Journal of Trace Elements in Medicine and Biology 54 (2019) 55–61

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Analytical methodology

Possibilities of single particle—ICP-MS for determining/characterizing titanium dioxide and silver nanoparticles in human urine

Kamala Badalova[a,b], Paloma Herbello-Hermelo[a], Pilar Bermejo-Barrera[a,*], Antonio Moreda-Piñeiro[a]

[a] Trace Element, Spectroscopy and Speciation Group (GETEE), Strategic Grouping in Materials (AEMAT), Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Chemistry. Universidade de Santiago de Compostela, Avenida das Ciencias, s/n. 15782, Santiago de Compostela, Spain
[b] Faculty of Pharmacy, General and Toxicological Chemistry Department, Azerbaijan Medical University, Bakihanov Street, 23. AZ1022, Baku, Azerbaijan

ARTICLE INFO

Keywords:
Silver nanoparticles
Titanium dioxide nanoparticles
Human urine
Single particle-inductively coupled mass spectrometry

ABSTRACT

Objective: The current use of nanoparticles in personal care and cosmetics, food safety, agriculture, medicine and pharmacy has led to a growing concern on the toxicity of these emerging materials to humans and also to the environment. Nanoparticles assessment (determination and size distribution) is a challenge mainly due to limitations of the current analytical instrumentation, but also because nanoparticles in foodstuff and environmental samples are usually found at low concentrations. The scenario is even more critical when dealing with clinical samples, mainly when trying to assess nanoparticles at basal levels in complex samples such as blood and urine. The aim of this paper is to find data regarding the presence of nanoparticles at basal levels in urine human samples.

Methods: The use of single particle – inductively couple plasma – mass spectrometry (sp-ICP-MS) has been explored to determine and characterize silver and titanium dioxide nanoparticles in human urine. Urine samples were directly diluted (1:5 to 1:10) with 1%(v/v) glycerol before sp-ICP-MS measurements, and efforts were made for validating the over-all procedure.

Results: The limit of detection and quantification for Ag NPs were 5.72×10^3 and 1.91×10^4 Ag NPs mL^{-1}, respectively; whereas, values for TiO_2 NP concentrations were 4.31×10^3 and $1.44 \times 10_4$ TiO_2 NPs mL^{-1}. The limit of detection in size after applying several methods (3σ/5σ criteria) was found to be within the 8–9 nm for Ag NPs, and from 15 to 18 nm for TiO_2 NPs. Within-batch precision for Ag NP concentration was 15% (11% for mean size of nanoparticle distributions). Repeatability for TiO_2 NPs was 25% (TiO_2 NP concentration) and 9% (TiO_2 NP mean size). Good analytical recovery rates were found for spiked experiments with Ag NP standards of 40 and 60 nm (values within the 104–106% range), and also for TiO_2 NPs of 50 and 100 nm (96–98%). Finally, basal levels of Ag NPs and TiO_2 NPs, as well as total Ag and Ti concentrations, in human urine were assessed. Low Ag and Ag NP concentrations were found. Ag NPs exhibited mean sizes of approximately 16–17 nm. Total Ti levels, however, were higher than total Ag concentration, and TiO_2 NP concentrations within the 1.56×10^4–2.80×10^4 NPs mL^{-1} range were measured (TiO_2 NP mean sizes were from 76 to 98 nm).

1. Introduction

Due to the completely different mechanical, chemical, electrical, magnetic, and optical properties of nanoparticles (NPs) from those of larger scale material with the same chemical composition, the manufacturing and exploration of applications of NPs have widely increased in recent years. More than 1600 nano-based products are currently available in the market [1]. Foodstuff and personal care products are important sectors in which NPs have found great applicability and use. US Regulatory Agencies as well as the European Commission have recognized the need for regulating the presence of these new nano-ingredients in consumer products. Some directives such as for the presence of NPs in cosmetics (Regulation (EC) No 1223/2009 [2]) and in materials for food packages (Regulation (EC) No 450/2009 [3] and Regulation (EC) 10/2011 [4]) have been developed in Europe. In addition, the increasing concern about the effect of nanoparticles (Nanotoxicity and Nanosafety) has recently led the European Food Safety Authority (EFSA) to publish guidance on the risk assessment of nanomaterials (NMs) in food and the feed chain (affecting human and animal health) [5]. NP assessment in biological fluids is becoming an

* Corresponding author.
E-mail address: pilar.bermejo@usc.es (P. Bermejo-Barrera).

https://doi.org/10.1016/j.jtemb.2019.04.003
Received 1 February 2019; Received in revised form 25 March 2019; Accepted 8 April 2019

important topic, and the development of reliable detection/quantification/characterization analytical methods is needed. Special considerations on relative low NP concentrations in biological fluids such as blood and urine are needed. Low NP levels in blood and urine can be expected because of the bioavailability/assimilation of these entities by the organism, and the possible biotransformation (degradation to dissolved ionic metal).

Although several instrumental techniques are available for chemical composition and particle size distribution (PSD), there is a gap in knowledge regarding the best approaches for an accurate and cost-effective detection, size measurement and characterization of NPs [6]. In addition to chemical composition and PSD, other NP characteristics such as agglomeration and surface area/functionality are also quite important, and these characteristics can be easily well-established for powders, but there are no straightforward techniques for NPs dispersed in a liquid. In this scenario, physico-chemical properties of NPs are context-dependent, and a distinction between *synthetic* and *biological* identity of NPs must be established [7]. *Synthetic* identity refers to the simplest characteristics of NPs (chemical, structural and compositional nature, and also surface coating, ligands or labelling molecules); whereas, *biological* identity refers to biomolecules that adsorbed onto NPs under specific conditions and the impact of these on the dispersion properties [8].

Imaging-based techniques, mainly scanning electron microscopy (SEM) and transmission electron microscopy (TEM); light scattering-based techniques, mainly dynamic light scattering (DLS), multi angle light scattering (MALS) and particle/nanoparticle tracking analysis (PTA/NTA); and separation techniques, such as centrifugal particle sedimentation (CPS), analytical ultracentrifugation (AUC) and flow field fractionation (FFF) techniques, can potentially be used for NP characterization [7,9,10]. In addition, other instrumental techniques have emerged and/or have been adapted recently for NP size fractionation, such as capillary electrophoresis (CE), ion mobility spectrometry (IMS), hydrodynamic chromatography (HDC), and single particle – inductively coupled plasma – mass spectrometry (sp-ICP-MS) [7,11–14]. When compared to other emerging techniques, sp-ICP-MS offers the possibility of distinguishing between dissolved and particulate analytes, in addition to the inherent capability of counting and sizing particles at very low concentrations. Despite of these advantages, sp-ICP-MS assume that measured NPs exhibit a spherical shape and they are totally made of the metal under study. Obviously, this assumption is right when analyzing synthetic NPs but it could not be true when assessing NPs in environmental, food and clinical materials. These limitations are fully discussed in recent reviews regarding sp-ICP-MS and also sp-ICP-MS hyphenated with specific separation methods for NP determination/characterization [15,16].

Available literature has focused on assessing NPs in environmental and foodstuff materials [7,9,12–17], and on NP assessment in biological fluids (blood) by sp-ICP-MS [18–20] as well as by ICP-MS hyphenated with size exclusion chromatography (SEC) [21]. Asymmetric flow field-flow fractionation (AF4) [22], and CE [23] have been recently discussed (the latter developments focused on assessing NP-protein complexes). Other approaches such as those developed by Chen et al. [24] have implied CPS for separating intact silica coated CdSeS quantum dots (QDs) and QD-protein complexes in blood and urine from mice before ICP-MS determination of Cd. Most of the developed methods [18–23], as well as other reports which use *in vitro* experiments with NPs and model plasma proteins [bovine serum albumin (BSA), globulin, or fibrinogen] [25–28], imply the assessment (determination and characterization) of NP standards that have been added to biofluids (serum or blood), and have been allowed to interact with proteins for a certain incubation time. These *in vitro* approaches, although useful for knowing/assessing NP-protein complexes, do not reflect the actual presence of NPs in biological fluids since NPs are previously added at a proper concentration to be easily measured by the selected analytical techniques.

The purpose of the current work has been to explore the possibilities of sp-ICP-MS for developing a reliable analytical method for the assessment of NPs containing Ag and NPs containing TiO_2 in human urine. Sample pre-treatment consists only in human urine dilution (1:5 to 1:10 with 1%(v/v) glycerol), and efforts have been made for validating the over-all analytical procedure and for assessing basal Ag NP and TiO_2 NP concentrations in urine from healthy volunteers.

2. Materials and methods

2.1. Instrumentation

Total Ag and Ti determinations were performed with a NexION® 300X ICP-MS (Perkin Elmer, Waltham, MA, USA) equipped with a nebulization system composed of a glass concentric nebulizer (PFA-ST), a PC3 glass cyclonic spray chamber termostatized by a Peltier refrigerator, and a SeaFast SC2 DX autosampler (Elemental Scientific, Omaha, NB, USA). The same ICP-MS instrument has been used for Ag NP and TiO_2 NP assessment (quantification and size distribution) by using the Syngistix™ Nano Application software version 1.1 (Perkin Elmer). Other devices were a Raypa UCI-150 ultrasonic water-bath from R. Espinar S.L (Barcelona, Spain) and a Laborcentrifugen 2K15 centrifuge from Sigma (Osterode, Germany).

2.2. Reagents

Ultrapure water (18 MΩ cm resistivity) was obtained from a AMilliQ Gradient A1 water purification system (Millipore, Bedford, MA, USA). NexIon Setup Solution (10 µg L^{-1} of U, Pb, Mg, Li, In, Fe, Ce, and Be in 1% HNO_3), multi-element calibration standard 3 (Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, and Zn, 10 mg L^{-1} in 5% HNO_3), germanium (1000 mg L^{-1} in H_2O/Tr F–), indium (1000 mg L^{-1} in 2% HNO_3), and scandium (1000 mg L^{-1} in n 2% HNO_3) stock standard solutions were from Perkin Elmer (Shelton, CT, USA). Titanium ($(NH_4)_2TiF_6$ in water, 1000 mg L^{-1}as Ti) and rhodium (1000 mg L^{-1} in 5% HNO_3) stock standard solutions were supplied by Merck (Darmstadt, Germany). Yttrium (Y_2O_3 in 2–5% HNO_3, 1000 mg L^{-1}as Y) stock standard solution was from Panreac (Barcelona, Spain). Ag NP standards (aqueous suspensions with sodium citrate as a stabilizer) of 60 nm (1.7×10^{13} NPs L^{-1}, 0.02 g L^{-1}) and 40 nm (5.7×10^{13} NPs L^{-1}, 0.02 g L^{-1}) were from Sigma-Aldrich (St. Louis, MO, USA). TiO_2 NP stock solutions were prepared in water (stock standard solutions of 100 mg L^{-1} as Ti) from TiO_2 nanopowder (rutile, 99.9%) of 50 and 100 nm purchased from US Research Nanomaterials (Houston, TX, USA). Certified reference material RM 8013 of gold nanoparticles (aqueous suspension with citrate as a stabilizer) of 60 nm nominal diameter was from the National Institute of Standards and Technology (Gaithersburg, MD, USA). Seronorm™ Trace Elements Urine L-2 (Ref. 210705, Lot. 1403081) was from Sero (Billingstad, Norway). Nitric acid 69% (Hyperpur) and n-butanol (HPLC grade) were supplied by Panreac. Glycerol (99.5%) and Triton X-100 were purchased from Sigma-Aldrich.

To avoid metal contamination, all glassware and plastic ware was washed and kept in 10% (v/v) nitric acid for 48 h, and rinsed several times with ultrapure water before use.

2.3. Urine samples

All urine samples used in the current research were from healthy volunteer adults (staff at the University of Santiago de Compostela). Sample collection was performed in accordance with the *Comité Ético de Investigación Clínica* – CEIC (Ethical Committee for Clinical Research) of Galicia (Registration Code: CEIC de Galicia 2010/372) which requires a written informed consent from all volunteers. Urine samples (10 mL) were collected in pre-cleaned polypropylene vials and were stored at 4 °C (for a maximum of 48 h) before analysis.

Table 1
ICP-MS and sp-ICP-MS instrumental parameters for total silver and titanium determination and for NPs containing Ag/TiO_2 determination/characterization.

Operating ICP-MS conditions		
Radiofrequency power		1600 W
Gas flows	Nebulization	0.95 mL min^{-1}
	Auxiliary	1.2 mL min^{-1}
	Plasma	16 mL min^{-1}
KED mode: He flow rate/ mL min^{-1}	1.0 (Ag, Cd, Mn, Pb, and Ti)	
	4.0 (Al, As, Co, Cu, Ni, Sb, Sn, V, and Zn)	
Analytes	Ag	(Ag 107) 106.905095
	Ti	(Ti 49) 48.947871
Internal standards	Rh	(Rh 103) 102.905503
	Ge	(Ge 74) 73.921179
Operating sp-ICP-MS conditions		
NPs containing Ag		
Mass (Ag)		(Ag 107) 106.905095
Density		10.49 g cm^{-3}
Mass fraction		100%
Ionization efficiency		100%
Sample flow rate		0.41 - 0.43 mL min^{-1}
Dwell time		100 μs
Sampling time		100 s
Mode		Standard
Number of readings		25000
Replicates		3
NPs containing TiO_2		
Mass (Ti)		(Ti 49) 48.947871
Density		4.23 g cm^{-3}
Mass fraction		59.9%
Ionization efficiency		100%
Sample flow rate		0.41 - 0.43 mL min^{-1}
Dwell time		100 μs
Sampling time		100 s
Mode		Standard
Number of readings		25000
Replicates		3

2.4. Total Ag and Ti determination by ICP-MS

Urine samples were 1:10 diluted with 1%(v/v) nitric acid before ICP-MS measurement. ICP-MS adjustment (nebulization gas flow, torch position, optical lens, and quadrupole voltages) was performed daily. The *m/z* ratios monitored were ^{107}Ag and ^{49}Ti. Internal standards were ^{103}Rh (10 μg L^{-1}) and ^{74}Ge (10 μg L^{-1}) for Ag and Ti, respectively. ICP-MS operating conditions are listed in Table 1. KED mode (1.0 mL min^{-1} He) was used for Ag and Ti determinations. A 1%(v/v) nitric acid matched calibration (concentrations within the 0–200 μg L^{-1} range) was used for measurements.

2.5. NPs containing Ag and NPs containing TiO_2 determination by sp-ICP-MS

Urine samples were sonicated for 5 min (water-bath 37 kHz) before 1:10 dilution with 1%(v/v) glycerol. Diluted urine samples were again sonicated (water-bath 37 kHz) for 5 min just before sp-ICP-MS (operating conditions listed in Table 1). ICP-MS operational conditions adjustment was performed daily. Sample flow rate and transport efficiency (TE%) assessment (method outlined by Pace et al. [29]) by using Au NPs aqueous suspension (particle concentration of 518 ng L^{-1} and 60 nm nominal diameter) was performed daily using the Syngistix™Nano Application software (TE% values close to 5%). Ionic silver and titanium aqueous calibrations (0–20 μgL^{-1}) were performed for NPs containing Ag and NPs containing TiO_2 size distribution assessment.

3. Results and discussion

3.1. Total Ag and Ti determination in urine

Preliminary studies based on direct urine sampling after dilution with several diluents (2%(v/v) HNO_3, 2%(w/v) Triton X-100, and 2%(v/v) n-butanol) were performed in order to choose the best sample pre-treatment (1:10 dilution) procedure. Selected diluents, all of them proposed in the literature for direct dilution of urine [30,31] and serum/plasma [32–35] before ICP-MS determinations, were used for preparing matched calibrations (Ag and Ti concentration levels from 0 to 50 μg L^{-1}) and further used to analyze a control sample (Seronorm™ Trace Elements Urine L-2). Three independent calibrations were performed in several days. In addition, three CRM aliquots were determined by using each matched calibration after 1:10 dilution with the selected diluent. As shown in Fig. 1, slopes for Ti aqueous calibration and Ti matched calibrations with 2%(v/v) HNO_3 and 2%(w/v) Triton X-100 are quite similar; whereas, slopes for 2%(v/v) n-butanol are slightly higher. Regarding Ag, slopes for 2%(v/v) HNO_3 and 2%(v/v) n-butanol matched calibrations have been found to be similar, and they are quite different from those obtained for Ag aqueous calibrations or when matching with 2%(w/v) Triton X-100.

Ti concentrations were found to be quite similar to the indicative Ti concentration for this control material (16.1 μg L^{-1}) after 1:10 dilution with 2%(v/v) HNO_3 (16.3 ± 0.70 μg L^{-1}) and 2%(v/v) n-butanol (16.5 ± 0.50 μg L^{-1}) as diluents and also as matching solvents, and also when using the standard addition technique (16.0 ± 0.70 μg L^{-1}). Accuracy for Ag, however, could not be verified because the indicative (additional approximate) Ag concentration in the analyzed control

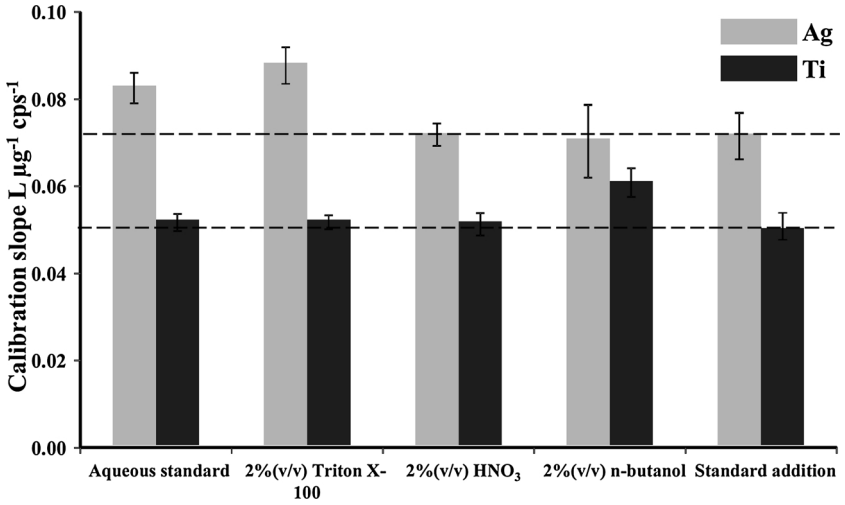

Fig. 1. Calibrations' slopes (n = 3) for Ag and Ti by using several matching solvents.

sample is lower than the LOD of the method. Other elements were also determined in the control sample (ICP-MS conditions listed in Table S1 in ESI section), and found concentrations (Table S2, ESI section) were found to be in good agreement with analytical values given in the control sample when using any of the proposed diluents. Based on these results, 2%(v/v) HNO_3 matched calibration was finally selected for total Ag and Ti assessment in urine samples. In addition, a standard addition method (1:10 dilution and using 2%(v/v) HNO_3 as a diluent) was performed in triplicate, and results (slopes of the standard addition graphs, Fig. 1) were similar to those obtained when preparing 2%(v/v) HNO_3 matched calibration. These findings, together with Ti (and other elements) concentrations similar to the informative (additional approximate) values/analytical values in the control sample (Table S2, ESI section), shows that determinations using 2%(v/v) HNO_3 matched calibration are free of matrix interferences.

The limit of detection (LOD) and the limit of quantification (LOQ), based on the 3σ/10σ criterion (σ is the standard deviation of eleven measurements of a blank), were found to be 0.34 and 1.12 μg L^{-1} for Ag, and 0.75 and 2.5 μg L^{-1} for Ti.

3.2. NPs containing Ag and NPs containing TiO_2 determination

3.2.1. Effect of centrifugation

Preliminary experiments based on a centrifugation stage for removing large biomolecules were carried out. After fixing the temperature at 4 °C and using a centrifugation time of 10 min, solid residues were not observed when applying centrifugation speeds lower than 3900 rpm (2364 *g*). After fixing the centrifugation speed at 4000 rpm, experiments for several centrifugation times were performed, and NPs containing Ag and NPs containing TiO_2 were assessed by sp-ICP-MS (1:10 dilution in 1%(w/v) glycerol) before and after centrifugation (several centrifugation times). Results show that NPs are partially lost during the centrifugation stage, even when using short centrifugation times (10 min). The effect is more important for NPs containing Ag ($7.5 \times 10^8 \pm 2.5 \times 10^7$ NPs L^{-1} without centrifugation, and $4.0 \times 10^8 \pm 1.3 \times 10^7$ NPs L^{-1} after centrifugation), and it is less significant for NPs containing TiO_2 ($1.3 \times 10^8 \pm 3.8 \times 10^6$ NPs L^{-1} without centrifugation, and $1.9 \times 10^8 \pm 4.5 \times 10^7$ NPs L^{-1} after centrifugation). Therefore, centrifugation was not considered any further.

3.2.2. Calibration, limits of detection and quantification, and limit of detection in size

Calibration was performed using aqueous ionic titanium and silver standards (concentrations within the 0–20 μg L^{-1} range). Patented routines in Syngistix™ Nano Application software has been demonstrated to offer accurate results for nanoparticle size assessment when using either dissolved or particle calibrations [36].

The 3σ/10σ criterion (σ is the standard deviation of eleven measurements of a blank) was used for establishing the limit of detection (LOD) and the limit of quantification (LOQ) for NP concentration (NPs containing Ag/TiO_2 L^{-1}). Blanks (1%(v/v) glycerol) were measured by sp-ICP-MS eleven times. Three times (LOD) or ten times (LOQ) the standard deviation of the measured NP concentrations were obtained. Calculated LOD and LOQ values referring to the urine sample (1:10 dilution for NPs containing TiO_2) were 4.31×10^3 and 1.44×10^4 NPs mL^{-1}, respectively. Values for NPs containing Ag (1:5 dilution) were 5.72×10^3 and 1.91×10^4 NPs $mL^{-1}$for LOD and LOQ, respectively.

Regarding the limit of detection in size (D_{min}), the 3σ and 5σ criterion (the standard deviation of counts in ultrapure water blanks analysed against calibration curves) were applied according to Eq. (1) [37,38]:

$$D_{min} = \sqrt[3]{\frac{6 \times 3\sigma_{DI}}{R \times f_a \times \rho \times \pi}} \quad (1)$$

where $3\sigma_{DI}$ (or $5\sigma_{DI}$) is three or five times the standard deviation of counts of ultrapure water blanks, R is the sensitivity of the detector (slope of the calibration curve of ionic standard solutions) for the element of the analyte (Ti), f_a is the mass fraction of analysed metallic element in the nanoparticles, and ρ is the density of the Ag/TiO_2 NPs.

Calculated D_{min}values were 15 nm (3σ criteria) and 18 nm (5σ criteria) for NPs containing TiO_2, and 8 nm (3σ criteria) and 9 nm (5σ criteria) for NPs containing Ag.

3.2.3. Within-batch precision

Precision of the method (Ag/TiO_2 NP concentration and average sizes) was assessed by the within-batch precision approach. Therefore, the proposed method was applied to eleven urine aliquots from a urine sample (1:10 dilution for TiO_2 NP assessment, and 1:5 dilution for Ag NP assessment). Regarding TiO_2 NP concentration ($1.41 \times 10^5 \pm 3.52 \times 10^4$ NPs mL^{-1}), a relative standard deviation (RSD) of 25% was assessed; whereas, the RSD when measuring the average size of the size distribution was 9% (average size of 84.3 ± 7.9 nm). The RSD value for Ag NP concentration was found to be 15% ($2.06 \times 10^4 \pm 3.15 \times 10^3$ NPs mL^{-1}), and the repeatability for Ag NP size was 11% (17.9 ± 2.0 nm).

3.2.4. Analytical recovery assays

Several spiking experiments (Ag and TiO_2 NPs exhibiting several size distributions) were performed to verify the accuracy of sp-ICP-MS determinations (analytical recovery assays). Three aliquots from urine samples were analyzed to determine Ag and TiO_2 NP concentrations. Three aliquots from the same urine sample were also spiked with each NP standard: Ag NPs (40 or 60 nm), or TiO_2 NPs (50 or 100 nm), and the mixtures were also analyzed by sp-ICP-MS. Regarding TiO_2 NPs, spiking solutions were prepared at a concentration level of 14 μg L^{-1} (as Ti); whereas, the Ag NP spiking solutions were fixed at 7.13×10^9, 8.50×10^8 (as Ag NPs L^{-1}) for 40, and 60 nm. These solutions were also measured by sp-ICP-MS at the same dilutions as those used when mixing with urine.

After measurements in triplicate, the Ag NP or TiO_2 NP concentrations found in the un-spiked urines were subtracted from the Ag NP or TiO_2 NP concentrations found in the spiked urine aliquots to calculate the concentrations of Ag NPs or TiO_2 NPs previously used for spiking (named as "found Ag NP or TiO_2 NP concentration"). Analytical recovery was then assessed by dividing the "found Ag NP or TiO_2 NP concentration" by the mean Ag NP or TiO_2 NP concentrations found in the aqueous Ag NP or TiO_2 NP solutions used for spiking (named as "added Ag NP or TiO_2 NP concentration") and multiplying by 100. Table 2 lists the mean analytical recovery values, which were found to be close to 100%. Fig. 2 shows some size distribution histograms obtained from spiked urine samples and aqueous NP standard solutions used for spiking. Two different distributions can be seen when using spiking solutions of larger size than those typically found in un-spiked urine samples. This is the case of NPs containing Ag, where mean sizes are around 20 nm when using a 60 nm Ag NP spiking solution (Fig. 2). Regarding NPs containing TiO_2, differences are not evident because NPs containing TiO_2 how a mean size close to 80 nm, and the spiking solutions (50 and 100 nm TiO_2 NPs) show size distributions which overlap with those exhibited by un-spiked urines.

Table 2
Analytical recovery of the method.

	Mean NPs diameter / nm	Analytical recovery (n = 9) /%
Ag NPs	40	106 ± 1
	60	104 ± 4
TiO_2 NPs	50	98 ± 4
	100	96 ± 4

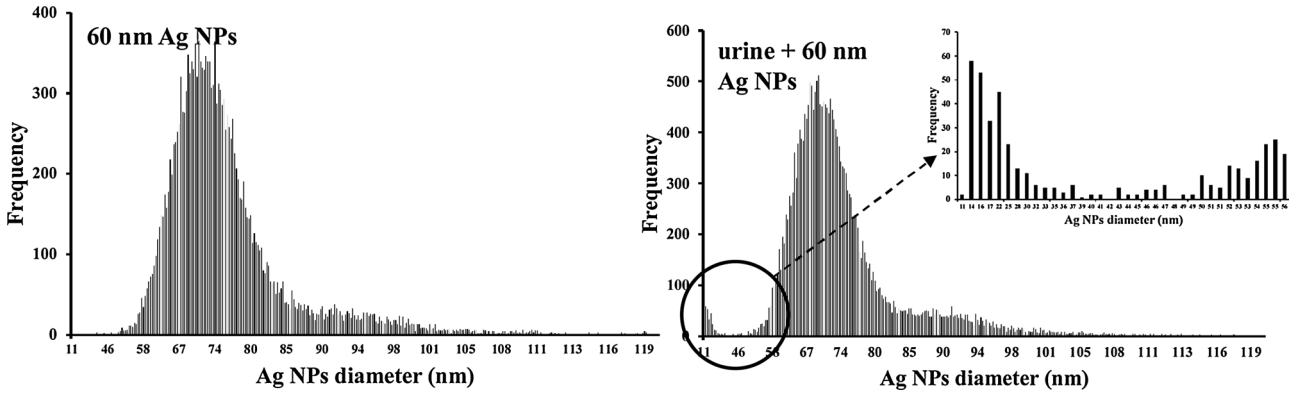

Fig. 2. Ag NPs size distribution from Ag NPs (60 nm, 8.50×10^8 NPs L^{-1}) standards and a human urine sample spiked with Ag NPs (60 nm, 8.50×10^8 NPs L^{-1}).

3.2.5. Applications

Eleven urine samples were analyzed for total Ag and Ti (ICP-MS) and for NPs containing Ag/TiO_2 (sp-ICP-MS). All urine samples, except one (concentration lower than 2.52 μg L^{-1}), were positive for Ti (concentrations ranging from 3.64 ± 0.45 μg L^{-1} to 15.1 ± 0.3 μg L^{-1}, Table 3). However, Ag was found to be lower than the LOD of the method (1.25 μg L^{-1}) in most of urine samples, and only two urine samples offered concentrations higher than the LOQ of the method (1.39 ± 0.13 μg L^{-1} and 1.53 ± 0.28 μg L^{-1}, Table 3).

Positive urine samples for total Ag and Ti were further analyzed by sp-ICP-MS. As listed in Table 3, NPs containing TiO_2 were quantified in only four urine samples from the ten urine samples containing total Ti. TiO_2 NP concentrations ranged from $1.56 \times 10^4 \pm 6.04 \times 10^3$ NPs mL^{-1} to $2.80 \times 10^4 \pm 2.55 \times 10^3$ NPs mL^{-1} (Table 3). The two positive urine samples for Ag showed Ag NP concentrations higher than the LOD of the method, and number of Ag NP as well as Ag NP distribution could be assessed (Table 3).

NPs containing TiO_2 and NPs containing Ag size distributions can be seen in Figs. 3 and 4, respectively; whereas, the mean values and most frequent sizes are listed in Table 3. Mean sizes for NPs containing TiO_2 varied from 76 to 98 nm (most frequent sizes from 70 to 86 nm). Similarity between the mean and most frequent size (Fig. 3 and Table 3) shows that NPs containing TiO_2 size distribution follow a lognormal distribution [39]. Regarding NPs containing Ag size distribution, lower sizes have been found, and the average and most frequent sizes were below 20 nm (Table 3). Moreover, NPs containing Ag size distributions appear to follow a lognormal distribution (Fig. 4).

4. Conclusions

Results suggest great potential for sp-ICP-MS for assessing low concentrations of NPs (also NPs of small sizes) such as NPs containing Ag and NPs containing TiO_2 in complex samples. The procedure requires minimum sample pre-treatment, and 1:5 to 1:10 dilution are adequate for accurate and precise NP measurements. The good sensitivity (NP concentration) offered by sp-ICP-MS allowed the assessment of NPs basal levels in urine. This fact is quite important since most of published literature deals with blood/serum samples which have been previously spiked with NPs (mainly Ag NPs and Au NPs) at concentrations higher than those expected as basal levels. NPs quantification/characterization in spiked samples is therefore simple using instrumental techniques such as sp-ICP-MS.

NPs containing TiO_2 (and also total Ti) were quantified in several urine samples, and size distribution showed sizes higher than the LOD (below 20 nm). However, NPs containing Ag (and also total Ag) were found at concentrations lower than the LOD (NP concentration) of the

Table 3
Total Ti and Ag contents (n = 3), Ag NP and TiO_2 NP concentrations (n = 9), and NPs containing Ag and NPs containing TiO_2 size distribution (n = 9) in urine samples.

			NPs containing Ag size distribution		
Code	[Ag] μg L^{-1}	NPs containing Ag mL^{-1}	Mean size nm	Most frequent size nm	Range size nm
U1	1.39 ± 0.13	$2.23 \times 10^4 \pm 7.73 \times 10^3$	16.4 ± 0.14	16.1 ± 0.10	11.2–28.7
U2	1.53 ± 0.28	$9.16 \times 10^4 \pm 2.06 \times 10^3$	16.7 ± 0.23	16.2 ± 0.11	11.2–29.3
U3–U11	< 1.12	—[a]	—[a]	—[a]	—[a]

			NPs containing TiO_2 size distribution		
Code	[Ti] μg L^{-1}	NPs containing TiO_2 mL^{-1}	Mean size nm	Most frequent size nm	Range size nm
U1	8.10 ± 0.48	$< 1.44 \times 10^4$	—[a]	—[a]	—[a]
U2	3.64 ± 0.20	$1.56 \times 10^4 \pm 6.04 \times 10^3$	96 ± 1	86 ± 4	70–150
U3	5.28 ± 0.16	$1.97 \times 10^4 \pm 3.93 \times 10^3$	94 ± 1	83 ± 1	70–146
U4	5.15 ± 0.24	$1.61 \times 10^4 \pm 1.07 \times 10^3$	98 ± 1	70 ± 2	61–148
U5	15.1 ± 0.27	$< 1.44 \times 10^4$	—[a]	—[a]	—[a]
U6	14.2 ± 0.65	$< 1.44 \times 10^4$	—[a]	—[a]	—[a]
U7	9.50 ± 0.31	$< 1.44 \times 10^4$	—[a]	—[a]	—[a]
U8	10.2 ± 0.24	$< 1.44 \times 10^4$	—[a]	—[a]	—[a]
U9	14.3 ± 0.36	$2.80 \times 10^4 \pm 2.55 \times 10^3$	76 ± 6	69 ± 4	61–137
U10	6.04 ± 0.19	$< 1.44 \times 10^4$	—[a]	—[a]	—[a]
U11	< 2.50	—[a]	—[a]	—[a]	—[a]

[a] Not determined.

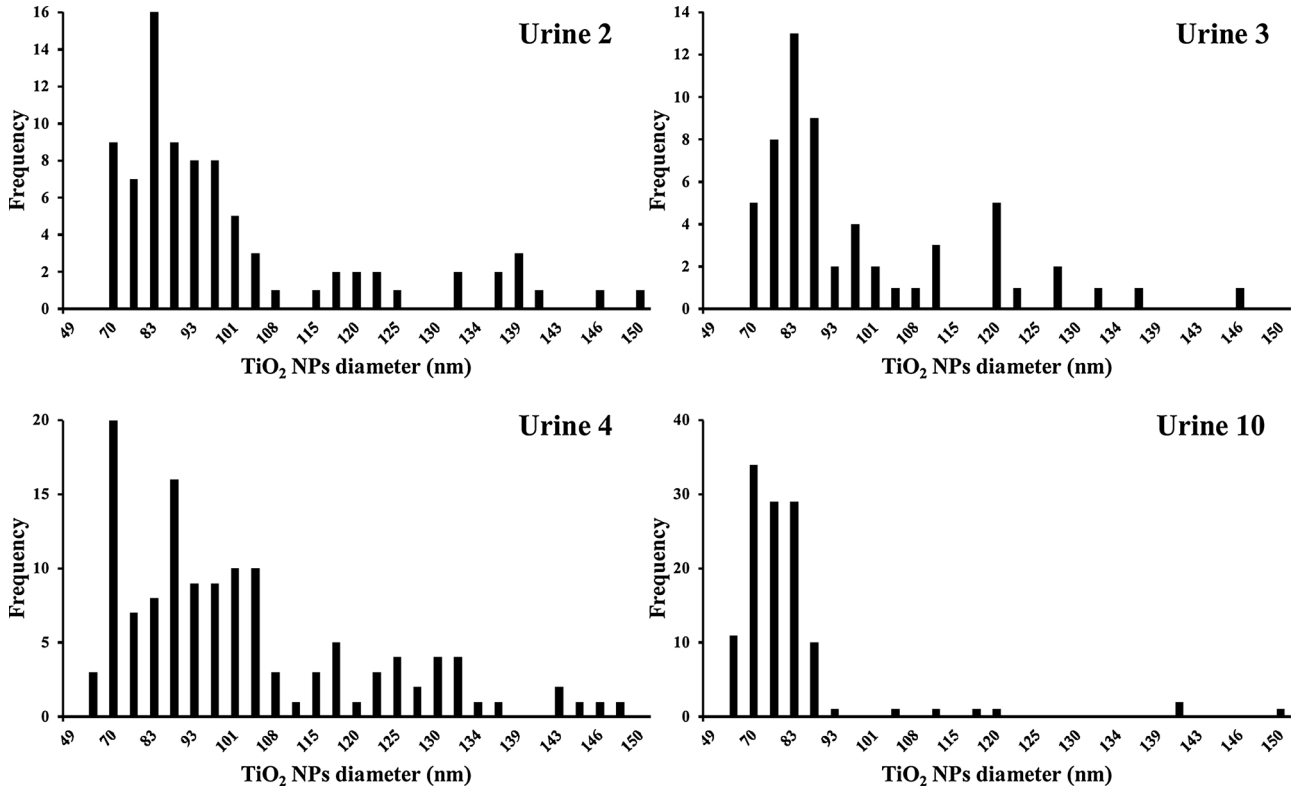

Fig. 3. NPs containing TiO_2 size distribution in some human urine.

method in most of samples. In addition, NPs containing Ag size distributions were close to the theoretical LOD (approximately 10 nm), and problems regarding integration/NPs counting (dissolved Ag integration) were observed when using the Syngistix™ Nano Application routine. Lower Ag NP concentrations and sizes in urine can be expected because NPs containing Ag are easily ionized (dissolved) under physiological conditions, including gastric-intestinal digestion (NPs uptake from water and foodstuff), and dermal absorption (NPs uptake from medical dressings, creams and ointments) processes.

Further work is therefore needed for developing pre-concentration methods for NPs such as Ag NPs, where basal levels in biofluids are quite low. In addition, improvements on counting methods are also needed to assess small NPs (also the case of NPs containing Ag in biological materials). Finally, research on assessing basal NPs-protein complexes in biofluids is also needed, and developments on new and advanced hyphenated techniques (NPs, proteins, and NP-protein complexes separation) are also required.

Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with ethical standards

The authors declare that the studies have been approved by the *Comité Ético de Investigación Clínica de Galicia* (registration code CEIC de Galicia 2010/372). The approved document by the CEIC de Galicia requires an informed consent from all volunteers who participated in the study. The authors declare therefore that all volunteers have signed an informed consent for allowing the use of the provided blood and urine samples in this study.

Acknowledgements

This work was supported by the Spanish Government (Ministerio de Economía y Competitividad, project CTQ2015-68094-C2-2-R); the

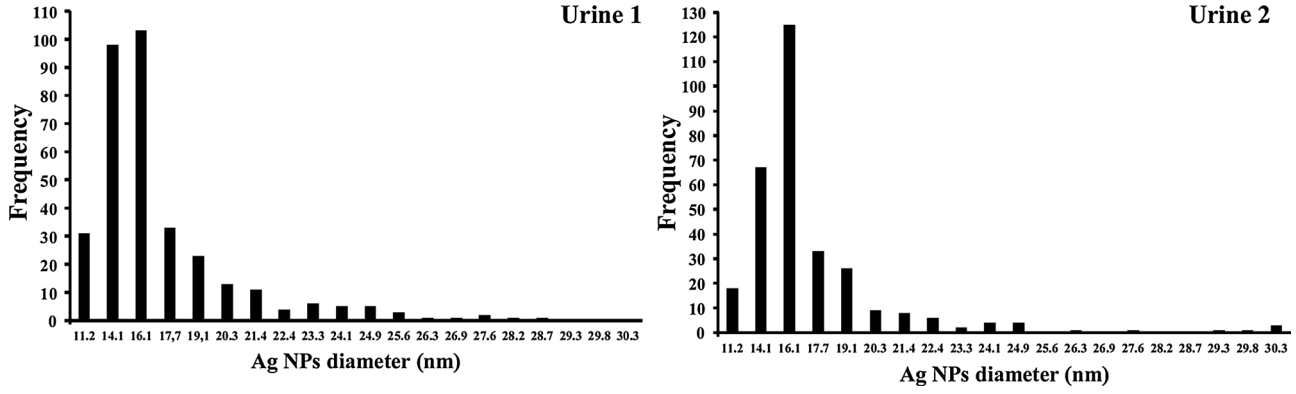

Fig. 4. NPs containing Ag size distribution in some human urine.

Dirección Xeral de I + D, Xunta de Galicia, Spain, Grupos de Referencia Competitiva (project number ED431C2018/19), and Development of a Strategic Grouping in Materials - AEMAT (grant ED431E2018/08); and European Regional Development Fund - FEDER – (project number GPC2014/035). K. Badalova is grateful to the Education, Audiovisual and Culture Executive Agency (EACEA) European Commission for an Erasmus Mundus Action Strand 1 MEDEA (project code 2013-2522/001-001-EMA2) research grant.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2019.04.003.

References

[1] Consumer Products Inventory. An Inventory of Nanotechnology-based Consumer Products Introduced on the Market, (2018) (Accessed on March 16th, http://www.nanotechproject.org/cpi.
[2] European Commission, Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products, Off. J. Eur. Union 22.12 (2009) (2009) L342/59–L342/209.
[3] European commission, Regulation (EC) No 450/2009 of 29 May2009 on active and intelligent materials and articles intended to come into contact with food, Off. J. Eur. Union 30.5 (2009) (2009) L135/3–L135/11.
[4] European Commission, Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food, Off. J. Eur. Union 15.1 (2011) (2011) L12/1–L12/89.
[5] Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: part 1, human and animal health, Eur. Food Saf. Authority, EFSA J. 16 (2018) 5327.
[6] K. Savolainen (coordinator), et al., Nanosafety in Europe 2015-2025: Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations, Finnish Institute of Occupational Health, 2013.
[7] C. Contado, Nanomaterials in consumer products: a challenging analytical problem, Front. Chem. 3 (2015) 48, https://doi.org/10.3389/fchem.2015.00048.
[8] M.P. Monopoli, D. Walczyk, A. Lowry-Campbell, G. Elia, I. Lynch, F. Baldelli Bombelli, K.A. Dawson, Physical-chemical aspects of protein corona: relevance to *in vitro* and *in vivo* biological impacts of nanoparticles, J. Am. Chem. Soc. 133 (2011) 2525–2534.
[9] I. de la Calle, M. Menta, F. Séby, Current trends and challenges in sample preparation for metallic nanoparticles analysis in daily products and environmental samples: a review, Spectrochim. Acta Part B 125 (2016) 66–96.
[10] M. Mattarozzi, M. Suman, C. Cascio, D. Calestani, S. Weigel, A. Undas, R. Peters, Analytical approaches for the characterization and quantification of nanoparticles in food and beverages, Anal. Bioanal. Chem. 409 (2017) 63–80.
[11] T.K. Mudalige, H. Qu, D. Van Haute, S.M. Ansar, S.W. Linder, Capillary electrophoresis and asymmetric flow field-flow fractionation for size-based separation of engineered metallic nanoparticles: a critical comparative review, Trends Analyt. Chem. 106 (2018) 202–212.
[12] F. Laborda, E. Bolea, G. Cepriá, M.T. Gómez, M.S. Jiménez, J. Pérez-Arantegui, J.R. Castillo, Detection, characterization and quantification of inorganic engineered nanomaterials: a review of techniques and methodological approaches for the analysis of complex samples, Anal. Chim. Acta 904 (2016) 10–32.
[13] J.M. Costa-Fernández, M. Menéndez-Miranda, D. Bouzas-Ramos, J. Ruiz Encinar, A. Sanz-Medel, Mass spectrometry for the characterization and quantification of engineered inorganic nanoparticles, Trends Analyt. Chem. 84 (2016) 139–148.
[14] A. López-Serrano, R.M. Olivas, J.S. Landaluze, C. Cámara, Nanoparticles: a global vision. Characterization, separation, and quantification methods. Potential environmental and health impact, Anal. Methods 6 (2014) 38–56.
[15] M. Mattarozzi, M. Suman, C. Cascio, D. Calestani, S. Weigel, A. Undas, R. Peters, Analytical approaches for the characterization and quantification of nanoparticles in food and beverages, Anal. Bioanal. Chem. 409 (2017) 63–80.
[16] B. Meermann, V. Nischwitz, ICP-MS for the analysis at the nanoscale – a tutorial review, J. Anal. At. Spectrom. 33 (2018) 1432–1468.
[17] C.-K. Su, Y.-C. Sun, Considerations of inductively coupled plasma mass spectrometry techniques for characterizing the dissolution of metal-based nanomaterials in biological tissues, J. Anal. At. Spectrom. 30 (2015) 1689–1705.
[18] M. Witzler, F. Küllmer, K. Günther, Validating a single-particle ICP-MS method to measure nanoparticles in human whole blood for nanotoxicology, Anal. Lett. 51 (2018) 587–599.
[19] K. Neubauer, C. Stephan, Determination of gold and silver nanoparticles in blood using single particle ICP-MS, Application note Perkin Elmer., 2019, https://www.perkinelmer.com/lab-solutions/resources/docs/APP_NexION-350D-Gold_Silver-Nanoparticles-in-Blood.pdf.
[20] C.-M. Cirtiu, N. Fleury, C. Stephan, Assessing the fate of nanoparticles in biological fluidsusing SP-ICP-MS, Application note Perkin Elmer, 2019, http://www.perkinelmer.com/lab-solutions//resources/docs/APP_012008_01-NexION-350S-Fate-of-NPs-in-Bio-Fluids.pd.
[21] X.-x. Zhou, J.-f Liu, G.-b. Jiang, Elemental mass size distribution for characterization, quantification and identification of trace nanoparticles in serum and environmental waters, Environ. Sci. Technol. 51 (2017) 3892–3901.
[22] K. Loeschner, C.F. Harrington, J.-L. Kearney, D.J. Langton, E.H. Larsen, Feasibility of asymmetric flow field-flow fractionation coupled to ICP-MS for the characterization of wear metal particles and metalloproteins in biofluids from hipreplacement patients, Anal. Bioanal. Chem. 407 (2015) 4541–4554.
[23] M. Matczuk, J. Legat, F. Scaletti, L. Messori, A.R. Timerbaev, M. Jarosz, The fate of differently functionalized gold nanorods in human serum: a response from capillary electrophoresis–inductively coupled plasma mass spectrometry, J. Chromatogr. A 1499 (2017) 222–225.
[24] Z. Chen, H. Chen, H. Meng, G. Xing, X. Gao, B. Sun, X. Shi, H. Yuan, C. Zhang, R. Liu, F. Zhao, Y. Zhao, X. Fang, Bio-distribution and metabolic paths of silica coated CdSeS quantum dots, Toxicol. Appl. Pharmacol. 230 (2008) 364–371.
[25] T.K. Mudalige, H. Qu, S.W. Linder, Asymmetric flow-field flow fractionation hyphenated ICP-MS as an alternative to cloud point extraction for quantification of silver nanoparticles and silver speciation: application for nanoparticles with a protein corona, Anal. Chem. 87 (2015) 7395–7401.
[26] P. Wimuktiwan, J. Shiowatana, A. Siripinyanond, Investigation of silver nanoparticles and plasma protein association using flowfield-flow fractionation coupled with inductively coupled plasma mass spectrometry (FlFFF-ICP-MS), J. Anal. At. Spectrom. 30 (2015) 245–253.
[27] M. Matczuk, J. Legat, S.N. Shtykov, M. Jarosz, A.R. Timerbaev, Characterization of the protein corona of gold nanoparticles by an advanced treatment of CE-ICP-MS data, Electrophoresis 37 (2016) 2257–2259.
[28] S. Jeon, D.R. Oberreit, G. Van Schooneveld, C.J. Hogan Jr, Liquid nebulization – ion mobility spectrometry based quantification of nanoparticle – protein conjugate formation, Anal. Chem. 88 (2016) 7667–7674.
[29] H.E. Pace, N.J. Rogers, C. Jarolimek, V.A. Coleman, C.P. Higgins, J.F. Ranville, Determining transport efficiency for the purpose of counting and sizing nanoparticles via single particle inductively coupled plasma mass spectrometry, Anal. Chem. 83 (2011) 9361–9369.
[30] G.H.O. Rocha, C. Steinbach, J.R. Munhoz, M.A.O. Madia, J.K. Faria, D. Hoeltgebaum, F. Barbosa Jr, B.L. Batista, V.C.O. Souza, S.B. Nerilo, E. Bando, S.A.G. Mossini, P. Nishiyama, Trace metal levels in serum and urine of a population in southern Brazil, J. Trace Elem. Med. Biol. 35 (2016) 61–65.
[31] M.R. Flórez, E. García-Ruiz, E. Bolea-Fernández, F. Vanhaecke, M. Resano, A simple dilute-and-shoot approach for the determination of ultra-trace levels of arsenic in biological fluids via ICP-MS using CH_3F/He as a reaction gas, J. Anal. At. Spectrom. 31 (2016) 245–251.
[32] R. Choi, H.-T. Kim, Y. Lim, M.-J. Kim, O.J. Kwon, K. Jeon, H.Y. Park, P.-H. Jeong, W.-J. Koh, S.Y. Lee, Serum concentrations of trace elements in patients with tuberculosis and its association with treatment outcome, Nutrients 7 (2015) 5969–5981.
[33] R. Gajek, K.-Y. Choe, Determination of ultra-trace elements in human plasma or serum by ICP-MS using sodium in the presence of carbon as a single calibration matrix-match component, J. Anal. At. Spectrom. 30 (2015) 1142–1153.
[34] Y. Lu, M. Kippler, F. Harari, M. Grandér, B. Palm, H. Nordqvist, M. Vahter, Alkali dilution of blood samples for high throughout ICP-MS analysis – comparison with acid digestion, Clin. Biochem. 48 (2015) 140–147.
[35] D.R. Jones, J.M. Jarrett, D.S. Tevis, M. Franklin, N.J. Mullinix, K.L. Wallon, C.D. Quarles Jr, K.L. Caldwell, R.L. Jones, Analysis of whole human blood for Pb, Cd, Hg, Se, and Mn by ICP-MS-DRC-MS for biomonitoring and acute exposures, Talanta 162 (2017) 114–122.
[36] C. Stephan, Accurate sizing and precise counting of 10 nm gold nanoparticles using the enhanced sensitivity of the NexION 2000 ICP-MS, Application note ICP- mass spectrometry. Perkin Elmer, 2019.
[37] S. Lee, X. Bi, R.B. Reed, J.F. Ranville, P. Herckes, P. Westerhoff, Nanoparticle size detection limits by single particle ICP-MS for 40 elements, Environ. Sci. Technol. 48 (2014) 10291–10300.
[38] A.R. Donovan, C.D. Adams, Y. Ma, C. Stephan, T. Eichholz, H. Shi, Single particle ICP-MS characterization of titanium dioxide, silver, and gold nanoparticles during drinking water treatment, Chemosphere 144 (2016) 148–153.
[39] F. Laborda, J. Jiménez-Lamana, E. Bolea, J.R. Castillo, Selective identification, characterization and determination of dissolved silver(I) and silver nanoparticles based on single particle detection by inductively coupled plasma mass spectrometry, J. Anal. At. Spectrom. 26 (2011) 1362–1371.