PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Preparation of snacks for repeated oral administration of nanomaterial to laboratory rats

This is a SOP recommended for external use by PATROLS

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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Reviewed by:

Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	DD/MM/YYYY	Initial Document	

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1 Introduction:

Domain: in vivo nanotoxicology

This SOP describes a method for repeated oral administration of a nanomaterial in a snack to rats, as an alternative to oral gavage. Although the agglomeration of nanomaterial is unknown in the snack, this dosage form could be preferred as a more relevant dosing regime for dietary exposure via food and from an animal welfare point of view. Furthermore, the method provides full control of the administered dose.

1.1 Scope and limits of the protocol

Oral administration of nanomaterial in a snack can be used for assessing oral toxicity or oral biodistribution of powder nanomaterials, especially for repeated or long-term oral toxicity studies.

The limitation of administration by snack dosing compared to by oral gavage is that there is no standard method for assessing the level of agglomeration of the nanomaterial and the size distribution of the agglomerates in the snack (solid food matrix). The snacks were prepared freshly every day to minimize the possible agglomeration of the nanomaterial before exposure.

1.2 Validation state of protocol

Level of advancement towards standardization	**Level reached** (please mark only one with "X")
Stage 1: Internal laboratory method under development	x
Stage 2: Validated internal laboratory method	
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	N
Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	N
Is the method included in an existing standard (or ongoing standardisation work)	N

If yes, specify		[standard reference number, eg. EN 17199-4]

2 Terms and Definitions:

Agglomerate
Collection of weakly or medium strongly bound *particles* where the resulting external surface area is similar to the sum of the surface areas of the individual components.

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.2]

Nanomaterial
Material with any external dimension in the *nanoscale* or having internal structure or surface structure in the nanoscale.

Note 1 to entry: This generic term is inclusive of *nano-object* and *nanostructured material*.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.4]

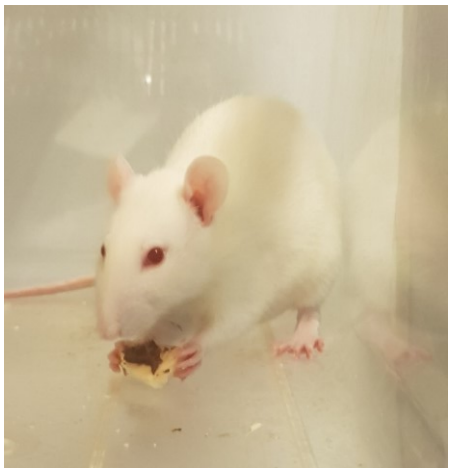
3 Abbreviations:

ICP-MS: Inductively coupled plasma-mass spectrometry

4 Principle of the Method:

Oral toxicity of nanomaterials depends on the uptake via the gastrointestinal tract. The principle of snack administration is that it 1) provides a more physiologically relevant exposure (not a bolus) and thus a better model for human oral exposure to nanomaterials in solid food than exposure via gavage providing a bolus dose dispersed in a vehicle, 2) stresses the animals less than administration by gavage and 3) it allows better control of the delivered dose than oral exposure through drinking water or feed. Here, the JRC reference nanomaterial cerium oxide (CeO_2 NM-212) is used as a model material for poorly soluble nanomaterials. Nanomaterial

powder was mixed with chocolate spread by the pharmaceutical volume doubling technique using a mortar and pistil to obtain a homogeneous mixture.



Female Sprague Dawley rat with a snack of nanomaterial in chocolate spread on a biscuit

5 Description of the Method:

5.1 Biological setting & test system used:

Not relevant.

5.2 Chemicals and reagents used:

- Nanomaterial in powder form (CeO_2 NM-212 nanomaterial, precipitated, uncoated, Fraunhofer IME, Schmallenberg, Germany)
- Chocolate spread (Nutella®: sugar, palm oil, hazelnuts, cocoa powder, skimmed milk powder, whey powder, soy lecithin, and vanillin)
- Biscuits (Marie biscuit®: wheat flour, sugar, vegetable oil)

5.3 Apparatus and equipment used:

- Porcelain mortars and pestles
- Metal spatulas
- Glass vials with lid for weighing and storing nanomaterial
- Glove box
- Analytical balances inside and outside glove box
- Weigh paper and weigh boats
- Dry ice in a flamingo box
- Tweezers for handling cooled portions of chocolate spread,

- Cages for single housing for rats when given a snack

5.4 Reporting of protected elements:

None

5.5 Health and safety precautions:

Prior to any use of this SOP a full risk assessment should be completed, considering all potential risks associated with chemicals equipment and use, in compliance with national regulation. Training of personnel should be completed before any person is working with the SOP.

5.6 Applicability:

The SOP applies to poorly soluble nanomaterials and was demonstrated for cerium oxide CeO_2 NM-212 powder.

5.7 Reagent preparation:

None

5.8 Procedure:

<u>Weighing nanomaterial and biscuits</u>

Nanomaterial

Weigh the amount of a nanomaterial needed for each day of administration into separate vials. Use an analytical balance placed inside a glove box to avoid human exposure to dry powder nanomaterial. Fill the vials containing the nanomaterial with argon, seal and store in a fume hood at room temperature until use.

Pieces of biscuits

Weigh pieces of biscuits of about 400-700 mg per piece and store at room temperature.

<u>Weighing of portions of chocolate spread for volume-doubling technique</u>

Weigh fresh portions of chocolate spread on every exposure day using an analytical balance outside the glove box (Figure 1). Chocolate spread is prepared for the highest concentration of nanomaterial needed for the oral exposure. For example, we administered 13 mg nanomaterial in 1 g of chocolate spread per animal per day and so the highest concentration needed was 13 mg nanomaterial per gram of chocolate spread.

- Place a piece of weigh paper on an analytical balance

- Weigh a small portion of chocolate spread of approximately 1 g. Use two small spatulas to handle the chocolate spread.
- On the same weigh paper without taring the balance, weigh another portion of chocolate spread of approximately twice the volume of the small portion e.g. 2 g.
- Continue to weigh portions of chocolate spread that is equal to the combined volume of what is already on the weight (e.g. portions of 1 g, 2 g, 3 g, 6 g…). Repeat until you reach the desired total mass of chocolate spread.
- Place the weigh paper with portions of chocolate spread on dry ice or at -20 °C to obtain a firm consistency. Cooling the chocolate spread allows handling the portions without loss of material.

Note: the combined weight of the portions must be accurate to achieve the highest concentration of nanomaterial needed for oral exposure, when mixed with the amount of nanomaterial weighed for that day.

Figure 1. Portions of chocolate spread prepared on an analytical balance (left) and cooled on dry ice (right).

<u>Mixing nanomaterial in chocolate spread by the volume doubling principle</u>

Volume doubling (aka geometric dilution or volume-to-volume mixing) is a pharmaceutical technique to prepare a homogenous mixture of for example a small amount of powder drug in a pharmaceutical cream. Apply the volume doubling technique using a mortar and pistil to obtain a homogeneous mixture of nanomaterial in the chocolate spread with the required concentration. Prepare a concentration by which 1g of mixture contains the highest daily dose of nanomaterial per rat.

- Place a flamingo box with dry ice and the weigh paper with prepared chocolate spread portions (Figure 1), tweezers for handling cooled portions of chocolate

spread, pre-weighed nanomaterial in sealed vial, and a mortar and pestle inside a glove box (Figure 2).

Figure 2. Mixing CeO2 in chocolate spread in a glove box using mortar and pistil. The empty glass vial is weighed to ensure that all nanomaterial has been transferred to the mortar.

- Transfer all the nanomaterial powder from the vial to the mortar. Check that all nanomaterial has been transferred by weighing the empty vial.
- Add the smallest portion of cooled chocolate spread to the mortar using tweezers. Let the spread adjust to room temperature and soft consistency. Grind thoroughly to make a homogenous mixture of the nanomaterial powder and the chocolate spread.
- Add the next portion of chocolate spread thereby doubling the volume and repeat the thorough mixing.
- Repeat above with all portions of chocolate spread each time doubling the volume in the mortar and mixing thoroughly.

<u>Dilution of chocolate/nanomaterial spread using the volume-doubling principle</u>

Dilute n-fold a fraction of the chocolate/nanomaterial spread prepared above, according to the dose range in your oral study design. Use mortar and pistil and the volume doubling technique.

- Place a piece of weigh paper on an analytical balance
- Weigh a small portion of the chocolate/nanomaterial spread prepared above (e.g. approximately 1 g). Note the exact weight.
- Place the weigh paper with the portion of chocolate/nanomaterial spread on dry ice or at -20 °C to obtain a firm consistency.
- Place a new weigh paper on the analytical balance and prepare portions of plain chocolate spread for the volume doubling principle. The first and smallest portion should be equal to the portion of chocolate/nanomaterial spread.

- Continue to weigh portions of plain chocolate spread on the same weigh paper that is equal to the combined volume of what is already on the weight and the chocolate/nanomaterial portion (e.g. plain portions of 1 g, 2 g, 4 g, 8 g…). Repeat until you reach the desired total mass of chocolate spread needed for the n-fold dilution.
- Place the weigh paper with portions of chocolate spread on dry ice or at -20 °C.

When the portion of chocolate/nanomaterial spread and the portions of plain chocolate spread are cooled to a firm consistency you can start the volume doubling.

- Transfer the cooled portion of chocolate/nanomaterial spread and the equal portion of plain chocolate spread to a mortar using tweezers. Let the spreads adjust to room temperature and a soft consistency. Grind thoroughly to make a homogenous mixture of the nanomaterial/chocolate spread and the plain chocolate spread.
- Add the next portion of plain chocolate spread thereby doubling the volume and repeat the thorough mixing.
- Repeat above with all portions of plain chocolate spread each time doubling the volume in the mortar and mixing thoroughly.

Preparation of chocolate spread without nanomaterial for control groups:

Use separate utensils, mortars and pistils for the control group and nanomaterial group to avoid contamination.

Calculate the total amount of control chocolate spread needed for each administration day, and weigh into smaller portions of approximately doubling volumes. Grind the portions of increasing size with mortar and pistil according to the volume doubling principle, but without adding nanomaterial.

Testing homogeneity and concentration of the nanomaterial in chocolate spread with ICP-MS

Store daily samples of the grinded chocolate spreads with and without nanomaterial at -20 °C for ICP-MS analysis, to assess the homogeneity and the concentration of nanomaterial (ICP-MS method is not described in this SOP).

Preparing snacks of 1 g chocolate spread per biscuit per animal

Snacks with nanomaterial should be prepared fresh every day. Exception can be done for the control snacks without nanomaterial, which can be prepared the day before the exposure and stored at 4 °C until administration.

- Place a weigh boat with a piece of biscuit on an analytical balance.

- Use two small spatulas to transfer the grinded chocolate spread to the biscuits, 1.01-0.99 grams per biscuit. Note the exact weight.
- Store the snacks in a box with animal numbers at 4 °C until the administration (Figure 3).

Figure 3. Snacks with 1g chocolate spread per biscuit. Snacks are placed in a box with double plastic weigh boats where the lower boat is glued to the box with the animal numbers and the upper boat can be transferred to the single housing.

Washing utensils

Wash mortars, pistils and spatulas with warm water and a dish brush and wipe with a towel. Use separate utensils for control snacks to avoid nanomaterial contamination.

Accustom laboratory rats to chocolate spread

Accustom laboratory rats to chocolate spread without nanomaterial by daily applying a small smear in the home cage for a week before the experiment. Perform a pilot rat study of the chocolate spread containing the highest concentration of nanomaterial needed in the main study to ensure that the rats voluntarily eat the daily snack. The rats can have access to feed and tap water *ad libitum* and fasting is not required.

Oral administration of nanomaterial in snacks to laboratory rats

- Transfer rats from the home cage to a single housing cage with no water or feed.
- Place a snack i.e. a biscuit with 1g chocolate spread with or without nanomaterial in the cage. The snack should be served cold for a firm consistency.
- Observe the rat eating the snack in order to confirm the dosing. The rats voluntarily eat the snack within few minutes (Figure 4).

- Return the rat to the home cage when all chocolate spread has been eaten.

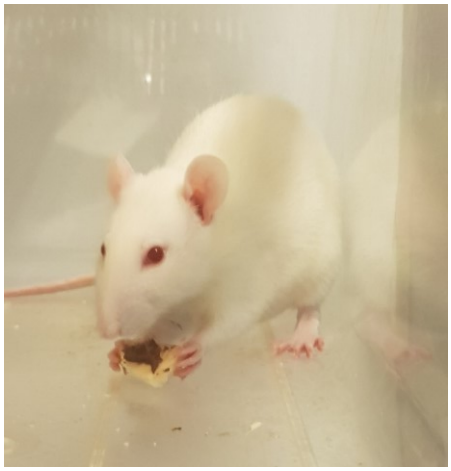


Figure 4. Female Sprague Dawley rat with a snack of CeO_2 nanomaterial in chocolate spread on a biscuit.

5.8.1 Testing for nanomaterial interference:

This SOP describes a method for oral administration of a nanomaterial. It is important to know the background exposure of the rats to the material from other sources (e.g from a bedding in the cages, the cages themselves, from bottles for water, or from the laboratory feed) or other exposure routes (e.g inhalation). For example in the case of cerium oxide nanoparticles, a low level of cerium has been detected by ICP-MS in a standard feed and in chocolate spread grinded in a porcelain mortar without addition of cerium oxide nanomaterial.

5.9 Quality control & acceptance criteria:

Quality control should if possible include checking the achieved concentration and homogeneity of nanomaterial in chocolate spread with and without nanomaterial added for example using ICP-MS.

The study should include vehicle control groups receiving snacks with plain chocolate spread grinded in a mortar without nanomaterial.

Note. Since we propose snack dosing as an alternative to oral gavage, we compared the oral uptake (cerium concentrations by ICP-MS) in liver, spleen, kidney, intestine, blood, and faeces, for the two types of oral exposure. There was no statistically significant difference in cerium content in liver or spleen after gavage compared to snack dosing (Berthing et al. manuscript in preparation).

6 Data Analysis and Reporting of Data:

Data analysis and reporting is outside the scope of this SOP.

7 Publications:

Berthing, Trine (1); Holmfred, Else (1,2); Vidmar, Janja (2); Hadrup, Niels (1); Mortensen, Alicja (1); Szarek, Józef (3); Löschner, Katrin (2); Vogel, Ulla (1, 2) Comparison of biodistribution of NM-212 CeO2 NPs after repeated oral administration by gavage or snack in rats, *manuscript in preparation*

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