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First steps towards a generic sample preparation scheme for inorganic engineered nanoparticles in a complex matrix for detection, characterization, and quantification by asymmetric flow-field flow fractionation coupled to multi-angle light scattering and ICP-MS†

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The applicability of a multi-step generic procedure to systematically develop sample preparation methods for the detection, characterization, and quantification of inorganic engineered nanoparticles (ENPs) in a complex matrix was successfully demonstrated. The research focused on the optimization of the sample preparation, aiming to achieve a complete separation of ENPs from a complex matrix without altering the ENP size distribution and with minimal loss of ENPs. The separated ENPs were detected and further characterized in terms of particle size distribution and quantified in terms of elemental mass content by asymmetric flow-field flow fractionation coupled to a multi-angle light scattering detector and an inductively coupled plasma mass spectrometer. Following the proposed generic procedure SiO_2-ENPs were separated from a tomato soup. Two potential sample preparation methods were tested these being acid digestion and colloidal extraction. With the developed method a complete SiO_2-ENPs and matrix separation with a Si mass recovery >90% was achieved by acid digestion. The alteration of the particle size distribution was minimized by particle stabilization. The generic procedure which also provides quality criteria for method development is urgently needed for standardized and systematic development of procedures for separation of ENPs from a complex matrix. The chosen analytical technique was shown to be suitable for detecting SiO_2-ENPs in a complex food matrix like tomato soup and may therefore be extended to monitor the existence of ENPs during production and safety control of foodstuffs, food labelling, and compliance with legislative limits.

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Introduction

Labelling of consumer products containing engineered nanoparticles (ENPs) will be a future legislative requirement in the EU ("EU recommendation on the definition of nanomaterials", 2011/696/EU) but also in many other countries which develop regulatory approaches for nanomaterials. Analytical methods to detect, characterize, and quantify these ENPs will therefore be required for the implementation and enforcement of such regulations.[1] Besides, such methods are also required for the detection and quantification of target ENPs in order to provide empirical data for risk assessments of ENPs released into the environment.[2] Generic procedures are not available yet. Therefore, they have to be developed in order to harmonize systematic method development procedures and apply uniform quality criteria for method optimization.

The ENPs in consumer products such as personal care products or foodstuffs are usually suspended or embedded in complex matrices containing particles of sizes and/or compositions similar to the ENPs which shall be quantified. Interactions between the matrix components and the ENPs and/or the lack of specificity in measurement techniques prohibit the direct use of available sizing techniques such as nanoparticle tracking analysis (NTA). In order to overcome this problem, von der Kammer *et al.*[3] suggested using a stepwise procedure (including several preparative and analytical steps) to obtain the

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desired information on particle sizes and concentrations. Following the stepwise procedure the complexity of the sample is decreased during sample preparation by separation of the ENPs from the matrix, without changing the properties of the ENPs. The separation can be based on differences between the chemical and physical properties of the ENPs and those of the matrix constituents. Quantitative information is subsequently required on particle sizes and concentrations (*i.e.* elemental mass concentration).

This paper extends this stepwise sample preparation by the introduction of quantitative quality criteria and it demonstrates its applicability by means of a case study. In principle this stepwise procedure can be considered as a generic methodology for development of sample preparation methods. The generic sample preparation for separation of inorganic ENPs from a complex matrix was demonstrated for a systematic method development for separation of engineered SiO_2 nanoparticles (SiO_2-ENPs) from a tomato soup matrix. For subsequent characterization and quantification of the separated SiO_2-ENPs a combination of field flow fractionation (FFF) coupled online to multi-angle light scattering (MALS) and inductively coupled plasma mass spectrometry (ICP-MS) detectors was selected. FFF is an analytical separation technique, which is both rapid and non-destructive. For complex samples containing natural nanoparticles FFF has been proven to be a powerful technique[4–6] and its application for ENP analysis in food or cosmetics has been shown to be promising[7] (TiO_2,[8,9] Ag,[10,11] SiO_2[12]). The most widely used FFF technique is currently asymmetric-flow FFF (AF^4) that only separates the particles according to their diffusion coefficient or hydrodynamic diameter. Therefore, AF^4 is typically coupled with online detectors such as UV-vis spectroscopy, MALS, and/or ICP-MS, in order to obtain information on the concentrations (or other characteristics) of particles eluting from the separation channel.[13–16] The presence of large particles (>1 μm) interferes with the desired normal mode of AF^4 separation and ENPs attached to large flocks or large particles must be removed from the sample. AF^4 therefore requires the ENPs to be separated from the matrix and the extracted ENPs to be stabilized in aqueous suspension. Several proof-of-concept demonstrations have been published for the separation of different inorganic nanoparticles from organic matrices (*e.g.* from sunscreen or rat lung tissue).[8–10,12,17–19] Methods for characterizing TiO_2 nanoparticles as an ingredient of sunscreens have been reported.[8,9,19] Recovery of spherical SiO_2 nanoparticles from rat lung tissue by enzyme digestion was demonstrated by Deering *et al.*,[17] but SiO_2 mass recovery was less than 30%. Tadjiki *et al.*[18] reported SiO_2 mass recoveries of between 25 and 79% from biological media through acid digestion. SiO_2-ENPs as a food additive were separated from coffee creamer by aqueous extraction and subsequent analysis by AF^4-ICP-MS revealed possible artifacts due to sample preparation.[12] The detection and characterization of Ag-ENPs in complex matrices (*e.g.* in wastewater) has been addressed by Poda *et al.*[20] and Hoque *et al.*[16] Loeschner *et al.*[10] demonstrated the extraction of Ag-ENPs from chicken meat and their subsequent size separation by AF^4. Their work revealed that the retention behaviour of the ENPs could be affected by the sample preparation; in this particular case changes in the surface properties of ENPs resulted in problems during the subsequent analysis by AF^4. Most of the reported data does not include any criteria for evaluating the quality of the method presented, or provide independent size information derived from online static or dynamic light scattering measurements following FFF that could validate the size distributions determined by AF^4. Only Contado & Pagnoni,[8] Loeschner *et al.*[10] and Heroult *et al.*[12] used EM (SEM or TEM) imaging of the eluting particles to verify their separation methods. None of them provided a generic procedure, which would allow translating sample preparation methods to other complex matrices. Therefore, the objectives of this study were (1) to test and verify the applicability of a generic sample preparation procedure to isolate ENPs from a complex food matrices using the case of SiO_2-ENPs contained in tomato soup, and (2) to identify and reduce artefacts of the sample preparation on the particle size distribution and particle mass recovery. These objectives were addressed by developing a method for food material, which was produced and carefully characterized in Grombe *et al.* (2014)[21] as a proof-of-concept food reference material containing engineered nanoparticles. This material was tomato soup spiked with SiO_2-ENPs. The choice of SiO_2-ENPs was based on their practical relevance as an approved food additive (anti-caking agent, E551, EU no. 1129/2011), while the choice of tomato soup was also made on their practical relevance and to provide a complex matrix.

Materials and methods

Chemicals

The Milli-Q water (MQ-water) used throughout the study was prepared using a Millipore Advantage A10 system (Millipore, Billerica, USA) equipped with a Bio-Pak™ ultrafilter (5000 g mol^{-1} molecular mass cut-off) for final purification. Ammonium carbonate (AC, analytical grade) and sodium chloride (analytical grade) were purchased from Sigma Aldrich. The commercial surfactant mixture used was Fisherbrand™ FL-70™ Concentrate, a biodegradable detergent from Thermo Fisher Scientific (USA, New Jersey). All solutions were prefiltered using Anodisc 0.02 μm membrane filters (Whatman, Maidstone, UK). The pH values were measured with a Metrohm 6.0234.100 electrode (Metrohm, Switzerland). Different concentrations of NaOH solution (0.01, 0.1, and 1 mol L^{-1} NaOH) were prepared from NaOH pellets (Merck, analytical grade, USA) and Milli-Q water which were used for pH adjustment. For acid digestion we used 65% HNO_3 (Merck, Suprapure®, USA) and 30% H_2O_2 (Merck, Suprapure®, USA) solutions. For total digestion tests 40% HF (Merck, Suprapure®, USA), 30% HCl (Merck, Suprapure®, USA), and H_3BO_3 (Merck, ACS reagent, USA) were purchased from Merck.

Samples

The method was developed for tomato soup containing SiO_2-ENPs. The material was designed and produced by Grombe *et al.*[21] as a proof-of-concept reference material for food

products containing ENPs. The material was produced to enable the control of the accuracy of analytical methods for characterization of inorganic ENPs in complex matrices such as food. For the sake of a homogeneous material with a natural composition of the matrix and a stable reference dispersion of the originally added ENPs a number of compromises had to be made. *E.g.* a liquid sample was produced instead of a powdered food material and a SiO_2-ENP suspension (not approved as food additive) instead of a SiO_2 powder (approved food additive) was selected as additive to the tomato soup. Detailed information on the sample production and sample characterization are given by Grombe *et al.*[21]

For development of the sample preparation in this study four types of samples were applied (Table 1). (1) Pure SiO_2-ENP suspension (Aerodisp® W7520 N, Evonik (Hanau, DE)) which was used to spike to tomato soup. The initial pure SiO_2-ENP suspension was characterized in terms of size and concentration (see ESI part 3†). This sample was used to identify the effect of sample preparation on the particle size distribution. Tomato soup without (2) and with SiO_2-ENPs (3) was used to demonstrate the potential of particle matrix separation and the selectivity of the detection method. Tomato soup samples (TS + SiO_2-ENP_{aged}) were spiked with the SiO_2-ENP suspension approximately one year prior to conducting the experiments, as described by Grombe *et al.* (2014) (where it is named Nano-Lyse10), in order to reflect realistic conditions since it is usually "aged" samples that are of interest in food control. (4) Blank tomato soup was spiked with a known amount of SiO_2-ENPs prior (*ca.* 30 minutes) to the experiment (TS + SiO_2-ENP), using SiO_2-ENPs from the same batch as used in (3) in order to identify effects of the ageing on the sample preparation procedure. Additionally, blank tomato soup samples were run in parallel in order to determine the background level of SiO_2-ENPs. The organic carbon concentration in all samples (except the pure particle suspension) was similar to that in the TS + SiO_2-ENP_{aged} sample. All samples were stored at 4 °C until analysis.

Generic sample preparation procedure

The tested generic procedure was based on von der Kammer *et al.*[3] and claims that ENP matrix separation can be achieved by stepwise sample preparation. The generic procedure was used in this study for the optimization and development of a sample preparation method for separation of SiO_2-ENPs from a food matrix (tomato soup). For this purpose additional quality criteria such as recovery and particle size distribution were included in the generic procedure in order to evaluate the development and optimization of the sample preparation. Besides the optimization of the sample preparation for separation of ENPs from the complex matrix the generic procedure includes tests with pure ENPs in order to identify possible alteration of the ENP size distribution due to the preparation procedure. The selected example of SiO_2 in tomato soup is regarded as a first proof-of-concept for this generic sample preparation procedure (Fig. 1). The procedure involved four steps prior to AF^4 analysis. These steps and the quality criteria can be considered as generic. However, in each step various treatments were tested and optimized based on test criteria which are described in detail in the ESI part 1†. These treatments are sample specific and have to be selected for depending on the properties (*e.g.* liquid or solid) of a sample. Fig. 1 summarizes the treatments which were tested for the separation of SiO_2-ENPs from tomato soup. To improve readability of the work, detailed descriptions of these treatments and their optimization were presented in the ESI (part 2†).

Step I: homogenization of the sample. The effects of manual agitation, heating to 50 °C for 30 minutes, and mechanical mixing were tested.

Step II: ENP separation from the matrix. Both acid digestion and colloidal extraction were investigated for the removal of the organic matrix. Based on physicochemical properties of SiO_2-ENPs and the tomato soup matrix both methods are potentially suitable to fully separate SiO_2-ENPs and tomato soup matrix. In case of ENPs (*e.g.* Ag ENPs) which are not stable at acidic conditions acid digestion would not be a suitable separation method. The efficiency of the sample preparation was evaluated after step II (test criteria A in Fig. 1). This evaluation was based on the calculation of bulk Si mass recovery ($rec_{Si,bulk}$ see ESI part 1† for detailed calculation) and the particle separation efficiency from the matrix. Sample preparation only continued if both criteria matched (see Fig. 1).

Step III: ENP enrichment. This step was required to increase the ENP concentration in order to obtain particle mass concentrations, which were suitable for the subsequent analysis by AF^4 coupled to MALS and ICP-MS detectors.

Step IV: ENP stabilization. Particles had to be stabilized in order to avoid aggregation, which would affect the particle size distribution. Subsequently, the stabilized particle suspension

Table 1 Stock samples used during method optimization (n.d. = not determined), concentration data was adopted from ref. 21

Sample type	Abbreviation	$c_{initial}(SiO_2)$ [g L^{-1}]	Description
1. SiO_2-ENP suspension in pure water (pH = 8)	SiO_2-ENPs	40.4 ± 0.6	No tomato soup matrix
2. Pure tomato soup	TS	0.23 ± 0.02	Blank sample of tomato soup
3. Tomato Soup spiked with SiO_2-ENPs (aged)	TS + SiO_2-ENP_{aged}	17.5 ± 2.3	Spiked with SiO_2-ENPs about 12 months prior to experiment
4. Tomato Soup spiked with SiO_2-ENPs (fresh)	TS + SiO_2-ENP	20.2 ± 0.6	Spiked with SiO_2-ENPs immediately prior to the experiment

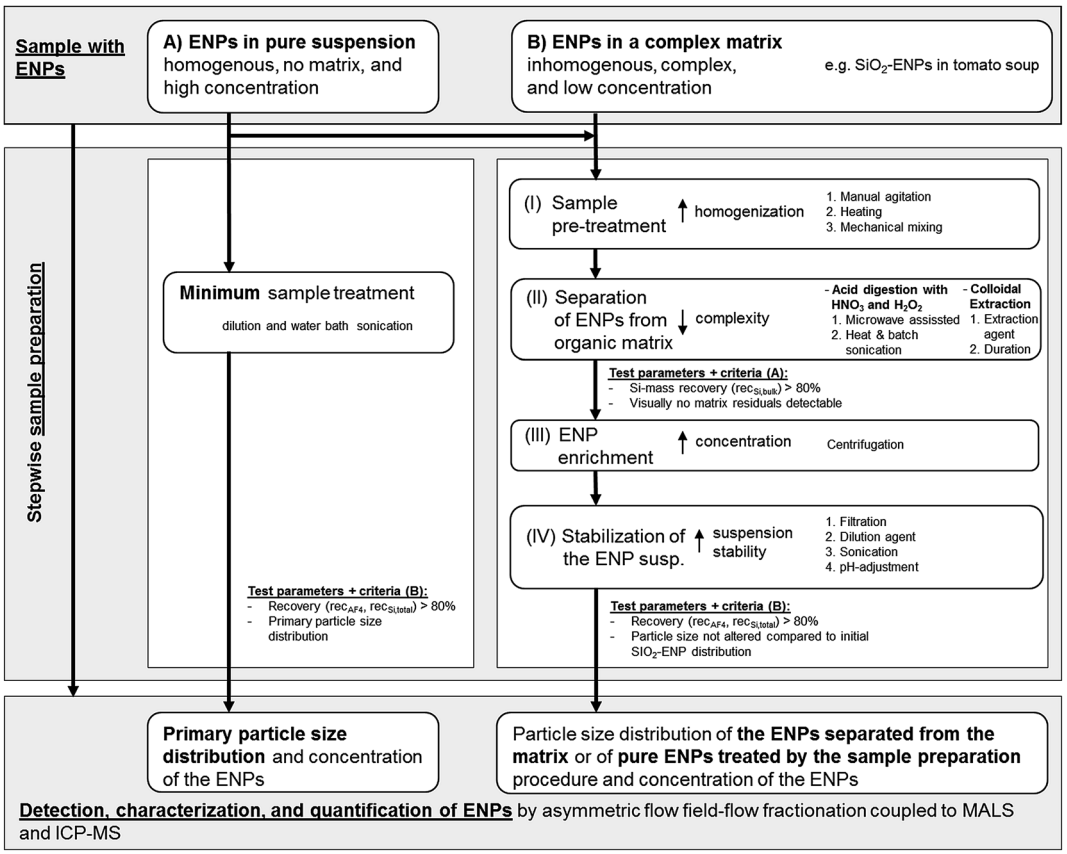

Fig. 1 Generic multi-step procedure for development of a sample preparation method to extract ENPs from a complex matrix. Specific details for the example separation of SiO_2-ENPs from tomato soup are given on the right side of the scheme (numbered sub-steps can be performed as stand-alone or in combination with other listed sub-steps).

was characterized using AF^4 coupled to MALS and ICP-MS detectors. Since details of the analytical method development has been described in von der Kammer *et al.*,[22] herein only the conditions are described. The efficiency of the total sample preparation was evaluated after step IV (test criteria B in Fig. 1). This evaluation was based on the particle size distribution, and the calculation of Si mass recovery of the entire sample preparation ($rec_{Si,total}$). For the example of SiO_2-ENPs separated from tomato soup, it was decided to additionally determine the recovery of the AF^4 separation method (rec_{AF^4}) based on the unspecific light-scattering signal in order to provide a measure for the quality of the separation which can be obtained easily (without ICP-MS instrument calibration which saves significant analysis time and resources). This approach, however, was only valid because the light scattering signal from a blank tomato soup (no SiO_2-ENPs were spiked) after extraction by acid digestion did not indicate the presence of any particles. In case particle impurities can be expected in the sample, it is recommended to calculate the AF^4 recovery not based on the MALS signal but on the element specific ICP-MS signal. Detailed calculation of $rec_{Si,total}$ and rec_{AF^4} are provided in ESI part 1.†

The application of the generic sample preparation procedure and its quality criteria requires knowledge about the target ENP (*i.e.* compound, size, and possibly concentration). In case these parameters are not know, which would be true for unknown ENPs, the effect of the sample preparation on the ENP size distribution cannot be identified based on the generic sample preparation. To identify and quantify "unknown" ENPs in a complex matrix an adapted generic sample preparation procedure has to be applied, which *e.g.* considers unique features of the target particles (*e.g.* elemental ratios, or homogeneity in elemental composition compared to matrix components).

Measurements and instrumentation

Initial total Si mass content after digestion by ICP-OES. Silica mass fraction for all acid digested samples was determined by inductively-coupled plasma optical emission spectrometry (ICP-OES; Optima 5300DV, PerkinElmer Inc., Waltham, USA) at a wavelength of 251.6 nm. Total digestion of SiO_2 particles was not necessary prior to ICP-OES analysis. ICP-OES analysis showed similar Si concentration with and without total digestion (data not shown). Total digestion tests were performed in a two-step microwave assisted digestion by HCl, HNO_3, and HF at a volumetric ratio of 0.5 : 4 : 2 : 1 (sample : HCl : HNO_3 : HF) followed by complexation of the remaining HF with H_3BO_3 (350 mg boric acid/15 mL of MQ-water).

Off-line particle characterization. For the pure particle suspension (100 mg L^{-1} diluted in MQ-water), the particle size distribution (based on hydrodynamic radius, r_h) and the zeta potential were determined by respectively dynamic light scattering (DLS) and Laser Doppler anemometry using a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK).

Particle separation by AF^4. The AF^4 separation techniques used for the particle size fractionation and the analytical techniques used for detection, characterization, and quantification were adapted from von der Kammer *et al.*[22] and the run specifications are briefly summarized in Table 2. Experiments were carried out using an Eclipse 3+ AF^4 system (Wyatt Technology, Dernbach, Germany). The sample was injected with a large volume injection loop with a maximum injection volume of 900 μL (Agilent G2260A, Agilent, USA). The separation channel in the AF^4 system had a length of 275 mm and was equipped with a 250 μm spacer and a 10 kDa regenerated cellulose membrane (Nadir, Wiesbaden, Germany). The applied constant cross flow rate was 0.75 mL min^{-1} during elution.

Online particle size characterization by MALS and AF^4 calibration. Two different approaches were used to determine the sizes of the SiO_2-ENPs separated by the AF^4. The first approach used MALS to determine the particle sizes (based on r_{rms}). The AF^4-system was coupled online with a MALS detector with 17 + 1 observation angles operated with a linear polarized laser at 658 nm (DAWN® HELEOS™, Wyatt Technology Europe GmbH, Dernbach, Germany). The data acquisition interval was set to 2 seconds. The calculation procedure of the particle sizes from the MALS data, and the discussion and limitation of approach 1 are beyond the scope of this work and were summarized in the ESI part 1.2.† In this work size data derived from MALS measurements was mainly applied as an independently acquired size distribution to prove the correctness of the particle fractionation in the AF^4. In the second approach the size distribution (based on r_h) was calculated from AF^4 calibrated with polystyrene latex beads as size standards (PS standards). AF^4 calibration was repeated regularly in order to check for changes in particle elution behaviour due to membrane ageing. Due to the fact that there is no size reference material for SiO_2-ENPs available size calibration of the AF^4 channel was done with PS standards. The size calibration of an AF^4 channel with material other than the sample is permissible as long as the elution behaviour of both, PS standard and ENPs is ideal *i.e.* the elution time of particles from the channel is solely determined by their diffusional behaviour. In order to ensure ideal elution behaviour the AF^4 run conditions have to be optimized separately for both PS standards and ENPs until conditions with maximum retention and maximum particle recovery are achieved. Since PS standards and SiO_2-ENPs have different properties (*e.g.* surface charge) the ideal AF^4 run conditions for both

Table 2 AF^4 and ICP-MS operational parameters used for SiO_2-ENP concentrations of 100 mg L^{-1}

	Unit	Value
AF^{4a}		
Tip to tip channel length	[cm]	27.5
Spacer	[μm]	250
Focus flow rate	[mL min^{-1}]	0.75
Injection flow	[mL min^{-1}]	0.1
Injection time	[min]	10
Focus time	[min]	2
Elution time	[min]	35
Detector flow rate	[mL min^{-1}]	1
Cross flow rate	[mL min^{-1}]	0.75
Membrane		Regenerated cellulose, 10 kDa, Nadir
Carrier[a]		Mixture of 0.025% (v/v) FL-70™ and 0.25 mM NaCl
Injection mass[a]	[μg]	5
ICP-MS parameters		
RF power	[W]	1600
Sample depth	[mm]	10
Gas flow rates		
Carrier	[L min^{-1}]	1.06
Dilution	[L min^{-1}]	0.35
Collision gas He	[mL min^{-1}]	4.0
Sample uptake rate	[mL min^{-1}]	0.3
Nebulizer		MICROMIST (glass expansion)
Spray chamber		Scott double-pass
Isotopes monitored		^{28}Si
Dwell time	[ms]	100

[a] Size calibrations of the AF^4 channel were performed under similar run conditions, with the only exception being for a carrier composition of 0.025% (v/v) FL-70™ and 3 mmol L^{-1} NaCl. As already pointed out by Neubauer *et al.*[6] in case that no particle size reference material of similar composition as the sample is available it might be necessary to run the AF^4 calibration with a different carrier composition as the sample. The mass of injected polystyrene latex beads (PS size standards 50, 100, and 150 nm) was 0.5, 0.25, and 0.1 μg, respectively.

differed. In general this means that run conditions in AF^4 separation for both calibration and measurement do not have to be the same. This fact has been addressed in literature and due to readability of this work the reader is referred for further information to *e.g.* Neubauer *et al.*,[6] who demonstrated the need of different run conditions for PS-standards and Fe-oxide particles. The ^{28}Si ICP-MS signal, which was recorded online by AF^4 following size separation, enabled a size distribution to be obtained based on particle mass for particles with a constant, known stoichiometry, as was the case for the SiO_2-ENPs used in this study.

The size distributions were evaluated using the modes and the medians (d_{50}) of the distributions. A mode/median ratio (peak shape factor) <1 indicates a tailing of the size distribution, while a ratio >1 indicates a fronting of the distribution. Where the ratio is equal to 1 the distribution is symmetric. The mode/median ratios were calculated for each sample and compared with each other. The independent determination of particle radii using MALS and hydrodynamic radii by AF^4 size calibration allowed us to calculate the ratio of the r_{rms} to r_h. This ratio is a direct expression of particle shape.[23] A solid, homogeneous, spherical shaped particle has an r_{rms}/r_h ratio of 0.775. Any deviation from such a spherical particle shape would cause the r_{rms}/r_h ratio to increase up to a maximum of 1 for oblate spheroids, and to a maximum of 2 for prolate spheroids (at an 1/100 aspect ratios).

Online Si mass quantification by ICP-MS. Online Si mass quantification of the fractionated samples was carried out using ICP-MS (Agilent 7700x, Agilent, USA). The methodology for the coupling of AF^4 with ICP-MS is described elsewhere[24] and briefly summarized herein. The ICP-MS run conditions are provided in Table 2. In order to establish a controlled, continuous, and reproducible mass flow in the ICP-MS nebulizer and to avoid a mass overload of the ICP-MS detector, the liquid flow from the online optical detectors was split using a peristaltic pump into two flows, one to the ICP-MS (30% or 0.3 mL min^{-1}) and the other to waste. Constant flow into the ICP-MS was verified by continuous monitoring of the flow using a flow meter (TruFlow Sample Monitor, Glass Expansion, Australia).

The ICP-MS measurements were calibrated using dissolved Si standards. According to Prestel *et al.*,[25] SiO_2-ENPs smaller than 500 nm are completely ionized within the plasma. By comparing the ICP-MS ^{28}Si signal intensities for 100, 500, and 1000 nm SiO_2-ENPs (Postnova, Landsberg am Lech, Germany) at identical mass concentrations (see ESI part 6†), even 1000 nm SiO_2-ENPs were shown to be quantitatively detected by the ICP-MS system used in this study. A background mixture of 0.025% (v/v) FL-70™ and 0.25 mmol L^{-1} NaCl were used during Si calibration of the ICP-MS in order to take into account possible interferences and matrix effects arising from the organic carbon content of the AF^4 carrier mixture when it contained FL-70™ surfactant. The Si calibration range was between 5 and 200 μg L^{-1}. The ICP-MS calibration was recorded using the full quantitative mode ($R^2 = 0.999$). Instead of using an internal standard the calibration was repeated at regular intervals following the sample analysis in order to check for any loss of sensitivity in the detection system. The detection limit (3× standard deviation of blank run) for Si analysis by ICP-MS was 2.60 μg L^{-1} (or 1.3×10^{-4} μg 50 μL^{-1}) in the measured solutions. The limit of quantification was 26 μg L^{-1} (10× standard deviation of blank run).

Results and discussion

From the regulatory point of view the analytical methodology has to provide size and concentration data of the primary ENPs added to the matrix of interest (*e.g.* foodstuff, information provision EU 1169/2011 and cosmetics, product regulation EU 1223/2009). Therefore, the developed method must be able to extract the particles without introducing artefacts by the sample preparation procedure, and be independent of any ageing of the ENPs in the complex matrix. The method development procedure must allow the identification of alterations of the ENPs concentration. Since current regulations demand number-based size distributions and the analytical methods applied in this study provide a mass-based particle size distribution a conversion algorithm has to be used to calculate number-based size distribution from mass-based input data. This conversion would result in false size distributions if the mass based signal is affected by artefacts from the sample preparation. Future work needs to focus on possible conversion algorithms and the error-prone of such conversions.

In the framework of the generic sample preparation many alternative sample preparation procedures were tested (Fig. 1). However, in the following section only the optimized sample preparation procedure is presented in detail *i.e.* both test criteria (A) and (B) were achieved and it is demonstrated which parameters had the most significant impact on Si bulk mass recovery or particle size distribution. Details on preparation procedures which did not pass the test criteria are summarized in the ESI part 4 and 5.† Main results and conclusions are shortly summarized at the end of this section.

Si mass recovery after step II (test criterion A)

For example in step II, several types of colloidal extraction, acid digestion assisted by heat and sonication (as described in Tadjiki *et al.*[18]) and acid digestion achieved by applying microwave-assisted pressurised digestion were evaluated. Prior to the extraction the tomato soup sample was pre-treated by heating and manual agitation. It was found that microwave-assisted pressurised acid digestion results in higher recovery rates ($rec_{Si,bulk}$ > 90%) and a more complete separation of SiO_2-ENPs from the tomato soup matrix compared to colloidal extraction ($rec_{Si,bulk}$ < 15%) (see ESI part 5†). However, acid digestion assisted by heat and sonication was not able to completely remove the tomato soup matrix, this was only achieved by applying microwave-assisted pressurised digestion (see ESI part 4†). Therefore, only microwave assisted acid digestion in combination with the various sample pre-treatment procedures (Fig. 1, step (I)) was tested in order to identify the optimum combination of pre-treatment procedure which yield maximum recovery and minimum alteration of the particle size distribution. For these tests the pristine particle suspension in

MQ-water (SiO_2-ENPs) and freshly spiked and aged SiO_2-ENPs in tomato soup were deployed. The pristine SiO_2-ENPs sample was included in the tests as a control, in order to understand the effect of sample preparation on the particles. The Si bulk recovery for SiO_2-ENPs was usually greater than 85% (Table 3) for all of the pre-treatment procedures tested. Similar results were obtained for tomato soup freshly spiked with SiO_2-ENP (TS + SiO_2-ENP), which yielded $rec_{Si,bulk}$ greater than 80% for each of the pre-treatment procedures. However, for the aged soup (TS + SiO_2-ENP_{aged}) the $rec_{Si,bulk}$ dropped to less than 10% when the sample was only agitated manually prior to acid digestion (procedure I.1 in Table 3). It only exceeded 50% when the sample pre-treatment also included heating of the sample at 50 °C for 30 minutes and mechanical homogenization (procedures I.2 and I.3 in Table 3) prior to acid digestion. The differences in recovery between the samples TS + SiO_2-ENP and TS + SiO_2-ENP_{aged} was likely to be due to the longer contact time between the SiO_2-ENPs and the tomato soup matrix in the aged samples (more than a year, compared to a few hours) causing changes in the ENP interaction with the matrix (organic fibers) or a change in the ENP surface properties. These changes in surface properties may have resulted in the formation of ENP aggregates or agglomerates greater than 1 μm, which were not available for ICP-OES analysis due to settling. The presence of large particles was suggested by qualitative DLS analysis, which indicated the presence of particles >3 μm. This effect was however not further investigated because it was beyond the scope of this study. A further increase in $rec_{Si,bulk}$ from 52% (I.2 + I.3) to 93% was achieved when additional tip sonication (I.2 + soni) of the particle suspension was applied after the acid digestion. The procedure I.2 + I.3 + soni was selected to provide sufficiently homogenized samples for sample preparation steps (III–IV).

Colloidal extraction aims at separating ENPs and matrix components by physical separation *e.g.* by centrifugation or filtration. Separation of SiO_2-ENPs from tomato soup resulted in lower recoveries and incomplete separation of ENPs and matrix compared to microwave assisted digestion. Silica recovery after colloidal extraction without any sample pre-treatment (I.1), $rec_{Si,bulk}$ values were greater than 85% from both SiO_2-ENPs and TS + SiO_2-ENPs samples for all of the extraction agents tested (see ESI, section 5.1†). There was virtually no recovery (1 ± 1%) from TS + SiO_2-ENP_{aged} samples with extraction for 30 min by MQ-water. In order to improve the Si mass recovery from TS + SiO_2-ENP_{aged} the extraction period was extended to 72 hours, but the maximum $rec_{Si,bulk}$ (20%) was already reached after 16 hours of agitation in 0.25 mM AC solution. Sample pre-treatment prior to liquid extraction was optimized through the use of mechanical homogenization (I.2) and heat treatment (I.3). Si mass recoveries from TS + SiO_2-ENP_{aged} increased to 40 ± 9% after applying the I.2 pre-treatment procedure. Where fatty constituents were dissolved or dispersed in the aqueous solution by the application of heat (I.3), the Si mass recovery was 10 to 40% lower than for the unheated sample. The surface area of the boundary layer between water and non-aqueous solution increased during heating, and particles tended to accumulate at this boundary or even to migrate into the fatty phase due to their hydrophobic properties. A well separated fatty phase reformed during the extraction, which was carried out at 20 °C. A considerable quantity of SiO_2-ENPs may remain at this boundary or within the fatty phase (which was not subsequently sampled), resulting in significantly lower recoveries. Generally, colloidal extraction yielded significantly lower Si mass recoveries and incomplete separation of SiO_2-ENPs and matrix (criteria A, for details see ESI, part 5†).

Table 3 Si mass concentrations, and mass recoveries depending on sample pre-treatment, both are given as the mean of triplicate measurements; errors are expressed as standard deviations from the mean value[a]

Sample	Pre-treatment	c(Si) [mg L^{-1}]	$rec_{Si,bulk}$ [%]
SiO_2-ENP	I.1	16.6 ± 4.1	86 ± 22
	I.2	17.4 ± 1.3	96 ± 9
	I.2 + I.3	20.4 ± 1.8	104 ± 9
	I.2 + soni	21.2 ± 0.3	114 ± 2
	I.2 + I.3 + soni	15.7 ± 0.8	84 ± 4
TS + SiO_2-ENP	I.1	14.5 ± 2.6	78 ± 14
	I.2	n/a	n/a
	I.2 + I.3	17.7 ± 2.8	95 ± 15
	I.2 + soni	21.8 ± 0.2	117 ± 3
	I.2 + I.3 + soni	16.8 ± 1.5	90 ± 8
TS + SiO_2-ENP_{aged}	I.1	1.3 ± 0.4	8 ± 2
	I.2	7.1 ± 0.3	44 ± 2
	I.2 + I.3	8.0 ± 1.0	52 ± 6
	I.2 + soni	15.2 ± 0.9	93 ± 5
	I.2 + I.3 + soni	13.2 ± 1.2	81 ± 7

[a] I.1: manual agitation; I.2: heating for 30 min; I.3: mechanical homogenisation; +soni: additional tip sonication of the sample prior to ICP-OES analysis.

Particle concentration enrichment (step III)

Since AF^4 separation has a broad operating range in terms of particle concentration, particle enrichment is only necessary for low concentrated samples. Particle enrichment can be achieved *e.g.* by centrifugation or cloud point extraction. Despite the high enrichment factors (up to 100) which can be achieved by cloud point extraction this methodology is strongly influence by matrix components and particle surface properties.[26] Therefore, it was not applied to enrich SiO_2-ENPs concentration, but it might be considered for other particle types and matrices. In the case of SiO_2-ENPs, enrichment of the particle concentration (III) was done immediately after microwave digestion by centrifugation (4500 rpm, 15 min) in order to reach concentrations which were suitable for further AF^4-MALS-ICP-MS analysis. The analysis of Si concentration in the supernatant and in the residual indicated that SiO_2-ENP concentration could be increased by the factor of 2.4 in the remaining solution, without significant loss of particles in the supernatant (<5% of the total SiO_2-ENP mass). However, particle enrichment by centrifugation introduces the risks of particle loss, due to incomplete sedimentation, or particle alteration. Considering that the enrichment step only increased the concentration by the factor of 2.4 alternatively the amount of sample injected in the AF^4 system could be increased. The AF^4 system equipped

Table 4 Peak evaluation parameters for acid digested samples (sonication after acid digestion for 90 seconds); uncertainty expressed as standard deviation from triplicate measurements. MALS 90° was used as concentration signal, the distributions are therefore intensity weighted

Sample	r_h (mode) [nm]	r_h (median) [nm]	Peak shape factor, [—]	Sample peak area [mV min]	Void peak area [mV min]	Release peak area [mV min]
SiO_2-ENP (no acid digestion)	63 ± 2	70 ± 5	0.90	0.33	4×10^{-3}	3.7×10^{-2}
SiO_2-ENP	76 ± 3	81 ± 6	0.94	0.34	5×10^{-3}	4.5×10^{-2}
TS + SiO_2-ENP	71 ± 3	76 ± 2	0.95	0.37	4×10^{-3}	3.9×10^{-2}
TS + SiO_2-ENP_{aged}	74 ± 11	81 ± 9	0.92	0.37	4×10^{-3}	4.0×10^{-2}

with the large volume injection loop allows injection volumes that range between 0.1 and 900 μL. An increase of the injection volume of the sample by the factor of 2.4, which means an injection of 120 μL instead of 50 μL, would substitute the particle enrichment by centrifugation. Increasing the injection volume results in both, a higher load of ENPs of interest as well as a higher load of possible remaining particles originated from the matrix. Generally, it is of course intended to remove most of the matrix components from the sample during sample preparation in order to avoid the injection of matrix components into the AF^4 channel. In case of SiO_2-ENPs in tomato soup it was demonstrated that blank tomato soup (no SiO_2-ENPs) did not exhibit any significant MALS signal after microwave assisted acid digestion (data not shown). The required pre-concentration can also be estimated by simple calculation which is done in the following for the sample TS + SiO_2-ENP_{aged}. For the suggested analytical procedure a SiO_2-ENPs concentration of >50 mg L^{-1} was required in suspension. The initial SiO_2-ENPs concentration in the presented example was 17.5 g L^{-1} (Table 1). Without particle enrichment (step III) this concentration was reduced by a factor of 500 during the sample preparation and stabilization (dilution factors: microwave assisted acid digestion 1 : 50; stabilization 1 : 10, see ESI part 2.2 and 2.5†) resulting in a concentration of 35 mg L^{-1}. For quantification of SiO_2-ENPs slightly higher SiO_2 concentration were required. Therefore, an increase in concentration or injection volume by the factor of 2 would result in sufficiently high SiO_2-ENP concentration (70 mg L^{-1}) for detection by MALS and ICP-MS.

Particle size distributions after step IV (test criterion B)

Several authors[8–10,19] previously stated that the final measured particle size distribution is strongly dependent on the sample preparation procedure and results presented herein support this statement. It is, however possible to minimize the effect by careful development of the sample preparation procedure, especially with respect to particle stabilization. A sequence of treatment steps is required in order to obtain an unaltered stable particle suspension for AF^4 separation and analysis. These steps (IV.2, IV.3 and IV.4) were essential in order to break down aggregates that were formed during digestion and to produce a particle suspension that would be stable for several days. After acid digestion the matrix was completely removed and filtration as suggested in Fig. 1 could be omitted. The acid digested sample was stabilized by pH adjustment in the range between 8 and 9 which equals the pH range of the original SiO_2-ENP suspension.[21] Furthermore, dilution in a suitable dilution agent was necessary (*e.g.* 0.025% FL-70™ as detergent or 0.25 mM ammonium carbonate as a buffer medium) in order to adjust the ionic strength. The authors refer to the ESI part 2† which depicts each single optimization step according to Fig. 1.

The described sample preparation procedure and subsequent analysis were applied to SiO_2-ENP, TS + SiO_2-ENP and TS + SiO_2-ENP_{aged} samples. Resulting size distributions were compared to the size distribution of the undigested SiO_2-ENPs (details on the characterization of undigested SiO_2-ENPs are summarize in ESI part 3†) in order to find out if the sample preparation procedure affects the size distribution and to quantify its bias (Table 4). In order to distinguish a possible effect of the tomato soup matrix from effects of sample preparation on the SiO_2-ENP size distribution particle size distribution obtained for TS + SiO_2-ENP and TS + SiO_2-ENP_{aged} were compared. Since SiO_2-ENPs were spiked shortly (*ca.* 30 minutes) prior to the sample preparation to TS + SiO_2-ENP sample it can be assumed that SiO_2-ENPs in the freshly spiked soup will not be altered by the matrix components.

The mode of r_h distribution derived from AF^4 calibration was slightly increased (maximum increase 21%) for all samples than for the undigested SiO_2-ENP sample. There was a less pronounced increase in median values (maximum increase 16%) resulting in less tailing and higher peak shape factors.

As for the intensity-based size distributions, the mass-based particle size distributions determined by AF^4 with the ICP-MS ^{28}Si signal intensity as a concentration signal, were shifted towards larger particle sizes for all digested samples relative to the size distribution of not digested SiO_2-ENPs (the mode of the size distribution of SiO_2-ENPs is indicated by a vertical line in Fig. 2 together with the SiO_2-ENP size distribution for pure particle suspension).

The r_{rms}/r_h ratios (*i.e.* the peak shape factor) remained stable at values close to 1 over the elution time irrespective of the sample type, indicating a small deviation from an ideal spherical particle,[4] which was expected since the particles in question are aggregates of smaller primary particles.[21] Data for the particles with r_h < 30 nm (based on MALS data) shows larger rms radii, indicating incomplete void peak separation. Due to limitations of the mathematical model, it is likely that the r_{rms} derived from MALS does not reflect the real particle size in this region of the fractogram, and the r_{rms}/r_h ratio can therefore, only be interpreted for radii between 40 and 120 nm.

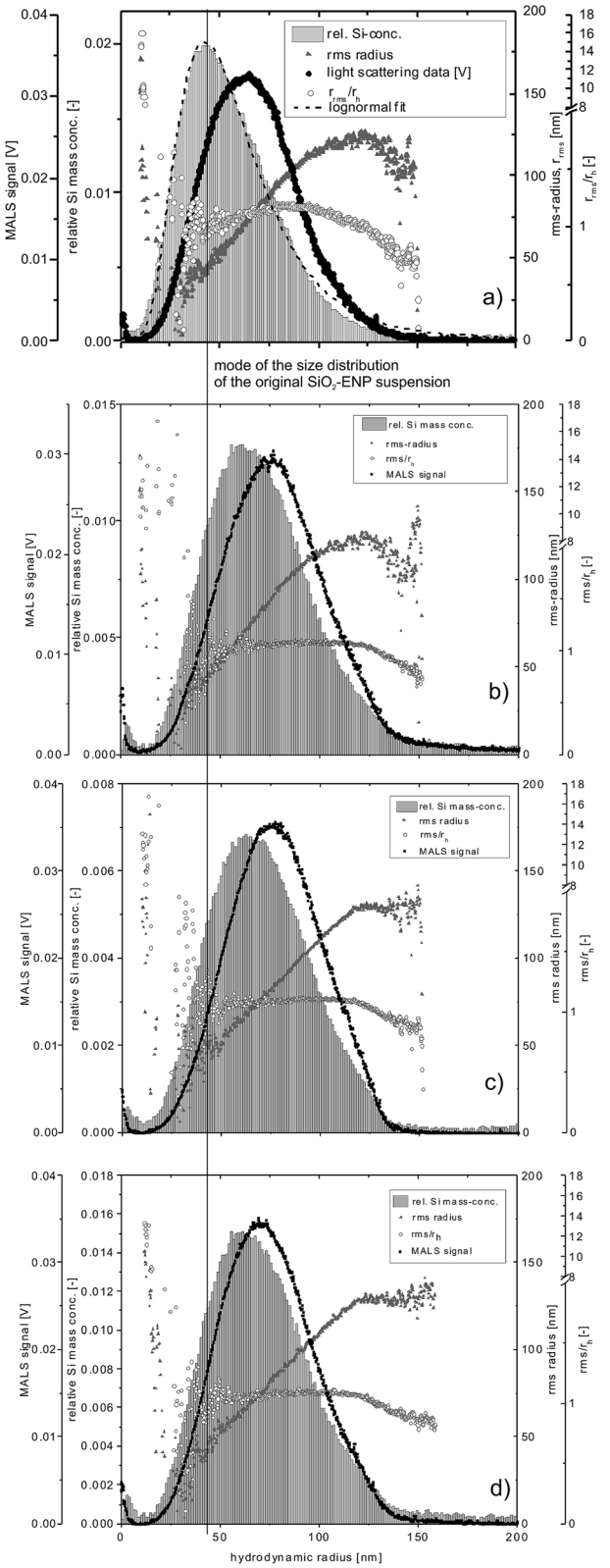

Fig. 2 Particle size distribution of (a) original SiO_2-ENP suspension and after digestion of samples (b) SiO_2-ENP, (c) TS + SiO_2-ENP, and (d) TS + SiO_2-ENP_{aged}, MALS data for a detector angle of 90°.

Despite the careful adjustment of the stabilization conditions a slight shift in the size distribution of SiO_2-ENPs was inevitable. In order to explain this shift, stabilization parameters such as energy input, ionic strength conditions and AF[4] separation have been considered. As a first indicator for the impact of acid digestion and the subsequent particle stabilization TEM images of the pure SiO_2-ENPs and the SiO_2-ENPs, extracted from the tomato soup with subsequent tip sonication, were recorded. The images indicated no alteration of the particle size distribution and particle shape (see ESI part 4†). However, TEM observation performed in this study were not appropriate to provide a quantitative particle size distribution. As an attempt to explain the slight shift in particle size distribution, the effects of energy input by sonication, ionic strength, and AF[4] separation conditions on the particle size distribution were investigated.

De-aggregation by energy input. The particle size distributions of the acid digested, pH stabilized samples dispersed in either 0.025% FL-70™ or 0.25 mmol L^{-1} AC differed from the initial size distribution if de-aggregation was not promoted by sonication (Fig. 3, black solid fractogram). The sample peak showed an intense fronting resulting in a peak shape factor >1, indicating the presence of large particles in the suspension (Fig. 3, ESI part 4.3†). These large particles were artefacts of the sample preparation and were most likely a result of agglomeration, which was induced by pH values in the range of the point of zero charge (PZC) of SiO_2 surfaces (PZC between 2.2 and 3.4 [27]) during acid digestion. The increase of the pH value to the alkaline range (pH between 8 and 9), where SiO_2-ENP are stable, did not lead to a break-down of the formed aggregates. Mechanical energy input in form of tip-sonication may support such a break-down. It was ensured that the primary SiO_2-ENP size distribution remained unaffected by tip sonication treatment by the similarity between size distribution patterns obtained from SiO_2-ENP sample following sonication for 135 seconds (calculated energy transfer 0.33 kJ mL^{-1}), and those obtained from the untreated sample (data not shown). Tip sonication of the SiO_2-ENPs extracted from the tomato soup resulted in a shift of the mode of the size distribution towards smaller sizes with increasing sonication time and the peak shape factor decreased from 1.09 to 0.95 (Fig. 3, ESI part 4.3†). Ninety seconds of sonication (calculated energy transfer 0.22 kJ mL^{-1}) provided sufficient energy input to re-establish a particle size distribution with similar patterns to the initial size distribution of SiO_2-ENPs (ESI part 4.3†). However, it was not possible to re-establish a completely similar size distribution applying mechanical energy input.

Aggregation due to ionic strength. One reason for the increase in particle size (Fig. 2) could be aggregation due to elevated ionic strength (IS) of 0.11 mol L^{-1}, which was induced by acidification (IS_{ACI} = 0.071 mol L^{-1}) and subsequent neutralization (IS_{NEUTR} = 0.039 mol L^{-1}). IS may exceed the critical coagulation concentration of SiO_2-ENPs (CCC_{SiO_2}). Stability tests using DLS measurements on SiO_2-ENPs suspended in 0.025% FL-70™ solution with ionic strengths increasing from 0.05 to 0.15 mol L^{-1} suggested that no aggregation occurred when IS values were below 0.1 mol L^{-1} (see ESI,

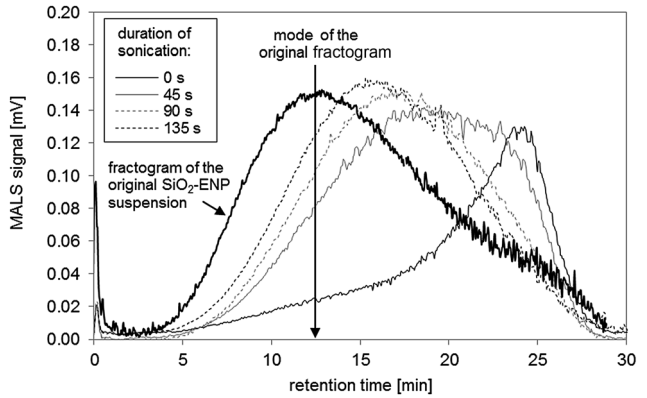

Fig. 3 Effect of increasing time of sonication after particle stabilisation in 0.025% FL-70™ and pH adjustment on AF^4 fractogram.

Table A-2†). Published data on the CCC_{SiO_2} for SiO_2-ENPs at a concentration of 0.25 wt% indicates CCC values of between 0.01 mol L^{-1} (pH 7) and 0.1 mol L^{-1} (pH 9).[28] According to the results of the stability tests and the CCC_{SiO_2} values reported in published literature,[28] it was concluded that aggregation was unlikely caused by elevated ionic strengths.

Does the elution behaviour of SiO_2-ENPs in AF^4 changes due to sample preparation? A change in surface chemistry (*e.g.* surface charge of SiO_2-ENPs) could have affected the elution behaviour of the SiO_2-ENPs separated in the AF^4 channel. This effect was observed for AF^4 separation of Ag-ENPs previously by Loeschner *et al.*[10] A positive shift in elution time might lead to a misinterpretation of the data towards too large particle sizes if based on external calibration of size. However, several lines of evidence tend to show that this was not the case here: (i) Zeta potential measurements of the stabilized particle suspensions revealed potentials <−30 mV that were independent of sample type and sample preparation. (ii) The MALS-derived r_{rms} increased linearly over the entire elution profile for all samples, indicating ideal elution behaviour during a constant cross flow field run (Fig. 2). (iii) The AF^4 recovery which was derived from the MALS signal was close to 100% (Table 5). (iv) Not more than 13% of the recovered Si mass was eluted in the void and the release peak of the SiO_2-ENP sample. (v) The total Si recoveries from the samples TS + SiO_2-ENP and TS + SiO_2-ENP_{aged} were within a similar range as the AF^4 recoveries (Table 5).

The AF^4 recoveries were greater than the total recoveries, which is reasonable because the total recoveries covered the complete sample preparation and analysis procedure (see eqn (3) in ESI, part 1.1†) whereas the AF^4 recoveries only covered mass loss during AF^4 procedure.

Table 5 AF^4 recoveries and total Si mass recoveries after sample pretreatment I.2 + I.3 + soni and subsequent acid digestion; recovery calculations based on duplicate measurements

Sample	rec_{AF^4} [%]	$rec_{Si,tot}$ [%]
SiO_2-ENP (no digestion)	90	97
SiO_2-ENP	87	82
TS + SiO_2-ENP	114	89
TS + SiO_2-ENP_{aged}	101	93

Conclusions

The generic concept of systematic method development was successfully tested for the analysis of SiO_2-ENPs in a complex matrix. The introduced and applied quality criteria proved to be applicable for the method development and optimization. As a next step in the direction of more routine method development the presented generic sample preparation procedure has to be transferred and tested for other ENP-matrix combination in order to prove its validity. As required by the generic procedure the method development for SiO_2-ENPs in a food matrix has been thoroughly tested in terms of nanoparticle size and concentration. For quality control, Si mass recovery data and an independently acquired SiO_2-ENP size distribution (*e.g.* using MALS) need to be determined for each run. Sample homogenization (step I) was identified as one of the most critical parameters for the recovery, while the stabilization procedure (step IV) is critical for the particle size distribution. As a result of the optimization procedure the following sample preparation is suggested: sample pretreatment (step I) by heating (60 °C) and mechanical mixing was required to sufficiently homogenize the soup. Successful SiO_2-ENP separation from the matrix (step II) was achieved by microwave-assisted acid digestion with HNO_3 and H_2O_2. After particle enrichment (step III) by centrifugation, particle stabilization is suggested (step IV) using an appropriate stabilizing agent (in this case 0.025% (v/v) FL-70™), pH adjustment to values between 8 and 9 and tip sonication for 90 seconds (0.22 kJ mL^{-1}). The slight shift of the size distribution after acid digested of SiO_2-ENPs was independent of the type of matrix (SiO_2-ENP, TS + SiO_2-ENP, TS + SiO_2-ENP_{aged}) and could not be explained by particle aggregation or a change in elution behaviour of SiO_2-ENPs. It remained unclear to what parameter this slight shift could be attributed.

The major difficulty for the direct application of this method on products, available on the market, is the lower ENP concentrations typically present in products. *E.g.* Dekkers *et al.*[29] estimated concentrations of nano-sized SiO_2-ENPs between <0.1 and 6.9 mg g^{-1}. Based on the generic sample preparation procedure, a sample preparation method for lower concentrations ranges can be designed and tested *e.g.* by increasing the enrichment factor after particle-matrix separation or simply increasing the injection volume in the AF^4.

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