BAM

Certification Report

Certified Reference Material BAM-N001

Particle Size Parameters of Nano Silver

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M. Menzel (coordinator), R. Bienert, W. Bremser, M. Girod, S. Rolf, A. F. Thünemann, F. Emmerling

BAM Federal Institute for Materials Research and Testing
Division 1.3 Structure Analysis
Richard-Willstätter-Str. 11

D-12489 Berlin, Germany

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Summary

This report describes preparation and certification of the certified reference material BAM-N001, including all analytical aspects and the proof of homogeneity and stability.

BAM-N001 is an aqueous suspension of silver particles certified for the particle size parameters d_{10}, d_{50}, and d_{90}. The d_{10}, d_{50}, and d_{90} values are specific particle diameters that correspond to 10, 50, and 90 % of the total particles in the cumulative undersize distribution. These diameters were determined for both the volume- and number-weighted distribution, using small-angle X-ray scattering. The certified values for d_{10}, d_{50}, and d_{90} are stated in the following table together with the expanded combined uncertainty of each of them.

Certified Values (volume-weighted)

Size Parameter*	Diameter** nm	Uncertainty *U*** nm
d_{10}	12.0	1.9
d_{50}	18.5	2.5
d_{90}	34.6	4.8

Certified Values (number-weighted)

Size Parameter*	Diameter** nm	Uncertainty *U*** nm
d_{10}	6.9	2.7
d_{50}	12.6	2.1
d_{90}	19.4	2.2

*Particle diameter corresponding to the cumulative size distribution

**The certified value is traceable to SI under the assumptions of the model used for interpretation of the SAXS data.

***The stated uncertainty is an expanded combined uncertainty consisting of contributions from method repeatability, measurement setup geometry, method bias, possible but undetected inhomogeneity and instability, and the model used, in particular binning, expanded by a factor of $k = 2$ corresponding to a confidence level of approximately 95 %.

CRM BAM-N001 is delivered as a set of 2 brown plastic bottles containing an aqueous solution having an approximate silver concentration of 100 µg/ml. Each bottle contains approximately 5 ml of the suspension.

BAM-N001 is intended to be used as a calibrant or, alternatively, a control sample for both precision and trueness estimation of particle-size determination methods, in particular of SAXS methods. The material is suitable for biomedical research purposes requiring silver particles in the corresponding range of the certified values suspended in aqueous solutions.

Minimum sample intake for a single measurement is 50 µl of suspension. Before aliquots are taken, the material has to be homogenised by shaking gently and thoroughly.

Provided the material is handled appropriately and stored at a temperature of (20 ± 3) °C in the dark, the validity of the certified values is

i) one day after opening of the bottle and
ii) maximum one year after dispatch of the material to the customer.

Abbreviations and symbols used

BAM	Federal Institute for Materials Research and Testing
CLS	Centrifuge Liquid Sedimentation
critical *F*	critical value of the Fisher distribution
CRM	Certified Reference Material
d	particle diameter
D_{SAXS}	intensity-weighted mean diameter determined by SAXS
E_n	“normalised error“, a measure of data compatibility
F statistic	Fisher statistic
HDPE	high density polyethylene
I	Intensity
n	number of replicate determinations
N	number of bottles
MSE	mean squared error
$MS_{between}$	variability between samples
MS_{within}	variability within samples
P>	excess probability
PTB	Physikalisch-Technische Bundesanstalt
q	magnitute of the scattering vector
R_G	Guinier radius
s	standard deviation
SAXS	Small-Angle X-ray Scattering
s_r	relative standard deviation
SSD	*s*um of squared deviations
TOC	total organic carbon (content)
TSEM	Transmission Scanning Electron Microscopy
u_{bb}	uncertainty caused by inhomogeneity
u_{bias}	uncertainty caused by bias
u_{bin}	uncertainty caused by binning
u_{geom}	uncertainty caused by instrument geometry
u_{lts}	uncertainty caused by instability
u_{rep}	uncertainty caused by repeatability
ν	degrees of freedom

1 Introduction and aim of the project

Often metallic nano particles reveal distinct changes of the physical and chemical properties in comparison to the compact material. The reason can be seen in the small size and, as a consequence, in the exceptional surface to volume ratio. This led to a wide application of nano materials [1]. It has been known for a long time that small silver particles are antibacterial [2]. Therefore colloidal silver has been in use in medicine for a long time. In the present time, the use of colloidal silver was extended. Today there are hundreds of products containing nano silver [3]. This and the increasing use of nano silver in future will supposedly cause an increasing release of silver into the environment. In this way, silver can be also incorporated into the human body and accumulated in different organs, which may be toxic or expose an unknown risk to human health [4].

Therefore, it is very important to study materials, their production, application in products and technical processes, and distribution as well as effects to humans and to nature.

To guarantee the traceability of measurements and to secure comparability of results of different analytical methods, certified reference materials (CRM) are essential.

The certification of the present nano silver reference material BAM-N001 opens the possibility to use this material to study technical processes or biomedical processes directly in living objects.

2 Production of the candidate material

2.1 Selection of the starting material

The starting material AgPURE™ W10 (Lot: A10 091015a) has been purchased from the manufacturer ras materials GmbH**,** Regensburg (Germany). AgPURE™ W10 is a silver particle suspension of 10 % (w/w) silver concentration. The silver particles have an average size of around 20 nm.

AgPURE™ W10 was stabilized against aggregation by adding both 4 % (w/w) polyoxyethylene glycerol trioleate (TAGAT™ TO) and 4 % (w/w) polyoxyethylene (20) sorbitan mono-laurate (TWEEN-20™) furthermore it contains 7.5 % (w/w) ammonium nitrate[1].

2.2 Preparation of the candidate material

The original stock solution as purchased has been diluted to an approximate silver concentration of 100 µg/ml in a two-step dilution procedure using Milli-Q ™ water (device type: Milli-Q Synthesis A10 from Millipore Corporation, 18.2 MΩ, TOC 3-6 ppb) as solvent. In the first step 20 g AgPURE™ W10 were pre-diluted with 380 g Milli-Q-water. From this suspension an aliquot of 50 g was taken and mixed with 2450 g Milli-Q water. After dilution, the suspension was filled into brown plastic bottles of 5 ml content each using a Dispensette ® III (type: Dig. Easy calibration 1-10 ml). The batch size of the lot is 500 bottles. This procedure has been performed twice to result in a whole set of 1000 bottles.

The used bottles are 8 ml HDPE Thermo Scientific Nalgene ™ quality amber narrow-mouth bottles. This material reduces UV-light transmission to protect light-sensitive liquids. The material has an excellent chemical resistance against most acids, bases and alcohols. It is reliable and durable for long-term use. The used closures are regular leak-proof screw caps.

The pH value of the suspension has been monitored. Although not critical, at the time of delivery the suspension has a pH value of around 5.4.

[1] Data as information from the producer.

2.3 Analytical method and instrumentation

Small-angle X-ray scattering was used for homogeneity and stability testing as well as for the characterisation of the material. For material characterisation, two independent reference equipments were used.

The instrument used at BAM is a SAXSess (Anton Paar, Graz, Austria). This Kratky type of camera is attached to a laboratory X-ray generator (PW3830, PANalytical), and was operated with a fine focus glass X-ray tube at 40 kV and 40 mA with a photon energy of 8.040 keV (wave length = 0.1542 nm). A focusing multi-layer optics and a block collimator provide a monochromatic primary beam with low background in line collimation.

Samples were filled in a reusable vacuum tight quartz glass capillary with an inner diameter of 1 mm to attain the same scattering volume and background contribution. Prior to use the bottles were softly shaken to homogenise the sample. The temperature was set to 20 °C with a TCS 120 sample holder (Anton Paar) with an accuracy of ± 0.2 °C. The intensity of the scattered radiation was recorded as a function of the scattering vector q with a CCD detection system at a distance of 0.309 m. Where q is defined as $q=(4\pi/\lambda)sin\theta$, where λ is the wavelength and θ is half of the scattering angle. The time of measurement was 20 min corresponding to the average of 120 experiments with an exposure time of 10 s. A typical scattering curve is shown in Figure 2.3-1. The two-dimensional intensity data were converted to one-dimensional data using the SAXSquant™ software (Anton Paar). After correcting the data for background scattering, taking a water-filled capillary into account, and for slit-smearing, the reduced data were converted into absolute intensities using water as reference [5].

To obtain the size distribution from small angle X-ray scattering data (q-range from 0.08 nm^{-1} to 1.2 nm^{-1}) the maximum entropy method with a constrained optimisation of parameters, implemented in the IGOR® macro "IRENA" was used [6].

This resulted in a histogram for the volume weighted size distribution with 100 columns of constant width, called bins, over a range of diameters from 3 nm to 80 nm. From this histogram the volume-weighted d_{10}, d_{50} (same as the median) and d_{90} were derived, defining the diameter where 10 %, 50 %, and 90 % of the particles' population is below the designated value. After transforming the volume (R^3)-weighted size distribution into a number (R)-weighted size distribution the corresponding size parameters were derived accordingly.

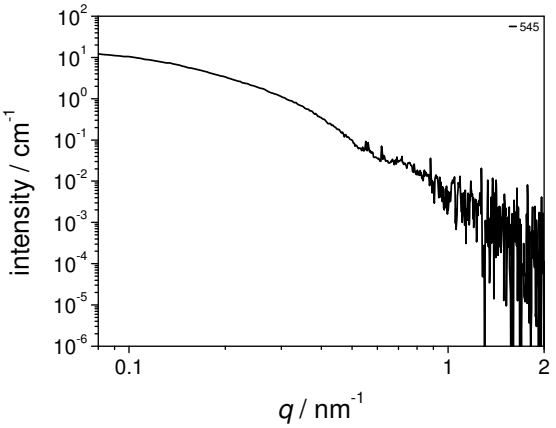

Figure 2.3-1 Typical small angle X-ray scattering curve for a sample of BAM-N001 taken with the SAXSess-System. The data are corrected for background scattering and slit smearing.

The instrument used at PTB is the SAXS setup of the Helmholtz-Zentrum Berlin für Materialien und Energie (HZB), installed at the PTB four-crystal monochromator beam line [7]. The liquid to be studied was injected in a glass capillary with an inner diameter of 1 mm and a wall thickness of 10 µm. Using a point focus with a photon energy of 8.000 keV (wave length = 0.1550 nm), the scattering patterns were registered, within 25 min, with a Pilatus detector at a distance of 2.2552 m. Measurements were performed under ambient conditions. The transmitted beam through the sample was blocked by a rectangular beam stop in front of the detector. The scattering pattern obtained from a capillary filled with

water was used to correct for background contributions. Data analysis was performed as described above. However, the data were not converted into absolute intensities.

2.4 Minimum sample intake

The minimum sample intake for a measurement to determine the particle diameter should be chosen in a way that no significant heterogeneity within the bottle is to be expected. For SAXS measurements, however, the sample intake is mainly determined by the size and capacity of the capillary, so no specific recommendation can be given in the certificate.

For the instruments used in this study, considerable homogeneity has been proven (see chapter 3), such that the dimensions of the capillary were suitable.

As a general recommendation, at least 50 µl of the suspension should be used for a single analysis.

3 Homogeneity study

Thirty units have been selected approximately equidistantly from the whole set of 1000 bottles. All units have been analysed in duplicate, each according to the SOP described in Chapter 2.3. Measurements have been carried out under repeatability conditions on consecutive days. One out of the 30 samples has been measured 6 times in a row providing a reliable estimate for the repeatability of the instrument used.

Means and standard deviations for the 30 samples and the volume-weighted size parameters are summarised in Figure 3-1. Measurement data and the results of the analysis of variance (ANOVA) are given in Annex A. No evidence suggesting a rejection of the hypothesis that the material is sufficiently homogeneous was observed. All data from the comprehensive homogeneity study may be pooled.

Thus, total mean values for the three size parameters and estimates of the uncertainty u_{bb} due to possible (undetected) inhomogeneity have been derived from the study. Values are given in Table 3-1.

Table 3-1: Results of the homogeneity study (total mean, uncertainty contribution due to possible inhomogeneity, and repeatability estimates) for volume- and number-weighted size parameters of BAM-N001

Summary	volume			number		
	d_10	d_50	d_90	d_10	d_50	d_90
status	homogeneous	homogeneous	homogeneous	homogeneous	(still) hom	homogeneous
value	11.9632813	18.544375	34.57	6.898125	12.5648438	19.3865625
u_bb	0.2178952	0.23576339	0.55291415	0.433125	0.26138566	0.19842446
u_bb_rel	1.82%	1.27%	1.60%	6.28%	2.08%	1.02%
repeatabili	0.62870237	0.80758281	1.64547156	1.23360312	0.79525258	0.56122912
repeat_rel	5.26%	4.35%	4.76%	17.88%	6.33%	2.89%

All except the uncertainty contribution due to inhomogeneity for number-weighted d_{10} have been calculated according to Eq. 3-1

$$u_{bb}^2 = \frac{MS_{within}}{n} \cdot \sqrt{\frac{2}{N \cdot (n-1)}} \tag{3-1}$$

while the contribution for number-weighted d_{10} shows a somewhat larger but still insignificant variability and has been calculated according to Eq. 3-2 [8]

$$u_{bb}^2 = \frac{MS_{between} - MS_{within}}{n} \tag{3-2}$$

where MS_{within} is the variability within samples, $MS_{between}$ the variability between samples, n the number of replicate determinations per sample, and N the number of bottles analysed.

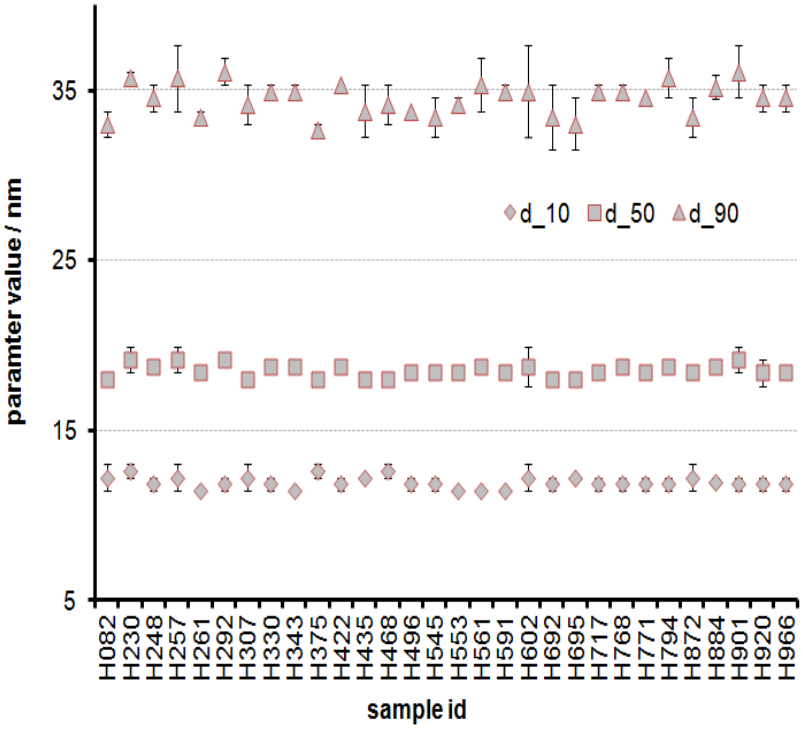

Figure 3-1: Results of the homogeneity study (sample means and standard deviations for the volume-weighted size parameters).

A worst-case estimate was used to determine the method repeatability value estimates as listed in Table 3-1. Therein, the estimate for method repeatability is either the average precision as calculated from the ANOVA or the standard deviation of the 6 measurements taken on one of the 30 selected samples, whichever is larger (worst-case estimate).

4 Stability study

4.1 Preliminary investigations

In a preliminary step, the robustness of the nano silver material against external influences was tested by:

- irradiation of the sample with a daylight lamp for 1 h or 1 day resp.
- irradiation of the sample with a Xenon lamp for 1 h or 1 day resp.
- freezing of the sample over a period of 23 days
- warming of the sample up to 72 °C over a period of 21 days
- sterilisation by ^{60}Co γ-irradiation (dose 25 kGy)
- addition of isotonic NaCl-solution (Ag-concentration: 0.1 g/l, 1 g/l) for 1 day

It could be shown that none of these influences caused any significant changes of the certified material parameters.

4.2 Initial stability study

After the exclusion of the above mentioned factors (Clause 4.1), a more detailed stability study was set up to test temperature-driven changes of the size parameters, which were to be expected from experience, at least at low temperatures.

Twenty-four units of the candidate material were submitted to an isochronous accelerated ageing at temperatures between 4 and 70 °C over periods of time between 4 and 24 weeks as shown in Table 4.2-1. After the respective periods of time individual units were stored at room temperature recommended also for storage at the user's side. All units were analysed in duplicate using the method described above under repeatability conditions, and the volume-weighted size parameters were determined. It can be assumed that proof of stability of the volume-weighted size parameters can be equally extended to the number-weighted parameters. This holds for any size distribution independent of its shape. Individual volume-weighted size parameters determined for the samples, and plots of the dependence of the three size parameters on time are given in Annex B.

Table 4.2-1: Number of bottles for the accelerated-ageing test of exposed samples at the various stress levels

Ageing [weeks]	+4 °C	+20 °C	+40 °C	+70 °C	Remarks
4	3	-	3	3	initial study
8	3	-	3	3	initial study
24	3	-	3	-	initial study
48	-	3	-	-	[1])
72	-	3	-	-	[1])

[1]) post certification monitoring

The dependence of temperature-driven changes upon time of the size parameters is widely unknown. At low temperatures, a trend towards particle agglomeration has to be expected, thus any freezing of the samples must be avoided. Similarly, some de-agglomeration should be expected at elevated (above room) temperatures. Figures B-1 to B-3 in Annex B do not provide a clear, systematic picture about any possible processes of degradation or agglomeration. Even at temperatures lower or higher than room temperature, no indication of significant degradation or agglomeration can be observed. Only two out of the nine regressions turned out to be significant (for d_{50} at 70 °C, and for d_{90} at 40 °C where it was not to be expected). However, all values remain well within the corresponding uncertainty band of the certified values over the 24 months duration of the study.

For further analysis, the slopes of the regression lines over time have been plotted against the inverse temperature (*1/T* in K). The result is displayed in Figure 4.2-1. Slopes for the stress levels 4 °C and 40 °C are indefinite, i.e. around zero for all three size parameters, not taking into account that d_{10} as the most susceptible parameter shows a tendency to decrease. All slopes for all three parameters are negative for a stress level of 70 °C, which might be interpreted as a certain de-agglomeration at this elevated temperature.

In order to obtain estimates for the thermal behaviour of the samples at the storage temperature, the slopes are approximated as a set by a part of an exponential function. Since agglomeration is rather expected at low temperatures, while strongly elevated temperatures may have the inverse effect, the saturation region of an exponential function of the general form $k_1 \cdot [1 - \exp(-k_2/T)]$ has been used in the regression. The function is also shown in Figure 4.2-1. Using this function, a prediction was done for the slope at the normal storage temperature of (20 ± 3) °C. With this estimate, and a pre-selected shelf life of 36 months, the possible change of the certified parameters has been calculated. This possible change is considered a contribution to measurement uncertainty and included in the total budget.

Parameters of the regression function and the value for the u_{lts} estimate are given in Table 4.2-2.

Table 4.2-2: Calculation of u_{lts} from the regression. Regression parameters are given on the left of the table. The estimated effective degradation rate (slope of the line) is taken at the recommended storage temperature (room temperature, corresponds to a value 1/T of 0.00338) and given in the table on the right. The relative change (the relative contribution to the total uncertainty) is calculated from the shelf life times the estimated rate.

exponential fit results					
constant	0.768090507		estimated rate is	0.00030662	per week
exponent	14.6044504		shelf life is	36	months
shift	0.76225508		u_lts_rel	0.04415359	

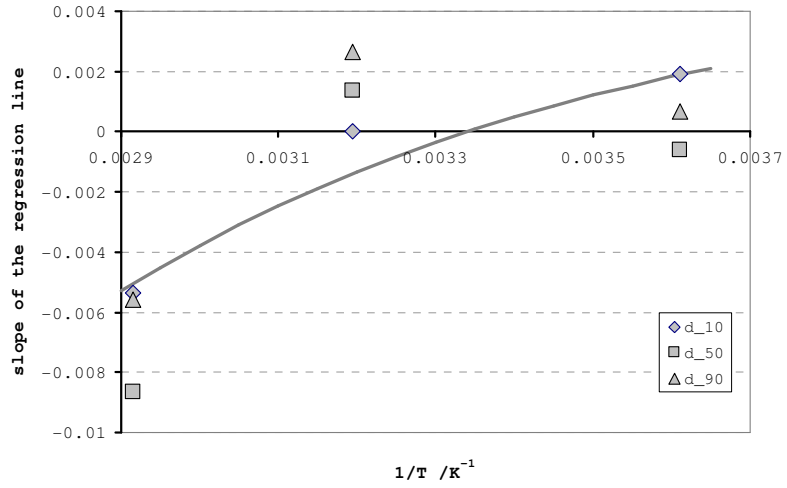

Figure 4.2-1: Stability study on BAM-N001: Dependence of the slopes of the regression lines on 1/T.

Although an allowance for a shelf life of 36 months has been made in the uncertainty budget, larger deviations from the recommended storage temperature may reduce the time of validity of BAM-N001. Therefore, a unique shelf life of **one year after delivery from storage** is established. Transportation/delivery time should be kept at the possible minimum and exposure to excess heat and/or cold should be avoided.

4.3 Post-certification stability monitoring

The first rough estimation of stability will be updated by further measurements of units stored at 20 °C over the period of availability of the material. The post-certification measurements will be conducted according to the information given in Table 4.2-1.

5 Certification study

5.1 Design of the study

The characterisation of BAM-N001 was entirely based on the instrumentation and the analytical method described in Clause 2.3. The method has thoroughly been validated, and full information on the total measurement uncertainty is available. For a detailed description of the uncertainty budget see Clause 5.2.4. Thirty samples have been selected randomly from the batch and measured in duplicate, for one out of the thirty, measurements were repeated six times.

Independent measurements of the size parameters have been carried out on four more samples using the SAXS instrumentation at the beam line of PTB.

For an experimental demonstration of traceability of the BAM equipment, measurements were carried out on the certified reference material ERM®-FD100 (colloidal silica in water). Data and the assessment of the comparison are given in Clause 5.2.3.

Supporting information was generated using size parameter determination based on different physical principles, namely Transmission Scanning Electron Microscopy (TSEM), and Centrifuge Liquid Sedimentation (CLS). Results and a brief discussion of the differences in the measurands are given in Clause 5.3.

5.2 Evaluation of results and certified values

5.2.1 Technical evaluation

All measurement results obtained on the 30 selected samples were scrutinised for technical validity. During SAXS measurement an increasing silver layer on the flow cell was observed. To account for this, the background was always measured prior to the sample measurement.

5.2.2 Statistical evaluation

The data set as given in Annex A for the measurement results has been subjected to a statistical analysis within which the following tests were carried out (at significance levels of 0.05 and 0.01):

- Grubbs test for the identification of outliers (single values)
- Kolmogorov-Smirnov Test (Lilliefors version) for the normality test
- Test for skewness and kurtosis
- Analysis of variance (ANOVA) for confirmation of absence of significant differences between units.

From the ANOVA, estimates for u_{bb} and the average repeatability were derived according to ISO Guide 35:2006 [8]. For all results of the above calculations see Annex A. Conclusions from the statistical tests were as follows:

- *Grubbs*-Test: No value had to be excluded from further consideration.
- *Kolmogorov-Smirnov* and skewness/kurtosis test: Based on the available data, the hypothesis of normality cannot be rejected.
- *ANOVA:* No significant inhomogeneity was detected for the three size parameters. Most often, variability between units was roughly equal or even smaller than the variability within units.

Thus, it may be assumed that all data obtained stem from one common distribution. The grand mean is used as the certified value for all three size parameters, both volume- and number-weighted.

Independent measurements had been carried out on four samples at the SAXS setup of the beam line of PTB. Figure 5.2.2.-1 depicts the SAXS curves obtained.

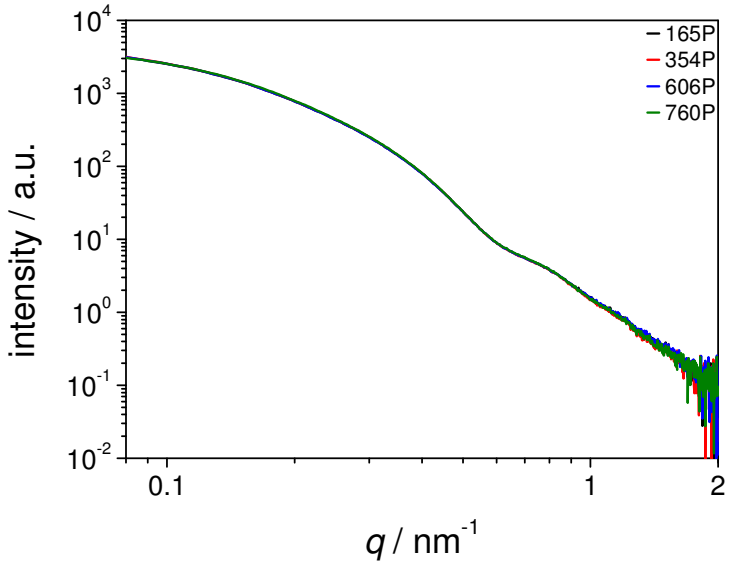

Figure 5.2.2.-1: SAXS data of four samples of BAM-N001 measured at the PTB beam line.

Curves were processed as described in Chapter 2.3. Results for the volume-weighted size parameters are given in Table 5.2.2-1.

Table 5.2.2-1: Results of the comparison with an independent SAXS instrument.

method	laboratory	parameter			weighting
		d_10	d_50	d_90	
SAXS	PTB beamlin	12.0475	16.86	32.8375	vol
SAXS	PTB beamlin	6.85	13.01	18.4	num
		parameter uncertainty			
		0.4304	0.3850	0.4304	vol
		0.3850	0.3850	0.3850	num
		E_n			
		0.0808	1.2733	0.7110	vol
		0.0339	0.3988	0.8422	num

The table also displays the E_n criterion for the comparison between the independently measured and the certified values. Note that the uncertainty assigned to the independent measurements includes repeatability only.

The E_n criterion given in the last rows of table 5.2.2-1 is a measure of the deviation of the value under consideration from the reference related to the combined uncertainty of that difference. Values are considered to deviate insignificantly if E_n is smaller than a value of two. As can be seen from the E_n criterion, the size parameters d_{10}, d_{50} and d_{90} are in excellent agreement.

5.2.3 Traceability

Measured values for the scattering vector q of a SAXS curve and their uncertainties are given by the geometry of the system which has been determined with due care. These values are directly traceable to the SI [9]

Direct traceability has also been established to ERM®-FD100 which is colloidal silica in water. Measurements have been taken on this CRM to determine the Guinier radius R_G, and results compared with the value and uncertainty given in the certificate of ERM®-FD100. Table 5.2.3-1 gives the results for the single measurements, the certificate values, and the E_n value calculated from the difference and its combined uncertainty. As can be seen, measured and certified values are fully consistent. Note

that, for this type of comparison, the uncertainty of the experimental mean value includes repeatability and the residual scatter from the model fits only.

Table 5.2.3-1: Mean diameter calculated from Guinier radius against ERM®-FD100 value

comparison with ERM-FD-100					
certified value:		21.8		measured value:	22.1633333
u(value):		0.35		u(value)	0.30717598
single results at SAXSess					
#	value	sd		E_n:	0.78022244
1	22.64	0.3648			
2	22.44	0.4112		conclusion:	consistent
3	21.98	0.4328		remaining bias:	0.18166667
4	22.06	0.4206		u_bias_rel:	0.00833333
5	21.96	0.608			
6	21.9	0.42			

The remaining bias, i.e. the uncertainty contribution due to evaluation with the CRM, is estimated as half of the difference between the certified and the measured value for ERM®-FD100, and expressed as a relative uncertainty which has been included in the total uncertainty budget.

The above calibration against a CRM confirms the correctness of the SAXS measurements from which the size parameters are calculated using a physical model. Given the above, it may be concluded that the values for the size parameters of BAM-N001 are traceable to the SI under the assumptions of the model used.

Following Eq. 5-1, the Guinier radius R_G was deduced from a weighted linear regression of the reduced, background corrected and deconvoluted (slit-length desmeared) scattering curves. The fit range was from 0.08 nm^{-1} to 0.15 nm^{-1}.

$$\ln[I(q)] = \ln[I_0] - \frac{1}{3}R_G^2 q^2 \tag{5-1}$$

The mean diameter was calculated according to $D_{SAXS} = 2\sqrt{\frac{5}{3}}R_G$.

5.2.4 Uncertainty evaluation

Different sources of uncertainty contribute to the total uncertainty of the certified values and have to be taken into account. Table 5.2.4-1 lists and explains the sources taken into account, and the ways of calculating the corresponding contributions.

Table 5.2.4-1: Uncertainty contributions and their estimation

Source	Symbol	Meaning and estimation
binning	u_{bin}	half of the binning width (see explanation), binning for number-weighted diameters correspondingly scaled
repeatability	u_{rep}	repeatability of a single measurement derived from the ANOVA analysis (see Chapter 3) as either the average repeatability of the full set or the repeatability of the 6 replicate measurements on one sample, whichever is larger
instrument geometry	u_{geom}	Average scatter vector *q* uncertainty due to finite capillary and detector size, and uncertainty of length measurements; taken from manufacturer's indication of a *q* uncertainty of not more than 0.002 nm^{-1} over the range between 0.08 to 1.2 nm^{-1}, corresponding to an average geometry uncertainty of 1.2 %
bias	u_{bias}	half of the difference of the comparison with ERM®-FD100 in the Guinier region, equally contribution from establishing traceability (see Clause 5.2.3)
(in)homogeneity	u_{bb}	inhomogeneity contribution from the homogeneity study as described in Chapter 3
(in)stability	u_{lts}	worst-case estimate of a contribution due to possible changes of the measurands derived from the stability study as described in Chapter 4

The binning contribution is a consequence of the model used for calculating the size parameters from the SAXS pattern. The IGOR® macro "IRENA" [6] generates a cumulative distribution of particles within a certain width of a histogram column called "binning". A pre-set value for the cumulative distribution (as e.g. 50 %, in the figure below bin *k + 1*) will normally not match with the ceiling of the bin but intersect at a certain distance. Thus, bin *k* under- and bin *k + 1* overestimates the pre-set value, on average by chance with equal expectation. Figure 5.2.4-1 displays this particular situation for two pre-set values of the cumulative distribution.

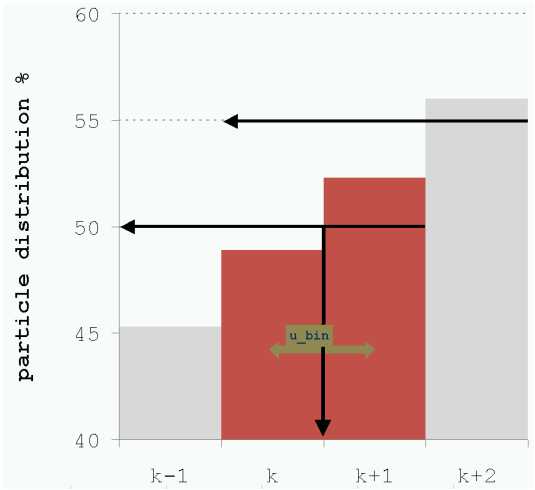

Figure 5.2.4-1: Binning uncertainty (scale resolution)

The pre-set value is characterised by the limiting line between the two bins, i.e. the lower left limit of the single histogram column exceeding the pre-set value. This value has an uncertainty, which is

characterised by half of the width of the column as the "scale resolution". In the tables, this uncertainty is denominated u_{bin}.

As a rule, uncertainty estimates have been derived from the experiments as described above in form of relative uncertainties, and then applied to the corresponding certified values. Table 5.2.4-2 displays the absolute values for the uncertainty contributions to the different size parameters, the combined uncertainty (both absolute and relative), and the expanded combined uncertainty. The combined uncertainty u_c was calculated as $u_c^2 = \sum_i u_i^2$ where the u_i are the different contributions as listed in the tables.

Table 5.2.4-2: Values of the uncertainty contributions and combined uncertainty of the certified values

in nm	volume-weighted			number-weighted		
parameter	d_10	d_50	d_90	d_10	d_50	d_90
value	11.96328	18.54438	34.57000	6.89813	12.56484	19.38656
uncertainties						
u_bin	0.38500	0.38500	0.38500	0.23408	0.23408	0.23408
u_rep	0.62870	0.80758	1.64547	1.23360	0.79525	0.56123
u_geom	0.14620	0.22663	0.42248	0.08430	0.15356	0.23693
u_bias	0.09969	0.15454	0.28808	0.05748	0.10471	0.16155
u_bb	0.21790	0.23576	0.55291	0.43313	0.26139	0.19842
u_lts	0.52822	0.81880	1.52639	0.30458	0.55478	0.85599
total	0.94937	1.26557	2.39851	1.36651	1.04779	1.10639
total_rel	0.07936	0.06825	0.06938	0.19810	0.08339	0.05707
total * k	1.89874	2.53115	4.79703	2.73302	2.09559	2.21277

Note that the bias contribution as listed in table 5.2.4-2 may be considered negligible in comparison with other uncertainty contributions. However, for the sake of completeness of the budget, this term is retained until the final rounding.

The certified values and their expanded uncertainties are given in the summary. The expansion factor for the expanded uncertainty is $k = 2$ throughout. The values and the expanded uncertainties are rounded according to the recommendations of [10].

5.3 Supporting measurements

Supporting information was generated using size parameter determination based on different physical principles, namely Transmission Scanning Electron Microscopy (TSEM), and Centrifuge Liquid Sedimentation (CLS). By TSEM, one sample was analysed. At a magnification of 150 000, 52 pictures have been taken, and 606 particles identified and their size parameters determined. From the number-weighted distribution, the corresponding d_{50} has been determined.

Two randomly selected samples have been investigated by Centrifuge Liquid Sedimentation (CLS). Table 5.3-1 displays the results of both supporting analyses together with the estimated uncertainties of the methods used, and the E_n values for the comparison with the values certified for BAM-N001. For CLS, the mean for the two samples is given.

Table 5.3-1 Results of the supporting analyses together with the estimated uncertainties of the methods

method	laboratory	parameter			weighting
		d_10	d_50	d_90	
CLS	LUM GmbH		28.5		vol
TSEM	PTB		14.07		num
		parameter uncertainty			
			33.6		vol
			0.95		num
		E_n			
			0.30		vol
			1.06		num

The number-weighted d_{50} parameter determined by TSEM is in good agreement with the number-weighted d_{50} value certified for BAM-N001, as can be seen from the corresponding E_n value. This confirms both the trueness of the SAXS results including the model, and the traceability statement (see Clause 5.2.3) since TSEM-measured values take their traceability directly from the SI unit metre.

The E_n value for the comparison of the certified volume-weighted d_{50} with the value determined by CLS also suggests good agreement. However, the uncertainty stated for the CLS measurements is quite large (leading to an underestimated E_n). Thus, the 28.5 nm median diameter determined by CLS is an estimate for the diameter range and its upper limit rather than a confirmation of the certified value. Difference in the measurands of a SAXS and a CLS method may play a major part in the occurrence of discrepancies, where the latter is a Stokes diameter determined from the dynamic properties of the particles in motion. These effects will be investigated further.

6 Information on the proper use of BAM-N001

6.1 Shelf life

From the initial stability study a preliminary shelf life of 36 months while stored at room temperature is estimated. Since the dispatch to the end user may occur at any time during this period the certified properties will be valid for 12 months beginning with the dispatch of the material from BAM. The validity of this information will be maintained by the post-certification monitoring.

6.2 Transport, storage and use

The stability of the certified size parameters allows the dispatch of the material at ambient temperature. On receiving, it has to be stored at (20 ± 3) °C in the dark. Before taking a sub-sample, the content of the bottle has to be homogenised by duly shaking. Thereafter, the bottle must be closed tightly and stored at (20 ± 3) °C.

Note that the general validity of the certified values is i) one day after opening of the bottle and ii) maximum one year after the material has been purchased and delivered to the customer

6.3 Safety instructions

No hazardous effect is to be expected when the material is used under conditions usually adopted for the analysis of materials displaying moderate bioactivity. It is, however, strongly recommended to dispose of the reference material in accordance with the guidelines for hazardous materials legally in force at the site of end use and disposal.

6.4 Legal notice

Neither the BAM Federal Institute for Materials Research and Testing nor any person acting on their behalf make any warranty or representation, express or implied, that the use of any information, material, apparatus, method or process disclosed in this document may not infringe privately owned rights,

or assume any liability with respect to the use of, or damages resulting from the use of any information, material, apparatus, method or process disclosed in this document.

7 References

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ANNEX A: Homogeneity assessment of silver samples, measured data and ANOVA results

Results of the homogeneity study on BAM-N001

	volume						number					
sample	d_10		d_50		d_90		d_10		d_50		d_90	
H082	11.47	13.01	18.4	17.63	33.8	32.26	6.85	9.16	12.24	13.78	19.17	19.17
H230	12.24	13.01	18.4	19.94	35.34	36.11	6.85	10.7	13.01	13.78	19.17	21.48
H248	11.47	12.24	18.4	19.17	33.8	35.34	6.08	6.85	11.47	12.24	18.4	19.94
H257	13.01	11.47	19.94	18.4	37.65	33.8	9.16	6.08	13.78	12.24	20.71	19.17
H261	11.47	11.47	18.4	18.4	33.03	33.8	6.08	5.31	12.24	12.24	19.17	19.17
H292	11.47	12.24	19.17	19.17	36.88	35.34	6.08	6.08	11.47	12.24	19.17	19.17
H307	11.47	13.01	18.4	17.63	35.34	33.03	6.08	8.39	12.24	13.78	19.17	19.17
H330	11.47	12.24	18.4	19.17	34.57	35.34	6.08	7.62	12.24	13.01	19.17	19.94
H343	11.47	11.47	18.4	19.17	34.57	35.34	6.08	6.08	12.24	12.24	18.4	19.94
H375	12.24	13.01	18.4	17.63	33.03	32.26	8.39	8.39	13.78	13.78	19.94	19.17
H422	11.47	12.24	19.17	18.4	35.34	35.34	6.08	6.85	12.24	12.24	19.17	19.17
H435	12.24	12.24	17.63	18.4	32.26	35.34	8.39	7.62	13.78	13.01	19.17	19.94
H468	12.24	13.01	18.4	17.63	35.34	33.03	7.62	8.39	13.01	13.78	19.17	19.94
H496	11.47	12.24	18.4	18.4	33.8	33.8	6.08	6.85	11.47	12.24	18.4	19.17
H545	11.47	12.24	18.4	18.4	34.57	32.26	6.08	8.39	12.24	13.78	19.17	19.94
H553	11.47	11.47	18.4	18.4	34.57	33.8	7.62	5.31	12.24	12.24	19.17	19.17
H561	11.47	11.47	19.17	18.4	36.88	33.8	6.08	5.31	11.47	12.24	19.17	19.17
H591	11.47	11.47	18.4	18.4	35.34	34.57	6.08	6.85	11.47	12.24	19.17	19.17
H602	11.47	13.01	19.94	17.63	37.65	32.26	5.31	9.16	11.47	13.78	19.17	19.94
H692	12.24	11.47	17.63	18.4	31.49	35.34	8.39	6.85	13.78	12.24	19.17	19.17
H695	12.24	12.24	17.63	18.4	31.49	34.57	8.39	6.08	13.78	12.24	19.17	19.17
H717	11.47	12.24	18.4	18.4	34.57	35.34	6.08	6.85	12.24	13.01	19.17	19.17
H768	11.47	12.24	18.4	19.17	34.57	35.34	6.08	6.85	12.24	12.24	19.17	19.94
H771	12.24	11.47	18.4	18.4	34.57	34.57	6.85	6.08	12.24	12.24	19.17	19.17
H794	11.47	12.24	19.17	18.4	36.88	34.57	6.85	6.85	12.24	12.24	19.94	19.17
H872	11.47	13.01	18.4	18.4	34.57	32.26	6.08	8.39	12.24	13.78	19.17	19.94
H884	12.24	12.24	18.4	18.4	34.57	33.8	6.85	6.08	12.24	13.01	19.17	19.17
H884	11.47	11.47	19.17	19.17	36.88	36.11	6.08	6.08	11.47	12.24	19.17	19.94
H884	11.47	13.01	19.94	17.63	36.88	33.03	6.08	9.16	12.24	13.78	19.94	19.94
H901	11.47	12.24	19.94	18.4	37.65	34.57	5.31	6.08	10.7	12.24	19.17	19.17
H920	11.47	12.24	17.63	19.17	33.8	35.34	6.08	6.85	12.24	13.01	19.17	19.94
H966	11.47	12.24	18.4	18.4	35.34	33.8	6.08	7.62	12.24	13.01	19.17	19.94

ANOVA results for the size parameters weighted by volume

source of variance	*SSD*	*ν*	*MSE*	*F* statistic	*P>*	critical *F*
d_{10} weighted by volume						
between groups	7.2538	31	0.2340	0.6161	0.9096	1.8104
within groups	12.1544	32	0.3798			
Total	19.4082	63				
s_{bb}			total mean	11.9633		
$s_{bb\text{-}min}$	0.2179		precision	0.6287		
u_{bb}	0.2179					
$u_{bb.\ relative}$	0.0182					
status:	homogeneous					
d_{50} weighted by volume						
between groups	9.3382	31	0.3012	0.6774	0.8594	1.8104
within groups	14.2296	32	0.4447			
Total	23.5678	63				
s_{bb}			total mean	18.5444		
$s_{bb\text{-}min}$	0.2358		precision	0.8076		
u_{bb}	0.2358					
$u_{bb.\ relative}$	0.0127					
status:	homogeneous					
d_{90} weighted by volume						
between groups	60.4758	31	1.9508	0.7976	0.7345	1.8104
within groups	78.2628	32	2.4457			
total	138.5080	63				
s_{bb}			total mean	34.57		
$s_{bb\text{-}min}$	0.5529		precision	1.6455		
u_{bb}	0.5529					
$u_{bb.\ relative}$	0.0160					
status:	homogeneous					

ANOVA results for the size parameters weighted by number

source of variance	*SSD*	*ν*	*MSE*	*F* statistic	*P>*	critical *F*
			d_{10} *weighted by number*			
between groups	39.5761	31	1.2766	0.8507	0.6729	1.8104
within groups	48.0249	32	1.5008			
total	87.6010	63				
s_{bb}			total mean	6.8981		
$s_{bb\text{-}min}$	0.4331		precision	1.2336		
u_{bb}	0.4331					
$u_{bb,\ relative}$	0.0628					
status:	homogeneous					
			d_{50} *weighted by number*			
between groups	20.2234	31	0.6524	1.1935	0.3105	1.8104
within groups	17.4906	32	0.5466			
total	37.7140	63				
s_{bb}	0.2300		total mean	12.5648		
$s_{bb\text{-}min}$	0.2614		precision	0.7953		
u_{bb}	0.2614					
$u_{bb,\ relative}$	0.0208					
status:	homogeneous					
			d_{90} *weighted by number*			
between groups	5.8919	31	0.1901	0.6034	0.9185	1.8104
within groups	10.0793	32	0.3150			
Total	17.6388	63				
s_{bb}			total mean	19.3866		
$s_{bb\text{-}min}$	0.19842		precision	0.4217		
u_{bb}	0.19842			75		
$u_{bb,\ relative}$	0.01024			0.56122912		
status:	homogeneous			6856		

where *SSD* is the sum of squared deviations, *ν* the number of degrees of freedom for the corresponding sum of squared deviations, and *P>* the excess probability.

ANNEX B: Stability assessment of silver samples, measured data and regression analysis results

d_{10}

Results of the accelerated aging study for the volume-weighted size parameter d_{10}. Values are relative to the reference value (total mean of the homogeneity study). Rows below the data columns display the parameters of the corresponding regression function over time. The result of the significance test for the regression is also given.

relative to reference			d_10						
4°C	time	value		40°C	time	value		70°C	time	value
	4	1.02313067			4	1.08749429			4	1.02313067
	4	1.08749429			4	1.02313067			4	1.02313067
	4	1.08749429			4	1.02313067			4	0.95876706
	4	1.08749429			4	1.08749429			4	1.02313067
	4	1.02313067			4	1.02313067			4	1.02313067
	4	1.02313067			4	1.08749429			4	1.02313067
	8	0.95876706			8	0.95876706			8	1.02313067
	8	0.95876706			8	0.95876706			8	1.02313067
	8	0.95876706			8	1.02313067			8	1.02313067
	8	1.02313067			8	1.02313067			8	0.95876706
	8	1.02313067			8	0.95876706			8	0.95876706
	8	0.95876706			8	1.02313067			8	0.95876706
	24	1.08749429			24	1.08749429				
	24	1.08749429			24	1.02313067				
	24	1.02313067			24	1.02313067				
	24	1.08749429			24	1.02313067				
	24	1.08749429			24	1.02313067				
	24	1.02313067			24	1.02313067				
slope	0.00191558	1.01087094	intercept		-7.571E-19	1.02670643			-0.0053636	1.03385794
u(slope)	0.0013392	0.01980322	u(inter)		0.00115664	0.01710373			0.00448754	0.02838169
r^2	0.11337868	0.04909578	stderr		1.206E-16	0.04240325			0.125	0.03109058
F	2.04603581	16	dof		1.9296E-15	16			1.42857143	10
significant:		no				no				no

Figure B-1 displays the normalised values of the replicate measurements in dependence on the time of subjection to temperature stress. Different symbols refer to the different temperature stress levels.

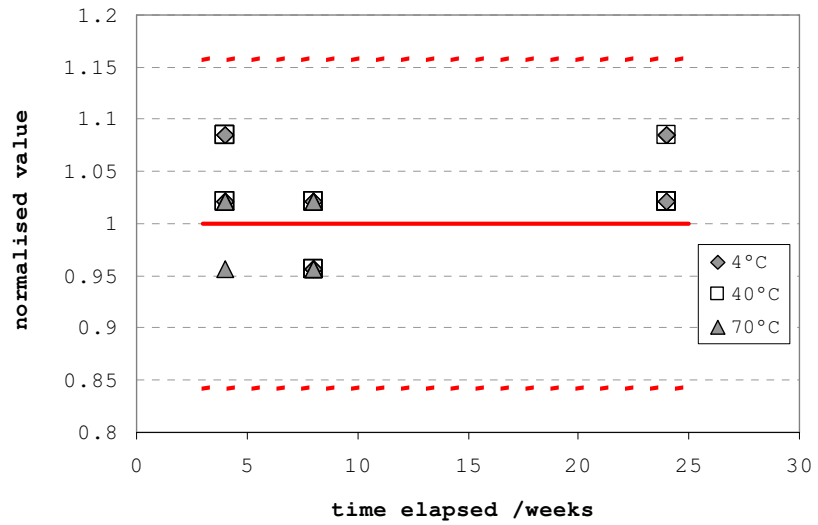

Figure B-1: Normalised values for d_{10} in dependence on the different stress levels, and the reference value and its expanded uncertainty band.

d_{50}

Results of the accelerated aging study for the volume-weighted size parameter d_{50}. Values are relative to the reference value (total mean of the homogeneity study). Rows below the data columns display the parameters of the corresponding regression function over time. The result of the significance test for the regression is also given.

relative to reference			d_50						
4°C	time	value		40°C	time	value		70°C	time	value
	4	0.99221462			4	0.99221462			4	1.03373665
	4	0.99221462			4	1.07525867			4	0.99221462
	4	0.99221462			4	1.03373665			4	1.03373665
	4	0.99221462			4	0.99221462			4	1.03373665
	4	1.07525867			4	1.03373665			4	1.07525867
	4	1.03373665			4	0.99221462			4	0.99221462
	8	1.03373665			8	0.9506926			8	0.99221462
	8	0.99221462			8	0.99221462			8	0.99221462
	8	0.99221462			8	0.99221462			8	0.99221462
	8	0.9506926			8	0.99221462			8	0.99221462
	8	0.9506926			8	0.99221462			8	0.99221462
	8	0.99221462			8	0.99221462			8	0.99221462
	24	0.99221462			24	1.07525867				
	24	0.9506926			24	1.03373665				
	24	1.03373665			24	0.99221462				
	24	0.9506926			24	1.03373665				
	24	1.03373665			24	1.03373665				
	24	0.99221462			24	1.03373665				
slope	-0.0006179	1.00424283	intercept		0.00135935	0.99666341			-0.0086504	1.061418
u(slope)	0.00095938	0.01418679	u(inter)		0.00085226	0.01260268			0.00319012	0.02017608
r^2	0.02526954	0.03517163	stderr		0.13718821	0.03124433			0.42372881	0.02210179
F	0.41479433	16	dof		2.54402102	16			7.35294118	10
significant:		no				no				yes

Figure B-2 displays the normalised values of the replicate measurements in dependence on the time of subjection to temperature stress. Different symbols refer to the different temperature stress levels.

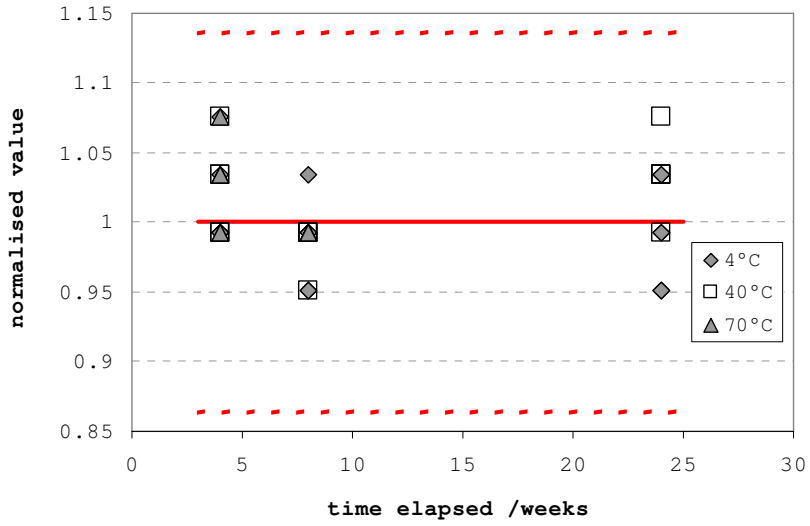

Figure B-2: Normalised values for d_{50} in dependence on the different stress levels, and the reference value and its expanded uncertainty band.

d_{90}

Results of the accelerated aging study for the volume-weighted size parameter d_{90}. Values are relative to the reference value (total mean of the homogeneity study). Rows below the data columns display the parameters of the corresponding regression function over time. The result of the significance test for the regression is also given.

relative to reference			d_90						
4°C	time	value		40°C	time	value		70°C	time	value
	4	0.97772635			4	0.93317906			4	1
	4	0.9554527			4	1.0445473			4	0.97772635
	4	0.93317906			4	1.0445473			4	0.97772635
	4	0.93317906			4	0.9554527			4	0.97772635
	4	1.06682094			4	1.02227365			4	1.06682094
	4	0.9554527			4	0.9554527			4	0.9554527
	8	1.11136824			8	0.97772635			8	0.9554527
	8	1			8	1.02227365			8	0.97772635
	8	1			8	0.9554527			8	0.97772635
	8	0.93317906			8	0.97772635			8	0.9554527
	8	0.91090541			8	0.97772635			8	0.97772635
	8	0.97772635			8	0.9554527			8	0.97772635
	24	0.93317906			24	1.06682094				
	24	0.93317906			24	1				
	24	1.0445473			24	1				
	24	0.93317906			24	1.0445473				
	24	1.06682094			24	1.0445473				
	24	1.02227365			24	1.06682094				
slope	0.00066291	0.97472118	intercept		0.00265162	0.97065535			-0.0055684	1.0148491
u(slope)	0.00163536	0.02418262	u(inter)		0.00101586	0.01502184			0.00415045	0.02624975
r^2	0.01016536	0.05995311	stderr		0.29865605	0.03724188			0.15254237	0.02875516
F	0.16431603	16	dof		6.8133428	16			1.8	10
significant:		no				yes				no

Figure B-3 displays the normalised values of the replicate measurements in dependence on the time of subjection to temperature stress. Different symbols refer to the different temperature stress levels.

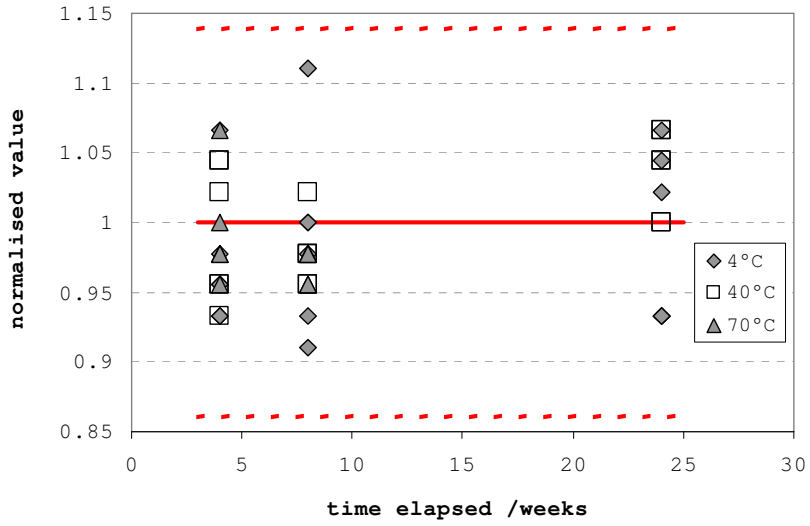

Figure B-3: Normalised values for d_{90} in dependence on the different stress levels, and the reference value and its expanded uncertainty band.