

Standard Operating Procedure

Title	Protocol for the measurement of water solubility
Subtitle	The following SOP describes the experimental set-up and sample preparation for evaluation of the revised OECD TG 105 (water solubility).
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TG 105 „Water Solubility“ using a modified flask method, LEITAT, UdL

The following SOP describes the experimental set-up and sample preparation for evaluation of the revised OECD TG 105 (water solubility). Measurements of the soluble fraction of NM-300K and NM-110 (JRCNM01100a) dispersed in purified water (or synthetic aqueous solutions) were performed by filtration/centrifugation experiments and ICP-MS analyses. For NM-300K, the results of one of the tested methods were compared with those obtained using Ag ion selective electrode (ISE) measurements. For NM-110, the results were compared with those obtained with AGNES measurements (without solid-liquid separation step).

Protocol for the measurement of water solubility

A. Materials to produce a stock dispersion for the NM water solubility experiment

- All equipment required for the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing
- Excel template for the probe-sonicator comparison 'Template for Probe Sonicator Calibration'
- All equipment required for the NANoREG ENPRA dispersion protocol
- DI water (Milli-Q)
- pH meter
- Closed bottles or vials
- Magnetic stirrers and stir bars
- Micropipettes and tips
- Temperature controlled, orbital shaker incubator

Materials to measure water solubility by ICP-MS

- ICP-MS
- Centrifuge
- Amicon® Ultra-15 Centrifugal Filter Device, cut off 3 kDa
- Microvials, 1 mL
- Micropipettes and tips

Materials to measure water solubility by Ag ISE

- Reference electrode and ISE for the ion under investigation
- AgNO₃ salts for the Ag ISE calibration curve
- Ionic strength adjuster (ISA) (5 M NaNO₃) for Ag ISE
- DI water
- Graduated flasks to prepare the standards for the ISE calibration curves
- Beakers, 30 mL
- Micropipettes and tips

Materials to measure water solubility by AGNES

- Potentiostat and polarographic stand
- Mercury capillary drop electrode
- Ag/AgCl reference and glassy carbon auxiliary electrodes
- Glass-jacketed thermostatic cell
- Water recirculating thermostatic bath
- pH meter
- water-saturated N₂/CO₂ (99.999% purity) gas mixtures for the removal of dissolved O₂
- Micropipettes and tips

B. Calibration

Probe-sonicator calibration

- Use the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing.
- Employ the Excel template for the probe-sonicator comparison 'Template for Probe Sonicator Calibration'.

ICP-MS calibration

- Use the NANoREG SOP for ICP-MS measurements.
- Important: Metals may be leached from glass by nitric acid. Store all standard solutions in polypropylene or other inert containers. If glassware is used, it should be cleaned with nitric acid and dedicated for trace metals analyses. Standards prepared in glassware should be used immediately or transferred to suitable containers for storage.
- Starting from commercial 1000 ppm standard solutions, intermediate standards of 5 ppm are prepared. From these, a set of final calibration solutions is prepared for constructing a multipoint standard curve covering the range of analyte concentrations anticipated in samples.
- Measurements are performed for each sample at least in triplicate.
- Reagent blanks (generally, nitric acid diluted in MilliQ water) are employed, where the blank signal shows in all samples and standard solutions.
- Preparation blanks are also recommended, where the blank is from the sample preparation process only and not present in the calibration solution/standards. In this particular case, preparation blanks were necessary to check for analyte adsorption on the Amicon® Ultra-15 Centrifugal Filters.

Ag ISE calibration

- Maintain a constant temperature and stirring conditions during both the ISE calibration and the sample measurements.
- Prepare a concentrated stock solution of a completely soluble salt of the element of interest and add 5 M NaNO₃ as an ISA (2% ISA, v/v). Obtain different standard solutions by serial dilutions of the stock solution with distilled water and the appropriate amount of 5 M NaNO₃ to have 2% ISA (v/v) in all standards.
- Obtain a calibration curve in the expected concentration range. A typical curve for the selective electrode is composed of a linear area that ranges from 1 to 1000 ppm and a non-linear area that ranges from 0.01 to 1 ppm. In the linear range, the theoretical slope of the calibration curve for monovalent cations is 59.16 ± 5 mV/pAg at 25 ± 5 °C. In the non-linear range, the calibration curve should include measurements for at least five standards and polynomial interpolation is required.
- Before the first measurement, rinse both the reference and ISE electrodes with DI water and dry them carefully, taking care not to touch the ISE membrane
- Measure the standards in ascending order of concentration.
- Between measurements, rinse both the electrodes with DI water and dry them carefully, taking care not to touch the ISE membrane.

AGNES calibration

- Use the NANoREG SOP for AGNES measurements.

C. Solubility Measurement

Planning the experiment and practical considerations

- Establish the NM concentration that will be used and the time points to measure.
- Based on the MNM concentration selected, the time points considered, and the technique/techniques that will be used to analyse the samples, estimate the total volume of stock dispersion that should be prepared.
- If the NM is UV light sensitive, suitable amber coloured glassware should be employed to prepare and store the samples. If amber coloured glassware is not available, the vials, flasks, or beakers should be covered with aluminium foil to protect the sample against UV degradation.

- If the NM is known to be sensitive to oxygen and light, different aliquots of identical (twin) samples should be prepared for measurements at each time point. This procedure ensures that the NM concentration does not change during tests and the same conditions are maintained for all the samples.
- Temperature control must be ensured during incubation of the samples, and time needed for the handling of samples prior to analytical measurement must be minimized.
- For the case of NMs that are very sensitive to pH changes (i.e., ZnO), a suitable control of eventual pH changes (e.g., due to variation in partial CO₂ pressure during experimental procedure) must be addressed (e.g., by use of a suitable buffer, continuous tracking of pH and/or use of correction factors, as described below).

Preparation of the stock dispersion

- Prepare a stock suspension of 2.56 mg particles/mL of MilliQ water, following the NANoREG ECOTOX dispersion protocol for producing reproducible dispersions of manufactured NM in environmental exposure media.
- Measure the size distribution of the stock dispersion using DLS according to the SOP developed in the NANoREG ECOTOX dispersion protocol.

Preparation of samples for NM water solubility testing

- Dilute the stock dispersion with water (or the desired test medium) to obtain the NM concentrations chosen for the water solubility tests.
- Transfer aliquots of the sample into closed (air-tight) bottles or vials, insert a stir bar, and leave the samples to stir, according to the time points selected. Alternatively, the sample vials can be stored in a temperature-controlled orbital shaker incubator. Check for pH changes before/after incubation.
- At the selected times, use the various aliquots of the sample to determine:
 - o the total NM concentration and ionic fraction content by digestion plus ICP-MS and centrifugation/filtration plus ICP-MS of the filtrate, respectively.
 - o the NM ionic fraction content by ISE measurement.
- For comparison (in the case of ZnO NMs), additional AGNES measurements can be performed with aliquots of the stock solution diluted directly in a suitable thermostated glass vessel containing the test media under intermittent stirring. This procedure allows measurement of kinetic dissolution rates up to a few hours. No solid-liquid separation step is necessary.

Determination of total NM content by ICP-MS

- Place a 1 mL aliquot of the sample in a 1 mL microvial, store in the fridge until use, measure the total NM content by ICP-MS, and calculate the percent dissolution based on the total mass of NM employed.

NOTE: A proper digestion protocol is required before ICP-MS analysis for NM dispersions.

Determination of NM dissolved fraction by filtration/centrifugation followed by ICP-MS of the filtrate

- Pre-rinse the Amicon® Ultra-15 Centrifugal Filter Device with 10 mL of DI water, employing similar conditions to those used in the filtration/centrifugation experiment. This operation is useful to check if the filter works correctly or if it has been damaged or has any manufacturing defects.
- Dry the filtration device carefully before the filtration/centrifugation experiment.
- Place a 10 mL aliquot of the sample in the filtration device, cap the device, insert it into the centrifuge, and spin the device at 4000 RCF (G) for 10 min. These conditions allow approximately 5 mL of filtrate to be obtained.
- Place 1 mL of the filtrate in a 1 mL microvial, store in the fridge until use, and use for ICP-MS analysis.

NOTE: If the NM does not contain particles smaller than 1.5–3 nm (~3 kDa cut-off), the filtrate should contain only ions and other low-molecular weight dissolved species; therefore, a digestion protocol is not required.

Determination of NM dissolved fraction by ISE measurement (NM-300K, Ag NM)

- Check the operating conditions of the ISE electrode, including the pH range in which the electrode can work, and potential interference from other molecules or ions present in the sample. For Ag^+ determination, the pH of the sample should be between 1 and 9 to avoid reaction between the Ag^+ and OH^- ions. Moreover, S^{2-} and Hg^{2+} ions and protein should be absent.
- Keep the temperature and stirring conditions constant during both the calibration and the sample measurements.
- Before the first measurement, rinse both the reference and SA electrodes with DI water and dry them carefully, taking care not to touch the ISE membrane.
- Place a 19 mL aliquot of the sample in a 30 mL beaker, and then add 1 mL of ISA while stirring.
- Immerse the reference and ISA electrodes in the sample, ensuring that no bubbles remain attached to the ISE membrane.
- Measure the Ag ion content in the sample three times, employing an already prepared calibration curve.
- Between measurements, rinse both electrodes with DI water and dry them carefully, taking care not to touch the ISE electrode membrane.