A simple analytical methodology for platinum nanoparticles control in complex clinical matrices via SP-ICP-MS

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Abstract

A simple method based on the use of inductively coupled plasma mass spectrometry in single particle mode (SP-ICP-MS) has been proposed, for the first time, for the study of platinum nanoparticles (PtNPs) in complex clinical matrices such as human urine and blood serum. Critical parameters for signal acquisition were optimized to achieve a correct and simultaneous sizing and counting (particle-based in particles L^{-1} and mass-based in ng L^{-1}) of 50 and 70 nm PtNPs. Different reagents, as tetramethylammonium hydroxide (TMAH) and/or Triton X-100, and concentrations have been tested to ensure an adequate stabilization and extraction of PtNPs. Finally, TMAH at 1% is demonstrated to be the best reagent to extract the NPs guaranteeing their integrity. No heating or any additional treatment was required, which allows sample preparation, and the overall process, to be simple and fast. Good precisions for size (2% RSD) and particle number and mass concentrations (<1% RSD), and limits of detection of 21.6 nm and $1.9x10^5$ particles L^{-1} were reported. The influence of matrix on the determination of PtNP sizes and number- and mass-based concentrations was evaluated. Particle sizes were in all cases in accordance with values determined by TEM or SEM, whereas recoveries of PtNPs in terms of concentration ranged between 92 and 101%. The stability of PtNP characteristics after 24 h was specifically studied in human urine spiked with PtNPs. Statistically significant differences were only reported for the particle number concentrations of 50 nm PtNPs in female urine samples. The present work will be relevant to understand the behaviour of PtNPs in body fluids and to take appropriate actions in future (pre)clinical trials.

Keywords: single particle; inductively coupled plasma mass spectrometry; platinum nanoparticles; complex clinical matrices; human urine; human blood serum.

1. Introduction

Inorganic nanoparticles (NPs) are promising nanomaterials with an important impact in many fields: i.e food technology, automotive industry, pharmaceutics production or biomedicine. Among them, platinum nanoparticles (PtNPs) have recently received considerable attention for biomedical applications due to their unique structural, optical and catalytic properties, which make them a promising candidate as antibacterial, antifungal, antioxidant, anticancer, anti-inflammatory, biosensor, drug delivery system, and for photothermal or radio therapy [1-3]. However, despite these interesting properties and applications, there is a growing concern about their potential risk for human health and studies about the toxicity of PtNPs should be performed [4].

For this purpose, methods enabling an appropriate identification, characterization, and quantification of these NPs in complex biological matrices are required. Up to now, microscopic (atomic force microscopy (AFM), transmission or scanning electron microscopy (TEM or SEM)) and spectroscopic (dynamic light scattering (DLS), multi-angle light scattering (MALS), X-Ray diffraction (XRD)) techniques have been widely applied [1]. These techniques provide mainly information about physical characteristics such as size, shape, agglomeration, surface area or zeta potential, but chemical composition and concentration are also demanded for a complete characterization. New analytical methods are required to properly provide this information. In this sense, inductively coupled plasma mass spectrometry in single particle mode (SP-ICP-MS) shows a great potential for concentration, not only based on mass but also in number of particles, of NPs suspensions in short times (about 1 minute) at very low concentrations. In addition, the core size of the NPs can be calculated if some additional information such as shape, composition, and density is available [5]. However, PtNPs behaviour has been scarcely studied by SP-ICP-MS until the moment and, as far as we know, it has only been reported in two model plants (*Lepidium sativum* and *Sinapis alba*) [6], in urban road dust leachates [7], in a Pt/silica nanocomposite decorated with ultra small PtNPs [8], and, recently, in fuel cells [9]. But, up to now, this approach has not been attempted the characterization and quantification of PtNPs in clinical matrices.

The study of PtNPs in these complex samples presents some limitations related to the extraction of the NPs preserving their native condition in the sample. Typical procedures based on acidic conditions are focused on the determination of total element content but could alter the NP properties [10]. Accordingly, alkaline or enzymatic digestions have been proposed in previous works to release metallic NPs from biological matrices guaranteeing the integrity of the analyte [11-17]. The most commonly used is the alkaline treatment with tetramethylammonium hydroxide (TMAH) at different concentrations. TMAH at 5% has been used to solubilize samples for the characterization of AuNPs in tissues from rats [11] and human umbilical vein endothelial cells [15] by several techniques including SP-ICP-MS. TMAH at 1% has also been extensively employed for the extraction of AgNPs or AuNPs (e.g., AgNPs in placenta tissues [12], AuNPs in cancer cell lines [13] or AuNPs and AgNPs in environmentally relevant biological tissues [16]). Alkaline solubilization has also been carried out combining TMAH with Triton-X 100, which is reported to have a stabilizing effect. Thus, Witzler et al. [14] diluted AuNP and AgNP suspensions in 2.5% TMAH and 0.1% Triton X-100 to achieve a correct metallic NP characterization. More recently, Bocca et al. (2020) [17] have also extracted AgNPs and AuNPs from human biofluids using TMAH (5%) and Triton X-100 (0.1%).

Therefore, this work is devoted to the development of a method based on SP-ICP-MS for the characterization of PtNPs, both in terms of size and mass- and particle-based concentration, in clinical samples such as human urine and blood serum with special attention to sample preparation.

2. Material and methods

2.1. Reagents

Ultrapure water (18.2 MΩ cm) from a Millipore Milli-Q Gradient A10 water purifier system was used for preparation of all solutions. All chemicals and reagents used in the present work were of analytical grade. Tetramethylammonium hydroxide (TMAH, 25% v/v in methanol) and Triton™ X-100 have been purchased from Aldrich Chemical Company (Milwaukee, USA). Platinum standard solution (1,025 μg mL^{-1} of Pt in 5% HCl)

has been acquired from Inorganic Ventures (Virginia, USA). Pt calibration solutions with concentrations of 500 - 5,000 ng L^{-1} have been freshly prepared daily by dilution of Pt standard in ultrapure water. Platinum nanospheres (PtNPs) suspended in aqueous solution and stabilized in 2 mM sodium citrate with nominal sizes of 50 and 70 nm and a particle number concentration in the suspension of $3.3x10^{13}$ particles L^{-1} and $1.2x10^{13}$ particles L^{-1}, respectively, have been acquired from nanoComposix, Inc. (San Diego, USA). They have been characterized in our laboratory in terms of size by SEM and the presence of monodispersive spherical PtNPs have been confirmed (Figure S1). The found values (50±4 nm and 74±3 nm, *n*=150) agreed with the report of TEM analysis provided by the supplier (nominal diameters of 51±7 nm and 72±4 nm). PtNP commercial suspensions were also characterized in terms of concentration, since the mass-concentration was determined experimentally by ICP-MS following a digestion protocol previously described [18]. Mass concentrations of 54±1 x 10^6 ng L^{-1} and 52±1 x 10^6 ng L^{-1} for 50 and 70 nm PtNP solutions, respectively, were found. These concentrations were consistent with those provided by the supplier (51 x 10^6 ng L^{-1} and 52 x 10^6 ng L^{-1}). In all experiments, PtNP solutions were stirred in a vortex mixer at 1,200 rpm during 1 min before dilution to prevent aggregation.

2.2. Apparatus and instruments

SP-ICP-MS and total concentration analyses have been performed with a Quadrupole ICP-MS Thermo X-Series II (Thermo Electron Corporation, Bremen, Germany) equipped with a Meinhard nebulizer. The raw ICP-MS data of the transient isotope Pt signal have been further processed using the XSeries PlasmaLab software. ICP-MS has been used in the time resolved analysis (TRA) mode and in normal resolution (millisecond integration time). The optimum SP-ICP-MS instrumental settings are summarized in Table S1. The sample flow rate was calculated daily by measuring the mass of water taken up by the peristaltic pump for 2 min. This measurement was performed by duplicate.

SEM images of 50 and 70 nm PtNPs were obtained from a Zeiss GeminiSEM 500 instrument (Oberkochen, Germany) with a maximum acceleration voltage of 30 kV, after deposition of these nano-sized solutions on silicon wafers.

An advanced ZX3 vortex mixer (Velp Scientifica, Italy) and an ultrasonic bath (Elmasonic S 30 H, Elma Schmidbauer GmbH, Germany) have been used.

2.3. Data treatment procedure

Raw signal data were processed manually using a dedicated Microsoft Excel® spreadsheet based on that obtained from the RIKILT website (https://www.wur.nl/en/show/Single-Particle-Calculation-tool.htm). Particle size and number- and mass-based concentrations were obtained assuming that NPs are spherical and solid and using the density of Pt and mass fraction of PtNPs. The data of a particle solution (50 nm PtNPs) in each series of measurements is needed to determine the transport efficiency (0.020±0.001 (2%)), and for the calculations of particle size and concentrations [19-21]. The response factor of the ionic analyte standard must also be known and was calculated using different standards (from 500 to 5,000 ng L^{-1}) of ionic Pt.

2.4. Samples

Human urine samples (*n*=10, 5 males and 5 females) were collected from healthy volunteers (staff at the Faculty of Environmental Sciences and Biochemistry, University of Castilla-La Mancha, Spain) using pre-cleaned polypropylene vials (Scharlab, Barcelona (Spain)). Urine samples were stored at 4°C during 24 h. Human blood serum samples (*n*=10) were provided by Hospital Virgen de la Salud (Toledo, Spain) and no personal information is given due to requirements for the personal data protection. Blood samples were collected with Vacuette® tubes (Sarstedt, Nümbrecht, Germany), centrifuged at 4,000 *g* for 10 min to obtain the human blood serum and stored at 4°C.

2.5. Sample preparation procedure for SP-ICP-MS analysis

Direct measurements of human urine and blood serum demonstrated that PtNPs were not initially occurring in the studied samples at detectable levels (Figure S2), where signals for spiked and unspiked urine samples are compared. Thus, both biofluids were spiked with 50 and 70 nm PtNPs at concentrations around 100 µg L^{-1}. TMAH at different concentrations, between 1 and 5%, and/or Triton™ X-100 at 0.1% were also added before incubation in order to evaluate the most suitable reagent for PtNP extraction and stabilization. After addition of these reagents, all samples were ultrasonicated for 1 min to obtain homogeneously disperse PtNPs. To check the stability in the NP sizes and concentrations after 24 h of exposure, these mixtures were analysed freshly prepared at the beginning of experiments (time 0 h) and after 24 h of incubation at 4ºC. Before the analysis by SP-ICP-MS, solutions were diluted to achieve PtNP concentrations around 300 ng L^{-1} in a final volume of 10 mL and ultrasonicated during 1 min. Human urine and blood serum without addition of PtNPs or reagents were used as control. Each analysis was conducted in 3 replicates.

3. Results and Discussion

3.1. Optimization of the SP-ICP-MS measurements

Several parameters, especially dwell and acquisition times, are critical to achieve a complete characterization of PtNPs in terms of particle size and concentration (number- and mass-based) by the SP-ICP-MS technique. These parameters were carefully studied in a previous work devoted to AuNPs in cell culture medium which was conducted with the same ICP-MS instrument [22]. In that study, different dwell (1-10 ms) and acquisition times (30-120 s) were assessed, and the optimum values were finally 5 ms and 60 s, respectively. Based on these data, operational conditions for the analysis of PtNPs were evaluated using dwell and acquisition times in the range of 3-5 ms and 60-75 s, respectively (Tables S2 and S3). Best results were obtained using 5 ms, which is consistent not only with our previous findings [22] but also with other works where AuNPs and AgNPs [13, 17] were characterised in biological matrices or PtNPs in environmental samples [7]. Regarding the acquisition time, comparable values were mostly reported for PtNPs sizing and counting when using 60 s and 75 s. Thus, in order

to minimize the time of analysis, 60 s were selected for further SP-ICP-MS measurements. Working under these optimum conditions (Table S1), measured particle sizes are comparable to those obtained by SEM and a good agreement with the nominal particle concentration (both number-based and mass-based) were also found with recoveries in the range of 92-101% (Table 1).

3.2. Analytical performance of the SP-ICP-MS method

A full analytical performance evaluation of the SP-ICP-MS approach has been carried out. The investigated parameters include the linear concentration range, the limit of detection for particle size (LOD_{size}) and particle number concentration (LOD_{NP}), and the precision in the determination of particle size and concentration (number and mass-based). Linear concentration range has been studied for 50 nm PtNPs between 50 to 500 ng L^{-1} with good linearity in this range (R^2=0.9904). The determination of limit of detection has been performed considering that the smallest NP detected is dependent on the sensitivity of the ICP-MS and the ability to differentitate NP signals from the background. Using the equations proposed by Laborda et al. in 2013 [23], the LOD_{size} was 21.63 nm and LOD_{NP} was $1.9x10^5$ particles L^{-1}. The precision in the determination of particle size, and concentration, expressed as the relative standard deviation (%RSD), have been calculated by replicate analysis (*n*=10) of a 50 nm PtNP solution in ultrapure water. Values of 2% for particle size and <1% for number- and mass-based concentration were found.

3.3. Study of PtNPs in complex clinical matrices by SP-ICP-MS

The developed SP-ICP-MS method was applied for the study of PtNPs in human urine and blood serum. Preliminary experiments were conducted by direct spiking PtNPs in these biofluids without any additional sample treatment. Changes in PtNP size were not observed. Stability on PtNP diameters was also found in both matrices after 24 h of exposure. However, problems related to reproducibility and stability on number- and mass-based concentrations after 24 h of incubation were reported. These drawbacks

revealed that it would be necessary to perform a convenient pretreatment of the sample to assure an adequate extraction of PtNPs before the SP-ICP-MS analysis, which is consistent with previous studies of metallic NPs in other complex biological matrices [11-14].

3.3.1. Evaluation of reagents for sample preparation

The reagent most commonly used for extraction of different metallic NPs in biological samples was TMAH [11-13, 15, 16], combined with Triton X-100 in some cases [14, 17], as previously described in the introduction. Accordingly, we have evaluated the effect of these reagents, both individually and combined. Initially, the tested concentrations were 0.1% for Triton X-100 and 5% for TMAH.

Regarding to particle size, measured values were in all cases in accordance with the data determined by SEM for both PtNP sizes, matrices, and incubation times. However, the recoveries in terms of particle mass and number concentration were considerably influenced by the reagent used for sample pretreatment. In the case of 50 nm PtNPs, quantitatives recoveries close to 100 % were achieved for particle mass concentrations when only TMAH is used (Figure 1). Intermediate values (60-80%) were obtained with Triton X-100 whereas with the use of a mixture of TMAH and Triton X-100 recoveries lower than 30% were reported. Similar behaviours were observed in both clinical samples. Comparable results were found for 70 nm PtNPs (Figure S3). These trends were also confirmed for recoveries in terms of particle number concentration so TMAH was chosen as the most adequate extractant for both sizes and matrices.

Then, the concentration of TMAH (1-5 %) was studied. All tested concentrations were suitable to achieve an adequate characterization of particle size. In the case of particle mass concentration (Table 2), the most adequate concentration was 1% with quantitative recoveries for both sizes, clinical samples, and times of incubation. These values are also consistent with those found in terms of particle number concentration. Therefore, the combination of TMAH (1%) addition to the sample, without any additional treatment, and the acquisition conditions optimized for SP-ICP-MS (Table S1)

are appropriate for the simultaneous sizing and counting of PtNPs in these complex clinical samples.

3.3.2. Monitoring of PtNPs in urine samples

In order to have a closer insight into significant factors which could affect the monitoring of PtNPs in humans, urine samples where information about gender was available, were specifically studied. These samples were analysed by SP-ICP-MS before and after addition of PtNPs using the proposed analytical strategy. Significance of statistical tests was established at $p < 0.05$.

The sizes determined by SP-ICP-MS were within their uncertainties in the range of the SEM values (Figure 2). We can observe a good agreement between the measured values for both techniques in samples from both female and male individuals. Moreover, measured particles sizes did not present statistically significant differences between genders for PtNPs with nominal sizes of 50 nm (p=0.935) or 70 nm (p=0.124) at initial conditions (time 0 h). This behaviour was also observed after 24 h of incubation (p=0.303 and 0.606 for 50 and 70 nm PtNPs, respectively), so stability on PtNP size after 24 h of exposure was proved.

Regarding the PtNP quantitation, recoveries from individuals of both genders are shown in Figures S4 (50 nm) and S5 (70 nm). Significant differences in particle number- and mass-based concentrations were not reported between genders (p values > 0.05 for both sizes and incubations times). Therefore, the gender appears not to have significant influence on PtNP characterization in biofluids by SP-ICP-MS, even though further experiments using a higher number of samples would be required to confirm these findings.

In addition, the stability of PtNP characteristics over time was also evaluated. Size distributions for PtNPs did not change remarkably along the 24 h exposure period (Figure S6). Statistically significant differences between results at 0 and 24 h were found neither in the determination of sizes and mass-based concentrations of 50 nm PtNPs nor in sizes and concentrations (mass- and number-based) of 70 nm PtNPs (p values > 0.05

in all cases) for male or female individuals. Statistically significant differences over time were, however, reported for particle number concentrations of 50 nm PtNPs but only if samples from female individuals are considered separately (p=0.006 and p=0.213 for females and males, respectively). Therefore, no degradation of original PtNP characteristics (size and concentrations) after 24 h of exposure were observed which suggests that typical processes such as NP sedimentation, aggregation or dissolution would not be relevant in these experiments. In addition, our results reveal that not only the complexity of the matrix but also some biological factors should be considered when monitoring the fate of metallic NPs in clinical samples.

3.3.3. Potential and limits of SP-ICP-MS for characterization of PtNPs in clinical samples

The knowledge about the fate and behaviour of PtNPs in biological systems is still limited and new analytical approaches are needed for the risk assessment of the potential impact of metallic NPs. In this context, SP-ICP-MS becomes a powerful analytical tool for its characterization at clinically relevant concentrations. It is remarkable that it is both a counting and sizing technique, which enables the simultaneous quantification of PtNP concentrations in number of particles and mass as well as the calculation of NP size after the application of some mathematical transformations. In this sense, particle number concentration is an especially valuable information that it is not usually provided by other sizing techniques (microscopic or spectroscopic ones). However, the NP diameter calculations in SP-ICP-MS are based on several assumptions related to element-specific density and particle geometry, which could limit the application of this approach [24].

In complex biological samples, the interactions between the biomolecules, such as proteins, and the surface of NPs can result in the potential formation of protein corona and changes in the observed size. Classical (DLS or UV-vis spectroscopy) or coupled techniques (HPLC-ICP-MS or AF4-ICP-MS) provide relevant information about the hydrodynamic volume of the new nanobioentities but it is also important to know about the metallic core size. This last information is achieved by SP-ICP-MS. The

combination of different approaches and tools would be required to achieve a complete comprehension of PtNP pathways in complex samples.

4. Conclusions

An analytical methodology has been proposed for the simultaneous sizing and counting of PtNPs in complex clinical matrices using SP-ICP-MS. A minimal sample preparation procedure based on the addition of TMAH (1%), without any heating, is required to obtain a correct PtNP characterization in terms of particle size and concentration (number- and mass-based). The applicability of the SP-ICP-MS approach for the analysis of human urine and blood serum samples spiked with PtNPs has been demonstrated. This work suggests the great potential of SP-ICP-MS to detect, identify and determine PtNPs in body fluids with a minimal sample pretreatment at low concentrations which it could be further applicable in (pre)clinical trials to understand the behaviour of these NPs in biological fluids.

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CRediT authorship contribution statement

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Conceptualization, Supervision, Visualization, Formal analysis, Methodology, Writing - review & editing

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Table 1. PtNPs characterization in terms of particle size and concentration, number and mass-based, by SP-ICP-MS (n=3) at optimal conditions (Table S1).

	Particle size (nm)		**Particle number concentration ($x10^6$ particles L^{-1})**			**Particle mass concentration (ng L^{-1})**		
Nominal size (nm)	Known[a]	Measured	Known[b]	Measured	Recovery (%)	Known[c]	Measured	Recovery (%)
50	50 ± 4	51.4 ± 0.4	183	168 ± 3	92 ± 2	317 ± 8	304 ± 6	96 ± 2
70	74 ± 3	73.3 ± 0.1	69	67 ± 3	97 ± 4	299 ± 7	302 ± 7	101 ± 2

[a]: Determined by SEM; [b]: Given by manufacturer; [c]: Determined experimentally by ICP-MS after extraction based on ref. [18].

Table 2. Effect of TMAH concentrations in the recoveries (%) in terms of particle mass concentration for 50 and 70 nm PtNPs (317 ± 8 and 299 ± 7 ng L^{-1}, respectively) at 0 h and 24 h in human urine and blood serum (n=3).

TMAH concentration		**1 %**		**2.5 %**		**5 %**	
	Nominal value (nm)	**0 h**	**24 h**	**0 h**	**24 h**	**0 h**	**24 h**
Human urine	**50**	95.7 ± 0.6	99.3 ± 0.4	77 ± 1	72.9 ± 0.6	94 ± 1	100 ± 6
	70	101 ± 2	103 ± 2	105 ± 1	94 ± 1	87.3 ± 0.1	99 ± 2
Blood serum	**50**	93.3 ± 0.3	101 ± 2	76 ± 2	78 ± 9	82.4 ± 0.3	107 ± 1
	70	99 ± 4	100 ± 2	68 ± 4	97 ± 4	92 ± 2	86.9 ± 0.3

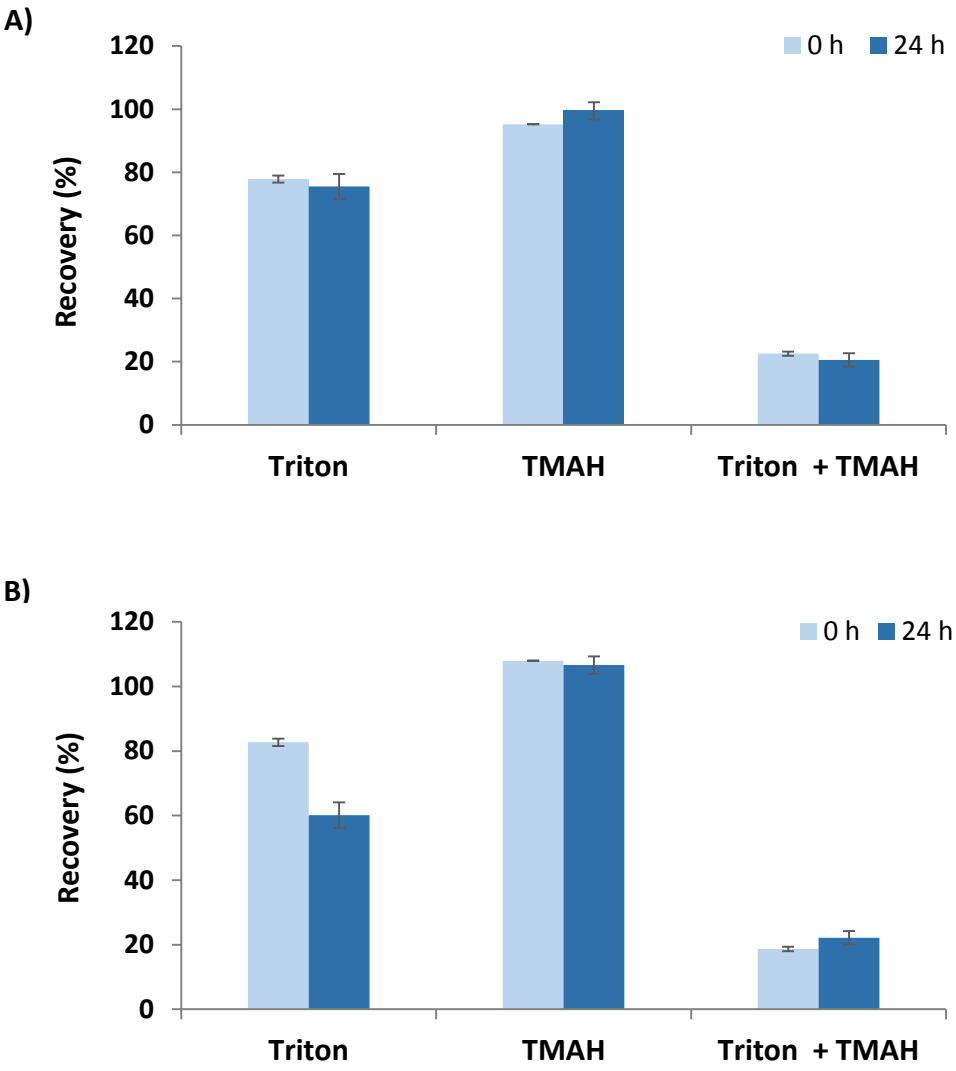

Figure 1. Effect of extractant (5% TMAH and/or 0.1% Triton X-100) on the recoveries (%) in terms of particle mass-based concentration for **50 nm PtNPs** (317 ± 8 ng L^{-1}) at 0 h (light) and 24 h (dark) incubation time in human urine (A) and serum (B).

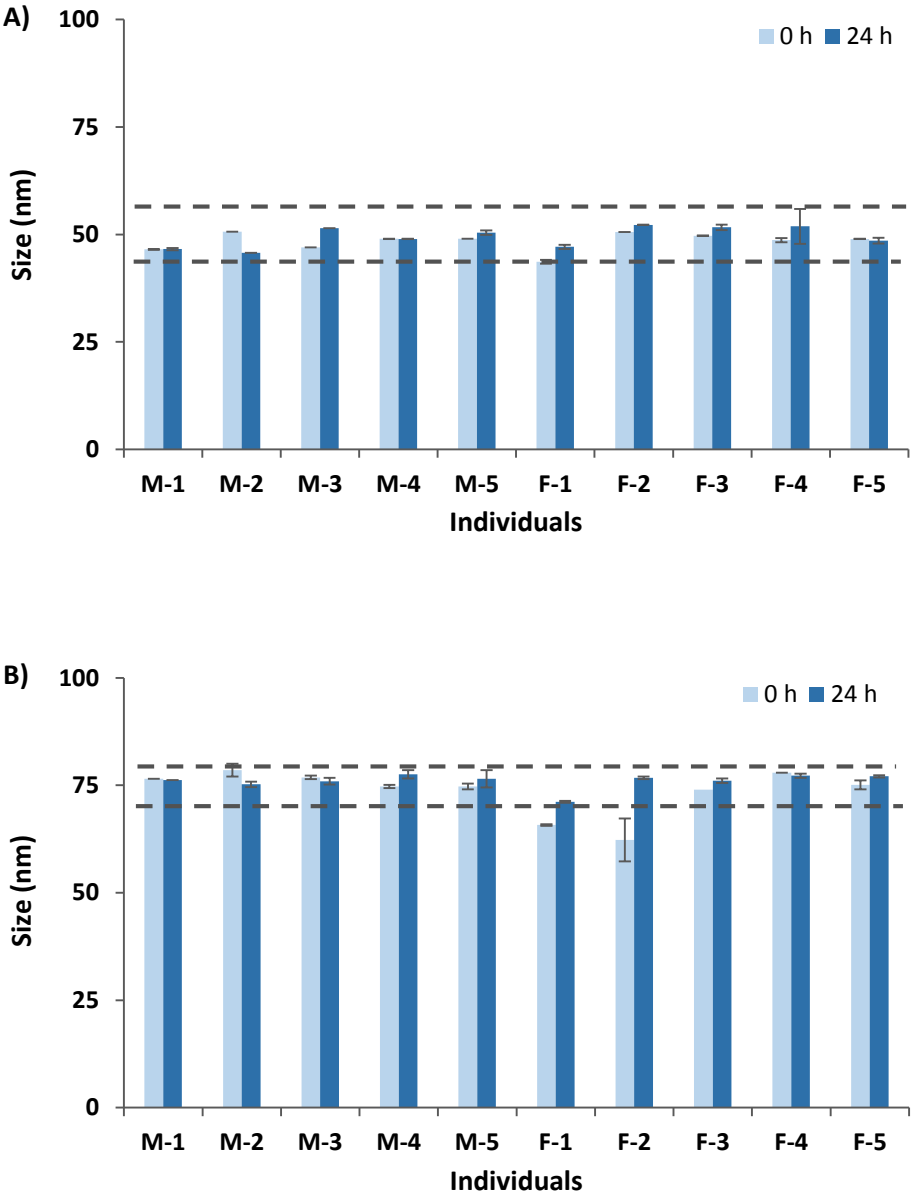

Figure 2. Particle size (nm) for 50 nm (A) and 70 nm (B) PtNPs at 0 h (light) and 24 h (dark) incubation time in human urine from individuals of both genders (M: Male; F: Female). The dashed lines represent values obtained by SEM for 50 nm (50 ± 4 nm) and 70 nm (74 ± 3 nm) PtNPs.