EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

# OPINION ON
# 1,3,5-Triazine, 2,4,6-tris[1,1'-biphenyl]-4-yl-

Scientific Committees
on consumer safety
on emerging and newly identified health risks
on health and environmental risks

The SCCS adopted this opinion at its 12th plenary meeting

of 20 September 2011

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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

SCCS

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

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## 1. Background

ETH 50 is a new notified substance to be used as an UV-filter in sunscreen products.

The first submission for this substance was received from the applicant in November 2005. An addendum was received in November 2006.

During review of the substance, it became apparent that it would be present in the form of nano-sized particles in the formulation to which the consumer is exposed. Therefore, further tests with this form of ETH50 were requested before the evaluation could be completed.

The present submission III provides an updated dossier including studies with the nano-sized form of ETH50.

## 2. Terms of reference

1. Does SCCS consider that the use of ETH50 as an UV-filter in cosmetic products in a concentration up to maximum 10.0% is safe for the consumers taken into account the scientific data provided?

2. Does SCCS have any other scientific concerns for the safe use of the new UV-filter ETH50 in finished cosmetic products?

## 3. OPINION

This opinion presents the safety assessment of 1,3,5-Triazine, 2,4,6-tris[1,1'-biphenyl]-4-yl- (trade name ETH50). Initially a set of studies were submitted in which ETH50 was used that had a particle size of d(0.5)= 15.4 µm. In some of the studies partially micronized ETH50 with a particle size of d(0.5)= 440 nm was used. However, in the intended commercial product ETH50 is further micronized to obtain particles with a d(0.5) = 100-110 nm. Upon request of the SCCS a new set of studies with the material to which the consumer would be exposed was provided in which ETH50 batches with a d(0.5)= 81, d(0.5)= 109 nm and d(0.5)=120 nm was used. In some of these studies a comparison was made between the nanosized and non-nanosized material. For this partially micronized material ETH50 with d(0.5) = 6 µm has been used.

Throughout this opinion the following terms are used to describe the different materials:
Non-micronized ETH 50: ETH50 with d(0.5) = 15.4 µm
Partially micronized (to a micron size) ETH 50: ETH50 with d(0.5) = 440 nm or 6 µm.
Fully micronized (to a nano size) ETH 50: ETH50 with d(0.5) = 81 nm, 109 or 120 nm.
The term nanosized refers to the fully micronized ETH50, whereas the term non-nanosized refers to both the non-micronized and the partially micronized ETH50.

### 3.1 Chemical and Physical Specifications

#### 3.1.1 Chemical identity

##### 3.1.1.1 Primary name and/or INCI name

1,3,5-Triazine, 2,4,6-tris[1,1'-biphenyl]-4-yl-

##### 3.1.1.2 Chemical names

1,3,5-Triazine, 2,4,6-tris[1,1'-biphenyl]-4-yl-

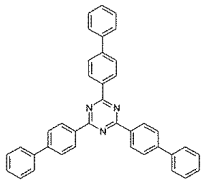
##### 3.1.1.3 Trade names and abbreviations

ETH50
C-801
FAT 65'080
FAT 65'080/A
FAT 65'080/B
FAT 65'082/B
COLIPA n°: /

##### 3.1.1.4 CAS / EC number

CAS: 31274-51-8
EC: not yet assigned

### 3.1.1.5 Structural formula



### 3.1.1.6 Empirical formula

Formula: $C_{39}H_{27}N_3$

### 3.1.2 Physical form

White solid

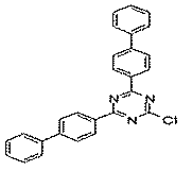
### 3.1.3 Molecular weight

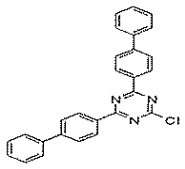
Molecular weight: 537.66 g/mol

### 3.1.4 Purity, composition and substance codes

Two different batches were used for studies with ETH50 and were shown to have a similar analytical profile. The purity was 98% (w/w) expressed as active molecule. In addition, each batch contained less than 0.1% of a known by-product and 0.32% defined as the sum of two unknown and non-coloured by-products; the balance of the composition comprised water, chloride, aluminium and residual solvents.
The samples were characterized by UV/VIS, IR and $^{1}H$ and $^{13}$-C-NMR spectroscopy.

The composition of the batches used for the toxicological assays is summarised in the table below:

| Batch | Measured purity active (% w/w) | Impurities | Water (%) | Others (%) |
|---|---|---|---|---|
| ETH 50/129B<br>(= FAT 65'080/A) | 98 ± 2 | **By-product 1, 0.099 area%:**<br>2-chlor-4,6-bis-(biphenyl)-triazine<br>  <br>**Sum of 2 non-coloured & unknown by-products, 0.32 area% (overestimated value):**<br>Structures will be similar to ETH50 and to by-product 1; they will be biphenyl-triazine derivatives as well. | 0.1 | <0.1 Dioxane |

| | | | | |
|---|---|---|---|---|
| KoC00050/004.E<br>(=FAT 65'080/B) | 98 ± 2 | **By-product 1, 0.099 area%:**<br>2-chlor-4,6-bis-(biphenyl)-triazine<br>  <br>**Sum of 2 non-coloured & unknown by-products, 0.32 area% (overestimated value):**<br>Structures will be similar to ETH50 and to by-product 1; they will be biphenyl-triazine derivatives as well. | 0.1 | 0.03 (xylene) |

Ref.: A, B (subm I)

For the assessment of ETH, six different batches (Lots) of ETH50 have been used.
The purity was determined by HPLC with an external-standard method, and ranged from 97.2% (w/w) to 98.5% (w/w) expressed as active molecule. The by-product profile of these batches are similar and are in total <1% area. Biphenyls were not detected (detection limit < 0.01%). The content of xylene was determined by GC and ranged from 0.7% to 2%.

Ref.: A, B (subm III)

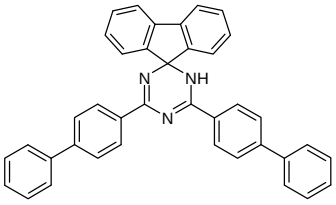
Table 1: Purity of batches used for the toxicological assays

| **Test Substance Denomination** | **Batches (Lots)** | **Measured purity active (% w/w)** |
|---|---|---|
| FAT 65080/D (*)<br>ETH50= Trisbiphenyltriazin | Lot 11103Cl4AA | 98.4 ± 2 |
| | Lot 11104Cl4AA | 98.5 ± 2 |
| | Lot 11105Cl4AA | 98.4 ± 2 |
| | Lot 11106Cl4AA | 98.5 ± 2 |
| | | |
| FAT 65080/E – C-801 [C-801/26 lot 5/50; KRG328-2 (Lot 04122FC7)(micronized, ETH50 (440)) | Lot 11106CL4AA | 98.5 ± 2 |
| | | |
| FAT 65080/F (**)<br>(Lot 04122FC7)(micronized, ETH50 (440)) | Lot 37874FC6 | 97.2 ± 2 |
| | Lot 37875FC6 | 97.4 ± 2 |
| (*) Mixture of 4 batches (Lots) produced within one production campaign according to the same production procedure.<br>(**) Active is a mixture of 2 batches (Lots), produced within one production campaign according to the same production procedure. | | |

## Analytical Profile and by-products in ETH50 (not-micronized and micronized) used for toxicity testing

A revised analytical method using LC-MS for the analysis of ETH50 in plasma samples was developed to give better detection sensitivity and as a result new information became available regarding by-products of ETH50 synthesis present in the various samples of test material used to support the safety dossier. According to the applicant, this by-product is formed during the synthesis process and has been part of each tested batch of ETH50. A recent re-analysis of the ETH50 batches has been conducted; the results for the isomer concentrations are summarized in the following Table 2.

Table 2: By-product in ETH50 used for toxicity testing

| **Test Substance Denominations (Batches)** | **Relative Ratio** (% Isomer as ETH50 peak area) | **By-Product Found** |
|---|---|---|
| FAT 65080/B (KOC0050/004E) | 0.008% | 4',6'-Bis-biphenyl-4''-yl-1'2'-dihydro-spiro[9H-fluorene-9,2'[1,3,5]triazine]  Molecular Weight =537.67<br>Exact Mass =537.22<br>Molecular Formula =C39H27N3 |
| FAT 65080/D (Mixture of 4 Lots: 11103CL4AA; 11104CL4AA; 11105CL4AA; 11106CL4AA) | 0.025% | |
| FAT 65080/E KRG 238.2 Liq. (Lot 11106CL4AA micronized) | 0.017% | |
| FAT 65080/F (Lot 04122FC7 – mixture of Lot 37874FC6 and Lot 37875FC6 ) | <0.003% | |

Ref.: C (subm III)

Particle Size determination and Characterization

Particle size ranges for ETH50 used in studies are summarized in Table 3. In several studies the not-micronized form of ETH50 was also used in separate animal groups as a reference point or bridge to earlier study results; these results provided a comparison for evaluating if the smaller sized particles changed the study's results and outcome.

Table 3: Summary of ETH50 batches and their particle size distributions used in the additional studies submitted

| **FAT 65'080 Suffix Used** | **Batch number** | **Particle size distribution*** | **Comment** |
|---|---|---|---|
| **/B** | KOC00050/004.E (purity 98%) | ♦ 5% < 1.3 µm ,<br>♦ 10% < 2.4 µm<br>♦ 19.49% < 5 µm<br>50 % < 15.4 µm. (MMD) | Not micronized; used for all the studies in Annex1, except the *in vivo* and *in vitro* absorption studies. |
| **/E** | KRG328-2 (concentration 51.4% ETH50) (from LOT11106CL4AA)= MUS/KRG328-2 | d(0.5) 81nm<br>d(0.9) 157 nm | This d(0.5) is a worst case distribution compared to the d(0.5)= 100-110 nm of the commercial product's specifications. |
| **/F** | Lot 04122FC7 (concentration 47.6% ETH50) from ETH50 lot 37874FC6 & lot37875FC6 | d(0.5) 109 nm<br>d(0.9) 175 nm | This d(0.5) is within the expected range for the commercial product's specifications. |
| ***** Particle size distribution measured by FOQELS (Fiber Optic Quasi Elastic Light Scattering) | | | |

Below is an example of a sample image. The majority of the particles in the image are below 100 nm, but also structures larger than 100 nm can be observed.

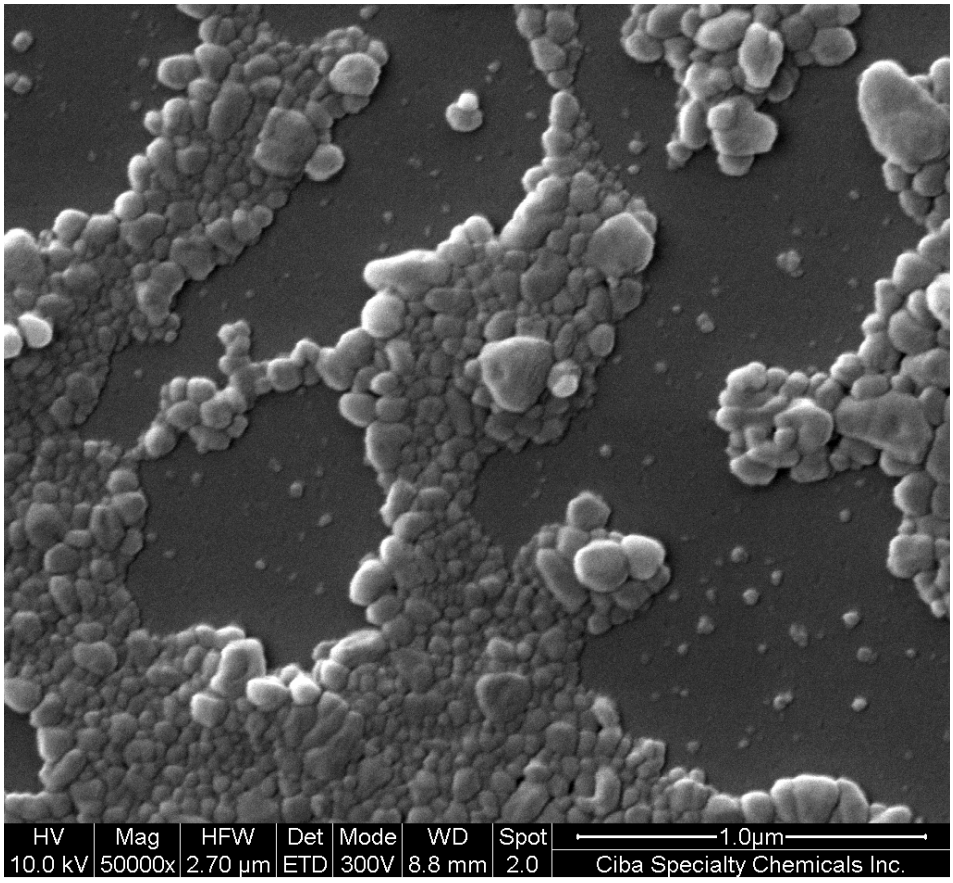



**Figure 1: SEM picture of a sample of Tinosorb A2B (active ingredient) OP Lot 07612FC7**

Particle Dosing Estimation

Physical characterization parameters of ETH-50 are summarized in Table 4.

Table 4: Particle parameters for ETH50

| **Parameter** | **Value*** |
|---|---|
| **Assumptions** | a. 30% concentration of mono-disperse particles of size d(0.5);<br>b. Values are representative for other ETH50 nano-sized dispersions used in toxicology studies. |
| d(0.5) | 8.7E-08 m |
| Surface Area | 2.38E-14 m²/particle |
| Volume | 3.45E-22 m³ |
| Density | 1256 kg/m³ |
| **Number particles per cm³** | **2.9E+15** |
| **Estimated Weight of one particle** | **4.33E-16 g** |
| **Specific Surface Area** | **54.9 m²/g** |
| Values prepared and summarized in appendix to submission III<br>Corrected values are provided in submission VI | |

Calculation of particle dosages or exposures calculated with these parameter values are assumed to be representative of the various batches of nano-sized ETH50 used in the toxicology studies reported herein. This is a conservative assumption because only one batch of test item approximated this median particle size; the other batches had a larger median particle size as summarized above in Table 3.

In the following study summaries, the particle doses are derived from the values shown in Table 4.

### 3.1.5 Solubility

Water solubility (of FAT 65080/B, non-micronized):

< 0.03µg/L at 21°C (OECD 105) insoluble

Ref.: E (subm I)

Other solvents: some examples

Ref.: H (subm I)

| Solvent (INCI Name) | Solubility |
|---|---|
| Cyclomethicone | < 0.001 % |
| $C_{12}$-$C_{15}$ Alkyl Benzoate | 0.029 % |
| Caprylic / Capric Triglyceride | 0.020 % |
| Propyleneglycol | < 0.001 % |
| Mineral Oil | 0.003 % |
| Coco Caprylate / Caprate | 0.019 % |
| Dicaprylyl Carbonate | 0.022 % |

### 3.1.6 Partition coefficient (Log $P_{ow}$)

Log $P_{ow}$ (FAT 65080/B): > 5.6 (calculated from the individual solubility in n-octanol and in water)
Log $P_{ow}$ (FAT 65080/B): 10.4 (calculated by a model calculation)

Ref.: E (subm I)

### 3.1.7 Additional physical and chemical specifications

Appearance: white solid
Melting point: 281.3 °C (OECD 102)
Vapour pressure: $4.15*10^{-21}$ Pa at 25°C**,** (calculated using the Modified Watson Correlation method; OECD 104)
Flammability: not considered as highly flammable
Explosive properties: Based on its chemical structure, FAT 65'080 is not considered as an explosive material.
Note: the physico-chemical specifications above are provided for ETH50 before the micronizing process.

Ref.: C, D, F, G, (subm I)

In the figure below, it is shown that, as the particle size of the ETH50 decreases there is a corresponding increase in the UV absorption by the smaller particles. C-801 is the marketed product.

UV-Spectrum: Absorption in UVA2 and UVB region increases with the decreasing particle size (Figure 2)

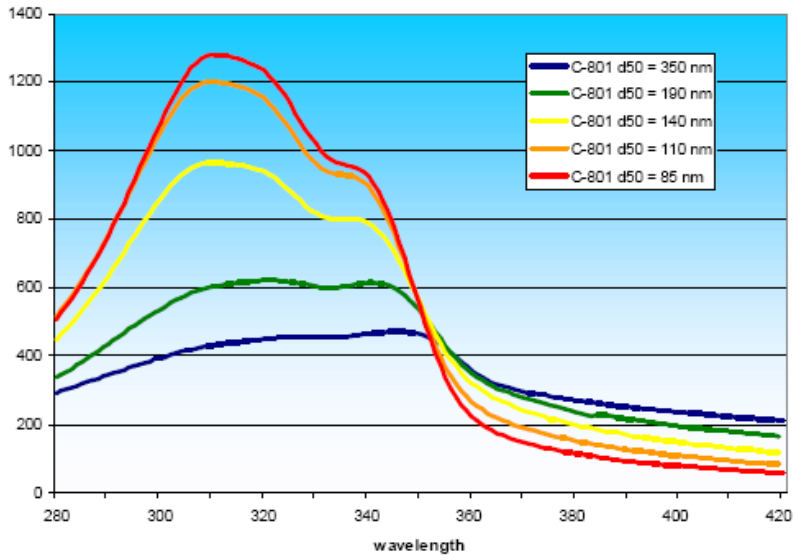



**Figure 2: UV-Spectrum**

Ref.: submission summary (subm V)

### 3.1.8 Stability

The substance suspended in 0.5% carboxymethylcellulose is stable and homogeneous in concentrations of 10 and 200 mg/ml over a 9-day storage period at +4 °C and protected from light.

Ref.: 3 (subm I)

The stability of FAT 65'080 in a cream formulation (FAT 65'082/B) used to test for dermal contact irritation, phototoxic, photoallergic and/or contact allergic reactions with normal topical use in human volunteers has been checked by HPLC- DAD with an external standard calibration method.

Ref.: 19 (subm I)

No significant differences of active content of FAT 65'080 were found without irradiation and after irradiation with 3MED of the sunscreen formulation; FAT 65'080 was therefore found to be stable during this study.

Stability of nanosized particles in commercial formulations: A representative commercial formulation, an oil-in-water type formulation, was prepared with a mixture of UV filters and excipients to deliver an SPF 50 sunscreen lotion.

Figure 3 shows the comparative Absorbance spectra for a sample after 8-months storage at room temperature (referred to as manufacture 1) and for a newly prepared formulation (manufacture 2). The shape of each UV absorption spectrum is similar thereby indicating particle agglomeration is not likely to have occurred. The samples showed good stability and representativeness of the UV absorption profile during the commercial preparation process and, for one sample, after 8-months storage at room temperature.

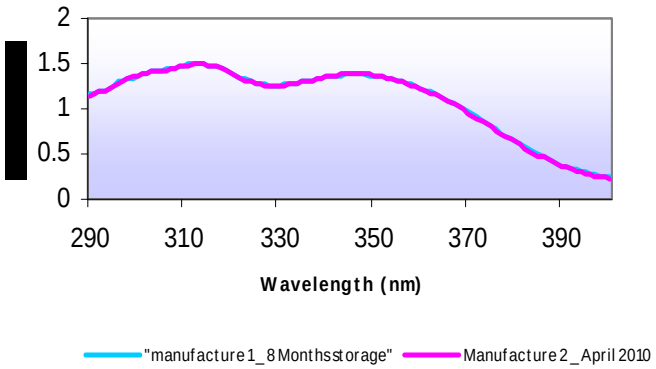



**Figure 3: Comparison absorption spectra of sample UV09071-2-7 "manufacture 1" after 8 month storage & "manufacture 2"**

Ref.: submission summary (subm V)

## 3.2 Function and uses

The substance is an active UV filter, intended to be used at concentrations of up to 10% in sunscreen products as skin protectant against UV-A2 and UV-B rays.

## 3.3 Toxicological Evaluation

The toxicity studies that were initially submitted were carried out with non-micronized material. However, the intended commercial product is micronized to ETH-50 with a d(0.5) of 100-110 nm (in this opinion referred to as nanosized). As the test substance used in toxicological testing should be representative of the marketed material, and moreover a significant fraction of the material is expected to be in the size range for nano-materials (i.e. below 100 nm), studies with nanosized ETH-50 were requested, which were provided in a later submission. In that same submission, additional studies with non-micronized material were provided to directly compare the nanosized and the non-micronized material.

From a safety assessment perspective the studies carried out with the nanosized material are the most relevant, including the studies that were submitted for reasons of comparison between nanosized and non-micronized material. Therefore these studies are described in the main part of the opinion. In Annex I the studies that were performed with the non-micronized and the micron-sized material, are described.

### 3.3.1 Acute toxicity (nano-sized material)

#### 3.3.1.1 Acute oral toxicity (nano-sized material)

| | |
|---|---|
| Guideline: | OECD 423 (2001) |
| Species/strain: | Sprague-Dawley rats |
| Group size: | Group 1: 3 females; Group 2: 3 females |
| Test substance: | FAT 65'080/E |
| Vehicle: | purified water |
| Batch: | KRG328-2 |
| Concentration: | 49.5 % of the active UV filter ETH50 |
| Particle size: | d(0.5) =81 nm |
| Dose level: | 2000 mg/kg bw (both groups) in 0.5% methylcellulose |
| Route: | Oral, gavage, administration volume 10 ml/kg bw |
| Observation: | 14 days |
| GLP: | In compliance |

| Dose in mg/kg bw | Total particles dosed (number) | Surface area of particles dosed ($m^2$) |
|---|---|---|
| **2000** | **$5.8 \times 10^{14}$** | **13.7** |

Corrected values provided in Submission VI.

Mortality and clinical signs of toxicity were not observed in any of the animals. In 2/6 animals, body weight was slightly reduced. The body weight gain of the other animals was not affected by treatment with the test item. At necropsy, no apparent abnormalities were observed in any animals.
Under the conditions of this study the median lethal dose of the test substance after oral dosing was found to be greater than 2000 mg test substance/kg bw for female rats, corresponding to greater than 1000 mg ETH50/kg bw.

Ref.: 1 (subm III)

#### 3.3.1.2 Acute dermal toxicity (nano-sized material)

No data submitted

### 3.3.1.3 Acute inhalation toxicity (nano-sized material)

| | |
|---|---|
| Guideline: | OECD 403 (1981) |
| Species/strain: | Wistar [HanRcc:WIST(SPF)] rats |
| Group size: | 15 males/15 females |
| Test substance: | FAT 65'080/FBatch:LOT04122FC7 |
| Concentration: | 47.6 % of the active UV filter ETH50 (actual concentration in the study: see below) |
| Particle size: | d(0.5)=109 nm |
| Duration: | 4 hours |
| Route: | Inhalation, nose only |
| Observation: | 14 days |
| GLP: | In compliance |

The test item and the placebo were prepared as aqueous dilutions at a ratio (w/w) of 20% FAT 65'080/F or 20% FAT 65'080-placebo plus 80% purified water, the target concentration as active ingredient of UV filter ETH50 was 10%.
Dose level: chemically determined mean aerosol concentration of 4.976 mg of a formulation containing 10% ETH50/L air (s.d. ± 0.513 mg/L air, n = 4), equivalent to a gravimetrically determined mean concentration of 0.602 mg ETH50/L air (s.d. ± 0.059 mg/L air, n = 4). Two gravimetric measurements of particle size distribution during exposure indicated mass median aerodynamic diameters (MMADs) and geometric standard deviations (GSD) of 1.23 µm (GSD 2.13) and 1.27 µm (GSD 2.19).

A group of 15 male and 15 female rats was exposed by nose-only, flow-past inhalation to an aqueous dilution of FAT 65'080/F at a chemically determined mean aerosol concentration of 4.976 mg formulation/L air (s.d. ± 0.513 mg/L air, n = 4), equivalent to a gravimetrically determined mean concentration of 0.602 mg/L air (s.d. ± 0.059 mg/L air, n = 4). Two gravimetric measurements of particle size distribution during exposure indicated mass median aerodynamic diameters (MMADs) of 1.23 µm (GSD 2.13) and 1.27 µm (GSD 2.19) (size of the aerosols). The particle size distribution was determined twice during each exposure using a Mercer 7 stage cascade impactor (Model 02-1300).
An additional group of 15 males and 15 females was exposed to an aqueous dilution of the control item, FAT 65'080 Placebo, at aerosol generation conditions similar to those used for the test item
The first satellite group was sacrificed about 14 hours post end of exposure for broncho-alveolar lavage fluid (BALF) and plasma sampling, the second group was assigned to interim pathology at approximately 24 hours post end of exposure (test day 2), and the third was assigned to pathology at 14 days post exposure (test day 15). All animals were observed for clinical signs and mortality during and following the inhalation exposure, i.e. until interim sacrifice or over a 15-day observation period. Body weights were recorded before exposure on test day 1 in all animals and during the observation period on test days 4, 8 and 15 in all animals assigned to be sacrificed on day 15.
The BALF examinations comprised total and differential cell counts and the determination of total protein, TNF-α and IL-6. In addition, total protein was determined in blood plasma from the animals assigned to BALF sampling. Pathology examinations comprised complete macroscopic pathology, the determination of lung weight and histopathology of the lungs and tracheobronchial lymph nodes on days 2 and 15.

| | MMAD | Median droplet volume $cm^3$ | No Particle a.i./L | Approx. Surface area ($m^2$) / L |
|---|---|---|---|---|
| **Test item** | 1.2 µm | 9.05E-19 | 1.15E+12 | 0.0275 |
| **Placebo** | 1.08 µm | 6.60E-19 | -- | -- |

Results
Clinical signs attributable to treatment with the aqueous placebo dilution or test item dilution did not occur and no premature deaths occurred during the study.

In BALF collected at about 14 hours after end of exposure the total cell count (mainly macrophage and neutrophil numbers), TNFα and total protein were considerably higher in both sexes of test item-treated animals than in placebo control animals, while total protein levels in plasma did not distinguish the two groups. The results are summarized in Table 5 below. The changes in BALF were consistent with the histopathology findings of granulocytic infiltration in alveolar wall and lumen, diffuse alveolar histiocytosis and alveolar lining cell activation seen in all test item-treated animals assigned to interim pathology at approximately 24 hours post end of exposure (test day 2).

**Table 5: BALF Cell Count and Morphology; Cytokines and Protein 24 hours after end of exposure.**

| Summary (mean ± std dev.) | | | | |
|---|---|---|---|---|
| **Parameter** | **Males** (N= 5) | | **Females** (N= 5) | |
| | **Placebo** | **Test Item** | **Placebo** | **Test Item** |
| **Total Cells (millions)** | 8.54 (3.03) | 62.41**(16.29) | 8.67 (0.79) | 34.98**(16.68) |
| **% of Total cells** | | | | |
| **Macrophages** | 95.8 (1.4) | 26.5 (1.3)[a] | 92.5 (3.6) | 36.7** (11.8) |
| **Eosinophils** | 0.2 (0.2) | 0.5 (0.7)[a] | 0.1 (0.1) | 0.3 (0.3) |
| **Lymphocytes** | 0.8 (0.4) | 0.5 (0.7)[a] | 0.4 (0.4) | 0.4 (0.4) |
| **Neutrophils** | 0.9 (0.4) | 71.0 (2.3)[a] | 2.4 (2.4) | 60.8**(12.1) |
| **Other cells** | 0.3 (0.3) | 0.9 (1.3)[a] | 0.3 (0.2) | 0.5 (0.5) |
| **Epithelial cells** | 2.0 (0.7) | 0.6 (0.8)[a] | 4.3 (1.4) | 1.2 (0.6) |
| **TNFα (pg/ml)** | <22.4 | 61 (17.73) | <22.4 | 46.2 (7.8) |
| **IL6 (pg/ml)** | 61[b] | 35; 226[c] | 283; 35[c] | 37; 72[c] |
| **Total Protein (g/l)** | 68.1 (24.38) | 317 (46) | 153 (72) | 243 (80.6) |
| **Plasma Protein (g/l)** | 57.45 (2.33) | 60 (1.61) | 62 (1.2) | 62 (2.04) |
| * / ** t-test based on pooled variance, significant at 5% or 1% level.<br>**a**. N = 2 animals; samples damaged. **b.** 4 rats <24 pg/ml **c.** 3 rats <24 pg/ml | | | | |

Increases of total cell count, TNFα and total protein in BALF and of absolute and relative lung weight, and the histopathology findings of granulocytic infiltration, diffuse alveolar histiocytosis and alveolar lining cell activation seen in the test item group on test day 2 were attributed to the treatment with the test item. Increase in neutrophil numbers in BALF on test day 2 was considered to be indicative of an inflammatory reaction.
The study authors considered the findings noted in test item-treated animals on test day 2 to represent an acute clearance reaction to the lung burden of test item. This is further supported by the absence of these findings by test day 15. This response is well documented for particle exposures to lung (Stone et al. 2007)[1] and the cellular response is not characteristic of immunologic response profiles reflected in the low numbers of lymphocytes at day 2 and their absence histologically at day 15 (Holt, P. et al. 2008)[2].
In the discussion of the study results, the applicant argued that such pulmonary inflammatory responses are not expected with use of ETH50 in sunscreens or other products from spray-on dispensers. Spray applicators are generally pump-type dispensers with some increase in marketed products using fine-spray aerosol type dispensers. In each of these applicators, the droplet sizes (aerosol) are designed to be at least 30 times larger than those used in this rat inhalation test. According to Durand et al. (2007)[3], sunscreen sprayable formulations should be dispensed with droplet size of more than 30 µm (MMAD) with not more than 1% of droplets of aerodynamic diameter at or below 10 µm.

The authors concluded that for nanosized ETH50 the inhalation $LC_{50}$ is greater than the highest technically achievable aerosol concentration level of 4.976 mg formulation/L air, or

1 Stone, V. et al. "Proinflammatory effects of particles on macrophages and epithelial cells." Ch 9 in Particle Toxicology, K. Donaldson & P. Borm editors. CRC Press, Boca Raton, FL 2007
2 Holt et al. Regulation of immunological homeostasis in the respiratory tract. Nature Reviews- Immunology. Vol. 8: 142-152, 2008
3 Durand et al. Influence of different parameters on droplet size and size distribution of sprayable sunscreen emulsions with high concentration of UV-filters. Int J Cosmetic Sci. vol 29: 461-471, 2007

greater than 0.4976 mg ETH50**/**L air, according to this study's results with MMAD of about 1.2 µm. A notable but reversible lung inflammatory response occurred which is considered a normal non-allergenic type response to particle exposures.

Ref.: 5 (subm III)

Comment
The observed effects are similar to those observed upon inhalation of particulate materials in general including some nano-particles. However, the massive influx of neutrophils and the increase of macrophages by approximately a factor 2 cannot be considered to be merely a mild inflammation after inhalation of particles. None of the results in the cited Reference Stone et al. 2007 supports this conclusion. In contrary, these results presented here clearly indicate a strong inflammatory response of the host. The fact that even on day 15 a few parameters differed from those of the controls confirms this strong adverse reaction after inhalation of ETH50 (109).

### 3.3.2 Irritation and corrosivity (nano-sized material)

#### 3.3.2.1 Skin irritation

Data generated with non-micronized test material only, see Annex I

#### 3.3.2.2 Mucous membrane irritation

Data generated with non-micronized test material only, see Annex I

### 3.3.3 Skin sensitisation

Data generated with non-micronized test material only, see Annex I

### 3.3.4 Dermal / percutaneous absorption (nano-sized material)

Additional data available for micron-sized test material ETH50 (440nm), see Annex I

***In vitro* percutaneous penetration study (human and rat skin)**

| | |
|---|---|
| Guideline | OECD 428 |
| Test substance: | Non-radiolabelled FAT 65'080 + [$^{14}$C]-labelled FAT 65'080, nanosized with surfactant (Plantacare®2000 UP), thickener (xanthan gum) and emulsifier (propylene glycol) to mixture of 90.88 mg [$^{14}$C]-labelled FAT 65'080/ml. Specific activity of the mixture: 1.07 µCi/mg. Concentration in the final formulation: 95.64 mg FAT 65' 080/ml. |
| Batch: | Non-radiolabelled: KOC00050/004E<br>Radiolabelled: 49336-1-61 |
| Purity: | Non-radiolabelled: 98%<br>Radiolabelled: >99% |
| Particle size: | d(0.5)= 86 nm (mean of 2 measurements, with values of 92 and 81 nm) |
| Dose applied: | 1.97 mg/cm² in 13 µl for 24 hours |
| Skin preparation: | Full thickness skin from rats and from humans, removed from subcutaneous fat and upper 200 µm from stratum corneal by dermatome. Pieces of 1.8 x 1.8 cm were mounted in flow-through diffusion cells, with 0.64 cm² of skin membrane exposed to the donor chamber. |
| Skin temperature: | ambient |
| Exposure period: | 24 hours |

Donor chamber: non-occluded
Receptor fluid: 6% (w/v) polyethoxyoleate (PEG 20 oleyl ether) in physiological saline (0.9% NaCl w/v). Solubility: 0.16 g FAT 65'080/ml
Control: No control was used
Skin integrity: 50 µL of tritium water was applied to the skin membrane surface in occluded donor chamber
GLP: in compliance

A target dose level of 2 mg/cm² was selected for both rat and human skin based on the topical application rate of final sunscreen formulations assumed to be used by humans. A 13 µL aliquot of the dosing solution, adjusted to pH 6.5 with 20% citric acid solution, was applied manually to each skin membrane preparation using a µL-syringe. The amount applied to each cell was shown to be 1224 µg/cell or 1912 µg/cm² by determination of the radioactivity content of three control doses taken prior to the first, in the middle, and after the last administration for each dose level. The penetration through the skin membranes was determined over a period of 24 hours under non-occluded conditions. The receptor fluid was delivered at a flow rate of about 3 mL/h during the testing period. The perfusate from each cell was collected separately at ambient temperature in 1-hour intervals for the 0 to 6 hour period (6 intervals), and in 2-hour intervals for the remaining exposure period (9 intervals).

Twenty-four hours after application the perfusate sampling was terminated and each skin membrane surface rinsed three times with about 0.5 mL shower gel (1%) in water and then with 0.5 ml tetrahydrofuran for each chamber. All skin membrane rinse fractions were combined according to the individual cells. The skin membranes were stripped until the stratum corneum was removed from the skin membrane, which were 6 to 8 strips per membrane. Up to five consecutive stripping tapes were combined into one specimen. The skin membranes remaining after stripping were digested in Solvable and the radioactivity was determined by LSC. The diffusion cells were then washed with 150 mL chloroform and the radioactivity in the cell wash was determined by LSC.

Results
Based on test item found in perfusate the percutaneous penetration rate in rat and human skin was very low or below reliably quantifiable concentrations (LQ) as shown in the following table.

| | **Rat Skin Membrane** | | **Human Skin Membrane** | |
|---|---|---|---|---|
| **Applied Dose [µg/cm²]** | 1971.7 | | 1912 | |
| **Applied Volume [µL]** | 13 | | 13 | |
| **Application Area [cm²]** | 0.64 | | 0.64 | |
| **Concentration [mg/cm³]** | 97 | | 94 | |
| **Penetration within** | % of dose | µg/cm² | % of dose | µg/cm² |
| **6 h** | < 0.01 | *0.193 | < 0.01 | *0.150 |
| **12 h** | 0.02 | *0.321 | < 0.01 | *0.246 |
| **24 h** | 0.02 | *0.486 | 0.02 | *0.373 |
| **Flux[µg/cm²/h]- measured** | 0.035 | | 0.026 | |
| **Flux[µg/cm²/h]- estimated⁺** | 0.044 | | 0.042 | |

* value calculated from the measured dpm, most of which are below LQ of 0.04 µg-equivalents
\+ estimated by replacing <LQ values with the LQ and calculating flux

Considering the solubility of FAT 65080 in the perfusate (0.16 µg/mL), the flow rate of the perfusate (about 3 mL/h), and the exposed skin membrane area (0.64 cm²) the limit for penetration rate due to the solubility of the test item in the perfusate was estimated to be 0.750 µg/cm²/h. This indicated that the perfusate solubility would not be expected to limit

the movement of test item through the skin. There is no information with regard to whether the penetrated substance is present in the form of particles or as solubilised material.

The estimated Flux for rat skin was 0.044 μg/cm²/h, defined as the penetration rate at steady state between 1-6 hours and calculated by using the corresponding LQ values instead of the measured values, most of which were below LQ. Similarly, the Flux for human skin was estimated to be 0.042 μg/cm²/h for 1-6 hours based on the LQ values.

| **Recovery [% of Dose]*** | | |
|---|---|---|
| **Skin Membrane:** | Rat | Human |
| Applied Dose [μg/cm²] | 1971 | 1912 |
| Perfusates<br>Remaining skin membrane | 0.02 (0.03) #<br>1.36 (1.7) | 0.02 (< 0.01) #<br>0.04 (0.07) |
| **Total absorbed (%)** | **1.38** | **0.06** |
| **As μg a.i./cm²** | **27.2** | **1.2** |
| Skin membrane rinse | 82.73 (8.1) | 94.49 (4.3) |
| Tape strips | 13.46 (4.84) | 3.73 (2.19) |
| Diffusion cell wash | 0.64 (0.16) | 0.66 (0.43) |
| **Recovery** | **98.2** (2.29) | **98.94** (3.04) |
| * values are the mean (± standard deviation)<br># calculated from measured dpm values, most of which were below LQ of about 0.4 μg a.i. equivalents | | |

Mean values for absorbed test item of 1.38% and 0.06% of the applied dose were obtained for rat and human skin, respectively.
The very low absorption and percutaneous penetration of the test item allowed only an estimation of a flux parameter because the majority of the values were below limits of quantification for the samples. The rat skin was somewhat more permeable to the test item and showed larger amounts of test item in the tape strips and the remaining skin compared to human skin, whereas from human skin almost 95% of the applied dose could be removed with surface wipe and membrane rinses. Both test systems showed recovery of more than 98% of applied dose and the test item was shown to remain stable during the 24-hour exposure period.

Based on these results, the test item did not penetrate through the skin membranes to a significant extent.
Given the large variability in the absorption values, the mean value ± 2 SD will be used for the calculation of the MOS. This results in a total absorption of $(1.38+ 2x\sqrt{(0.03^2+1.7^2)} = 4.78$ % for the rat and $(0.06+2x\sqrt{(0.01^2 +0.07^2)}= 0.20\%$ for human skin.

Ref: 2 (subm III)

Comment:
Since most of the measured values were below the LQ, the calculation of the absorption value can be considered conservative.

***In vitro* penetration study with pre-damaged human skin:**

| | |
|---|---|
| Guideline | OECD 428 |
| Test substance: | Non-radiolabelled FAT 65'080 + [$^{14}$C]-labelled FAT 65'080, nanosized with surfactant (Plantacare®2000 UP), thickener (xanthan gum) and emulsifier (propylene glycol). Concentration in final formulation: 95.64 mg [$^{14}$C]-labelled FAT 65'080/ml. Specific activity of the mixture: 1.07 μCi/mg |
| Batch: | Non-radiolabelled: KOC00050/004E<br>Radiolabelled: 49336-1-61 |
| Purity: | Non-radiolabelled: 99.1%<br>Radiolabelled: >99% |
| Particle size: | ($d_{0.5}$) = 120 nm |

Dose applied: 2.05 mg/cm² in 13 µl for 24 hours
Skin preparation: The subcutaneous fat was carefully removed from the full thickness skin and pieces of about 4 x 5 cm² were stretched evenly over a cork block, with stratum corneum uppermost. Before sectioning three consecutive tape strips were performed upon each of the two skin samples. Skin sections of 200 µm thickness were cut off from the top using a dermatome. Pieces of 1.8 x 1.8 cm were mounted in flow-through diffusion cells, with 0.64 cm² of skin membrane exposed to the donor chamber.
Skin temperature: ambient
Exposure period: 24 hours
Donor chamber: non-occluded
Receptor fluid: 6% (w/v) polyethoxyoleate (PEG 20 oleyl ether) in physiological saline (0.9% NaCl w/v).
Control: none
Particle size: d(0.5)= 120 nm
Skin integrity: 50 µL of tritium water was applied to the skin membrane surface in occluded donor chamber
Recovery: 107.15%
GLP: in compliance

Results

The mean particle size of the test item in this study was ($d_{0.5}$) = 120 nm, which is larger than in the first study with normal skin ((d0.5) = 86 nm). The test item was shown to remain stable during the exposure period. Six of 9 human skin membranes demonstrated acceptable permeability coefficients (mean $K_p$= 2.69 $\pm$ 0.19) and were used for the study.
Based on test item found in perfusate the percutaneous penetration rate in pre-damaged human skin was very low or below reliably quantifiable concentrations as shown in the following table.

| | **Pre-damaged Human Skin Membrane** | |
|---|---|---|
| **Applied Dose [µg/cm²]** | 2053 | |
| **Applied Volume [µL]** | 13 | |
| **Application Area [cm²]** | 0.64 | |
| **Concentration [mg/cm³]** | 101 | |
| Penetration within | % of dose | µg/cm² |
| 6h | * 0.75 | * 15.315 |
| 12h | * 0.75 | * 15.483 |
| 24h | * 0.76 | * 15.658 |
| **Flux[µg/cm²/h]- measured*** | **0.281** | |
| * calculated with values below LOQ (~0.05 µg-equivalents) | | |

Considering the solubility of FAT 65080 in the perfusate (0.16 µg/mL), the flow rate of the perfusate (about 3 mL/h), and the exposed skin membrane area (0.64 cm) the limit for penetration rate due to the solubility of the test item in the perfusate was estimated to be 0.750 µg/cm²/h. This indicated the perfusate solubility would not be expected to limit the movement of test item through the skin.
The estimated Flux was 0.281 µg/cm²/h, defined as the penetration rate at steady state between 1-8 hours and calculated by using the measured values, most of which were below LOQ.

| **Recovery [% of Dose]*** | |
|---|---|
| **Skin membrane:** | **Result #** |
| Applied dose [µg/cm²] | 2053 |
| Perfusates | 0.76 (1.67) |
| Remaining skin membrane | 0.05 (0.08) |
| **Total absorbed** | **0.81** |

| **As µg a.i./cm²** | **~15** |
|---|---|
| Skin membrane rinse | 101.14 (16.68) |
| Tape strips | 3.4 (5.21) |
| Diffusion cell wash | 1.79 (3.0) |
| **Recovery** | **107.15 (9.27)** |
| * Values are mean ( ± standard deviation)<br># Calculated from measured dpm values, most of which were below LQ of 0.04-0.05 µg a.i. equivalents. Results for Cell 6 are excluded from calculations | |

The very low absorption and percutaneous penetration of the test item allowed only an estimation of a flux parameter because the majority of the values were below limits of quantification for the samples. As shown, all (101%) of applied was removable in surface wipe and membrane rinses. Test item recovery was complete and the test item was shown to remain stable during the 24-hour exposure period.
Based on these results, the test item with particle mean diameter of 120 nm did not penetrate through the skin membranes to a significant extent and the damaged stratum corneum did not result in a significantly increased penetration rate of the nanosized test item.

Ref.: 1 (subm IV)

### 3.3.4.1 Combined repeated dose toxicity study/ Reproduction/Developmental Toxicity Screening Test (nano-sized material)

**Oral**

Guideline: OECD 422
Species/strain: Sprague-Dawley, Crl CD® (SD) IGS BR rats
Group size: 10 males + 10 females per dose (11 weeks old at start of study)
Test substance: FAT 65'080/F
Batch: LOT04122FC7
Concentration: 47.6 %
Particle size: d(0.5) =109 nm
Dose levels: 100, 500 and 1000 mg ETH50/kg bw/day in purified water at 5 ml/kg bw/day
Route: oral, gavage
Exposure period: from before mating until day 4 post partum during lactation period
GLP: In compliance

| **Test Item Dose as mg a.i./kg bw** | **Total particles dosed (number)** | **No. Particles/kg bw** | **Surface area of particles dosed ($m^2$)** |
|---|---|---|---|
| 100 | 5.8E+13 | 2.31E+14 | 1.37 |
| 500 | 2.8E+14 | 1.15E+15 | 6.86 |
| 1000 | 5.8E+14 | 2.31E+15 | 13.7 |

Daily dosing, at approximately the same time each day, was by gastric intubation as follows:

For the males:
- for 14 days before mating,
- during the 2 weeks mating and the 2 weeks post-mating periods until sacrifice (maximum of 6 weeks in total).

For the females:
- 14 days before mating,
- during the mating period (maximum of 14 days),

- during pregnancy and lactation, until day 4 *post-partum* inclusive, or until sacrifice for un-mated and non-pregnant females.

Day 1 corresponded to the first day of treatment period.

A placebo group was used to identify any effects related to the excipients; accordingly, a mixture of decyl glucoside, silicon defoamer, xantham gum, and butylene glycol was prepared and labelled FAT 65'080/E-placebo. The placebo was diluted in distilled water and administered at a dose of 420 mg/mL, which was equivalent to that of the group receiving the highest dose of test item.

Clinical signs and mortality were checked daily. Body weight and food consumption were recorded weekly. The animals were paired for mating and the dams were allowed to litter and rear their progeny until day 4 *post-partum*. The total litter sizes and numbers of pups of each sex were recorded after birth, pup's clinical signs were recorded daily and pup body weights were recorded on days 1 and 4 *post-partum*. Parameters for pre- and post-implantation loss, mating, fertility, and gestation were recorded. In parent animals from each group, haematology and blood biochemistry investigations were performed in five male and five female animals at terminal sacrifice. In addition, urinalysis was carried out in five males at terminal sacrifice.

The parent males were sacrificed 2 weeks after the end of the mating period. The body weight and principal organ weights (adrenals, brain, epididymis, heart, kidneys, liver, spleen, testes, and thymus) were recorded, a complete macroscopic *post-mortem* examination was performed and selected organs/tissues were preserved. A microscopic examination was performed on selected organs for five males in control and high-dose groups, with particular attention paid to the male gonads for spermatogenesis staging and morphological structure.
The parent females were sacrificed on day 5 *post-partum* (or on day 25 *post-coitum* for females which did not deliver or 24 days after the end of the pairing period for unmated females) and a complete macroscopic examination was performed. A microscopic examination was performed on selected organs of five delivered females in control and high-dose groups.
The litters were sacrificed on day 5 *post-partum* and were carefully examined for gross external abnormalities and a macroscopic *post-mortem* examination was performed.

Results
No test item- related mortality or clinical signs were observed.
Clinical chemistry, haematology, and urinalysis results showed effects in some parameters of the test-item treated groups when compared to the placebo control group; however, when compared to the vehicle control group, these differences were not statistically or biologically significant and were considered as not attributable to the test item treatment.

| **Dose-level (mg/kg bw/day)** | **0 Purified water** | **0 Placebo (a)** | **100** | **500** | **1000 (b)** |
|---|---|---|---|---|---|
| Number of pregnant females | 9 | 7 | 9 | 10 | 9 |
| Number of females surviving delivery | 8 | 7 | 9 | 10 | 9 |
| Mean duration of gestation (days) | 21.1 | 21.1 | 21.3 | 21.3 | 21.2 |
| Mean number of *corpora lutea* | 19.0 | 16.6 | 19.0 | 17.0 | 17.0 |
| Mean number of implantations | 16.9 | 15.7 | 16.6 | 16.0 | 15.6 |
| Mean number of pups delivered | 16.1 | 14.1 | 15.9 | 15.3 | 13.4 |
| Mean number of live pups on day 1 *p.p.* | 15.8 | 14.0 | 15.4 | 15.1 | 13.3 |

| Dose-level (mg/kg bw/day) | 0 Purified water | 0 Placebo (a) | 100 | 500 | 1000 (b) |
|---|---|---|---|---|---|
| The statistical analysis was performed first between placebo controls and purified water controls and then between placebo controls and dose-levels of 100, 500 and 1000 mg/kg bw/day.<br>(a): one female (N27410) excluded due to absence of evidence of mating.<br>(b): one female (N27440) excluded due to unknown male. | | | | | |

Reproductive data evaluation showed that neither the mating nor the fertility parameters were adversely affected by the test item treatment or administration of the placebo. However, in the placebo control females, a slightly low mean number of *corpora lutea*, and slightly low mean number of implantations, pups delivered and live pups on day 1 *post-partum* were obtained. The results for all groups are summarized in the table.
The observation of the pups after birth did not reveal any increased incidence of pups dying, adverse effects on the pup body weight gains or influence on the pups' sex ratio; furthermore, gross malformations were not found in any of the pups.
At the *post-mortem* examinations of the F0 generation parent animals, test item treatment-related macroscopic observations were not revealed. None of the differences in organ weights noted between the placebo and control groups or between the test item-treated and the placebo groups were considered to be of toxicological importance.
Qualitative staging for testis did not indicate any abnormalities in the integrity of the various cell types present within the different stages of the spermatogenic cycle. The oestrous stages were not affected by test item or placebo treatments, and microscopic abnormalities were not revealed in the evaluation of the ovarian follicles and *corpora lutea* or in the evaluation of the uterus.
Overall, nanosized ETH50 did not show adverse effects to the systemic toxicity endpoints, mating and reproduction parameters, offspring survival to 5 days of age, or to grossly and microscopically evaluated organs and tissues.
No Observed Adverse Effect Level is 1000 mg ETH50/kg bw/day, the highest dose tested.
Ref.: 6 (subm III), 2 (subm IV)

### 3.3.4.2 Sub-chronic (90 days) dermal toxicity (nano-sized material)

**Dermal**

| | |
|---|---|
| Guideline: | OECD 411 |
| Species/strain: | Wistar Han Crl: WI (GLX/BRL/Han) IGS BRO |
| Group size: | 10 males + 10 females per dose. Five males and 5 females added to untreated and placebo control and both high-dose groups for treatment-free recovery period. Three males and 3 females were added to each test group for toxicokinetics investigations. |
| Test substance: | FAT 65'080/F |
| Batch: | LOT04122FC7 |
| Concentration: | 47.6% of the active UV filter ETH50 |
| Dose levels: | 150, 500 and 1000 mg ETH50/kg bw/day in a mixture of 80% Base ointment, hydrophilic and 20% of 0.5% carboxymethylcellulose (w/v) in purified water |
| Exposed area: | 10% of the clipped body surface area (45-50 cm² in males, 30- 35 cm² in females according to their age/growth) |
| Route: | dermal |
| Exposure period: | 13 weeks |
| GLP: | In compliance |
| Particle size: | d(0.5)= 109 nm |

The animals were housed individually with free access to food and water.
Based on results of a 14-day dermal dose range finding study dosages for the 13-week study were set at the dose-levels as active ingredient of 150, 500 and 1000 mg of ETH50/kg bw/day; the study design is shown in the following table.

| Group No. | Treatment | | Number of animals | Dose-level[▽] (mg/kg bw/day) | Dose-level[#] (mg/kg bw/day) | Concentration of excipients (mg/mL) | Concentration of Active (mg/mL) |
|---|---|---|---|---|---|---|---|
| 1 | Control (untreated) | Principal | 15 M<br>15 F | - | - | - | |
| 2 | Control (placebo/vehicle)[(a)] | Principal<br>Satellite | 15 M<br>15 F<br>3 M<br>3 F | 0 | 0 | 440 | 0 |
| 3 | Low-dose | Principal<br>Satellite | 10 M<br>10 F<br>3 M<br>3 F | 150 | 315 | 66 | 60 |
| 4 | Mid-dose | Principal<br>Satellite | 10 M<br>10 F<br>3 M<br>3 F | 500 | 1050 | 220 | 200 |
| 5 | High-dose I | Principal<br>Satellite | 15 M<br>15 F<br>3 M<br>3 F | 1000 | 2100 | 440 | 400 |
| 6 | High-dose II (with collar) | Principal<br>Satellite | 15 M<br>15 F<br>3 M<br>3 F | 1000 | 2100 | 440 | 400 |

M: male; F: female.
(a): placebo was diluted in the vehicle at the concentration of 840 mg/mL (taking into account the density).
[▽]: expressed as active component FAT 65'080.
[#]: expressed as test item as received (FAT 65'080/F).
-: untreated control group.

For animals of groups 2 to 5, no dressing or protective plastic collar was used. At least 6 hours after each application the dose site was cleaned using purified water and dried with a cotton pad. Animals of group 6 wore a protective plastic collar for a period of at least 6 hours after each application in order to prevent ingestion of the test item. The collar was removed after each exposure period and the application site was cleaned using purified water and dried with a cotton pad. A constant dosage-volume of 2.5 mL/kg bw/day was used.

| **Mass dosed: mg a.i./kg bw/ day** | **% a.i. applied** | **mg a.i. / cm² on dose site** | **Number a.i. Particles/ kg bw/d** | **Number a.i. particles/ cm²** | **surface area (m²) dosed/cm²** |
|---|---|---|---|---|---|
| 150 | 6 | 1.5 | 3.46E+14 | 3.46E+12 | 0.08 |
| 500 | 20 | 5 | 1.15E+15 | 1.15E+13 | 0.275 |
| 1000 | 40 | 10 | 2.31E+15 | 2.31E+13 | 0.549 |
| 1000 | 40 | 10 | 2.31E+15 | 2.31E+13 | 0.549 |
| Estimated Human exposures | 10 | 0.2 | 6.93E+13 | 4.62E+11 | 0.011 |

Animals of group 1 (untreated control group) received neither treatment nor rinsing but clipping of the application site was conducted as for the other groups. The animals of group 2 (placebo/vehicle control group) received the placebo diluted in the vehicle. The dosage forms were stirred continuously throughout the dosing procedure.

The test item was applied daily for a period of at least 13 weeks (*i.e.* 91 to 92 days according to the necropsy schedule). At the end of the treatment period, the principal animals of each group were humanely sacrificed, except the first five surviving animals of each sex in groups 1, 2, 5 and 6, which were kept for a 2-week treatment-free period. The satellite animals were allocated to toxicokinetics investigations and those animals in groups 5 and 6 were kept for the 2-week treatment-free period.

Results
No effect was seen with regard to mortality, food consumption, ophthalmology, in clinical examination, haematology, blood biochemistry, urinalysis, and in the Functional Observation Battery

Scabs were noted at the application site in about half of the animals in the high dosed group. Clinical signs related to pain, such as abnormal vocalization and/or hyperactivity were observed, mainly from week 5, within the 30-minute period after treatment and generally lasted for less than 30 minutes, in animals given the test item at the high dose-level of 1000 mg/kg bw/day, but mainly in the high-dose group with no protective collar.

In all males given 1000 mg/kg bw/day (groups 5 and 6) a statistically significantly ($p<0.05$) lower mean body weight gain was recorded, principally from week 2 and during the whole study period. This effect was reversed during the treatment-free period. Mean body weight and mean body weight gains for females of both high dose groups (groups 5 and 6) were similar during the full study period.

All group 3 to 6 animals showed quantifiable amounts of the active ingredient of the test item in the plasma during the study period, suggesting a systemic exposure occurred although no time (duration of exposure) or dose-related patterns were demonstrated. The analytic level of quantification was 0.8 ng/ml. Only parent ETH50 was found in the analyzed samples. The results are summarized in the following table.

**Plasma ETH50 Concentrations (ng/ml) in 13-Week Dermal Rat Study**

| | **Male** | | | | | **Female** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group Number | 2 | 3 | 4 | 5 | 6 | 2 | 3 | 4 | 5 | 6 |
| Dosage (mg/kg bw/day) | 0 | 150 | 500 | 1000 High-dose I | 1000 High-dose II | 0 | 150 | 500 | 1000 High-dose I | 1000 High-dose II |
| **Day 8** | 0 | 0.79 | 1.36 | 4.17 | 1.21 | 0 | 0.36 | 5.62 | 3.04 | 1.39 |
| **Week 13** | 0 | 2.39 | 3.48 | 2.36 | 2.47 | 0 | 2.05 | 9.82 | 2.10 | 11.81 |
| **Week 15** (after reversibility) | *n.a* | *n.a* | *n.a* | 1.98 | 0.39[▽] | *n.a* | *n.a* | *n.a* | 0.39 | <0.8[#] |

[▽]: mean concentration with exclusion of the N26873 value (16.8 ng/mL), considered to be aberrant
*n.a*: not applicable.
[#] : missing values for animals N27054 and N27055

Organ weight changes were recorded in the thymus (decrease) of group 5 males, and the adrenal glands (increase) of group 5 females. Although no histopathologic changes were observed in these tissues in rats of this dose group, the changes were considered as non-specific indicators of a stress-related response.

Epidermal hyperplasia and associated hyperkeratosis were noted in rats from groups 2, 5 and 6, which received similar doses of excipients either directly or mixed with test item. These findings were considered likely to reflect mild non-specific irritant-effects related to the mechanical preparation of the application site and repeated treatments with equal excipient concentrations to the application site. Minimal increased lymphocytolysis was recorded in a few rats of groups 5 and 6 (*i.e.* mainly females of group 5). This finding may reflect a minimal non-specific stress-related response related to the treatment procedure.

At the dose-levels of 150 and 500 mg/kg bw/day, no signs of local or systemic toxicity were noted.

Under the experimental conditions of this study, the No Observed Adverse Effect Level (NOAEL) of ETH50 (FAT 65'080) is 1000 mg/kg bw/day as active ingredient given by cutaneous application to rats of the FAT 65'080/F test item form during 13 weeks.

Ref.: 7 (subm III), 3 (subm IV)

Comment
The SCCS considers the decrease in body weight in the high dose group as an adverse effect, and therefore sets the NOAEL of ETH50 at 500 mg/kg bw/day.
The animals were suffering pain, indicated by 'Clinical signs related to pain, such as abnormal vocalization and/or hyperactivity'. It is not clear what the reason for this pain was.
It is remarkable that in all dose groups ETH50 was detected in the blood, also in the high dose group animals that wore a collar. Although there is no clear dose-relationship, there is an indication of dermal and/or oral absorption. Although the levels are low and variable, comparison of the plasma levels after day 8 and week 13, and the low levels still present 2 weeks after the end of exposure (week 15), might suggest accumulation of the substance/particles.

### 3.3.5 Toxicokinetics (nano-sized and non-nanosized material)

**_In vivo_ oral absorption, distribution, and elimination- rat**

| | |
|---|---|
| Guideline | OECD 417 |
| Test substance: | Non-radiolabelled FAT 65'080 + [$^{14}$C]-labelled FAT 65'080, nanosized with surfactant (Plantacare®2000 UP), thickener (xanthan gum) and emulsifier (propylene glycol) to mixture of 90.88 mg [$^{14}$C]-labelled FAT 65'080/ml. specific activity of the mixture: 1.07 µCi/mg. 0.8 mL was diluted in 2.4 mL purified water to prepare the oral gavage mixture dosed at 1.0 mL per animal |
| Batch: | Non-radiolabelled: KOC00050/004E<br>Radiolabelled: 49336-1-61 |
| Purity: | Non-radiolabelled: 98%<br>Radiolabelled: >99% |
| Dose: | 100 mg/kg bw |
| Species/strain: | Rats, HanRcc:WIST (SPF): Wistar rats |
| Group size: | four males |
| Route: | oral, gavage |
| Particle size: | d(0.5)= 86 nm (mean of 2 measurements) |
| GLP: | in compliance |

This study was conducted immediately after the in vitro percutaneous penetration study and used the same test item and dosing mixtures.

| Dose in mg a.i./kg bw | Total particles dosed (number) | No. Particles/kg bw | Surface area of particles dosed ($m^2$) |
|---|---|---|---|
| 113 | 5.22E+13 | 2.61E+14 | 1.24 |
| 117 | 5.40E+13 | 2.70E+14 | 1.28 |

The nominal dose was 100 mg test item per kg body weight.

Urine and faeces samples for each of four 24-hour periods were collected individually and separately per metabolism cage; urine was collected into containers on dry ice, faeces at room temperature and the daily collections stored frozen until analysis. Study termination

at 96-hours after dosing was by $CO_2$ anaesthesia and exsanguinations, during which blood was collected from each animal.

Cages were rinsed separately. Samples of liver, kidney, renal fat, muscle were taken, and as the remaining carcass, analysed for radioactivity.

Results
All 4 animals survived the study period, gained weight, and did not show signs of toxicity or adverse effects. Administered doses ranged from 113 to 117 mg ETH50 /kg bw with mean of 115.5 mg/kg bw and 912.98 kBq/animal.

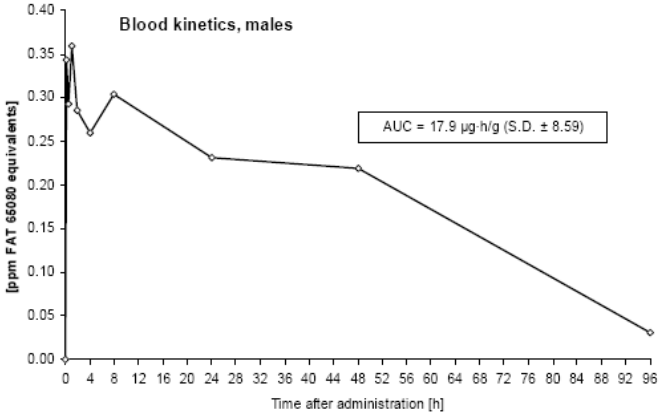



**Figure 4: blood kinetics**

Absorption into systemic circulation based on urinary excretion was 0.06% of applied dose. The maximum concentration level in blood was achieved 1 hour after administration, accounting for 0.360 µg FAT 65080 equivalents/g. This concentration level remained almost constant until 8 hours post dosing. Thereafter the concentration decreased with a terminal half-life of about 31 hours. The AUC values, being an index of bioavailability, were calculated to be 17.9 µg·h/g for blood. Figure 4 shows the time course for the radioactivity administered with the test item.

Blood and plasma did not show differences in distribution of the test item. Radiolabelled test item did not exceed LOQ in any tissue or organ at 96-hours after dosing; the LOQ was about 0.091 µg-equivalents per gram except for fat, which was 0.182 µg-equivalents/g. Only the remaining carcass (LOQ of 0.012 µg-equivalents/g) showed measurable quantities of test item that approximated 0.07 ($\pm$ 0.01)% of the applied dose

| **Excretion** | | |
|---|---|---|
| **Urine** | Time period | [% of dose] |
| | 0 – 24 h | **0.04** |
| | 24 – 48 h | **0.01** |
| | 48 – 72 h | **<0.01** |
| | 72 – 96 h | **<0.01** |
| | *Subtotal* | **0.06** |
| **Faeces** | | |
| | 0 - 24 h | **92.27** |
| | 24 - 48 h | **1.06** |
| | 48 - 72 h | **<0.01** |
| | 72 - 96 h | **<0.01** |
| | *Subtotal* | **93.34** |
| **Cage wash** | | **0.10** |
| **Total excretion** | | **93.49** |

Elimination was almost fully via faeces and accounted for 93.34% of administered dose or 99.8% of the recovered excreted radioactive material. Urine, representing absorbed radioactive material, was 0.06% of the total amount administered.

Total recovery was 93.56% of administered radioactivity during the study period.

The study authors concluded that there was only minimal absorption for the nano sized (d(0.5) = 86 nm) ETH50 from the gastro-intestinal tract.

Ref.: 3 (subm III)

Comment
Although not specifically required by OECD 417, it would have been informative to analyse other major organs like spleen, lung and brain for radio-activity.
The applicant assumed that the absorption was mainly due to impurities present in the radio-labelled product. Although co-labelling of impurities indeed cannot be excluded, no reason was presented by the applicant why impurities should have a preferred higher absorption than ETH-50 itself. Therefore it is assumed that the measured radioactivity is derived from the absorbed radiolabelled active ingredient.

For comparison between the toxicokinetic behaviour of the particles in nano- and non-nano size, also a ADME study in male rats with particles with a mean size of 6 µm has been carried out:

### *In vivo* oral absorption, distribution, and elimination- rat

| | |
|---|---|
| Guideline | OECD 417 |
| Test substance: | FAT 65'080 + [$^{14}$C] labelled FAT 65'080; micronised and filled up with CMC solution (= 0.5% carboxymethylcellulose and 0.4% Tween 80). The final micro suspension had a concentration of 17.4 mg/test item/ml suspension |
| Particle size: | $d_{0.5}$ =6 µm |
| Batch: | Non-radiolabelled: KOC00050/004E<br>Radiolabelled: 49336-1-61 |
| Purity: | Non-radiolabelled: 98%<br>Radiolabelled: >99% |
| Dose: | 100 mg/kg bw |
| Species/strain: | Rats, HanRcc:WIST (SPF): Wistar rats |
| Group size: | 2 groups, group 1 mass balance: 4 rats, group 2 blood kinetics: 9 rats |
| GLP: | in compliance |
| Route: | oral, by gavage |

[$^{14}$C] FAT 65080 (d(0.5) = 6 µm) was administered to male rats at a nominal dose level of 100 mg/kg bw. The excretion of radioactivity in urine and faeces was measured in daily intervals up to 96 hours after administration. The concentration of radioactivity in blood, plasma, liver, kidneys, muscle, and fat was determined 96 hours after administration. The blood and plasma kinetics after oral administration was investigated. Additionally the urinary and faecal metabolite pattern was established.
After oral administration the test item was very poorly absorbed from the gastro intestinal tract into system circulation. The extent of absorption, calculated based on the urinary excretion, accounted for 0.73% of the administered dose. Almost the complete dose was excreted unabsorbed with the faeces as unchanged parent compound, accounting for 97.2 % of dose within 48 hours after administration.
The maximum concentration of radioactivity in blood and plasma was achieved 1 hour after administration, accounting for 2.463 and 4.359 µg FAT 65080 equivalents/g, respectively. This plateau level remained constant until 8 hours post dosing. Thereafter the concentrations in blood and plasma decreased with a half-life (8-48 h) of about 13 hours. The AUC values (0-96 h) were calculated to be 65.2 and 114.3 µg·h/g for blood and plasma, respectively.

The tissue residues, 96 hours after administration, were consequently very low. The highest concentration was found in abdominal fat accounting for 1.712 µg FAT 65080 equivalents/g. All other selected tissues and organs revealed concentrations below 0.110 µg FAT 65080 equivalents/g.
The urinary metabolite pattern investigated revealed 7 metabolite fractions. However, the major fraction (U7) represented only 0.19 % of the dose. All other fractions were below 0.1 % of the dose. The faecal metabolite pattern consisted essentially of unchanged FAT 65080.
In summary, after oral administration of [14C] FAT 65080 only a very low amount of radioactivity was absorbed from the gastrointestinal tract and almost the complete dose was excreted unabsorbed as unchanged parent with the faeces.

Ref.: 4 (subm III)

## 3.3.6 Mutagenicity / Genotoxicity

### 3.3.6.1 Mutagenicity / Genotoxicity *in vivo* (nano-sized and non-nanosized material)

**Unscheduled DNA synthesis in hepatocytes, rat**

| | |
|---|---|
| Guideline: | OECD 486 |
| Species/strains: | Fischer rat from Charles River France (males) |
| Test substance (nanosized): | FAT 65'080/E (49.5% ETH50, batch number KRG328-2, prepared by micronizing ETH50 LOT11106CL4AA in decyl glucoside, xanthan gum, and butylene glycol; subsequent analysis of the test item sample indicated a concentration of 50.6% a.i) particle median diameter d(0.5)= 81 nm. |
| Test substance (non-micronised): | FAT 65'080/B (ETH50 batch No. KOC00050/004.E, purity: 98%); the median particle size, d(0.5) =15.4 µm. |
| Test substance (placebo): | a mixture of decyl glucoside, xantham gum, and butylene glycol |
| Batch: | see above |
| Vehicle: | water |
| Expression times: | 2-4h and 12-16h after dosing |
| Concentrations: | nanosized: 0, 500, 1000, 2000 mg/kg bw; 0, 253, 506, 1012 mg a.i./kg bw |
| Non nanosized: | 2000 mg a.i./kg bw |
| Route: | oral, by gavage |
| Solubility: | Precipitation of the test substance was found from about 500 µg/plate onward |
| GLP: | yes |

| Test Item Dose as mg a.i./kg bw | Total particles dosed (number) | No. Particles/kg bw | Surface area of particles dosed ($m^2$) |
|---|---|---|---|
| 500 | 2.31E+14 | 1.15E+15 | 5.49 |
| 1000 | 4.62E+14 | 2.31E+15 | 10.98 |

Doses were selected based on a preliminary toxicity test to define maximum tolerated doses. Dosing was once by oral gavage at 10 ml/kg bw and each group consisted of 3 males. Positive control substances were dimethylhydrazine at 10 mg/kg bw for the 2-4 hour expression time and 2-acetamidofluorene at 25 mg/kg bw for the 12-16 hour expression time. Blood samples were collected after sacrifice for determination of test item concentrations. Hepatocytes were collected after liver perfusion and removed to culture well-plates for radiolabelling; 12 culture wells per animal were prepared as slides.

Autoradiography was conducted with 6 slides, 6 were held as backup if needed, and where possible 50 cells per slide from 3 slides per animal were evaluated for grain counting classified as nuclear (NC) or cytoplasmic (CC) grain counts, and calculation of net nuclear grains per cell (NNG = NC-CC).

Results

**Results of UDS Assay with ETH50 (FAT 65'080)**

**Assay I: Expression time 12-16 hours**

| 12-16 hours expression time | DOSES in mg/kg | Net Nuclear Grain Count NNG | Net Nuclear Grain Count of cells in repair NNG≥5 | % cells in repair NNG≥5 | % cells in S-Phase |
|---|---|---|---|---|---|
| | | Mean | Mean | Mean | Mean |
| Vehicle control | 0 | -1.65 | 5.65 | 1.49 | 0.14 |
| FAT 65'080 | 2000 | -1.77 | 5.63 | 1.81 | 0.09 |
| | 1000 | -1.83 | 6.19 | 1.78 | 0.14 |
| FAT 65'080/E - Placebo | 1000 | -1.69 | 5.82 | 2.17 | 0.00 |
| FAT 65'080/B | 2000 | -1.78 | 5.59 | 1.55 | 0.16 |

**Assay II: Expression time 2-4 hours**

| 2-4 hours expression time | DOSES in mg/kg | Net Nuclear Grain Count NNG | Net Nuclear Grain Count of cells in repair NNG≥5 | % cells in repair NNG≥5 | % cells in S-Phase |
|---|---|---|---|---|---|
| | | Mean | Mean | Mean | Mean |
| Vehicle control | 0 | -1.37 | 5.53 | 1.38 | 0.07 |
| FAT 65'080 | 2000 | -1.09 | 5.60 | 1.59 | 0.08 |
| | 1000 | -1.28 | 6.08 | 1.69 | 0.09 |
| FAT 65'080/E - Placebo | 1000 | -1.31 | 6.53 | 1.47 | 0.25 |
| FAT 65'080/B | 2000 | -0.92 | 6.12 | 1.98 | 0.10 |

All three tested items (micronised ETH-50, non-micronised ETH-50, placebo) did not cause increased net nuclear grain counts, did not increase the frequency of cells in repair, or induce cellular proliferation as seen in frequency of cells in S-phase. The viability of the hepatocytes was not affected by the in vivo treatment. The positive control items each gave responses indicating a responsive test system.
The genotoxic response from the nano-ETH50 was not different to that of non-nano ETH50. Under the conditions of this study, ETH50, regardless of its particle size, is not mutagenic or genotoxic.

Ref.: 9 (subm III)

Comment
Given the low oral absorption (and dosing by oral gavage), it is not clear whether the hepatocytes have actually been exposed. Therefore, this study is of limited relevance.

3.3.6.2. Mutagenicity / Genotoxicity *in vivo* (nano-sized and non-nano-sized material)

**Bone Marrow Micronucleus test**

| | |
|---|---|
| Guideline: | OECD 474 |
| Species/strains: | Mice, Swiss Ico: OF1 (IOPS Caw) mice |
| Test substance (nano): | FAT 65'080/E. (49.5% ETH50, batch number KRG328-2, prepared by micronizing ETH50 LOT11106CL4AA in decyl |

| | |
|---|---|
| | glucoside, xanthan gum, and butylene glycol; subsequent analysis of the test item sample indicated a concentration of 50.6% ETH50); particle median diameter d(0.5) = 81 nm |
| Test substance (non-nano): | FAT 65'080/B (ETH50 batch No. KOC00050/004.E, purity 98%); median particle size d(0.5) = 15.4 µm. |
| Test substance (placebo): | a mixture of decyl glucoside, xantham gum, and butylene glycol |
| Batch: | see above |
| Vehicle: | water |
| Route: | i.p. |
| Concentrations: | 250, 500 and 1000 mg/kg bw/day active ingredient (nano-sized) and 2000 mg/kg bw/day active ingredient (non nanosized) |
| Solubility: | Precipitation of the test substance was found from about 500 µg/plate onward |
| GLP: | yes |

| Test Item Dose as mg a.i./kg bw | Total particles dosed (number) | No. Particles/kg bw | Surface area of particles dosed ($m^2$) |
|---|---|---|---|
| 250 | 1.73E+14 | 5.77E+14 | 0.41 |
| 500 | 3.46E+14 | 1.15E+15 | 0.82 |
| 1000 | 6.93E+14 | 2.31E+15 | 1.65 |

A preliminary toxicity test was performed to define the dose-levels to be used for the cytogenetic study. In the main study, one group of five males and five females received the positive control test item (Cyclophosphamide) once by oral route at the dose-level of 50 mg/kg bw. Three groups of five males and five females mice were given intraperitoneal administrations of ETH50 (nano), placebo, or ETH 50 (non-nano) at dose levels cited above. The high dose ETH 50 (nano and non-nano) group retained satellite groups of 3 male and 3 female mice for blood sampling after dosing. Blood samples for these determinations were taken from 3 mice per sex at 1 hour (satellite animals) and 24 hours (at terminal sacrifice on 3 out of 8 animals of each sex) after the second treatment. At the time of sacrifice, all the animals were killed by $CO_2$ inhalation. The femurs of the animals were prepared and the slides were scored for the number of the micronucleated polychromatic erythrocytes (MPE) in 2000 polychromatic erythrocytes.

Results
No clinical signs and no mortality occurred in the animals of both sexes either given placebo mix (1000 mg/kg bw/day) or test item at 250 and 500 mg/kg bw/day. At 1000 mg/kg bw/day, the ETH 50 (nano) treated group showed hypoactivity and piloerection. The ETH 50 (non-nano) group at 2000 mg/kg bw/day showed hypoactivity, piloerection and soft faeces.
The ETH50 treated males and females had mean values of MPE and a PE/NE ratio that were equivalent to those of the vehicle control group. Also for either placebo or ETH50 (non nano) treated groups, the mean values of MPE were equivalent to those of the vehicle control group. The PE/NE ratio of females treated with the reference item was significantly lower than that of the vehicle control group. Blood samples showed measurable concentrations of test item, both in the nano and in the non-nano groups, indicating that the bone marrow was exposed to the test item.
Cyclophosphamide induced a significant increase in the frequency of MPE, indicating the sensitivity of the test system under the experimental conditions.
In conclusion: the test item, regardless of its particle size did not induce damage to the chromosomes or the mitotic apparatus of mice bone marrow cells after two i.p. administrations at dose levels of 500-1000 and 2000 mg/kg bw/day. The test item in vivo is not clastogenic or aneuploidic under the conditions of the study.

Ref.: 8 (subm III)

### 3.3.7 13-Week Phototoxicity study in hairless mice (nano-sized material)

| | |
|---|---|
| Guideline: | - (The requirements of the U.S. Food and Drug Administration (FDA) were used as the basis for study design) |
| Species/strains: | Mouse/Crl:SKH1-*hr* (m/f) |
| Test substance (non-nano): | FAT 65'080/E; d(0.5)= 81 nm |
| Vehicle: | Aloe Vera Lotion |
| Route: | dermal |
| Duration | 13 weeks (range finding study) |
| Concentrations: | 0, 25, 50, 100 and 200 mg a.i./g vehicle (0, 80, 160, 325, 650 mg a.i./kg bw/day) |
| GLP: | in compliance |

Eighty four male and eighty four female albino hairless Crl:SKH1-hr mice were randomized to fourteen groups, six mice per sex per group, as outlined in the below study design table.

| Group | Description | Formulation Concentration (mg/g) | UVR Exposure per Week (RBU) | Administration Volume (mcl/mouse) |
|---|---|---|---|---|
| 1 | No Administration | Not Applicable | None | None |
| 2 | Vehicle[a] | 0 | None | 100 |
| 3 | FAT65'080/E | 25 | None | 100 |
| 4 | FAT65'080/E | 50 | None | 100 |
| 5 | FAT65'080/E | 100 | None | 100 |
| 6 | FAT65'080/E | 200 | None | 100 |
| 7 | No Administration | Not Applicable | 600 | None |
| 8 | Vehicle[a] | 0 | 600 | 100 |
| 9 | No Administration | Not Applicable | 1200 | None |
| 10 | Vehicle[a] | 0 | 1200 | 100 |
| 11 | FAT65'080/E | 25 | 1200 | 100 |
| 12 | FAT65'080/E | 50 | 1200 | 100 |
| 13 | FAT65'080/E | 100 | 1200 | 100 |
| 14 | FAT65'080/E | 200 | 1200 | 100 |

[a] Aloe Vera Lotion
Abbreviations : UVR : Ultraviolet Radiation ; RBU : Robertson-Berger Units

The ultraviolet radiation (UVR) source used was a 6.5 kilowatt xenon long arc, water cooled burner vertically-suspended within an octagonal metal frame holding one optical filter on each side. Each filter (15 cm by 15 cm, 1 mm thick; Schott WG 320 doped glass) was held approximately 20 cm from the burner. The racks holding the mouse cages were located approximately 2.25 meters from the UVR source during exposure. Each rack of cages was irradiated through one filter; all racks of cages are irradiated simultaneously from one xenon arc. Each rack of animal cages was monitored by a customized detector that records both intensity and UVR dosage in Robertson-Berger Units (RBU). The RBU is a measure of skin response to UVR; 400 RBU approximates one minimal erythema dose (MED) on previously untanned human skin.
The test item was ETH50 (LOT11106CL4AA) nanosized in decyl glucoside, xanthan gum, and butylenes glycol and labelled as FAT 65'080/E (KRG328-2); after this study was completed the test item sample was again evaluated for purity (50.6% active ingredient) and particle size distribution characterized as d(0.5)=81 nm; d(0.9)=157 nm. Dosages are expressed as active ingredient (a.i.) and summarized in the following table.

| Mg a.i./g dose formulation | % a.i. | mg a.i. / cm² on dose site | Approx. mg a.i./kg bw/ day | Approx. No. A.i. particles/ cm² | Specific surface area (m²)/cm² |
|---|---|---|---|---|---|
| **25** | 2.5 | 0.1 | 80 | 2.3E11 | 0.005 |
| **50** | 5.0 | 0.2 | 160 | 4.6E11 | 0.010 |
| **100** | 10 | 0.4 | 325 | 9.2E11 | 0.020 |
| **200** | 20 | 0.8 | 650 | 18.4E11 | 0.040 |
| **Approximate Human Exposures** | 10 | 0.2 | 60 | 4.6E11 | 0.011 |

The test article formulations and/or vehicle were administered at a dosage volume of 100 ul/mouse and the appropriate mice were irradiated once daily, 5 days per week, for 13 weeks as outlined in the Study Design Tables. Formulations were administered to the back and sides (approximately 25 cm²) of appropriate mice before daily UVR exposure on Monday, Wednesday and Friday and after UVR exposure on Tuesday and Thursday. On Monday, Wednesday and Friday, UVR exposure began no later than 15 minutes after the completion of formulation administration for each group. On Tuesday and Thursday, the duration of time between the completion of UVR exposure and the start of formulation administration for each group of mice was no longer than 15 minutes.

Clinical observations and skin observations were recorded at least once weekly. Erythema, oedema, flaking, or any other abnormal findings were recorded when observed and the intensity of these observations is described in terms of an internationally accepted standard, which is a modified Draize system. Body weights were recorded at least weekly throughout the study. Skinfold thickness for each mouse was measured using a thickness gauge before administration of any formulation (0 week) and in weeks 4, 8 and 13.

After completion of the 13-week dosage period, the mice were sacrificed and a gross necropsy of the thoracic, abdominal, and pelvic viscera was performed. Gross lesions found were retained in neutral buffered 10% formalin for possible histopathological examination. Samples of skin from the site of formulation administration for each mouse (or the equivalent anatomical location for untreated mice) were processed for possible histopathological examination. These histopathological examinations were not performed; the remainder of the carcass was discarded without evaluation.

Results
Test article residue was observed in all groups of male mice, without or with UVR exposure administered the FAT65'080/E formulations, as compared with the group of mice not administered any formulation. This finding is a characteristic of the test article and not considered adverse.

All male and female mice survived to scheduled sacrifice. The only necropsy finding in male mice was a tan mass in one mouse not administered any formulation and exposed to 200 RBU/week of UVR and one male mouse administered 50 mg/g FAT 65'080/E and exposed to 1200 RBU/week of UVR. These findings were considered not related to the UVR exposure or FAT 65'080/E administration. All other tissues appeared normal.

Over the course of the study, no significant differences in group mean body weight occurred across the groups of male or female mice administered the vehicle formulation or the FAT 65'080/E formulations, without or with UVR exposure, as compared with groups not administered any formulation or administered vehicle formulation, without or with UVR exposure. Instances of significant changes (increases and reductions) in group mean body weight occurred, as compared with groups not administered any formulation or administered vehicle formulation, without or with UVR exposure. These changes in group mean body weights were not considered related to formulation administration and/or UVR exposure or biologically important because: 1) the occurrences were not administration

volume-dependent; 2), the changes were both increases and reductions; and/or 3) the occurrences were intermittent.

For male and female mice throughout the study, there were scattered instances of significant changes in mean skinfold thickness changes in both male and female mice administered the formulations, without or with UVR exposure, as compared with groups not administered any formulation without or with UVR exposure. These significant changes were not considered adverse because of the lack of dose and time-dependence and lack of a pattern of response that would indicate a clear association of the test item with this clinical endpoint.

The topical administration of nanosized ETH50 with and without UV radiation did not adversely affect oedema formation, wrinkling, or skin fold thickness in any group of males or females; these endpoints showed an increase in male and female mice treated with the vehicle alone or with UVR alone. This indicates ETH50 in nanosized formulation does not generate alternative toxic forms or increase the adverse effects of UV irradiation and leads to a decreased incidence of markers of UV radiation damage indicative of an efficacious protective effect.

Ref.: 10 (subm III)

### 3.3.8 Human data (nano-sized material)

**Human phototoxicity and photoallergenicity test**

| | |
|---|---|
| Guideline: | / |
| Subjects: | 57 subjects, of which 53 completed the study: 35 women and 18 men, age 19 - 56 years, mean age 35.1 years (SD = 10.9) |
| Test substance: | 1. CD05-123-01 (Cream formulation containing 9.9% FAT 65'082/B)<br>2. CD05-123-02 (Placebo cream)<br>3. CD05-123-03 (FAT 65'082/B Placebo) |
| Batch: | Unknown |
| Concentration: | 9.9% |
| Particle size: | d(0.5)= 90 nm |
| UV radiation: | UVA+UVB from a 150 watt xenon arc solar simulator (Solar Light Company, Philadelphia, PA), equipped with a Schott WG320 UVC blocking filter, a heat-rejecting dichroic mirror and a visible and infrared blocking UG-11 filter. A UVB blocking filter was used for UVA doses. The lamp beam was uniform, as evidenced by uniform erythema across exposed sites, with a continuous spectrum that was free from substantial peaks. Less than 0.01% of total lamp energy was contributed by wavelengths shorter than 290 nm. The irradiation doses were 10 Joules/cm² of UVA followed by 0.5 MEDs of UVA/UVB radiation. |
| Dose levels: | 20 mg in Finn chambers (0.5 cm²) on midback secured with tape. Subsequent application of 2 µl/cm² directly on skin. |
| Route: | topical |
| Observation period: | Immediately, 24±4, 48±4 and 72±4 hours after each application / irradiation |
| GCP: | In compliance |

The design of the study was an open-label, controlled study, consisting of six duplicate, occluded exposures to test products in Finn Chambers, followed by UVR administration to half the sites, over a two-week Induction Phase; a 9-14 day Resting Phase and a single 24 hour application of the test products in a Challenge Phase. In the Challenge Phase, test products were applied to sites not previously treated, half the sites were irradiated with UVR and responses were evaluated 48 and 72 hours after removal of patches.

Thirteen subjects reported 16 adverse experiences, none of which were considered related to the test substance as that were similar to both controls. Based on these results, the study authors considered that the test substance showed no significant potential for phototoxicity or photoallergenicity.

Ref.: 19 (subm I)

### 3.3.9 Special investigations

***Exposure Studies***

The purpose of this investigation was to evaluate the influence of different formulation types and technological parameters such as spray type (propellant or pump spray) or propellant gas concentration on the size and size distribution of droplets in a sunscreen emulsion containing nanosized ETH50 as the only UV filter in the formulation.
Two different types of fluid o/w cosmetic emulsions were prepared containing nanosized ETH50 (mean diameter 124 nm) at a high concentration of 8 and 10%, respectively (formulation names: GEUV10079-1-2 and GEUV10079-2-2). In addition, a third formulation was prepared as a variation of the second. Only Magnesium aluminium silicate was omitted in this emulsion (GEUV10079-2-3).

These formulations were incorporated in aerosol cans with gas (a blend of propane and butane). The propellant was added at a final concentration of 30 and 40%, respectively. The size and the particle size distribution of the droplets were analysed using a Malvern Mastersizer (Malvern, UK). The first and the second formulation were also incorporated into cosmetic pump spray bottles. The type of aerosol valves and spray heads were of typical quality and specification for cosmetic aerosols, e.g. hair sprays (supplier: Precision Company, USA). The particle size was characterized by the mass median diameter d(0.5). The measuring distance of droplets was set at 30 cm, because it was considered as a relevant distance under normal use conditions.

Results
The results of the spraying experiments with the ETH50 formulations are summarized in the table below.

Influence of the formulation type:
Formulation 1 produced larger droplets than formulation 2 and 3 (e.g. the d(0.1) values were about five times higher compared to formulation 2 and 3 for the propellant sprays).

Influence of the propellant concentration:
As it could be expected, 40% propane/butane lead to finer aerosols compared to a concentration of 30% propellant.

Influence of the spray type (pump spray versus propellant spray):
The pump spray containing formulation 1 produced smaller droplets than the propellant spray with the same formulation. As expected, the pump spray containing formulation 2 generated larger droplets compared to the propellant spray.
For both pump sprays, the droplet fraction below 10 µm was below 1%. For the propellant spray with formulation 2 and 3 this fraction was well below 10%.

**Table 6: Droplet sizes for different propellant and pump sprays using laser diffraction (single measurements for pump sprays and propellant sprays 40%, duplicates for propellant sprays 30%)**

| Formulation | o/w Emulsion (GEUV10079-1-2) | | | o/w Emulsion GEUV10079-2-2 | | | o/w Emulsion GEUV10079-2-3 |
|---|---|---|---|---|---|---|---|
| Conc. Butane/Propane | 30% | 40% | 0% Pump-spray | 30% | 40% | 0% Pump spray | 40% |
| Conc. ETH50 in formulation | 8% | 8% | 8% | 10% | 10% | 10% | 10% |
| Mean Diameter (D0.5, volume distribution) | 210/229 µm | 204 µm | 132 µm | 82 / 78 µm | 57 µm | 68 µm | 50/52 µm |
| Mean Diameter (D0.1, volume distribution) | 110/128 µm | 100 µm | 53 µm | 29 / 29 µm | 21 µm | 40 µm | 16/19 µm |
| Max. fraction of droplets below 10 µm | <1% | <1% | <1% | < 10% | < 10% | <1% | < 10% |

Conclusion:
The mean droplet size (d(0.5); volume distribution) for all spray formulations (propellant sprays and pump sprays) using laser diffraction methodology (Malvern Mastersizer) was found to be about 50 µm or above. The droplet fraction below 10 µm was found to be well below 10% (propellant sprays) and 1% (pump sprays), respectively.

Ref.: 2 (subm VI)

The release of aerosols from 3 pressurised ETH50 spray formulations was investigated with a Scanning Mobility Particle Sizer (SMPS) regarding their particle size distribution in the sub-micron range range between 10 and 600 nm. The atomisation process was operated in an enclosed box to get constant conditions and a particle free background. An SMPS equipped with a sample holder for TEM-grids was used in order to sample particle fractions and perform an element analysis by a Transmission Electron Microscope (TEM) and EDX (Energy Dispersive X-Ray)-spectroscopy. A SMPS for online recording of particle distributions was used, and a second CPC to monitor the particle concentration in the glove box during the whole measurement time. A closed environment with minimal particle background is needed to perform reliable spray experiments. The closed glove box setup ensured stable conditions and avoided external influences such as air flow. The setup assured a minimal particle background environment with maximum of 10 particles per $cm^3$ in the measured size range between 10 nm and 600 nm. The particle evacuation after a spray experiment required around 5 minutes of flushing the glove box with nitrogen until reaching acceptable background levels. Before each experiment, the spray cans were shaken 20-times by hand and a 2-minute measurement period with the SMPS followed. The recording time was divided into 1 minute of measurement with spraying followed by 1 minute of measurement without spraying.

The investigated formulations were the same as for the Mastersizer experiments. However, only the 40% propane/butane sprays were tested in the SMPS experiments as they were considered a worst case scenario based on the laser diffraction data.

The first formulation (GEUV10079-1-2) showed particle number concentrations lower than 1000 particles/cm³.
The two other formulations showed particle concentrations above 1·106 particle/cm³. These differences might be explained by the different composition of the three formulations. The third sample (GEUV 10079-2-3) was investigated after the second formulation (GEUV 10079-2-2) with the aim to elucidate the increased particle number concentration seen with the second formulation. The signals detected might have been caused by tiny particles of Magnesium aluminium silicate, which was contained as an ingredient in this formulation. Therefore, the third formulation was prepared without Magnesium aluminium silicate. However, the submicron fraction was still observed with this formulation.

In order to identify the chemical nature of the submicron particles or droplets detected with formulation 2 and 3, two particle size fractions of about 120 nm and 370 nm were then collected on a TEM grid sampler that was connected to the SMPS. The TEM-images and EDX-spectra were taken from the third formulation only, as the composition of the second and the third formulation was very similar except the presence of Magnesium aluminium silicate in the second formulation (GEUV 10079-2-2).

The TEM-grids were prepared in deep frozen sample holders (-180 °C) to avoid a sublimation or vaporisation of low volatile components under high vacuum. The results showed that no organic particles were present on the TEM-grid. ETH50 was not found in the EDX-spectra. Magnesium aluminium silicate was also not identified. The submicron droplets or particles detected by the sensitive SMPS have therefore to be of volatile nature whereas ETH50 has a high molecular weight (MW 538) and a very low volatility (calculated: 4.15x10-21 Pa at 25 °C).
One explanation for the observation of volatile droplets or particles in the submicron range may provide the findings of Chen et al. (1995) who reported that nanoparticles in a range of around 10 nm can be generated through water nucleation in a saturated atmosphere by number concentrations of cN≥1051/cm³ is therefore considered possible. It could be speculated that a coagulation of low volatile nanoparticles (liquid or solid) in the range between 10 nm to 600 nm might have occurred with the o/w sunscreen emulsions, which was then detected by the sensitive SMPS system.

In conclusion: The SMPS results showed that the presence of submicron ETH50 particles or agglomerates could not be detected in any of the spraying experiments. By EDX and TEM analysis, it could be demonstrated that the submicron fraction, which was observed with one of the formulation types, consisted of volatile molecules or particles. They were most likely formed by water/oil nucleation processes during spraying or either could originate from impurities from the cosmetic raw materials used.

Ref.: 3 (subm VI)

Exposure calculations (inhalation exposure):

The applicant has provided an exposure assessment using the ConsExpo model. The current models of ConsExpo has not yet been validated for the use of nanomaterials in spray products. However, the applicant has performed a number of exposure studies in which they have determined relevant exposure parameters for a pump spray and a propellant spray containing nano-sized ETH50.

In addition, an exposure calculation was performed in which it was assumed that particles dry after spraying (thus increasing the number of inhalable particles).

**ConsExpo estimations for pump and propellant sun sprays containing 10% ETH50 in a sunscreen formulation (see ref 1, 4 and 5 (subm VI))**

| Exposure | Pump Spray | Propellant Spray | Propellant Spray (worst case, dried particles) |
|---|---|---|---|
| Mean event concentration (5 minutes) | 0.32 mg/m$^3$ | 1.26 mg/m$^3$ | 5.47 mg/m$^3$ |
| Acute internal dose by inhalation | 0.000607 mg/kg bw | 0.00238 mg/kg bw | 0.0104 mg/kg bw |

The cut-off level for inhalation was set at 15 µm. In the worst case scenario (dried particles) the inhalable fraction (fraction particles <15 µm) was larger compared to the 'normal' situation, therefore the mean event concentration is accordingly higher.

See Annex 2 for the ConsExpo calculations

### 3.3.10 Safety evaluation (including calculation of the MoS)

***Dermal exposure***

ETH50 has low oral bioavailability From the in vivo ADME study with ETH50, particle size d(0.5) = 86 nm, it was shown that absorption after oral exposure was < 1% of the administered dose. Therefore, for a risk assessment based on route-to-route extrapolation, the NOAEL from the oral 13 week rat study has to be re-calculated to an internal dose, which would lead to a MOS value below 100.

The SCCS is of the opinion that in the case of substances with very low bioavailability, and in addition the absence of an effect at the highest dose tested, route-to-route extrapolation is not an appropriate approach and prefers to use the dermal 90 day study for the calculation of the Margin of Safety.

**Comparative MoS calculations for ETH50 based on human skin in vitro study results for nanosized (80 nm) and micronized particle size (440 nm) on normal skin**

| Parameter | ETH50 d(0.5) = 80nm | ETH50 d(0.5) = 440nm |
|---|---|---|
| **Adult Body weight** | 60 kg | 60 kg |
| **Body surface area** | 17.500 cm² | 17.500 cm² |
| **Sunscreen applied** (if at 1 mg/cm²) | 18 g | 18 g |
| **ETH50 applied** (10%) | 1800 mg | 1800 mg |
| **Skin absorption (human)** RCC B236 24 April '07 RCC A00112 August '05 | 0.20% of applied dose | 0.57% of applied dose |
| **Systemic Exposure Dose (human)** | 0.06 mg/kg bw/day | 0.171 mg/kg bw/day |

| | | |
|---|---|---|
| **NOAEL**<br>Rat 13-wk dermal study (CIT 32404 TCR, Aug' 08) | 500 mg/kg bw/day | Not available; applicant used 1000 mg/kg bw/day for study performed with d(0.5)= 15 µm |
| **Skin absorption (rat)**<br>(RCC B23624 April '07) | 4.28% of applied dose | 12.77% of applied dose |
| **Systemic Exposure Dose (rat)** | 21.4 mg/kg bw/day | 127.7 mg/kg bw/day |
| SED Rat / SED Human | **357** | **746** |

Based on the comparison of the internal dose between rat and man, the MoS is 357 for ETH50 with a d(0.5)= 80 nm.
It should be noted that the above calculations are very conservative, in particular with regard to the skin absorption value used. Most values in the dermal absorption assay were below the limit of quantification, but used for the calculation of the penetration. Moreover, the majority of the dose was recovered from the skin compartment, rather than the receptor fluid.

***Exposure via inhalation***

The applicant has provided a calculation of the margin of exposure. The basis for this calculation is an exposure assessment using ConsExpo. The current models of ConsExpo has not yet been validated for the use of nanomaterials in spray products. However, the applicant has performed a number of exposure studies in which relevant exposure parameters for a pump spray and a propellant spray-can have been determined.

**Calculation of Margin of Exposure by comparing the lung burdens in rat and human**

| **Parameter** | **Pump spray** | **Propellant spray** | **Propellant Spray (dried particles)** |
|---|---|---|---|
| Mean event concentration (5 min) | 0.32 mg/$m^3$ | 1.26mg/$m^3$ | 5.47 mg/$m^3$ |
| Human ventilation rate | 33 $m^3$/d= 1.375 $m^3$/h | 33 $m^3$/d= 1.375 $m^3$/h | 33 $m^3$/d= 1.375 $m^3$/h |
| Exposure time consumer | 5 min | 5 min | 5 min |
| Inhaled alveolar amount | 0.32 mg/$m^3$ x 5 min/60 min x 1.375 $m^3$/h = 0.0367 mg | 1.26 mg/$m^3$ x 5 min/60 min x 1.375 $m^3$/h = 0.144 mg | 5.47 mg/$m^3$ x 5 min/60 min x 1.375 $m^3$/h = 0.627 mg |
| Human lung weight | ca. 1 kg | ca. 1 kg | ca. 1 kg |
| Human lung burden (per g lung) | 0.0367 mg/kg lung = 0.0367 µg/g | 0.144 mg/kg lung = 0.144 µg/g | 0.627 mg/kg lung = 0.627 µg/g |
| Rat lung burden (per g lung)<br>(see also chapter 1 A) | 1.17 mg/g | 1.17 mg/g | 1.17 mg/g |
| **Margin of Exposure**<br>(lung burden rat / lung burden human) | 1.17 mg/g/ 0.0367 µg/g<br>**= 31 880** | 1.17 mg/g/ 0.144 µg/g<br>**= 8 125** | 1.17 mg/g/ 0.627 µg/g<br>**= 1866** |

The magnitude of the MoE was calculated in the presented user scenarios by comparing the modelled air concentrations or the internal doses or the lung burdens after the potential inhalation of ETH50. In particular, a comparison of the lung burden gave a MoE of 31880 for pump sprays and 8125 for propellant sprays. If a complete evaporation of the volatile components with a subsequent shrinking of the droplets is assumed, a MoE of 1866 can be calculated for the propellant spray. This MoE is calculated using an effect level (serious lung effects after acute inhalation for 4 hours). It should be noted that a conservative exposure scenario was used (5 minutes exposure, sprayed towards a person (scenario of a hairspray), sprayed in a small room, low air ventilation) and ETH50 was not identified in two measured aerosol fractions.

### 3.3.11 Discussion

This dossier presents toxicology studies for ETH50. ETH50 is intended to be used in commercial preparations in a nanosized form with a mean particle size around 100 nm.

The studies in the first submission, except for the dermal absorption studies, were carried out with a suspension of non-nanosized ETH50. The dermal absorption studies were carried out with so-called 'micronized' ETH50 having a mean particle size of about 440 nm. Upon specific enquiry, it was confirmed that the mean particle size of 'non-micronised' ETH50 was about 15 µm, but in the formulation to which the consumer is to be exposed the mean particle size would be about 80-100 nm.
Subsequently, new studies were submitted, which were carried out with nanosized ETH50 (d(0.5) of around 80-110 nm), equivalent to the commercialised material. These studies comprised an acute oral and inhalation study, a screening repeated dose/ repro/ developmental toxicity study, a dermal penetration study, an oral ADME study, a 13-week dermal toxicity study and genotoxicity studies. In the toxicological assessment, the results of studies with non-nano material will be compared to the studies carried out with the nano-materials.
It should be noted that the risk assessment for ETH50 is based on mass- dose metrics, and not on particle number or surface area. At this moment the most adequate dose metric for hazard and exposure characterisation for nanoparticles is still under discussion. However, as cosmetic ingredients are usually assessed and regulated based on the concentration in the finished product, this dose metric is maintained in this opinion.

*Acute toxicity*
In an acute inhalation study with ETH50 (d(0.5) = 109 nm) no lethality was observed. However, a strong inflammatory response was seen in the lung of exposed animals.
The acute oral toxicity of 49.5% nano-ETH50 d(0.5) = 81 nm was >2000 mg/kg bw (corresponding to >1000 mg ETH50/kg bw).

The oral and the dermal toxicity of non-nanosized ETH50 (d(0.5) = <15.4 µm) was >2000 mg/kg bw.

*Irritation /sensitisation*
ETH50 (d(0.5) = <15.4 µm) does not show irritating properties to the eyes or to the skin. It is not sensitising in an LLNA test. No studies were performed with nanosized ETH50.
Given the very limited systemic exposure, the fact that there are no indications for irritation from the dermal 90 day study carried out in rats with nanosized ETH50, and there were no signs of irritation and photoallergenicity in the human study it is not considered necessary to request new irritation/sensitization studies with nanosized ETH50.

*Dermal absorption*
In an *in vitro* dermal penetration study with nanosized particles (d(0.5)= 86nm), the mean absorption for rat skin was 1.38% or 27.2 µg/cm², and for human skin these values were 0.06% or 1.2 ug/cm². Given the large variability seen in these studies, the mean + 2 SD

were used for the calculation of the MoS. This results in a total absorption of 4.78% for the rat and 0.20% for human skin (it should be realized that these are very conservative values, given the low dose (levels around the limit of quantitation) that could be recovered in the perfusate).
An additional study was performed, in which ETH50 (d(0.5)= 120 nm) was applied to pre-damaged human skin. Again, the concentrations that could be measured were very low (below the limit of quantitation). The mean *in vitro* dermal absorption under these conditions was 0.81% or 15 µg ETH50/cm².
Although under conditions of damaged skin, an increase of dermal absorption can be noted, given the very low levels this is not interpreted as a concern.

The penetration of nanosized ETH50 seemed about a factor 10 higher through damaged skin compared to the penetration through intact skin. However, the results of both tests are rather uncertain, since the majority of the values used for the calculation were below the limit of quantification. For the conventional assessment of UV filters dermal absorption is tested on intact skin only. Given the conservative estimate of the dermal absorption, the uncertainty in the penetration values, the severe damage to the human skin in the in vitro experiment and the fact that in the exposure assessment it is assumed that the total body surface area is exposed, the results of the damaged skin are not taken into account for the calculation of the MoS.

In an *in vitro* study using rat skin and human skin (see annex), dermal absorption of non-nanosized ETH50 (d(0.5)= 440 nm) was higher that the absorption with the nanosized ETH50. Using the mean absorption + 2 SD results in a dermal absorption for the rat of 12.77% and for human skin of 0.57%. It should be noted that these are a very conservative values.

*Repeated dose toxicity*
In a study in which rats were dermally exposed to ETH50 (d(0.5)= 109 nm) for 13 weeks no signs of local or systemic toxicity were noted at dose-levels of 150 and 500 mg ETH50/kg bw/day. In the highest dose group (1000 mg ETH50/kg bw) a decrease in body weight gain was noted. In view of that, the SCCS considers 500 mg ETH50/kg bw/day to be the NOAEL in this study.
In a combined oral repeated dose/repro-/developmental toxicity screening study, no adverse systemic effects were observed after exposure to 1000 mg ETH50/kg bw/day (d(0.5)=109 nm).
In a 90 day oral gavage study (see annex) in rats that were exposed to non-nano sized ETH50, d(0.5) = <15.4 µm, the NOAEL (No Observed Adverse Effect Level) for systemic repeated dose toxicity under the experimental conditions of the study was 1000 mg/kg bw/day.

*Mutagenicity/Genotoxicity*
An UDS test in rat hepatocytes and an in vivo mouse bone marrow assay, using ETH50, d(0.5)= <15.4 µm, and ETH50, d (0.5)= 81 nm, did not show genotoxicity. However, given the low oral absorption (and dosing via gavage), is was not clear whether in the UDS test the cells were adequately exposed.
An Ames test, an in vitro mammalian chromosome aberration assay and a mouse TK locus in vitro mutation assay, carried out with non-nanosized ETH50 d(0.5)=<15.4 µm did not show genotoxicity.
ETH50 (d(0.5)= 15.4 µm) did not result in photo-genotoxic effects in bacteria and in V79 Chinese Hamster cells.
Overall, the data indicate no concern with regard to potential genotoxicity of ETH50.

*Carcinogenicity*
No study available

*Reproductive and developmental toxicity*
In a combined repeated dose/repro-/developmental toxicity screening study, no adverse systemic effects were observed after exposure to 1000 mg ETH50/kg bw/day d(0.5)=109 nm.
In a developmental toxicity study in rats, non-nanosized ETH50 (d (0.5)= 15µm) did not result in maternal or developmental toxicity up to 1000 mg/kg bw/day.

*Toxicokinetics and metabolism*
Two *in vivo* ADE studies were carried out in rats dosed with ETH50, one with a particle size of d(0.5)=87 nm, the other with d(0.5)=6 µm. The study with the 6 µm radiolabelled particles showed a higher absorption percentage of radioactivity, and higher levels of radioactivity in various tissues. However, the observed absorption was low. In the study using nanosized particles, about 0.06% of the dose was recovered in urine, and only the remaining carcass showed ETH50 above the LOQ (levels in carcass were about 0.07% of the applied dose). From the results presented, accumulation cannot completely be excluded, since half life time seems to be longer than 24 h. Also in the 13 week dermal study, an increase in blood levels is observed over time. It is not clear in which organs the particles accumulate. Unfortunately not all organs have been analysed for residues. For the larger particles (d(0.5)=6 um) 0.73% of the dose was recovered in the urine. Radioactivity was recovered in a number of organs at low levels, however only levels in fat (0.01% of the dose) and the remaining carcass (0.25% of the dose) could be reliably quantified.
In a dermal *in vivo* study (particle size d(0.5)=440 nm, see Annex) absorption was estimated to be about 0.15% of the applied dose.

*Phototoxicity*
In a dermal 13 week toxicity study, hairless mice were exposed to dose levels of 80, 160, 325 and 650 mg ETH50/kg bw/day (d(0.5)=81 nm) and were daily exposed to UV light. This dose was about 4 times higher than anticipated human topical dose rates from sunscreen application. No increase in oedema formation, wrinkling, or skin fold thickness was observed, compared to control animals.

In a human study, 57 subjects were exposed (six cycles of application and irradiation) to a cream formulation containing 10% ETH50 (d(0.5)=100 nm) and were irradiated with UV light. Phototoxicity or photosensitization responses did not occur.

*Inhalation*
Inhalation exposure to ETH50 was calculated using ConsExpo. However, this model has not been validated yet for applications with nanosized particulate material. Moreover, only an acute inhalation study (one dose level) was available. From this study and the exposure calculations it could be concluded that there is a relative large margin of exposure between the lung load of the rat and humans. However, no information is available on the effects of ETH50 after repeated exposure to lower doses via inhalation. From other inhalation studies using nano-materials, it is known that the nano-material is not cleared from the lung in the same way as is the case for 'conventional' substances. In addition, the effect of the formulation on the behaviour of the ETH50 particles in the human lung is not known.
Therefore, at the moment, it cannot be concluded that the use of 10% ETH50 in spray formulations is safe, because of the uncertainties associated with the repeated exposure of the lung to low doses of ETH50.

In view of the noted effects in the lung, to ensure the safe use in spray application the following information is needed: a 28-day repeated dose inhalation study, including tissue distribution and systemic toxicity, determination of an LOAEL and NOAEL and possible recovery.

Accumulation of ETH50 in the 13-week dermal study could not be excluded. Given the very low levels (around the limit of quantification), the exaggerated exposure scenario and the

relative large difference in internal dose between man and rat, this is not considered as a concern for ETH50.
However, it is recommended that detailed studies be carried out to characterise the physico-chemical form of the substance (solubilised on in nano form) in tissues and blood, and to investigate possible accumulation in tissues and organs.

The suitability of the currently available toxicological testing methods for nano-materials is still under discussion. For some of the standard methods employed for ETH50, the relevance of the results might be debatable (e.g. *in vivo* UDS test with oral administration, where exposure of the target tissue has not been demonstrated). In addition, the test materials in the assays (*in vitro* and *in vivo*) have not been characterised in the various vehicles and body fluids. Therefore it is not clear to which material (nanoparticles or aggregates/agglomerates or solubilized material) the cells and/or the animals were exposed. However, despite the possible shortcomings of the methodologies, and in view of the very low levels of absorption of ETH50 through dermal route, the SCCS is of the opinion that the submitted data provides sufficient basis for assessment of the safety of ETH50.

It is, however, of note that the potential ecotoxicological impacts of ETH50 when released into the environment have not been considered in this opinion.

## 4. CONCLUSION

1. *Does the SCCS consider the use of 1,3,5-Triazine, 2,4,6-tris[1,1'-biphenyl]-4-yl- in a concentration up to 10% w/w in sunscreen products safe for the consumer?*

The SCCS is of the opinion that:

Dermal exposure to formulations containing the UV-filter ETH50 with a mean particle size of 81 nm, as described in the dossier, results in low absorption of ETH50. Also after oral exposure, absorption of ETH50 is low. No systemic effects are observed after oral or dermal exposure up to 500 mg/kg bw/day. Due to the low bioavailability of ETH50, a risk assessment based on a NOAEL from oral studies and applying route-to-route extrapolation was not considered appropriate. Based on comparison of the internal dose in man and rat (resulting in a MoE of 357) and comparison of the NOAEL in the 13 week dermal study in the rat and the human systemic exposure dose it was concluded that the use of 10% ETH50 can be considered safe for dermal application.

The risk assessment of nanomaterials is evolving. It should be noted that the testing of the substance and the present assessment are based on methodologies initially developed for toxicity testing of substances in non-nano form and current knowledge. From this perspective it is concluded that the use of 10% ETH50 can be considered safe for dermal application. This assessment, however, is not intended to provide a blue-print for future assessments, where depending on the developments in methodology and risk assessment approaches and probable development of nano-specific testing requirements, additional/different data could be required and/or requested on a case-by-case basis.

2. *Does the SCCS foresee any other restrictions to the safe use of 1,3,5-Triazine, 2,4,6-tris[1,1'-biphenyl]-4-yl-?*

At this moment there is too much uncertainty to conclude about safe use of 10% ETH50 in spray applications, because of concerns over possible inhalation exposure.
Therefore, the SCCS concludes that spray products containing ETH-50 cannot be recommended until additional information on safety after repeated inhalation is provided.

## 5. MINORITY OPINION

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**Annex 1: Studies performed with ETH50, mean particle size 15 µm, unless stated otherwise.**

| 3.3.1. | Acute toxicity |
|---|---|

| 3.3.1.1. | Acute oral toxicity |
|---|---|

| | |
|---|---|
| Guideline: | OECD 423 (1996) |
| Species/strain: | Sprague-Dawley rats |
| Group size: | Group 1: 3 males; Group 2: 3 males + 3 females (approx. 8 weeks old at start of study) |
| Test substance: | FAT 65'080/A |
| Batch: | ETH 50/129B |
| Purity: | > 98% |
| Dose level: | 200 and 2000 mg/kg bw (group 1 and 2, respectively), in 0.5% methylcellulose |
| Route: | Oral, gavage, administration volume 10 ml/kg bw |
| Observation: | 14 days |
| GLP: | In compliance |

At 200 mg/kg bw, no clinical signs were observed. At 2000 mg/kg bw, piloerection and dyspnea, together with hypoactivity in females, were observed in all animals on day 1.
When compared to historical control animals, a slight reduced body weight gain was recorded in 2/3 males given 200 mg/kg bw during the first or second week of the study. At 2000 mg/kg bw, a reduced body weight gain was noted in 1/3 females during the second week of the study. The overall body weight gain of the other animals was not affected by the treatment with the test substance.

Under the conditions of this study the median lethal dose of the test substance after oral dosing was found to be greater than 2000 mg/kg bw for male and female rats.

Ref.: 1 (subm I)

| 3.3.1.2. | Acute dermal toxicity |
|---|---|

| | |
|---|---|
| Guideline: | OECD 402 (1987) |
| Species/strain: | Sprague-Dawley rats |
| Group size: | 5 males + 5 females (approx. 8 weeks old at start of study) |
| Test substance: | FAT 65'080/A |
| Batch: | ETH50/129B |
| Purity: | > 98% |
| Dose level: | A single dose of 2000 mg/kg bw placed on a hydrophilic gauze pad, applied to an area of the skin representing approximately 10% of the total body surface, under semi-occlusive dressing and restraining bandage |
| Route: | Topical |
| Exposure period: | 24 hours |
| Observation: | 14 days |
| GLP: | In compliance |

A white coloration of the skin was noted in all animals on day 2. No other cutaneous reactions were recorded during the study.

Under the experimental conditions of this study, the median lethal dose of the test substance after topical dosing is higher than 2000 mg/kg bw in rats. No signs of toxicity were observed at this dose.

Ref.: 2 (subm I)

### 3.3.1.3. Acute inhalation toxicity

No data submitted

### 3.3.2. Irritation and corrosivity

### 3.3.2.1. Skin irritation

| | |
|---|---|
| Guideline: | OECD 404 (1992) |
| Species/strain: | New Zealand White Rabbits |
| Group size: | 3 males (2-4 months of age) |
| Test substance: | FAT 65'080/A |
| Batch: | ETH50/129B |
| Purity: | 98% |
| Dose level: | Single topical application of 0.5 g to intact skin under semi-occlusion for 4 hours |
| Route: | Topical |
| Exposure period: | 4 hours |
| Observation: | 72 hours |
| GLP: | yes |

In the first instance, the test item was applied for periods of 3 minutes and 4 hours to a single male New Zealand White rabbit. Since the test item was not severely irritant on this first animal, it was then applied for 4 hours to two other animals.
A single dose of 500 mg of the test item in its original form was applied to the closely-clipped skin of one flank.
The test item was held in contact with the skin by means of a semi-occlusive dressing. Cutaneous reactions were observed approximately 1 hour, 24, 48 and 72 hours after removal of the dressing.
The mean values of the scores for erythema and oedema were calculated for each animal.

Results
After a 3-minute exposure (one animal), a very slight erythema was observed on days 1 and 2. After a 4-hour exposure (three animals), except for a very slight erythema noted in 1/3 animals one hour after the removal of the dressing, no cutaneous reactions were recorded during the study.
Mean scores over 24, 48 and 72 hours for each animal were 0.0, 0.0 and 0.0 for erythema and 0.0, 0.0 and 0.0 for oedema.

Conclusion
Under the experimental conditions, the test item FAT 65'080/A is non-irritant when applied topically to rabbits.

Ref.: 4 (subm I)

### 3.3.2.2. Mucous membrane irritation

| | |
|---|---|
| Guideline: | OECD 405 (1987) |
| Species/strain: | New Zealand White albino rabbits |
| Group size: | 3 males |
| Test substance: | FAT 65'080 |
| Batch: | ETH50/129B |
| Purity: | > 98% |

| | |
|---|---|
| Dose level: | Single application of 0.1 g of the test article as a powder in the conjunctival sac of the left eye, lower and upper eyelids were held together for about 1 second. 24 hours after application, eye was rinsed |
| Route: | Ocular |
| Exposure period: | 24 hours |
| Observation: | 72 hours |
| GLP: | In compliance |

The test substance was administered as a powder by placing 0.1 g of the powder into the conjunctival sac of the left eye and the lower and upper eyelids were held together for about 1 second. The right eye served as the untreated control. The eyes of the three animals remained unrinsed for approximately 24 hours after instillation of the test article. Indication of pain did not occur in any animal upon instillation of the test article or shortly thereafter. Residual test article was not noted in the treated eyes of any of the animals at the 1-hour observation interval.

A very slight or slight chemosis (grade 1 or 2), a very slight or slight redness of the conjunctiva (grade 1 or 2) and/or a clear discharge, were observed in all animals from day 1 up to day 2 or 3. A slight iritis was also noted in 1/3 animals on day 1. Mean scores calculated for each animal over 24, 48 and 72 hours were 0.3, 0.0 and 0.3 for chemosis, 1.0, 0.3 and 0.0 for redness of the conjunctiva, 0.0, 0.0 and 0.0 for iris lesions and 0.0, 0.0 and 0.0 for corneal opacity.
The substance was slightly irritant to the eye under the conditions of the test

Ref.: 5 (subm I)

### 3.3.3. Skin sensitisation

**Local Lymph Node Assay in mice**

| | |
|---|---|
| Guideline: | OECD 429 (2002) |
| Species/strain: | CBJ/A mouse |
| Group size: | 4 females (age ±8 weeks) |
| Test substance: | FAT 65'080/A |
| Batch: | ETH50/129B |
| Purity: | >98% |
| Dose level: | Positive control: 25% α-hexacinnamaldehyde |
| Vehicle: | propylene glycol (due to unsatisfactory solubility of the test item in acetone/olive oil and in dimethylformamide)<br>Test substance: 0.5, 1.0, 2.5, 5.0 or 10% |
| Application: | Quantity of test formulation applied: 25 µl |
| Treatment scheme: | Test item was applied to the dorsum of both ears daily for 3 consecutive days followed by 2 days without treatment. On days 1, 2 and 3 as well as on day 6 (after sacrifice), thickness of the left ear of each animal of the vehicle and treated groups was measured using a micrometer. Any irritation reaction (erythema and oedema) was recorded in parallel. |
| Preparation procedure: | Termination: On day 6, the mice were injected intravenously with 3H-TdR, and five hours later the mice were sacrificed, and the auricular lymph nodes were excised. |
| Isolation lymph nodes: | Excised and pooled for each experimental group by mechanical disaggregation in Petri dishes with the plunger of a syringe. Cell suspensions were washed with 15 mL of 0.9% NaCl, pellets re-suspended in 0.9% NaCl for numeration of lymphocytes and determination of viability. Centrifuged and precipitated with 5% TCA (+4 °C) overnight. Centrifuged and precipitated with 5% TCA, addition of 3 mL Ultima GoldxR scintillation fluid (Packard). |
| GLP: | In compliance |

This study was based on the design adopted by ICCVAM (Interagency Coordination Committee on the Validation of Alternative Methods, ICCVAM 1999) and ECETOC (Technical Report No. 78 Skin sensitization Testing: Methodological Considerations, Brussels, December 1999), with the addition of the evaluation of local irritation.

A homogeneous dosage form preparation was obtained at the maximum concentration of 10%. No cutaneous reactions and no increase in ear thickness were observed in the animals of the treated groups. A positive lymphoproliferative response (SI >3) was noted at the concentration of 0.5%. However, as no positive response and no dose-response relationship were observed at the highest tested concentrations, this positive response could not be considered as biologically relevant. It is concluded that under these experimental conditions, the test item FAT 65'080/A does not induce delayed contact hypersensitivity in the murine Local Lymph Node Assay.

Ref.: 6 (subm I)

### 3.3.4. Dermal / percutaneous absorption

***In vitro* Percutaneous absorption – rat skin, human cadaver skin ex vivo**

| | |
|---|---|
| Guideline: | OECD 428 (2004) |
| Test substance: | Non-radiolabelled FAT 65'080 + [$^{14}$C]-labelled FAT 65'080, specific activity 2.11 µCi/mg, micronized with surfactant (Plantacare®2000 UP), thickener (xanthan gum) and emulsifier (propylene glycol) to mixture of 90.88 mg [$^{14}$C]-labelled FAT 65'080/ml |
| Particle size: | d(0.5) = 440 nm |
| Batch: | Non-radiolabelled: KOC00050/004E<br>Radiolabelled: 49336-1-61 |
| Purity: | Non-radiolabelled: 98%<br>Radiolabelled: >99% |
| Dose applied: | 2 mg/cm² in 13 µl for 24 hours |
| Skin preparation: | Full thickness skin from rats and from humans, removed from subcutaneous fat and upper 200 µm from stratum corneal by dermatome. Pieces of 1.8 x 1.8 cm were mounted in flow-through diffusion cells, with 0.64 cm² of skin membrane exposed to the donor chamber. |
| Skin temperature: | / |
| Exposure period: | 24 hours |
| Donor chamber: | non-occluded |
| Receptor fluid: | 6% (w/v) polyethoxyoleate (PEG 20 oleyl ether) in physiological saline (0.9% NaCl w/v). Solubility: 0.16 g FAT 65'080/ml |
| Control: | No control was used |
| Skin integrity: | 50 µL of tritium water was applied to the skin membrane surface in occluded donor chamber |
| Recovery: | 95.48 ± 1.49 (rat skin) 91.83 ± 5.38 (human skin) |
| GLP: | in compliance |

The skin membranes were set up in flow-through diffusion cells, the formulated [$^{14}$C] FAT 65080 was applied onto the skin membranes at a finite dose of 13 µL/cm² and the perfusates collected at defined time intervals.
The formulated [$^{14}$C] FAT 65080 was applied onto skin membranes of 200 µm thickness at a concentration of 94 mg/cm³ leading to an area concentration of 1912 µg/cm² (reflecting a concentration of 10 % test item in the final formulation).
For each species 7 replicates were used, one cell with human skin was excluded because of a kp > 2.5X10$^{-3}$ cm/hr. The exposure of the test item was performed under non-occluded conditions over an exposure time of 24 hours. During the exposure period the receptor fluid

(6% (w/v) polyethoxyoleate (PEG 20 oleyl ether) dissolved in physiological saline (0.9% NaCl w/v)) and was collected in hourly intervals between 0-6 hours and thereafter in 2 hours intervals until the end of the experiments. At the end of the experiment the remaining [$^{14}$C] FAT 65080 was removed from the skin membranes by rinsing the skin membranes three times with a mild soap solution. The skin membranes were removed from the diffusion cell and consecutively stripped until the stratum corneum was removed from the skin membrane.

During 24 hours of exposure only 0.12% of the applied dose penetrated through rat skin membranes into perfusate. At the end of exposure the bulk of the applied test item could be washed off from the skin membranes, accounting for 70.57% of the dose. After skin membrane rinsing 20.92% of the dose remained in/on the skin membrane. The major part of this remaining test item was located in the stratum corneum (tape strips), accounting for 15.97% of the dose, and 4.95% of the dose was found in the remaining skin membrane after tape stripping, indicating that the applied test item entered the skin membrane but it did not penetrate through the membrane to a significant extent. The mean flux value was calculated to be 0.209 µg/cm²/h. The flux value was about 3 times lower than the estimated penetration rate limit caused by the very limited solubility of the test item in the perfusate, i.e. 0.750 µg/cm²/h.

For the human skin membrane the penetration of the test item resembles very closely to that observed in rat skin membrane. Within 24 hours of exposure only 0.10% of the applied dose penetrated totally through human skin membranes into perfusate. Also for human skin membranes the bulk of applied test item could be washed off 24 hours after start of exposure, i.e. 73.18% of dose. In stratum corneum 15.38% of the dose was found and 0.18% of the dose was found in the remaining skin membrane after tape stripping, which was significantly lower than observed in rat skin membranes. The mean flux value was calculated to be 0.178 µg/cm²/h.
The amount of test item in lower skin layers was significantly lower in human skin membranes as compared to rat skin membranes. Therefore the total absorption, based on the amount penetrated through the skin membrane (perfusate) and the amount measured in the remaining skin membrane layers after tape stripping, was 5.07% and 0.28% of the applied dose, or 96.9 µg-eq/cm² and 5.4 µg-eq/cm² for rat and human skin membranes, respectively.
Based on the flux values a human/rat ration of 1:1.2 was calculated for dermal penetration.

In conclusion, micronized FAT 65080, applied to rat and human skin membranes, penetrated at an extremely low rate and to a very limited extent through the skin membranes. The penetration through rat split-thickness skin membranes was slightly higher than through human split-thickness skin membranes. Although, the test item entered the skin membrane after exposure, it did not penetrate through the membrane to a significant extent. The penetration into lower skin layers (below stratum corneum) was more pronounced in rat skin membrane.

Ref.: 12

Comment
FAT 65080 was micronized: The mean particle size of the test item was found to be d(0.5) = 440 nm

Table 7 Recovery of radioactivity following application of [$^{14}$C]-FAT 65080 (ETH50) to rat skin membranes (Group Q1, Formulation A1)

| [% of Dose] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Dose applied [µg·cm$^{-2}$] | 1912 | | | | | | | | |
| Cell No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Mean | SD |
| **Dislodged Dose** | | | | | | | | | |
| Membrane Rinse | 50.33 | 59.83 | 57.54 | 85.49 | 62.27 | 86.29 | 92.27 | **70.67** | 16.86 |
| **Perfusates** | | | | | | | | | |
| **0-24 h** | **0.16** | **0.15** | **0.16** | **0.09** | **0.10** | **0.11** | **0.08** | **0.12** | **0.03** |
| **Remaining Dose** | | | | | | | | | |
| Tape Strips I | 25.38 | 25.62 | 29.06 | 6.72 | 13.94 | 8.17 | 2.92 | **15.97** | 10.60 |
| Remaining Skin Membrane | 10.02 | 4.98 | 5.85 | 2.48 | 9.73 | 1.50 | 0.08 | **4.85** | 3.90 |
| Cellwash | 8.65 | 3.49 | 2.30 | 2.21 | 8.26 | 1.94 | 0.19 | **3.86** | 3.29 |
| **Recovery** | **94.63** | **94.07** | **94.89** | **98.99** | **94.31** | **98.01** | **96.63** | **95.48** | 1.49 |

Given the large variation in the data, the dermal in vitro absorption value for the rat is set as the mean absorption + 2SDs. The mean absorption is calculated by adding the concentration in the perfusate plus the concentration in the remaining skin membrane. This results in an dermal absorption for the rat of 0.12+ 4.85 + 2x√( 0.032 + 3.92) = 12.77%. It should be noted that this is a very conservative value, especially since this value is driven by the large dose remaining on the skin membrane.

Table 8 Recovery of radioactivity following application of [$^{14}$C]-FAT 65080 (ETH50) to human skin membranes (Group Q2, Formulation A1)

| [% of Dose] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Dose applied [µg·cm$^{-2}$] | 1912 | | | | | | | |
| Cell No. | 8 | 10 | 11 | 12 | 13 | 14 | Mean | SD |
| **Dislodged Dose** | | | | | | | | |
| Membrane Rinse | 68.92 | 85.49 | 83.00 | 59.76 | 64.42 | 77.49 | **73.18** | 10.41 |
| **Perfusates** | | | | | | | | |
| **0-24 h** | **0.04** | **0.02** | **0.14** | **0.12** | **0.20** | **0.11** | **0.10** | 0.07 |
| **Remaining Dose** | | | | | | | | |
| Tape Strips I | 25.44 | 6.46 | 10.12 | 17.50 | 21.62 | 11.13 | **16.38** | 7.35 |
| Remaining Skin Membrane | 0.31 | < 0.01 | 0.19 | 0.06 | 0.19 | 0.31 | **0.18** | 0.13 |
| Cellwash | 0.20 | 3.13 | 2.82 | 4.77 | 2.42 | 4.62 | **2.99** | 1.67 |
| **Recovery** | **94.91** | **95.11** | **96.28** | **82.21** | **88.84** | **93.86** | **91.83** | 5.38 |

Given the large variation in the data, the dermal in vitro absorption value for the human skin is set as the mean absorption + 2SDs. The mean absorption is calculated by adding the concentration in the perfusate plus the concentration in the remaining skin membrane. This results in an dermal absorption for human skin of 0.10+ 0.18 + 2x√( 0.072 + 0.132) = 0.57%. It should be noted that this is a very conservative value.

### *In vivo* rat dermal absorption study

Guideline: OECD 427
Species/strain: Wistar HanBrl:WIST (SPF) rats
Group size: 4 males per group (one dose, 4 sacrifice time points)
Test substance: Non-radiolabelled FAT 65'080 + [$^{14}$C]-labelled FAT 65'080, specific activity 2.11 µCi/mg, micronized with surfactant (Plantacare®2000 UP), thickener (xanthan gum) and emulsifier (propylene glycol) to mixture of 90.88 mg [$^{14}$C]-labelled FAT 65'080/ml
Batch: Non-radiolabelled: KOC00050/004E

| | |
|---|---|
| | Radiolabelled: 49336-1-61 |
| Purity: | Non-radiolabelled: 98% |
| | Radiolabelled: >99% |
| Particle size: | d(0.5) = 440 nm |
| Dose level: | Single topical application of 2 mg/cm² (nominal) or 1829 µg/cm² (actual) in 200 µl on 10 cm² shaved, intact skin for 6 hours under semi-occlusive conditions |
| Route: | topical |
| Exposure period: | 6 hours |
| Sacrifice time points: | 6, 24, 48, or 72 hours after application |
| Recovery: | 92.76%, 94.36%, 97.00%, 95.11% |
| GLP: | in compliance |

The formulated test item was dermally applied at a nominal dose level of 1829 µg/cm² to a skin area of 10 cm². The dermal absorption of the test item during a 6 hour exposure period was determined. Furthermore, the amount remaining in/on the skin at the application site after washing was determined at three additional time points of 24, 48 and 72 hours after application of the test item in order to estimate the depletion of the dose associated with the application site. The association of the remaining test item in/on the skin at the application site was investigated by skin stripping in order to separate the stratum corneum from the epidermis. Urine and faeces were collected up to 72 hours after administration. Residue levels in blood, plasma, liver and kidneys were also measured up to 72 hours after application.
During 6 hours exposure to formulated [Triazine-U-$^{14}$C] FAT 65080 (ETH50), only 0.11% of the dose was systemically absorbed. The penetration rate was calculated to be 0.3345 µg·cm$^{-}$2·h$^{-}$1.
The highest blood and plasma concentrations were found at the end of exposure (6 h), accounting for 0.1272 and 0.2327 ppm FAT 65080 equivalents, respectively. Thereafter the concentration in blood and plasma decreased reaching the limit of quantification within 48 hours. The dermally absorbed FAT 65080 and/or its radiolabelled metabolites were exclusively present in plasma. The highest residues in liver and kidneys were also found 6 hours after start of exposure, accounting for 0.1812 ppm in liver and 0.1051 ppm in kidneys.
The depletion kinetics of the residues was very similar to that observed in blood.
At the end of exposure period 90-92% of the dose could be dislodged from the application site. After the washing procedure, 6 hours after start of exposure, 2.1-4.4% of the dose remained in/on the treated skin area located almost exclusively in/on the stratum corneum.
Less than 0.1% of the dose was found in the lower skin layers. During the 3 days after exposure the remaining amount of FAT 65080 (ETH50) in/on the treated skin after washing lead only to a very low increase of the systemic absorption. The amount totally absorbed accounted for 0.15% of the applied dose, indicating that the remaining amount of FAT 65080 in/on the skin was not available for dermal absorption.
The systemically absorbed test item was slowly excreted with the urine and the faeces. Within 72 hours 0.05% and 0.03% of the dose were excreted with the urine and faeces, respectively.
Total recovery was 92-97% of the applied radioactivity in each subgroup.

Ref.: 13 (subm I)

### 3.3.5. Repeated dose toxicity

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

No data submitted

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

Oral

| | |
|---|---|
| Guideline: | OECD 408 |
| Species/strain: | Sprague-Dawley, Crl CD® (SD) IGS BR rats |
| Group size: | 10 males + 10 females per dose (6 weeks old at start of study) |
| Test substance: | FAT 65'080, d(0.5) = <15.4 µm |
| Batch: | KOC00050/004.E |
| Purity: | 98% |
| Dose levels: | 50, 250 and 1000 mg/kg bw/day in 0.5% carboxymethylcellulose (w/v) in purified water |
| Route: | oral, gavage |
| Exposure period: | 13 weeks |
| GLP: | In compliance |

Clinical examination revealed piloerection in males treated at 250 or 1000 mg/kg bw/day, which was probably treatment related, but was considered not adverse. The finding was infrequent (1/10 at mid-dose, 3/16 at high-dose) and had always disappeared by week 10.
Other clinical findings included alopecia, differences in body weight and unilateral linear chorioretinopathy, but were considered not treatment related. The changes were minor, occurred at similar incidence in control animals and there was no dose-relationship.

Clinical pathology showed no treatment related effects.

Laboratory investigations revealed slight to moderate differences in white blood cell counts and biochemistry, which were considered not treatment related. Values were within normal ranges and there was no dose-relationship.
On week 6, dose related changes in testosterone, oestrogen and progesterone were observed. The variations were considered to be in relation with oestrous stages (which were not affected by treatment) and therefore to reflect biological variability rather than any treatment-related effect.
There was a dose-related reduction of mean urine volume at 250 (females) or 1000 (females and males) mg/kg bw/day, compared to controls. In the absence of biochemical or histological signs of renal dysfunction, these differences were not considered as biologically relevant.
Due to high inter-individual variations, the changes in white blood cell counts, sexual hormones and urine volumes were not statistically significant.

When measured on day 2 (24 hours after dosing on Day 1) and on weeks 6 and 13 for groups 2, 3 and 4, which received the test item at 50, 250 and 1000 mg/kg bw/day, plasma levels of the test substance were not quantifiable for all animals at any time-point.

When male and female rats were treated with the test substance by daily oral gavage at dose-levels up to 1000 mg/kg bw/day for 13 weeks, followed by a 4-week recovery period, no adverse effects were noted. Therefore, the NOAEL (No Observed Adverse Effect Level) after oral gavage under the experimental conditions of the study was determined to be 1000 mg/kg bw/day.

Ref.: 3 (subm I)

Comment
Piloerection could be a sign of neurotoxicity and, although effects were seen only in a few animals and had disappeared after ten weeks, it should be considered adverse. A functional observation battery was performed (at week 12 only) but this only considers functional effects. The piloerection appears to be an acute effect, and was also seen in the acute oral toxicity study.

### 3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

### 3.3.6. Mutagenicity / Genotoxicity

### 3.3.6.1. Mutagenicity / Genotoxicity in vitro

**Bacterial Reverse Mutation Test**

| | |
|---|---|
| Guideline: | OECD 407 (1997) |
| Species/strains: | Salmonella typhimurium TA98, TA100, TA102, TA1535 and TA1537 and Escherichia coli strain WP2uvrA |
| Test substance: | FAT 65'080, d(0.5) = <15.4 µm |
| Batch: | ETH50/129B |
| Purity: | >98% |
| Replicates: | 2 |
| Concentrations: | 62.5, 125, 250, 500, 1000 µg/plate in DMSO |
| Test conditions: | Direct plate incorporation method with and without metabolic activation (Aroclor-induced rat liver S9-mix) and preincubation method (60 minutes, 37 ºC) with metabolic activation. |
| Solubility: | Precipitation of the test substance was found from about 500 µg/plate onward |
| GLP: | in compliance |

Results:
A moderate to marked precipitate was observed in the test dishes when scoring the revertants at dose-levels ≥ 500 µg/plate.
No toxicity was noted towards all the strains used, both with and without S9 mix. The test item did not induce any significant increase in the number of revertants, both with or without S9 mix, in any of the five strains.
Under the conditions of the test, ETH50 did not show mutagenic activity in the bacterial reverse mutation test with Salmonella typhimurium and Escherichia coli.

Ref.: 14 (subm I)

***In vitro* Mammalian Chromosome Aberration Test**

| | |
|---|---|
| Guideline: | OECD 473 (1997) |
| Species/strains: | Human lymphocytes |
| Test substance: | FAT 65'080, d(0.5) = <15.4 µm |
| Batch: | KOC00050/004.E |
| Purity: | 98.8% |
| Cell system: | Heparinized whole blood from two healthy donors, added with culture medium containing the mitogen phytohemagglutinin and incubated at 37ºC for 48 hours |
| Concentrations: | Vehicle: DMSO<br>1st experiment<br>3 h exposure, 20 h harvest time, with or without S-9-mix: 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250 and 500 µg/mL.<br>2nd experiment:<br>20 h exposure, 20 h harvest time without S-9-mix: 15.6, 31.3, 62.5, 125, 250, 500 µg /mL<br>44 h exposure, 44 h harvest time without S-9-mix: 15.6, 31.3, 62.5, 125, 250, 500 µg /mL |

| | |
|---|---|
| | 3 h exposure, 20 h harvest time with S-9-mix: 15.6, 31.3, 62.5, 125, 250, 500 µg /mL<br>3 h exposure, 44 h harvest time with S-9 mix: 15.6, 31.3, 62.5, 125, 250, 500 µg /mL |
| Test conditions: | see below |
| GLP: | in compliance |

In the first experiment, lymphocyte cultures were exposed to the test or control items (with or without S9 mix) for 3 hours then rinsed. Cells were harvested 20 hours after the beginning of treatment, corresponding to approximately 1.5 normal cell cycles.
The second experiment was performed as follows: without S9 mix, cells were exposed continuously to the test or control items until harvest, with S9 mix, cells were exposed to the test or control items for 3 hours and then rinsed.

Cells were harvested 20 hours and 44 hours after the beginning of treatment, corresponding to approximately 1.5 normal cell cycles and 24 hours later, respectively. One and a half hours before harvest, each culture was treated with a colcemid solution (10 µg/mL) to block cells at the metaphase-stage of mitosis. After hypotonic treatment (KCl 0.075 M), the cells were fixed in a methanol/acetic acid mixture (3/1; v/v), spread on glass slides and stained with Giemsa. All the slides were coded for scoring. The dose-levels of the positive controls were as follows: without S9 mix, Mitomycin C: 3 µg/mL (3 hours of treatment) or 0.2 µg/mL (continuous treatment), with S9 mix, Cyclophosphamide: 12.5 and 25 µg/mL

Results:
In the culture medium, the dose-level of 500 µg/mL showed a moderate precipitate. A slight to moderate precipitate was observed at the end of the treatment period, generally at dose-levels ≥ 250 µg/mL.

Experiments without S9 mix:
Cytotoxicity:
Following the 3-hour treatment, a slight to marked decrease in mitotic index was noted without any clear evidence of a dose relationship (26-62% decrease). Following the 20-hour treatment, a slight decrease in mitotic index was noted at 500 µg/mL (33% decrease). Following the 44-hour treatment, no decrease in mitotic index was noted at any dose-level.

Metaphase analysis:
No noteworthy increase in numerical aberrations was noted throughout the study.
No significant increase in the frequency of cells with structural chromosomal aberrations was noted after 3-, 20- as well as 44-hour treatments.

Experiments with S9 mix:
Cytotoxicity:
At the 20-hour harvest time in the first experiment, a slight to moderate decrease in mitotic index was noted without any clear evidence of a dose relationship (26-43% decrease). At the 20-hour harvest time in the second experiment, a moderate decrease in mitotic index was noted at 500 µg/mL (45% decrease). At the 44-hour harvest time, a slight to moderate decrease in mitotic index was noted without any clear evidence of a dose relationship (25-39% decrease).

Metaphase analysis:
No noteworthy increase in numerical aberrations was noted throughout the study.
No significant increase in the frequency of cells with structural chromosomal aberrations was noted in either experiments and at either harvest times.

In conclusion: Under the conditions of the test, ETH50 did not induce chromosome aberrations in cultured human lymphocytes. No noteworthy increase in numerical aberrations was noted throughout the study.

Ref.: 15 (subm I)

**Mouse lymphoma (TK locus) *in vitro* mutation**

| | |
|---|---|
| Guideline: | OECD 476 (1997) |
| Species/strains: | mouse lymphoma L5187Y cells |
| Test substance: | FAT 65'080, d(0.5) = <15.4 µm |
| Batch: | KOC00050/004.E |
| Purity: | 98.8% |
| Concentrations: | |
| | 1st experiment<br>3 h exposure, 15.6, 31.3, 62.5, 125, 250 and 500 µg/mL with or without S9-mix<br>2nd experiment<br>24 hour exposure: 6.25, 12.5, 25, 50, 100, and 200 µg/mL, without S9-mix<br>3 h exposure: 6.25, 12.5, 25, 50, 100, and 200 µg/mL, with S9-mix |
| Vehicle: | DMSO |
| Positive controls: | -S9:methylmethane sulfonate (MMS); +S9: cyclophosphamide (CPA) |
| GLP: | in compliance |

For the 3-hour treatment, approximately 0.5 x 106 cells/mL in 20 mL culture medium were exposed to the test or control items, at 37 °C. For the 24-hour treatment, approximately 0.15 x 106 cells/mL in 20 mL culture medium (RPMI 5) were exposed to the test or control items, at 37 °C.

The numbers of mutant clones (differentiating small and large colonies) were checked after the expression of the mutant phenotype.

The cloning efficiencies CE2 and the mutation frequencies of the vehicle and positive controls met the study acceptance criteria and it was therefore considered valid.

Since the test item was poorly soluble and non-toxic in the preliminary assay, the highest dose-level used for treatment in the main test was limited by precipitate in the culture medium.

Experiments without S9 mix: The selected treatment-levels were:
- 15.6, 31.3, 62.5, 125, 250 and 500 µg/mL, for the first experiment (3-hour treatment), and
- 6.25, 12.5, 25, 50, 100 and 200 µg/mL for the second experiment (24-hour treatment).

A slight to marked precipitate was observed at the end of the treatment period at dose-levels ≥ 50 µg/mL. No noteworthy toxicity was noted at any dose-level, in either experiment. The test item did not induce any significant increase in the mutation frequency in either experiment. The ratio of small and large colonies was not adversely affected.

Experiments with S9 mix: The selected treatment-levels were (3-hour treatment)
- 15.6, 31.3, 62.5, 125, 250 and 500 µg/mL, for the first experiment, and
- 6.25, 12.5, 25, 50, 100 and 200 µg/mL, for the second experiment.

A slight to marked precipitate was observed at the end of the treatment period at dose-levels ≥ 50 µg/mL. No noteworthy toxicity was noted at any dose-level, in either

experiment. The test item did not induce any significant increase in the mutation frequency in either experiment.
Under the experimental conditions, ETH50 did not show any mutagenic activity in the mouse lymphoma assay.

Ref.: 16 (subm I)

### 3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

No data submitted

### 3.3.7. Carcinogenicity

No data submitted

### 3.3.8. Reproductive toxicity

### 3.3.8.1. Two generation reproduction toxicity

No data submitted

### 3.3.8.2. Teratogenicity

**Prenatal Developmental Toxicity Study in Sprague-Dawley Rats - Oral Administration (Gavage)**

| | |
|---|---|
| Guideline: | OECD 414 |
| Species/strain: | Sprague-Dawley, Crl CD® (SD) IGS BR |
| Group size: | 24 mated females per dose (10-11 weeks old at beginning of treatment period) |
| Test substance: | FAT 65'080, d(0.5) = <15.4 µm |
| Batch: | KOC00050/004.E |
| Purity: | 98% |
| Dose level: | 0, 100, 300, 1000 mg/kg bw/d in 0.5% carboxymethylcellulose in purified water |
| Route: | Oral (gavage, 5 ml/kg bw/d) |
| Exposure period: | Day 6 - 19 p.c. |
| GLP: | In compliance |

The test substance administered daily by gavage to pregnant Sprague-Dawley rats from day 6 to day 19 p.c. at dose-levels of 100, 300 or 1000 mg/kg bw/day did not elicit any signs of maternal toxicity.

At 1000 mg/kg bw/day, one foetus presented a mandibular micrognathia associated with soft tissue malformations: microglossia and dilated cerebra. As only one foetus was affected, even though it was in the high-dose group, it was considered not to be related to treatment.
At 300 mg/kg bw/day, two foetuses also presented soft tissue malformations: dilated renal pelvis and/or dilated ureter. As this also occurred in the control group, at higher incidence, this was considered not to be related to treatment.
Some skeletal variations were observed, but these were minor, not dose-related and didn't reach statistical significance and/or were not observed at the high dose-level; they were consequently considered not to be treatment-related.

Consequently, under these experimental conditions, the No Observed Effect Level (NOEL) for both the maternal and the prenatal developmental toxicity is set at 1000 mg/kg bw/day.

Ref.: 11 (subm I)

### 3.3.9. Toxicokinetics

No data submitted

### 3.3.10. Photo-induced toxicity

#### 3.3.10.1. Phototoxicity / photoirritation and photosensitisation

**Phototoxic and photoallergenic potential by cutaneous route in guinea pigs**

| | |
|---|---|
| Guideline: | / |
| Species/strain: | Male guinea pigs, Hartley Crl: (HA) BR |
| Group size: | 5 or 10 animals per group |
| Test substance: | FAT 65'080/A, d(0.5) < 15um |
| Batch: | - Study plan: none<br>- Labelling: ETH 50/129B |
| Purity: | > 98% |
| UV irradiation: | Toxicotronic 312/365 nm (Vilbert/Lourmat, Marne-la-Vallée, France). The lamp consists of two groups of three fluorescent tubes producing either UV A (365 nm) or UV B (312 nm). The irradiation was performed in two stages, first irradiation with UV B and then irradiation with UV A, at an infra-erythematogenic irradiation dose (score of erythema ≤ 0.5). The irradiation doses were approximately 9 joules/cm² for UV A and 0.1 joule/cm² for UV B. |
| Dose level: | 0.1 ml of the test substance in propylene glycol at the concentration of 10% (w/w) on area of 9 cm² (interscapular region). |
| Groups: | Group 1: irradiation control group (5 animals)<br>Group 2: test item control group (five animals)<br>Group 3: test group (ten animals)<br>Group 4: vehicle control group (five animals) |
| Route: | Topical |
| Observation period: | 1, 4, 24 and 48 hours after the challenge application and/or irradiation |
| GLP: | In compliance |

The design of the study was based on the method published by Unkovic et al., Sci. Tech. Ani. Lab 8-3: 149-160 (1983).

The photoallergenic potential of the test item was assessed as follows:

- during an induction period of 8 days, six topical applications and/or UV A+UV B irradiation (including that for phototoxic potential assessment) were performed on the anterior scapular area of animals of all groups,
- during a rest period of 20 days, the animals received no treatment and no irradiation,
- on day 29, a challenge phase was performed by topical application and/or irradiation to the posterior area of the right (UV A) and left (UV B) flanks of the animals.

For each treatment, a dose-volume of 0.1 mL of the test item at the concentration of 10% (w/w) in propylene glycol was applied by cutaneous route. The irradiation doses of UV A and UV B were infra-erythematogenic. The cutaneous reactions were evaluated at the treatment sites.
At the end of the study, animals were killed without examination of internal organs. No skin samples were taken from the challenge application sites.

No clinical signs and no deaths were noted during the study. The body weight gain of the treated animals was similar to that of the control animals.

Phototoxic potential
The cutaneous reactions observed at the 1-, 4- and 24-hour readings were questionable to moderate erythema and were of similar incidence in control and treated groups. No cutaneous reactions which could be attributed to a photoirritant effect of the test substance were recorded.

Photoallergenic potential
The cutaneous reactions observed at the 1-, 4-, 24- and 48 hour readings remained in the range of a local reaction at an infra-erythematogenic irradiation dose (questionable to discrete) and were of similar incidence in the control and treated groups. No cutaneous reactions which could be attributed to a photoallergenic effect of the test substance were observed.

Ref.: 7 (subm I)

Comment
Under the experimental conditions, two very specific wavelengths of UV radiation were used without information of the absorption spectra of the substance. Broadband UVA and UVB irradiation would more appropriately mimic the intended use of this cosmetic UV-filter.

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

**Photo-mutagenicity in Bacteria**

| | |
|---|---|
| Guideline: | |
| Species/strains: | Salmonella typhimurium TA 1537, TA98, TA100 and TA 102 |
| Test substance: | FAT 65'080, d(0.5) = <15.4 µm |
| Batch: | KOC00050/004.E |
| Purity: | 98% |
| Replicates: | 3 plates per test |
| Concentrations: | 33 – 5000 µg/plate (in acetone) |
| UV irradiation: | Source of light: Xenon-lamp (Sunset CPS, ATLAS) emitting a continuous spectrum of simulated sunlight. UV dose: |
| GLP: | |

The test material FAT 65'080 (batch No. KOC00050/004.E; purity 98%) was dosed as a suspension in acetone. The irradiation was performed with a Xenon-lamp (Sunset CPS, ATLAS, D-63558 Gelnhausen) that emits a continuous spectrum of simulated sunlight. The intensity of irradiation was 0.1 – 0.3 mW/cm² and each bacterial strain received its respective amount of tolerable UVA and UVB exposure.
The assay was performed in two independent experiments. Each concentration, including the controls, was tested in triplicate. Metabolic activation with rat S9 was also applied in each experiment. Based on absence of cytotoxicity in the preliminary experiment the test item was dosed at the following concentrations: 33, 100, 333, 1000, 2500, or 5000 µg/plate No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation. The plates incubated with the test item showed normal background growth up to 5000 µg/plate in all strains used. No substantial increase in revertant colony numbers of any of the four tester strains was observed following treatment with FAT 65´080/B under irradiation with artificial sunlight at any dose level. The test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.
Under the conditions of the test, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Ref.: 17 (subm I)

**Photo-clastogenicity assay with V79 Chinese Hamster cells in vitro**

| | |
|---|---|
| Guideline: | OECD 473 |
| Species/strains: | V79 cells derived from Chinese Hamster |
| Test substance: | FAT65'080, d(0.5) = <15.4 µm |
| Batch: | KOC00050/004.E |
| Purity: | 98% |
| Concentrations: | 1.3 – 15 µg/mL in tetrahydrofuran |
| Cell system: | |
| Light source: | Xenon-lamp (Suntest CPS, ATLAS) with an additional special filter glass emitting visible UVA/UVB light > 290 nm |
| UV doses: | 125 mJ/cm² UVA (Exp. I and II) or 200 mJ/cm² UVA (Exp. II) |
| Positive controls: | without irradiation: EMS; with irradiation: 8-MOP |

The light source was an Atlas Suntest CPS, a xenon burner with an additional special filter glass, emitting visible light and UVA/UVB light (ratio: about 30:1) > 290 nm. Cultures were pre-incubated with the test item for 30 minutes where after the cultures were exposed to 125 mJ/cm² UVA (Exp. I and II) or 200 mJ/cm² UVA (Exp. II). Three hours after start of treatment, the cultures were washed. Corresponding cultures with the test item were kept in the dark for the 3 hrs exposure period. The chromosomes were prepared 18 hrs (Exp. I) and 28 hrs (Exp. II) after start of treatment with the test item.

In the cytogenetic experiments for each experimental group two parallel cultures were set up. Per culture at least 100 metaphase plates were scored for structural chromosome aberrations.

Dose selection for the cytogenetic experiments was performed considering the occurrence of test item precipitation and included test item concentrations between 0.12 and 15 µg/mL (with and without irradiation). For both experiments the concentrations used were 1.3 up to 15 µg/ml and due to precipitation, the endpoints evaluation was based on concentrations of 1.3, 2.5, and 5.0 µg/ml.

In both experiments, no toxic effects indicated by reduced mitotic indices or cell numbers of below 50% of control were observed. In Experiment I and II, in the absence and the presence of irradiation, no biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item. However, in Experiment I, in the absence of irradiation, two statistically significant increases (2.5% and 2.0%, respectively) were observed, but were within the historical control data range for non-irradiated cultures (0.0–4.0% aberrant cells, exclusive gaps) and, therefore, are regarded as being biologically irrelevant.

In conclusion, it can be stated that under the experimental conditions reported, the test item FAT 65´080/B did not induce structural chromosome aberrations in V79 cells (Chinese hamster cell line) after irradiation with artificial sunlight when tested up to precipitating concentrations.

Ref.: 18 (subm I)

### 3.3.11. Human data

No data submitted

### 3.3.12. Special investigations

***In vitro* androgen receptor binding assay**

| | |
|---|---|
| Guideline: | / |
| Species/strains: | cytosolic preparations of prostate gland tissue of Alderley Park rats treated with GnRH antagonist (Antarelix) |

| | |
|---|---|
| Test substance: | FAT 65'080/B, d(0.5) = <15.4 µm |
| Batch: | KOC0005/004E |
| Purity: | 98% |
| Concentrations: | vehicle: DMSO<br>positive control: methyl trienolone, $5x10^{-6}$M, $5x10^{-7}$M, $5x10^{-8}$M, $5x10^{-9}$M and 5x10-$^{10}$M<br>Test substance: $5x10^{-4}$M, $5x10^{-5}$M, $5x10^{-6}$M, $5x10^{-7}$M, $5x10^{-8}$M, $5x10^{-9}$M and $5x10^{-10}$M |
| GLP: | In compliance |

The design of the study was based on the method published by Ashby et al. (2001) Replacement of surgical castration by GnRH inhibition for rat prostrate androgen receptor preparations. J. Appl. Toxicol. 21, 353-354.
Phase I of the study consisted of dosing 8 male Alderley Park rats with the GnRH antagonist (Antarelix) and harvesting the prostate gland cytosol 24 hours later, prior to conducting the in vitro phase of the study (Phase II). In Phase II, cytosolic preparations of prostate gland tissue were incubated with the test substance (FAT 65'080/B) at a range of concentrations (0.5 nM to 0.5 mM) and a fixed concentration (5 nM) of the radiolabelled androgen, methyl trienolone ($^{3}$H-R1881 (~0.2µCi ≅ $5x10^{-9}$ M R1881) in order to determine the ability of the test substance to displace the $^{3}$H-R1881. Preparations were incubated with the test substances for approximately 17 hours at approximately 4 ºC. Quantitation of displacement was used to determine the intrinsic activity of the test substance to interact with the androgen receptor (AR). Two independent assays with duplicates in each assay were performed.
The test substance exhibited no displacement of 3H-R1881 at any of the concentrations tested. Displacement of $^{3}$H-R1881 was observed following incubation with increasing concentrations of non-radioactive R1881 (positive control) but not with DMSO (vehicle control). Consistent results were obtained between duplicates within each assay and across both assays.
In conclusion, the inability to displace $^{3}$H-R1881 from cytosolic preparations of rat prostate gland tissue, indicated that, at concentrations up to $5x10^{-4}$ M, the test substance does not possess intrinsic potential to interact with the rat androgen receptor in the in vitro androgen receptor binding assay.

Ref.: 8 (subm I)

***In vitro* estrogen receptor binding assay**

| | |
|---|---|
| Guideline: | / |
| Species/strains: | cytosolic preparations of uterine tissue of immature Alpk:APfSD (Wistar derived) rats |
| Test substance: | FAT 65'080/B (technical), d(0.5) = <15.4 µm |
| Batch: | KOC0005/004E |
| Purity: | 98% |
| Concentrations: | Vehicle: DMSO<br>Positive control: Estradiol, $5x10^{-6}$M, $5x10^{-7}$M, $5x10^{-8}$M, $5x10^{-9}$M and $5x10^{-10}$M<br>Test substance: $5x10^{-4}$M, $5x10^{-5}$M, $5x10^{-6}$M, $5x10^{-7}$M, $5x10^{-8}$M, $5x10^{-9}$M and $5x10^{-10}$M |
| GLP: | In compliance |

The design of the study was based on the method published by Ashby et al. (2001) Lack of binding to isolated oestrogen or androgen receptors, and inactivity in the immature rat uterotrophic assay, of the ultraviolet sunscreen filters Tinsorb M-Active and Tinsorb S. Regul. Toxicol. Pharmacol. 34, 287-291.

The study consisted of harvesting the uteri from 40 immature Alderley Park rats. Freshly prepared cytosolic preparations of uterine tissue were then incubated with the test substance at a range of concentrations (0.5 nM to 0.5m M) and a fixed concentration (5n M) of the radiolabelled oestrogen, 3H-estradiol in order to determine the ability of FAT 65'080/B to displace the $^{3}$H-estradiol. Preparations were incubated with the test substances for approximately 17 hours at approximately 4 ºC. Quantitation of displacement was used to determine the intrinsic activity of the test substance to interact with the oestrogen receptor (ER). Two independent assays with duplicates in each assay were performed.
The results for the test substance showed a good correlation in both assays and between duplicates in each assay, with no increase in radioactivity displacement at the concentrations of test substance tested in both assays.

In conclusion, the inability to displace $^{3}$H-Estradiol from cytosolic preparations of rat uterine tissue, indicated that, at concentrations up to $5x10^{-4}$ M, the test substance (FAT 65'080/B) does not possess intrinsic potential to interact with the rat oestrogen receptor in the in vitro oestrogen receptor binding assay.

Ref.: 9 (subm I)

**Uterotrophic assay in immature female rats**

| | |
|---|---|
| Guideline: | / |
| Species/strain: | Immature female Alpk;APfSD rats (Wistar derived) |
| Group size: | 10 females, 19-20 days old at start of experiment |
| Test substance: | FAT 65'080B, d(0.5) = <15.4 µm |
| Batch: | KOC0005/004 |
| Purity: | 98% |
| Dose level: | Positive control: Estradiol 0.4 mg/kg bw/day<br>Test substance: 250, 500 or 1000 mg/kg bw/day<br>Daily administration of 10 ml/kg bw in 0.5% carboxymethylcellulose, for three consecutive days |
| Route: | Oral, by gavage |
| Exposure period: | Three days |
| GLP: | In compliance |

Groups of ten immature female rats received a single oral dose of 0 (vehicle control), 250, 500 or 1000 mg/kg bw of the test substance once a day for 3 consecutive days. As a positive control, one group of rats received a single oral dose of 0.4 mg β-estradiol/kg bw once a day for 3 consecutive days. The vehicle used for the test substance and β-estradiol was 0.5% carboxymethylcellulose.
The bodyweight of each rat was recorded daily, and detailed clinical observations were made at the same time. At the end of the study (approximately 24 hours after administration of the final dose), all of the animals were killed. The uterus was removed from each animal and trimmed of any fat and adhering non-uterine tissue. The uterus was then opened with a small incision, squeezed and blotted onto filter paper to remove any excess fluid and the uterine wet weight was recorded.

Initial analysis of study dose preparations gave poor results with regard to suspension, low achieved concentration and poor homogeneity. Samples from residues from the animal rooms were analysed and results indicated that the original initial concentrations of these dosing preparations were likely to be correct and that the stability of the dosing preparations was likely to be acceptable.

There were no clinical signs observed during this study for correctly dosed animals. Two animals died soon after dosing, (500 and 1000 mg/kg bw/day) and one animal required euthanasia during the study (1000 mg/kg bw/day). Post-mortem examination revealed dosing accidents to be the cause of death. One animal showed some adverse clinical signs

on day 2 (1000 mg/kg bw/day); on termination examination revealed a dosing accident had occurred.

There was no effect of the test substance on bodyweight or on uterus weight. As expected, oral gavage administration of β-estradiol to the immature rat for three consecutive days resulted in a marked increase in uterine weight, demonstrating a positive uterotrophic response with this substance.
In conclusion, there was no evidence of an uterotrophic response to this test substance.

Ref.: 10 (subm I)

## Annex 2: Exposure calculations using ConsExpo

**Product**
ETH50 Pump Spray (10% ETH50)

**Compound**

| | | |
|---|---|---|
| Compound name | ETH50 | |
| CAS number | 31274-51-8 | |
| molecular weight | 538 | g/mol |
| vapour pressure | 4.15E-21 | Pascal |
| KOW | 10.4 | 10Log |

**General Exposure Data**

| | | |
|---|---|---|
| exposure frequency | 75 | 1/year |
| body weight | 61 | kilogram |

**Inhalation model: Exposure to spray**

| | | |
|---|---|---|
| weight fraction compound | 10 | % |
| exposure duration | 5 | minute |
| room volume | 10 | $m^3$ |
| ventilation rate | 2 | L/hr |
| mass generation rate | 1 | g/sec |
| spray duration | 0.5 | minute |
| airborn fraction | 0.2 | fraction |
| weight fraction non-volatile | 10 | % |
| density non-volatile | 1.5 | $g/cm^3$ |
| room height | 2.5 | meter |
| inhalation cut-off diameter | 15 | micrometer |
| cloud volume | 0.0625 | $m^3$ |
| non-respirable uptake fraction | 1 | % |
| Spraying towards exposed person | | |

**Initial particle distribution:**

| | | |
|---|---|---|
| Distribution function | normal | |
| Median | 60 | micrometer |
| s.d. | 18 | micrometer |

**Uptake model: Fraction**

| | | |
|---|---|---|
| uptake fraction | 1 | fraction |
| inhalation rate | 33.3 | $m^3$/day |

**Dermal model: Direct dermal contact with product: instant application**

| | | |
|---|---|---|
| weight fraction compound | 10 | % |
| exposed area | 1,75E4 | $cm^2$ |
| applied amount | 18 | gram |

**Uptake model: fraction**

| | | |
|---|---|---|
| uptake fraction | 0,06 | % |

**Output**

**Inhalation (point estimates)**

| | | |
|---|---|---|
| inhalation mean event concentration | 0,32 | $mg/m^3$ |
| inhalation mean concentration on day of exposure | 0,00111 | $mg/m^3$ |
| inhalation air concentration year average | 0,000228 | $mg/m^3$/day |

| | | |
|---|---|---|
| inhalation acute (internal) dose | 0,000607 | mg/kg |
| inhalation chronic (internal) dose | 0,000125 | mg/kg/day |
| **Dermal: point estimates** | | |
| dermal load | 0,103 | mg/cm² |
| dermal external dose | 29,5 | mg/kg |
| dermal acute (internal) dose | 0,0177 | mg/kg |
| dermal chronic (internal) dose | 0,00364 | mg/kg/day |
| **Oral non-respirable: point estimates** | | |
| oral external dose | 0,000443 | mg/kg |
| oral acute (internal) dose | 0,000443 | mg/kg |
| oral chronic (internal) dose | 9,09E-5 | mg/kg/day |
| **Integrated (point estimates)** | | |
| total external dose | 29,6 | mg/kg |
| total acute dose (internal) | 0,0188 | mg/kg |
| total chronic dose (internal) | 0,00385 | mg/kg/day |

Ref.: 4 (Sub. VI)

**Product**

ETH50 Propellant Spray (10% ETH50, 40% Propane/Butane)

| | | |
|---|---|---|
| **Compound** | | |
| Compound name | ETH50 | |
| CAS number | 31274-51-8 | |
| molecular weight | 538 | g/mol |
| vapour pressure | 4,15E-21 | Pascal |
| KOW | 10.4 | 10Log |
| **General Exposure Data** | | |
| exposure frequency | 75 | 1/year |
| body weight | 61 | kilogram |
| **Inhalation model: Exposure to spray** | | |
| weight fraction compound | 10 | % |
| exposure duration | 5 | minute |
| room volume | 10 | m³ |
| ventilation rate | 2 | L/hr |
| mass generation rate | 1 | g/sec |
| spray duration | 0.5 | minute |
| airborn fraction | 0.5 | fraction |
| weight fraction non-volatile | 10 | % |
| density non-volatile | 1.5 | g/cm³ |
| room height | 2.5 | meter |
| inhalation cut-off diameter | 15 | micrometer |
| cloud volume | 0.0625 | m³ |
| non-respirable uptake fraction | 1 | % |
| Spraying towards exposed person | | |
| **Initial particle distribution:** | | |
| Distribution function | Normal | |
| Median | 50 | micrometer |
| s.d. | 15 | micrometer |

**Uptake model: Fraction**

| | | |
|---|---|---|
| uptake fraction | 1 | fraction |
| inhalation rate | 33.3 | m³/day |

**Dermal model: Direct dermal contact with product : instant application**

| | | |
|---|---|---|
| weight fraction compound | 10 | % |
| exposed area | 1.75E4 | cm² |
| applied amount | 18 | gram |

**Uptake model: fraction**

| | | |
|---|---|---|
| uptake fraction | 0.06 | % |

**Output**

**Inhalation (point estimates)**

| | | |
|---|---|---|
| inhalation mean event concentration | 1.26 | mg/m³ |
| inhalation mean concentration on day of exposure | 0.00437 | mg/m³ |
| inhalation air concentration year average | 0.000897 | mg/m³/day |
| inhalation acute (internal) dose | 0.00238 | mg/kg |
| inhalation chronic (internal) dose | 0,000489 | mg/kg/day |

**Dermal: point estimates**

| | | |
|---|---|---|
| dermal load | 0.103 | mg/cm² |
| dermal external dose | 29.5 | mg/kg |
| dermal acute (internal) dose | 0.0177 | mg/kg |
| dermal chronic (internal) dose | 0,00364 | mg/kg/day |

**Oral non-respirable: point estimates**

| | | |
|---|---|---|
| oral external dose | 0.00127 | mg/kg |
| oral acute (internal) dose | 0.00127 | mg/kg |
| oral chronic (internal) dose | 0.000261 | mg/kg/day |

**Integrated (point estimates)**

| | | |
|---|---|---|
| total external dose | 29.6 | mg/kg |
| total acute dose (internal) | 0.0214 | mg/kg |
| total chronic dose (internal) | 0.00439 | mg/kg/day |

Ref.: 5 (Sub. VI)

**Product**

ETH50 Propellant Spray (10%), particles after drying

**Compound**

| | | |
|---|---|---|
| Compound name | ETH50 | |
| CAS number | 31274-51-8 | |
| molecular weight | 538 | g/mol |
| vapour pressure | 4.15E-21 | Pascal |
| KOW | 10.4 | 10Log |

**General Exposure Data**

| | | |
|---|---|---|
| exposure frequency | 75 | 1/year |
| body weight | 61 | kilogram |

**Inhalation model: Exposure to spray**

| | | |
|---|---|---|
| weight fraction compound | 10 | % |

| | | |
|---|---|---|
| exposure duration | 5 | minute |
| room volume | 10 | m³ |
| ventilation rate | 2 | 1/hr |
| mass generation rate | 1 | g/sec |
| spray duration | 0.5 | minute |
| airborn fraction | 0.5 | fraction |
| weight fraction non-volatile | 30 | % |
| density non-volatile | 1.5 | g/cm³ |
| room height | 2.5 | meter |
| inhalation cut-off diameter | 15 | micrometer |
| cloud volume | 0.0625 | m³ |
| non-respirable uptake fraction | 1 | fraction |
| Spraying towards exposed person | | |

**Initial particle distribution:**

| | | |
|---|---|---|
| Distribution function | Normal | |
| Median | 37 | micrometer |
| s.d. | 12 | micrometer |

**Uptake model: Fraction**

| | | |
|---|---|---|
| uptake fraction | 1 | fraction |
| inhalation rate | 33.3 | m³/day |

**Dermal model: Direct dermal contact with product: instant application**

| | | |
|---|---|---|
| weight fraction compound | 10 | % |
| exposed area | 1.75E4 | cm² |
| applied amount | 18 | gram |

**Uptake model: fraction**

| | | |
|---|---|---|
| uptake fraction | 0.06 | % |

**Oral model: Oral exposure to product:**

| | | |
|---|---|---|
| weight fraction compound | 10 | % |

**Uptake model:**

**Output**

**Inhalation (point estimates)**

| | | |
|---|---|---|
| inhalation mean event concentration | 5.47 | mg/m³ |
| inhalation mean concentration on day of exposure | 0.019 | mg/m³ |
| inhalation air concentration year average | 0.0039 | mg/m³/day |
| inhalation acute (internal) dose | 0.0104 | mg/kg |
| inhalation chronic (internal) dose | 0.00213 | mg/kg/day |

**Dermal: point estimates**

| | | |
|---|---|---|
| dermal load | 0.103 | mg/cm² |
| dermal external dose | 29.5 | mg/kg |
| dermal acute (internal) dose | 0.0177 | mg/kg |
| dermal chronic (internal) dose | 0.00364 | mg/kg/day |

**Oral: point estimates**

| | | |
|---|---|---|
| oral external dose | - | mg/kg |
| oral acute (internal) dose | - | mg/kg |
| oral chronic (internal) dose | - | mg/kg/day |

| | | |
|---|---|---|
| **Oral non-respirable: point estimates** | | |
| oral external dose | 0.153 | mg/kg |
| oral acute (internal) dose | 0.153 | mg/kg |
| oral chronic (internal) dose | 0.0314 | mg/kg/day |
| **Integrated (point estimates)** | | |
| total external dose | 29.7 | mg/kg |
| total acute dose (internal) | 0.181 | mg/kg |
| total chronic dose (internal) | 0.0372 | mg/kg/day |

Ref.: 1 (Sub. VI)