

NANoREG

Grant Agreement Number 310584

Deliverable D 2.04

Protocol for quantitative analysis of inorganic and organic MNM surface coatings

Due date of deliverable: 2015/03/31

Actual submission date: 2015/11/09

Author(s) (Beneficiary(ies)):	NRCWE
Work package/task:	WP2 / Task 2.3n
Document status:	draft/ <u>final</u>
Confidentiality:	confidential / restricted / <u>public</u>
Key words:	

DOCUMENT HISTORY

Version	Date	Reason of change
1	2015/03/25	First draft by NRCWE
2	2015/11/07	Final draft send to WP2 leader
3	2015/11/09	Draft revised by WP2 leader and submitted to coordinator
4	2017/03/07 2017/06/21	Project Office harmonized lay-out

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*This project has received funding from the European Union
Seventh Framework Programme (FP7/2007-2013)
under grant agreement no 310584*



Lead beneficiary for this deliverable: Joint Research Centre, JRC, partner number 2

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A. D2.4 in the NANoREG context

Description of task 2.3n: SOPs for identification and quantification of chemical composition, inorganic and organic NM surface coatings

Validated procedures are necessary for identification and quantification of organic and inorganic chemical modifications of MNM. Accidental or deliberate organic surface coatings or functionalization of commercial MNM is common and there is a need to establish guidelines and standard operation procedures (SOPs) that can enable reliable identification and quantification of these chemical modifications to properly categorize such higher generation MNM for potential further assessment of their toxicological properties as well as their role in the analysis of particle characteristics and fate.

Description of the work done and main achievements

Summary

This deliverable presents a Technical Guideline and SOPs for identification and quantification of chemical surface modifications on MNM. The methods address both inorganic and organic surface coatings and functionalizations and can also be used to identify impurity phases and additives in MNM samples.

The key aim of the Technical Guideline in this deliverable is to establish a methodology that can enable identification and quantification of coatings and functionalizations on MNM. The analytical procedures support characterization needs in D2.2, developments on categorization and classification of MNM in a regulatory context in D2.5, data generation for grouping and read-across in WP1 and WP5. It can also support analysis required for assessment of safety by design in WP6 and the methods suite in the NANoREG Tool Box.

The Technical Guideline is constructed as screening procedure where initial analysis are made using relatively simple and inexpensive bulk methods to assess the potential presence of organics and inorganic compounds or foreign impurity elements. The presence of organics is screened by oven and hot furnace tests (loss-on-ignition; LOI) or thermogravimetric analysis (TGA). The presence of inorganic elements or compounds is screened using non-destructive bulk inorganic elemental analysis (here wave-dispersive X-Ray Fluorescence; WD-XRF). Mass-loss on LOI not ascribed to adsorbed water and carbonates and/or non-stoichiometric compositions of the MNM triggers more advanced analysis to determine the identity and amounts of the “impurities”. The advanced analysis includes a SOP for HAADF-STEM analysis to identify inorganic coatings, a SOP for characterization of non-covalently bound organic surface coatings, and a SOP for analysis of functional groups containing oxygen on CNT by TGA-MS. In the future the TG should be further expanded with SOPs to cover specific other chemical groups.

Background of the task

MNM may often be chemically modified by doping, coating, or functionalization or added chemical agents to improve e.g., their stability or dispersibility in liquids or solid matrices. Currently the 20 wt% impurity is accepted in the substance definition of REACH. Even-though, this impurity acceptance may be established

for pragmatic reasons considering e.g., impurities from processing or processing of natural substances, 20 wt% is far beyond concentration levels where deliberate chemical modification of MNM will or can change dramatic change in behavior and (eco-)toxicological effects.

Regulatory questions are concerned with tailored properties of higher-generation MNM and on how surface modifications should be considered in regards to grouping, categorization, and finally risk assessment. To enable an answer to these questions, it is necessary to first enable the ability to identify, quantify and group chemical modifications according to their composition, extent and structural location. Whereas D2.5 address the system for categorization and naming, this D2.4 is developing a first Technical Guideline with several analytical SOPs that can be used for identification and quantification inorganic and organic chemical modifications, including doping and surface coatings and screen MNM to identify chemical modifications of MNM from presence of from additives and associated impurities.

The deliverable is an output from Task 2.3 and belongs to the group of activities considering methods that can be proposed as new OECD Technical Guidelines. D2.4 will together with other feed into D2.12 as the final outcome from WP2 as well as all NANoREG activities considering definition and categorization of MNM, read-across, grouping, and risk assessment. The proposed SOPs are also considered important and suitable for quality control of MNM for characterization and toxicological studies in general and are therefore also of interest for the NANoREG Toolbox.

Description of the work carried out

A first proposal for general Technical Guideline and associated SOPs for identification and quantification of chemical surface modifications MNM has been established. The guideline and SOPs is intended to be proposed by NANoREG as a future work item for the OECD Working Party on MNM.

The Technical Guideline consists of an initial screening procedure using relatively simple methods to first identify MNM, which may have been chemically modified by doping and/or surface coating and/or functionalization, which is then followed by more advanced analysis using different proposed SOPs depending on the type(s) of possible modifications observed .

The Technical Guideline is primarily described for analysis of unknown MNM, however, the SOPs can naturally also be applied for quality control and quantification of chemical modifications and surface coatings of MNM with known chemical modifications. In such cases the low-cost simpler screening procedures could be sufficient to identify and quantify the chemical modifications of the MNM.

Even-though it was attempted several times, the Technical Guideline and associated SOPs have not been subject to interlaboratory comparisons or full internal validation. The NANoREG core MNM does not include a sufficient number and variety of chemically modified MNM to allow validation. Therefore, further work should involve testing of the proposed Technical Guideline and SOPs, interlaboratory comparison studies, and validation after SOP revisions as required.

Results

The Technical Guideline and SOPs are described in full hereafter in Part B and associated SOPs.

Evaluation and conclusions

This Technical Guideline is to our knowledge the first, which attempts to establish a systematic procedure for identification and quantification of chemical modifications and surface modifications on MNM by which higher generation MNM can be identified and distinguished from first generation MNM. We believe that the procedures are of high quality while also considering the pragmatic needs on quality to allow general implementation.

This deliverable establishes the basic tool in support of the characterization needs in D2.2 and the categorization and classification procedures in D2.5. Last, but not least, the D2.4 also establish the ability for the physicochemical characterization that can enable future analysis of grouping and read-across between first and higher generation MNM with different types and levels chemical modifications. The Technical guideline and SOPs are therefore also of relevance for the NANoREG Toolbox.

Deviations from the work plan

The deliverable has been subject to a number of delays due to technical issues in SOP developments, but also human resource issues, which made it impossible to keep the planned deliverable dates.



A common European approach to the regulatory testing of nanomaterials

B. Technical Guidance Document for

Quantitative analysis of inorganic and organic MNM surface coatings

*This project has received funding from the European Union
Seventh Framework Programme (FP7/2007-2013)
under grant agreement no 310584*



1. Background

Manufactured nanomaterials (MNM) are often chemically surface modified in order to tailor their physico-chemical properties for specific applications. For example, lipophilic surfaces are needed for incorporation of MNM in polymers whereas hydrophilic surfaces are used in water-based paints. The modifications are made by different classes of small molecules (such as silanes, small linkers enabling Click-chemistry, amino acids, etc.). Their presence may strongly influence the surface chemical nature as well as the external surface charge and size of nanoparticles both in water and in biological media. Therefore these surface modifications may influence the toxicological and environmental properties of the MNM. They are often trade secrets and their exact composition and formulation is not disclosed. The possible modifications of the surface of MNM can cause numerous interactions that affect their physico-chemical properties and play an important role in their toxicological behaviour. Specifically, a surface modification by organic moieties can cause changes in their water solubility, agglomeration state, surface charge and area, hydrophilic/hydrophobic character and specific surface area as well as in their interaction with biological entities or living organisms (Rivera Gil et al., 2010). Their nature (*e.g.* amines, carboxylic or hydroxyl chemical groups) will directly influence many relevant aspects of MNM lifecycle ranging from the interactions of nanoparticles with the bio-macromolecules present in biological matrices and cells, to cellular uptake and internal dose. Inorganic coatings are broadly used both during colloidal synthesis of nanoparticles and for their post-synthesis functionalization. For inorganic oxide MNM deposition of inorganic coatings and layers on inorganic cores is typically considered the best approach to modify the surface properties of this type of MNM. Surface modification has a major influence on surface reactivity, by enhancing or depressing the pristine features, especially when catalytically active nano-sized materials are concerned (Dal Santo et al., 2012). Hence, the characterization and the quantification of the surface chemistry are central to highlight the dynamic interactions of nanoparticles with biological systems as recommended in several review papers (Bouwmeester et al., 2011; Stone et al., 2010; Hassellöv et al., 2008).

The strategy for the conjugation of coating/ligand-molecules to nanoparticles generally falls into four classes according to Sperling and Parak (Sperling and Parak, 2010): 1) ligand-like binding by chemisorption, 2) electrostatic adsorption, 3) covalent binding 4) non-covalent, affinity-based receptor-ligand systems (adsorption). The stronger chemical bonds such as the covalent bonds may require special chemical detachment to be characterized quantitatively whereas more loosely adsorbed coating may be quantified by simple extraction. Few papers describe quantitative methods to characterize organic surface coatings of MNM. Thermogravimetric analysis (TGA) is often an important part of the quantification of both covalent bound organic coating as *e.g.* in carbon nanotubes (CNT) (Wepasnick et al., 2010; Jackson et al., 2015) and more loose bound coatings as *e.g.* polyvinylpyrrolidone coated on nanosilver particles (Nymark et al., 2013). Zhang and Yan (2010) reviewed characterization of the surface chemistry of nanoparticles with nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FTIR), mass spectrometry (MS), x-ray photoelectron spectroscopy (XPS), and combustion elemental analysis of C, H, N, O, and S, though the detection limit and standard deviation of this technique sometimes is too high to give a proper quantification. Liu and Yan (2011) reviewed the same methods for characterizing surface chemistry of nanoparticles and pointed out that they in many cases will be similar to methods used for solid-phase synthesis samples. Baer et al. (2010) reviewed the application of surface chemical analysis methods for characterization of especially the coating of MNM. Wepasnick et al. (2011) reviewed methods for characterization of surface chemical and structural changes that occur to CNT as a consequence of covalent functionalization. Du et al. (2009) reviewed NMR methods for characterization of organic coatings of MNM. In addition, XPS, coupled with other techniques such as transmission electron microscopy (TEM), high resolution transmission electron microscopy (HRTEM), Raman and scanning electron microscopy (SEM), has also been used to investigate the surface chemistry of several nanomaterials ranging from gold (Au)

nanoparticles (Techane et al., 2011), to CNT (Yang et al., 2005), and from polymers (Sun et al., 2008), to metal oxides (Baer et al., 2008). But these surface spectroscopy techniques have their main limitation in the need of high-vacuum conditions reducing the knowledge of interfacial physico-chemical processes under actual environmental conditions. Only a few studies describe the use of MS methods for quantitative characterization of organic surface coatings despite of the obvious advantages.

Inorganic surface coatings modify completely the chemical nature and morphology of nanosized particles and it is a major challenge to identify and characterize heavier elements and metals at the nanoscale. Characterization of the surface chemistry of such nanoparticles requires therefore analysis using spectroscopy, such as XPS and Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS) (Zorn et al., 2011). Atomic-scale techniques, such as conventional TEM, although powerful, are limited by the extremely small amounts of material that are examined. However, recent advances in electron microscopy provide a number of new analytical techniques that expand its application in studies of nanoparticles, particularly those concerning heavy metals. High-angle annular dark field scanning TEM (HAADF-STEM), STEM-energy-dispersive X-ray spectrometry (EDX), and energy-filtered TEM (EFTEM) can be effectively used to identify and characterize nanoparticles (Utsunomiya and Ewing, 2003).

2. Aim

One of the key requirements in comparative testing is that all preparation and subsequent characterization procedures are as harmonized and inter-calibrated as possible. Toxicity and fate assessment are key elements in the evaluation of the environmental, health and safety risks of MNM. While significant effort and resources have been devoted to the toxicological evaluation of many MNM, obtaining conclusive and reproducible results continues to be a challenge. This can be traced in part to the lack of standardized protocols for characterization of MNM. This technical guidance document describes proposals for analytical standard operating procedures (SOP) that are needed to reliably enable detection and quantification of unknown surface modifications on MNM.

Three SOPs for characterization of unknown MNM with unknown surface coating on the screening level was developed. The first one is a method using TGA for determination of the amount of a possible organic coating on an inorganic core particle or the amount of carbon in a possible carbon based MNM (Appendix I). The second is a SOP for determination of water, organic and carbonate content (loss-on-ignition: LOI) using weighing and heating in drying oven and laboratory furnace and serves as a low cost alternative to TGA (Appendix II). The third one is a method using wavelength dispersive x-ray fluorescence spectroscopy (WDXRF) to determine the elemental composition and thus indicates a possible organic coating or a possible carbon based MNM (Appendix III).

An approach for specific characterization of organic surface coatings on MNM based on TGA, several extraction techniques and off-line MS techniques was developed (Appendix IV). This is a further development of a TGA method, without, or in combination with MS detection of effluent gasses (Clausen et al. 2014), and several extraction and off-line MS techniques for characterization of extractable and non-covalently bound organic coatings of MNM (Clausen et al., 2015).

An approach for specific characterization of inorganic coatings was developed (Appendix V). This approach involves an evaluation the total elemental composition of an inorganic MNM using WDXRF and specific analysis of a possible inorganic surface coating using electron microscopy (EM). The fundamental WDXRF data are matched and compared to those obtained by EM that can be used to successfully establish the

nature of inorganic coating in some specific cases. This is possible by using HAADF-STEM technique in combination with STEM-EDX to discern the nature of the core MNM and the coating around it.

Finally, an approach for specific characterization of oxygen containing functional groups attached to the outer walls of CNT was developed (Appendix VI). This approach is based on the ability of oxygen containing functionalities on the CNT surface to decarboxylate, decarbonylate and/or dehydrate during heating (Kundu et al., 2008; Datsyuk et al., 2008). The method was developed for TGA with MS detection of decarboxylated CO₂, decarbonylated CO and/or dehydrated water intended for future peer-reviewed publication (Clausen, 2015).

3. Scope

The proposed procedures are developed for the purpose of characterization the surface modifications/coatings of MNM and not the core MNM itself, though it may be deduced from the generated results. There exist an infinite number of possible combinations of surface coating molecules, core MNM and binding types, e.g. electrostatic adsorption or covalent binding. Therefore it is not possible to cover all combinations. Here we present procedures that relative easy can indicate the presence of an inorganic, organic or no coating or whether we have to do with a carbon based material such as the CNT. The further procedures that can actually identify an organic or inorganic coating are complicated and are only applicable in specific cases e.g. only applicable for extractable organic compounds i.e. non-covalently bound organic compounds. In the case of CNT a procedure which can determine the amount of oxygen containing functional groups on the surface of CNT is presented.

In all cases the proposed SOPs should be used together with other characterization techniques such as EM and collection of all possible information on the MNM under investigation.

4. Overview of the procedures

The overall procedure consists of six SOPs of which three SOPs are for the screening level and three are on the specific level (see Figure 1).

The first step to be carried out before any of the SOPs are considered is collection all possible information on the MNM of interest from manufacturer and other sources. This includes all available analysis results such as for example EM in reports within the NanoReg consortium, from other projects, and in the peer-reviewed literature.

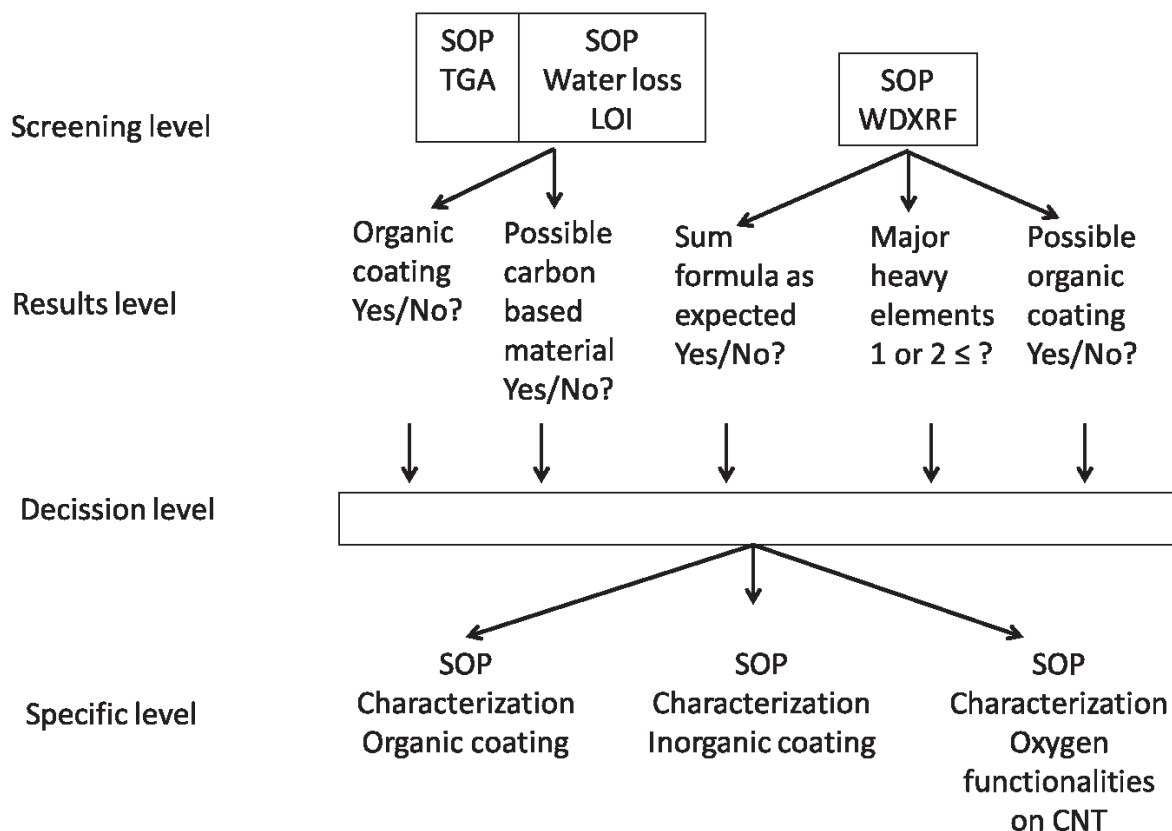


FIGURE 1. FLOWCHART AND OVERVIEW OF THE PROCEDURES.

Analysis on the screening level includes TGA (Appendix I) and WDXRF (Appendix III). LOI and water loss on heating (Appendix II) may be used as a simpler alternative to TGA if such instrumentation is not available. TGA with an IR or MS detector will increase the application range by indications of the composition of the effluent gasses.

Decisions on further analysis depend on the results obtained on the screening level. If the results clearly point to an inorganic MNM coated with an organic surface (Appendix I or Appendix II and Appendix IV), an inorganic MNM coated with an inorganic coating (Appendix III and Appendix V), or a carbon based MNM (Appendix I or Appendix II and Appendix VI) it is not difficult to decide to go further with the respective SOP. It may happen that the results are not conclusive, but here you have to go further with the type of MNM that are most likely.

After analysis on the specific level a combined interpretation of the screening and specific level results may be carried out to conclude on the identity and amount of surface coating on the MNM of interest. It may well happen that it is not possible to draw a final conclusion. Then it has to be decided on further analysis with methods not covered by this guidance document. There may be several reasons for such unsuccessful results. For inorganic MNM with organic surface coating reasons include covalently bound coating or a very complex structure of the MNM. Here not only surface techniques such as IR, XPS, and MALDI-TOF-MS may be considered, but also elemental analysis such as combustion elemental analysis (CEA) to determine the content of carbon, hydrogen, nitrogen and oxygen. For inorganic MNM with possible inorganic coating reasons include unknown, unusual, or complex crystal structure of the core MNM, of the coating or of both.

Supplementary methods to be applied include x-ray diffraction to uncover the crystal structure. For carbon based materials the reasons include functionalizations containing elements other than oxygen and here CEA would be a help. Other important properties of carbon based MNM includes the content of amorphous carbon which can be deduced using Raman spectroscopy.

5. Illustrative examples of the procedures

5.1 MNM with organic coating

In the following the procedure for identification of organic surface coating on MNM by applying different extraction techniques and mass spectrometry methods is illustrated by a case example on nano-sized silver particles (nano-Ag) which were shown to consist of 85 % (w/w) silver by WDXRF and to be associated with 15 % (w/w) organic compounds by TGA (Nymark et al., 2013). These results were in accordance with the information on surface coating of the nano-Ag given by the manufacturer.

Without knowing that the finally identified surface coating was broken down at 200 °C the first extraction method applied was pressurized liquid extraction (PLE) with methanol at 200 °C. The extract was centrifuged to remove all residual nano-Ag which was not filtered off in the PLE process. The supernatant in different volumes (1-40 µl) was injected onto adsorbent tubes containing Tenax TA and analyzed with TD-GC-MS. This showed that the extract only contained one compound, namely 2-pyrrolidone.

The idea that 2-pyrrolidone might be a thermal degradation product emerged and consulting the literature confirmed that both 2-pyrrolidone, and N-vinyl-2-pyrrolidone may be thermal degradation products of polyvinylpyrrolidone (PVP) and that PVP often is used in synthesis mixtures of nano-Ag.

Now we had to prove that the coating consisted of PVP. For this purpose the nano-Ag was subjected to ultrasonic extraction in methanol at ambient temperature. The extract was centrifuged to sediment all nano-Ag and the supernatant analyzed by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) (see Figure 2).

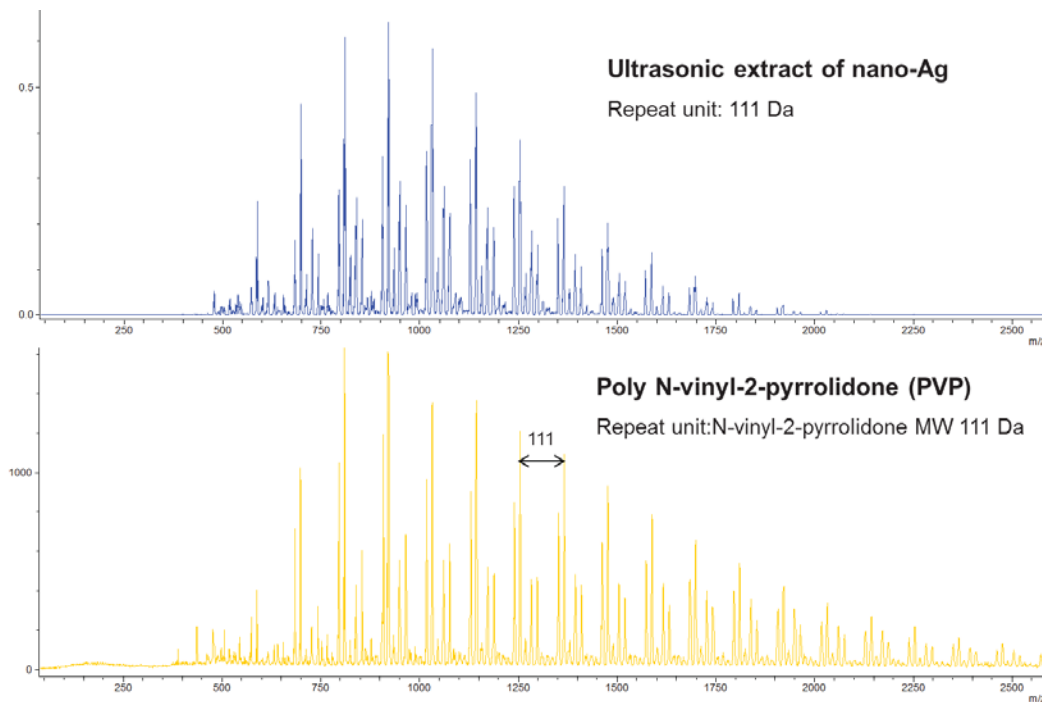


FIGURE 2. MALDI-TOF-MS MASS SPECTRA OF THE ULTRASONIC EXTRACT OF NANO-AG (UPPER PANEL), AND A STANDARD OF PVP WITH AVERAGE MW 40,000 (LOWER PANEL).

Though the distributions of masses in the two mass spectra shown in Figure 2 are not exactly identical, the pattern with the repeat unit of 111 Da reveals that the coating is PVP. The differences may be due to different average MW of the PVP on the nano-Ag and the PVP standard.

As a final proof that the coating on the nano-Ag consisted of PVP only, it was decided to quantify the amount of PVP on the nano-Ag using the ability of PVP to thermally degrade to 2-pyrrolidone and N-vinyl-2-pyrrolidone in reproducible manner. Thus the ultrasonic extracts of nano-Ag which contained the extracted PVP was injected onto adsorbent tubes with Tenax TA and analyzed with thermal desorption combined with gas chromatography and mass spectrometry (TD-GC-MS) to measure the formation of N-vinyl-2-pyrrolidone from thermal degradation of PVP during the TD process at 250 °C. A calibration curve was made in a similar manner by injections of standard solutions of PVP in methanol on Tanax TA adsorbent tubes and analyzed by TD-GC-MS to measure the formation of N-vinyl-2-pyrrolidone. The reading on this curve for the formed N-vinyl-2-pyrrolidone from the extracts of nano-Ag showed a content of 23 % (w/w) of PVP on the nano-Ag. A little overestimated compared to the 15 % (w/w) determined by TGA but precise enough to conclude that the surface coating of the nano-Ag consisted solely of PVP.

5.2 MNM with inorganic coating

In the following the procedure for identification of inorganic surface coating on MNM by applying WDXRF to get the bulk elemental composition and the scanning transmission electron microscopy (STEM) technique, HAADF-STEM (high angular annular dark field), to identify the inorganic coating, is illustrated by a case example on JRCNM01003a/NM-103, which is a Al-coated TiO₂ MNM. The WDXRF analysis showed a content of 96 % (w/w) TiO₂ and 3 % (w/w) Al₂O₃.

The HAADF-STEM technique is particularly important in the study of inorganic coatings on the surface of MNMs. In this technique one uses the Annular Dark Field (ADF) detector to collect the signal that produces

the image. With the annular detector, only the electrons scattered more than a minimum angle θ are able to reach the detector and contribute to the image. At high angles (more than 50 mrad), electrons are scattered incoherently, with essentially no Bragg scattering present. The image intensity is then mostly due to the individual contribution of each atom to the scattering. It has been previously demonstrated that the signal collected by the HAADF detector will have an intensity directly related to the scattering cross-section, and thus it will depend on the atomic number of the atoms in the specimen. Due to this dependence on the atomic number (Z) of the atoms on the specimen, the contrast in this imaging technique is strongly dependent on the chemical composition, and for this reason it is called Z-contrast imaging. The Z-contrast technique works remarkably well with different Z materials, because the intensity dependence is close to $Z^{3/2}$, with minimum dependence on microscope defocus. This is definitely different from what is expected of bright field (BF) imaging, where the signal varies weakly and non-monotonically with Z . The Z dependence technique allows identifying easily the elements present in the sample. Moreover, as the electron beam interacts with the sample, the electrons are elastically or in-elastically scattered. The signals of those elastically scattered electrons are the sources of STEM images that can be recorded either in HAADF or ADF detectors. The signals of in-elastically scattered electrons are the sources of chemical information present in the sample. This in combination with analytical techniques such as EDS (energy-dispersive X-ray spectroscopy) or EELS (electron energy loss spectroscopy) can provide very clear and unambiguous information especially in the present example related to the fact that MNMs have inorganic coated species around it. Figure 3, shows a typical BF-TEM and HAADF-STEM images of the TiO_2 NPs (JRCNM01003a). A closer imaging and analysis of the TiO_2 NP – AlO_x inorganic coating by EDS chemical analysis is provided in Figure 4 – Figure 6. The TiO_2 nanoparticle is crystalline whereas the outer inorganic coating (AlO_x) is amorphous. It is also important to note here that the analysis is not only qualitative but more importantly quantitative information can be extracted easily in EDS (or EELS) (See Table 1 and 2) from the respective analytical signals; EDS in this case.

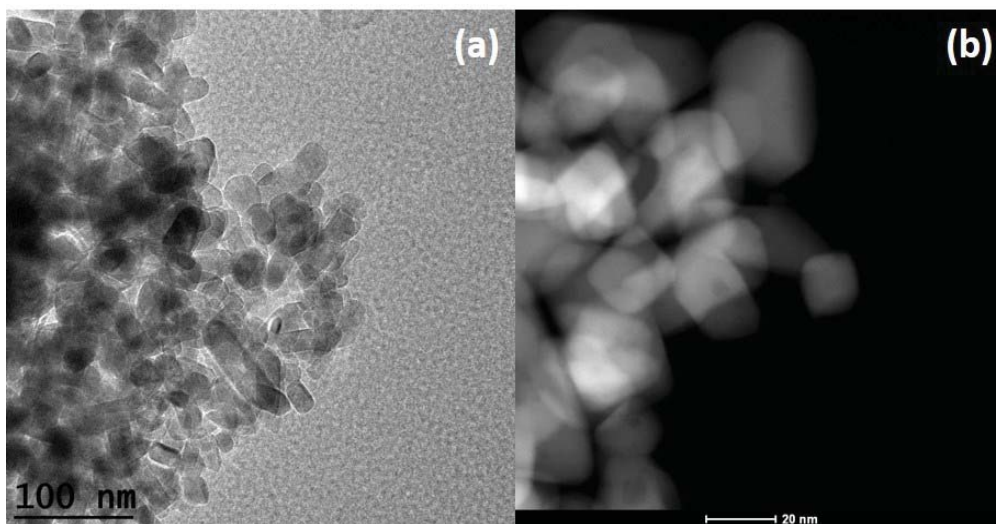


FIGURE 3. (A) BF-TEM AND (B) HAADF-STEM IMAGES OF THE TiO_2 NANOPARTICLES (JRCNM01003A).

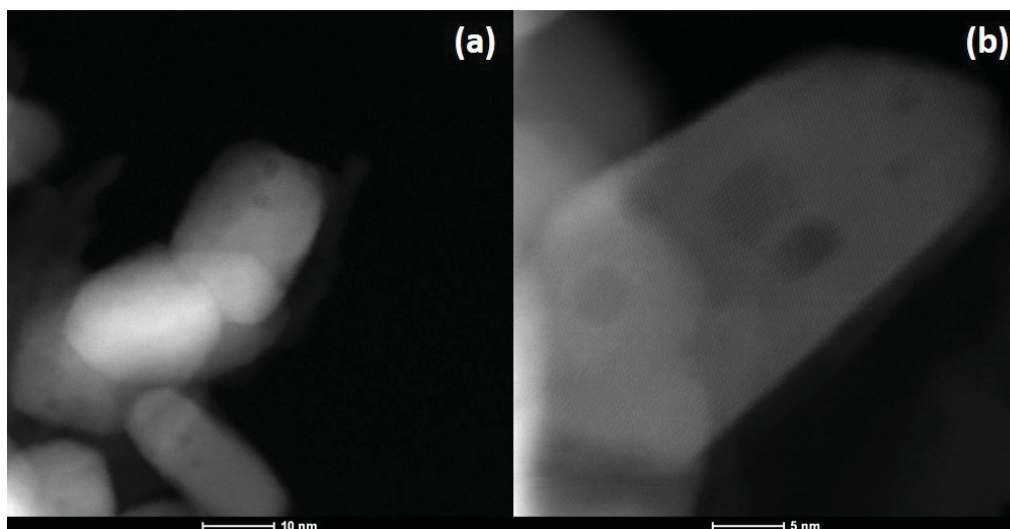


FIGURE 4. HAADF-STEM IMAGES OF (A) COLLECTION OF TiO_2 NANOPARTICLES AND (B) INDIVIDUAL TiO_2 NANOPARTICLE. SINCE THE INTENSITY ALPHA Z^2 ($Z_{\text{Ti}} > Z_{\text{Al}}$, $Z(\text{Ti}) - 22$, $Z(\text{Al}) - 13$) IT IS POSSIBLE TO SEE THE DIFFERENCE IN CONTRAST BETWEEN THE TiO_2 NANOPARTICLE AND THE AMORPHOUS INORGANIC COATING (AlO_x) AROUND IT.

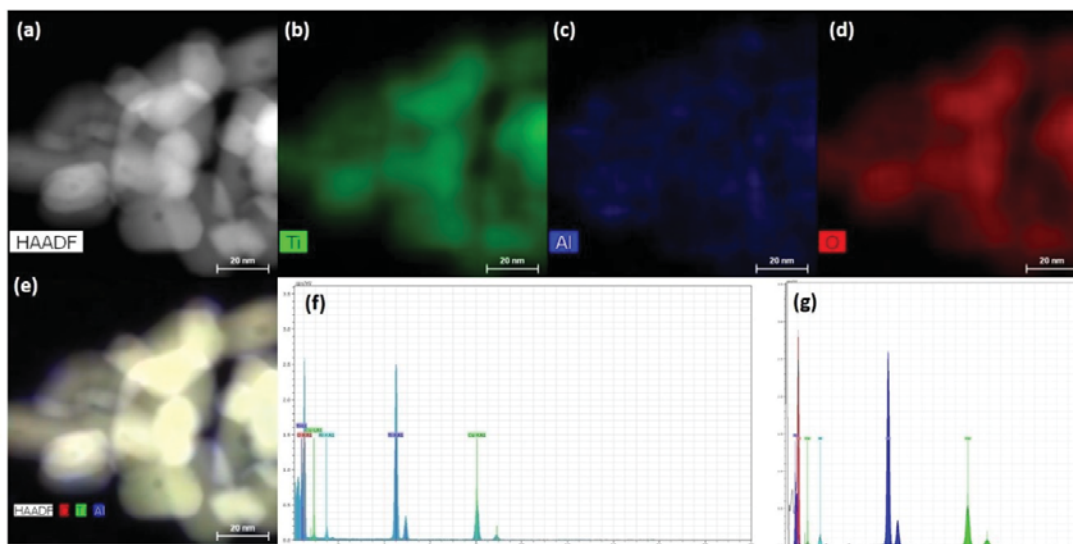


FIGURE 5. (A) HAADF-STEM IMAGES OF A COLLECTION OF TiO_2 NANOPARTICLES. (B) – (D) CORRESPONDING EDS MAPS OF THE NANOPARTICLE AND THE INORGANIC COATING SEEN WITH THE ELEMENTS CORRESPONDING TO TI, AL AND O. (E) COMBINED EDS MAP OF THE ELEMENTS. (F) EDS SPECTRUM OBTAINED FROM THE SAME REGION (G) DECONVOLUTION OF THE PEAKS OF THE EDS SPECTRUM AND QUANTIFICATION OF THE VARIOUS ELEMENTS (TI, AL, O) CARRIED OUT (TABLE 1).

TABLE 1. QUANTIFICATION OF THE ELEMENTS PRESENT IN THE NANOPARTICLE-INORGANIC COATING (TiO₂-AlO_x) IN A COLLECTION OF NANOPARTICLES. NOTE: CU SIGNAL ARISING FROM THE GRID WAS USED ONLY FOR THE DECONVOLUTION AND NOT FOR THE QUANTIFICATION.

Element	series	Net	[wt.%]	[norm. wt.%]	[norm. at.%]	Error in wt.% (3 Sigma)
Oxygen	K-series	41380	46,31496	46,31496	71,64216	4,887759
Copper	K-series	28002	0	0	0	0
Titanium	K-series	96556	52,16472	52,16472	26,96335	4,800459
Aluminium	K-series	3261	1,520315	1,520315	1,394497	0,233702
		Sum:	100	100	100	

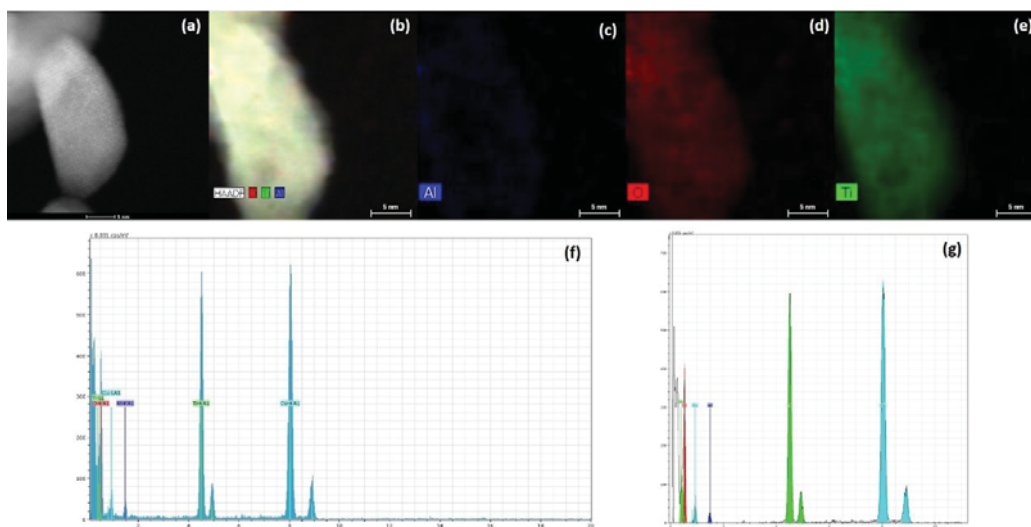


FIGURE 6. (A) HAADF-STEM IMAGES OF A SINGLE TiO₂ NANOPARTICLE. (C) – (E) CORRESPONDING EDS MAPS OF THE NANOPARTICLE AND THE INORGANIC COATING SEEN WITH THE ELEMENTS CORRESPONDING TO Ti, Al AND O. (B) COMBINED EDS MAP OF THE ELEMENTS. (F) EDS SPECTRUM OBTAINED FROM THE SAME REGION (G) DECONVOLUTION OF THE PEAKS OF THE EDS SPECTRUM AND QUANTIFICATION OF THE VARIOUS ELEMENTS (Ti, Al, O) CARRIED OUT (TABLE 2).

TABLE 2. QUANTIFICATION OF THE ELEMENTS PRESENT IN THE NANOPARTICLE-INORGANIC COATING (TiO₂-AlO_x) OF A SINGLE NANOPARTICLE. NOTE: CU SIGNAL ARISING FROM THE GRID WAS USED ONLY FOR THE DECONVOLUTION AND NOT FOR THE QUANTIFICATION.

Element	series	Net	[wt.%]	[norm. wt.%]	[norm. at.%]	Error in wt.% (3 Sigma)
Oxygen	K-series	2353	34,90844	34,90844	61,23624	4,265045
Titanium	K-series	8898	63,75088	63,75088	37,3692	6,206788
Aluminium	K-series	217	1,340675	1,340675	1,394564	0,375251
Copper	K-series	12455	0	0	0	0
		Sum:	100	100	100	

Based on the screening level WDXRF and the specific level HAADF-STEM analysis it may be concluded that it is an inorganic core MNM consisting of about 96%w/w TiO₂ with an inorganic coating consisting of about 3%w/w Al₂O₃.

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7. Appendix I - VI

Appendix I. SOP for determination of organic content in MNMs by thermogravimetric analysis (TGA)

SOP for determination of organic content in MNMs by thermogravimetric analysis (TGA)

Version 1.0

Work in progress (revision is being tested)

Date: November 5, 2015

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Tested and commented by NANOREG Deliverable D2.4, Task 2.3n participants:

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Foreword

This SOP (Standard Operating Procedure) is one in a series of protocols resulting from a collaboration of the partners in the NanoReg project. The original version of this protocol (Ver. 1.0) is stored in the NANoREG area on CIRCABC. Updates to this protocol may be released in the future and within the duration of the NANoREG project, such updates will also be stored on CIRCABC. The latest version of the SOP will always be available from the NANoREG homepage.

Please visit <http://nanoreg.eu/> for updates and to retrieve the latest version of the protocol as well as other NANoREG methods for MNM characterization.

The NANoREG consortium is interested in soliciting feedback on this method. We value user comments and suggestions to improve or further validate this protocol. Please send your name, email address and comments/suggestions to Per Axel Clausen (pac@nrcwe.dk). We also encourage users to report citations to published work in which this protocol has been applied.

Certain commercial entities, equipment or materials may be identified in this document in order to describe an experimental procedure or concept adequately. Such identification is not intended to imply recommendation or endorsement by the National Research Centre for the Working Environment (Denmark) and the NANoREG project team, nor is it intended to imply that the entities, materials or equipment are necessarily the best available for the purpose.

1. Introduction

1.1 Background

One of the key requirements in comparative testing is that all preparation and subsequent characterization procedures are as harmonized and inter-calibrated as possible. Toxicity and fate assessment are key elements in the evaluation of the environmental, health and safety risks of manufactured nanomaterials (MNMs) and nano-sized engineered solids. While significant effort and resources have been devoted to the toxicological evaluation of many MNMs, obtaining conclusive and reproducible results continues to be a challenge. This can be traced in part to the lack of standardized protocols for characterization of MNMs.

The present protocol describes a simple and robust procedure to quantify the amount of volatile and/or decomposable organic matter present on the surface of and/or in a MNM using thermogravimetric analysis (TGA). TGA is a technique that measures the change in weight of a sample when it is heated, cooled or held at constant temperature. Its main use is to characterize materials with regard to their composition. When performed under oxidizing conditions (*i.e.*, under air or pure oxygen flow), TGA provides useful information about the content of oxidizable compounds (e.g., hydrocarbons, organic compounds, coke). With regard to investigation of possible unknown surface coatings of unknown MNM the application range of TGA can be strongly extended by use of an infrared (IR) or mass spectrometry (MS) detector to determine the composition of the effluent gasses from the TGA instrument.

1.2 Purpose and background for the SOP

The purpose of this SOP is to describe in detail a simple and robust procedure to quantify the amount of volatile, oxidizable and/or decomposable organic matter present on the surface of and/or in a MNM using TGA. This SOP complies with the ASTM standard E1131 and is consistent with the ASTM standards E2040 and E2402, and with the International Confederation for Thermal Analysis and Calorimetry (ICTAC) recommendations of the International Union of Pure and Applied Chemistry (Lever et al. 2014); it was prepared in collaboration with all partners participating in deliverable D2.4 of the NANoREG project.

2. Principles and scope

TGA is a technique that measures the change in weight of a sample when it is heated, cooled or held at constant temperature in a controlled atmosphere. Its main use is to characterize materials with regard to their composition. In particular, TGA is used to determine a material thermal stability and its fraction of volatile and/or decomposition components by monitoring the weight change that occurs as a sample/specimen is heated. The measurement is normally carried out in air or in an inert atmosphere, such as N₂, He or Ar, and the weight is recorded as a function of increasing temperature.

A TGA instrument consists of a sample pan (crucible) that is supported by a precision balance. This pan resides in a furnace with an accurate temperature control and is heated or cooled during the experiment. The weight of the sample is monitored during the experiment. A sample purge gas flow controls the sample environment, whereas a balance purge gas flow protects the weighing system from contamination due to heavy by-products in evolved flow.

The sample purge gas may be an inert (typically, He, N₂ or Ar) or reactive gas (typically, air, synthetic air or pure O₂) that flows over the sample and exits through an exhaust. For some specific purposes, the analysis may also be performed under lean oxygen atmosphere (1 to 5

vol% O₂ in He, N₂ or Ar) to slow down the oxidation process. Other reactive gases may be used providing they do not react with furnace components, thermocouple or other analyzer components which the gases come in contact with.

The recommended balance purge gas is an inert gas such as N₂ or Ar.

While TGA may not be the primary technique for structure determination, it often plays an important role in pre-evaluating materials prior to other material characterization techniques. It can be used as a part of the physico-chemical characterization of MNMs required as prior knowledge for toxicological testing and environmental compatibility assessment. By heating milligram-scale quantities of sample under a defined protocol, including time-temperature profile and sample gas composition, the TGA can detect and quantify the presence of volatiles and/or adsorbates (e.g., water, solvents) and decomposable organic matter by accurately measuring the weight loss the sample undergoes during heating. The temperature over which volatilization, decomposition or combustion occurs provides information about the volatilized/decomposed/oxidized species and this helps in the qualitative identification and quantitative characterization of these species.

The task for the TGA operator is to obtain reliable weight losses and onset temperatures using a prescribed temperature and sampling gas program. Obtaining consistent, high-quality TGA data does not require the dedicated presence of a research-level expert, however the operator should carry out the TGA experiments following a clear, simple and robust SOP.

3. Terminology and abbreviations

The terminology and abbreviations used in this protocol are consistent with the International Confederation for Thermal Analysis and Calorimetry (ICTAC) recommendations of the International Union of Pure and Applied Chemistry (Lever et al.2014).

TGA —thermogravimetric analysis

N₂— nitrogen

O₂— oxygen

He— helium

Ar— argon

MNM—manufactured nanomaterial

vol% - volumetric percentage (in gas composition)

For more information on the terminology and abbreviations in the TGA field, see (Lever et al. 2014).

4. Equipment, materials and chemicals

4.1 Equipment

Semi-micro-analytical balance (4 decimal digits) for weighing the samples.

Platinum sample pan (crucible).

Spatula.

Tweezers.

TGA thermogravimetric analysis instrument. We used a Perkin-Elmer TGA 7 HT Thermogravimetric Analyzer equipped with a High-Temperature Furnace (50 °C – 1500 °C)

and platinum hangdown parts. Perkin-ElmerPyris 700 was used as thermal analysis system software.

4.2 Materials

MNM approved by NANoREG Management Committee.

4.3 Chemicals

High-purity (99.999 vol%, i.e. 5.0 purity) dry inert gas (N₂, He, Ar, etc.) or oxidizing gas (air, O₂ in inert mixture, etc.) is recommended as sample purge gas.

Pure (minimum 99.9 vol%, i.e. 3.0 purity) dry inert N₂ or Ar gas is recommended as balance purge gas.

The use of oil-lubricated valves, joints or gas pipelines has to be carefully avoided, in order to minimize contamination from the flowing gas.

5. Preparations

5.1 Cleaning, validation, and calibration

To ensure that previous experiments and measurements have not left behind semi-volatile decomposition deposits, it is highly recommended to carry out a rapid high-temperature conditioning run with empty holder and crucible and oxidizing gas purge (typically, dry air). If the TGA has not been used for some time, it is also advisable to make a weight and/or temperature validation run with a known material and - if needed - perform a complete calibration.

5.2 Sample purge gas control

One source of variation in TGA results arises from incomplete control of sample purge gas flow rate and composition in the furnace chamber during analysis. Analogously, the flow rate of the sample purge must be less than the flow rate of the balance purge, in order to avoid that decomposition by-products may be deposited in the balance chamber and eventually damage the weighing apparatus. These critical aspects require operator diligence to check the gas purge flow and ensure that it meets the specification. Changes in source pressure or line leakage can lead to inconsistent data that would be impossible to reproduce in subsequent experiments. Purge gas related issues are virtually eliminated when the purge flow is determined by digital mass flow controllers (rather than by line pressure) and the flow rates are under computer control using settings specified by the selected TGA method. Excessive purge gas flow should be avoided since this may introduce buoyancy effects and temperature gradients.

5.3 Weight loss validation

Validation is a process designed to demonstrate that an instrument works as intended. Validation tests yield figures-of-merit data for repeatability, linearity, detection limit, quantification limit and bias when running standard tests on a series of well-characterized materials. Such a standardized test for mass loss validation has been under development by ASTM standard E2402 (ASTM E2402). The test materials consist of blends of two materials that boil at quite different, elevated temperatures. These pre-blended reference materials are available from TA Instruments. They can be used to validate that a TGA system is capable of generating accurate data, or they can be used on a regular basis to generate figure-of-merit data as a means of tracking instrument performance.

In addition, in special cases (especially, when very small samples are analyzed or fast gas flows are used in particular instrument geometries), blank correction experiments for buoyancy effects may need to be performed. Buoyancy effects can cause apparent increase or decrease

in weight, which is not related to chemical or physical changes in the sample, but rather to a combination of factors, not directly linked to the nature of the specimen. Such factors may include: genuine buoyancy effects (due to the change of analysis gas density during the temperature programme), onset of convection currents within the measurement section of the instrument, changes in gas flows or density as a function of the temperature during the analysis as well as direct thermal effects on the balance mechanism (depending of the geometrical and instrumental characteristics of each instrument). In the analysis software of most TGA instruments, data for buoyancy run can be stored and subtracted from the actual experiment runs to give actual weight changes.

5.4 Balance calibration

Balance calibration usually is an instrument-provided routine that uses a calibration weight (typically, 100 mg, class M, ASTM) that is supplied with the instrument. A standardized test for weight scale calibration validation can be found in ASTM Standard E2040. It is performed after the balance calibration routine cited above and involves weighing a reference material of known mass (traceable to a national standards laboratory) with TGA balance and comparing the result with the certified material value.

5.5 Furnace calibration

Furnace calibration usually is an instrument-provided routine that allows the operator to match the thermocouple temperature measurement with the furnace temperature in a desired temperature range.

5.6 Temperature calibration

The best standard for TGA temperature calibration is the use of ferromagnetic standards. These materials undergo a reversible, second-order phase transition from the ferromagnetic to the paramagnetic state when a critical temperature (Curie temperature) is exceeded and lose the strong magnetization typical of ferromagnetic materials. A magnet appropriately placed above or below the furnace provides the magnetic field that attracts the reference materials placed in the sample holder. When going through the Curie transition the reference material magnetization vanishes resulting in an apparent weight change.

Each ferromagnetic material undergoes the ferromagnetic-paramagnetic transition at a reproducible, characteristic temperature that is only determined by the chemical composition of the ferromagnetic sample. A working group under the auspices of the International Congress for Analysis and Calorimetry (ICTAC) selected a series of alloys having Curie temperatures approximately every 200 °C between 152 and 1116 °C and the Curie temperatures of these reference materials were determined in an interlaboratory test (Gallagher, 2003). These materials (alloys of cobalt and nickel) are available from TA Instruments. Alternatively, Curie Point calibration kits spanning between 163°C (Alumel alloy) and 1000°C (Hisat-50 alloy) are available from Perkin-Elmer.

5.7 Final recommendation

While cleaning, gas purge control, validation, and calibration are highly advisable steps to ensure that trustable results are obtained. These steps are time-consuming and are often omitted by less experienced operators for the sake of saving time. We recommend that these procedures are carried out when appropriate.

How often should calibration be done? This depends on the nature of the samples to be measured. If the samples are relatively clean and do not coat the furnace or the furnace tube, then it is recommended to check daily, weekly, or somewhere in between. Once the operator gains an understanding of the nature of the robustness of the calibration and how often it changes, it is then possible to change the checking interval too; for instance, every second day. If the TGA instrument is moved from one place to another, then a new calibration is strongly advisable. Again, if the instrument is shut down for a long period of time, a calibration check is necessary.

6. Procedure

6.1 Preparation of the TGA

Set the flowrate of the sample purge gas at 40 ml min⁻¹ and the flow rate of the balance purge gas, at 60 ml min⁻¹.

6.2 Weighing the MNM sample

1. Weigh the appropriate MNM amount (see below) using an analytical balance under a fume hood.
 2. Transfer the weighed MNM sample into the sample crucible using a spatula under a fume hood. Hold the crucible with tweezers. It is essential to avoid any contamination of the sample and of the platinum tools (for instance, crucible and hanging stirrup) by skin moisture and/or grease.
- The amount of MNM should be between 2 and 50 mg. If you have only a tiny amount of sample, measure at least 1 mg. When expecting significant weight loss > 50 %, 5 mg of powder is appropriate; if expecting 5-20 % weight loss, then 10-20 mg of powder is appropriate.
 - If the material decomposes giving large quantities of gas, it could lead to an expulsion of the sample from the crucible. In this case, one must fill the crucible not more than one third of the global crucible capacity.
 - If possible, entirely cover the bottom of the pan with the sample material. Many small pieces of sample are better than one large chunk. It is better to have a large surface area exposed to the purge gas.
 - Once you have decided on the sample amount, then use approximately the same sample amount for all experiments to ensure reproducibility.

6.3 Performing the TGA

1. Using the tweezers, place the crucible in the TGA instrument measuring space.
2. Set the time – temperature profile as follows
 - a. Heat from room temperature up to 50 °C at rate 10 °C min⁻¹
 - b. Hold at 50 °C for 1 min
 - c. Heat up to 100 °C at rate 2.5°C min⁻¹
 - d. Hold at 100 °C for 10 min
 - e. Heat up to 800 °C at rate 2.5°C min⁻¹
 - f. Hold at 800 °C for 1 min
 - g. Cool down to room temperature

Total duration of the analysis program (from 50 °C): 312 min
3. Save experimental results recording the percent weight vs. sample temperature curve (that is, the actual temperature measured at the thermocouple)
 - Step 2.d is of particular importance when liquid, solution, dispersion or suspension samples are analyzed

6.4 Sample removal and disposal

1. Ensure that the furnace is close to room temperature
2. Remove the crucible and empty it under the fume hood
3. Remove the residue from the crucible using the spatula
4. Clean the crucible using clean paper
5. Dispose of the residue and dirty paper following your Institution's procedures for wastes

In case of residues particularly difficult to remove, the platinum sample crucible can be washed with concentrated (ca. 68% in water) nitric acid and then thoroughly rinsed with deionized water. The crucible must be subsequently dried carefully to remove any trace of water or moisture.

7. Expected outcome and interpretation of results

7.1 Residual mass and volatilization/decomposition/oxidation temperature

In most cases, TGA is performed in an oxidative atmosphere (air or oxygen/inert gas mixtures) with a linear temperature ramp. The maximum temperature is selected so that the sample weight is stable at the end of the experiment, implying that all chemical reactions, including volatilization and decomposition, are completed. For nominally inorganic MNMs this usually means that metal oxides are left; in particular cases pure metals (e.g. Au, Pt). This approach provides the residual weight (W_{res}) and allows one to get more information about the nature of the organics by determining the volatilization/decomposition/oxidation weight losses (W_i) and the correspondent temperatures (T_i).

The evaluation of W_{res} is an important parameter to estimate the extent of a possible organic coating or functionalization and the presence of organic moieties/species/compounds on the MNM. For organic-containing material, it is recommended (ASTM E1131) that W_{res} is determined at a temperature higher than 750 °C (minimum) or 900-100 °C (recommended) so that volatilization and combustion (oxidation) of organics is complete.

In parallel, a qualitative evaluation of the thermoanalytical curve and a semi-quantitative estimation of the weight loss onset temperatures for the various species are powerful tools to determine the presence of low- or medium-molecular weight organics, the thermal stability of the MNM and the resistance to oxidation. ASTM Standard E1131 indeed suggests, for several classes of organic material (coals, rubbers, lubricants, etc.), the temperature ranges in which the weight loss can be attributed to various components (highly-volatile matter, medium-volatile matter, oxidize-able material,). In brief, one first has to discern the different weight losses, if present, by looking for weight loss plateaus or minima in the derivative dW/dT plot. Weight losses up to 100-200 °C are ascribed to highly-volatile matter (e.g., solvents); weight losses between 100-200 °C and 500-600 °C to medium-volatile matter; weight losses between 500-600 °C and 900-1000°C are ascribed to oxidize-able (combustible) material. We however refer the reader to the ASTM Standard E1131 for a detailed description of this procedure.

Based on these combined observations, it is possible to evaluate whether the MNM is to be subjected to identification of the organic compounds/functional groups/coatings associated with it, according to NanoReg SOP for the identification of organic coatings on MNM.

7.2 Sources of error

The residual weight (w_{res}) might increase at high temperature if metals are present since the latter might be slowly oxidized. Similarly, other inorganic compounds may result in weight decrease at high temperature because of decomposition (eg. carbonates may release CO_2). Under such conditions, a specific temperature must be identified for the measurement of these components.

At a heating rate of $2.5^{\circ}C\ min^{-1}$ and with initial sample masses above 10 mg, the measurements show an adequate reproducibility and two replicates of the same specimen may be sufficient in order to achieve a confident result.

When performing TGA on MNMs with minimal M_{res} ($< 3\ %$), it may occur that the experimentally determined M_{res} is negative. This may occur even after fresh calibration of the instrument. The typical long-term stability of a TGA instrument zero (over a 3-hour run) is within 20-40 μg , which constitutes 1-2 % of the initial 2-4 mg sample. For samples with very small M_{res} , this amount gives rise to a fairly large error.

There may be a time lag in the sample temperature and the oven temperature which can be reduced by lowering the rate of the heating program.

8. Validation of method

The method has been (partly) validated by the partners in deliverable D 2.4. Further documentation of the procedures will be presented in Clausen et al. (2015). In addition, an interlaboratory study (ILS) should be performed among the D 2.4 partners to document the robustness of the method.

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Appendix II. SOP for assessment of the contents of water, organic compounds, and degradable inorganics in MNMs using a drying oven and a laboratory furnace

SOP for assessment of the contents of water, organic compounds, and degradable inorganics in MNMs using a drying oven and a laboratory furnace

Version 1.0

Work in progress (revision is being tested)

If in trouble, please send feed-back to

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Date: October 29, 2015

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Tested and commented by NANoREG Deliverable D2.4, Task 2.3n participants:
NRCWE, INL, IIT, CNR

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Foreword

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Please visit <http://nanoreg.eu/> for updates and to retrieve the latest version of the protocol as well as other NANoREG methods for MNM characterization.

The NANoREG consortium is interested in soliciting feedback on this method. We value user comments and suggestions to improve or further validate this protocol. Please send your name, email address and comments/suggestions to Per Axel Clausen (pac@nrcwe.dk). We also encourage users to report citations to published work in which this protocol has been applied.

Certain commercial entities, equipment or materials may be identified in this document in order to describe an experimental procedure or concept adequately. Such identification is not intended to imply recommendation or endorsement by the National Research Centre for the Working Environment (Denmark) and the NANoREG project team, nor is it intended to imply that the entities, materials or equipment are necessarily the best available for the purpose.

DRAFT - NANOREG RESPECTED

1. Introduction

1.1 Background

One of the key requirements in comparative testing is that all preparation and subsequent characterization procedures are as harmonized and inter-calibrated as possible. Toxicity and fate assessment are key elements in the evaluation of the environmental, health and safety risks of manufactured nanomaterials (MNMs) and nano-sized engineered solids. While significant effort and resources have been devoted to the toxicological evaluation of many MNMs, much less strategic effort has been set on the development of tested and harmonized MNM characterization methods. This may be one of the reasons that it still is a challenge to obtain conclusive and reproducible results on how physicochemical properties may relate to and modify toxicological effects.

The present protocol describes a simple and robust procedure to quantify the content of water as well as volatile and/or decomposable organic and inorganic matter associated with MNM by sequentially measuring weight loss in MNM samples after heating at selected temperatures. The method is usually performed under oxidizing conditions (*i.e.*, under access of air), and thus can provide useful information about the content of oxidizable organic compounds (e.g., hydrocarbons, polymers, coke, etc.).

It should be noted that knowledge about the thermal degradation temperatures of the MNM phase to be tested is advantageous to select the most appropriate test temperatures and make proper conclusion from the results. This is because some materials degrade within temperature ranges for general determination of high-volatile organic compounds. For example kaolinite ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$) rapidly dehydroxylates between 400 and 600°C in air (e.g. Hurst and Kunkle (1985)). For comparison bentonite dehydroxylates between 500 and 700°C in air (e.g. Yilmaz et al. (2013)). Another issue is that if water is trapped in material porosities it is known to be released at temperatures higher than the normal boiling point and can therefore be misinterpreted as organics. Therefore the SOP is proposed for screening for possible presence of coatings, functionalizations and associated degradable compounds associated with MNM.

1.2 Purpose and background for the SOP

This SOP facilitates a simple and robust procedure to identify MNM that may contain volatile, oxidizable and/or decomposable organic and inorganic matter, which can be present on the surface of and/or within porosities of a MNM or other individual phases in mixture (e.g., additives, dispersants and process impurities). The procedure is based on weighing and heating in a drying oven and a laboratory furnace. The SOP serves as a low cost alternative to thermogravimetric analysis (TGA) and builds on several laboratory procedures developed over time considering several procedures for mineral, rock, and material analysis. The specific description in this SOP, was based on the loss-on-ignition (LOI) method applied on sediments by Heiri et al., (2001). The SOP was prepared in collaboration with all partners participating in deliverable D2.4 of the NANoREG project.

2. Principles and scope

The associated water, volatile, oxidize-able and/or decomposable organic and inorganic matter are estimated by sequentially measuring weight loss in MNM samples after heating at selected temperatures. Weight loss is measured after heating at 110°C overnight to remove water and other low-volatile compounds, at 550°C for at least four hours to remove organic matter, and at 1050°C overnight to remove carbonates as well as to decompose carbon-based materials such as carbon black and carbon nanotubes. Higher temperatures may be required in special cases if analyses are performed on some highly resistant phases and information on e.g., crystal-bound OH, F, CO, SO or other species.

Based on the results from different standard heating temperatures, a simple modal compositional profile can be generated rapidly and for very low cost. This profile is sufficient to develop a general sense of main composition of the MNM. The results are relatively accurate for content of water, organic matter and carbonate. Here, it should however be noted that oxidation of polyvalent elements (e.g., Fe^{2+} to Fe^{3+}) result in a weight increase (e.g., FeO to Fe_2O_3) and may need to be accommodated in the analysis.

While the drying oven and laboratory furnace method for determination of water content and LOI is not the primary technique for structure determination, it may play an important role in pre-evaluating materials prior to other material characterization techniques. It can be used as a part of the physico-chemical characterization of MNMs required as prior knowledge for toxicological testing and environmental compatibility assessment. If materials are not stored at similar relative humidity conditions, important differences (up to ca. 20 wt% for e.g., ZnO and a HNO_3 stabilized TiO_2) can be observed in weight as recently shown by Levin et al. (2015). Such differences cause errors in the comparative dose measures when dosing is based on weighing. The temperature over which volatilization, decomposition or combustion occurs provides information about the volatilized/decomposed/oxidized species and this helps in the qualitative identification and quantitative characterization of these species. If high precision is needed, or if MNM is in short supply, thermogravimetric analysis (TGA) is recommended.

LOI is often used as an estimate of the content of non-volatile organic matter in the sample by the weight of the annealing residue. However, it should be noted that inorganic substances or decomposition products (e.g. H_2O , CO_2 , SO_2 , O_2) may be released (weight loss) or absorbed (weight gain) and some inorganic substances are volatile (e.g., K^+ and Na^+) under these high temperature conditions.

The task for the operator of the drying oven and laboratory furnace method is to obtain reliable weight losses and onset temperatures using the prescribed time and temperature program of the method in the SOP.

3. Terminology and abbreviations

This protocol complies with the definition of a manufactured nanomaterials as proposed by the EC as well as ISO TS 80004-1 (ISO, 2010) and ASTM standard E2456 (ASTM, 2006).

nanomaterial— a nanomaterial consists of particulate materials (as individual, agglomerated and aggregated) among which 50% of the primary particulate entities by number are between 1 and 100 nm in diameter along their shortest dimension. Graphene, fullerene and single-walled carbon nanotubes are automatically defined as nanomaterials irrespective of the fact that their shortest diameters may be smaller than 1 nm. The primary particles in the nanoscale are also referred to as “nano-objects” in ISO TS 80004-1 and part of the ISO nanomaterial system.

primary particle — the smallest discrete identifiable entity associated with a particle system; in this context, larger particle structures (e.g., aggregates and agglomerates) may be composed of primary particles.

aggregate — a discrete assemblage of primary particles strongly bonded together (i.e., fused, sintered and with chemical bonds exceeding e.g., van der Waals and hydrogen binding forces).

agglomerate — assemblages of particles (including primary particles and/or smaller aggregates) held together by relatively weak forces (e.g., van der Waals, capillary or electrostatic), that may break apart into smaller particles upon further processing.

LOI — loss-on-ignition

TGA — thermogravimetric analysis

MNM— manufactured nanomaterial

4. Equipment, materials and chemicals

4.1 Equipment

- Semi-micro-analytical balance (4 decimal digits) placed in glove or exhaust box for weighing the samples.
- Drying oven with a reasonable accuracy and precession of $110^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- Muffle furnace capable of reaching and keeping temperatures with a reasonable accuracy and precession of 550°C and $1050^{\circ}\text{C} \pm 10^{\circ}\text{C}$.
- Weighing glasses with ground glass stoppers for determination of adsorbed water. The reason for using ground glass stoppered weighing glasses is that they are relatively tight and that dried powders including MNM may strongly adsorb water from air (hygroscopic) after dehydration.
- Flat bottom sample pans (crucibles) with lids suitable for temperatures up to at least 1050°C , e.g. made of nickel, ceramics, silica, or platinum.
- Spatula.
- Tweezers or flat-nose pliers for handling the crucibles.
- Desiccator with active drying agent such as yellow silica gel.
- Ethanol for cleaning the outer surface of the crucibles for MNM adhered during weighing.
- Temperature gauge (either build-in or externally mounted) suitable for the temperature range $110\text{-}1050^{\circ}\text{C}$ and with a reasonable accuracy and precession $\pm 1^{\circ}\text{C}$, e.g. Testo 735-2 Multichannel thermometer and Testo Comfort Software X35 for data logging.
- Container with lid for storing and transport of the samples.

4.2 Materials

MNM approved by NANoREG Management Committee.

5. Preparations

5.1 Balance, drying oven and furnace calibration

Make sure that the balance is well calibrated. Use the thermometer to make sure that the drying oven and the furnace can reach and maintain the respective temperatures at a reasonable accuracy and precession.

6. Procedure

6.1 Summary of the procedure

MNM samples are placed in weighed crucibles and weighed. Weight loss is measured after heating at 110°C overnight to remove water, at 550°C for at least four hours to remove organic matter which may constitute the surface coating of the MNM, and at 1050°C for overnight to remove carbonates and/or combust carbonaceous material such as carbon nanotubes (CNT). After each heating step, the crucibles are allowed to cool completely placed in a desiccator. Samples must be cool so that convection currents do

not affect the balance, and kept in desiccator so that they do not absorb atmospheric water. Samples must not be placed in a non-venting desiccator when too warm. A brick tray may be used to handle several crucibles.

Ash left at the end of the procedure can be saved for analysis of remaining elements as oxides.

6.2 The detailed procedure

Only one heating step can be accomplished each day, because the 110°C drying time, and the ramp-up and cool-down times of the furnaces are all >8-10 hours. The user should thus plan seven days consecutively or do the water determination and LOI in parallel:

Day 1 Weighing glasses with ground glass stoppers askew are dried 1 hour at 110 ° C, and cooled down to room temperature in a desiccator. Never touch weighing glasses with your hands. Skin oils will add weight and introduce error to your results. Always use a pair of tweezers or flat-nose pliers when handling the weighing glasses.

Day 2 Weigh glasses with ground glass stoppers (mg), add 2 g sample, and weigh in the glove box (msw); clean the weighing glasses on the outer surface for MNM adhered during weighing before taken out of the glove box; place the weighing glasses containing the samples with the ground glass stoppers askew in drying oven at 110°C for 12-24 h (allow several hours for these steps).

Day 3 Turn off and open oven, replace immediately the glass stoppers from askew to closed position before the weighing glasses are taken out of the oven; let samples cool down to room temperature in desiccator (10 min), weigh (mw0) within 15-20 min (water loss: $mw = msw - mw0$).

Day 4 Crucibles with the lid askew are dried 1 hour at 110 ° C, and cooled down to room temperature in a desiccator. Never touch crucibles with your hands. Skin oils will add weight and introduce error to your results. Always use a pair of tweezers or flat-nose pliers when handling crucibles.

Day 5 Weigh crucibles (mc), add 2 g sample, and weigh in the glove box (msLOI); clean the weighing glasses on the outer surface for MNM adhered during weighing before taken out of the glove box; place crucibles with lids askew in a cold furnace and heat up to 550°C for at least 4 hrs. After heating turn off and open furnace, replace immediately the lids from askew to closed position before the crucibles are taken out of the oven and placed on brick trays; let samples cool down to room temperature in desiccator (10 min), weigh (morg0) within 15-20 min (organic matter loss: $morg = msLOI - morg0$); place crucibles with lids askew in furnace at 1050°C for at least 14 hrs.

Day 6 After heating to 1050 °C turn off and open furnace, replace immediately the lids from askew to closed position before the crucibles are taken out of the oven and placed on brick trays; let samples cool down to room temperature in desiccator (10 min), weigh (mCO20) within 15-20 min (carbonate loss: $mCO2 = morg0 - mCO20$), discard or save sample residues, wash crucibles, place crucibles in furnace at 1000°C for 2 hours to completely clean the crucibles.

Day 7 Make sure crucibles are cool, remove from oven, cover with foil, and place in desiccator for reuse (1-2 hr). Label these as clean and ready to be reused.

7. Expected outcome and interpretation of results

7.1 Residual masses after volatilization/ oxidation/ decomposition

$$\text{Water content: } m_w(\%) = 100\% \cdot (m_{sw} - m_{w0}) / (m_{sw} - m_g)$$

$$\text{Organic content: } m_{org}(\%) = 100\% \cdot (m_{sLOI} - m_{org0}) / (m_{sLOI} - m_c)$$

$$\text{Carbonate content: } m_{CO_2}(\%) = 100\% \cdot (m_{org0} - m_{CO_20}) / (m_{sLOI} - m_c)$$

Based on these combined observations, it is possible to evaluate whether the MNM is to be subjected to identification of the organic compounds/functional groups/coatings associated with it, according to NANoREG SOP for the identification of organic coatings on MNM.

7.2 Sources of error

The residual weight might *increase* at high temperature if polyvalent metals are present since the latter might be slowly oxidized. Similarly, other inorganic compounds may result in weight *decrease* at high temperature because of decomposition, e.g., release of H₂O, CO₂, SO₂ or some inorganic substances are volatile under the temperature conditions used.

8. Validation of method

The method has been (partly) validated by the partners in deliverable D 2.4. In addition, an interlaboratory study (ILS) should be performed among the D 2.4 partners to document the robustness of the method.

9. References and Bibliography

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Appendix III. SOP for determination of inorganic content in MNMs by Wavelength dispersive X-ray fluorescence (WDXRF)

DRAFT - NANOREG RESTRICTED

SOP for determination of inorganic content in MNMs by Wavelength dispersive X-ray fluorescence (WDXRF)

Version 1.0

Work in progress (revision is being tested)

Please send feed-back to

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Date: November 5, 2015

Kirsten Inga Kling (NRCWE)

Tested and commented by NANoREG Deliverable D2.4, Task 2.3n participants:
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DRAFT - NANOREG RESPECTED

1. Introduction

1.1 Background

One of the key requirements in comparative testing is that all preparation and subsequent characterization procedures are as harmonized and inter-calibrated as possible. Toxicity and fate assessment are key elements in the evaluation of the environmental, health and safety risks of manufactured nanomaterials (MNMs) and nano-sized engineered solids. While significant effort and resources have been devoted to the toxicological evaluation of many MNMs, obtaining conclusive and reproducible results continues to be a challenge. This can be traced in part to the lack of standardized protocols for characterization of MNMs.

The present protocol describes a simple and robust procedure to quantify the amount of inorganic matter present in and/or on a MNM using Wavelength dispersive X-ray fluorescence (WDXRF). X-ray fluorescence (XRF) is the emission of characteristic "secondary" (or fluorescent) X-rays from a material that has been excited by bombarding with high-energy X-rays. It is a non-destructive method used in chemical analyses of rocks, metals, powders, liquids and filters.

1.2 Purpose and background for the SOP

The purpose of this SOP is to describe in detail a simple and robust procedure for semi-quantitative determination of the chemical composition of nanomaterials collected on a filter, dispersed in a liquid or as a powder.

2. Principles and scope

WDXRF is a technique that measures the elemental composition in a controlled atmosphere. Its main use is to characterize materials in regards to their composition; in particular, the relative abundance of elements in the bulk. Measurements can be run automated and a large number of samples can be analyzed in a short amount of time. The measurement duration normally varies from ca. 1 minute to 18 minutes depending on the sample type and detection limits required. Sample preparation is usually simple; in many cases, material can be observed directly.

WDXRF instruments rely on diffractive optics to give them high spectral resolution. WDXRF instruments use a x-ray tube source to directly excite the sample. Wavelength-dispersive spectrometers employ diffraction by a single crystal to separate characteristic wavelengths emitted by the sample. A single crystal of known interplanar spacing d is used to disperse the collimated polychromatic beam of characteristics wavelengths that is coming from the sample, such that each wavelength λ will diffract at a specific angle θ , given by Braggs law:

$$n\lambda = 2d \cdot \sin\theta$$

where n is an integer number denoting the order of the diffracted radiation. A goniometer is used to maintain the required $\theta/2\theta$ relationship between sample and crystal/detector. A diffraction device, usually a crystal or multilayer, is positioned to diffract x-rays from the sample toward the detector. Diffracted wavelengths are those that satisfy the Braggs equation. Other wavelengths are scattered very inefficiently. Collimators are normally used to limit the angular spread of x-rays, to further improve the effective resolution of the WDXRF system. For the detection of light elements a proportional counter and for the heavier elements a scintillation counter is used. Both detectors are perfectly suited to the respective energy ranges. All the components can be fixed to form a fixed single WDX channel that is ideal for analyzing a single element. One element after another may be measured in sequence.

An instrument consists of a loader for subsequent analysis of up to 60 samples. Samples can be either liquid or solid, pressed in pellets, fused to beads or loose powders.

The detector purge gas is typically P10 gas (10 % methane, 90 % Ar). Analysis takes place under vacuum or a protective He-atmosphere. The He should be of High-purity (99.999 vol%, i.e. 5.0 purity).

While WDXRF may not be the primary technique for structure determination, it often plays an important role in pre-evaluating materials prior to other material characterization techniques. It can be used as a part of the physico-chemical characterization of MNMs required as prior knowledge for toxicological testing and environmental compatibility assessment. By applying a semi-quantitative detection method based on internal standards, a relative composition of the sample down to detection limits of 0.05% can be achieved in samples of some hundred mg in mass. Depending on the element of interest and matrix of the sample, and given the right standards are available, the instrument can be calibrated to detect few ppm (e.g. 1µg in 1ml sample fluid) of MNMs, and ng of MNM, or even coatings can be detected if samples are optimal.

Obtaining consistent, semi-quantative high-quality WDXRF data does not require the dedicated presence of a research-level expert, however the operator should carry out the WDXRF analysis following a clear, simple and robust SOP.

3. Terminology and abbreviations

WDXRF — Wavelength dispersive X-ray fluorescence

N₂ — nitrogen

O₂ — oxygen

He — helium

Ar — argon

MNM — manufactured nanomaterial

ppm – parts per million. 1ppm = 0,0001%

4. Equipment, materials and chemicals

4.1 Equipment

XRF sample cups or filters (e.g. Whatmann, Teflon max. 40mm)

XRF foil (e.g. Mylar 3µm, Prolene or Polypropylene 4µm)

Cotton wool

Semi-micro-analytical balance (4 decimal digits) for weighing the samples before analysis

Tweezers

WDXRF: We used a Bruker Tiger S8 equipped with PET, LIF200 and XS55 crystals and SPECTRAplus installed on a support PC, which is a standard setup offered by Bruker. The package solution is similar to other suppliers for XRF instruments.

1.1 Materials

MNM approved by NANoREG Management Committee.

1.2 Chemicals

High-purity (99.999 vol%, i.e. 5.0 purity) dry inert gas (N₂ or He) is recommended as sample purge gas.

P10 gas (10 % methane, 90 % Ar) is recommended for the flow proportional detector.

5 Preparations

5.1 Cleaning, validation, and calibration

The semi-quantitative approach suggested here relies on internal standards and calibration provided by the manufacturer of the XRF-instrument, and does not require any calibration or cleaning procedure.

5.2 Sample preparation for powder samples

Samples should be weighed and mounted carefully into the instrument according to the following procedure.

- Wear gloves! Take sampling cup part I and sampling cup part II, the lid (all in 40 mm) and a foil out of the boxes in the cabinet. Put equipment on a clean support. Place foil between cup part I and cup part II and push parts gently towards each other. Check foil: it must have no crinkles, edges and holes after mounting.
- Place mounted cup on a clean support on the scale. Tare, then fill the amount of material (powder) you want to measure. Note amount. Powder has to cover all of the foil. Tare, and add cotton wool. Note amount. Remove the cup from the scale and place it on a clean support. Close cup with lid. If your gloves got in contact with powder or any other contaminant, change them immediately, before you touch any outer region of the sampling cup and lid!!!
- Mount a sample holder (34 mm) with a distance ring at the bottom (ring has to be mounted so that the sample can slide in) and place the prepared sample cup in the sample holder. Place sample holder at a position in the instrument and note down position.
- Take off your gloves.

5.3 Sample preparation for liquid samples

Samples should be weighed and mounted carefully into the instrument according to the following procedure.

- Wear gloves! Take sampling cup part I and sampling cup part II, the lid (all in 40 mm) and a foil out of the boxes in the cabinet. Put equipment on a clean support. Place foil between cup part I and cup part II and push parts gently towards each other. Check foil: it must have no crinkles, edges and holes after mounting.
- Fill liquid until approximately 10 mm height in the sampling cup. Close the cup with a lid.
- Place the sampling cup on a clean non-contaminating paper substrate to check for leaks (leaks are detected by color change of the substrate as sign of wetting). Wait for 10 min. and check for leaks in the foil. Make sure there is absolutely no liquid outside the sampling cup!!! If your gloves got in contact with liquid or any other contaminant, change them immediately, before you touch any outer region of the sampling cup and the lid!!!
- Mount a sample holder (34 mm for 36-40 mm sampling cup) with a distance ring at the bottom (ring has to be mounted so that the sample can slide in) and place the prepared sample cup in the sample holder. Place sample holder at a position in the machine and note down position.
- Take off your gloves.

5.4 Sample preparation for filter samples

- Make sure the filter weight is measured and noted before and after sampling!
- Prepare sample holder (top and bottom) and filter clamp.
- To mount the filter clamp, fix the spring into the notch in the sample holder.
- Place the filter carefully (don't touch!!!) on the filter clamp and cover with a foil, so that the foil overlaps the filter on its edges.
- Mount and fix the sample holder bottom part.
- Place sample holder in the changer and close the cover. Note down position on the Sample positioning sheet.

6 Procedure

6.1 Preparation of the WDXRF

The WDXRF instrument should be turned on permanently. If the instrument is not running permanently, we recommend 12-24 hours conditioning time before the first measurement.

6.2 Analysis of powder samples

- Check if X-rays are "on" and machine is ready. Open He-valve.
- Press "Measurement" in the SpectraPlus Launcher, or go to "Loader" window. Select tag "quant express". Enter sample name at sample position. Use logic naming, it is convenient if you use a date-code in the sample name already, and in consistency with previous and later samples! Choose "material" (oxides, elements. Can be changed in Eval later on). Choose "preparation". Choose "mode": He. Choose "diameter" 34 mm. Choose "method of analysis".
- Mark your sample(s) and press "send selected samples".
- Wait until measurement is finished.
- Press "Evaluation" in the SpectraPlus Launcher. Press "default search". Select your sample and press either "interactive quant" or "interactive qual". Results will be displayed as list, if necessary add matrix compound and recalculate.

6.3 Analysis of liquid samples

- Check if X-rays are on and machine is ready. Open He-valve.
- Press "Measurement" in the SpectraPlus Launcher, or go to "Loader" window. Select tag "quant express". Enter sample name at sample position. Use logic naming, it is convenient if you use a date-code in the sample name already, and in consistency with previous and later samples! Choose material (oxides, elements, can be changed in "Eval" later on). Choose preparation. Choose mode: "He". Choose diameter 34 mm. Choose method of analysis.
- Mark your sample(s) and press "send selected samples".
- Wait until measurement is finished.
- Press "Evaluation" in the SpectraPlus Launcher. Press "default search". Select your sample and press either "interactive quant" or "interactive qual". Results will be displayed as list, if necessary add matrix compound and recalculate.

6.4 Analysis of filter samples

- Filter samples, when covered by a foil, can be analyzed in vacuum mode.
- On the XRF computer: open the loader, choose the tab "_Routine-Vac" and type in sample names. Choose a method indicated with "_Filter". Make sure no other samples are neither on the list (any tab in the loader) nor in the instrument.

- Press "send all samples". Measurement should start running now. When finished, the samples should be marked "OK".
- Take samples out after successful measurement.
- Press "Evaluation" in the SpectraPlus Launcher. Press "default search". Select your sample and press either "interactive quant" or "interactive qual". Results will be displayed as list, if necessary add matrix compound and recalculate.

7 Expected outcome and interpretation of results

7.1 Calculation of concentration for unknown samples

In most cases, WDXRF is performed on mixed mineral samples with a complete inorganic content (100%). In some cases it might be that the sum is not reached by the elements measured. This might be a hint on carbon (or other light elements) being present in the matrix. If this is the case, enter C as the matrix in the evaluation and the concentration of elements will be recalculated. If the amount of C (and other light elements) has been determined by a method like TGA, the value can also be entered as fixed.

7.2 Sources of error

We strongly recommend to perform each measurement three times, and calculate the mean of the three measurement results. If the deviation between the measurements is significant (> 3%), the sample preparation should be reviewed. Check if the variation is systematic. Drying the sample might be appropriate. If the measurements still vary it might be that the sample in its present form is not suitable for XRF analysis.

The resulting concentrations should also be checked by reviewing the scan. Line overlap may occur, and detection limits can be higher for some elements due to the presence of radiation from the X-ray-tube (rhodium) itself. In this case, the method used has to be reviewed and a calibration for the elements of interest has to be performed.

7.3 Standard operation procedure for making a calibration curve

Sample preparation

PRECAUTIONS:

- Wear fresh gloves and change them regularly, when handling liquids.
- Choose elemental standards and matrix carefully: note that water (e.g. nanopure) can contain contaminations in the range of concentrations, you want to calibrate. Same goes for support film.
- Make sure your support film has chemical resistance against all chemicals you intend to use. If you are in doubt, test film by putting on the liquid in the highest concentration for some hours and check for damage afterwards.
- Always use exact same type and thickness of support film.
- Work carefully and accurate, avoid cross contamination and spill.
- Reserve enough time: make sure your solutions are freshly prepared when it comes to measurements!

Prepare standards by mixing n solutions with increasing concentration of the element of interest in a constant matrix. For example: 0, 5, 10, 25, 50, 100 ppm of an element of interest in weak HNO₃ solution, or other. Make sure your matrix doesn't contribute with contamination of the same element.

Wear gloves! Take sample cup part I, sample cup part II, the lid (all in 40 mm) and a foil out of the boxes in the cabinet. Put equipment on a clean support. Place foil between cup part I and cup part II and push parts gently towards each other. Check foil: it must have no crinkles, edges and holes after mounting.

For each concentration: Place mounted cup on a clean support. Fill liquid until approximately 10 mm height in the sample cup. Close cup with lid. Remove the cup from the scale and place it on to a clean support, which changes color when it takes up liquid. Wait for 10 min. and check for leaks in the foil. Make sure there is absolutely no liquid outside the sample cup!!! If your gloves got in contact with liquid or any other contaminant, change them immediately, before you touch any outer region of the sample cup and the lid!!!

Mount a sample holder (34 mm) with a distance ring at the bottom (ring has to be mounted so that the sample can slide in) and place the prepared sample cup in the sample holder. Place sample holder at a position in the machine and note down position.

Take off your gloves.

Check if X-rays are turned on and instrument is ready. Open He-valve.

Make sure a SpectraPlus manual is available: find a pdf-copy of the English and French version in folder "Manuals" on the Desktop.

Press "Application" in the SpectraPlus Launcher. Click on "New Application". Enter a name containing the element you want to calibrate, concentration range and date. Click "OK". Use the arrows in the tool box to navigate and follow instructions on the screen and consult the manual chapter "Application Wizard". Make sure to follow all instructions precisely!

Save all steps you make!

Remove the used samples from the machine immediately after measuring. Remove sample cups from sample holders and check for spills of liquid. Clean the sample cups with a dry tissue and a gentle stream of compressed air after use. Pack sample holders away and dispose your samples according to regulations and your Institution's procedures for wastes.

8 Validation of method

Multi walled carbon nanotubes (NRCWE_040-049, NM 400-403) have been measured according to the procedure described under 5.2 and 6.2. The results have been compared with TGA analysis for the derived calculated carbon content and proofed to be in accordance. Thereby, the impurity content has been characterized for these 14 MNM (Jackson et al. 2015).

The method has been (partly) validated by the partners in deliverable D 2.4 using JRCNM01003a/NM-103, which is Al-coated TiO₂ MNM. Table 1 displays the results for these two elements, assuming Al to be present as oxide.

Table 1 WDXRF – best detection results in weight % for JRCNM01003a/NM-103 for Al and Ti, assuming those to be present as Oxides.

Formula	Z	Concentration	Status	Line 1	Net int.	Calc. concentration	Stat. error
TiO ₂	22	95,66%	XRF 1	Ti KA1-HR-Tr	2907	95,66	0,06%
Al ₂ O ₃	13	3,12%	XRF 1	Al KA1-HR-Tr	41,18	3,124	0,51%

9 References and Bibliography

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DRAFT - NANOREG REGISTERED

Appendix IV. SOP for characterization of non-covalent bound organic surface coating on MNM by extraction and mass spectrometry

DRAFT - NANOREG RESTRICTED

SOP for characterization of non-covalent bound organic surface coating on MNM by extraction and mass spectrometry

Version 1.0

Work in progress (revision is being tested)

If in trouble, please send feed-back to

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Date: November 05, 2015

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Tested and commented by NANOREG Deliverable D2.4, Task 2.3n participants:

INL, IIT, CNR



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Foreword

This SOP (Standard Operating Procedure) is one in a series of protocols resulting from a collaboration of the partners in the NanoReg project. The original version of this protocol (Ver. 1.0) is stored in the NANoREG area on CIRCABC. Updates to this protocol may be released in the future and within the duration of the NANoREG project, such updates will also be stored on CIRCABC. The latest version of the SOP will always be available from the NANoREG homepage.

Please visit <http://nanoreg.eu/> for updates and to retrieve the latest version of the protocol as well as other NANoREG methods for MNM characterization.

The NANoREG consortium is interested in soliciting feedback on this method. We value user comments and suggestions to improve or further validate this protocol. Please send your name, email address and comments/suggestions to Per Axel Clausen (pac@nrcwe.dk). We also encourage users to report citations to published work in which this protocol has been applied.

Certain commercial entities, equipment or materials may be identified in this document in order to describe an experimental procedure or concept adequately. Such identification is not intended to imply recommendation or endorsement by the National Research Centre for the Working Environment (Denmark) and the NANoREG project team, nor is it intended to imply that the entities, materials or equipment are necessarily the best available for the purpose.

DRAFT - NANOREG RESOURCES

1. Introduction

1.1 Background

One of the key requirements in comparative testing is that all preparation and subsequent characterization procedures are as harmonized and inter-calibrated as possible. Toxicity and fate assessment are key elements in the evaluation of the environmental, health and safety risks of manufactured nanomaterials (MNM). While significant effort and resources have been devoted to the toxicological evaluation of many MNM, obtaining conclusive and reproducible results continues to be a challenge. This can be traced in part to the lack of standardized protocols for characterization of MNM. This protocol describes procedures to obtain information on the identity of organic compounds that can be extracted from MNM which in turn has been shown to be associated with organic substances by thermogravimetric analysis (TGA) and/or x-ray fluorescence (XRF) analysis. It is up to an assessment whether the extracted organic substances can be assumed to be intentional added to form a non-covalent surface coating.

1.2 Purpose and background for the SOP

The purpose of this SOP is to enable reliable procedures for identification of organic substances which can be extracted from MNM and analyzed with mass spectrometry (MS). The SOP do not describe an easy-to-use procedure since the often unknown organic coating can consist of anything from relative small volatile molecules to polymeric compounds which may be bound by a range of different forces such as Van der Waals forces, electrostatic forces, and ionic bindings. The procedures described in the SOP require an operator with great experience in the techniques used. The procedures describes in a general manner how the extractions and analyzes can be performed. The SOP is based on results in Nymark et al. (2013) and a publication in preparation (Clausen et al., 2015) and is developed in collaboration with the partners in deliverable D 2.4 in the NANoREG project. It is a prerequisite for this SOP that TGA and/or XRF have indicated organic substance in association with the MNM under investigation. The amount of the organic compounds associated with the MNM is determined by TGA.

2. Principles and scope

The intention with this protocol is to provide a validated method for identification of organic substances which can be extracted from MNM and analyzed with MS. More specifically said this is a protocol that describes procedures to obtain information on the identity of organic compounds which can be extracted from MNM, which in turn has been shown to be associated with organic substances by TGA. It is up to an assessment whether the extracted organic substances can be assumed to be intentional added to form a non-covalent surface coating. The amount of the organic compounds associated with the MNM is determined by TGA. The method is not suitable for covalently bound surface coating of MNM. This may require chemical decoupling of the attached surface modification.

TGA is used to determine whether an MNM has organic coating or not and quantify the amount of coating. The following types of MNM with more than 1 % apparent coating were tested with the protocol: Organoclays, graphite, synthetic amorphous silica, titanium dioxide, silver, calcium carbonate, iron oxide, and nickel-zinc-iron oxide. The MNM are extracted with either ultrasonic extraction with suitable solvent at ambient temperature, pressurized liquid extraction (PLE) using methanol at 200 °C, or thermal desorption (TD) at 250 °C. Volatile organic compounds in the extracts are analysed with MS combined with on-column gas chromatography (GC-MS) or TD. Non-volatile and polar organic compounds in the extracts are analysed with liquid chromatography (LC-MS) or direct infusion combined with quadruple time-of-flight MS using either electrospray ionization or atmospheric pressure chemical ionization. Tandem MS based on collision induced dissociation is used for structure determination. Matrix- or nanostructured surface-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS or NALDI-TOF-MS) is used for characterization of polymeric compounds. The identification

of the organic compounds from the MALDI/NALDI-TOF-MS results is based on pattern recognition, literature, and GC-MS data.

The method can be used as a part of the characterization of MNM for chemical and industrial use and for toxicological testing. Other methods such as electron microscopy and x-ray fluorescence spectroscopy are important for the full characterization of MNM.

3. Terminology and abbreviations

This protocol complies with the definition of a manufactured nanomaterials as proposed by the EC as well as ISO TS 80004-1 (ISO, 2010) and ASTM standard E2456 (ASTM, 2006).

nanomaterial— a nanomaterial consists of particulate materials (as individual, agglomerated and aggregated) among which 50% of the primary particulate entities by number are between 1 and 100 nm in diameter along their shortest dimension. Graphene, fullerene and single-walled carbon nanotubes are automatically defined as nanomaterials irrespective of the fact that their shortest diameters may be smaller than 1 nm. The primary particles in the nanoscale are also referred to as “nano-objects” in ISO TS 80004-1 and part of the ISO nanomaterial system.

primary particle — the smallest discrete identifiable entity associated with a particle system; in this context, larger particle structures (e.g., aggregates and agglomerates) may be composed of primary particles.

aggregate — a discrete assemblage of primary particles strongly bonded together (i.e., fused, sintered and with chemical bonds exceeding e.g., van der Waals and hydrogen binding forces).

agglomerate — assemblages of particles (including primary particles and/or smaller aggregates) held together by relatively weak forces (e.g., van der Waals, capillary or electrostatic), that may break apart into smaller particles upon further processing.

PLE — pressurized liquid extraction

GC-MS — gas chromatography combined with mass spectrometry

LC-MS — high performance liquid chromatography combined with mass spectrometry

MALDI-TOF-MS — matrix-assisted laser desorption ionization time-of-flight MS

NALDI-TOF-MS — nanostructured surface-assisted laser desorption ionization time-of-flight MS

TD — thermal desorption

TGA — thermogravimetric analysis

MS — mass spectrometry

MNM — manufactured nanomaterials

XRF — x-ray fluorescence spectroscopy

VOC — volatile organic compound

SVOC — semi-volatile organic compound

4. Equipment, materials and chemicals

4.1 Equipment

Various small scale laboratory equipment such as glassware, pipettes, and ultrasonic bath.

Injection manifold to inject methanol solutions onto adsorbent tubes for TD-GC-MS and subsequent purge off.

Glove box equipped with an analytical balance for weighing the MNM samples.

Fume hood for extractions with solvents.

The following large scale laboratory equipment was used in the development of the protocol: PLE: ASE 200 (Dionex/Thermo Scientific); GC-MS and TD-GC-MS: Turbomatrix 350 (Perkin Elmer) – Scion TQ (Bruker) or ATD 400 (Perkin Elmer) – Autosystem XL GC (Perkin Elmer) – Turbomass MS (Perkin Elmer); LC-MS: Series 1200 HPLC (Agilent Technologies) – microTOF-Q (Bruker); MALDI/NALDI-TOF-MS: Autoflex III (Bruker).

4.2 Materials

Vials, septa, and syringes for the autosamplers on GC-MS and LC-MS instruments. Adsorbent tubes with 200 mg Tenax TA and end-caps for TD-GC-MS. Various vials, septa, filters, O-rings and filler sand for PLE. Steel target plates and NALDI plates for MALDI/NALDI-TOF-MS.

4.3 Chemicals

Samples of the MNM to be tested. Solvents for extraction and analysis, in particular methanol which is suitable for injection on adsorbent tubes for TD-GC-MS and subsequent purge off. Pure standards of the organic compounds in question and matrix compounds for MALDI-TOF-MS.

5. Procedure

5.1 Prerequisites for performing the procedure

It is a prerequisite for performing the procedure described below that TGA and/or XRF have indicated organic substance in association with the MNM under investigation. The amount of the organic compounds associated with the MNM is determined by TGA.

5.2 Extraction of organic compounds from the MNM

Consult the instruction manual of the actual instrumentation for specific recommendations on preparation and settings.

We propose to use different extraction methods with different properties and strengths, namely thermal desorption (TD) at 250 °C, ultrasonic extraction with suitable solvent at ambient temperature, and pressurized liquid extraction (PLE) using methanol at 200 °C or similar techniques. The reason not to use the most harsh and efficient method (PLE) from the beginning is that large organic molecules may be degraded to smaller ones. If a TD-GC-MS system is available it is easy to use for gas phase extraction (evaporation) of volatile organic compounds (VOC) directly from the MNM under investigation. If no VOC are detected by TD-GC-MS, the MNM can be subjected to ultrasonic extraction with suitable solvent at ambient temperature and extract analysed with the methods described below. If none of the analytical methods below show any VOC, semi-VOC (SVOC) or polymeric compounds in the ultrasonic extract, PLE can be used as a last possibility.

5.3 Analysis directly of the MNM

If techniques such as TD-GC-MS, MALDI-TOF-MS or other similar techniques are available at the laboratory it is in some cases possible directly to liberate the organic compounds from the surface, ionize and lead them into the mass spectrometer. Consult the instruction manual of the actual instrumentation for specific recommendations on preparation and settings.

5.4 Analysis of extracts of MNM

TD-GC-MS can be used to analyze methanol extracts from both ultrasonic extraction and PLE since the methanol can be purged off from the adsorbent tube and leave the organic compounds concentrated on the adsorbent. The advantage of using TD is that possible insoluble or high molecular weight compounds are trapped on the adsorbent in the adsorbent tube and only relative volatile VOC and SVOC are desorbed from the adsorbent during analysis.

The extracts can also be analyzed using GC-MS by solvent injection and here it is required to use a solvent suitable for GC-MS analysis, e.g. cyclohexane. This method is suitable for both VOC and SVOC.

For non-volatile, high molecular weight and polar organic compounds LC-MS or direct infusion combined with quadrupole time-of-flight MS using either electrospray ionization or atmospheric pressure chemical ionization is required and extraction is recommended to be performed with methanol or acetonitrile. Tandem MS based on collision induced dissociation is used for structure determination.

High molecular weight and polymeric compounds may be analyzed with MALDI/NALDI-TOF-MS. Here a solvent suitable for co-crystallization with the matrix compound, e.g. dihydroxybenzoic acid, on the MALDI steel target is required. The identification of the organic compounds from the MALDI/NALDI-TOF-MS results is based on pattern recognition, literature, and GC-MS data.

5.5 Sample removal and disposal

Dispose residues of samples and solvents following your Institution's procedures for wastes.

6. Expected outcome and interpretation of results

The coating of MNM may consist of one or more organic compounds and/or homologous series of compounds. The observation of small very volatile compounds may be due to degradation of larger and/or polymeric compounds.

In the following the procedure is illustrated by a case example on nano-sized silver particles (nano-Ag) which were shown to consist of 85 % (w/w) silver by XRF and to be associated 15 % (w/w) organic compounds by TGA (Nymark et al., 2013). These results were in accordance with the information on surface coating of the nano-Ag given by the manufacturer.

Without knowing better the first extraction method applied was PLE with methanol at 200 °C. The extract was centrifuged to remove all residual nano-Ag which was not filtered off in the PLE process. The supernatant in different volumes (1-40 µl) was injected onto adsorbent tubes containing Tenax TA and analyzed with TD-GC-MS. This showed that the extract only contained one compound, namely 2-pyrrolidone.

The idea that 2-pyrrolidone might be a thermal degradation product emerged and consulting the literature confirmed that both 2-pyrrolidone, and N-vinyl-2-pyrrolidone may be thermal degradation products of polyvinylpyrrolidone (PVP) and that PVP often is used in synthesis mixtures of nano-Ag.

Now we had to prove that the coating consisted of PVP. For this purpose the nano-Ag was subjected to ultrasonic extraction in methanol at ambient temperature. The extract was

centrifuged to sediment all nano-Ag and the supernatant analyzed by MALDI-TOF-MS (see Figure 1).

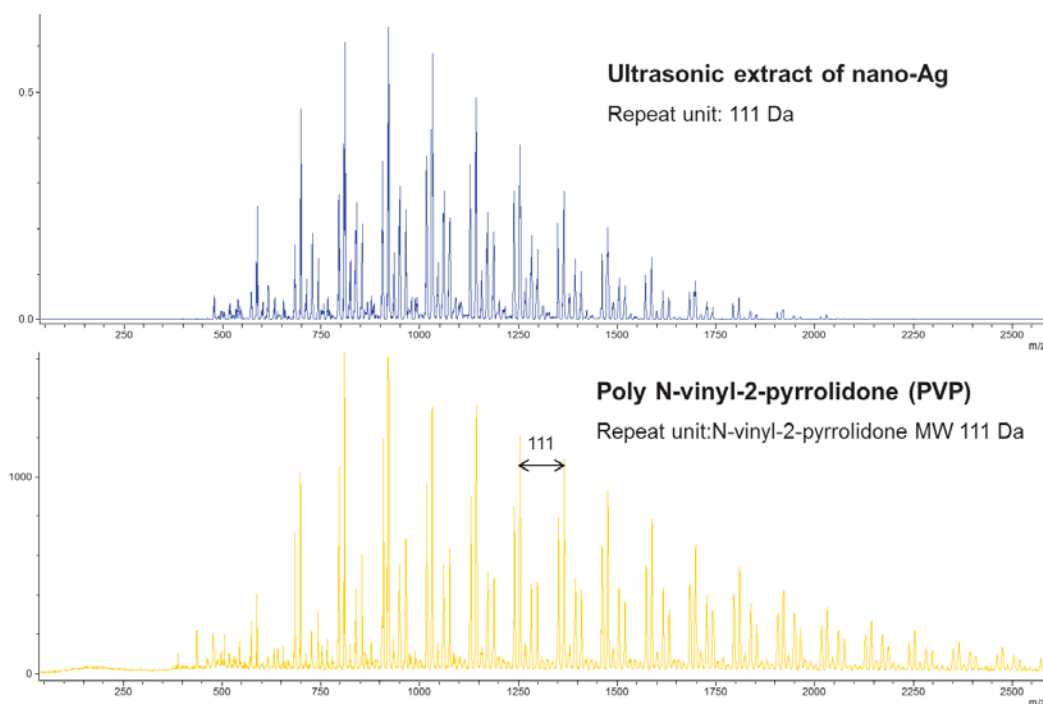


Figure 1. MALDI-TOF-MS mass spectra of the ultrasonic extract of nano-Ag (upper panel) and a standard of PVP with average MW 40,000 (lower panel).

Though the distributions of masses in the two mass spectra shown in Figure 1 are not exactly identical the pattern with the repeat unit of 111 Da reveals that the coating is PVP. The differences may be due to different average MW of the PVP on the nano-Ag and the PVP standard.

As a final proof that the coating on the nano-Ag consisted of PVP only, it was decided to quantify the amount of PVP on the nano-Ag using the ability of PVP to thermally degrade to 2-pyrrolidone and N-vinyl-2-pyrrolidone in reproducible manner. Thus the ultrasonic extracts of nano-Ag which contained the extracted PVP was injected onto adsorbent tubes with Tenax TA and analyzed with TD-GC-MS. Standard solutions of PVP in methanol were also injected on Tenax TA adsorbent tubes and analyzed by TD-GC-MS to make a calibration curve for the formation of N-vinyl-2-pyrrolidone from thermal degradation of PVP during the TD process at 250 °C. The reading on this curve of the formation N-vinyl-2-pyrrolidone from the extracts of nano-Ag showed a content of 23 % (w/w) of PVP on the nano-Ag. A little overestimated compared to the 15 % (w/w) determined by TGA but precise enough to conclude that the surface coating of the nano-Ag consisted solely of PVP.

7. Validation of method

The method has been (partly) validated by the partners in deliverable D 2.4, NanoReg. Further documentation of the procedures will be presented in Clausen et al. (2015). In addition, an interlaboratory study (ILS) should be performed among the D 2.4 partners to document the robustness of the method.

8. References

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DRAFT - NANOREG RESTRICTED

**Appendix V. SOP for determination of inorganic coating in MNMs by
Transmission Electron Microscopy**

DRAFT - NANOREG RESTRICTED

SOP for determination of inorganic coating in MNMs by Transmission Electron Microscopy

Version 1.0

Work in progress (revision is being tested)

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Foreword

This SOP (Standard Operating Procedure) is one in a series of protocols resulting from a collaboration of the partners in the NanoReg project. The original version of this protocol (Ver. 1.0) is stored in the NANoREG area on CIRCABC. Updates to this protocol may be released in the future and within the duration of the NANoREG project, such updates will also be stored on CIRCABC. The latest version of the SOP will always be available from the NANoREG homepage.

Please visit <http://nanoreg.eu/> for updates and to retrieve the latest version of the protocol as well as other NANoREG methods for MNM characterization.

The NANoREG consortium is interested in soliciting feedback on this method. We value user comments and suggestions to improve or further validate this protocol. Please send your name, email address and comments/suggestions to Per Axel Clausen (pac@nrcwe.dk). We also encourage users to report citations to published work in which this protocol has been applied.

Certain commercial entities, equipment or materials may be identified in this document in order to describe an experimental procedure or concept adequately. Such identification is not intended to imply recommendation or endorsement by the National Research Centre for the Working Environment (Denmark) and the NANoREG project team, nor is it intended to imply that the entities, materials or equipment are necessarily the best available for the purpose.

DRAFT - NANOREG RESOURCES

1. Introduction

1.1 Background

One of the key requirements in comparative testing is that all preparation and subsequent characterization procedures are as harmonized and inter-calibrated as possible. Toxicity and fate assessment are key elements in the evaluation of the environmental, health and safety risks of manufactured nanomaterials (MNM). While significant effort and resources have been devoted to the toxicological evaluation of many MNM, obtaining conclusive and reproducible results continues to be a challenge. This can be traced in part to the lack of standardized protocols for characterization of MNM.

This protocol describes a procedure to obtain information on the localization and description of secondary phase/coating etc. on the surface of MNMs. This also includes non-constituting elements or non-stoichiometric compositions. The identification and detailed analysis required for such surface specific coatings is provided by TEM/STEM, HAADF-STEM or Analytical TEM: EDS, EELS etc. for the localization, identification and description of the impurity, secondary phase and/or coating on the surface of the MNMs. These methods can be both qualitative and quantitative.

1.2 Purpose and background for the SOP

The purpose of this SOP is to enable reliable procedures for identification and quantification of inorganic coatings on the surface of MNMs by Transmission Electron Microscopy (TEM). The SOP do not describe an easy-to-use procedure since the often unknown inorganic coating can consist of almost any element and/or combinations of elements. The procedures described in the SOP require an operator with great experience in the techniques used. The SOP is prepared in collaboration with the partners in deliverable D 2.4 in the NANoREG project. It is a prerequisite for this SOP that XRF have identified two or several elements in amounts that indicate a possible inorganic coating on the MNM under investigation.

The primary purpose of Scanning Transmission Electron Microscopy (STEM) is to identify and quantify inorganic coating/secondary phases that can be found in MNMs, both by carrying out imaging (TEM/STEM) as well as by employing analytical methods (EDS, EELS etc.).

2. Principles and scope

The intention with this protocol is to provide a validated method for qualitative and quantitative estimation of inorganic coatings on the surface of MNMs. This can be obtained both by qualitatively (TEM/STEM) imaging where the nature of the coating on the surface of the MNM can be identified, as well as quantitatively wherein analytical-TEM (EDS, EELS) can be employed to enable quantification of the coating species/substance. Thus in the case of the coatings depending on the Z (atomic number) of the species that can coat the MNM, employing HAADF-STEM imaging technique within STEM, it can be observed that since Intensity $I \propto Z^2$ in this technique, the species that coats the MNM can be identified (If Z of the coating \neq Z of the MNMs). Hence depending on the variation in the atomic number of the MNM and the corresponding species that coats the material the two species can be differentiated by imaging. Subsequently by employing Energy Dispersive X-ray Analysis (EDS)/Electron Energy Loss Spectroscopy (EELS) quantification can be obtained for the species that coats the MNM.

TEM remains an important characterization technique in the perspective of NM characterization. Other imaging techniques such as Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) are possible alternatives with each of their complementary strengths and weaknesses. The regulatory definition of manufactured nanomaterials (MN) according to the EC definition says that a nanomaterial is solid particulate compound where at least 50% of the particle number is between 1 and 100 nm along at least one dimension, but it is agreed that such particulate MN are minute pieces of matter with defined physical boundaries. In aggregates and agglomerates they are referred to as primary particles. The physical and chemical properties of a NM can be different from the properties of the corresponding bulk material because of quantum and surface effects which are size dependent. The influence of a NM on an organism or cell depends on the characteristics of its aggregates or agglomerates as well as on the size of its primary particles. The size of aggregates and agglomerates but also their morphology and the charge, coating and reactivity of their surface were shown to influence their interactions with biological systems.

Due to its high resolution and wide-spread use, TEM remains an important characterization technique in the perspective of NM characterization and supporting on e.g. the EC definition. For characterization at least one method, like TEM, should be applied that takes in account the 'dimensionality' of a NM. The EC definition explicitly states 'in one dimension'. Techniques based on scattering, like DLS, and on the measurements of the hydrodynamic radius, like centrifugal sedimentation, reduce 3D information to 1D (e.g. radius of hypothetical sphere) which in unequiaxial NM, like fibers, might lead to erroneous conclusions. Taken in account the 1-nm-resolution is aimed for NM characterization, TEM is one of the few techniques, in addition to SEM and in specific cases AFM, with sufficient resolution. TEM yields number-based results, allows size measurements but also specific shape measurements and characterization of surface topologies on a number basis (per particle), it allows making a distinction between the characterizations of primary particles and of aggregates/agglomerates. Qualitative TEM analysis allows visual evaluation of possible measurement artifacts or bias. It was essential to judge the relevance/suitability of quantitative analyses by TEM and by diffraction based techniques (DLS, PIXE) and differential centrifugation. TEM is further used to determine the primary particle characteristics. These are relatively robust parameters, which compared to aggregate/agglomerate size, are less influenced by environmental conditions like pH, solvent, sonication, presence of proteins). They correlate well with volume-specific surface area (VSSA) and nano-specific properties. Primary particle size is measured manually.

Compared to TEM, scanning transmission electron microscope (STEM), which is distinguished from conventional transmission electron microscopes (CTEM) by focusing the electron beam into a narrow spot which is scanned over the sample in a raster. The rastering of the beam across the sample makes these microscopes suitable for analysis techniques such as mapping by energy dispersive X-ray (EDX) spectroscopy, electron energy loss spectroscopy (EELS) and annular dark-field imaging (ADF). These signals can be obtained simultaneously, allowing direct correlation of image and quantitative data. By using a STEM and a high-angle detector, it is possible to form atomic resolution images where the contrast is directly related to the atomic number (z-contrast image). The directly interpretable z-contrast image makes STEM imaging with a high-angle detector appealing. This is in contrast to the conventional high resolution electron microscopy technique, which uses phase-contrast, and therefore produces results which need interpretation by simulation. There is a wide range of possible signals available in the STEM, but the commonly collected ones are the following: (i) Transmitted electrons that leave the sample at relatively low angles with respect to the optic axis (smaller than the incident beam convergence angle). This mode is referred to as bright field (BF). (ii) Transmitted electrons that leave the sample at relatively high angles with respect to the optic axis (usually at an angle several times the incident beam convergence angle). This mode is referred to as annular dark field (ADF). (iii) Transmitted electrons that have lost a measurable amount of energy as they pass through the sample. Forming a spectrum of these electrons as a function of the energy loss leads to electron energy loss spectroscopy (EELS). (iv) X-rays generated from electron excitations in the sample (EDS).

Documentary standards ISO 13322 and ISO 9276 describe instructions for sample preparation, instrument operation, and image analysis (ISO 13322-1 2004; ISO 9276-3 2008; ISO 9276-1 1998). However, detailed procedures with respect to the choice of magnification, calibration, and data binning are not included in these standards.

In the present SOP qualitative as well as quantitative analysis of coatings/secondary phases etc. by TEM/STEM imaging as well as by analytical methods are described with an example material (JRCNM01003a – TiO₂ NPs) ordered from the JRC Repository. During the course of search for already existing documentary standards it was found that there exists previous documentary standards which include ISO 9220:1988 Metallic coatings – Measurement of coating thickness – Scanning electron microscope method. ISO 1463:2003 Metallic and oxide coatings – Measurement of coating thickness – Microscopical method. ISO 2064:1980, Metallic and other non-organic coatings — Definitions and conventions concerning the measurement of thickness.

3. Terminology and abbreviations

This protocol complies with definitions relevant to nanotechnology as set forth in the ASTM standard E2456 (ASTM, 2006).

nanomaterial— a nanomaterial consists of particulate materials (as individual, agglomerated and aggregated) among which 50% of the primary particulate entities by number are between 1 and 100 nm in diameter along their shortest dimension. Graphene, fullerene and single-walled carbon nanotubes are

automatically defined as nanomaterials irrespective of the fact that their shortest diameters may be smaller than 1 nm. The primary particles in the nanoscale are also referred to as “nano-objects” in ISO TS 80004-1 and part of the ISO nanomaterial system.

primary particle — the smallest discrete identifiable entity associated with a particle system; in this context, larger particle structures (e.g., aggregates and agglomerates) may be composed of primary particles.

aggregate —particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components [ISO/TS 27687:2008]

agglomerate — collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components [ISO/TS 27687:2008] Particle: minute piece of matter with defined physical boundaries [ISO 14644-6:2007, definition 2.102]

MNM — manufactured nanomaterials

TEM – Transmission Electron Microscopy

STEM – Scanning Transmission Electron Microscopy

EDX – Energy Dispersive X-ray Analysis

EDS – Energy Dispersive Spectroscopy

EELS – Electron Energy Loss Spectroscopy

HAADF – High Angle Annular Dark Field

BF – Bright Field

DF – Dark Field

For more terminology in Scanning/Transmission Electron Microscopy, see “Scanning Transmission Electron Microscopy, Imaging and Analysis, S. J. Pennycook and P. D. Nellist (Eds.).

4. Equipment, materials and chemicals

4.1 Equipment

During the development of the present protocol TEM/STEM imaging as well as EDS was carried out with the FEG-Titan ChemiSTEM 80-200 kV (FEI), equipped with the Probe Cs corrector. The BF and DF images were acquired simultaneously together with the HAADF image. The BF/DF on-axis detector consists of three solid-state detectors; 1x bright field (BF) and 2x dark field (DF2 and DF4). The EDX system includes the Super-X EDX consisting of an integration of four windowless Silicon Drift Detector (SDD) symmetrically placed around the sample offering 0.7 srad collection angle and 120 mm² detector size provides the ideal EDX detector for use with probe corrector. This also includes an (Electron Energy Loss Spectroscopy (EELS) / Energy Filtered TEM (EFTEM) set-up with a Gatan Energy Filter, Quantum and the EFTEM, EELS module included.

The main optical specifications in STEM for the Titan ChemiSTEM at 200 kV

- STEM 0.08 nm
- TEM point resolution 0.24 nm
- TEM Information limit 0.11 nm

4.2 Materials

The current protocol is developed using the NM (JRCNM01003a – TiO₂ NPs) approved by the NANoREG Management Committee. The materials have been produced and supplied by the Joint Research Centre (JRC) of the European Commission (Italy). The materials have been certified in a transparent, detailed, and metrologically rigorous manner, and are primarily intended for quality control in daily laboratory practice, method development, and proficiency testing schemes.

4.3 Chemicals, reagents, small equipment and laboratory materials.

4.3.1 Small Laboratory Material

- Pioloform-coated and carbon-shaded copper grids 400 mesh (Agar Scientific Ltd., G2400C). Both home-made and commercially available grids can be used.
- 120 mm diameter polyethylene petridish
- 10 ml syringe (BD Plastipak, Becton Dickinson S.A. Madrid, Spain)
- 100 nm syringe filter (Minisart 0.10 μm , Sartorius AG, Vilvoorde, Belgium)
- pipette tips of 200 μl (Gilson Diamond) and 200-1000 μl (VWR)
- Tweezers
- Filter papers \varnothing 70 mm (Whatman, 54 hardened).
- Scotch tape to fix a filter paper in the petri dish.
- 4" Parafilm M (American National Can, Freewich, CT06830)
- Permanent, waterproof marker or a ball point to indicate references on filter paper

4.3.2 Specific Reagents

- Double distilled water
- Saturated Alcian blue stock solution: 2 % (w/w) in double distilled water
- Alcian blue working solution: Dilute the Alcian blue stock solution with an equal amount of water to obtain a 1 % working solution. The latter is at least stable for 1 month at 4 °C.

4.3.3 Small Equipment

- Micropipettes of 20 μl , 200 μl and 1000 μl (Gilson,)
- EasiGlow glow discharge device (Pelco, Redding, CA, USA)

5. Procedure

5.1 Preparation of TEM grids

Dispersed NM are brought in contact with an EM-grid and are allowed to interact with its surface. When excess fluid is drained and grids are air-dried, a fraction of the NM remains attached to the grid by different types of interactions (electrostatic, apolar, van der Waals, ...). The concentrations of NM, and the type and charge of the grid are chosen such that the fraction of nanoparticles attached to the grids optimally represents the dispersed NM, and that the particles of interest can be detected individually.

5.1.1 (Optional) Adaptation of the charge of the EM grid to the NM charge

- Introduction of positive charges to the EM-grid by pre-treating them with Alcian blue.
 - Fix a strip of parafilm to a flat and clean surface by wetting the surface with some drops of water, press the parafilm with the film side to the surface and remove the protective cover carefully.
 - Place an EM grid with the carbon-coated side on a drop of about 20 μl Alcian blue working solution and incubate for 1 to 10 minutes. Avoid air drying of the grids.
 - Grip the grid carefully with a pair of tweezers and wash most of the blue stain away by transferring it to 5 drops of water placed on the parafilm. Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.
 - Use the grids immediately in the next step.

- Introduction of negative charges to the EM-grid by glow discharging.
 - Grids are glow discharged using the EasiGlow glow discharge device according to the manufacturer's instructions.
 - Use the grids immediately in the next step.

The interaction of NM with an EM-grid is strongly determined by the charge of the grid. In general, freshly prepared carbon-coated grids are hydrophobic or lightly negatively charged. Dependent on the preparation and storage time of the grid these charges tend to disappear leaving a hydrophobic surface. For non-charged particles and hydrophobic media, non-treated grids are advised. For charged particles in polar media, hydrophilic grids are required. The hydrophilicity of EM-grids can be increased by pre-treating them with Alcian blue pre-treatment. The resultant positive charge strongly increases attachment of negatively charged NM. Alternatively, negative charges can be introduced by glow discharging strongly increasing attachment of positively charged NM. In case the charge of the NM is unknown, it is wise to evaluate the different approaches.

5.1.2 Coating the NM on the grid

- Homogenize the dispersion by shaking, vortexing, stirring or pipetting.
- (Optional) Dilute the dispersed NM in a suitable dilution medium.

The concentration of NM should be chosen such that the number of particles per micrograph is optimal for later analysis. It should be taken into account that the particles do not touch or overlap each other. Optimal concentrations vary from sample to sample as illustrated in following examples.

- A typical concentration for a colloidal, spherical, unaggregated silica NM is 0.05 mg/ml.
- For colloidal aggregated silica NP a concentration up to 0.5 mg/ml can be used.
- For non-colloidal, aggregated, silica NM a concentration of 2.5 to 25 mg/ml can be used.

For polydisperse samples, the grid-on-drop method might result in a preferential binding of the fraction of smaller particles, whereas the drop-on-grid method might result in a preferential binding of the fraction of larger particles.

- Allow the NM to interact with the grid surface by either the drop-on-grid method, or the grid-on-drop method
 - Grid-on-drop method: Place the EM-grid on a droplet of dispersed NP.
 - Fix a strip of parafilm to a flat and clean surface by wetting the surface with some drops of water, press the parafilm with the film side to the surface and remove the protective cover carefully.
 - Place a droplet of 2 to 50 μ l of dispersed NM on the parafilm.
 - Transfer the grid to the droplet and indicate the appropriate references with a waterproof marker.
 - Float the grids, with coated surfaces down, on the droplets for 1 to 10 minutes.
 - Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.
 - Drop-on-grid method: Place a droplet of dispersed NP on an EM-grid.
 - Put the grid on the parafilm and then pipette a droplet of 2 to 50 μ l of dispersed NM on the grid. Leave this for 1 to 10 minutes.
 - Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.
- (Optional) Rinse step
 - Rinse the grids, with coated surfaces down, by placing them on a droplet of water for 30 seconds to remove excess salts and contaminating material.
 - Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.

The additional washing step reduces the background signal of the grid by removing excess salts and contaminating material. Extensive washing might result in the selective loss of the larger particles from the grid which should be avoided.

5.1.3 Storage of the grids

- Place the grids in a grid box or on a filter paper in a petri-dish.
- Make sure to always note the appropriate references in order to be able to retrieve specific grids later on.

5.1.4 Specific safety measures

- Because of the possible toxicity of the reagents and NM and the possible presence of unknown contaminants, it is highly recommended to wear gloves during this procedure.
- All waste materials generated during this procedure should be disposed in the suitable container for chemical waste.

5.2 Description of the conditions of the acquisition of the STEM images and EDS Analysis

- STEM Images were collected with the HAADF Detector and acquired with the Tia Software.
- Point EDS Analysis, Line Scan Analysis and chemical maps were acquired with the Super-X EDX Detector and analyzed with the corresponding software.
- EDS Spectrum and line scan analysis on individual nanoparticles were collected for 5 min and chemical maps were acquired for 5 min.
- EDS Spectrum Quantification was carried out by using Cliff-Lorimer method of quantification. (Cu signal was used for deconvolution only and not for quantification.).
- Point EDS Analysis, Line Scan Analysis on individual nanoparticles and EDS maps were collected over a significant set of Nanoparticles.

6. Expected outcome and interpretation of results

The HAADF-STEM technique is particularly important in the study of inorganic coatings on the surface of MNMs. In this technique one uses the Annular Dark Field (ADF) detector to collect the signal that produces the image. With the annular detector, only the electrons scattered more than a minimum angle θ are able to reach the detector and contribute to the image. At high angles (more than 50 mrad), electron are scattered incoherently, with essentially no Bragg scattering present. The image intensity is then mostly due to the individual contribution of each atom to the scattering. It has been previously demonstrated that the signal collected by the high angle annular dark field (HAADF) detector will have an intensity directly related to the scattering cross-section, and thus it will depend on the atomic number of the atoms in the specimen. Due to this dependence on the atomic number (Z) of the atoms on the specimen, the contrast in this imaging technique is strongly dependent on the chemical composition, and for this reason it is called Z -contrast imaging. The Z -contrast technique works remarkably well with different Z materials, because the intensity dependence is close to $Z^{3/2}$, with minimum dependence on microscope defocus. This is definitely different from what is expected of bright field imaging, where the signal varies weakly and non-monotonically with Z . The Z dependence technique allows identifying easily the elements present in the sample. Moreover, as the electron beam interacts with the sample, the electrons are elastically or inelastically scattered. Those elastically scattered electrons are the sources of STEM images that can be recorded either in HAADF

or ADF detectors. The signals of inelastically scattered electrons are the sources of chemical information present in the sample. This in combination with analytical techniques such as EDS or EELS can provide very clear and unambiguous information especially in the present example related to the fact that MNMs have inorganic coated species around it. Figure 1, shows a typical BF-TEM and HAADF-STEM images of the TiO_2 NPs (JRCNM01003a). A closer imaging and analysis of the TiO_2 NP – AlO_x Inorganic Coating by EDS Chemical Analysis is provided in Figs. 2 – 4. The TiO_2 nanoparticle is crystalline whereas the outer inorganic coating (AlO_x) is amorphous. It is also important to note here that the analysis is not only qualitative but more importantly quantitative information can be extracted easily in EDS (or EELS) (See Table 1 and 2) from the respective analytical signals; EDS in this case.

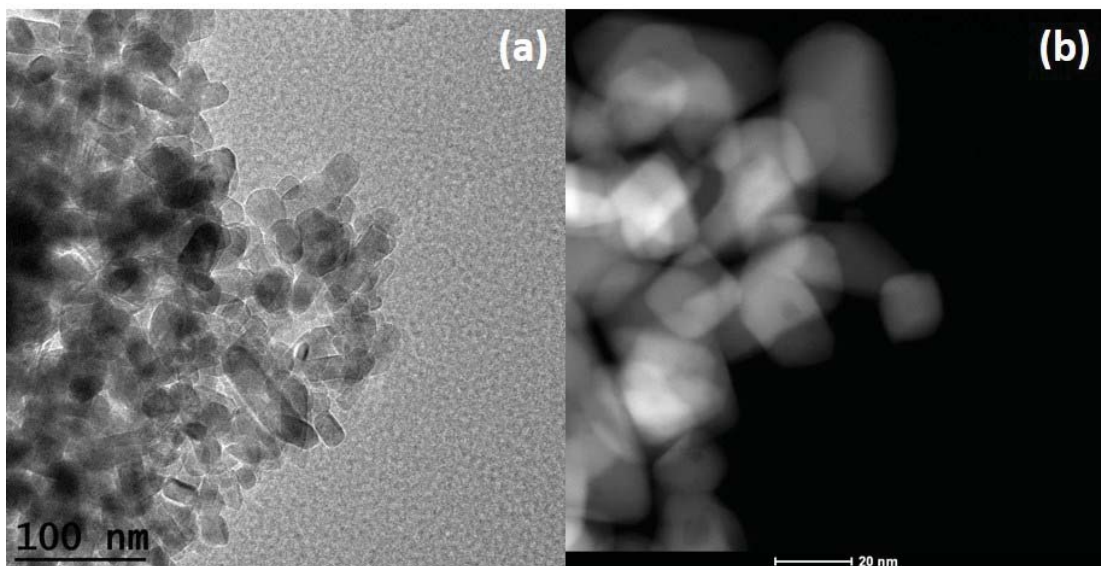


Figure 1. (a) BF-TEM and (b) HAADF-STEM images of the TiO_2 Nanoparticles (JRCNM01003a).

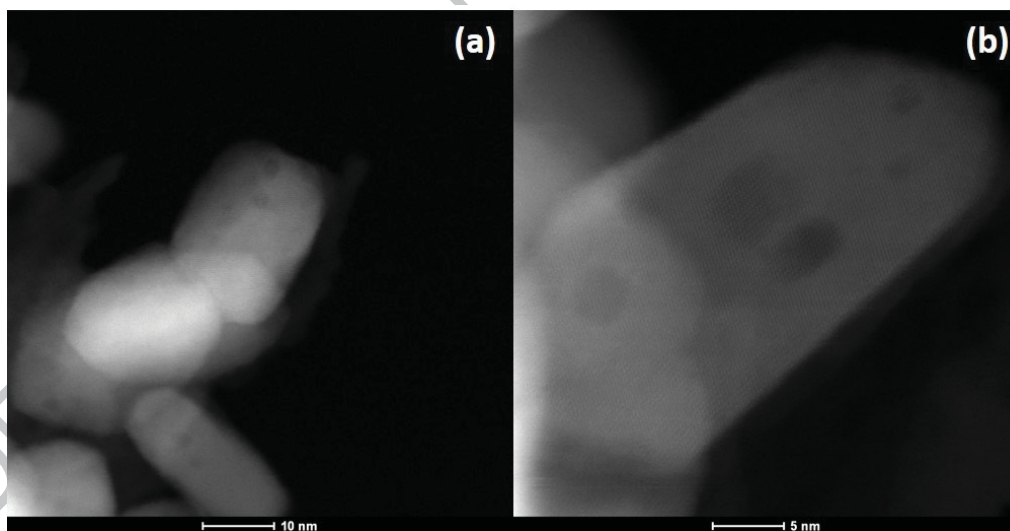


Figure 2. HAADF-STEM images of (a) collection of TiO_2 Nanoparticles and (b) Individual TiO_2 Nanoparticle. Since Intensity $\propto Z^2$ ($Z_{\text{Ti}} > Z_{\text{Al}}$, $Z(\text{Ti}) - 22$, $Z(\text{Al}) - 13$) it is possible to see the difference in contrast between the TiO_2 nanoparticle and the amorphous inorganic coating (AlO_x) around it.

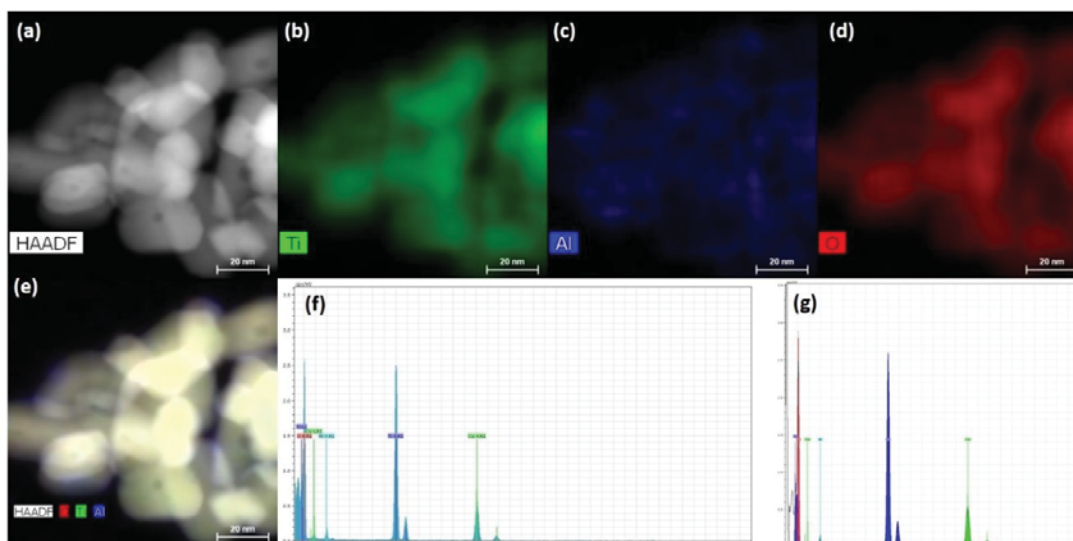


Figure 3. (a) HAADF-STEM images of a collection of TiO₂ Nanoparticles. (b) – (d) Corresponding EDS Maps of the nanoparticle and the inorganic coating seen with the elements corresponding to Ti, Al and O. (e) Combined EDS Map of the elements. (f) EDS Spectrum obtained from the same region (g) Deconvolution of the peaks of the EDS spectrum and quantification of the various elements (Ti, Al, O) carried out (Table 1).

Table 1: Quantification of the elements present in the nanoparticle-inorganic coating (TiO₂-AlO_x). Note: Cu signal arising from the grid was used only for the deconvolution and not for the quantification.

Element	series	Net	[wt.%]	[norm. wt.%]	[norm. at.%]	Error in wt.% (3 Sigma)
Oxygen	K-series	41380	46,31496	46,31496	71,64216	4,887759
Copper	K-series	28002	0	0	0	0
Titanium	K-series	96556	52,16472	52,16472	26,96335	4,800459
Aluminium	K-series	3261	1,520315	1,520315	1,394497	0,233702
Sum:			100	100	100	

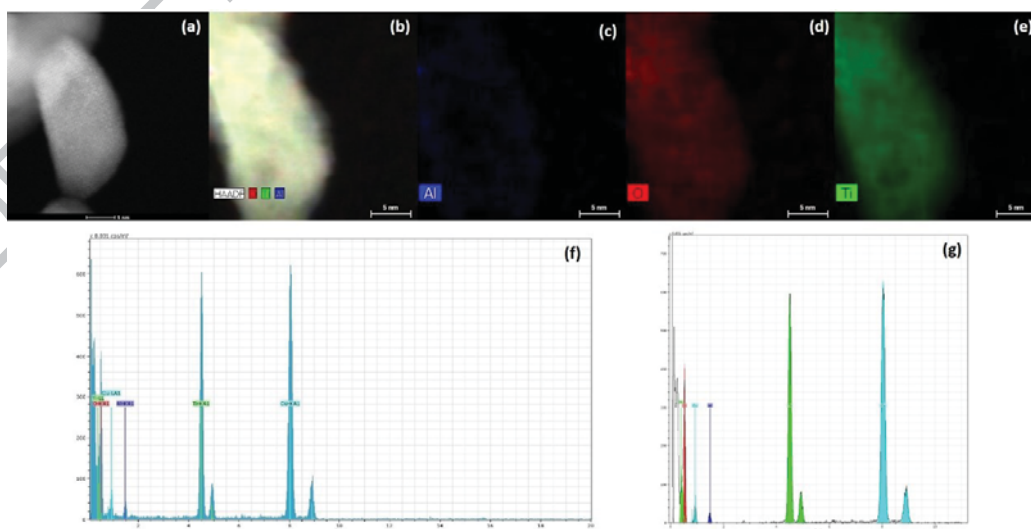


Figure 4. (a) HAADF-STEM images of a single TiO₂ Nanoparticle. (c) – (e) Corresponding EDS Maps of the nanoparticle and the inorganic coating seen with the elements corresponding to Ti, Al and O. (b) Combined EDS Map of the elements. (f) EDS Spectrum obtained from the same region (g) Deconvolution of the peaks of the EDS spectrum and quantification of the various elements (Ti, Al, O) carried out (Table 2).

Table 2: Quantification of the elements present in the nanoparticle-inorganic coating (TiO₂-AlO_x). Note: Cu signal arising from the grid was used only for the deconvolution and not for the quantification.

Element	series	Net	[wt.%]	[norm. wt.%]	[norm. at.%]	Error in wt.% (3 Sigma)
Oxygen	K-series	2353	34,90844	34,90844	61,23624	4,265045
Titanium	K-series	8898	63,75088	63,75088	37,3692	6,206788
Aluminium	K-series	217	1,340675	1,340675	1,394564	0,375251
Copper	K-series	12455	0	0	0	0
		Sum:	100	100	100	

Based on the screening level WDXRF which showed a content of 96 % (w/w) TiO₂ and 3 % (w/w) Al₂O₃, and the specific level HAADF-STEM analysis it may be concluded that it is an inorganic core MNM consisting of about 96%w/w TiO₂ with an inorganic coating consisting of about 3%w/w Al₂O₃.

7. Validation of method

The method has been (partly) validated by the partners in deliverable D 2.4, NanoReg. In addition, an interlaboratory study (ILS) should be performed among the D 2.4 partners to document the robustness of the method.

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Appendix VI. SOP for determination of oxygen containing functional groups on CNT by decarbonylisation, decarboxylation and dehydration in inert atmosphere using TGA-MS

DRAFT - NANOREG RESTRICTED

SOP for determination of oxygen containing functional groups on CNT by decarbonylisation, decarboxylation, and dehydration in inert atmosphere using TGA-MS

Version 1.0

Work in progress (revision is being tested)

If in trouble, please send feed-back to

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Foreword

This SOP (Standard Operating Procedure) is one in a series of protocols resulting from a collaboration of the partners in the NanoReg project. The original version of this protocol (Ver. 1.0) is stored in the NANoREG area on CIRCABC. Updates to this protocol may be released in the future and within the duration of the NANoREG project, such updates will also be stored on CIRCABC. The latest version of the SOP will always be available from the NANoREG homepage.

Please visit <http://nanoreg.eu/> for updates and to retrieve the latest version of the protocol as well as other NANoREG methods for MNM characterization.

The NANoREG consortium is interested in soliciting feedback on this method. We value user comments and suggestions to improve or further validate this protocol. Please send your name, email address and comments/suggestions to Per Axel Clausen (pac@nrcwe.dk). We also encourage users to report citations to published work in which this protocol has been applied.

Certain commercial entities, equipment or materials may be identified in this document in order to describe an experimental procedure or concept adequately. Such identification is not intended to imply recommendation or endorsement by the National Research Centre for the Working Environment (Denmark) and the NANoREG project team, nor is it intended to imply that the entities, materials or equipment are necessarily the best available for the purpose.

DRAFT - NANOREG RESOURCES

1. Introduction

1.1 Background

One of the key requirements in comparative testing is that all preparation and subsequent characterization procedures are as harmonized and inter-calibrated as possible. Toxicity and fate assessment are key elements in the evaluation of the environmental, health and safety risks of manufactured nanomaterials (MNM). While significant effort and resources have been devoted to the toxicological evaluation of many MNM, obtaining conclusive and reproducible results continues to be a challenge. This can be traced in part to the lack of standardized protocols for characterization of MNM. This protocol describes a relatively simple procedure to obtain information on the amount of oxygen containing functional groups on the surface of carbon nanotubes (CNT) that can be decarbonylated (release of CO), decarboxylated (release of CO₂) or dehydrated by heating in an inert atmosphere (He).

1.2 Purpose and background for the SOP

The purpose of this SOP is to enable an easy-to-use and still reliable procedure for quantification of oxygen containing functional groups on the side-walls of CNT using thermogravimetric analysis (TGA) with a detector suitable for CO and CO₂, and H₂O. The SOP is based on results in Jackson et al. (2015) and a publication in preparation (Clausen, 2015) and developed in collaboration with the partners in deliverable D 2.4 in the NanoReg project.

The primary purpose of the thermogravimetric analysis (TGA) is to measure the amount of carbon monoxide that can be decarbonylated and carbon dioxide (CO₂) that can be decarboxylated and water (H₂O) that can be dehydrated during heating in a non-oxidizing He atmosphere.

2. Principles and scope

The intention with this protocol is to provide a validated method for quantitative estimation of oxygen containing functional groups covalently bound to the side-walls of CNT. More correctly it is a quantitative estimation of the CO, CO₂, and H₂O, that is released from CNT during heating in an inert atmosphere, and which contain the oxygen (O) bound in oxygen containing functional groups. Then from the released CO/CO₂/H₂O the total amount of O bound in oxygen containing functional groups can be calculated. The study of O containing surface functionalities of carbon material has mainly been performed on oxidized activated carbon and the method used termed temperature programmed desorption (TPD) (Figueiredo et al., 1999; Szymanski et al., 2002; Kundu et al., 2008). Figure 7 shows possible oxygen containing functional groups on a carbon surface and their decomposition by heating into CO/CO₂. Dehydration may occur when e.g. adjacent carboxylic acid groups are exposed to heat.

TGA is a technique that combines an oven and a balance. Heating the sample in a crucible in the oven the weight (loss) can be monitored as a function of temperature. Usually samples contain water which is desorbed at low temperatures but also organic compounds adsorbed to the surface of MNM may be desorbed during heating in the TGA thus resulting in weight loss of the sample. To be sure which weight loss is associated with release of CO/CO₂/H₂O a detector is required at the outlet of the TGA. In this SOP a method using a mass spectrometer as the detector is described. However, an infrared spectrometer (FTIR) could be used as well if the detection ability is sufficient.

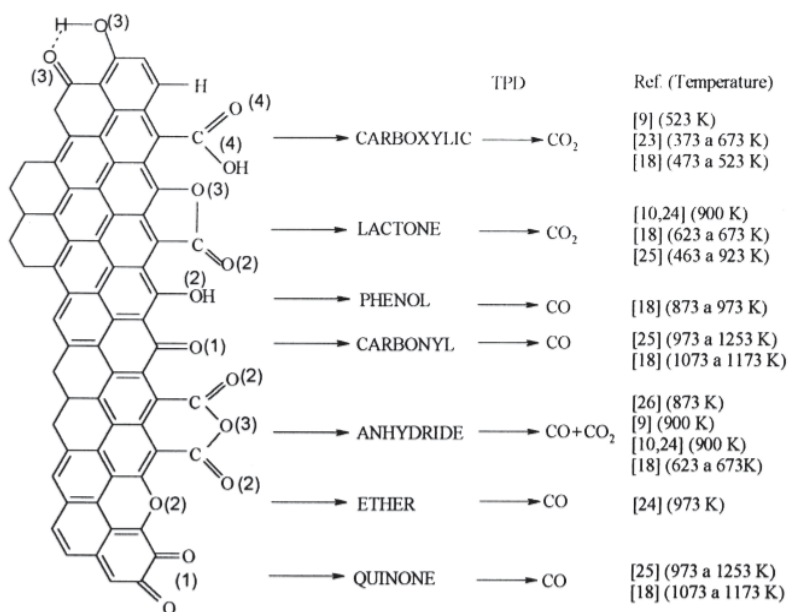


FIGURE 7. POSSIBLE OXYGEN CONTAINING FUNCTIONAL GROUPS ON CARBON SURFACE AND THEIR DECOMPOSITION BY HEATING INTO CO/CO₂. ADOPTED FROM FIGUEIREDO ET AL. (1999).

The method can be used as a part of the characterization of CNT for chemical and industrial use and for toxicological testing.

3. Terminology and abbreviations

This protocol complies with the definition of a manufactured nanomaterials as proposed by the EC as well as ISO TS 80004-1 (ISO, 2010) and ASTM standard E2456 (ASTM, 2006).

nanomaterial— a nanomaterial consists of particulate materials (as individual, agglomerated and aggregated) among which 50% of the primary particulate entities by number are between 1 and 100 nm in diameter along their shortest dimension. Graphene, fullerene and single-walled carbon nanotubes are automatically defined as nanomaterials irrespective of the fact that their shortest diameters may be smaller than 1 nm. The primary particles in the nanoscale are also referred to as "nano-objects" in ISO TS 80004-1 and part of the ISO nanomaterial system.

primary particle — the smallest discrete identifiable entity associated with a particle system; in this context, larger particle structures (e.g., aggregates and agglomerates) may be composed of primary particles.

aggregate — a discrete assemblage of primary particles strongly bonded together (i.e., fused, sintered and with chemical bonds exceeding e.g., van der Waals and hydrogen binding forces).

agglomerate — assemblages of particles (including primary particles and/or smaller aggregates) held together by relatively weak forces (e.g., van der Waals, capillary or electrostatic), that may break apart into smaller particles upon further processing.

pristine CNT — CNT without intentional functionalization, though commercial available CNT usually contain COOH and OH groups covalently bound to walls as a consequence of e.g. acid treatment after synthesis.

Side-wall of CNT — the outer tubular network of CNT consisting of carbon atoms connected in 5- and 6-membered rings.

functionalized CNT (f-CNT) — pristine CNT with functional groups attached to the side-wall. There are three main approaches: 1) covalently bound surface groups generated through acid-induced oxidation of the carbon nanotube surface; 2) direct addition to the carbon nanotube side-walls by covalent binding; 3) groups attached to e.g. polycyclic aromatic hydrocarbons that are immobilised to the carbon nanotube surface through Van der Waals forces. The last group includes a vast variety of substances that are able to interact with CNT side-walls through Van der Waals forces.

single-walled CNT (SWCNT) — a tube with a one-carbon-atom thick wall

multi-walled CNT (MWCNT) — consist of a number of concentric SWCNT inside each other (from 2 to several)

CNT — carbon nanotube

TGA — thermogravimetric analysis

TPD — temperature programmed desorption

CO — carbon monoxide

CO₂ — carbon dioxide

MS — mass spectrometry

O — oxygen

MNM — manufactured nanomaterials

He — helium

XPS — x-ray photoelectron spectroscopy

4. Equipment, materials and chemicals

4.1 Equipment

Glove box equipped with an analytical balance for weighing the CNT samples.

TGA combined with mass spectrometry (TGA-MS) or other detector suitable for CO/CO₂/H₂O. During development of the present protocol TGA-MS was performed using a Netzsch STA 449 F3 TGA coupled to a Varian CP-3800 GC and a Varian 1200 L triple quadrupole MS with electron ionization. The GC was equipped with a short piece of deactivated capillary column as transfer-line to the MS, but was intended for future experiments on trapping simultaneously released degradation products of surface modifications and subsequent chromatographic separation with an active column.

4.2 Materials

Large TGA crucible with lid to ensure that the CNT sample stays in the crucible. The large crucible is required due to the very low bulk density of CNT.

4.3 Chemicals

A sample of the CNT to be tested. CNT may be considered carcinogenic (NIOSH, 2013) and must be treated as such. If preferred, to obtain a more accurate result, the detector (MS) may be calibrated with suitable standards of CO/CO₂/H₂O diluted in an inert gas and with a certified concentration.

5. Procedure

5.1 Preparation of the TGA

Consult the instruction manual of the actual TGA instrument for specific recommendations on preparation and settings. Adjust the TGA sample flow of dry He to 25 ml/min, the balance purge flow of He to 25 ml/min, and adjust the flow to the MS using a restrictor column to fit a suitable pressure in the ion source. Set the temperatures in all transfer lines and GC to 230°C. Air as TGA gas cannot be used in connection with MS since the filament in the ion source of the mass spectrometer will burn in the presence of oxygen. In the presence of oxygen in the TGA the carbon backbone of CNT may burn and develop CO₂ which cannot be distinguished from decarboxylated CO₂ originating from O containing groups on the sidewalls of the f-CNT. Set TGA temperature program: 50 to 1150°C at 20° C/min. Run the temperature program with an empty crucible to be able to correct for buoyancy.

5.2 Preparation of the CO/CO₂/H₂O detector (MS)

Ensure that the MS is mass calibrated. Use full scan mode and single ion monitoring (SIM) e.g. m/z 40-500 and SIM for m/z 18 (water), m/z 28 (CO), and m/z 44 (CO₂). Extinguish signals from He (m/z 2). The TGA-MS system may be calibrated with a pure solid that release CO₂ at thermal decomposition e.g. CaCO₃ (Slager and Prozonic, 2005; Biswas et al., 2007).

5.3 Weighing the CNT sample

Transfer about 120 mg of the CNT sample from the sample container to the clean crucible using a glove box equipped with an analytical balance for weighing the sample. Remember to put the lid on to prevent that the CNT "fly" out of the crucible due to their possibly electrostatic charging. Hold the crucible with tweezers. It is essential to avoid any contamination of the sample, crucible and TGA by skin moisture and/or grease.

5.4 Performing the TGA

1. Insert the crucible with the CNT sample into the TGA oven using tweezers and close it
2. Set the time – temperature profile as follows
 - a. Start temperature 50 °C
 - b. Heat up to 1150 °C at rate 20 °C min⁻¹
 - c. Hold at 1150 °C for 0 min?
 - d. Cool down to room temperature

Total duration of the analysis program (from 50 - 1150 °C): approx. 55 min + about 1 hour for cool down
3. Start the TGA and the MS simultaneously.
4. Save TGA experimental results recording the percent weight vs. sample temperature curve (that is, the actual temperature measured at the thermocouple). Save the MS recording thus the TGA weight curve and the traces can be compared.

5.5 Sample removal and disposal

1. Ensure that the furnace is close to room temperature
2. Remove the crucible and empty it in the glove box
3. Remove the residue from the crucible using the spatula
4. Clean the crucible using clean paper and solvent followed by e.g. ultrasonic cleaning
5. Dispose of the residue and dirty paper with solvent following your Institution's procedures for hazardous wastes

6. Expected outcome and interpretation of results

In the following the procedure is illustrated by a case example on *f*-CNT functionalized only with carboxylic acid (COOH) groups and thus only releases CO₂. When the investigated *f*-CNT contain enough COOH groups to produce CO₂ sufficiently to get above the detection limit a peak may appear in the mass trace *m/z* 44 as shown in **FIGURE 8**.

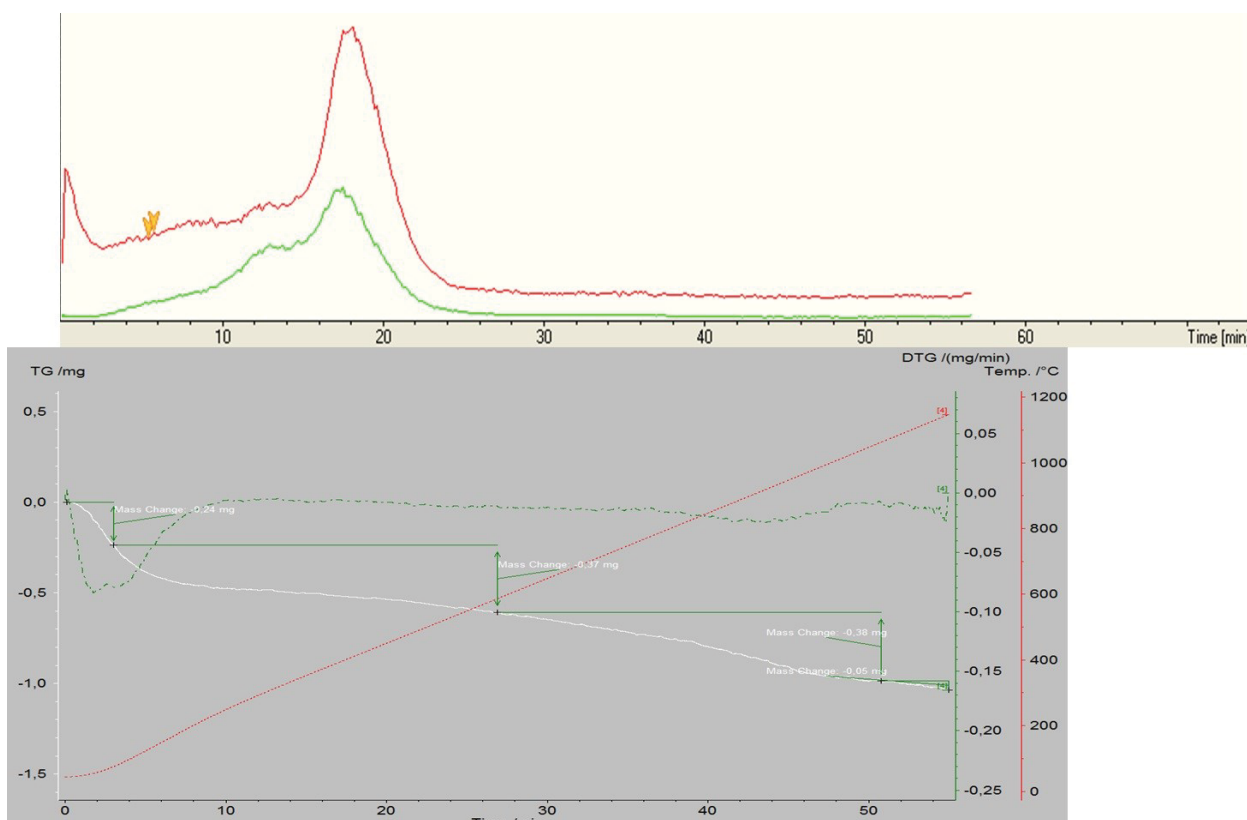


FIGURE 8 EXAMPLE OF TGA-MS ANALYSIS OF *f*-CNT INTENTIONALLY FUNCTIONALIZED WITH COOH. THE UPPER PANEL SHOWS MS TRACES (TIC AND CO₂ (*m/z* 44)) AND THE LOWER PANEL SHOWS THE TGA CURVE.

The way to derive the amount of CO₂ produced by the CNT sample is to transfer the time range for CO₂ release detected by the MS (approximately 3 – 28 min in the upper panel in Figure 1) to the TGA curve (marked with green vertical arrows in lower panel in Figure 1) and calculate the weight loss during this period. In the present case this amounts to 0.37 mg CO₂. Assuming that no other compounds are released in this period all weight loss can be attributed to release of CO₂. Furthermore assuming that all released CO₂ originates from COOH groups the content of COOH in the *f*-CNT amounts approximately 0.28 mmol/g *f*-CNT. The prerequisites for this result is that no other compounds than CO₂ is released in the actual time range and that all O released as CO₂ is bound in COOH groups. None of these prerequisites holds in reality but the question is to what extent it is true. The conclusion is that using the described method the result may be reported as maximal content of COOH groups in the *f*-CNT sample is 0.28 mmol/g *f*-CNT or < 0.28 mmol/g *f*-CNT.

To get the exact mass of CO₂ that is released the MS has to be calibrated for CO₂ with a pure solid that release CO₂ at thermal decomposition e.g. CaCO₃.

The method may be supplemented by other analytical techniques that can measure carboxylic acid groups. The simplest method is acid-base titration but this method is not very precise

(about \pm 50%) (Jackson et al., 2015). A more advanced method is XPS which can distinguish and quantify all types of bound O.

7. Validation of method

The method has been (partly) validated by the partners in deliverable D 2.4, NANoREG, using combustion elemental analysis, acid-base titrations and x-ray photoelectron spectroscopy (XPS) in addition to more general characterization methods such as electron microscopy as presented in Clausen et al. (2015). These methods may be applied to obtain more accurate results. In addition, an interlaboratory study (ILS) should be performed among the D 2.4 partners to document the robustness of the method.

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