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TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION

# Challenges in developing a testing programme following the EFSA Guidance on risk assessment of nanomaterials

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# Titanium dioxide ($TiO_2$)

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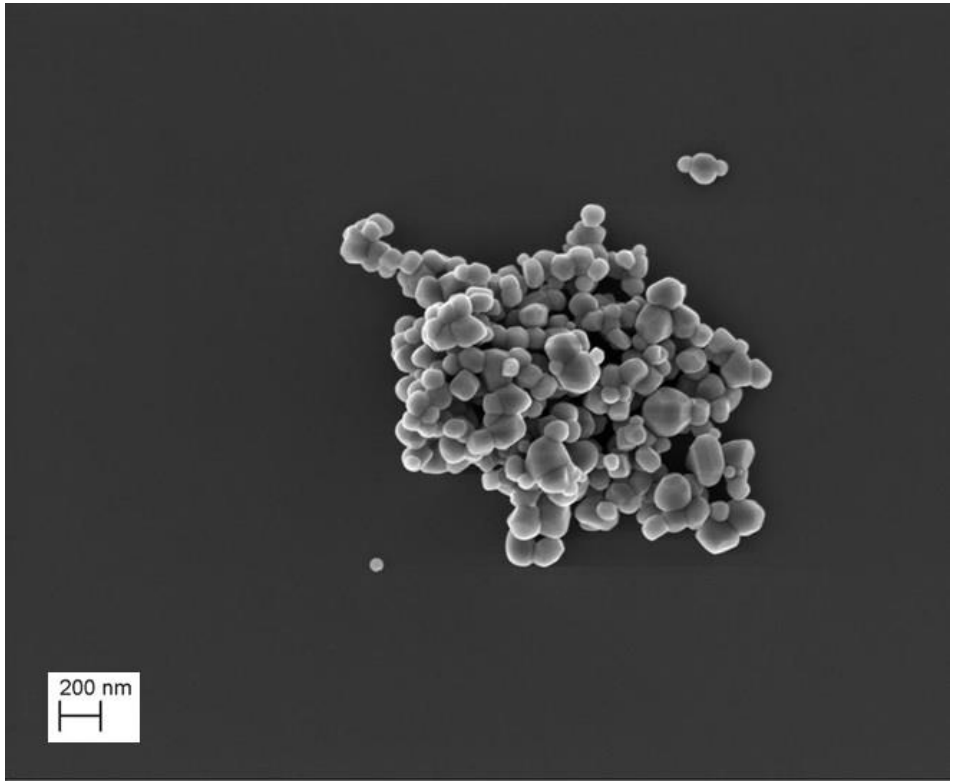
- Used as a pigment in food due its whiteness and high opacity – E 171
- Pigmentary $TiO_2$ has a median constituent particle size above 100 nm
  - Less than 50% of particles smaller than 100 nm by number
  - Not a nanomaterial according to the European Union recommendation
- Particles less than 100 nm do not scatter light and have no value as a pigment
- Is highly agglomerated
  - Constituent particles bond together




E 171-E Pigmentary $TiO_2$ anatase by scanning electron microscopy

# Situation

- May 2021 - European Food Safety Authority (EFSA) Opinion concluded that due to uncertainty about genotoxicity with respect to titanium dioxide ($TiO_2$) nanoparticles, they could not conclude that $TiO_2$ was safe.
  - EFSA Guidance on risk assessment of nanomaterials in food was applied in the assessment
- As a result, the Titanium Dioxide Manufacturers Association (TDMA) set up a $TiO_2$ Expert Panel chaired by David Kirkland to carry out a comprehensive independent external review of all studies and literature related to the genotoxicity of $TiO_2$

# Expert panel conclusions

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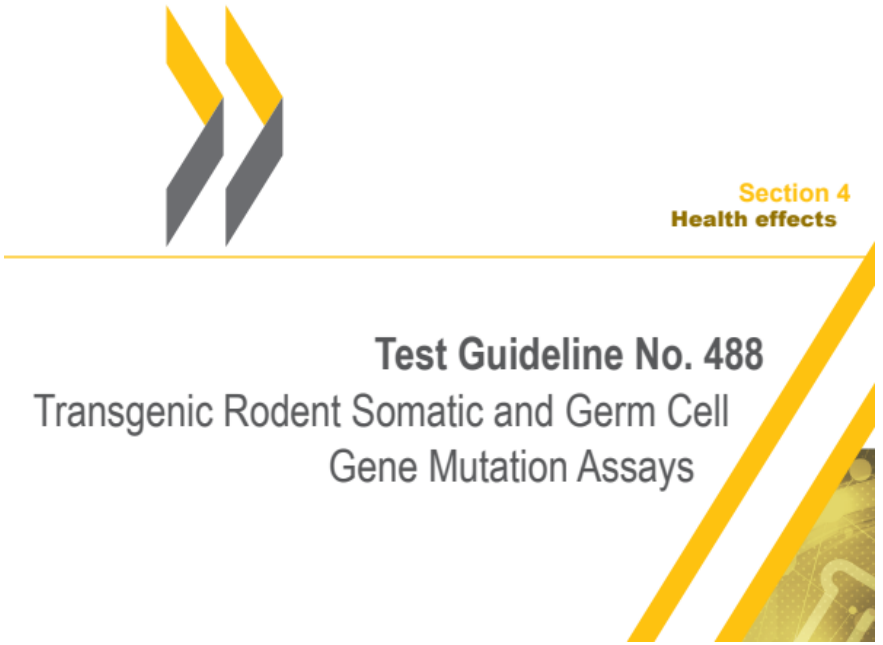
- There are many academic studies on the safety of $TiO_2$
- The Panel did not find convincing evidence of direct genotoxicity but recommended
  - Carefully designed studies …. following OECD recommended methods, performed with well characterised preparations of $TiO_2$, would allow firmer conclusions to be reached.
  - Published in Regulatory Toxicology and Pharmacology in September 2022
- September 2022 – Slough, UK workshop including experts from academia, industry and regulatory bodies
  - Recommended a transgenic rodent gene mutation (TGR) study by the oral route
- The TDMA developed a science programme to address the uncertainty

# Objectives of the programme

- Address concerns related to the genotoxicity of $TiO_2$ with focus on:
  - Food applications outside the European Union
  - Non-food applications influenced by the EFSA Opinion
- Example of impact for medicines
  - Approximately 91,000 human medicinal products contain $TiO_2$
  - $TiO_2$ has unique properties including non-interference with and UV protection of the active substance
  - Removal of $TiO_2$ would have very large impacts on medicine availability in Europe (source European Medicines Agency)

# Transgenic rodent gene mutation (TGR) study

- Gold standard assay for gene mutation testing
  - OECD Test Guideline No. 488
- Uses transgenic rats and mice
  - Segment of DNA containing a gene sequence that has been isolated and introduced into a different organism
- Allows detection of various types of mutations induced by test chemicals
- Combined with duplex sequencing of cells in the small intestine to evaluate genotoxicity



# Challenges for study validity

The animals were not exposed to nanoparticles

The dose was too low

The negative result is not valid as uptake could not be demonstrated

The route of exposure was not relevant

It was the wrong test item

The dose was too high resulting in highly agglomerated particles and no exposure to nanoparticles

How is the low bioavailability of $TiO_2$ considered?

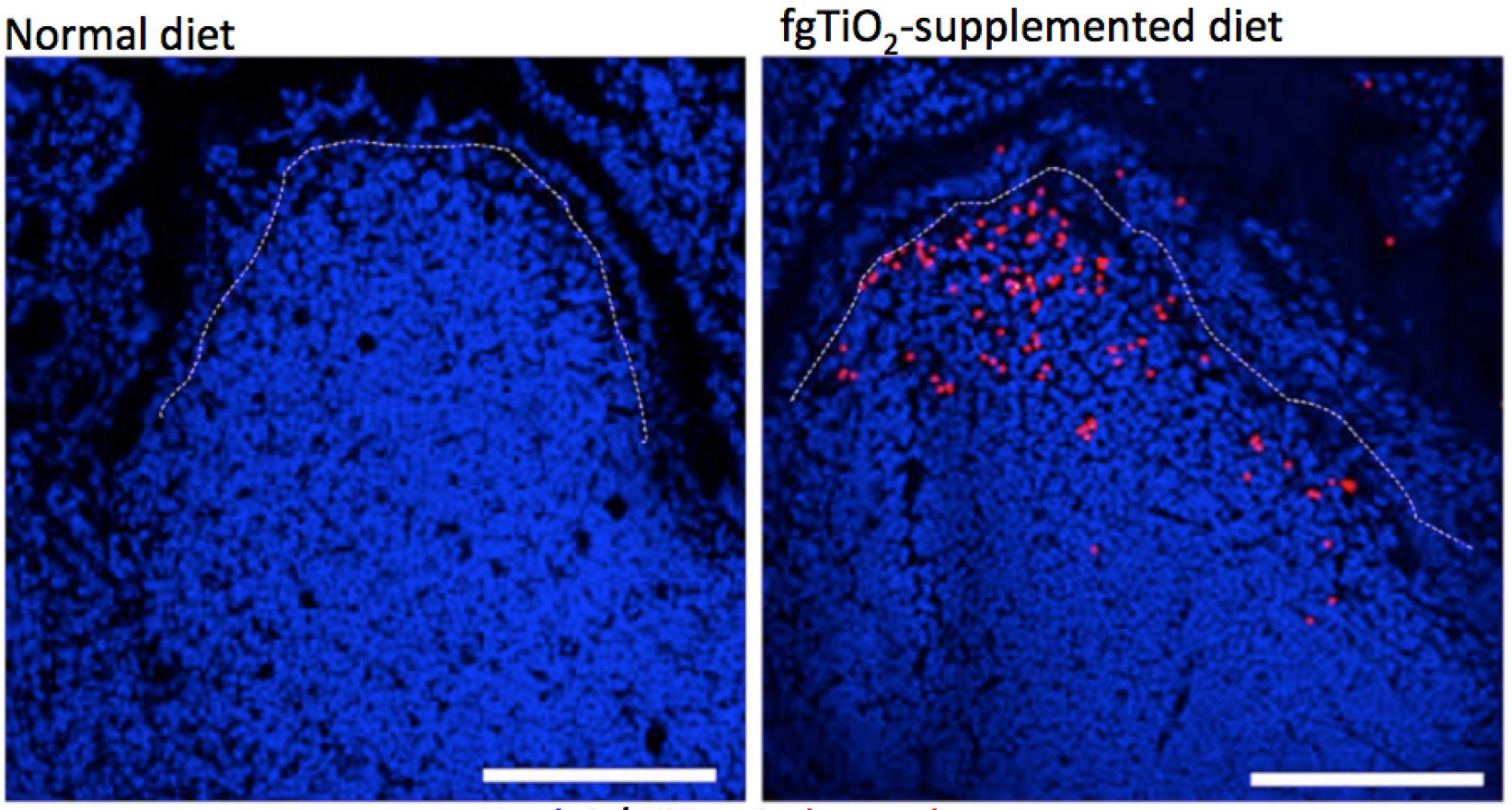
# Range finding study (RF1)

- Determine the differences in uptake by species and oral delivery route
  - Rats and mice
  - Solid diet, gavage and drinking water
  - Impact of dispersion including sonication
- Identify the delivery method that gives the highest tissue exposure with a preparation showing good dispersion
  - Apply the same mg/kg bw/day to all cases
  - Probably 500 mg/kg bw/day
- Evaluate uptake in tissues and blood
- The main transgenic rodent study (TGR) study to be performed with several dose levels (up to 1000 mg/kg bw/day) using the selected species and optimum delivery method

# Peyer's patches

- Lymph nodes embedded in the wall of the small intestine
- Mice and humans exposed by the oral route contain significant amounts of $TiO_2$ particles
- Appear to persist for long periods
- Will be used to evaluate uptake

## Mouse intestinal Peyer's patch tissue

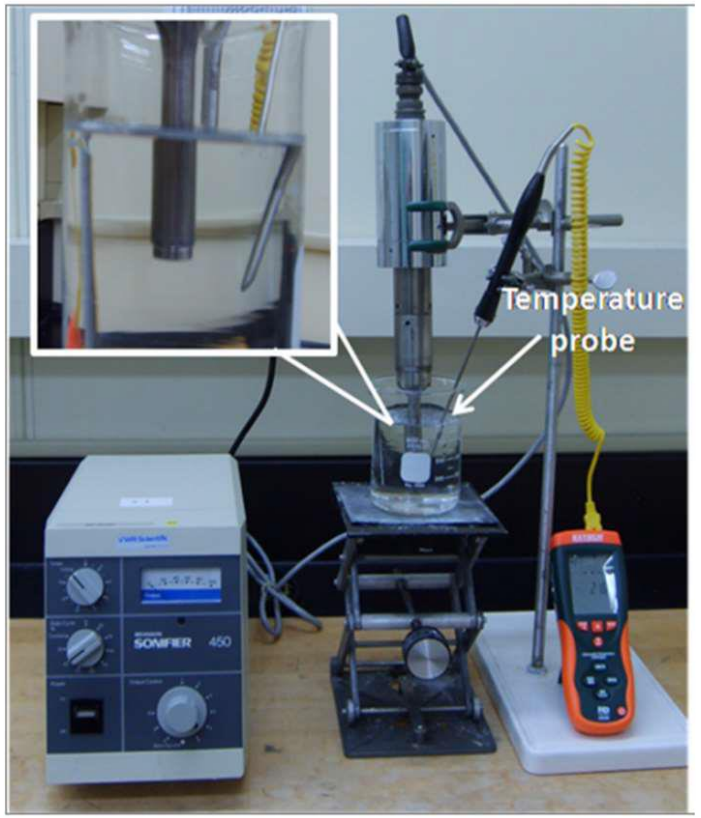



Nuclei / $TiO_2$-circle markers

**Uptake of food-grade titanium dioxide into mouse small intestinal Peyer's patches after dietary supplementation.** $FgTiO_2$ was detected using reflectance confocal microscopy. To aid visualisation, red circle-markers were placed on the reflectant foci demarking $fgTiO_2$ events using image analysis. *Scale bars = 150 microns.*

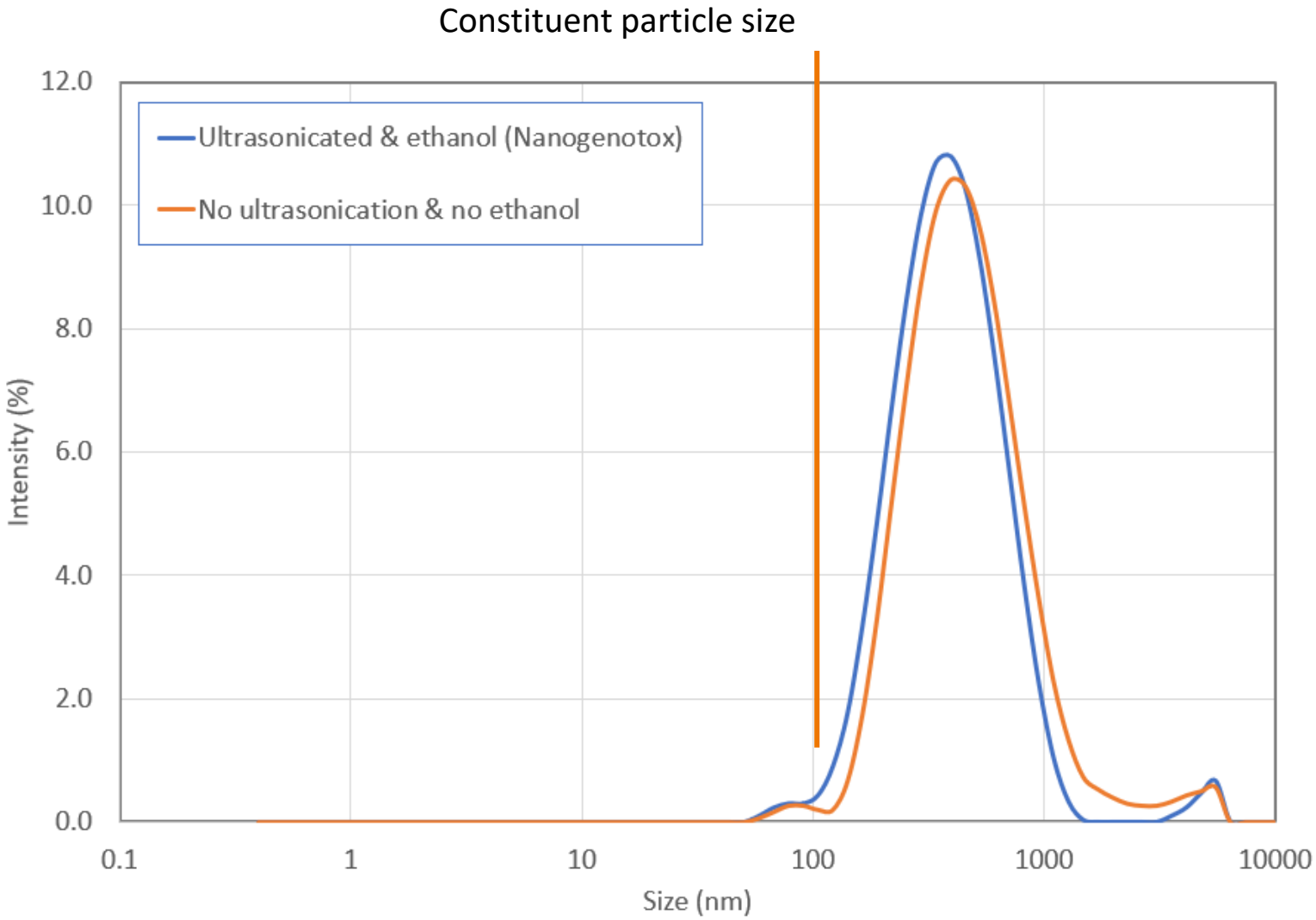
# Dispersion and sonication

- Preparation of $TiO_2$ dispersions following the Nanogenotox protocol
  - Main reference in the EFSA Guidance
    - *Proper dispersion of particulate materials prior to characterisation and application in toxicological tests is essential to achieve valid and comparable results. A dispersion protocol can be considered effective if it yields samples which consist as much as possible of non-agglomerated/non-aggregated particles.*
- High energy probe sonication with addition of dispersant
- Energy is significant and results in:
  - High temperature (compensated with ice bath)
  - Potential erosion of metals from the probe tip
  - Potential for radical formation (sonolysis)



# Sonication

- Agglomerate size measured by dynamic light scattering (DLS)
- $TiO_2$ is dense and sedimentation occurs rapidly
- Results indicate only a limited change in agglomerate size



# Gavage

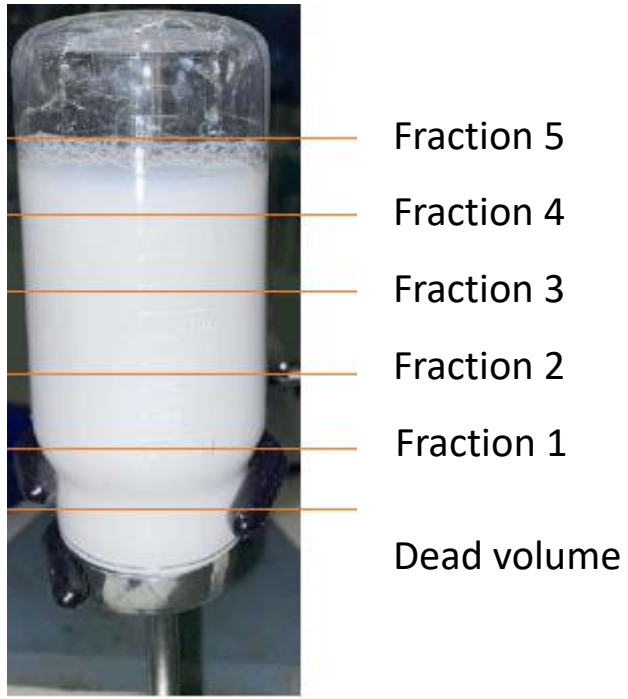
- Preparation of $TiO_2$ dispersions following the Nanogenotox protocol
- High dose rates requires $TiO_2$ concentrations much higher than the protocol
  - 10% solution of $TiO_2$ for 1000 mg/kg body weight limit dose
- Not possible to use dynamic light scattering (DLS)
- Due to settling, daily preparations are required

# Solid diet

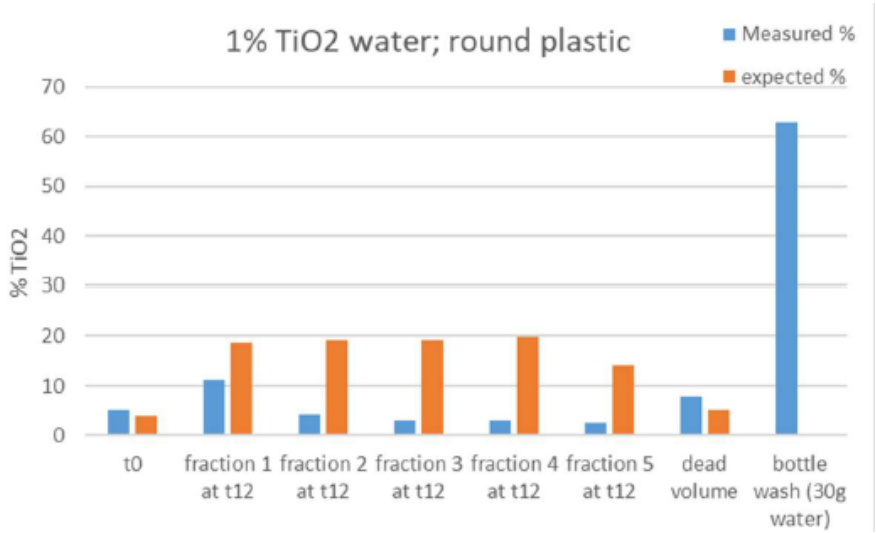
- Diet mixture prepared by directly adding $TiO_2$ powder to a solid feed mixture
- Deviates from the EFSA Guidance as want to study a wide cross section
  - *Diet should be first suspended in a liquid medium by using an appropriate dispersion protocol, and then, the resulting suspension should be thoroughly blended into the feed matrix to ensure a homogeneous mixture*
- Checking dispersions in the diet
  - Macroscopically with X-ray Fluorescence (XRF) and ICP-MS
  - Microscopically with scanning electron microscopy (SEM) combined energy-dispersive X-ray spectroscopy (EDX) and cryogenic electron microscopy

Scanning electron micrograph(SEM) showing a particle of $TiO_2$ in yellow box on a particle of food.

# Drinking water




- Most challenging oral delivery route
- Potential palatability issues
- Dispersion needs to be maintained over an extended period to allow drinking
  - Sedimentation occurs quickly
  - Settling of particles into drinking spout may restrict water consumption and may cause dehydration
- Determination of actual $TiO_2$ consumed likely to be inaccurate
- Will probably require a separate study



# In vitro studies

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- Parallel studies to provide high quality in vitro gene mutation data to understand the difference between different $TiO_2$ forms
- *In vitro* mammalian cell *Hprt* gene mutation OECD 476 studies with
  - Additional biomarkers of oxidative stress
  - Uptake analysis by electron microscopy
- Aligned with the recent draft advice of the Scientific Committee on Consumer Safety (SCCS) related to $TiO_2$ in cosmetics

# Summary

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- The TDMA is committed to address the uncertainty identified by EFSA Opinion
- The TDMA science programme is designed to address the many questions including species differences, oral delivery route and uptake
- Applying the EFSA Guidance has raised many challenges which the TDMA are trying to address
- The TDMA welcomes feedback on this work and approach


THANK
YOU!