Identification of nanomaterials: Anticipating and addressing the main difficulties during dimensional analysis by microscopy of particulate substances

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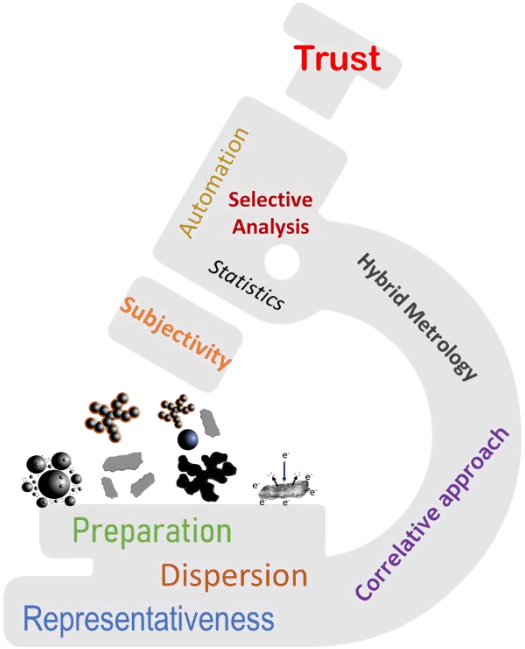

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"Identification of nanomaterials: Anticipating and addressing the main difficulties during dimensional analysis by microscopy of particulate substances" – **version December 11, 2024**

INTRODUCTION

This note aims to enable any player (regulatory or otherwise), not necessarily having analytical expertise, to **anticipate and address the difficulties they might encounter when confronted with a request for an analysis** to determine number size distribution of the constituent particles **in particulate substances within a regulatory context for the identification of nanomaterials.**

Various complexity classes are introduced and illustrated with the help of concrete examples of electron micrographs to clarify the difficulties most commonly encountered during the analysis of materials composed of particles that may have sizes within the nanoscale range. This note, aimed at practical application and intentionally concise, aims to help various players classify their substances according to complexity categories. This classification will facilitate communication with raw material suppliers, analytical laboratories, and public authorities.

More extensive information on the existing analysis technologies and potential areas for consideration is also available in a companion document, drawn up as a guide for analytical experts. This more detailed document aims to **develop the most appropriate analytical strategy** to increase confidence in the data produced on such complex materials.

CONTEXTUAL ELEMENTS

The revision of the European recommendation on the definition of nanomaterials (2022/C229/01), in 2022, has launched a new round of technical discussions on the analytical approaches to implement. If this new definition recommendation, as it currently stands, is not currently binding, it nevertheless raises a number of questions regarding the interpretation, in certain cases, of the dimensional characteristics of particulate materials potentially considered as nanomaterials. In this way, establishing the number size distribution of the constituent particles based on their smallest external dimension may prove to be relatively complex, depending on particles.

At the European level, and to assist the implementation of this definition, the JRC: Joint Research Centre has published a guide[1] that includes a decision tree based on two analysis strategies to be considered depending on the materials involved. This methodology includes a *screening* step possibly followed by *confirmation* tests when the *screening* step would not have led to a conclusion on the categorization as a **Nanomaterial**. The *confirmation* step is based on direct analytical methods, such as scanning or transmission electron microscopy (SEM or TEM) as well as atomic force microscopy (AFM), which make it possible to determine the number size distribution of the particles.

If microscopy methods are considered as references in a regulatory context[2] for identifying the *Nanomaterial* status of particulate substances, it is necessary to develop and optimise analysis strategies for each type of material in order to rationalise their analysis cost and avoid performing unnecessary or unsuitable tests. Furthermore, the degree of applicability of these methods is limited for materials with levels of complexity that differ significantly from the samples used to validate these same methods.

To date, no document illustrates the challenges associated with materials with specific physico-chemical properties that can make it difficult to identify, through microscopy methods, whether they qualify as nanomaterials within a regulatory framework, especially when "screening" techniques are inconclusive. This document lists specific cases of these difficulties. It helps regulatory stakeholders anticipate and address the issues, work with expert laboratories for proper analysis, and engage with authorities on a solid foundation of shared understanding. **NanoMesureFrance declines all responsibility for the use and interpretation of this document.**

[1] H. Rauscher, V. Kestens, K. Rasmussen, T. Linsinger, E. Stefaniak, Guidance on the implementation of the Commission Recommendation 2022/C 229/01 on the definition of nanomaterial, Publications Office of the European Union, Luxembourg, 2023, doi:10.2760/143118, JRC132102.

[2] https://www.echa.europa.eu/documents/10162/17250/how_to_register_nano_en.pdf/f8c046ec-f60b-4349-492b-e915fd9e3ca0

OBJECTIVES AND COMPLEXITY CLASSES

This note discusses the difficulties that various stakeholders (*producer/distributor and user of nanomaterials, analysis laboratory*) **may face as they endeavour to produce data on the dimensional characteristics of particulate materials through microscopy approaches.**

To do this, the difficulties have been categorised into classes based on the inherent complexities of the materials involved. For illustration purposes, microscopy images will be shown for each case.

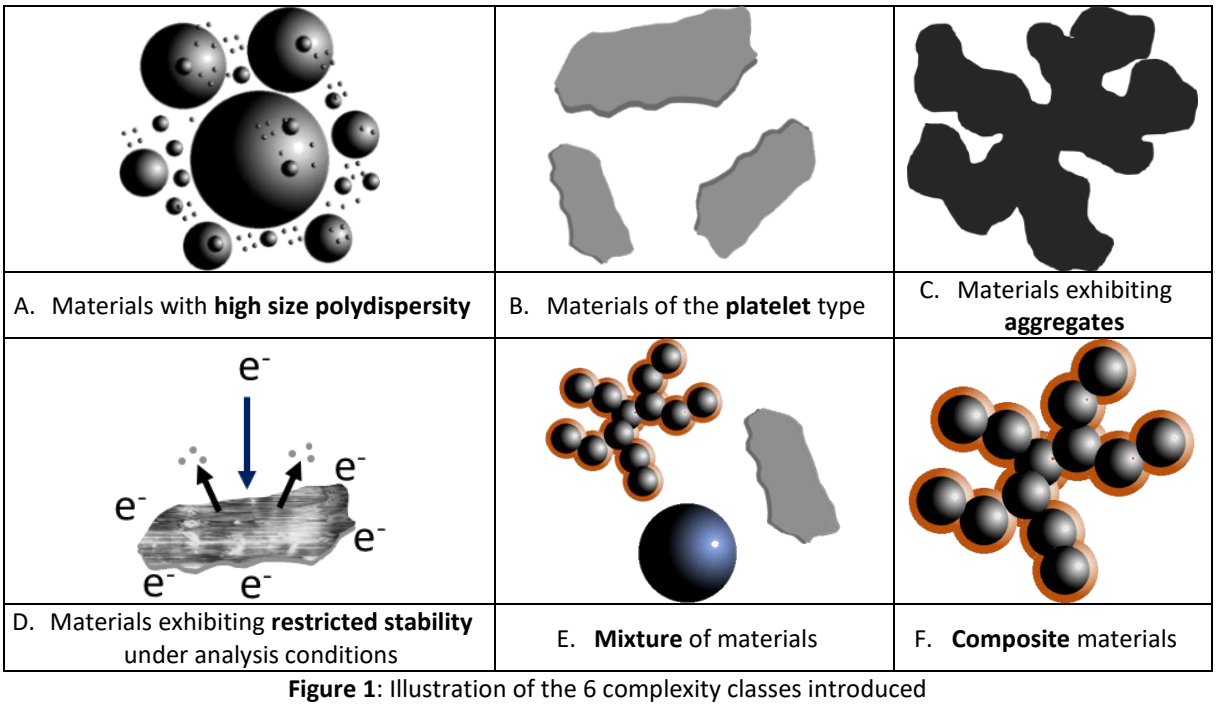
This note:

- Defines **complexity classes** based on feedback from NanoMesureFrance members;
- **Discusses the difficulties** inherent in these classes **on the basis of concrete cases** of common materials;
- **Illustrates these difficulties using SEM / TEM / AFM** micrographs of such materials.

Six complexity classes have been defined based on the feedback provided by the NanoMesureFrance association (Figure 1). The complexities inherent to these various classes can trace their origin to:

1. A **size distribution** spanning **hundreds of nanometres, or even micrometres**;
2. A **shape very different from that of a sphere**: platelets, tubes, or rods;
3. A strong interaction between particles leading to **aggregation phenomena**;
4. A chemical nature that causes **instability of the particle during its analysis**;
5. A **mixture of various materials** possibly in the form of **(6) composite materials**.

This document is currently based on feedback from the members of the NanoMesureFrance association and does not aim to be exhaustive. The reader is invited to contact the association at (contact@nanomesurefrance.fr) if they wish to provide insights regarding their own experiences.



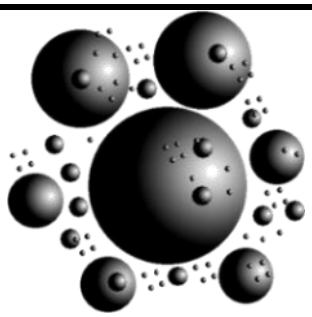
A. Materials with **high size polydispersity**	B. Materials of the **platelet** type	C. Materials exhibiting **aggregates**
D. Materials exhibiting **restricted stability** under analysis conditions	E. **Mixture** of materials	F. **Composite** materials

Figure 1: Illustration of the 6 complexity classes introduced

ILLUSTRATION OF THE TYPES OF DIFFICULTY ENCOUNTERED

A. Materials exhibiting high size polydispersity

SUMMARY



A “material exhibiting high size polydispersity” is a material composed of particles with a size distribution featuring several distinct peaks (or modes) or covers a wide continuous range spanning several orders of magnitude (ranging from 1 nm to 100 µm).

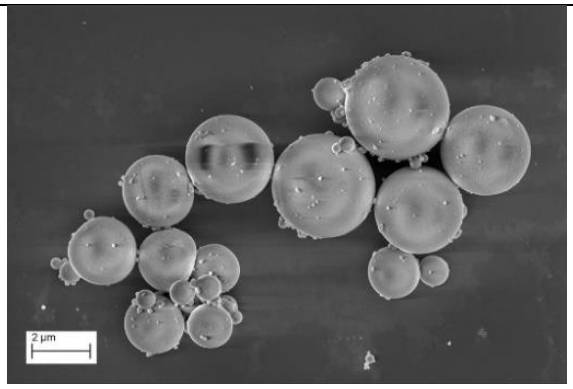
To date, no *screening* technique is capable of determining the number size distribution of a population of particles over such a wide range because they all tend to underestimate the proportion of the smallest particles. Only microscopy techniques are capable of gathering accurate information.

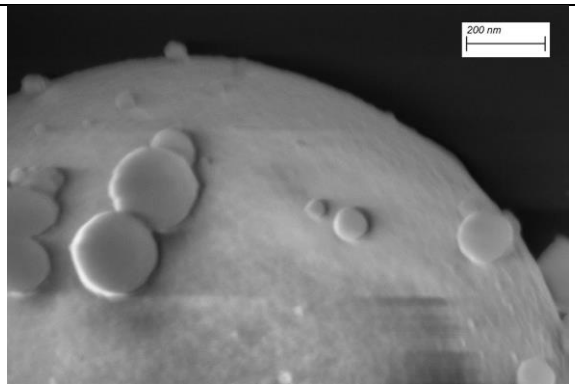
The main difficulty in analysing such samples lies in the limited resolution of the images, which hinders observing the smallest and largest particles using a single magnification. Furthermore, the tendency of nanoparticles to agglomerate on the surface of the largest particles severely limits the use of AFM, and to a lesser extent TEM.

SEM proves to be the most capable of imaging all the particles present in such samples. The evolution of technologies for the automated acquisition and processing of the images should make such analyses routine in the short term.

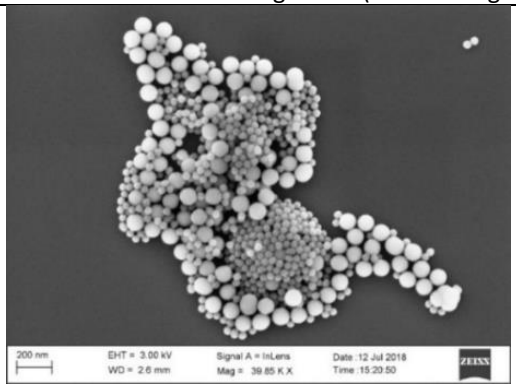
However, **particular attention must be paid to the validation of sample preparation protocols** as well as the **establishment of counting rules** specifying the number of images and the resolutions to be considered in such situations.

Table 1: Illustration of the difficulties associated with class "*A. Materials exhibiting high size polydispersity*"





silica - Illustration of the difficulties associated with the high polydispersity spanning several orders of magnitude (several magnifications are necessary)



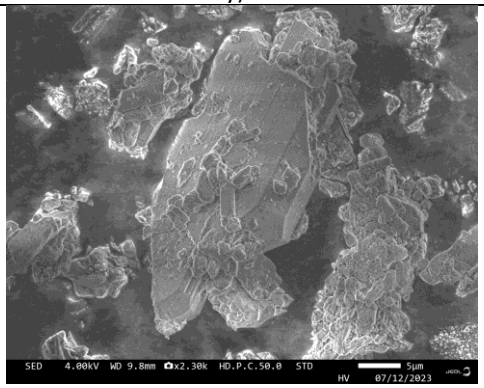


Illustration of the phenomenon of “sticking” of the smallest particles to the largest (case of a silica sample on the left, and of a Bauxite sample on the right)

B. Platelet-type materials

SUMMARY

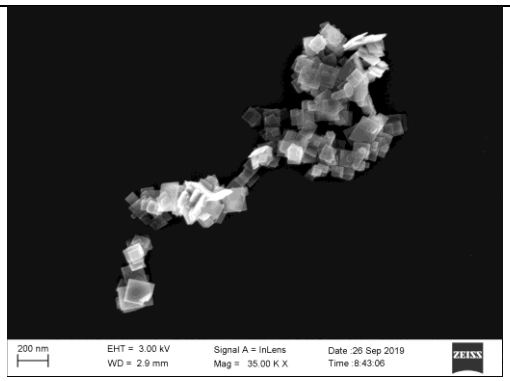
A nanoplate is a nano-object that has a thickness of less than 100 nm and lateral dimensions that can exceed 100 nm. Therefore, the smallest dimension to measure is the **thickness**.

No screening technique is able to determine the thickness of a material in platelet form because all of these techniques use models that assume spherical particles. It is also difficult, even impossible, to determine the thickness of a platelet using electron microscopy techniques.

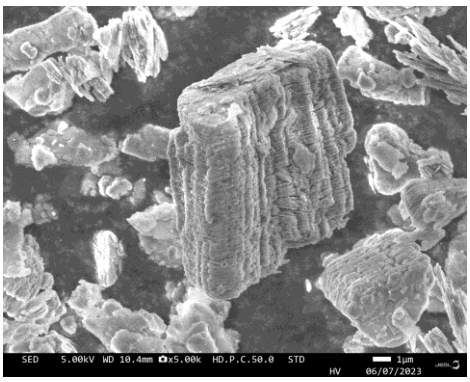
If platelets are deposited on an analysis substrate from a colloidal suspension, their larger surface area will naturally adhere to the substrate, causing them to appear flat and obscuring their edges. This behaviour is more pronounced in the case of nanoplatelets. One possible approach is to **embed the platelets in resin** and surface the resulting block after the resin has hardened, making it possible to observe the thickness of the platelets with electron microscopy.

In theory, the only technique really capable of reliably determining the thickness of a platelet in contact with the analysis substrate, **is atomic force microscopy (AFM)**. However, **special attention must be devoted to the preparation of the sample for analysis** to ensure that the platelets do not overlap, which can be a real challenge. The main current obstacle therefore concerns the difficulty of deagglomerating these platelets in the colloidal suspension before deposition.

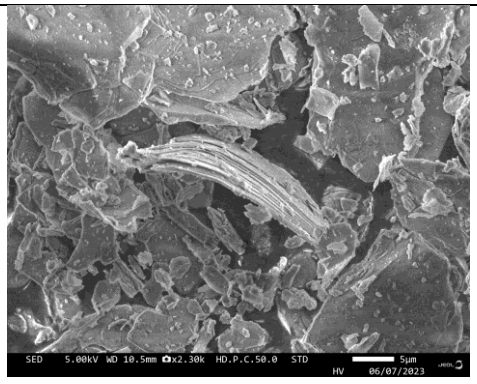
Table 2: Illustration of the difficulties associated with class "*B. Platelet-like materials*"



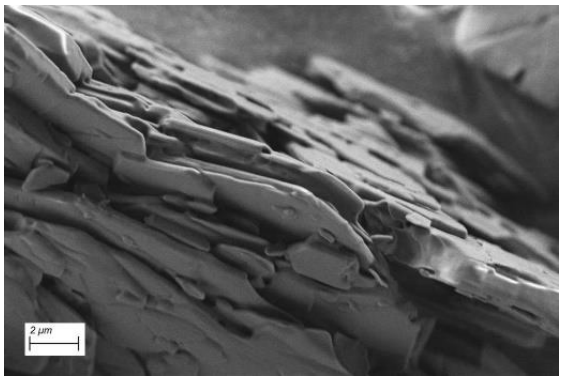
Titanium dioxide – illustration of the platelet stacking phenomenon, where even after ultrasound treatment, they remain strongly attached to each other



Mica - illustration of the platelet stacking phenomenon, and the difficulty in identifying the constituent particles and their outlines



Mica – Illustration of the difficulty in imaging platelet slices and identifying the presence of constituent particles and their outlines. Since this is a polycrystalline material, the difficulties inherent to class C also apply



Lauroyl Lysine - Illustration of the difficulty in imaging platelet slices and identifying the constituent particles and their outlines

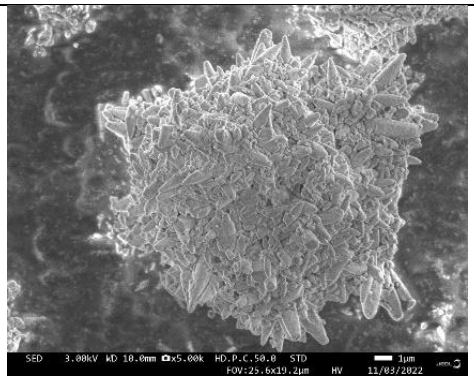
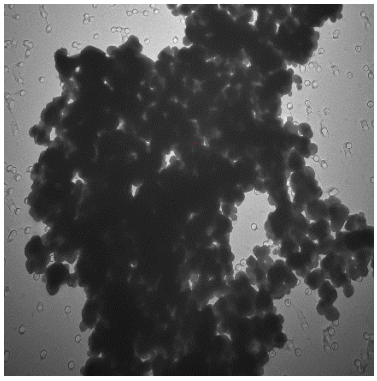
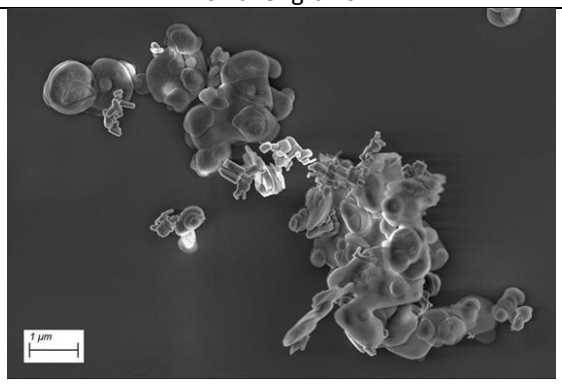
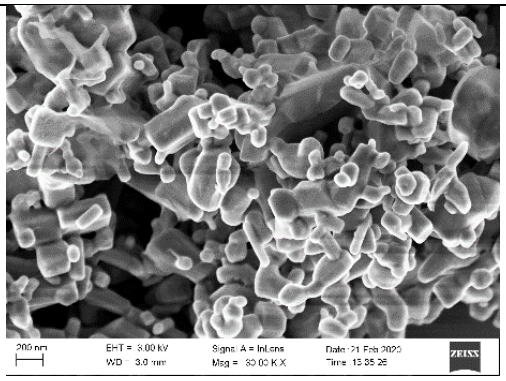
C. Materials with aggregates

SUMMARY

Nano-objects can be found in aggregated/agglomerated form following synthesis. A specific sample preparation step can make it possible to de-agglomerate the particles to access the constituent particles. For aggregates, this will be very difficult, if not impossible. The distinction between agglomerates and aggregates is difficult to make because no technique is capable of quantitatively measuring the force of cohesion between nano-objects forming a cluster. In the specific case of a substance in powder form, measurement of the specific surface by the BET method can nevertheless provide information on the state of aggregation (strongly bonded or fused particles) by comparison with electron microscopy results. *Screening* methods are not able to distinguish aggregates of constituent particles of similar size, nor the constituent particles within aggregates.

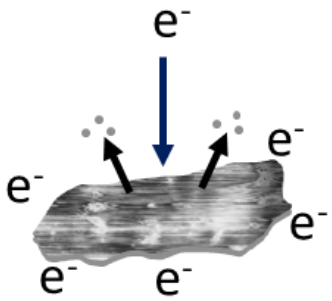
The difficulties in the measurement, by electron microscopy (TEM or HR-SEM), of the size of constituent particles within an aggregate lie in the fact that their outlines are difficult to distinguish when they are fused together. **This introduces a degree of subjective assessment in the definition of the object considered when determining the size and thus leads to discrepancies between operators and laboratories.** Atomic force microscopy (AFM) is unable to measure the constituent nanoparticles at the core of aggregates.

Table 3: Illustration of the difficulties associated with class "*C. Materials presenting aggregates*"

 Precipitated calcium carbonate - mineral material that should not be interpreted as aggregates of particles, but as polycrystalline particles made up of smaller grains	 Titanium dioxide - Illustration of the difficulty in imaging nanoparticles that may be masked within aggregates
 Boron nitride/Zinc oxide - Illustration of the difficulty in identifying the outline of constituent particles	 Zinc oxide - Illustration of the difficulty in identifying the outline of constituent particles

D. Materials exhibiting restricted stability under analysis conditions

SUMMARY



This complexity class brings together two cases:

- Materials whose chemical properties can cause a change in their size and/or shape under the electron beam of SEM, TEM, and STEM microscopes;
- Materials whose mechanical properties can cause them to deform, thereby altering their size and shape under the AFM tip.

No *screening* method is available or recommended to anticipate and address this issue of sample instability during analysis by SEM/TEM/STEM/AFM.

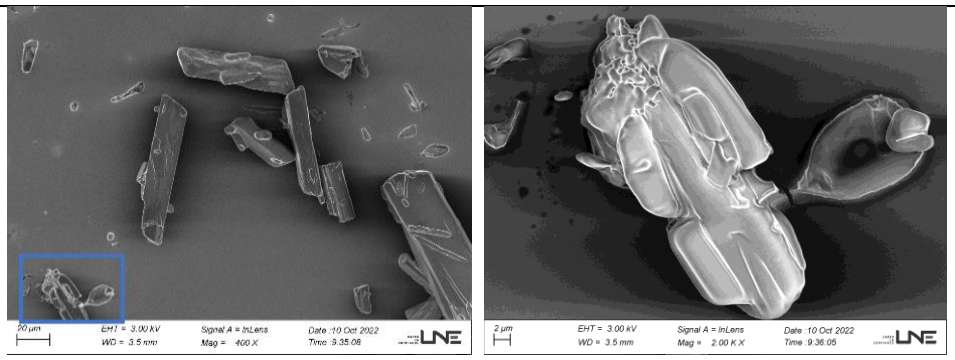
In electron microscopy (SEM/TEM/STEM), particularly for non-conducting materials or those with an organic phase, electron bombardment may cause particles to change size and/or shape, leading to a drift in the measured values of their smallest dimension.

For AFM, particle deformation may lead to an underestimation of their height.

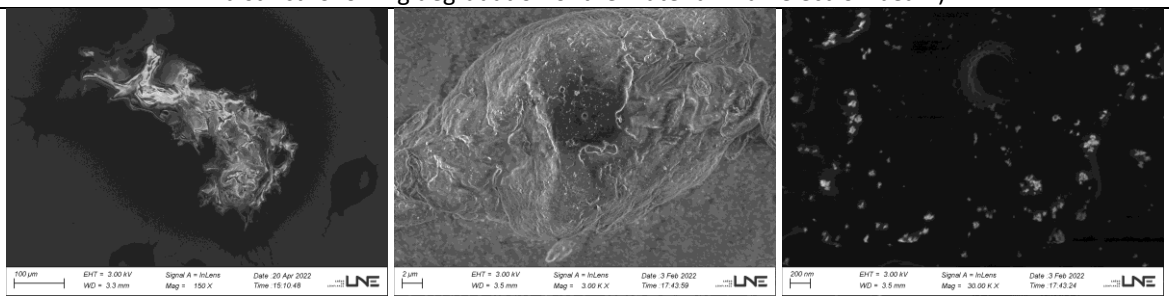
As it currently stands, no recommendations or specific analytical strategies are available and only the expertise of laboratories on this type of sample makes it possible to best adapt the analysis conditions to limit bias.

Harmonisation of the methods is therefore necessary, by proposing an experimental strategy (additional *screening* methods, choice of substrate and analysis conditions) to identify, anticipate, and mitigate the difficulties inherent in this complexity class.

Table 4: Illustration of the difficulties associated with class "*D. Materials exhibiting limited stability under analysis conditions*"



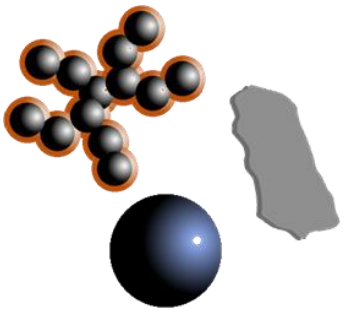
CBD isolate – Illustration of the degradation of the sample during analysis (Left: low magnification image of constituent particles of CBD isolate / Right: enlarged view of the particle framed in blue; the outlines are less distinct following degradation of the material in an electron beam)



Hydroxypropyl methylcellulose – Illustration of the difficulty in imaging a non-conductive sample (Left: material after attempted dispersion in a solvent. The surface of the particle is completely degraded / Middle: observation of the raw powder / Right: enlarged view of the central section of the middle image; because the sample is insulating, charge accumulation prevents further zooming-in)

E. Mixtures of materials

SUMMARY



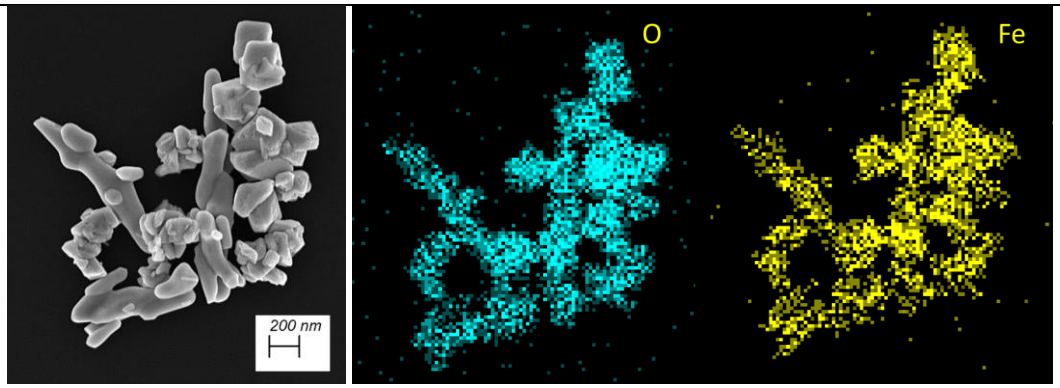
This complexity class includes mixtures of multiple materials that do not exhibit strong mutual interactions. The complexity here is directly related to the need to determine the number size distribution of particles in the mixture for each material present, thus requiring analytical techniques capable of discriminating between each material.

Several levels of complexity exist with mixtures of particles of different elemental chemical compositions or others with the same elemental chemical composition but different chemical formulas, particle shapes, or crystallographic structures.

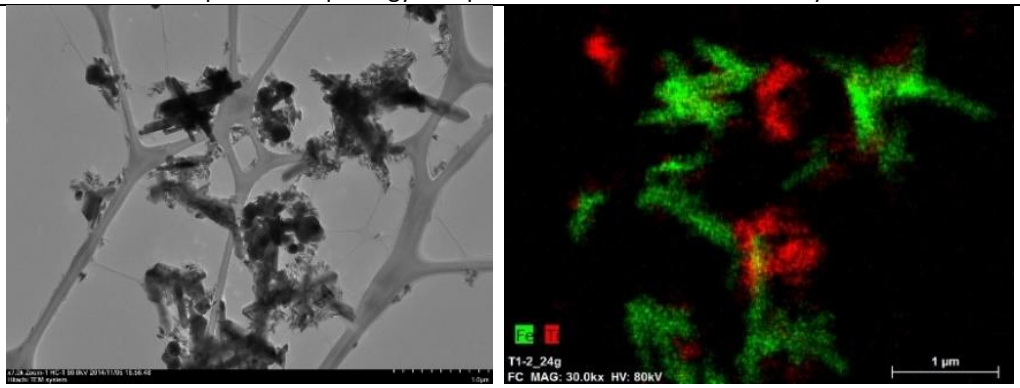
Most *screening* techniques are not able to produce a size distribution according to chemical or elemental composition. Only sp ICP-MS (depending on the chemical composition of the particles) or the coupling of A4F with mass spectrometry can provide answers in this case. Furthermore, *screening* techniques will be unable to take into account the differences in particle shapes or their crystallographic structures.

The use of electron microscopy approaches (SEM/TEM) coupled with specific detectors (EDX/EBSD/Raman) is therefore to be preferred.

Table 5: Illustration of the difficulties associated with class *"E. Case of mixtures of materials"*



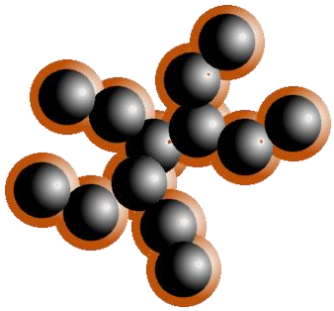
Mixture of 2 oxides of iron - Illustration of the difficulty in identifying a mixture of two types of particle with the same elemental composition. We observe various morphologies without being able to attribute a specific morphology to a particular material with certainty



A mixture of titanium dioxide and iron oxide - Illustration of the resolution limits of EDX detectors available to identify the elemental composition (TEM analysis (left) and EDX (right) for the identification of the elements Fe and Ti)

F. Composite materials

SUMMARY

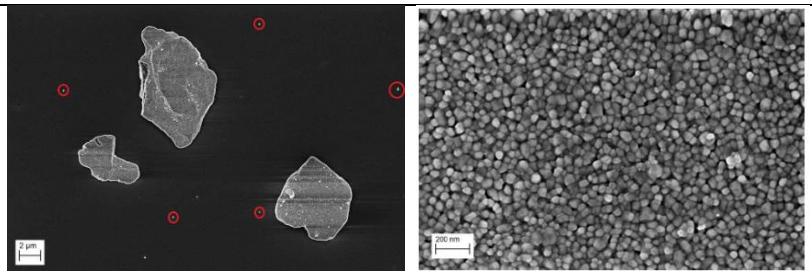
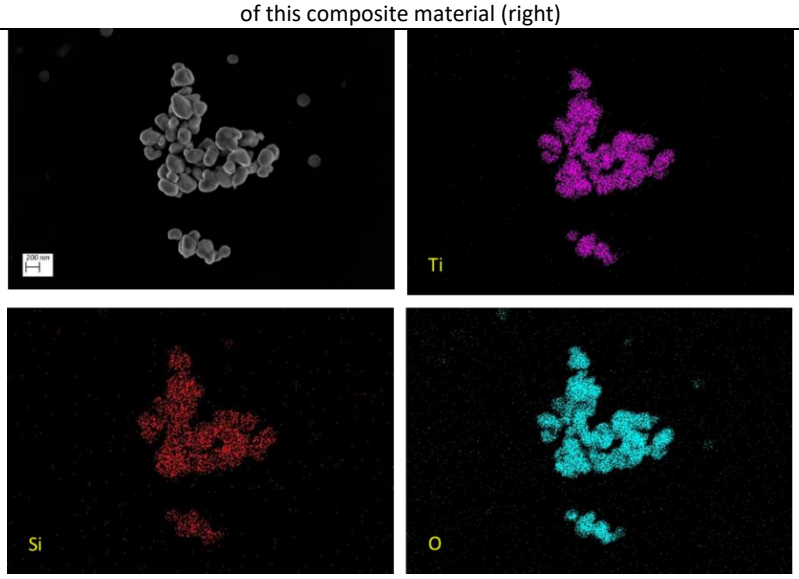


This complexity class groups together **materials composed of a substrate, sometimes on a micrometre scale, onto which a coating is bonded through strong interactions to achieve specific properties** (optical, affinity for certain matrices). The chemical compositions of the substrate and coating may be similar or totally different.

Preamble (11) of the European Commission's definition recommendation 2022/C229/01, excludes large composite materials from its framework, even if they have an internal or surface structure at the nanoscale, such as coatings, certain ceramic materials, and complex nanocomponents, including nanoporous materials **and nanocomposites.** Some of these products or components may have been manufactured using nanomaterials and may even still contain them. The JRC[1] guide also specifies in paragraph 2.10 that "***even if a product is designed to release nanomaterials, or releases nanomaterials in the form of wear debris*** *during its use or aging,* ***the product of origin still does not become a nanomaterial***".

It is therefore necessary to keep in mind that **such composite materials are to be considered as an inseparable whole "substrate + coating" and that it is of course for this whole that the smallest dimension should be determined in order to assess its potential nanomaterial status.**

Table 6: Illustration of the difficulties associated with class *"F. Composite materials"*

 Pearlescent Pigments Substrate/Coating: Mica / titanium dioxide - illustration of the presence of nanofragments (circled in red in the left image), and enlarged view on the coating of this composite material (right)	**Interpretation:** the presence of debris in nanomaterial form (ch. 2.10, JRC guide) or a structuration of the coating surface at the nanoscale (preamble (11), 2022/C229/01) do not call into question the status of the composite material if the smallest dimension of the "substrate + coating" assembly is not at the nanoscale.
 Titanium dioxide rods coated with silica - the difficulty in describing the nature of the composite material lies in distinguishing the silica coating from the titanium dioxide substrate via EDX analysis	**Interpretation:** here the elemental analysis highlights the presence of a coating on the entire substrate surface. It is therefore necessary to determine the smallest dimension of the identifiable constituent particles in this image without separating the coating from the substrate. If this smallest dimension is at the nanoscale, the composite material can be considered to be a nanomaterial. This interpretation is consistent with the presence of silica-coated titanium dioxide.

GLOSSARY

A4F: Asymmetric Flow Field Flow Fractionation: a separation technique which uses a transverse flow field applied perpendicularly to the channel flow to achieve separation based on the diffusion coefficient or the size of the analyte (XP CEN ISO/TS 80004-6:2021)

AFM: Atomic Force Microscopy: a surface imaging method by mechanical scanning of surface contours where the positioning of a sharp force-sensing tip mounted on an adapted cantilever, is precisely controlled (XP CEN ISO/TS 80004-6:2021)

Agglomerates: set of loosely or moderately bonded particles, the resulting external surface area of which is similar to the sum of the surface areas of the individual components (ISO 26824:2022)

Aggregates: particle composed of other strongly bonded or fused particles, the resulting external surface of which is significantly smaller than the sum of the surface areas of the individual components (ISO 26824:2022)

BET: the Brunauer-Emmett-Teller method: a method for determining the total specific external and internal surface area of dispersed powders and/or porous solids by measuring the amount of physically adsorbed gas, using a model developed by Brunauer, Emmet, and Teller for interpreting gas adsorption isotherms (XP CEN ISO/TS 80004-6:2021)

Constituent particles: smallest individual particles, identifiable within an agglomerate or aggregate (JRC[3])

D50: the median diameter derived from the particle size distribution

EBSD: Electron BackScatter Diffraction: a diffraction phenomenon that occurs between backscattered electrons and the atomic planes of a highly inclined crystalline sample struck by a fixed incident electron beam (XP CEN ISO/TS 80004-6:2021)

EDX: Energy Dispersive X-ray spectroscopy: spectroscopy of X-rays in which the energies of individual photons measured by a parallel detector (an array of detectors) are used to construct a histogram representing the distribution of X-rays as a function of energy (XP CEN ISO/TS 80004-6:2021)

ICP-MS: Inductively Coupled Plasma–Mass Spectrometry

Material: any type of material (including materials composed of particles) without making assumptions about, for example, its origin, chemical composition, or morphology

Measurand: quantity to be measured (International Metrology Vocabulary – Fundamental and General Concepts and Associated Terms, JCGM 200:2012, International Bureau of Weights and Measures)

Mode of a particle size distribution: the mode is the diameter of the most populated class (having the highest value) within the entire particle size distribution or a distinct part of it

Nanofibre: a nano-object with two external dimensions at the nanoscale and the third dimension at a significantly larger scale (NF EN ISO 80004-1:2023-08)

Nanomaterial: a material having one external dimension at the nanoscale or having an internal or surface structure at the nanoscale (NF EN ISO 80004-1:2023-08)

Nano-object: a discrete piece of material of which one, two, or all three external dimensions are at the nanoscale (NF EN ISO 80004-1:2023-08)

Nanoparticle: a nano-object, all external dimensions of which are at the nanoscale (ISO 26824:2022)

Nanoplate: a nano-object having an external dimension at the nanoscale and the other two external dimensions significantly larger (NF EN ISO 80004-1:2023-08)

Nanorod: a solid nanofibre (NF EN ISO 80004-1:2023-08)

Nanoscale: a length scale ranging from approximately 1 nm to 100 nm (NF EN ISO 80004-1:2023-08)

Nanotube: a hollow nanofiber (ISO 26824:2022)

Particle: a tiny portion of matter with well-defined physical boundaries (ISO 26824:2022)

Particulate material: material composed of particles that may all be of the same type or may differ in their chemical and/or structural composition (JRC[3])

Particle size distribution: distribution of the quantity of particles according to their size (XP CEN ISO/TS 80004-6:2021)

Polycrystalline material: a solid material composed of numerous small crystals (the "grains"). Grains are separated by grain boundaries and usually have random crystallographic orientations. The grain size can vary from a nanometre to a millimetre (from the Encyclopedia of Biomedical Engineering[4])

Raman Spectroscopy: a spectroscopic method that uses the Raman effect (emitted radiation coming from molecules illuminated by monochromatic radiation, and which is characterised by a loss or gain of energy from vibrational or rotational excitations) for investigating molecular energy levels (XP CEN ISO/TS 80004-6:2021)

Screening metrology: the first step in establishing the presence of a substance in a population for risk estimation purposes (EFSA glossary: https://www.efsa.europa.eu/en/glossary/screening-method)

SEM: Scanning Electron Microscopy: a method by which the surface of a sample is scanned by an electron beam thereby generating after-effects (such as secondary electrons, backscattered electrons, absorbed electrons and x-rays) from which physical information can be used to determine the structure, composition and topography of the sample (XP CEN ISO/TS 80004-6:2021)

sp ICP-MS: single particle Inductively Coupled Plasma–Mass Spectrometry: a method using inductively coupled plasma-mass spectrometry in which a dilute suspension of nano-objects is analysed and ICP-MS signals are collected with high temporal resolution, allowing one to perform particle-by-particle detection at specific mass peaks and number concentrations, and to determine the particle size and particle size distribution (XP CEN ISO/TS 80004-6:2021)

Specific surface area per unit volume: absolute surface area of the sample divided by its volume (XP CEN ISO/TS 80004-6:2021)

STEM: Scanning Transmission Electron Microscopy: a method for producing magnified images or diffraction patterns of the sample, using a finely focused electron beam scanning the sample surface, and thereby traversing the sample and interacting with it (XP CEN ISO/TS 80004-6:2021)

TEM: Transmission Electron Microscopy: a method for producing magnified images or diffraction patterns of the sample using an electron beam to traverse the sample and interact with it (XP CEN ISO/TS 80004-6:2021)

[3] https://publications.jrc.ec.europa.eu/repository/handle/JRC132102
[4] https://doi.org/10.1016/B978-0-12-801238-3.99860-X