

1 **Uncertainties of Size Measurements in Electron Microscopy**

2 **Characterization of Nanomaterials in Foods**

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14 **Abstract**

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

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

26

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

29 **1. Introduction**

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

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

54 water content, and EM instruments operate under high vacuum. This means that samples at
55 least need to be dehydrated for analysis. The EFSA acknowledges that sample preparation
56 and in particular matrix removal can introduce changes to the original state of ENMs in the
57 sample and thus preparation protocols involving minimal processing should be applied.
58 Additionally only small sample volumes (order of pL) can be used during EM analysis, thus
59 limiting the number of measured ENMs and affecting statistical reliability (Linsinger et al.,
60 2013).

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

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

76

77 **2. Experimental design**

78 **2.1 Materials**

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

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

96 Prior to the study, Soup COM and SAS COM were suspended in aqueous media using a
97 magnetic stirrer. Soup COM was mixed at a ratio of 11:100 with boiling tap water. The SAS
98 COM was mixed at a ratio 2:98 with borate buffer at pH 8.0 of composition 0.05M H₃BO₃,
99 0.05M KCl, 0.004M NaOH (BB 8.0).

100 **2.2 Electron microscopy and energy dispersive x-ray** 101 **spectroscopy**

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

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

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

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

119 **2.3 Data acquisition and image analysis**

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

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

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

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

143 **2.4 Quantification of uncertainty in particle size measurements**
144 **related to measured sample number and broadness of the size**
145 **distribution**

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

168 RSD_{pn} values were plotted against the $IQR\%$ values for each particle sampling number (Fig.
169 1A). In the following, the obtained dependencies of RSD_{pn} from $IQR\%$ were further used to
170 fit a phenomenological equation (Eq. 11) for calculation of standard relative uncertainty related
171 to measured number of ENMs.

172 **2.5 Intermediate precision and expanded uncertainty of particle** 173 **size measurements**

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

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

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

$$RSD_r = \frac{100 \times \sqrt{MSW}}{s} \quad \text{Eq. 1}$$

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

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

188 Where:

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

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

191 *s*- mean ECD of the median measurements between replicates

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

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

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

199 The expanded uncertainty as described in (ISO/IEC Guide 98-3:2008) gives a measure of an
200 interval where the value is confidently within, and is obtained by combining all the sources of
201 measurement uncertainty and multiplying by the coverage factor-*k* (*k*=2 for approximately
202 95% confidence interval). In this study the expanded uncertainty (*U_{exp}*) was derived
203 combining *RSD_{ip}* and goodness of instrumental calibration (*RU_t*) according to Eq. 4.

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

204

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

209 **2.6 Influence of data acquisition stages on intermediate precision**

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

- 216 1) Sampling - 10 different portions of a sample were prepared on the same day and imaged
217 within one day;
- 218 2) Sample preparation - 10 replicates of the same subsample were prepared on the same day,
219 then imaged within a day;
- 220 3) Imaging - a single replicate was imaged on 10 different days; and
- 221 4) Image analysis – the same set of 10 images was analyzed 10 times (returning image
222 analysis settings to default every time).

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

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

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

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

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

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

231 **3. Results and discussion**

232 **3.1 Uncertainty in particle size measurements related to** 233 **measured sample number and broadness of the size distribution**

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

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

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

240

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

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

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

250 Using Eq. 11 for calculation of N for samples with different $IQR\%$, and RSD_{pn} at the level of
251 5 and 1%, results shown in Table 3 were obtained.

252 This shows that, under the assumption that the size distribution of the particle population is
253 sufficiently narrow, the minimum number of measured particles required to achieve RSD_{pn} of
254 5% may be much smaller than the 500 particles previously recommended for reliable
255 measurement (Linsinger et al., 2013). Nevertheless to achieve a lower uncertainty of 1%,
256 particle numbers need to be typically higher than 500. The acceptability of the RSD_{pn}
257 threshold will ultimately depend on other contributing factors during data acquisition. This is
258 further discussed in subsequent sections.

259 **3.2 Intermediate precision, expanded uncertainty and trueness of** 260 **particle size measurements**

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

263 **3.2.1 Number of measured particles and intermediate precision**

264 The RSD_{pn} for all measured samples was significantly lower (1-7%) than RSD_{ip} (5-21%) (F
265 test, $p < 0.05$). This is in agreement with the published data on characterization of the
266 reference materials for ENMs measurement. For example in the study of Braun et al. (2012),
267 ENM with $IQR\% \sim 20$ and 500 particles measured per replicate was characterized by EM in
268 11 different facilities. The RSD_{ip} measured between the laboratories ranged from 1.2 to 8.5
269 whereas calculated for this material from Eq. 11, $RSD_{pn}=0.6$. The result suggests that factors
270 other than particle size distribution broadness and measured particle number must affect the
271 measurement uncertainty.

272 **3.2.2 Food matrix presence and intermediate precision**

273 For samples containing SAS, the presence of the soup matrix significantly increased the
274 uncertainty of the measurements (RSD_{ip} ranging 13-21%) when compared to the stock
275 dispersions ($RSD_{ip} \sim 5\%$) (F test, $p < 0.05$). Contrary to this result, the RSD_{ip} were similar for
276 AgNPs in stock and in meat at respective concentrations, i.e. 21-22% for the lower
277 concentration and 8-10% for the higher one (F test, $p > 0.05$). Therefore the presence of the
278 matrix hampered reproducibility of measurement of ENMs only in soup samples. The
279 uncertainty increase for the measurement of SAS in soup seemed to depend on the nature of
280 the sample. SAS in the Soup COM were measured with 13% RSD_{ip} , whereas for Soup 1 and
281 2 RSD_{ip} exceeded 20%. For Soup 1 and 2, only one jar of the sample for the 10 testing days
282 spread over period of four weeks was used. Nevertheless, there was no observable trend of
283 changing particle size toward smaller or larger values with sampling time (Supplementary
284 data, section 1, Fig. A2). Thus either a) subsamples taken at the same time point had a higher
285 chance of being closely related by size, or b) imaging of the samples on different days
286 introduced a major error to the measurement. This was further investigated in section 3.3.

287 **3.2.3 Measurement uncertainties introduced by electron microscopy in** 288 **comparison to other measurement methods**

289 **3.2.3.1 Nanomaterials in stock dispersions**

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

307 **3.2.3.2 Nanomaterials in food matrices**

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

312 plasma-mass spectrometry detection (AF4-ICP-MS) and AgNPs in Meat 1 and 2 by means of
313 single particle-inductively coupled plasma-mass spectrometry (SP-ICP-MS). Methods used
314 by the authors for the preparation of the reference materials for AF4-ICP-MS and SP-ICP-
315 MS analyses were based on matrix digestion (either by acid or enzymes according to
316 protocols described by: Loeschner et al., 2013; Peters, Rivera, van Bommel, Marvin, Weigel
317 & Bouwmeester, 2014; Grombe et al., 2014). Digestion most likely allowed better
318 homogenization of the samples prior to measurements compared to the sample preparation
319 applied here, which aimed at retaining ENMs within the matrix for EM analysis. It was thus
320 expected that ENMs measurements obtained by EM in this study were characterized by a
321 higher uncertainty than ones generated by AF4-ICP-MS and SP-ICP-MS in (Grombe et al.,
322 2014 and In press). As expected AgNPs in meat were measured with better precision by SP-
323 ICP-MS (*RSD* of 5% for Meat 1 and 3% for Meat 2) than TEM (*RSD* of 19% for Meat 1 and
324 10% for Meat 2). Nevertheless SAS in Soup 2 was measured with similar precision by AF4-
325 ICP-MS and SEM (21 and 20% respectively). These high standard deviations indicate either
326 undetected effects in one of the steps of the analytical process or intrinsic inhomogeneity of
327 the sample.

328 **3.2.4. Trueness**

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

337 well as different measurement expressions (ECD for SEM, and hydrodynamic diameter for
338 AF4-ICP-MS) being comparable in theory only for perfectly spherical ENMs (Bowen, 2002).
339 Median diameters of AgNPs in Meat 1 and Meat 2 characterized by SP-ICP-MS (51 and 50
340 nm respectively; Grombe et al., In press) were nearly twice as large as those measured by
341 TEM (27 and 26 nm respectively) in this study. Nevertheless, in previous work where authors
342 measured AgNPs 1 and freshly spiked them into blank chicken meat matrix (Loeschner et al.,
343 2013) SP-ICP-MS revealed AgNPs median diameter between 30-35 nm, regardless of the
344 matrix presence which is closer related to the TEM measurements reported in Table 1 (26-32
345 nm for AgNPs in meat and stock dispersions). In this case it seems like ageing of AgNPs in
346 the meat matrix affected the size reported by the SP-ICP-MS method.

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

353 ***3.3 Influence of data acquisition stages on the intermediate*** 354 ***precision***

355 The results presented in section 3.2 suggested that sample homogeneity might have been a
356 major cause for increase of ENMs size measurement uncertainty in foods. As we have shown
357 this was the case not only for EM but also for methods which were expected to be more
358 robust, such as AF4-ICP-MS. To test if this was the case further experiments on the
359 uncertainty level introduced by individual stages in the analysis process were performed on
360 chosen reference materials (SAS 1, Soup 1, AgNPs 2 and Meat 2) as described in section 2.6.
361 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

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

390 **3.3.1 Combined uncertainty of data acquisition stages and intermediate** 391 **precision**

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

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

408 **4. Conclusions**

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

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

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

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

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

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

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

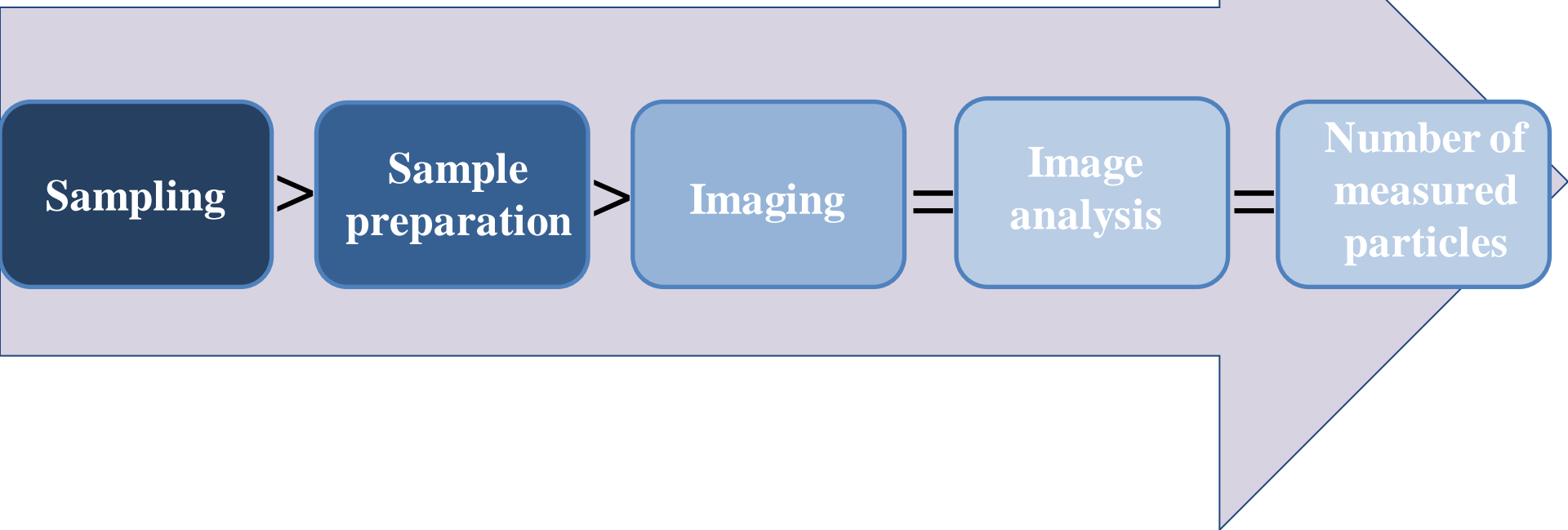
Sampling

Sample preparation

Imaging

Image analysis

Number of measured particles





	Sampling	Sample preparation	Imaging	Image analysis	Particle number	Total
Group 1	11%	7%	2%	2%	2%	14%
AS 1	1%	1%	6%	1%	2%	7%
Heat 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]

^aInterquartile range, ^bvalues 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), ^cPolyvinylpyrrolidone, ^dstatic light scattering, ^erefers to powder, measured using ICP-MS Thermo Axiom instrument at Food and Environment Research Agency, UK..

Table 2 Data acquisition parameters

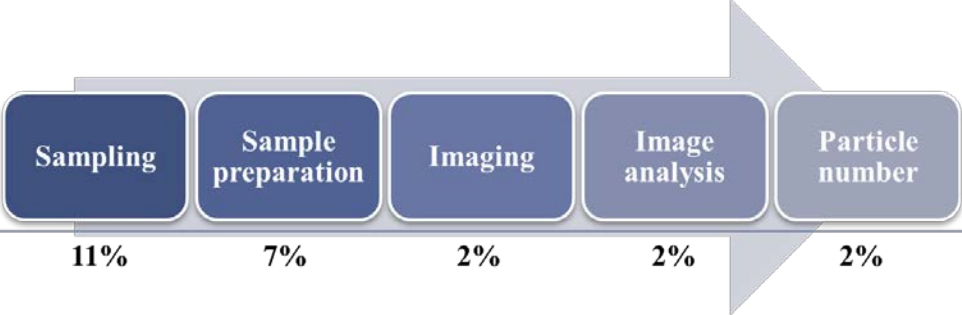
Technique	Area of a single image ($\mu\text{m} \times \mu\text{m}$)	Pixel size (nm)	Smallest particle area (no. of pixels)	Smallest particle ECD (nm)	No. of images analysed per replicate	Volume analyzed per replicate (mL)
SEM	6.3 x 4.73	8.7	15	30	10	Cannot be specified
TEM	1.6 x 1.6	1.6	80	16	10	2.8×10^{-9}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

^arefers to the volume of Meat 1 and 2 sample with a density of 1.0 g/mL

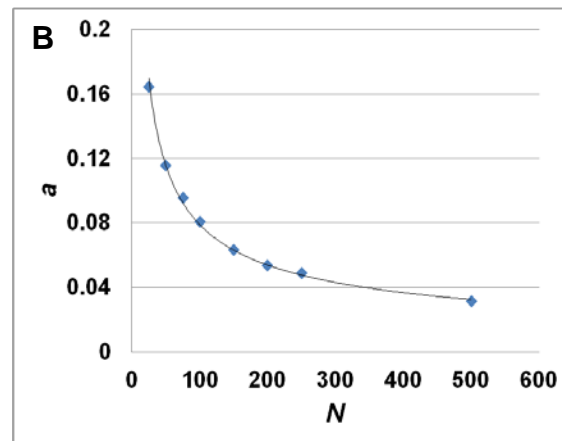
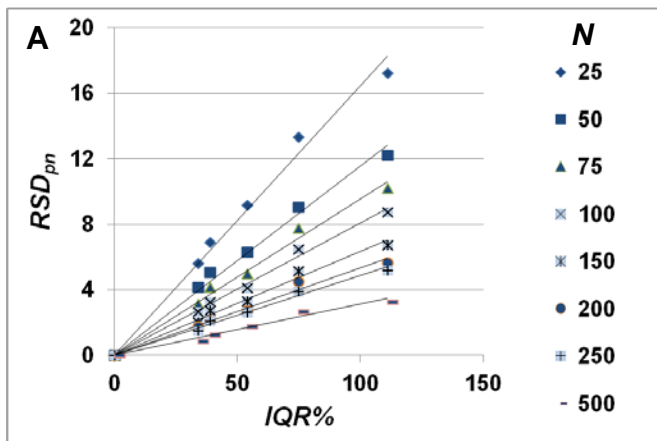
Table 3 The smallest number of particles necessary to obtain a desired level of RSD_{pn} of the median ECD for particle populations with known $IQR\%$ according to Eq. 11

$IQR\%$	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}



	Sampling	Sample preparation	Imaging	Image analysis	Particle number	Total
Soup 1	11%	7%	2%	2%	2%	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%



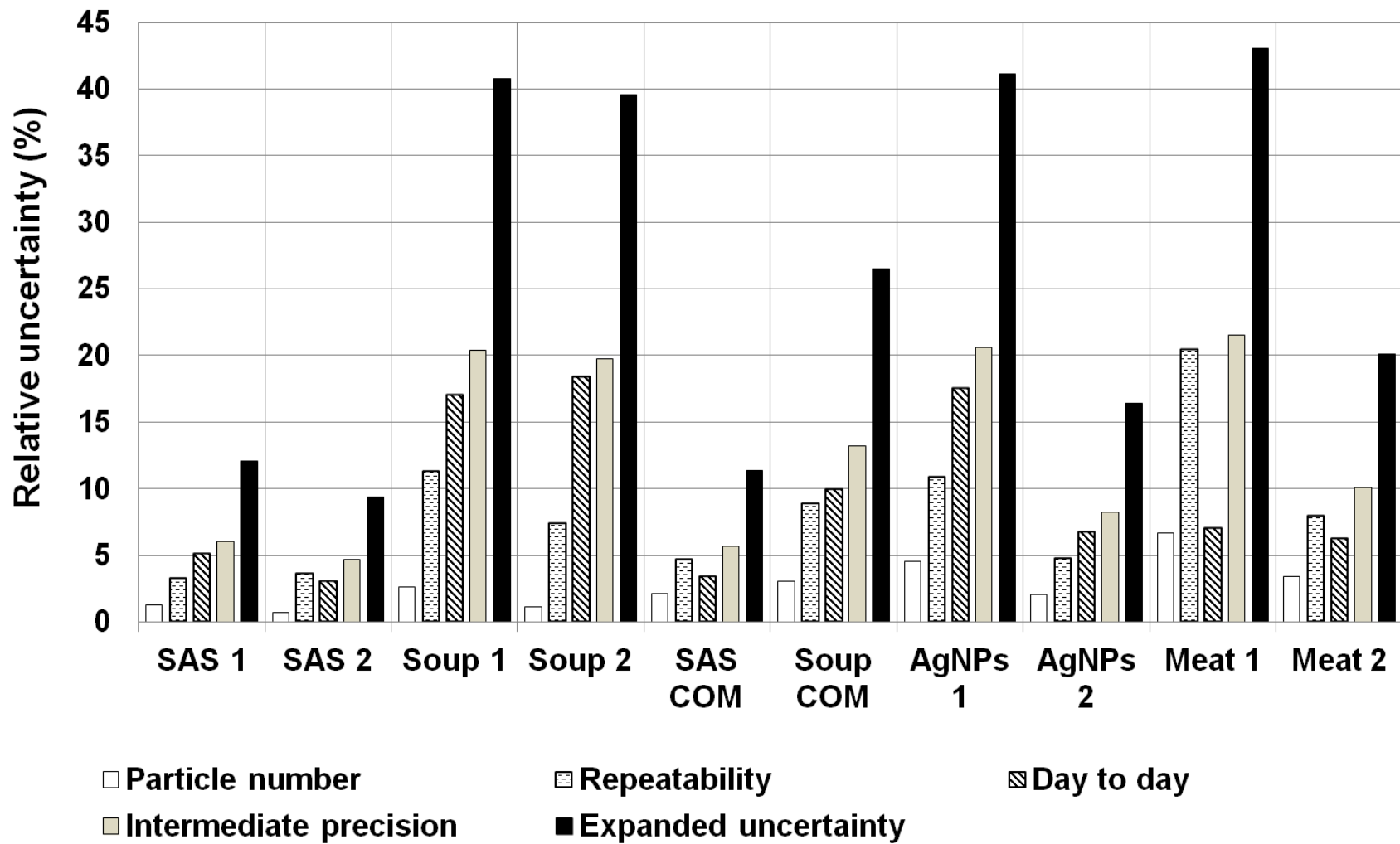


Fig. 1. (A) Dependence of median size measurement RSD_{pn} of the sample size N to $IQR\%$ and (B) Relationship between slope coefficient a of Eq. 11 and N .

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