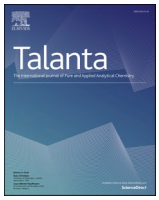
Talanta 247 (2022) 123556

Contents lists available at ScienceDirect

Talanta

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



Ultrasonication followed by enzymatic hydrolysis as a sample pre-treatment for the determination of Ag nanoparticles in edible seaweed by SP-ICP-MS

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ARTICLE INFO

Keywords:
Seaweed
Silver nanoparticles
Ultrasonication
Enzymatic hydrolysis
Single-particle-ICP-MS

ABSTRACT

Seaweed can bioaccumulate nanomaterials that would be transferred to the trophic chain. This work describes the optimization of a method for the separation of silver nanoparticles (AgNPs) from seaweed using an ultrasound-assisted enzymatic hydrolysis method and ulterior determination by single particle inductively coupled plasma mass spectrometry (SP-ICP-MS).

The following parameters affecting the isolation of AgNPs were optimized using a *Palmaria palmata* (red seaweed) sample previously exposed to AgNPs: type of sonication (bath vs. ultrasonic probe), ultrasound amplitude, sonication time, sonication mode (pulsed vs. continuous sonication), concentration of the enzymes mixture (Macerozyme R-10®), and enzymatic hydrolysis time. The stability of AgNPs during extraction was tested by transmission electron microscopy (TEM) and using a standard of 15 nm of polyvinylpyrrolidone (PVP)-coated AgNPs analyzed by SP-ICP-MS. The analytical performance was evaluated with good results. For total Ag determination, the limits of detection and quantification were 2.2 and 7.7 ng g^{-1}, respectively; and for AgNPs determination, the limits of detection in size and number were 14 nm and 4.34 × 10^7 part g^{-1}, respectively. Besides, the matrix effect, the repeatability and the analytical recovery were also studied. Finally, the method was applied to the analysis of several red (*Palmaria palmata*) and green (*Ulva* sp.) seaweed samples.

1. Introduction

Natural nanomaterials have been present on earth since the beginning of time, mainly due to volcanic eruptions and hydrothermal processes. However, since the beginning of the 21st century, due to the expansion of Nanotechnology [1] many nanomaterials [2] found in the environment are of anthropogenic origin, either accidental or manufactured (engineered nanomaterials, ENMs). Silver nanoparticles (AgNPs) have special antibacterial, optical, electrical and thermal properties [3] that imply many applications [4] including catalysis, medical, domestic and industrial product manufacturing, health care, biosensing, electronics [5], photonics [6] food industry (food packaging, food additive E−174, etc), and even in the agriculture sector [3,7]. The environmental exposure to nanoparticles can originate from their synthesis, manufacturing, distribution, the final use of the product, and end-of-life disposal [8]. AgNPs can change their properties, conformation, and chemical composition when they are present in the environment. Nanoparticles can be suspended, agglomerated or aggregated, dissolved, or even they can react with several species in the aquatic environment [8] depending on factors like pH, ionic strength, organic matter content, coating, and ligands (complexing substances) concentration.

The European population has increased the consumption of seaweed due to its high nutritional value. The presence of vitamin B-12, selenium, omega-3, dietary fibre, fatty acids, and iodine, together with changes in European lifestyles and a growing interest in a healthier, more sustainable, and environmentally friendly diet, have all contributed to this trend [9]. Seaweeds are essential marine primary producers, having a direct impact on the trophic chain as well as on the qualitative end state of many coastal environments [4]. Besides, seaweeds are commonly used for bioremediation of marine pollution [10–12] due to their capacity for biosorption and accumulation of heavy metals and trace

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https://doi.org/10.1016/j.talanta.2022.123556
Received 8 April 2022; Received in revised form 13 May 2022; Accepted 14 May 2022
Available online 18 May 2022

elements [9,13]. Although there is a European recommendation (European Commission, 2018) on the monitoring of iodine and metals such as Cd, Pb, As, and Hg in seaweed [14], there is no legislation about the presence of ENMs in seaweed.

The study of the presence and bioaccumulation of nanoparticles in complex biological samples, such as seaweed, demands the use of methodologies that can offer information on the element's existence in nanoparticulated and dissolved form, as well as the concentration and size of nanoparticles at environmentally real concentrations. Single-particle inductively coupled mass-spectrometry (SP-ICP-MS) is the most promising technique that allows the simultaneous determination of particle concentration, size and size distribution, as well as the detection of the dissolved element. SP-ICP-MS has been used in several kinds of biological and environmental matrices. Thus, several authors have analyzed NPs in animal tissues, e.g. Au NPs in rat spleen [15], Au and AgNPs in *Daphnia Magna and Lumbriculus variegatus* [16], and AgNPs in chicken [17]. Other authors have also studied the uptake of NPs in plant tissues, such as Dan et al. [18,19] for CeO_2 NPs uptake and accumulation in soybean, pumpkin, tomato, and cucumber, and Au NPs uptake by tomato plants, Jiménez-Lamana et al. [20] for Pt NPs accumulation in *Sinapsis alba* and *Lepidium sativum,* and Kińska et al. [21] for Pd NPs uptake by *Sinapsis alba*. Wojcieszek et al. [22] have also determined the CeO_2 NPs uptake by *Raphanus sativus* L, and Bao et al. [23] the AgNPs internalization in *Arabidopsis thaliana*. Finally, Nath et al. [24] have synthesized isotopically-labelled Ag NPs, Cu NPs and ZnO NPs to assist the traceability of the interactions between NPs and plants.

One of the biggest challenges in the characterization of NPs is their extraction from a complex matrix using mild conditions, without changing their size, conformation, and composition. Consequently, the use of strong acids should be avoided, and therefore, several authors employed basic extractions, and extractions based on enzymes. Tetramethylammonium hydroxide (TMAH) was used for alkaline solubilization of biological tissues before NPs assessment [15,16,25]. In addition, several authors have used mixtures of enzymes to isolate NPs from complex biological matrices. In the case of tissues with high content of protein and fat, a pancreatin-lipase combination has been proposed [26,27], but Macerozyme R-10® (a mixture of cellulase, hemicellulase, and pectinase) is more adequate to destroy the vegetal cell walls previously to the determination of NPs by SP-ICP-MS [20,23,24,28,29].

The aim of this work is the development of an ultrasound-assisted enzymatic methodology for the extraction of Ag NPs from seaweed samples with a mixture of enzymes (Macerozyme R-10®), and the application to some red and green seaweed exposed to Ag NPs. ICP-MS and SP-ICP-MS were respectively used for quantifying the total amount of silver, and for the determination of Ag NPs concentration and size distributions.

2. Experimental

2.1. Instrumentation

Total silver determination was performed using an inductively coupled plasma-mass spectrometer NexIon® 2000 (PerkinElmer, Waltham, MA, USA) equipped with an ESI Single Cell Micro DX (PerkinElmer, Shelton, USA) autosampler. AgNPs data acquisition and management were performed with the help of the SP-ICP-MS Syngistix™ Nano application software (PerkinElmer).

An Ethos Plus microwave lab station (Milestone, Bergamo, Italy) was used for sample digestion. The microwave is equipped with ten Teflon vessels (100 mL), Teflon covers, HTC adapter plate, HTC safety springs (Milestone), and pressure and temperature controller probe.

Scanning transmission electron microscopy (STEM) with a probe-corrected FEI Titan G2 80–200 kV ChemiSTEM (FEI, Hillsboro, USA), was used for the acquisition of AgNPs images present in cleaned enzymatic extracts.

A Basic20 pH meter (Crison, Barcelona, Spain) was used for buffer pH adjustments. The VibraCell™ VCX 130 V (Sonics Newtown, CT, USA) ultrasound probe was used to homogenize the seaweed samples previous to the enzymatic extraction. A VWR Ultrasonic Cleaner (Barcelona, Spain) was used to disperse the nanoparticles before their use. AgNPs isolation from seaweed was performed with a Boxcult temperature-controlled incubation chamber (Stuart Scientific, Surrey, UK) equipped with a Rotabit orbital-rocking platform shaker (J.P. Selecta, Barcelona, Spain). A Laborcentrifugen 2K15 centrifuge (Sigma, Osterode, Germany) was used for cleaning the enzymatic extracts before the STEM analysis. Other instrumentation to perform the analysis includes an analytical balance ML204 (Mettler Toledo, Barcelona, Spain), and a Reax top vortex vibrational shaker (Heidolph, Schwabach, Germany).

2.2. Reagents

Macerozyme R-10® (Merck, Darmstadt, Germany) was used for AgNPs isolation from seaweed matrix. This mixture of enzymes is obtained from *Rhizopus* sp., and it is formed by cellulase (0.1 units per mg), hemicellulase (0.25 units per mg), and pectinase (0.5 units per mg). Citric acid for analysis (ACS, Panreac, Barcelona, Spain), and trisodium citrate di-hydrated for analysis (Merck) were used for buffer preparation and extracts homogenization.

Ionic silver stock solution (1000 mg L^{-1}) in 2% HNO_3 (PerkinElmer, Shelton, CT, USA) was used to prepare the ionic silver standards; whereas, ionic Rhodium stock solution (1000 mg L^{-1}) in 2% HCl (PerkinElmer) was used as an internal standard (5 μg L^{-1} in 1% nitric acid). Nitric acid 69% v/v (SUPRAPUR®, Sigma Aldrich, Darmstadt, Germany) and hydrogen peroxide 33% v/v ACS (Panreac, Barcelona, Spain) were used for the seaweed microwave-assisted acid digestion. A reference material PEG-COOH Gold Nanospheres (49.6 nm and 9.89×10^6 particles mL^{-1}) were from NanoComposix (San Diego, CA, USA), and an ionic gold stock standard (1000 mg L^{-1}) in 2 mol L^{-1} HCl (Merck) were used to calculate the transport efficiency in SP-ICP-MS. A suspension of polyvinylpyrrolidone (PVP) coated silver nanopowder of 15 nm and a concentration of 5.4×10^{14} particles mL^{-1} were from SkySpring Nanomaterials, Inc. (Houston, TX, USA), and were used for analytical performance check.

Other reagents were glycerol for analysis (Merck), absolute ethanol (PanReac, AppliChem, Barcelona, España), and NexIon Setup Solution (1.0 μg L^{-1} of Be, Ce, Fe, In, Li, Mg, Pb, and U in 1% of HNO_3) from PerkinElmer. Ultrapure water (18 MΩcm of resistivity) was obtained from a Milli-Q® purification system (Millipore Co., Bedford, MA, USA). Argon with a 99,999% of purity was from Nippon Gases (Madrid, Spain).

Other consumables were Minisart NML hydrophilic non-sterile 5.0 μm filters (Sartorius, Goettingen, Germany) for filtrating the enzymatic extracts, and regenerated cellulose Amicon Ultra- 0.5 mL centrifugal filter units (30 kDa nominal molecular weight limit, NMWL) from Merck for enzymatic extracts cleaning for STEM analysis. Plastic material (pipette tips, syringes, tubes …) was discarded after use, and all the glassware material was submitted to a decontamination procedure to avoid ultra-trace metal contamination. Glasswares were washed three times with ultrapure water and kept in nitric acid (10% v/v) for 48 h, then washed several times with ultrapure water, dried in an oven and stored until the next use.

2.3. Seaweed samples harvesting and exposure trial

Seaweed samples used for the optimization of the procedure were from Indigo Rock Marine Research Station (Cork, Ireland) and they consist of five Dulse (*Palmaria palmata*) and five sea lettuce (*Ulva* sp.) samples. Seaweeds were harvested in 40 L tanks at 16 ± 1 °C with periods of 12 h of white light and 12 h of darkness and exposed to AgNPs for twenty-eight days. Available fresh seaweed samples were washed in the laboratory with ultrapure water (salts and other contaminants removal) and were manually homogenized before storing in polyethylene tubes at −18 °C.

Table 1
Operating conditions for ICP-MS and SP-ICP-MS measurements.

ICP-MS	
Parameter/Component	Type/Value/Mode
Nebulizer	Meinhard CT R^+
Nebulizer Chamber	5 °C refrigerated glass cyclone chamber with Peltier PC^{3X}
Cone material	Nickel/aluminium
Plasma gas flow	15 L min^{-1}
Auxiliary gas flow	1.2 L min^{-1}
Nebulizer gas flow	1.15 mL min^{-1}
Power RF	1600 W
Integration time	1000 ms
Replicates per sample	3
Operation mode	KED (4.5 mL min^{-1})
Isotopes	^{107}Ag,^{109}Ag, and^{103}Rh (internal standard)
SP-ICP-MS	
Parameter	Value
Sample flow rate	0.19–0.21 mL min^{-1}
Dwell time	50 μs
Sampling time	100 s
Number of readings	2.000.000
RPq	0.25
Operation mode	Standard
Isotope	^{107}Ag,^{197}Au (for TE)

2.4. Microwave assisted acid digestion

Microwave assisted acid digestion was performed by weighing 1.0000 g of homogenized seaweed samples and mixing with 4.0 mL of ultrapure water, 3.0 mL of 69% (v/v) HNO_3, and 1.0 mL of 33% (v/v) H_2O_2 in Teflon vessels. The microwave digestion program lasts 34 min at 850 W. The first step consists of heating from room temperature to 90 °C (ramp of 4 min), then heating from 90 to 140 °C (ramp of 5 min), then from 140 to 200 °C (ramp of 5 min), and finally maintaining the reactors at 200 °C for 20 min. After cooling, the acid digests were made up to 25 mL with ultrapure water and stored before ICP-MS analysis. Each seaweed sample was subjected to the microwave assisted acid digestion in triplicate and two reagent blanks were prepared for each set of sample pre-treatment.

2.5. Enzymatic hydrolysis for AgNPs extraction

A mass of 0.0500 g of seaweed sample was introduced in a 10 mL polyethylene tube with 7 mL of 2 mM/2 mM citric acid/trisodium citrate buffer (pH = 4.5). The mixture was sonicated in an ice bath for 2.5 min with intermittent 1.0 s pulses (total time: 5 min) using the ultrasound probe operating at 20% of amplitude. Then 2.0 mL of a 25 g L^{-1} Macerozyme R-10® solution was added. After extraction in a Boxcult temperature-controlled incubation chamber at 150 rpm and 37 °C for 6.0 h, the extracts were filtered with 5.0 μm cellulose syringe disks and were diluted with 1.0% (v/v) glycerol before SP-ICP-MS analysis.

2.6. Total Ag determination by ICP-MS

The total content of Ag in the acid digests was determined by ICP-MS under the conditions listed in Table 1. Standard addition calibration method in an Ag concentration range from 0 to 15.0 μg L^{-1} was used. Isotopes ^{107}Ag and ^{109}Ag were monitored and ^{103}Rh was used as an internal standard. The Kinetic Energy Discrimination (KED) mode was used to reduce the polyatomic interferences of silver, introducing a continuous flow of 4.5 mL min^{-1} of He in the collision cell of the instrument.

2.7. AgNPs analysis by SP-ICP-MS

Enzymatic extracts were diluted at least 100 times with 1.0% (v/v) glycerol and submitted to bath-sonication for 1.0 min previous to their analysis by SP-ICP-MS under the conditions listed in Table 1. The Syngistix™ Nano Application Software needs to perform different measurements to calculate the efficiency with which the NPs reach the nebulizer (transport efficiency, TE). These measurements include: (1) the calculation of the sample introduction flow rate through the peristaltic pump by aspirating a volume of ultrapure water for 1 min in triplicate, (2) the analysis of the Au nanospheres (49.6 nm in size) reference material at a concentration of 9.89×10^4 particles mL^{-1}, (3) the measurement of gold ionic standards of 0, 1.0, 2.0, and 3.0 μg L^{-1}. The TE is measured in the same operational conditions as silver (Table 1) but using the *m/z* 197 of the gold isotope. The experimental TE was approximately 9 ± 1%. Once the Syngistix™ Nano Application Software obtains the TE, a silver ionic calibration with standards of 0, 0.5, 1.0, 2.5, and 5 μg L^{-1} was carried out. Finally, the AgNPs concentration and size distributions in the diluted extracts were directly obtained from the software after analysis.

2.8. Scanning transmission electron microscopy analysis of macerozyme R-10® extracts

An enzymatic extract cleaning and NPs pre-concentration are necessary before STEM analysis of seaweed enzymatic extracts. The procedure consists of filtration by ultracentrifugation using Amicon® Ultra 30 KDa centrifugal filter units. Therefore, the cleaning process starts by filling the units with 0.5 mL of extract and centrifuging at 14,000 g and 4 °C for 20 min. The process was repeated five times by refilling the units with 0.5 mL of ultrapure water. Then, a volume of 0.5 mL of absolute ethanol was added and shaken with the extract for 1.0 h to eliminate the excess of organic matter. Finally, the cleaning process using ultrapure water was repeated five more times. The clean and pre-concentrated extract (approximately 25 μL) was collected and five drops of 3.0 μL were deposited on a Ti grid for increasing the concentration of particles during microscopic analysis. These drops were successively deposited and allowed to dry at room temperature before inserting the grid in the STEM sample holder.

3. Results and discussion

3.1. Selection of ultrasound-assisted enzymatic hydrolysis conditions for AgNPs extraction

An ultrasound-assisted enzymatic hydrolysis method for silver nanoparticles analysis was properly optimized and validated. Ultrasonic energy was used to improve the extraction procedure, and parameters related to sonication, such as ultrasound amplitude, sonication time, presence vs. absence of pulses during sonication, and the use of a probe vs. an ultrasonic bath were evaluated. Furthermore, the effect of the concentration of Macerozyme R-10® and the incubation (extraction) time were studied. All experiments were performed in triplicate with three blanks per condition. The experiments carried out during the optimization process were performed with portions of 0.0500 g of a pool from *Palmaria palmata* samples exposed to 1.0 mg L^{-1} of AgNPs, and an ionic silver calibration (0–5.0 μg L^{-1}) was used for AgNPs concentration determination by SP-ICP-MS.

3.1.1. Ultrasound amplitude

Ultrasound amplitude is correlated with the power of the ultrasonic probe. For extraction of AgNPs the amplitude cannot be very high due to the possible destruction, or ionization of the particles. Therefore, different amplitudes of the vibration movement of the probe (20, 30, and 40%) were checked. For each amplitude value, a mass of 0.0500 g of red seaweed sample was mixed in a polyethylene tube with 7 mL of the sodium citrate/citric acid 0.2 mM/0.2 mM (pH = 4.5) buffer and submitted to sonication for 2.5 min with intermittent 1.0 s pulses. Each tube was placed in an ice bath to prevent the sample from heating up due to the ultrasound energy. After sonication, a volume of 2.0 mL of the 25 g L^{-1} Macerozyme R-10® solution was added and then incubated in a

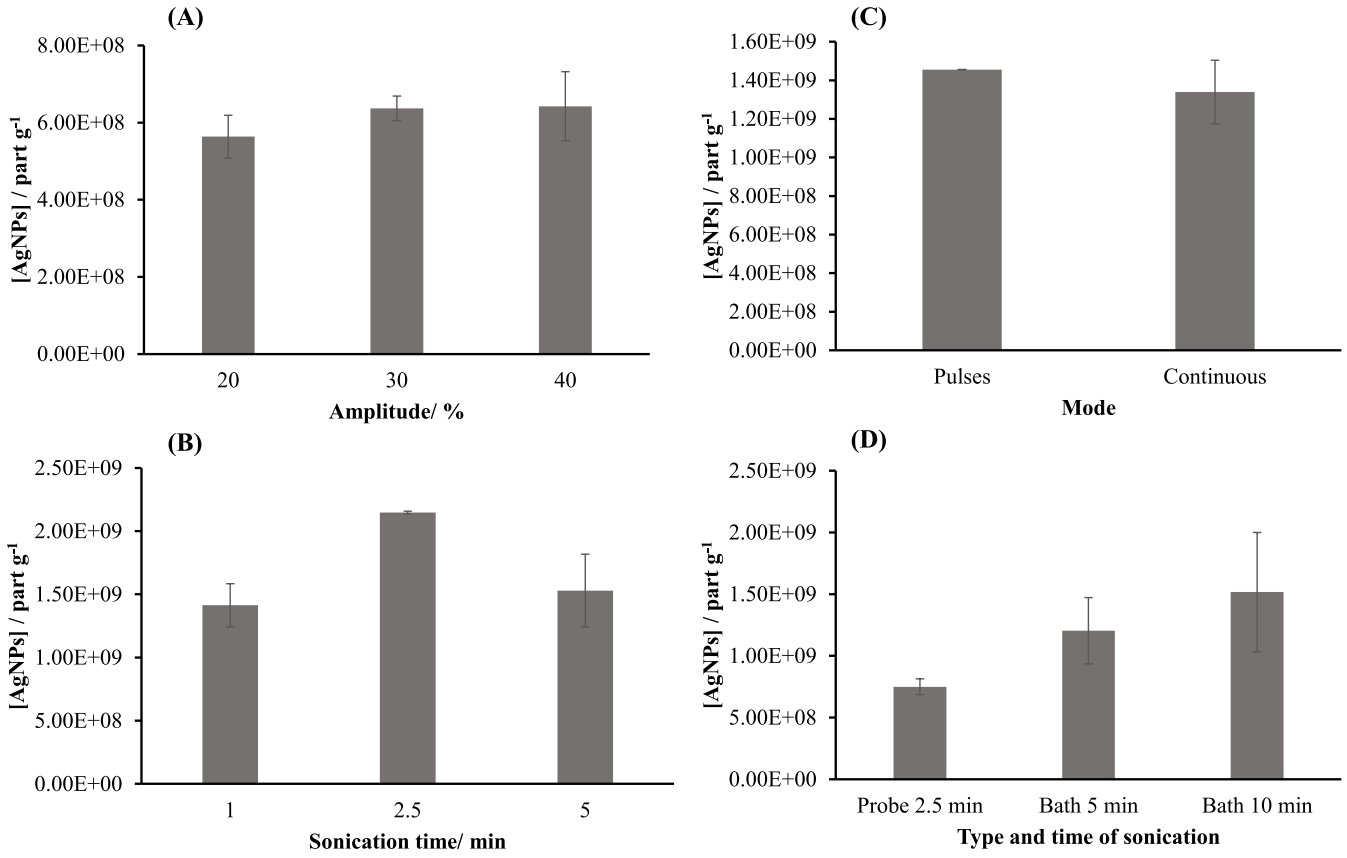

Fig. 1. Optimization of parameters affecting sonication: (A) amplitude, (B) sonication time, (C) mode, and (D) type of sonication.

Boxcult incubation chamber overnight at 37 °C and 150 rpm. The number of AgNPs extracted from the sample was between $5.64 \times 10^8 \pm 5.55 \times 10^7$ part g^{-1} (mean ± standard deviation, for 20% amplitude) and $6.42 \times 10^8 \pm 8.97 \times 10^7$ part g^{-1} (for 30% amplitude) (Fig. 1A). Regarding the AgNPs size, no significant differences were shown for the amplitudes studied, and the most frequent size was 19 nm. Then, a 20% amplitude value was selected to minimize nanoparticle degradation.

3.1.2. Sonication time

Once the amplitude of sonication was set at 20%, the sonication time was the next parameter studied. Different sonication times (1.0, 2.5, and 5.0 min using intermittent 1.0 s pulses) were selected considering previous studies reported in the literature [20,21]. Results for these conditions are plotted in Fig. 1B. The highest AgNPs concentration in the enzymatic extracts was achieved when 2.5 min of ultrasound energy was used. Particle concentration and the most frequent size were $2.15 \times 10^9 \pm 1.04 \times 10^7$ Ag part g^{-1} and 18 ± 1 nm, respectively, for 2.5 min sonication time (total time of 5.0 min). It seems that an increase in sonication time might degrade the nanoparticles and produce a rise of the temperature in the extract.

3.1.3. Pulsed vs. continuous sonication

Once the sonication time was selected, the continuous sonication and pulsed sonication modes were compared (Fig. 1C). The number of nanoparticles measured in the enzymatic extracts after pulsed and continuous sonication was $1.64 \times 10^9 \pm 1.34 \times 10^6$ and $1.34 \times 10^9 \pm 1.78 \times 10^8$ part g^{-1}, respectively. The most frequent sizes were the same for both types of sonication (18 ± 1 nm). There were no significant differences between the concentrations obtained by sonication with pulses and using continuous sonication. However, the relative standard deviation was lower for pulsed mode (0.1%) than for continuous sonication (12%). Then, the pulsed sonication mode was selected.

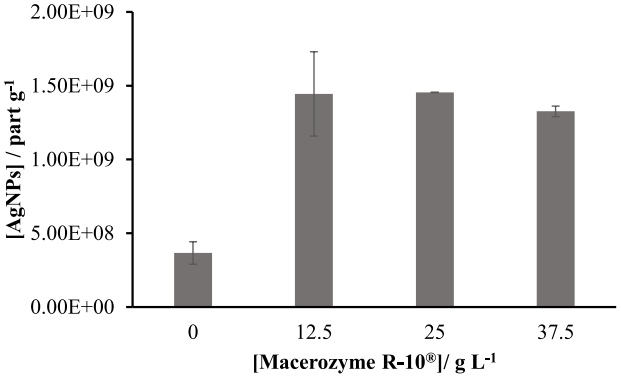

Fig. 2. Optimization of Macerozyme R-10® concentration.

3.1.4. Type of sonication (bath vs. probe)

Regarding sample preparation, sonication in an ultrasound bath would be easier than using an ultrasound probe (a sample each time). Therefore, the use of an ultrasound bath was evaluated. This experiment was performed using the enzymatic extraction with the previously optimized parameters for the probe (20% of amplitude, 2.5 min of sonication with pulses), and using an ultrasound bath for 5.0 and 10 min. Results for these conditions are shown in Fig. 1D. The concentration of nanoparticles extracted using the ultrasound probe (2.5 min) and the ultrasound bath (5 min and 10 min) were $7.49 \times 10^8 \pm 6.44 \times 10^7$,

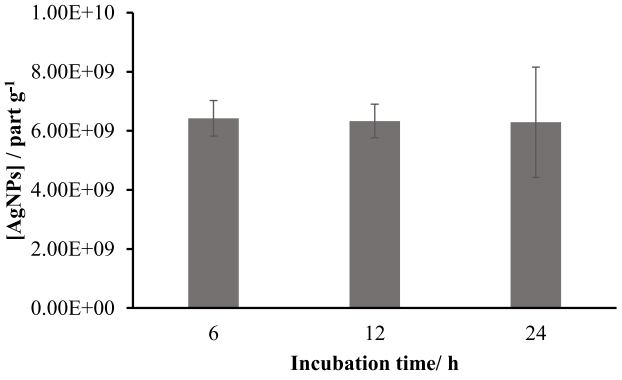

Fig. 3. Optimization of incubation time.

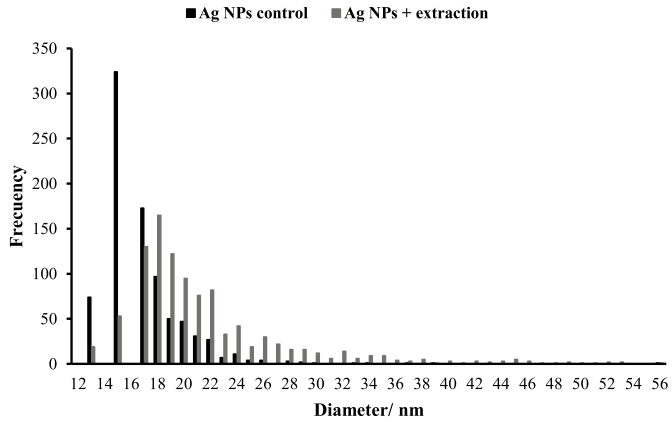

Fig. 4. SP-ICP-MS histograms of AgNPs control and AgNPs after extraction (sonication + enzymatic hydrolysis).

$1.29 \times 10^9 \pm 2.68 \times 10^8$, and $1.52 \times 10^9 \pm 4.83 \times 10^8$ part g^{-1}, respectively. Although the mean concentration of particles extracted from the sample was higher when using the ultrasonic bath, an ANOVA test (Statgraphics XVIII, Warrenton, USA) showed that there was no significant statistical differences ($P > 0.05$) between the ultrasound bath and the ultrasound probe. Finally, due to the higher relative standard deviations obtained with the ultrasound bath (RSD> 22%), the decision was to continue the research with the ultrasound probe.

3.1.5. Macerozyme R-10® concentration

Macerozyme R-10® concentration was studied within the 0–37.5 g L^{-1} of enzyme range. Results for these experiments are plotted in Fig. 2. The concentration of AgNPs obtained with the enzymatic extraction using 0, 12.5, 25, and 37.5 g L^{-1} of Macerozyme R-10® solution were $3.66 \times 10^8 \pm 7.60 \times 10^7$, $1.44 \times 10^9 \pm 2.86 \times 10^8$, $1.46 \times 10^9 \pm 1.34 \times 10^6$, and $1.33 \times 10^9 \pm 3.58 \times 10^7$ part g^{-1}, respectively. The most frequent sizes remained constant with the different concentrations of enzymes added (18 ± 1 nm). Considering the extracted particle concentration, and the lower relative standard deviation (0.1%), a concentration of 25 g L^{-1} of Macerozyme R-10® was selected as the optimum concentration for AgNPs isolation from seaweed.

3.1.6. Incubation (extraction) time

Overnight extractions are highly time-consuming in terms of sample preparation. Therefore, the effect of the incubation time in the extracted AgNPs was studied. Several incubation times (6, 12, and 24 h) were evaluated. Results for this experiment are shown in Fig. 3. The number of AgNPs extracted from seaweed were $6.42 \times 10^9 \pm 6.05 \times 10^8$, $6.33 \times 10^9 \pm 5.69 \times 10^8$, and $6.29 \times 10^9 \pm 1.87 \times 10^9$ for 6, 12, and 24 h, respectively. No statistically significant differences were found for the three different incubation times. Therefore, 6 h was chosen due to a decrease in the sample preparation time.

3.2. Stability of AgNPs size under the selected extraction conditions

The stability of the nanoparticles during the extraction procedure was tested after comparing control AgNPs standards (un-treated suspensions) and the same AgNPs standards submitted to the whole procedure (sonication + enzymatic hydrolysis). A 15 nm AgNPs standard (5.4×10^{14} part mL^{-1}) was diluted with ultrapure water, subjected to the optimized enzymatic extraction and diluted with glycerol 1% (v/v) to a final theoretical concentration of 6×10^7 part mL^{-1} before SP-ICP-MS measurements. The control AgNPs standard (without the enzymatic extraction) was diluted in the same way to the same final concentration of 6×10^7 part mL^{-1}. This final concentration was selected to obtain a histogram with approximately one thousand single peaks, to avoid double events. Both experiments were performed in triplicate. No significant changes were observed in the most frequent sizes, taking into account that those values are close to the limit of detection in size of the technique. The sizes reported were 15 ± 1, and 18 ± 1 nm for the AgNPs control, and the AgNPs submitted to the enzymatic extraction, respectively. Fig. 4 shows two overlapping histograms, corresponding to the AgNPs control and the AgNPs subjected to enzymatic extraction. The superimposed histograms show that enzymatic extraction does not affect the size of the AgNPs.

3.3. Efficiency of AgNPs extraction

The efficiency of silver extraction was checked by comparing the Ag content in the digested samples and the total amount of Ag extracted from a seaweed sample. This study was carried out using a *Palmaria palmata* sample submitted to the microwave acid digestion method and the enzymatic extraction method in triplicate. Blanks of the sample were also prepared in duplicate by both methods. Finally, the total content of Ag, in the digested sample and the extract was analyzed by ICP-MS. The achieved average total concentration found were 0.69 ± 0.25 μg g^{-1}, and 0.57 ± 0.10 μg g^{-1} for the total silver in the microwave acid digest, and the enzymatic extract, respectively, providing an 80% of the recovery percentage for the total silver extraction.

3.4. Analytical characteristics of the method

3.4.1. Matrix effect evaluation

For ICP-MS measurements, a silver ionic calibration prepared in HNO_3 1% (v/v) was compared to a standard addition silver calibration prepared with a pool of seaweed digested samples in the same dilution (1:25) as the samples using ultrapure water. For both calibrations, the concentrations of ionic silver were 0, 0.01, 0.05, 0.1, 1.0, 5.0, 10.0, and 15.0 μg L^{-1}. Good linearities were obtained for both calibration graphs (R^2 of 0.998 and 0.999, respectively). The slopes for the external calibration and the standard addition graphs were 8855.6 L μg^{-1} and 9848.7 L μg^{-1}, respectively. As the slopes were statistically different (*t*-test, 95% confidence level), the standard addition calibration method was used to measure total Ag in the digests by ICP-MS and correct the matrix effect.

For SP-ICP-MS measurements, an aqueous silver ionic calibration was compared to a silver ionic calibration matched with the enzymatic solution prepared at the same dilution (1:100) than the samples using 1.0% (v/v) glycerol. For both calibrations, the concentrations of ionic silver were 0, 0.5, 1.0, 2.5, and 5.0 μg L^{-1}. Good linearities were obtained for both calibration graphs (R^2 values 0.993 and 0.999, respectively). The slopes of the aqueous calibration and the matrix-matched calibration resulted in 2.01 L μg^{-1} and 1.94 L μg^{-1}, respectively. Both slopes were statistically similar (*t*-test, 95% confidence level), and then the matrix effect can be assumed as negligible. Therefore, the aqueous

Table 2
Precision of the method for AgNPs determination by SP-ICP-MS. RSD (%) are included in brackets.

	Palmaria palmata	Ulva sp.
Most frequent size (nm)	26 ± 1 (4%)	26 ± 1 (4%)
Mean size (nm)	30 ± 2 (5%)	33 ± 3 nm (8%)
NPs concentration (part g^{-1})	$1.56 \times 10^9 \pm 2.45 \times 10^8$ (15%)	$2.82 \times 10^8 \pm 5.04 \times 10^7$ (18%)

ionic silver calibration can be used in the analysis of AgNPs in enzymatic extracts from seaweed by SP-ICP-MS.

3.4.2. Sensitivity

The limit of detection and quantification were calculated using the 3 × SD/slope and 10 × SD/slope criterion (SD: standard deviation of ten measurements of a blank) and considering the treatment of the sample. For total Ag determination by ICP-MS in the digested seaweed samples, the isotope ^{107}Ag was selected due to its higher relative abundance and the lower limit of detection (LOD = 2.2 ng g^{-1}) and quantification (LOQ = 7.7 ng g^{-1}) achieved. Limits of detection in size (LOD_{size}) and particle concentration (LOD_{number}) for the analysis by SP-ICP-MS were calculated using the Laborda et al. [30] Microsoft Excel tool. The 5-sigma threshold is used to avoid the occurrence of false positives. Thus, the LOD_{size} was calculated using Equation (1), where σ_B is the standard deviation of the mean baseline signal, w is the peak width at microsecond times, ρ and F_p are the density of the particle analyte (10.49 g cm^{-3} for AgNPs), and the mass fraction of the particle, respectively, and t_{dwell} is the dwell time for the analyte (μs). K_{ICPMS} is the detection efficiency, or the number of ions detected relative to the number of analyte atoms introduced into the ICP-MS, and K_M (=AN_{av}/M_M) is a factor dependent on the element measured (A, N_{AV}, and M_M are the atomic abundance of the selected isotope, the Avogadro number, and the atomic mass of the selected element, respectively).

$$LOD_{size} = X_D^{size} = \left(\frac{30\,\sigma_B}{\frac{2}{w}\pi\rho F_p K_{ICPMS} K_M t_{dwell}} \right)^{1/3} \quad (1)$$

The LOD_{number} was calculated using Equation (2), where $Y_{N,B}$, η_{neb}, Q_{sam}, and t_i are the mean number of particles counted in a blank, the transport efficiency of the ICP-MS, the flow rate introduction of the sample solution through the peristaltic pump, and the total acquisition time, respectively.

$$LOD_{number} = X_D^{number} = \frac{\sqrt[5]{Y_{N,B}} + 3}{\eta_{neb} Q_{sam} t_i} \quad (2)$$

The SP-ICP-MS limits of detection were calculated using a blank submitted to the whole extraction procedure and diluted 100 times in 1% (v/v) glycerol: LOD_{size} = 14 nm (5 sigma criteria; 12 nm using the less restrictive 3 sigma criteria), and $LOD_{number} = 2.40 \times 10^6$ part L^{-1} (instrumental limit) or 4.34×10^7 part g^{-1} (referred to the wet seaweed sample). Bao et al. [23] characterized AgNPs in plant tissues using a Macerozyme enzymatic extraction by SP-ICP-MS and they obtained a similar LOD_{size} (10 nm), but they did not provide concentration detection limit results. In fact, to the best of our knowledge, there are no reports about ultrasound-assisted enzymatic methods to determine AgNPs from seaweed using SP-ICP-MS.

3.4.3. Repeatability and analytical recovery

The repeatability assay of the ultrasound-assisted enzymatic isolation method was estimated using both types of seaweed samples. A *Palmaria palmata* sample and an *Ulva* sp. sample, both exposed to 1.0 mg L^{-1} of AgNPs, were submitted to the optimized enzymatic extraction method in quintuplicate. For five measured replicates of each specimen of seaweed, the most frequent sizes, the mean sizes, and the AgNPs concentration were obtained. The relative standard deviations are shown in Table 2. The RSD for the most frequent sizes and the mean sizes were lower than 10% in both kinds of seaweed, which confirms the good repeatability of the method. However, for the AgNPs concentration in *Palmaria palmata* and *Ulva* sp., the RSD were 15 and 18% respectively. These RSD values were considered adequate considering the complexity of the matrix and the overall enzymatic extraction process.

Table 3
Total Ag and AgNPs concentration and mean size (n = 3) in some seaweed samples.

	[Ag]/μg g^{-1}	[AgNPs]/part g^{-1}	Mean size/nm	Most frequent size/nm
Palmaria palmata 1 [b]	0.34 ± 0.05	$8.38 \times 10^8 \pm 7.27 \times 10^7$	29 ± 1	22 ± 3
Palmaria palmata 2 [b]	0.72 ± 0.09	$1.36 \times 10^9 \pm 1.74 \times 10^8$	26 ± 1	22 ± 2
Palmaria palmata 3 [b]	0.51 ± 0.06	$8.03 \times 10^8 \pm 5.00 \times 10^7$	24 ± 2	20 ± 1
Palmaria palmata 4	0.021 ± 0.002	$<4.34 \times 10^7$	-[a]	-[a]
Palmaria palmata 5	0.011 ± 0.002	$<4.34 \times 10^7$	-[a]	-[a]
Ulva sp. 1 [b]	0.20 ± 0.03	$6.96 \times 10^8 \pm 1.20 \times 10^8$	28 ± 3	21 ± 3
Ulva sp. 2 [b]	0.20 ± 0.03	$8.12 \times 10^8 \pm 7.25 \times 10^7$	32 ± 4	22 ± 2
Ulva sp. 3 [b]	0.17 ± 0.02	$7.44 \times 10^8 \pm 1.20 \times 10^8$	28 ± 1	20 ± 1
Ulva sp. 4	0.017 ± 0.001	$<4.34 \times 10^7$	-[a]	-[a]
Ulva sp. 5	0.010 ± 0.001	$<4.34 \times 10^7$	-[a]	-[a]

[a] Not evaluated.
[b] Seaweed exposed to 1 mg L^{-1} PVP-AgNPs.

The accuracy of the extraction method was also guaranteed with the analytical recovery assay. An SP-ICP-MS analysis was performed in triplicate using a *Palmaria palmata* sample submitted to the Macerozyme enzymatic extraction and spiked with 15 nm PVP-AgNPs with a final concentration of 6.0×10^7 part mL^{-1}. The un-spiked Macerozyme enzymatic extract and the 15 nm PVP-AgNPs suspension of 6.0×10^7 part mL^{-1} used for the spiking experiments were also analyzed in triplicate by SP-ICP-MS. The analytical recovery was calculated using Equation (3), where $[Ag]_{spiked}$ is the PVP-AgNPs concentration (part mL^{-1}) in the seaweed Macerozyme enzymatic spiked extract, $[Ag]_{un\text{-}spiked}$ is PVP-AgNPs concentration (part mL^{-1}) in the seaweed Macerozyme enzymatic extract, and $[Ag]_{suspension}$ is the PVP-AgNPs concentration (part mL^{-1}) of the standard used for spiking the extract.

$$AR = \frac{[Ag]_{spiked} - [Ag]_{un-spiked}}{[Ag]_{suspension}} \times 100 \quad (3)$$

The average analytical recovery obtained by SP-ICP-MS for this experiment was close to 100% (112 ± 12%, expressed as mean ± SD), and was considered adequate.

3.5. Applications

The optimized and validated proposed methodology was applied to ten *Palmaria palmata* and *Ulva* sp. seaweed samples from the Atlantic area, some of them exposed to Ag NPs (sample numbers 1, 2, and 3). The samples were subjected to microwave acid digestion and the enzymatic extraction methods described in sections 2.6 and 2.7 and then, analyzed by ICP-MS and SP-ICP-MS.

Table 3 shows the total concentration of Ag in the samples, the concentration of AgNPs, the mean size and the most frequent size. The total basal silver in unexposed seaweed (*Palmaria palmata* 4 and 5 and *Ulva* sp. 4 and 5) varies between 0.011 ± 0.002 and 0.021 ± 0.002 μg g^{-1} for *Palmaria palmata,* and between 0.010 ± 0.001 and 0.017 ±

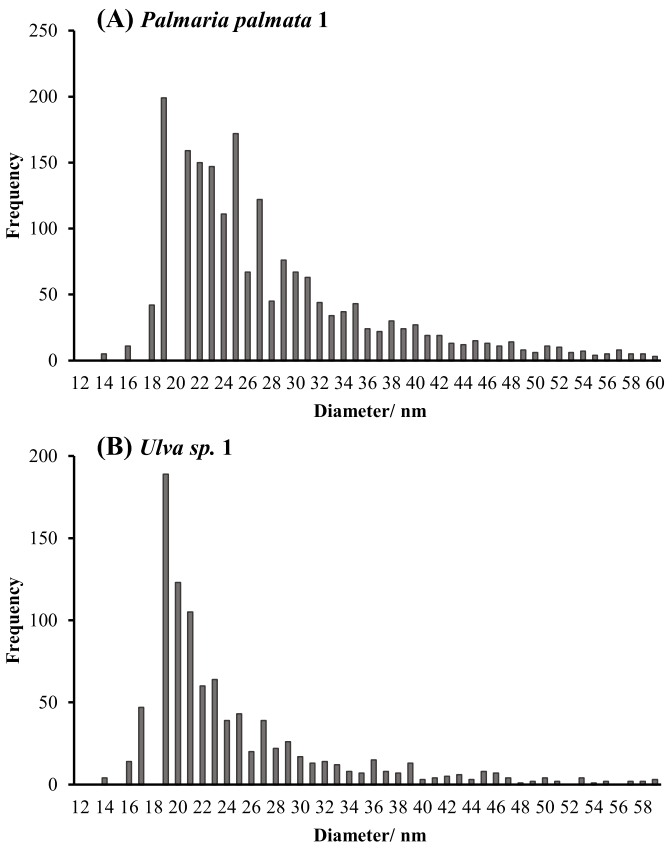

Fig. 5. AgNPs size distribution in: (A) *Palmaria palmata* (B) Ulva sp.

0.001 μg g^{-1} for *Ulva* sp. This low basal concentration does not allow the detection of AgNPs by SP-ICP-MS. Although total Ag concentration in unexposed seaweed was in the same magnitude order for *Palmaria palmata* and *Ulva* sp., total Ag concentrations in exposed *Palmaria palmata* were higher (between 0.34 ± 0.05 and 0.72 ± 0.09 μg g^{-1}) than for exposed *Ulva* sp. (between 0.17 ± 0.03 and 0.20 ± 0.03 μg g^{-1}). In the same way, the AgNPs concentration was higher for exposed *Palmaria palmata* (between $8.03 \times 10^8 \pm 5.00 \times 10^7$ and $1.36 \times 10^9 \pm 1.74 \times 10^8$ AgNP g^{-1}) than for exposed *Ulva* sp. (between $6.96 \times 10^8 \pm 1.20 \times 10^7$ and $8.12 \times 10^8 \pm 7.25 \times 10^7$ AgNP g^{-1}). The most frequent sizes were very similar in both kinds of exposed seaweed, between 20±1–22 ± 3 nm, and 20±1–22 ± 2 nm for *Palmaria palmata* and *Ulva* sp., respectively. Similarly, mean sizes were also similar, within the 24±2–29 ± 1 nm and 28±1–32 ± 4 nm ranges for *Palmaria palmata* and *Ulva* sp., respectively.

Fig. 5 shows the histograms corresponding to *Palmaria palmata* sample 1 (Fig. 5A) and *Ulva* sp. sample 1 (Fig. 5B).

Using the procedure described in section 2.8., a Macerozyme enzymatic extract from *Palmaria Palmata* was cleaned, pre-concentrated and deposited on a Ti grid to visualize AgNPs using STEM. Fig. 6 shows the STEM images obtained working in TEM mode (Fig. 6A) and STEM mode (Fig. 6B and 6C). These images confirm the presence of the spherical AgNPs in the enzymatic extracts.

The histogram in Fig. 7 shows the polydispersity sizes of 200 particles measured with Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA software. The mean size measured in TEM was 24 nm; this value agrees with the mean size obtained for *Palmaria palmata* by SP-ICP-MS (between 24 ± 2 and 29 ± 1 nm). The mean sizes observed in SP-ICP-MS and TEM were higher than the original 15 nm nanoparticles used for exposure. That could indicate that seaweed can aggregate the AgNPs inside of the organism to decrease toxicity that seems to be size-dependent [31]. This result is very similar to that obtained by Bao et al. [23] that exposed *Arabidopsis* plant cells to 10 nm silver nanoparticles and after SP-ICP-MS analysis observed average diameters of 26–27 nm. Similarly, Li et al. [29] reported an increase from 17-18 nm to 36–48.9 nm in leaves of soybean and rice plants, after foliar or root exposure. More studies about bioaccumulation are planned to be carried out in the future in our research group to deepen our knowledge about seaweed metabolism and detoxification mechanisms.

4. Conclusions

There is a certain concern about the accumulation of nanomaterials by biota in the aquatic media. In this work, an ultrasound-assisted enzymatic extraction method was developed to analyze AgNPs by SP-ICP-MS. The extraction conditions (affecting sonication and enzymatic hydrolysis with the Macerozyme R-10® enzyme complex) were optimized. The analytical performance was evaluated with good

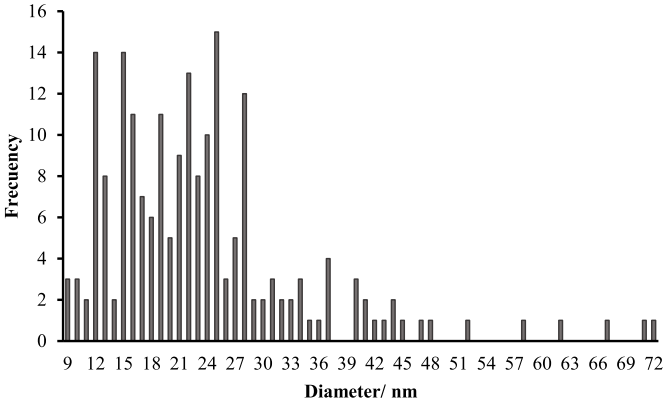

Fig. 7. AgNPs size distribution histogram in *Palmaria palmata* (data from TEM images, n = 200 nanoparticles).

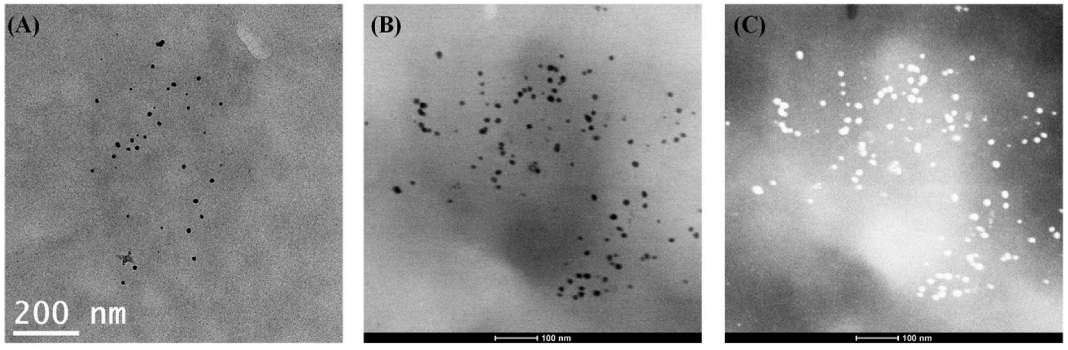

Fig. 6. Images of AgNPs in the enzymatic extracts obtained operating in mode: (A) TEM (B) STEM, bright field (C) STEM, high angle annular dark field.

repeatability, sensitivity, and analytical recovery for SP-ICP-MS measurements. Then the optimized method was applied to the detection of AgNPs in red and green seaweed samples without significantly size-changing distribution of AgNPs, as it was confirmed by TEM.

Credit authors statement

Juan José López-Mayán: Methodology, Formal analysis, Investigation, Validation, Visualization, Writing – original draft preparation. Blanca Álvarez Fernández: Formal analysis, Investigation, Validation. Elena Peña-Vázquez: Methodology, Supervision, Validation, Writing-Reviewing and Editing, Visualization.María Carmen Barciela-Alonso: Methodology, Data curation, Supervision, Validation, Visualization. Antonio Moreda-Piñeiro: Writing- Reviewing and Editing, Supervision, Writing- Reviewing and Editing, Project administration, Funding acquisition.Pilar Bermejo- Barrera: Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to acknowledge the financial support of Ministerio de Economía y Competitividad (project INNOVANANO, reference RT2018-099222-B-100), European Union (INTERREG Atlantic Area, project NANOCULTURE, reference EAPA590/2018), and Xunta de Galicia (Grupo de Referencia Competitiva, grant number ED431C2018/19). The authors also wish to thank Dr. Mick Mackey and Dr. Julie Maguire from Indigo Rock Marine Research Station (Cork, Ireland) for seaweed harvesting and exposure experiments, Daniel Lage Cacheda (undergraduate student from University of Santiago de Compostela) for his collaboration in ICP-MS measurements, and Monica Quarato (International Laboratory of Nanotechnology, INL, Braga, Portugal) for her assistance during TEM analysis.

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