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Requirements to Measurements of Nanomaterials and Nanoproducts

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Editor:
Jørgen Garnæs and Lars Nielsen, Danish Fundamental
Metrology

Eva Jacobsen, Morten Køcks, Peter Bøgh Pedersen and
Pia Wahlberg, Danish Technological Institute

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Preface

The project “Requirements to Measurements of Nanomaterials and Nanoproducts” has been carried out from September 2015 to December 2015.

The report describes the project results regarding the requirements to validation of measurements of nanomaterials and nanoparticles relevant in the regulatory context.

The project has been carried out by Danish Fundamental Metrology with Technological Institute as a subcontractor. From EPA the project was headed by PhD Flemming Ingerslev and Anne Mette Zenner Boisen, Section of Chemicals attended the steering group.

The project was financed by the Danish EPA as a part of the activities on the national action plan on nanomaterials and their safety which was agreed in 2012 by the Social Democrat led coalition government in agreement with the Red-Green Alliance. The action plan has been carried out between 2012 and 2015.

Summary and conclusion

The possible impact of nanoparticles on human health and the environment is a concern that is getting increasing attention and the first regulatory decisions has been made. However, to address the concern, there is still a need for better test and measurement methods that can support decision making further. So far, the performance of analytical methods to support these requirements is not sufficiently documented.

The overall objective of the project is to establish a set of validation parameters based on the relevant international standards and the scientific literature, which can be used especially to document the performance of measurement methods to detect and quantify nanoparticles. The core of the report is to specify, interpret and clarify this set of validation parameters tailor-made for the requirements that are relevant for nanomaterials in the regulatory context. Based on relevant international standards and the scientific literature this report also outlines the physical and chemical character of nanomaterials.

The report proposes to specify a measurement split into the specimen to be measured (e.g. textile with silver nanoparticles), measurement method, measurand[1] and possible special measurement conditions. It is *suggested* that the validation of the measurement capability is split into

- selectivity (capability to measure analyte without interference from other components)
- limit of detection and quantification (lowest quantity that can be distinguished)
- working range, (range for which the method is applicable)
- precision, (closeness between replicated measurements)
- traceability (the result can be related to a reference)
- measurement uncertainty (observed dispersion of the quantity being measured).
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Each "parameter" shall document the methods performance regarding 1) the chemical identity, 2) particle size and mass or particle number concentration. Ideally the measurement method shall tell the chemical identity and number of particles as function of particle size in different matrixes, including different coatings or surface treatment of the nanoparticles. The report *conclude* that in general these tasks are not always possible to do with reference to methods described in international standards or the scientific literature. The task is more complicated than chemical measurements of concentration.

The report focuses on the sizing and counting of nanoparticles which is an aspect of nanomaterial measurements that is new in comparison with analysis of chemicals. The report examines as an example the counting and sizing from images from electron microscopes as a methods which could potentially be validated and give the number of particles as function of the diameter and the associated measurement uncertainty. *It is concluded that* the suggested validation parameters can be used to give evidence if requirements are fulfilled for these imaging method. The report clarifies in details the interpretation of the validation parameters for the imaging methods and gives examples of reasonable numerical values for some parameters. The most important validation parameters is the selectivity of the method to the nanoparticles, the limit of detection, the limit of quantification, the working range, the precision, the traceability and the measurement uncertainty. For the chemical analysis set of validation parameters are well established.

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[1] The quantity intended to be measured

For nanoparticles the basic, but concurrent also "new" measurand, is the particle size distribution, that is, the total number of particle split into small discrete size ranges. The report goes into depth with the interpretation of this measurand and how to interpret the combined measurement uncertainty[2] when there is an uncertainty on both the estimated size and the estimated number of particles in each small discrete size ranges. *It is found* that such an analysis is necessary in any regulatory compliancy assessment to e.g. prove that a material shall be categorized as a nanomaterial according to e.g. the EU definition of nanomaterial. *The report delivers* a complete example of the calculation of the nanomaterial fraction including the associated measurement uncertainty. This can be used to determine if a material shall be classified as a nanomaterial at a certain threshold. The calculation is based on a tailor-made excel template. The example and calculation is the fundament for the reports interpretation of measurements results and the proposed validation parameters.

There are many instrumental methods, which are used to quantify different chemical species in a mixture and these are also suitable for determination of the chemical composition of the nanoparticles, the chemical composition of the matrix or the chemical composition of the material (i.e. nanoparticles and matrix as a whole). The report focuses on the analysis of inorganic nanoparticles using inductively coupled plasma mass spectroscopy detection (ICP-MS) as an example in the discussion of the validation parameters.

To further clarify and interpret the validation parameters four realistic but fictitious cases are defined and discussed. The cases cover two generic issues which are concentration of silver nanoparticles in a consumer product and the release of nanoparticles from crayon and two common but very difficult issues which are handling of agglomeration and nanoparticles which are coated.

The measured size and concentration of silver nanoparticles in a consumer product can e.g. be based on analysis of electron microscope images following ISO standards. Important validation parameters are clarified as the methods selectivity of silver nanoparticles, working range, that is, the size range of nanoparticles for which the measurement procedure is applicable, and traceability to measurements on reference materials. It is concluded that a validation of the measurement procedure can potential be performed based on the suggested validation parameters. Validation parameters are also clarified for the size distribution of released nanoparticles or nanoaerosols in air for a particular measurement method based on the aerodynamic properties of the nanoaerosols. The measurand is the particle *mobility* diameter and particle number concentration. Important validation parameters are clarified as the working range, that is, the size rage of nanoparticles for which the measurement procedure is applicable, precision related to a background measurement of ambient air and traceability to measurements on reference materials. It is concluded that in some cases method defined approaches and standard procedures constitute an acceptable solution. Agglomeration and aggregation are discussed and a dispersion protocol is proposed together with a method to identify and quantify the aggregation or agglomeration based on the findings in the report. A full validation of any method to assess issues about coating of e.g. titanium nanoparticles is missing in the literature and is further complicated because of the lack of any reference material.

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[2] The combined measurement uncertainty is obtained using the individual standard measurement uncertainties associated with the input quantities in a measurement model for the measurement process. The measurement model is the mathematical relation among all quantities known to be involved in a measurement (ISO/TR 13014:2012)

Summary in Danish

Nanopartiklers mulige påvirkning af menneskers sundhed og miljøet er en bekymring der har fået stigende opmærksomhed og den første lovgivningen er implementeret. For at adressere denne bekymring, er der fortsat et behov for bedre test- og målemetoder, som kan understøtte beslutningsprocesserne yderligere. Hidtil er korrektheden og præstationen af de analysemetoder der skal understøtte disse krav dog ikke tilstrækkeligt dokumenteret.

Det overordnede formål med projektet er at etablere et sæt af valideringsparametre baseret på relevante internationale standarder og den videnskabelige litteratur, som kan bruges specifikt til at dokumentere korrektheden og præstationen for målemetoder til påvisning og kvantificering af nanopartikler. Rapportens kerne er at specificere, fortolke og klarlægge dette sæt af valideringsparametre skræddersyet til de krav, der er relevante for nanomaterialer i lovgivning. Baseret på relevante internationale standarder og den videnskabelige litteratur skitserer rapporten også den fysiske og kemiske karakter af nanomaterialer.

Rapporten foreslår at specificere en måling opdelt i den type prøve der skal måles (f.eks. tekstil med sølvnanopartikler), målemetoden, målestørrelsen[3] og eventuelle særlige målebetingelser. Det foreslås, at valideringen af måleevnen opdeles i

- Selektivitet (evne til at måle analyten uden interferens fra andre komponenter)
- Detektionsgrænse og kvantificering (laveste mængde, der kan skelnes)
- Måleområde, (interval, hvor metoden kan bruges)
- Præcision, (overensstemmelse mellem gentagne målinger)
- Sporbarhed (resultatet kan relateres til en reference)
- Måleusikkerheden (den observerede spredning af den størrelse der udmåles).

Hver "parameteren" skal dokumentere metodens korrekthed og præstation vedrørende 1) den kemiske identitet, 2) partikelstørrelse og masse eller partikelantals-koncentrationen. Ideelt skal målemetoden skelne den kemiske identitet og antallet af partikler som funktion af partikelstørrelse i forskellige matricer, herunder forskellige belægninger eller overfladebehandling af nanopartiklerne. Rapporten konkluderer, at disse måleopgaver i almindelighed ikke altid kan løses med reference til målemetoder beskrevet i internationale standarder eller den videnskabelige litteratur. Opgaven er mere kompliceret end kemiske målinger af kun koncentration.

Rapporten fokuserer på størrelse og optælling af nanopartikler, som er et nyt aspekt af nanomaterialemålinger, i forhold til analyse af kemikalier. Rapporten undersøger som et eksempel optælling og størrelsesopmåling fra billeder fra elektronmikroskoper som en metode, der potentielt kan valideres og give antallet af partikler som funktion af diameteren inklusiv den tilhørende måleusikkerhed. *Det konkluderes*, at de foreslåede valideringsparametre kan bruges til at eftervise, om givne krav er opfyldt for disse afbildningsmetoder. Rapporten klarlægger i detaljer fortolkningen af valideringsparametre for afbildningsmetoderne og giver eksempler på passende numeriske værdier for nogle af parametrene. De vigtigste valideringsparametre er selektiviteten af metoden mht. nanopartiklerne, detektionsgrænsen, kvantifikationsgrænsen, måleområdet, præcisionen, sporbarheden og måleusikkerheden. For den kemiske analyse er sættet af valideringsparametre veletablerede.

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[3] Den egenskab der skal måles

For nanopartikler er den grundlæggende, men samtidigt "nye" målestørrelse, partikelstørrelsesfordelingen, dvs. det samlede antal partikel størrelsesopdelt i små diskrete intervaller. Rapporten går i dybden med fortolkningen af denne målestørrelse og hvordan den kombinerede måleusikkerhed fortolkes, når der er en usikkerhed på både bestemmelsen af størrelsen og bestemmelsen af antal af partikler i hver lille diskrete størrelse intervalle. *Rapporten finder*, at en sådan analyse er nødvendig i enhver lovgivningsmæssig sammenhæng f.eks. for at bevise om et materiale skal kategoriseres som et nanomateriale efter f.eks EU-bekendtgørelsen om definitionen af nanomateriale. *Rapporten leverer* et komplet eksempel på beregning af nanomaterial-fraktionen herunder den tilhørende måleusikkerhed. Dette kan bruges til at afgøre om et materiale skal klassificeres som et nanomateriale for en givet grænseværdi. Beregningen er baseret på en skræddersyet Excel-skabelon. Eksemplet og beregning er fundamentet for rapportens fortolkning af måleresultater og valideringsparametre.

Der er mange metoder, basseret på forskellige instrumenter, som kan anvendes til at kvantificere forskellige kemiske elementer i en blanding, og disse er også egnet til bestemmelse af den kemiske sammensætning af nanopartiklerne, den kemiske sammensætning af matricen eller den kemiske sammensætning af materialet (dvs. nanopartikler og matrice som en helhed). Rapporten fokuserer på analysen af uorganiske nanopartikler og bruger induktivt koblet plasma masse spektroskopi detektion (ICP-MS) som et eksempel i diskussionen af valideringsparametrene.

For yderligere at anskueliggøre og fortolke valideringsparametre defineres og diskuteres fire realistiske, men fiktive eksempler (”cases”). Eksemplerne dækker to generiske emner, koncentrationen af sølvnanopartikler i et forbrugerprodukt og frigivelsen af nanopartikler fra farvekridt samt to almindelige, men meget vanskelige emner som er håndteringen af agglomerering og overfladebelagte nanopartiker.

Den målte partikelstørrelse og koncentration af sølvnanopartikler i et forbrugerprodukt kan f.eks. findes ud fra analysen af elektronmikroskopi billeder efter ISO-standarder. Vigtige valideringsparametre afklares og udpeges. Disse er målemetodens selektivitet for sølv nanopartikler, måleområde, dvs., størrelsesintervallet for nanopartikler, som målemetode kan anvendes til, og sporbarhed til målinger på referencematerialer. Det konkluderes, at det er muligt at udføre en validering af måleproceduren baseret på rapportens foreslåede valideringsparametre. Valideringsparametrene præciseres også for størrelsesfordelingen af frigivne nanopartikler eller nanoaerosoler i luft for en udvalgt målemetode baseret på de aerodynamiske egenskaber af nanoaerosoler. Målestørrelsen er partiklernes mobilitets diameter og partikelkoncentrationen. Vigtige valideringsparametre præciseres som størrelsesintervallet for nanopartikler, hvor målemetoden kan anvendes, præcision relateret til en baggrundsmåling af den omgivende luft og sporbarhed til målinger på referencematerialer. Det konkluderes, at i nogle tilfælde udgør metode definerede fremgangsmåder og standardmetoder en acceptabel løsning. Agglomerering og aggregering diskuteres og basseret på rapportens resultater foreslås en fortyndingsprotokol som en metode til at identificere og kvantificere agglomerering og aggregering. En fuld validering af en metode til at vurdere belægning af f.eks. titanium nanopartikler mangler i litteraturen og kompliceres yderligere på grund af manglende referencematerialer.

1. Introduction to the report

For the measurements of nanomaterials a number of different and often advanced physical and chemical methods are used. As the area is new the development of suitable methods is some time lacking behind the needs or laboratories must develop tailor-made non-standardized methods to fulfil measurement requirements. Most methods are therefore not so well established, as seen in, for example standard chemical analysis[4]. The consequence is that it is difficult or impossible to specify the requirements to measurements, interpret the results, and secure the validity of the measurement results. Questions where clarification and guidelines are required could be:

1. How to interpret the nanomaterial analysis when the quantity to be measured is the size distribution of particle, their number or mass concentration?
2. How to specify measurements and judge if quantification offered by a laboratory is sufficiently documented and thereby fit for the intended purpose?
3. How to prove that a material shall be categorized as a nanomaterial based on measurement of the particle size distribution?

A barrier that makes it difficult to answer such questions is that uncertainty, variability, detection limits, etc. is not properly described for the measurement of potential nanomaterials.

1.1 Objective and structure of the report

The overall objective of the project is to establish a way to specify the requirements of measurement methods, which can be used especially to specify and interpret measurements of nanomaterials. For this purpose a set of validation parameters is proposed as a part of this project. To be able to interpret the validation parameters the report needs to give first an overview of how to characterize nanomaterials and nanoparticles (**chapter 2**). The main focus will be the aspects that distinguishes nanomaterials from ordinary chemicals, i.e. particle size and particle size distribution.

The core of the report is to specify, interpret and clarify a set of validation parameters aimed for the requirements that are relevant in the regulatory context[5]. The specification and interpretation of the validation parameters will be outlined both in general (**section 3.1**) and with special emphasis on the new type of quantification of nanomaterials, that is, with emphasis on particle size and particle size distributions (**section 3.2**).

The report will then give an overview of some selected types of measurements instruments used in the analysis of these parameters including a discussion of their validation parameters **(chapter 4)**

To better understand and interpret the validation parameters four realistic but fictitious cases will be defined and discussed (**chapter 5**). The cases shall cover two generic issues which are concentration of nanoparticles in a consumer product and concentration of nanoparticles in air

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[4] In this report a standard chemical analysis is a measurement of the concentration of a chemical substance with a unique molecular formulation.

[5] In Denmark, e.g., an “Order on a register of mixtures and articles that contain nanomaterials as well as the requirement for producers and importers to report to the register” has been in force from 13/06/2014 (Order 644)

and two common but very difficult issues which are handling of agglomeration and nanoparticles which are coated.

1.2 Method

The report is based on relevant international standards such ISO standards and relevant technical and scientific papers. Particular relevant standards identified were:

- Nanotechnologies - Guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment[6]
- Nanomaterials -- Quantification of nano-object release from powders by generation of aerosols[7]

In reference [6] some standards relevant in nanotechnology in general are listed[8].

The validation parameters and discussion in **Chapter 3** is based on generally acknowledged principles in analytical chemistry as laid down in international guidelines, taking into consideration potential issues arising from the particulate matter of nanomaterials.

1.3 The nanomaterial definition

In practice it is difficult to define nanoparticles in an unambiguous way and therefore a more or less ad-hoc based definition that match the specific purpose must often be used, see also the following discussion in **Chapter 2** and in particular the discussion in section 2.1.1. ISO has devoted a standard to the classification and categorization of nanomaterials[9] and reference [10] is devoted to the discussion of more than 10 national or international nanomaterial definitions for safety considerations.

In this report the definition of nanomaterial given in the EU commission recommendation[11] is used when possible. It states as follow[12]:

1) *Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm.*

To determine the size distribution and weather a material fulfils the recommended definition one has to determine if the material consist of particles, determine the constituent particles, the external dimensions of the (constituent) particles and finally the median value of the particle size distribution based on the external dimension. The EU commission recommendation is supplemented with the report from the Joint Research Centre about the requirements on measurements for the implementation of the EU definition[13]. It should be noted that a critical review of the EU definition has been made[14].

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[6] *Nanotechnologies - Guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment*, ISO/TR 13014:2012

[7] *Nanomaterials -- Quantification of nano-object release from powders by generation of aerosols* ISO/TS 12025:2012

[8] A relevant document announced *after* the project has ended but before publication of this report is *Guideline for the validation of physico-chemical analytical methods* CEN/TS 16800:2015

[9] *Nanotechnologies - Methodology for the classification and categorization of nanomaterials* DS/ISO/TR 11360:2010

[10] *Comparative assessment of nanomaterial definitions and safety evaluation considerations*. Regul Toxicol Pharmacol. 2015 Oct;73(1):137-50. doi: 10.1016/j.yrtph.2015.06.001. Epub 2015 Jun 23

[11] *Commission Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU)*

[12] The definition also discuss fullerenes, graphene flakes and single wall carbon nanotubes but this report is limited to other particles than these highly specialized engineered particles

[13] *Requirements on measurements for the implementation of the European Commission definition of the term "nanomaterial"*, T. Linsinger, G. Roebben, D. Gilliland, L. Calzolai et al., JRC Reference Report EUR 25404 EN

[14] *Towards a review of the EC Recommendation for a definition of the term "nanomaterial"* Part 1, 2 and 3, Report EUR 26567 EN, Report EUR 26744 EN and EUR 27240 EN

2. Characterizing nanomaterials and nanoproducts

Based on the relevant international standards and the literature this chapter clarify the special physical and chemical properties of nanoparticles and defines the measurand for each of these properties. It is divided into two parts one about the physical characterization discussing the size and number and one part briefly discussing chemical identification. In general many terms are also defined in ISO vocabulary standards[15]

2.1 Physical characterizers

2.1.1 Size and shape

Particles are defined as "minute pieces" of matter with defined physical boundaries"[16] To determine if a material is a nanomaterial according to the EU definition it must be in the form of a powder or a suspension, that is, dispersed in a liquid. This report will also discuss nanosized aerosols as this also is relevant in regulatory context and in risk assessment.

The EU definition of nanomaterial does not use the word "diameter" or "particle size" but use instead "external dimensions". For an ideal round and hard particle the "external dimensions" is equal to the diameter of the physical boundaries of the matter. However, most particles will have irregular shaped bodies and thus the issue is not as easy to understand – see **Figure 1**. A simple external dimension is the smallest perpendicular distance x_{Fmin} between the two parallel planes restricting the particles.

Measurand: The external dimension in the unit [m]. In this report it will pragmatically often be named the diameter. A size-independent description can be added such as a ration to describe the relation between length and possible circular diameter of rod-like particles.

2.1.2 Size distribution

The particle size distribution is the cumulative distribution of particle concentration as function of particle size. The particle size distribution is a key parameter for the determination of their function and properties. A particulate material will almost always consist of particles with various sizes, called polydisperse size distribution. This is contrary to an idealized material consisting of only particles with the same size called a monodisperse size distribution – see **Figure 2** . The histogram in **Figure 2** b) shows how many particles have sizes between 10 nm and 20 nm, 20 nm and 30 nm and so on. The number of particles in each "size bin" is depicted as a rectangular with an area proportional to the number of particles in each size bin. According to the EU definition of nanomaterial "50% or more of the particles in the number size distribution" shall have an external

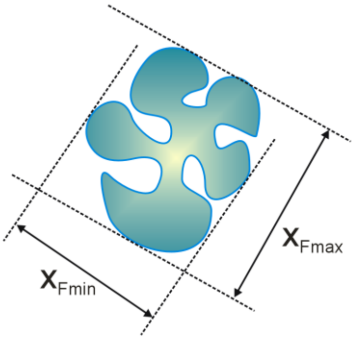

FIGURE 1
SCHEMATIC ILLUSTRATION OF THE INTUITIVE EXTERNAL DIMENSION OF AN IRREGULAR PARTICLE FROM A 2D PROJECTION OF THE PARTICLE.

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[15] *Nanotechnologies - Vocabulary* DS/CEN ISO/TS 80004-1 to 7

[16] *Nanotechnologies – Terminology and definitions for nano-objects – Nanoparticles, nanofibre and nanoplates*, ISO/TS 27687:2008, ISO, Geneva, 2008. After the end of the project this standard has been reversed and is now *Nanotechnologies -- Vocabulary -- Part 2: Nano-objects* ISO/TS 80004-2:2015

dimension in the size range 1 nm to 100 nm. In **Figure 2** b) 63% of the number size distribution of the particles is in the size range 1 nm to 100 nm and the particulate material is thus positively identified as a nanomaterial.

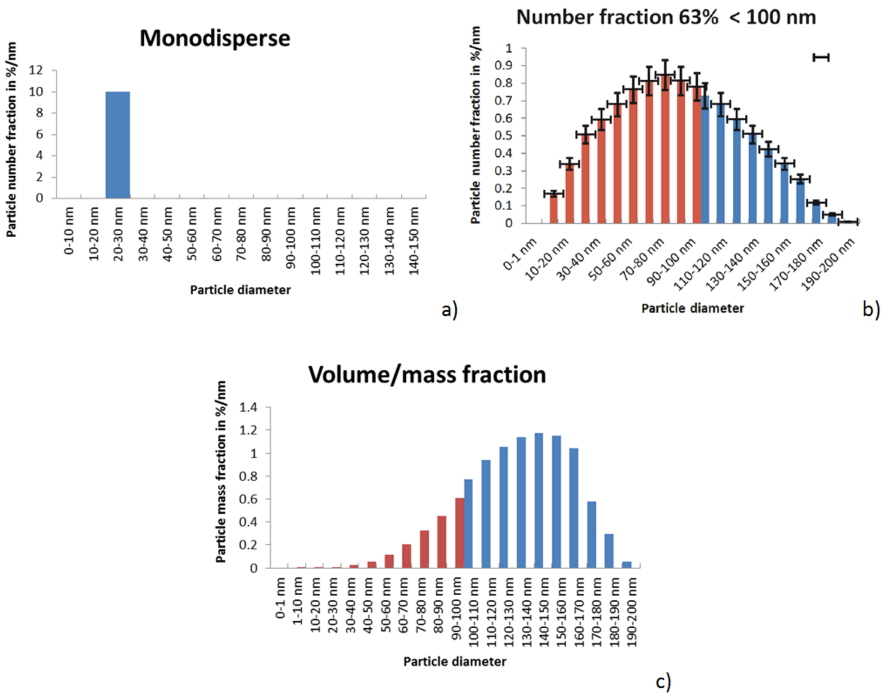

FIGURE 2

HISTOGRAM REPRESENTING THE NUMBER-BASED SIZE DISTRIBUTION. A) IS A MONODISPERSE MATERIAL. B) IS A NUMBER-BASED POLYDISPERSE DISTRIBUTION. THE CROSSES REPRESENT FICTITIOUS UNCERTAINTIES ON THE OBSERVED PARTICLE DIAMETER AND THE NUMBER FRACTION. C) IS THE VOLUME OR MASS-BASED SIZE

A quantification of size distribution can be achieved by various methods some of which are discussed in more details in **chapter 4**. The conceptual simplest method is *counting methods*, which create separate signals for each measured particle directly creating a number size distribution. The main focus of this report will be on such methods. More complicated to interpret are the *methods*, which detect and analyse a collective signal such as light reflected or scattered from all particles in the sampled volume (sometimes called "ensample methods"). The size distributions deduced from such data rely on – often complicated - convolution steps in an analysis algorithm.

Fullerenes, graphene flakes and single wall carbon nanotubes are nanomaterial and require a separate testing. A specific surface area larger than 60 m²/cm³ can also be used to positively classify a material as a nanomaterial. Neither are relevant for the cases and will not be discussed further in this report.

Measurand: The measured number q_k of particles in a "size bin" k is depicted as a rectangle with a width equal to the width of the size bin and an area proportional to the measured number q_k of particles in each size bin. The unit of the measured number q_k per size bin is [m^{-1}].

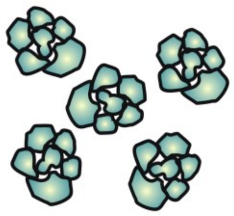

FIGURE 3

MATIC DRAWING OF FIVE AGGREGATIONS. THE EU DEFINITION OF NANOMATERIALS IS BASED ON THE SIZE OF THE 25 CONSTITUENT PARTICLES.

In general the rules and nomenclature for the graphical representation of particle size analysis data in histograms and density distributions is specified in an ISO standard[17]

2.1.3 Aggregation and agglomeration

The EU definition of nanomaterial explicitly states that the principle for the size estimation is the size of the constituent particles even if they are only present as aggregates or agglomerates, see **Figure 3**. Agglomerate is particles hold together by weak forces, for example van der Waals forces, or simple physical entanglement [16]. Aggregates are particles hold together by strong forces, for example covalent bonds, or those resulting from complex physical entanglement [16]. Nearly no methods can determine the size of the constituent particles in agglomerate or aggregation and therefore these has to be broken up before the measurements. This is in general not possible but some weakly-bound particles can be broken by standard ultrasonic cleaning procedures. Some information about the relative binding strength between the constituent particles can be achieved from such ultrasonic cleaning but absolute values are difficult to obtain as the breaking power depends on temperature, solvent, time applied, frequency and energy transferred.

Measurand: The size of the aggregates/agglomerates, unit [m] or [nm], number of aggregated particles compared to the total number of particles [number/number] and the number and distribution of constituent particles per aggregate.

2.1.4 Coating and surface chemistry

Coating and surface chemistry is informally used about the chemical nature, including composition, of the outermost layer of the nanoparticles. Molecules adsorbed to the surface alter the surface chemistry and dictate the functionality in many contexts.

Measurand: chemical composition and thickness of fixed layers, unit [m]. Standard chemical reaction rate concepts [mole/(dm^3)] preferably of a (toxicological) interest or its surrogate[6].

2.1.5 Concentration and fractions of nanoparticles

A nanomaterial can be characterized either qualitatively (nanoparticles are present) or quantitatively (which type of nanoparticles are present and what is the concentration and particle size distribution of each type of nanoparticles). For material containing nanoparticles in a matrix, it is possible to talk either about the presence of nanoparticles, the concentration of nanoparticles in the matrix or the particle size distribution of the nanoparticles (i.e. nanoparticles in the matrix as a whole).

Measurand:

The *concentrations* of nanoparticles can be stated in absolute terms with units, e.g., c mg/kg, c mol/kg etc. where c is the total mass of the nanoparticles.
The amount of nanoparticles can also be stated as the *nanoparticle fractions* f_n of the total number of particles with no unit. Fractions always sum to 1 (or 100 %) and is normally based on the number of particles or their mass.

This basic discussion about the concentration and fraction of nanoparticles is similar to the discussion of the chemical characterization in **Section 2.2.** Mass concentration of nanoparticles can also be determined by direct measurements of collected particles on a filter or by determining the added mass on a resonator crystal (peizobalance) which is very sensitive to small masses.

The particle size distributions can be differently weighted. In **Figure 2 c)** the particle size distribution is given by their mass fraction in %/nm and in **Figure 2 b)** by their particle number fraction in %/nm for the same particle size distribution. It is seen that large particles weight more

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[17] *Representation of results of particle size analysis — Part 1: Graphical representation* ISO 9276-1

and that the maximum fraction of the mass in %/nm is around 1.1%/nm in the narrow size range from 140-150 nm while the maximum particle number fraction is around 0.9 %/nm for the narrow size range from 70-80 nm. For broader size distributions this shift in maximum fraction is much larger.

For idealized particles with perfect spherical shape, homogeneous density and optical properties etc. the concentration of nanoparticles can in principle be calculated as the mass of the nanoparticles divided by the volume of the suspension media in the unit of e.g. µm/litter. For most real particles in e.g. nanoproducts these assumptions are fare from correct. If the particle size distribution is measured based on e.g. the intensity of the light scattered which scale by the power of 6 a conversion from intensity to mass and number are not very accurate.

If – and only if – the total mass of the particles are known it is possible to calculate the concentration of the nanoparticles in e.g. mg/kg from the particle size distribution which is found by nearly all particle measuring systems. The total mass of the particles can be found directly by weighing or indirectly such as relative to the total amount of the matrix including the nanoparticles. However this conversion from to concentration is only meaningful for idealized particles with perfect spherical shape, homogeneous density and if the size distribution include all particles and in particular the larges particles. For most real particles in nanoproducts these assumptions are far from correct.

2.1.5.1 Compliancy with permitted limits

In any regulatory context dealing with e.g. concerns for the environment, health or safety the basic question is to assess the amount of nanomaterial either in the form of the concentration or number based fraction – see the above **section 2.1.5** for a clarification of concentration, fraction and the conversion between concentration and fraction.

In regulatory context the most important task will be to assess compliancy with permitted limits for the amount of nanoparticles based on concentration or number based fraction. This is not straight forward as nearly all particle measurement give a particle size distribution with some uncertainty associated with both the size of the particles and the observed number (or deduced fraction) of each narrow size range. This is informally[18] shown in **Figure 2** b) by the error bars.

Estimation of uncertainty is a pre-request for the assessment of compliance or non-compliance with any permitted limit such as the 50% nanomaterial fraction in the EU Commission Recommendation[11]. Measurement results without statement of measurement uncertainties are of little worth, certainly in a legal situation. Based on these considerations it is concluded that this report – as an example - should contain a thorough clarification of how to determine compliancy for the EU suggested limit of 50% for the amount of nanoparticles in terms of the size fraction. This issue is not directly clarified in the ISO standards about representation of results of particle size analysis[19].

The following part of the section will thus clarify how to determine a particular size fraction with diameter smaller than 100 nm based on the measurement of the particle size distribution. The particular nanomaterial fraction (in %) and the associated uncertainty shall be determined from the observed size distribution of all the particles, see the definition in **section 2.1.2** and **Figure 2 .**

Expressed simple it is much more complex to assess the 50% nanomaterial fraction in the EU Commission Recommendation compared to the measurement of a classical chemical

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[18] The word “informally” should be noted; from a mathematical point of view it is *not* possible to associate an uncertainty with e.g. the centre value of a size bin

[19] *Representation of results of particle size analysis*. ISO 9276 part 1 to 6

concentration such as the concentration of mercury in a sample of earth. The measurement of the concentration of e.g. mercury involves only the estimation of one parameter that is the amount of mercury. However, when assessing the nanomaterial fraction the result of the measurement process is a set of two very different parameters which is a number of particles and the associated bin sizes. This imply that the estimation of the combined measurement uncertainty of the nanomaterial fraction also become much more complex as it must take into account both the uncertainty in the measured size and the measured numbers for each size bin.

To clarify the above issues about interpreting the measured particle sized distributions and estimation of the measurement uncertainties a simple example has been analysed at a level comparable to the level found in written international standards such as the ISO standards about the representation of results of particle size analysis[17].

2.1.5.2 Summary of example of determination of size fraction

The most simple and direct method to estimate a particle size distribution and the easiest method to understand is to directly measure the diameter of a number of particles and give the size distribution and size fraction *f* based on these measurements.

Below is a summary of the example developed and analysed in this project. The full example with all details and mathematical clarifications are given in **Appendix 1**. The example measures the size nanomaterial fraction *f* with an associated uncertainty. From images of the particles their size is measured according to the definitions and discussion given above in this chapter. All measured diameter is then sorted into the correct bin number and counted in the bin. However, there is an uncertainty on the measurement of the size and some particles will therefore not be put in the correct bin. This measured size and associated uncertainty can be described with a simple model function which takes into account the calibration of the measurement system. Similar for the counting of the particles it is never certain that all particles are counted correctly. Small particles may be difficult to identify and not counted in the correct bin. In the example the counting efficiency as function of the particle size and the associated uncertainty is described in a simple analytical model function. Finally only a (small) number of particles counted in the actual measurement process (perhaps less than ten thousand) are only a small selection of the large number of particles in the sample examined (which could perhaps consist of trillions of particles). If fewer particles are counted the uncertainty of the estimated size fraction *f* will increase. This selection and counting process can be described by associating each counted value in each bin with an associated uncertainty based on the counting process.

When calculating the uncertainty of a classical chemical concentration it is nearly always possible to make a simple analytical model function of the measurement process which describe the concentration as function of the measured quantities such as the measured mass, the mass of the matrix and other (possibly unwanted) influence parameters. Similar it would have been nice if it was possible from the measurement uncertainty of the particle size and the measurement uncertainty of the numbers of particles in each bin to calculate an approximate measurement uncertainty on the nanomaterial fraction based on a simple or intuitive analytical formula describing the nanomaterial fraction as function of the particle number, the bin size and the (small) number of particles actually counted. However, it is a finding of this report that this is *not* possible. The only possible assessment of the uncertainty of the nanomaterial fraction is based the more complex statistical simulation of the full measurement procedure described in the Appendix 2 as the “Monte Carlo procedure”.

One way to express the final result of the measurement in the example is as

(56 ± 6) % of the particles have a size between 1 nm and 100 nm

In this formulation of the result the measured value (here 56%) has an expanded measurement uncertainty of 6%. This means there is only a very small chance that the nanomaterial fraction *f* is smaller than 50% and the material examined is categorized as a nanomaterial.

2.1.5.3 Conclusion of the example

Appendix 1 and Appendix 2 deliver a complete example and calculation of the nanomaterial fraction including the associated measurement uncertainty, which can be used to determine if a material shall be classified as a nanomaterial at a certain confidence level. The calculation is based on a tailor-made excel template. *It is found* that such an analysis is necessary in any regulatory compliancy assessment to e.g. prove from a measurement of the particle size distribution that a material shall be categorized as a nanomaterial according to e.g. the EU definition of nanomaterial. The calculation and analysis is also necessary for the specification, understanding and interpretation of compliancy measurements in general.

2.2 Chemical characterization

Numerous guidelines exist for the validation of methods for chemical parameters in matrices. Some influential ones are the Eurachem Guide “Fitness for purpose of analytical methods”[20], the International Union for Pure and Applied Chemistry’s (IUPAC) report on single method validation[21] and the European Commission Decision on method validation for contaminants[22].

The chemical composition of a system can be characterised either qualitatively (which chemical species are present) or quantitatively (what is the concentration of each species). The system must be defined, thus for a material containing nanoparticles in a matrix, it is possible to talk either about the chemical composition of the nanoparticles, the chemical composition of the matrix or the chemical composition of the material (i.e. nanoparticles and matrix as a whole). Concentrations can be stated in absolute terms (e.g. mg/kg, mol/kg etc.) or as fractions (e.g. for a system containing species A, B and C, the fractions are A = 0.24, B = 0.45 and C = 0.31). Fractions always sum to 1 (or 100 %) and can be based either on mass or amount of substance (mole fractions) or volume.

There are many instrumental methods, which are used to determine quantity of different chemical species in a mixture. The methods can be consisting of a separation technique combined with a detection technique, e.g., mass spectroscopy (MS). Some a priori knowledge on the chemical identity of the species present is often needed to fully interpret the results. Examples of separation techniques are gas chromatography (GC), high performance liquid chromatography (HPLC) and inductively coupled plasma (ICP). The first two are used mostly for analysis of organic species whereas the last is used for elemental analysis, predominantly of metallic elements.

The report will focus on inorganic nanoparticles and inductively coupled plasma mass spectroscopy (ICP-MS) has been chosen as an example in the cases and in the discussion of the validation.

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[20] *The fitness for purpose of analytical methods – A laboratory guide to method validation and related topics*. D. Holcombe, (Ed.) (1998), Teddington (UK): LGC. ISBN 0-948926-12-0. <http://www.eurachem.org/guides/pdf/valid.pdf>

[21] *Harmonized guidelines for single laboratory validation of methods of analysis* (IUPAC technical report), M. Thompson, S. L. R. Ellison, and R. Wood, (2002), Pure and Applied Chemistry, 74, 835–855

[22] *Commission decision 2002/657/EC* of 12 August 2002 implementing council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, European Commission. (2002).

3. Describing the requirement to analytical results

In most cases European regulatory authorities will only accept test or calibration results from a lab that is accredited according to the international standard[23] describing the general requirements to testing laboratories which include chemical analysis. This international standard summarize the definition of validation as follow:

"Validation is the conformation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled."

The international standard requires that the procedure and results of the validation are *recorded*, and a statement as to whether the method is fit for the intended use.

This chapter starts by suggesting some generic validation parameters suitable for nanomaterial analysis and intended to validate *both* the particle properties and the chemical content. The chapter continue by clarifying the interpretation of the parameters with focus on the new parameters, that is, the parameters describing the particle size and the particle size distribution.

3.1 Generic validation parameters

This section *suggests* and outlines a generic approach for the validation of methods for detection and quantification of nanoparticles and nanoparticles in matrixes. The suggested specification of the measurand and the validation parameters are based on the scientific literature, relevant international standards and the conclusion from a resent EU project about methods for detection and characterisation of nanoparticles in food[24, 25]. The suggested validation parameters are summarized in specify what is measured. The interpretation of the items "specimen", "instrument type or method" and "Measurand: determination of" is clarified in **section 2.1.1** to **section 2.1.5**. Under the item "measurand" the physical or chemical quantity which is measured shall be specified with units. The measurand is the quantity intended to be measured.

Measurement conditions shall specify the range of independent variables necessary for the size measurements. The measurement of the particle size distribution in a suspension will often be possible only for a certain concentration range if the measurement uncertainty should be valid.

Selectivity: The selectivity is defined as[26] "the extent to which other substances interfere with the determination of a substance according to a given procedure"[27]. The performance of the selectivity should be documented for different samples of the same *matrix components* such as different textiles with no silver nanoparticles. If no particles are present in a sample ideally no particles should be detected. Particles of *different chemical species* should be validated if the

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[23] ISO/IEC 17025:2005 General requirements for the competence of testing and calibration laboratories

[24] European Union's Seventh Framework Programme (FP7/2007-2013) NanoLyse, *Nanoparticles in Food: Analytical methods for detection and characterisation*

[25] *Validation of methods for the detection and quantification of engineered nanoparticles in food*, T.P.J. Linsinger et al. Food Chemistry 138 (2013) page 1959–1966

[26] *IUPAC compendium of chemical terminology* (2nd ed.) Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997). XML on-line corrected version: http:// goldbook.iupac.org created by M. Nic, J. Jirat, B. Kosata; updates compiled by A. Jenkins. ISBN 0-9678550-9-8. doi:10.1351/goldbook.

[27] Selectivity of a measuring system is also defined in section 4.13 in *International vocabulary of metrology -- Basic and general concepts and associated terms (VIM)* ISO/IEC Guide 99:2007

method can select between them. The selectivity for *chemical equivalent particles*, but from different producers or with e.g. different shape or surface properties, should be determined.

The limits of detection (LOD) and limit of quantification (LOQ) can be calculated relative to a blank sample, that is, by multiplying the standard uncertainty of blank with a reasonable factor. This holds for both the concentration chemical substance, the number of particles and the size. A blank sample is a sample with no sampled particulate material.

To determine the **working range** the particle size range for which the method is applicable must be established.

Precision is a description of the level of the deviation of measurement results when repeated under specified conditions such as the same sample and lab, but different operators and different instruments[28]. The sampled used could be a sample spiked with relevant nanoparticles, that is, a known amount of nanoparticles have been added to the sample.

(Metrological) Traceability is the property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations [6]. In general traceability can be achieved from certified reference materials (preferred), reference material,

Specification of measurand	
Specimen	- Aerosol, granulate or suspension
Instrument Type or Method	- SEM, TEM, AFM, SMPS, DLS, ISO, CEN, DIN, EC, ASTM
Measurand	- Chemical species - Average diameter, minimum external dimension, number size, volume fraction, counting, ensample*
Measurement conditions or independent variables	- Concentration range, possible aggregation and agglomerations level

Validation parameter	
Selectivity	- Matrix components - Particles of different chemical species - Chemically equivalent particles from different producers or batches
Limit of detection	- Mass/number fraction - Particle size
Limit of quantification	- Mass/number fraction - Particle size
Working range/linearity	- Mass/number fraction range -Particle size range
Precision (repeatability, intermediate precision)	- Mass/number fraction - Particle size
Traceability	- Chemical identity - Mass/number fraction - Particle size
Measurement uncertainty	- Mass/number fraction - Particle size

TABLE 1
MODIFIED AND SIMPLIFIED FROM REFERENCE [33]. ALL TERMS ARE EXPLAINED IN THE TEXT.
*METHODS, WHICH DETECT AND ANALYSE A COLLECTIVE SIGNAL SUCH AS LIGHT REFLECTED OR SCATTERED FROM ALL PARTICLES IN THE SAMPLED VOLUME – SEE SECTION 3.3.

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[28] Defined in section 2.15 in *International vocabulary of metrology -- Basic and general concepts and associated terms (VIM)* ISO/IEC Guide 99:2007

reference measurement procedures, or by spiked samples which is acceptable if reference material is not available.

Uncertainty[29]: The evaluation of the measurement uncertainty is dependent on the specification of the measurand and the reported measurement result. The combined measurement uncertainty can in a simple approach include the combination of the repeatability standard deviation, the uncertainty from different operators, different instruments, long separation in time, different calibration and the uncertainty of systematic errors.

Reporting the result
The test report should include all relevant information for the interpretation of the special issues regarding nanoparticles including – if relevant - sample preparation, methods for extraction of the particles, handling, transportation, storing and preparation, state of agglomeration/aggregation, impurities.

3.2 Validation of counting /imaging methods

In this section the interpretation of some of the validation parameters are clarified and interpreted for the counting type instruments which are the main physical measurement method this report describes.

The report focuses on the new aspect of nanomaterial measurements, that is, the sizing and counting of nanoparticles. The report suggest the counting and sizing from images from scanning electron and transmission electron microscopy (SEM and TEM) as methods which could potentially give the number of particles as function of the diameter and the associated measurement uncertainty. Scanning mobility particle sizing (SMPS) is a standard method to measure airborne particle size[30] distributions. It is not a counting method in the same sense as SEM and TEM but some of the principles might be used in a simplified analysis of scanning mobility particle sizing.

3.2.1 Specimen

The particulate material can be present as aerosol, as a granulate or in a suspension

3.2.2 Instrument type or method

Typical the method can be identified by the name of the measuring instrument such as SEM, TEM and scanning mobility particle sizer (SMPS). Standard methods such as ISO, CEN, DIN, EC, ASTM should be stated and used if possible.

3.2.3 Measurand

For the measurement of the *particle size distribution* by a counting methods the measurand can probably be expressed as

counts Q_k in diameter size bins number $1 < k < k_{\max}$

where: $k_{\max}$ is the total number of bins.

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[29] The measurement uncertainty is defined as a "non-negative parameter characterizing the dispersion of the quantity values being attributed to the measurand, based on the information used" according to section 2.26 in *International vocabulary of metrology -- Basic and general concepts and associated terms (VIM)* ISO/IEC Guide 99:2007

[30] The (electrical) mobility (equivalent) diameter is the diameter of a sphere carrying a single elementary charge with the same drift speed in an electrical field as the particle under prevailing conditions of temperature and pressure

The size measured should be specified e.g. as the average external dimension, the smallest external dimension, the average diameter from a circle having the same area as a 2D projection of the particle or a diameter calculated from the average volume.

For the measurement of the nanomaterial fraction f the result can be expressed as

$$\boldsymbol{f \pm U(f)\ \%}$$

where f it is the number size distribution in % of particles with diameters x in the range from 1 nm to 100 nm relative to the number of particles in the range from 1 nm to X_{MAX} nm and $U(f)$ is the associated expanded uncertainty at a confidence level of e.g. 95%. The maximum diameter X_{MAX} with 1 µm << X_{MAX} << 100 µm should also be specified.

3.2.4 Measurement conditions for size measurements

Any additional measurement condition should be stated. Additional measurement condition could be the concentration range of the particulate material in the suspension, the possible material type of the particles material such a heavy ions or organic material and for example that the particulate material has to be free of aggregation and agglomerations.

3.2.4.1 Counting efficiency and selectivity

In some simple cases the performance characteristic of the quantification of the particle number in presence of an interference can be approximated by the counting efficiency. Some particles with indicated diameter I_i are not detected, measured and counted in the bin number k, even if $b_{k-1} < I_i \leq b_k$. This can be described by a *counting efficiency* $e(x)$ defined as the probability that a particle of diameter x is detected by the counting and the associated uncertainty $u\big(e(x)\big)$. For an SMPS a particle will not be counted if it is not charged correctly and not all particles are charged correctly leading to a counting efficiency $e(x) < 1$ with an associated standard uncertainty $u(e(x))$ due to e.g. random variations in the charging process.

3.2.5 Number size distribution threshold X_{MAX}

For the measurement of the number size distribution f of particles with diameters x in the range from 1 nm to 100 nm relative to the number of particles in the range from 1 nm to X_{MAX} nm the maximum diameter 1 µm << X_{MAX} << 100 µm should be specified.

3.2.6 Working range

The minimum and maximum diameter

$$x_{\mathrm{min}} < x < x_{\mathrm{max}}$$

between which the particle diameter x will be counted shall be specified as diameter x, $x_{\mathrm{min}} < x < x_{\mathrm{max}}$.

A key parameter for the quality of the measurement is the magnitude of this uncertainty and it is recommended that the validation of the measurement method specifies the following

$$\min u(x) \cong u(x_{min})$$
$$\max u(x) \cong u(x_{max})$$

where $\min u(x)$ is the minimum value of $u(x), x_{min} < x < x_{max}$, $\max u(x)$ is the maximum value of $u(x), x_{min} < x < x_{max}$, $u(x_{min})$ is the uncertainty associated with the diameter x_{min} and $u(x_{max})$ is the uncertainty associated with the diameter x_{max}

3.2.7 Measurement uncertainty

The uncertainty $u(x)$ shall be stated for all diameter x in the range $x_{\mathrm{min}} < x < x_{\mathrm{max}}$ and is related to the confidence of centre value of each diameter size bin.

Nanomaterial number size distribution fraction, f: It is not possible in general to state meaningful limits for the associated standard uncertainty $u(f)$ as it is difficult to identify the particle size distribution which will give the largest standard uncertainty $u(f)$. For regulatory purpose, it is recommended to specify the maximum acceptable measurement uncertainty level of the fraction f in the range from 5 to 20 %.

Diameter: The lab shall as part of the validation estimate the uncertainty associated with the measured diameter $u(x)$ in the range $x_{\min} < x < x_{\max}$ as the confidence related to the centre value of each diameter size bin. For SEM and TEM the uncertainty associated with the measured diameter $u(x)$ could include random variation due to image interpretation and the scaling or calibration of the images. For SMPS the measured diameter $u(x)$ is influenced by e.g. the accuracy of the voltage generation to bend the motion of the particles in the laminar airflow.

Counting efficiency: For SEM and TEM some particles might be overlooked leading to a counting efficiency $e(x) < 1$ with an associated standard uncertainty $u(e(x))$ due to random variations in the measuring process. Furthermore as counting in general only estimate the number of counts Q_k in bin number k based on a small fraction of the total number of particles in the potential nanomaterial to be investigated. Thus there is also a standard uncertainty $u(Q_k)$ associated with the best estimate Q_k for the counts falling in bin number k
A key parameter for the quality of the measurement is the magnitude of this uncertainty relative to the number of counts Q_k, that is

$$u(Q_k)/Q_k$$

It is recommended that the validation of the measurement method specify the relative uncertainty of the counting efficient $u(Q_k)/Q_k$ as part of the measurement capability.

3.2.8 Conclusion

Based on the mathematical clarification of the size distribution (section 2.1.5.2) and the above interpretation of the validation parameters it is concluded that the suggested validation parameters can be used to examine and give evidence if requirements are fulfilled for imaging method such as TEM and SEM.. The validation of SMPS can to some extend also follow some of the described approach.

3.3 Validation of sizing methods not based on counting/imaging

Many sizing methods are not based on counting and imaging. Ensample methods are in this report DLS - Dynamic Light Scattering and e.g. SAXS - Small-Angle X-ray Scattering. The size distributions deduced from such measurement does not have a real "counting efficiency" equivalent to the relative number fraction as function of the particle diameter but rely on – often complicated - convolution steps in an analysis algorithm – see section 2.1.2. It will thus be very difficult to determine the uncertainty on a broad particle size distribution using the approach outlined in section 2.1.5.1. A third category of methods for measuring particle size is fractionation methods which separate the sample into monodisperse fractions prior to sizing with other methods. This is a potential accurate method but they are complex and it will be very difficult to determine the uncertainty on a broad particle size distribution using the approach outlined in section 2.1.5.1.

3.4 Validation of chemical analysis

In general, it is currently in many cases not possible to carry out a full validation of a method for chemical analysis of nanoparticles and the reasons are described in this section. In this report only inductively coupled plasma mass spectrometry (ICP-MS) is described in details, see **section 4.1**. It is recommended[31] that the validation parameters for methods for chemical analysis include limit of detection, limit of quantification, linearity/working range, trueness/recovery, precision,

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[31] *Validation of Analytical Procedures: Text and Methodology* ICH Guidelines, Q2(R1)

selectivity and ruggedness.[32,33]. According to section 3.1 each “result” must include information about the chemical identity, particle size and mass or particle number concentration. However, in general it is not possible to identify the chemical composition of each nanoparticle together with its actual size and mass. The reason for this is discussed below and guidelines for the validation are clarified.

Carrying out a full validation of a chemical analytical method requires reference material or the possibility to use the actual nanoparticles from the manufacturers to spike the samples. Development of reference material is neither feasible nor affordable and only few are commercially available. Therefore, spiked samples must be used for several parts of the validation studies if it is possible to obtain the relevant nanoparticles. It is also important to change the nanoparticles as little as possible during the preparation of the spiked validation test material.[33]

Microscopic methods such as electron microscopy can in principle determine the basic chemical composition of each nanoparticle with sizes larger than approximately 1 micro metre, but only at the level of elements (not molecules etc.)[33]. The chemical composition of nanoparticles can in general not be determined. ICP-MS can measure the total content of an element, e.g., silver after digestion with acid from the samples. The mass fraction of, e.g., silver nanoparticles can never be larger than the total mass fraction of silver in a given sample.

Nanoparticles are by definition particles, and their discrete, particulate nature could cause problems when sampling, because only a small fraction of the analytical portion is studied.[33] There could also be a problem about sampling depending on how the particles is distributed in the matrix, e.g. in a liquid it may be homogeneous, but in a textile it may not. Many subsamples therefore can be required to ensure the correct results.

A second potential problem is that nanoparticles and matrix may undergo changes during sample preparation and final quantification. The changes can causes such as agglomeration, disagglomeration or dissolution. One example could be the digesting of a sample before analysing by ICP-MS, where any nanoparticles and the matrix will be destroyed to their elements, and therefore it is not possible to determine if any elements is derived from the nanoparticles or is from the matrix. Another example could be the isolation of nanoparticles from a complex matrix, e.g., sunscreen, with different kind of solvents that can cause changes in the organic coating of the nanoparticles. The method validation therefore needs to demonstrate integrity of the particles throughout the analytical process. [33] Agglomeration and aggregation is discussed further in section 5.3.

A third issue, which is unique for nanoparticles, is that particles of the same chemical identity and size may exist in different stabilising agents. This is a distinct difference to classical analytical chemistry. Properties of nanoparticles may also vary from manufacturer to manufacturer and even from batch to batch. Therefore, it must be ensured that methods respond equally to nanoparticles coming from different manufacturers and production processes; otherwise, a precise identification of the source of the nanoparticle is necessary before a quantitative result can be produced.[33]

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[32] *Chemical analysis, requirements to documentation of the method*, Danish Environmental Protection Agency

[33] *Validation of methods for the detection and quantification of engineered nanoparticles in food* T.P.J Linsinger et al. Food Chemistry 138 (2013) 1959-1966,.

4. Methods for analysis of nanomaterials

No single particle sizing method is fully validated and covers the full range of diameters necessary from 1 nm to several micrometres. The focus in this report will be counting methods as they are conceptual most easy to understand and interpret and therefore more suited for a validation. It is also noted that counting methods were the core methods used by e.g. NIST for the certification of gold particles with nominal diameter in the range from 10 to 60 nm in their well accepted reference material RM 8013[34].

4.1 Physical methods

4.1.1 Electron microscopy (TEM, SEM)

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are important method for the cases. SEM examine in its basis configuration images obtained by generating a focused electron beams scanning the surface of the sample in order to determine the structure. Additional information about the chemical composition can be obtained. TEM is a very high resolution microscopy method that produces images or diffraction patterns of a thin sample by a focused electron beam which passes through the sample and interacts with it. SEM and TEM trace back to 1937 and much literature describe their applications.

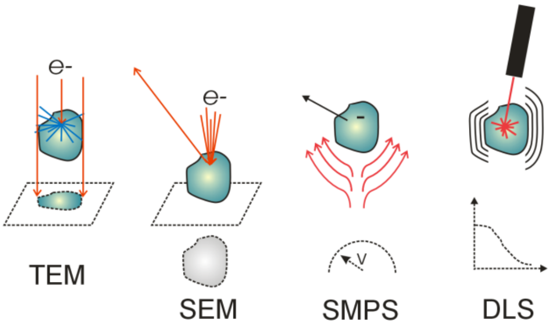

FIGURE 4
THE DIFFERENT PHYSICLA MEASUREMENT METHODS. TRANSMISSION ELECTRON MICROSCOPY (TEM), SCANNING ELECTRON MICROSCOPU (SEM), SCANNING MOBILITY PARTICLE SIZER (SMPS) AND DYNAMIC LIGHT SCATTERING (DLS)

4.1.1.1 Technical details – TEM, SEM

4.1.1.2 Validation parameters – SEM

Instrument Type or Methods: SEM, analysis methods for dedusing the shape from the image is described in an ISO standard[35]

Specimen: Granulate

The measurand. Size-specific number concentration

Measurement conditions: less than 1µg. Particles must be prepared ideally as a monolayer and be stable under an electron beam and a (high) vacuum

FIGURE 5
EXAMPLE OF SEM MICROSCOPE

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[34] *Report of Investigation, Reference Material 8013, Gold Nanoparticles, Nominal 60 nm Diameter*, NIST - National Institute of Standards & Technology, Gaithersburg, MD 20899, Robert L. Watters, Jr., Chief, Report Issue Date: 13 December 2007

[35] *Particle size analysis - Image analysis methods - Static image analysis methods* DS/ISO 13322-1:2014 which give guidance for a measurement description and its validation when determining particle size by image analysis

Selectivity:

Limits of quantification and detection: 5 nm

Limit of detection, quantification and working range: Typical 1 nm to 6 µm. The exact limits depend on material and must be validated.

Working range: 10 nm to several µm

Precision: It is possible to perform a minimum of six analyses on the same sample to reveal the instrument repeatability uncertainty, as the analysis is non-destructive

Traceability: To e.g. monodisperse gold nanoparticles with diameter from 10 nm to 60 nm.. ISO 16700:2004[36] specifies a method for calibrating the magnification of images generated by a scanning electron microscope (SEM) using an appropriate reference material but is not tailor-made to critical dimension such as the diameter of a nanoparticles. Reference materials are available in terms of gratings.

Measurement uncertainty: As for TEM – see below.

Additional remarks: None

4.1.1.3 Validation parameters – TEM

Instrument Type or Methods: TEM
Specimen: Granulate

The measurand: Size-specific number concentration, projected area of particles

Measurement conditions: less than 1µg. Particles must be prepared ideally as a monolayer adhering to a reference surface (grid) and be stable under an electron beam and a (high) vacuum

Selectivity: Can to some extend identify different chemical specimens. Resent results in [37] suggest that a careful sample preparation protocol using active deposition of gold nanoparticles by ultracentrifugation enables a recovery in the range 80–100% from a suspensions in water within the concentration range of ca. 0.2–30 µg L^{-1}.

Limits of quantification and detection: 1 nm

Working range: Larger than 1 nm

Precision: It is possible to perform a minimum of six analyses on the same sample to reveal the instrument repeatability uncertainty, as the analysis is non-destructive

Traceability: To e.g. monodisperse gold nanoparticles with diameter from 10 nm to 60 nm. [38]. ISO 29301:2010[39] specifies calibration procedure applicable to images recorded over a wide

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[36] *Microbeam analysis -- Scanning electron microscopy -- Guidelines for calibrating image magnification* ISO 16700:2004

[37] *An electron microscopy based method for the detection and quantification of nanomaterial number concentration in environmentally relevant media* A. Prasad , J.R. Leada, M. Baalousha Science of the Total Environment 537 (2015) 479–486

[38] •RM 8011, Gold Nanoparticles, Nominal 10 nm Diameter, •RM 8012, Gold Nanoparticles, Nominal 30 nm Diameter and •RM 8013, Gold Nanoparticles, Nominal 60 nm Diameter, available from NIST

[39] *Microbeam analysis -- Analytical transmission electron microscopy -- Methods for calibrating image magnification by using reference materials having periodic structures* ISO 29301:2010

magnification range in a TEM but does not apply to the dedicated critical dimension measurement. Reference materials are available in terms of scales and crystals.

Measurement uncertainty: Major uncertainty come from the magnetic hysteresis of the electro-magnetic lenses [39] and lack of knowledge about the how even the distribution of the nanoparticles is. It is unlikely that small and large particle have even chance for sticking to the substrate[13].

Additional remarks: None

4.1.2 Scanning Mobility Particle Sizer (SMPS)

Many systems for determining size distributions in the submicron range are based on mobility analysis or impaction. The most commonly used on-line state-of-the-art techniques are the scanning mobility particle sizer[40] and the electrical low pressure impactor[41].

Mobility analysis allows a very good size resolution; one scan covers about two orders of magnitude in size from a few nm to about 700 nm depending on the choice of instruments and flow rates. SMPS is an important method for the case about release of nanoparticles from crayon.

4.1.3 Technical details - SMPS

The SMPS (Scanning Mobility Particle Sizer) can select particles according to their size (mobility diameter). The particles are counted in a CPC (Condensation Particle Counter) and therefore it is possible to determine both the number concentration (number/cm^3) and the size distribution (dN/dlogDp) of the particles. The instrument is commercially available.

When relevant the international standard about determination of particle size distribution with differential electrical mobility analysis for aerosol particles should be used[42]

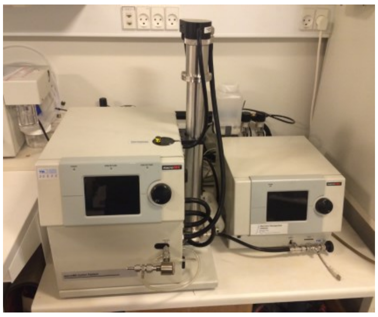

FIGURE 6
SCANNING MOBILITY PARTICLES SOZER (SMPS)

It is possible to insert dilution equipment (e.g., rotating disc diluter) before the SMPS, allowing measurements to be performed on high-emission sources (e.g., smoke from wood-burning stoves or engine emissions).

Size (DMA): The measurement principle for SMPS is based on physical laws. A SMPS measurement will show the particle sizes (mobility diameter) depending on the voltage and airflow in the instrument. A calibration of the instrument will be closer to a *validation* of the instrument's measurement precision. In praxis, this means that a specific voltage on the DMA in connection with a specific airflow (in theory) will allow a specific particle size to be selected in the DMA.

Counting (CPC): The counting of the particles takes place in the CPC. Calibration of the CPC is carried out by separate calibration of the smallest detectable particle size, counting efficiency and linearity of concentrations, respectively[43].

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[40] SMPS; Wang and Flagan, 1990

[41] ELPI; Keskinen et al., 1992

[42] *Determination of particle size distribution -- Differential electrical mobility analysis for aerosol particles*, ISO 15900:2009

[43] PowerPoint from TSI

In their guidelines, the Danish Environmental Protection Agency suggests that the method robustness (precision) should be tested by performing a sixfold determination on a single sample. In many cases, it may be possible to perform a sixfold determination on a single sample, but depending on the sample, it is estimated that the variation found will be a reflection of the natural variation of the particle emission from the sample and not the precision of the instrument itself. Therefore, it is assessed that the calibration of the instrument can substitute the precision measurement proposed.

4.1.4 Validation parameters - SMPS

Specimen: Aerosols

Measurand: Particle size (mobility diameter) and particle number concentration. The results are given as the number of particles/cm^3. In addition, measurement data can be graphically displayed as number of measured particles/cm^3 (dN/dlogDp) as a function of time.

Selectivity: All particles are counted with SMPS and no chemical information is obtained. It is possible to add equipment (catalytic stripper or thermodenuder) in front of the SMPS, which removes volatile and semi-volatile compounds, with the solid particle fraction left. However, it should be noted that diffusion losses in the system introduce a bias in the data, which subsequently must be corrected. This is particularly relevant for the smallest particles.
Limits of quantification and detection: 2.5 to 1,000 nanometre (mobility diameter) and ~10^2 to 10^7 particles/cm^3 distributed in up to 167 size channels.

Working range: For instance 2.5 to 1,000 nm (mobility diameter), with the specific size range depending on instrument, airflow and time resolution. The particles are size fractionated in several size channels, depending on instrument, e.g., 167 size channels.

The concentration range of the SMPS can be from, e.g., ~10^2 to 10^7 particles/cm^3, depending on the instrument. Dilution equipment can extend this range. Using the CPC alone can result in a concentration range of, e.g., 0 - $3\text{x}10^5$ particles/cm^3 (no size information).

Precision: *Repeatability:* Not available. Depends on a stable particle flow. *Zero measurement:* A zero measurement is performed at the start-up of the instrument to ensure that the system is sealed (using HEPA filter). *Background measurement:* A background measurement can be performed on the air in the room ("blind") before the actual measurement is initiated, if referring to a specific emission source. Moreover, a background measurement can be performed at the end of the measurement, to ensure that the measurement can be corrected for the background level of particles from the environment, which is of special relevance for low emission sources. Failure to do so could lead to a relatively large bias in the results.

Traceability: Suggested reference material: For instance polystyrene latex (PSL) of relevant sizes. *Calibration of equipment:* There are two areas that should be taken into account when calibrating the instrument: Size separation and counting.

- The instrument is calibrated/validated by following the manufacturer's instructions, by which the measurement range and recovery/correctness are verified. Internal comparisons (ring calibration) of different relevant particle measurement equipment can be performed at suitable intervals.

- Instead of using ring calibration or letting the manufacturer calibrate the instrument, it is possible to calibrate/validate the instrument by using, e.g., an electrospray aerosol generator. In this type of calibration/validation, e.g., spherical polystyrene latex (PSL) particles (with a well-defined particle size) are sprayed and the size of the generated particles is verified with SMPS.

The instrument and the measurement method are well-documented and described in the peer reviewed scientific literature and international standards, and they are also widely recognized in the particle industry. The method is often used as reference method for other particle measurement techniques.

Measurement uncertainty: Measurement setup, procedure and fluctuations in, e.g., background level will almost always have the highest influence on the measurement uncertainty.

Additional remarks: Nanoparticles are defined as particles less than 100 nm in all three dimensions, and due to the high resolution in respect to size in the SMPS it is possible to obtain a separation of particles above and below 100 nm (mobility diameter), respectively. *Limitations:* The method cannot distinguish particles of different types, such as volatile, semi-volatile or solid particles. For instance, one titanium-dioxide based particle and one carbon based particle will both be counted and so will a drop of oil. Therefore, three particles will be counted in this scenario.

4.2 Dynamic light scattering (DLS)

DLS will bean important method for the case about nanomaterial with different coatings.

4.2.1 Technical details - DLS

Dynamic light scattering (DLS) can be used to determine the size distribution of nanoparticles in a liquid medium. Typically, a few mL of the liquid sample is required, depending on the particle concentration. The measurement principle is based on ultra-fine particles in a liquid medium always being in motion as the fluid "nudges" the particles (Brownian motion, the Stokes-Einstein equation: $d(H) = kT/(3\pi\eta D)$, where d(H) is the hydrodynamic diameter, D is the translational diffusion coefficient, k is Boltzmann's constant, T is the absolute temperature and η the viscosity).

4.2.2 Validation parameters - DLS

This section will interpret the validation parameters and suggest some reasonable values. International standard about particle size analysis with photon correlation spectroscopy [44] and dynamic light scattering[45] should be followed. Additional description and discussion is given in **Appendix 3** *Additional discussion of DLS*
Instrument Type or Methods*:* ISO 22412:2008[45]

Specimen: Suspension of particles

FIGURE 7
EXAMPLE OF INSTRUMENTATION TO MEASURE DYNAMIC LIGHT SCATTERING (DLS)

The measurand. Measurement of particle size distribution in fluid represented as a table. (Mass and concentration *cannot* be determined.) The measured diameter is the hydrodynamic diameter which is an effective hydrated diameter in solution. The hydrodynamic diameter gives information of the particle diameter and the solvent layer attached to the particle as it moves under the influence of Brownian motion.

Measurement conditions: The measurement of the size distribution requires that the concentration of particles is within certain limits.

Selectivity: The instrument cannot differentiate between different materials and it only measures the particle size distribution (PSD).

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[44] *Photon correlation spectroscopy* ISO 13321:1996 Particle size analysis
[45] *Particle size analysis - Dynamic light scattering (DLS)* ISO 22412:2008

Limit of detection, quantification and working range: Typical 1 nm to 6 µm (hydrodynamic diameter). The exact limits depend on the material and must be validated. Influence parameters to be examined include e.g. the particles optical properties such as refractive index and absorption (imaginary refractive index) and the solutions refractive index influences the measurements quality.

Precision: It is possible to perform a minimum of six analyses on the same sample to reveal the instrument repeatability uncertainty, as the analysis is non-destructive. The uncertainty must be within ± 5%[45] for monodisperse particle size distributions.

Traceability: The measured particle size distribution is based on modelling of the recorded light signal and can in principle not be calibrated. It is possible to *validate* that the instrument measures correctly within the given permitted limit of e.g. a PSL standard from NIST with a deviation no more than ±2% between the individual measurements of monodisperse particle size distributions. If the instrument does not, it is necessary that the supplier carries out service on the instrument.

Measurement uncertainty: The uncertainty for monodisperse particle size distributions must be within ±5%. It is not possible to state measurement uncertainties for polydisperse particle size distributions, as the instrument cannot measure these distributions.

Additional remarks: *Limitations:* Too low sample concentration. Too high sample concentration (it is possible to measure on diluted sample). It is necessary that the sample can be suspended (in either water or other organic fluid). Sample refractive index (RI) to close to fluid RI disturbs the measurement. Too high imaginary refractive index (IRI) influences the measurement

4.3 Chemical methods

4.3.1 Inductively coupled plasma mass spectrometry (ICP-MS)

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) is an important and well accepted method to measure small amounts of matter relevant in many cases involving nanoparticles and the ICP-MS is used in the case about silver nanoparticles in socks in **section 5.1**.

4.3.1.1 Technical details - ICP-MS, nitric acid destruction

ICP-MS is a mass spectrometry, which is capable of detecting metals and several non-metals. That is achieved by ionizing the sample with inductively coupled plasma and then using a mass spectrometer to separate and quantify the ions. The method is well documented for determination of trace elements in water and wastewater (EPA method 200.8).[46]

The sample is digested with acids and introduced via a sample introduction system to plasma, having a temperature of 5000 - 9000 ° C. This produces free atoms and free ions. The free atoms in the exhaust air are sucked out, while the free ions are passed through the interface to the mass spectrometer where the ions separated according to m/z ratios. In the quadruple, the ion current is passed on to the detector in which ions are converted into electrical pulses, which are proportional to the concentration of the metal in the given sample. An ICP-MS can analyse the majority of elements in the periodic

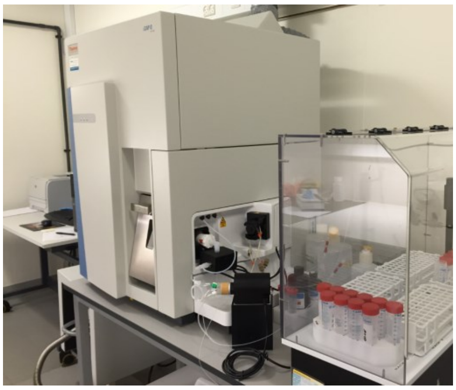

TABLE 2
INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS)

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[46] *Determination of trace elements in waters and wastes by inductively coupled plasma - mass spectrometry*, EPA method 200.8

table, with the following general exceptions: H, He, C , N, O , F, Ne, Ar, Kr. [47]

An example is to determine the total content of silver, including the content of silver nanoparticles.[49] All of the requirements of the Danish Environmental Protection Agency regarding documentation of the method for determination of total content of silver in textiles was fulfilled by carrying out the necessary analyses for determination of precision, blank value, linearity, working range and detection limit.[32] The expanded uncertainty of the method was calculated on the basis of the performed analyses. An inhomogeneity contribution coming from a possible irregular distribution of the silver in the textile sample should be included. The contribution was determined by separate measurements on an appropriate number of samples of the textile containing silver.

4.3.1.2 Validation parameters – ICP-MS, nitric acid destruction

Instrument Type or Method: EPA Method 200.8

Measurand: Total determination of the amount of the element, e.g. silver. Amount of elements e.g., silver per g or kg textile.

Measurement conditions for sizing: Not relevant

Selectivity: With collision cell, ICP-MS is selective for detection of most elements.

Limits of quantification and detection: 0,5 ng/mL in the digested sample corresponding to 0,5 ng/g solid sample. Determined from blanks and low standards. *Zero measurement*: Blank samples are analysed. *Background measurement:* Blank samples are analysed.

Working range: 0,5 ng/mL- 250 ng/mL in the digested sample. Linearity during calibration is determined with 6 measuring points of the relevant elements, e.g., $r^2 > 0.995$.

Precision: Repeatability: Std. dev. on 6 repeated analyses, e.g., < 10%

Traceability: Suggested reference material: E.g., NIST RM 8017 in the case of silver nanoparticles. Calibration of equipment: Carried out as part of the analysis.

Measurement uncertainty: Calculated from repeatability measurements and measurements on reference material, e.g. < 15% relative.

Additional remarks: Measures the element, e.g., cannot determine if the silver is silver nanoparticles. If the amount of silver nanoparticles is to be determined, the particles must be extracted from the textile before the analysis. If the elements are inhomogeneously distributed in the sample, e.g., textile, determinations must be carried out on at least 5 sub samples.

4.3.2 Single particle ICP-MS

In a special installation of the ICP-MS instrument the amount and mass distribution of nanoparticles e.g. AgNP can be determined in a liquid sample. The mass distribution can be converted to a particle size distribution. A brief description appears in: *"ICP-MS: a promising tool in the detection and analysis of nanoparticles"*[48]. The field flow fractionation (FFF) is a fractionation or separation technique. It separates particles based on their hydrodynamic size and the separation process is similar to chromatography, except that the separation is based on

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[47] *Analyse teknik, Instrumentering og metoder*, Helle Jeppesen, Merete Norsker Bergsøe, Flemming Simonsen, Nyt Teknisk Forlag, 4. udgave

[48] http://www.afsca.be/laboratories/labinfo/_documents/2014_02_labinfo11_p18_en.pdf
ICP-MS: a promising tool in the detection and analysis of nanoparticles. Retrieved December 2015

physical forces as opposed to chemical interaction. A lower limit of 10 – 20 nm is stated for the particles that can be determined. The limit originates from analyses at a Thermo X-series 2 instrument. Thermo iCAPc instrument is of a newer generation and might be able to detect 5 – 10 nm AgNP.

In order to analyse AgNP in textiles they must be extracted from the textile above an aqueous solution. The method description must be prepared and in the method documentation that comprises all the previously mentioned elements the inhomogeneity contribution can be omitted by extracting the entire textile, but a contribution from the extraction efficiency must be included. Textiles with a known content of AgNP are necessary for determination hereof.

4.3.2.1 Validation parameters – Single particle ICP-MS

Reference method: None. The method is described in articles in chemical scientific journals.

Instrument type or method: Determination of number of particles and mass distribution of elements in particles.

Measurand: Number and mass distribution. Size distribution is calculated.

Selectivity: ICP-MS with collision cell is selective for detection of silver

Limits of quantification and detection: Size: 10 nm, 50 particles. *Zero measurement*: Blank samples are analysed. *Background measurement*: Blank samples are analysed.

Working range: 10 – 1000 nm, In principle, few particles can be determined, but at least 50 are necessary to obtain a distribution.

Precision: Repeatability: Std. dev. on 6 repeated analyses < 15% rel.

Traceability: Suggested reference material: Textile with known content of silver nanoparticles. *Calibration of equipment:* Carried out as part of the analysis.

Measurement uncertainty: < 20% rel. Calculated from repeatability measurements and measurements on reference material.

Additional remarks: 1) The measurement takes place on liquid sample, and therefore silver particles must be extracted from the textile. The extraction efficiency is determined by means of the reference material that must be representative of the textiles to be measured. 2) When calculating the size distribution on the basis of the mass distribution, it is necessary to know/anticipate the particle geometry. If the silver particles are inhomogeneously distributed in the textile, determinations must be carried out on at least 5 sub samples of the textile.

5. Measurement of nanoparticles in four cases

The overall objective of the report is to set up requirements for the validation parameters of measurement methods to ensure reliable results of e.g. toxicological assessment in actual cases. In this chapter four realistic cases are defined and discussed. They cover two generic issues which are concentration of nanoparticles in a consumer product (nanoparticle of silver) and concentration of nanoparticles in air and two common but very difficult cases which are handling of agglomeration and assessing nanoparticles which are coated.

TEM: Specification of measurand	
Specimen	- granulate < 1 µg
Instrument Type or Method	- TEM, tailor- made procedure, - Image analysis methods DS/ISO 13322-1:2014 - If relevant ISO 9276 1-6 for representation of the size results shall be followed
Measurand	- Size-specific number concentration - projected area of particles
Measurement conditions or independent variables for size measurements	- less than 1 µg. Particles must be prepared ideally as a monolayer

TEM; Validation parameter	
Selectivity	- Matrix components: - Particles of different chemical species: Can to some extend identify different chemical specimens. - Chemically equivalent particles from different producers or batches
Limit of detection: smallest detectable	- Mass/number fraction: Larger than 1 nm - Particle size: Larger than 1 nm
Limit of quantification:	- Mass/number fraction: Larger than 1 nm - Particle size: Larger than 1 nm
Working range/linearity	- Mass/number fraction range -Particle size range
Precision (repeatability, intermediate precision)	- Perform a minimum of six analyses
Traceability	- Mass/number fraction: Certified monodisperse gold nanoparticles with diameter from 10 nm to 60 nm - published procedure from NIST - silver nanoparticle BAM-N001 CRM - Particle size: method for calibrating the magnification ISO 16700:2004
Measurement uncertainty	- Mass/number fraction - Particle size: Uneven distribution of the nanoparticles

TABLE 3
SUMMARY OF VALIDATION PARAMETERS FOR PARTICLE PROPERTIES

5.1 Silver nanoparticles in socks

Silver is well known for its antiseptic qualities, attributed to the surface oxidation of metallic silver followed by the generation of silver ions, which can be toxic to many bacteria and fungi. This quality has allegedly led to the incorporation of silver nanoparticles into textiles as an anti-microbial agent. Another possibility is incorporation of AgCl nanoparticles. Their small size and large surface area to volume ratio results in increased rates of oxidation and subsequent dissolution compared to larger-scale forms of silver. However, the usage of silver nanoparticles has led to concerns regarding risk for the environment and this concern is the basis for a recent report from EAP [49]

In order to address the above concern a fictitious, but realistic, investigation is done to find the content of silver nanoparticles in the textile cotton from some socks. The investigation shall elucidate if nanoparticles are present based on representative samples collected from a batch of socks. This could involve taking small parts from different positions on different socks. The sampling is important and needs to be done in a well-designed and controlled manner by trained and approved personnel.

Specification of measurand	
Specimen	Textile with silver nanoparticles
Instrument Type or Method	ICP-MS, EPA Method 200.8
Measurand	Total content of silver
Measurement conditions or independent variables for size measurements	If the amount of silver nanoparticles is to be determined, the particles must be extracted from the textile before the analysis

Validation parameter	
Selectivity	ICP-MS can determine the total content of silver in the sample, but it cannot determine if the silver is silver nanoparticles
Limit of detection	Determined from blanks and a low standard of silver
Limit of quantification	Determined from the lowest standard of silver in the working range: 0,5 ng/mL in the digested Using sample size and volume of nitric acid this corresponding to: 0,5 ng/g textile sample
Working range/linearity	Determined from at least 5 standards with silver in the concentration area: 0,5 ng/mL- 250 ng/mL in the digested sample
Precision (repeatability; intermediate precision)	At least 6 repeated analysis of a reference material: < 10 %
Traceability	Analysis of a reference material: NIST RM 8017
Measurement uncertainty	Determined from at least 6 repeatability measurements of the reference material: < 15% relative

TABLE 4
VALIDATION PARAMETRES FOR ICP-MS ANALYSIS OF SILVER

Ideally the measurements should in a regulatory context prove, if nanoparticles are present in the collected sample (particle size smaller than 100 nm), what kind of particles (chemical identity), how much nanomaterial is in the sample (mass), the particle size distribution and if the material fulfil the EU definition of a nanomaterial based on the particle size distribution.

Clarification: A possible measurement procedure is outlined in [49] and [50] consisting of a quantitative analysis of the silver content of the textile products with ICP-MS (Inductive Coupled Plasma-Mass Spectrometry) and quantitative analysis of the particle size distribution using SEM

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[49] *Kortlægning af tekstiler med nanosølv på det danske marked*, Johnny Rodam, to be published by EAP

[50] *Anbefalinger om kvalitetskrav til identifikation og kvantifikation af Ag nanopartikler i tekstiler*, confidential concultancy report to EAP

(Scanning Electron Microscopy) coupled with in situ chemical analysis with EDS (Energy Dispersive Spectrometry).

5.1.1 Validation parameters and interpretation

The validation parameters outlined in section 3.1 could be use in the case about silver nanoparticles. To estimate the particle size distribution using TEM the general approach outlined in **section 3.2** could be use. For the case of silver nanoparticles an attempt is done to specify the measurand and validation parameters and give examples of possible values in **Table 3** and **Table 4**

Clarification: To validate the parameters and interpret the measurement result a reference material is the most important element. However, no reference material of silver nanoparticle loaded cotton threads is commercial readily available. Therefore spiked samples should be used for the validation where necessary. A series of silver nanoparticle loaded cotton threads could be prepared as a spiked validation material. The loading of cotton threads by of silver nanoparticle could be done following the published procedure from NIST[51]. The spiking should be done with nanoparticles with known properties. Here the test material would probably be silver nanoparticle BAM-N001 CRM, a monodisperse colloidal silver, which carries certified values for d10, d50 and d90 of its number based size distribution. BAM-N001 is characterised with SAXS[52]. These particles are non-agglomerated.

The counting of particles and determination of their size could be validated using monodisperse colloidal silver loaded to cotton threads as described above.

5.2 Release of nanoparticles from crayon

When determining the number concentration and size distribution of released nanoparticles or nanoaerosols at e.g. workplaces from products the following issues are often difficult to assess

- To determine whether the particles released is actually nanoparticles and their number concentration.
- Different measurement methods give different number concentration for the same measured size fraction.

One example of a possible release of nanoparticles or nanoaerosols is the release of dust from crayons during drawing on a black board in a classroom or similar. A possible release of nanoparticles could be enhanced by some handling of the black board like a drop. The release of airborne particles could be measured with a SMPS. However, also other methods could be used including SEM and TEM and methods based on the particles optical properties.

Clarification: Strictly the EU-definition of nanomaterial does not cover airborne particles or aerosols – see also section 2.1. However, the EU-definition can pragmatically be extended to a mixture of nanomateial and gasses. An ISO standard also defines nano-aerosols as a gaseous matrix and at least one liquid or solid nanophase[53].

It is commonly agreed between scientist working with accurate measurements of nanoparticles that “the measurand is often difficult to define, and that measurement techniques which claim to perform the same measurement can give different results because they actually measure different

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[51] *Preparation of silver nanoparticle loaded cotton threads to facilitate measurement development for textile applications*, Version 1.0, J. M. Gorham, K. Murphy, J. Liu, D. Tselenchuk et al. NIST Special Publication 1200-8, downloaded 2015-12-10 from: http://dx.doi.org/10.6028/NIST.SP.1200-8

[52] *Certification Report of CRM BAM-N001, Particle size parameters of nano silver*, M. Menzel, R. Bienert, W. Bremser, M. Girod, S. Rolf, A. F. Thuenemann, F. Emmerling, 2013

[53] *Nanotechnologies - Vocabulary* ISO/TS 80004-4:2011

properties”[54]. This is particularly the case for size measurements, where it is acknowledged that the term “size” is not precise enough[54]. Put in other words different measurement techniques measure different aspects of particle dimensions[55]: TEM e.g. measures a projected geometric area by a focused electron beam which passes through the nanoparticle and interacts with it while SMPS measures a mobility of the nanoparticles which is then converted to a diameter. Even when measuring gold nanoparticles of high quality intended as reference material different measurement methods give different certified diameter[38]. However, for an ideal round particle, with uniform density and no surface coating or contamination we would like that different measurement methods *should* give the same results. But in most nanomaterials and nanoproducts these assumptions are fare from correct and therefore different kind of measurements will give different results particularly for size measurements.

The above task is difficult to handle but it is commonly agreed between scientist working with accurate measurements of nanoparticles that in some cases “method defined approaches and standard procedures constitute an acceptable solution”[54].

5.2.1 Validation parameters for scanning mobility particle sizers

The specification of the measurand, validation parameters and some typical values give in section 4.1.4. The principles outlined in section 2.1.5.1 can be used to determine a nanoparticle fraction with expanded uncertainty. A particular relevant standard about the characterization of ultrafine aerosols and nanoaerosols in workplace atmospheres should be followed[56, 57].

5.3 Aggregation and agglomeration of nanoparticles

An implantation of the EU definition of nanomaterials requires a determination of the size of the constituent particles of the potential nanomaterial. This is in general not possible but some weakly-bound particles can be broken by standard ultrasound procedures before measuring the particle size distribution. In regulatory context an important question is therefore how to handle the measurement of the particle size distribution of materials which potential has agglomerations or aggregates.

Clarification: Sample preparation and the issue of aggregation and agglomeration is discussed in an international standard[58] about the dispersing procedures for powders in liquids and the general recommendations herein should be followed.

Particles in powder form are well-known for having a great capacity to agglomerate and aggregate. There are several different agglomeration mechanisms. The particles can agglomerate due electric charges (e.g. Van der Waals bonds), magnetic properties (ferromagnetism); physical-locking (e.g. entanglement) or bridge-binding (e.g. liquid film or greasy coatings). Some of these bonds can be broken relatively easily by mechanical action, while others require great energy.

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[54] *Report on the BIPM workshop on metrology at the nanoscale* Sèvres, 18-19 February 2010 A.G. Steele, J. Viallon, P. Hatto, T.J.B.M. Janssen, A. Knight, L. Locascio, J.R. Miles, V. Morazzani, S. Prins, W. Unger BIPM retrieved 2015-12-20 from http://www.bipm.org/utils/common/pdf/rapportBIPM/RapportBIPM-2010-06.pdf

[55] *Diameter measurements of polystyrene particles with atomic force microscopy* J. Garnaes, Meas. Sci. Technol. 22 (2011) 094001 (8pp)

[56] *Workplace atmospheres -- Characterization of ultrafine aerosols/nanoaerosols -- Determination of the size distribution and number concentration using differential electrical mobility analysing systems* ISO 28439:2011

[57] After the project has ended further clarification has become available in the ISO Draft *Workplace exposure - Metrics to be used for the measurements of exposure to inhaled nanoparticles (nano-objects and nanostructured materials) such as mass concentration, number concentration and surface area concentration* ISO 16966:2016

[58] *Sample preparation -- Dispersing procedures for powders in liquids*, ISO 14887:2000

Possible approach - dispersion protocol: In principle the constituent particles would have to be present in the measuring phase, that is, the agglomerates or aggregates have to be broken via a certain dispersion protocol, specifying exactly which dispersants to use, the nature and amount of external energy input etc. Such a dispersion protocol could be necessary to develop and validate in connections to measurement procedure. It could – more pragmatic - be included in an international standard. A dispersion protocol could require the nanoparticles to be prepared in three different dispersants, one nonpolar (e.g. ethanol), one polar-aprotic (e.g. acetone) and water to cover the three large solvent classes[59]. In case that a constituent particles cannot by isolated a pragmatic adjustment of the measurand could be to specified the procedures that have to be follow in an attempt to break the particles into constituent particles and the pragmatic conclude that the measurement result is a "de facto" unbreakable particle. These aggregates may have biological properties that are effectively represented by larger-scale or bulk forms of the same material. These concepts of how easy the aggregates are to break down will be considered and will be important in reviewing the EU definition[60]. For a further discussion of this approach also see reference [59].

Case – A practical approach to identifying agglomeration or aggregation: The observed size distribution of a dispersion of nanoparticles in a liquid is not only a function of the concentration but also depend on the history of how the concentration has been achieved. If aggregation and agglomeration has been established previously e.g. at a high concentration of dispersed nanoparticles or under other specific conditions the observed size distribution of dispersed nanoparticles can be different for a dispersion which have the same mass concentration of nanoparticles but have underwent a different history. To estimate aggregation of a dispersion of nanoparticles in a liquid for a particular process, such as aging or dilution of the dispersion, the size distribution of the particles should be measured before and after the process.

The nanoparticle size fraction including the expanded uncertainty should be calculated before and after the process suspected to change the agglomeration or aggregation as outlined in in section 2.1.6. By subtracting comparing the two nanoparticle size fraction, taking the expanded uncertainty into account, it can be determined if the process has changed the size fraction of nanoparticles significantly and the possible aggregation or agglomeration process can be quantified including expanded uncertainty.

5.4 Nanomaterial with different coatings

The case will examine analytical methods used to identify coated nanoparticles in products, with focus on sunscreen. The light scattering and absorption properties of TiO_2 nanoparticles can be used for UV protection in sunscreens as an alternative to existing chemical UV absorbers.[61] While the application properties of coated nanoparticles are discussed in many papers, only few deal with the chemical identity and analyses of coated nanoparticles from commercially available products, and even fewer with healthcare products. Many different commercial types of coated nanoparticles are available, and they are used in a broad variety of products. For sunscreen products few types of coated nanoparticles are used, but several commercial manufacturers exist.

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[59] *Towards a review of the EC Recommendation for a definition of the term "nanomaterial"* e.g. page 23 in Part 3, Edited by Hubert Rauscher and Gert Roebben, 2015 EUR 27240

[60] *Comparative assessment of nanomaterial definitions and safety evaluation considerations* Darrell R. Boverhof , Christina M. Bramante , John H. Butala, Shaun F. Clancy et al. Regulatory Toxicology and Pharmacology 73 (2015) 137e150

[61] *Sunscreens with titanium dioxide (TiO2) Nano-Particles: A Societal Experiment*, Johannes F. Jacobs, Ibo van de Poel, Patricia Osseweijer, Nanoethics (2010) 4:103-113

Examples of coated nanoparticles used in sunscreen are:

- TiO_2, rutile, coated with silicon and alumina (hydrophilic)
- TiO_2, rutile, coated with silicone oil (lipophilic)
- UV-Titan M161: TiO_2, rutile, coated with alumina and stearic acid
- UV-Titan M262: TiO_2, rutile, coated with alumina, silicone
- ZnO, coated with 3-aminopropyltriethoxysilane (KH550) (hydrophilic/oleophilic).[62]

It's difficult to describe TiO_2 nanoparticles for sunscreen applications as the following factors have to be considered:

- Particle sizes and distribution
- Agglomeration and aggregation
- Morphology of the particle
- Crystal structure
- Purity and doping
- Use of coatings
- Surrounding matrix.[61]

The overall challenge when analysing the coating materials is that not one analytical principle can cover the most frequently applied coating materials, even if the coated nanoparticles are in pure form and not part of a skincare product. Added to that comes the problem of either isolating the nanoparticles before any chemical analysis or analysing the nanoparticles directly in the product.

Isolating nanoparticles is difficult, as it has to be ensured that the nanoparticle coating is intact after the isolation procedure. For most types of coating on nanoparticles (e.g., titanium nanoparticles) the mechanism of interaction between the coating material and the nanoparticle is unknown. Hydrous silica and titanium particles form a TI-O-Si bond on the surface[63], whereas nanoparticles coated with stearic acid are converted to CO_2 when exposed to infrared light[64]. This means that for some coating materials, isolating the nanoparticles might not be a problem. For others, the coating material could be removed during the isolation process. Not only the type of coating material influences the stability of the coating, but also the thickness of the coating can have an impact on the properties and stability of the coating. However, analysing nanoparticles and their coating directly in the product without isolation can give rise to other types of problems, as an element analysis (e.g., ICP-MS) of the product only describes the elements in the product and not if, e.g., any aluminium present in the product is associated with the coating of nanoparticles.

10 different commercial sunscreens were tested for the structure, composition, and dimension of TIO_2 and ZnO. Depending on the analytical method, three different sample preparations were used with different degrees of impact with the nanoparticles in the products. They ranged from sample spread on a glass plate to simple dilution with water or organic solvents followed by centrifugation. The subsequent analyses performed were SEM, TEM, EDS[65], XRD[66] and BET[67]. By using this analytical setup, it was possible to acquire information about the inorganic composition of the nanoparticles, and the physical characterization was described as particle length, width, crystal structure, the crystallite size and surface area. This study produced a method for comparing different types of sunscreen, and identified sunscreen that contained nanoparticles.

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[62] *Dermal Absorption of Nanomaterials Titanium Dioxide and Zinc Oxide Based Sunscreen Role of Size and Surface Coating*, Ministry of Environment and Food, Environmental project No. 1736, 2015

[63] *Surface characteristics of hydrous silica-coated TiO2 particles*, Yu-Lan Lin, Ting-Jie Wang, Yong Jin, Powder Technology 123 (2002) 194-198.

[64] *TiO2 Photocatalysis: A historical overview and future prospects*, K. Hashimoto, H. Irie, A. Fujishima, Japanese Journal of Applied Physics, Vol. 44, No. 12, 2005, pp. 8269-8285.

[65] EDS - Energy dispersive X-ray spectroscopy was used to analysed for their elemental composition.

[66] XRD - X-ray diffraction was used to probe the crystal structure of the inorganic sunscreen pigments.

[67] BET - Brunauer-Emmett-Teller surface area analysis.

Moreover, as part of the conclusion of the study it was emphasized that no coating of nanoparticles deriving from the sunscreen could be identified, even though it was present. The coating could only be positively identified by TEM in the form of pure powder from coated nanoparticles.[68]

Spectroscopic analyses of nanoparticles of TiO2 coated with silicon have shown that a chemical symmetry of the Si-O tetrahedron is broken under the influence of the Ti atom, constructing a Ti-O-Si bond. This change in the bond vibration, compared with non-coated TiO2, from the non-infrared to the infrared spectrum, leads to a new absorption band, clearly visible with FTIR[69] and XPS[70].[63] It will probably not be possible to transfer this specific means of identification of a TiO2 silicon-coated particle as an identifier method for silicon coated nanoparticles in products (e.g., sunscreen), due to the influence of the remaining chemical ingredients of the product, that most likely affect the bond vibration. If a sample preparation is used to isolate the nanoparticles, there will again be the risk that the coating layer will be destroyed or affected. Methods developed on coated nanoparticle powders can therefore not solve the challenge of analysing nanoparticles directly in the product.

FTIR has been used to compare the interaction between a TiO_2 core and a BTSE[71] shell, but FTIR did not reveal any bonding between lauric acid and the coating (TiO_2O-BTSE-LA, e.g., Si-O-C(=O)-). This kind of "diluted" covalent bonding can be detected by TG-DTA[72].[73]

Another method used in the identification of coated nanoparticles is the combination of FFF-ICP-MS. The FFF can separate macromolecules, colloids, nanoparticles and microparticles according to their size or mass. On-line FFF-ICP-MS can be used to provide size-resolved elemental composition of the nanoparticles.[74] This method can give information on a nanoparticle, which coating includes Al. This type of coating is used together with either silicon or stearic acid. Neither can be identified by using ICP-MS. The method provides information on which fraction, and thereby the size of particles that contain Ti together with Al. If both Ti and Al are present in the same fraction, it is an indication of Al coating of nanoparticles. Again, this method is only applicable for a small part of the coating types found on the commercial market.

5.4.1 Examples of validation parameters

For ICP-MS, SEM and TEM the validation parameters outlined in section 3 and 4 can be used. The other methods like FFF-ICP-MS, FTIR, EDS, XRD, BET, XPS and TG-DTA are not included in this report.

If it is possible to identify an applicable method for analysing a specific coated nanoparticle, then it will be possible to validate the method if actual nanoparticles from the manufacturers can be obtained as reference. For products (e.g., sunscreen) a complete validation requires a reference material or it must be possible to spike samples with the actual nanoparticles. The use of spiked samples is limited by the matrix because it depends on whether it is possible to spike the samples so they look like the real samples, e.g., spiking nanoparticles to a liquid matrix is easier than

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[68] *The structure, composition, and dimensions of TiO2 and ZnO nanomaterials in commercial sunscreens*, Zuzanna A. Lewicka, Angelo F. Benedetto, Denise N. Benoit, William W. Yu, John D. Fortner, Vicki L. Colvin, J Nanopart Res (2011) 13:3607-3617

[69] FTIR - Fourier transform infrared spectrum

[70] XPS - X-ray photoelectron spectroscope

[71] BTSE - Bis-1 2-(Triethoxysilyl) Ethane

[72] TG-DTA - simultaneous thermogravimetry/differential thermal analysis

[73] *Suspensions of modified TiO2 nanoparticles with supreme UV filtering ability*, T. Ukmar, A. Godec, U. Maver, O. Planinsek, M. Bele, J. Jamnik and M. Garberscek, J. Mater. Chem., 2009, 19, 8176-8183

[74] *Improved sample preparation and quality control for the characterization of titanium dioxide nanoparticles in sunscreens using flow field flow fractionation on-line with inductively coupled plasma mass spectrometry*, Volker Nischwitz and Heidi Goenaga-Infante, J. Anal. At. Spectrom., JAAS, www.rsc.org/jaas, DOI: 10.1039/c2ja10387g

spiking to a solid matrix. A second potential problem could be that particles may undergo changes during sample preparation and final quantification.

Conclusion: Literature that describes the identification and analysis of the coating of a nanoparticle is very limited. The literature concentrates most on the determination of particle sizes. The chemical analyses were carried out by ICP-MS, which cannot distinguish between any element derived from the matrix, or nanoparticles, and which cannot analyse any organic coating. A full validation of any method is complicated because of the lack of any reference material.

6. Conclusion

The report proposes to specify a *description* of the measurement split into the specimen to be measured (e.g. nanoparticles present as granulate), measurement method, measurand and possible special measurement conditions. It is suggested that the validation of the measurement capability is split into selectivity, limit of detection and quantification, working range, precision, traceability and measurement uncertainties. Each “parameter” shall document the methods performance regarding

- selectivity (capability to measureanalyte without interference from other components)
- limit of detection and quantification (lowest quantity that can be distinguished)
- working range, (range for which the method is applicable)
- precision, (closeness between replicated measurements)
- traceability (the result can be related to a reference)
- measurement uncertainty (observed dispersion of the quantity being measured).

Ideally the measurement method shall tell the chemical identity and number as function of particle size in different matrixes, including different coatings or surface treatment of the nanoparticles. The report *conclude* that in general these tasks are not always possible to do with reference to methods described in international standards or the scientific literature and the task is more complicated than chemical measurements of concentration.

It is concluded that the suggested validation parameters can be used to examine and give evidence if requirements are fulfilled for measurement method. and the detailed interpretation is discussed for sizing from images from fictitious scanning electron and transmission electron microscopy measurements. The report clarifies in details the interpretation of the validation parameters for the methods and gives examples of reasonable numerical values for some parameters.

For nanoparticles the basic and “new” measurand is the particle size distribution, that is, the total number of particle split into small discrete size ranges. The report goes into depth about the basic interpretation of this measurand and how to interpret the combined measurement uncertainty[75] when there is an uncertainty on both the estimated size and the estimated number of particles in each small discrete size ranges.

It is found that a thorough description of particle size distributions and an interpretation of the associated uncertainty are necessary in any regulatory compliancy assessment. *The report deliver* a complete example and calculation of the nanomaterial fraction including the associated measurement uncertainty, which can be used to determine if a material shall be classified as a nanomaterial at a certain threshold and confidence level. The calculation is based on a tailor-made excel template. The example and calculation is the fundament for interpreting measurements results and validation parameters.

In relation to chemical identity of the nanomaterial, the report focuses on inorganic nanoparticles. A particular type of mass spectrometry which is capable of detecting metals and several non-

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[75] The combined measurement uncertainty is obtained using the individual standard measurement uncertainties associated with the input quantities in a measurement model for the measurement process. The measurement model is the mathematical relation among all quantities known to be involved in a measurement (ISO/TR 13014:2012)

metals at concentrations as low as one part in 10^{15} (ICP-MS) has been chosen as an example in the discussion of the validation parameters.

Based on the report's findings validation parameters are suggested for measurements of nanoparticles in textiles. The particle size distribution measured in this fictitious case is e.g. the size-specific number concentration deduced from the projected area. Some key validation parameters for this measurement are the selectivity to silver particles when particles with different chemical composition are presents, the limit of detection for the size of the particles and the traceability to a reference material. Is *concluded* that a validation of the measurement method could potentially be performed based on the suggested validation parameters but at high cost. Also based on the report's findings validation parameters are suggested for the size distribution of released nanoparticles or nanoaerosols at e.g. workplaces from products using a specific measurement method based on the aerodynamic properties of the nanoaerosols. Key validation parameters are working range, that is, the size rage of nanoparticles for which the measurement procedure is applicable, precision related to a background measurement of ambient air and traceability to measurements on reference materials. An issue is that different measurement methods give different number concentration for the same measurement size fraction. Based on scientific literature and current state-of-the-art it is *concluded* that in some cases method defined approaches and standard procedures constitute an acceptable solution. A dispersion protocol for nanoparticles is *proposed* together with a method to identify and quantify the aggregation or agglomeration based on the findings in the report. It is *concluded* that a full validation of any method to assess issues about coating of e.g. titanium nanoparticles is missing in the literature and is further complicated because of the lack of any reference material.

Appendix 1 Example of particles with indicated diameter

Let there be given a fictitious, but realistic, measurement of the particle size distribution based on electron microscopy images of a small amount of powder adsorbed on a surface, which potentially could be a nanomaterial. The particle size distribution is given as the observed number of particles with diameter between 0 nm and 5 nm, between 5 and 10 nm and so on. The number of particles in 61 "size bins" is given in Table 6 (page 45) and in **Figure 8** (page 42)**.** The following will give guidelines which in a regulatory context can prove, at a confidence level of 95%, if the potential nanomaterial complies with the EU definition of a nanomaterial based on the particle size distribution. However, the method described is used for any confidence level and size fraction.

To give the above guidelines it is necessary to clarify how such a measurement is done and make a simple, but realistic, mathematical model of the measurement process. From images of the potential nanomaterial, N = 1479 particles are sampled and measured at random. The (smallest) diameter x_i of particle number i in the sample is measured as outlined in **Figure 1** (page 12) and the general discussion of the determination of particle size in **section 2.1.1**.

The measured distance on the image provides an indication I_i that is related to the diameter x_i through a calibration of the measuring system. The simplest possible relation is

$$x_i = aI_i,$$

where a is a calibration constant with an associated standard uncertainty $u(a)$. The calibration constant a can e.g. be determined from images of a reference standard with certified dimensions and the associated standard uncertainty $u(a)$ is due to e.g. the uncertainty of the certified dimensions.

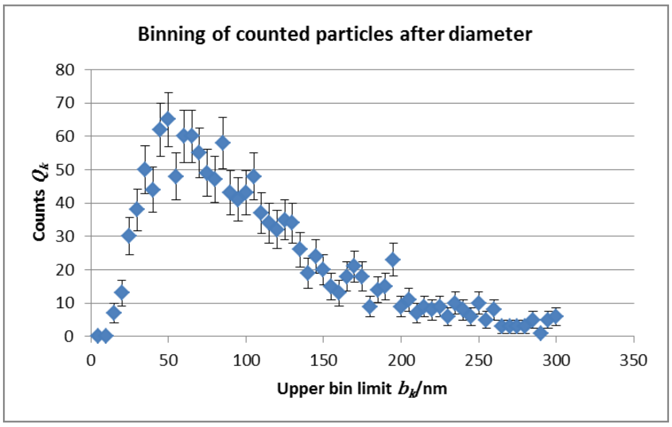

FIGURE 8
COUNTS Q_K OF PARTICLES, TO BE FOUND ON THE AVERAGE IN A SAMPLE OF 1479 PARTICLES TAKEN FROM THE POTENTIAL NANOMATERIAL, WITH INDICATED DIAMETER I_I IN THE RANGE $B_{K-1} < I_I \leq B_K$, WHERE B_0 = 0 NM, B_{K-1} = 300 NM, AND $B_K - B_{K-1}$ = 5 NM. THE ERROR BARS INDICATE STANDARD UNCERTAINTIES.

The measured distance I_i on the image related to the diameter x_i could be the reading of the distance between two cursers on the screen using the microscope manufactures (uncorrected) image processing software. The indication I_i itself also has an associated standard uncertainty $u(I_i)$, which is given by the repeatability of the measuring process. The repeatability is e.g. influenced by the limited image resolution of the edge of the particle and the lack of knowledge of where to measure the diameter if the particle is not round.

Based on the indications, the observed N = 1479 particles are binned to form a histogram with bin width of 5 nm and bin limits $b_0 < b_1 < \ldots < b_K$, where K = 61 is the number of size bins. A particle with indicated diameter I_i is counted in bin number k, if $b_{k-1} < I_i \leq b_k$. For example will a particle with the indicated diameter of 83 nm be counted in the bin 80 nm to 85 nm. The number of counts in bin number k is denoted q_k. As counting in general follows a so-called Poisson process, the best estimate Q_k and associated standard uncertainty $u(Q_k)$ of the counts falling in bin no. k in an average sample of N particles taken from the same material is

$$Q_k = q_k + 1, \qquad u(Q_k) = \sqrt{Q_k},$$

see ref.[76] section 6.4.11.

Figure 8 shows the example, where N=*1479* particles were sampled and binned into K = 61 bins. The first 60 bins have equal width $b_k - b_{k-1}$ = 5 nm; the first lower bin limit is b_0 = 0 nm, and the last lower bin limit is b_{K-1} = 300 nm. The last bin no. K has upper bin limit b_K = 5000 nm; the counts falling in that bin is not shown in the figure. The error bars indicates the standard uncertainties $u(Q_k)$ of the counts.

As small particles are difficult to detect, some of the smallest particles in the sample are not detected, measured and counted. On a microscopy image one reason could be that small particles can hide behind a bigger particle. For particle counters this is described by a *counting efficiency* $e(x)$ defined as the probability that a particle of diameter x is detected by the counter.

A simple model for $e(x)$ could be

$$e(x) = A\left(1 - \exp\left(-\frac{x}{x_c}\right)\ln(2)\right),$$

where the constant A is the limiting counting efficiency for large particles, and x_c is the 50 % counting efficiency point defined by the equation $e(x_c)$ = 0.5. Both quantities A and x_c have non-negligible standard uncertainties $u(A)$ and $u(x_c)$ that propagate to a combined standard uncertainty $u(e(x))$ of the counting efficiency $e(x)$. An example of a counting efficiency curve is shown in **Figure 9**. The corresponding relative standard uncertainty $u(e(x))/e(x)$ is shown in **Figure 10** in **Appendix 1**.

In principle, the counting efficiency could be taken into account in two different ways. It could be done after binning by dividing the number Q_k by an average counting efficiency e_k valid for bin no. k. Alternatively, it could be done before binning in the following way: For particle no. i in the sample, calculate the diameter x_i from the indication I_i and replace “1 particle” with “$1/e(x_i)$ particle” when counting particles in the relevant bin. For sufficiently small bin widths, the two alternatives are equivalent. Note, however, that for the corrected counts Q_k^c, the relation $u(Q_k) = \sqrt{Q_k}$ does no longer apply.

In order to determine if the material, from which the sample was taken, is a nanomaterial, the nanomaterial fraction f of particles with diameters x in the range from 1 nm to 100 nm relative to the number of particles in the range from 1 nm to 5000 nm has to be determined. This requires an upper bin limit b_K = 5000 nm, and in order to minimise the uncertainty of the nanomaterial fraction f, the bin widths should be relatively small for diameters close to 1 nm and 100 nm.

The probability density function (PDF) for the nanomaterial fraction f is found by “Monte Carlo simulation” and described in detail in **Appendix 2**. The nanomaterial fraction f is given as

$$f = \frac{n_{1-100}}{n_{1-5000}}$$

where n_{1-100} is the number of particles with size between 1 nm and 100 nm and n_{1-5000} is the total number of particles with size between 1 nm and 5 000 nm. By the “Monte Carlo simulation”, a PDF for the nanomaterial fraction f is determined, including the expectation value of f and the associated standard uncertainty $u(f)$. By counting the number of times M_{nano} that the nanomaterial fraction calculated by the “Monte Carlo simulation” satisfies the inequality $f \geq 0.5$, the probability p_{nano} that the sample was taken from a nanomaterial can be calculated from the equation

$$p_{\text{nano}} = \frac{M_{\text{nano}}}{M}$$

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[76] *Evaluation of measurement data* — Supplement 1 to the “Guide to the expression of uncertainty in measurement” — Propagation of distributions using a Monte Carlo method, JCGM 101:2008

If $p_{\mathrm{nano}} \geq 0.95$, the material can be classified as a nanomaterial at a 95 % level of probability. If $p_{\mathrm{nano}} \leq 0.05$, the material can be classified as not being a nanomaterial at a 95 % level of probability. The exact definition of the nanomaterial fraction f and the probability p_{nano} that the sample was taken from a nanomaterial is given in **Appendix 2**. The excel macro and the excel template which can do the described "Monte Carlo simulation" and calculate the probability p_{nano} which can be used to classify a material as being a nanomaterial has been developed in the project.

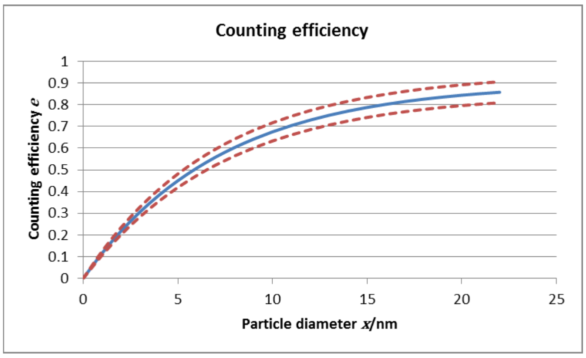

FIGURE 9

COUNTING EFFICIENCY E OF A PARTICLE COUNTER AS A FUNCTION OF PARTICLE DIAMETER X. THE DOTTED CURVES INDICATE THE STANDARD UNCERTAINTY ASSOCIATED WITH THE COUNTING EFFICIENCY. THE LIMITING COUNTING EFFICIENCY IS A = 0.9 WITH STANDARD UNCERTAINTY U(A) =0.05; THE 50 % COUNTING EFFICIENCY POINT IS $X_C = 5$ NM WITH STANDARD UNCERTAINTY $U(X_C) = 0.3$ NM.

Using the values and associated uncertainties listed in **Table 5** for the calibration constant a, the limiting counting efficiency A, the 50 % counting efficiency point x_c, the relative standard uncertainty $u(I_i)/I_i$ associated with the diameter measurings system, and the counts and associated standard uncertainties shown in **Figure 8**, the "Monte Carlo procedure" described in **Appendix 2** provides a nanomaterial fraction $f = 0.56$ with standard uncertainty $u(f) = 0.03$, and a probability $p_{\mathrm{nano}} = 0.97$ that the sample analysed was taken from a nanomaterial. The conclusion of the measurement is thus that the material can be categorized as a nanomaterial according to the EU definition at a confidence level of 95%. The level of confidence of 95% is a usual level in conformity assessment.

a	$u(a)$	A	$u(A)$	x_c/nm	$u(x_c)$/nm	$u(I_i)/I_i$	f	$u(f)$	p_{nano}
1	0.05	0.9	0.05	5	0.3	0.1	0.56	0.03	0.98

TABLE 5
VALUES AND ASSOCIATED UNCERTAINTIES FOR INPU PARAMETERS TO CALCULATE THE NANOMATERIAL FRACTION $F = 0.56$ WITH EXPANDESTANDARD UNCERTAINTY $U(F)$=0.03

After a recalculation the result can also be expressed as

(56 ± 6) % of the particles have a size between 1 nm and 100 nm

In this formulation of the result the measured value (here 56%) has an expanded measurement uncertainty of 6% equal to two times the standard uncertainty of 3% assuming a normal distribution. The analysing lab states that it is sure that between 50% and 62% of the particles have a size between 1 nm and 100 nm. This statement is made with a certain degree of confidence which in this case is approximately 95%. This means there is only a 5 % chance that the nanomaterial fraction *f* is not within the stated limits and only 5%/2 = 2,5% chance that the nanomaterial fraction *f* is smaller than 50%.

b_k /nm	b_{k-1} /nm	b_k /nm	q_k	Bin k	b_{k-1} /nm	b_k /nm	q_k	Bin k	b_{k-1} /nm	b_k /nm	q_k
1	0	5	0	**21**	100	105	48	**41**	200	205	11
2	5	10	0	**22**	105	110	37	**42**	205	210	7

3	10	15	7	**23**	110	115	34	**43**	210	215	9
4	15	20	13	**24**	115	120	32	**44**	215	220	8
5	20	25	30	**25**	120	125	35	**45**	220	225	9
6	25	30	38	**26**	125	130	34	**46**	225	230	6
7	30	35	50	**27**	130	135	26	**47**	230	235	10
8	35	40	44	**28**	135	140	19	**48**	235	240	8
9	40	45	62	**29**	140	145	24	**49**	240	245	6
10	45	50	65	**30**	145	150	20	**50**	245	250	10
11	50	55	48	**31**	150	155	15	**51**	250	255	5
12	55	60	60	**32**	155	160	13	**52**	255	260	8
13	60	65	60	**33**	160	165	18	**53**	260	265	3
14	65	70	55	**34**	165	170	21	**54**	265	270	3
15	70	75	49	**35**	170	175	18	**55**	270	275	3
16	75	80	47	**36**	175	180	9	**56**	275	280	3
17	80	85	58	**37**	180	185	14	**57**	280	285	5
18	85	90	43	**38**	185	190	15	**58**	285	290	1
19	90	95	41	**39**	190	195	23	**59**	290	295	5
20	95	100	43	**40**	195	200	9	**60**	295	300	6
								61	300	5000	76

TABLE 6
THE COUNTED NUMBER q_k OF PARTICLES WITH INDICATED DIAMETER IN THE RANGE $b_{k-1} < l_i \leq b_k$. THE BEST ESTIMATE OF THE NUMBER IS $Q_k = q_k + 1$, I.E. THE COUNTED NUMBER PLUS ONE, WITH STANDARD UNCERTAINTY $u(Q_k) = \sqrt{Q_k}$.

Appendix 2 Mote Carlo simulations of the probability density function

The probability density function (PDF) for the nanomaterial fraction f is found by Monte Carlo simulation as described in reference [77] The first step is to identify the input quantities on which the output f depends and to assign PDFs that describes the possible values that these input quantities might take given the information available:

To the quantity $Q_k, k = 1, \dots, K$ a gamma distribution $\mathrm{G}(Q_k, 1)$ is assigned, cf. reference [77], section 6.4.11; this PDF has expectation Q_k and variance Q_k. Note that Q_k was defined above as the counted number **plus 1** in order to be equal to the expectation of the assigned PDF.

To the indicated diameter $I_i, i = 1, \dots, N$, a Gaussianl distribution $\mathrm{N}(I_i, u^2(I_i))$ with expectation I_i and standard deviation $u(I_i)$ is assigned; for simplicity it is assumed that the relative standard deviation $s_r = u(I_i)/I_i$ is constant.

To the calibration constant a, a Gaussian distribution $\mathrm{N}(a, u^2(a))$ with expectation a and variance $u^2(a)$ is assigned.

To the limiting counting efficiency A, a Gaussian distribution $\mathrm{N}(A, u^2(A))$ with expectation A and variance $u^2(A)$ is assigned.

To the 50 % counting efficiency point x_c, a Gaussian distribution $\mathrm{N}(x_c, u^2(x_c))$ with expectation x_c and standard deviation $u(x_c)$ is assigned.

The calculation of the nanomaterial fraction f is complicated by the fact, that once the particles have been binned, the information about the individual measured diameters I_i is lost; the only information left is that $b_{k-1} < I_i \leq b_k$. The bin limits are assume to have zero uncertainty, but due to the uncertainty $u(I_i)$ associated with the indicated diameter I_i, a particle might be put into the wrong bin, particularly if I_i is close to a bin limit. In order to take that uncertainty contribution into account the following un-binning procedure is proposed:

Set numbers $n_{1-100} = n_{1-5000} = 0$. For each bin no. k, repeat the following procedure Q_k times:

1. Draw an indication I from a uniform distribution over the interval $b_{k-1} < I \leq b_k$.
2. Draw an indication I_i with repeatability noise from a Gaussian distribution $\mathrm{N}(I, \sigma^2)$ with expectation I and standard deviation $\sigma = s_r I$, where $s_r = u(I_i)/I_i$ is a specified constant.
3. Calculate the diameter $x_i = aI_i$.
4. If $1\text{ nm} \leq x_i \leq 100\text{ nm}$, increase the number n_{1-100} by $1/e(x_i)$.
5. If $1\text{ nm} \leq x_i \leq 5000\text{ nm}$, increase the number n_{1-5000} by $1/e(x_i)$.

When this un-binning procedure has been completed, the nanomaterial fraction f is given by

$$f = \frac{n_{1-100}}{n_{1-5000}}$$

If the un-binning procedure is repeated a large number of times, a distribution of the nanomaterial fraction f is obtained, which encapsulates the binning error due to the uncertainty of the diameter measurement and the information loss due to binning. In order to take into account all uncertainty components, the un-binning procedure is combined with a Monte Carlo simulation in accordance with reference [77]0 in the following way:

1. For each bin no. k, draw a count Q'_k from the gamma distribution $\mathrm{G}(Q_k, 1)$ and round it to an integer number.

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[77] *Evaluation of measurement data — Supplement 1 to the "Guide to the expression of uncertainty in measurement" — Propagation of distributions using a Monte Carlo method*, JCGM 101:2008

2. Draw a calibration constant a' from a Gaussian distribution $\mathrm{N}(a, u^2(a))$ with expectation a and standard deviation $u(a)$.
3. Draw a limiting counting efficiency A' from a Gaussian distribution $\mathrm{N}(A, u^2(A))$ with expectation A and standard deviation $u(A)$.
4. Draw a 50 % counting efficiency point x_c' from a Gaussian distribution $\mathrm{N}(x_c, u^2(x_c))$ with expectation x_c and standard deviation $u(x_c)$.
5. Apply the un-binning procedure using the drawn values $Q_k', k = 1, \ldots, K, a', A'$, and x_c' as input.
6. Calculate the nanomaterial fraction f.

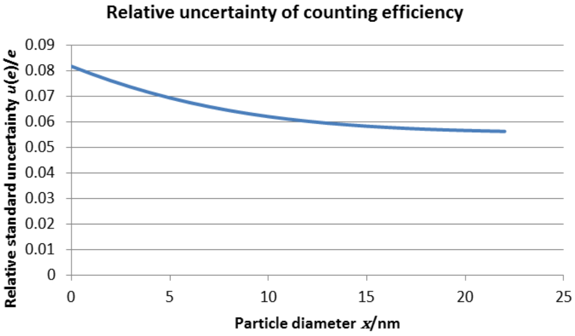

FIGURE 10

THE RELATIVE STANDARD UNCERTAINTY U(E)/E CORRESPONDING TO THE COUNTING EFFICIENCY CURVE SHOWN

By repeating this procedure a large number M of times, a PDF for the nanomaterial fraction f is determined, including the expectation value of f and the associated standard uncertainty $u(f)$. By counting the number of times M_{nano} that the nanomaterial fraction calculated in step 6 satisfies the inequality $f \geq 0.5$, the probability p_{nano} that the sample was taken from a nanomaterial can be calculated from the equation

$$p_{\mathrm{nano}} = \frac{M_{\mathrm{nano}}}{M}$$

If $p_{\mathrm{nano}} \geq 0.95$, the material can be classified as a nanomaterial at a 95 % level of probability. If $p_{\mathrm{nano}} \leq 0.05$, the material can be classified as not being a nanomaterial at a 95 % level of probability.

The excel macro and the excel template which can do the described “Monte Carlo simulation” and calculate the probability p_{nano} which can be used to classify a material as being a nanomaterial has been developed in the project.

Appendix 3 Additional discussion of DLS

Some instrument detects at an angle of e.g. 173°, and therefore it is the backscatter radiation that is detected, while other instruments detect the scattered light at an angle of 90°. Calculation of the particle size is achieved by using the Mie theory. The instruments will automatically attenuate the light intensity to match the current sample; the transmission ranges is typical from 100% - 0.0003%. By relating the degree of movement of a particle in the fluid over time, information about the particle size is obtained; therefore the name: dynamic light scattering.

An ISO standard is available for DLS measurements, which can be followed to ensure the measurement quality (ISO 22412). All future considerations presented in this part are based on the Zetasizer Nano ZS instrument, which is commercially available and is used for sizing of various particles in liquid media. Other instruments may have different size distribution ranges and other detection limits.[78, 79] The validation process is instrument independent, as it is based on the measurement of a standard sample.

Calibration and validation: Basically, a light scattering instrument consists of a laser, optics (lenses and mirrors), a sample holder, and a detector and signal-processing unit. All these parts are physical devices, which are aligned and therefore there is nothing on the instrument that can be calibrated. The instrument's performance is checked by a validation where a standard dispersion of latex particles is measured. If the measured result is consistent with the manufacturer's specifications, then the instrument is expected to work within the specifications. However, if there is discrepancy, there might be a problem with either the particle dispersion, the preparation of the sample or the instrument, and that must be investigated further. Instrument validation should be performed regularly. The standard sample is a dispersion of polystyrene latex particles with a narrow size distribution and a mean size of typically 100 nm as measured by DLS. The standard deviation of the measured size distribution must be +/- 2% of the specified size, and the PDI (polydispersity index) must be less than 0.1. Such a standard can be purchased from NIST.

Detection limit, measurement interval and measurement uncertainty: Repeatability of the average particle size should be better than 5%[80]. In the settings of the measurement, the number of repetitions, for example 10, can be set to ensure that the measurements are correctly performed.

Detection limits and measurement ranges may vary with both instrument and sample. Generally, the more powerful the laser, the lower the detection limit. A sample with a low particle concentration, which can be measured on one instrument, cannot necessarily be measured by using another instrument. However, many commercially available instruments have rather similar detection limits and measurement ranges (see the table below). Particles with a refractive index (RI) that is very different from the solvent, can be detected in lower concentrations than particles with a refractive index close to that of the solvent.

Therefore, there can be considerable differences in the detection limit from sample to sample, as the detection limit is material and solvent dependent.

Example of detection limits and size ranges based on information from the webpages of the respective distributers.

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[78] R.C. Murdock el al. Toxicol. Sci 101(2), 239-253 (2008)

[79] D.B. Warheit et al. Toxicology 230(1), 90-104 (2007)

[80] *Particle size analysis -- Dynamic light scattering (DLS)* cf. ISO-22412

Instrument	Specification
Zetasizer Nano ZS from Malvern Instruments	Particle size distribution with a lower detection limit of 0.01%* and a size range of 0.3 nm - 10 µm
DynaPro NanoStar from Wyatt Technology	Particle size distribution with a lower detection limit of 0.01%* and a size range of 0.4 nm - 5 µm
NanoBrook 90Plus Particle Size Analyzer from Brookhaven Instruments Corporation	Particle size distribution with a lower detection limit of 2 ppm and up to 50 mg/mL, dependent on the refractive index§, and a size range of 2 nm – 6 µm

TABLE 7
EXAMPLES OF INSTRUMENTS

*Based on 14 kDa lysozyme.
§It is not stated what refractive index is necessary to obtain a detection limit of 2 ppm.

Preparation for measurements: Depending on the particle concentration it may be necessary to dilute the sample prior to measuring, as concentrated samples give rise to multiple scattering, which distorts the measurement or makes it impossible.

On a regular basis, measurements are carried out on certified polystyrene latex particles to verify the instrument's performance.

Results: The results are given as a mean particle size in nanometres or as a particle size distribution in a graph.

Limitations of the method: The method cannot distinguish different types of particles. That means that the method does not distinguish, e.g., carbon based and titanium-dioxide based particles, or solid particles and air bubbles. Therefore, it is necessary that a highly skilled specialist performs the measurements.

For optimal measurement performance, the refractive index (RI) of the particles to be investigated must be stated as a predetermined parameter prior to the measurements. If there are multiple types of particles, the stated RI will be a weighted average or a guesstimate. Following this, an uncertainty of the measurement is present. RI is required to perform calculations on the basis of the scattered light and to relate this to the particle size. RI is material specific and can be determined by using a different analytical method.

Additional possibilities when using the method: It is possible to measure the particle size as a function of pH using an associated automated titration unit. It is also possible to measure the particle surface charge (zeta potential) by using the instrument. Here, the zeta potential can be determined as a function of the pH-value of the solution.

Appendix 4 Danish translation of the validation parameters

Specifikation af målestørrelsen	
Emne	- Aerosol, granulat eller opslæmning
Instrumenttype eller metode	- SEM, TEM, AFM, SMPS, DLS, ISO, CEN, DIN, EC, ASTM
Målestørrelse	- Kemisk identitet - Middeldiameter, minimum ekstern dimension, størrelsesantal, volumenfraktion, antal
Målebetingelser eller uafhængige variable for størrelsesmålinger	- Koncentrationsinterval, mulig aggregering- og agglomereringsniveau
Valideringsparametre	
Selektivitet	- Matricekomponenter - Partikler af forskellige kemiske elementer - Kemisk ækvivalente partikler fra forskellige producenter ellers partier
Detektionsgrænse	- Mass/antalsfraktion - Partikelstørrelse
Kvantifikationsgrænse	- Masse/antalsfraktion - Partikelstørrelse
Måleområde/linearitet	- Masse/måleområde for antalsfraktion - Måleområde for partikelstørrelse
Præcision (repeterbarhed;)	- Masse/antalsfraktion - Partikel størrelse
Sporbarhed	- Kemisk identitet - Masse/antalsfraktion - Partikelstørrelse
Måleusikkerhed usikkerhed	- Mase/antalsfraktion - Partikelstørrelse

TABLE 8
DANISH TRANSLATION OF THE VALIDATION PARAMETERS MODIFIED FROM REFERENCE [33]

Requirements to Measurements of Nanomaterials and Nanoproducts

The objective of this project was to establish a set of validation parameters which can be used to document the performance of measurement methods to detect and quantify nanoparticles. The core of the report has been to specify, interpret and clarify this set of validation parameters so they meet the requirements that are relevant for nanomaterials in the regulatory context.

Environmental
Protection Agency
Strandgade 29
DK-1401 København K

www.mst.dk