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Analytical Methods

# In-house validation of a method for determination of silver nanoparticles in chicken meat based on asymmetric flow field-flow fractionation and inductively coupled plasma mass spectrometric detection

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ABSTRACT

Nanomaterials are increasingly used in food production and packaging, and validated methods for detection of nanoparticles (NPs) in foodstuffs need to be developed both for regulatory purposes and product development. Asymmetric flow field-flow fractionation with inductively coupled plasma mass spectrometric detection ($AF^4$-ICP-MS) was applied for quantitative analysis of silver nanoparticles (AgNPs) in a chicken meat matrix following enzymatic sample preparation. For the first time an analytical validation of nanoparticle detection in a food matrix by $AF^4$-ICP-MS has been carried out and the results showed repeatable and intermediately reproducible determination of AgNP mass fraction and size. The findings demonstrated the potential of $AF^4$-ICP-MS for quantitative analysis of NPs in complex food matrices for use in food monitoring and control. The accurate determination of AgNP size distribution remained challenging due to the lack of certified size standards.

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## 1. Introduction

With the increasing use of nanotechnology in food and consumer products, there is a need for accurate and precise detection and characterization methods for nanoparticles (NPs) in complex matrices. Asymmetric flow field-flow fractionation ($AF^4$) coupled to inductively coupled plasma mass spectrometry (ICP-MS) is a highly promising method for this purpose (Dubascoux et al., 2010; von der Kammer, Legros, Larsen, Loeschner, & Hofmann, 2011). In (Linsinger et al., 2013) a generic approach for the validation of methods for detection and quantification of nanoparticles in food samples was described. It was concluded that validation of methods for detecting and quantifying NPs in food must answer three questions: (1) Are there *nano*particles in the sample (size identity), (2) if yes, what kind of particles (chemical identity) and (3) how much nanomaterial is in the sample (mass or number fraction). The use of spiked samples for validation studies was recommended and a number of rules were derived, including the use of non-agglomerated particles with known properties (i.e. particle size distribution and concentration) for spiking and the analysis of the spiked samples as quickly as possible to avid changes of the particles (Linsinger et al., 2013).

In a previous report, silver nanoparticle (AgNPs) spiked chicken meat was studied as a relevant model system to investigate the potential of $AF^4$-ICP-MS for detection and characterization of inorganic NPs in a complex food matrix (Loeschner et al., 2013a).

AgNPs were selected as an example of inorganic NPs because they are presently one of the most frequently used nanomaterials in products related to food, such as food storage containers and dietary supplements (nanotechproject.org, 2011). Chicken meat was chosen as an example of a complex food matrix, which illustrated a scenario where AgNPs may migrate from a bacteriostatic food contact material into meat. Recent studies showed that a fraction of Ag was released from food storage containers in the form of AgNPs (Echegoyen & Nerín, 2013; Goetz et al., 2013).

Before analyzing AgNPs in chicken meat, an $AF^4$ method was developed and optimized for the aqueous suspension of the AgNPs (Loeschner et al., 2013b). Later the same type of AgNPs was incorporated into chicken meat and a sample preparation method based on enzymatic digestion was developed (Loeschner et al., 2013a).

Here we present the results of an in-house validation study which evaluates the performance of the $AF^4$-ICP-MS method for AgNPs in chicken meat. The method parameters selectivity,

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linearity/working range, trueness/recovery, precision and limit of detection/limit of quantification were assessed following as close as possible the recommendations given in (Linsinger et al., 2013). In contrast to usual analytical methods, not only the presence and amount of a substance needed to be determined, but also the size of the particles to determine whether they are nanoparticles or not.

## 2. Experimental

### 2.1. Standards and reagents

Ultrapure water (18.2 MΩ/cm), which was obtained from a Millipore Element apparatus (Millipore, Milford, MA, USA), was used throughout the work. Polyvinylpyrrolidone (PVP)-stabilized AgNPs in aqueous suspension (NGAP NP Ag-2103) were purchased from Nanogap (Milladoiro, Spain) and characterized by the Institute for Reference Materials and Measurements (IRMM) as detailed elsewhere (Loeschner et al., 2013b). Briefly, the nominal size stated by the supplier was 42 ± 10 nm. The mass fraction of Ag determined by inductively coupled plasma optical emission spectrometry (ICP-OES) was 197.4 ± 0.6 μg/g (Loeschner et al., 2013a). The Ag mass fraction in filtrates obtained by ultrafiltration (cut-off 5 kDa) was 0.38 ± 0.04 μg/g. PVP K10 was used as steric stabilizer at a concentration of 3 μg/g. The zeta potential of the AgNPs was −37.9 ± 1.0 mV (pH 7). Lean chicken meat paste was produced by the Institute for Reference Materials and Measurements (IRMM) on the basis of 6 kg of fresh chicken breast filet obtained from a local butcher (Geel, Belgium). After cutting, freezing with liquid nitrogen and cryo-milling the material was allowed to thaw. The resulting product appeared as a homogenous meat paste. The meat paste was mixed with deionized water in a ratio 2:1 (m/m).

Additionally, a potential reference material of AgNP containing meat paste (referred to as "NanoLyse13") was produced by mixing meat paste with diluted AgNP suspension (NGAP NP Ag-2103) in a ratio 2:1 (m/m) to achieve a final nominal Ag mass concentration of 0.1 mg/g. The Ag mass fraction in NanoLyse13 samples was 105 ± 4 μg/g as determined by k0-neutron activation analysis. All samples were filled into 2 mL Nunc™ plastic cryo-vials and shock-frozen above liquid nitrogen. Dispatch was done on dry ice and the samples were stored at −80 °C: NanoLyse13 samples were stored for 10 months before analysis.

For the enzymatic digestion the commercial protease Proteinase K from *Engyodontium album* was used (Sigma–Aldrich St. Louis, MO, USA). ReagentPlus sodium dodecyl sulfate (SDS) with ⩾98.5% purity and sodium azide ($NaN_3$) with ⩾98% purity were purchased from Sigma–Aldrich (St. Louis, MO, USA). Nitric acid (67–69%) of PlasmaPURE quality and single element PlasmaCAL standards of Ag and rhodium (Rh, used as internal standard) at 1 mg/mL were obtained from SCP Science (Quebec, Canada). $AF^4$ carrier liquid was produced by dissolving ammonium bicarbonate $NH_4HCO_3$ (ReagentPlus, Sigma–Aldrich, St. Louis, MO, USA) in ultrapure water to a final concentration of 0.5 mM and adjusting the pH to 7.4 by adding nitric acid. As accumulation wall in the $AF^4$ channel polyether sulfone (PES) membranes (Nadir®, Lot no. 213150) with a molecular weight cut-off of 10 kDa were used and purchased from Wyatt Technology (Dernbach, Germany). For size calibration of the $AF^4$ channel 40 nm Nanosphere™ polystyrene nanoparticles (PSNPs) with an average hydrodynamic particle diameter of $d_h$ = 41 ± 1.8 nm (NIST™ traceable size standard) from Thermo Fischer Scientific (Fremont, CA, USA) were used.

### 2.2. Sample preparation procedure

The detailed sample preparation procedure has been described earlier (Loeschner et al., 2013a). Briefly, a portion of 0.25 g thawed blank chicken meat paste was spiked with a volume of 125 μL (low level), 250 μL (medium level) or 375 μL (high level) of the AgNP suspension to achieve AgNP mass fractions in the samples of 65.8, 98.7, and 118.4 μg/g. An Eppendorf Multipette® Xstream with a 500 μL combitip was used for spiking (accuracy 0.483% and precision 0.468% for pipetting volume of 100 μL). The mixture was vortexed for 1 min at 2500 rpm and 5 mL of the Proteinase K solution (3 mg/mL Proteinase K in 50 mM $NH_4HCO_3$ buffer at pH 7.4 containing 5 mg/mL SDS and 0.2 mg/mL $NaN_3$) were added. The mixture was incubated at 37 °C in a water bath using continuous stirring for 40 min. A volume of 10 μL was injected into the $AF^4$ channel. Blank meat without AgNPs was processed using the same procedure. Instead of AgNPs an equal volume of ultrapure water was added. NanoLyse13 samples were thawed and carefully agitated with a disposable polypropylene spatula before addition of the Proteinase K solution. A probe sonicator (Microson XL 2000, QSonica, LLC) operating at 20 kHz, and equipped with a P1-probe of 3.2 mm diameter, 127 mm length and a maximum amplitude of 180 μm was applied for probe sonication of the NanoLyse13 samples after enzymatic digestion. The electrical input power was set to 5 W (the point shortly before foaming of the sample occurred). The volume of the sample was 2.5 mL.

### 2.3. Sample analysis

The instrumentation as well as the separation and detection methods are described in more detail elsewhere (Loeschner et al., 2013a, 2013b). The $AF^4$ system used in this study consisted of an Agilent 1200 series autosampler (G1329A), a high performance liquid chromatography pump (G1311A) (Agilent Technologies, Santa Clara, CA, USA), an Eclipse™ 3 $AF^4$ flow control module, and a short channel-type $AF^4$ separation channel (Wyatt Technology Europe GmbH, Dernbach, Germany) with a 350 μm spacer. The $AF^4$ separation program used a detector flow rate of 1.0 mL/min and a constant cross flow rate of 1.0 mL/min (40 min elution with cross flow after sample injection and focusing). Retention times $t_r$ were converted to $d_h$ by calibration using PSNPs (for details see (Loeschner et al., 2013b)). For each analytical sequence, two PSNP samples were analyzed.

Following separation by $AF^4$, a series 1200 diode array detector (Agilent G1315A, DAD) was used to record absorption spectra in the wavelength range of 191–949 nm (steps of 10 nm) every two seconds. As the final detector in the hyphenated system an ICP-MS instrument (ICP-MS 7500ce, Agilent Technologies, Japan) was used. External calibration with internal standardization (10 ng/mL Rh added to carrier liquid and standards) was applied to quantify the Ag mass concentration in the eluate following $AF^4$ separation (for details see (Loeschner et al., 2013a)). The $^{107}Ag$ and $^{103}Rh$ signal intensities were recorded. An external calibration curve was established based on analysis of six concentration levels of certified silver standard in 2% v/v $HNO_3$, which were introduced off-line using a peristaltic pump. The external mass concentration standard curve was established in the beginning and in the end of the each sequence. Integration of selected peaks or of the whole fractogram resulted in the Ag mass per peak or total recovered Ag mass, respectively (ng). Finally, the Ag mass fraction in the chicken meat samples was calculated taking the injection volume, the dilution factor and the density of the analyzed sample (=1 g/cm$^3$) into account.

### 2.4. Transmission electron microscopy (TEM)

The pristine AgNP suspension as well as the enzymatically digested meat sample containing AgNPs were diluted twice with ultrapure water. A volume of 10 μL of the diluted suspension was applied to a 200 mesh Formvar/carbon-coated copper grid. The

particle size and shape were observed by transmission electron microscopy (TEM) using a CM 100 BioTwin instrument (Philips, Eindhoven, The Netherlands) operated at 80 kV accelerating voltage.

### 2.5. Data analysis

Unless stated otherwise, results based on repeated measurements are given as mean ± one standard deviation. The number of repetitions N is stated in parentheses. Data were statistically analyzed by using one-way Analysis of variance (ANOVA) in Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA). Peak areas, modes and medians were determined by using the Peak Analyzer in the software OriginPro 9.0 (OriginLab Corporation, MA, USA).

## 3. Results and discussion

### 3.1. Method validation procedure

Because not reference material exists for AgNPs in chicken meat or any other food matrix, spiked samples were used throughout the validation study (Linsinger et al., 2013). Spiking was performed on the day of analysis and the samples were analyzed as quickly as possible to avoid changes of the particles.

For determination of trueness, repeatability and in-house reproducibility the following strategy was chosen: AgNP size and mass fraction results were collected from analysis of three independent analytical series. Each series included seven replicate analyses at three concentration levels (65.8, 98.7, and 118.4 μg/g). The three series were analyzed on three different days by the same operator using for each a new ampoule of AgNP suspension, new enzyme solutions, carrier liquids and standard solutions and a new PES membrane for the $AF^4$ cell taken from the same batch. Otherwise the same instrumentation and instrumental settings were used. To determine the selectivity against matrix constituents, at total of six blank meat samples (matrix samples) were analyzed. The sample sequence of a series and an overview of the experimental design for the validation study are presented as Electronic Supplementary Material S1. The measurement data were evaluated for AgNP size, shape of the size distribution and mass fraction using one-way ANOVA. The number of samples that could be analyzed during one day was limited by the measurement duration of 60 min per sample.

### 3.2. Selectivity

As recommended in (Linsinger et al., 2013), at first the ability of the method to discriminate against usual matrix constituents particles was assessed by analyzing blank chicken meat samples. The position of the blank samples ($N = 2$) in the analytical sequence can be seen in Electronic Supplementary Material S1. The detected Ag was caused by carry-over, as AgNPs are not naturally contained in chicken meat. Method development had shown that carry-over of Ag could not be avoided (Loeschner et al., 2013a). This was explained by the general tendency of NPs to adhere to surfaces because of their high surface energy and the strong affinity between Ag and organic constituents of the matrix.

Secondly, the specificity of the method for certain types of NPs needed to be evaluated (Linsinger et al., 2013). The ICP-MS instrument is selective to Ag, with a mass to charge ratio ($m/z$) of 107 corresponding to the most abundant Ag isotope. Consequently, NPs of other chemical identity will not be detected. Additionally, the method was selective against dissolved Ag. The elution of Ag as AgNPs in the "nanoparticle peak" eluting at $t_r = 5$–40 min (Fig. 1) was confirmed during method development by fraction collection and following single particle ICP-MS analysis (Loeschner et al., 2013a), whereas dissolved Ag (associated with organic constituents of the enzymatic digest) eluted in the "early eluting peak" at $t_r < 5$ min. A further proof for the elution of AgNPs was the detection of an absorbance maximum at a wavelength of 400 nm in the recorded optical absorbance spectra (caused by the surface plasmon resonance (SPR) of the AgNPs). Ag atoms or ions do not absorb light at this wavelength. Examples of absorbance spectra recorded at the peak maximum of the nanoparticle peak are presented as Electronic Supplementary Material S2.

### 3.3. Calibration, linearity and working range

#### 3.3.1. AgNP size

For each series, size calibration with a 40 nm PSPN (N = 2) was used to convert $t_r$ into $d_h$. A two point calibration ($t_r$ of the void peak = 0 nm, $t_r$ at peak maximum for PSNP standard = 41 nm) was performed. This was assumed to be sufficient because previous results had shown a linear relationship between $t_r$ and diameter of PSNP size standards up to at least 100 nm, which corresponded to a $t_r \approx 20$ min (Loeschner et al., 2013b). The slopes of the calibration curve ($t_r$ in min vs. $d_h$ in nm) were 0.252 (series 1), 0.250 (series 2) and 0.248 (series 3). Based on the size calibration, AgNPs in the size range up to 160 nm could theoretically be separated with the applied separation program, which covered the full nanoscale range from 1 to 100 nm. The upper limit was restricted by the time when the cross flow rate was set to zero, i.e. the separation stopped ($t_r = 40$ min). $AF^4$ in normal mode can typically separate NPs in the size range of one nanometer up to one micrometer (von der Kammer et al., 2011). The exact value for the upper and lower limit of detection (LOD) of particle diameter depends mainly on the applied separation program (e.g. cross flow rate, channel dimensions).

#### 3.3.2. AgNP mass fraction

A certified silver standard, which was based on silver nitrate, was used for mass concentration calibration of the ICP-MS because

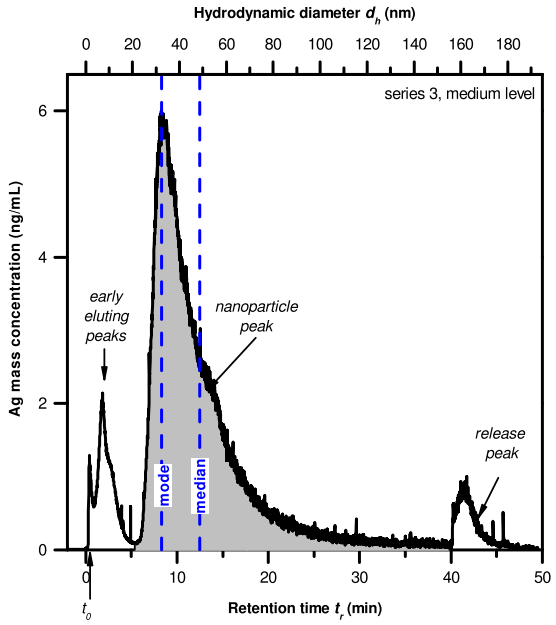


**Fig. 1.** Fractogram for a chicken meat sample with a medium level of AgNPs. The peak corresponding to AgNPs is marked gray. The upper scale presents the hydrodynamic diameter $d_h$ which was derived from retention time $t_r$ by size calibration with PSNPs. The dashed vertical lines indicate the mode and median of the mass concentration-based particle size distribution.

no AgNP-based certified standards were available. Preliminary experiments showed that the signal intensity for a given mass concentration of ionic silver standard and of 20, 60 and 100 nm AgNPs were the same (results not presented). Therefore, a conventional silver standard could be used for quantification of AgNPs by ICP-MS. No matrix matched calibration was performed because of the high dilution of the injected chicken meat sample during separation (10 μL injected sample eluting in a volume of 40 mL). The standard curves (ICP-MS signal ration $^{107}Ag/^{103}Rh$ vs. Ag concentration in μg/L) were linear with $R^2$ values > 0.999. The slopes were 0.089 (series 1), 0.090 (series 2) and 0.086 (series 3). The differences between slopes of the external mass concentration standard curves at the beginning and the end of the each sequence were always <5% and the slope of the first calibration curve was used for data analysis.

### 3.4. Trueness and recovery

#### 3.4.1. AgNP size

No AgNP reference material with a certified size/size distribution was available. Thus, the trueness of the determined size values could only be evaluated by comparison with size information from other methods. Single particle ICP-MS and TEM were applied in a previous study to determine the number-based particle size distribution of the samples (Loeschner et al., 2013a). The mode of this size distribution, as determined by both methods, was between 30 and 35 nm. The AF$^4$-ICP-MS results (Table 2) had a comparable modal diameter of 37 nm.

Previous investigations had shown an earlier elution of the extracted AgNPs in comparison to the pristine AgNPs (in aqueous suspension) in AF$^4$, which corresponded to a difference of the hydrodynamic diameter of approximately 10 nm (Loeschner et al., 2013a). TEM and single particle ICPMS analysis, however, showed that no change of the particle size distribution had occurred. Consequently, the observed shift in the retention times was rather caused by a different separation behavior of AgNPs in the presence of the enzymatically digested meat and of the same AgNPs in aqueous suspension. The comparable modal particle diameters for extracted AgNPs as determined by AF$^4$-ICP-MS (~37 nm), TEM and single particle ICP-MS (between 30 and 35 nm with both methods) indicate that the changed separation behavior does not have a significant influence on the obtained size information. However, it has to be taken into account that the compared methods provide different types of size information. When using AF$^4$-ICP-MS hydrodynamic diameters are determined and the size distribution is based on mass concentration. In contrast, spICP-MS and TEM determine geometric diameters and the size distribution is based on number concentration.

Consequently, any direct (quantitative) comparison of the obtained values for size and size distribution from AF$^4$-ICP-MS was not possible as for example also discussed in (Linsinger et al. (2012). An influence of the separation behavior, e.g. by changes in the particle-membrane interaction due to the presence of enzymatically digested meat, cannot be excluded.

#### 3.4.2. AgNP mass fraction

The trueness of the determined Ag and AgNP mass fraction was evaluated by comparison with the expected mass fraction in the spiked samples, as no certified AgNP reference material was available. This approach had to rely on the measured Ag concentration of the pristine AgNP suspension, the accuracy of the spiked volume of suspension and the accuracy of the weighted mass of chicken meat.

Total Ag recoveries (integration of the whole fractogram area) and AgNP recoveries (integration of the AgNP peak, Fig. 1) were determined. The results are presented in Table 1. The average recoveries of total Ag and AgNPs were not significantly different for any of the three spike levels of AgNPs (ANOVA, $p < 0.05$). The average total Ag recoveries of the samples were within 80–110% of the injected masses of AgNPs. The total Ag recoveries of single samples were within this range for 54 out of 60 samples. The average AgNP recoveries were below 80% except for the medium level of series 2 (83%). The AgNP recoveries of single samples varied between 50% and 93%. Possible sources for the variation were the sample preparation method and the separation process. However, that topic needs further investigation.

AgNP recoveries were lower than total Ag recoveries because of the partial elution of Ag in the early eluting peaks and in the release peak (Fig. 1). The former peaks originated from Ag ions (already present in the AgNP suspension and released during sample preparation), which were most likely bound to molecules. The latter fractogram peak was caused by strongly retained AgNPs, which did not elute until the cross flow rate was set to zero. Method development showed that these effects could not be avoided (Loeschner et al., 2013a).

### 3.5. Precision

#### 3.5.1. AgNP size

The relative standard deviation under repeatability conditions ($RSD_r$) and in-house reproducibility conditions ($RSD_{IR}$) for the AgNP modal diameter were calculated (Table 2). $RSD_r$ and $RSD_{IR}$ represent the variation between repeated enzymatic digestion and analysis within days and between days, respectively. Four samples were excluded from data analysis: the first sample of series 1, the first and second sample of series 2 and the first sample of series 3, respectively (all medium level). For all four samples the shape of the fractogram peak was different from all the other fractograms, which was indicative of particle-membrane interactions.

The concentration level in the samples did not have a statistically significant ($p > 0.05$) influence on the determined modal diameters. This proved that the obtained size information was not influenced by the concentration of AgNPs in the samples.

There was a statistically significant difference ($p < 0.01$) of the mean modal diameters between days for all analyzed levels. Size calibration with PSNPs should have accounted for possible day-to-day differences between the membranes (channel height, membrane surface properties). While no explanation for this between-series variation can be given, it should be noted, that such a variation is rather common in chemical analysis.

**Table 1**
Total Ag and AgNP recoveries (mean ± 1 s.d.).

| Level | Total Ag recovery (%) | | | AgNP recovery (%) | | |
|---|---|---|---|---|---|---|
| | Series 1 | Series 2 | Series 3 | Series 1 | Series 2 | Series 3 |
| Low | 83 ± 7 | 92 ± 7 | 90 ± 9 | 62 ± 11 | 76 ± 10 | 72 ± 13 |
| Medium | 93 ± 10 | 96 ± 5 | 92 ± 10 | 78 ± 14 | 83 ± 5 | 80 ± 14 |
| High | 85 ± 8 | 92 ± 4 | 96 ± 5 | 65 ± 13 | 75 ± 5 | 76 ± 7 |
| All | 91 ± 8 | | | 74 ± 12 | | |

**Table 2**
Performance of the AF$^4$-ICP-MS method for the determination of the AgNP modal diameter (sizes in nm).

| Level | Low | Medium | High | All |
|---|---|---|---|---|
| Observations (N) | 21 | 17 | 21 | 59 |
| Mean | 36.6 | 37.3 | 36.9 | 36.9 |
| Range | 31.7–40.2 | 32.9–40.6 | 32.9–40.6 | 31.7–40.6 |
| Repeatability $RSD_r$ | 3.4% | 3.0% | 4.8% | 4.7% |
| In-house reproducibility $RSD_{IR}$ | 10.3% | 8.5% | 8.1% | 9.1% |

For describing the asymmetric shape of the nanoparticle peak, the ratio between peak median (corresponds to 50% of the peak area) and peak mode were determined (Fig. 1, dashed vertical lines). A ratio median/mode > 1 indicates a tailing of the size distribution while ratios < 1 are indicating a fronting of the distribution. Table 3 shows the measured ratios between peak median and peak mode. There was no statistically significant difference ($p > 0.05$) between days with respect to the ratio between peak median and peak mode of the peak. The repeatability and in-house reproducibility of the peak shape indicates a repeatable and reproducible separation of the AgNPs with a constant degree of peak tailing.

### 3.5.2. AgNP mass fraction

The results for the determined AgNP mass fractions are presented in Table 4. $RSD_{IR}$ and $RSD_r$ were in the range of approximately 15% which was satisfactory taking the complexity of the AgNP-containing sample and the sample preparation as well as the separation method into account. Subsampling of the spiked chicken meat could be excluded as a source for the variation, as the complete spiked sample was digested.

## 3.6. Limit of detection and limit of quantification

### 3.6.1. AgNP size

For the studied samples, the early eluting peaks eluted typically at $t_r < 5$ min corresponding to $d_h < 20$ nm. Consequently, AgNPs with $d_h < 20$ nm (lower LOD) would co-elute with this peak and could not be differentiated from Ag bound to organic molecules (lower limit of detection). The theoretical size range for particle separation offered by the separation program could not be exploited due to the specific sample properties, i.e. the presence of further Ag species than AgNPs.

$AF^4$-ICP-MS analysis of the pristine AgNPs showed that AgNPs < 20 nm were not present in measurable levels (Loeschner et al., 2013b). Therefore, it is not likely that any significant fraction of AgNPs co-eluted with the early eluting peak. The upper limit was restricted by the time when the cross flow rate was set to zero, i.e. the separation stopped. This time ($t_r = 40$ min) corresponded to $d_h \approx 160$ nm (upper LOD).

### 3.6.2. AgNP mass fraction

Two approaches were applied to determine the LOD for the AgNP mass fraction. The first approach was based on the aforementioned carry-over of Ag in blank meat samples (Fig. 2). The carry-over was between 0.6 and 1.4 ng in the range where the AgNP peak eluted (approximately $t_r = 5$–40 min). This corresponded to 0.7–1.6% of the injected mass of the sample which was separated before (medium level, $m_{inj} = 89.7$ ng). An injected mass of AgNPs equal to or below this value could not be distinguished from the carry-over. For the low level samples ($m_{inj} = 45.9$ ng), the determined LOD and LOQ values were 1.2 and 3.5 μg/g, respectively (assuming a carry-over of 1.6% of $m_{inj}$ and taking average recovery of 70%, sample dilution rate and injection volume into account).

As a second approach for determination of the LOD, the more classical method based on the standard deviation (s.d.) of the baseline was applied (Linsinger et al., 2013). For calculation of the LOD 2.33 times the standard deviation of the blank meat signal in the range $t_r = 30$–40 min (Fig. 2) was determined (mean + 2.33 * s.d. = 0.09 ± 0.05 ng/mL, $N = 6$). This time range of the fractogram was chosen because it was least affected by carry-over, and thus presenting the baseline value. However, the observation of "spikes" in the ICP-MS trace showed that carry-over of AgNPs still influenced the signal. For conversion of the mass fraction information into an injected mass value, the average ratio between $m_{inj}$ (in ng) and peak height (in ng/mL) was calculated for the low level samples. This ratio was 11.1 ± 3.4 ($N = 21$) and allowed to convert the 2.33 * s.d. value (ng/mL) into $m_{inj} = 1.0 \pm 0.6$ ng ($N = 6$). Taking the average recovery for the low level samples of 70%, sample dilution and injection volume into account, the sample LOD was 1.6 ± 1.0 μg/g and the LOQ (based on mean + 10 * s.d.) was 5.0 ± 3.5 μg/g. Both approaches for determination of LOD/LOQ gave similar values. The LOD was sufficiently low for quantitative detection of AgNP even at the lowest spiked concentration at 65.8 μg/g.

The AgNP concentrations after a possible migration from a food contact material into food could be lower than the determined LOD. Thus, a reduction of the LOD is desirable. A decrease of the analytical LOD is expected when in general samples with lower Ag concentrations are analyzed. This would result in less adsorption/desorption of Ag/AgNPs to the inner surfaces of the analytical system. During method development it was observed that carry-over decreased during consecutive injections of the blank meat (Loeschner et al., 2013a). This was attributed to gradual removal of Ag and/or AgNPs associated with surfaces in the analytical

**Table 3**
Performance of the $AF^4$-ICP-MS method expressed as the ratio between peak median and peak mode.

| Level | Low | Medium | High | All |
|---|---|---|---|---|
| Observations (N) | 21 | 17 | 21 | 59 |
| Mean | 1.6 | 1.5 | 1.6 | 1.6 |
| Range | 1.4–1.9 | 1.4–1.7 | 1.4–1.7 | 1.4–1.9 |
| Repeatability $RSD_r$ | 9.2% | 7.0% | 7.1% | 8.1% |
| In-house reproducibility $RSD_{IR}$ | 10.7% | 7.1% | 7.2% | 8.8% |

**Table 4**
Performance of the $AF^4$-ICP-MS method for the determination of the mass fraction of AgNPs (unit: μg/g).

| Level | Low | Medium | High |
|---|---|---|---|
| Spike level of Ag in the samples | 65.8 | 98.7 | 118.4 |
| Observations (N) | 21 | 17 | 21 |
| Mean | 45.9 | 79.4 | 85.4 |
| Range | 33.0–56.5 | 59.4–91.2 | 58.5–103.0 |
| Repeatability $RSD_r$ | 15.6% | 14.4% | 12.6% |
| In-house reproducibility $RSD_{IR}$ | 17.5% | 15.5% | 14.3% |

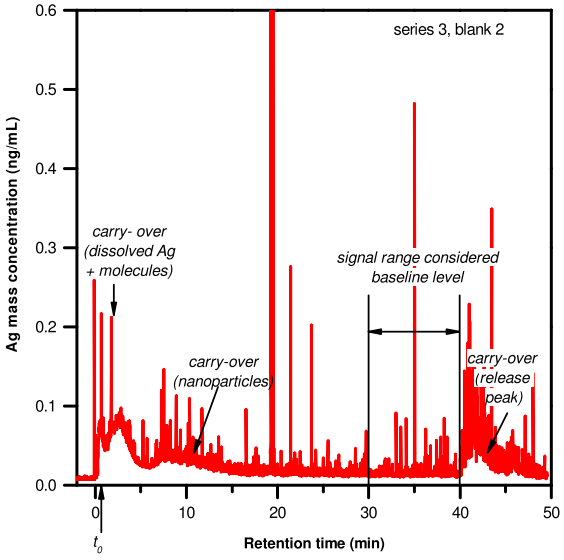


**Fig. 2.** Fractogram of a blank meat sample presenting the peak caused by carry-over of AgNPs and the signal range which was considered baseline level.

system and could be used as a cleaning procedure for the analytical system.

The sample LOD could further be decreased by reducing the sample dilution during preparation (which was already relatively low with a dilution factor of approximately 10) or AF$^4$ separation and by increasing the injection volume. The instrumentation used in this work would allow an injection volume of up to 500 µL (suggesting a 50-fold decrease of the LOD). However, it is possible that remaining matrix constituents in the enzymatically digested matrix restricts the useful injectable amount of sample. At too high injected masses (of the organic material) channel overloading effects may occur. Consequently, a maximum decrease in LOD of approximately 500-fold would theoretically be possible by optimizing dilution factor and injection volume.

### 3.7. Sample stability

In the so far presented experiments the contact time between AgNPs and meat after spiking was approximately one minute before the enzyme solution was added. In a potential reference material the contact time would be much longer. For this purpose the NanoLyse13 samples were prepared and analyzed after 10 months storage at −80 °C. In contrast to the freshly spiked samples, efficient separation of AgNPs after enzymatic digestion was not possible by AF$^4$-ICP-MS for these samples. Besides the void peak, the resulting fractograms contained one early eluting (“unknown”) Ag-containing peak (Fig. 3a, black solid line). The retention time of this peak was much lower than the retention time of the peak corresponding to the elution of AgNPs (“nanoparticle peak”) from the spiked samples (Fig. 3a, gray dotted line). The total Ag recovery was 55 ± 4% and the AgNP recovery 7 ± 0% ($N$ = 2).

The changed elution behavior could be caused by several reasons, like dissolution, agglomeration/aggregation and chemical conversion of AgNPs in the meat matrix during sample freezing, storage or transportation. To test this hypothesis several additional investigations were performed. The presence of intact AgNPs in NanoLyse13 samples after enzymatic digestion was confirmed by TEM (Electronic Supplementary Material S3) which excluded complete dissolution as a cause for the absence of the AgNP peak in the fractograms. Partial dissolution and agglomeration/aggregation could not be excluded based on the qualitative TEM investigations. Single particle ICP-MS of the enzymatic digests of NanoLyse13 (Electronic Supplementary Material S4, Table 1) confirmed a reduced AgNP mass fraction in comparison to the expected value. The determined AgNP mass fraction was 19 µg/g, i.e. 20% of the AgNP mass fraction of NanoLyse 13. Fraction collection followed by spICP-MS analysis as described in (Loeschner et al., 2013a) (Electronic Supplementary Material S4, Table 2) showed that the “unknown peak” of the NanoLyse13 samples in Fig. 3a did not contain AgNPs. Thus, the detected Ag was most likely ionic Ag associated with matrix molecules which indicated (partial) dissolution of AgNPs.

Probe sonication of the samples after enzymatic treatment and prior to AF$^4$-ICP-MS led to release of AgNPs, most likely from agglomerates. It was, however, not possible to obtain a similar AgNP recovery for the frozen stored samples as that found for the spiked samples. The relative area of the nanoparticle peak in comparison to the area of the whole fractogram was between 10% and 17% (at total recoveries for the whole fractogram of 63 ± 8, $N$ = 5) while the largest fraction of Ag (67–76% of the total area) still eluted at $tr$ < 5 min (Fig. 3b). The peak was most pronounced for a 20 min sonication treatment. Longer sonication (40 min) did not result in any further increase in peak area.

Similar observations were described for 60 nm AgNPs spiked to chicken meat and analyzed by spICP-MS after enzymatic digestion (Peters et al., 2014). Samples were either processed directly after addition of the spike or processed after intervals of 2, 24 and 48 h storage at 4 °C in the dark. A decrease of particle size was detectable already after 24 h storage indicating the dissolution of AgNPs. Additionally, a significant decrease in particle mass concentration by 60% was described which could not solely be explained by particle dissolution. As a potential mechanism the formation of insoluble silver salts like AgCl or $Ag_2S$ was discussed which could agglomerate, deposit and not be detected in the chemical analysis. The formation of $Ag_2S$ was confirmed by energy dispersive X-ray spectrometry.

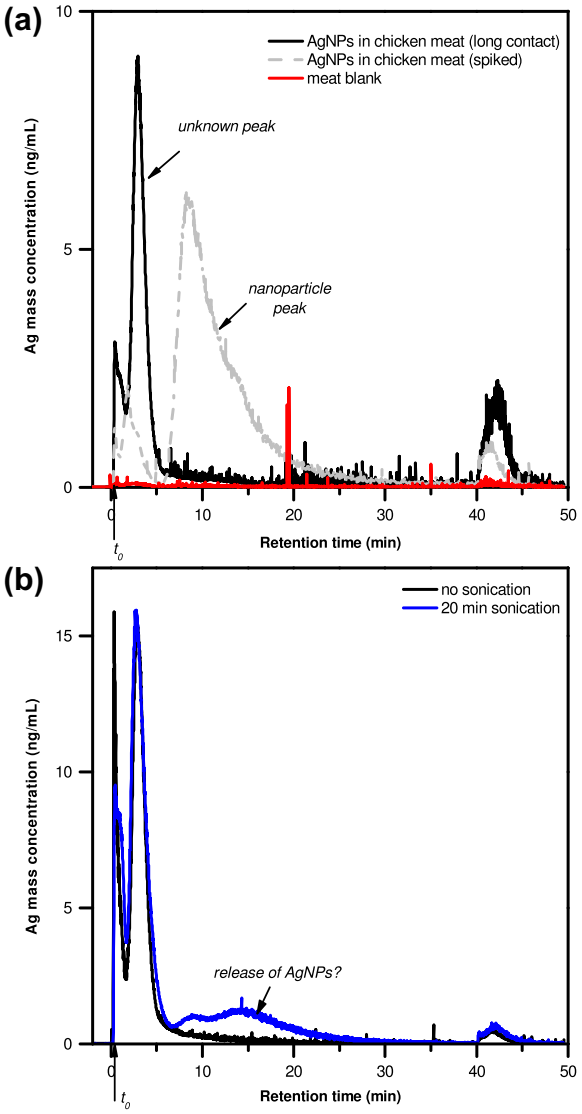


**Fig. 3.** (a) AF$^4$-ICP-MS fractograms obtained after long contact between AgNPs and chicken meat (NanoLyse13 sample). For comparison the fractogram of a spiked sample (low level) is given. The injected mass of Ag was $m_{inj}$ = 51 and 46 ng, respectively. (b) Influence of probe sonication (20 min) prior to separation for a sample with long contact time between AgNPs and chicken meat ($m_{inj}$ = 90 ng).

A combination of dissolution and chemical transformation could also explain the described results for the NanoLyse13 samples. Chemical reactions between silver and sulfur are likely to occur. Ionic Ag is known to have a strong affinity to thiol groups (Liu, Sonshine, Shervani, & Hurt, 2010) and thiol-containing biomolecules are abundant in tissues. Furthermore, hydrogen sulfide is released as a metabolite by microorganisms present in fresh chicken meat (McMeekin, Gibbs, & Patterson, 1978). The limited stability of AgNPs in chicken meat even when stored at −80 °C

limits the potential of the samples as a future reference material. If AgNPs migrate from food contact materials in meat a partial or complete transformation to Ag salts is possible. $AF^4$-ICP-MS will be able to detect the remaining (free) AgNPs.

## 4. Conclusions

The coupled $AF^4$-ICP-MS instrumental system was useful for quantitative analysis of AgNPs in a chicken meat matrix following enzymatic sample preparation. $AF^4$-ICP-MS fulfilled the requirements for determination of NPs in a food matrix (Linsinger et al., 2013). (1) The particle size distribution was determined based on the hydrodynamic particle diameter (size identity). (2) The chemical information was obtained by coupling to ICP-MS and makes the method specific to AgNPs (chemical identity). (3) The AgNP mass fraction in the samples was measured by ICP-MS.

For the first time an analytical validation of AgNPs in a food matrix has been carried out by $AF^4$-ICP-MS and the results showed that repeatable and intermediately reproducible determination of AgNP size and mass fraction in this food matrix was possible. The accurate determination of AgNP size remained challenging for several reasons: No certified size standards of AgNPs were available to test the accuracy and consequently separation artefacts by $AF^4$ cannot be excluded. The diameters determined by independent instrumental techniques like electron microscopy and $AF^4$ could not directly be compared because of different physical principles and the nanoparticles sizes they are able to determine. No AgNPs could be separated from long-term frozen chicken meat with AgNP. This was most likely related to the instability of the AgNPs in terms of dissolution, chemical transformation and agglomeration/aggregation.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.02.033.

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