

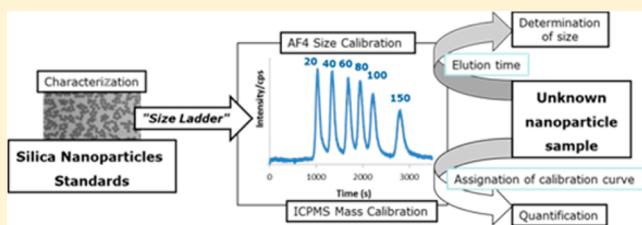
Simultaneous Determination of Size and Quantification of Silica Nanoparticles by Asymmetric Flow Field-Flow Fractionation Coupled to ICPMS Using Silica Nanoparticles Standards

Francisco Barahona, Otmar Geiss, Patricia Urbán, Isaac Ojea-Jimenez, Douglas Gilliland, and Josefa Barrero-Moreno*

European Commission, Joint Research Centre, Institute for Health and Consumer Protection, Via E. Fermi 2749, 21027 Ispra, Varese Italy

S Supporting Information

ABSTRACT: This work proposes the use of multimodal mixtures of monodispersed silica nanoparticles ($\text{SiO}_2\text{-NPs}$) standards for the simultaneous determination of size and concentration of $\text{SiO}_2\text{-NPs}$ in aqueous suspensions by asymmetric flow field-flow fractionation (AF4) coupled to inductively coupled plasma mass spectrometry (ICPMS). For such a purpose, suspensions of $\text{SiO}_2\text{-NPs}$ standards of 20, 40, 60, 80, 100, and 150 nm were characterized by transmission electronic microscopy (TEM), centrifugal liquid sedimentation (CLS), dynamic light scattering (DLS) and by measuring the Z-potential of the particles as well as the exact concentration of NPs by offline ICPMS. An online AF4-ICPMS method which allowed the separation of all the different sized $\text{SiO}_2\text{-NPs}$ contained in the mixture of standards was developed and the analytical figures of merit were systematically evaluated. The method showed excellent linearity in the studied concentration range ($0.1\text{--}25 \text{ mg L}^{-1}$), limits of detection between 0.16 and 0.3 mg L^{-1} for smaller and greater particles, respectively, besides a satisfactory accuracy. AF4 calibration with particles with identical nature to those to be analyzed, also permitted accurate size determination in a pragmatic way. Similarly, by using prechannel calibration with NPs for mass determination it was possible to overcome common quantification problems associated with losses of material during the separation and size-dependent effects. The proposed methodology was successfully applied to the characterization in terms of size and concentration of aqueous test samples containing $\text{SiO}_2\text{-NPs}$ with monomodal size distributions.



Engineered nanoparticles (ENPs), including silica nanoparticles ($\text{SiO}_2\text{-NP}$), can be produced using a wide variety of well-established synthetic techniques with precise control over their physical and chemical characteristics. Because of their particular properties, ENPs offer technical or economic benefits so that the interest in the applications of such materials is continuously growing.¹ $\text{SiO}_2\text{-NPs}$ have been developed for a wide range of applications in different fields of research such as sample preparation,² development of sensors³ and biomedical applications, including imaging contrast agents, controlled-release delivery vehicles^{4,5} or intracellular sensing.^{6,7} Nano-medicine-related applications of $\text{SiO}_2\text{-NPs}$ have been widely investigated and outcomes are frequently reviewed.⁸

In addition to this, synthetic amorphous silica has been used in food processing, food and feed applications for decades as anticaking agent in powdered food, in clearing processes of beverages or fragrance and flavor carriers.^{9,10} The material, registered as food additive E551, often contains primary particles and aggregates with sizes smaller than 100 nm,¹¹ which is the size-threshold of nanomaterials according to the European Commission Recommendation on the definition of nanomaterial.¹² However, as it is the case of biomedical applications, there is still insufficient knowledge about potential

health and environment risks.¹³ In this regard, biosafety evaluations of $\text{SiO}_2\text{-NPs}$ have revealed that the *in vivo* behavior of NPs are strongly related to their preparation procedures, particle size and shape, surface chemistries, dosing parameters, and administration routes.¹⁴

Currently, there is a lack of well documented and validated test methods to detect, characterize and quantify NPs in general and $\text{SiO}_2\text{-NPs}$ in particular for environmental, biomedical or food applications.¹⁵ As a consequence, there is widespread agreement that efforts should be dedicated to develop accurate analytical methods allowing comprehensive characterization of nanomaterials. Furthermore, since no universally applicable measurement method is available, any method development must be carefully targeted toward specific but relevant materials. In this regard, hydrodynamic size-based separation techniques, such as hydrodynamic chromatography (HDC), field-flow fractionation (FFF) based methods, such as sedimentation field-flow fractionation (SdFFF) and asymmetric

Received: December 17, 2014

Accepted: January 27, 2015

Published: January 27, 2015

flow field-flow fractionation (AsFFFF or also cited as AF4), are powerful separation techniques¹⁶ well suited for the characterization of NPs in biological,¹⁷ environmental^{18–21} and food samples.^{22–24} Although, in principle, AF4 theory^{25,26} could be used to obtain NPs size information from the calibration with size standards regardless the nature of the particles, in practice the interactions between NPs and the permeation membrane within the separation channel can lead to errors in the calculated particle dimensions.^{27,28} In the same manner, losses of material in AF4 separation often make quantification difficult²⁹ and recovery issues must be addressed either by a *case-by-case* (and *size-by-size*) basis³⁰ or via a multidetector approach.³¹ Both HDC and FFF-based separation techniques have often been coupled to inductively coupled plasma mass spectrometry (ICPMS),^{32,33} thus combining the advantage of AF4's ability to separate NPs according to their hydrodynamic diameter with the excellent sensitivity and selectivity of ICPMS detection.

Despite these promising features, only a very few publications actually report on the application of hydrodynamic size-based separations coupled to ICPMS for the determination of size and quantification of SiO₂-NPs. This can be partially attributed to the fact that SiO₂-NPs standards suited to the development and verification of such analytical methodologies are also still scarce and in general insufficiently documented. For instance, although there are some examples of certified SiO₂-NPs suspensions for size (Institute for Reference Materials and Measurements ERM FD100, ERM FD102, and ERM FD304), they do not cover an adequate range of size yet. In addition, there are currently no certified materials for SiO₂-NPs concentration. HDC-ICPMS has been employed^{34,11} to assess on the presence of SiO₂ in the nanoform in food products using particles in the size range of 32–500 nm to calibrate the HDC column and estimate the NP size. However, no detailed description of SiO₂-NPs size distribution was made nor was a systematic evaluation of the analytical performance of the method, including possible differences in the recovery related to the size, undertaken. This last issue plays a crucial role in the development of accurate methods for NP quantification, as it was observed by Heroult and co-workers who found clear bias in the quantification of SiO₂-NPs in food matrix by AF4-ICPMS using postchannel calibration with ionic silicon (direct injection into the ICPMS), which were attributed to unexplained losses of material in the separation channel.³⁵ These difficulties can be potentially overcome by using prechannel calibration with NPs of similar nature to the test NPs. In prechannel calibration the NPs standards are introduced before the separation system so that they undergo the same conditions as NPs present in the test samples. Tadjiki and co-workers used prechannel calibration with single-sized SiO₂-NPs standard of 70 nm for the quantification of SiO₂-NPs by SdFFF coupled to UV and Light Scattering detectors,¹⁷ but different recoveries in the size range under study (70–250 nm) were not considered and the analytical performance of the proposed method was only partially evaluated.

In summary, either the recovery determination for each specific case or alternative methodologies involving the use of multiple techniques have been proposed. The latter requires multiple high-cost technologies and results in long, tedious and not pragmatic methodologies. The prechannel calibration approach with SiO₂-NPs proposed in this work addresses these important drawbacks allowing the simultaneous determination of size and quantification of SiO₂-NPs by AF4-ICPMS.

For this purpose, a set of dispersions of SiO₂-NPs in the range 20–150 nm have been comprehensively characterized for their application as size and concentration standards in the form of a multimodal SiO₂-NPs standard mixture, functioning as a *size-ladder*. An AF4-ICPMS method has been developed, subjected to in-house validation and systematically documented in this article. To test the applicability of the methodology presented, various samples containing monodispersed SiO₂-NPs from diverse sources and prepared for different purposes, were also analyzed.

■ EXPERIMENTAL SECTION

Chemicals. The following chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, USA): ammonium carbonate (product code 379999), sodium dodecyl sulfate (SDS, product code 436143), hydrofluoric acid (HF, product code 339621), nitric acid (product code 84385), yttrium, and silicon standards for ICPMS (codes 01357 and 08729, respectively). Sodium hydroxide (product code 383042500) was purchased from Acros Organics (Thermo-Fischer Scientific Group, Belgium). Hydrochloric acid (product code 100317) was purchased from Merck (Merck KGaA, Germany). Water used in all the experiments was Milli-Q ultrapure grade water.

Nanoparticles. SiO₂-NPs (NanoSilica Size Standards) with 20, 40, 60, 80, 100, and 150 nm nominal diameters, respectively, were purchased from MSP Corporation (Shoreview, MN, USA). SiO₂-NPs (NanoXact Silica) with 20 and 80 nm, here named NC-20 and NC-80 respectively, were purchased from NanoComposix (San Diego, CA, USA). Polystyrene size standards (3000 Series Nanosphere Size Standards) with 20, 40, 60, 81, and 102 nm nominal diameter were obtained from Thermo (ThermoFischer Scientific, Waltham, MA, USA) and used in the development of the AF4 method. Aqueous SiO₂ suspensions labeled as NS1–8 were synthesized in a laboratory with a different research purpose, and donated as test samples to evaluate the performance and applicability of the method.

Stock suspensions were stored in dark and following the supplier's recommendations. Dilutions and working suspensions from the stock materials were freshly prepared.

Characterization of Silica Standards by Transmission Electron Microscopy. For transmission electron microscopy (TEM) analysis, each sample (5 μL) was dropped onto ultrathin Formvar-coated 200-mesh copper grids (Ted Pella, Inc.) and air-dried. Images were acquired with a JEOL 2100 electron microscope (Japan) at an accelerating voltage of 200 kV. For each sample, the size of at least 300 particles was measured to obtain the average size using ImageJ software.

Dynamic Light Scattering, Z-Potential, and Centrifugal Liquid Sedimentation measurements. A Zetasizer Nano-ZS, (Malvern, England) was used to perform dynamic light scattering (DLS) and Z-potential measurements. Direct dilutions of the stock suspensions were prepared in ammonium carbonate 0.25 mM and measurements were performed at 25 °C in triplicate. Sodium hydroxide 0.1 M or hydrochloric acid 0.1 M was added to the suspensions when pH adjustment was required.

Diameter of the particles has been evaluated by centrifugal liquid sedimentation (CLS) in sucrose gradient 8–24% (w/w) with a CPS disc centrifuge model DC24000 (CPS Instruments Europe, Netherlands) running at 22000 rpm. The instrument was calibrated before each measurement using an aqueous

reference solution of 377 nm diameter poly(vinyl chloride) spheres. All samples were vortexed for 30 s prior to injection. Parameters of sample (solid) density 1.9 g cm⁻³, media refractive index 1.344 and sample refractive index 1.45 were assumed in the measurement.

Elemental Analysis of Silica Nanoparticles by ICPMS.

The elemental analysis was performed using a PerkinElmer Nexlon 300D quadrupole ICPMS, equipped with a SC Fast peristaltic pump, a Meinhard concentric nebulizer, a glass cyclonic spray chamber and a standard quartz torch (2.5 mm i.d.). The system operated in standard mode monitoring isotopes *m/z* 28 and 29 for Si with dwell time of 50 ms and integration time of 1 s. The quantification was performed by external calibration with silicon standards for ICP (TraceCert, Sigma-Aldrich). Reported values were the average result of 5 measurements.

Elemental Analysis of Digested Silica by ICPMS.

SiO_2 -NPs were digested by mixing 100 μL of each SiO_2 suspension with a determined volume of concentrated hydrofluoric acid (HF) in 10 mL polypropylene tubes, which were subsequently filled up to 1 mL with water. A volume of 50 μL of HF ensured the dissolution of 1 mg of SiO_2 contained in the suspensions and this proportional ratio was kept constant. The tubes were sealed and placed in an ultrasound bath thermostated at 60 °C for 1 h. Prior to ICPMS analysis the solutions were diluted with water to produce a final HF concentration level of approximately 0.05%. It should be noted that HF is a dangerous substance and it must be handled with caution and following the appropriate environmental, health and safety recommendations. The content of silicon was determined using an ICPMS HF-compatible configuration, which consisted of a Teflon PFA-ST nebulizer, a Teflon PFA cyclonic spray chamber, a 1.8 mm sapphire injector and a ceramic torch. The system operated with identical settings to previous section. The quantification was also performed by external calibration with ionic silicon standards for ICP, under identical settings.

Online AF4-MALS-ICPMS Coupling: Separation and Elemental Analysis of Silica Nanoparticles. A SiO_2 -NPs suspension volume of 50 μL was injected into the AF4 system for separation. The AF4 instrument comprised an Eclipse Dualtec Separation System from Wyatt Technology Europe and an Agilent 1260 Infinity high performance liquid chromatograph (HPLC) equipped with a degasser (G1322A); an Isocratic pump (G1310B); an autosampler (G1329B); a multi wavelength detector (G1365C), from Agilent Technologies. A DAWN 8⁺ Heleos II multiangle light scattering (MALS) detector operating with a 658 nm laser (Wyatt Technology) was coupled to the fractionation system and the 90° angle was used to monitor the signal. The MALS detector flow cell was subjected to a weekly ultrasound cleaning using a COMET cleaning system (Wyatt Technology). A pre-cut 10 kDa regenerated cellulose membrane and a 350 μm height spacer were assembled inside the Eclipse SC channel 153 mm effective length and 22 mm maximum spacer width, which was located inside a Thermos[PRO] thermostatic unit, all from Wyatt Technologies. The temperature was set at 25 °C. The eluent consisted of a 0.25 mM ammonium carbonate aqueous solution which was prepared fresh every day. A Solaris UV lamp (Wyatt Technology) was placed into the eluent bottle to avoid algal and bacterial growth. Separation settings are summarized in Table 1. The duration of a representative AF4 run was 70 min, which included 3 min of additional focus and 50 min of elution.

Table 1. Settings for AF4 Separation, Where F_{ch} , F_{cr} , and F_f Stand for Channel Flow, Cross-Flow, and Focusing Flow, Respectively

step	time (min)	F_{ch} (mL min ⁻¹)	F_{cr} (mL min ⁻¹)	F_f (mL min ⁻¹)
pre-elution	1	0.5		1
focus	2	0.5		1
focus + injection	3	0.5		1
add. focus	3			1
1st elution	<i>a</i>	0.5		1
2nd elution	10	0.5		
3rd elution	1	0.5		1

^aTime for the first elution step (*) varied among experiments, allowing total elution of injected materials.

The AF4 detector flow-outlet tube was connected directly to the ICPMS. A 25.0 $\mu\text{g L}^{-1}$ yttrium aqueous solution (1% nitric acid content) was used as internal standard to correct signal variations. The internal standard solution was aspirated through a 0.38 mm internal diameter tube by the SC Fast peristaltic pump operating at 10 rpm (0.33 mL min⁻¹), and connected to the detector flow via a “T-piece” junction, located prior the nebulizer inlet. Isotopes monitored were Si *m/z* 28 and Y *m/z* 89 with dwell time of 50 ms and integration time of 1 s. The raw data were exported and converted into .xlsx file for subsequent integration of peak areas using the Peak Analyzer tool present in OriginPro8 software (OriginLab Corporation, USA).

Recovery Experiments. For recovery experiments, post-channel injections of particles were performed directly into the ICPMS using a manual Rheodyne injection valve equipped with a 50 μL sample loop. The detector flow coming from the AF4 was connected to the Rheodyne valve and used as carrier (0.5 mL min⁻¹, 0.25 mM ammonium carbonate). Recovery was calculated dividing the average ($n = 5$) peak areas obtained by prechannel injection with those obtained with postchannel injections of particles. Saturation of the membrane was performed first by consecutive injections of a fix volume of SiO_2 -NPs standards until increase of peak area was not observed anymore.

Application to Test Samples. The particle size in the test samples was determined from the time of elution by interpolation of the size calibration curve. Concentrations were calculated by prechannel calibration with SiO_2 -NPs standards. A 200- and 1000-fold dilution factor was applied to samples NS1–8 and NC20–80, respectively, prior to AF4-ICPMS analysis.

RESULTS AND DISCUSSION

As mentioned previously in the introduction, one of the main problems for the characterization and analysis of nanomaterials, it is the lack of certified reference materials, reference materials and, in general, appropriate standards of size and concentration in the nanorange. Thus, in this work monodisperse NanoSilica Size Standards (MSP Corp.) have been well characterized both in terms of size and concentration before being used as standards in the proposed AF4-ICPMS method. Suspensions of SiO_2 -NPs standards showed good stability over at least 8 weeks at least as verified by periodic analysis by DLS and AF4-MALS.

TEM Characterization of Silica Nanoparticles Standards. Micrographs of different sized SiO_2 -NPs from MSP

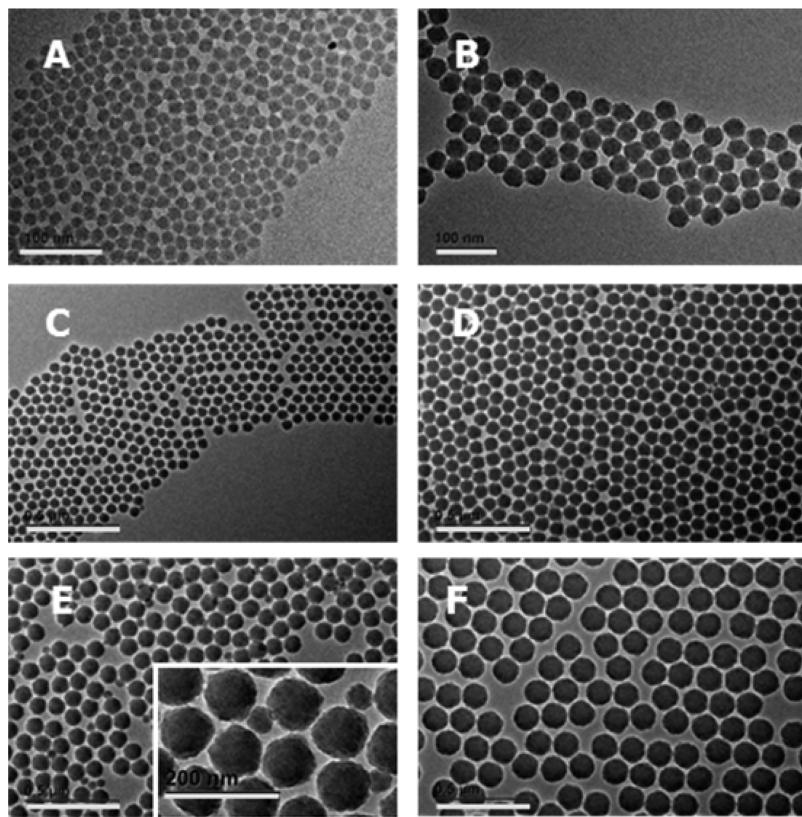


Figure 1. TEM images of SiO_2 -NP from MSP Corp.: (A) 20, (B) 40, (C) 60, (D) 80, (E) 100 (inset shows detail), and (F) 150 nm diameter. The scale bar is 100 nm for A and B and 500 nm for C–F.

Table 2. Characterization of SiO_2 -NP Suspensions Purchased from MSP Corp.

particle mobility spectrometry ^b			TEM ^c		DLS ^d		CLS ^e		Z-potential	
nominal size (nm)	<i>d</i> (nm)	fwhm (nm)	<i>d</i> (nm)	RSD (%)/N	Z-average (nm)	PDI	<i>d</i> (nm)	PDI	% mass ^f	mV
20	20.2	2.4	19.4	10.7/300	23.6	0.212	19.6	<i>a</i>	0.1	-50.5
40	39.1	2.4	38	8.5/778	40.3	0.015	39.1	1.107	0.4	-50.6
60	61.6	2.3	53.7	17.9/620	64	0.011	62.6	1.03	0.3	-53.8
80	79.1	3.2	70.6	4.7/1245	81	0.009	81	1.032	0.4	-54.2
100	99.3	3.7	91.1	7.5/848	100.8	0.012	101.9	1.014	0.4	-55.2
150	148	4.4	135.1	10.7/520	149.3	0.021	151.1	1.011	0.5	-56.7

^aBelow the limit of detection of the instrument. ^bSize measured by particle mobility spectrometry, as declared by the supplier; Full-width at half-maximum (fwhm) is included as indicator of dispersity. ^cSize measured by TEM; N is the number of particles counted. ^dZ-average and polydispersity index (PDI) obtained by DLS cumulants analysis. ^eSize measured by CLS; PDI defined as $D(w)/D(n)$, where $D(w)$ is the mean particle size expressed in a weight basis while $D(n)$ is the mean particle size expressed in a number basis. ^fApproximate content of SiO_2 -NP in 0.25 mM ammonium carbonate solution.

were taken by TEM and they are displayed in Figure 1. Particles of all different sizes were close to spherical in shape. It is noteworthy the presence of a secondary minority population of smaller particles for 60, 80, and 100 nm. The contribution in mass of this secondary population of particles was studied for the 100 nm particle suspension, where the presence of the second population was more noticeable (see inset detail in Figure 1E). After evaluation by AF4-MALS and TEM, it was confirmed that the 100 nm suspension also contained a lesser quantity of particles with a size of approximately 50 nm. However, the contribution in mass to the total mass of SiO_2 was estimated to be lower than 2% and hence negligible. Mean diameters measured by TEM are included in Table 2. The results are in agreement with the measurements obtained by complementary techniques also included in Table 2. The relative standard deviations (RSDs) of the TEM mean diameter

oscillated between 4.7% and 17.9%, suggesting good monodispersity.

Dynamic Light Scattering and Centrifugation Liquid Sedimentation. All the suspensions of SiO_2 -NPs were analyzed by DLS, in order to provide representative homogeneous values for hydrodynamic size. Ammonium carbonate 0.25 mM was selected as dispersant as it was the same solution acting as carrier in the AF4 system. To verify that the concentration of nanoparticles was not affecting the quality of the measurements, SiO_2 -NPs suspensions at different values of concentration (0.01%, 0.1%, and 1%, respectively) were also measured. As Figure 2A illustrates, size characterization by DLS was performed within a range of concentration where size measurements are independent of the NPs content.

With an identical aim, measurements of the particle size were also performed by CLS. Results of DLS and CLS measurements

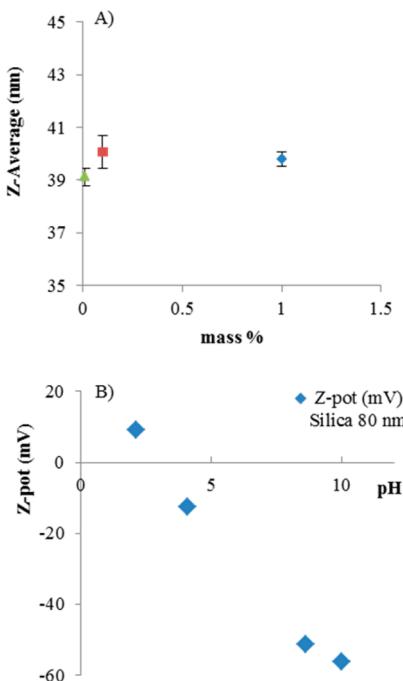


Figure 2. (A) DLS measurement of 40 nm SiO_2 –NP at 0.01, 0.1, and 1% solid mass content, in ammonium carbonate 0.25 mM; B) Measurements of the Z-potential of 80 nm nanosilica suspensions (approximate concentration 0.08%, solid mass content) at different pH. In all cases, $n = 3$.

are summarized in Table 2 together with the diameters measured by particle mobility spectrometry, according to the information provided by the supplier. CLS measurements were performed assuming the manufacturer declared value of particle density (1.9 g cm^{-3}).³⁶ The corresponding polydispersity indexes (PDI) for both DLS and CLS measurements are also reported in the table. All results suggest that the materials are highly monodispersed.

Z-Potential Characterization. For better characterization of the particles, the Z-potential of the particles was measured. Values obtained for the Z-potential of the SiO_2 suspensions in ammonium carbonate solution (pH of 9, approximately) are also included in Table 2. Data show negative values for all different sizes, ranging between -50.5 mV for the smaller and -56.7 mV for the bigger particles. Since the pH of the suspension directly affects the Z-potential of the particles and this may influence not only colloidal stability but also interactions with the permeation membrane, the Z-potential of 80 nm silica suspensions at different pH were measured and the results are depicted in Figure 2B. From this it can be seen that the isoelectric point of the material is to be found in the pH range from 2 to 4. Furthermore, it can be seen that the Z-potential values of the particles in ammonium carbonate fall into a region where the surface charge is strongly negative and varies little with pH so favoring an electrostatically stabilized colloid.

Quantification of Silica Nanoparticles Standards. To quantify SiO_2 –NPs in unknown samples, the first step is the precise determination of the exact concentration in the standards. Thus, measurements of the silicon content of the NP size standards were performed by ICPMS, operating in batch-mode. Quantification was carried out using external calibration with ionic silicon, followed by stoichiometric

correction. Prior to the measurements of SiO_2 –NPs, the method for ionic silicon was optimized by varying the flow rate of He in the collision chamber (Dynamic Reaction Cell mode) between 0.1 and 2 mL min⁻¹. The signal/background ratio for the three different silicon isotopes was then evaluated. Since no signal/background-ratio improvement was observed the standard mode without helium for Si m/z 28 was finally selected to quantify the NP suspensions. Similarly, no signal/background-ratio improvement was observed when using the HF-compatible configuration in the ICPMS system. To assess the efficiency of the ionization of the SiO_2 –NPs standards, an aliquot of the original suspension was digested using HF and measured by ICPMS. The signal was compared to that obtained from undigested SiO_2 , at similar concentration level (see Supporting Information Figure S-1). The ratio between the signal of digested and undigested particles ranged between 88% and 102% across the different sizes, leading to the conclusion that direct quantification of the SiO_2 –NPs is possible and reliable in the size range considered this work. These observations are in good agreement with Prestel and co-workers, who pointed out that the atomization was complete with monodisperse SiO_2 colloids with particle diameters $<500 \text{ nm}$.³⁷ The obtained final concentrations (see Supporting Information Table S-1) were subsequently used for further quantification purposes.

AF4 Method Development and Evaluation of SiO_2 –NPs as Size Standards. To evaluate the suitability of the SiO_2 suspensions as size standards for AF4 separation, mixtures containing all the different particle sizes (20, 40, 60, 80, 100, and 150 nm) were prepared in water and injected in the AF4-MALS system under different separation conditions. The effects of the carrier chemical composition and different cross-flow rates on the separation and peak resolution were investigated.

Analyses by AF4-MALS of a mixture containing all the six different SiO_2 size standards were performed with different eluents under identical separation conditions. SDS 0.05% (pH = 7.3) and ammonium carbonate 0.25 mM (pH = 8.9) were tested as AF4 carriers. SDS (0.05% aqueous) was tested as AF4 eluent, as it is a well-established carrier solution for polystyrene size standards. The pH of the fresh solution was slightly above 7 (7.3). It is noteworthy that despite of the fact that the pH of the SDS solution was close to the pH of ultrapure water, the introduction of low concentrations of surfactant in the carrier solution leads to a proper separation of the SiO_2 particles. Different phenomena might contribute to explain this behavior, being the electrostatic mechanism generally used to explain the stabilization of nanoparticles by anionic surfactants.¹⁹ Fractograms obtained using SDS as eluent exhibited well resolved peaks but a noisy background signal (see Supporting Information Figure S-2).

Separation of SiO_2 size standards with ammonium carbonate 0.25 mM as carrier was also studied, as it is a highly volatile buffer that offers a mild ionic content and a controlled pH suited for the stabilization of the SiO_2 –NPs. Fractograms (displayed in Supporting Information Figure S-2) showed well-defined peaks for all different particle sizes with good resolution and smooth baseline. Figure 3 shows the size calibration curves constructed by plotting the hydrodynamic diameters of the SiO_2 –NPs standards and polystyrene size standards measured by DLS, versus their respective time of elution in both SDS and ammonium carbonate-based eluents. All curves exhibited good linearity (R^2 greater than 0.998), as well as very similar slopes. This behavior, in which two different kinds of particles differing

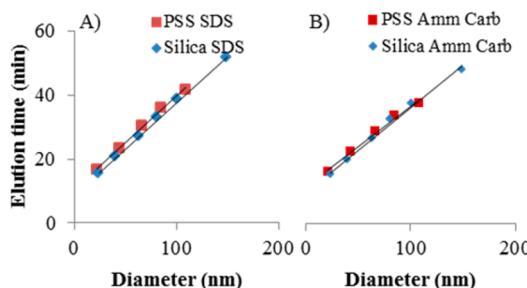


Figure 3. Size calibration curves obtained after injection of polystyrene standards ($2 \mu\text{g mL}^{-1}$ each) and SiO_2 -NP standards (concentrations between $10 \mu\text{g mL}^{-1}$ and $1.0 \mu\text{g mL}^{-1}$, for small and greater particles, respectively): (A) using SDS 0.05% as carrier and (B) using ammonium carbonate 0.25 mM as carrier. Diameters were determined by DLS (Z-average).

in physical properties such as core density, behave similarly through the separation channel, can be considered close to ideal and consistent with the FFF theory. Thus, under these conditions either polystyrene or SiO_2 size standards could be potentially useful to convert fractograms into intensity size distributions according to an external size calibration. Both for SiO_2 and polystyrene spheres, the times of elution were slightly shorter when using ammonium carbonate 0.25 mM compared to those obtained with SDS 0.05% (or equivalent 1.7 mM). This reduction in the retention time can be attributed to a lower ionic strength of the media. However, the use of diluted ammonium carbonate is more advantageous for the online AF4-ICPMS coupling due to its greater volatility and better background signal, preventing the introduction of potential interferences, such as carbon derivate (e.g., carbon monoxide, m/z 28), as well as to reducing contamination of the ICPMS cones.

The impact of varying the ionic strength on the retention time of the particles was also studied by comparing fractograms obtained using ammonium carbonate buffers at different concentrations (0.10, 0.25, and 0.50 mM). It was confirmed that as a result of a weaker repulsion between particles and membrane, the retention time of the particles shifted to longer times when higher salt concentrations were used. The same phenomenon was observed when injecting polystyrene NPs (see Supporting Information Figure S-3). The use of high concentration buffers also resulted in broader and less resolved peaks impairing peak resolution. Therefore, an intermediate concentration of 0.25 mM ammonium carbonate was selected as a compromise.

To have an optimum separation of SiO_2 -NPs standards in the size range of interest in a convenient and practical time of analysis, different cross-flow rate settings were applied, using 0.25 mM ammonium carbonate as eluent. Fractograms obtained with 0.75, 1.0, and 1.5 mL min^{-1} cross-flow rate values showed good peak resolution under the three different conditions (Supporting Information Figure S-4). A mild cross-flow rate of 0.75 mL min^{-1} enabled the separation of all the peaks in a shorter time. However, in order to avoid overlapping of the early eluting particles with the void peak, longer retention times might be desirable while keeping a reasonable time of analysis. On the other hand, peak tailing appeared when applying high cross-flow rate, suggesting that the particles are forced to interact with the membrane. Thus, a value of 1.0 mL min^{-1} was finally selected as a suitable cross-flow rate.

Evaluation of the Analytical Performance of the AF4-ICPMS Method: Recovery Experiments and Analytical Figures of Merit.

In contrast to postchannel mass-calibration with ionic silicon, prechannel mass-calibration with particles takes into account possible losses of analyte particles in the whole system due to interactions between the NPs and the permeation membrane or other wetted surfaces in the AF4 system. The losses of material were investigated using monomodal suspensions of 20, 60, and 100 nm SiO_2 -NPs at two different concentration levels. Samples were analyzed according to the AF4-ICPMS method described in the Experimental Section and recovery was calculated by comparing the peak areas obtained with postchannel injection of particles. Calculated recoveries, which ranged between 75% for the smallest size (20 nm) and 53% for the greatest size (150 nm), are shown in Figure 4. Data showed great repeatability at

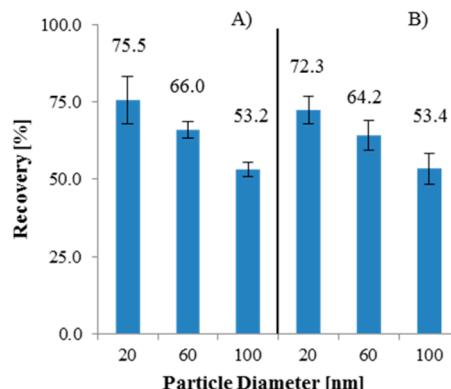


Figure 4. Recoveries of SiO_2 -NP, as the ratio of peak areas (postchannel/prechannel injections) measured by ICPMS ($n = 5$) at two different concentration levels: (A) 0.8 and (B) $5.0 \mu\text{g g}^{-1}$.

the two different levels of concentration under study. Taking into account that particles of different size exhibited a similar ionization efficiency in the ICPMS plasma, an important contribution to the lower recovery rate of bigger particles could be attributed to the fact that larger particles, being confined closer to the accumulation wall, have a greater possibility to interact irreversibly with the membrane. This is in agreement with previously reported results on Ag-NPs.³⁰ However, such an explanation should be considered as hypothesis that could be confirmed in future experiments replacing the separation channel with a bypass connector. As a result of the observed differences in the recovery rates which vary with the particle size, it is important to note that quantification of samples containing SiO_2 -NPs by prechannel injection of NPs standards with a different particle size to that of the analyte sample, could lead to under/overestimate the concentration in test samples. In this regard, and following the approach here proposed, concentrations should be then calculated using the corresponding prechannel calibration curve assigned to the standard with the closest time of elution.

The analytical performance of the developed AF4-ICPMS method was systematically studied by evaluating the resulting analytical figures of merit. Additionally, the method was applied for the characterization and evaluation of different test samples.

A seven point calibration was performed in triplicate by injecting into the AF4 separation channel a fixed volume of mixtures of the different SiO_2 -NPs standards included in this work in the concentration range of $0.1\text{--}25 \text{ mg L}^{-1}$. The

Table 3. Calculated Limits of Detection and Determination of Trueness and Precision, at Two Different Concentration Levels ($n = 5$)

nominal size (nm)	LOD (mg L ⁻¹)	trueness and precision					
		concentration level ^a			concentration level ^b		
found (mg L ⁻¹)	RSD (%)	t-value ^c	found (mg L ⁻¹)	RSD (%)	t-value ^c		
20	0.16	0.82	3.1	1.488	7.75	2.7	2.745
40	0.18	0.82	1.7	2.712	7.59	2.3	5.310
60	0.16	0.82	3.9	1.708	7.65	2.1	4.926
80	0.20	0.76	6.1	2.099	7.87	1.4	2.416
100	0.22	0.79	8.8	0.209	7.96	1.5	0.713
150	0.3	0.77	2.6	3.574	7.88	1.5	2.142

^a0.8 mg L⁻¹. ^b8.0 mg L⁻¹. ^cTheoretical t-value ($n = 5$, $p = 95$) = 2.776.

calibrations showed good linearity, and the regression coefficients obtained were greater than 0.999 in every case. The limits of detection (LOD), calculated as three times the average signal of the background noise, are summarized in Table 3. The LODs obtained were slightly lower than the value reported by Dekkers and co-workers³⁴ for SiO₂ aqueous suspensions (1 mg L⁻¹), and in general higher than values reported for the detection of NPs of a different nature by ICPMS, such as Ag-NPs^{38,30} or AuNPs,¹⁸ usually in $\mu\text{g L}^{-1}$ level. The relatively high LOD for SiO₂ is an inherent consequence of the monitoring of isotope *m/z* 28, which is hampered by the natural occurrence of interfering species such as carbon monoxide and nitrogen. However, according to the concentrations measured in publications reporting on the presence of SiO₂-NPs in a variety of applications, such as SiO₂-NPs for toxicological studies,³⁹ exposure assessment^{11,34} or occurrence in food,³³ the calculated LODs of the proposed method make it well suited for the detection and quantification of SiO₂-NPs in these types of matrixes.

The repeatability which is summarized in Table 3 was studied in terms of the relative standard deviations (RSDs) of the concentration values found experimentally for each size at two different concentration levels. The calculated intraday RSDs were always below 9% and 3% for the lower and higher concentration levels, respectively, proving excellent repeatability. In addition, trueness was determined applying the *t*-test ($p = 95\%$) at two different concentration levels. Obtained concentrations of SiO₂-NPs for each size were compared with the theoretical expected concentration value. The following equation allowed the calculation of the *t*-values included in Table 3:

$$t = \frac{|\bar{X} - \mu|}{s} \sqrt{n} \quad (1)$$

where \bar{X} is the average concentration of the five replicates, μ is the theoretical "should"-value, s is the standard deviation of the 5 replicates, n is the number of replicate measurements (5 in this case). The resulting *t*-values were compared with theoretical value for a confidence interval of 95%, and $n - 1$ degrees of freedom. The calculated *t*-values were within the range of acceptance in 9 of 12 measurements, suggesting thus an overall acceptable accuracy.

Applicability to Silica Nanoparticles Samples. The AF4-ICPMS method developed in this study has been applied to SiO₂-NPs-based test samples and used to simultaneously determine both the NP size and concentration. Test samples of SiO₂-NPs from commercial sources as well as materials tailor-made in-house for toxicological studies of SiO₂ in the nanoform

and are used here to illustrate the applicability of the method. Mixtures containing SiO₂-NPs standards of different sizes at different concentration levels were used to perform the corresponding size and concentration calibration curves as described in Experimental Section. Figure 5B shows a

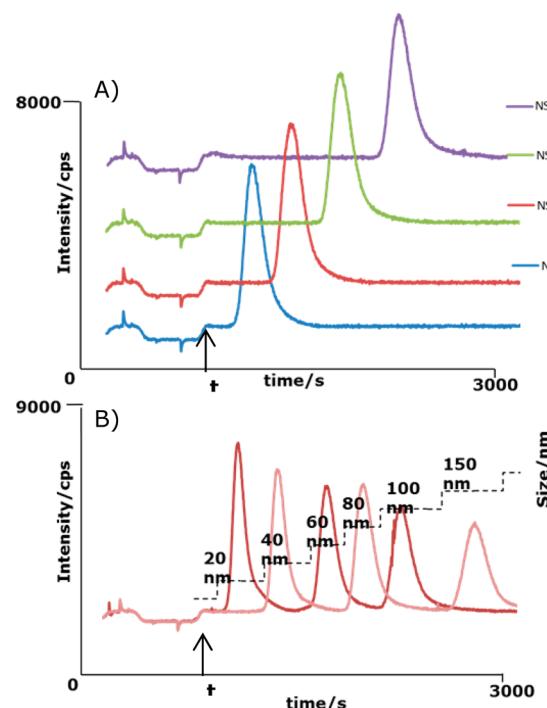


Figure 5. (A) Representative fractograms of samples NS1–4 according to the developed AF4-ICPMS method. (B) Illustrative elution profile of the SiO₂-NPs standards (size-ladder), obtained after injecting mixtures of standards with different sizes at 10 $\mu\text{g mL}^{-1}$ into the AF4-ICPMS system. The length of the segments representing the SiO₂-NPs standards size-ladder is proportional to the width of the elution peaks.

representative elution profile of the SiO₂-NPs standards that enabled the determination of size in test suspensions of NPs. Concentrations were then calculated using the corresponding prechannel calibration curve assigned to the standard with the closest time of elution. Figure 5A shows fractograms corresponding to test samples NS1–4, randomly selected as illustrative examples. The elution profiles showed single defined peaks that suggested the presence of monomodal size distributions. As it can be observed in the data included in Table 4, the calculated diameters using AF4 calibration are in

Table 4. Application of the AF4-ICPMS Method to the Determination of Size and Quantification of Nano-Silica in Test Samples

sample	size			concentration			
	DLS	AF4 calibration		offline ^a	calculated	online ^b	
	diameter (nm)	diameter (nm)	RSD (%)	measured (mg/L) × 10 ⁻³	(mg/L) × 10 ⁻³	RSD (%)	t-value ^c
NS-1	28.9	28	0.8	2.1	2.1	0.5	0.139
NS-2	39.7	46.4	0.4	2.6	2.5	2.1	5.587
NS-3	66.7	69.8	0.2	2.3	2.4	2	3.701
NS-4	103.6	100.6	1	2.5	2.5	0.8	1.087
NS-5	27.6	25	1.5	2.6	2.3	3.7	6.236
NS-8	89	90.9	2.3	2.5	2.3	6.3	3.311
NC-20	24.9	29.2	0.7	11.6 ^d	13. 9	1	
NC-80	98	96.7	0.3	10.3 ^d	10.4	1	
NS-6	39.9	37.5	n.a. ^e	2.6	3.3	n.a. ^e	
NS-7	62.7	55.6	n.a. ^e	2.3	2.6	n.a. ^e	

^aBatch ICPMS analysis. ^bOnline AF4-ICPMS analysis. ^cTheoretical t-value ($n=3$, p 95) = 4.3. ^dNot measured. Concentration declared by the manufacturer. ^en.a. = Not applicable.

good agreement with the values obtained by off-line DLS measurements. Similarly, Table 4 also includes the concentration values determined in triplicate by the online AF4-ICPMS, together with their RSDs and the expected concentration values as measured by off-line ICPMS analysis. To assess the accuracy of the measurements, the resulting experimental t-values were calculated for samples with at least three replicates and a reference concentration value. As in the case of the size determination, it is remarkable that quantifications following the proposed *size-ladder* approach using SiO₂-NPs standards were in general accurate and reliable. In contrast to off-line quantification by ICPMS, the online AF4-ICPMS method is selective for silicon in particulate form. The results demonstrate that the presence of silica in nonparticulate form was negligible in the samples analyzed.

CONCLUSIONS

This work has proposed an approach based on the use of multimodal mixtures of monodispersed SiO₂-NPs standards in an online AF4-ICPMS method for the simultaneous determination of size and concentration of SiO₂-NPs in aqueous suspensions.

After physical characterization and evaluation, the commercially available SiO₂-NPs standards under study were found to be well suited for use as size and concentration standards for AF4-ICPMS. A major drawback of the methodology presented here is the need to determine the exact concentration of the standard materials by off-line ICPMS analysis before being used as AF4-ICPMS standards. In this regard, the existence of SiO₂-NPs reference materials that could be used for simultaneous calibration of both size and concentration would be desirable.

The developed method was subjected to in-house validation by determining analytical figures of merit, such as recovery, precision, trueness, linearity, and limits of detection. The results suggested that the method performed in an accurate and reliable manner. According to the limits of detection obtained, and taking into account measured concentrations of SiO₂-NPs reported in scientific literature, the method shows a broad field of applicability.

The proposed methodology was successfully applied to the characterization in terms of size and concentration of aqueous test samples containing SiO₂-NPs with monomodal size distributions. However, for elution profiles without regular peak-shape, such as plateau-type or strongly overlapping peaks,

the applicability of the proposed method was not tested and therefore cannot be guaranteed. The application to more complex samples must be further investigated.

It has been proved that by using prechannel calibration with NPs of identical nature to those to be analyzed, it was possible to overcome common quantification problems associated with size dependent losses of material in the instrument prior to the ICPMS sample-introduction system. The proposed method permitted accurate size and mass determination of SiO₂-NPs in a pragmatic way.

ASSOCIATED CONTENT

S Supporting Information

Table S-1 and Figures S-1–4. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

REFERENCES

- Project on Emerging Nanotechnologies. Consumer Products Inventory, 2014. <http://www.nanotechproject.org/cpi> (accessed October 2014).
- Pyrzynska, K. *TrAC, Trends Anal. Chem.* **2013**, *43*, 100–108.
- Montalti, M.; Rampazzo, E.; Zaccheroni, N.; Prodi, L. *New J. Chem.* **2013**, *37*, 28–34.
- Jaganathan, H.; Godin, B. *Adv. Drug Delivery Rev.* **2012**, *64*, 1800–1819.
- Duan, R.; Xia, F.; Jiang, L. *ACS Nano* **2013**, *7*, 8344–8349.
- Schulz, A.; McDonagh, C. *Soft Matter* **2012**, *8*, 2579–2585.
- Ruedas-Rama, M. J.; Walters, J. D.; Orte, A.; Hall, E. A. H. *Anal. Chim. Acta* **2012**, *751*, 1–23.
- Libermana, A.; Mendez, N.; Troglerb, W. C.; Kummelb, A. C. *Surf. Sci. Rep.* **2014**, *69*, 132–158.
- EU Official Journal of the European Union. Regulation (Ec) No. 1333/2008 of the European Parliament and of the Council of 16 December 2008 on Food Additives, 2008.
- Peters, R.; Brandhoff, P.; Weigel, S.; Marvin, H.; Bouwmeester, H.; Aschberger, K.; Rauscher, H.; Amenta, V.; Arena, M.; Botelho Moniz, F.; Gottardo, S.; Mech, A. Inventory of Nanotechnology applications in the agricultural, feed and food sector, CFT/EFSA/FEED/2012/01.
- Peters, R.; Kramer, E.; Oomen, A. G.; Herrera Rivera, Z. E.; Oegema, G.; Tromp, P. C.; Fokkink, R.; Rietveld, A.; Marvin, H. J. P.; Weigel, S.; Peijnenburg, A. A. C. M.; Bouwmeester, H. *ACS Nano* **2012**, *6*, 2441–2451.

- (12) EC Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU), Official Journal of the European Union, L275/38.
- (13) Dekkers, S.; Bouwmeester, H.; Bos, P. M. J.; Peters, R. J. B.; Rietveld, A. G.; Oomen, A. G. *Nanotoxicology* **2013**, *7*, 367–377.
- (14) Chen, Y.; Chen, H.; Shi, J. *Adv. Mater.* **2013**, *25*, 3144–3176.
- (15) Alvarez, P. J. J.; Codin, V.; Lead, J.; Stone, V. *ACS Nano* **2009**, *3*, 1616–1619.
- (16) Gigault, J.; Pettibone, J. M.; Schmitt, C.; Hackley, V. A. *Anal. Chim. Acta* **2014**, *809*, 9–24.
- (17) Tadjiki, S.; Assemi, S.; Deering, C. E.; Veranth, J. M.; Miller, J. D. *J. Nanopart. Res.* **2009**, *11*, 981–988.
- (18) Tiede, K.; Boxall, A. B. A.; Tiede, D.; Tear, S. P.; David, H.; Lewis, J. *J. Anal. At. Spectrom.* **2009**, *24*, 964–972.
- (19) Dubascoux, S.; Von Der Kammer, F.; Le Hécho, I.; Potin Gautier, M.; Lespes, G. *J. Chromatogr. A* **2008**, *1206*, 160–165.
- (20) Jiménez, M. S.; Gómez, M. T.; Bolea, E.; Laborda, F.; Castillo, J. *Int. J. Mass Spectrom.* **2011**, *307*, 99–104.
- (21) Baalousha, M.; Stolpe, B.; Lead, J. R. *J. Chromatogr. A* **2011**, *1218*, 4078–4103.
- (22) Contado, C.; Ravani, L.; Passarella, M. *Anal. Chim. Acta* **2013**, *788*, 183–192.
- (23) Von Der Kammer, F.; Legros, S.; Larsen, E. H.; Loeschner, K.; Hofmann, T. *TrAC, Trends Anal. Chem.* **2011**, *30*, 425–436.
- (24) Gray, E. P.; Bruton, T. A.; Higgins, C. P.; Halden, R. U.; Westerhoff, P.; Ranville, J. F. *J. Anal. At. Spectrom.* **2012**, *27*, 1532–1539.
- (25) Wahlund, K. G.; Giddings, J. C. *Anal. Chem.* **1987**, *59*, 1332–1339.
- (26) Giddins, J. C. *Science* **1993**, *260*, 1456–1465.
- (27) Hagendorfer, H.; Kaegi, R.; Traber, J.; Mertens, S.; Scherrers, R.; Ludwig, C.; Ulrich, A. *Anal. Chim. Acta* **2011**, *706*, 367–378.
- (28) Gigault, J.; Hackley, V. A. *Anal. Bioanal. Chem.* **2013**, *405*, 6251–6258.
- (29) Ulrich, A.; Losert, S.; Bendixen, N.; Al-Kattan, A.; Hagendorfer, H.; Nowack, B.; Adlhart, C.; Ebert, J.; Lattuada, M.; Hungerbühler, K. *J. Anal. At. Spectrom.* **2012**, *27*, 1120–1130.
- (30) Geiss, O.; Cascio, C.; Gilliland, D.; Franchini, F.; Barrero-Moreno, J. *J. Chromatogr. A* **2013**, *1321*, 100–108.
- (31) Hagendorfer, H.; Kaegi, R.; Parlinska, M.; Sinnet, B.; Ludwig, C.; Ulrich, A. *Anal. Chem.* **2012**, *84*, 2678–2685.
- (32) Pornwilard, M.-M.; Siripinyanond, A. *J. Anal. At. Spectrom.* **2014**, *29*, 1739–1752.
- (33) Dubascoux, S.; Le Hécho, I.; Hassellöv, M.; Von Der Kammer, F.; Potin Gautier, M.; Lespes, G. *J. Anal. At. Spectrom.* **2010**, *25*, 613–623.
- (34) Dekkers, S.; Krystek, P.; Peters, R. J.; Lankveld, D. P.; Bokkers, B. G.; van Hoeven-Arentzen, P. H.; Bouwmeester, H.; Oomen, A. G. *Nanotoxicology* **2011**, *5*, 393–405.
- (35) Heroult, J.; Nischwitz, V.; Bartczack, D.; Goenaga-Infante, H. *Anal. Bioanal. Chem.* **2014**, *406*, 3919–3927.
- (36) Kimoto, S.; Dick, W. D.; Syedain, Z.; Pui, D. Y. H.; Roberts, D. L. 2014 International Aerosol Conference, Aug. 28–Sep. 2 2014, Effective Density of Silica Nanoparticle Size Standards.
- (37) Prestel, H.; Schott, L.; Niessner, R.; Panne, U. *Water Res.* **2005**, *39*, 3541–3552.
- (38) Bolea, E.; Jimenez-Lamana, J.; Laborda, F.; Castillo, J. R. *Anal. Bioanal. Chem.* **2011**, *401*, 2723–2732.
- (39) Slomberg, D. L.; Schoenfisch, M. H. *Environ. Sci. Technol.* **2012**, *46*, 10247–10254.