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RESEARCH PAPER

Production of reference materials for the detection and size determination of silica nanoparticles in tomato soup

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Abstract A set of four reference materials for the detection and quantification of silica nanoparticles (NPs) in food was produced as a proof of principle exercise. Neat silica suspensions were ampouled, tested for homogeneity and stability, and characterized for total silica content as well as particle diameter by dynamic light scattering (DLS), electron microscopy (EM), gas-phase electrophoretic molecular mobility analysis (GEMMA), and field-flow fractionation coupled with an inductively coupled mass spectrometer (FFF-ICPMS). Tomato soup was prepared from ingredients free of engineered nanoparticles and was spiked at two concentration levels with the silica NP suspension. Homogeneity of these materials was found sufficient to act as reference materials and the materials are sufficiently stable to allow long-term storage and distribution at ambient temperature, providing proof of principle of the feasibility of producing liquid food reference materials for the detection of nanoparticles. The spiked soups were characterized for particle diameter by EM and FFF-ICPMS (one material only), as well as for the total silica content. Although questions regarding the trueness of the results from EM and FFF-ICPMS procedures remain, the data obtained indicate that even assigning values should eventually be feasible. The materials can therefore be regarded as the first step towards certified reference materials for silica nanoparticles in a food matrix.

Keywords Engineered nanoparticles · Colloidal silica · Reference material production

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Introduction

Nanotechnology holds great promise for conservation of resources and better products, but—like any new technology—has potential risks connected to it. The European Commission has stepped forward to propose a definition of "nanomaterial" to be used in legislative context [1] and legislation requiring food producers to inform consumers if ingredients are present in the nano-form [2] has been passed. According to [1], a nanomaterial is defined as a material where 50 % of the number of particles have external diameters between 1 and 100 nm. The definition explicitly mentions that this criterion applies not to the overall size of agglomerates or aggregates, but to the constituent particles of potential agglomerates and

aggregates. Implementation of this legislation requires reliable methods to detect and quantify nanomaterials in foodstuff. The project NanoLyse, funded under the European Union's 7th Framework Programme (grant agreement 245162), aims to provide proof of principle that such methods are feasible, as there are currently no validated methods for this purpose available.

Reference materials play a crucial role in the development and validation of analytical methods. Two sorts of reference materials exist:

- Pure calibration standards, where the analyte is present either in its pure form or is dispersed in a solvent.
- Matrix reference materials, where the analyte in question is contained in the type of material to be measured (food, soil, human serum etc.).

Calibration standards are used to establish traceability and are also often used for preliminary method development, as they are the simplest conceivable samples. In a second step, matrix reference materials are required for establishing precision during method validation, and if a certified value is available, also for demonstrating method trueness and method proficiency. Therefore, the project also foresaw development of reference materials along with method development for specific nanoparticle–food combinations, as currently no certified nanoparticle reference materials with particles having a broad size distribution in complex matrices exist [3].

Development of certified reference materials for emerging measurands is a classic case of a chicken and egg problem: reliable analytical methods are required to assess not only homogeneity and stability of the candidate certified reference materials, but also to characterize them in a way that a reliable value can be assigned in a metrologically valid way. At the same time, exactly such certified reference materials are required for the development and validation of reliable methods. It is therefore acknowledged that production of certified reference materials and method development have to be approached in a stepwise fashion, starting with certified reference materials that are not perfect but allow development of the first methods, which are in turn used to develop and characterize certified reference materials of a better quality. The very first step in such an iterative process is the production of non-certified reference materials, i.e., materials for which homogeneity and stability have been assessed, but for which no value has been assigned. Reaching this stage is usually easier than production of certified RMs, as homogeneity and stability assessment can be done on a relative basis and does not require absolute method trueness. This manuscript describes the first step of such an iterative process for the development of reference materials for silica nanoparticles in food, namely the production of non-certified RMs and indicates that even the production of certified RMs should be eventually feasible.

Concept

Silicon dioxide ("silica") is together with carbon black the nanomaterial currently used in the highest quantities [4]. As silica is also an approved food additive in Europe (E551), silica was chosen as one of the nanomaterials to be investigated. One of the uses of silica is as anticaking agent, i.e., to ensure that dried powders remain free-flowing. For this reason, vegetable soup was chosen as matrix for the determination of the silica NPs.

The project foresaw development of one screening method (electron microscopy) and one confirmation method (field-flow fractionation coupled to an inductively coupled plasma mass-spectrometer (FFF-ICPMS)) for silica particles in soup. As it was deemed unlikely that an unambiguous value assignment could be achieved using the results from these methods alone, a characterization approach was necessary that allowed assessment of the particle diameters in the matrix independently from the analytical measurements on the matrix. Determination of particle sizes in pure suspensions is analytically much less challenging than in matrices. It was therefore decided to produce matrix materials by spiking a blank matrix with characterized pure suspensions. In this way the particle size distribution in the matrix should resemble the one in the suspension, allowing checking for biases in the particle size distribution and for recovery.

For this approach to be successful, silica particles must not dissolve in the food matrix. In addition, particles in the pure suspensions should (ideally) be not agglomerated or aggregated. If aggregation is not avoidable, the aggregates should not disaggregate in the food matrix, so that pure suspensions and food matrix still contain the same particles. Therefore, the following choices were made:

- A suspension rather than a dry powder was used as source material for the nanoparticles. In this way problems of incomplete deagglomeration and disaggregation can be minimized or avoided.
- Fumed silica was preferred over precipitated silica. While this material is certainly aggregated, aggregation at high temperatures should prevent disaggregation in suspension or in the food matrix. The high temperature of synthesis should also prevent dissolution in the matrix.
- Liquid soup was preferred over powdered soup, again to prevent formation of agglomerates that are difficult to resuspend.
- The liquid soup had to be prepared from base ingredients to avoid incidental presence of silica from bouillon stock etc.

Processing

Aqueous silica suspensions

Aqueous silica dispersion Aerodisp® W7520 N was purchased from Evonik (Hanau, Germany). Two custom-made batches of 30 kg each were obtained, subsequently named NanoLyse01 and NanoLyse02, having silica mass fractions of 10 and 40 g/kg, respectively. The suspensions were adjusted from pH 9 to pH 8 to fit better the pH of the foodstuff to be spiked. The suspensions were ampouled as received into precleaned and dried amber glass ampoules on an automatic ampouling machine (ROTA Verpackungstechnik, Wehr, Germany). Each ampoule contained 25 mL suspension in a sealed ampoule flushed with Ar to minimize degradation. The ampoules were labeled displaying a unique sample number in the order of the filling sequence.

Silica spiked tomato soup

Tomato soup was produced from fresh ingredients in order to obtain a food matrix with low silica nanoparticle background. Therefore, a 30-L tomato soup was produced by a local catering service according to IRMM's specifications (adopted from [5]):

Thirty-kilogram beef bones were added to 70 L water, brought to boil and emerging foam was skimmed off. Two-kilogram onions were cut in half, roasted with a little fat until brown and added to the boiling water/bone mixture. After another 1.5 h, 1.4 kg of carrots and some salt was added. The soup was left to boil for another hour, then onions, bones, and carrots were filtered off and the volume was adjusted to 30 L. This bouillon was further processed with 50 kg fresh tomatoes (non-processed) and 5 L cream. In order to minimize heterogeneity within the soup matrix, fat was skimmed from cold bouillon, seeds were removed from the tomatoes, and no additional spices were added. A corresponding flow chart is depicted in Fig. 1.

Tests were performed to find the right conditions to obtain a homogenous spiked material. Adjusting the pH was considered the most important parameter affecting inhomogeneity. Rapid precipitation upon spiking of small test batches with silica suspension was observed in all cases irrespective of pH adjustment or formulating strategy. As there are several reasons for such behavior like matrix clearing due to agglomeration or segregation of fatty from non-fatty parts, at least pH induced agglomeration was prevented by adjusting 30 L soup to pH 8 by means of addition of 140 mL of a 12.5-M NaOH solution.

The pH-adjusted soup matrix was filtered (1.4 mm, Analysensieb, Haver&Boecker, Oelde, Germany) before blending with the aqueous silica dispersions also used for the preparation of the materials NanoLyse01 and NanoLyse02. Aerodisp® W7520 N dispersions of 10 and 40 g/kg were added over 30 min to the soup matrix under constant stirring at 50 revolutions per min (rpm), using a laboratory stirrer designed for simple stirring tasks of up to 10 L with a speed range from 40 to 1,200 rpm. The resulting materials were filled into 25 mL clear glass jars on an automated filling machine (All-Fill model SHA, Allfill, Sandy, United Kingdom) and sealed with a twist-off lid on an automated filling and sealing machine (model TO-05-06, Lensen Vul en Sluittechniek, Sevenum, the Netherlands). The spiked soup was continuously stirred during the filling process to avoid segregation and the mass of the filled jars was monitored to ensure constant amounts per jar. Closing of jars was performed under a constant steam flow and a slight vacuum kept the lid tightly closed. The flowchart in Fig. 1 shows the processing steps of the material labeled NanoLyse09. An equivalent scheme was followed for the production of NanoLyse10. In addition to the spiked material, non-spiked tomato soup was filled for blank determinations and spiking experiments.

The samples needed to be sterilized to prevent matrix degradation induced by bacterial contamination. Steam water spray sterilization was not suitable as silica nanoparticles were observed to change slightly in size upon heat treatment (see the "Assessment of stability" section). Thus, the samples were γ-irradiated on a GS6000 pallet irradiator at SynergyHealth Ede (Etten-Leur, the Netherlands) with an average dose of 10.8 kGy within 24 h after processing. The success of the irradiation was checked by means of plating *Escherichia coli* CIP 106878 spiked samples for viable bacteria. There was no bacterial activity detectable after the irradiation. As a consequence, users have two ways of checking for the validity of the material: (a) the dark color of the glass indicating a successful sterilization by means of γ-irradiation and (b) the typical sound of vacuum sealed jars during opening.

Measurement and statistical methods

Determination of dissolved silica

Ultrafiltration was the method chosen as it is straightforward and readily available compared to approaches such as cloud point extraction [6]. Potential biases from this method come from adsorption of dissolved silica to the filter as well as migration of silica nanoparticles through the filter. Nevertheless, the method should give a sufficiently accurate estimate of dissolved silica.

The determination of dissolved silica in the dispersions was done in two steps:

1. Separation of solute from colloids using ultrafiltration

For the separation of dissolved silica, three samples of each material were randomly chosen from stock and aliquoted (5 mL, two of each sample) into ultrafiltration spin columns

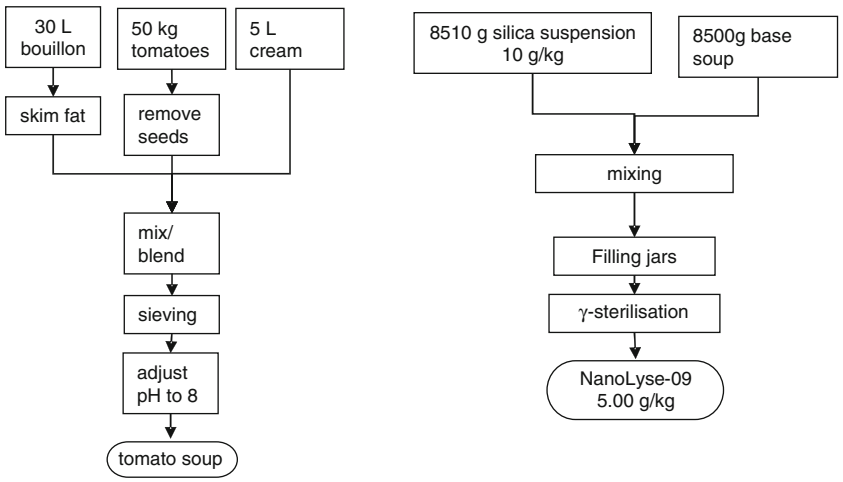

Fig. 1 Flowchart of the processing of tomato soup (*left*) and silica doped tomato soup NanoLyse09 (*right*)

(VS0611, 5,000 g/mol MWCO PES, Sartorius, Göttingen, Germany). The tubes were centrifuged at 5,000 rpm (20 °C) for 15 min.

2. Analysis of the filtrate of NanoLyse01 and NanoLyse02 by ICP-OES (described below).

Dynamic light scattering (DLS)

DLS measurements for homogeneity and stability of the aqueous suspensions were performed on a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The scattering angle was 173°, the measuring position was 4.65 mm inside the cuvette, and the measuring temperature was set to 25 °C. The liquid viscosity was set to 0.89 mPa·s (25 °C) and the liquid refractive index 1.33 (25 °C). The silica nanoparticle refractive index and absorption was set to 1.46 and 0.00, respectively. For homogeneity and stability, three replicate measurements using the cumulants method were performed.

For characterization, additional measurements on a Malvern 41700C (Malvern Instruments, Malvern, United Kingdom), an LS Spectrometer (LS Instruments, Fribourg, Switzerland) both with samples in NMR or Pyrex tubes, and a Horiba LB-550 (Horiba, Longjumeau Cedex, France) were performed.

Zeta potential by electrophoretic light scattering (ELS)

ELS measurements were performed on a Malvern Zetasizer Nano ZS. The scattering angle was 13°, the measuring position was 2.00 mm inside the cuvette, and the measuring temperature was set to 25 °C. The liquid viscosity used was 0.89 mPa·s (25 °C) and the liquid refractive index 1.33 (25 °C). The dispersant dielectric constant was 78.5 (25 °C). The zeta potential values were obtained from 3 consecutive measurements. Measurement uncertainties are given as standard deviations.

Inductively coupled plasma optical emission spectroscopy (ICP-OES)

The mass fraction determination of the silica dispersions was performed at Solvias (Kaiseraugst, Switzerland). Thirty to 40 mg of two duplicate samples was digested using the Wurzschmitt procedure (fusion melting with Na_2O_2 with subsequent dissolution of the digest in water [7]). The silicon mass fraction of the resulting solution was quantified on a Thermo Iris Intrepid II ICP-OES.

Scanning electron microscopy (SEM)

The silica dispersions and soup samples were investigated by means of SEM (FEI Sirion S FEG, Hillsboro, OR, USA). The microscope was equipped with a lens detector used for the image acquisition in this study and energy dispersive X-ray spectroscopy system (Thermo Fisher NS7 system with NSS112E NORAN operating software). All the samples were diluted (200-fold-particle suspensions, 100-fold-soup reference materials) with a 0.05-M borate buffer at pH 8 (BB8.0). Diluted samples were equilibrated at this pH for 6 h by mixing with a magnetic stirrer. Particles from the samples were then transferred onto the formvar-carbon-coated TEM grids (Agar Scientific, Stansted, UK) by means of electrostatic attraction. The protocol comprised of four stages:

1. Grid placed for 5 min floating on the drop of freshly prepared gelatin (Gelatin from porcine skin Type A, Sigma-Aldrich, St. Louis, MO, USA) 0.1 % solution in demineralized water (DMW);
2. Excess of gelatin removed by blotting with the filter paper and surface of the grids washed with a drop of DMW

three times followed by blotting off the moisture with the filter paper;
3. Grid placed floating on the drop of the sample for 2 min;
4. Sample blotted off and grid rinsed in two drops of DMW followed by blotting off the moisture with the filter paper;

The prepared grids were attached to standard SEM aluminum stubs using carbon tape and coated with a nominal 10 nm layer of Pt/Pd using 2300HR High Resolution Fine Coater with a JEOL FC-TM20 Thickness Controller. The coating was used in order to improve the imaging conditions—they reduce charging and increase particle contrast but also increased the size of the particles given in this study as equivalent circle diameter (ECD). Calibration on spherical mercaptoundecanoic acid coated gold ENPs (University of Alberta, Edmonton, Canada) by imaging particles before and after coating indicated that this increase was close to 8 nm. Therefore, this value was subtracted in all of the particle measurements.

The imaging was carried out using the same micrograph size for all of the samples (6.3 μm×4.73 μm). For sizing of the particles, object based image analysis software (eCognition Architect, version 8.7.2, Trimble, Munich, Germany) with a specially designed application facilitating the measurement of NPs in complex matrices (Centre for GeoInformatics, Paris Lodron University of Salzburg, Salzburg, Austria) was used. The accepted limit for the particle size measurement from the image was 15 pixels which was equivalent of 30 nm particle ECD after subtraction of the coating thickness.

Solid content determination (dry mass, ashing)

The silica mass fractions of NanoLyse01 and NanoLyse02 were determined gravimetrically. Duplicates of 10 samples of each material were dried at 85 °C for 4 h in a drying oven (Heraeus model WV 6100, Hanau, Germany). The samples were weighed after cooling to room temperature in a dessicator. Absence of residual moisture was confirmed by additional drying of the samples for 1 h at 105 °C and additional 30 min at 170 °C. The dry mass also includes reagents for adjusting the pH value (NaOH). However, the purpose of homogeneity testing is to demonstrate equivalence of each ampoule, so that a slight analytical bias is acceptable.

The silica mass fractions of NanoLyse09 and NanoLyse10 were determined gravimetrically after sample ashing [8] on 10 different jars with three independent subsamples per sample. Samples were placed in a pre-heated Carbolite furnace (RWF 1100, Sheffield, United Kingdom) and kept at 550 °C for 2 h. The resulting samples were stored at room temperature to cool down and weighed afterwards. An identical procedure was applied to blank tomato soup (three determinations) to determine its solid content. Ashing accounts for all mineralic substances in the soup, but it is still suitable for demonstrating the homogeneity of the jars. Ash content was corrected for the ash content of the blank soup to be able to compare the target silica mass fraction with the measured mount of non-combustible solids.

FFF-ICPMS

The system consisted of an Eclipse Dualtec AF^4 flow control module with a flat AF^4 separation channel (Superon, Dernbach, Germany, length 275 mm, wide spacer). A 350-μm spacer was used and a 10-kg/mol nominal cut-off regenerate cellulose membrane (Millipore, Billerica, MA, USA) was used as the accumulation wall. Detector flow was set to 1 mL/min and cross flow was set to 0.6 mL/min. Flows were controlled using an Agilent Technologies 1200 Series quaternary pump equipped with a micro-vacuum degasser. The detection chain consisted of a diode-array ultraviolet/visible detector (UV-DAD, Agilent Technologies 1200 Series, primary detection wavelength λ=254 nm), a multi-angler laser light scattering detector MALS (Dawn Heleos II, Wyatt Technology, Santa Barbara, CA, USA) and an inductively coupled plasma mass spectrometer ICP-MS (Agilent Technologies 7700x). All injections were performed using an autosampler (Agilent Technologies 1200 Series, large volume kit); injection volumes were 50 μL. As carrier liquid a mixture of 0.025 % FL70 (alkaline detergent; Fisher Scientific, Waltham, MA, USA) and 0.25 mM NaCl was used. The method was calibrated with latex beads as size standards (Thermo Fisher Scientific, Dreieich, Germany), themselves calibrated against NIST certified reference materials . The reported size distributions from the FFF-ICPMS method are ^{28}Si-concentration particle mass-based size distributions assuming a constant stoichiometry of the SiO_2 particles.

Silica nanoparticles were isolated from tomato soup applying a multiple step sample preparation, consisting of heating the soup for 30 min at 50 °C, homogenization in a glass beaker (Ultra Turrax, IKA-T10; 30 s at 20,000 to 25,000 rpm), removal of the organic material by acid digestion and stabilization of the remaining particle suspension by pH adjustment and probe sonication [9]. Subsequently separation by FlowFFF was used for the determination of size distributions.

Gas-phase electrophoretic molecular mobility analysis (GEMMA)

Measurements on the neat silica NP suspensions were performed by GEMMA [10] using a nano electrospray/charge reduction (Po-210) unit type 3480 (TSI, Shoreview, MN, USA) coupled to a nano DMA unit type 3080 (TSI, Shoreview, MN, USA) and an ultralow condensation particle counter type 3025A (TSI, Shoreview, MN, USA). The electrospray unit was run in the positive ion mode with a mixture of 0.5 L/min compressed air supplied by a table top

compressor (Dürr-Technik, Bietigheim-Bissingen, Germany) and 0.1 L/min CO_2 (99.995 %, Air Liquide, Schwechat, Austria) and an applied spraying voltage of 2.5 kV resulting in an electrical current of 360–470 nA. A cone-tipped-fused silica capillary with 40 μm inner diameter was used and the pressure difference along the capillary was 4 psid. The nano DMA was run with a sheath flow of 3 L/min and the condensation particle counter used butanol in the high flow mode. Samples were diluted prior to analysis in 20 mM CH_3COONH_4.

Three measurement series consisting of 4–10 measurements each were performed for NanoLyse01 (24 measurements in total) and 5 measurement series consisting of 5–10 measurements each (36 measurements in total) were performed for NanoLyse02.

Density of silica particles

The density of silica particles was determined by isopycnic centrifugation performed by Dr. Lerche (Berlin, Germany) [11].

Study designs and statistcial methods

Assessment of homogeneity

Nanomaterial related measurands are method-defined, so each method, while analyzing the same particles, measures different material properties (sedimentation velocity, transmission of electrons etc.). This means that a material could be homogeneous for one method but not for another method. Ideally, homogeneity would therefore be assessed using a number of methods to obtain method independent information on the homogeneity. However, such practice was not feasible within the frame of the project. Hence, it was decided that homogeneity data on mass fraction and particle size should be obtained from one appropriate method each.

Homogeneity was tested on 10 samples using drying/ashing and DLS and on 9 samples using FFF-ICPMS. Results of the homogeneity tests were evaluated using one-way analysis of variance (ANOVA) as described in [12]. Standard deviations within ampoule (s_{wb}, repeatability) and between ampoule variation (s_{bb}) were calculated. In addition, the maximum heterogeneity that could be hidden by method repeatability (u^*_{bb}), the "limit of detection of the homogeneity study," was calculated as described in [13].

Assessment of stability

Stability testing was performed to assess stability during transport (short-term stability) and stability during storage (long-term stability). As the transport time is very limited (1–2 days in Europe, 3–4 days world-wide), stability studies for assessing transport stability only need to be of a short duration. On the other hand, transport conditions such as temperature may vary. Therefore, a typical short-term stability study lasts 4 weeks, but includes the conditions that cover the "worst-case" scenario (low and high temperatures). The goal of the study is to identify transport conditions where a potential change during transport is absent or negligible.

Analytical variation should be reduced to a minimum when assessing stability. Therefore, stability studies were carried out using an isochronous design [14]. In that approach, samples randomly selected from the stock are stored for a certain time at different temperature conditions. After each time point, the samples are moved to conditions where further degradation can be assumed to be negligible ("reference conditions"), effectively "freezing" the degradation status of the materials. The definition of such conditions is difficult for the characterization of nanoparticles, as little information on their stability is available so far, but all possible degradation processes will lead to different rates of change at different conditions. Absence of any change of the measurand (here particle diameter) is therefore a clear indication of stability. At the end of the isochronous storage, the samples are analyzed simultaneously under repeatability conditions eliminating day-to-day variations. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

Regarding the methods employed, ideally each stability parameter would be assessed using a number of methods. However, that would cause an unrealistic work load. Stability was therefore assessed using only one method per parameter and material.

The results were evaluated by trend analysis as described in [15]: Linear regressions were performed and the slopes of the regressions were tested for significance. If the slope was found insignificant, uncertainties of long-term stability (u_{lts}) were calculated from the standard uncertainty of the slope for a shelf life of 2 years.

Value assignment

As the materials are not certified reference materials, value assignment is in principle not required. However, using the value obtained in the assessment of homogeneity and stability and the data provided for characterization allows an indication about the feasibility of the production of CRMs in the future.

As is common practice for certified reference materials (CRMs) [16], uncertainty contributions of homogeneity (u_{bb}), long-term stability (u_{lts}) and characterization (u_{char}) were combined and multiplied with a coverage factor of $k=2$ to obtain an idea of which uncertainties would be achievable for an assigned value of a CRM. The following data were used:

All uncertainties of homogeneity (u_{bb}) were based on the larger value of s_{bb} or u^*_{bb} for each of the suspensions. The

homogeneity data for NanoLyse10 from FFF-ICPMS was also used as homogeneity contribution to the median diameter by EM for both soup materials.

The uncertainty of long-term stability for particle size was in all cases based on the u_{lts} estimated for NanoLyse01 and NanoLyse02 for a storage period of 2 years. This could be an underestimation for the soup material, but, given the high homogeneity contribution, should not influence the result too much. Based on the high chemical stability of silica, uncertainties for the time variation of silica mass fractions are assumed to be negligible. Uncertainties of characterization were in all cases estimated as the standard error of the mean of results.

Results and discussion

Assessment of homogeneity

Aqueous suspensions NanoLyse01 and NanoLyse02

Homogeneity of the silica mass fraction was determined gravimetrically by oven drying. Homogeneity in terms of particle size was determined by DLS on a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, United Kingdom). The results were calculated using the cumulants method [17]. One replicate measurement per ampoule of NanoLyse01 and NanoLyse02 was performed, as the measurements were repeatable enough to set narrow limits for repeatability. For NanoLyse02, data were also available from the validation of the FFF-ICPMS method. On three different days, seven measurements were performed on three units each.

The results of the homogeneity assessment (s_{wb}, s_{bb}, and u^*_{bb}) are shown in Table 1. Relative variations (s_{wb}[%], s_{bb}[%], u_{bb}[%]) of NanoLyse01 were smaller than 1 %. For NanoLyse02, the relative between bottle variation s_{bb} % and the relative hidden uncertainty u^*_{bb} for the dry mass and the diameter as determined were below or around 1 %, respectively. Between-unit standard deviation as obtained by FFF-ICPMS was with 4.6 % significantly higher [18]. The results show that the between-unit variations are sufficiently small to make NanoLyse01 and NanoLyse02 suitable as reference materials.

Spiked soups NanoLyse09 and NanoLyse10

Homogeneity of silica mass fractions was determined by ashing at 550 °C. Homogeneity of particle size distribution of NanoLyse 10 was assessed by FFF-ICPMS on 9 jars, with 7 replicate determinations performed on each jar. The results for s_{wb}, s_{bb}, and u^*_{bb} are shown in Table 1.

Blank-corrected ash mass fractions of 5.4±0.2 and 19.2± 0.2 g/kg (uncertainties are single standard deviations) were measured for NanoLyse09 and NanoLyse10, respectively. The within and the between bottle variations of NanoLyse09 are below 2 %. For NanoLyse10, a within bottle variation of 3.5 % was obtained. This, as well as the lower between bottle variation matches the homogeneity characteristics of NanoLyse01/02. Such match indicates an insignificant heterogeneity contribution of the soup as far as mass fractions are concerned. The total silica mass fraction of NanoLyse09 and NanoLyse10 was also determined in duplicate using ICP-OES. The obtained values were 6.2 and 19.3 g/kg, respectively, which agree with the values obtained by ashing within the respective uncertainties of the measurement results.

The median particle diameter as measured by FFF-ICPMS scatters significantly more: a between-unit variation of 16 % was observed [18]. Nevertheless, the variation is still significantly smaller than method reproducibility and the material is therefore fit for purpose as reference material.

Assessment of stability

Aqueous suspensions NanoLyse01 and NanoLyse02

For the aqueous silica nanoparticle systems (NanoLyse01, NanoLyse02), the short-term stability was assessed using an isochronous scheme with testing times of 0, 1, 2, and 4 weeks at 4 °C and 60 °C. The materials were measured by DLS using the cumulants method with relative standard deviations for the silica dispersions were below 1 %, thus allowing a good assessment of the stability of the materials.

A statistical analysis of the data revealed that the slopes of the regression lines for NanoLyse01 (both temperatures) and of NanoLyse02 (4 °C) were not different from zero on a 95 % confidence level. Although the slope of the regression line for NanoLyse02 (60 °C) differed from zero on a 95 %, but not on a 99 % confidence level, the extent of the potential change during transport even at 60 °C (observed change: 0.02 % per week) is technically negligible. The long-term stability data demonstrate that the materials remain stable when stored at ambient temperatures. Uncertainties of stability for storage of 2 years at 18 °C were calculated as 0.43 % (NanoLyse01) and 0.99 % (NanoLyse02) as described in [15].

DLS measurements on autoclaved samples (20 min at 121 °C on an AR092, JBTC, Sint Niklaas, Belgium) of NanoLyse01 and NanoLyse02 showed a reduction of the particle diameter to 85 and 95 nm, respectively. Thus, a thermal sterilization of materials containing silica nanoparticles is inappropriate.

Additionally, the silica dispersions were frozen at −70 °C for 10 min causing irreversible sedimentation (data not shown). The samples therefore should not be exposed to such condition. The results of all stability studies are summarized in Table 2.

Table 1 Homogeneity data on a) silica mass fraction of NanoLyse01 and NanoLyse02 by dry mass, silica mass fraction of NanoLyse09 and NanoLyse10 by ashing; b) silica nanoparticle size of NanoLyse01 and NanoLyse02 by DLS, silica nanoparticle size of NanoLyse09 and NanoLyse10 by FFF-MALLS; n.c.—not calculated as only one replicate analysis per sample was performed

		NanoLyse01	NanoLyse02	NanoLyse09	NanoLyse10
[a)] Mass fraction	Nominal [g/kg]	10	40	5	20
	Average [g/kg]	10.4	40.5	5.4	19.2
	s_{wb} [%]	0.97	2.2	1.9	3.5
	s_{bb} [%]	0.85	0.63	1.8	1.0
	u^*_{bb} [%]	0.46	1.04	0.6	1.1
[b)]Diameter	Average [nm]	135	135	Not measured	103
	s_{wb} [%]	n.c.	n.c.		15
	s_{bb} [%]	0.27	0.59		16
	u^*_{bb} [%]	n.c.	n.c.		2.3

Spiked soups NanoLyse09 and NanoLyse10

Only a tentative assessment of the stability of the soups can be given, as no real reference conditions for long-term stability are available, precluding therefore the use of an isochronous design. Nevertheless, several independent indications are available supporting the assumption of sufficient stability of the nanoparticles in soup:

- Addition of the silica to the soup led to a rapid precipitation of soup components. Particle size distributions obtained by FFF-ICPMS (see section below) after storage of the soup for several months still show similar particle size distributions for aqueous suspensions and soup, indicating reversible agglomeration and no severe ageing effects.
- Measurements by electron microscopy also show no reversible agglomeration after several months, also indicating absence of severe ageing.

The fact that addition of the silica led to precipitation is certainly a major drawback, but, as explained in the Section "concept", using suspensions is currently the only realistic way to allow assessment of trueness of methods. The results from the characterization (see below) show that the precipitate is re-suspendible by heating and ultrasonication. It is therefore possible to re-create the soup in its initial state. Unfortunetaly, the measurement methods are currently not accurate enough to assess whether this is also true after extended storage periods. While there is little doubt about the chemical stability of silica, the stability of the particle size distribution still requires further studies with more precise methods. Nevertheless, despite of the absence of any measurements shortly after processing, there is indication that the materials are unchanged after several months of storage at 4 °C.

Characterization

Two parameters (mass fraction, particle size) were characterised in dedicated studies. The mass fraction was determined by ICP-OES for NanoLyse01 and NanoLyse02 as well as NanoLyse09 and NanoLyse10.

Aqueous silica suspensions NanoLyse01 and NanoLyse02

Total and dissolved silica mass fractions For the aqueous silica nanoparticle systems (NanoLyse01, NanoLyse02), the total silica mass fraction as determined by ICP-OES was 11.9 ±1.4 and 41.2±1.4 g/kg (uncertainties are single standard deviations) for NanoLyse01and NanoLyse02, respectively. This is in accordance with the results of the gravimetrical determination. Therefore, the standard error of the dry mass determinations were adopted as uncertainty of characterization (u_{char}).

Ultrafiltration gave clear and colorless filtrates. Their SiO_2 concentrations as determined by ICP-OES were 90.3±2.0 and

Table 2 Summary of the results of the stability studies of NanoLyse01 and NanoLyse02

Storage condition	NanoLyse01	NanoLyse02
<0 °C	Irreversible sedimentation	Irreversible sedimentation
4 °C (4 weeks)	Stable	Stable
60 °C (4 weeks)	Stable	Particle diameter decreases by 0.02 % per week
18 °C (46 weeks)	Stable	Stable
	$u_{lts,2\ years}$ is 0.4 %	$u_{lts,2\ years}$ is 1.0 %

90.6±0.8 mg/L (uncertainties are single standard deviations) for NanoLyse01 and Nanoyse02, respectively. These values are negligible compared to the total silica mass fractions. Therefore, practically all silica is present in particulate form.

Particle size by DLS Different methods (cumulants, frequency, cross-correlation) at three different laboratories were used to conduct DLS measurements. On each of 3 days, one ampoule of NanoLyse01 and NanoLyse02 were measured in duplicate. A certified reference material (ERM-FD100; IRMM, Geel, Belgium) was measured on the first day to establish traceability and to demonstrate proper functioning and calibration of the equipment used. Representative particle diameter distribution graphs for silica dispersions as obtained by DLS are shown in Fig. 2 and the characterization results are shown in Table 3.

For NanoLyse01 and NanoLyse02, the results from Malvern Zetasizer using cumulants analysis differ significantly from results obtained with the other DLS instruments (see Table 3). Particle-particle interactions may partly explain this difference: measurements were performed undiluted, but also diluted to 2.5 g/kg. There was no difference for NanoLyse01, but a particle diameter of about 142 nm on the undiluted suspension of NanoLyse02 was obtained. However, the main reason for the difference is most likely the mode of evaluation, which yields different results from the cumulants method, frequency analysis and cross-correlation analysis as they show different sensitivities for deviation from monodispersity. While this may be surprising, as the analytical instruments are based on the same measurement principle and all report the hydrodynamic diameter, this observation has been already reported previously [19]. However, as the results differed not too widely (difference max-min is about 15 %), a general overall average over all methods was calculated. Also frequency analysis as performed by the Horiba LB-550 gave results that agreed with the other measurements and the results were therefore pooled with those from correlation analysis. Using the standard error of the mean of means as estimate of u_{char}, relative uncertainty values of 2.2 % (NanoLyse01) and 2.3 % (NanoLyse02) were obtained for the intensity weighted harmonic mean hydrodynamic diameter.

Particle size by FFF-ICPMS The size information for NanoLyse02 is again derived from the measurements obtained in the method validation study [18]. On three different days, three different samples were analysed in 7 replicates each, yielding 9 datasets of 7 individual results. An average mass-based median diameter of 58.1 nm was obtained for the average over all samples and days. The variation between datasets was significantly larger than the one within each dataset, so it was decided to base an uncertainty estimation on the variation of the means of datasets. The standard deviation of the mean results of the 9 datasets was 2.7 nm. Using the standard error of the mean values, a u_{char} of 1.5 % is obtained.

Particle size by electron microscopy The SEM images show irregular particles in various agglomeration/aggregation states, which may be due to the sample preparation (Fig. 3). The higher concentration of NanoLyse02 is reflected in a higher particle density on the grid.

The median diameter of NanoLyse01 and NanoLyse02 was obtained by performing duplicate SEM measurements on each of the two materials on 10 different days (20 results per material). Average median particle diameters of 57.2±3.4 and 59.9±2.7 nm were obtained for NanoLyse01 and NanoLyse02 (uncertainties are single standard deviations). Using the standard error of the mean as estimate of u_{char}, uncertainty contributions of 1.3 % and 1.0 % were obtained for NanoLyse01 and NanoLyse02. These low uncertainties stem from the fact that because the grand mean value is based on a high number of individual measurement averages (20), so the contribution of repeatability and between-run variation to the measurement uncertainty becomes very small. This estimate most likely underestimates the true uncertainty, as only one laboratory performed the characterization measurements, which by definition excludes any possibility to quantify a between-laboratory effect.

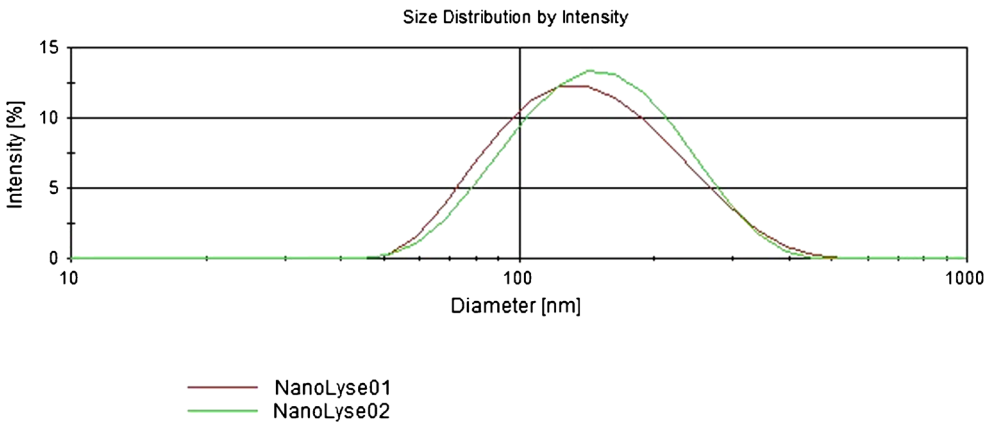

Fig. 2 Representative DLS intensity-weighted size distribution graphs for NanoLyse01 and NanoLyse02

Table 3 Results of the DLS characterisation study of the neat dispersions (NanoLyse01, NanoLyse02)

DLS method	Instrument	NanoLyse01	NanoLyse02
Correlation analysis, cumulants method	Malvern Zetasizer	133.2±1.3	133.3±1.4
	Nano ZS	134.2±1.7	135.5±1.2
	Malvern 41700C	152.1±5.8	142.3±2.9
Cross-correlation	LS Spectrometer 3D; NMR tubes	149.3±1.9	154.1±1.8
	LS Spectrometer 3D; Pyrex tubes	145.5±3.6	150.4±2.5
Frequency analysis	Horiba LB-550	146.1±6.6	142.9±6.7
	Average	143.4±7.9	143.1±8.1

The certified value for ERM-FD100 is 19.0±0.7 nm (cumulants analysis). Size is diameter average; uncertainties are single standard deviations

Particle size by GEMMA Particle diameters determined by GEMMA are based on sizing of several ten thousand particles at minimum. Mean number-based particle diameter as determined by GEMMA were 61.4±1.7 nm for NanoLyse01, 58.8±1.8 nm for NanoLyse02 (uncertainties are single standard deviations). The two results are not statistically significantly different on a 95 % confidence level. The diameters as determined at atmospheric pressure (no vacuum at all) by GEMMA agree very well with the results obtained by SEM. Using the standard error of the mean as estimate of u_{char}, uncertainty contributions of 1.6 % and 1.4 % were obtained for NanoLyse01 and NanoLyse02. Representative GEMMA spectra of NL01 and NL02 can be seen in Fig. 4.

Zeta potential and particle density The zeta potential of the silica colloidal dispersions were analyzed by means of ELS. NanoLyse01 and NanoLyse02 had a zeta potential of −44.7± 0.7 and −46.2±0.7 mV, respectively (uncertainty are single standard deviations).

Measurements of the density of 18 subsamples gave an average density of 2.010±0.042 g/cm^3 (uncertainty is a single standard deviation).

Soup materials NanoLyse09 and NanoLyse10

Particle size by electron microscopy Images by SEM clearly show increased agglomeration of silica NPs (Fig. 3), but a large number of NPs is still present as individual particles. Measurements by SEM indicate a shift of the number-based particle diameter distribution towards smaller particles with, compared to the pure suspensions, more particles in the range of 30–50 nm and fewer particles above 60 nm (Dudkiewicz et al., manuscript submitted). This results in lower median particle diameters for the silica in soup than in the neat suspension as shown in Fig. 5.

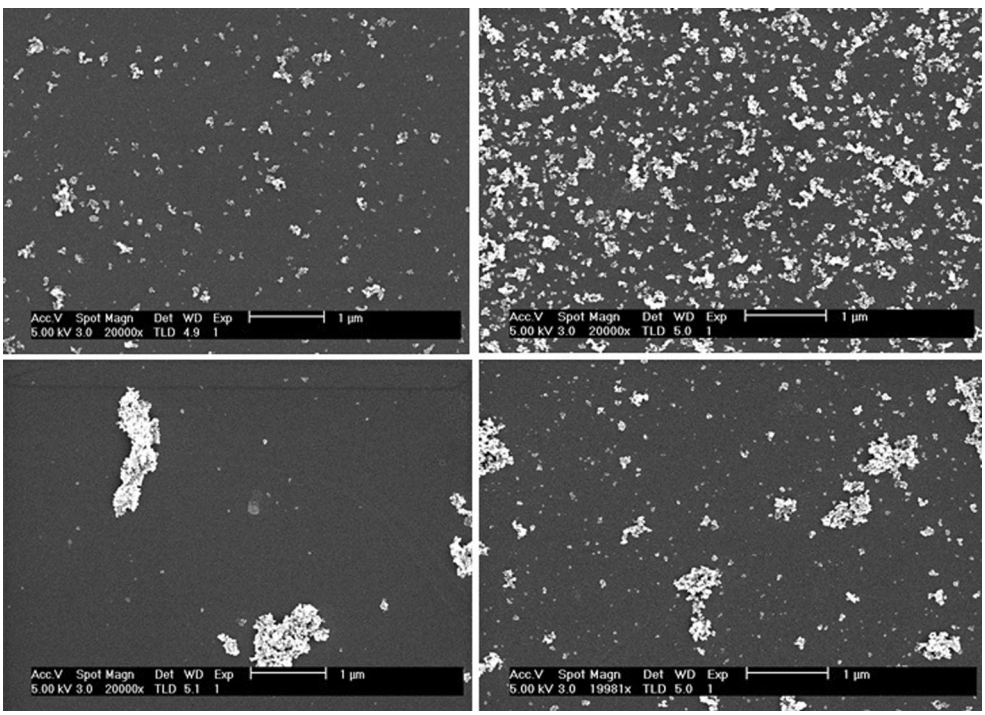

Fig. 3 SEM images of NanoLyse01 (*top left*), NanoLyse02 (*top right*), NanoLyse09 (*bottom left*), and NanoLyse10 (*bottom right*). *Size bars* are 1 µm

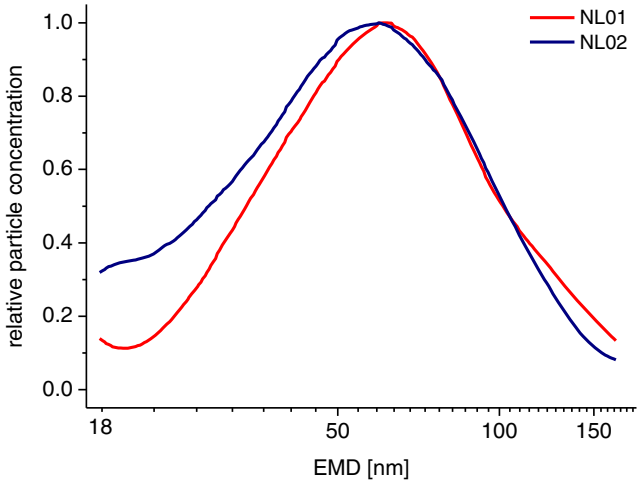

Fig. 4 GEMMA spectra of NanoLyse01 and NanoLyse02

The data from the method validation study were used for a tentative characterization of particle diameter by SEM. The average number-based median diameters of 20 replicate determinations were 43.7 (NanoLyse09) and 43.5 nm (NanoLyse 10) with standard deviations of 8.7 and 8.0 nm, respectively. These values are roughly in agreement with the number-weighted distributions as obtained by DLS for the spiking suspensions, especially taking into consideration the rather low reliability of the conversion between intensity to number based distributions for polydisperse, non-spherical particles. Using the standard error of the mean as estimate of u_{char}, uncertainty contributions of 4.5 % and 4.1 % were obtained for NanoLyse09 and NanoLyse10. Again, very low uncertainties were obtained due to the high number of average results on which the grand mean is based (20 individual median sizes).

Particle size by FFF-ICPMS The data from the homogeneity testing were also used for characterization of NanoLyse10 [18]. The average median particle diameter of the 63 measurements was 207.6 nm with a standard deviation of 42 nm. Again, using the standard error of the mean as estimate of u_{char}, an uncertainty contribution of 2.6 % was obtained.

The sizes obtained for NanoLyse10 differ significantly from those obtained on the neat suspension. This difference is an effect of the sample preparation by acid digestion. It is hypothesized that the time-lab between digestion and measurement is important with longer time-lags resulting in smaller differences, but also lower recoveries.

As is the case for EM, also here the high number of measurements result in a very small standard error of the mean, which is an underestimation of the true uncertainty. As there is currently no possibility to assess a potential between-laboratory effect, this is the best estimate available.

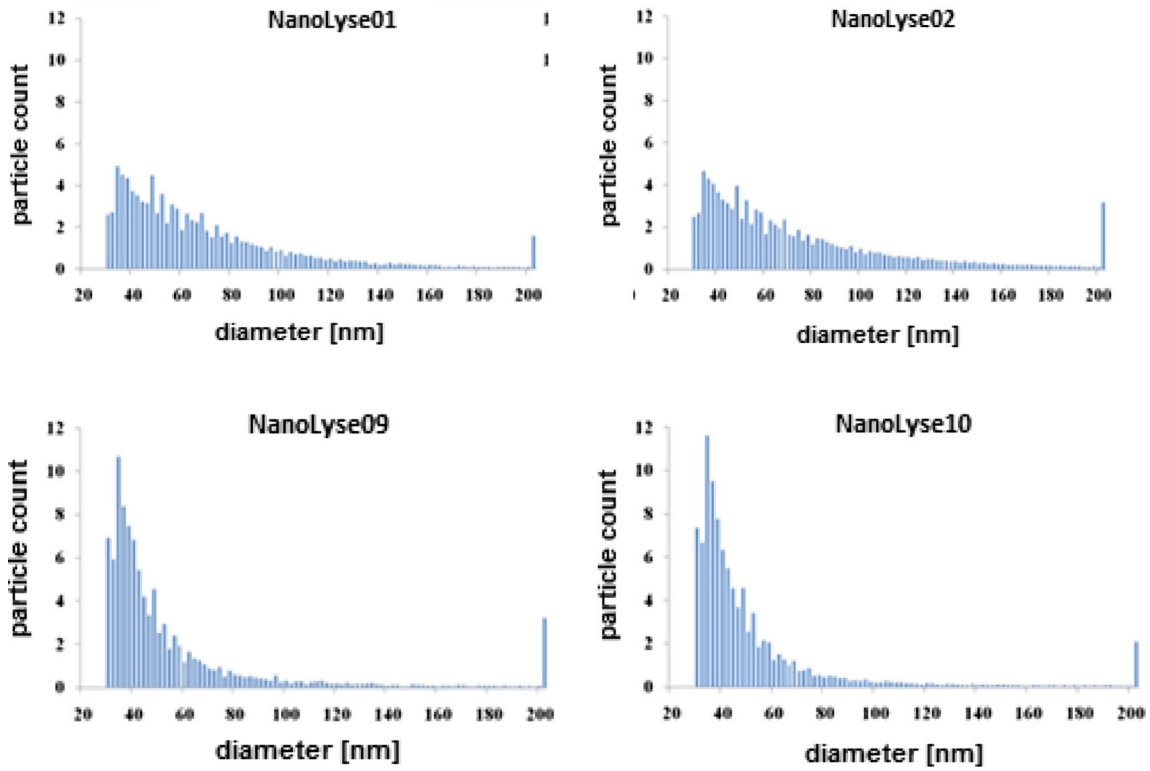

Fig. 5 Particle diameter distribution as measured by EM for neat suspensions (NanoLyse01, NanoLyse02) and silica in tomato soup (NanoLyse09, NanoLyse10) from. Size-cut off of the measurements is 30 nm

Tentative value assignment

The uncertainty budgets and assigned values are shown in Table 4. For the neat suspensions NanoLyse01 and NanoLyse02, the median diameters obtained by SEM, GEMMA, and FFF-ICPMS agree with each other. This agreement is surprising, as these methods are based on completely different method principles. FFF-ICPMS and GEMMA are influenced by the movement of the particles in a liquid or gas phase, whereas EM evaluates a static image. GEMMA and EM produce number based median diameters, whereas FFF-ICPMS produced mass-based median diameters. Finally, GEMMA is run at atmospheric pressure whereas EM under vacuum.

The results from these three methods differed from the average diameter obtained by DLS. This is expected, as the intensity-weighted mean value as obtained by DLS is much more influenced by a few large particles than the median diameters obtained by the other methods. Using the instrument software to convert the intensity-weighted diameters into number-weighted diameters gives averages of about 65 nm, i.e., in much closer agreement with the results obtained from the other methods (data not shown).

The work described here shows that the production of RMs, eventually even CRMs is feasible if the remaining issues regarding mainly stability and characterization are solved:

With respect to stability, the uncertainty of stability for the soup materials, currently used from the neat suspension, is only a first estimation. Measurements with more precise methods are required to assess whether the particles remain re-suspendable for extended time periods.

The second major question concerns the trueness of results. Data for GEMMA, FFF-ICPMS, and SEM were obtained by one laboratory only. The detection of unrecognized laboratory bias is the reason why many reference materials are characterized by a collaborative study involving multiple laboratories performing independent measurements [16]. The situation is less severe for the neat suspensions, where the agreement of the data from SEM and GEMMA indicates absence of major bias, but no such check is available for the soup materials. It is therefore not clear whether the change in the median particle diameter found by FFF and SEM is due to a method bias or due to real changes. Repeated measurements in several laboratories would be required to clarify this issue.

The values listed in Tables 3 and 4 therefore are tentative only and they must not be considered certified or even indicative values. This tentative nature of the these values has important consequences for the use of the materials: As no values are assigned, the materials cannot be used as ultimate proof of method accuracy. It should, however, be pointed out that the aim of this part of the project was a proof of principle of RM production and not the actual production of certified reference materials. The materials produced demonstrate the possibility of producing reference materials and indicate that even the production of certified RMs should be feasible. In addition, in the absence of any other RMs for nanoparticles in complex matrices, they do allow laboratories to compare their values with those obtained by other methods and laboratories. The materials therefore form the first step in the iterative process of improving analytical methods and reference materials.

Conclusion

This work highlighted some of the problems in developing reference materials for NPs in complex matrices. A major

Table 4 Uncertainty budget and assigned values for NanoLyse01, NanoLyse02, NanoLyse09, and NanoLyse10

		u_{bb} [%]	u_{lts} [%]	u_{char} [%]	Assigned value[e]
NanoLyse01	Mass fraction	0.89	0	0.29	10.4±0.2 g/kg
	Mean diameter by DLS[a]	0.3	0.4	2.2	135±7 nm
	Median diameter by SEM[b]	0.3	0.4	1.3	57±2 nm
	Median diameter by GEMMA[c]	0.3	0.4	1.6	61±2 nm
NanoLyse02	Mass fraction	1.0	0	0.5	40.5±1.0 g/kg
	Mean diameter by DLS[a]	0.6	1.0	2.3	135±7 nm
	Median diameter by SEM[b]	0.6	1.0	1.0	60±2 nm
	Median diameter by GEMMA[c]	0.6	1.0	1.4	59±2 nm
	Median diameter by FFF-ICPMS	4.6	1.0	1.5	58±6 nm
NanoLyse09	Mass fraction	1.8	0	0.47	5.4±0.2 g/kg
	Median diameter by SEM[b]	16	0.4	4.5	44±15 nm
NanoLyse10	Mass fraction	1.1	0	0.66	19.2±0.5 g/kg
	Median diameter by SEM[b]	16	1.0	4.1	44±15 nm
	Median diameter by FFF-ICPMS[d]	16	1.0	2.6	208±68 nm

[a] Intensity-weighted harmonic mean diameter as determined by DLS

[b] Number-weighted median diameter as determined by SEM

[c] Number weighted mean electrophoretic mobility diameter as determined by GEMMA

[d] Mass-weighted hydrodynamic diameter as determined by FFF-ICPMS

[e] Uncertainties are expanded uncertainties with a coverage factor $k=2$ corresponding to a level of confidence of about 95 %

issue for food materials is homogeneity, as silica acts as clarifying agent, leading to considerable precipitation of food components. Processing of the materials was made more difficult by the lack of methods for suitable process control. Existence of analytical methods are expected to lead to improved materials in the future.

Information on stability is limited, based on the only recently developed analytical methods. In addition, the application of isochronous measurements is not straightforward, as little information is currently available on what would be suitable reference conditions for nanoparticles in food. Also here, further knowledge will lead to more robust assessments of stability.

Finally, more extensive data on the reliability of methods for the characterization of nanoparticles in food matrices are necessary. Interlaboratory comparisons should help establishing reproducibility limits for the individual methods, allowing more reliable characterization.

Despite of these challenges, the work has demonstrated that development and characterization of reference materials for the detection and quantification of silica nanoparticles in liquid food is possible and that it should be feasible to assign values with acceptable uncertainties. The materials developed in this project might be the first step in the iterative improvement of methods and RMs for the analysis of nanoparticles in complex matrices.

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