Application of SS-CS-HR-AAS Measurements for the Detection of Ag Nano-particles in Marine Invertebrates

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Abstract

The aquatic fate of NPs governs the ecotoxicity in the aquatic environment and marine organisms. The present study describes the development of fit for purpose analytical procedure for the determination of Ag NPs in different marine organisms by Solid Sampling Continuous Source High Resolution Atomic Absorption Spectrometry (SS-CS HR-AAS). The detection is based on the observation of the Ag absorption peak and its atomization delay t_{ad} , *i.e.*, the time at which the maximum absorbance is observed. Indeed, the presence of Ag NPs is easily detected through the analysis of t_{ad} as Ag ions and Ag NPs have different interactions with the sample matrix and different t_{ad} . The method was first developed using biota CRMs (DORM-3 and SRM 2976) spiked with Ag NPs standard solutions of different sizes (10, 20, 40 and 60 nm) or ionic Ag (Ag^+) at the same concentrations. The temperature program was optimized in order to achieve the maximum difference between the t_{ad} obtained for Ag^+ and the one obtained for Ag NPs (Δt_{ad}). Then, laboratory experiments were performed on mussels and marine sponges, two different marine invertebrates. The results showed that the developed methodology is suitable for the detection of Ag NPs for both groups of organisms, showing Δt_{ad} up to 3.1 s. The behavior can be slightly different from matrix to matrix but a clear separation between ionic Ag and Ag NPs is always visible. The developed analytical method is therefore a promising tool to assess the presence of Ag NPs in marine invertebrates.

1. Introduction

Nanomaterials are defined as materials of size between 1 and 100 nm (Handy et al. 2008). Several types of nanomaterials are manufactured and commercially available: carbon based (e.g., carbon nanotubes) or metal based (Ag, Cu but also metal oxides as TiO_2) for instance. In particular Ag nanoparticles (NPs) have found numerous applications due for instance to their promising antibacterial properties, e.g. as disinfectant of manufacturing areas including those in contact with water (Adamek et al. 2018). Other applications include antibacterial uses in water treatment, fabric softener, clothing, soft toys, wound dressing, kitchen utensils and appliances, among others (Shaw and Handy 2011). An important application of Ag NPs is represented by their use in food packaging and recent studies have shown that Ag is potentially transferred from the packaging to the food in the form of NPs, under different experimental conditions (Echegoyen and Nerín 2013; von Goetz et al. 2013; Jokar and Rahman 2014; Artiaga et al. 2015; Ntim et al. 2015).

The rapid development and application of nanoparticles in all aforementioned sectors, have resulted in increased input of these materials in the environment, including aquatic and marine ecosystems. Their widespread use has raised concern about their potential toxicity for different aquatic organisms. Recent findings suggest that the introduction of manufactured NPs into the environment can influence other contaminant and pathogen behavior towards living organisms, causing increasing concern (Lead and Wilkinson 2006). It has also been proved that exposure to Ag and other metallic nanoparticles can have negative effects on fish health, affecting growth and survivability (Handy et al. 2008; Shaw and Handy

2011). In this context, the need of fast and reliable methods for the screening and detection of Ag NPs in environmental matrices is becoming acute.

The most recent analytical development for the detection, quantification and characterization of metallic NPs is the inductively coupled plasma mass spectrometry operating in single particle mode (sp-ICPMS) sometimes supported by the use of field flow fractionation system (AF⁴-ICP-MS) to first separate the NPs from the remaining matrix before its analysis (Loeschner et al. 2013; Lee et al. 2014; Navratilova et al. 2015; Peters et al. 2015). This technique proved to be very promising for the analysis of various metallic NPs. Gagné et al. (2012) reported the first application of atomic absorption spectrometry to discriminate between Ag NPs and ionic form of Ag in acidified homogenates of rainbow trout's liver exposed to these contaminants. The main limitations of these techniques are the time consuming sample preparation and the optimization of multi-step procedures including partial digestion of the complex matrix (usually consumer products, food, environmental samples) and selective separation of NPs prior to element-specific instrumental detection (Wigginton et al. 2007; Tiede et al. 2009; Blasco and Picó 2011; Silva et al. 2011). Additionally, transformation of Ag NPs can occur during the multi-step sample preparation, causing biased results (Silva et al. 2011).

Recently, High Resolution Continuous Source Atomic Absorption Spectrometry (HR-CS-AAS) has proven to be a powerful analytical tool in the study of the presence of metallic nanoparticles. Recent studies have shown that this technique is capable to monitor AuNPs and to distinguish between different sizes (Resano et al. 2010, 2016; Leopold et al. 2017). Other studies have been focused on the use of detection and characterization of Ag NPs by graphite furnace AAS often with the use of HR-CS-AAS (Resano et al. 2013). Many studies involve the use of solid sampling (SS) devices, allowing the rapid detection of Ag NPs directly on solid state samples of different origins. Studies involving Ag detection using SS-HR-CS-AAS were recently published for various matrices such as different food samples (Feichtmeier et al. 2016), commercially available spiked dried parsley (Feichtmeier and Leopold 2014), small aquatic invertebrate exposed to Ag NPs (Resano et al. 2013). The use of direct methods has drastically reduced the analysis time, as no sample preparation is required, thus it and they now represent suitable alternatives for the fast screening of Ag NPs in environmental monitoring. Most of these works based the detection of Ag NPs and/or the distinction from ionic form of Ag on the observation of the absorbance peak and specifically of the time at which the maximum absorbance is observed. This strategy was first proposed by Feichtmeier et al. (2014) for the distinction of Ag NPs from Ag⁺, and then further applied to different matrices.

Only a very limited number of the studies contain experiments involving Ag⁺ and Ag NPs exposure in living organisms, while many are focused on the analysis of dried samples subsequently mixed with different Ag solutions. To the best of our knowledge no literature is available on the accumulation and detection of Ag NPs in marine sponges. In this context the aim of the present work was 1) to develop a method for the detection of AgNPs in marine invertebrates, by SS-HR-CS-AAS and 2) to apply the developed methodology to marine mussels and sponges, preliminary exposed to the ionic form of Ag and Ag NPs with different size. The proposed method is suitable for the distinction of Ag NPs and Ag⁺ in

marine organisms exposed to these two different Ag forms. The method proved to be very appropriate for fast screening of the Ag NPs in the environmental and seafood monitoring studies.

2. Materials And Methods

2.1. Chemicals

MilliQ water with resistivity $\geq 18 \text{ M}\Omega$ was used throughout the entire experimental work to dilute standard solutions and to rinse the injection system. The Ag nanoparticles dispersions (sizes 10, 20, 40 and 60 nm) in aqueous solution at concentration of 20 mg L^{-1} as well as the Ag ionic solution at 1000 mg L^{-1} were obtained from Sigma Aldrich. For optimization of temperature programs, the solutions were diluted to a final concentration of about $3 \mu\text{g L}^{-1}$ using MilliQ water. The Certified Reference Materials (CRM) SRM 2976 (mussels' tissue, purchased by NIST, USA) and DORM-3 (dogfish muscle, from NRCC, Canada) were used during the experiments and optimization of the temperature program.

2.2. Instrumentation

Ag detection was carried out using a High-Resolution Continuum Source Atomic Absorption Spectrometer (ContrAA 700, Analytic Jena, Germany). This instrument is equipped with graphite furnace atomizer, a Xenon short-arc lamp (GLE, Berlin, Germany) operating in "hotspot" mode as radiation source, a high-resolution double echelle monochromator (DEMON) and a linear CCD array detector with 588 pixels, 200 of which are used for monitoring the analytical signal and performing background corrections. The HR CS AAS instrument operates with a transversely heated graphite tube atomizer and an automated solid sampling accessory (SSA 600). The solid sampling device incorporates a microbalance with a declared precision of 0.001 mg. The samples and standards were introduced using solid sampling graphite platforms. Argon with a purity of 99.999% (Airliquide, France) was used as purge and protective gas. The most sensitive Ag line at 328.07 nm was used for atomic absorption of silver.

2.3. Spike experiments

Two spike experiments on living organisms were carried out: one on mussels and one on sponge samples. Mussels (*Mytilus edulis*) were manually collected in the Arcachon bay (Atlantic southwest coast of France). Sponges (*Acanthella acuta*) were collected by scuba diving in the Bay of Villefranche sur Mer (French Mediterranean coast). All the organisms were transported to the lab and transferred alive in aquarium where they were left 15 days for acclimatation under flowing seawater. Both sponges and mussels were fed twice per day during the acclimatation and the spike experiments.

The experiments for each of the investigated species - mussels and sponges, involved 4 different aquaria: aquarium with a control samples, aquarium spiked with Ag^+ solution, and aquaria spiked with 10 nm and 60 nm Ag NPs solutions respectively. The 10 and 60 nm sizes were selected because they showed the biggest difference (one from each other) during preliminary experiments involving liquid standards. Between 3 and 6 specimens of mussel or sponges were placed in closed circuit aquaria and included for

each experiment. The spike concentration for each aquarium was set as $5 \mu\text{g L}^{-1}$ and the exposure experiment was carried out as follows: each aquarium was spiked with the mentioned Ag^+ or AgNPs concentration for 12 hours, then the organisms were fed with phytoplankton, the seawater was replaced with a clean portion and the $5 \mu\text{g L}^{-1}$ spike was renewed. This cycle was repeated twice per day and the total time of exposure was set at 96 hours.

Then, the organisms were carefully rinsed with clean seawater and euthanized by deep freezing (-18°C). Subsequently samples were freeze-dried, manually ground in a mortar, and analyzed by HR-CS-AAS. This experimental set up guaranteed the exposure of sponges and mussels to a constant concentration of the respective contaminant, preventing possible decrease in bioavailability. Additionally, we prevent possible Ag depletion as a consequence of accumulation by phytoplankton, used to feed the sponges.

2.4. Interpretation of spectra

Following the concept previously proposed by Feichtmeier and Leopold (Feichtmeier and Leopold 2014) and Feichtmeier et al. (Feichtmeier et al. 2016), the main parameter considered in the interpretation of our results was the so called “atomization delay” (t_{ad}), i.e. the time at which the maximum absorbance is registered for each peak. In theory, Ag^+ and Ag NPs will show measurably different t_{ad} , which will make possible the differentiation between these two silver forms. The difference was defined as:

$$\Delta t_{ad} = t_{ad}(\text{Ag}^+) - t_{ad}(\text{AgNP})$$

(1)

where $t_{ad}(\text{Ag}^+)$ and $t_{ad}(\text{AgNP})$ are the atomization delays (in s) measured for samples containing Ag^+ and Ag NPs, respectively. The differences were expected to be visible in liquid standards but also in spiked samples, as the interaction between the analytes and the sample matrix should influence the atomization delay.

3. Results And Discussion

First, the temperature program was developed and optimized using liquid standard solution containing Ag^+ and Ag NPs of different sizes. Then, some biota CRMs in which the Ag content was not detectable using the developed program, were spiked with Ag^+ and Ag NPs in order to observe a possible matrix effect. This was a necessary step as no CRM certified for Ag NPs is available on the market. The third and final step of the experimental setting involved the spiking of living organisms (mussels and sponges) with Ag NPs solutions (10 and 60 nm), Ag^+ and the respective analyses.

3.1. Optimization of temperature program

As previously detailed, the principle followed in this study is that Ag NPs can be distinguished by Ag^+ when observing the different t_{ad} of the different Ag forms. Therefore, the main objective during the

optimization of temperature programs, was to maximize the observed difference in t_{ad} , Δt_{ad} . The optimized temperature programs used in this study are presented in Table 1.

Table 1
Optimized temperature program used for the detection of ionic and nanoparticle Ag. A measurement delay of 5s was applied, therefore the reading time was set as 15.6s.

Step	Temp (°C)	Ramp (°C/s)	Hold (s)	Step time (s)
Drying 1	70	10	15	18.7
Drying 2	110	10	20	24.0
Drying 3	150	10	20	24.0
Pyrolysis	300	300	15	15.5
Gas adaption	300	0	5	5.0
Atomize	1000	1200	20	20.6
Clean	2450	500	4	6.9

As expected, the use of a matrix modifier had an influence on the results. Specifically, through the thermal stabilization of the analyte, even a permanent modifier such as Ir, often used in GF AAS, had the negative result of reducing the Δt_{ad} . Therefore, the experiments were constantly carried out without the use of matrix modifiers and using graphite platforms not pre-treated with Ir coating.

Another factor which appeared to improve the separation between the two t_{ad} was the atomization temperature. Specifically, the results showed that a better separation between the two peaks was obtained when decreasing the atomization temperature. Fig. 1 and 2 show the peaks obtained when analyzing the Ag^+ and Ag NPs solutions using 2000°C and 1000°C atomization temperatures, respectively.

This trend was already observed by (Feichtmeier et al. 2016) who already studied the positive effect of reducing atomization temperature on the Δt_{ad} values. In the study, the Δt_{ad} determined for a series of different matrices ranged from -0.77 to 1.39 s, while in the present study it was possible to obtain values up to -2.9 s. When working at 2000°C atomization temperature, change in pyrolysis temperature did not significantly improve the differentiation between peaks. Therefore, an optimum pyrolysis temperature of 300°C was selected. This observation was also in agreement with a previous study showing that pyrolysis temperature above 400°C resulted in insignificant differences and generally lower Δt_{ad} for different biological matrices (Feichtmeier et al. 2016). Figure 2 shows that the Δt_{ad} values were different

for different Ag NPs size. Specifically, the lowest Δt_{ad} was obtained when analyzing solutions of Ag NPs of 10 nm while the highest Δt_{ad} was obtained when measuring Ag NPs of 60nm, even if a clear trend was not observed.

To check the possible interactions of Ag^+ , Ag NPs with the biological matrix, some experiments were carried out on solid biota spiked with 20 μL of the solutions. The selected biota CRMs had Ag content below the detection limit of the technique, meaning that the solid itself, when analyzed by SS-HR-CS-AAS, did not show any detectable Ag peak. These materials were therefore suitable to be spiked, and to mimic the behavior of the biological matrix during the analysis. The spike with either Ag^+ or Ag NP solution was performed just before the analysis directly on the graphite platform containing a certain amount of solid sample. The results obtained for these two materials are shown in Fig. 3a and 3b

As expected, the behavior of the peak varied with respect to the analyzed matrix, meaning that the obtained Δt_{ad} for the SRM2976 were different from those measured for DORM-2. The behavior of Ag was much more similar to the liquid standard when placed with SRM2976. The only exception was the 10 nm mix which, combined with SRM2976, gave a Δt_{ad} much lower than the value obtained for the liquid standard alone. On the other hand, the material DORM-3 mixed with solutions containing Ag NPs generally led to much lower Δt_{ad} when compared with liquid standards. It was already shown that the t_{ad} can greatly vary depending on the studied matrix. Specifically, the increasing protein content led to increased atomization delays (Feichtmeier et al. 2016) which, in turn, led to more negative Δt_{ad} . Tissues of animal origin with high protein content (e.g., mussels) retain more Ag, causing higher atomization delays. In the case of DORM-3, the Δt_{ad} obtained for Ag NPs were in all cases positive, and the atomization delays were generally lower than for the mussel spiked with the same solution. One exception was the solid spiked with Ag^+ , which presented much higher t_{ad} for DORM than for SRM2976. These data confirm that the interaction with different matrices, even belonging to the same family (i.e., marine organisms) can affect the atomization delay of Ag. Even considering these differences, the method developed in the present study was always capable to distinguish between Ag^+ and Ag NPs spiked samples, proving its robustness and flexibility for a rapid screening of these analytes. On the other hand, this type of experiment cannot guarantee that the homogeneity of the analyzed solid-liquid mixture is good enough for an accurate evaluation of the results. For this reason, spike experiments were then carried out on living organisms, during which the organism has the time to physically incorporate the different Ag forms.

3.2. Results for mussels

A spike experiment was first carried out on mussels. At least 4 specimens of mussels were subjected to one of the following conditions: control (absence of any spike), Ag^+ , Ag 10 nm and Ag 60 nm spikes. The silver solutions were added to the aquaria in order to have a final exposure concentration of 5 $\mu g L^{-1}$. The analyses were then performed on the freeze-dried solid mussel samples duly grinded in a fine powder.

The control mussels did not show any detectable Ag peak when analyzed, meaning that the Ag content naturally present in the collected organisms was not detectable using the developed method (Fig. 1S, supplementary data). Peaks obtained for mussels spiked with Ag⁺ and Ag NPs are shown in Fig. 4.

The repeatability of the obtained t_{ad} was determined by measuring at least three times the same mussel specimen and the calculated RSD was between 0.9 and 1.5%, regardless of the considered spiked sample. The reproducibility was also evaluated by measuring several specimens exposed to the same condition; this was also a way to evaluate the so-called intra-specie variability. The latter was again calculated as RSD and it was found to be less than 2% for each studied condition.

As a first conclusion, the application of the developed method is valid when a reference measurement of an individual specimen spiked with Ag⁺ is available for comparison. This first Ag⁺ spike represents some sort of one-point calibration for the provided qualitative method, which is in fact based on the measurement of a relative parameter, the Δt_{ad} . The most important conclusion that can be drawn from the results obtained for mussels is that it is possible to easily distinguish between specimens spiked with Ag⁺ and Ag NPs. Specifically, in these samples the t_{ad} found for mussels spiked with Ag NPs are consistently higher than those found for Ag⁺, resulting in negative values of Δt_{ad} . This result is consistent with previous findings, suggesting that Ag was efficiently taken up in the form of NPs and did not undergo any transformation within the mussel tissue (Feichtmeier et al. 2016). The difference of behavior between Ag 10 nm and 60 nm was not so evident as in the experiment carried out with liquid standard. This might be linked to the specific interaction with the biological matrix during the time at which mussels were exposed to Ag NPs. As expected, the behavior observed for the mussel CRM (SRM 2976) spiked with Ag⁺ and different sizes of Ag NPs, was rather different. This is easily explained considering the possible low homogeneity and low interaction efficiency observed for the spiked solid powder, in comparison with the living mussels. The Δt_{ad} calculated for the spike CRM are closer to those obtained for liquid standards than to those obtained for spiked mussel samples. Even though the Δt_{ad} obtained for spiked mussels and for SRM 2976 were consistently negative, meaning that Ag NPs always showed higher t_{ad} than Ag⁺, the numerical values obtained for the CRMs were generally different. Also, the difference between different Ag NPs sizes was more evident in the results obtained for the CRMs than in the spiked mussels. Not only the low homogeneity but also the insufficient mixing contact between the Ag solution and the solid sample could be responsible for this difference. Therefore, the importance of real exposure experiment involving living organisms is particularly evident in this context. In fact, the exposure experiment likely gives more accurate results, as it mimics a probable environmental exposure during which the contaminant has the time to be incorporated and possibly bio-transformed by the organism itself.

3.3. Results for Sponges

Specimens of the Mediterranean sponge *Acanthella acuta* were selected for another spike experiment analogous to the one carried with mussels. The major difference encountered when analyzing sponges was the already high Ag content in control samples. As a consequence, in order to provide reasonable

and comparable graphs, the samples were analyzed as slurries, in order to “dilute” the Ag content and be able to provide comparable peaks. The slurries were prepared by adding a volume of about 10 mL of MilliQ water to the freeze-dried sponge samples. The sample mass and the added water were adjusted in order to obtain comparable absorbance during the experiment. This adjustment was necessary because the total integrated absorbance might influence the peak shape, which in turn could influence the t_{ad} . To avoid this possible effect, the absorbances were kept close to each other, thus the effect of the peak shape minimized in the evaluation of Δt_{ad} .

An aliquot of 20 μ L of slurry was manually injected and analyzed. The repeatability was determined by measuring at least three times the same slurry solutions: the calculated RSD was generally below 1%, regardless of the considered spiked sample. The reproducibility or intra-specie variability was found to be not more than 6% for each studied condition. A graph summarizing the obtained results is presented in Figure 5.

Interestingly, the t_{ad} determined for control samples was very close to the one measured for Ag^+ , meaning that the high Ag peak observed in control samples is likely ascribable to Ag in its ionic form rather than NPs. Nevertheless, in all cases it was possible to clearly distinguish the peaks of sponges spiked with either Ag^+ or Ag NPs. This result proves the robustness of the developed method, which was capable to detect a certain Δt_{ad} for different biological matrices. The differences were significant in all investigated matrices and therefore indicative of the presence/absence of Ag NPs. The main difference in the case of the sponge *A. acuta* is that unlike the mussels, even control samples show a very high silver content, not quantified as it was not the object of the present study, but definitely detectable with the optimized method. The sponge *A. acuta* had previously been object of studies involving its great capability to specifically accumulate Ag (Genta-Jouve et al. 2012; Orani et al. 2018). The accumulation kinetics of Ag in this sponge was previously studied with the use of a radiotracer and showed an exponential model while the depuration process was found to be extremely long with respect to other elements and/or species. The affinity of this sponge for Ag is probably associated to its metabolism. It was hypothesized that the secondary metabolites composed of isonitrile terpenoids produced by this organism can have high affinity for Ag (Genta-Jouve et al. 2012). The present study confirmed this special affinity of the sponge for silver ions and give additional information, as it was proved that also Ag in the form of NPs is highly accumulated in these organisms. This aspect certainly deserves further studies to better understand the Ag accumulation cycle and the potential of sponges as bio-remediation organisms also for the pollution related to nanoparticles.

4. Conclusions

In conclusion the present work provides a fast and fit-for-purpose method for the distinction and detection of Ag^+ and Ag NPs by SS-CS-HR-AAS in different marine biological matrices. The method was based on the observation of the t_{ad} (time at which the maximum absorbance is observed) and it was at first optimized using liquid standard. The decrease of atomization temperature had a decisive role in the

obtention of maximum difference between t_{ad} determined for Ag^+ and Ag NPs of different sizes. The developed method was further applied to solid CRMs (mussel and fish homogenate) spiked with Ag^+ and Ag NPs solutions and in all cases, it was possible to easily distinguish between peaks obtained for the two Ag forms. Additionally, spike experiments on living organisms (mussels and sponges) were carried out. The obtained results were very promising, showing that the method can be successfully applied to distinguish Ag^+ from Ag NPs for the study of different organisms. Particularly sponges, which had never been used for accumulation studies of Ag NPs, proved to be very promising biological tools for the bioremediation of marine areas contaminated with Ag NPs.

Declarations

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Author contribution

All authors contributed to the study conception and design. Material preparation and analysis were performed by AMO under the supervision of EV. The first draft of the manuscript was written by AMO and EV and OT reviewed and corrected it. All authors read and approved the final manuscript before submission.

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Competing interest

The authors have no relevant financial or non-financial interests to disclose.

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Figures

Figure 1

Peaks obtained for liquid standard solutions containing $3 \mu\text{g L}^{-1}$ of Ag^+ and Ag NPs obtained applying an atomization temperature of 2000°C . The differentiation of the peaks is practically impossible, with a Δt_{ad} of only 0.1s.

Figure 2

Peaks obtained for liquid standard solutions ($n=3$) containing $3 \mu\text{g L}^{-1}$ of Ag^+ and different sizes of Ag NPs, obtained applying the final optimized temperature program. The obtained Δt_{ad} were much higher than those determined when applying a higher atomization temperature, with values up to -2.9.s.

a)

b)

Figure 3

Peaks obtained for the CRM materials a) SRM2976 and b) DORM-3 spiked with 3 μg L⁻¹ of either Ag⁺ or Ag NPs of different sizes.

Figure 4

Peaks obtained for mussel specimens spiked with Ag⁺, Ag NPs 10nm and 60 nm.

Figure 5

Peaks obtained for sponge specimens spiked with Ag⁺, Ag NPs 10nm and 60 nm

Supplementary Files

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