Uncertainties of Size Measurements in Electron Microscopy Characterization of Nanomaterials in Foods

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Abstract

Electron microscopy is a recognized standard tool for nanomaterial characterization, and recommended by the European Food Safety Authority for the size measurement of nanomaterials in food. Despite this, little data have been published assessing the reliability of the method, especially for size measurement of nanomaterials characterized by a broad size distribution and/or added to food matrices. This study is a thorough investigation of the measurement uncertainty when applying electron microscopy for size measurement of engineered nanomaterials in foods. Our results show that the number of measured particles was only a minor source of measurement uncertainty for nanomaterials in food, compared to

the combined influence of sampling, sample preparation prior to imaging and the image analysis. The main conclusion is that to improve the measurement reliability, care should be taken to consider replications and matrix removal prior to sample preparation.

Keywords: Nanomaterials, Electron Microscopy, Food, Measurement Uncertainty, Minimal Sample Intake.

1. Introduction

Engineered nanomaterials (ENMs) are increasingly finding new applications in the food industry. Some food additives already used for decades (Dekkers et al., 2010) might be classified as nanomaterials, e.g. synthetic amorphous silica (SAS). Others as for instance silver ENMs are applied in food packaging (Chaudhry et al., 2008). The potential risks posed by the presence of ENMs in foods and food contact materials is an area of major interest because of the current uncertainties in relation to the potential consumer exposure to ENMs through food, and the fate and effects of the orally ingested ENMs in the body (Dudkiewicz, Luo, Tiede, & Boxall, 2012). In order for studies on ENMs to provide meaningful and accurate data to assess exposure appropriately developed and validated methods are required (Joner, Hartnik & Amundsen, 2008; Calzolai, Gilliland, & Rossi, 2012; Hassellöv, Readman, Ranville, & Tiede, 2008).

Electron microscopy (EM) is one of the standard methods that are currently used for ENM measurement (Calzolai et al., 2012) and also recommended for such use by the European Food Safety Authority (EFSA) in a guidance document (EFSA Scientific Committee, 2011). In the guidance document EM is listed as a method of first choice for ENM measurement in foods along other complementary methods. Nevertheless so far no validation of this technique for the characterization of ENMs has been presented. Only a few studies have assessed the uncertainty of ENMs size measurement by EM using spherical ENMs characterized by a narrow size distribution and in pristine dispersions e.g. (Braun, Kestens, Franks, Roebben, Lamberty & Linsinger, 2012; Lamberty, Franks, Braun, Kestens, Roebben & Linsinger, 2011). The presence of the food matrix in the sample is however expected to introduce difficulties during sample preparation and analysis (Tiede, Boxall, Tear, Lewis, David & Hassellöv, 2008; Dudkiewicz et al., 2012; Dudkiewicz et al., 2011) and is likely to affect the ENM measurement uncertainty. Food samples are usually characterized by a high

water content, and EM instruments operate under high vacuum. This means that samples at least need to be dehydrated for analysis. The EFSA acknowledges that sample preparation and in particular matrix removal can introduce changes to the original state of ENMs in the sample and thus preparation protocols involving minimal processing should be applied. Additionally only small sample volumes (order of pL) can be used during EM analysis, thus limiting the number of measured ENMs and affecting statistical reliability (Linsinger et al., 2013).

This paper presents an evaluation of EM procedures for the measurement of ENMs in foods using simple sample preparation methods which allow to retain ENMs in the food matrices. This study relies on two examples of reference materials, namely spherical silver nanoparticles (AgNPs) in meat and SAS in tomato soup covering narrow (AgNPs) and broad (SAS) size distributions. Both of these reference materials were produced within an EU FP7 funded project “NanoLyse” on the development and validation of analytical methodologies for ENMs in foods. The choice of ENMs reflects realistic scenarios in which humans could be exposed to ENMs that are applied in food packaging, potentially migrating to food (AgNPs) and ENMs readily applied as a food additive (SAS). The robustness of the obtained data from SAS containing reference materials was tested by analyzing a commercially available food product with declared content of SAS.

The study addressed three main questions: 1) how many ENMs need to be measured in order to obtain a reliable measure of size; 2) what is the precision of ENM measurement by EM; and 3) which step(s) within the procedure, including sampling, sample preparation, imaging and image analysis, contribute most to the measurement uncertainty?

2. Experimental design

2.1 Materials

The materials included in the study as well as characterization information provided by the manufacturer or determined in our laboratories are listed in Table 1. Two groups of reference food materials spiked with ENMs were used: These were chicken paste (Meat 1, Meat 2 and Meat Blank), and tomato soup (Soup 1, Soup 2 and Soup Blank). Meat reference materials contained AgNPs and soup reference materials contained SAS at the spiked concentrations listed in Table 1. These reference materials were developed by the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre (JRC-IRMM, Geel, Belgium). The development of soup and meat reference materials was described in (Grombe et al., 2014 and In press).

Along with the reference materials, the JRC-IRMM also provided pure suspensions of the respective ENMs that had been used in the preparation of these reference materials. The suspensions were also studied to provide information on the original characteristics of ENMs prior to spiking into foods as recommended (EFSA Scientific Committee, 2011). Additionally, a commercial soup powder (Soup COM) with a declared content of SAS- E551 was obtained from a local supermarket. As a control for the Soup COM, SAS powder (SAS COM)- NM203 from the JRC, Institute for Health and Consumer Protection, Nanomaterial Repository for Toxicology Testing (Ispra, Italy) was used.

Prior to the study, Soup COM and SAS COM were suspended in aqueous media using a magnetic stirrer. Soup COM was mixed at a ratio of 11:100 with boiling tap water. The SAS COM was mixed at a ratio 2:98 with borate buffer at pH 8.0 of composition 0.05M H_3BO_3, 0.05M KCl, 0.004M NaOH (BB 8.0).

2.2 Electron microscopy and energy dispersive x-ray spectroscopy

Two different EM methods were selected for imaging depending on the sample's matrix type (solid/liquid) and chemistry of the ENMs. The SAS has generally weak contrast in EM, however for imaging in scanning electron microscopy (SEM), samples can be coated with a nanometric layer of metal to improve contrast and minimize charging. AgNPs could be best visualized using TEM as these ENMs were embedded in a layer of the meat sample. Therefore for imaging of SAS and AgNPs containing samples, SEM and TEM were selected respectively.

Samples were prepared for analysis as described in Supplementary data section 2 and (Lari & Dudkiewicz, 2014). The preparation methods were developed and evaluated in our laboratories before use in this study. In course of this evaluation we have found that these sample preparation methods allowed to limit agglomeration of the ENMs (a typical artifact hampering image analysis) and recover sufficient number of ENMs for imaging and measurements.

The SEM images were taken using an FEI Sirion S field emission gun SEM equipped with a through the lens detector and operating at a voltage of 5 kV and spot size 3.

The TEM images were acquired with a JEOL JEM 2011 TEM operating at 200 kV and using a digital camera (Gatan 794).

2.3 Data acquisition and image analysis

All provided particle size measurements refer to the equivalent circle diameter (ECD) which is the diameter of the circle with the same surface area as projected in the 2D image of the ENMs. The data acquisition parameters used in this study were summarized in Table 2.

The images were taken from randomly selected places (predetermined coordinates) in the grid. SEM and TEM image area sizes were adjusted to capture and measure the maximal number of particles for the respective sample types (imaging at relatively low magnifications). As a result, the micrograph area was relatively large in proportion to the measured ENMs size. Hence, it was necessary to estimate a size cut-off point for the smallest measurable size of a particle. For SEM images with good contrast and large pixel size of 8.7 nm, the smallest measurable particle size (Table 2) was estimated experimentally (based on the evaluation by our laboratories using repetitive imaging and image analysis of mono-dispersed gold nanoparticles at decreasing magnification). For TEM images with poor contrast and small pixel sizes (1.6 nm) the smallest measurable particle size (Table 2) was chosen so as to minimize background interference during image analysis.

The acquired images were analyzed using object based image analysis (OBIA) software. A software solution within the eCognition® Architect framework (version 8.7.2, Trimble Geospatial) was specifically developed for semi-automated image analysis of ENMs in complex matrices by the Centre for Geoinformatics, University of Salzburg in Austria.

The levels of matrix interference (natural or contaminating nanomaterials) were investigated prior to analyses of food spiked with ENMs reference materials using blank food matrices provided also by JRC IRMM. The results proved that the contribution of interfering natural or contaminating nanomaterials to the measurement results was negligible in the blank with the selected cut-off values.

2.4 Quantification of uncertainty in particle size measurements related to measured sample number and broadness of the size distribution

A simulated approach previously applied for estimation of influence of the number of samples to precision of microbiological counts (Jarvis & Hedges 2011) was used to derive the dependence of ECD measurement uncertainty on the number of measured particles in the sub-set. This approach was based on re-sampling without replacement from large dataset (population) multiple sub-sets of data with given number of elements. Subsequently the measurement uncertainty was estimated based on variance of means from the obtained sub-sets featuring same number of re-sampled elements. Jarvis & Hedges (2011) showed that the variance between the means of data subsets was slightly and possibly not significantly larger in case of sampling without replacement compared to sampling with replacement (bootstrap). We preferred a more conservative estimate of the minimum required number of counted ENM to achieve a given measurement uncertainty and thus also chose re-sampling without replacement. Five of the samples listed in Table 1 (Meat 1, AgNPs 1, Soup 1, SAS 1, and SAS COM) were selected to cover different interquartile ranges of particle size distributions (given as relative to median *IQR%*). For each of these samples, 200 images recorded as part of the intermediate precision study (section 2.5) were used. For each sample, 1388 particles were randomly selected from 200 images. These 1388 particles from each sample were used to create a population and subjected to simulations. The simulations were based on random selection without replacement of either 25, 50, 75, 100, 150, 200, 250 and 500 particles from the population of each sample, and the process was repeated 500 times for each sample and particle sampling number. Median particle sizes and relative standard deviations (RSD_{pn}) between them were then estimated from the 500 sets for each sample and particle number. In order to investigate the magnitude of RSD_{pn} increase with increase of *IQR%*, the obtained

RSD_{pn} values were plotted against the *IQR%* values for each particle sampling number (Fig. 1A). In the following, the obtained dependencies of RSD_{pn} from *IQR%* were further used to fit a phenomelogical equation (Eq. 11) for calculation of standard relative uncertainty related to measured number of ENMs.

2.5 Intermediate precision and expanded uncertainty of particle size measurements

The materials listed in Table 1 were used to determine the intra-laboratory reproducibility (intermediate precision) of size measurement. The study setup was based on the routine protocol for analytical method validation as described in (Boque, Maroto, Riu, & Rius, 2002). For this, samples were prepared and imaged in duplicate on 10 different days spread through a period of four weeks.

Different vials of Meat 1 and 2 were prepared and analyzed every day. For Soup 1 and 2 it was decided to use only 1 jar over the 10 testing days due to the variability of the pH in between received jars (5.2-6.5), which could potentially affect particle size distribution. The opened jars were not refrigerated for the duration of the test. The Soup COM was freshly prepared on each day. Respective particle stock dispersions were sampled from one bottle during the whole test.

Data acquired from this test were used to calculate relative standard deviation (*RSD*) of the median particle ECD measurements for repeatability (RSD_r), day to day variation (RSD_{dd}), and intermediate precision (RSD_{ip}) according to equations (Eq.) 1-3:

$$RSD_r = \frac{100 \times \sqrt{MSW}}{s} \qquad \textbf{Eq. 1}$$

$$RSD_{dd} = 100 \times \frac{\sqrt{\frac{(MSB - MSW) + MSW}{n} \times e^{-\frac{MSB}{MSW}}}}{s} \quad \textbf{Eq. 2}$$

$$RSD_{ip} = \sqrt{RSD_r^2 + RSD_{dd}^2} \quad \textbf{Eq. 3}$$

Where:

MSW- median ECD mean squares of replicates measured on the same day

MSB- median ECD mean squares of replicates of all 10 days

s- mean ECD of the median measurements between replicates

The *MSW* and *MSB* were calculated by using the output from the "one way ANOVA function" available in Microsoft Office Excel 2007.

Eq. 2 was adapted from (Federer, 1968) as suggested in (Linsinger, Pauwels, van der Veen, Schimmel, & Lamberty, 2001) to allow calculation of RSD_{dd} for results, where *MSW*>*MSB*.

The RSD_r and RSD_{ip} obtained for two levels of concentrations of ENMs in the reference materials and relevant stock dispersions were compared using the F-test with significance level (p) of 0.05.

The expanded uncertainty as described in (ISO/IEC Guide 98-3:2008) gives a measure of an interval where the value is confidently within, and is obtained by combining all the sources of measurement uncertainty and multiplying by the coverage factor-*k* (*k*=2 for approximately 95% confidence interval). In this study the expanded uncertainty (U_{exp}) was derived combining RSD_{ip} and goodness of instrumental calibration (RU_t) according to Eq. 4.

$$U_{exp} = k \times \sqrt{RSD_{ip}^2 + RU_t^2} \quad \textbf{Eq. 4}$$

The RU_t values were 1.4% and 1.9% for TEM and SEM respectively and were calculated using the procedure described in the (Linsinger, 2010). The RU_t was determined by the measurement of ENMs reference material (NIST 30 nm gold nanoparticles, manufacturer's id: 8012).

2.6 Influence of data acquisition stages on intermediate precision

As the data acquisition from EM is more complex than in many other analytical methods, estimation of the relative uncertainty for each of the stages in the process was of interest. This was tested by using four selected reference materials: for SEM: SAS 1, Soup 1, and for TEM: AgNPs 2 and Meat 2. Four separate experiments were performed to assess *RSD* attributed to sampling (RSD_s), sample preparation (RSD_{sp}), imaging (RSD_i) and image analysis (RSD_{ia}). The following experiments were performed:

1) Sampling - 10 different portions of a sample were prepared on the same day and imaged within one day;

2) Sample preparation - 10 replicates of the same subsample were prepared on the same day, then imaged within a day;

3) Imaging - a single replicate was imaged on 10 different days; and

4) Image analysis – the same set of 10 images was analyzed 10 times (returning image analysis settings to default every time).

Experiments 1-3 resulted in *RSD* values (RSD_1, RSD_2 and RSD_3 respectively). Obtained this way *RSD* values represented uncertainty of several factors combined and not only the sought individual uncertainty contribution. Therefore to calculate individual *RSD* contributions, we used the root-sum-square manner subtraction Eq. 5-7 of inclusive uncertainties from RSD_1, RSD_2 and RSD_3 as proposed in (Boque et al., 2002).

$$RSD_s = \sqrt{RSD_1^2 - (RSD_{sp}^2 + RSD_{ia}^2 + RSD_{pn}^2)} \quad \textbf{Eq. 5}$$

$$RSD_{sp} = \sqrt{RSD_2^2 - (RSD_{ia}^2 + RSD_{pn}^2)} \quad \textbf{Eq. 6}$$

$$RSD_i = \sqrt{RSD_3^2 - (RSD_{ia}^2 + RSD_{pn}^2)} \quad \textbf{Eq. 7}$$

To validate values determined for contributing uncertainties their sum was calculated using Eq.8 and compared against intermediate precision values determined previously (as described in section 2.5).

$$RSD_{total} = \sqrt{RSD_s^2 + RSD_{sp}^2 + RSD_i^2 + RSD_{ia}^2 + RSD_{pn}^2} \quad \textbf{Eq. 8}$$

3. Results and discussion

3.1 Uncertainty in particle size measurements related to measured sample number and broadness of the size distribution

Linear relationships were obtained between *IQR%* and RSD_{pn} of median ECD measurements depending on measured number of particles (*N*) (Fig. 1A). Fits between R^2= 0.973 to 0.997 were achieved with an preset intercept of 0.0 and were described using Eq. 9. The slope coefficient *a* in Eq. 9 clearly depended on the number of particles, therefore dependence of *a* to *N* was shown in Fig. 1B. This dependence followed a power curve and was well described (R^2=0.998) by Eq. 10.

$$RSD_{pn} = a \times IQR\% \quad \textbf{Eq. 9}$$

$$a = 1.0071 \times N^{-0.553} \quad \textbf{Eq. 10}$$

The expected measurement uncertainty for samples with known *IQR%* and a defined sample size can be calculated as:

$$RSD_{pn} = 1.0071 \times N^{-0.553} \times IQR\% \qquad \textbf{Eq. 11}$$

Eq. 11 can be compared to a theoretically derived equation (Supplementary data, section 3, equation A1) adapted from work of Professor Hideto Yoshida, Hiroshima University, Japan in ISO standard draft (Draft ISO/WD 14411-2, Unpublished results). The comparison shows that both approaches do not give significantly different level of the RSD_{pn} for a given sample. Nevertheless, as the empirical Eq. 11 does not assume any particular particle size distribution and theoretical one refers to special case of normal distribution, Eq 11 is considered more practical for the ENMs studied here.

Using Eq. 11 for calculation of *N* for samples with different *IQR%*, and RSD_{pn} at the level of 5 and 1%, results shown in Table 3 were obtained.

This shows that, under the assumption that the size distribution of the particle population is sufficiently narrow, the minimum number of measured particles required to achieve RSD_{pn} of 5% may be much smaller than the 500 particles previously recommended for reliable measurement (Linsinger et al., 2013). Nevertheless to achieve a lower uncertainty of 1%, particle numbers need to be typically higher than 500. The acceptability of the RSD_{pn} threshold will ultimately depend on other contributing factors during data acquisition. This is further discussed in subsequent sections.

3.2 Intermediate precision, expanded uncertainty and trueness of particle size measurements

The intermediate precision (Eq. 3), expanded uncertainty (Eq. 4) and RSD_{pn} (calculated according to Eq. 11 and *N* and *IQR%* values from Table 1) were summarized in Fig. 2.

3.2.1 Number of measured particles and intermediate precision

The RSD_{pn} for all measured samples was significantly lower (1-7%) than RSD_{ip} (5-21%) (F test, $p<0.05$). This is in agreement with the published data on characterization of the reference materials for ENMs measurement. For example in the study of Braun et al. (2012), ENM with *IQR%* ~ 20 and 500 particles measured per replicate was characterized by EM in 11 different facilities. The RSD_{ip} measured between the laboratories ranged from 1.2 to 8.5 whereas calculated for this material from Eq. 11, $RSD_{pn}=0.6$. The result suggests that factors other than particle size distribution broadness and measured particle number must affect the measurement uncertainty.

3.2.2 Food matrix presence and intermediate precision

For samples containing SAS, the presence of the soup matrix significantly increased the uncertainty of the measurements (RSD_{ip} ranging 13-21%) when compared to the stock dispersions (RSD_{ip} ~5%) (F test, $p<0.05$). Contrary to this result, the RSD_{ip} were similar for AgNPs in stock and in meat at respective concentrations, i.e. 21-22% for the lower concentration and 8-10% for the higher one (F test, $p>0.05$). Therefore the presence of the matrix hampered reproducibility of measurement of ENMs only in soup samples. The uncertainty increase for the measurement of SAS in soup seemed to depend on the nature of the sample. SAS in the Soup COM were measured with 13% RSD_{ip}, whereas for Soup 1 and 2 RSD_{ip} exceeded 20%. For Soup 1 and 2, only one jar of the sample for the 10 testing days spread over period of four weeks was used. Nevertheless, there was no observable trend of changing particle size toward smaller or larger values with sampling time (Supplementary data, section 1, Fig. A2). Thus either a) subsamples taken at the same time point had a higher chance of being closely related by size, or b) imaging of the samples on different days introduced a major error to the measurement. This was further investigated in section 3.3.

3.2.3 Measurement uncertainties introduced by electron microscopy in comparison to other measurement methods

3.2.3.1 Nanomaterials in stock dispersions

Previously published data indicate that EM may offer similar or better uncertainties in measurement of ENMs in pristine dispersions compared to other techniques, such as e.g. dynamic light scattering (DLS), gas electrophoretic mobility molecular analyzer (GEMMA), centrifugal liquid sedimentation, or small angle neutron x-ray scattering (Braun et al., 2012; Braun et al. 2011; Kaiser & Waters, 2007a; Kaiser & Waters, 2007b; Small & Waters, 2012). Same ENMs dispersions as studied here were characterized also by Grombe et al. (2014 and In press) using dynamic light scattering (DLS) and GEMMA. Authors obtained similar uncertainties (*RSD* calculated from data given in cited publications as standard deviations of the median or mean measurements between replicates, corresponding to RSD_{ip}) for SAS 1 and 2 using GEMMA and DLS (3-6%) as SEM in this study (5 and 6%). Nevertheless, AgNPs 1 and 2 were measured with higher uncertainty by TEM (21 and 8% respectively) compared to GEMMA (8.2 and 2.7% respectively), but similar to DLS (measurements of these samples were carried out on 7 different instruments and the uncertainty values were ranging between these instruments from 2-16%). The low precision of TEM sizing of AgNPs in aqueous dispersion and especially AgNPs 1 could be an effect of sample inhomogeneity, sample preparation, or other problem with data acquisition, since similar uncertainty values were also obtained for AgNPs in Meat 1 and 2 samples.

3.2.3.2 Nanomaterials in food matrices

Recently publications on characterization of the studied here reference materials of SAS in Soup and AgNPs in Meat appeared (Grombe et al., 2014 and In press). In both cited studies authors used state of the art analytical methodologies. Reference material of SAS in Soup 2 was measured by means of asymmetric flow field-flow fractionation with inductively coupled

plasma-mass spectrometry detection (AF4-ICP-MS) and AgNPs in Meat 1 and 2 by means of single particle-inductively coupled plasma-mass spectrometry (SP-ICP-MS). Methods used by the authors for the preparation of the reference materials for AF4-ICP-MS and SP-ICP-MS analyses were based on matrix digestion (either by acid or enzymes according to protocols described by: Loeschner et al., 2013; Peters, Rivera, van Bemmel, Marvin, Weigel & Bouwmeester, 2014; Grombe et al., 2014). Digestion most likely allowed better homogenization of the samples prior to measurements compared to the sample preparation applied here, which aimed at retaining ENMs within the matrix for EM analysis. It was thus expected that ENMs measurements obtained by EM in this study were characterized by a higher uncertainty than ones generated by AF4-ICP-MS and SP-ICP-MS in (Grombe et al., 2014 and In press). As expected AgNPs in meat were measured with better precision by SP-ICP-MS (*RSD* of 5% for Meat 1 and 3% for Meat 2) than TEM (*RSD* of 19% for Meat 1 and 10% for Meat 2*).* Nevertheless SAS in Soup 2 was measured with similar precision by AF4-ICP-MS and SEM (21 and 20% respectively). These high standard deviations indicate either undetected effects in one of the steps of the analytical process or intrinsic inhomogeneity of the sample.

3.2.4. Trueness

Measurement trueness can only be estimated when a true value of the measured property is known. The reference materials used here were characterized by a range of different analytical techniques in Grombe et al., (2014; and In press). Previously Grombe et al. (2014) showed the SAS in Soup 2 measured by AF4-ICP-MS had nearly five-fold larger diameter compared to that measured by SEM here (208 and 44 nm respectively). It is expected that several factors contribute to the measurement discrepancies: differences in sample preparation (only dilution in case of SEM and matrix acid digestion for AF4-ICP-MS), size distribution being expressed either per particle number (SEM) or weight (AF4-ICP-MS) as

well as different measurement expressions (ECD for SEM, and hydrodynamic diameter for AF4-ICP-MS) being comparable in theory only for perfectly spherical ENMs (Bowen, 2002). Median diameters of AgNPs in Meat 1 and Meat 2 characterized by SP-ICP-MS (51 and 50 nm respectively; Grombe et al., In press) were nearly twice as large as those measured by TEM (27 and 26 nm respectively) in this study. Nevertheless, in previous work where authors measured AgNPs 1 and freshly spiked them into blank chicken meat matrix (Loeschner et al., 2013) SP-ICP-MS revealed AgNPs median diameter between 30-35 nm, regardless of the matrix presence which is closer related to the TEM measurements reported in Table 1 (26-32 nm for AgNPs in meat and stock dispersions). In this case it seems like ageing of AgNPs in the meat matrix affected the size reported by the SP-ICP-MS method.

Overall it becomes clear that estimation of the measurement trueness for ENMs in foods is a challenge, as all methods have their inherent bias and measured properties are often not the same. It is therefore difficult to assess which result should be trusted over others. Factors such as procedural/instrumental interferences, size measurement expression, cut-off points and limits of detection for the particle size all affect median size value and result interpretation.

3.3 Influence of data acquisition stages on the intermediate precision

The results presented in section 3.2 suggested that sample homogeneity might have been a major cause for increase of ENMs size measurement uncertainty in foods. As we have shown this was the case not only for EM but also for methods which were expected to be more robust, such as AF4-ICP-MS. To test if this was the case further experiments on the uncertainty level introduced by individual stages in the analysis process were performed on chosen reference materials (SAS 1, Soup 1, AgNPs 2 and Meat 2) as described in section 2.6. The results were summarized in Table 4.

362 The highest uncertainty in measurement of ENMs in food samples was attributed to the
363 sampling (for Meat 2 and Soup1 RSD_{sp}=8 and 11% respectively). At the same time the
364 sampling was affecting the measurement uncertainty of ENMs in stock dispersions very little
365 (RSD_s up to 1%).

366 Such results were partly expected. The EMs can analyze only a very small volume (in the
367 order of a few pL) of the sample at a time, and it seems that it is not possible to make food
368 products so homogenous as to ensure representativeness of such small sample volume.

369 The imaging, sample preparation, and image analysis were each expected to influence the
370 measurement uncertainty of the AgNPs in meat. This is because the particles were suspended
371 in meat matrix at different depths and it was not possible to fully focus on all of the particles
372 within the field of view. Additionally, the sample layer obtained in the preparation procedure
373 was thick (approximately 100 nm) and not uniform (up to 33 % *RSD* of the sample thickness
374 between different images- based on Lari & Dudkiewicz, 2014). This inevitably affected the
375 definition of particle boundaries and consequently the results of image analysis. It also means
376 that the instrumental performance had limited influence on the RSD_i of AgNPs in meat. An
377 interesting result is the better performance of sample preparation for AgNPs in meat
378 (RSD_{sp}=3%) than respective stock dispersion (RSD_{sp}=9%), which suggests that the presence
379 of the meat matrix may have prevented random ENMs clustering in course of sample
380 preparation. Agglomeration to an extent could be noted in stock dispersions of AgNPs
381 (Supplementary data, section 1, Fig. A1).

382 Imaging of the SAS in stock dispersion, yielded higher uncertainty (RSD_i=6%) than in soup
383 (RSD_i=2%). It is possible that for this sample the instrumental or operator performance on a
384 day-to-day basis and certain particle features (shape, size) may have had a significant impact
385 on the measurements. As with the increase of the size (on median particles in SAS 1 were
386 characterized by larger ECD than in Soup 1- Table 1), the particle perimeter increases, the

possible instrumental or operator variations in alignment, noise from the microscope surroundings (stage drifting), may cause a shift in the particle boundaries and affect size measurement more than in case of small, nearly spherical particles.

3.3.1 Combined uncertainty of data acquisition stages and intermediate precision

In theory the RSD_{total} (Eq. 8) should be equal to RSD_{ip} (Eq. 3) if all contributing elements were included in Eq. 8. Indeed the RSD_{total} was very similar to RSD_{ip} (Table 4 and Fig. 2, a difference of 1 %) for all the samples, with the exception of Soup 1. The estimated RSD_{total} for Soup 1 (14%) had values closer to the previously estimated RSD_{ip} of Soup COM (13%) rather than of Soup 1 (20%). It is hypothesized that the degradation of liquid soup matrix over the precision test duration (four weeks) caused dynamic changes in the particle size. Particles' random agglomeration and release from complexes with soup solids due to the bacterial/ oxidative activity, pH and ionic strength changes could result in a very high day-to-day size measurement variation. The result also emphasizes robustness of derived RSD_{ip} value for the measurement of SAS in very different food matrices (fully liquid reference material, and commercially processed powder).

The SAS as E551 food additive is mainly used in food powders and therefore RSD_{ip} derived for Soup COM relates to the case of this additive better than Soup 1 and 2. Nevertheless, for other types of ENMs, the obtained information in study of Soup 1 and 2 might be useful in relation to liquid foods, where the matrix changes will have to be considered as one of the factors that might influence particle size and measurement uncertainty.

4. Conclusions

In our study a partial validation of the two main electron microscopy methods - SEM and TEM - for the measurement of ENMs in solid and liquid food matrices was achieved. In the process, we addressed the issues of measurement uncertainty and minimal sample size required for adequate EM measurements.

We found that the EM methods were able to measure ENMs in food with typically an expanded uncertainty of around 21-27% accounting for different samples (solid and liquid food matrix, ENMs with narrow and broad size distribution, different imaging conditions and sample preparation methods). This study will therefore be useful in predicting uncertainties associated with the measurement of ENMs in complex matrices by EM, where the ENMs are relatively stable. For samples containing particles that are undergoing constant transformation e.g. aggregation and/or dissolution, much greater expanded uncertainties may be expected. For example, an expanded uncertainty of 43% was derived in this study for liquid soup samples containing SAS that were analyzed at different time points.

The study also showed that a number of factors can influence uncertainties in the particle size measurements by EM methods. The results have indicated that the number of measured particles and small sample intake were only secondary contributors to the ENMs size measurement uncertainty in foods. The major factor was the sampling step. Most food samples are inherently inhomogeneous, and cannot be homogenized to the nanoscale. As a result, different sub-samples of the same sample may vary a lot in terms of particle size. To overcome the sampling issue a viable option may be to digest the food matrix or extract the particles, instead of the homogenization steps tested in this study. However, such pretreatment is likely to change particle characteristics and in consequence lead to inaccurate results. Furthermore comparison of the measurement uncertainties related to EM against

other analytical techniques also suggested that if ENMs undergo dynamic changes in the food sample, even matrix removal will not improve measurement precision.

Alternative possibility for improvement of particle size measurement precision is to increase the sample replication during routine analysis. As it is shown here, the particle quantities necessary to obtain reliable data on median size measurement would depend on broadness of the size distribution and the desired measurement confidence level, which can be calculated from a simple dependence as outlined in Eq 11. Therefore cutting the number of measured particles to an essential minimum, and increasing the number of replication instead, would allow acquisition of more precise information on the particle size and a better characterization of the sample.

In summary, with few considerations EM can be successfully applied for the measurement of ENMs in foods. Nevertheless further work is required to address few existing issues, such as measurement trueness of ENMs especially characterized by a broad size distribution and non-spherical shape as studied here example of SAS. For this further developments allowing cross comparison of the data outputs from EM and other techniques or/ and reference materials are needed.

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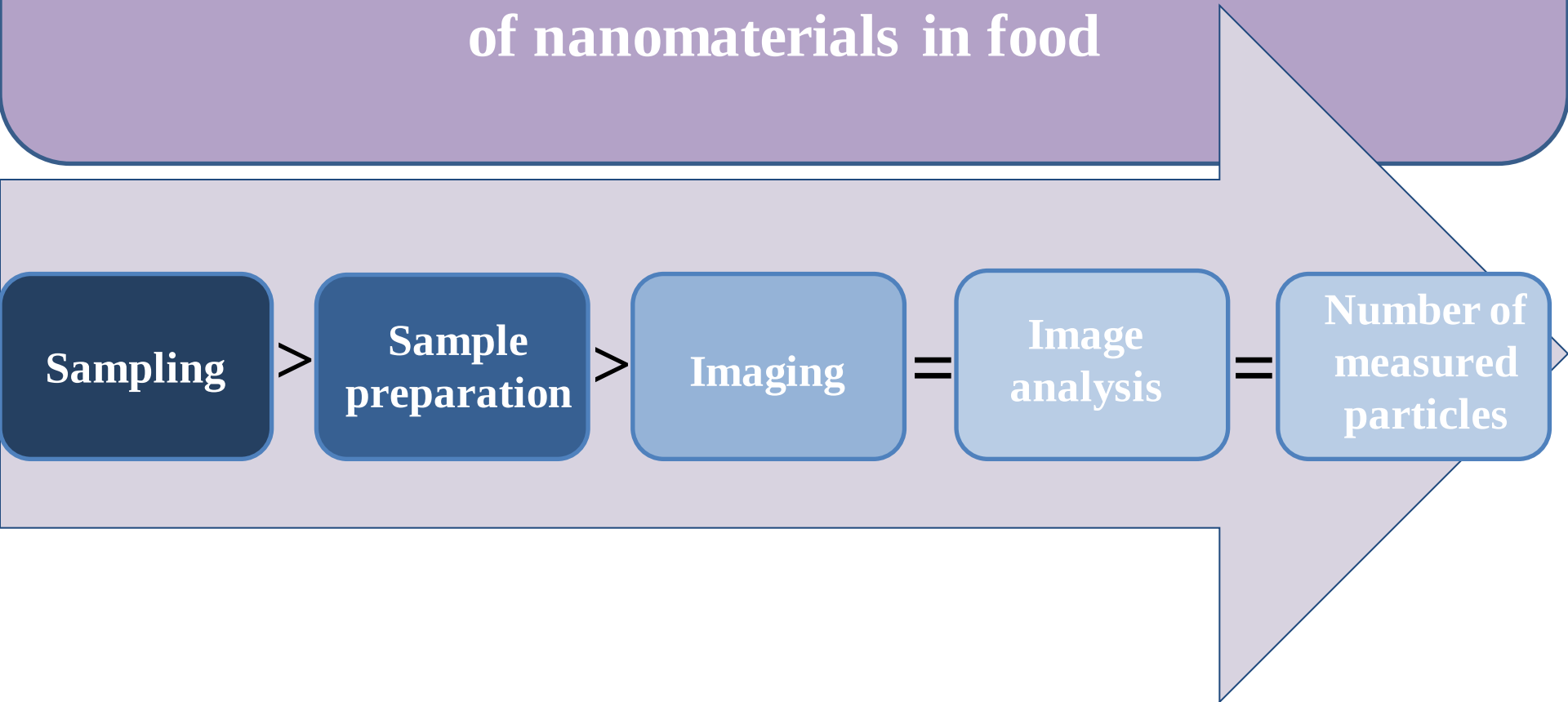
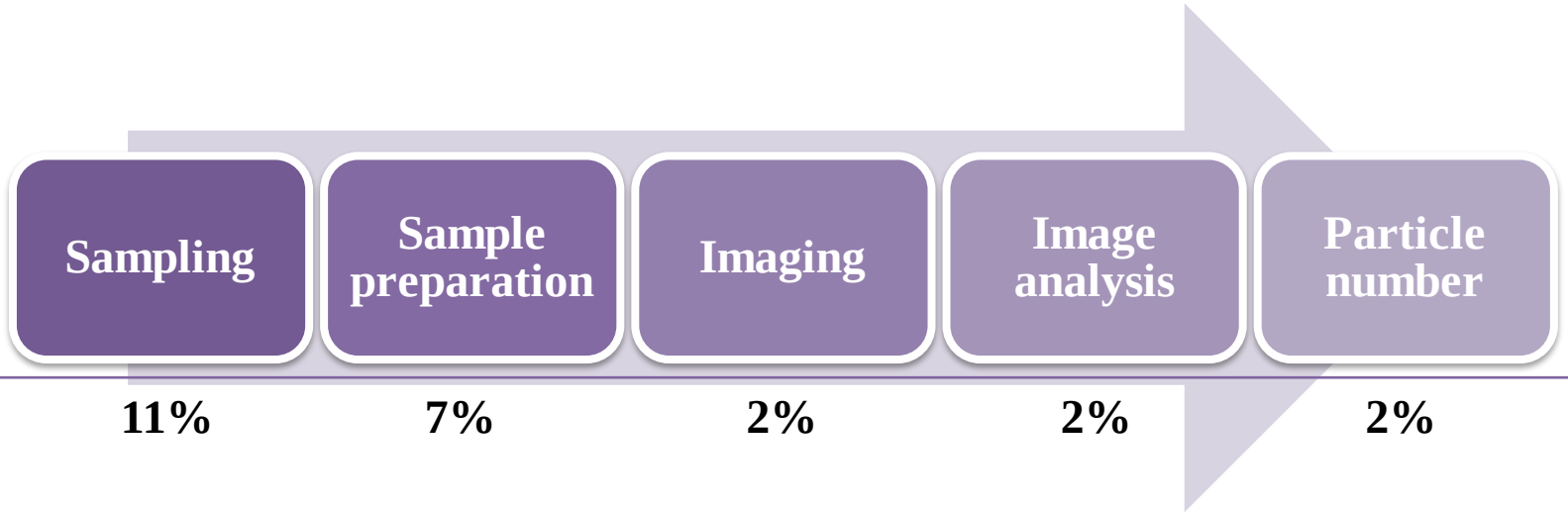
Importance of stages in data acquisition to uncertainty of electron microscopy measurements of nanomaterials in food





oup 1	11%	7%	2%	2%	2%	Tota 14%
AS 1	1%	1%	6%	1%	2%	7%
Ieat 2	8%	3%	3%	3%	3%	10%
gNPs 2	negligible	9%	negligible	2%	3%	9%

Table 1 List of the materials used. NanoLyse labeling from Grombe et al. (2014 and In press) provided to allow comparison of data

Sample	Type of particles	Concentration of core particle % w/w	Declared average particle size	Median [IQR][a]	
				size (nm)[b]	number[b]
Meat 1 (NanoLyse13)	Ag coated with PVP[c]	0.01	-	27 [12]	32 [24]
Meat 2 (NanoLyse14)		0.05	-	26 [10]	83 [87]
AgNPs 1 (NanoLyse03)		0.02	42±10 nm by TEM	30 [11]	47 [29]
AgNPs 2 (NanoLyse04)		0.1	42±10 nm by TEM	32 [11]	163 [35]
Soup 1 (NanoLyse09)	Synthetic amorphous SiO_2 stabilized with NaOH	0.5	-	42 [24]	264 [493]
Soup 2 (NanoLyse10)		2	-	41 [21]	909 [987]
SAS 1 (NanoLyse01)		1	120 nm by SLS[d]	57 [40]	1361 [770]
SAS 2 (NanoLyse02)		4	120 nm by SLS[d]	60 [49]	5640 [951]
SAS COM	Synthetic amorphous SiO_2 (E551)	~2	-	53 [57]	1190 [463]
Soup COM		0.28[e]	-	57 [40]	305 [528]

[a]Interquartile range, [b]values for ENMs size and number of particles counted (per replicate- 1 EM grid) obtained by characterization with transmission electron microscopy (TEM)- AgNPs containing samples, and scanning electron microscopy (SEM)- SAS containing samples based on intermediate precision study data (for full size distribution and EM images see Supplementary data, Fig. A1), [c]Polyvinylpyrrolidone, [d]static light scattering, [e]refers to powder, measured using ICP-MS Thermo Axiom instrument at Food and Environment Research Agency, UK..

Table 2 Data acquisition parameters

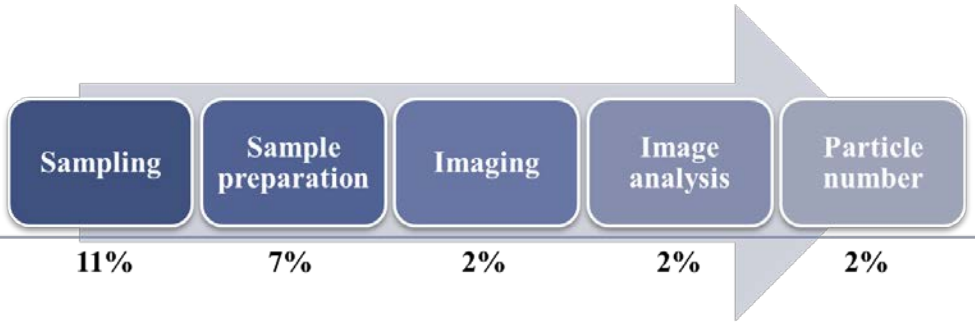
Technique	**Area of a single image (µm x µm)**	**Pixel size (nm)**	**Smallest particle area (no. of pixels)**	**Smallest particle ECD (nm)**	**No. of images analysed per replicate**	**Volume analyzed per replicate (mL)**
SEM	6.3 x 4.73	8.7	15	30	10	Cannot be specified
TEM	1.6 x 1.6	1.6	80	16	10	2.8×10^{-9} [a]

[a]refers to the volume of Meat 1 and 2 sample with a density of 1.0 g/mL

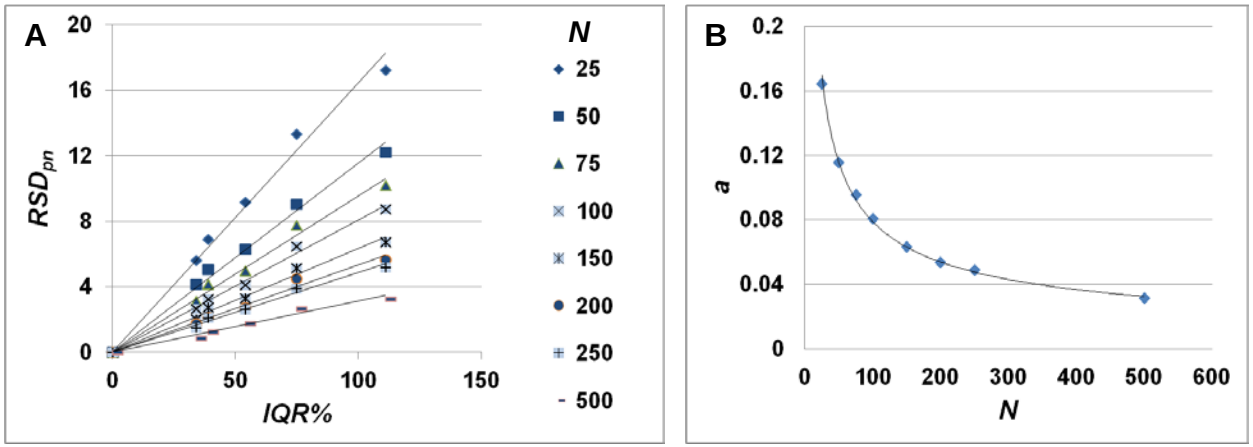
Table 3 The smallest number of particles necessary to obtain a desired level of RSD_{pn} of the median ECD for particle populations with known *IQR%* according to Eq. 11

IQR%	**Numbered of particles needed for targeted RSD_{pn}**	
	RSD_{pn}=5	**RSD_{pn}=1**
34	38	994
39	49	1630
54	91	5260
75	170	17166
111	359	70424

Table 4 The contribution of the stages in the data acquisition process to the RSD_{total}



Soup 1	**11%**	**7%**	**2%**	**2%**	**2%**	**Total 14%**
SAS 1	**1%**	**1%**	**6%**	**1%**	**2%**	**7%**
Meat 2	**8%**	**3%**	**3%**	**3%**	**3%**	**10%**
AgNPs 2	**negligible**	**9%**	**negligible**	**2%**	**3%**	**9%**


A
20
16
12
8
4
0
RSD_{pn}
0
50
100
150
IQR%
N
25
50
75
100
150
200
250
500
B
0.2
0.16
0.12
0.08
0.04
0
a
0
100
200
300
400
500
600
N

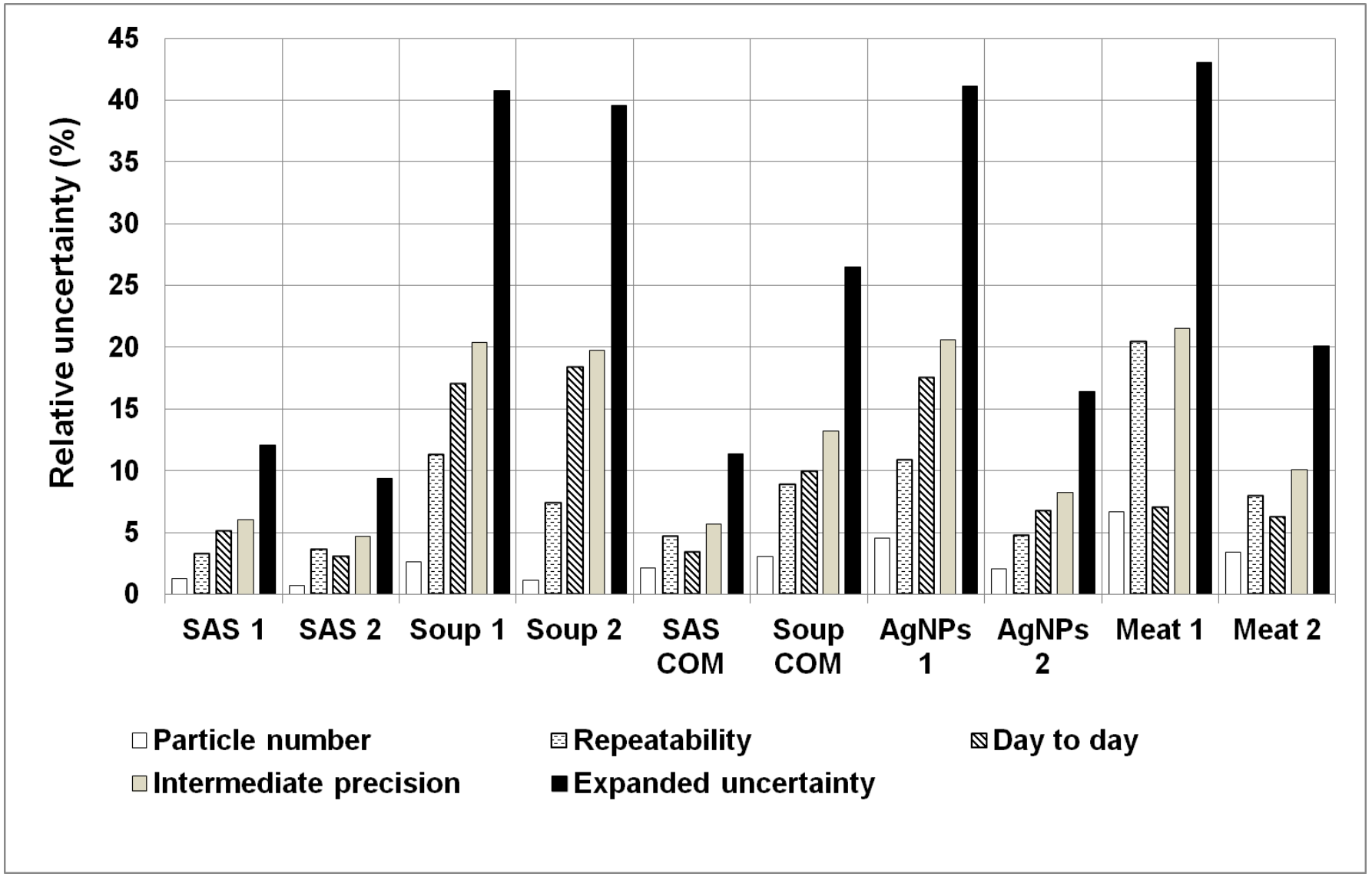

Relative uncertainty (%)
45
40
35
30
25
20
15
10
5
0
SAS 1
SAS 2
Soup 1
Soup 2
SAS COM
Soup COM
AgNPs 1
AgNPs 2
Meat 1
Meat 2
Particle number
Repeatability
Day to day
Intermediate precision
Expanded uncertainty

Fig. 1. (**A)** Dependence of median size measurement RSD_{pn} of the sample size *N* to *IQR%* and (**B)** Relationship between slope coefficient *a* of Eq. 11 and *N*.

Fig. 2 The median ECD particle number, repeatability, day to day, intermediate precision and expanded uncertainty for ENMs measured in respective samples.