# Optimization and evaluation of asymmetric flow field-flow fractionation of silver nanoparticles

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## ABSTRACT

Asymmetric flow field-flow fractionation (AF$^4$) in combination with on-line optical detection and mass spectrometry is one of the most promising methods for separation and quantification of nanoparticles (NPs) in complex matrices including food. However, to obtain meaningful results regarding especially the NP size distribution a number of parameters influencing the separation need to be optimized. This paper describes the development of a separation method for polyvinylpyrrolidone-stabilized silver nanoparticles (AgNPs) in aqueous suspension. Carrier liquid composition, membrane material, cross flow rate and spacer height were shown to have a significant influence on the recoveries and retention times of the nanoparticles. Focus time and focus flow rate were optimized with regard to minimum elution of AgNPs in the void volume. The developed method was successfully tested for injected masses of AgNPs from 0.2 to 5.0 μg. The on-line combination of AF$^4$ with detection methods including ICP-MS, light absorbance and light scattering was helpful because each detector provided different types of information about the eluting NP fraction. Differences in the time-resolved appearance of the signals obtained by the three detection methods were explained based on the physical origin of the signal. Two different approaches for conversion of retention times of AgNPs to their corresponding sizes and size distributions were tested and compared, namely size calibration with polystyrene nanoparticles (PSNPs) and calculations of size based on AF$^4$ theory. Fraction collection followed by transmission electron microscopy was performed to confirm the obtained size distributions and to obtain further information regarding the AgNP shape. Characteristics of the absorbance spectra were used to confirm the presence of non-spherical AgNP.

## 1. Introduction

Because of their proposed antimicrobial properties [1], AgNPs are presently one of the most frequently used nanomaterials in consumer products related to food, such as food storage containers and dietary supplements [2–4]. Validated methods for the detection and characterization of nanoparticles in food are however, not available. One of the most promising candidate methods for future validation is based on asymmetric flow field-flow fractionation (AF$^4$) coupled to inductively coupled plasma mass spectrometry (ICP-MS) [5–7].

For successful separation by AF$^4$ the NPs have to be in liquid suspension, analyte particles should be stably dispersed and large particles (>1 μm) must be absent or removed before analysis to allow interference-free separation [8]. Regarding NPs in a complex food matrix like for example meat or soup it remains a challenge

to prepare the sample in such a way that a stable suspension of NPs contained in the sample can be obtained, and which is still representative for the NPs in the original sample. Possible sample preparation methods include enzymatic [9], alkaline [10] or acid digestion [11]. However, before development and optimization of a suitable sample preparation method for extracting NPs from a food matrix is feasible an AF$^4$ separation method for the NPs is required. This method needs to be optimized with respect to recovery, separation efficiency and run time and can be developed by using the virgin NPs in suspension and later be applied as a starting point for analyzing the more complex NP-containing extract obtained after sample preparation.

In most cases a new separation method needs to be developed for each type of NP depending on its composition, surface properties, average size, size distribution and shape as well as on the sample matrix. Therefore it is of advantage to identify and optimize the most important parameters that influence the separation.

In this paper we present the development and optimization of a separation method for AgNPs stabilized by polyvinylpyrrolidone (PVP). The influence of several parameters of the AF$^4$ separation

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including carrier liquid composition, membrane material, cross flow rate, spacer height, focus flow rate, focus time and injected mass were tested. Furthermore, a variety of detectors used on-line with $AF^4$ for obtaining fractograms and the possibilities for determining the particle size distribution were evaluated. The scope of the paper is to pinpoint the need for a careful study of the influence of a variety of parameters influencing the separation by $AF^4$ and to understand the advantages and limitations of various detectors before accurate information regarding the size distribution of nanoparticles contained in the samples can be obtained.

## 2. Material and methods

### 2.1. Materials

Ultrapure water was obtained from a Millipore Element apparatus (Millipore, Milford, MA, USA) and used throughout the work. Nitric acid (67–69%) of PlasmaPURE quality, which was used to adjust the pH value of the carrier liquid, was obtained from SCP Science (Quebec, Canada). Nitric acid for acid digestion of AgNPs and filtrates as well as CertiPUR certified Ag reference standard of 1000 μg/ml in 2–3% nitric acid were obtained from Merck (Darmstadt, Germany).

$AF^4$ carrier liquids were produced by dissolving either ammonium carbonate $(NH_4)_2CO_3$ (Puratonic, Alfa Aesar, Ward Hill, MA, USA) or ammonium bicarbonate $NH_4HCO_3$ (ReagentPlus, Sigma–Aldrich, St. Louis, MO, USA) in ultrapure water. Before usage, the pH of the $NH_4HCO_3$ solution was reduced from 8 to 7.4 by adding a few drops of nitric acid.

For $AF^4$ size calibration Nanosphere™ polystyrene nanoparticle size standards (PSNPs) with diameters of 20 nm ($21 \pm 1.5$ nm), 40 nm ($41 \pm 1.8$ nm), 60 nm ($59 \pm 2.5$ nm), 80 nm ($81 \pm 2.7$ nm) and 100 nm ($97 \pm 3$ nm) in diameter were obtained from Thermo Fischer Scientific (Fremont, CA, USA). The respective NIST traceable diameters as determined either by DLS (20 and 40 nm particles) or TEM (60–100 nm particles) are given in parenthesis.

PVP-stabilized AgNPs in aqueous suspension NGAP NP Ag-2103 were purchased from Nanogap (Milladoiro, Spain). The nominal mass concentration of silver was 0.2 g/kg and the nominal size was $42 \pm 10$ nm. PVP K10 was used as stabilizer with a concentration of $3 \times 10^{-4}$%, m/m. From a large pool of amber glass ampoules containing the AgNP suspension samples needed for testing were randomly chosen. The suspensions were stored at room temperature and protected from light.

For filtration of the AgNP suspension ultrafiltration spin columns with a PES filter membrane and a molecular weight cut-off (MWCO) of 5 kDa (VS0611, Sartorius, Göttingen, Germany) were used. As accumulation wall in $AF^4$ NADIR® regenerated cellulose membranes with MWCO = 10 kDa and PES membranes with MWCO = 10 kDa were purchased from Wyatt Technology (Dernbach, Germany). GE Osmonics® polyvinylidene difluoride (PVDF) membranes with MWCO = 30 kDa were ordered from Sterlitech (Kent, WA, USA).

### 2.2. Methods

A description of the determination of the silver concentration and the Zeta potential as well as of the imaging by transmission electron microscopy can be found in supplementary materials.

#### 2.2.1. NP separation and characterization by $AF^4$

The $AF^4$ system used in this study consisted of a high performance liquid chromatography pump (G1311A), an Agilent 1200 series autosampler (G1329A) equipped with 900 μl injection loop (Agilent Technologies, Santa Clara, CA, USA), an Eclipse™ 3 $AF^4$ flow control module, and an short channel-type $AF^4$ separation channel (Wyatt Technology Europe GmbH, Dernbach, Germany).

**Table 1**
Typical ICP-MS settings for coupling to $AF^4$.

| | |
|---|---|
| RF power | 1550 W |
| Plasma gas flow rate | 15 l/min |
| Carrier gas flow rate | 0.98 ml/min |
| Makeup gas flow rate | 0.19 ml/min |
| Nebulizer | Agilent Micro Flow (model no. G3139A-100) |
| Spray chamber | Scott double-pass |
| Isotopes monitored | $^{103}$Rh, $^{107}$Ag, $^{109}$Ag |
| Integration time per isotope | 300 ms |

The channel had a trapezoid shape with a length from inlet to outlet of 172 mm, a length from inject port to outlet of 152 mm and a width of 24.0 mm at the inlet and 3 mm at the outlet port. The width at the sample inject port was 21.5 mm. The length of the injection capillary tubing was 280 mm with i.d. 0.12 mm. The area of the accumulation wall was 2363 mm$^2$.

After changing the membrane or when the carrier liquid composition was changed the system was flushed with a flow of 1 ml/min for 60 min to allow equilibration. A series of typically five AgNP injections ($m_{inj} = 2$ μg) was performed to saturate the new membrane until the relative recovery did not further increase. The time $t_{extra}$ required for the sample to travel through external tubing was subtracted from the apparent retention time. The value $t_{extra}$ was determined based on the length and inner diameter of the external tubing and the applied detector flow rate.

Following separation various detectors were used to collect information about the eluting fractions. A series 1200 diode array detector (Agilent G1315A, DAD) was used to record the absorbance signal at pre-defined wavelengths. Additionally, absorption spectra were recorded in the wavelength range of 191–949 nm (steps of 10 nm) during the separation every 2 s. Relative recoveries were determined by integrating fractograms obtained by recording the 400 nm DAD absorbance signal of the $AF^4$ effluent. A 100% recovery was assumed for flow injections of samples through the $AF^4$ channel (no cross flow).

A DAWN® HELEOS™ (Wyatt Technology Europe GmbH, Dernbach, Germany) multi angle light scattering (MALS) detector with 17 + 1 observation angles operated with a linear polarized laser light at 658 nm was used to record the light scattering signal. The MALS detector was set to a sampling time interval of 1 s per data point. A dynamic light scattering (DLS) detector at angle 99° of the DAWN® HELEOS™ light scattering cell was used for on-line determination of the hydrodynamic diameter of the AgNPs. Data from the light scattering detectors was processed using the ASTRA V software (version 5.3.2.15, Wyatt Technology Corporation, Santa Barbara, CA, USA). As the final detector in the hyphenated system an ICP-MS instrument (ICP-MS 7500ce, Agilent Technologies, Japan) was used. The typical instrumental settings are presented in Table 1.

#### 2.2.2. 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 single factor ANOVA in Microsoft Office Excel 2007 (Microsoft, Redmond, WA, USA). For conversion of retention times into diameters estimated on the basis of $AF^4$ theory the software ISIS 1.0.0 (Wyatt Technology Europe GmbH, Dernbach, Germany) was used. Peak areas and maxima were determined by using the Peak Analyzer in the software OriginPro 8.1 (OriginLab Corporation, MA, USA). The same software was used for linear regression and peak fitting.

To evaluate the separation of two peaks the resolution $R_S$ was determined as [12]:

$$R_s = \frac{2|(t_{r,1} - t_{r,2})|}{W_1 + W_2}$$