Mérieux NutriSciences

Better Health. Better World.

Sustainable Innovation

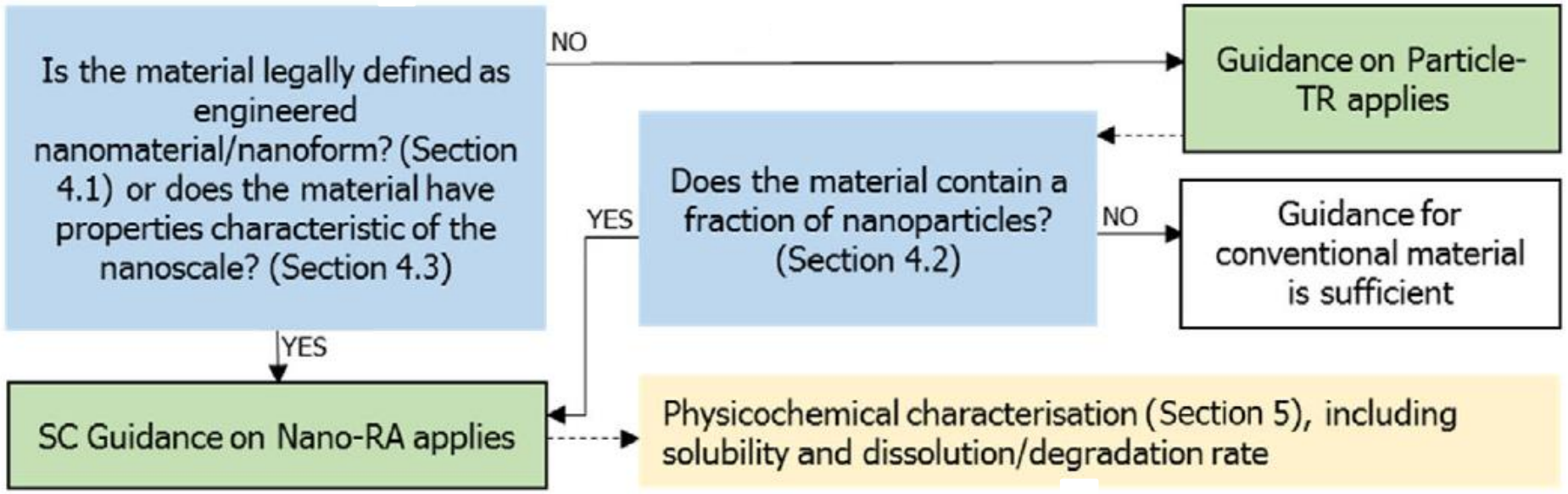
Physico-chemical characterization of Novel Foods

the ECAMRICERT SRL testing lab facility experience

Federico Benetti

ECSIN LAB, Executive Director

Why particle size?



Particle size distribution

When the material is not expected to have a fraction at the nanoscale, the applicant should demonstrate that the particles are equal to or larger than 500 nm after a proper dispersion of the test material. **The method(s) used for this assessment should provide convincing evidence that the material contains less than 10% particles (number-based) with at least one dimension smaller than 500 nm.**

Electron microscopy is proposed as the recommended method to determine the size distribution of the fraction of small particles.

The applicant should justify the use of other methods and provide sufficient evidence on the limit of particle size detection and the level of dispersion avoiding the aggregation/agglomeration of the particles.

Analytical techniques

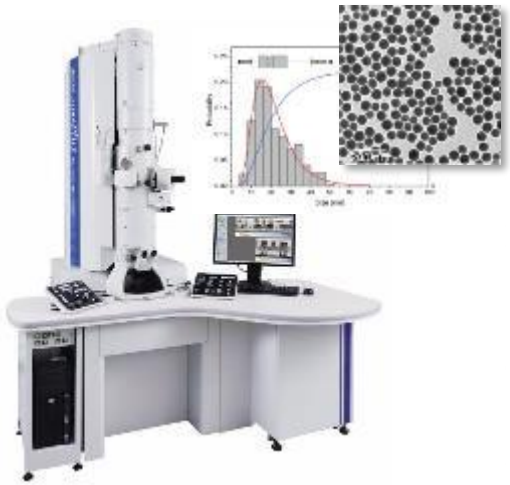
- Electron microscopy (EM)
- Centrifugal liquid sedimentation (CLS)
- Dynamic light scattering (DLS)
- Particle tracking analysis (PTA)
- Asymmetric Flow Field Flow Fractionation (AF4) associated to suitable detection methods
- Filtration complemented with chemical analysis

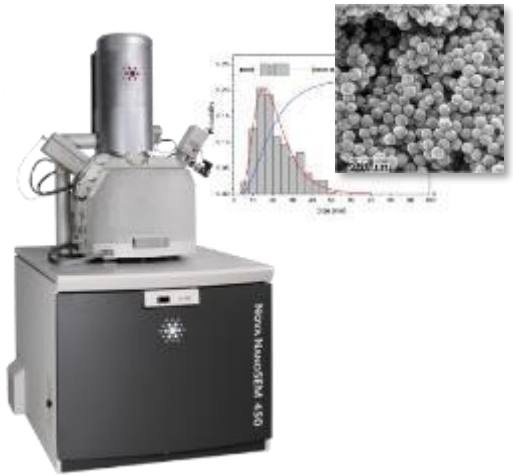
Electron microscopy
FOR PARTICLE SIZE DETERMINATION

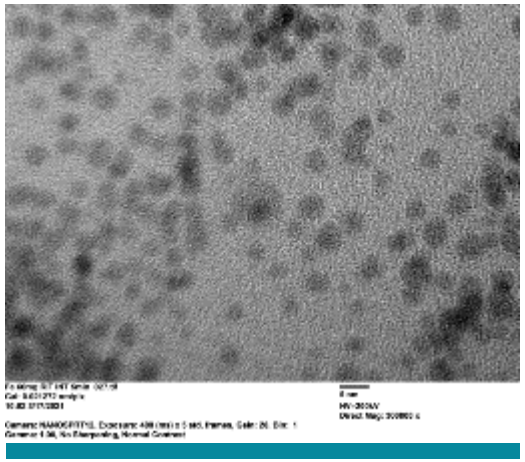
Particle size distribution

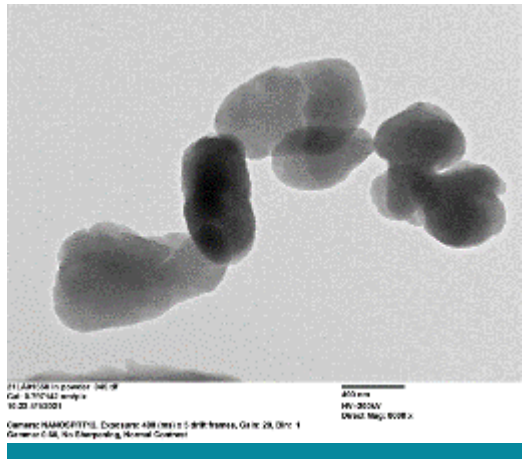
ELECTRON MICROSCOPY (EM)

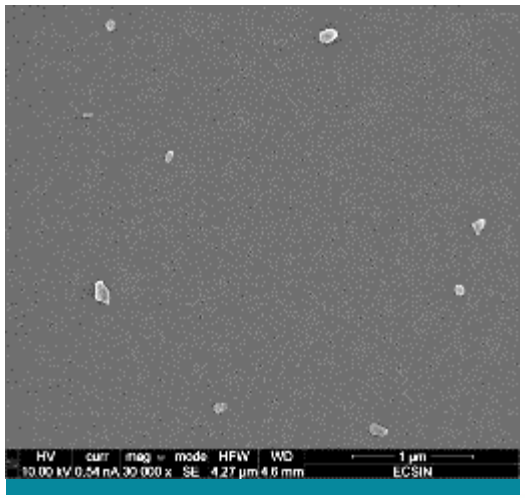
Electron microscopy allows to **visualize and identify individual objects of interest based on specific criteria** such as size, shape, crystallographic structure, and elemental composition. It is one of the few methods that, to some extent, allow to identify constituent particles in aggregates and agglomerates.

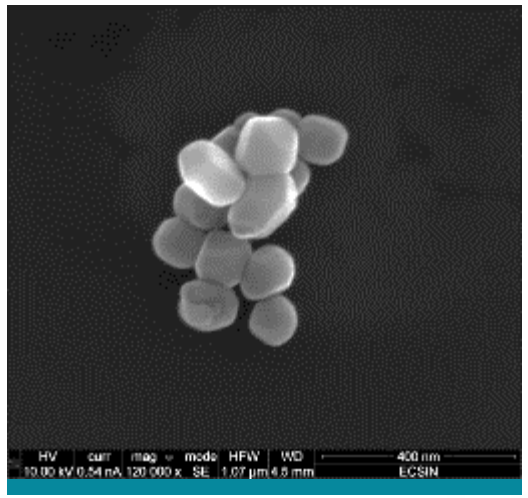












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Why disperse particles?

A **good level of dispersion** avoids the aggregation/agglomeration of the particles allowing the detection of smallest primary particles.

A **dispersion protocol** can be considered effective if it yields samples which consist as much as possible of non-agglomerated/non-aggregated particles.

The **dispersion procedure** used may influence the particle size distribution measurements. For material characterization, the final liquid dispersion of the material should result in a particle size distribution that consists of the smallest dispersible particles.

Dispersion protocol
FOR SAMPLE PREPARATION

Particle size distribution

DISPERSION PROTOCOL

To monitor the effectiveness of a protocol, analytical methods, which can reliably distinguish constituent particles from agglomerates and aggregates, are required. Suitable methods are those based on EM techniques, such as SEM or TEM.

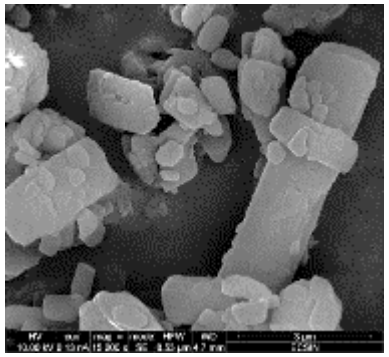
THERE IS NO UNIVERSALLY APPLICABLE TEST PROTOCOL

FOR PREPARING STABLE DISPERSIONS OF MATERIALS WITH A FRACTION OF SMALL PARTICLES

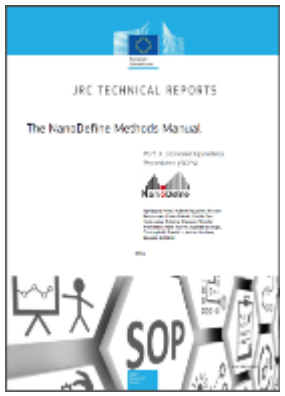
A systematic approach has been proposed in the **NanoDefine EU-project**, where a specific optimised dispersion protocol was developed for a number of **priority nanomaterials** (Pigment Yellow 83, $BaSO_4$, MWCNT, nanosteel, $CaCO_3$, Kaolin, coated TiO_2, basic methacrylate copolymer, zeolite) and laid down in the form of **SOPs**.

The NanoDefine Methods Manual is a good starting point for development of "*ad hoc*" dispersion protocols that exploit chemical or morphological similarities between the NF and the priority nanomaterials

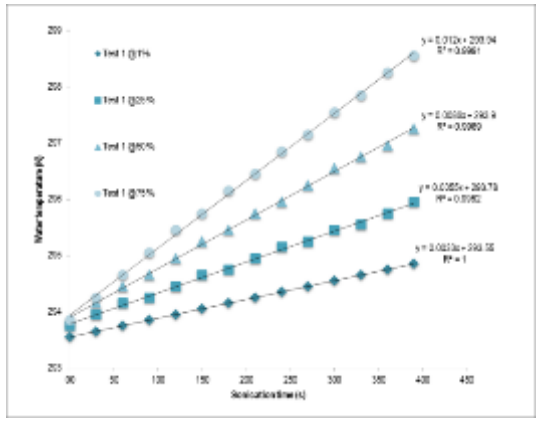
Dispersion protocol development

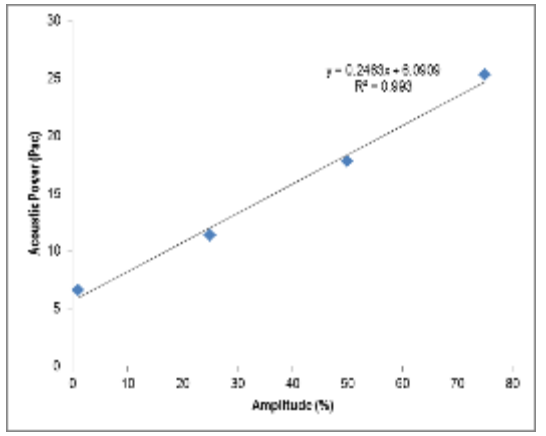


Powder

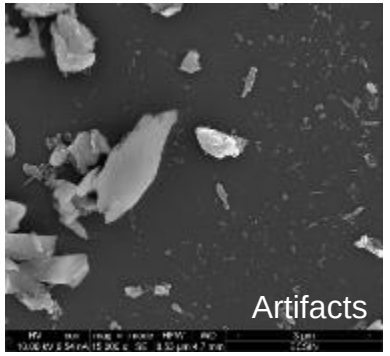


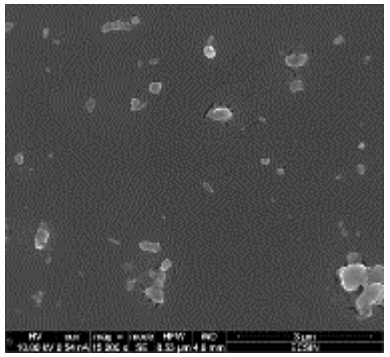
Platelet-like particles
Dispersion in water
Probe sonication: 25 KJ





Calibration of probe sonicator



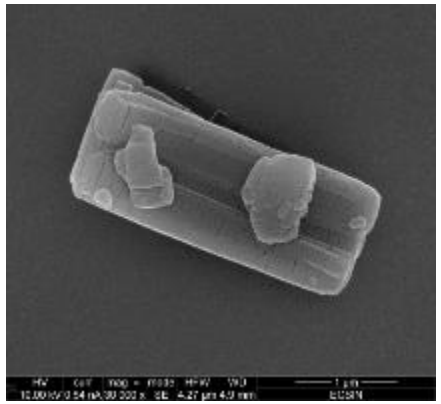


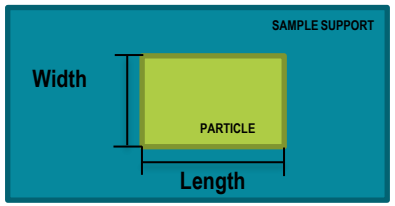
Dispersion with different sonication energies and different media (water/water + 0.01% SDS)

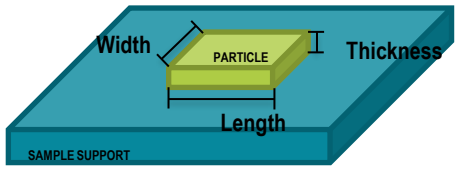
Particle size
ANALYSIS

Particle size distribution

NOT NANOMATERIAL



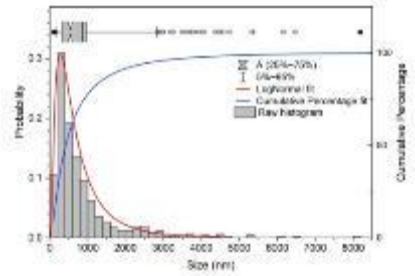


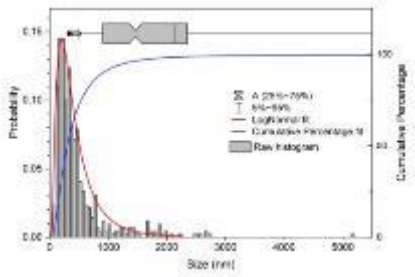


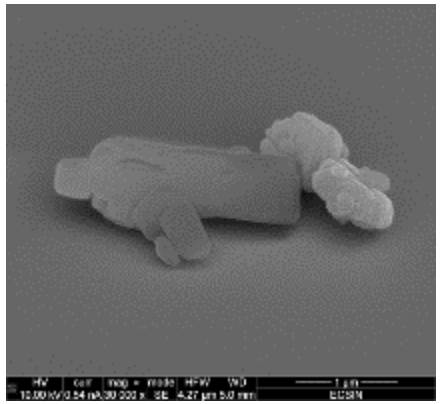
Horizontal view: Length and width are visible, thickness can only be perceived

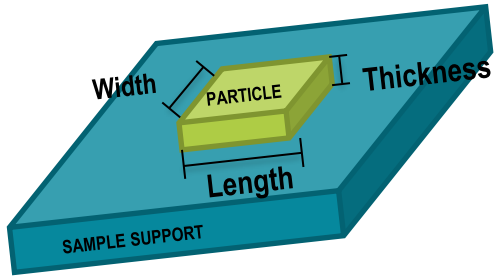
Dimensional Parameters - Length	Value
Analyzed particles	500
Minimum size (nm)	121 ± 16
First quartile (nm)	301 ± 22
Median (nm)	552 ± 12
MAD (nm)	281 ± 3
Average (nm)	885 ± 32
Standard deviation (nm)	1006 ± 14
Third quartile (nm)	940 ± 20
Maximum size (nm)	7925 ± 1822
D10 (nm)	1911
D50 [median] (nm)	552
D90 (nm)	2236

Dimensional Parameters - Width	Value
Analyzed particles	500
Minimum size (nm)	80 ± 9
First quartile (nm)	199 ± 12
Median (nm)	364 ± 13
MAD (nm)	141 ± 2
Average (nm)	471 ± 13
Standard deviation (nm)	500 ± 164
Third quartile (nm)	524 ± 9
Maximum size (nm)	3149 ± 2103
D10 (nm)	146
D50 [median] (nm)	364
D90 (nm)	996



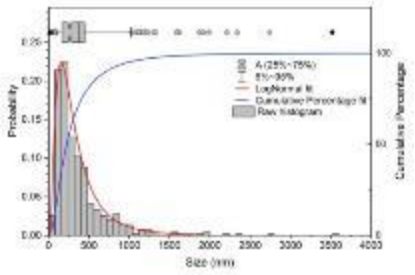






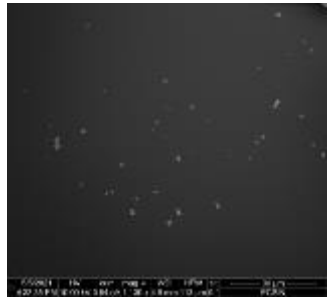
Tilted view: Thickness is visible and can be suitably measured

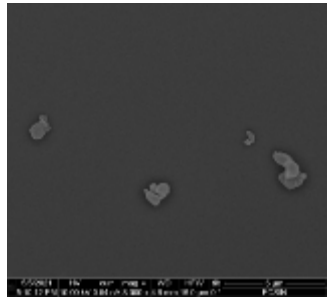
D10 < 500 nm
Fraction of small particles

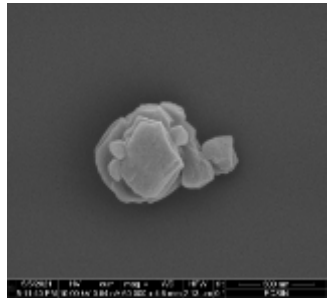


Dimensional Parameters - Length	Value
Analyzed particles	508
Minimum size (nm)	15
First quartile (nm)	159
Median (nm)	284
MAD (nm)	114
Average (nm)	382
Standard deviation (nm)	376
Third quartile (nm)	458
Maximum size (nm)	3680
D10 (nm)	118
D50 [median] (nm)	284
D90 (nm)	806

Particle size distribution





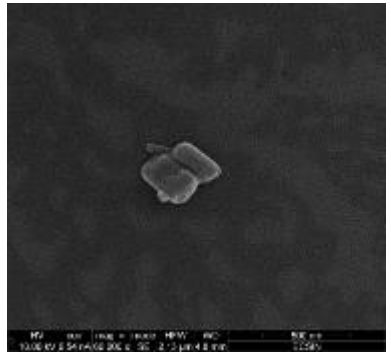


Sample preparation	Powder quartering, sample dispersion in isopropyl alcohol and deposition on a silicon wafer
Magnification range	250x-60000x
Image analysis	Manual measurement carried out by operators with ImageJ software. Minimum number of analyzed particles: 500.
Impurities	None
Aggregation/agglomeration state	Particles are well dispersed even though few aggregates/agglomerates (from a few to tens of particles) were detected.
Agglomerates shape	Ellipsoidal
Primary particles shape	Smaller particles are mainly polyhedrons with rounded edges, some bigger particles are spheroidal. Surface of particles appears regular.
Particle size distribution	**LENGTH** ▪ broad distribution (min-max: 74 - 33256 nm) ▪ median: 200 – 822 nm ▪ D10: 186 - 263 nm ▪ D50: 200 – 222 nm ▪ D90: 1396 – 3874 **WIDTH** ▪ broad distribution (min-max: 62 - 24358 nm) ▪ median: 332 – 527 nm ▪ D10: 125 - 203 nm ▪ D50: 332 – 527 nm ▪ D90: 930 – 3241 nm

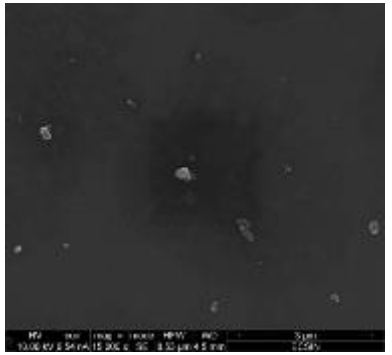
Particle size distribution < 500 nm

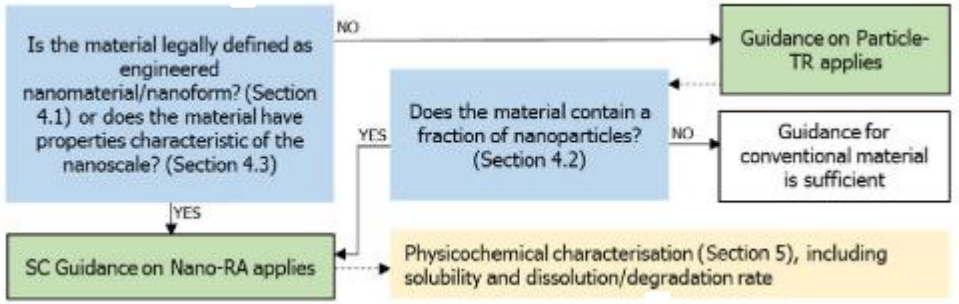
Characterization of the fraction of small particles, calculation of the D10 value and comparison to the threshold of 250 nm.

If D10 < 250 nm a fraction of particles at the nanoscale is confirmed.

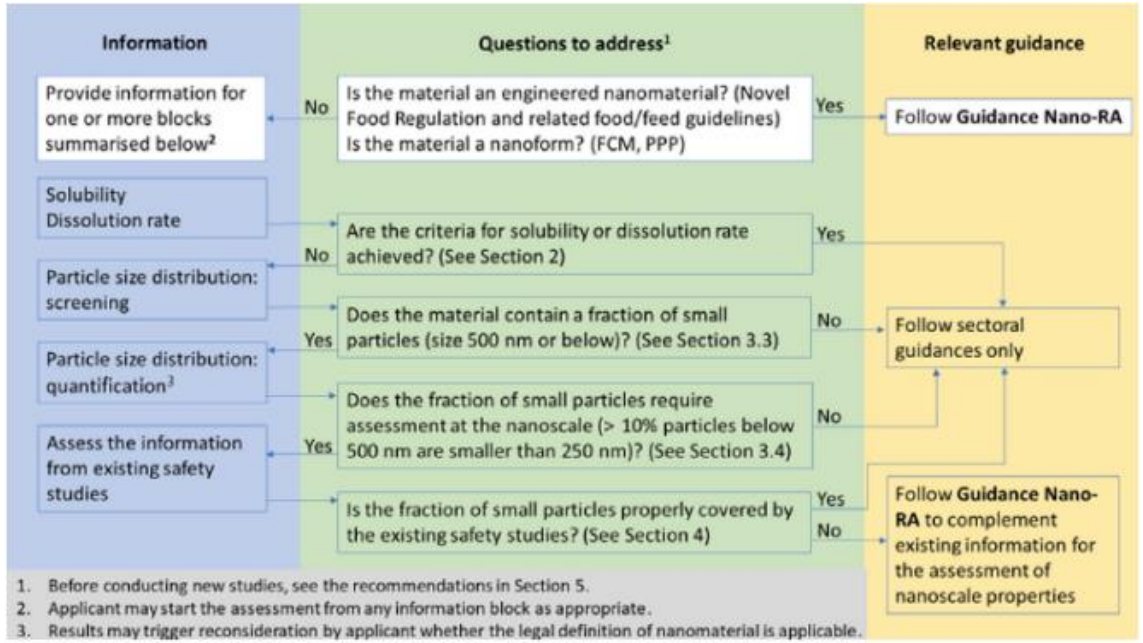


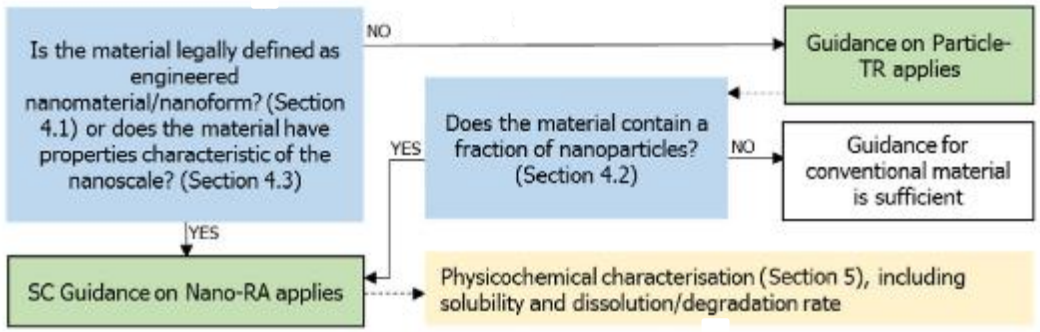
Batch #	Length (nm)	Width (nm)	Thickness (nm)
1	271	158	142
2	308	169	156
3	285	143	138
4	307	186	176
5	292	172	167
6	315	213	195



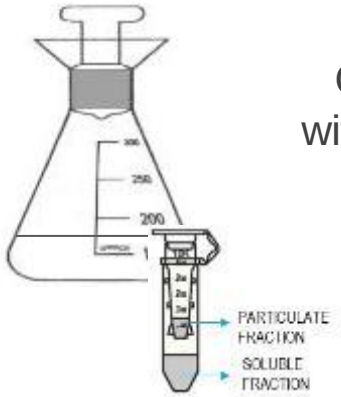


What's the next?





Solubility/dissolution rate



OECD TG 105
with **ultrafiltration**
3-10 KDa

Solubility ≥ 33.3 g/L

Max consumer exposure

4 time points (up to 60 min)

pH (depend on substance)

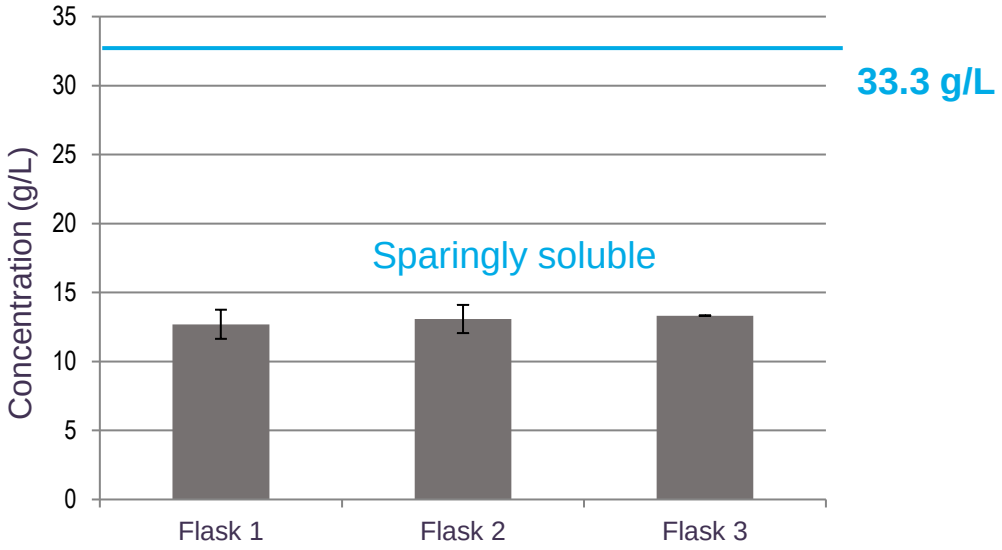
≤ 12% (mass based) of particles remains after 30 min (half-life = 10 min)

CRITERIA SATISFIED

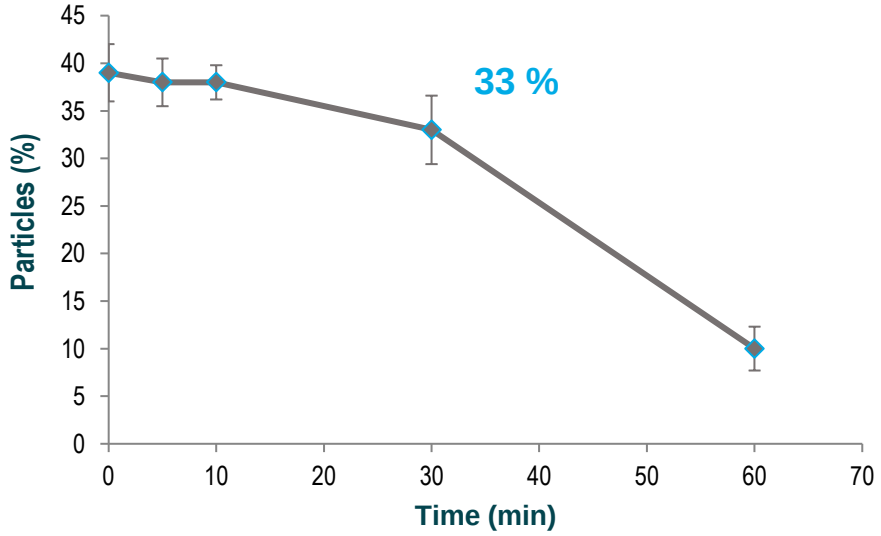
NO ADDITIONAL ASSESSMENTS FOR THE FRACTION OF SMALL PARTICLES IS NEEDED

Solubility/dissolution rate

SOLUBILITY IN WATER



DISSOLUTION RATE



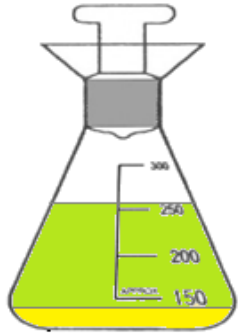
SOLUBILITY < SPARINGLY SOLUBLE: RELEVANT MEDIA (e.g. liquid matrices, matrices used before consumption)

Solubility/dissolution

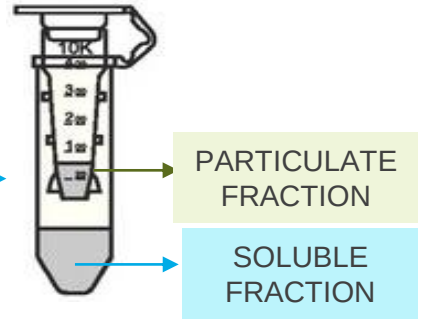
IN THE MARKETED PRODUCT OR IN FOOD

Solubility is tested in non-aqueous matrix or in a relevant solvent.

No reference methods are reported in the guidance.



NF with liquid fat/ relevant solvent



Ultrafiltration of the fat phase

Quantification of soluble fraction by suitable analytical technique (ICP-MS, HPLC…)

IF THE SUBSTANCE AT THE EXPECTED MAXIMUM LEVELS IS FULLY DISSOLVED OR RESIDUES IN FOOD ARE BELOW THE REPORTED/RELEVANT SOLUBILITY LIMIT NO ADDITIONAL ASSESSMENTS FOR THE FRACTION OF SMALL PARTICLES IS NEEDED

Solubility/dissolution

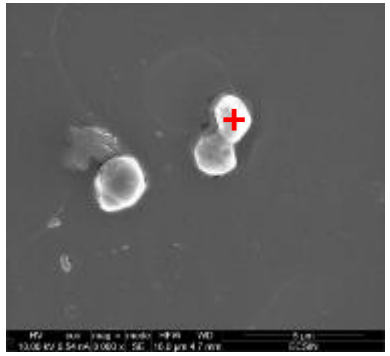
IN THE MARKETED PRODUCT OR IN FOOD

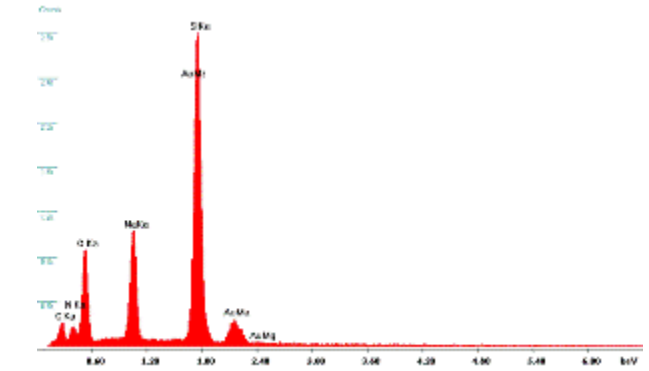
Solvent or lipophilic media could damage ultrafiltration device

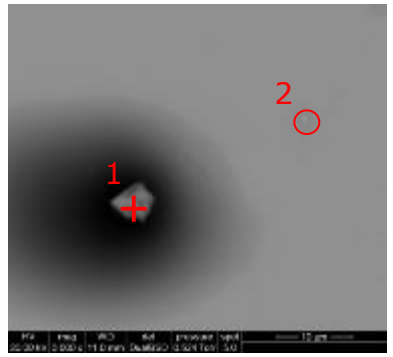
BEFORE
ULTRAFILTRATION

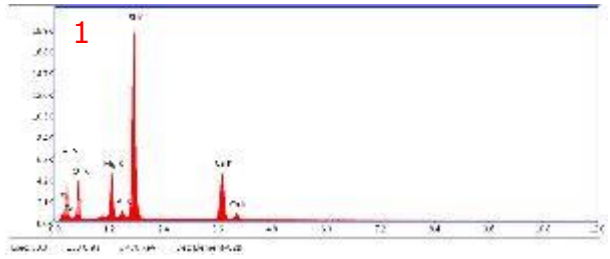
AFTER
ULTRAFILTRATION

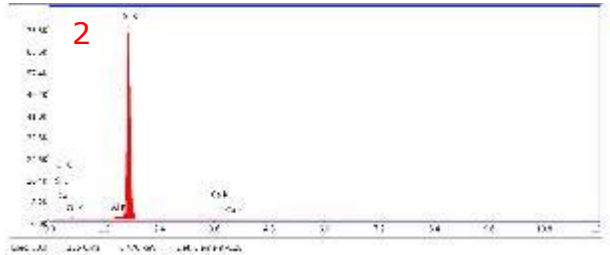
Electron microscopy coupled with EDX

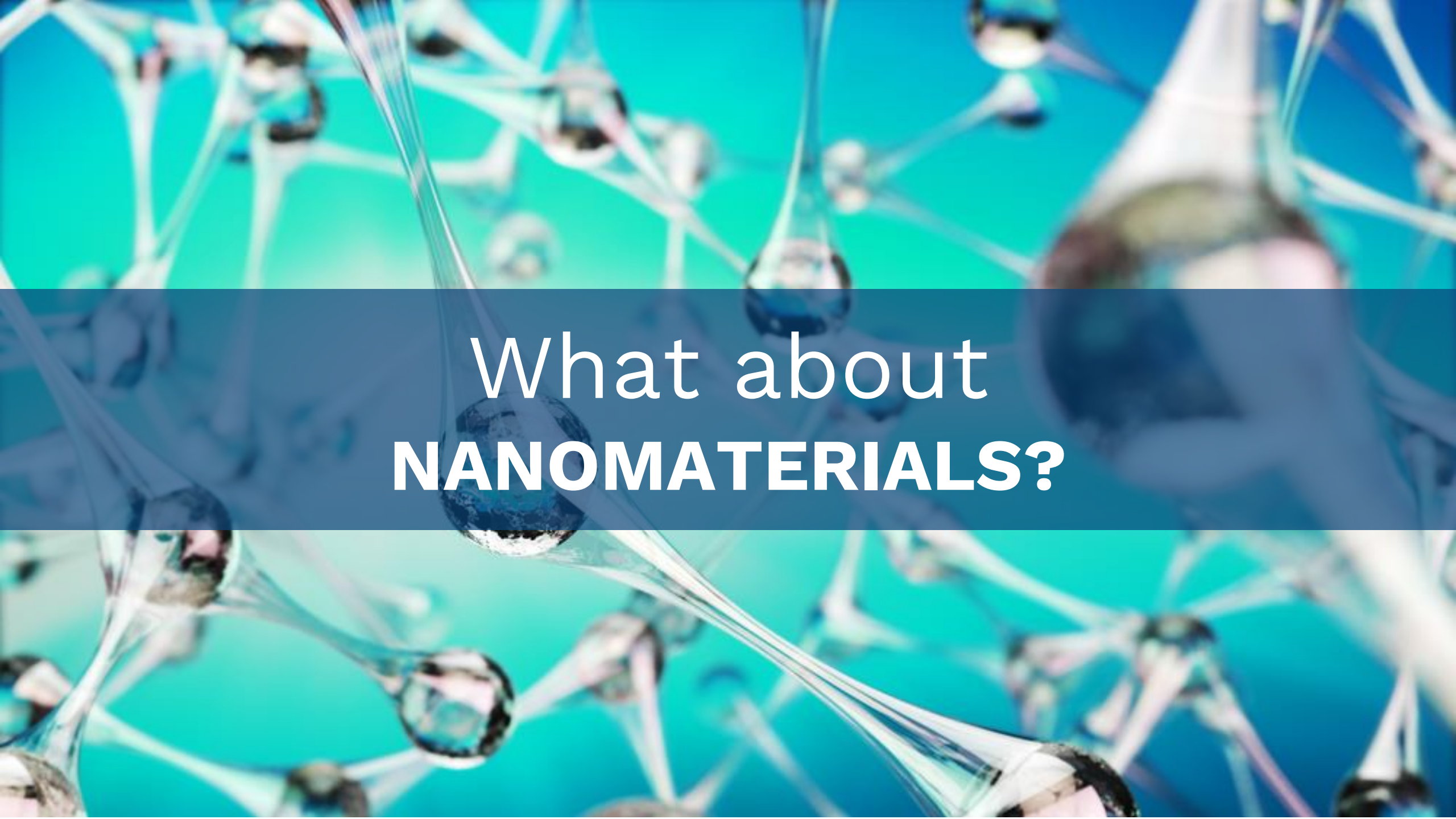






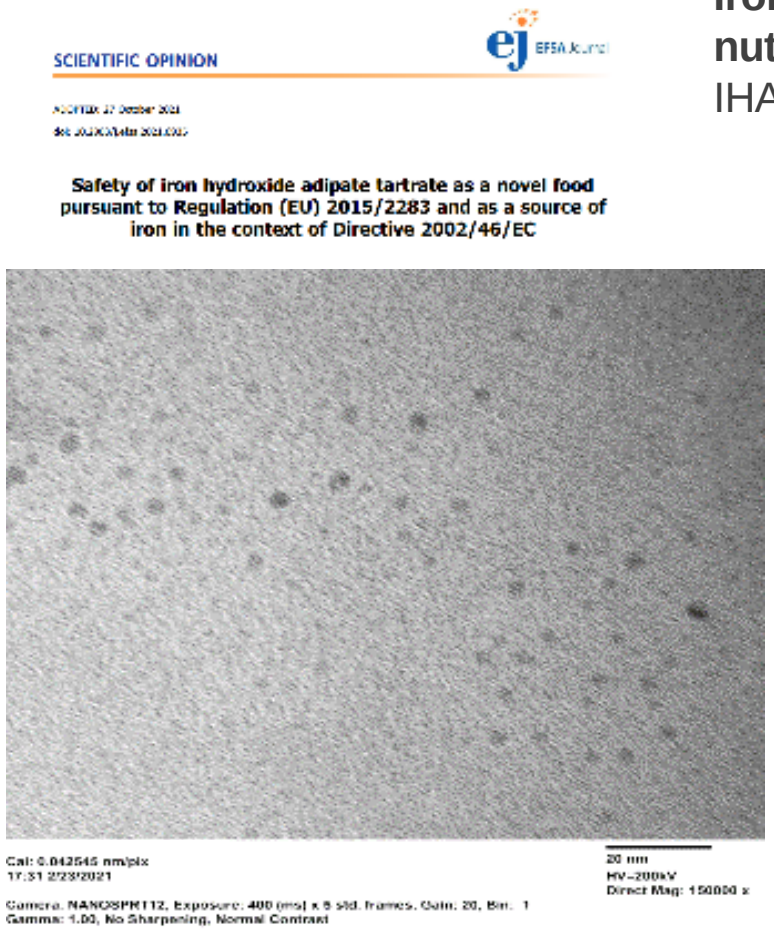





What about
NANOMATERIALS?

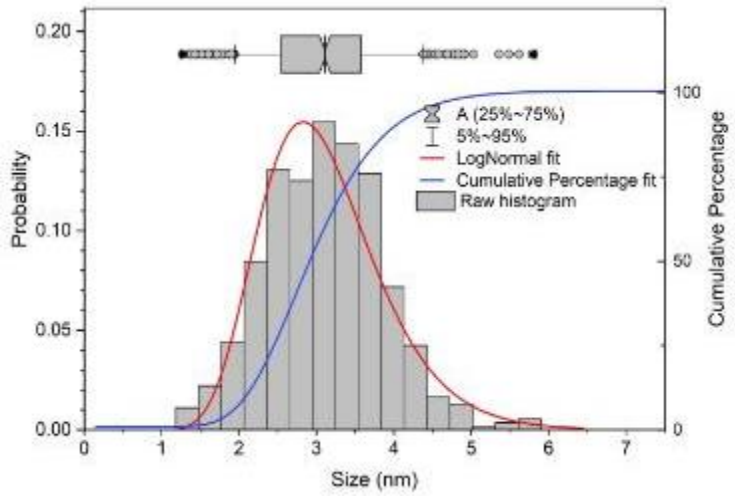
Particle size distribution

NANOMATERIAL


SCIENTIFIC OPINION

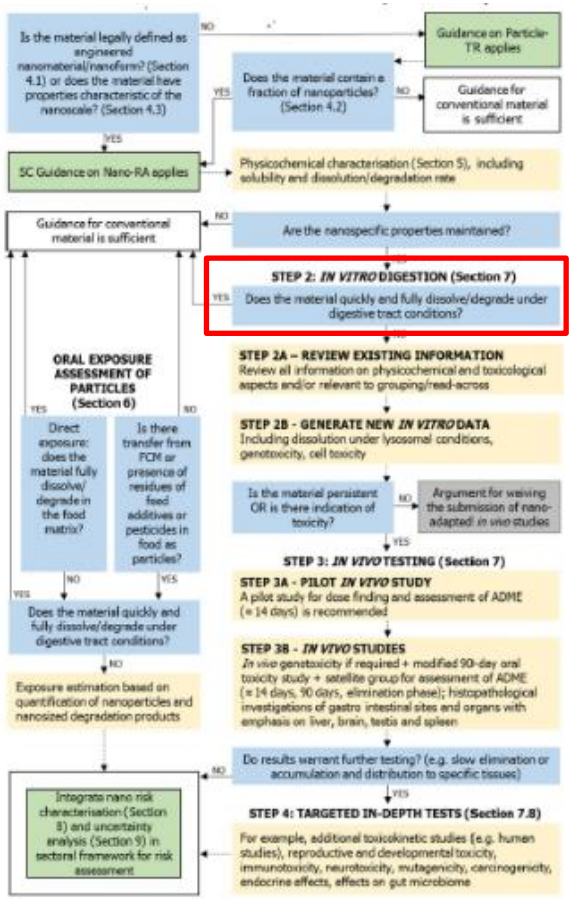
Safety of iron hydroxide adipate tartrate as a novel food pursuant to Regulation (EU) 2015/2283 and as a source of iron in the context of Directive 2002/46/EC

Iron Hydroxide Adipate Tartrate (IHAT) is requested from **Nemysis Limited** for use as **nutritional substance in food supplements**, in agreement with **Directive 2002/46/EC**. IHAT is a well-absorbed ferritin mimetic, able to preserve the gut microbiome.

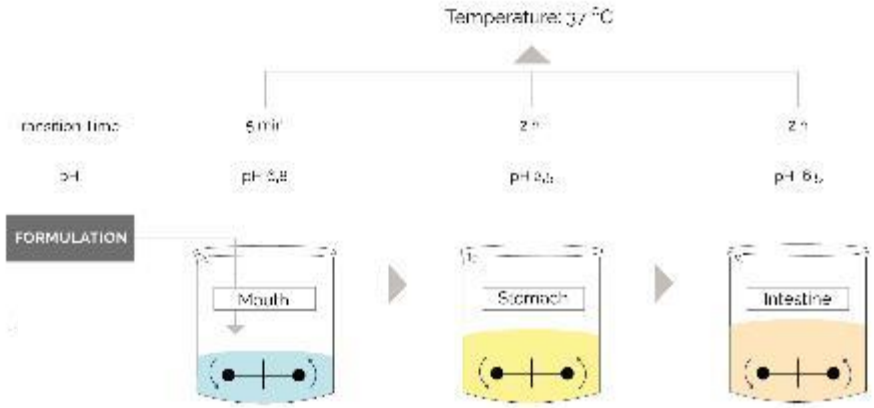


Dimensional Parameters	Value
Analyzed particles	543
Minimum size (nm)	1.3 ± 0.2
First quartile (nm)	2.5 ± 1.5
Median (nm)	3.1 ± 2.4
MAD (nm)	0.5 ± 0.8
Average (nm)	3.1 ± 1.8
Standard deviation (nm)	0.8 ± 0.2
Third quartile (nm)	3.6 ± 2.8
Maximum size (nm)	5.8 ± 2.0
D10 (nm)	2.2
D50 [median] (nm)	3.1
D90 (nm)	4.0

Nanomaterial



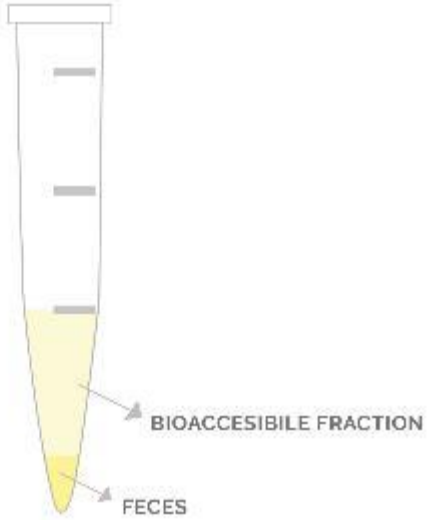
In vitro gastrointestinal digestion



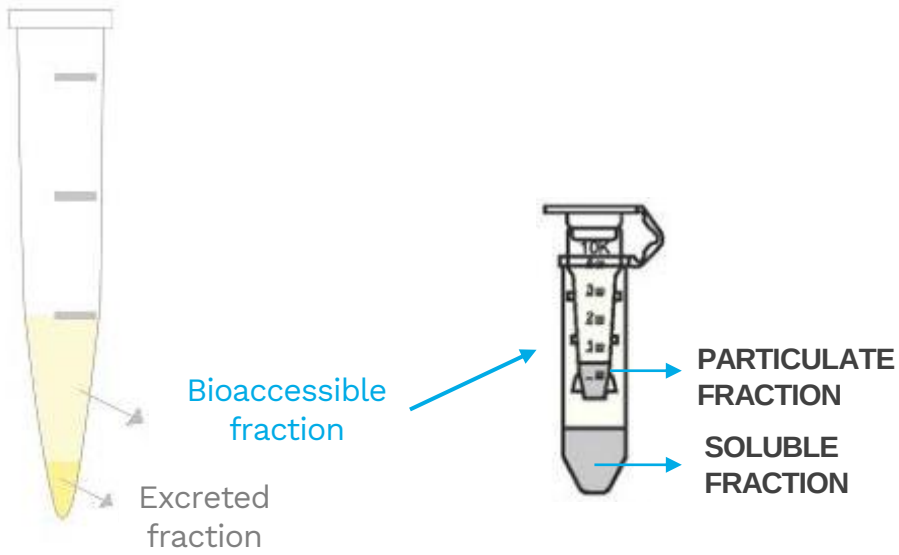
3 CONCENTRATIONS

4 TIME POINTS
IN THE INTESTINAL PHASE

DUPLICATE



Nanomaterial



Looking for

Bioaccessible fraction
Check for particles: EM (TEM-EDX/SEM-EDX)
Quantification: ICP-MS, HPLC, ...

Particulate fraction
Check for particles: EM (TEM-EDX/SEM-EDX), DLS, AF4-MALLS
Quantification: ICP-MS, HPLC, …

Soluble fraction
Quantification: ICP-MS, HPLC, …

CRITERIA

- Particles **clearly decrease** over time in the intestinal phase (no plateau)

and

- **≤ 12%** (mass based) of **particles after 30 min** of **intestinal digestion** (half-life=10 min)

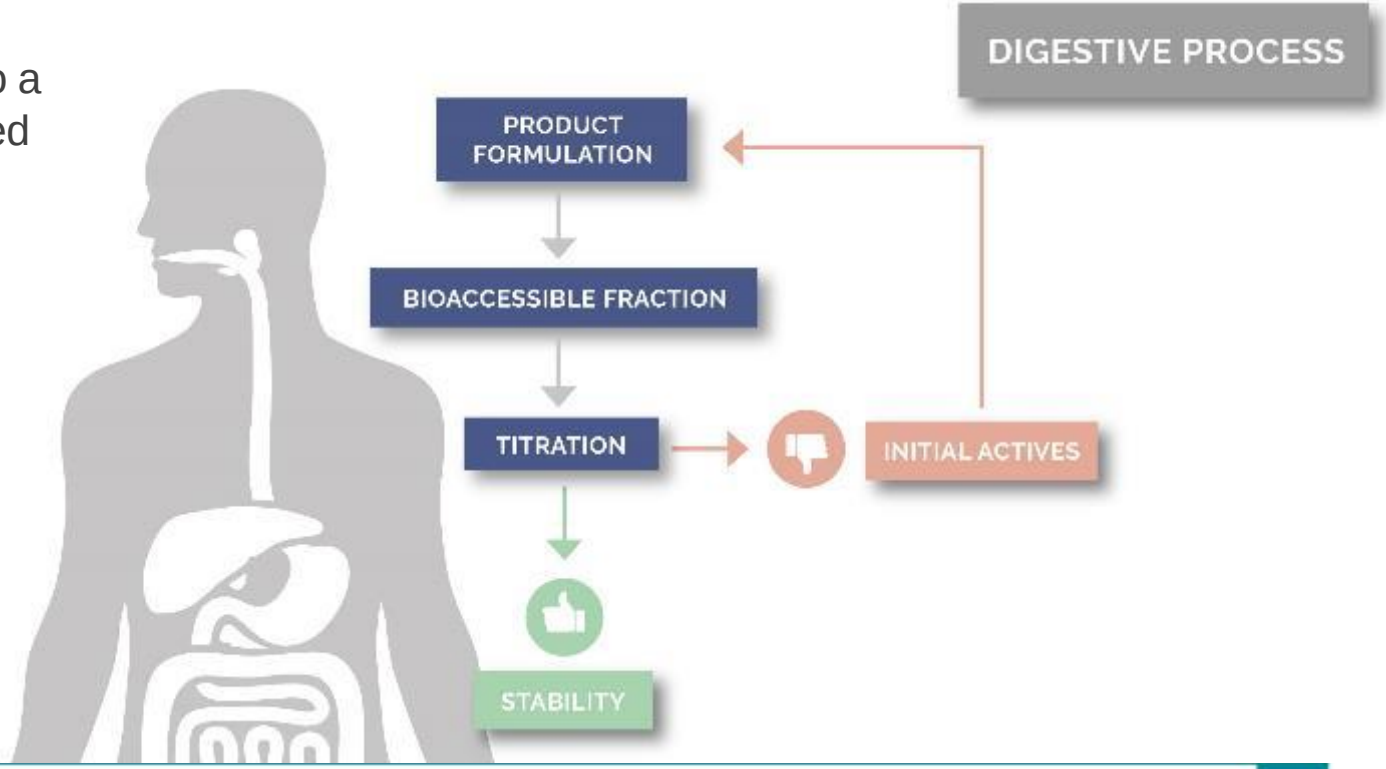
→ **NM with high dissolution rate/**
NM dissolves quickly
Nanospecific risk assessment is not required

Nanomaterial

IHAT *IN VITRO* DIGESTION

In vitro simulated GI digestion study of the NF in fed conditions, according to Minekus *et al.* (2014), and fasted condition as well:

- the model food used was equivalent to 1 g of chicken meat;
- three concentrations corresponding to correspond to a daily iron intake of 15 mg, 30 mg (i.e. the NF expected intake based on use levels) and 60 mg;
- ionic control (ferrous chloride)



Nanomaterial

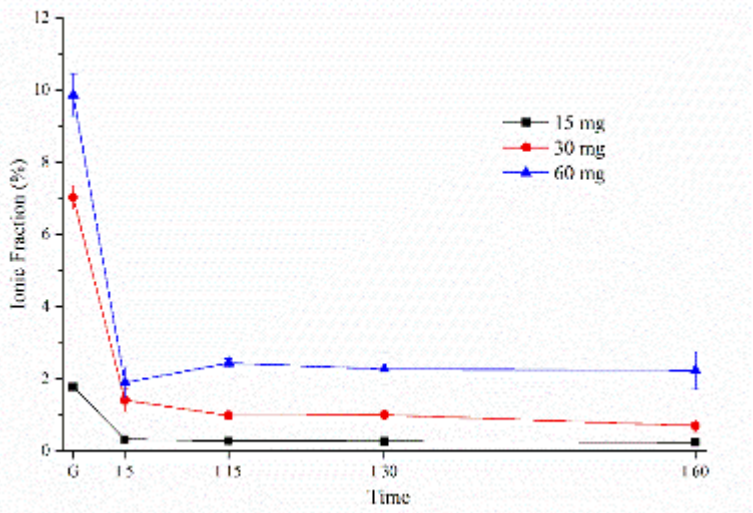
IHAT *IN VITRO* DIGESTION

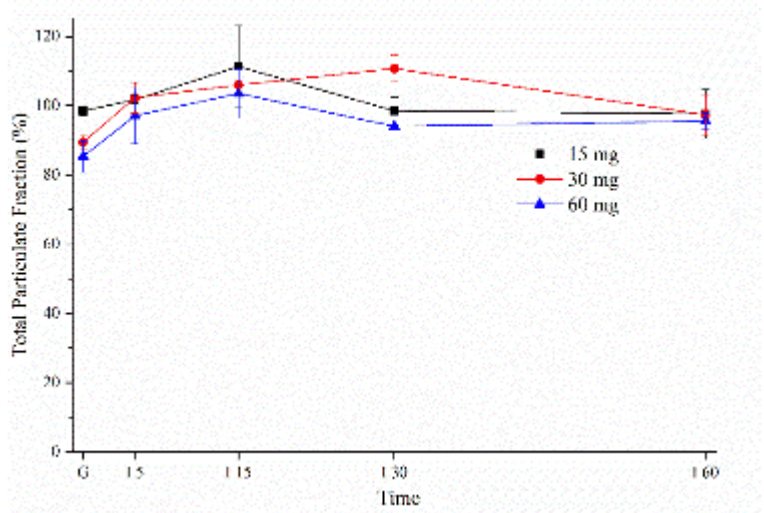
Percentage of soluble iron for IHAT

	15 mg			30 mg			60 mg		
	Percentage (%)			Percentage (%)			Percentage (%)		
Stomach – 2 h	1.78	±	0.06	7.03	±	0.33	9.87	±	0.59
Intestine - 5 min	0.32	±	0.05	1.41	±	0.32	1.89	±	0.44
Intestine - 15 min	0.28	±	0.06	0.99	±	0.10	2.44	±	0.12
Intestine - 30 min	0.27	±	0.03	1.00	±	0.09	2.27	±	0.42
Intestine - 60 min	0.25	±	0.05	0.70	±	0.17	2.24	±	0.53

Percentage of particulated iron for IHAT

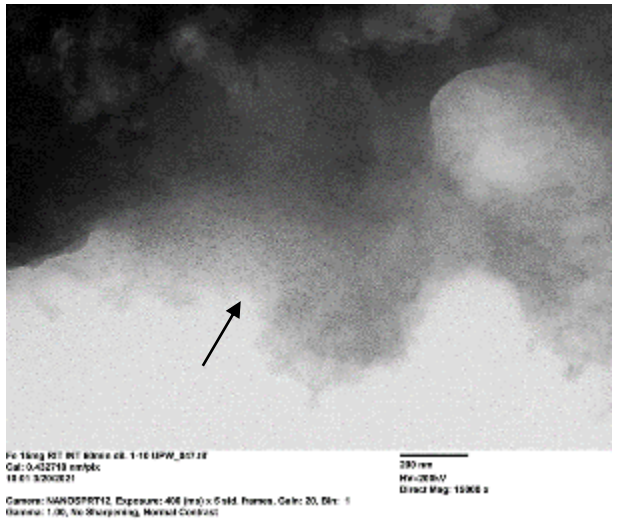
	15 mg			30 mg			60 mg		
	Percentage (%)			Percentage (%)			Percentage (%)		
Stomach – 2 h	98	±	1	89	±	2	85	±	5
Intestine - 5 min	102	±	1	102	±	4	100	±	6
Intestine - 15 min	111	±	12	106	±	1	103	±	7
Intestine - 30 min	99	±	4	113	±	2	94	±	0
Intestine - 60 min	98	±	7	101	±	3	96	±	2

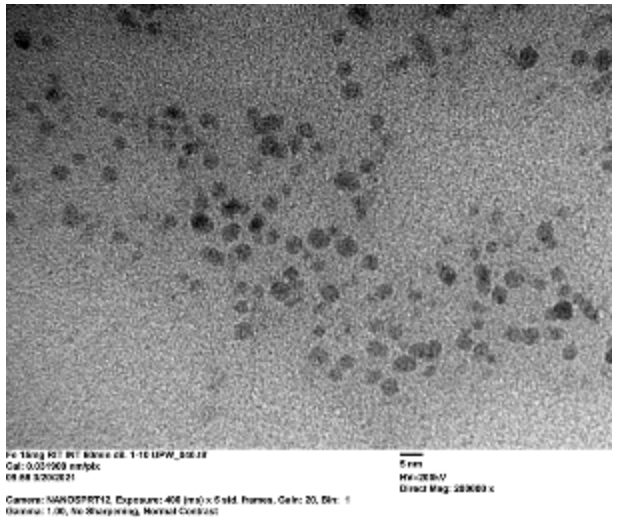


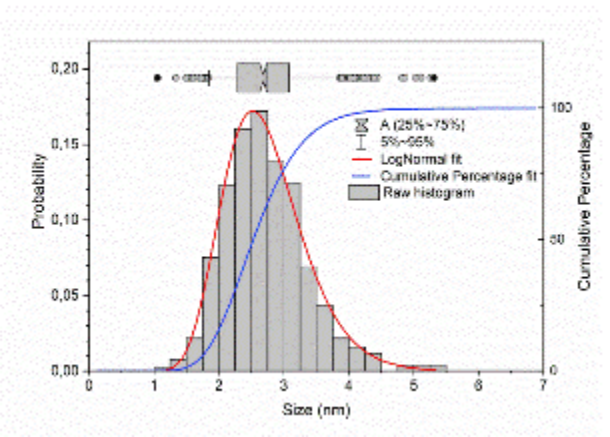


Nanomaterial

IHAT *IN VITRO* DIGESTION





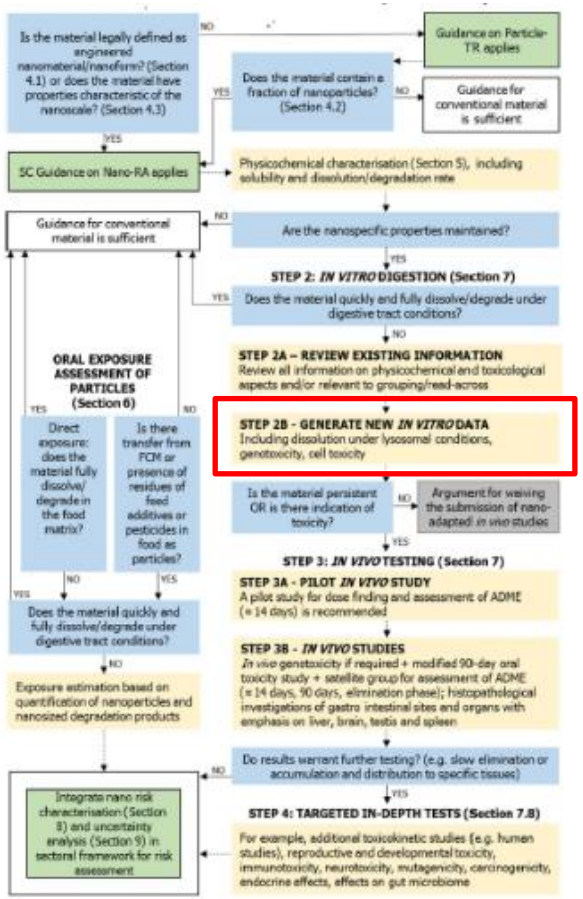


Dimensional Parameters	Value
Analyzed particles	505
Minimum size (nm)	1.1 ± 0.1
First quartile (nm)	2.3 ± 1.5
Median (nm)	2.7 ± 2.4
MAD (nm)	0.4 ± 0.8
Average (nm)	2.7 ± 1.8
Standard deviation (nm)	0.6 ± 0.2
Third quartile (nm)	3.1 ± 2.8
Maximum size (nm)	5.3 ± 1.9
D10 (nm)	2.0
D50 [median] (nm)	2.7
D90 (nm)	3.5

The material does not quickly and fully dissolved under digestive tract conditions

Nanomaterial

IHAT LYSOSOMAL CONDITIONS



Stability in lysosomal fluid

Evaluation of the biopersistence and intracellular accumulation

3 CONCENTRATIONS

4 TIME POINTS (UP TO 72 OR 96 H)

DUPLICATE

CRITERIA

≤ 12% (mass based) of particles at 72 hours

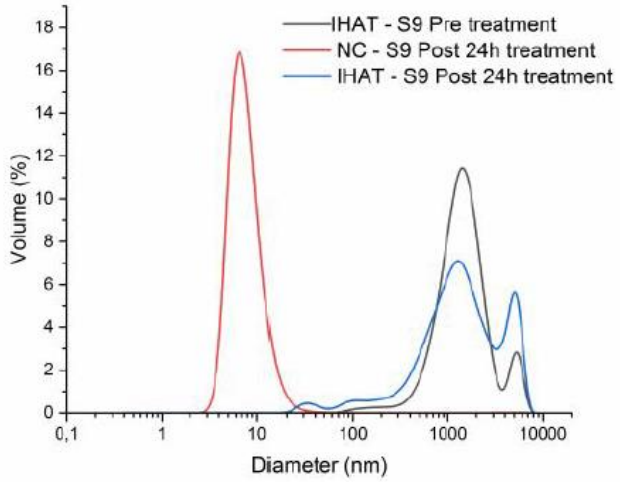
(half-life= 24 hours)

NM with high dissolution rate/ in lysosomal fluid

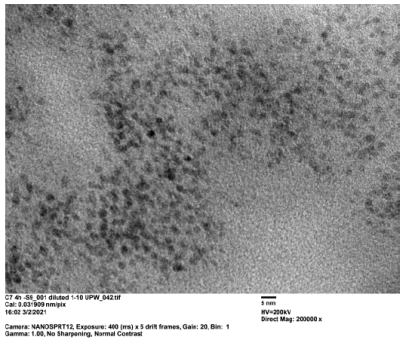
Adaptation of Test Guidelines and test designs

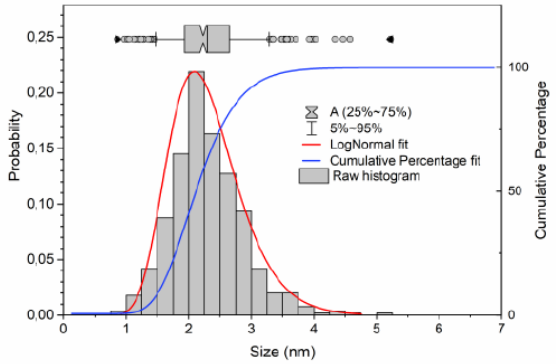
FOR TOXICITY TESTING OF NANOMATERIAL (OECD 487 - MN)

Stability in OECD 487 – MN test conditions



DLS – Dynamic Light Scattering



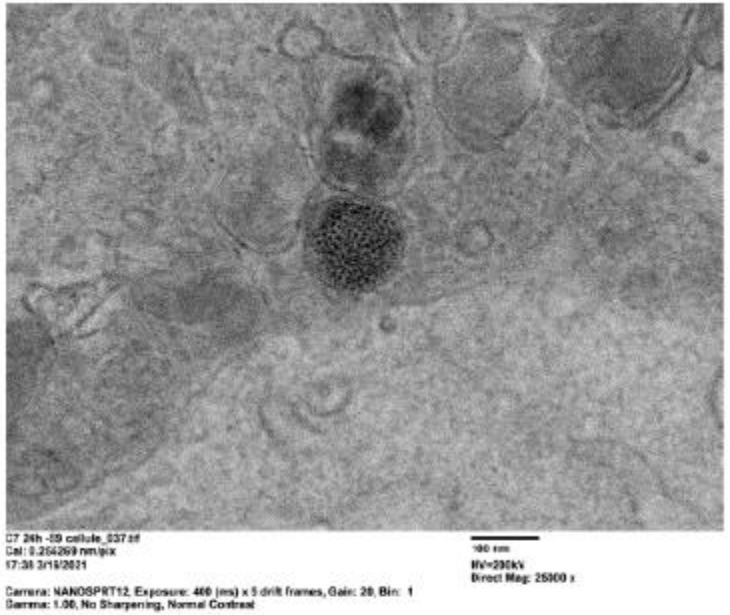


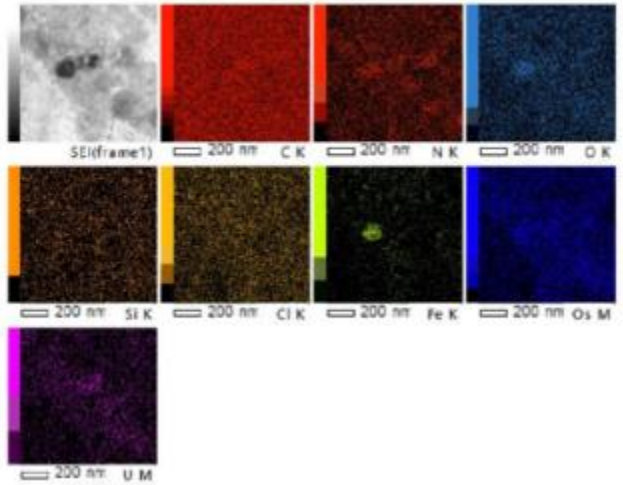
Dimensional Parameters	Value
Analyzed particles	502
Minimum size (nm)	0.9 ± 0.1
First quartile (nm)	1.9 ± 1.5
Median (nm)	2.2 ± 2.4
MAD (nm)	0.4 ± 0.8
Average (nm)	2.3 ± 1.8
Standard deviation (nm)	0.6 ± 0.2
Third quartile (nm)	2.6 ± 2.8
Maximum size (nm)	5.2 ± 1.8
D10 (nm)	1.6
D50 [median] (nm)	2.2
D90 (nm)	3.0

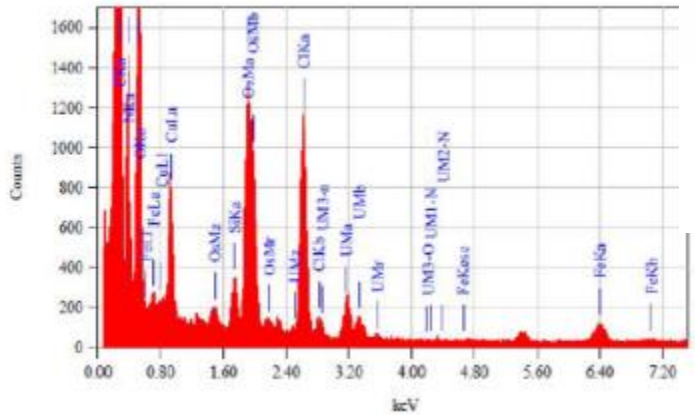
Adaptation of Test Guidelines and test designs

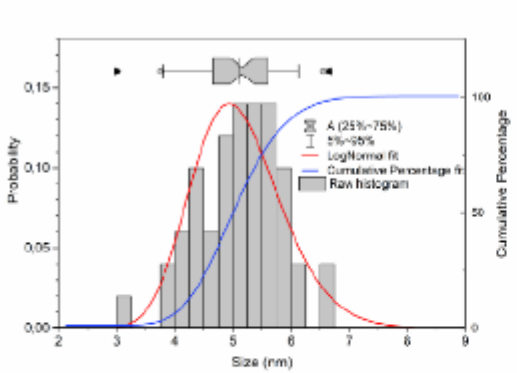
FOR TOXICITY TESTING OF NANOMATERIAL (OECD 487 – MN)

CELLULAR UPTAKE









Dimensional Parameters	Value
Analyzed particles	50
Minimum size (nm)	3.0 ± 0.4
First quartile (nm)	4.7 ± 1.6
Median (nm)	5.2 ± 2.5
MAD (nm)	0.5 ± 0.8
Average (nm)	5.1 ± 1.9
Standard deviation (nm)	0.7 ± 0.2
Third quartile (nm)	5.6 ± 2.9
Maximum size (nm)	6.7 ± 2.3
D10 (nm)	4.2
D50 [median] (nm)	5.2
D90 (nm)	5.9

Our testing lab facility experience

ECAMRICERT SRL, PART OF MÉRIEUX NUTRISCIENCES GROUP

EM is the most powerful technique to characterize primary particles of single substances or mixtures from qualitatively (shape and morphology) to quantitatively point of views (particle size distribution)

Dispersion protocol is crucial to better characterize small particles, but:

- no SOP for real samples are available and ad hoc protocols need to be developed;
- it could introduce artifacts, so comparison with pristine material is essential;
- Solvent used for particle dispersion should be carefully evaluated to avoid small particle solubilisation.

Solubility in lipophilic media is challenging since the recommended method based on ultrafiltration cannot be always applied.

Validated methods with well-defined performances **permit to obtain reliable quantitative data** and avoid any artifacts or useless data.

Participation to an international network permits to continuously improve our expertise and knowledge of the topic.

To properly **adapt toxicity testing of nanomaterials and small particles**, characterization techniques (i.e., EM) coupled with chemical identification techniques (e.g., EDX) should be always used.

Thank you

www.ecsin.it/en
ecsin@ecamricert.com
Corso Stati Uniti, 4 35127 – Padova (PD) Italy
Phone: 00 39 0425 377501