The calculated solubility in water at 25°C is 7.42 ng/L (water-phase of water-octanol), based on measured values in octanol (of octanol-water phase) and octanol-water partition coefficient. Solubilities in various solvents at 25 °C range from ethanol (1.4 mg/L) to water-saturated toluene (2430 mg/L) and toluene (3000 mg/L), (Jafvet *et al.,* 2008). Water solubility is also reported to be greatly increased by the addition of hydroxyl groups either to the cage (giving fullerenols) or having them present in addends (Li *et al.*, 2013).

3.1.7 Partition coefficient (Log P_{ow})

Fullerenes:
Log $P_{O/W}$: /

Fullerene C60 log $K_{o/w}$ = 6.67
Fullerene C60 toluene-water partition coefficient, log $K_{T/W}$: 8.44

Ref: https://www.bioactivec60.com/wp-content/uploads/2016/06/Fullerene-C60-C60-PubChem.pdf; Jafvert CT, Kulkarni PP; Environ Sci Technol 42: 5945-5950 (2008)

Hydroxylated fullerenes: /

Hydrated forms of Hydroxylated Fullerenes:
Octanol/water partition coefficient: $P_{O/W}$ = 0.18941
Log $P_{O/W}$ = -0.72

Ref: 281_spec_file_2020-2-28-19-37-53

SCCS comment
Log $P_{O/W}$ values for hydroxylated fullerenes should be provided.

3.1.8 Additional physical and chemical specifications

Fullerenes:

Table 10. Additional physicochemical properties of Fullerene C60 and Fullerene C70.

	Fullerene C60	**Fullerene C70**	**Ref.**
Molecular Structure [nm]	0.704 (Frame) 1.002 (Electron Cloud)	0.796 (Transverse Diameter) 0.712 (Conjugate Diameter)	Ahmad *et al.*, 1999.
Electron Affinity [eV]	2.65	2.72	
Melting Point [ºC]	1180	No data	Beckhaus *et al.*, 1992.
Electric Conductivity (300K) [S/cm]	10^{-8} ~10^{-14}	No data	Arai *et al.*, 1992; Mort *et al.*, 1992.
Sublimation Heat [kcal/mol]	40, 38	43, 45	Pan *et al.*, 1994.
Vapor Pressure [Torr]	1.9 x 10^{-5} (400 ºC) 5 x 10^{-4} (500 ºC) 1 x 10^{-3} (600 ºC)	1.4 x 10^{-5} (430 ºC) 2 x 10^{-4} (500 ºC) 7 x 10^{-3} (600 ºC)	Abrefah *et al.*, 1992

According to one of the Notifiers, the appearance of Fullerene C60 (Lot 040406) was as a black powder.

Ref: Risk_Assessment_-_Fullerenes_NEDO_Oct_16_2009

Hydroxylated fullerenes:

Melting point: 101.59 ± 0.14 ºC (374.74 K)
Colour: The test substance is nearly colourless with a yellow shine, not comparable with RAL colour.
Determination of Odour: No odour was detectable.
Flash point: No flash point could be detected up to 100 °C. Therefore, no flash point could be established.
Viscosity: 1.005 ± 0.004 mPa·s at 20.00 ± 0.02 °C

Ref: 05 Viscosity HF; 02 Boiling point HF; 02 Colour, odour and physical state HF; 04 Flash point HF

Hydrated forms of Hydroxylated Fullerenes:

According to the Notifier, the Hydrated forms of Hydroxylated Fullerenes are formed by mixing the hydroxylated fullerene [$\boldsymbol{C6}$O($\boldsymbol{OH}$)$\boldsymbol{x}$] with ultrapure water (grade 2), and then water layers are generated and stabilised by oscillatory magnetic field: [$\boldsymbol{C60(OH)x@(H2O)nlf}$] (n is number of water molecules, l is number of water layers and f is number of frequency modes). Before mixing with water, hydroxylated fullerene is pre-treated with heating (drying) and the UV-Vibro apparatus (prevention of agglomeration and aggregation process).

Melting point: 102.07 ± 0.14 ºC (375.22 K)
Colour: The test substance is nearly colourless with a yellow shine, not comparable with RAL colour.
Determination of Odour: No odour was detectable.
Flash point: No flash point could be detected up to the boiling stage of 102.4 °C in the pre-test. Therefore, no flash point could be established.
Viscosity: 1.007 mPa·s at 20.00 ± 0.02 °C

Ref: 281_spec_file_2020-2-28-19-37-53; Risk_Assessment_-_Fullerenes_NEDO_Oct_16_2009; The Regulatory Company - 3HFWC data submission main document; 01 Boiling point 3HFWC; 02 Colour, odour and physical state 3HFWC; 04 Flash point 3HFWC; 05 Viscosity 3HFWC

3.1.9 Particle size

Fullerenes*:
The following data were submitted by the Notifier:
Lowest cut-off level (nm):
Volume weighed median: 0.7 nm (C60)
Number weighed median: 0.7 nm (C60)

* Since fullerene is a molecule, the primary particle size is the same as the molecular size.

According to one of the Notifiers, Fullerene C70 (Table 1) is a rugby-ball shaped particle with Major axis 0.8 nm and minor axis 0.7 nm.

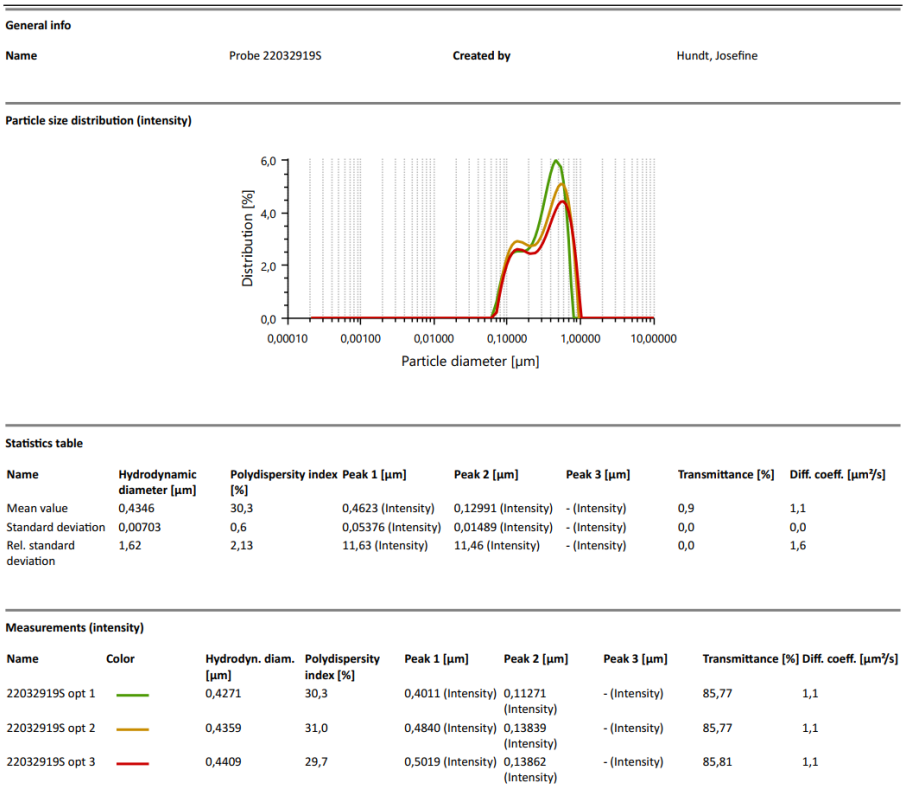
Ref: NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2; https://pubchem.ncbi.nlm.nih.gov/compound/123591

Hydroxylated fullerenes: 2.0 ± 0.6 nm

According to one of the Notifiers, hydroxylated fullerenes can be clustered, forming large agglomerates.

Ref: The Regulatory Company - 3HFWC data submission main document; 10 Characterization HF

Dynamic light scattering
Dynamic Light Scattering for hydroxylated fullerenes is presented in the next Figure, as given by the Notifier.



General info

Name	Probe 22032919S	**Created by**	Hundt, Josefine

Particle size distribution (intensity)

Statistics table

Name	Hydrodynamic diameter [µm]	Polydispersity index [%]	Peak 1 [µm]	Peak 2 [µm]	Peak 3 [µm]	Transmittance [%]	Diff. coeff. [µm²/s]
Mean value	0,4346	30,3	0,4623 (Intensity)	0,12991 (Intensity)	- (Intensity)	0,9	1,1
Standard deviation	0,00703	0,6	0,05376 (Intensity)	0,01489 (Intensity)	- (Intensity)	0,0	0,0
Rel. standard deviation	1,62	2,13	11,63 (Intensity)	11,46 (Intensity)	- (Intensity)	0,0	1,6

Measurements (intensity)

Name	Color	Hydrodyn. diam. [µm]	Polydispersity index [%]	Peak 1 [µm]	Peak 2 [µm]	Peak 3 [µm]	Transmittance [%]	Diff. coeff. [µm²/s]
22032919S opt 1		0,4271	30,3	0,4011 (Intensity)	0,11271 (Intensity)	- (Intensity)	85,77	1,1
22032919S opt 2		0,4359	31,0	0,4840 (Intensity)	0,13839 (Intensity)	- (Intensity)	85,77	1,1
22032919S opt 3		0,4409	29,7	0,5019 (Intensity)	0,13862 (Intensity)	- (Intensity)	85,81	1,1

Figure 5: Dynamic Light Scattering for hydroxylated fullerenes.

Particle Size Distribution

- Particle size distribution by scanning transmission electron microscopy (STEM) method:

As reported by the Notifier, High-angle annular dark-field (HAADF) scanning transmission electron microscopy (STEM) carried out on a FEI Osiris ChemiSTEM microscope at 200 keV was employed for investigation of the size, the shape and the chemical composition of the test item (Hydroxylated Fullerene).

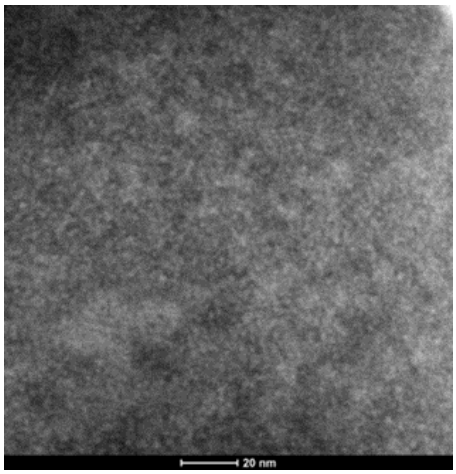


Figure 6: Images of the test item Hydroxylated Fullerenes captured with a HAADF-STEM at 200 keV

According to the Notifier, the constituent particles of the test item are expected to have a diameter between 0.7 and 1.4 nm. It was not possible to clearly identify single constituent particles due to blurred boarders of the particulate structures. The particles were expected to

be clustered, forming large agglomerates. The visible nanostructures have diameters of approximately 2.0 ± 0.6 nm, which is above the expected size, but in the same order of magnitude.

Ref: LAUS, Report Aug.2022.

Zeta potential by electrophoretic light scattering (ELS)

ELS data are presented in the table below as given by the Notifier.

Table 11: ELS data for hydroxylated fullerene

Test item	Temperature [°C]	Zeta potential [mV)]	Electrophoretic mobility [µm/s)/(V/cm]	Conductivity [mS/cm]
Hydroxylated Fullerene	25 °C	-25.85 ± 1.71	-2.01 ± 0.13	0.18

Ref: 10. Characterization HF

Hydrated forms of Hydroxylated Fullerenes:

Primary particle size, as given be the Notifier

1. Lowest cut-off level (nm) value: 6 nm.
2. Volume weighted median (nm) min: 8.66 nm; max: 18.06 nm
3. Number weighted median (nm) min: 8.66 nm; max: 18.06 nm

Secondary particle size

There is no secondary particle size

Ref: 281_spec_file_2020-2-28-19-37-53

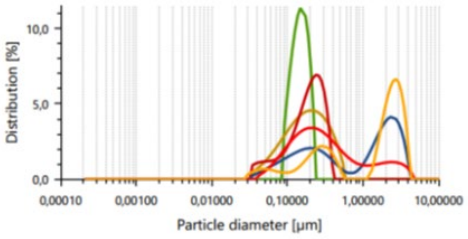
Dynamic Light Scattering

Dynamic Light Scattering for 3HFWC is presented in the next Figure, as given by the Notifier.

General info

Name	Probe 220329165	Created by	Hundt, Josefine

Particle size distribution (intensity)



Statistics table

Name	Hydrodynamic diameter [µm]	Polydispersity index [%]	Peak 1 [µm]	Peak 2 [µm]	Peak 3 [µm]	Transmittance [%]	Diff. coeff. [µm²/s]
Mean value	5,933	37,5	0,9335 (Intensity)	0,9358 (Intensity)	0,05277 (Intensity)	0,9	0,7
Standard deviation	12,019	17,4	1,1305 (Intensity)	1,2073 (Intensity)	- (Intensity)	0,0	0,8
Rel. standard deviation	202,56	46,38	121,11 (Intensity)	129,02 (Intensity)	- (Intensity)	0,0	105,1

Measurements (intensity)

Name	Color	Hydrodyn. diam. [µm]	Polydispersity index [%]	Peak 1 [µm]	Peak 2 [µm]	Peak 3 [µm]	Transmittance [%]	Diff. coeff. [µm²/s]
220329165 opt 1		30,433	71,9	0,14971 (Intensity)	- (Intensity)	- (Intensity)	86,24	0,0
220329165 opt 2		0,2537	29,3	0,2051 (Intensity)	- (Intensity)	- (Intensity)	86,24	1,9
220329165 opt 3		0,3575	28,3	0,19275 (Intensity)	- (Intensity)	- (Intensity)	86,29	1,4
220329165 opt 4		1,2911	27,2	2,189 (Intensity)	0,2060 (Intensity)	- (Intensity)	86,30	0,4
220329165 opt 5		1,4139	29,5	0,2868 (Intensity)	2,329 (Intensity)	- (Intensity)	86,29	0,3
220329165 opt 6		1,8508	38,7	2,577 (Intensity)	0,2720 (Intensity)	0,05277 (Intensity)	86,29	0,3

Figure 7. Dynamic Light Scattering for 3HFWC

Particle Size Distribution

-**Wet dispersion cell**

First measurements: As reported by the Notifier, during the initial studies, 3HFWC was filled by a pipette into the tank of the wet dispersion cell (SUCELL), and no increase of the obscuration was observed. When more test item was filled into the tank of the SUCELL, no increase of the obscuration was observed. Therefore, no measurement could be taken. No increase of the obscuration showed that no aggregates and agglomerates or particles in the measuring range above 100 nm could be detected.

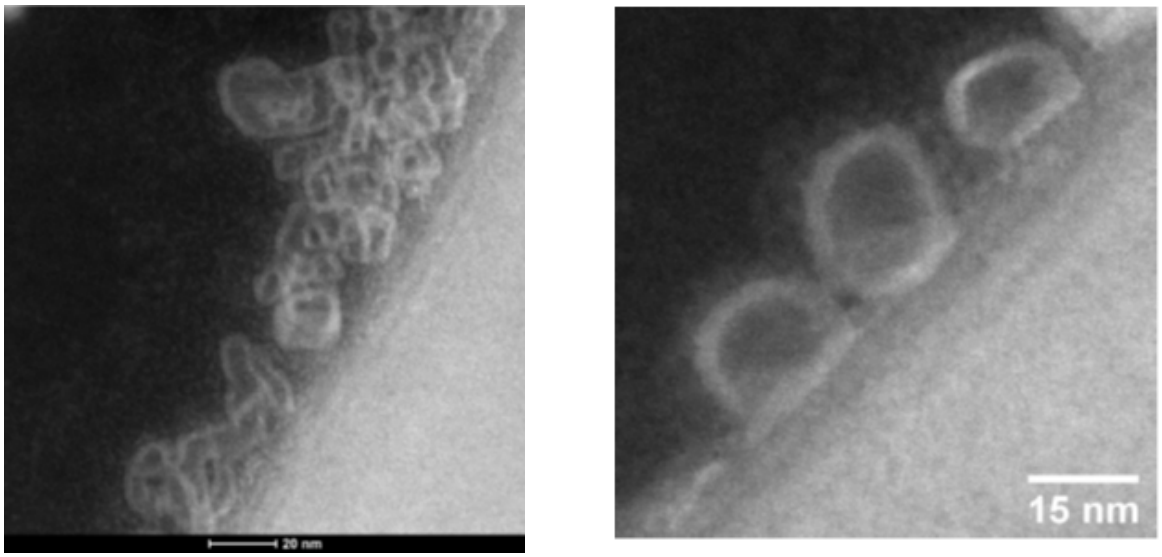
Repetition of measurements: The SUCELL was then filled with 400 mL of water for the blank measurement and then drained. In the next step, 400 mL of the liquid sample were inserted into the tank and measured twice - with and without sonification (ultrasound 100% for seconds before the measurement). Very large values resulted, which exceeded the range 5 (maximum range for our SUCELL), meaning that particles larger than 850 µm can be found.

Conclusion of the wet dispersion with and without ultra-sonic: According to the Notifier, particles in the range from 5 µm up to 850 µm were observed, which is the limitation of the feasibility.

Ref: LAUS, Report Aug. 2022.

-Particle size distribution by scanning transmission electron microscopy (STEM) method

As reported by the Notifier, High-angle annular dark-field (HAADF) scanning transmission electron microscopy (STEM); carried out on a FEI Osiris ChemiSTEM microscope at 200 keV electron energy was employed for investigation of the size, the shape and the chemical composition of test item.



a **b**

Figure 8. Images of the test item 3HFWC captured with a HAADF-STEM at 200 keV, **a)** 20 nm, and **b)** 15 nm.

Evaluation of the observed HAADF-STEM images: According to the Notifier, the constituent particles of 3HFWC are expected to have a diameter between 0.7 and 1.4 nm. It was not possible to identify single constituent particles. The particles were expected to be clustered, forming micelles and "chains". If that has happened the maximum particle size can be assumed to be equivalent to the diameter of walls of the visible structures, which is approximately 2.3 nm above the expected size, but in the same order of magnitude.
Notifiers' conclusions: The evaluation of the results was performed by the Notifier according to the NanoDefine approach: https://ec.europa.eu/jrc/en/publication/nanodefine-methods-manual by using the particle size laser, particles in the range from 5 µm up to 850 µm, which is the limitation of the feasibility. Using Dynamic light scattering yielded no results and particles in the range between 0-100 nm, as the concentration of the test item "3HFWC" was too low**.** The diameters of the "circles" in HAADF-STEM images are ~20 nm, and according to the literature and the information provided, the fullerenes should be ~1 nm in size. It can be assumed that the observed structures are "chains, tubes" in the form of a circle of functionalised fullerenes that have formed a kind of micelle. The wall thickness diameter of the "circles" was 2 to 4 nm. Hydrodynamic diameter of 3HFWC was reported as 5.933 ± 12.019 µm.

Zeta potential by electrophoretic light scattering

ELS data for 3HFWC are presented in the table below, as given by the Notifier.

Table 12: ELS data for 3HFWC

Test item	Temperature [°C]	Zeta potential [mV)]	Electrophoretic mobility [µm/s)/(V/cm]	Conductivity [mS/cm]
HFWC	25 °C	-43.29 ± 1.23	-3.37 ± 0.10	0.17

According to the Notifier, zeta potential was measured as an indicator of the stability of a particle system. According to substance categorization stated in the report, substances with zeta potential values higher than +30 mV or lower that -30 mV are considered stable. The

experimental value of zeta potential for 3HFWC is -43.29 mV (table 12) and it can enable the classification of this substance into the group of stable substances.

Ref: 10. Characterization HFWC;
The Regulatory Company - 3HFWC data submission main document;
LAUS, Report Aug. 2022.

SCCS comment

Although a few electron microscopy (EM) images have been provided for fullerenes C60, hydroxylated fullerenes and 3HFWC, a more detailed quantitative EM analysis is needed for accurate size measurement of the particles in the nano-scale. A proper dispersion of the samples is also essential, and it is not clear whether this was carried out as part of the sample preparation for electron microscopy. Detailed guidance on the use of EM for characterising nanoparticles, including sample preparation, EM imaging, image analysis, is provided in a recent EFSA Guidance (EFSA, 2021). The level of magnification and pixel size for EM imaging should be determined based on the criterion of Merkus (2009), and suitability of the imaging settings can be evaluated on the basis of the simplified criterion that requires the minimal external dimension of the smallest detected particle to be at least 10 pixels. With respect to fullerenes, it is of note that it is not the Notifier's intention to market C60 as such, but as a mixture of C60 and C70.

3.1.10 Crystal structure

Fullerenes:

Crystalline shape: Irregular, as given by the Notifier.

Table 13:

	Fullerene C60	**Fullerene C70**
Crystal structure	Face-Centered Cubic Lattice (>260K) Simple Cubic Lattice.	Face-Centered Cubic Lattice, Trigonal Lattice, and Hexagonal Close-Packed Lattice at at Transitional Phase

Ref: NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2; Risk_Assessment_-_Fullerenes_NEDO_Oct_16_2009; Lichtenberger *et al.*, 1992; Beckhaus *et al.*, 1992.

Hydroxylated fullerenes: /

Hydrated forms of hydroxylated fullerenes: /

SCCS comment

Information indicating the shape, aspect ratio and agglomeration/ aggregation state of the hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes should be provided.

3.1.11 UV absorption

Fullerenes:

Fullerene C60 exhibits strong absorption bands at 213, 257 and 329 nm.

Ref: Cadek M *et al.* (1999-2013)

Hydroxylated Fullerenes:
According to one of the Notifiers, the UV-Vis spectrum of a solution of the test item (hydroxylated fullerene) showed a high absorption at 243.5 nm in neutral medium which increased by addition of basic medium to a maximum at 244 nm and is the same by addition of acidic medium to a maximum at 243.5 nm (Figure 9). No extinction coefficients could be calculated as the molecular mass of the test item is unknown.

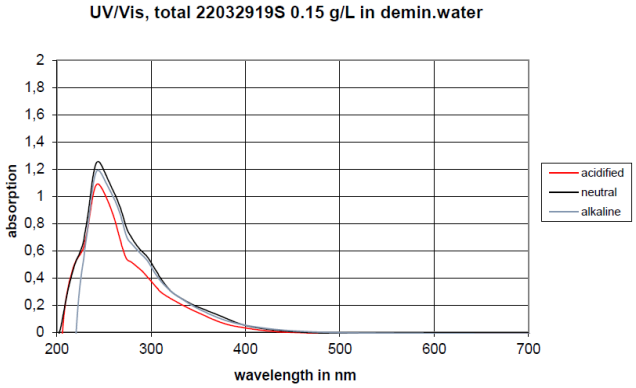


Figure 9. UV spectra of hydroxylated Fullerene, as given by the Notifier.

Ref: 10 Characterization HF

Hydrated forms of hydroxylated fullerenes:
According to one of the Notifiers, in the UV-Vis spectrum, the test item solution (3HFWC) showed a high absorption at 200 nm in neutral medium which increased by the addition of basic medium to a maximum at 235.5 nm and by the addition of acidic medium to a maximum at 211 nm (Figure 10).

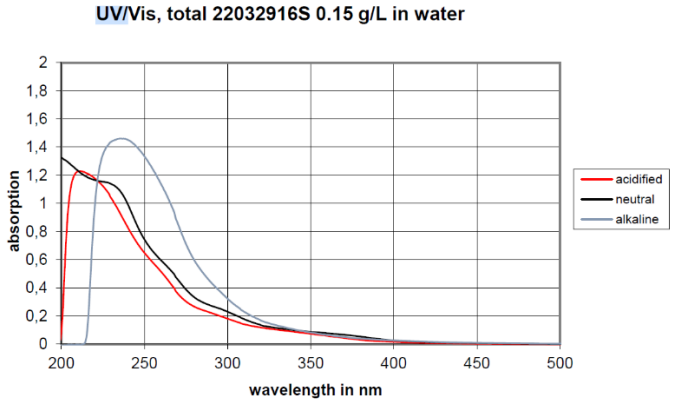


Figure 10. UV spectra of 3HFWC as given by the Notifier.

Ref: 10 Characterization HFWC

3.1.12 Surface characteristics

The following data on surface characteristics were provided by the Notifiers

Fullerenes:

Surface charge (mV): No data
According to the Notifier, Fullerene is a strong hydrophobic substance. Surface charge is unmeasurable because it is not dispersed in water.
Surface modifications or functionalization: No
Coating: None

Ref: NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2

Hydroxylated fullerenes:
Surface charge (zeta potential, mV) value: /

Hydrated forms of Hydroxylated Fullerenes:
Surface charge (zeta potential, mV) value: 50-70 mV
According to the Notifier, at the surface of the 3HFWC substance, there is a positive charge which depends on the number of hydrogen atoms. The zeta potential depends on the number of water layers and the diameter of the sphere.
Surface modifications or functionalization: No
Coating: None

Ref: 281_spec_file_2020-2-28-19-37-53

SCCS comment
Data on the surface charge of hydroxylated fullerenes should be provided.

3.1.13 Droplet size in formulations

Fullerenes: /

Hydroxylated fullerenes: /

Hydrated forms of Hydroxylated Fullerenes: /

SCCS comment
Data were not provided.

3.1.14 Homogeneity and stability

Fullerenes: /

Hydroxylated fullerenes: /

Hydrated forms of Hydroxylated Fullerenes:
As reported by the Notifier, determination of pH-dependent hydrolysis in water of 3HFWC was conducted according to OECD Guideline 111 and EU Method C.7. The test item is a fullerene with a 30 – 50 covalently attached hydroxyl groups and further coordinated with 144 – 2528 water molecules. Experiments were performed by a partner laboratory using gel permeation chromatography where molecules are separated using their apparent size, including the solvation shell. A size distribution was found corresponding to the range 10314 – 48213 g/mol. However, this technique is not suitable for monitoring the hydrolysis process due to an expected very small change in molecular mass during the reaction. Due to the practical and scientific challenges pointed out above, the performance of the study pH-dependent hydrolysis of Hyperharmonized hydroxylated fullerene water complex (3HFWC) is concluded to be technically not feasible.

Ref: 07 3HFWC Hydrolysis Statement

SCCS comment
Detailed information on homogeneity and stability of fullerenes, hydroxylated fullerenes and the hydrated forms of hydroxylated fullerenes should be provided.

3.1.15 Other parameters of characterisation

The following data were provided by the Notifier(s)

Fullerenes:

Density/porosity (for granular materials): /

Mass density: Fullerene C60: 1.729 g/cm^3(5K Calculated value),
Fullerene C70: 1.6926 g/cm^3(Ambient Temperature).

Molecular density: Fullerene C60: 1.44 x 10^{21} molecule/cm^3,
Fullerene C70: no data

Ref: NANOMATERIALS SPECIFICATIONS_ENGLISH_Fullerene-V2; Risk_Assessment_-_Fullerenes_NEDO_Oct_16_2009; Heiney *et al.*, 1991.

Hydroxylated fullerenes:

Mass density: 0.9982 g/cm^3 at 20.0 ± 0.4 °C

Ref: 03 Density HF

Hydrated forms of Hydroxylated Fullerenes:

Mass density: 0.9984 ± 0.0004 g/cm^3 at 20.0 ± 0.4 °C

Ref: 03 Density 3HFWC

SCCS comment
The mass density values should be provided for hydroxylated fullerenes and hydrated forms of hydroxylated Fullerenes, as the currently provided values are in fact for the density of water.

3.1.16 Summary on supplementary physicochemical characterisation

/

SCCS general comment on the physicochemical part

- The nanomaterial notified as raw fullerene powder is a mixture of fullerenes C60 and C70. The measured values for the contents in five batches have shown that the C60 content ranges from 70% to 80%. Data on the exact content of fullerene C70 have not been provided but could be deduced to range between 20-30%.
- A clarification is needed on the exact degree of hydroxylation for hydroxylated fullerenes and their hydrated forms.
- In this Opinion, the SCCS has considered 3HFWC as hydrated form of hydroxylated fullerene - similar to other hydroxylated fullerenes dispersed in aqueous media – because of the absence of reasonable scientific explanation for the nature of bonding between hydroxylated fullerenes and water molecules (other than hydrogen bonding), and other possible reactions/transformations of the starting materials (hydroxylated fullerene, hydrogen peroxide) during the manufacturing process.
- Based on the submitted studies, no analytical method was reliable for quantitative determination of 3HFWC. The composition of 3HFWC is provided by the Notifier as measured content of hydroxylated fullerenes and water, and this further supports the SCCS conclusion that, in terms of chemical composition, 3HFWC is a hydrated form of hydroxylated fullerene - similar to other hydroxylated fullerenes dispersed in aqueous media.

- The Notifiers should provide the following for fullerenes (C60 and C70), hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes:
    - detailed information on the levels of impurities, heavy metals, accompanying contaminants and organic solvents, along with detailed information on the methods of manufacturing (synthesis route, solvent removal, and any co-synthesized by-products).
    - Quantitative EM analysis for accurate size measurement of the particles in the nanoscale.
    - Detailed information on homogeneity and stability of the notified nanomaterials
- The Notifiers should also provide information indicating the shape, aspect ratio and agglomeration/ aggregation state of hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes and data on the surface charge of hydroxylated fullerenes.
- The SCCS needs more data/information to exclude the potential formation of free oxyradicals by the notified nanomaterials when used in cosmetics.

3.2 TOXICOKINETICS

3.2.1 Dermal / percutaneous absorption

Fullerenes:
According to one of the Notifiers, data analysis in general allows assumption of fullerenes' negligible dermal bioavailability during the cosmetics application. Available *in vitro* data shows its penetration ability only to the stratum corneum. Fullerenes were not detected in the dermis (one publication describes its detection in the epidermis in high-dose tests, but the test was performed only for 3 skin samples).

Ref: FULLERENES toxicity profile

The following two studies are reported by this Notifiers for dermal/ percutaneous absorption:

Based on the *in vivo* skin penetration studies of Xia et. al 2010 in Yorkshire weanling pigs and *in vitro* studies using skin discs from the same pig strain using powdered fullerene (99.5 % purity) in different solvents (chloroform, toluene, cyclohexane and mineral oil), penetration depth into stratum corneum was dependent on the solvent used. In the *in vitro* part of the study, fullerenes were not detected in the receptor fluid, but there was no report on epidermis or dermis.

Ref: Xia et. al 2010

SCCS comment to the study by Xia *et al.,* 2010
The study by Xia *et al.* 2010 was cited by one of the Notifiers and is presented as an article from public literature. The original study report was not available for evaluation of the study quality. The reported results indicated that by applying fullerenes *in vivo* and *in vitro*, the depth of penetration into stratum corneum is solvent dependent and that distribution of fullerene C60 into the stratum corneum was not only at the superficial layers, but also into deeper layers of the stratum corneum. In *in vitro* experiments using flow-through diffusion cells, for each of the organic solvents used, fullerene C60 could not be detected in the receptor fluid. This is, however, an exploratory study, not performed according to the SCCS requirements, especially for the flow-through experiments, the amounts in epidermis and dermis were either not measured or not presented. The study material used was Fullerene C60 at 99.5% purity, while the notified material (raw Fullerene powder) consists of a mixture of Fullerene C60 and Fullerene C70. It is also not clear what receptor fluid has been used. Therefore, the study cannot be considered for safety assessment.

Another *in vitro* study of Kato *et al.,* 2009 using human skin and Fullerene C60 in squalene showed that, after 24h Fullerene C60 was not detected in the dermis. Some amount was detected only in the epidermis with the highest dose tested. Only 3 skin samples were used.

Ref: Kato *et al.,* 2009.

SCCS comment to the study by Kato *et al.,* 2009

The study is described in a publication from open literature. The original study report was not available for evaluation. Moreover, it was performed using the test material in an organic solvent and not in a representative formulation, and therefore the findings of the study cannot be used for safety assessment.

One of the Notifiers stated that they do not have data on the skin and percutaneous absorption of Fullerenes C60 and C70 in accordance with the guidelines. In addition, they have not evaluated skin permeability using cosmetic formulations containing the fullerenes. Therefore, the Notifier agrees with SCCS recommendation to use a default 50% dermal absorption value in safety assessment.

Ref.: 20220627 supplemental document SCCS interim feedback.pdf

SCCS overall comment on dermal absorption of fullerenes

Studies on dermal penetration of fullerenes (a mixture of C60 and C70) have been described in the open literature. However, the studies were not performed in line with the current OECD test guidelines and/or the SCCS basic requirements for dermal penetration studies. Moreover, the published studies have indicated that dermal penetration of fullerenes is influenced by the solvents used in the test. It is not clear whether and to what extent the materials used in the published literature refer to the notified substances. Therefore, dermal penetration studies should be provided on the notified ingredients and performed in line with the SCCS requirements as detailed in the SCCS Notes of Guidance (SCCS/1628/21). In the absence of sound experimental data on the notified ingredients, it cannot be assumed that there is no dermal penetration of the nanoparticles, and therefore, the SCCS will use the default value of 50% for dermal absorption in safety assessment.

Hydroxylated fullerenes

Hydroxylated Fullerenes, as large water soluble (hydrophilic) molecules, with MW > 500 Da, are generally not expected to pass the skin barrier easily. A molecular dynamics study by Oiao *et al.* 2007, on translocation of Fullerene C60 and Hydroxylated Fullerene (C60(OH)20) across a model cell membrane of di-palmitoyl-phosphatidylcholine showed that the molecule of Hydroxylated Fullerene can barely penetrate the bilayer. The mean translocation time via diffusion for the Hydroxylated Fullerene molecule was several orders of magnitude longer than for the Fullerene C60. It was also determined that the two different forms of fullerenes, when adsorbed into/onto the bilayer, affected the membrane structure differently. This study offers a mechanistic explanation of that difference and for the reduced acute toxicity of functionalized fullerenes.

Ref: Qiao et. al. 2007.

SCCS overall comment on dermal absorption of Hydroxylated fullerenes

The study provided on dermal penetration of hydroxylated fullerenes does not meet the SCCS basic requirements as laid out in the SCCs Notes of Guidance (SCCS/1628/21). In the absence of sound experimental data, dermal penetration of hydroxylated fullerenes cannot be excluded. Unless experimental data on dermal penetration are provided, the SCCS will use the default value of 50% for dermal absorption in safety assessment.

Hydrated forms of Hydroxylated Fullerenes

The Notifier cites the study by Kato *et al.*, reported above for LipoFullerenes. According to the Notifier, based on the available studies indicating limited to negligible percutaneous absorption of Fullerenes, and in particular that of water-soluble functionalised derivatives like fullerenol, it can be concluded that the percutaneous absorption of Hyperharmonised Fullerenol-Water Complex (HFWC), with its additional stable water layers surrounding the fullerenol core, will be very low (practically negligible).

Ref: Kato *et al.,* 2009; 281_safety_file_2020-3-12-18-44-18.pdf

The Notifier submitted the following OECD TG 428 *in vitro* dermal absorption studies using cosmetic products:

Skin Absorption Assay V07 (Ref: VT_DA-PVA_664_22_001):

Guideline:	OECD 428 Guideline
Test system:	Human skin explants. Fresh abdomen skin collected from surgery and frozen.
Number of donors:	2 samples from 4 donors (2 Caucasian Females, 1 African Female and 1 Caucasian male).
Skin preparation:	200 µm thick prepared with a dermatome
Membrane integrity:	Not provided
Test substance:	Hyper-Harmonized Hydrolylated fullerene water complex (3HFWC)
Test item:	La Danza Hyperlight Fusion Anti-Aging Essential Complex. A cream containing 16% 3HFWC substance (formed from fullerenol at 0.15 g/L concentration). Initial dose of hydroxylated fullerene in cream is 14.9 mg/l.
Batch:	69226016
Purity:	Unknown
Dose applied:	2.5 mg
Exposed area:	Unknown
Study period:	24 hours
Assay conditions:	32°C±1°C. and 50% relative humidity
Sampling:	at 4 hours and 24 hours
Receptor fluid:	Phosphate Buffer Saline (PBS)
Solubility in receptor fluid:	Not provided
Mass balance analysis:	Not provided
Tape stripping:	No
Method of analysis:	LC-MS
GLP:	No
Period:	16/03/22 – 29/08/22

The test item investigated was a cosmetic cream (La Danza Hyperlight Fusion Anti-Aging Essential Complex Cream) containing 16% of 3HFWC substance (formed from fullerenol at 0.15 g/L concentration). The reconstructed skin was maintained overnight with maintenance medium at assay conditions before the application of the product. Fresh receptor solution was put in the receptor chamber avoiding the formation of air bubbles below the membrane. The incubation time with product started once the product was applied on the surface of the skin. Once the time was over, samples were taken from the receptor chamber, donor chamber, and skin, and analysed to obtain the absorbed amount of each analyte. The LC-MS analyses carried out to date allow the adequate determination of the analyte hydroxylated fullerene reliably and accurately in the expected real samples.

Results

The concentration of analyte detected and quantified in the donor chamber is below the limit of quantification of the analytical method used for analyte determination.

A mean percentage of 44.97% (± 22.62) of the analyte retained on human skin is observed.

In the receptor chamber, after 4 hours of contact, the concentration obtained was not measurable (out of the limit of detection and quantification).

After 24 hours of contact, the evaluated analyte was not detected in most of the analysed replicates, with the exception of one replicate, in which a concentration of 2.6 mg/L was quantified.

The detection of an amount of analyte in one replicate, in contrast to the 7 replicates where it cannot be quantified, may be due to the variability of the absorption system itself when using human skin from 4 different donors, which may result in anomalous values or outliers.

Conclusions

The concentration obtained after the absorption through human skin after application of "La Danza Hyperlight Fusion anti-aging Essential Complex" for the analyte Hydroxylated Fullerene is as follows:

Table 14:

Initial quantity	Quantity of unabsorbed dose	Quantities absorbed on/in the skin	Quantities that pass the skin after 4 hours	Quantities that pass the skin after 24 hours
14.9	< 2.8 mg/L	6.7 mg/L	< 1.38 mg/L	< 1.8 mg/L
100%	< 18.792%	44.97%	< 9.23%	< 12.33%

Ref: Skin Absorption test 16 V7 OECD 428

SCCS comment

According to the Notifier, the purpose of this study was to estimate the skin absorption of 3HFWC. However, the concentration of hydroxylated fullerene was measured by LC-MS in the cosmetic product and in the donor and receptor chambers, without measuring the concentration of the test material (3HFWC). Human skin from 4 different donors was used. Skin samples were not separated into epidermis and dermis, therefore it remains unclear how much material was present in living skin layers, which has to be included in the amounts considered absorbed. A proper mass balance is not possible, as concentrations in donor chambers and receptor fluid were below LoQ. Also, the fact that amounts in donor chamber were below LoQ puts the study results into question. However, based on the amounts determined in/on the skin, it can be assumed that the material becomes systemically available by the dermal route.

Skin Absorption Assay V08 (Ref: VT_DA-PVA_664_21_004):

Guideline:	OECD 428 Guideline
Test system:	Human skin explants. Fresh abdomen skin collected from surgery and frozen.
Number of donors:	2 samples from 4 donors (2 Caucasian Females, 1 African Female and 1 Caucasian male).
Skin preparation:	200 µm thick prepared with a dermatome

Membrane integrity:	Not provided
Test substance:	Hyper-Harmonized Hydrolylated fullerene water complex (3HFWC)
Test item:	Hyperlight Fusion anti-aging essential complex containing 71,517% 3HFWC substance (formed from fullerenol at 0.15 g/L concentration).
Batch:	210825.005
Purity:	Unknown
Dose applied:	2.5 mg
Exposed area:	Unknown
Study period:	24 hours
Assay conditions:	32°C±1°C. and 50% relative humidity
Sampling:	at 4 hours and 24 hours
Receptor fluid:	Phosphate Buffer Saline (PBS)
Solubility in receptor fluid:	Not provided
Mass balance analysis:	Not provided
Tape stripping:	No
Method of analysis:	LC-MS
GLP:	No
Period:	16/03/22 – 30/08/22

The test item investigated was a cosmetic cream (Hyperlight Fusion anti-aging essential complex) containing 71,517% of 3HFWC substance (formed from fullerenol at 0.15 g/L concentration).
The skin absorption study is performed using a semipermeable membrane such as reconstructed skin or skin explants. The membrane is located between the (i) donor and the (ii) receiver chambers. The product is applied on the stratum corneum exposed in the donor chamber. Below the membrane, the receptor chamber contains tissue culture media or a solution that simulates the physiological conditions and where the tested substances are highly soluble.
Fresh receptor solution was put in the receptor chamber avoiding the formation of air bubbles below the membrane. The incubation time with product started once the product was applied on the surface of the skin. Once the time was over, samples were taken from the receptor chamber, donor chamber, and skin, and analysed to obtain the absorbed amount of each analyte.
The LC-MS analyses carried out to date allow the determination of the analyte hydroxylated fullerene reliably and accurately in the expected real samples.

Results
The concentration of analyte detected and quantified in the donor chamber is below the limit of quantification of the analytical method used for analyte determination.
A mean percentage diffusion of 40.02% (± 14.75) of the analyte retained on human skin is observed.
In the receptor chamber, after 4 hours of contact, the concentration obtained for most of the analysed replicates was not measurable (out of the limit of quantification), except one replicate, which showed 5.6 mg/L.
After 24 hours of contact, the evaluated analyte was detected in all analysed replicates, in which a mean concentration of 4.8 mg/L was detected.
The detection of an amount of analyte in one replicate, in contrast to the 7 replicates where it cannot be quantified, may be due to the variability of the absorption system itself when using human skin from 4 different donors, which may result in anomalous values or outliers.

Conclusions
This skin absorption assay, based on the OECD 428 Guideline for the testing of chemicals "Skin absorption: *in vitro* method", was conducted to determine the skin and trans-dermal absorption of the Hyper-harmonized Hydroxylated fullerene water complex (3HFWC) using a nanosubstance (Hydroxylated Fullerene) as reference molecule used in cosmetic products to measure the diffusion of chemicals into and across human skin from 4 different donors.

The concentration obtained after the absorption through human skin after application of "Hyper-harmonized Hydroxylated fullerene water complex (3HFWC)" for the analyte Hydroxylated fullerene is as follows:

Table 15:

Initial quantity	Quantity of unabsorbed dose	Quantities absorbed on/in the skin	Quantities that pass the skin after 4 hours	Quantities that pass the skin after 24 hours
13.9	< 2.8 mg/L	5.56 mg/L	< 2.28 mg/L	4.75 mg/L
100%	< 20.14%	40.02 %	< 16.40 %	34.17%

Ref: Skin Absorption test 71,517 V8 OECD 428

SCCS comment

According to the Notifier, the purpose of this study was to estimate the skin absorption of 3HFWC, however, the concentration of hydroxylated fullerene was measured by LC-MS in the cosmetic product and in the donor and receptor chambers without measuring the concentration of the test material (3HFWC). Human skin from 4 different donors was used. Skin samples were not separated into epidermis and dermis; therefore, it remains unclear how much material was present in living skin layers, which has to be included in the amounts considered absorbed. A proper mass balance is not possible as concentrations in donor chambers and receptor fluid were below LoQ. Also, the fact that amounts in donor chamber were below LoQ puts the study results into question. However, based on the amounts determined in/on the skin, it can be assumed that the material becomes systemically available by the dermal route.

Skin Absorption Assay V04

Guideline:	OECD 428 Guideline
Test system:	Human skin explants. Fresh abdomen skin collected from surgery and frozen.
Number of donors:	2 samples from 4 donors (2 Caucasian Females, 1 African Female and 1 Caucasian male).
Skin preparation:	200 µm thick prepared with a dermatome
Membrane integrity:	Not provided
Test substance:	Hyper-Harmonized Hydrolylated fullerene water complex (3HFWC)
Test item:	Hyperlight Fluid Fusion Subcellular Essential Complex (aqueous solution 0.15 g/L)
Batch:	22DHA002/21
Purity:	Unknown
Dose applied:	2.5 mg
Exposed area:	0.38465 cm^2
Study period:	24 hours
Assay conditions:	32°C±1°C. and 50% relative humidity
Sampling:	at 4 hours and 24 hours
Receptor fluid:	Phosphate Buffer Saline (PBS)
Solubility in receptor fluid:	Not provided
Mass balance analysis:	Not provided
Tape stripping:	No
Method of analysis:	LC-MS
GLP:	No
Period:	2/08/22 – xx/08/22

Results

-Qualitative and quantitative analysis on donor chamber:
In the donor chamber samples, the concentration obtained is below the limit of quantification of the analytical method used for analyte determination.

-Qualitative and quantitative analysis on skin:
In the skin samples, the concentration obtained is below the limit of quantification of the analytical method used for analyte determination.

-Qualitative and quantitative analysis on receptor chamber after 4 hours:
In the receptor chamber, after 4 hours of contact, the concentration obtained is below the limit of quantification of the analytical method used for analyte determination.

-Qualitative and quantitative analysis on receptor chamber after 24 hours:
In the receptor chamber, after 24 hours of contact, the concentration obtained is below the limit of quantification of the analytical method used for analyte determination.

Notifiers' conclusions

The concentration obtained after the absorption through human skin after application of "Hyperlight Fluid Fusion Subcellular Essential Complex" for the analyte Hydroxylated fullerene is as follows:

Table 16:

	Unabsorbed dose	Absorbed on/in the skin	Doses that pass the skin after 4 hours	Doses that pass the skin after 24 hours
Concentration detected	< 2.5 mg/L	< 2.5 mg/L	< 2.5 mg/L	< 2.5 mg/L

In the receptor chamber, after 4 hours of contact, the concentration obtained is below the limit of quantification of the analytical method used for analyte determination of Hydroxylated fullerene. Moreover, after 24 hours of contact, the concentration obtained is below the limit of quantification of the analytical method used for analyte determination of Hydroxylated fullerene.

Ref: Skin Absorption test 100 V4 OECD 428 not signed

SCCS comment

The Notifier also submitted the above unsigned skin absorption study, where in the receptor chamber, after 4 and 24 hours of contact, the concentration obtained was below the limit of quantification of the analytical method used for analyte determination.

SCCS overall comment on dermal absorption of Hydrated forms of Hydroxylated Fullerenes

For the hydrated forms of hydroxylated fullerenes (3HFWC), the studies provided on *in vitro* dermal penetration are not in line with the OECD guidelines and/or the SCCS Notes of Guidance (SCCS/1628/21). In addition, there are various uncertainties concerning the results Nevertheless, based on the amounts determined in/on the skin, it can be inferred that the material becomes systemically available by the dermal route. Unless sound experimental data on dermal penetration are provided, the SCCS will use the default value of 50% for dermal penetration.

3.2.2 Other studies on toxicokinetics

Fullerenes

According to one of the Notifiers, published data show low oral bioavailability of C60 fullerene.

Ref: FULLERENES toxicity profile

***In vivo* Studies**

Inhalation

The following studies were referenced by the Notifier:

Taken from OECD 2016 (ENV/JM/MONO(2016)21):
To estimate the clearance rate and deposition fraction of C60 from inhalation exposure, the Fullerene C60 burden in the lungs, liver and brain of rats was determined after intratracheal instillation and inhalation (Shinohara *et al.,* 2010). In this study, male Wistar rats (6 rats/dose/observation period) were intratracheally instilled of a Fullerene C60 (Nanom Purple) suspension prepared with Tween 80 at the dose of 0.1, 0.2 and 1 mg/rat or exposed to a Fullerene C60 aerosol prepared with nebulizer at a concentration of 0.12 +/-0.03 mg/m^3 of the particle weight concentration in the exposure chamber (Morimoto *et al.,* 2010; Ogami *et al.,* 2011; Fujita *et al.,* 2009). Animals were sacrificed at 3 days, 1 month and 3 months after the end of exposure. Fullerene C60 burdens in the lungs, liver and brain was determined at various points (1 h to 6 months) by sensitive HPLC with UV detection. Inhaled Fullerene C60 clearance from the lung was evaluated using a 2-compartment model, fast clearance after deposition on lung surface and slow clearance after retention in the epithelium. Pulmonary Fullerene C60 burden decreased with time and depend on the Fullerene C60 concentration administered. The concentration of Fullerene C60 in the liver and brain was below the detection limit: 8.9 ng/g tissue after intratracheal instillation and inhalation. The half-life in the lung of intratracheally instilled Fullerene C60 was 15-28 days. Mode evaluation revealed that most instilled particles could be eliminated by the fast clearance pathway. This finding was consistent with the transmission electron microscopy finding that many particles were present in alveolar macrophages.

Ref: Shinohara *et al.* 2010

A study by Naota *et al.,* 2009, cited by Hendrickson *et al.,* 2014, investigated the translocation pathway of intratracheally instilled fullerene C60 particles from the lung into the blood circulation in the mouse. Using light microscopy, aggregated particles of fullerene were observed in the capillary lumen in the lung and the pulmonary lymph nodes immediately after instillation. Electron microscopic analysis demonstrated an increased number of pinocytotic vesicles (caveolae) of various sizes in the type 1 alveolar epithelial cells and endothelial cells; occasional caveolae containing some particulate substances were observed. In addition, particles of various sizes were observed throughout the structure of the air-blood barrier. These findings suggest that fullerene particles may pass the air-blood barrier by both diffusion and caveolae-mediated pinocytosis, resulting in immediate translocation into the systemic circulation.

Ref: Naota *et al.* 2009

IV administration

The following study was referenced by the Notifier:
Taken from OECD 2016 (ENV/JM/MONO(2016)21)
Biodistribution of C60 (Nanom Purple) in male Wistar rats (5 rats/time point) after tail vein administration (5 mg/kg bw/injection x 4 times) was examined using LC-MS/MS (Kubota *et al.,* 2011). Fullerene C60 was detected in various tissues, such as brain, kidneys, liver, lungs,

and spleen of male Wistar rats. On the other hand, no Fullerene C60 was found in blood. The highest Fullerene C60 concentration was observed in the lungs, followed by spleen, liver, kidneys and brain. These results suggested that Fullerene C60 injected in the tail vein could be filtered by lung capillary vessels and accumulate in the lungs prior to being distributed to other tissues. Furthermore, Fullerene C60 not being detected in the blood indicated that clearance of Fullerene C60 from the blood by filtration might effectively occur in the lungs. The time-dependent variation in the biodistribution of Fullerene C60 was evaluated. A time-dependent decrease in Fullerene C60 concentrations was observed in all tissues, except spleen. Moreover, a decreasing trend of Fullerene C60 levels differed among tissues, which could be due to differences in accumulation.

Ref: OECD 2016 (ENV/JM/MONO(2016)21)

Hydroxylated fullerenes

In-silico ADME prediction – toxicokinetics modelling

The Notifier conducted in-silico assessment of the ADME properties of Hydroxylated Fullerenes $C_{60}(OH)_x$. According to the Notifier 'Hydroxylated Fullerene $C_{60}(OH)_{30\text{-}50}$ is experimentally obtained nanomaterial which contains 40 hydroxyl groups. Therefore, selected substances, $C_{60}(OH)_{30}$, $C_{60}(OH)_{40}$ and $C_{60}(OH)_{50}$, as well as related Hydroxylated Fullerenes from the literature, $C_{60}(OH)_{24}$ and $C_{60}(OH)_{60}$ were analysed by in-silico methodology. Additionally, Fullerene, C60 was tested, as a substance insoluble in water. The available online servers ADMETlab, admetSAR 2.0, ALOGPS 2, Molinspiration, pkCSM, and SwissADME were used for in-silico ADME prediction. Contradictory results were obtained for the assessment of intestinal resorption in the gastrointestinal tract.
According to one in-silico tool and for all tested substances, Hydroxylated Fullerenes are poorly resorbed (HIA and Lipinski parameters), while the results of the other two in-silico tools indicated good intestinal resorption for most of the tested substances. In-silico prediction of volume of distribution factors for tested Hydroxylated Fullerenes showed low value (<0.6 L/kg) and average value (0.6 < Vd < 5.0 L/kg). The predicted results for the binding potential of the investigated substances to bind to plasma proteins and BBB permeability are radically different depending on the applied software. For most of the tested Hydroxylated Fullerenes used in-silico tools showed that they do not inhibit CYP enzymes. Also, the majority of the investigated substances are not substrates for CYP enzymes according to the used online software. The obtained in-silico results about skin permeability indicating that Hydroxylated Fullerenes $C_{60}(OH)_x$ have no potential for permeability through the skin.
In context of the obtained opposite results, it is not clear how the results obtained by two or more models/systems should be interpreted where the estimates are widely different or contradicting. In general, taking into account the many different available in-silico tools, systemic exposure Hydroxylated Fullerenes $C_{60}(OH)_x$, as cosmetic ingredients, via oral (in-silico prediction) and dermal absorption (in-silico prediction of skin permeability) is expected to be minimal. But, according to the Notifier these in-silico ADME results should be taken into account with all the uncertainly of in-silico analysis, especially when it comes to nanomaterials and applicability of tools'.

Ref: 3HFWC data submission main document

Another study by Ji *et al.* (2009) examined the biodistribution and tumour uptake of hydroxylated fullerenes (C60(OH)x) in five mouse-bearing tumour models. The results showed that the intravenously administered ^{125}I-labelled fullerenol [^{125}I–$C_{60}(OH)_x$] at dose of 10 µg per mouse is distributed in all organs of rats, except for the brain. One hour after the administration, labelled fullerenol was accumulated mainly in the liver, spleen, and bone tissues and was also detected in the stomach and blood. After 6 h, the character of the distribution of ^{125}I–$C_{60}(OH)x$ changed and the level decreased in the blood and increased in the liver, spleen, kidney, and bone tissues. After 72 h, the compound was completely absent in the tissues and 92% of the particles were excreted in the urine and 8% in the faeces.

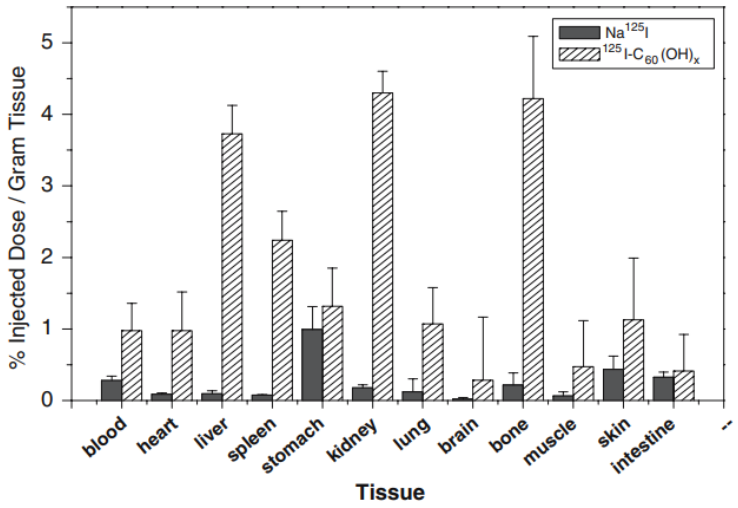


Figure 11: Comparison of the biodistribution between $^{125}I–C_{60}(OH)x$ and $Na^{125}I$ in normal Kunming mice at 6 h post dosing (Ji *et al.* 2006).

Figure 11 shows the distribution of $^{125}I–C_{60}(OH)x$ in normal mice, at 6 h post-dosing the levels increased in the liver, spleen, kidney, and bone tissues. These finding indicate that systemically available hydroxylated fullerenes can be distributed to various organs in the body.

Hydrated forms of hydroxylated fullerenes

No studies were submitted for the hydrated forms of hydroxylated fullerenes.

SCCS overall comments in toxicokinetics

Fullerenes

Oral route

According to the studies reviewed by Hendrickson *et al.* (2014), systemically available fullerenes have been found in liver, kidney, and spleen after oral exposure of test animals. Fullerenes were mainly excreted in faeces. However, there are no data to allow estimation of the bioavailability of the nanoparticles from the oral exposure.

Inhalation route

The study by Shinohara *et al.* (2010) was not designed to estimate the absorption by inhalation, but only for the deposition and clearance from the lung. According to the data presented by the authors, lung accumulation of fullerene C60 has been demonstrated. In a mouse study, Noata *et al.* (2009) suggested that inhaled fullerene could translocate into the systemic circulation by diffusion at the air-blood barrier.

IV route

According to the studies reviewed by Hendrickson *et al.* (2014), the liver is the main target organ and the site of accumulation of fullerenes after intravenous administration.

In summary, the limited toxicokinetics data indicate that systemically available fullerenes will be well distributed to various organs in the body (including foetal tissues), with potential for accumulation in the lungs and the liver.

Hydroxylated Fullerenes

For the hydroxylated fullerenes, the Notifier reported that *in-silico* assessment was carried out of the ADME properties of fullerenes, and hydroxylated fullerenes C60(OH)x [x=24,30,40,50,60]. Although the Notifier had indicated that details were provided in a report, this information could not be found in any of the submitted documents.

From the brief available summary of the *in-silico* assessment, the SCCS has noted that:

- in-silico ADME assessment was not performed for 3HFWC due to the lack of SMILES identifiers.
- the results of the assessment carried out for fullerenes and hydroxylated fullerenes showed contradictory results, where one *in-silico* tool predicted hydroxylated fullerenes to be poorly resorbed, and two other tools indicated good intestinal resorption for most of the tested substances.

The SCCS also noted that, for the *in silico* ADME assessment, the Notifier had considered fullerenes and hydroxylated fullerenes as chemical substances. Considering that these materials also have a particle nature, the SCCS is of the view that the *in silico* tools used to predict ADME properties are not appropriate as they have been developed and tested for predicting ADME behaviour of chemicals, not that of (nano)particles. Also, in view of the contradictory results from different *in silico* tools, the SCCS considers that the information from *in silico* assessment is not relevant for safety assessment of fullerenes and hydroxylated fullerenes.

3.3 EXPOSURE ASSESSMENT

3.3.1 Function and uses

Data on function and uses were not provided by the Notifiers.

SCCS comment
Detailed data on function and uses for fullerenes, hydroxylated fullerenes and the hydrated forms of hydroxylated fullerenes must be provided.

The SCCS has retrieved the following information from 19 notifications uploaded on the CPNP portal by the Notifiers:

Notification No.	Ingredient/CAS No	Cosmetic Product	Concentration	Exposure route
1003493	(C60-Ih)[5,6]fullerene/ 99685-96-8	1279 RF 1 Face Cream	0.0002 % w/w	Dermal/ Leave on
1003557	C60-Ih)[5,6]fullerene/ 99685-96-8	1280 RF 2 Face Cream	0.0002 % w/w	Dermal/ Leave on
1003558	C60-Ih)[5,6]fullerene/ 99685-96-8	1281 RF 3 Face Cream	0.0002 % w/w	Dermal/ Leave on
1003559	C60-Ih)[5,6]fullerene/ 99685-96-8	1282 RF 4 Face Cream	0.0002 % w/w	Dermal/ Leave on
1003560	C60-Ih)[5,6]fullerene/ 99685-96-8	1283 RF 5 Face Cream	0.0002 % w/w	Dermal/ Leave on

1003561	C60-Ih)[5,6]fullerene/ 99685-96-8	1285 RF 1 Face Serum	0.0002 % w/w	Dermal/ Leave on
1003562	C60-Ih)[5,6]fullerene/ 99685-96-8	1285 RF 2 Face Serum	0.0002 % w/w	Dermal/ Leave on
1003563	C60-Ih)[5,6]fullerene/ 99685-96-8	1287 RF 1 Face Mask	0.0002 % w/w	Dermal/ Leave on
1003564	C60-Ih)[5,6]fullerene/ 99685-96-8	1287 RF 2 Face Mask	0.0002 % w/w	Dermal/ Leave on
1003565	C60-Ih)[5,6]fullerene/ 99685-96-8	Face care products other than face mask/ 1288 RF 1 Skin Lightening	0.0002 % w/w	Dermal/ Leave on
1003566	C60-Ih)[5,6]fullerene/ 99685-96-8	Face care products other than face mask/ 1289 RF 2 Skin Lightening	0.0002 % w/w	Dermal/ Leave on
1003567	C60-Ih)[5,6]fullerene/ 99685-96-8	1277 RF 1 Eye Contour	0.0002 % w/w	Dermal/ Leave on
1003568	(C60-Ih)[5,6]fullerene/ 99685-96-8	1278 RF 2 Eye Contour	0.0002 % w/w	Dermal/ Leave on
1004108	Hydroxylated Fullerenes/ not reported	Face care products/ Anti-Ageing Essential Complex	0.0024 % w/w	Dermal/ Leave on
1004204	Hydroxylated Fullerenes/ not reported	Other skin care products/ Hyperlight Fusion - Intensive Body Sculptor- Anti-cellulite body lotion	0.0024 % w/w	Dermal/ Leave on
1004546	(C60-Ih)[5,6]fullerene/ 99685-96-8	Global Anti-ageing Face Cream	0.0002 % w/w	Dermal/ Leave on
1004547	(C60-Ih)[5,6]fullerene/ 99685-96-8	Illuminating Eye Contour Cream	0.0002 % w/w	Dermal/ Leave on
1004548	(C60-Ih)[5,6]fullerene/ 99685-96-8	Neck & Décolleté Firming Cream	0.0002 % w/w	Dermal/ Leave on
1004864	Hydroxylated Fullerenes/ not reported	Body care products/ Hyperlight Fluid Fusion - Subcellular Essential Complex - Personal Care Nanolotion	0.015 % w/w	Dermal/ Leave on

From the received notifications, it is not clear whether the concentration of 0.0002 % w/w is related to fullerene C60 or to "raw fullerene powder", which is a mixture of fullerene C60 and fullerene C70. It is also not clear whether the concentrations of 0.0024 % w/w and 0.015 % w/w are related to hydroxylated fullerenes or to their hydrated forms (3HFWC).

3.4 TOXICOLOGICAL EVALUATION

Fullerenes

As reported by the Notifier on Fullerenes, the raw fullerene powder provided by them is a mixture of Fullerene C60 and Fullerene C70. The content of Fullerene C60 ranges approximately from 70 to 80% and the concentration of other fullerenes such as Fullerene C82 and oxygenated fullerene is less than 1%. As shown in Table 1, both Fullerene C60 and C70 particles are composed only of carbon atoms, and their physical properties such as solubility are similar. Based on the chemical similarity between C60 and C70, the Notifier speculates that C70 possesses the same physiological activity, transdermal absorption, and safety as C60. The Notifier also stated that the National Institute of Advanced Industrial Science and Technology in Japan reported in a study published by Horie *et al.* 2013 that the safety of Fullerene C60 and Fullerene C70 are equivalent (Table 17).

Table 17: *In vitro* evaluation of cellular influences induced by stable fullerene C70 medium dispersion: Induction of cellular oxidative stress (Horie *et al.*, 2013)

Comparison of cellular influences induced by C_{60} and C_{70}.

	HaCaT				A549			
	C_{60}[a]		C_{70}		C_{60}[a]		C_{70}	
Concentration of fullerene (μg mL^{-1})	14.2	6.6	13.4	5.4	14.2	6.6	13.4	5.4
MTT conversion (24 h) (% of control)	104.9 ± 4.6	108.4 ± 6.4	96.8 ± 9.1	102.4 ± 6.2	86.4 ± 3.5	97.3 ± 8.9	90.4 ± 8.1	96.6 ± 7.6
Colony forming ability (% of control)	107.8 ± 13.6	97.8 ± 11.3	59.6 ± 11.1**	90.2 ± 15.0	71.1 ± 11.4**	80.9 ± 12.7**	77.3 ± 16.6**	84.1 ± 6.9**
Intracellular ROS level (24 h)	1.9 ± 0.3	1.25 ± 0.14	2.13 ± 0.1**	1.40 ± 0.04**	1.22 ± 0.24	0.95 ± 0.08	0.99 ± 0.04	1.22 ± 0.1**
Intracellular lipd peroxidation level (24 h)	1.97 ± 1.0*	2.31 ± 1.0*	1.4 ± 0.3*	1.9 ± 0.02**	2.02 ± 0.3**	2.16 ± 0.5**	1.85 ± 0.2**	2.09 ± 0.2**

The value of the intracellular ROS level and lipid peroxidation level were indicated as a relative value to the unexposed cells.
[a] These values were reported previously except lipid peroxidation level. Horie et al. (2010).
* $P < 0.05$ (vs. unexposed cells, Dunnett, ANOVA).
** $P < 0.01$ (vs. unexposed cells, Dunnett, ANOVA).

All safety evaluation studies submitted by the Notifier were conducted using raw fullerene powder (mixture of Fullerene C60 and Fullerene C70), while the safety evaluations reported in the externally cited references mainly used Fullerene C60. However, based on the equivalence between C60 and C70 mentioned above (Table 17), the Notifier decided to use equally both internal and external safety data in the evaluation of safety of fullerenes.

SCCS comment

Considering the similarities between fullerenes C60 and C70 in terms of chemical composition, close structural analogy, and toxicological aspects tested via *in vitro* assays, the SCCS has accepted the Notifier's justification for data read-across between the two fullerenes.
In this regard, the SCCS is also aware of two studies that reported a disparity between C60 and C70 fullerenes in terms of the potential to induce reactive oxygen species (ROS) in exposed cell lines *in vitro* (Proskurnina *et al.*, 2021), and ROS production and photoinduced cleavage of supercoiled plasmid pBR322 DNA (Liosi *et al.*, 2021). The study by Liosi *et al.* (2021) used a conjugate of fullerene-polyethylene glycol, and not (neat) fullerenes that are under current assessment. However, both *in vitro* studies reported that C60 is more active in inducing ROS production, and eliciting DNA damage, than C70. These findings further support the SCCS consideration of an equivalence for data read-across between C60 and C70 because they indicate that the worst case from a risk assessment point of view will be covered for a fullerene mixture that is typically composed of C60 (70-80%) and C7 (20-30%) fullerenes.

3.4.1 Acute toxicity

3.4.1.1 Acute oral toxicity

Fullerenes

The following reports and studies were provided by the Notifier(s):

In a study by Mori *et al.,* Fullerenes (mixture of C60 and C70, fullerite, sublimed technical grade, purity: 99.5%, supplied by one of the Notifiers) were administered once orally at a dose level of 2000 mg/kg to male and female Sprague–Dawley rats. The study was conducted in compliance with the guiding principles for the care and use of laboratory animals by the Japanese Pharmacological Society. No deaths were observed and the body weights in both sexes of the 2000 mg/kg group increased in a similar pattern to the control group.

LD50> 2000 mg/kg.

Ref: Mori *et al.*, 2006.

A Single Dose Oral Toxicity Study of Fullerenes in Rat

Guideline:	Non-Guideline study - conducted following Standards for Conduct of Nonclinical Studies on the Safety of Drugs (MHW, Ordinance No. 21, March 26, 1997)
Species/strain:	Rats/ CD(SD)IGS, 6 weeks old at the time of administration
Group size:	2 groups of 5 males and 5 females
Test substance:	Fullerene (powder)
Batch:	Lot No. 040406
Purity:	66.4 ± 0.78 % (impurities not mentioned)
Vehicle:	Water containing 0.5% carboxymethylcellulose-sodium salt and 0.1% Tween 80
Dose levels:	2000 mg/kg bw; 10 ml/kg body weight
Administration:	Oral gavage (single)
GLP:	In compliance
Study period:	21 May – 30 November 2004

Results: A single dose of fullerene (powder) suspended in water was administered via oral gavage at 2000 mg/kg bw (10 ml/kg bw) to two groups of 3 females each. No animal died during the 14-day post-administration observation period. Body weights were comparable to the control group. Necropsy did not show any abnormal findings in any of the animals. Coloured stool was noted on day-1 in both sexes and day-2 in one male, which was attributed to excretion of the test substance. It was concluded that fullerene has no acute toxicity and the lowest lethal dose is above 2000 mg/kg bw in both sexes.

Ref: B040373: A Single Dose Oral Toxicity Study of Fullerenes in Rat

Acute Oral Toxicity Study of Water-Soluble Fullerenes in Rat

Guideline:	OECD Guideline no. 423:2001
Species/strain:	Rats/ CD(SD)IGS, 8-week-old
Group size:	2 groups of 3 females each

Test substance:	mentioned as 'water-soluble fullerenes (synonym fullerene)'
Batch:	Not given
Purity:	Not given. The composition of test substance is mentioned as to contain 0.365% C60 fullerene in excipient polyvinylpyrrolidone (PVP)
Vehicle:	Water
Dose levels:	2000 mg/kg bw; 10 mL/kg body weight
Administration:	Oral gavage (single)
GLP:	In compliance
Study period:	24 February – 12 July 2005

Results: A single dose of fullerene suspended in water was administered via oral gavage at 2000 mg/kg bw (10 ml/kg bw) to two groups of 3 female rats each. The animals were fasted from the evening before administration. No dead animals were recorded during the 14-day post-administration observation period. Body weights showed normal growth. Necropsy did not show any abnormal findings in any of the animals. Coloured stool was noted on day2, which was attributed to excretion of the test substance. It was concluded that the test substance has no acute toxicity under the test conditions, and hence can be regarded as category 5 (unclassified) in regard to acute oral toxicity.

Ref: B040965: Acute Oral Toxicity Study of Water-Soluble Fullerenes in Rat; SDS_Radical Sponge170331; FULLERENES toxicity profile

SCCS comment

The raw fullerene powder used in the single dose oral toxicity study of Fullerenes contains ~66% C60 fullerene; from the test reports it is unclear whether the remaining content is C70 or another material. In view of the results from this study, the SCCS agrees with the Notifier that raw Fullerene powder is not acute toxic via the oral route.

The Notifier submitted an additional acute oral toxicity study of water-soluble fullerenes in rat (compliant with OECD Guideline 423) which was conducted by using the formulation (Radical sponge®) and therefore it will not be used in this safety evaluation.

Hydroxylated fullerenes

The following reports and studies were provided by the Notifier(s):

An *in-vivo* study on acute toxicity of $C_{60}(OH)_{30}$ from 2012, after intravenous administration to female Sprague-Dawley rats observed no clinically significant chemistry changes after IV treatment with 10 mg/kg dose. These experiments suggest that fullerenol is well tolerated after IV administration to rats (administered dose was 10 mg/kg).
According to the Notifier, based on the available studies, it can be concluded that the applied concentrations and exposure (potentially achievable biological exposure) in practice is far below the demonstrated tolerated acute dose in rodents.

Ref: Monteiro-Riviere *et al.*, 2012; 281_safety_file_2020-3-12-18-44-18

Study: Acute toxicity study of Hydroxylated Fullerene $C_{60}(OH)_{30-50}$

NOTE: Certified translation from Serbian into English.

Study number:	LMEM-AT-03/2022
Guideline:	Study performed according to OECD TG no 423, EU Directive 2010/63/EU, and ISO 10993-2:2006 Animal welfare requirements.
Species/strain:	Mouse, NMRI HAN, 5 weeks of age

Group size:	2 groups of 6 experimental and 6 controls (animals of both genders were used.
Test substance:	Hydroxylated Fullerene $C_{60}(OH)_{30-50}$
Batch:	Laboratory sample
Purity:	Not given. The composition of test substance is mentioned with a concentration of 0.15 g/L
Vehicle:	Not given
Dose levels:	7.5 mg/kg
Administration:	Gastric probe (1 mL in two applications of 0.5 mL in 24 hours)
GLP:	-
Study period:	10 May – 24 May 2022

Results: Treated animals did not show signs of intoxication immediately upon administration, or later during the period of observation. Treated experimental animals behaved quite normally (just like the control group). Behaviour was also normal on intentional standard provocation tests. No neurological misbehaviour was noticed. Hygienic behaviour was normal. Eyes were clear and clean; the nostrils and other natural orifices were clean.
Experimental animals did not exhibit any abnormal reactions in relation to food and water. They ate and drank water in a normal manner. No animals died during the experimental period.
After day 14 of the experiment, all animals were sacrificed and pathoanatomical examination was performed. Macroscopic examination of organs and tissues (liver, spleen, kidney, stomach, small intestines, lungs, heart, and brain) did not show any pathological changes in any animal.
Based on clinical observation of the experimental animals during the 14-day period and on subsequent pathoanatomical examination, it was concluded that the test substance Hydroxylated Fullerene $C_{60}(OH)_{30-50}$ applied at a dose of 7.5 mg/kg does not cause any toxic effects in test animals.

Ref: Acute toxicity – ENG Report HF

SCCS comment

According to the Notifier, the data produced in this study is not specifically intended for demonstrating the safety of substance for use in cosmetics, but is part of substance evaluation for medical application, yet the Notifier did not specify the exact regulation for which the study was performed. In the absence of that information, the Notifier cannot use it to demonstrate safety of the material for cosmetic purposes. The study by Monteiro-Riviere *et al.*, 2012, on the other hand, indicated a lack of acute toxicity of hydroxylated fullerenes at the oral dose of 7.5 mg/kg. The SCCS has noted that the evaluated hydroxylated fullerenes used in the study were prepared "in house" by the authors based on fullerenes from a US supplier.

Hydrated forms of Hydroxylated Fullerenes:

The following report was provided by the notifier(s)

Investigation of acute toxicity of 3HFWC

Study number:	LMEM-AT-01/2022
Guideline:	Study performed according to OECD TG no 423, EU Directive 2010/63/EU, and ISO 10993-2:2006 Animal welfare requirements.
Species/strain:	Mouse, NMRI HAN, 5 weeks of age
Group size:	2 groups of 6 experimental and 6 controls (animals of both genders were used).
Test substance:	Hyper-Harmonized Hydroxylated Fullerene Water Complex-3HFWC
Batch:	Laboratory sample
Purity:	Not given. The composition of test substance is mentioned with a concentration of 0.15 g/L
Vehicle:	Not given

Dose levels: 7.5 mg/kg
Administration: Gastric probe (1 mL in two applications of 0.5 mL in 24 hours)
GLP: -
Study period: 10 May – 24 May 2022

Results: Treated animals did not show signs of toxic reaction immediately after application, or in the later course of observation. They behaved normally in accordance with what is expected for their species, gender, age and environment. The reaction of animals to provoked behaviour was normal and expected. No signs of neurological deficits were observed. The hygienic behaviour of the animals was normal. The eyes were clear and clean, the nostrils and other natural orifices were clean.
Experimental animals did not exhibit any eating disorders. They ate and drank water as usual. No animals died during the experimental period.
After 14 days from the start of the experiment, all animals were sacrificed, and a macroscopic examination was performed. Macroscopic examination of organs and tissues (liver, spleen, kidney, stomach, intestines, lungs, and heart) did not reveal any changes in any animal.
Based on clinical observation of the experimental animals and the macroscopic examination of the organs after 14 days from the start of the experiment, it was concluded that the investigated product Hyper Harmonized Hydroxylated Fullerene Water Complex-3HFWC at a dose of 7.5 mg/kg does not cause toxic effects in tested animals.

Ref: Acute toxicity – ENG Report 3HFWC.

SCCS comment
According to the Notifier, the data produced in this study is not specifically intended for demonstrating the safety of substance for use in cosmetics, but is part of substance evaluation for medical application, but the exact regulation for which the study was performed has not been specified. In the absence of this information, the study cannot be used to demonstrate safety of the material when used for cosmetic purposes.

SCCS overall comment on acute oral toxicity
The limited available information indicates that C60/C70 fullerenes and hydroxylated fullerenes may not be acute toxic.
Various acute oral toxicity studies were provided by one of the Notifiers for hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes which were claimed to have been performed for medical application. However, the exact regulation(s) for which these studies were performed were not given. In the absence of this information, the studies cannot be used for assessment safety of the material when used as cosmetic ingredients.

3.4.1.2 Acute inhalation toxicity

Fullerenes:
According to the Notifier, considering the nature of the used cosmetic material (the Fullerene water dispersion) the inhalation route of exposure is out of concern, thus the available data about the inhalation toxicity were not analysed.

Ref: FULLERENES toxicity profile

SCCS comment
The SCCS has noted the Notifiers' reasoning for not carrying out inhalation exposure assessment and has therefore not considered the use of the materials in inhalable products in this safety assessment.

3.4.2 Irritation and corrosivity

Fullerenes

Skin irritation

The following two reports were provided by the notifier(s):

1. **Primary dermal irritation study of fullerenes in rabbits**

Guideline: /
Substance: 0.5 g fullerenes moistened with 0.3 ml propyleneglycol
Lot: 040406
Application: 0.5 g per test site 24 hrs on intact and abraded skin
Animals: 3 Japanese white rabbits
Results: No skin reactions upon removal of patches and at 48 and 72 hrs.
Year: 2004

Ref: Primary dermal irritation study in rabbits. Mitsubishi Chemical Safety Institute 2004, B040374

2. **A 14-day cumulative skin irritation study of fullerenes in rabbits**

Guideline: /
Substance: 10% fullerenes w/v in propyleneglycol
Lot: 040406
Application: 0.2 mL per test site without occlusion, daily 14 days
Animals: 5 Japanese white rabbits
Results: No skin reactions.
Year: 2004
Ref: A 14-day cumulative skin irritation study of fullerenes in rabbits Mitsubishi Chemical Safety Institute 2004, B0403745

SCCS comment

The raw fullerene powder consists of about 66% fullerene C60. From the test reports, it is unclear whether the remaining 34 % is Fullerene C70 or another material. The primary test was performed in 3 animals; therefore, it can be regarded as a preliminary test, *i.e.* only indicative of the absence of irritation potential. The cumulative test indicates absence of potential for skin irritation.

3. **Occlusive human patch-test study, 24 hours, in 20 subjects**

Guideline: /
Test material: Suspension of 3% natural fullerene in petrolatum
Control: saline and white petrolatum
Product ID: 10970, Product code 080508-01
Application: 1 cm^2 under occlusion, during 24 hours in Finn chamber
Reading: 2 hrs and 24 hrs after patch removal
Subjects: 20 humans (2 m, 18 f)
Year: 2020
Study No: NDR-0007236 and 4200162
Results: None of the subjects presented clinical signs.

Ref: NDR-0007236NF Human Patch Test

SCCS comment

From page 3 onwards in the study report, a different Study Number is listed at the top of each page.

The following published studies were referenced by the notifier(s):

1. Dermal skin irritation study with Fullerene C60
A Guinea pig skin irritation study according to OECD Guideline 404 with 10% fullerenes in olive oil showed no skin reactions.
Ref: Ema *et al.*, 2013. (study conducted in 2010)

2. Primary and cumulative skin irritation tests according to the Draize method
The tests were conducted with highly purified fullerenes (a mixture of C60 and C70 fullerite) in 3 resp. 5 rabbits. Dose: 0.5 g in 0.3 ml propyleneglycol (PG) for the primary test and 20 mg in 0.2 ml PG for the cumulative test. Results: no skin reactions.
Ref: Aoshima 2009

3. Human patch test study
A 24-hour patch test (Finn chamber) with 0.01 g highly purified fullerenes on the upper arms in 55 human volunteers showed no skin reactions.
Ref: Aoshima 2009

4. Human patch-test study.
A brief study report on a 24-hour patch-test in 55 human volunteers with 0.01 g fullerene (no further specification) showed no skin reactions.
Ref: Nichimoko No. 16027

SCCS comment
It seems that the Aoshima (2009) publication presents the same data as the studies presented in the B040374, B040375 and Nichimoko 16027 reports (see above). Overall, the studies indicate that the test material is not a skin irritant.

Eye irritation
The following study was provided by the notifier:

Primary eye irritation study in rabbits

Study nr:	B040376-1
Guideline:	Standard for conduct of nonclinical studies on the safety of drugs, Japan (MHW, Ordinance No 21, March 26, 1997), Draize method.
Test material:	Fullerene, lot nr 040406, black powder, purity 66.4 %.
Animals:	6 male Japanese White rabbits
Schedule:	3 animals' right eye exposed without washing after application, 3 animals right eye exposed followed by rinsing after 30 seconds
Application:	0.1 g test substance in lower conjunctival sac
Assessments:	1, 24, 48, 72 hrs and 4 days after application
Scoring:	Draize method
Date:	2004
Results:	Weighted mean score (Draize) 6.0, indicating eye irritating potential attributable to physical effects from powder.

Ref: Primary eye irritation study in rabbits. Mitsubishi Chemical Safety Institute 2005

SCCS comment
From the test report, the composition of the Fullerene powder is unclear. From other reports, it can be deduced that it is about 66% Fullerene C60, but it is unclear whether the remaining 34% is Fullerene C70.

Hydroxylated fullerenes

Skin irritation:
The following information was provided by the notifier:
An OECD compliant study on reconstructed human skin with hydroxylated fullerene powder (c60(OH)n, n=30-60) showed no skin irritation potential

Guideline:	OECD 439
Test material:	Hydroxylated fullerene $C60(OH)_n$, n=30-50, as 99.9% pure beige/yellow powder
Batch:	21C0226
Control:	DPBS buffer (neg contr) and SDS 5% aq (pos control)
Tissue:	human epidermis.
Nr:	3 tissues for main test, 3 tissues for neg control, 3 tissues for pos control
Historic data:	negative and positive controls compatible with current test results
Exposure:	60 minutes
Result:	Tissue viability (optical density MTT) was 85%, indicating non-irritant.

Ref: Laus version2 21102502G840 (2021)

Eye irritation:
The following information was provided by the notifier:
An OECD compliant study on reconstructed human cornea-like epithelium (RhCE) with hydroxylated fullerene powder (c60(OH)n, n=30-60) showed that the test item is an eye irritant

Guideline:	OECD 492
Test material:	Hydroxylated fullerene $C60(OH)_n$ (n=30-50) 99.9% pure beige/yellow powder
Batch:	21C0226
Control:	Sterile demi water (neg) and methyl acetate
Tissue:	Reconstructed human corneal epithelium
Nr:	2 tissues for main test, 2 tissues for neg control, 2 tissues for pos control
Historic data:	negative and positive controls compatible with current test results
Exposure:	6 hours
Result:	Tissue viability (optical density, MTT) reduced to 8.1%, indicating irritant

Ref: Laus version2 21102502G891 (2022)

Hydrated forms of hydroxylated fullerenes
The following information was provided by the notifier:

An OECD compliant study on reconstructed human skin with HFWC shows no skin irritation potential.

Guideline:	OECD 439
Test material:	Hydroxylated fullerene $C60(OH)_{30\text{-}50}$ @$(H_2O)_{144\text{-}2528}$ 0.015% in water
Batch:	01-2021-07-13
Control:	DPBS buffer (neg contr) and SDS 5% aq (pos control)
Tissue:	Reconstructed human epidermis.
Nr:	3 tissues for main test, 3 tissues for neg control, 3 tissues for pos control
Historic data:	Negative and positive historical controls compatible with current test results
Exposure:	60 minutes
Result:	Relative tissue viability (optical density MTT) 122%, indicating non-irritant.
Date:	2022

Ref: Laus version1 21092301G840 (2022)

Eye irritation:
The following information was provided by the notifier:
An OECD compliant study on reconstructed human cornea-like epithelium (RhCE) with HFWC shows that the test item is not an eye irritant.

Guideline:	OECD 492
Test material:	Hydroxylated fullerene $C60(OH)_{30-50}$ @$(H_2O)_{144-2528}$ 0.015% in water
Batch:	21C0226
Control:	Sterile demi water (neg) and methyl acetate
Tissue:	Reconstructed human corneal epithelium
Nr:	2 tissues for main test, 2 tissues for neg control, 2 tissues for pos control
Exposure:	28 minutes
Result:	Mean relative tissue viability (optical density, MTT) 103%

SCCS overall comment on skin and eye irritation
For raw fullerene powder (mixture of C60 and C70) and hydroxylated fullerene, the tests showed no skin irritation potential. The eye irritation from raw fullerene powder is likely due to physical effects of the powder.
The raw fullerene powder contains about 66% C60 fullerene; from the test reports it is unclear whether the remaining content is C70 or another material.
Hydroxylated fullerene showed eye irritation potential.
Hydrated forms of Hydroxylated Fullerenes (HFWC) showed no skin and eye irritation at the relatively low tested concentration (0.015%).

3.4.3 Skin sensitisation

Fullerenes
The following information was provided by the notifier:
A Guinea pig skin sensitisation study according to OECD Guideline 406 with 10% fullerenes in olive oil showed no skin reactions.

Ref: Ema *et al.*, 2013. (study conducted in 2010)

Guinea pig adjuvant and patch test study

Guideline:	/
Test material:	Raw fullerenes powder 50% w/v in propyleneglycol (PG) for induction. Raw fullerenes powder 25% w/v in propyleneglycol for challenge. Propyleneglycol (PG) for control induction DNCB 0.05% w/v in acetone as positive control substance FCA as adjuvant intradermally on each induction site
Lot:	040406: raw fullerene powder containing 66.4% C60
Animals:	30 male guinea pigs, Hartley strain
Schedule:	10 animals induced with fullerenes, 10 with propyleneglycol, 5 with DNCB and 5 with acetone on day 1 and 9. 10% SLS patch on all induction sites on day 8. Challenge on day 22
Year:	2004
Results:	No skin reactions on the sites challenged with fullerenes or PG. Skin reactions in all animals on the sites challenged with DNCB.

Ref: study report B040377

Human repeat patch-test study

Test material:	Suspension of 3% natural fullerene in petrolatum
Control:	saline

Product ID:	10970, Product code 080508-01
Application:	as is, 1 cm2 under occlusion
Subjects:	107 humans (21 m, 86 f) enrolled, 54 completed the study
Induction	3x per week same spot during 48 hrs on the back during 3 weeks.
Challenge:	10 days after the last induction, application on a site that had not been used for induction.
Year:	2020
Results:	None of the subjects presented clinical signs at the challenge site.

Ref: NDR-0006973

SCCS comment
The Guinea pig adjuvant test showed that the tested fullerenes are not sensitisers. The raw fullerene powder used in this study contains about 66% C60 fullerene; from the test reports it is unclear whether the remaining content is C70 or another material.

The repeat human patch-test study is a modification of the existing HRIPT protocols, the use of which is considered by the SCCS as unethical. Because the high number of subjects who did not complete the study raises uncertainties in the interpretation of the results, the SCCS considers the results as inconclusive. While the test report does not specify the composition of the raw fullerene, the Notifier stated that it was derived from a plant and contained about 66% C60. The chemical nature of the remaining content is unclear.

Hydroxylated fullerenes
The following information was provided by the notifier:

Are-Nrf-2 Luciferase test (Keratinosens)

Guideline:	OECD 442 D
Test material:	$C_{60}(OH)_n$, n=30-50
Concentrations:	3.91 - 8000µM
Batch:	21C0226
Date:	2021
Ref:	Zurko-version2 – VT_SEG-ARE.NRF2-01_664__21_002 (2021)
Result:	inconclusive because of no clear dose-response and because the viability at max concentration did not reach cytotoxicity.

Direct Peptide Reactivity Assay (DPRA)

Guideline:	OECD 442 C
Test material:	Hydroxylated fullerene c60(OH)n, n=30-60 powder 8000 µMol
Concentration:	471 mg powder in 3 ml water
Depletion:	Cys peptide 29.98%, Lys peptide 3.36%
Result:	Positive, low reactivity.
Ref:	Laus-version2 21102502G875 (2022)

SCCS comment
The low reactivity and the turbidity in the Cys sample cast doubt on the DPRA result.

Hydrated forms of hydroxylated fullerene-Water Complex
The following information was provided by the Notifier:

Are-Nrf-2 Luciferase test (Keratinosens)

Guideline:	OECD 442D
Test material:	Hydroxylated fullerene C60 ($C_{60}(OH)_{30-50}$) 10 g ad ultra-pure water 10 L, called Hyperharmonised Fullerene-Water Complex (HFWC)
Batch:	01-2021-12-07

Concentration: range from 0.49 µg/ml to highest concentration 1000 µg/ml
Date: 2022
Test result: Negative
Ref: Zurko-version1 (2022) 22032914G888

h-CLAT test
Guideline: OECD 442E
Test material: Hydroxylated fullerene $C60(OH)_{30-50}$ @$(H_2O)_{144-2528}$ 0.015% in water
Batch: 01-2021-10-14
Concentr: highest concentration tested 1.5 µg/mL
Date: 2022
Result: Negative - no upregulation of markers at the highest test concentration
Ref: Laus-version1 22032914G888 (2022)

Direct Peptide Reactivity Assay (DPRA)
Guideline: OECD 442 C
Test material: Hydroxylated fullerene $C60(OH)_{30-50}$ @$(H_2O)_{144-2528}$ 0.015% in water
Concentration: 100 mM
Reactivity: Cys peptide 100%, Lys peptide 3.68%
Result: Positive
Ref: Laus-1 21092301G875 (2022)

SCCS comment
The description of the test material used in the Are-Nrf2 Luciferase test (Keratinosens) report seems to refer to hydroxylated fullerene and not to the hydrated forms of hydroxylated fullerene (3HFWC).
The test concentration used in the DPRA is not clear in the absence of a well-defined specification for the molecular weight.
The test concentrations used in the hCLAT tests appear to be too low.
The Notifier reported that a human patch test on 20 volunteers was conducted for skin sensitisation of 3HFWC, however, the study report was not provided to the SCCS for assessment of study quality.

SCCS overall comment on sensitisation
A Guinea pig study indicates the absence of sensitisation potential of fullerenes. For hydroxylated fullerene and hydrated forms of hydroxylated fullerenes, the test results do not clearly exclude a sensitising potential.

3.4.4 Repeated dose toxicity

Fullerenes
As reported by one of the Notifiers, repeated-dose toxicity studies with the raw fullerene powder have never been conducted in accordance with the guidelines. On the other hand, in external references, there are two important reports of the repeated dose studies conducted by affiliated organizations of the Japanese Government. The first study by Shinohara *et al.* (2010), covered repeated inhalation safety evaluation of Fullerene C60 using rats conducted by The National Institute of Advanced Industrial Science and Technology (AIST) belonging to the Japanese government. The second study by Takahashi *et al.* (2012) covered repeated oral safety evaluation of Fullerene C60 using rats conducted by National Institute of Health Sciences belonging to the Japanese government. These reports are also cited in the OECD Document ENV/JM/MONO(2016)21.
In the study by Takahashi *et al.* (2012), a repeated oral toxicity study on Fullerene C60 was conducted using rats in accordance with the test guideline of the Japanese Chemical Control Act. In this study, the NOAEL was reported to be 1000 mg/kg-bw /day because the maximum dose of 1000 mg/kg-bw/day was not toxic after oral administration at 1, 10, 100, and 1000

mg/kg bw/day for 29 days. However, dose-independent results showed increased urinary ketones, decreased lymphocyte ratio, and increased eosinophil ratio in the 10 mg/kg-bw/day group, as well as increased blood creatinine and increased relative weight of the thymus gland in the 100 mg/kg/day males. Based on these results, the Notifier determined that a dose of 1 mg/kg-bw/day, which showed similar safety data to the control, was the non-toxic dose for this evaluation.

SCCS comments

The Notifier has quoted two repeat-dose toxicity studies from the open literature that have also been described in the OECD Document ENV/JM/MONO(2016)21. However, the original study reports were not made available to the SCCS. The inhalation study by Shinohara *et al.* (2010) was not accessible to the SCCS, but it is described by the OECD Document ENV/JM/MONO(2016)21 and it is also fully described under section 3.2 of this Opinion. The conclusions of the study on repeated dose toxicity are as follows: "Based on data for histopathological examination, BALF (bronco-alveolar lavage fluid) examination, chemokine analysis in lung tissue and DNA microarray analysis, it was suggested that C60 fullerene might not have a severe pulmonary toxicity after 4 weeks inhalation exposure in rats. Although slight inflammatory response was observed in the lungs, no histopathological abnormalities were observed in the liver, kidney, spleen, cerebrum, cerebellum, testis, or nasal cavity tissues in C60 inhalation group."

Details on Takahashi study

Takahashi *et al.* (2012) on the oral repeated dose toxicity study, that was used by the Applicant to derive the NOAEL, could be retrieved from the open literature as follows:

Guideline:	Test Guideline of the Japanese Chemical Control Act for 28 d Test
Species/strain:	Rat, Crl: CD(SD), 4 weeks old
Group size:	10/sex in controls and highest dose; 5/sex for the other doses
Test substance:	Fullerene C60 (Nanom Purple SU, 0.71 nm in diameter, black powder, CAS 99685-96-8)
Batch:	10B0098-A
Purity:	99.9 %
Vehicle:	Olive Oil
Dose levels:	0, 1, 10, 100, and 1000 mg/kg bw/d
Administration:	Oral gavage (10 ml/kg bw)
Duration:	29 d treatment, 14 d recovery
GLP:	In compliance
Study period:	2010-2011

Rats were given Fullerene C60 by gavage once daily at the doses given in the table above. One day after the last dosing, five animals/sex/dose were euthanised for the assessment of haematology, blood biochemistry, organ weights, macroscopic and microscopic findings. The remaining five animals from the control and high dose group were kept without treatment for 14 days and examined thereafter. Functional observation battery (FOB) was investigated during the 4th week of treatment. Clinical signs, body weight and food consumption were monitored on a regular basis. Urine was collected for urinalysis during the 4th week of treatment. At the end of treatment and after recovery, concentrations of C60 fullerenes were determined in liver (median lobe), right and left kidneys and spleen from male control and high dose animals.

Results:

No deaths or clinical signs of toxicity occurred. In high-dose animals, blackish faeces was observed at the highest dose starting from dosing day 4 (until day 1 of the recovery period). Urinalysis revealed increased incidences of ketone bodies in male animals at 10 and 1000 mg/kg bw/d. In male animals, there was an increase in the differential eosinophil ratio at 10 mg/kg bw/d and a decrease in the differential lymphocyte ratio at the end of treatment, but not after recovery. Haematology revealed a statistically significant ($p<0.01$) increase in creatinine in 100 mg/kg bw/d males and a decrease in albumin ($p<0.05$) at the highest dose

at the end of treatment but not after recovery. In high-dose females, protein was statistically significantly ($p<0.05$) increased after recovery. No changes from controls were observed for serum levels of triiodothyronine, thyroxine and thyroid stimulating hormone. At the end of the treatment period, but not after recovery, relative thymus weights were increased in females at 100 mg/kg bw/d and in males, relative kidney weights were decreased ($p<0.05$). After recovery both absolute and relative liver weights as well as absolute spleen weight were increased ($p<0.05$ each). There were no histopathological findings and the concentrations of fullerene C60 were below detection limit in the tissue samples investigated.

Further studies cited by the Notifier(s)

In a study by Shipelin *et al.*, male Wistar rats (n=24), peroral administration of dispersion of nano-sized (31 nm) multimolecular fullerene C60 particles in doses of 0.1, 1.0, and 10 mg/kg body weight over 92 days. No noted physiological, biochemical, hematological and immunological changes which can be addressed with Fullerene C60 toxicity. However, the highest doses (1 and 10 mg/kg bw) increased population and modified distribution of hepatic CD106+ cells; also resulted in accumulation of cytoplasmic granules presumably identified as Kupffer macrophages without any signs of visible inflammation or necrotic areas. In the authors' opinion, it is a proof of the beginning of a hepatotoxic effect.

Ref: Shipelin *et al.*, 2015.

In a study by Baati *et al.* (2012), rats, oral administration of C(60) dissolved in olive oil (0.8 mg/ml) at reiterated doses (1.7 mg/kg of body weight) for 7 months (dosing schedule: each day for first 7 days; once a week till the end of 2nd month; once every 2 weeks till the end of experiment). Effects in rats – not only does not entail chronic toxicity but it almost doubles their lifespan.

Ref: Baati *et al.*, 2012.

Ref: FULLERENES toxicity profile [67051_spec_file_2019-4-17-12-4-16.zip]

SCCS comment

The study of Takahashi *et al.* (2012) was not performed according to an OECD test guideline, because a lower number of animals was used. The Notifier indicated a NOAEL of 1 mg/kg bw/d from the Takahashi *et al.* study. However, another study by Shipelin *et al.* (2015) points to a lower NOAEL. A third study by Baati *et al.* (2012) is not considered relevant for this safety assessment.

Hydroxylated Fullerenes

Study: Subacute systemic toxicity study of the product Hydroxylated Fullerene $C_{60}(OH)_{30-50}$

NOTE: Certified translation from Serbian into English as provided by the notifier.

Study number:	LMEM-SAT-03/2022
Guideline:	Study performed according to OECD TG no 407, EU Directive 2010/63/EU, and ISO 10993-2:2006 Animal welfare requirements.
Species/strain:	Mouse, NMRI HAN, 5 weeks of age
Group size:	total number of animals 40 (30 experimental and 10 control animals, 10 animals per group). Animals of both genders were used (5 males and 5 females per group).
Test substance:	Hydroxylated Fullerene $C_{60}(OH)_{30-50}$
Batch:	Laboratory sample
Purity:	Not given. According to the manufacturer the composition of test substance has a concentration of 0.15 g/L
Vehicle:	Not given
Dose levels:	0.75 mg/kg, 2.25 mg/kg, 3.75 mg/kg. Experimental group: 0.1 mL/mouse; 0.3 mL/mouse; 0.5 mL/mouse (every day for 28 days)

	Control group: 0.5 mL of purified water (every day for 28 days)
Administration:	Gastric probe
GLP:	-
Study period:	10 May – 6 June 2022

Results: Treated animals did not demonstrate signs of toxic reactions immediately upon application, as well as in the later course of observation. They acted normal, in conformity with the expected for their species, sex, age and environment. Reaction of animals to the provoked behaviour was normal and expected. No signs of neurological misbehaviour were noticed. Hygienic behaviour of the animals was normal. Eyes were clear and clean, nostrils and other natural openings were clean.
There were no significant differences in body weight gain of experimental compared to control animals. The experimental animals did not demonstrate any nutritional disorders. They were feeding and drinking water in the customary manner. Food and water consumption did not significantly differ between experimental and control animals.
During the experimental period, there were no fatalities of experimental and control animals. After 28 days since the beginning of the experiment, all animals were sacrificed and a pathoanatomical examination was performed. Macroscopic examination of organs and tissues did not show any pathological changes in experimental and control groups.

Based on clinical monitoring of the experimental animals and on performed macroscopic examination of the organs after 28 days since the commencement of the experiment, it was concluded that the tested Hydroxylated Fullerene $C_{60}(OH)_{30\text{-}50}$ applied at doses of 0.75, 2.25, and 3.75 mg/kg of body weight does not cause any toxic effects on tested animals.

Ref: Subacute (28d) toxicity -ENG report HF.

SCCS comment
According to the Notifier, the data produced in this study are not specifically intended for demonstrating the safety of hydroxylated fullerene for use in cosmetics, but it is part of substance evaluation for medical application. The Notifier did not specify the exact regulation for which the study was performed. In the absence of this information, the study cannot be used to demonstrate safety of the material when used for cosmetic purposes. Furthermore, the parameters reported are considered insufficient to address repeated dose toxicity and the study was not performed according to OECD TG.

Hydrated forms of Hydroxylated Fullerenes

The following information was provided by the notifier:
By reviewing mentioned literature different, even contradictory results of fullerene/fullerol toxicity investigations. Many studies do not clearly state which substance was investigated, the manner of obtaining the substance, if the presence of impurities was established, etc. In accordance with results of some of the mentioned studies, the Notifier expresses the opinion that it is exactly the presence of solvent residues and other impurities that are the cause of undesirable/toxic effects of the substance, but also the reason for the absence of expected positive effects. For this reason, during 3HFWC production special attention is devoted to removing solvent residues and obtaining a quality, safe and efficient cosmetic ingredient.

Ref:281_tox_profile_2020-3-12-18-44-18

Study: Subacute systemic toxicity study of 3HFWC

NOTE: Certified translation from Serbian into English as provided by the notifier.

Study number:	LMEM-SAT-01/2022
Guideline:	Study performed according to OECD TG no 423, EU Directive 2010/63/EU, and ISO 10993-2:2006 Animal welfare requirements.
Species/strain:	Mouse, NMRI HAN, 5 weeks of age
Group size:	total number of animals 40 (30 experimental and 10 control animals, 10 animals per group). Animals of both genders were used (5 males and 5 females per group).
Test substance:	Hyper-Harmonized Hydroxylated Fullerene Water Complex-3HFWC
Batch:	Laboratory sample
Purity:	Not given. According to the manufacturer the composition of test substance has a concentration of 0.15 g/L
Vehicle:	Not given
Dose levels:	0.75 mg/kg, 2.25 mg/kg, 3.75 mg/kg. Experimental group: 0.1 mL/mouse; 0.3 mL/mouse; 0.5 mL/mouse (every day for 28 days) Controls: 0.5 mL of purified water (every day for 28 days)
Administration:	Gastric probe
GLP:	-
Study period:	10 May – 6 June 2022

Results: No treated animals in any of the groups showed signs of a toxic reaction immediately after application, or in the later during observation. Animals behaved normally in accordance with what is expected for their species, gender, age and environment. The reaction of animals to provoked behaviour was normal and as expected. No signs of neurological deficits were observed. The hygienic behaviour of animals was normal. Their eyes were clear and clean, nostrils and other natural orifices were clean.
There were no significant differences in weight gain of experimental compared to control animals. They ate and drank water in the usual manner. Food and water consumption of experimental and control animals did not differ significantly.
During the experimental period there were no deaths of experimental or control animals. After 28 days from the beginning of the experiment, all animals were sacrificed and a pathoanatomical examination was performed. Macroscopic examination of organs and tissues did not reveal any changes in any animal, both in the treated and control group.
Based on clinical observation of the experimental animals and the macroscopic examination of the organs after 28 days from the commencement of the experiment, it was concluded that the investigated product Hyper Harmonized Hydroxylated Fullerene Water Complex-3HFWC at doses of 0.75, 2.25, and 3.75 mg/kg did not cause toxic effects in tested animals.

Ref: 3HFWC data submission main document.pdf; FULLERENES toxicity profile; Subacute (28d) toxicity -ENG report 3HFWC

SCCS comment

According to the Notifier, the data produced in this study is not specifically intended for demonstrating the safety of 3HFWC for use in cosmetics, but it is part of substance evaluation for medical application. The Notifier did not specify the exact regulation for which the study was performed. In the absence of this information, the study cannot be used to demonstrate safety of the material when used for cosmetic purposes. Furthermore, the parameters reported are considered insufficient to address repeated dose toxicity and the study was not performed according to a published OECD TG.

SCCS overall comments on repeated-dose toxicity

The studies were not performed with the fullerenes that have been notified in the CPNP. Most studies on fullerene C60 were cited in literature overviews, and full study reports were not provided. Data on fullerene C70 were not provided in any of the submitted studies.

For hydroxylated fullerenes and their hydrated forms, the Notifier has provided results from two *in vivo* toxicity studies performed in the context of medical application. However, the exact regulations, for which these studies were performed were not given. Without such information the studies cannot be used for the evaluation of the safety of the materials for use as cosmetic ingredients.

3.4.5 Mutagenicity/genotoxicity

Following the mandate Fullerenes, Hydroxylated Fullerenes and Hydrated forms of Hydroxylated Fullerenes were evaluated for genotoxicity. Radical Sponge® and LipoFullerene® were excluded from the evaluation.

1. Fullerenes

Following information on Fullerenes was provided by Notifier(s):

Data presented in ENV/JM/MONO(2016)21:

- OECD 471: negative with and without metabolic activation
- OECD 473 and Japanese Guideline (Chemical Substances Control Law of Japan): negative
- Chromosomal aberration, DNA damage and/or repair in vivo: no effects

Ref.: ENV/JM/MONO(2016)21

Several full reports and two publications were further provided and analysed by SCCS.

Bacterial Reverse Mutation test

Several reports and publications on Bacterial Reverse Mutation tests have been submitted:

1. Ames test (with and without metabolic activation)
Mori *et al.* (2006): Fullerenes (the mixture of C60 and C70, fullerite), sublimed technical grade, purity: 99.5%, were supplied from Vitamin C60 BioResearch Corp. (Tokyo, Japan) – result negative.
Shinohara *et al.* (2009): Commercially available C60, 500-mg Nanom purple, refined by sublimation, C60 purity >99.5%; Frontier Carbon Co., Ltd., Japan – result negative

Ref: Mori *et al.* (2006); Shinohara *et al.* (2009)

2. Ames test (with and without metabolic activation)
Study report: "Fullerene raw powder"; probably 66.4% fullerene C60, with the rest being mainly Fullerene C70 – result negative.

Ref.: Bacterial Reverse Mutation Study of Fullerenes. Final Report #B040380, Mitsubishi Chemical Safety Institute, Ltd. 2004a

SCCS comment
As explained in the SCCS Guidance on Nanomaterials (SCCS/1611/19), the bacterial gene mutation test is not suitable for testing nanoparticles for gene mutation, and thus it was not included in the evaluation of mutagenicity of fullerenes.

Chromosomal aberration test in Cultured Mammalian Cells

Guideline:	Chromosomal aberration test Guidelines on Genotoxicity Tests of Pharmaceuticals (Notification No.1604 of the Evaluation and Licensing Division, MHW'S PMSB dated November 1, 1999)
Test system:	CHL/IU lung Chinese hamster cells
Replicates:	2 replicates
Test substance:	Water-Soluble Fullerenes 0.365% of C60, amorphous granule
Batch (Purity):	Lot 041206
Vehicle:	water
Assay medium:	MEM Eagle
Concentrations:	313, 625, 1250, 2500 and 5000 µg/mL
Treatment:	experiment I: 6 h exposure, without and with metabolic activation; experiment II; 24h exposure, only without metabolic activation.
S9	phenobarbital induced rat liver
Positive controls:	Mitomycin C (MMC) 0.1 µg/mL without S9 and Benzo[a]pyrene with S9 20 µg/mL
Negative control:	Vehicle
Statistics:	None
GLP:	Yes
Study period:	2005

The confirmation of the stability and contents of the test substance solutions (vehicle DMSO) was measured by HPLC before the experiments in another study.

An *in vitro* chromosomal aberration study of Water-Soluble Fullerenes was conducted using CHL/IU cells derived from the lungs of female Chinese hamsters as the indicator cells.
Based on the result of a preliminary test, the cell growth inhibition test was conducted at 313, 625, 1250, 2500 and 5000 µg/mL in the short-term treatment assay for 6 hours in the absence of S9 mix (-S9 mix assay) and the presence of S9 mix (+S9 mix assay), and in the continuous treatment assay for 24 hours (24-hour assay). As a result, cell growth was not inhibited more than 50% in any treatment condition. Based on the result of the cell growth inhibition test, the chromosomal aberration test was conducted at 1250, 2500 and 5000 µg/mL in each treatment condition. 100 cells per plate (200 cells per concentration) have been assessed for chromosomal aberrations. Incidences of cells with structural and numerical chromosome aberrations were less than 5.0% in all the treatment conditions. In conclusion, Water-Soluble Fullerenes was considered not to have the ability to induce chromosomal aberration under the conditions employed in the present study.

Ref. Final report B040967, Mitsubishi Chemical Safety Institute, Ltd. 2005

SCCS comment
The SCCS considers the study inconclusive, as the uptake of fullerene by CHL/IU cells was not demonstrated. Also, information on historical positive and negative control was not provided. Characterisation of fullerene in dispersion for size and size distribution was not performed. It is not clear which form of fullerene was tested, as it was mentioned that it was water soluble fullerene, which suggests that it might have been Radical Sponge®. Radical Sponge® was not evaluated in this Opinion.

Chromosomal Aberration Study in Cultured Mammalian Cells

Guideline:	Chromosomal aberration test Guidelines on Genotoxicity Tests of Pharmaceuticals (Notification No.1604 of the Evaluation and Licensing Division, MHW'S PMSB dated November 1, 1999)
Test system:	CHL/IU lung Chinese hamster cells
Replicates:	2 replicates
Test substance:	Fullerenes (synonym: Fullerene) purity 66.4%, powder
Batch (Purity):	Lot 040406
Vehicle:	DMSO
Assay medium:	MEM Eagle
Concentrations:	313, 625, 1250, 2500 and 5000 µg/mL
Treatment:	experiment I: 6 h exposure, without and with metabolic activation; experiment II; 24h exposure, only without metabolic activation.
S9	phenobarbital induced rat liver
Positive controls:	Mitomycin C (MMC) 0.1 µg/mL without S9 and Benzo[a]pyrene with S9 20 µg/mL
Negative control:	Vehicle
Statistics:	None
GLP:	Yes
Study period:	2005

Fullerene was suspended in DMSO. The confirmation of the stability and contents of the test substance solutions was measured by HPLC before the experiments in another study. The confirmation of contents and homogeneity of the test substance suspension was done in testing facility. The highest and lowest concentrations in the same dilution series of the chromosomal aberration test (500 and 31.3 mg/mL) were analysed by HPLC. The contents (average of the measured values, n=3) of the test substance suspensions ranged 100.8 % - 102.9% of the nominal concentrations and were within the laboratory criterion (90% - 110%).

An *in vitro* chromosomal aberration study of Fullerenes was conducted using CHL/IU cells derived from the lungs of female Chinese hamsters as the indicator cells. Based on the result of a preliminary test, the cell growth inhibition test was conducted at 156, 313, 625, 1250, 2500, and 5000 µg/mL in the short-term treatment assay for 6 hours in the absence of S9 mix (-S9 mix assay) and the presence of S9 mix (+S9 mix assay), and in the continuous treatment assay for 24 hours (24-hour assay). As a result, the concentrations producing 50% inhibition in cell growth were estimated to be 2317 µg/mL in the +S9 mix assay and 564 µg/mL in the 24-hour assay. Cell growth was not inhibited more than 50% in -S9 mix assay. Based on the result of the cell growth inhibition test, the chromosomal aberration test was conducted at 625, 1250, 2500, and 5000 µg/mL in the -S9 mix assay and +S9 mix assay, as well as at 313, 625, 1250, 2500, and 5000 µg/mL in the 24-hour assay. As a result, the incidences of cells with structural and numerical chromosome aberrations were less than 5.0% in all the treatment conditions. In conclusion, Fullerenes were considered not to have the ability to induce chromosomal aberration under the conditions employed in the present study.

Ref.: Final Report #B040381, Mitsubishi Chemical Safety Institute, Ltd. 2004b

SCCS comment

The SCCS considers the study inconclusive, as the uptake of fullerene by CHL/IU cells was not demonstrated. Cytotoxicity after 24h exposure exceeded the recommended cytotoxicity range in all tested concentrations (cell growth index was 23-44%). Also, information on historical positive and negative controls was not provided. Additionally, characterisation of fullerene in dispersion for size and size distribution was not performed. The raw fullerene powder consists of about 66 % fullerene C60. From the test reports it is unclear whether the remaining 34 % is Fullerene C70.

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Chromosomal aberration test in Cultured Mammalian Cells
Chromosomal aberration test in cultured Chinese hamster lung (CHL/IU) cells *in vitro* was reported by Mori *et al.* (2006). Fullerenes (the mixture of C60 and C70, fullerite), sublimed technical grade, purity: 99.5%, supplied from Vitamin C60 BioResearch Corp. (Tokyo, Japan)– result negative.

Ref: Mori *et al.* (2006)

SCCS comment
The study is of limited reliability for the following reasons: no physicochemical analysis (e.g. TEM, stability of nanoparticle suspension before and after dilution in culture medium, etc.) of the in-laboratory synthetised C60 was performed; no demonstration of cell internalisation of C60 has been provided; for chromosomal aberration test no data on historical negative and positive controls have been provided; the study was not performed under GLP conditions.
The results are identical with those reported in the final report B040381, but with incorrect transposition of the data for structural and numerical aberrations after continuous treatment assay. Also, in the publication by Mori *et al.* (2006) referred to fullerenes (the mixture of C60 and C70, fullerite), sublimed technical grade, purity: 99.5%, which is probably not identical with the fullerene used in the B040381 study report, in which fullerenes purity 66.4% is reported.

Chromosomal aberration test in Cultured Mammalian Cells
Chromosomal aberration test in cultured Chinese hamster lung (CHL/IU) cells *in vitro* was reported by Shinohara *et al.* (2009) who used commercially available C60 (500-mg Nanom purple, refined by sublimation, C60 purity >99.5%; Frontier Carbon Co., Ltd., Japan; mixed with carboxymethylcellulose sodium. The material was tested with and without metabolic activation – with negative results.

Ref: Shinohara *et al.* (2009)

SCCS comment
The study is of limited reliability for the following reasons: no physicochemical analysis (e.g. stability of nanoparticle suspension before and after dilution in culture medium, etc.) of the C60 was performed; no demonstration of cell internalisation of C60 has been provided; no data on historical negative and positive controls have been provided; the study was not performed under GLP conditions.

Bone marrow micronucleus test *in vivo*
Shinohara *et al.* (2009) reported bone marrow micronucleus test *in vivo* using a stable C60 nanoparticle suspension (commercially available C60 (500-mg Nanom purple, refined by sublimation, C60 purity >99.5%; Frontier Carbon Co., Ltd., Japan; mixed with Tween 80) on ICR mice with negative results. In this study male mice were twice administrated with doses of 22, 45, and 88 mg/kg C60 by gavage with a stomach tube at 24-h intervals.

Ref: Shinohara *et al.* (2009)

SCCS comment
Although the MN result was negative, there is no proof of systemic availability/distribution of the test material after oral administration (including to bone marrow). Hence, the SCCS considers the study result inconclusive.

Conclusion from the Notifier
According to the Notifier #1, based on the above studies *in vitro* and some *in vivo* tests confirms lack of fullerene genotoxic potential.

Ref: FULLERENES toxicity profile [CPNP data/ 67051_spec_file_2019-4-17-12-4-16.zip]

Overall SCCS comment on genotoxicity of fullerene

Having considered all the available data, the SCCS cannot conclude on the genotoxicity of fullerenes (C60 and C70) for following reason:

From the information provided by the Notifiers, it is not clear if the physicochemical characteristics of the test items used in the biological studies cited were the same as those nanomaterials notified for this assessment. To enable the SCCS to assess the relevance of the submitted genotoxicity studies, a detailed comparative analysis of the physicochemical characteristics of the tested nanomaterials with those produced by the Notifiers is required.

The study on chromosomal aberration with negative results has limited value, as uptake of fullerene by CHL/IU cells was not provided. *In vivo* micronucleus study results are considered inconclusive due to the lack of proof of systemic exposure.

Additionally, valid data on gene mutation endpoint (mammalian gene mutation test) are missing. It is generally recommended for regulatory safety assessments, as in the SCCS Guidance on Nanomaterials (SCCS/1611/19), that bacterial gene mutation tests are not suitable for testing the genotoxic potential of nanomaterials. Therefore, the SCCS did not consider studies on bacterial model in the evaluation of genotoxicity of fullerenes.

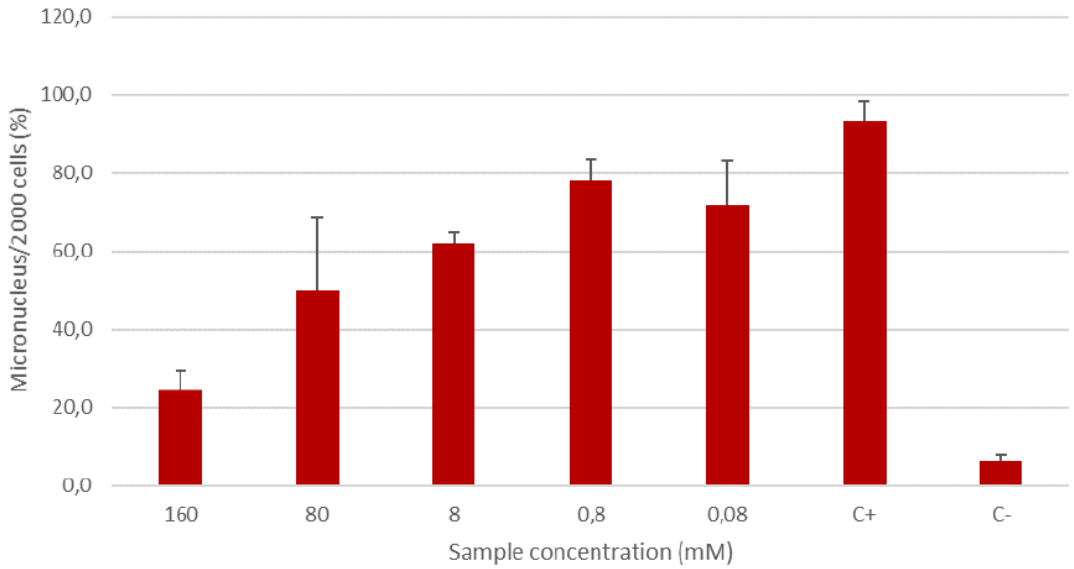
2. Hydroxylated Fullerenes

In vitro Mammalian Cell Micronucleus Test

Guideline:	Micronucleus™ instaCELL Micronucleus Assay Kit,
Test system:	V79 Chinese hamster cells
Replicates:	3-well chamber slides from Ibidi®, 2 replicates
Test substance:	Hydroxylated fullerenes, C60(OH)n (n=30-50)
	MW=1332g/mol
Batch (Purity):	21C0226
Vehicle:	assay buffer,
Assay medium:	DMEM+20%FBS
Concentrations:	160, 80, 8, 0.8 and 0.08 mM
Treatment:	16h
Positive controls:	Mitomycin C (MMC): 4.7 µg/mL (4 h), 0.02 µg/mL (24 h)
Negative control:	Vehicle
Statistics:	Student t-test
GLP:	No
Study period:	November 03-17, 2021

The aim of this study was to determine the genotoxic potential of the product HYDROXYLATED FULLERENES (reference: -, batch: 21C0226) according to the micronucleus assay. Assay was performed with instaCELL Micronucleus Assay Kit according to the protocol. To set up the assay, 3-well chamber slides from Ibidi® were used. Within the slides, cells can be cultured, treated, fixed and mounted without transfer. One vial of Assay Ready V79 was thawed, and the cells were seeded into 3-well chamber slides at a density of 10.000 cells per well. The cells were incubated at 37°C for 24h to allow them to attach to the glass slide. The next day, medium was removed and the cells were treated with test fullerenes dilutions for 16h. After incubation, the cells were fixed with mixture of acetic acid and methanol (1:4), washed and stained with DAPI. 2.000 cells per well were analysed by fluorescence microscopy counting the cells with and without micronuclei. The criteria used to determine whether a test sample is positive or negative for genotoxicity were based on the OECD 487. All 5 concentrations of test sample induced significant increase of micronuclei with respect to the negative control. Inverse dose-dependent effect was observed. The authors noted that the reason to the results obtained *i.e.* at higher concentrations the sample dispersion might be less stable and form aggregates that decrease the bioavailability of the product and therefore, reduce the

differences with the negative control. The lowest 2 concentrations tested were similar, however only 2 values are not sufficient to evaluate the trend adequately. Repetition of the test with lower concentrations is recommended. The authors concluded that under the experimental conditions adopted and taking into account the defined procedure, the test is considered inconclusive.



Percentage of micronucleate cells from 2000 cells after the treatment with Hydroxylated fullerenes. Positive control (C+) MMC.

Study ref. VT_SEG-GEN.MN_664_21_002

SCCS comment

The study was performed on a commercially available Micronucleus Assay Kit and it was not done under GLP conditions. Basic data on test substance, how concentrations were calculated, dispersion procedure, vehicle and characterisation of hydroxylated fullerenes in dispersion (size, size distribution, agglomeration) were not provided. Information on the uptake of hydroxylated fullerenes by V79 cells was not provided. Historical positive and negative controls data were also missing. The test substance was clearly positive in all 5 tested concentrations.

Based on all the shortcomings identified, the SCCS considers the study as not acceptable for evaluation of genotoxicity of hydroxylated fullerenes.

Chromosome aberration assay and the cytokinesis-block micronucleus test *in vitro*

Mrdanovic *et al.* (2009) in their *in vitro* study on CHO-K1 cells, analysed the genotoxic and antigenotoxic potential of fullerenol C60(OH)24. The results show that fullerenol does not induce genotoxic effects in a wide range of concentrations (11-221 μM), and that it protects both non-damaged and MMC-damaged CHO-K1 cells.

Ref: Effects of fullerenol C60(OH)24 on the frequency of micronuclei and chromosome aberrations in CHO-K1 cells, Mrdanovic *et al.* 2009; Mutation Research 680 (2009) 25–30

SCCS comment

The study is not reliable for the following main reasons: no physicochemical analysis was provided of the in-laboratory synthetised $C_{60}(OH)_{24}$ (e.g. TEM, stability of nanoparticle suspension before and after dilution in culture medium, etc.); no demonstration of cell internalisation of $C_{60}(OH)_{24}$ has been provided; for micronuclei and chromosomal aberration tests, no data on historical negative and positive controls have been provided; studies were not performed under GLP conditions.

Bacterial gene mutation test
Internal company study (unpublished data) showed absence of mutagenicity alert in Ames test (reference test result data included in references file).

SCCS comment
Bacterial gene mutation test is not suitable for testing nanoparticles for gene mutation and thus it was not included in the evaluation of mutagenicity of fullerene.

***In vitro* comet assay**
Internally available study from 2018 (unpublished data from *in vitro* comet assay; University of Belgrade, Faculty of Veterinary Medicine) showed that the tested substance (fullerenol) exhibited genotoxic effects solely at concentrations > 150 µg/ml. Consequently, the authors concluded that the substance is potentially genotoxic at high doses, but not in potentially achievable biological exposure concentrations.

SCCS comment
The information provided by Notifiers has limited value, as the *in vitro* comet assay results can be considered only as supportive in the overall weight of evidence.

Conclusion from the Notifier
Based on the above referred data, it can be concluded that the substance does not show genotoxic or mutagenic potential in potentially achievable biological exposure concentrations as used in cosmetic products.

Ref: 281_safety_file_2020-3-12-18-44-18

Overall SCCS comment on Hydroxylated Fullerenes
The SCCS cannot conclude on the genotoxicity of hydroxylated fullerenes due to the lack of data on gene mutation and valid data on chromosomal aberrations, and high uncertainty related to missing information on characterisation and uptake of hydroxylated fullerenes.

3. Hydrated forms of Hydroxylated Fullerenes

In vitro Mammalian Cell Micronucleus Test

Guideline:	Micronucleus™ instaCELL Micronucleus Assay Kit,
Test system:	V79 Chinese hamster cells
Replicates:	3-well chamber slides from Ibidi®, 2 replicates
Test substance:	Hyper Harmonized Hydroxylated Fullerene Water Complex (3HFWC), C60;C= 0.15 g/L, Ultra-pure water (0,055 µS/cm)
Batch (Purity):	/
Vehicle:	assay buffer
Assay medium:	DMEM+20%FBS
Concentrations:	15.0, 7.50, 3.75 ,1.88 and 0.94 µg/mL
Treatment:	16h
Positive controls:	Mitomycin C (MMC): 4.7 µg/mL (4 h), 0.02 µg/mL (24 h)
Negative control:	Vehicle
Statistics:	Descriptive analysis, t-test, central tendency, variance, Linear mixed effects models, Wilcoxon Signed Rank test.
GLP:	No
Study period:	August 10-19, 2022

The aim of this study was to determine the genotoxic potential of the Hyper Harmonized Hydroxylated Fullerene according to the micronucleus assay. Assay was performed with instaCELL Micronucleus Assay Kit according to the protocol. To set up the assay, 3-well chamber slides from Ibidi® were used. Within the slides, cells can be cultured, treated, fixed and mounted without transfer. One vial of Assay Ready V79 was thawed, and the cells were

seeded into 3-well chamber slides at a density of 10.000 cells per well. The cells were incubated at 37°C for 24h to allow them to attach to the glass slide. The next day, medium was removed and the cells were treated with test fullerenes dilutions for 16h. After incubation, the cells were fixed with mixture of acetic acid and methanol (1:4), washed and stained with DAPI. 2.000 cells per well were analysed by fluorescence microscopy counting the cells with and without micronuclei. The test sample concentrations 3.75µg/mL, 1.88µg/mL and 0.94µg/mL did not induce a significant increase of micronuclei with respect to the negative control. The tested concentrations 15.0µg/mL and 7.50µg/mL of the product clearly show a statistically significant increase compared to the negative control. Authors concluded that under the experimental conditions adopted and taking into account the defined procedure, according to the criteria determining whether a test sample is positive or negative for genotoxicity, based on OECD 487, they shall consider that: The tested concentrations 15.0µg/mL and 7.50µg/mL of the product clearly show a statistically significant increase compared to the negative control. Therefore, the response is considered to be positive, the test chemical is then considered able to induce chromosome breaks and/or gain or loss in this test system under the experimental conditions examined.

Study ref. VT_SEG-GEN.MN_664_22_002

SCCS comment

The study was not performed according to OECD TG 487 and it was not under GLP conditions. Basic data on test substance, how concentrations were calculated, dispersion procedure, vehicle, and characterisation of hydrated forms of hydroxylated fullerenes in dispersion (size, size distribution, agglomeration) are not provided.
Information on the uptake of hydrated forms of hydroxylated fullerenes by V79 cells was not provided. Historical positive and negative controls data were missing. Based on all the shortcomings identified. the SCCS considers this study as not acceptable for evaluation of the genotoxicity of hydrated forms of hydroxylated fullerenes.

In vitro Mammalian Cell Micronucleus Test

Guideline:	OECD TG 487, EU B.49
Test system:	Human peripheral lymphocytes in whole blood culture
Replicates:	2 replicates
Test substance:	Hyper Harmonized Hydroxylated Fullerene Water Complex (3HFWC), C60; C= 0.15 g/L, Ultra-pure water (0.055 µS/cm) 99.985 %
Batch (Purity):	01-2021-10-14
Vehicle:	water (ROTIPURAN® Ultra)
Assay medium:	Lymphogrow Medium with FBS
Concentrations:	15.0, 7.50, 3.75 µg/mL (for cytotoxicity from 0.24-15 µg/mL)
Treatment:	experiment I: 4 h exposure, without and with metabolic activation; experiment II; 23.5 h exposure, only without metabolic activation.
S9	rat liver induced by Phenobarbital/5,6-Benzoflavone
Positive controls:	Mitomycin C (MMC): 0.3 µg/mL and colchicine 0.035 µg/mL without S9 mix and Cyclophosphamide mono-hydrate (CPA) with S9mix
Negative control:	Vehicle
Statistics:	Descriptive analysis, Fisher's exact test
GLP:	Yes
Study period:	Ju 13- August 24, 2022

The study was performed to assess the potential of Hyperharmonized hydroxylated fullerene water complex (3HFWC) to induce formation of micronuclei in human lymphocytes cultured *in vitro* in absence and presence of an exogenous metabolic activation system in two valid experiments. In deviation from OECD TG 487, testing of test item was started with 15.0 µg/mL as highest concentration, based on the specification of the sponsor. Precipitation or

turbidity of the test item was not visible in all experimental parts at any of the concentrations tested. Human peripheral blood lymphocytes in whole blood culture were stimulated to divide by phytohaemagglutinin. All cell cultures were set up in duplicates. The cytokinesis-block proliferation index (CBPI) was calculated for all evaluable cultures to assess cytotoxicity. Three highest concentrations were selected to determine the proportion of binucleated cells containing micronuclei.
In experiment I as well as in experiment II, no relevant cytotoxic effects were observed up to me maximum test item concentration (15.0 µg/mL).
In experiment I with metabolic activation, the concentration 3.75 µg/mL showed a statistically significantly increased value of binucleated cells with micronuclei compared with the concurrent solvent control above the 95.5% control limits and also slightly above the range min – max of the historical data for solvent controls. No dose-response relationship was found. Therefore, in experiment I with metabolic activation two criteria (out of three) for a positive result are fulfilled.
In experiment I without metabolic activation, the value of micronuclei was also slightly (but not statistically significantly) increased at the concentration 3.75 µg/mL lying above the 95.5% control limits but still inside the range min – max of the historical data for solvents. No dose-response relationship was found. That means, one criterion for a positive result is met.
In experiment II (extended exposure, only without metabolic activation), the highest test item concentration (15.0 µg/mL) showed a statistically significantly increased value ($p = 0.039$) of binucleated cells with micronuclei. This value also lay above the historical laboratory data for solvents, both above the range min – max and the 95.5% control limits. The micronucleus rates of the two lower test item concentrations (7.5 µg/mL and 3.75 µg/mL) did not show a statistically significant difference compared to the solvent control. A clear dose-dependency was observed as well but did not reach statistical significance. Nevertheless, this effect was declared as biologically relevant since the values were increased at higher dose(s) but since the values of the test item concentrations 7.5 µg/mL and 3.75 µg/mL were already in the range of the solvent control, the fulfilment of the dose dependence criteria must be taken with limited relevance. Therefore, all three criteria for a positive result are fulfilled and the result of experiment II is considered "positive". All positive control compounds caused large, statistically significant increases in the proportion of binucleate cells with micronuclei, demonstrating the sensitivity of the test system.
In conclusion, under the experimental conditions reported, Hyperharmonized hydroxylated fullerene water complex (3HFWC) is able to induce the formation of micronuclei in human lymphocytes *in vitro*.

Study ref. No. 22032914G86LAUS GmbH, 2022

SCCS comment

The SCCS is of the opinion that Hyper Harmonized Hydroxylated Fullerene is positive in micronucleus assay. The study was performed according to OECD TG 487 under GLP conditions. Characterisation of Hyperharmonised hydroxylated fullerene in dispersion (size, size distribution, agglomeration) was not provided. Information on the uptake of Hyper Harmonized Hydroxylated Fullerene by human peripheral blood mononuclear cells was also not provided.

Additional information from the Notifier

The information provided by Notifiers indicates that Hyperharmonised Fullerenol-Water Complex (3HFWC) was tested in the bacterial genotoxicity test (Ames test) and the in vitro comet assay on human peripheral blood lymphocytes.

SCCS comment

Bacterial gene mutation tests are not recommended for testing genotoxic potential of nanomaterials (SCCs Guidance on Nanomaterials SCCS/1611/19), and the *in vitro* Comet assay results can be considered only as supportive in the overall weight of evidence.

Overall SCCS comments on mutagenicity/genotoxicity of Hydrated forms of Hydroxylated Fullerenes

The SCCS considers hydrated forms of hydroxylated fullerenes not safe due to the indication of positive chromosomal aberration results in both *in vitro* micronucleus studies and high uncertainty due to missing information on characterisation and cellular uptake of hydrated forms of hydroxylated fullerenes. Additionally, the Notifier did not provide valid data on mammalian gene mutation endpoint. Although in the submission the Notifier reported that a study on mouse lymphoma assay on Hydrated forms of Hydroxylated Fullerenes was submitted to the SCSS (3HFWC data submission main document, page 33 of 40), the study report could not be found in the dossier or the associated submitted files.

Overall SCCS comments on mutagenicity/genotoxicity on fullerenes, hydroxylated fullerenes and Hydrated forms of Hydroxylated Fullerenes

Having considered all the information provided by the Notifiers, the SCCS cannot conclude on the genotoxicity of fullerenes (C60, C70) and hydroxylated fullerenes. The SCCS considers the hydrated forms of hydroxylated fullerenes potentially genotoxic.

To exclude the genotoxicity potential of the notified materials, the Notifiers need to provide valid information (data) on mammalian cell gene mutation assays and a micronucleus test performed with the nanomaterials indicated above, either based on published literature with these nanomaterials, or from experimental studies. Cellular uptake of the nanoparticle also needs to be confirmed.

Physicochemical characterisation data for the test materials should be provided, e.g. quantitative TEM analysis, description of the dispersion method used, and the measurement of stability of nanoparticle suspensions in the culture media (SCCS/1611/19).

3.4.6 Carcinogenicity

Fullerenes:

There are no data about the carcinogenic properties of fullerenes.

Ref: FULLERENES toxicity profile

Hydroxylated fullerenes

Data were not provided.

Hydrated forms of Hydroxylated Fullerenes

Data were not provided.

SCCS comment

Data on carcinogenicity were not provided on any of the materials assessed in this Opinion. As described in the SCCS Guidance on Nanomaterials (SCCS/1611/19), information on carcinogenicity is required if significant systemic exposure or genotoxicity cannot be excluded. The SCCS considers that the currently available information is not sufficient to exclude both systemic availability via the relevant uptake route(s), and genotoxicity, to allow discounting the need for information on carcinogenicity. Data on carcinogenicity potential will therefore be needed if further evidence cannot exclude systemic availability and/or genotoxicity of the material included in this safety assessment.

3.4.7 Reproductive toxicity

Fullerenes

According to the Notifier, some toxicity data show reprotoxic properties of Fullerene C60, however, those kinds of effects are not expected after dermal application (the effects were noted after injection or intraperitoneally administered fullerenes).

Ref: FULLERENES toxicity profile

Hydroxylated fullerenes

Data were not provided.

Hydrated forms of Hydroxylated Fullerenes

Data were not provided.

SCCS comment

Information on reproductive toxicity of the Notified materials was not provided. As described in the SCCS Guidance on the Safety Assessment of Nanomaterials in Cosmetics (SCCS/1611/19), information on reproductive toxicity is required if systemic exposure cannot be excluded.

3.4.8 Photo-induced toxicity

Fullerenes

The following information was provided by the notifier:

1. Skin photosensitization study in Guinea pigs

Guideline:	/
Test material:	Raw Fullerenes powder 50% w/v in propyleneglycol (PG) for induction. Raw Fullerenes powder 25% w/v in propyleneglycol for challenge. (PG) for control induction (TBS) 2% w/v in acetone as positive control FCA as adjuvant intradermally on each induction site
Lot:	040406: raw Fullerene powder containing 66.4% Fullerene C60
Animals:	30 male guinea pigs, Hartley strain
Irradiation:	Long-wave UV, 10 J/cm^2
Schedule:	10 animals induced with fullerenes in PG and UV, days 1-5 5 with TBS in acetone with UV on days 1-5 with FCA and UV on days 1-5 Challenge on day 23
Year:	2004
Results:	No skin reactions on the sites challenged & irradiated with fullerenes or PG. Skin reactions on all the sites challenged with TBS and UV.

Ref: study report B040378

2. Skin phototoxicity study in Guinea pigs

Guideline:	/
Test material:	raw Fullerenes powder 25% w/v in propyleneglycol (PG). 8-Methoxypsoralen 0.005% in 70% ethanol as positive control. Propylene glycol as negative control
Lot:	040406: raw Fullerene powder containing 66.4% Fullerene C60 (same lot as the combined study B040378 mentioned above)

Animals: 10 male guinea pigs, Hartley strain
Irradiation: Long-wave UV, above 320 nm, 11.2 J/cm^2
Schedule: Irradiation started 30 mins after application of the test articles
Reading of test sites at 24, 48 and 72 hrs after irradiation
Year: 2004
Results: No skin reactions on the irradiated sites treated with fullerene powder or suspension or propyleneglycol. Skin reactions on all the irradiated sites treated with 8-Methoxypsoralene (pos control).

Ref: study report B040379

3. Combined phototoxicity and photosensitization patch-test study in humans

Guideline: /
Test material: raw Fullerene powder 3% w/v dispersed in petrolatum.
Control: Saline.
Lot: product ID 10970, code 080508001
Subjects: 36 humans enrolled, 28 completed
Irradiation: Induction: UV-A and UVB 6.2 J. Challenge: UVA 6.4 J
Schedule: Induction: 2x/week for 3 wks test material 24hrs, followed by irradiation.
Challenge: after 10 days rest, patch-test 24 hrs, followed by UVA irradiation.
Year: 2020
Results: No skin reactions on the sites challenged & irradiated with fullerenes or PG.
Skin reactions on all the sites challenged with the positive control and UV.

Ref: study report NDR-0006972

SCCS comment

The design of the study in humans is a modification of an HRIPT. While conclusions about photosensitisation cannot be drawn, the study points towards the absence of phototoxicity by the test material (fullerene). From the test report, the exact composition of the test material (described as natural fullerene) is unclear; according to the Notifier's report it is about 70-70% fullerene C60 and 20-30% fullerene C70.
The Guinea pig studies indicate absence of phototoxic potential.

Hydroxylated fullerenes:

/

Hydrated forms of Hydroxylated Fullerenes:

The following information was provided by the notifier:

In vitro phototoxicity test

Test system: 3T3 NRU phototoxicity test
Guideline: OECD 432
Test material: Transparent yellow liquid stock solution 1 g/L
Batch: 01-2021-12-07
Test concentrations: 0.39 – 50 µg/mL from stock solution
Date: 2022
Result: PIF 7.9, indicating phototoxicity

Ref: ENAC – Instit Val Micr TX/22/088 (2022)

SCCS comment

The study shows that hydrated forms of hydroxylated fullerenes are phototoxic. Although a clear specification of the test material was not provided in the report, it can be assumed that

it is the same material that was used in the Are-Nrf-2 Luciferase test for sensitisation. In a subsequent response letter to queries from the SCCS, the Notifier stated that, based on the cell viability data, the results of phototoxicity test and PIF calculation should be taken with caution.

SCCS overall comment on phototoxicity

Hydroxylated fullerene and its aggregates absorb both UVA and VIS radiation and have the potential to act phototoxically on the skin and the eye. Hydroxylated fullerene exposed to UV or VIS radiation has been shown to be phototoxic to human keratinocytes *in vitro* (Zhao 2008). Hydroxylated fullerene has also been reported to cause phototoxic damage to epithelial cells of the human lens *in vitro* (Roberts 2008), and the pigment epithelial cells of the retina (Wielgus, 2010). Therefore, hydroxylated fullerenes can be regarded as phototoxic. Similar concerns about phototoxicity are also applicable to hydrated forms of hydroxylated fullerenes.

3.4.9 Human data

Skin sensitisation

Fullerenes:

The relevant skin irritation and sensitisation studies in humans are covered in the section 3.4.2

Hydroxylated fullerenes: /

Hydrated forms of hydroxylated fullerenes:

One Notifier has submitted clinical studies with formulated cosmetic products containing HFWC. The SCCS does not consider studies on products for assessment of the safety of a specific ingredient.

3.3.10 Special investigations

Information from the Notifier

In-vitro cytotoxicity and in-vivo (Drosophila) toxicity

An *in-vitro* study on effect of fullerenols (C60(OH)20, C60(OH)24 and C60(OH)30) on human skin cells by Saathof in 2011 showed that the tested substances had no effect on HEK viability, suggesting they are not toxic to HEK at concentrations up to 8,5 µg/ml. Only at highest concentration C60(OH)30 tested, 42.5 µg/mL, significant decrease in cell viability was noted at 24 h. By 48 h, however, the cells appeared to recover from the treatment. Characterisation studies suggest that fullerenol agglomeration increased with concentration and decreased with hydroxyl groups at 8.5 and 42.5 µg/mL, indicating a possible relation between agglomeration at very high concentrations and observed effect. These effects are only seen at high concentrations, which may exist outside of any potentially achievable biological exposure.

Ref: *In vitro* toxicity assessment of three hydroxylated fullerenes in human skin cells; J.G. Saathof, Toxicology in Vitro 25 (2011) 2105–2112

A recent study from 2019 by O. Bolshakova et.al studied *in-vitro* toxicity on Chinese hamster lung fibroblasts (cell line V79) and HeLa cells (human cervix carcinoma cells) and *in-vivo* toxicity on Drosophila wild type Canton-S (alternative model study). The results showed that C60(OH)30 and C70(OH)30 at concentrations 0.1 mg/mL, 0.5 mg/mL and 1 mg/mL are non-toxic for cells and that even high concentrations, such as 1mg/ml for C60(OH)30 and C70(OH)30 did not cause a significant increase in the level of apoptosis in cells compared to

control. *In-vivo* study on Drosophila Canton-S line flies (a model system for evaluating the toxicities of artificial nanomaterials) showed that the studied compounds administered in dose at 2 mg/mL (the flies were fed with yeast that contained fullerenols during the duration of their life) did not cause the decrease in the life span and did not change the form of the survival curve. The results of this study indicate that studied fullerenols are of very low toxicity.

Ref: *In vitro* and *in vivo* study of the toxicity of fullerenols C60, C70 and C120O obtained by an original two step method; O. Bolshakova et.al. Materials Science and Engineering: C Volume 104, November 2019, 109945

Notifiers Conclusions: Based on the above referred studies, it can be concluded that *in-vitro* studies and an *in-vivo* alternative study on Drosophila indicate very low toxicity of Hyperharmonised Fullerenol-Water Complex (HFWC). The lowest No Observed Effect Level (NOEL) of 8.5 µg/ml is significantly higher than the potentially achievable biological concentrations when used in cosmetic products (0.0006 µg/g bw/day, which equals the average tissue concentration of 0,0006 µg/mL).

Ref: 281_safety_file_2020-3-12-18-44-18

SCCS comment

The SCCS considers that this study is more relevant to environmental risk assessment and not suitable for deriving a PoD for assessment of risk to human health.

3.5 SAFETY EVALUATION (INCLUDING CALCULATION OF THE MOS)

SCCS comment

The SCCS has noted that Notifiers have provided calculations for the Margin of Safety (Mos) for fullerenes and hydrated forms of hydroxylated fullerenes. However, the SCCS considers that calculation of MoS is not possible because genotoxicity potential of any of the materials considered in this Opinion cannot be excluded on the basis of the available data.

3.6 DISCUSSION

The information provided by three Notifiers through CPNP on the materials considered in this Opinion was assessed by the SCCS, and further clarifications were asked where appropriate. Additionally, a call for information was made and a literature search performed by the Commission to obtain further information from other sources. In developing this Opinion, the SCCS has taken into account the responses received from the Notifiers, the information received from the Commission's call for information, and the results of the open literature search.

Having considered all the available information, the SCCS is of the view that the information available at present is insufficient to allow drawing conclusions on the safety of fullerenes, hydroxylated fullerenes, and the hydrated forms of hydroxylated fullerene.

- According to two Notifiers, the raw fullerene powder is a mixture of C60 and C70, and the content of C60 measured in five batches ranges approximately from 70 to 80%. Considering that there are similarities between fullerenes C60 and C70 in terms of chemical composition, structural features, and toxicological aspects tested via *in vitro* assays, the SCCS has accepted the Applicant's justification for data read-across between the two fullerenes.
- In the absence of reasonable scientific explanation for the nature of bonding involved between hydroxylated fullerenes and water molecules, the SCCS has considered in this

Opinion the hydrated form of hydroxylated fullerene as similar to other hydroxylated fullerenes dispersed in aqueous media.

- In the absence of sound experimental data on the dermal absorption of the notified nanomaterials, the SCCS will consider the use of default value of 50%.

The following information/data should be provided to enable safety assessment:

- Detailed information on the levels of impurities, heavy metals, accompanying contaminants and organic solvents, along with detailed information on the methods of manufacturing (synthesis route, solvent removal, and any co-synthesised by-products) for fullerenes (C60 and C70), hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes.
- Detailed quantitative EM analysis for accurate size measurement of the particles in the nanoscale.
- Information indicating the shape, aspect ratio and agglomeration/ aggregation state of the hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes and data on the surface charge of hydroxylated fullerenes.
- Detailed information on homogeneity and stability of the notified nanomaterials.
- Information/data on the function and uses.
- Data on the systemic availability for fullerenes (C60 and C70), hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes.
- The Notifiers should provide valid information (data) on mammalian cell gene mutation assays and micronucleus test performed with the notified nanomaterials either from published literature with these nanomaterials, and/or experimental studies. Cellular uptake of the nanoparticles needs to be confirmed.
- In view of the phototoxic potential, hydroxylated fullerenes, including hydrated forms of hydroxylated fullerenes (HFWC), are of concern for consumer safety when used in leave-on products that are applied to (sun)light exposed skin.

In Annex-I, the SCCS has provided more detailed views about concerns that the use of these materials in cosmetic products can pose risks to the consumer.

4. CONCLUSION

1. In view of the above, and taking into account the scientific data provided, does the SCCS consider Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes safe when used in cosmetic products according to the maximum concentrations and specifications as reported via CPNP, taking into account reasonably foreseeable exposure conditions?

Having assessed the information provided by the Notifiers, and the information available from published literature, the SCCS has not been able to conclude on the safety of fullerenes and (hydrated) hydroxylated forms of fullerenes due to a number of uncertainties and data gaps in regard to physicochemical, toxicokinetic and toxicological aspects. These uncertainties and data gaps have been indicated in relevant sections of the Opinion and must be addressed by the Notifiers to enable a conclusion on the safety of the materials for use in cosmetic products.

In particular, the SCCS has not been able to conclude on the genotoxicity potential of fullerenes (C60 and C70). The available evidence indicates that hydrated forms of hydroxylated fullerenes are genotoxic and hence SCCS considers them as not safe for use in cosmetic products. In view of equivalence as discussed before (see section 3.1.1.5), the same concerns over genotoxicity potential also apply to hydroxylated fullerenes.

2. Based on the currently available scientific literature and SCCS' expert judgement, the SCCS is requested to assess any further scientific concerns with regard to the use of Fullerenes, Hydroxylated Fullerenes and hydrated forms of Hydroxylated Fullerenes in cosmetic products and whether a potential risk to human health can be identified according to Article 16(6) Reg.1223/2009.

In Annex-1 of this Opinion, the SCCS has noted the basis for concerns over risks that the use of fullerenes, hydroxylated fullerenes, and hydrated forms of hydroxylated fullerenes in cosmetic products may pose to the consumer. In brief, the SCCS has a concern in regard to:

- the potential presence of impurities, heavy metals, accompanying contaminants and/or organic solvents in the notified nanomaterials. Lack of data on stability of hydroxylated fullerenes and their hydrated forms.
- the potential ability of fullerenes and derivatives to induce production of free oxyradicals when used in cosmetic products.
- phototoxicity of hydroxylated fullerenes – with similar concerns for the hydrated forms of hydroxylated fullerenes.
- sensitising potential of hydroxylated fullerenes.
- dermal absorption and systemic availability of the nanoparticles after use in cosmetic products.
- distribution of systemically available fullerenes to various organs in the body and potential accumulation of the nanoparticles in certain organs – such as lungs and liver.
- the available information does not allow the SCCS to exclude genotoxic/carcinogenic potential of any of the materials assessed in this Opinion.

5. MINORITY OPINION

None.

6. REFERENCES

Abrefah J. and Olander D.R. Vapor pressure of Buckminsterfullerene. Applied Physics Letters 1992; 60: 1313-1314. https://doi.org/10.1063/1.107327

Ahmad S., FIETE. Carbon Nanostructures Fullerenes and Carbon Nanotubes. IETE Technical Review 1999; 16(3-4): 297-310. https://doi.org/10.1080/02564602.1999.11416845

Aoshima H., Saitoh Y., Ito S., Yamana S., Miwa N. Safety evaluation of highly purified fullerenes (HPFs): Based on screening of eye and skin damage. J. Toxicol. Sci. 2009;34(5):555–562, http://dx.doi.org/10.2131/jts.34.555

Arai T., Murakami Y., Suematsu H., Kikuchi K., Achiba Y., Ikemoto I. Resistivity of single crystal C60 and effect of oxygen. Solid State Communications 1992; 84: 827-829. https://doi.org/10.1016/0038-1098(92)90099-U

Baati T., Bourasset F., Gharbi N., Njim L., Abderrabba M., Kerkeni A. i wsp. The prolongation of the lifespan of rats by repeated oral administration of [60]fullerene. Biomaterials 2012;33(19):4936–4946, http://dx.doi.org/10.1016/j.biomaterials.2012.03.036.

Beckhaus H.D., Rüchardt C., Kao M., Diederich F., Foote C.S., l. The Stability of Buckminsterfullerene (C60): Experimental Determination of the Heat of Formation. Angewandte Chemie-International Edition in English, 1992; 31: 63-64. https://doi.org/10.1002/anie.199200631

Cadek M *et al.* Carbon, 7. Fullerenes and Carbon Nanomaterials. Ullmann's Encyclopedia of Industrial Chemistry. 7th edition (1999-2013). New York, NY: John Wiley & Sons. Online Posting Date: Jan 15, 2010, from HSDB.

Cataldo F., and Braun T. The solubility of C60 fullerene in long chain fatty acids esters, Fullerenes, Nanotubes and Carbon Nanostructures, 2007; 15:5, 331–339 http://dx.doi.org/10.1080/15363830701512450

EFSA Scientific Committee, More, S., Bampidis, V., Benford, D., Bragard, C., Halldorsson, T., Hernández-Jerez, A., Bennekou, S. H., Koutsoumanis, K., Lambré, C., Machera, K., Naegeli, H., Nielsen, S., Schlatter, J., Schrenk, D., Silano , V., Turck, D., Younes, M., Castenmiller, J., Chaudhry, Q., Cubadda, F., Franz, R., Gott, D., Mast, J., Mortensen, A., Oomen, A. G., Weigel, S., Barthelemy, E., Rincon, A., Tarazona, J., & Schoonjans, R. (2021). Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles. EFSA Journal, 19(8), e06769. doi: https://doi.org/10.2903/j.efsa.2021.6769

Ema M., Matsuda A., Kobayashi N., Naya M., Nakanishi J. Dermal and ocular irritation and skin sensitization studies of fullerene C60 nanoparticles. Cutan. Ocul. Toxicol. 2013;32(2):128–134, http://dx.doi.org/10.3109/15569527.2012.727937

Fujita, K. *et al.* Gene expression profiles in rat lung after inhalation exposure to C60 fullerene particles, Toxicol., 2009; 258: 47-55.

Hendrickson O.D., Zherdev A.V., Gmoshinskii I.V., Dzantiev B.B. Fullerenes: *In Vivo* Studies of Biodistribution, Toxicity, and Biological Action, Nanotechnologies in Russia, 2014; 9(11–12), 601–617. https://doi.org/10.1134/S199507801406010X

Heiney P.A., Fischer J.E., McGhie A.R., Romanow W.J., Denenstein A.M., McCauley Jr. J.P., Smith A.B., Cox D.E. Orientational ordering transition in solid C60. Physical Review Letters 1991; 2911-2914. https://doi.org/10.1103/PhysRevLett.66.2911

Hirsch A., Brettreich M. Fullerenes: chemistry and reactions, J. Am. Chem. Soc. 2005, 127, 33, 11876. https://doi.org/10.1021/ja059725z

Horie M., Nishio K., Kato H., Shinohara N., Nakamura A., Fujita K., Kinugasa S., Endoh S., Yoshida Y., Hagihara Y., Iwahashi H. In vitro evaluation of cellular influences induced by stable fullerene C70 medium dispersion: Induction of cellular oxidative stress. Chemosphere, 2013, 93(6), 1182-1188.

Ji Z.Q, Sun H., Wang H., Xie Q., Liu Y., and Wang Z., Biodistribution and tumor uptake of $C_{60}(OH)_x$ in mice, J. Nanoparticle Res. 8 (1), 53–63 (2006).

Kato S., Aoshima H., Saitoh Y., Miwa N. Biological safety of lipofullerene composed of squalane and fullerene-C60 upon mutagenesis, photocytotoxicity, and permeability into the human skin tissue. Basic. Clin. Pharmacol. Toxicol. 2009; 104(6): 483–487, http://dx.doi.org/10.1111/j.1742-7843.2009.00396.x

Kubota R., Tahara M., Shimizu K., Sugimoto N., Hirose A., Nishimura T. Time-dependent variation in the biodistribution of C60 in rats determined by liquid chromatography-tandem mass spectrometry, Toxicol. Lett., 2011; 206: 172-177.
http://dx.doi.org/10.1016/j.toxlet.2011.07.010

Li F.B., Wang G.W. Fullerenes. Kirk-Othmer Encyclopedia of Chemical Technology (1999-2013). New York, NY: John Wiley & Sons. Online Posting Date: 19 Apr 2013 DOI: 10.1002/0471238961.0621121220012512.a01.pub2

Liosi, K., Stasyuk, A. J., Masero, F., Voityuk, A. A., Nauser, T., Mougel, V., Solà, M. & Yamakoshi, Y. (2021). Unexpected Disparity in Photoinduced Reactions of C60 and C70 in Water with the Generation of O2•- or $^1O_2$. JACS Au, 1(10), 1601–1611.
https://doi.org/10.1021/jacsau.1c00239

Mrdanovic J., Solajic S., Bogdanovic V., Stankov, K., Bogdanovic G., Djordjevic A. Effects of fullerenol $C_{60}(OH)_{24}$ on the frequency of micronuclei and chromosome aberrations in CHO-K1 cells. Mutation Research 680 (2009) 25–30

Merkus H.G. (2009). Particle size measurements: fundamentals, practice, quality, Springer Science & Business Media, ISBN: 978-1-4020-9015-8.

Monteiro-Riviere N.A., Linder K.E., John A.O., Saathoff G., Xia X.-R., Riviere J.E. Lack of Hydroxylated Fullerene Toxicity after Intravenous Administration to Female Sprague-Dawley Rats; J Toxicol Environ Health A. 2012; 75(7): 367–373

Mori T., Takada H., Ito S., Matsubayashi K., Miwa N., Sawaguchi T. Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis, 2006, Toxicology 225, 48–54, https://doi.org/10.1016/j.tox.2006.05.001

Morimoto, Y. *et al.* Inflammogenic effect of well-characterized fullerenes in inhalation and intratracheal instillation studies, Part. Fibre. Toxicol.,2010; 7:4.

Mort J., Machonkin, M., Ziolo R. Temperature dependence of photoconductivity in buckminsterfullerene films. Appl. Phys. Lett. 1992; 60: 1735-1737.
https://doi.org/10.1063/1.107201

Naota M, Shimada A., Morita T., Inoue K., Takano H. Translocation Pathway of the Intratracheally Instilled C60 Fullerene from the Lung into the Blood Circulation in the Mouse: Possible Association of Diffusion and Caveolae-mediated Pinocytosis, Toxicologic Pathology, 2009; 37, 456-462.

OECD 2016: ENV/JM/MONO(2016)21: Environment Directorate Joint Meeting Of The Chemicals Committee And The Working Party On Chemicals, Pesticides And Biotechnology Fullerenes (C60): Summary Of The Dossier Series on the Safety of Manufactured Nanomaterials No. 69.

Qiao R., Roberts A.P., Mount A.S., Klaine S.J., Ke R.CH. Translocation of C60 and Its Derivatives Across a Lipid Bilayer, Nanoletters, 2007. 7(3), 614-619. https://doi.org/10.1021/nl062515f

Ogami, A. *et al.* Pathological features of rat lung following inhalation and intratracheal instillation of C60 fullerene, Inhal. Txicol., 2011; 23(7): 407-416.

Pan C., Sampson M.P., Chai Y., Hauge R.H., Margrave J.L. Heats of sublimation from a polycrystalline mixture of carbon clusters (C60 and C70). J Phys Chem 1991; 95: 2944-2946. https://pubs.acs.org/doi/pdf/10.1021/j100161a003

Proskurnina E.V., Mikheev I.V., Savinova E.A., Ershova E.S., Veiko N.N., Kameneva L.V., Dolgikh O.A., Rodionov I.V., Proskurnin M.A., Kostyuk S.V. Effects of Aqueous Dispersions of C60, C70 and Gd@C82 Fullerenes on Genes Involved in Oxidative Stress and Anti-Inflammatory Pathways. Int. J. Mol. Sci. 2021, 22, 6130. https://doi.org/10.3390/ijms22116130

Ryan J.J., Bateman H.R., Stover A., Gomez G., Norton S.K., Zhao W., Schwartz L.B., Lenk R., Kepley C.L. Fullerene nanomaterials inhibit the allergic response. J Immunol. 2007 Jul 1;179(1):665-72. doi: 10.4049/jimmunol.179.1.665

Roberts J.E., Wielgus A.R., Boyes W.K., Andley U., Chignell C.F. Phototoxicity and cytotoxicity of fullerol in human lens epithelial cells. Toxicol Appl Pharmacol 2008; 228: 49–58.

Shinohara N., Matsumoto K., Endoh S., Maru J., Nakanishi J. In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles. Toxicol. Lett. 2009;191(2–3):289–296, http://dx.doi.org/10.1016/j.toxlet.2009.09.012

Shinohara N., Nakazato T, Tamura M., Endoh S., Fukui H., Morimoto Y., Myojo T., Shimada M., Yamamoto K., Tao H., Yoshida Y., Nakanishi J. Clearance kinetics of fullerene C_{60} nanoparticles from rat lungs after intratracheal C_{60} instillation and inhalation C_{60} exposure. Toxicol Sci. 2010 Dec;118(2):564-73. doi: 10.1093/toxsci/kfq288.

Shipelin V.A., Smirnova T.A., Gmoshinskii I.V., Tutelyan V.A. Analysis of toxicity biomarkers of fullerene C60 nanoparticles by confocal fluorescent microscopy. Bull. Exp. Biol. Med. 2015;158(4):443–449, http://dx.doi.org/10.1007/s10517-015-2781-4.

Sivaraman N., Dhamodaran R., Kaliappan I., Srinivasan T. G., Vasudeva Rao P. R. P., Mathews C. K. C. Solubility of C70 in Organic Solvents, Fullerene Science and Technology 1994; 2 (3): 233-246. https://doi.org/10.1080/15363839408009549

Taylor R; Fullerenes. Kirk-Othmer Encyclopedia of Chemical Technology. (2001). New York, NY: John Wiley & Sons. Online Posting Date: Sept 20, 2002 from HSDB.

Takahashi M., Kato H., Doi Y., Hagiwara A., Hirata-Koizumi M., Ono A., Kubota R., Nishimura T., Hirose A. Sub-acute oral toxicity study with fullerene C60 in rats, J. Toxicol. Sci., 2012; 37(2): 353-361.

Wielgus A.R., Baozhong Z., Chignell C.F., Hu D.N., Roberts J.E. Phototoxicity and cytotoxicity of fullerol in human retinal pigment epithelial cells. Toxicology and Applied Pharmacology 2010; 242: 79-90

Xia X.R., Monteiro-Riviere N.A., Riviere J.E. Skin penetration and kinetics of pristine fullerenes (C60) topically exposed in industrial organic solvents, Toxicol. Appl. Pharmacol. 2010; 242(1):29-37.
http://dx.doi.org/10.1016/j.taap.2009.09.011

Zhao B., He Y.Y., Bilski P.J., Chignell C.F. Pristine (C60) and hydroxylated [C60(0H)24] fullerene phototoxicity towards HaCaT keratinocytes: type I vs type II mechanisms. Chem. Res. Toxicol.2008; 21(5): 1056-1063.

Annex 1

Safety concerns for Fullerenes and Hydroxylated/Hydrated form of Hydroxylated Fullerenes

As indicated in this Opinion, the data/information provided by the Notifiers were not sufficient to enable a safety assessment. In view of this, the SCCS obtained and considered additional information on fullerenes, hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes from the published literature.
Although safety of the materials could still not be concluded on the basis of evaluation of all the available data/information, the SCCS has noted the following scientific aspects that raise a concern over the potential risk to consumer's health from the use of the materials in cosmetic products:

PHYSICOCHEMICAL ASPECTS

Fullerenes are lipophilic molecules that exist in the form of extremely small particles (below 1 nm), made of carbon lattice. Fullerenes are practically insoluble in water, whereas derivatives of fullerene with added hydroxyl (-OH) groups – such as hydroxylated fullerenes and their hydrated forms - are water-soluble. This makes them potentially useful for a variety of applications, including cosmetics. Surface chemistry, such as the degree of hydroxylation of fullerenes and concentration, may affect the degree of agglomeration and thus biological effects (Saathoff *et al.*, 2011). The presence of certain impurities, residual solvents, and heavy metals in fullerenes and their derivatives is a common concern since they can affect their properties and behaviour, and potentially alter their efficacy and toxicity (Zhang *et al.* 2009). It is, therefore, important to ensure the highest purity of fullerenes and their derivatives and minimise the potential presence of impurities that may affect their safety and efficacy. In this regard, the SCCS has a concern that a proper evaluation of safety may not be possible in the absence of detailed information on the levels of impurities, heavy metals, accompanying contaminants and organic solvents for the notified nanomaterials. Similar issues may arise from degradation of hydroxylated fullerenes and their derivatives (Rodrıguez-Zavala *et al.*2006, Xing *et al.*2006, and Kong *et al.*2009). Therefore, stability data of hydroxylated fullerene and hydrated forms of hydroxylated fullerenes are also important for a conclusive safety evaluation.
Due to their unique physicochemical properties, fullerenes and their hydroxylated derivatives have been reported to act both as pro-oxidants as well as antioxidants (Marcovic *et al.* 2008 cited by Savinova *et al.*, 2023; Markelic *et al.*, 2022). It is well known that free radicals of oxygen are highly reactive and can cause oxidant damage to the exposed cells and tissues in the body. The SCCS has a concern in this regard for consumer safety and needs evidence to exclude the potential formation of free radicals by these ingredients when used in cosmetics.

TOXICOLOGICAL ASPECTS

Cytotoxicity in vitro

Fullerene C60 derivative coupled to a heptapeptide could elicit an inflammatory response, indicated by an increase in interleukin (IL)-6, IL-8, and IL-1b production in HEK keratinocytes (Rouse *et al.*, 2006). The fullerene ultimately initiated dose-dependent cytotoxicity via a necrotic mechanism. Harhaji *et al.* (2008) observed that C60/C70 and polyhydroxylated fullerene preparations (up to 250 µg/ml for 24 h, dispersed in serum-containing cell culture medium) were cytotoxic to the mouse L929 fibroblast cell line but that C60/C70 was more potent. Sayes *et al.* (2005) demonstrated that C60 fullerene (0.00024–2.4 µg/mL) exerted cytotoxicity that was mediated through enhanced ROS production, lipid peroxidation, and membrane damage in a variety of cell lines (dermal fibroblasts, hepatocytes, and astrocytes).

The SCCS is aware of other studies that have indicated negative cytotoxicity results, but considers that the available data are not sufficient to convincingly exclude the potential cytotoxicity of the fullerenes and fullerene-derivatives assessed in this Opinion under some exposure conditions of the materials.

Skin Sensitisation

The test results available for hydroxylated fullerene and hydrated forms of hydroxylated fullerenes do not clearly exclude a sensitising potential.
While the *in-vivo* tests with fullerenes indicate absence of skin sensitising potential, an *in-vitro* experiment on FullereneC60 did not exclude such a potential (Bezerra, 2021).

Phototoxicity

The available scientific literature indicates that hydroxylated fullerene as such, and in aggregated form, absorbs both UVA and VIS light, and has the potential to cause phototoxicity to skin and eyes. Hydroxylated fullerene exposed to UV or VIS radiation has been shown to be phototoxic to human keratinocytes in vitro (Zhao, 2008). In vitro, hydroxylated fullerene has also been shown to cause phototoxic damage to epithelial cells of the human lens (Roberts, 2008), and the pigment epithelial cells of the retina (Wielgus, 2010).

Without excluding the phototoxicity potential of hydroxylated fullerenes and hydrated forms of hydroxylated fullerenes, these nanomaterials cannot be considered safe in cosmetic products intended for use on the skin exposed to sunlight.

Induction of lung inflammatory reaction

Fullerene
Gene expression profiles in the rat lung, after inhalation exposure to C60 fullerene, revealed that few genes involved in the inflammatory response, oxidative stress, apoptosis, and metalloendopeptidase activity were up-regulated at both 3 days and 1-month post-exposure (Fujita *et al.*, 2009). C60 fullerene after intratracheal instillation in mice induced an increase in sub G1 and G1 arrest in BAL cells, an increase in proinflammatory cytokines such as IL-1, TNF-α, and IL-6, and an increase of Th1 cytokines such as IL-12 and IFNr in BAL fluid (Park *et al.*, 2010). Cell infiltration and expression of tissue damage related genes in lung tissue were constantly observed during the experiment period. In addition to the effects on pulmonary responses (Sayers *et al.*, 2016; Pinheiro *et al.*, 2021), fullerenes were also reported to modulate the immune system (e.g. induction of splenic inflammatory process) (Ding *et al.*, 2011).

Hydroxylated fullerene
Xu *et al.* (2009) showed that the polyhydroxylated derivative of fullerene [C60(OH)x] was not able to induce adverse pulmonary pathological changes but elicited dose-dependent inflammation (increase in %neutrophils, IL-1β, TNF-α and IL-6) in BAL supernatants, associated with the nitric oxide synthase-dependent induction.
Intratracheal exposure to fullerols at a dose of 200 µg (equivalent to 10 mg/kg) elicited a neutrophil-driven pulmonary inflammatory response, which was associated with increased macrophage inflammatory protein-2 production (Roursgaard *et al.*, 2008).
Although, the exposure route via lung is not relevant to the current submission, the SCCS is of the opinion that it might be of importance for other submissions where the materials are intended to be used in inhalable products that could lead to exposure of the consumer's lung (such as powders, sprayable products).

Genotoxicity/mutagenicity

Analysis of the currently available information from published literature has yielded both negative and positive results for genotoxicity of fullerenes and fullerene-derivatives. For example, a dose-dependent increase in micronucleus frequencies by fullerene C60 was observed in A549 cells (Totsuka *et al.*, 2009), as well as in CHO, HeLa, and HEK293 cell lines after long-term incubation with C60(OH)24 at picogram per mL concentrations (Niwa *et al.*,

2006).

Comet assay using stable aqueous suspensions of colloidal fullerenes C60 prepared by two methods - ethanol to water solvent exchange (EthOH/nC60 suspensions) and extended mixing in water (aqu/nC60 suspensions) - demonstrated genotoxicity potential for both types of suspensions. There was a strong correlation between the genotoxic response and the nC60 concentration, with genotoxicity observed at concentrations as low as 2.2 µg/L for aqu/nC60 and 4.2 µg/L for EtOH/nC60 (Dhawan *et al.*, 2006). Jacobsen *et al.* (2008) also reported an increase in FPG sensitive sites/oxidised purines in fullerenes C60 exposed FE1-Muta™ Mouse lung epithelial cells, as revealed by the Comet assay. In another study, in Comet assay C60 and $C60(OH)_{24}$ showed DNA damaging effect on HepG2 cells and human peripheral blood mononuclear cells (Vesterdal *et al.*, 2014; Sharoyko *et al.*, 2021).

Fullerene C60 has also been reported to induce DNA damage *in vivo* in the lungs of C57BL/6J mice, measured by Comet assay. Moreover, single, or multiple instillations of fullerenes C60 increased gpt mutant frequencies in the lungs of gpt delta transgenic mice (Totsuka *et al.*, 2009).

The SCCS is aware of other studies that have indicated negative genotoxicity results (see Section 3.4.5 of the Opinion) but considers that the currently available weight of evidence is not sufficient to exclude the genotoxicity potential of the materials assessed in this Opinion.

Systemic Toxicity

Fullerenes
In a developmental toxicity study (Tsuchiya *et al.*1996 reviewed by Nielsen *et al.* 2008 and Snyder *et al.*2015), fullerene C60/PVP was administered to pregnant SLC mice on gestational day (GD) 10 by intraperitoneal injection. The administered doses ranged from 25 to 137 mg/kg, and the effects were monitored at 18 hours following administration. At a dose of 137 mg/kg, all the embryos died and showed severe abnormalities. At a dose of 50 mg/kg, C60 was clearly distributed into the embryos based on the characteristic colour development of C60, and caused abnormalities, especially around the head region and tail. At 25 mg/kg, abnormal enlargement of the head was reported in one embryo. This study by Tsuchiya *et al.* (1996), however has certain shortcomings in that the number of animals per exposure group was low, the route of administration was unusual, and the study covered only a small part of the pregnancy period.
Fullerene C60 given intratracheally to mice (1.0 mg/kg bw) which were tested at 12, 24, 72 and 96 h thereafter, worsened the spermatic parameters in the animals over the whole study period (Pinheiro *et al.*, 2021).

Although the analysed studies have some limitations, they indicate potential developmental effects and therefore the need for further investigation to exclude the reproductive/developmental effects of fullerenes and the hydroxylated/hydrated derivatives.

Exposure Aspects

Although the exposure calculations by the Notifier(s) have indicated that the amount to be used in cosmetics will be very small, considering the extremely small particle size of fullerenes and fullerene-derivatives, these amounts still represent very large number of particles.

Dermal penetration

Fullerenes
Some studies have suggested that fullerenes can penetrate the skin, particularly if they are formulated in a way that enhances dermal penetration. A study by Martins *et al.* (2017) showed that 14% of fullerene C60 dispersed in a solution of fatty acids was able to cross the intact skin into the receptor compartment. In this study, the localisation and permeation extent of fullerene C60 was depicted by TEM analysis that clearly showed the presence of

fullerene C60 aggregates in the skin sample. Another study investigated the dermal penetration of a fullerene C60 derivative (fullerene coupled to a heptapeptide) in flexed and unflexed porcine skin (Rouse *et al.*, 2007 reviewed by Nielsen *et al.*, 2008). The results of this study showed that the fullerene particles could penetrate to the dermis. Therefore, it can be inferred that systemic availability of fullerenes after dermal administration is possible. There are indications that skin penetration of pristine fullerenes C60 will be dependent of the solvent used (Xia *et al.*, 2010), and that dermal penetration of fullerenes and their derivatives may be modulated by the formulations they have been added to. This means that certain (lipophilic) formulations may enable them to cross the dermal barrier to reach other organs in the body.

Toxicokinetics/distribution

Fullerene

Information from the available literature so far has indicated systemic bioavailability of fullerenes via the oral route. Systemically available fullerenes will be well distributed to various organs in the body and may accumulate in certain organs – such as lungs and liver (Hendrickson *et al.*2014). In studies using parenteral administration, approximately a quarter of a pristine Fullerene C60 suspension was found accumulated in the liver (mainly in Kupffer cells), where their levels remained constant for about one week (Gharbi *et al.,* 2005 reviewed by Nielsen *et al.*, 2008).

A study by Sumner *et al.* (2010) determined the distribution of ^{14}C-labelled fullerene C60 in the pregnant rat and foetuses, and in the lactating rat and offspring after i.v. administration of the radiolabelled fullerene C60 suspended in PVP. The results of this study indicated distribution to the placenta, foetuses, and to the milk and offspring of the exposed lactating dam. Another study by Snyder *et al.* (2015) investigated the distribution of [^{14}C(U)]C60 (in 5% PVP-saline suspension) in pregnant and lactating rat exposed by the i.v. route at different developmental time points, and at different time points post administration. Radioactivity was distributed from mothers to their offspring both during pregnancy through the placenta to foetuses, and via milk to lactating pups. The distribution and organ specific distribution were different in pregnant and lactating rats. In the case of pregnant dams, maternal-fetal transfer depended on both the stage of gestation and the elapsed time between exposure and termination.

Hydroxylated fullerene and their hydrated forms

A study by Ji *et al.*, 2006 used ^{125}I–labelled hydroxy-fullerenes, administered i.v. to mice that had been implanted subcutaneously with various tumours. These mouse models were used to study the accumulation of ^{125}I–C60(OH)$_x$ [x= ~ 24]. The results showed that ^{125}I–labelled hydroxy-fullerenes distributed to all major organs and accumulated mostly in liver, spleen, kidney, and bone tissues. In the same study, the distribution of ^{125}I–C60(OH)$_x$ in normal Kunming mice showed similar results.

It should also be kept in mind that the toxicokinetics of fullerenes derivatives may be influenced by surface modifications (Aschberger *et al.*, 2010).

Conclusions

- The SCCS has a concern over the safety of the notified nanomaterials in regard to the potential presence of impurities, heavy metals, accompanying contaminants and organic solvents, and more information on impurity profile of the materials will be needed to exclude this concern. Stability data of hydroxylated fullerenes and their hydrated forms are also important for effective evaluation of the notified nanomaterials.
- The available information indicates that fullerenes may become systemically available after dermal administration. The limited available information also indicates that systemically available fullerenes and hydroxylated fullerenes will be widely distributed to various organs in the body and may accumulate in certain organs – such as lungs

and liver. Such information is not available for the hydrated forms of hydroxylated fullerenes.

- The SCCS has a concern in regard to the potential ability of systemically available fullerenes and derivatives to induce production of free oxyradicals when used in cosmetic products.
- The results of the tests provided for this opinion do not clearly exclude a sensitising potential of hydroxylated fullerenes.
- The available information suggests that hydroxylated fullerenes are phototoxic. Similar concerns for phototoxicity are also applicable to the hydrated forms of hydroxylated fullerenes.
- The available information does not allow the SCCS to exclude genotoxic/carcinogenic potential of any of the materials assessed in this opinion.

References for Annex I

1. Aschberger K, Johnston HJ, Stone V, Aitken RJ, Tran CL, Hankin SM, Peters SAK, Christensen FM, Review of fullerene toxicity and exposure--appraisal of a human health risk assessment, based on open literature. Regul Toxicol Pharmacol 2010;58(3):455-73. doi: 10.1016/j.yrtph.2010.08.017.
2. Bezerra SF, Rodrigues B dos S, Silva ACG, Ávila RI, Brito HRG, Cintra ER, … Valadares MC. (2020). Application of the adverse outcome pathway (AOP) framework for investigating skin sensitization potential of nanomaterials using new approach methods. Contact Dermatitis. doi:10.1111/cod.13669
3. Damgård Nielsen GD, Roursgaard M, Jensen KA, Alstrup K, Poulsen SS, Seier S, Larsen ST, In vivo, Biology and Toxicology of Fullerenes and Their Derivatives, Basic & Clinical Pharmacology & Toxicology, 2008; 103, 197–208. (Doi: 10.1111/j.1742-7843.2008.00266.x).
4. Dhawan A, Taurozzi JS, Pandey AK, Shan W, Miller SM, Hashsham SA & Tarabara VV. (2006). Stable Colloidal Dispersions of C60 Fullerenes in Water: Evidence for Genotoxicity. Environmental Science & Technology, 2006 40(23), 7394–7401. doi:10.1021/es0609708.
5. Ding N, Kunugita N, Ichinose T, Song Y, Yokoyama M, Arashidani K, & Yoshida Y. (2011). Intratracheal administration of fullerene nanoparticles activates splenic CD11b+ cells. Journal of Hazardous Materials, 194, 324–330. doi:10.1016/j.jhazmat.2011.07.101
6. Franskevych D, Palyvoda K, Petukhov D, Prylutska S, Grynyuk I, Schuetze C, … Ritter U. (2017). Fullerene C60 Penetration into Leukemic Cells and Its Photoinduced Cytotoxic Effects. Nanoscale Research Letters, 12(1). doi:10.1186/s11671-016-1819-5.
7. Fujita K, Morimoto Y, Ogami A, Myojyo T, Tanaka I, Shimada M, … Nakanishi J. (2009). Gene expression profiles in rat lung after inhalation exposure to C60 fullerene particles. Toxicology, 258(1), 47–55. doi:10.1016/j.tox.2009.01.005
8. Gharbi N, Pressac M, Hadchouel M, Szwarc H, Wilson SR, Moussa F. [60]Fullerene is a powerful antioxidant in vivo with no acute or subacute toxicity. Nano Lett 2005;5:2578–85
9. Hendrickson O.D., Zherdev A.V., Gmoshinskii I.V., Dzantiev B.B. Fullerenes: In Vivo Studies of Biodistribution, Toxicity, and Biological Action, Nanotechnologies in Russia, 2014; 9(11–12), 601–617. https://doi.org/10.1134/S199507801406010X
10. Jacobsen NR *et al.*, Genotoxicity, cytotoxicity, and reactive oxygen species induced by single-walled carbon nanotubes and C60 fullerenes in the FE1-MutaTM Mouse lung epithelial cells, Environ. Mol. Mutagen. 49 (6) (2008) 476–487 (doi: 0.1002/em.20406).
11. Ji Z.Q, Sun H., Wang H., Xie Q., Liu Y., and Wang Z., Biodistribution and tumor uptake of C60(OH)x in mice, J. Nanoparticle Res. 8 (1), 53–63 (2006).

12. Kong L, Tedrow O, Chan YF, Zepp RG, Light-Initiated Transformations of Fullerenol in Aqueous Media, Environ. Sci. Technol. 2009, 43, 24, 9155–9160. https://doi.org/10.1021/es901839q
13. Markelic, M.; Draca, D.; Krajnovic, T.; Jovic, Z.; Vuksanovic, M.; Koruga, D.; Mijatovic, S.; Maksimovic-Ivanic, D. Combined Action of Hyper-Harmonized Hydroxylated Fullerene Water Complex and Hyperpolarized Light Leads to Melanoma Cell Reprogramming In Vitro. Nanomaterials 2022, 12, 1331. https://doi.org/10.3390/nano12081331
14. Markovic, Z.; Trajkovic, V. Biomedical potential of the reactive oxygen species generation and quenching by fullerenes (C60). Biomaterials 2008, 29, 3561–3573
15. Martins M., Azoia N.G., Melle-Franco M., Ribeiro A., Cavaco-Paulo A. Permeation of skin with (C60) fullerene dispersions. Eng Life Sci. 2017 17(7): 732–738.
16. Niwa Y and Iwai N. Genotoxicity in Cell Lines Induced by Chronic Exposure to Water-Soluble Fullerenes Using Micronucleus Test. Environmental Health and Preventive Medicine 2006, 11, 292–297.
17. Park EJ, Kim H, Kim Y, Yi J, Choi K, & Park K. (2010). Carbon fullerenes (C60s) can induce inflammatory responses in the lung of mice. Toxicology and Applied Pharmacology, 244(2), 226–233. doi:10.1016/j.taap.2009.12.036
18. Pinheiro FG, Moreira-Gomes MD, Machado MN, Almeida T dos S, Barboza P da PA, Silva Oliveira LF, … Zin WA. (2021). Eugenol mitigated acute lung but not spermatic toxicity of C60 fullerene emulsion in mice. Environmental Pollution, 269, 116188. doi:10.1016/j.envpol.2020.116188.
19. Roberts JE, Wielgus AR, Boyes WK, Andley U, Chignell CF. Phototoxicity and cytotoxicity of fullerol in human lens epithelial cells. Toxicol Appl Pharmacol 2008; 228: 49–58.
20. Rodríguez-Zavala JG, Guirado-López R.A. Stability of Highly OH-Covered C60 Fullerenes: Role of Coadsorbed O Impurities and of the Charge State of the Cage in the Formation of Carbon-Opened Structures. J. Phys. Chem. A 2006, 110, 30, 9459–9468. https://doi.org/10.1021/jp061855m
21. Rouse JG, Yang J, Barron AR, and Monteiro-Riviere NA. Fullerene-based amino acid nanoparticle interactions with human epidermal keratinocytes. Toxicol. In Vitro 2006; 20, 1313–1320.
22. Rouse JG, Yang JZ, Ryman-Rasmussen JP, Barron AR, Monteiro-Riviere NA. Effects of mechanical flexion on the penetration of fullerene amino acid-derivatized peptide nanoparticles through skin. Nano Lett 2007; 7: 155–160.
23. Roursgaard M, Poulsen SS, Kepley CL, Hammer M, Nielsen GD, and Larsen ST. (2008). Polyhydroxylated C60 fullerene (fullerenol) attenuates neutrophilic lung inflammation in mice. Basic Clin. Pharmacol. Toxicol. 103, 386–388.
24. Saathoff J.G., Inman A.O., Xia X.R., Riviere J.E., Monteiro-Riviere N.A., In vitro toxicity assessment of three hydroxylated fullerenes in human skin cells. Toxicology in Vitro, 2011; 25(8) 2105-2112. https://doi.org/10.1016/j.tiv.2011.09.013
25. Sayes CM, Gobin AM, Ausman KD, Mendez J, West JL, and Colvin VL. (2005). Nano-C60 cytotoxicity is due to lipid peroxidation. Biomaterials 26, 7587–7595.
26. Sayers BC, Germolec DR, Walker NJ, Shipkowski KA, Stout MD, Cesta MF, … Smith MJ. (2016). Respiratory toxicity and immunotoxicity evaluations of microparticle and nanoparticle C60 fullerene aggregates in mice and rats following nose-only inhalation for 13 weeks. Nanotoxicology, 10(10), 1458–1468. doi:10.1080/17435390.2016.1235737
27. Savinova, E.A.; Salimova, T.A.; Proskurnina, E.V.; Rodionov, I.V.; Kraevaya, O.A.; Troshin, P.A.; Kameneva, L.V.; Malinovskaya, E.M.; Dolgikh, O.A.; Veiko, N.N.; *et al.* Effect of Water-Soluble Chlorine-Containing Buckminsterfullerene Derivative on the Metabolism of Reactive Oxygen Species in Human Embryonic Lung Fibroblasts. Oxygen 2023, 3, 1–19. https://doi.org/10.3390/oxygen3010001
28. Sharoyko VV e al. In Vitro and In Silico Investigation of Water-Soluble Fullerenol C60(OH)24: Bioactivity and Biocompatibility. J. Phys. Chem. B. 2021, 125, 9197−9212, (doi: 10.1021/acs.jpcb.1c03332).

29. Snyder RW, Fennell TR, Wingard CJ, Mortensen NP, Holland NA, Shannahan JH, Pathmasiri W, Lewin AH, Sumnera SCJ, Distribution and Biomarker of Carbon-14 Labeled Fullerene C60([^{14}C(U)]C60) in Pregnant and Lactating Rats and their Offspring after Maternal Intravenous Exposure. J Appl Toxicol. 2015 Dec; 35(12): 1438–1451. (doi: 10.1002/jat.3177)
30. Sumner SC, Fennell TR, Snyder RW, Taylor GF, Lewin AH, Distribution of carbon-14 labeled C60 ([14C]C60) in the pregnant and in the lactating dam and the effect of C60 exposure on the biochemical profile of urine, J Appl Toxicol 2010 May;30(4):354-60. (doi: 10.1002/jat.1503).
31. Totsuka Y *et al.* Genotoxicity of nano/microparticles in in vitro micronuclei, in vivo comet and mutation assay systems. Part Fibre Toxicol 2009 Sep 3; 6:23 (doi: 10.1186/1743-8977-6-23).
32. Tsuchiya T., Oguri I., Yamakoshi Y.N., Miyata N., Novel harmful effects of [60]fullerene on mouse embryos in vitro and in vivo. FEBS Lett 1996 9;393(1):139-45. (doi: 10.1016/0014-5793(96)00812-5).
33. Vesterdal LK, Danielsen PH, Folkmann JK, Jespersen LF, Aguilar-Pelaez K, Roursgaard M, … Møller P. (2014). Accumulation of lipids and oxidatively damaged DNA in hepatocytes exposed to particles. Toxicology and Applied Pharmacology, 274(2), 350–360. doi:10.1016/j.taap.2013.10.001
34. Wielgus AR, Baozhong Z, Chignell CF, Hu DN, Roberts JE. Phototoxicity and cytotoxicity of fullerol in human retinal pigment epithelial cells. Toxicology and Applied Pharmacology 2010; 242: 79-90
35. Xia X.R., Monteiro-Riviere N.A., Riviere J.E. Skin penetration and kinetics of pristine fullerenes (C60) topically exposed in industrial organic solvents, Toxicol. Appl. Pharmacol. 2010; 242(1):29-37. http://dx.doi.org/10.1016/j.taap.2009.09.011
36. Xing G, Zhang J, Zhao y, Tang J, Zhang B, Gao X, Yuan H, Qu L, Cao W, Chai Z, Ibrahim K, Su R, Influences of Structural Properties on Stability of Fullerenols, J. Phys. Chem. A 2006, 110, 9459-9468. https://doi.org/10.1021/jp0487962
37. Xu JY, *et al.* Pulmonary responses to polyhydroxylated fullerenols, C60 (OH)x, J. Appl. Toxicol. 29 (7) (2009) 578–584 (doi: 10.1002/jat.1442).
38. Zhang B., Cho M., Fortner JD, Lee J, Huang CH, Hughes JB, Kim JH, Delineating Oxidative Processes of Aqueous C60 Preparations: Role of THF Peroxide, Environ. Sci. Technol. 2009, 43, 1, 108–113
39. Zhao B, He YY, Bilski PJ, Chignell CF. Pristine (C60) and hydroxylated [C60(0H)24] fullerene phototoxicity towards HaCaT keratinocytes: type I vs type II mechanisms. Chem. Res. Toxicol.2008; 21(5): 1056-1063.