Annex D - Statement on the safety of Titanium Dioxide (E171) as a Food Additive

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Studies used to review the toxicokinetics and absorption of the nanoparticle form of TiO2

Studies in rats and mice

Disdier et al., (2015)

1. The test material used was 75% anatase, 25% rutile titanium dioxide nanoparticles 21.5 ± 5 nm.

2. Adult Fischer rats were treated with 1 mg/kg titanium dioxide or saline buffer (control); no dispersion protocol was applied for the in vivo experiment. The dose was administered intravenously, and samples were taken at 30 minutes, 1, 2, 6 and 24 hours and 7-, 28-, 90- and 365-days post treatment from blood, liver, brain, spleen, kidney and lungs. Blood and brain samples were additionally collected at 5 and15 minutes post injection. Titanium concentrations were determined by ICP-MS.

3. The authors reported that titanium burdens in the liver, spleen and lungs of the treated group were significantly higher for all time points post injection, however the levels declined over time. Levels in the liver were higher than the spleen and lungs. Titanium burden after a year remained high, suggesting biopersistence (approximately 33% of the titanium burden of the early time points). The titanium burden in the kidneys increased significantly from 30 minutes to 24 hours but decreased significantly 7 days after i.v. administration.

No statistically significant results were reported for the blood samples. For the time points before 24 hours, there was a statistically significant increase if titanium concentrations in the brain. After 24 hours, titanium content did not differ from controls. No further details were given in the text, but graph 2 provides more details on the levels of titanium in the different organs. The authors estimated that they recovered approximately 44 % of the administered dose in the liver, 10 % in lungs and 2 % in spleen 6 hours post administration.

Kreyling et al., (2017a)

4. The test item used was titanium dioxide anatase nanoparticles, 7-10 nm.

5. Female Wistar- Kyoto rats were dosed with 10-20 µg of radiolabelled titanium dioxide given as a single i.v. injection of nanoparticles suspended in water.

6. The animals were terminated at 1, 4, 24 hours and 7and 28 days post administration (n=4 per time point). All organs as well as blood and all excretions of the animals were collected. The results of a separate intravenous study performed to investigate the absorption and biodistribution of soluble ionic48V were used to correct48V release from [48V] titanium dioxide nanoparticle.

7. The highest [48V] titanium dioxide accumulations were found in liver (95.5%ID on day 1), followed by spleen (2.5%), carcass (1%), skeleton (0.7%) and blood (0.4%). Detectable nanoparticle levels were found in all other organs. The [48V] Titanium dioxide NP content in blood decreased rapidly after 24h while the distribution in other organs and tissues remained rather constant until day-28. Particularly, 4 hours post, administration, 99.5% of the radioactive dose was found in the liver and at 28 days 88.9% of the dose was detected in the liver. The spleen and the kidneys contained: spleen between 2.5% and 4%, and kidneys between 0.05% and 0.2%. All other tissues had lower contents. The bones (including the marrow) and the remaining tissues contained 1% and 0.7%. The radiolabelled compound was excreted in urine and within 28 days the excretion amounted to roughly 1%. Highest excretion occurred on day 1. Excretion by the faeces, indicative of biliary excretion, amounted to 3% over 28 days.

Kreyling et al., (2017b)

8. A similar experiment to the one described above was also carried out, with the animals exposed via the oral route (a single dose of an aqueous, radiolabelled-nanoparticle suspension by intra-esophageal instillation) in female Wistar-Kyoto rats.

9. Titanium concentrations were determined at five retention time points 1 h, 4 h, 24 h, 7 d and 28 d after gavage in four rats for each time point. However, after observing in the seven-day experiment that fecal excretion of the test item was complete after 4–5 days, no further animals were terminated for a 28-day biodistribution.

10. Blood, all organs, tissues and excreta were collected, and the concentration of radioactive titanium dioxide was measured. Most of the radioactivity was excreted in the faeces. Absorption was calculated as the fraction of the dose that could not be accounted for by the radioactive content of the intestinal tract plus faeces. Approximately 0.6% of the dose was absorbed within an hour post-treatment. Seven days post treatment, roughly 0.05% of the dose administered was still present.

11. The authors noted that the distribution patterns between animals were variable and that several data were below the LOD during the first 4 hours. The spleen, kidneys, heart and uterus contained detectable levels even after 4 hours post treatment. Maximum retention was reached in the spleen, kidneys and heart at 24 hours post-treatment. In the liver, lung and blood, nanoparticle retention declined from 4 hours to 7 days. In the brain, uterus and kidneys, the highest concentrations were observed at day 7. The peak concentration in liver and spleen was 12.5% (4 hours) and 2.6% (24 hours) of the absorbed dose, respectively. According to the authors, due the slow excretion kinetics, accumulation of systemically circulating particles in specific cells and organs is likely to occur in subjects chronically exposed to titanium dioxide nanoparticles. When comparing the biodistribution of the radioactive titanium dioxide nanoparticles retained after oral administration with the results obtained after intravenous injection (Kreyling et al., 2017a), the authors concluded that the kinetics patterns are very different and intravenous injection does not appear an adequate surrogate for assessing the biodistribution occurring after oral exposure to titanium dioxide nanoparticles.

Geraets et al., (2014)

12. In the EFSA 2021 Opinion, only the i.v. experiment was discussed; however, the paper also contains the result of an oral administration experiment.

13. The test items used were NM-100, NM101, NM-102, NM-103 and NM-104. More information is provided on these in paragraph 46.

14. In the oral study, the single dose groups received a gavage dose of 2.3 mg/rat corresponding to 6.8-8.6 mg/kg bw. The repeat dose groups received five consecutive daily administrations of 2.3 mg titanium dioxide in one mL per rat resulting in a cumulative dose range of 34.1- 42.4 mg/kg bw for male rats and 54.5- 59.9 mg/kg bw for female rats.

15. In the i.v. study, the suspensions prepared contained 2.3 mg titanium dioxide/mL. The single i.v. dose treated rats received a dose of 8.4-9.8 mg/kg bw and 12.4- 14.1 mg/kg bw for male and female rats respectively, via the tail vein. The repeated dose treated rats received a cumulative dose that ranged from 42.3-49.4 mg/kg bw and 61.2-71.9 mg/kg bw for male and female rats, respectively. Thus, the actual dose in mg/kg bw depended on the weight of the rats.

16. After repeated oral exposure (overall dose of 11.5 mg Titanium dioxide) titanium levels were near or below the detection limit in liver and spleen, indicating a very low absorption. In two out of 30 liver/spleen samples of exposed animals (for NM-102 and NM-103) titanium levels were above the LOD, whereas all mesenteric lymph nodes (MLN) samples (including controls) contained titanium amounts above LOD. Only a small increase in titanium content was observed, because the background levels in MLN were 2–3 times the LOD. MLN from control rats contained 0.14 µg titanium whereas the highest titanium average was 0.36 µg and was located in MLN from NM-104 exposed rats. This gives an increase of 0.226 µg titanium in MLN or 0.003% of the 6895 µg Titanium exposure in the dose. The total recovery of dosed titanium in all tested organs (expressed as % of the total dose) was estimated to be approximately 0.02%.

17. In the i.v. study, the highest levels were observed in the liver, but redistribution to the spleen was observed over the 90-day post-exposure period Day 2/Day 6 and Day 90). Redistribution to remaining tissues was not identifiable. The authors hypothesised that release of particles from liver and possibly other organs may be responsible for the increase in spleen levels. Titanium was detected in all investigated tissues in the present study, i.e., blood, liver, spleen, kidney, lung, heart, brain, thymus and reproductive organs.

18. Both after single and repeated i.v. exposure, blood titanium levels in blood decreased rapidly during the first minutes after which the titanium levels slowly decreased and approached the limit of detection at 24 hours post exposure.

19. Based on the available data, the authors concluded that elimination of total Titanium dioxide has a long half-life. For the liver, which was considered the main

target organ, the estimated half-life was 28–248 days.

20. The authors considered that the data showed that at the long run Titanium dioxide particles will accumulate in the spleen. Finally, they noted that the expected accumulation with daily exposure as a consequence of the negligible elimination might indicate a potential concern for human health risk.

Hendrickson et al., (2016)

21. The test materials used were NM-101 (5-10 nm) and NP-25 (20-25 nm).

22. Male Sprague Dawley rats were treated with 250 mg/kg bw/d of either one of the test materials, dispersed in an aqueous starch solution containing 0.1% Tween-80 and sonicated via intragastric administration for 28 days.

23. Within a day of the last exposure, the animals were terminated and blood samples as well as samples from the lungs, liver, spleen, testes, small intestine heart, stomach and kidneys were harvested.

24. For animals treated with NM-101, titanium dioxide nanoparticles were detected in all organs and tissues. The organs with the highest concentrations were the spleen (0.227 µg/g) and liver (0.147 µg/g). In the kidneys, small intestine and testicles similar amounts of nanoparticles were detected (0.092, 0.098 and 0.089 µg/g respectively). Titanium dioxide nanoparticles were detected at 0.028 µg/g in the heart, 0.04 µg/g in the lungs and 0.049 µg/g in the brain.

25. In NP-25 treated animals' accumulation of titanium dioxide nanoparticles were detected in the small intestines and liver (0.29 µg/g in the liver) and at low levels (0.01 µg/g) in the kidneys. In the spleen, it was detected at levels of 0.11 µg/g of the organ. No titanium dioxide nanoparticles were detected in the lungs, brain, testicles, heart or blood.

26. The authors concluded that biodistribution differs between smaller (NM-101) and larger (NP-25) titanium dioxide nanoparticles, with the smaller particles showing a greater distribution spread and accumulation in all organs. The larger particles exhibited reduced but similar tendency. The main difference was that the larger nanoparticles could not overcome the blood brain barrier and penetrate the brain.

27. Due to the fact that the detected levels accounted for less than 1% of the administered dose, the authors concluded that the data was evidence of the limited bioavailability and efficient excretion of titanium dioxide.

Ammendolia et al. (2017)

28. The test material used was titanium dioxide nanoparticles (anatase, primary size 25nm BET) surface area 45-55 m^2/g, purity 99%, suspended; the suspensions were sonicated.

29. Sprague-Dawley rats (10/sex/group) were treated with 1 or 2 mg/kg bw/d titanium dioxide nanoparticles or vehicle only (ultrapure water) via gavage for 5 consecutive days.

30. Twenty-four hours after the last dose, the animals were terminated, and the small intestine was excised. A piece of jejunum was used for histological analysis and the remaining part of small intestine was sampled for studying either tissue accumulation of titanium dioxide nanoparticles, determined as titanium by ICP-MS.

31. Titanium was detected in small intestine tissue at 0.08 ± 0.02 lg/g in the control, 0.09 ± 0.02l g/g in the low dose group and at 0.13 ± 0.03 lg/g at the high dose group.

Hendrickson et al., 2020

32. The test material used was titanium dioxide nanoparticles, rutile, rod/ needle like shape, 5 x 30 nm.

33. Wistar rats were treated with 50 mg/kg bw titanium dioxide using an isolated intestinal loop technique.

34. Three hours post treatment, the isolated loop was cut out. The liver and spleen were collected.

35. The presence of particles in tissues was studied by TEM and diffraction analysis. Loose agglomerates (100 nm and larger) were detected. Diffraction analysis was used to confirm that the particles were titanium dioxide. Titanium dioxide nanoparticles were detected on the surface and between the microvilli of the mucosal cells of the small intestine and also in the mucosal tissue. Nanoparticles were detected in the Peyer’s patches, both as single nanoparticles and agglomerates of sizes ranging between 20 and 60 nm. In the liver, parenchymal tissue aggregates of titanium dioxide nanoparticles (150–200 nm) and up to 300 nm were seen. In the spleen red pulp, single nanoparticles (20–30 nm), agglomerates (up to 100 nm) and conglomerates (up to 800 nm) were observed.

Chen et al., 2020a

36. The test material used was titanium dioxide nanoparticles anatase, 29 nm (SEM).

37. Sprague- Dawley rats were dosed with 0, 2, 10, 50 mg/kg bw by oral gavage for 90 days. The test material was sonicated prior to treatment.

38. The tissue distribution of the titanium dioxide nanoparticles was evaluated by determining the titanium content in blood and tissues including liver, stomach, small intestine, colon, spleen, heart, lung, kidneys and testicles by high resolution ICP-MS.

39. Significantly increased titanium dioxide nanoparticle levels were only detected in the colon of rats exposed to 50 mg/kg test material, compared with the control group. There was no dose-response relationship, however. The authors hypothesised that the significant increase of titanium dioxide in colon tissue was due to the titanium dioxide nanoparticles attaching on the surface of the colonic mucosa tissue and not in mucosa cells. As most of orally ingested test material was excreted through feces, it resulted to long-term retention in large intestine. The titanium dioxide nanoparticles did not enter the colon epithelial cells and were mainly deposited in the intestinal cavity or between villi. The content of Ti in all tissues was very low, which was approximately 0.0001%-0.00001% or 100-1000 ng/g tissue, except for the colon of the high dose group. All spleen and heart tissue samples from rats contained very low titanium levels, which were below the LOD of 0.032 µg/g. Finally, they concluded that the results indicate that the absorption and distribution of titanium dioxide nanoparticles was very low after low-dose and long-term oral administration.

Warheit, Boatman and Brown, 2015

40. The COT noted that TiO_2 is able to move into the body through the GI tract. Warheit, Boatman and Brown (2015) evaluated the potential maternal or developmental toxicity of six different forms of predominantly NP form of TiO_2 in pregnant rats (22 or 23 rats per group). The dose levels for each form of TiO_2 were 0, 100, 300, and 1000 mg/kg bw per day. The TiO_2 formulations were in sterile water and delivered via oral gavage. This study noted that in a set of companion analyses, one was of the pigment-grade (pg) TiO_2 and the other was of the ultrafine (uf) TiO_2 test material (BET (Brunauer-Emmett-Teller) and the surface areas of pg and uf samples ranged from 7-17 m^2/g and 50-82 m^2/g respectively). Each material was evaluated for potential systemic

exposure/uptake from the GI tract to the blood for circulation.

41. In three studies, Warheit, Boatman and Brown (2015) dosed time-mated pregnant Sprague–Dawley, Crl:CD(SD), rats (n=22 per group), daily with TiO2 by gavage on Gestational Days 6 to 20. Three additional studies included pregnant Wistar rats (n = 22 – 23 per group) exposed daily to TiO2 by gavage from Gestational Days 5 to 19. The dose levels used in the studies were 0, 100, 300 or 1,000 mg/kg bw per day. The dose volume was 5 mL/kg bw per day with the TiO2 specifications detailed below:

i. anatase/rutile (89/11%)(uf-1), d50 = 43 nm (XSDC), d50 = 23 nm (TEM), irregular,

ii. anatase (100% nano) (uf-2), d50=42 nm (XSDC), d50=19 nm (TEM), irregular,

iii. rutile (100% nano) (uf-3), d50=47 nm (XSDC),d50=22 nm (TEM), rod-like,

iv. 4)anatase (27% nano) (pg-1), d50=153 nm (XSDC), d50=120 nm (TEM), irregular,

v. rutile (11% nano) (pg-2), d50=195 nm (XSDC), d50=165 nm (TEM), irregular.

42. No dose-dependent increase in TiO2 was observed in the rat blood at 48 or 72 hours, or in the liver at 72 hours following a single exposure (2000 mg/kg bw TiO2). These results suggested that there was little to no absorption of particles from the GI tract into the blood, which conflicted results from a previous study by Tassinari *et al*., 2014.

Tassinari et al., 2014

43. In the Tassinari *et al*. (2014) study, rats were dosed with distilled water (controls) or TiO2 NPs (anatase, primary size 25nm, BET surface area 45–55 m2 /g, purity 99%) by gavage for 5 consecutive days at 0, 1, or 2 mg/kg bw/day (7 rats/sex/group). Twenty-four hours after the last treatment (day 6), the animals were terminated, and the uterus, ovary, testes, thyroid and adrenals were excised and weighed. The spleen was sampled both for histopathological examination and for studying tissue deposition of Titanium dioxide nanoparticles.

44. The highest total titanium concentration was detected in the thyroid. However, the difference in levels was not of statistical significance between the treated animals and the controls (0.24 ± 0.09 mg/g and 0.22 ± 0.04 mg/g of fresh thyroid weight in animals dosed with 2 mg/kg bw/day and controls, respectively). A significant increase was observed in the ovaries of animals dosed with 2 mg/kg bw/day TiO2 NPs compared with control animals (0.28 ± 0.07 vs. 0.12 ± 0.04 mg/g fresh weight, respectively). Levels in uteri were low (0.051 ± 0.006 vs. 0.49 ± 0.04 mg/g fresh weight). A significant increase in the concentration of titanium in the spleen was observed in animals dosed with 2 mg/kg bw per day when compared to controls. (Tassinari *et al*. 2014). Overall, the authors considered that these results indicated the potential for titanium dioxide bioaccumulation. The COT considered that the strength of evidence for the absorption of TiO2 from the GI tract into the blood was not clear.

Bettini et al., 2017

45. In addition to the rats dosed with E171 as described in paragraphs xx-xx, Bettini et al also assessed TiO2-NPs using NM-105. As described previously, rats (10 per group) were dosed daily by gavage with NM-105 (10 mg/kg bs/day) for 7 days (the control group was given water). As described previously the NanoSIMS analyses of subcellular TiO2 NM-105 showed a similar distribution in the immune cells of Peyer’s patches, with the greatest concentration of particles in the central zones of the Peyer’s patches which have a high concentration of immune cells. Ti was also located in the cytoplasm and nuclei of the Peyer’s Patches cells.